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MECANISMOS DE TRANSPORTE E ACUMULAÇÃO DO COBRE COMO BIOMARCADORES DE CONTAMINAÇÃO EM **Callinectes sapidus** *RATHBUN, 1896.*

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RESUMO

O objetivo desta Tese foi realizar estudos *in vitro* para identificar os principais mecanismos de transporte iônico envolvidos na acumulação e toxicidade do cobre em células branquiais e hepatopancreáticas isoladas de *Callinectes sapidus*, e sua aplicação como biomarcadores da contaminação do cobre em ambientes estuarinos e marinhos. Foram utilizadas técnicas de cultura celular associadas a ferramentas fluorimétricas e farmacológicas. Os resultados indicam que células branquiais de *C. sapidus* são boas indicadoras dos níveis de cobre presente no meio. A acumulação de cobre nas células branquiais é influenciada pelo nível de cobre intracelular pré-existente e pela salinidade de aclimatação. Células branquiais de *C. sapidus* acumulam mais cobre e são mais sensíveis ao metal do que as células hepatopancreáticas. A cinética de acumulação do cobre em função do tempo de exposição é diferente em células branquiais (linear) e hepatopancreáticas (saturação), mas ambos os tipos celulares apresentaram uma relação linear positiva de acumulação de cobre em função da concentração do metal no meio de incubação. Além disso, foi observada uma relação entre a acumulação de cobre e a viabilidade de células branquiais, indicando que estas são órgãos-alvo da toxicidade do cobre e podem servir como modelo no desenvolvimento de uma versão *in vitro* do Modelo do Ligante Biótico (BLM) para condições marinhas e estuarinas. O bloqueio dos transportadores de Na^+ e Cl^- ou a inibição da atividade das enzimas a eles associadas caracteriza a competição entre cobre e o Na^+ pelos mesmos sítios de ligação na membrana da célula branquial. Esta situação indica que a toxicidade do cobre em células branquiais de *C. sapidus* pode estar relacionada ao desequilíbrio iônico e osmótico, com um possível impacto sobre a regulação osmótica e iônica intracelular.

Palavras-chaves: *Callinectes sapidus*, cobre, acumulação, toxicidade, células isoladas

ABSTRACT

This Thesis aimed to identify *in vitro* the mechanisms of ionic transport involved in copper accumulation and toxicity in isolated cells from gills and hepatopancreas of the blue crab *Callinectes sapidus*. The application of these mechanisms as biomarkers of contamination by copper in estuarine and marine environments was also evaluated. To assess these objectives, techniques of cell culture were associated to fluorescence and pharmacological tools under environmental and experimental conditions. Results obtained indicate that cells isolated from the posterior gills of the blue crab *C. sapidus* are good indicators of the copper level in the surrounding medium. Copper accumulation in gill cells is influenced by the level of copper pre-exposure in the field and the acclimation salinity in the laboratory. Results also showed that gill cells are more sensitive and accumulate more copper than hepatopancreatic cells. Accumulation kinetics of copper over the time of exposure depends on the type of cell, following a linear and saturation model in gill and hepatopancreatic cells, respectively. Both cells showed a linear relationship between copper accumulation and the metal concentration in the incubation media. It was also observed a relationship between cell copper accumulation and cell viability, indicating that gill cells are targets for copper toxicity and could be used as a model in the development of an *in vitro* Biotic Ligand Model (BLM) version to estuarine and marine conditions. The blockage of Na^+ and Cl^- transporters or the inhibition of the enzymes activity related to them characterize the competition between copper and Na^+ for the same binding sites in the gill cell membrane. This finding indicates that copper toxicity in gill cells of *C. sapidus* could be related to ionic and osmotic imbalances, with a possible impact on intracellular ionic and osmotic regulation.

Key-words: *Callinectes sapidus*, copper, accumulation, toxicity, isolated cells

1. INTRODUÇÃO

Os estuários estão localizados em regiões costeiras e devido as suas características geomorfológicas podem ser considerados como receptores de uma variedade de substâncias químicas provenientes dos sistemas terrestres, atmosférico e oceânico, bem como das ações humanas. Estas substâncias, quando causadoras de processos de poluição, são denominadas de "xenobióticos" e suas quantidades e variedades estão em contínuo aumento (Livingstone, 1993). Uma clara distinção entre o meio marinho de maior profundidade e os ambientes estuarino-lagunares, é que nos últimos há uma maior interação entre a coluna d'água e o meio bentônico, a qual atua como um forte agente estruturador, regulando ou modificando a maioria dos processos físicos, químicos e biológicos em sistemas estuarinos (Day *et al.*, 1989).

A introdução de metais nos sistemas aquáticos ocorre naturalmente através de processos geoquímicos, intemperismos e através do despejo de fontes antropogênicas (Santore *et al.*, 2000). Cabe ressaltar que a presença destes íons metálicos é vital aos ciclos biológicos.

