Universidade Federal do Rio Grande - FURG

Instituto de Ciências Biológicas - ICB

Pós Graduação em Ciências Fisiológicas – Fisiologia Animal Comparada

Tese de Doutorado

MECANISMOS DE TRANSPORTE IÔNICO E DE ACUMULAÇÃO E TOXICIDADE AGUDA DO COBRE EM CÉLULAS DO MANTO DO MARISCO BRANCO *Mesodesma mactroides* Deshayes, 1854

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SUMÁRIO

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RESUMO

Moluscos bivalves acumulam o cobre (Cu) de forma proporcional à concentração do metal na água. Em animais dulcícolas, a toxicidade aguda do Cu é caracterizada por distúrbios na regulação iônica dos fluídos corporais. Em animais marinhos, esta toxicidade parece estar relacionada a distúrbios no balanço ácido-base e na excreção de amônia. Assim, o objetivo do presente estudo foi avaliar a importância do manto como via de acumulação do Cu, bem como estudar o envolvimento dos principais mecanismos de transporte iônico envolvidos neste processo e o mecanismo de toxicidade aguda deste metal no marisco *Mesodesma mactroides*. Para isso, a acumulação *in vivo* do Cu foi determinada em diferentes tecidos (manto, brânquias, glândula digestiva e hemolinfa) de mariscos expostos (96h) ao Cu (CL_{20} = 5 µM). Os resultados apontaram o manto como sendo o tecido que mais acumulou Cu. Assim, o envolvimento das proteínas transportadoras de íons na acumulação de cobre e o mecanismo de toxicidade do metal foram analisados em células isoladas do manto. Com base nos resultados obtidos, foi proposto que o influxo de íons nestas células estaria associado à atividade das seguintes proteínas: trocadores Na^+/H^+ e Cl^-/HCO_3^-, cotransportadores $Na^+/K^+/2Cl^-$ e Na^+/Cl^-, canal de Na^+, Na^+/K^+-ATPase e anidrase carbônica. Por sua vez, o efluxo de íons envolveria as seguintes proteínas: Na^+/K^+-ATPase e H^+-ATPase e os canais de K^+ e Cl^-. Com base nestas informações, o efeito do Cu sobre o conteúdo iônico foi avaliado em células do manto expostas ao Cu por 1 h (5 µM) e 3 h (2.5 e 5 µM). Os resultados indicaram uma redução no conteúdo de Cl^- nas células expostas a 2.5 µM e de Na^+, K^+ e Cl^- naquelas expostas a 5 µM. Para análise da acumulação *in vitro* de Cu, as células do manto foram expostas (1 e 3 h) ao Cu (0.5, 1.0, 2.5 e 5.0 µM). Houve acumulação significativa de Cu nas células expostas a 2.5 e 5 µM, em ambos os tempos de exposição. Portanto, o envolvimento dos diferentes mecanismos de transporte iônico na acumulação de Cu foi avaliado em células expostas a 2.5 µM de Cu por 1 h, utilizando-se ferramentas farmacológicas. A inibição da anidrase carbônica ou do canal de Cl^- aumentou o conteúdo celular de Cu. Por outro lado, a inibição do cotransportador $Na^+/K^+/2Cl^-$ reduziu a acumulação de Cu. Portanto, conclui-se que o manto é uma importante via de acumulação de Cu no marisco *M. mactroides*, sendo o cotransportador $Na^+/K^+/2Cl^-$ a principal proteína envolvida na acumulação deste metal. Conclui-se ainda que o mecanismo de toxicidade aguda do Cu está associado a um desequilíbrio da regulação iônica nas células do manto.

INTRODUÇÃO

Nas últimas décadas, os níveis dos metais têm aumentado nos estuários e nas regiões costeiras como consequência de processos geoquímicos, intemperismo e através do despejo proveniente de fontes antrópicas (Santore *et al*., 2000). Quanto ao cobre (Cu), estima-se que do total anual lançado nos oceanos, grande porcentagem seja resultante de processos naturais, principalmente através do intemperismo (Pedroso e Lima, 2001). Assim, a ocorrência deste metal no meio ambiente pode ser resultante não apenas da ação humana, mas ser considerada como um fenômeno natural. Cabe ressaltar que a presença deste íon metálico no ambiente é vital para os ciclos biológicos.

O cobre é considerado um micronutriente essencial, participando de uma série de funções fisiológicas que ocorrem nos organismos aquáticos (Morgan, 2000). Este metal é componente essencial da estrutura de enzimas e proteínas, atua como co-fator enzimático e está envolvido na respiração celular (Bury *et al*., 2003; Arredondo e Núñez, 2005). Por outro lado, o cobre pode ser tóxico aos organismos aquáticos quando presente em elevadas concentrações na água, sendo que as formas iônicas deste metal são consideradas de maior toxicidade.

O cobre possui dois estados de oxidação, o íon cúprico (Cu^{2+}) e o íon cuproso (Cu^{+}). Em sistemas biológicos, o cobre é encontrado principalmente na forma de Cu^{2+}, já que na presença de oxigênio, o Cu^{+} é rapidamente oxidado a Cu^{2+} (Arredondo e Núñez, 2005). O íon cúprico ou bivalente (Cu^{2+}) reage preferentemente com ligantes inorgânicos, tais como H_2O_2, OH^-, CO_3^- e SO_4^{-2} via oxigênio, e a compostos orgânicos através dos grupos fenólicos e carboxílicos (Barceloux, 1999). Visto que os organismos aquáticos são expostos diretamente ao cobre dissolvido na água, elevadas concentrações deste metal podem levar ao seu acúmulo em vários tecidos (Romeo e Gnassia-Barelli,

1995; Chavez-Croocker *et al*., 2003; Grosell *et al*., 2002, 2004; Morales-Hernandez *et al*., 2004).

Os efeitos biológicos e a captação dos metais são regulados pela concentração do íon livre. Entretanto, estudos indicam que substâncias de baixo peso molecular, lipofílicas, complexadas ao cobre, possam difundir-se pela membrana celular e causar toxicidade (Phinney & Bruland 1994). Neste contexto, o Modelo do Ligante Biótico (BLM) foi desenvolvido com o propósito de avaliar quantitativamente a maneira pelas quais as características físico-químicas da água afetam a especiação química e a biodisponibilidade do metal em sistemas aquáticos. Este modelo considera a especiação e a complexação do metal dissolvido e a ligação competitiva entre o metal e outros cátions pelo sítio de ligação no órgão-alvo, formando o complexo "metal-ligante biótico". Portanto, a premissa do BLM é que exista uma forte correlação entre a concentração do metal no/ou sobre o alvo biológico e sua subseqüente toxicidade (Di Toro et al., 2001; Paquin et al., 2002). Paralelamente ao desenvolvimento deste modelo, estudos fisiológicos vêm sendo desenvolvidos visando aperfeiçoar o entendimento a respeito das interações dos metais com os organismos aquáticos (Bianchini & Wood, 2002; Bianchini et al., 2002; Grosell et al., 2002, 2004).

O mecanismo de toxicidade do cobre em animais aquáticos difere entre espécies dulcícolas e marinhas (Blanchard e Grosell, 2006). Isto se deve à diferença na dinâmica relacionada com a competição iônica, as relações entre acumulação e toxicidade, bem como a toxicidade das diferentes formas de cobre encontradas nos dois ambientes (Paquin *et al.*, 2002). O cobre pode competir com outros cátions como Na^{+}, Ca^{2+} e Mg^{2+} pelos sítios de incorporação destes elementos em nível branquial (Grosell *et al*., 2002; Bianchini e Wood, 2002). Em diversos organismos dulcíaquicolas, a toxicidade aguda do cobre é observada pela perda da capacidade iônica e osmorregulatória. Este metal

inibe a atividade da Na^+/K^+-ATPase, induzindo a um desequilíbrio no balanço iônico, que por sua vez é caracterizado por uma reduzida concentração de Na^+ nos fluídos corporais (Grosell *et al.*, 2002, 2004). Já em peixes e invertebrados marinhos, a toxicidade do cobre parece estar relacionada com distúrbios no balanço ácido-base e na excreção de amônia (Grosell *et al.*, 2007). De modo geral, assume-se que a toxicidade do cobre ocorre como resultado da reação do metal livre com sítios de ligação fisiologicamente ativos nas brânquias de animais de respiração aquática (Di Toro *et al*., 2001; Paquin *et al*., 2002).

Moluscos são ótimos bioindicadores de contaminação por metais, servindo como monitores biológicos da qualidade da água (Salánki *et al.*, 2003). Além disso, Roesijadi (1980) relatou que os bivalves acumulam cobre de forma proporcional à concentração deste no ambiente. Dados sobre a acumulação do cobre em moluscos sob exposição aguda ao metal indicam que este contaminante é preferencialmente acumulado nas brânquias, seguido por uma acumulação na glândula digestiva (Romeo e Gnassia-Barelli, 1995; Gu Jing *et al.*, 2006). A exposição de moluscos ao cobre (80 a 500 µg Cu L^{-1}) induz a uma variedade de efeitos tais como redução na taxa de filtração (Manley, 1983), alterações no metabolismo de proteínas (Viarengo *et al.*, 1981) e redução na homeostase do cálcio (Gnassia-Barelli *et al.*, 1995; Viarengo *et al.*, 1996). Já em baixas concentrações (10 µg Cu L^{-1}), o cobre reduz a atividade de enzimas antioxidantes (Isani *et. al.*, 2003) e afeta a taxa de crescimento (Munari e Mistri, 2007).

Os invertebrados estuarinos e costeiros são freqüentemente expostos a variações na salinidade ambiental e com isso seus epitélios devem enfrentar elevadas mudanças nas composições iônicas e osmóticas do meio externo. A osmorregulação em animais aquáticos compreende uma série de estratégias, as quais atuam em conjunto para manutenção do volume celular (Gilles e Péqueux, 1983; Péqueux, 1995). Para suportar

as variações do ambiente, os organismos aquáticos apresentam diferentes mecanismos de regulação iônica e osmótica, podendo manter a concentração osmótica do fluído extracelular diferente do meio em que vivem, e por isso são chamados osmorreguladores, ou então variar a concentração osmótica de seus fluídos corporais em função da variação da concentração osmótica do meio externo, e por isso são chamados osmoconformadores.

Por sua vez, as células animais usam estratégias osmorregulatórias que preservam seu volume e concentração de eletrólitos durante as variações da salinidade ambiental. Neste caso, a resposta osmorregulatória em nível celular inclui a regulação do volume celular, regulação adaptativa de permeabilidade à água e aos íons, regulação adaptativa do transporte iônico e a proteção de estruturas e funções de macromoléculas (Kültz, 2001).

A membrana plasmática é uma barreira seletivamente permeável entre a célula e o ambiente externo. Movimentos de todas as moléculas e íons através da membrana celular são mediados por proteínas transportadoras embebidas na bicamada lipídica (Lodish *et al.*, 2005). De forma geral, os sítios de incorporação podem ser proteínas carreadoras e proteínas canais. As proteínas carreadoras ou transportadoras se ligam a ao soluto específico a ser transportado e sofrem uma série de mudanças conformacionais, de modo a transferir o soluto através da membrana, sendo que este transporte pode ser ativo ou passivo. Quanto às proteínas canal, estas formam poros hidrofílicos que permitem o movimento passivo, normalmente de pequenos íons de carga e tamanhos conhecidos. Se a molécula a ser transportada não possuir carga, o transporte será em razão do gradiente de concentração.

A correlação entre os transportadores iônicos na manutenção do metabolismo celular pode ser observada, por exemplo, nas células responsáveis pela secreção do

excesso de íons que os animais marinhos acumulam (Hiroi e McCormick, 2007). O mecanismo de secreção consiste na ação cooperativa de três moléculas essenciais: a $Na^{+/}K^{+}$-ATPase, o co-transportador $Na^{+}/K^{+}/2Cl^{-}$ e os canais de Cl^{-}. Além disso, outra correlação existe também como conseqüência do gradiente químico gerado pela Na^{+}/K^{+}-ATPase. Em função deste gradiente, o K^{+} se movimenta do líquido intracelular para o ambiente extracelular através de canais de K^{+}. Por conseguinte, este movimento de K^{+} gera um potencial de membrana que favorece o movimento de Cl^{-} do líquido intracelular para o ambiente extracelular através de canais de Cl^{-}, sendo este íon mantido no líquido extracelular pelo potencial de membrana que é eletropositivo em relação ao meio intracelular (Alberts *et al.*, 2005).

Por sua vez, a anidrase carbônica promove a hidratação do CO_2, liberando substrato tanto para o trocador Na^{+}/H^{+} quanto para o trocador Cl^{-}/HCO_3^{-}. A inibição desta enzima pode reduzir a atividade da V-ATPase, a qual promove o bombeamento do H^{+} do citosol com utilização de ATP, em função da depleção do substrato. A atividade desta bomba protônica pode estar associada ou não a um canal apical de Na^{+}, podendo este estar ou não relacionado com o transporte de Na^{+}. Por exemplo, a manutenção da baixa concentração de Na^{+} em células epiteliais do mexilhão *Mytilus galloprovincialis* é regulada pela extrusão de Na^{+} através da bomba Na^{+}/K^{+}-ATPase e sua captação através do canal de Na^{+} (Ottaviani *et al.*, 2002). Por outro lado, a captação de Na^{+} pode se dar através da atividade do trocador Na^{+}/H^{+}, que acopla um influxo de Na^{+} à extrusão de H^{+}. Quanto maior a acidez intracelular, maior será a extrusão de H^{+} e maior será o influxo de Na^{+}, elevando assim o pH intracelular (pHi) e o aumento do Na^{+} intracelular. Quanto ao trocador Cl^{-}/HCO_3^{-}, este aumenta a velocidade de extrusão de HCO_3^{-} e de influxo de Cl^{-} quando o pH intracelular está elevado, atuando, portanto, no sentido de

diminuir o pH intracelular sempre que o citosol se torne muito alcalino (Alberts *et al.*, 2005).

Moluscos são conhecidos como animais osmoconformadores, ou seja, ficam isosmóticos em relação ao meio, apresentando assim um alto grau de tolerância osmótica celular. Animais osmoconformadores, tais como os moluscos, são excelentes bioindicadores de acumulação de metais (Hamed e Emara, 2006). Porém, pouco se sabe ainda a respeito dos mecanismos de acumulação e toxicidade do cobre nestes organismos, o que leva à necessidade de estudos que visem uma melhor compreensão dos efeitos causados por esse metal.

Em moluscos, a maioria dos estudos de acumulação e toxicidade de metais indica as brânquias e glândula digestiva como sendo as principais vias de absorção (Simkiss e Mason, 1983; Romeo e Gnassia-Barelli, 1995; Bonneris *et al.*, 2005). Porém, estudos prévios realizados por nosso grupo de pesquisa demonstraram que o manto é capaz de acumular cobre, tanto quanto as brânquias (Lima e Bianchini, 2006). De fato, o manto de moluscos bivalves pode atuar como um sítio adicional de trocas gasosas (Barnes, 1996). Portanto, o manto, assim como a brânquia, pode representar uma importante via de entrada e acumulação de metais.