Segundo Morgan (2000), o cobre é considerado um micronutriente essencial pois participa de uma série de funções fisiológicas que ocorrem nos organismos aquáticos. Este metal é componente essencial na estrutura de algumas enzimas e proteínas, atua como cofator enzimático e está envolvido na respiração celular (Dameron & Howe, 1998; Bury *et al*., 2003). Entretanto, o cobre é tóxico aos organismos aquáticos quando presente em altas concentrações na água, sendo que as formas iônicas deste metal são consideradas de maior toxicidade (Di Toro *et al*., 2001). Estas contrastantes considerações a respeito da essenciabilidade, biodisponibilidade e toxicidade do cobre são importantes fatores responsáveis pelas conseqüências da presença deste metal nos ecossistemas aquáticos.

O estuário da Lagoa dos Patos (31º 50'- 32º 05' S e 52º 15' – 52º 00' W) destaca-se tanto por sua importância ecológica, sendo zona de produção biológica e de biodiversidade, quanto por sua relevância sócio-econômica, já que reúne atividades portuárias, industriais, agrícolas, pesqueiras e turísticas (Asmus & Tagliani, 1998). No entanto, o equilíbrio ecológico desta região está ameaçado pelo contínuo aumento da poluição orgânica e inorgânica devido ao crescimento populacional e a expansão do pólo industrial (Santos *et al*.,1997; Baumgarten & Niencheski, 1998). Estudos realizados neste estuário indicaram um aumento no fator de enriquecimento de alguns metais, incluindo o cobre, no material em suspensão (Niencheski & Baumgarten, 1998; Niencheski & Baumgarten, 2000) e nos sedimentos (Rosa Filho, 2001; Niencheski *et al*., 2006) da região. Cabe ressaltar a importância destes dados para a trama trófica do estuário, já que o detrito orgânico constitui um importante componente da dieta de várias espécies de invertebrados da macrofauna, inclusive na dieta de crustáceos decápodes (Kapusta & Benvenuti, 1998). Na biota, a concentração de cobre nas folhas e rizomas da macrófita *Spartina alterniflora*, outro importante item alimentar de decápodes como *Neohelice granulatus*, é maior em ambientes impactados por ações humanas (Rosa, 2002).

Desta forma, o cobre, quando presente em concentrações elevadas, pode representar um perigo para a biota, devendo seu lançamento para o ambiente estar sob controle. Por exemplo, concentrações de cobre entre 0,9 e 42 μg Cu/L já foram registradas no estuário da Lagoa dos Patos (Baumgarten & Niencheski, 1998). Cabe ressaltar que estes valores não ultrapassaram o limite máximo estipulado pela legislação da época (50 μg Cu total/L: CONAMA, 1986 e FEPAM, 1995), porém estão acima das concentrações atualmente vigentes para esta categoria ambiental (5 μg Cu dissolvido/L: CONAMA, 2005).

Os programas de avaliação e monitoramento da contaminação aquática de muitos países, inclusive no Brasil (CONAMA; FEPAM), consideram os níveis dos principais tipos de contaminantes na água e no sedimento. Contudo, apesar da fundamental importância deste

tipo de informação, organizações internacionais e agências ambientais reconheceram nos últimos anos, a necessidade crescente de se detectar e avaliar o efeito tóxico provocado pelos contaminantes nos organismos, e não somente avaliar a qualidade do ambiente em que eles vivem (Paquin *et al*., 2002; Allen & Moore, 2004). Neste contexto, o Modelo do Ligante Biótico (BLM) foi desenvolvido com o propósito de avaliar quantitativamente como 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 (Paquin *et al*., 2002; Di Toro *et al*., 2001). Paralelamente ao desenvolvimento deste modelo, estudos fisiológicos vêm sendo desenvolvidos com o objetivo de aperfeiçoar o entendimento a respeito das interações dos metais com os organismos aquáticos (Bianchini *et al.*, 2002; Bianchini & Wood, 2002; Grosell *et al.*, 2002; 2004).

Visto que os organismos aquáticos são expostos diretamente ao cobre dissolvido na água, elevadas concentrações deste metal podem levar ao acúmulo de cobre em vários tecidos, incluindo as brânquias e o hepatopâncreas (Chavez-Croocker *et al.*, 2003; Ahearn *et al.*, 2004; Grosell *et al.*, 2004; Morales-Hernandez *et al.*, 2004).

A acumulação de cobre nas brânquias parece ser o fator controlador da toxicidade, já que o cobre é considerado um tóxico osmorregulatório que induz o desequilíbrio no balanço iônico dos animais aquáticos, incluindo crustáceos, podendo levá-los à morte (Laurén & McDonald, 1985; Bianchini *et al.*, 2002; Bianchini & Wood, 2002; Grosell *et al.*, 2002; 2004;).

A osmorregulação compreende uma série de estratégias desenvolvidas pelos organismos, as quais atuam em conjunto para manutenção do volume celular frente às variações de salinidade do ambiente. Em crustáceos, a capacidade de tolerar variações de salinidade é uma característica essencial para o estabelecimento e manutenção destes organismos nos estuários. Para tanto, os crustáceos eurialinos utilizam dois mecanismos básicos, assegurando um balanço de água e solutos: (1) regulação anisosmótica do fluído extracelular (RAFE), que mantém o fluído extracelular constante independente da salinidade do meio circundante; e (2) regulação isosmótica do fluído intracelular (RIFI), que mantém o fluído intracelular isosmótico em relação ao fluído extracelular, que por sua vez varia de acordo com as variações do meio externo (Gilles, 1982; Péqueux, 1995). As espécies osmorreguladoras dependem principalmente da RAFE, enquanto as osmoconformadoras dependem, sobretudo, da RIFI.

O transporte iônico transepitelial existente nas brânquias posteriores de crustáceos, um processo que faz parte dos mecanismos osmorregulatórios, reflete a presença de proteínas de transporte na membrana celular, tais como enzimas, canais e transportadores iônicos. Estas proteínas estão localizadas tanto no domínio apical quanto basolateral e intervêm no transporte de íons através da membrana quando o animal sofre algum tipo de estresse osmótico (choque hipo ou hiperosmótico).

O primeiro passo na captação dos íons consiste nas trocas independentes de Na^+ por H^+ e Cl^- por HCO_3^- na membrana apical do epitélio branquial, processos estes que envolvem a ação da anidrase carbônica (Mantel e Farmer, 1983; Péqueux, 1995). Esta enzima catalisa a hidratação do CO_2 gerando H^+ e HCO_3^-, os quais são trocados por Na^+ e Cl^-. A atividade desta enzima aumenta significativamente em brânquias de crustáceos eurialinos transferidos de altas para baixas salinidades (Henry, 1996). Outro mediador da captação de Na^+ é o co-transportador $Na^+/K^+/Cl^-$, identificado na membrana basolateral de epitélios de absorção e

excreção de sais. Além disso, pode ser observada a presença da V-H^+-ATPase, uma enzima que possui extrema importância na captação iônica branquial de espécies animais que toleram baixa salinidade, como o caranguejo *Eriocheir sinensis*. Esta enzima é uma proteína altamente conservada, que transporta H^+ contra seu gradiente eletroquímico através da membrana apical da brânquia, gerando energia que é utilizada para captação de Na^+ pelos canais apicais para este íon (Towle e Wethauch, 2001). Entretanto, a significância desta proteína em espécies eurialinas é pouco clara. Por sua vez, a Na^+/K^+-ATPase é uma enzima localizada exclusivamente na membrana basolateral da brânquia, sendo responsável pelo transporte ativo de Na^+ da célula branquial para a hemolinfa. Esta enzima requer energia na forma de ATP, que pode ser obtida através do processo de fosforilação oxidativa na mitocôndria ou menos comumente pelo processo de respiração anaeróbica (Towle *et al*., 1976; Towle e Kays, 1986; Péqueux, 1995). Em brânquias de *Callinectes sapidus*, estudos eletrofisiológicos revelaram a importância central do trocador apical Na^+/H^+ e da Na^+/K^+-ATPase na captação de Na^+ do meio ambiente para a hemolinfa (Burnet e Towle, 1990).

A toxicidade do cobre é assumida por ocorrer como resultado da reação do metal livre com os sítios de ligação fisiologicamente ativos no sítio de ação, as brânquias de animais de respiração aquática (Paquin *et al*., 2002; Di Toro *et al*., 2001). Ambas as formas do cobre, monovalente e divalente, são transportadas através do epitélio branquial através de, pelo menos, três transportadores: canal apical de Na^+ para o cobre monovalente, transportador tipo CTR1 e via transportador metal divalente para o cobre divalente. Outros componentes do transporte de Na^+ branquial incluem a enzima Na^+/ K^+-ATPase, o trocador apical Na^+/H^+ e a enzima anidrase carbônica (Handy *et al*., 2002; Grosell *et al*., 2002; 2004). Neste contexto, é importante ressaltar que uma característica típica dos transportadores de metal é a sua conservação ao longo da evolução e o fato de serem responsáveis pela biodisponibilidade do metal. Estas considerações, provavelmente, são responsáveis pela similaridade dos efeitos

encontrados sobre a biodisponibilidade de um dado metal tanto em dafinídeos como em peixes (Bury *et al.*, 2003). Portanto, considerando que a depleção do Na^+ extracelular é a causa primária da toxicidade do cobre em água doce, a inibição ou a competição com qualquer um dos transportadores mencionados acima poderá influenciar o transporte de Na^+ através das brânquias (Bianchini & Wood, 2002).