Anatomicamente, o manto é composto de dois epitélios, um voltado para a concha, chamado de epitélio externo, e outro voltado para a massa visceral, chamado de epitélio interno. Estes epitélios são separados por seios hemolinfaticos e tecido conectivo (Neff, 1972; Lemaire-Gony e Boudou, 1997). O epitélio externo separa a hemolinfa do fluido extrapaleal, ou seja, o fluído presente entre o epitélio externo e a concha, e está envolvido na formação da concha. O epitélio interno está em contato com o meio ambiente e separa a hemolinfa do animal da água que banha a cavidade do manto.

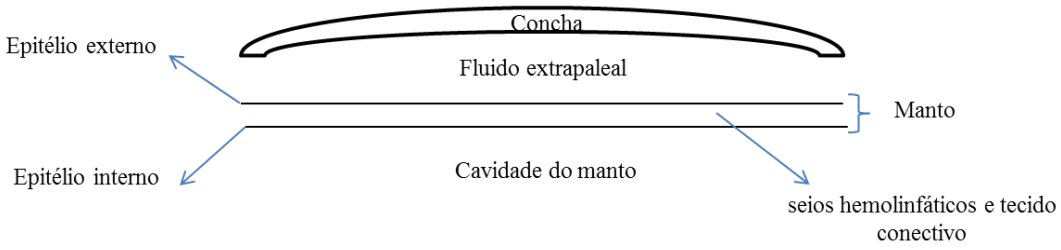

Fig. 1: Desenho esquemático do manto de moluscos bivalves.

Considerando o exposto acima, é necessária a obtenção de informações relacionadas aos mecanismos de transporte iônico envolvidos na absorção e acumulação do cobre, para um melhor entendimento dos processos de acumulação e toxicidade deste metal em organismos osmoconformadores, bem como uma possível utilização destes mecanismos como biomarcadores de contaminação ambiental pelo cobre.

Um biomarcador pode ser definido como uma alteração na resposta biológica, indo do nível molecular ao comportamental, sendo que esta pode estar relacionada à exposição ou ao efeito de contaminantes ambientais (Livingstone, 1993; Depledge *et al.*, 1995; Walker *et al.*, 1996). O uso de biomarcadores tem sido indicado pelos programas de monitoramento ambiental por serem considerados uma ferramenta sensível na avaliação da qualidade ambiental e dos efeitos biológicos dos poluentes (Cajaraville *et al*., 2000; Paquin *et al*., 2002; Allen e Moore, 2004). Outra característica importante dos biomarcadores moleculares e celulares é o potencial de antecipar mudanças em níveis organizacionais mais altos, como populações, comunidades e ecossistemas. Este aviso antecipado oferecido pelos biomarcadores pode ser usado como ferramenta preventiva, permitindo que estratégias de biorremediação sejam tomadas, antes que ocorram danos ecológicos irreversíveis (Cajaraville *et al*., 2000).

Considerando a célula como um sítio de acumulação, metabolismo e toxicidade de contaminantes como o cobre, certas respostas celulares têm sido utilizadas como biomarcadores de exposição em programas de monitoramento ambiental (Cajaraville *et al.*, 2000; Allen e Moore, 2004). Estudos baseados na toxicologia *in vitro* foram introduzidos nas metodologias de quantificação de biomarcadores, possibilitando a integração de respostas celulares com diferentes níveis de organização biológica e também contribuindo para o desenvolvimento dos estudos a respeito da toxicidade dos contaminantes (Fletcher, *et al.*, 2000; Kelly *et al.*, 2000; Taylor *et al.*, 2002; Dissanayake e Galloway, 2004).

Para a realização deste trabalho foi utilizado o molusco bivalve *Mesodesma mactroides*, o qual pertence à família Mesodesmatidae. É classificado como sendo suspensívoro, entretanto, quando há escassez de alimento em suspensão na coluna da água, este passa a se alimentar de detritos contidos no sedimento (Defeo & Scarabino, 1990). É popularmente conhecido como marisco branco ou sernambi. Esta espécie se distribui desde o Rio de Janeiro, no Brasil, até a Bahia Blanca, na Argentina (Rios, 1994). Este bivalve é dominante na área que habita, devido ao seu grande tamanho e suas altas densidades populacionais (Olivier *et al.*, 1971). Segundo alguns autores, os fatores que mais influenciam na distribuição destes animais são a morfologia da praia, a composição do substrato, a presença de cursos de água doce e a superexploração (Olivier *et al.*, 1971; Bastida *et al.*, 1991; Defeo *et al.*, 1992)

Considerando-se o exposto acima, o estudo ao nível celular permite a investigação e a compreensão dos mecanismos tóxicos de metais, como o cobre, uma vez que reproduz *in vitro* situações de exposição aos contaminantes nos ambientes aquáticos (Castaño et al., 2003) De fato, as células são as estruturas básicas de toda forma de vida, e representam o nível básico de organização. O conhecimento dos

mecanismos celulares comuns facilita a extrapolação dos impactos de contaminantes ambientais entre diferentes espécies, que são um dos maiores desafios para a ecotoxicologia. Por outro lado, a escassez de dados sobre os mecanismos de transporte iônico em invertebrados eurialinos, especialmente os osmoconformadores, bem como os efeitos do cobre sobre estes mecanismos e também a participação do manto como via de entrada e acumulação do cobre, torna o presente estudo inovador e de extrema importância para o avanço do conhecimento na área e avaliação da possibilidade de aplicação destes mecanismos como indicadores de problemas biológicos relacionados à contaminação ambiental pelo cobre.

Quanto à espécie a ser estudada, o marisco *M. mactroides* foi selecionado, tendo em vista sua distribuição e importância ecológica, seu hábito alimentar, sua capacidade de acumulação de cobre, seu comportamento osmoconformador, bem como sua fácil coleta, manuseio e manutenção em cativeiro.

OBJETIVOS

Objetivo geral

Avaliar a importância do manto no marisco branco *Mesodesma mactroides* como via de entrada e acumulação de cobre a partir da fase dissolvida, bem como estudar o envolvimento dos principais mecanismos de transporte iônico nestes processos.

Objetivos específicos

- Determinar *in vivo* os níveis de acumulação de cobre em diferentes tecidos (manto, brânquias, hemolinfa e glândula digestiva) a partir da fase dissolvida.
- Estudar *in vitro* o efeito do cobre sobre o conteúdo intracelular de Na^+, K^+, Ca^{2+} e Cl^- em células isoladas do manto.
- Estudar *in vitro* os principais mecanismos de transporte de íons, especialmente Na^+, em células isoladas do manto.
- Avaliar *in vitro* o envolvimento dos principais mecanismos de transporte de íons, especialmente do Na^+, na acumulação de cobre no manto.

ARTIGO 1

Ion transport in isolated mantle cells of the marine clam *Mesodesma mactroides*

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Abstract

Mechanisms involved in ion transport in isolated mantle cells of the clam *Mesodesma mactroides* were investigated using pharmacological tools. Mantle cells were isolated and incubated in saline solution of ionic composition similar to the clam hemolymph. Cells were incubated (1 h, 20°C) in the absence (control) and the presence of different drugs: phenamil (10 μM; Na^+ channel blocker, quinine sulfate (50 μM; K^+ channel blocker), furosemide (100 μM; $Na^+/K^+/2Cl^-$ blocker), amiloride (100 μM; Na^+/H^+ exchanger blocker), SITS (500 μM; Cl^-/HCO_3^- blocker), EIPA (0.02 μM; Na^+/H^+ exchanger blocker), ouabain (100 μM; Na^+/K^+-ATPase inhibitor), benzthiazide (100 μM; Na^+/Cl^- cotransporter blocker), bafilomycin A_1 (1 μM; V-ATPase inhibitor), and acetazolamide (1 mM; carbonic anhydrase inhibitor). Results were expressed as percentage of the cell ion (Na^+, K^+, and Ca^{2+}) content, considering the mean values for control cells as 100%. Based on results obtained, a functional hypothetical model depicting the major mechanisms involved in ion transport in mantle cells of *M. mactroides* was generated. According to the proposed model, the influx of ions would occur via the following mechanisms: Na^+/H^+ and Cl^-/HCO_3^- exchangers, $Na^+/K^+/2Cl^-$ and Na^+/Cl^- cotransporters, Na^+ channels, and Na^+/K^+-pump. The H^+ and HCO_3^- for operation of the Na^+/H^+ and Cl^-/HCO_3^- exchangers would be provided by the carbonic anhydrase activity. In turn, the efflux of ions would involve the following mechanisms: Na^+/K^+- and H^+-pump, and K^+ and Cl^- channels. Results also indicate that the Ca^{2+} transport across the mantle cells is strongly dependent on the transport of monovalent (Na^+, K^+, and/or Cl^-) ions.

Key words: clam, ion transport, isolated cell, mantle, *Mesodesma mactroides*, seawater.

Introduction

Marine mollusks like the clam *Mesodesma mactroides* are osmoconformers, *i.e.*, alter the osmotic concentration of their body fluids according to changes in ambient salinity, being isosmotic with the surrounding environment (Schimidt-Nielsen 2002). They also show high osmotic cell tolerance (Randall et al. 2000). In general, mollusks are excellent indicators of metal accumulation (Hamed and Emara 2006). However, there is little information on the mechanisms involved in metal accumulation and toxicity in these organisms, especially in marine mollusks.

In bivalve mollusks, the mantle is an important route of ionic exchange with the external medium, as observed in the gills. Therefore, it can be also involved in metal accumulation and toxicity. In fact, the mantle of all bivalve mollusks is considered as an additional site of gas exchange (Barnes and Ruppert 1996). Anatomically, the mantle is composed by two different epithelia. The inner epithelium covers the mantle cavity, surrounding the animal's body. Therefore, it is in contact with the external medium. In turn, the outer epithelium covers the animal's shell. It is a tiny layer that separates the hemolymph from the extrapaleal fluid, *i.e.*, the fluid present between the outer epithelium and the shell. The outer epithelium is involved in shell formation (Neff 1972).

Oliveira et al. (2008) proposed a functional model of ion transport across the mantle outer epithelium of the freshwater mollusk *Anodonta cygnea*. According to this model, the following mechanisms of ion transport are present in the basolateral side of the epithelium facing the hemolymph: the Na^+/K^+-ATPase pump and the Na^+/H^+, Cl^-/HCO_3^-, $Cl^-/NvAc^-$, and $Na^+/HCO_3^-/Cl^-$ exchangers. These authors also proposed the presence of Na^+, K^+, Cl^- and HCO_3^- channels. In the apical side of the epithelium facing

the extrapaleal fluid, it was suggested the presence of the H^+-ATPase pump, the K^+/Cl^- cotransporter and the Na^+, K^+ and Cl^- channels. This model also included the presence of the carbonic anhydrase, which catalyze the CO_2 hydration, generating H^+ and HCO_3^-, which are exchanged by Na^+ and Cl^-, respectively. As it can be seen, the mechanisms involved in ion transport in the mantle outer epithelium are well described in freshwater mollusks. However, it is not known if the same mechanisms that are found in freshwater mollusks are also present in marine ones.

In light of the above, the aim of the present study was to identify the main mechanisms involved in ion transport in isolated mantle cells from the marine clam *Mesodesma mactroides* using pharmacological tools.

Material and methods

Clam collection and acclimation

Clams (M. mactroides; length = 36.4 □ 3.1 mm; wide = 19.6 □ 1.5 mm; height = 9.3 □ 0.9 mm) were collected at the Mar Grosso Beach (São José do Norte, RS, Southern Brazil), immediately transferred to the laboratory, and acclimated to in a plastic tank containing seawater (30 ppt salinity) continuously aerated and in the absence of sediment for at least 5 days. Temperature (20°C) and photoperiod (12L:12D) were fixed. The tank was covered with a black plastic to minimize stress. The acclimation medium was renewed three times a week. Clams were fed the diatom *Thalassiosira weissflogii* ($2x10^4$ cells/L). After acclimation, clams were crioanesthetized and had their mantle dissected.

Mantle cell isolation

Briefly, the mantle epithelium of 6 clams was dissected, pooled, sliced in small pieces, and incubated in a calcium-free phosphate solution (342 mM NaCl; 20 mM Na_2HPO_4; 1.7 mM K_2HPO_4; 16 mM KCl; 780 mOsmol/Kg H_2O; pH 7.5; 20^oC), and shacked (100 rpm; Certomat-MO-II; Sartorius Stedim Biotech GmbH, Göttingen, Germany) for 40 min. After incubation, mechanically dissociated cells were filtered (30 μm-mesh nylon filter) to remove the non-dissociated tissue and large debris. The filtered solution containing isolated cells was transferred to plastic tubes and centrifuged (360 x *g*; Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA) for 3 min. The pellet obtained was resuspended in 1 mL of saline solution of similar ionic composition to the clam hemolymph (350 mM NaCl; 9 mM KCl; 30 mM $MgCl_2$; 9 mM $CaCl_2$; 2 mM $NaHCO_3$; pH 7.6; 20^oC).

Isolated cells showed a mean diameter and volume of 10.94 ± 0.51 μm and 773.09 ± 110,91 μm^3, respectively. Cell counting and viability, *i.e.*, the percentage of viable cells, were checked using the Tripan Blue (0.08%) exclusion assay. Only preparations showing more than 80% viable cells were used in the experiments.

Ionic content measurements

Cell ion content was measured using $5x10^5$ mantle cells incubated (1 h) in 180 μL of saline solution without blockers (control) or with blockers. After incubation, an aliquot of cells was collected to determine cell viability using the Tripan Blue exclusion method. The remaining mantle cells were centrifuged (1,800 x g) for 3 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA). The supernatant was discarded and the pellet was quickly washed with a sucrose solution of osmolality similar to the hemolymph osmotic concentration (850

mOsmol/Kg H_2O). The centrifugation procedures were repeated and the new supernatant was discarded. Cell samples were then dried (60°C; 24 h), digested with 50 µl of HNO_3 (Suprapur®, Merck, St. Louis, MO, USA) and diluted with 500 µL Milli-Q water. Sample aliquots were collected and properly diluted for Na^+, K^+, and Ca^{2+} cell content measurements. Ion concentrations were measured by atomic absorption spectrophotometry (AAS 932 Avanta Plus, GBC, Hampshire, IL, USA), as previously described (Pinho et al. 2007; Pedroso et al. 2007; Paganini and Bianchini 2009). Results were expressed as µg/5x10^5 cells.

In all cases, three different and independent mantle cell preparations were tested for each experimental condition (n = 3). Each mantle cell preparation was tested in triplicate.