Entretanto, apesar dos mecanismos de toxicidade aguda do cobre serem semelhantes em organismos de água doce e marinhos, existe uma grande variabilidade quanto à tolerância ao metal nos diferentes grupos animais (Bianchini & Wood, 2002). O transporte dos íons Na^+ e Cl^- através do epitélio opercular é inibido pelo cobre no peixe estuarino *Fundulus heteroclitus*, quando adaptado a água marinha (Crespo & Karnarky, 1983). Em peixes marinhos expostos ao cobre, a atividade da anidrase carbônica intestinal também é inibida (Grosell *et al.*, 2004). Porém, ao contrário dos peixes de água doce, a atividade da Na^+/K^+-ATPase não é claramente influenciada pela exposição ao cobre nos tecidos osmorregulatórios dos peixes marinhos, apesar da substancial acumulação do metal (Grosell *et al.*, 2002; 2004). Entretanto, os processos ionorregulatórios são afetados, desestabilizando assim o equilíbrio iônico e osmótico dos organismos. Estes resultados indicam a possibilidade de que outros componentes do mecanismo de transporte iônico sejam os alvos do distúrbio osmorregulatório induzido pelo cobre. Neste contexto, a caracterização dos mecanismos de transporte iônico branquial dos principais íons envolvidos na regulação iônica e osmótica hemolinfática em invertebrados estuarinos e marinhos é essencial para o entendimento da contribuição destes mecanismos na acumulação e toxicidade de metais, incluindo o cobre, nestes organismos. Quanto aos crustáceos, estudos demonstraram que a regulação do Na^+ é afetada pelo cobre no caranguejo *Carcinus maenas* (Lawson *et al*., 1995) e no camarão *Peneaus japonicus* (Bamhang *et al*., 1995). O cobre também é responsável pela inibição da anidrase carbônica branquial em *Neohelice granulatus* (Vitale *et al*., 1999). De acordo com

Bianchini *et al*. (2003; 2004; 2005), invertebrados marinhos, em geral, são osmoconformadores, podendo agir como ionorreguladores e esta situação pode tornar a brânquia o sítio-alvo para a toxicidade do cobre também nestes organismos. Estas diferenças de tolerância ao cobre podem ser um reflexo de diferentes formas de interação do cobre com os mecanismos de transporte iônico branquial e, conseqüentemente, diferentes níveis de acumulação e distribuição do cobre nos diversos grupos animais em função de suas habilidades de regulação iônica e osmótica.

Por outro lado, o hepatopâncreas dos crustáceos é considerado um órgão multifuncional, responsável pelo seqüestro e detoxificação de metais. Este órgão controla a entrada de cátions divalentes como cálcio e cobre, provenientes do trato gastrointestinal, podendo tanto acumular estes cátions no epitélio hepatopancreático, como transferi-los para a hemolinfa, onde serão distribuídos para o organismo (Ahearn *et al.*, 2004). Os mecanismos de detoxificação epitelial conhecidos em invertebrados incluem as metalotioneínas (MTs) e o acúmulo de metal em organelas celulares (Chavez-Croocker *et al.*, 2002). Segundo Klein & Ahearn (1999), organelas hepatopancreáticos são capazes de acumular cobre, limitando a concentração citoplasmática e hemolinfática deste metal. Quanto as MTs, sua indução tem sido demonstrada em organismos expostos ao cobre, evidenciando a alta afinidade destas proteínas com os sítios de ligação catiônicos, os quais são sintetizados em resposta ao aumento da concentração do metal na célula (Roesijadi, 1992). O envolvimento das MTs na diminuição da disponibilidade do metal é mais evidente nas brânquias e hepatopâncreas, refletindo a importância destes órgãos na captação, acúmulo e excreção do cobre (Cajaraville *et al.*, 2000).

Entretanto, apesar das diferenças na quantidade de possíveis ligantes para o cobre (Cl^-, $S0_4^{2-}$, matéria orgânica, $S_2O_3^{2-}$, sulfeto, Br^-, e $B(OH)_4^-$) ou competidores (Na^+, Mg^{2+}, Ca^{2+}, K^+, e Sr^{2+}) na água doce, estuarina e marinha, estudos sobre os mecanismos de toxicidade do

cobre em invertebrados marinhos e estuarinos são menos abundantes quando comparados com os estudos realizados com animais de água doce.

Considerando o exposto acima, informações relacionando mecanismos de transporte iônico branquial envolvidos na absorção e acumulação do cobre tornam-se necessárias para um maior esclarecimento a respeito da toxicidade do metal nestes organismos estuarinos, bem como uma possível utilização destes mecanismos como biomarcadores de contaminação ambiental pelo cobre. Atualmente, o Conselho Internacional para a Exploração do Mar tem recomendado a utilização de metodologias complementares nos novos programas de monitoramento ambiental, como o estudo de biomarcadores. O uso de biomarcadores tem sido indicado por ser considerado uma ferramenta sensível na avaliação da qualidade ambiental e dos efeitos biológicos dos poluentes (Cajaraville *et al.*, 2000).

Biomarcadores podem ser definidos como alterações biológicas em nível molecular, celular e fisiológico, as quais expressam a exposição e os efeitos tóxicos induzidos por xenobióticos presentes no ambiente (Walker *et al*., 1996). Livingstone (1993) considera como biomarcadores os fluídos corpóreos, as células e/ou os tecidos que indicam, em termos bioquímicos ou celulares, a presença de contaminantes. Portanto, os biomarcadores selecionados para um programa de monitoramento ambiental devem detectar a exposição do organismo aos contaminantes através de qualquer alteração biológica mensurável (biomarcadores de exposição) e/ou a magnitude de resposta do organismo aos contaminantes (biomarcadores de efeito). Existem assim biomarcadores moleculares, celulares ou organísmicos, sendo alguns deles específicos para determinados contaminantes (Livingstone, 1993; Walker *et al.*, 1996). Uma das características mais importantes dos biomarcadores moleculares e celulares é o potencial de antecipar mudanças em níveis de organização biológica mais altos, como populações, comunidades ou ecossistemas. Portanto, o aviso antecipado oferecido pelos biomarcadores pode ser usado de uma maneira preventiva,

permitindo que estratégias de biorremediação possam ser desenvolvidas antes que ocorram danos ambientais e ecológicos irreversíveis (Cajaraville *et al*., 2000).

Neste contexto, como a célula é o sítio de acumulação, metabolismo e toxicidade de contaminantes como o cobre, certas respostas celulares são consideradas como biomarcadores de exposição e estão sendo utilizadas em programas de monitoramento ambiental (Cajaraville *et al.*, 2000; Allen & Moore, 2004). Diferentes métodos estão disponíveis para a quantificação de cada biomarcador. Recentemente, 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 & Galloway, 2004). Um dos mais importantes resultados destas investigações com inúmeras aplicações para a toxicologia foi a reconstrução do tecido epitelial da brânquia de peixes dulciaqüícolas (Fletcher, *et al.*, 2000; Kelly *et al.*, 2000). A cultura primária de tecidos permite a investigação e a compreensão dos mecanismos tóxicos envolvidos em nível celular causados por metais, os quais não podem ser analisados por métodos *in vivo*. Assim, esta ferramenta fornece a possibilidade de reproduzir, em um sistema *in vitro*, situações de exposição aos contaminantes nos ambientes aquáticos, possibilitando a caracterização das vias de entrada e cinética de transporte de metais, bem como o envolvimento dos mecanismos básicos de transporte iônico celular nestes processos. Niyogi & Wood (2004) apontam que a melhor caracterização das propriedades dos ligantes bióticos em nível molecular, o desenvolvimento de versões do BLM a partir de estudos *in vitro*, bem como a extensão deste modelo para diferentes grupos animais e ambientes estuarinos e marinhos, são algumas das necessidades atuais para o refinamento do BLM, visando sua aplicação para definição de critérios de qualidade de água para metais.

Inserido neste contexto, o presente estudo verificou, em nível celular, os mecanismos de transporte branquial dos principais íons envolvidos na regulação iônica e osmótica de invertebrados estuarinos osmorreguladores, para uma melhor compreensão dos mecanismos de acumulação e toxicidade do cobre e sua possível aplicação como biomarcador de exposição a este metal no estuário da Lagoa dos Patos. Para este fim, foram realizados experimentos *in vitro* abordando aspectos fisiológicos, toxicológicos e farmacológicos utilizando culturas primárias do epitélio branquial e hepatopancreático do siri azul *Callinectes sapidus.*

O siri azul *C. sapidus* é uma espécie bentônica residente no estuário, que influencia decisivamente na transferência energética ao longo da cadeia trófica (Bemvenuti, 1987). Este decápodo eurialino possui uma marcada variação ontogenética na dieta: exemplares recém recrutados nas enseadas, alimentam-se do filme superficial na vegetação ou no epistrato (Geraldi, 1997), enquanto que os juvenis de *C. sapidus* atuam como consumidores secundários, pois além de macrófitas e detritos, consumem peracáridos epifaunais e juvenis da infauna (Kapusta & Bemvenuti, 1998). Os juvenis de maior porte ingerem detritos e ampliam o consumo da macrofauna bentônica com a inclusão de exemplares maiores, capturando outros decápodes e peixes. Já os pré-adultos e adultos de *C. sapidus* adotam uma estratégia alimentar tipicamente oportunista-generalista, caracterizada pelo amplo espectro trófico (Bemvenuti, 1998; Kapusta & Benvenuti, 1998). A reconhecida plasticidade da dieta de invertebrados de grande mobilidade, como *C. sapidus*, permite que esta espécie atue em mais de um nível trófico.