Identification of the mechanisms of ion transport

The involvement of different mechanisms of ion transport and associated enzymes was analysed using the same procedures described above, but associated with several pharmacological tools. The mechanisms evaluated were the Na^+, K^+, and Cl^- channels, the $Na^+/K^+/2Cl^-$ and Na^+/Cl^- cotransporters, and the Na^+/Ca^{2+}, Cl^-/HCO_3^- and Na^+/H^+ exchangers. The enzymes studied were the Na^+/K^+-ATPase, V-ATPase, and carbonic anhydrase. The following ion transport blockers and enzyme inhibitors were employed: phenamil (10 µM; Na^+ channel blocker; Wood et al. 2002), quinine sulfate (50 µM; K^+ channel blocker; Imai et al. 1999), furosemide (100 µM; $Na^+/K^+/2Cl^-$ blocker; Ross et al. 2007), amiloride (100 µM; Na^+/H^+ exchanger blocker; Onken and Riestenpatt 2002), 4-acetamido-4'-isothiocyano-2,2'-stilbene disulphonic acid (SITS; 500 µM; Cl^-/HCO_3^- blocker; Song et al. 1998), etil-isopropil-amiloride (EIPA; 0.02 µM; Na^+/H^+ exchanger blocker; Dailianis and Kaloyianni 2004), ouabain (100 µM; Na^+/K^+-

ATPase inhibitor; Balestrino et al. 1999), benzthiazide (100 μM; Na^+/Cl^- cotransporter blocker; Ross et al. 2007), bafilomycin A_1 (1 μM; V-ATPase inhibitor; Herak-Kramberger et al. 2000), and acetazolamide (1 mM; carbonic anhydrase inhibitor; Parkkila et al. 2000). SITS and ouabain were purchased from Fluka Chemie (Buchs, SG, Switzerland). The other drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Results were expressed in $\mu g/5x10^5$ cells.

Statistical analysis

Data were expressed as mean ± standard error. Percentages of change in cell ionic content in the presence of the pharmacological tools were calculated considering the mean value for control cells as 100%. Student's t test was used for comparisons among the cells non-exposed to drugs (control cells) and those exposed to the different drugs. In all cases, the significance level adopted was 95% ($\alpha = 0.05$).

Results

Ionic (Na^+, K^+, and Ca^{2+}) contents of isolated mantle cells kept under control conditions (non-exposed to drugs) are shown in figure 1.

A significant increase in the Na^+ content of isolated mantle cells was observed after incubation with acetazolamide (54%) and quinine sulphate (59%). On the other hand, a significant decrease in Na^+ content was observed in isolated mantle cells incubated with amiloride (24%), bafilomycin A_1 (11%), and phenamil (32%) (Fig. 2).

The K^+ content significantly increased in cells incubated with furosemide (62%), benzthiazide (85%), bafilomycin A_1 (108%), and quinine sulphate (26%). On the other

hand, it significantly decreased in cells incubated with acetazolamide (47%), ouabain (61%), and SITS (22%) (Fig. 3).

The Ca^{2+} content significantly increased in isolated mantle cells incubated with any of the tested drugs: amiloride (501%), furosemide (343%), benzthiazide (142%), bafilomycin A_1 (76%), acetazolamide (340%), ouabain (496%), quinine sulphate (288%), phenamil (192%), SITS (478%), and EIPA (573%) (Fig. 4).

Discussion

Studies using the mantle outer epithelium of freshwater mollusks indicate the presence of the H^+-pump (V-ATPase activity) in the apical membrane and the HCO_3^- /Cl^- and Na^+/H^+ exchangers in the basolateral membrane. They also reported that a carbonic anhydrase activity is linked to the operation of these mechanisms, providing H^+ and HCO_3^- (Coimbra and Machado 1988; Machado et al. 1990; Hudson 1992, 1993; Rebelo da Costa et al. 1999b; Oliveira et al. 2004). In turn, Saintsing and Towle (1978) observed an increased Na^+/K^+-ATPase activity in the mantle of the mollusk *Rangia cuneata* acclimated to low salinity. In agreement with this finding, Rebelo da Costa et al. (1999a) reported that ouabain, a specific inhibitor of the Na^+/K^+-ATPase activity, was effective on the short-circuit current generated by the mantle outer epithelium in the mollusk *Anodonta cygnea* only when applied to the hemolymph side, indicating the presence of the enzyme in the basolateral membrane of the cells. In the present study with the marine clam *Mesodesma mactroides*, the inhibition of the Na^+/K^+-ATPase activity by ouabain significantly reduced the cell K^+ content, which is explained by a lowered influx of this ion. However, an increase in the cell Na^+ content would be expected, but no significant change was observed. This is likely due to a reduced activity of a Na^+/H^+ exchanger with a consequent reduction in Na^+ influx to compensate

the lowered efflux of this ion associated with inhibition of the Na^+/K^+-ATPase activity induced by ouabain. In fact, the presence of a Na^+/H^+ exchanger has been reported in several cell types of both vertebrates and invertebrates (Rothstein 1989).

To verify the possible involvement of the Na^+/H^+ exchanger and the epithelial Na^+ channel in the ionic regulation in mantle cells of the marine clam *M. mactroides*, the effects of the following drugs on cell ion content were tested: amiloride, phenamil and EIPA. Amiloride is an inhibitor of the Na^+/H^+ exchanger (Grinstein and Smith 1987; Mahnensmith and Aronson 1985). It can also inhibit the epithelial Na^+ channels (Benos et al. 1987). Results from the present study with amiloride showed a significant reduction in the cell Na^+ content. To investigate which mechanism would be involved in this response, phenamil, a Na^+ channel selective blocker (Reid et al. 2003), and EIPA, a selective Na^+/H^+ exchanger inhibitor (Dailianis and Kaloyianni 2004), were tested. A significant reduction in the cell Na^+ content was observed when the Na^+ channels were blocked. However, the Na^+/H^+ blockade with EIPA did not significantly alter the cell Na^+ content. However, the involvement of the Na^+/H^+ exchanger in the reduced cell Na^+ content in the presence of amiloride cannot be ruled out, since EIPA concentration tested is generally lower than other reported in the literature. However, it must be pointed out that a higher concentration of EIPA than that tested in the present study showed to be very toxic to isolated cells of the marine clam *M. mactroides*. Total cell mortality was observed after exposure to higher concentrations of EIPA (data not shown). This finding indicates an extremely important role also of the Na^+/H^+ exchanger in mantle cell physiology.

Acetazolamide is an inhibitor of the carbonic anhydrase activity (Parkkila et al. 2000). Carbonic anhydrase provides H^+ and HCO_3^- for the Na^+/H^+ and Cl^-/HCO_3^- exchangers, respectively. In the presence of this drug, isolated mantle cells of the

marine clam *M. mactroides* showed a significant increase in the Na^+ content and a significant decrease in K^+ content. This response is likely a result from a combined effect of a reduced activity of the Na^+/H^+ exchanger with a consequent reduction in the activity of the Na^+/K^+ pump to compensate a lower influx of Na^+. In fact, a decreased Na^+/K^+-ATPase activity would induce to an increased cell Na^+ content and a reduced cell K^+ content, as observed. The reduced activity of the Na^+/H^+ exchanger is associated with the lower amount of H^+ available after the carbonic anhydrase inhibition by acetazolamide. In this case, a lower amount of HCO_3^- would be also available and a reduced activity of a Cl^-/HCO_3^- exchanger would be expected, resulting in a reduction cell Cl^- content. Unfortunately, the cell Cl^- content was not analyzed in the present study. However, the effect of an inhibitor of the Cl^-/HCO_3^- exchanger (SITS) was tested.

Mantle cells exposed to SITS showed a significantly reduced cell K^+ content. Blockade of the Cl^-/HCO_3^- exchanger by SITS would increase the intracellular HCO_3^- concentration. The reduced efflux of HCO_3^- would lead to a decreased intracellular H^+ concentration, a reduced activity of the Na^+/H^+ exchanger and a lower Na^+ influx. This lower Na^+ influx would induce a reduced activity of the Na^+/K^+-ATPase, thus explaining the decreased cell K^+ content after exposure to SITS.

The observed increase in cell K^+ content induced by quinine sulphate indicates the involvement of K^+ channels in the ion regulation in isolated mantle cells of the marine clam *M. mactroides.* The increased cell K^+ content would induce a reduced activity of the Na^+/K^+-ATPase, thus explaining the increased Na^+ content observed in the presence of quinine sulphate. Also bafilomycin A_1, a specific inhibitor of the V-ATPase (Drose and Altendorf 1997), increased the cell K^+ content, but without significant changes in the cell Na^+ content. It can be suggested that the bafilomycin-induced blockade of the H^+ pump would result in an increased cell H^+ content. To avoid

intracellular acidosis, the increased H^+ concentration would be compensated by an increased activity of the Na^+/H^+ exchanger, resulting in a higher Na^+ influx, which would be compensated by a higher Na^+ efflux through the Na^+/K^+-pump to maintain the intracellular level of Na^+. Therefore, a higher Na^+/K^+-ATPase would be observed, leading to a higher K^+ cell content, as observed in mantle cells in the presence of bafilomycin A_1.

It is known that the $Na^+/K^+/2Cl^-$ cotransporter plays a major role in the secretion of the excess of ions and the cell volume regulation in marine animals (Hiroi and McCormick 2007). In the presence of furosemide ($Na^+/K^+/2Cl^-$ cotransporter blocker) or benzthiazide (Na^+/Cl^- exchanger blocker), a significant increase in cell K^+ content without significant changes in cell Na^+ content was observed. This response could be a result of a reduced influx of ions via the $Na^+/K^+/2Cl^-$ cotransporter and an increased Na^+/K^+-ATPase activity to compensate the consequent lower influx of osmolytes in order to maintain the cell volume. This situation would lead to the increased K^+ observed after mantle cell incubation with furosemide. In turn, the blockade of the Na^+/Cl^- exchanger by benzthiazide would reduce the influx of Na^+ and Cl^-, inducing a higher activity of the $Na^+/K^+/2Cl^-$ cotransporter to increase the influx of osmolytes in order to maintain the cell volume regulation. This response could explain the increased cell K^+ content observed after incubation with benzthiazide.

Regarding the intracellular Ca^{2+} regulation, it is reported that the shell formation in mollusks is a result from the deposition of $CaCO_3$ on an organic matrix. The carbon supply for the carbonate present in the shell seems to be originated from the CO_2 and/or HCO_3^- resulting from cell metabolism. In turn, Ca^{2+} can be absorbed directly from the external medium or from $CaCO_3$ stocks present in the soft tissues (Wheeler 1992). The Ca^{2+} transport from the hemolymph to the extrapaleal fluid seems to occur by diffusion

across the mantle epithelium (Coimbra and Machado 1988; Wheeler 1992). In the present study, all drugs tested significantly increased the cell Ca^{2+} content. It is interesting also to note that all drugs tested had influence either on Na^+ or K^+ cell content. These findings indicate that any alteration in the transport of Na^+ and/or K^+ can affect Ca^{2+} transport across the mantle epithelium, suggesting that this transport is dependent on Na^+ and/or K^+ movements. This evidence might explain why some aquatic contaminants inducing ionic disturbances such as copper have important impacts on mollusk survival and growth (Nebeker et al. 1986; Penã and Pocsidio 2007).

Based on findings reported in the present study, a functional hypothetical model depicting the mechanisms of ion transport involved in the ionic regulation in isolated mantle cells of the marine clam *M. mactroides* is shown in figure 5.

According to the proposed model, Na^+ and Cl^- would enter the mantle cells through Na^+/H^+ and Cl^-/HCO_3^- exchangers, $Na^+/K^+/2Cl^-$ and Na^+/Cl^- cotransporters and Na^+ channels. The inward movements of Na^+ and Cl^- through the Na^+/H^+ and Cl^-/HCO_3^- exchangers and the $Na^+/K^+/2Cl^-$ and Na^+/Cl^- cotransporters would be driven by a Na^+ gradient existing between the extracellular and the intracellular media, which would be generated by the Na^+,K^+-ATPase activity. The substrates (H^+ and HCO_3^-) for the operation of the Na^+/H^+ and Cl^-/HCO_3^- exchangers would be provided by the CO_2 hydration catalyzed by the carbonic anhydrase. The protons generated would be also extruded from the cell via the proton pump (V-type H^+-ATPase). The H^+ efflux generated by the H^+-ATPase would generate a local electrical gradient that would favor the Na^+ influx through the Na^+ channel. Therefore, the intracellular acid-base balance would be dependent on the Na^+/H^+ and Cl^-/HCO_3^- exchangers, as well as the proton pump activity (Fig. 5).

Regarding Na^+ and Cl^- extrusion from the cell, effluxes of these ions would be dependent on the Na^+/K^+-ATPase activity. The Na^+/K^+ pump would be responsible for an active efflux of Na^+ paralleled with an active influx of K^+, generating the Na^+ gradient between the extracellular and the intracellular media mentioned above, as well as a K^+ gradient between the intracellular and the extracellular media. An efflux of K^+ would then occur through K^+ channels, generating a local electrical gradient that would favor a Cl^- efflux via Cl^- channels (Fig. 5).

Acknowledgments

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Figure Captions

Fig. 1 Ion content of isolated mantle cells of the marine clam *Mesodesma mactroides*. Data are expressed as mean ± standard error.

Fig. 2 Changes in Na^+ content of isolated mantle cells of the marine clam *Mesodesma mactroides* after incubation with different drugs. Data are expressed as percentage considering the mean value of the control cells (non-exposed to drugs) as 100%. * indicates significant difference from the control ($p<0.05$).

Fig. 3 Changes in K^+ content in isolated mantle cells of the marine clam *Mesodesma mactroides* after incubation with different drugs. Data are expressed as percentage considering the mean value of the control cells (non-exposed to drugs) as 100%. * indicates significant difference from the control ($p<0.05$).

Fig. 4 Changes in Ca^{2+} content in isolated mantle cells of the marine clam *Mesodesma mactroides* after incubation with different drugs. Data are expressed as percentage considering the mean value of the control cells (non-exposed to drugs) as 100%. * indicates significant difference from the control ($p<0.05$).

Fig. 5 Functional hypothetical model depicting the mechanisms of ion transport in isolated mantle cells of the marine clam *Mesodesma mactroides*. See text for further details. CA = carbonic anhydrase.

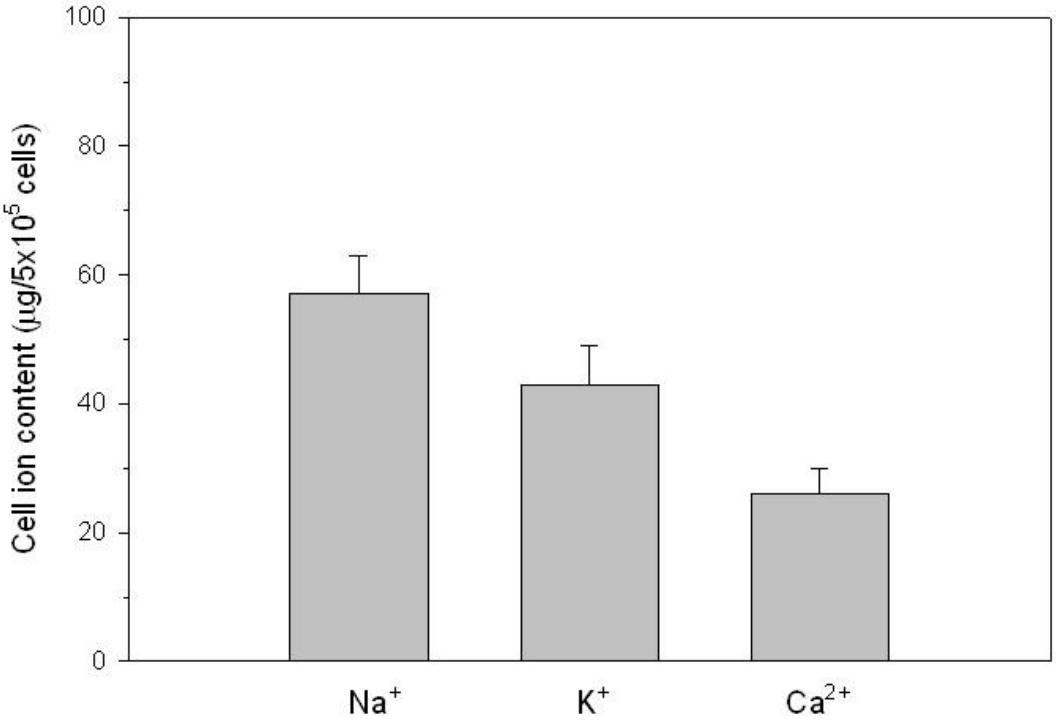
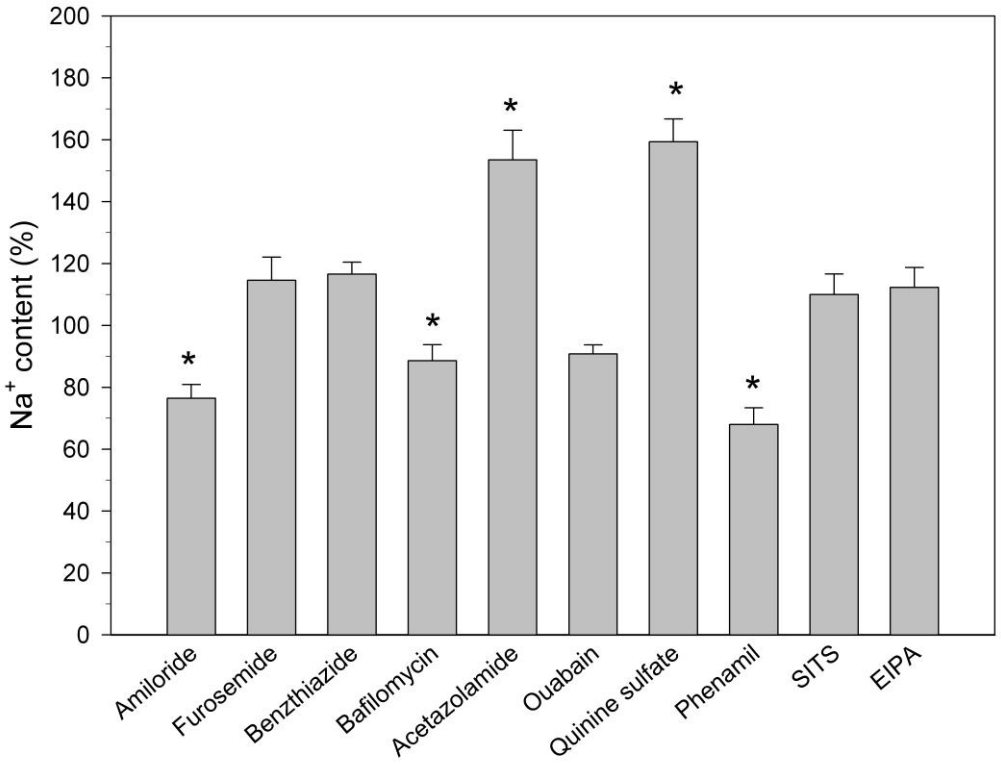


Figure 1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Na+ content (%)
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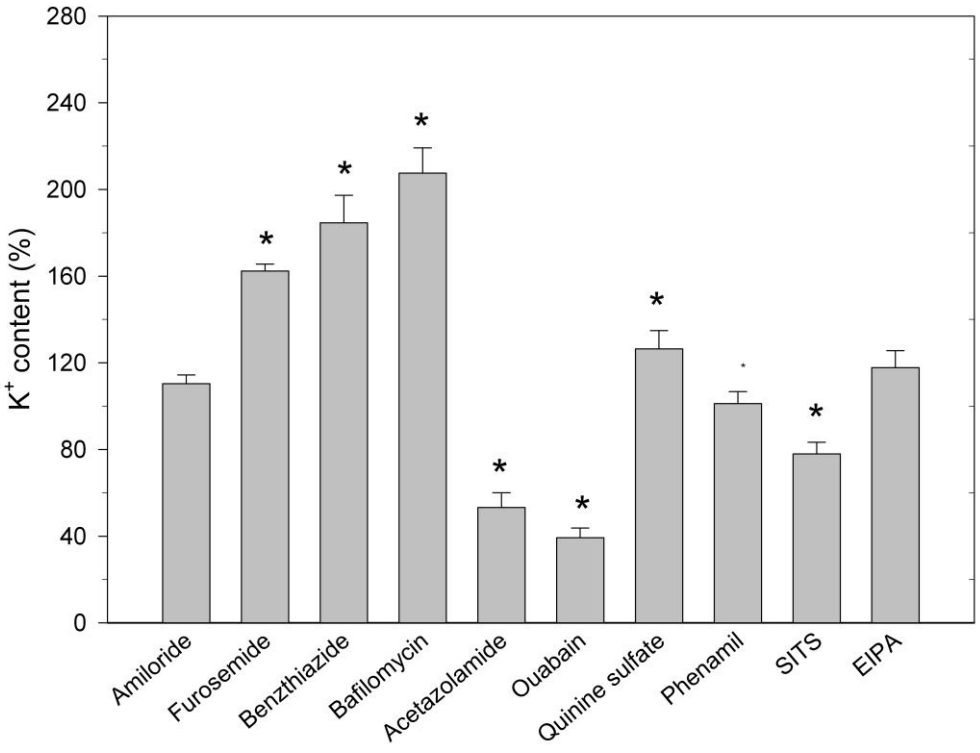
Benzthiazide
Bafilomycin
Acetazolamide
Ouabain
Quinine sulfate
Phenamil
SITS
EIPA

Figure 2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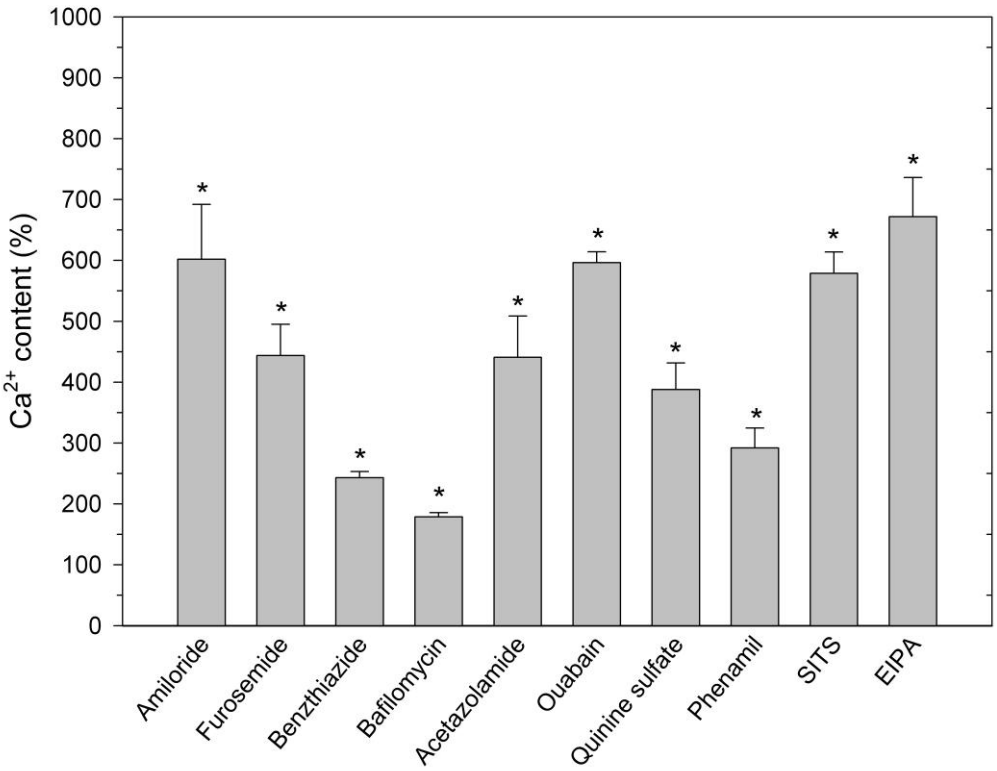
Phenamil
SITS
EIPA

Figure 3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Ca2+ content (%)
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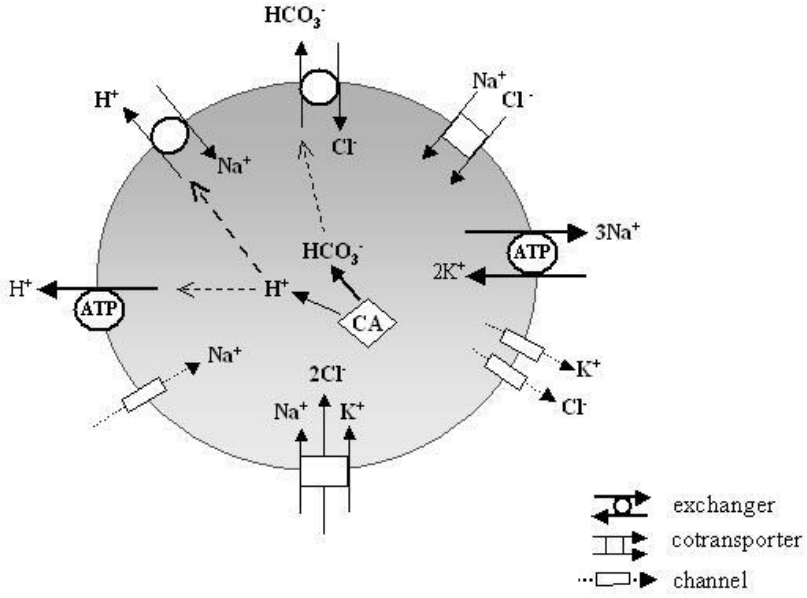
Acetazolamide
Ouabain
Quinine sulfate
Phenamil
SITS
EIPA

Figure 4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ATP
2K+
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ATP
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Na+
2Cl-
K+
Cl-
Na+
K+
exchanger
cotransporter
channel

Figure 5

ARTIGO 2

Effect of copper on ion content in isolated mantle cells of the marine clam *Mesodesma mactroides*

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Effect of copper on ion content in isolated mantle cells of the marine clam *Mesodesma mactroides*

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Abstract

In the present study, the copper effect on ion content (Na^+, K^+, Ca^{2+} and Cl^-) was evaluated in isolated mantle gills of the marine clam *Mesodesma mactroides*. Clams were collected at the Mar GrossoBeach (Rio Grande, RS, Southern Brazil), crioanesthetized, and had their mantle dissected. Mantle cells were isolated and incubated in a calcium-free phosphate solution without (control) or with copper ($CuCl_2$). Cells were exposed to copper for 1 (5 µM) or 3 h (2.5 and 5 µM). In cells incubated with 2.5 µM copper, a significant decrease in intracellular Cl^- content was observed. However, in cells incubated with 5.0 µM copper, significant reductions in Na^+, K^+ and Cl^- intracellular content were observed. Considering the mechanisms involved in ion transport in mantle cells of the marine clam *M. mactroides*, findings described in the present study suggest that copper exposure inhibits carbonic anhydrase and Na^+/K^+ATPase activity. Also, it can be suggested that copper is competing with Na^+ for the same mechanisms of ion transport in the cell membrane such as the Na^+ channels and the $Na^+/K^+/2Cl^-$ cotransporter. Results from the present study also clearly indicate that processes involved in cellular anion regulation are more sensitive to copper exposure than those associated with the cellular cation regulation. Findings reported in the present study are extremely valuable for the development of a Biotic Ligand Model version for marine and estuarine waters.

Keywords: Clam, Copper, Mantle Cells, *Mesodesma mactroides*, Ion Content

Introduction

Copper is an essential micronutrient for aquatic animals, being involved in several physiological functions. This metal is an essential structural component of enzymes and other proteins. It is also acts as an enzymatic co-factor and is involved in cellular respiration [1,2]. However, copper can be toxic to aquatic animals when in elevated concentrations in the water. In turn, it has been demonstrated that water chemistry significantly alters copper toxicity. To quantitatively evaluate how the water chemistry affects the speciation and bioavailability of copper in aquatic systems and, therefore, to predict copper toxicity in freshwater organisms, the Biotic Ligand Model was developed [3]. Parallel to the development of this model, the physiological effects of copper in freshwater animals have been determined to describe more precisely the site of metal toxic action [4].

More recent studies show that the mechanism of copper toxicity in aquatic animals is different in freshwater and marine species [5]. In several freshwater animals, the acute copper toxicity is characterized by a lost ability to regulate íon and osmotic concentration in body fluids. The metal inhibits the Na^+/K^+-ATPase activity, inducing to an ionic imbalance due to a reduced Na^+ concentration in body fluids [4,6]. In estuarine and marine crustaceans acclimated to low salinity, a copper effect on Na^+ and Cl^- balance has also been described [7,8]. However, copper toxicity in marine fish and invertebrates seems not to be related to imbalances in extracellular ion regulation, but to effects on acid-base equilibrium and ammonia excretion [7,8,9].

In mollusks, responses to high copper concentrations (80-500 µg Cu/L) exposure are diverse. Reduced filtration rates [10], changes in protein metabolism [11] and disturbances in calcium homeostase have been described [12,13]. At lower copper

concentrations (10 µg Cu/L), copper exposure reduces the activity of antioxidant enzymes [14] and affects the growth rate [15].

Marine mollusks are known as osmoconformers, i.e., they change the osmotic concentration of their body fluids to be isosmotic with the external medium [16]. Consequently, they show high levels of osmotic tolerance at the cellular level [17]. It is also well known that mollusks are good bioindicators of metal accumulation [18]. However, information on the precise mechanism(s) of cell copper accumulation and toxicity in these animals is still lacking.