Na região estuarina da Lagoa dos Patos, a ação do vento, a precipitação pluvial e o longo e estreito canal de desembocadura determinam uma intensa hidrodinâmica, instabilidade do substrato que, na maioria das vezes, não permitem a formação de gradientes estáveis de salinidade. Assim, o grau de amplitude, as bruscas flutuações e a baixa

previsibilidade das variações de salinidade, devem representar um considerável fator de perturbação para a macrofauna bentônica. Mesmo nas enseadas, onde estes efeitos são tamponados pelo maior tempo de residência da água, a salinidade influencia na estruturação das comunidades faunísticas (Bemvenuti, 1987; 1998). Assim, a capacidade de suportar as freqüentes mudanças de salinidade é essencial para o estabelecimento das populações de *C. sapidus* neste habitat, sendo este considerado um osmoconformador em altas salinidades e hiperosmorregulador em baixas salinidades (Mangum *et al*., 1985). Além disso, o ciclo reprodutivo de *C. sapidus* está relacionado à ampla faixa de variação de salinidade dos estuários que estes animais habitam (Williams, 1974; Capitoli *et al*., 1978). Após o acasalamento no estuário, as fêmeas se dirigem durante a maré baixa para a desembocadura do estuário e costa adjacente do oceano, onde desovam até 4 milhões de larvas por indivíduo; enquanto que os machos adultos do siri costumam distribuir-se para o interior do estuário em locais de baixa salinidade (Bemvenuti, 1998).

Visto o exposto acima, os resultados obtidos no presente trabalho certamente permitirão uma melhor compreensão dos mecanismos de transporte, acumulação e toxicidade do cobre em animais aquáticos eurialinos, bem como fornecerão subsídios para uma possível aplicação destes mecanismos como biomarcadores de exposição deste metal em ambientes estuarinos e marinhos.

1.1. HIPÓTESES

1. Exemplares de *C. sapidus* coletados em diferentes áreas do estuário da Lagoa dos Patos apresentarão distintos padrões de acumulação de cobre em células branquiais.

2. A exposição prévia ao cobre poderá induzir modificações no padrão de acumulação nas células branquiais de *C. sapidus*.

3. A salinidade atuará como fator de proteção contra a toxicidade do cobre em células branquiais do siri-azul *C. sapidus.*

4. As brânquias (via de absorção) e o hepatopâncreas (órgão de acumulação e detoxificação) serão órgãos-alvo da toxicidade do cobre em *C. sapidus*.

5. Os mecanismos de transporte de Na^+ em células isoladas de *C. sapidus* estarão envolvidos na acumulação do cobre nas brânquias posteriores, indicando que o desequilíbrio da regulação iônica seja o principal mecanismo de toxicidade do cobre no siri-azul.

6. O cobre afetará diferentemente os mecanismos de entrada e saída de Na^+ em células branquiais isoladas do siri-azul *C. sapidus* aclimatado às salinidades 30 e 2, indicando que estas respostas poderão ser utilizadas como biomarcadores de exposição ao metal.

1.2. OBJETIVOS

Objetivo Geral:

O objetivo central deste trabalho foi realizar estudos *in vitro* utilizando células isoladas das brânquias e hepatopâncreas do crustáceo eurialino *Callinectes sapidus* visando fornecer subsídios para uma melhor compreensão dos mecanismos de acumulação e toxicidade deste metal em animais aquáticos, bem como avaliar a possibilidade de aplicação destes mecanismos como biomarcadores de exposição ambiental ao cobre em regiões estuarinas e marinhas.

Objetivos Específicos

1. Determinar *in situ* e *in vitro* a influência da pré-exposição ao cobre na acumulação do metal em células branquiais isoladas do siri-azul *Callinectes sapidus* proveniente do estuário da Lagoa dos Patos. (anexo 6.1)

2. Determinar *in vitro* a influência da salinidade de aclimatação na acumulação do cobre em células branquiais isoladas do siri-azul *C. sapidus*. (anexo 6.1)

3. Determinar a cinética de transporte e acumulação do cobre em células isoladas da brânquia e hepatopâncreas do siri-azul *C. sapidus*. (anexo 6.2)

4. Avaliar a toxicidade do cobre na viabilidade de células branquiais e hepatopancreáticas isoladas do siri-azul *C. sapidus*. (anexo 6.2)