In mollusks, most studies on metal accumulation and toxicity suggest that gills and digestive gland are the main routes of absorption [19,20,21]. However, a previous study from our laboratory with the marine clam *Mesodesma mactroides* has shown that mantle is also able to accumulate copper at similar levels of gills. Therefore, mantle could be also considered as a major route for copper entry in mollusks. In addition, our previous study showed that the acute copper toxicity is not associated with an imbalance in the ionic content of body fluids [22], as reported for freshwater or marine invertebrates acclimated to low salinity. This finding suggest that the mechanism of acute copper toxicity in the marine clam *M. mactroides* could be associated to metal effects at a cellular level.

Considering the background described above, the main objective of the present study was to evaluate the effect of copper exposure on cellular ion (Na^+, K^+, Ca^{2+} and Cl^-) content in isolated cells from the mantle of the marine clam *M. mactroides*. Results from the present study are of major importance for a better characterization of the biological site targeted by copper and the mechanism involved in metal toxicity in marine invertebrates. Consequently, they are valuable for the development of a Biotic Ligand Model version for marine waters.

Material and Methods

Clam collection and acclimation

Clams (*M. mactroides*; length = 36.4 ± 3.1 mm; wide = 19.6 ± 1.5 mm; height = 9.3 ± 0.9 mm) were collected at the Mar Grosso Beach (São José do Norte, RS, Southern Brazil), immediately transferred to the laboratory, and acclimated to seawater (30 ppt salinity) continuously aerated and in the absence of sediment, for at least 5 days. Room temperature (20°C) and photoperiod (12L:12D) were fixed. The acclimation tank was covered with a piece of black plastic to minimize stress. The acclimation medium was renewed three times a week. Clams were fed the diatom *Thalassiosira weissflogii* ($2x10^4$ cells/L). After acclimation, clams were crioanesthetized and had their mantle dissected.

Mantle cell isolation

Briefly, the mantle epithelium of 6 clams was dissected, pooled, sliced in small pieces, and incubated in a calcium-free phosphate solution (342 mM NaCl; 20 mM Na_2HPO_4; 1.7 mM K_2HPO_4; 16 mM KCl; 780 mOsmol/Kg H_2O; pH 7.5; 20^oC), and shacked (100 rpm; Certomat-MO-II; Sartorius Stedim Biotech GmbH, Göttingen, Germany) for 40 min. After incubation, mechanically dissociated cells were filtered (30 μm-mesh nylon filter) to remove the non-dissociated tissue and large debris. The filtered solution containing isolated cells was transferred to plastic tubes and centrifuged (1,800 x *g*; Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA) for 3 min. The pellet obtained was resuspended in 1 mL of saline solution of similar ionic composition to the clam hemolymph (350 mM NaCl; 9 mM

KCl; 30 mM $MgCl_2$; 9 mM $CaCl_2$; 2 mM $NaHCO_3$; pH 7.6; 20^oC). The population of cells obtained showed a mean diameter and volume of 10.94 ± 0,512 µm and 773.09 ± 110,91 μm^3, respectively. Cell counting and viability, i.e., the percentage of viable cells, were checked using the Tripan Blue (0.08%) exclusion assay. Only preparations showing more than 80% viable cells were used in the experiments.

Copper effect on cellular ionic content

To evaluate the copper effect on cellular ion content, $5x10^5$ cells were incubated with 2.5 µM of copper for 3 h or to 5.0 µM of copper for 1 and 3 h. Cells non-exposed to copper (control) were also analyzed. Copper concentrations tested were selected based on previous studies from our laboratory showing that low mortality rates of *M. mactroides* were observed after *in vivo* exposure to 2.5 µM (<10%) and 5.0 µM (20%) copper. The exposure times (1 and 3 h) were selected based on the fact that cells in suspension should be tested within 5 h after isolation [23].

After incubation, an aliquot of cells was collected to determine cell viability using the Tripan Blue exclusion method (0.08% Trypan Blue). The remaining mantle cells were centrifuged (1,800 x g) for 3 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA). The supernatant was discarded and the pellet was quickly washed with a sucrose solution of osmolality similar to the hemolymph osmotic concentration (850 mOsmol/Kg H_2O). The centrifugation procedures were repeated and the new supernatant was discarded. Cell samples were then dried (60^oC; 24 h), digested with 50 µl of HNO_3 (Suprapur®, Merck, St. Louis, MO, USA) and diluted with 500 µL Milli-Q water. Sample aliquots were collected and properly diluted for Na^+, K^+, Ca^{2+} and Cl^- cell content measurements. Cation (Na^+, K^+ and Ca^{2+}) concentrations were measured by atomic absorption spectrophotometry (AAS

932 Avanta Plus, GBC, Hampshire, IL, USA), as previously described [8]. The Cl^- cell content was measured using a commercial reagent kit (Visocolor ECO; Macherey-Nagel 1-60mg/L Cl^-).

In all cases, experiments were performed in triplicate for each mantle cell preparation. Three different and independent mantle cell preparations were tested for each experimental condition (n = 3). Results were expressed in µg/5x10^5 cells.

Statistical analysis

Data were expressed as mean ± standard error. Ionic contents in the cells incubated without (control) or with copper were compared using one-way analysis of variance (ANOVA) followed by the Tukey's. Assumptions of the analysis of variance (data normality and homogeneity of variances) were checked. The significance level adopted was 95% ($\alpha = 0.05$).

Results

In mantle cells exposed to 2.5 µM of copper for 3 h, no significant change in cation (Na^+, K^+, and Ca^{2+}) content was observed. However, a significant reduction (~30%) in Cl^- cellular concentration was observed (Fig. 1). In mantle cells exposed to 5.0 µM copper, significant reduction in cellular ion content was observed. After 1 h of exposure, approximately 30, 70, and 35% decrease in cellular Na^+, K^+, and Cl^- content was observed, respectively. After 3 h of exposure, reductions were of approximately 45, 60 and 60%, respectively.

Discussion

Data from the present study show that exposure to high concentrations of copper (5.0 µM) reduces both cation (Na^+ and K^+) and anion (Cl^-) content in isolated mantle cells of the marine clam *M. mactroides*. However, no significant effect on cellular Ca^{2+} content was observed. These findings could be explained considering the potential effect of copper on the activity of major ion-transporting proteins involved in ionic regulation in the studied species and the role played by the mantle in mollusk shell formation.

Copper effects on cellular Na^+, K^+ and Cl^- content can be explained considering the potential effect of copper on Na^+/K^+ATPase and carbonic anhydrase activities, as well as in the Na^+ channel and the $Na^+/K^+/Cl^-$ cotransporter. In fact, the involvement of these proteins in ion regulation in isolated mantle cells of *M. mactroides* was previously reported by our research group [24].

In freshwater fish and crustaceans, copper inhibits the activity of the Na^+/K^+ATPase present in cell membranes [4]. In marine mollusks, an inhibition of the enzyme activity was also observed in the gill of the bivalve *Mytilus galloprovincialis* exposed *in vivo* to subletal concentrations of copper [13]. The Na^+/K^+ATPase is responsible for the maintenance of the ionic gradient existing between the body fluids and the external medium. This enzyme actively extrudes Na^+ from the cell while pumps K^+ from the extracellular medium to the intracellular medium. Another enzyme inhibited by copper in aquatic invertebrates is the carbonic anhydrase [25]. This enzyme catalises the CO_2 hidration generating H^+ and HCO_3^-, which are substrates used by ion exchangers to transport Na^+ and Cl^- into the cell, respectively.

In light of the above, the observed reductions in cellular K^+ and Cl^- contents in isolated mantle cells of *M. mactroides* incubated with 5 µM copper could be explained

by a copper-induced inhibition of both Na^+/K^+-ATPase and carbonic anhydrase. The significant decrease in cellular Cl^- content in mantle cells exposed to 2.5 µM of copper for 3 h associated with a lack of effect on cellular cation content clearly indicate that the anion regulation processes are more sensitive to copper exposure than those related to cation regulation. In turn, the reduced Na^+ content observed in mantle cells exposed to 5.0 µM copper for 1 or 3 h would be associated with a competition between Cu^+ and Na^+ for the same sites of incorporation in the mantle membrane such as the Na^+ channel and the $Na^+/K^+/Cl^-$ cotransporter. In fact, it has been shown that copper can compete with other cations like Na^+, Ca^{2+} and Mg^{2+} for the sites of cellular incorporation of these ions at the gill level [4,26].

The lack of copper effect on cellular Ca^{2+} content observed in isolated mantle cells of *M. mactroides* could be explained considered the major role of the mantle in mollusk shell formation [27]. In fact, large deposits of calcium carbonate have been described in the extrapaleal fluid, i.e. the fluid present in between the mantle and the shell. In turn, the high Ca^{2+} concentration in the extrapaleal fluid is a consequence of the diffusive movement of the ion through the mantle cells [28]. Therefore, eventual changes in Ca^{2+} content in mantle cells induced by copper exposure would be in fact difficult to detect using the methodology employed in the present study.

In conclusion, data reported in the present study clearly indicate that copper is also an ionoregulatory toxicant in the marine clam *M. mactroides*. However, metal toxicity is associated to an imbalance in ionic regulation at the intracellular level rather than at the extracellular level, as previously shown for freshwater invertebrates and fish. They also clearly indicate that mechanisms involved in intracellular anion (Cl^-) regulation are more sensitive to copper exposure than those involved in intracellular cation (Na^+ and K^+) regulation. Finally, findings reported in the present study suggest

that mantle is a target for copper toxic action in marine mollusks and should be considered in the development of a Biotic Ligand Model version for marine waters.

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Figure legends

Figure 1. Cellular ion (Na^+, K^+, Ca^{2+} and Cl^-) content in mantle cells isolated from the marine clam *Mesodesma mactroides* kept under control conditions (black bars) or exposed to 2.5 µM copper for 3 h (white bars). * indicates significant difference from the control ($p<0.05$).

Figure 2. Cellular ion (Na^+, K^+, Ca^{2+} and Cl^-) content in mantle cells isolated from the marine clam *Mesodesma mactroides* kept under control conditions (black bars) or exposed to 5 µM copper for 1 h (white bars) and 3 h (stripped bars). * indicates significant difference from the control ($p<0.05$).

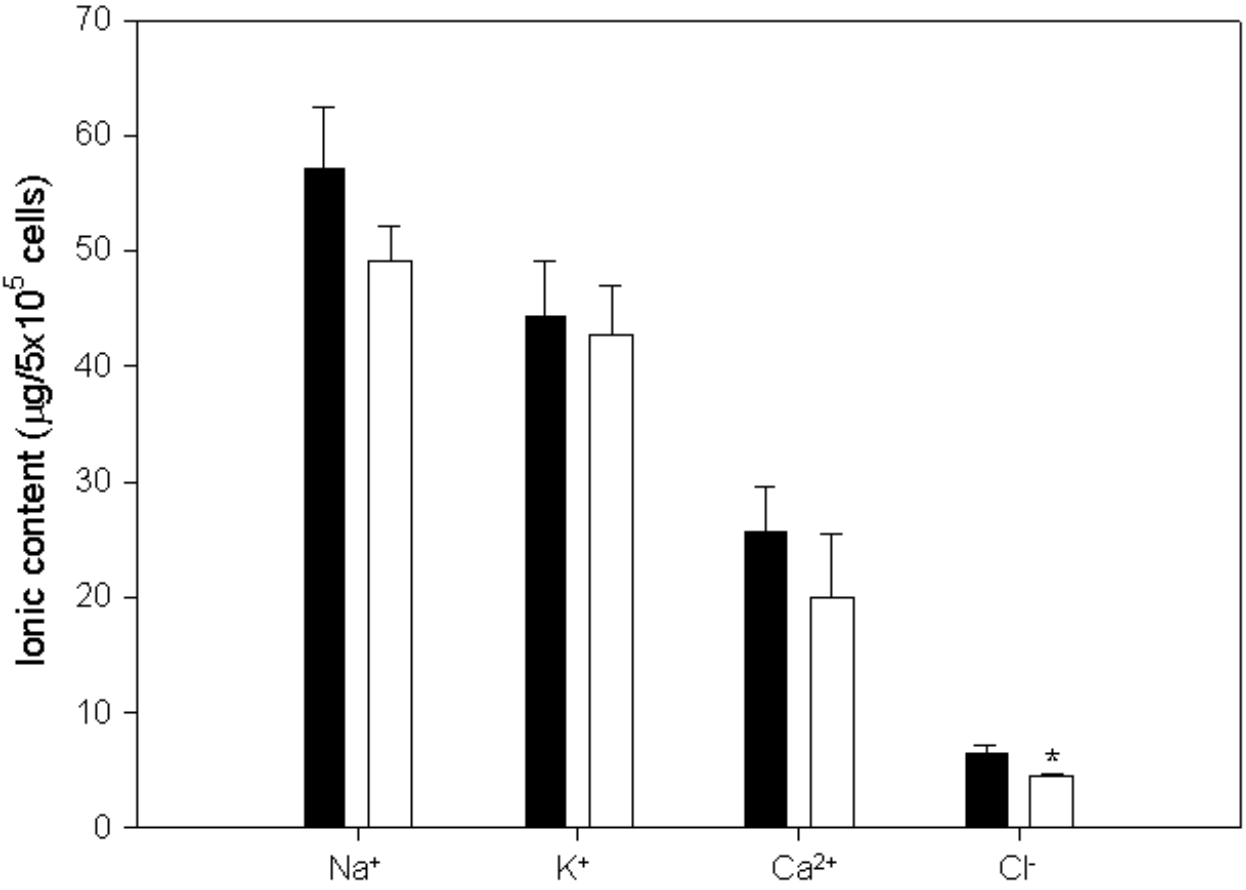

Figure 1

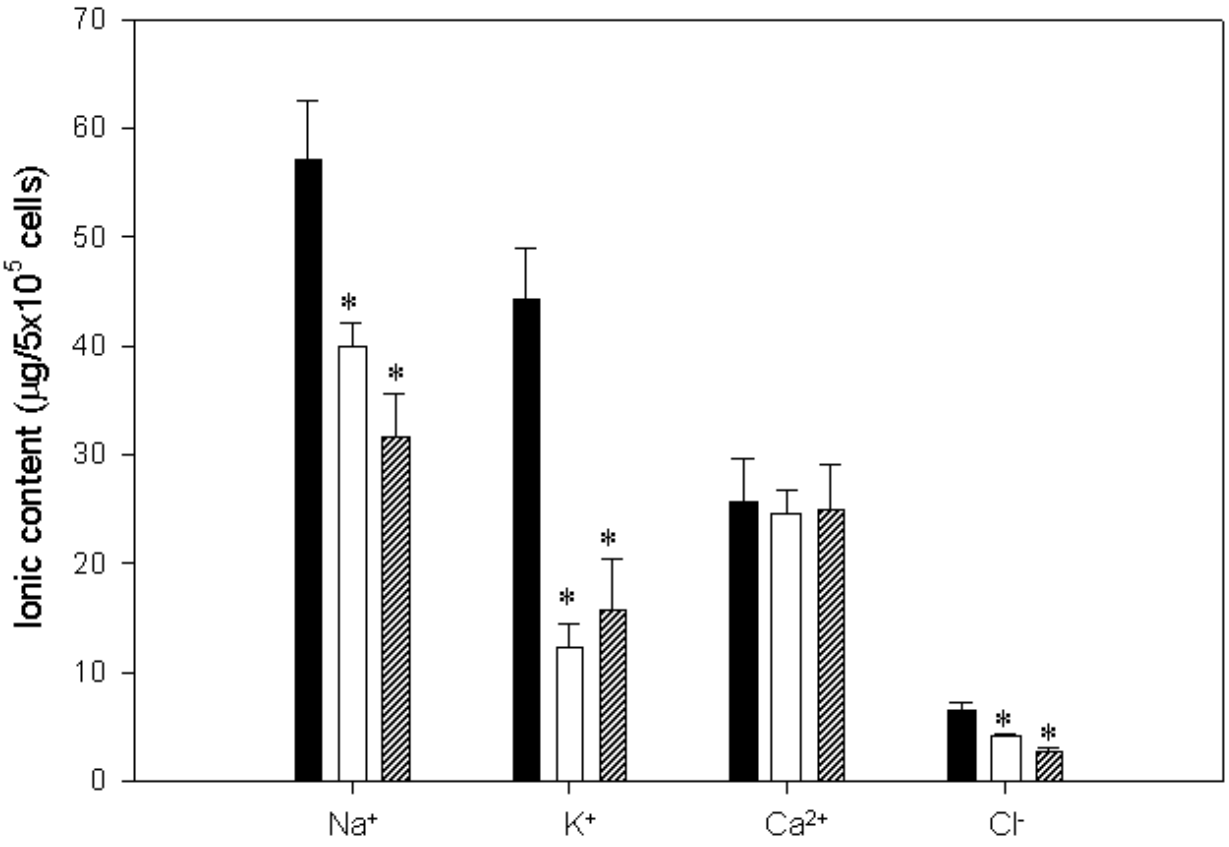

Figure 2

ARTIGO 3

Mechanisms of copper accumulation in isolated mantle cells of the marine clam *Mesodesma mactroides*

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Abstract

The involvement of major ion-transporting mechanisms in copper accumulation was evaluated in isolated mantle cells of the marine clam *Mesodesma mactroides*. In vivo copper accumulation was determined in tissues (mantle, gills, digestive gland, and hemolymph) following exposure (96 h) to copper (5 μM) for up to 96 h. Mantle was the tissue that most accumulated copper, followed by gill, digestive gland, and hemolymph. Therefore, in vitro copper accumulation was evaluated in isolated mantle cells exposed to 0.5, 1.0, 2.5 and 5.0 μM Cu for 1 and 3 h. After both exposure times, no significant change in cell viability was observed for all the different copper concentrations tested. However, a significant copper accumulation was observed in cells exposed to 2.5 and 5.0 μM Cu. Cell exposure to 2.5 μM Cu for 1 h did not affect the ionic (Na^+, K^+, Ca^{2+} and Cl^-) content of isolated mantle cells. Therefore, the involvement of different ion-transporting proteins (Na^+, K^+, and Cl^- channels, Na^+/K^+2Cl^- and Na^+/Cl^- cotransporters, Na^+/Ca^{2+}, Cl^-/HCO_3^- and Na^+/H^+ exchangers, Na^+/K^+-ATPase, V-ATPase, and carbonic anhydrase) in copper accumulation was evaluated in mantle cells exposed to 2.5 μM Cu for 1 h. Isolated cells were pre-exposed (30 min) to specific blockers or inhibitors of the ion-transporting proteins and then exposed to copper in the presence of the drug. A significant increase in copper accumulation was observed after cell incubation with acetozalamide (carbonic anhydrase inhibitor) and NPPB (Cl^- channels blocker). On the other hand, a significant decrease in copper accumulation was observed after incubation with furosemide ($Na^+/K^+/2Cl^-$ blocker). Taken altogether, these findings indicate the mantle as an important route of copper entry in *M. mactroides*, pointing the cotransporter $Na^+/K^+/2Cl^-$ as a major mechanism of copper accumulation in mantle cells of the clam.

Keywords: accumulation, clam, copper, ion transport, mantle, *Mesodesma mactroides*.

Introduction

Copper is an essential micronutrient to aquatic animals. It is involved in several physiological functions as enzyme cofactor. It also participates in cellular respiration [1,2]. However, copper is toxic when in elevated concentrations in the water. The monovalent copper (Cu^{+}) is considered the most toxic copper species [3].

In biological systems, copper is found mainly as divalent copper (Cu^{2+}), since Cu^{+} is readily oxidized to Cu^{2+} in the presence of oxygen [4]. Cu^{2+} reacts preferentially with inorganic ligands such as H_2O, OH^{-}, CO_3^{-} and SO_4^{-2} via oxygen, and with organic compounds through the phenolic and carboxylic groups [5].

Since aquatic mollusks are directly exposed to dissolved copper in the water, they can accumulate this metal in several tissues such as gills and digestive gland [3,6-8]. In fact, mollusks are known as good indicators of metal accumulation, being widely used as biological monitors of water quality [9,10]. Furthermore, copper accumulation in tissues of bivalve mollusks has shown to be proportional to the metal concentration in the water [11].

In mollusks, copper is preferentially accumulated in gills, followed by the digestive gland [12-16]. However, previous studies from our laboratory have shown that mantle of the marine clam *Mesodesma mactroides* accumulates copper at levels similar to those found in gills [17]. In fact, a relatively well-developed circulatory way through the mantle is found in all bivalve mollusks [18]. Therefore, the mantle can be considered as an important route of copper entry and accumulation in bivalve mollusks. However, no information is available on the cellular mechanisms involved in copper accumulation in these animals. In freshwater fish and invertebrates, it has been shown that copper can compete with other cations like Na^{+}, Ca^{2+} and Mg^{2+} for the sites of cellular incorporation of these ions at the gills [3].

Considering the background described above, in vivo copper accumulation was determined in different tissues of the marine clam *M. mactroides*. Also, in vitro copper accumulation and the involvement of the major mechanisms of ion transport and associated enzymes in copper accumulation was evaluated in isolated mantle cells of *M. mactroides*. Results from the present study are of major importance for a better characterization of the biotic ligand for copper, as well the mechanism of metal toxicity in marine invertebrates. Consequently, they are of interest for the development of a Biotic Ligand Model version for marine waters.

Material and Methods

Clam collection and acclimation

Clams (*M. mactroides*; length = 36.4 ± 3.1 mm; wide = 19.6 ± 1.5 mm; height = 9.3 ± 0.9 mm) were collected at the Mar Grosso Beach (São José do Norte, RS, Southern Brazil), immediately transferred to the laboratory, and acclimated for at least 5 days in a plastic tank containing seawater at salinity 30 ppt continuously aerated and in the absence of sediment. Room temperature (20°C) and photoperiod (12L:12D) were fixed. The acclimation tank was covered with a black plastic to minimize stress. The acclimation medium was renewed three times a week, when clam were fed with the diatom *Thalassiosira weissflogii* ($2x10^4$ cells/L).

In vivo copper accumulation in tissues

Acclimated clams (n = 24) were individually exposed (up to 96 h) to 5 µM Cu in glass beakers containing 200 mL of seawater at salinity 30 ppt (copper-exposed clams). Also, acclimated clams (n = 6) were individually kept under control conditions without

addition of copper into the water (control clams). Copper concentration tested (5 μM) was shown to correspond to the 96-h CL_{20} to the marine clam *M. mactroides* after 96 h of exposure to copper (96-h CL_{50}: 5.8 μM total copper; 5.2 μM dissolved copper) at the same experimental conditions employed in the present study [17]. Copper as $CuCl_2$ (Merck, St. Louis, MO, USA) was added to the water 3 h prior clam's introduction in the experimental medium. Every 24 h, the experimental medium was 100% renewed by a fresh medium prepared as described above.

Before and after exposure, non-filtered and filtered (0.45-μm mesh filter) water samples (10 mL) were collected and acidified (0.5%) with HNO_3 (Suprapur®, Merck, St. Louis, MO, USA) for total and dissolved copper concentration analysis, respectively. Also, clams (n = 6) were collected and crioanesthetized at the different times of exposure (0, 24, 48, 72 and 96 h). Hemolymph was collected by puncture of the foot hemolymph sinus. Mantle, gill and digestive gland were dissected from each clam and quickly washed with saline solution (see composition below) containing EDTA (12 mM) to remove the experimental medium and excess of hemolymph. Also, this procedure was performed to remove the copper loosely bound on the tissue surface. Samples were dried (48 h; 60°C), digested with 500 μL of HNO_3 (Suprapur®, Merck, St. Louis, MO, USA), and properly diluted with Milli-Q water. Copper concentration in water and digested tissue samples was measured by atomic absorption spectrophotometry (AAS 932 Avanta Plus, GBC, Hampshire, IL, USA), as previously described [19-21]. Results were expressed as μg Cu/g dry weight.

Mantle cells isolation

Each cell suspension was prepared as follow. Mantle epithelium of 6 clams was dissected, pooled, sliced in small pieces, and incubated in a calcium-free phosphate

solution (342 mM NaCl; 20 mM Na_2HPO_4; 1.7 mM K_2HPO_4; 16 mM KCl; 780 mosmol/Kg; pH 7.5; 20^oC) and shaked (100 rpm; Certomat-MO-II; Sartorius Stedim Biotech GmbH, Göttingen, Germany) for 40 min. After incubation, dissociated cells were filtered (30 μm-mesh nylon filter) to remove the non-dissociated tissue and large debris. The filtered solution containing isolated cells was transferred to plastic tubes and centrifuged 360 x g for 3 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA). The pellet was resuspended in 1 mL of saline solution of similar ionic composition to the clam hemolymph (350 mM NaCl; 9 mM KCl; 30 mM $MgCl_2$; 9 mM $CaCl_2$; 2 mM $NaHCO_3$; pH 7.6; 20^oC).

Isolated cells showed a mean diameter and volume of 10.94 ± 0.51 μm and 773.09 ± 110,91 μm^3, respectively. Cell counting and viability (% of viable cells from the total number of cells) was checked using the Tripan Blue exclusion assay (0.08% Trypan Blue). Only preparations showing more than 80% viable cells were used in the experiments.

In vitro copper accumulation and toxicity in isolated mantle cells

Copper accumulation and toxicity was measured in $5x10^5$ mantle cells incubated for 1 and 3 h in the saline solution without copper (control) or with copper at different concentrations (0.5, 1.0, 2.5 and 5.0 μM Cu). Copper was added as $CuCl_2$ (Merck, St. Louis, MO, USA). Three independent mantle cell preparations obtained as described above were tested (n = 3). Each preparation was tested in triplicate.

After exposure (1 or 3 h), one aliquot of the test medium containing cells was sampled to determine cell viability using the Tripan Blue exclusion method (0.08% Trypan Blue). Remaining mantle cells were centrifuged (360 x g) for 3 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA).

The supernatant was discarded and the pellet was quickly washed with a 12 mM EDTA solution to remove the loosely bound copper on the cell surface. The washing and centrifugation procedures were performed two times. The pellet was resuspended in a fresh copper-free saline solution. Cell samples were then dried (60^{o}C; 24 h), digested with 50 µl of HNO_3 (Suprapur®, Merck, St. Louis, MO, USA), and diluted with 500µL Milli-Q water. Copper concentration in the digested samples was analyzed by atomic absorption spectrophotometry (AAS 932 Avanta Plus, GBC, Hampshire, IL, USA), as previously described [19-21].

Copper toxicity in mantle cells was expressed in percentage of viable cells considering viability of control cells as 100%. Copper content in mantle cells was expressed as µg Cu/5x10^5 cells, considering the amount of "new copper" accumulated by the cells and the respective cell viability in each experimental condition. The "new copper" accumulated was calculated subtracting the copper concentration measured in mantle cells exposed to copper from that obtained in the respective control mantle cells for each preparation.

As mentioned above, the higher copper concentration tested (5.0 µM Cu) was shown to correspond to the 96-h LC_{20} in the marine clam *M. mactroides* [17]. The exposure times (1 and 3 h) were selected based on the fact that cells in suspension can be used within the next 5 h after isolation [22]. In fact, no significant change in cell viability was observed after 1 or 3 h of exposure to the copper concentrations tested (see Results section).

Ionic content in isolated mantle cells exposed to copper

Ionic content of isolated mantle was measured using 5x10^5 cells kept under control conditions (control cells) or after exposure to 2.5 µM Cu for 1 h. Three

independent cell preparations were tested (n = 3). For each preparation, tests were performed in triplicate. Cell isolation and copper exposure were performed as described above for the in vitro experiment.

After incubation, cells were centrifuged (1,800 x g) for 3 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA). The supernatant was discarded and the pellet was quickly washed with a sucrose solution of osmolality similar to the osmotic concentration of the clam hemolymph (850 mOsmol/Kg H_2O). Centrifugation procedures were repeated and the new supernatant was discarded. Cell samples were then dried (60^oC; 24 h), digested with 50 µl of HNO_3 (Suprapur®, Merck, St. Louis, MO, USA) and diluted with 500 µL Milli-Q water. Sample aliquots were collected and properly diluted for cell Na^+, K^+, Ca^{2+} and Cl^- content measurements. Cation concentration (Na^+, K^+ and Ca^{2+}) was measured by atomic absorption spectrophotometry (AAS 932 Avanta Plus, GBC, Hampshire, IL, USA), as previously described [19]. Anion (Cl^-) content was measured using a commercial reagent kit (Visocolor ECO, Macherey-Nagel, Düren, Germany).

Mechanisms of copper accumulation in isolated mantle cells

Isolated mantle cells from three independent preparations (n = 3) were incubated (30 min) with a specific blocker or inhibitor of the ion-transporting proteins evaluated and then exposed to 2.5 µM Cu for 1 h in the presence of the drug. These experimental conditions were selected based on the results obtained in the in vitro copper accumulation study described above. For each isolated cell preparation, tests were performed in triplicate. After exposure, aliquots of cells were sampled for cell viability measurement using the Trypan Blue (0.08%) exclusion method. Remaining mantle cells

were collected and prepared for copper determination as described above for the in vitro study.