5. Determinar o grau de participação das principais moléculas (transportadores e enzimas) envolvidas no transporte iônico sobre a cinética de transporte e acumulação do cobre em células branquiais do siri-azul *C. sapidus*. (anexo 6.3)

2. MATERIAL E MÉTODOS: aspectos gerais

2.1.Avaliação do padrão de acumulação de cobre em *Callinectes sapidus* de diferentes áreas do estuário da Lagoa dos Patos

2.1.1. Metodologia de campo

Exemplares juvenis de *Callinectes sapidus* (Decapoda: Portunidae) foram coletados durante a primavera de 2007 em diferentes áreas do estuário da Lagoa dos Patos (Fig. 1). Os siris foram coletados em área considerada não contaminada (Ilha dos Marinheiros – ponto 1), bem como em áreas próximas à fontes de contaminação: Saco do Rio Grande (entre Ilha das Pombas e Yatch Clube – ponto 2), Porto Velho (região industrial – ponto 3), e Canal de Acesso (próximo ao Super Porto – ponto 4). Os pontos de amostragem foram selecionados considerando-se as indicações de estudos prévios quanto aos seus níveis de impacto antropogênico e de contaminação (Baumgarten & Niencheski, 1998; e Projeto RECOS, 2004). No mínimo, 20 exemplares de siris foram coletados em cada ponto amostral, através

de arrastos de fundo de 5 minutos de duração. Os siris capturados foram mantidos em tanques com água do local devidamente aerada, até o transporte dos animais para o Departamento de Ciências Fisiológicas (DCF) da Fundação Universidade Federal do Rio Grande (FURG). Em todas as coletas realizadas foram determinados parâmetros ambientais, como temperatura e salinidade.

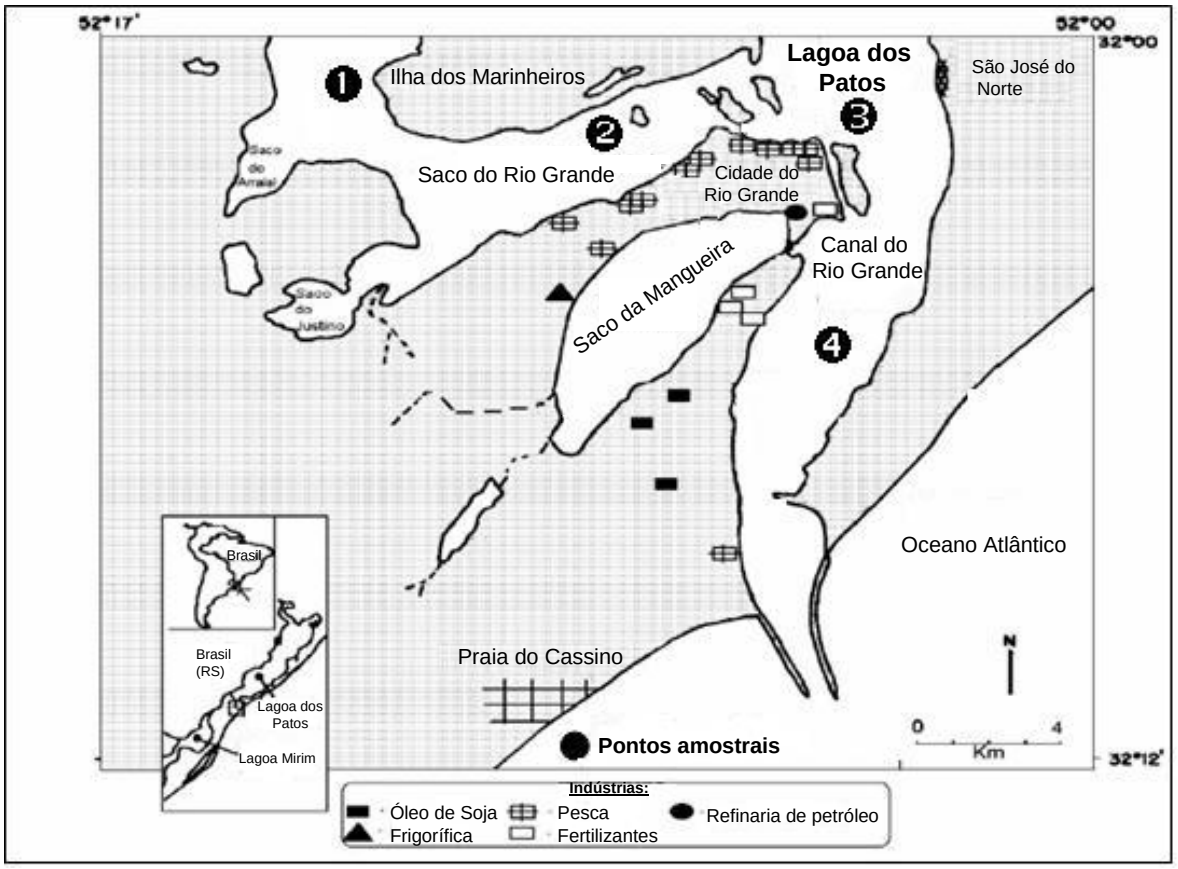

Figura 1: Mapa da porção sul do estuário da Lagoa dos Patos mostrando a localização dos pontos de coleta (Modificado de Niencheski &Baumgarten, 2000).