The involvement of different mechanisms of ion transport and associated enzymes in copper accumulation was analyzed using several pharmacological tools. The mechanisms evaluated were: Na^+, K^+, and Cl^- channels, Na^+/K^+2Cl^- and Na^+/Cl^- cotransporters, and Na^+/Ca^{2+}, Cl^-/HCO_3^- and Na^+/H^+ exchangers. The enzymes studied were: Na^+/K^+-ATPase, V-ATPase, and carbonic anhydrase. The following ion transport blockers and enzyme inhibitors were employed: phenamil (10 μM; Na^+ channel blocker [23]), quinine sulfate (50 μM; K^+ channel blocker [24]), furosemide (100 μM; $Na^+/K^+/2Cl^-$ blocker [25]), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB; 100 μM; Cl^- channel blocker [26]), amiloride (100 μM; Na^+/H^+ exchanger blocker [27]), 4-acetamido-4'-isothiocyano-2,2'-stilbene disulphonic acid (SITS; 500 μM; Cl^-/HCO_3^- blocker [28]), etil-isopropil-amiloride (EIPA; 0.02 μM; Na^+/H^+ exchanger blocker [29]), ouabain (100 μM; Na^+/K^+-ATPase inhibitor [30]), benzthiazide (100 μM; Na^+/Cl^- cotransporter blocker [25]), bafilomycin A_1 (1 μM; V-ATPase inhibitor [31]), and acetazolamide (1 mM; carbonic anhydrase inhibitor [32]). It is important to note that the concentrations of the drugs used were previously employed to characterize the major mechanisms of ion transport in isolated mantle cells of *M. mactroides* [33]. SITS and ouabain were purchased from Fluka Chemie (Buchs, SG, Switzerland). Other drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Copper accumulation was expressed in μg Cu/$5x10^5$ cells, considering the amount of "new copper" accumulated and the respective cell viability in the experimental condition. The "new copper" accumulated was calculated as described above. Results were expressed as percentage of new copper accumulated after drug

incubation and copper exposure, considering the amount of copper accumulated in the absence of the drug as 100%.

Statistical analysis

All data were expressed as mean ± standard error. Mean values were compared using analysis of variance followed by the Tukey's test (in vivo copper accumulation and in vitro copper accumulation and toxicity data) or using the Student-*t* test (effects of drugs on copper accumulation and effects of copper on cellular ion content data). Analysis of variance and Student-*t* test assumptions (data normality and homogeneity of variances) were previously checked. Kinetics of in vivo (mantle tissue) and in vitro (isolated mantle cells) copper accumulation was determined using linear regression analysis. In gill, kinetics of in vivo copper accumulation was determined using non-linear regression analysis (one binding-site saturation kinetics). In all cases, the significance level adopted was 95% ($\alpha = 0.05$).

Results

In vivo copper accumulation in tissues

No copper was detected in the control medium (saline solution without copper addition; AAS detection limit: 0.16 μM Cu). Total and dissolved concentrations (mean ± SEM) in the experimental medium were 4.5 ± 0.7 and 3.8 ± 0.5 μM Cu, respectively. Total and dissolved copper concentrations were not significantly different ($p>0.05$).

No clam mortality was observed over 72 h of exposure to 5 μM Cu. As expected, one out of six clams (17%) died after 96 h of exposure to copper. No significant "new copper" accumulation was observed in the hemolymph over the

exposure period. However, a significant accumulation of "new copper" was observed in the digestive gland, but only at 96 h of exposure to copper. On the other hand, gill and mantle showed significant accumulation of "new copper" at the first sampling time (24 h). Furthermore, additional significant copper accumulation was observed over time along the 96-h exposure period in both tissues. However, significant differences in the kinetics of "new copper" accumulation were observed between gill and mantle tissue. Accumulation in mantle was significantly higher than in gill. Furthermore, "new copper" accumulation in mantle increased linearly over time ($y = 4.75 + 5.46x$; $r^2 = 0.99$) while it followed a saturation-type kinetics in gill [$y = 420.3(1-e^{-0.014x})$; $r^2 = 0.95$] (Fig. 1).

In vitro copper toxicity and accumulation in isolated mantle cells

No significant change in viability of isolated mantle cells was observed after exposure to 0, 0.5, 1.0, 2.5 and 5.0 µM Cu for 1 and 3 h, respect to that of control cells (Fig. 2). Significant accumulation of "new copper" was observed only in isolated mantle cells exposed to 2.5 and 5.0 µM Cu for 1 and 3 h. As observed in vivo, a linear increase in "new copper" accumulation was observed in isolated mantle cells exposed in vitro to copper for 1 h ($y = 0.0020 + 0.0059x$; $r^2 = 0.94$) and 3 h ($y = 0.0017 + 0.0064x$; $r^2 = 0.98$). No significant difference was observed between cells exposed for 1 and 3 h ($p>0.05$) (Fig. 3).

Mechanisms of copper accumulation in isolated mantle cells

Exposure of isolated cells to copper in the presence of the different blockers and inhibitors of ion-transporting proteins did not significantly change mantle cells viability

(always >80%). Also, single exposure to 2.5 µM Cu for 1 h did not significantly affect the ionic (Na^+, K^+, Ca^{2+} and Cl^-) content of isolated mantle cells (Fig. 4).

No significant change was observed in "new copper" accumulation in isolated mantle cells exposed to 2.5 µM Cu for 1 h in the presence of amiloride, benzthiazide, bafilomycin A_1, ouabain, quinine sulfate, phenamil, SITS and EIPA, respect to the control cells. However, a significant decrease in "new copper" accumulation was observed after exposure to copper in the presence of furosemide (48,2%). Also, a significant increase was observed after exposure to copper in the presence of NPPB (24.3%) and acetazolamide (29.1%) (Fig. 5).

Discussion

In control clams (non-exposed to copper), data from the in vivo experiment showed a higher background of copper burden in the digestive gland than in the other tissues analyzed (Fig. 1). This finding can be explained by the key role of the digestive gland in the metabolism of essential metals in mollusks. In fact, this tissue acts as a site of storage of essential metals and detoxification of non-essential metals [13,16,34,35].

In bivalve mollusks, the visceral mass is in contact with the environmental water entering the mantle cavity through the inhalant siphon [18]. Therefore, contaminants present in the water like copper are in direct contact with this organ. The fact that mantle significantly accumulated more copper than the other tissues after 96 h of in vivo exposure to 5 µM Cu indicates that this tissue is an important route of copper accumulation in the marine clam *M. mactroides*. The linear kinetic of copper accumulation observed in the mantle cells after both in vivo (Fig. 1) and in vitro (Fig. 2) exposures to the metal also supports this idea (Fig. 1). In this case, copper would be trapped at different metal-binding sites such as metalothioneins that are available in

mantle cells, thus reducing copper assimilation to the hemolymph and its subsequent distribution to other tissues. In turn, the lower copper level observed in hemolymph when compared to the other tissues analyzed (mantle, gill and digestive gland) and the lack of additional copper accumulation over the exposure time (Fig. 1) suggest that mantle could be also an important barrier for copper assimilation into the hemolymph when the metal is present at elevated concentrations in the water. The lower level of copper in the hemolymph and the lack of additional accumulation of copper over the exposure period can also be explained by the role played by hemolymph in tissue nutrition.

For a better understanding of the mechanisms involved in copper transport at the cell membrane level, the in vitro copper accumulation was measured in isolated mantle cells either in the absence or in the presence of different pharmacological tools. Cells were exposed to a sublethal concentration of copper (2.5 μM) for 1 h. This statement is based on the facts that no significant changes in cell viability (always >80%) and ion content (Na^+, K^+, Ca^{2+} e Cl^-; Fig. 3) were observed after exposure to 2.5 μM for 1 h. However, it is important to note that a significant copper accumulation was observed in isolated mantle cells after in vitro exposure to 2.5 μM for 1 h (Fig. 2). These finding suggest that the experimental conditions employed were "ideal" to test the involvement of the major ion-transporting proteins in copper accumulation in isolated mantle cells of the marine clam *M. mactroides*.

In isolated mantle cells exposed to copper in the presence of NPPB, a higher copper accumulation was observed (Fig. 4). This finding suggests that the intracellular excess of negative charges would be driven the copper entry in mantle cells. This statement is based on the fact that NPPB is a specific blocker of Cl^- channels [26]. NPP-induced blockade of these channels would lead to an increased concentration of Cl^-

inside the cells, thus increasing the internal electronegativity and consequently the driving force for the influx of copper. It is important to note that Cl^- channels are in fact involved in ionic regulation in isolated mantle cells of the marine clam *M. mactroides* [33].

Inhibition of carbonic anhydrase activity by acetazolamide [32] also led to an increased accumulation of copper in isolated mantle cells of *M. mactroides* (Fig. 4). It was previously shown that exposure of these cells to acetazolamide at the same concentration employed in the present study (1 mM) led to an increased intracellular Na^+ content [33]. This finding was explained considering an increased activity of the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+ channels to maintain the adequate levels of Na^+ and Cl^- in isolated mantle cells of the marine clam *M. mactroides*. The increased activities of these ion-transporting proteins would be compensating the decreased activities of the Na^+/H^+ and Cl^-/HCO_3^- exchangers associated with the inhibition of the carbonic anhydrase activity by acetazolamide [33]. Considering that copper was shown to compete with other cations, including Na^+, by the same binding sites of incorporation in gills and intestine of aquatic animals [3,36,37], the higher intracellular accumulation of copper observed in the presence of acetazolamide in the present study suggests that this metal would be entering the mantle cells through the Na^+ channel and/or the $Na^+/K^+/2Cl^-$ cotransporter. However, no significant change in copper accumulation was observed after inhibition of the Na^+ channels by both amiloride and fenamil (Fig. 4). On the other hand, a reduced copper accumulation was observed in isolated mantle cells exposed to furosemide (Fig. 4). Taken altogether, these findings indicate that copper is entering the mantle cells of the marine clam *M. mactroides* through the $Na^+/K^+/2Cl^-$ cotransporter. It was previously shown that this ion-transporting protein plays an

essential role in intracellular ion regulation in the mantle cell of the marine clam *M. mactroides* [33], as well as in other marine animals [38].

In summary, data from the present study indicate that mantle is an important route of copper accumulation in the marine clam *M. mactroides* both in vivo and in vitro. They also show that cellular copper level is a good indicator of the metal concentration in the dissolved phase, suggesting that mantle cell is good biological tool to evaluate the degree of water contamination by copper in monitoring programs. Finally, it is suggested that isolated mantle cell of the marine clam *M. mactroides* could be considered as the biotic ligand for copper in the development of a Biotic Ligand Model for marine environments. This statement is based on the fact that copper toxicity is well related to its accumulation in the isolated mantle cells of *M. mactroides*, being the mechanism of toxicity associated with disturbances in intracellular ionic content [39].

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Figure legends

Figure 1. In vivo copper accumulation in tissues of the marine clam *Mesodesma mactroides* after exposure to 5 µM Cu for 96 h. (●) mantle; (▲) gills; (■) digestive gland; (♦) hemolymph. Data are expressed as mean ± standard error (n = 6). Different lower letters indicate significant different mean values within exposure times for the same tissue (p<0.05). Different capital letters indicate significant different mean values within tissues for the same time of exposure (p<0.05).

Figure 2. Viability of isolated mantle cells of the marine clam *Mesodesma mactroides* exposed to 0.5, 1.0, 2.5 and 5.0 µM Cu for 1 (closed bars) and 3 h (open bars). Data are expressed as mean ± standard error (n = 3). For both exposure times, no significant difference was observed between treatments (p>0.05).

Figure 3. In vitro copper accumulation in isolated mantle cells of the marine clam *Mesodesma mactroides* exposed to 0.5, 1.0, 2.5 and 5.0 µM Cu for 1 (closed circles) and 3 h (open circles). Data are expressed as mean ± standard error (n = 3). Small and capital letters indicate significant difference between mean values for 1 h and 3 h of exposure, respectively (p<0.05). No significant difference was observed between the two times of exposure at the different copper concentrations tested (p>0.05).

Figure 4. Ion content in isolated mantle cells of the marine clam *Mesodesma mactroides* kept under control conditions (closed bars) and in vitro exposed to 2.5 µM Cu for 1 h (open bars). Data are expressed as mean ± standard error (n = 3). No significant difference was observed between control and copper-exposed mantle cells (p>0.05).

Figure 5. Percentage of copper accumulation in isolated mantle cells of the marine clam *Mesodesma mactroides* after exposure to 2.5 µM Cu for 1 h in the presence of different drugs. Data are expressed as mean ± standard error (n = 3). AM: amiloride; NP: NPPB; FU: furosemide; BE: benzthiazide; BA: bafilomycin A_1; AC: acetazolamide; OU: ouabain; QS: quinine sulfate; PH: phenamil; SI: SITS; EI: EIPA. Data are expressed as percentage of the control (cells non-exposed to drugs and copper). * indicates significant difference from the control (p<0.05).

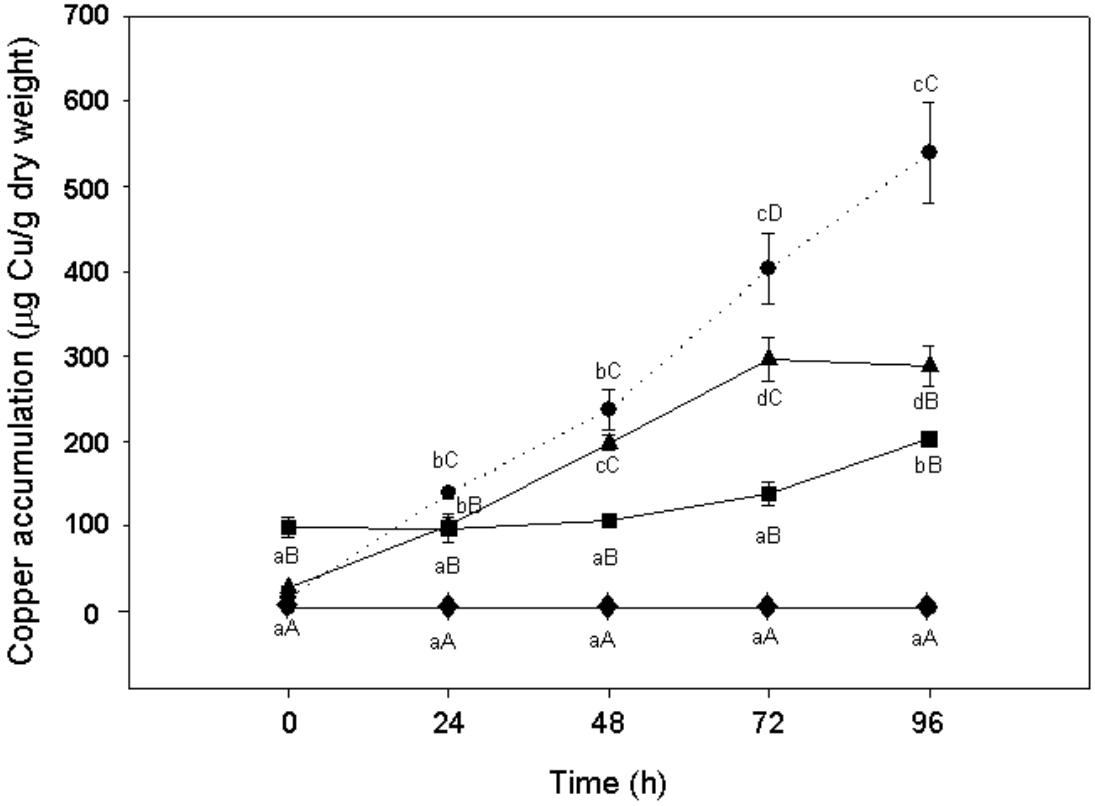
Copper accumulation (µg Cu/g dry weight)
700
600
500
400
300
200
100
0
0
24
48
72
96
Time (h)
cC
cD
bC
dC
dB
bC
bB
cC
bB
aB
aB
aB
aB
aA
aA
aA
aA
aA

Figure 1

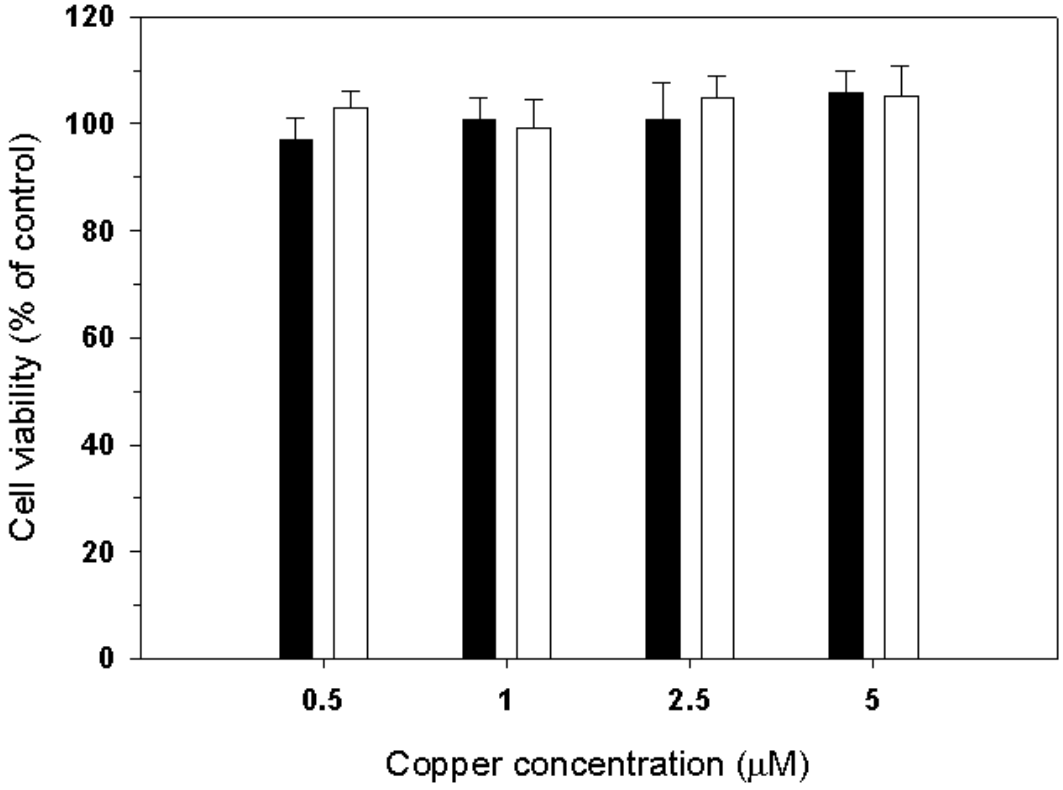
120
100
80
60
40
20
0
Cell viability (% of control)
0.5
1
2.5
5
Copper concentration (μM)

Figure 2

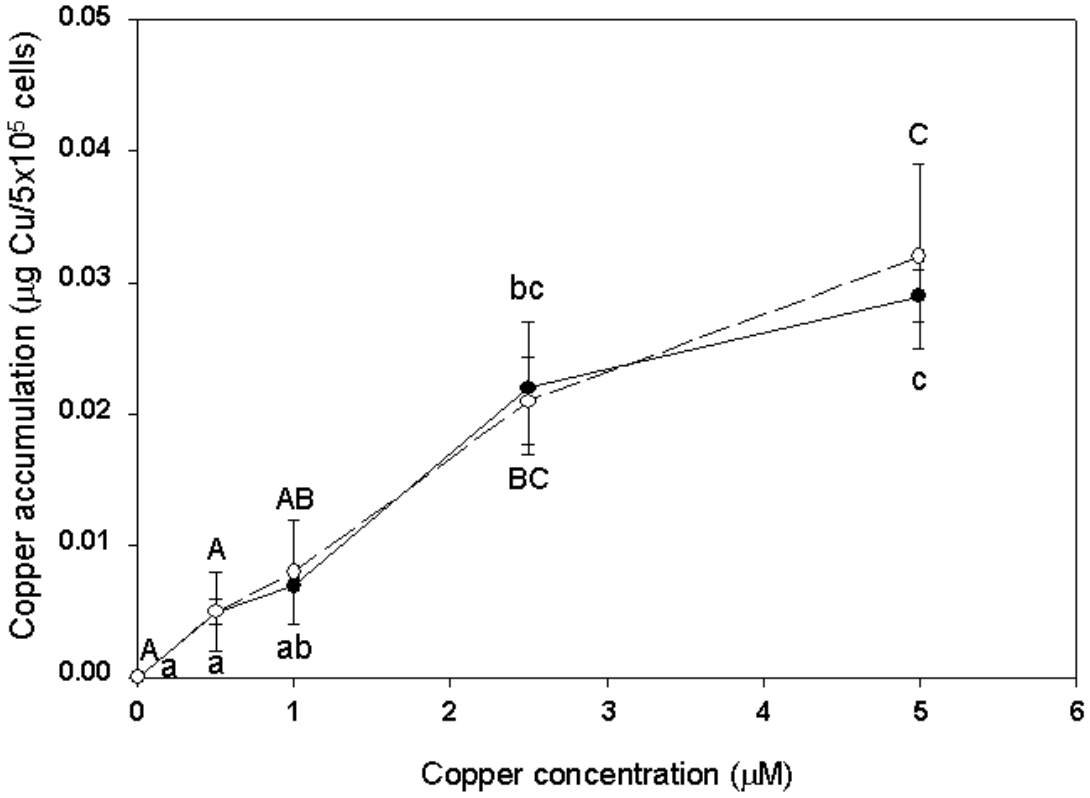
Copper accumulation (µg Cu/5x10^5 cells)
0.05
0.04
0.03
0.02
0.01
0.00
0
1
2
3
4
5
6
Copper concentration (µM)
A
a
A
AB
ab
bc
BC
C
c

Figure 3

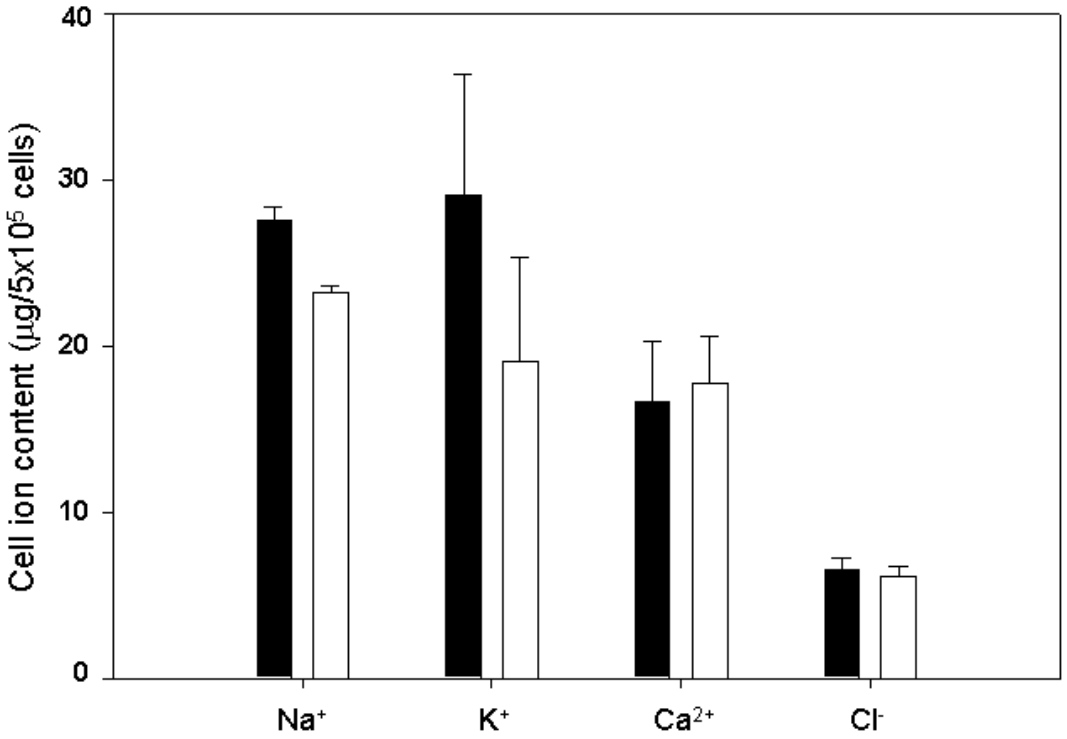

Figure 4

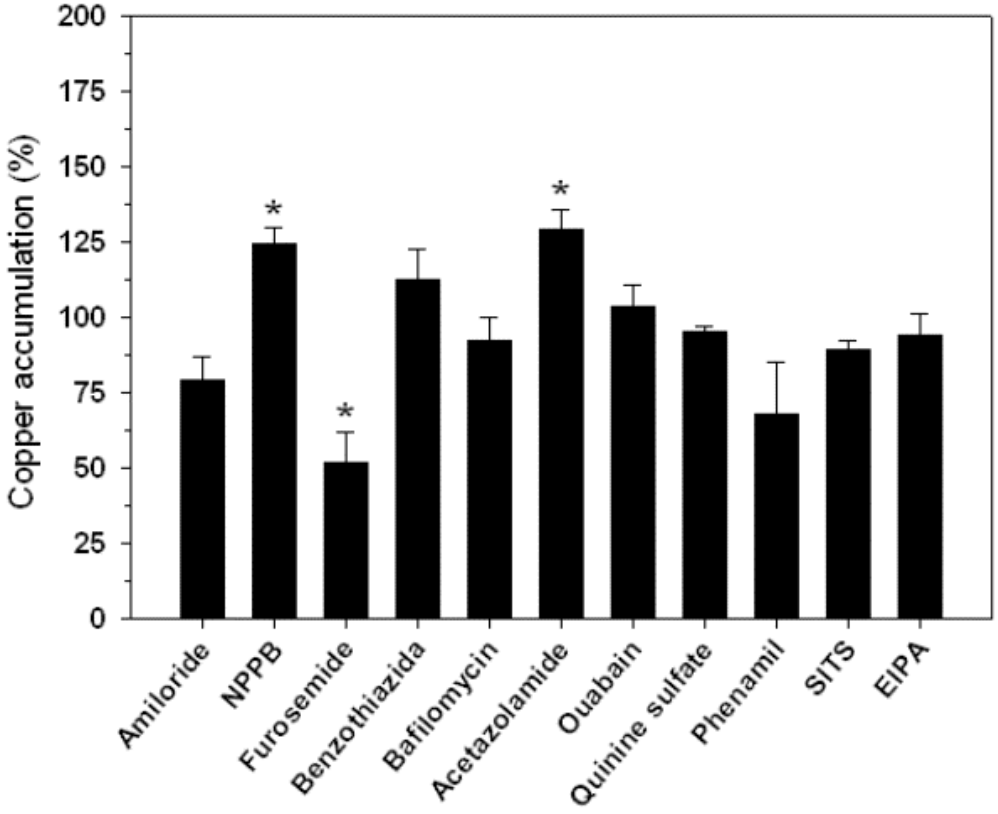
Copper accumulation (%)
200
175
150
125
100
75
50
25
0
*
*
*
Amiloride
NPPB
Furosemide
Benzothiazida
Bafilomycin
Acetazolamide
Ouabain
Quinine sulfate
Phenamil
SITS
EIPA

Figure 5

CONCLUSÕES GERAIS

Baseado nos resultados obtidos nesta tese, um modelo hipotético dos mecanismos de transporte iônico nas células isoladas do manto do marisco *Mesodesma mactroides* foi proposto. De acordo com o modelo proposto a entrada de Na^+ e Cl^- nestas células pode ocorrer através dos trocadores Na^+/H^+ e Cl^-/HCO_3^-, dos cotransportadores $Na^+/K^+/2Cl^-$ e Na^+/Cl^- e dos canais de Na^+. O movimento de entrada de Na^+ e Cl^- através dos trocadores Na^+/H^+ e Cl^-/HCO_3^- e dos cotransportadores $Na^+/K^+/2Cl^-$ e Na^+/Cl^- pode ser dirigido pelo gradiente de Na^+ existente entre o meio extra e intracelular, que pode ser gerado pela atividade da Na^+,K^+-ATPase. Os substratos (H^+ e HCO_3^-) para a operação dos trocadores Na^+/H^+ e Cl^-/HCO_3^- pode ser provido pela hidratação do CO_2 catalisado pela enzima anidrase carbônica. Os prótons gerados podem ser retirados também via bomba protônica (V-tipo H^+-ATPase). O efluxo de H^+ gerado pela H^+-ATPase pode gerar um gradiente elétrico local que pode favorecer o influxo de Na^+ através dos canais de Na^+. Portanto, o balance ácido-base intracellular pode ser dependente dos trocadores Na^+/H^+ e Cl^-/HCO_3^- bem como da atividade da bomba protonica. O efluxo de Na^+ e Cl^- destas células pode ser dependente da atividade da Na^+/K^+-ATPase. A bomba Na^+/K^+ pode ser responsável por um ativo efluxo de Na^+ paralelo com um ativo influx de K^+, gerando um gradient de Na^+ entre o meio extra e intracelular já mencionado acima, bem como um gradiente de K^+ entre o meio intra e extracelular. Um efluxo de K^+ pode ocorrer através dos canais de K^+, gerando um gradiente elétrico local que pode favorecer o efluxo de Cl^- via canais de Cl^-.

Nossos resultados permitem concluir ainda que o cobre também pode ser considerado um tóxico ionoregulatório em moluscos marinhos, sendo este o possível mecanismo de toxicidade aguda do cobre nas células isoladas do manto do marisco *M. mactroides*. Além disso, os mecanismos envolvidos na regulação aniônica (Cl^-)

intracelular parece ser mais sensível a exposição ao cobre do que as envolvidas na regulação catiônica (Na^+ and K^+) intracelular.

Os dados do presente estudo indicam também que o manto é uma importante rota de acumulação de cobre no molusco marinho *M. mactroides* tanto "in vivo" quanto "in vitro". O nível de cobre intracelular também mostrou ser um bom indicador da concentração do metal na fase dissolvida, sugerindo que o manto é uma boa ferramenta biológica na avaliação do grau de contaminação da água por este metal em programas de monitoramento. Ainda pode ser concluído que o cotransportador $Na^+/K^+/2Cl^-$ parece ser o principal mecanismo envolvido na acumulação de cobre nestas células. Finalmente, é sugerido que as células isoladas do manto do molusco marinho *M. mactroides* pode ser considerado como um ligante biótico para o cobre no desenvolvimento do Modelo do Ligante Biótico para ambientes marinhos.

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