2.1.2. Determinação *in vitro* da acumulação de cobre

Imediatamente após a chegada dos siris ao laboratório, foram realizados os procedimentos para a obtenção da cultura primária das células branquiais. Os siris das diferentes áreas foram divididos em grupos de, pelo menos, cinco animais para a realização de distintas dissociações celulares (n = 4). A concentração de cobre nas células branquiais foi determinada por espectrofotometria de absorção atômica (AAS 932 Plus - GBC, IL, USA).

Um segundo experimento foi realizado com o objetivo de verificar o padrão de acumulação de cobre nas células branquiais pré-expostas ao metal proveniente de siris de áreas com distintos históricos de contaminação. Para tal, células foram expostas a 100 μM de cobre e a concentração de cobre intracelular foi determinada após 1 h de exposição ao metal. A concentração celular de cobre foi determinada após lavagem das células em solução salina (sem cobre) e digestão do precipitado contendo as células com HNO_3 (Merck Suprapur). A determinação da concentração de cobre nas amostras digeridas foi realizada em espectrofotômetro de absorção atômica (AAS 932 Plus - GBC, IL, USA). A acumulação do cobre nas células foi expressa em ng Cu/10^5 células. Os procedimentos para a obtenção das células estão descritas a seguir.

2.2. Influência da salinidade e do choque osmótico na acumulação de cobre em células branquiais de *Callinectes sapidus*

2.2.1. Coleta e manutenção dos animais experimentais

Com base nos resultados obtidos nos experimentos de acumulação, exemplares de *C. sapidus* (Fig. 2) foram coletados na Ilha dos Marinheiros para todos os demais experimentos. Os animais foram transferidos para o DCF-FURG, onde foram aclimatados por, no mínimo, 15 dias à salinidade experimental (2 ou 30), em temperatura constante (20ºC) e fotoperíodo fixo (12L:12E). A aclimatação foi realizada em tanques equipados com filtro biológico e aeração constante. Os animais foram alimentados três vezes por semana com filé de peixe congelado. Após a aclimatação, os animais foram crioanestesiados e os tecidos dissecados (Fig. 3).

Figura 2: Exemplar adulto de *Callinectes sapidus*.

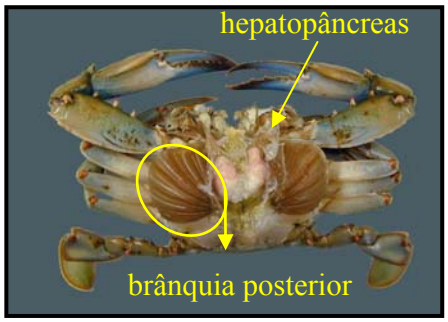

Figura 3: Foto de *Callinectes sapidus* indicando os órgãos utilizados no presente estudo.

2.2.2. Salinidade de aclimatação

Experimentos foram realizados com células branquiais isoladas. Para tanto, cada preparação celular (n = 4) foi dividida em três alíquotas. A fim de verificar a influência das técnicas utilizadas nos resultados, duas alíquotas foram separadas e expostas por 1 h a 100 µM de cobre ($CuCl_2$) em solução isosmótica. Após a exposição, a concentração de cobre nas células de cada alíquota foi verificada através da espectrofotometria de absorção atômica (descrita anteriormente) e da técnica de fluorescência. Esta técnica utiliza um marcador fluorescente sensível ao cobre (Phen Green) realizada de acordo com a metodologia descrita por Chavez-Croocker *et al.* (2001).

Para comparar os resultados obtidos com as duas técnicas, AAS e fluorescência, a acumulação de "novo cobre" foi calculada para células branquiais de cada tratamento (animais aclimatados a salinidade 2 ou 30). Este valor foi obtido subtraindo a média da concentração de cobre presente nas células branquiais antes da exposição ao cobre realizada *in vitro* por AAS do valor obtido após a exposição ao metal. O cálculo do "novo cobre" acumulado foi necessário, pois a medida de fluorescência pelo Phen Green considera o "quenching" de fluorescência induzido apenas pelo "novo cobre" acumulado nas células branquiais.

2.2.3. Choque osmótico

Para verificar a influência do choque osmótico na acumulação de cobre intracelular, células branquiais da terceira alíquota também foram expostas a 100 μM de cobre por 1 h. Porém neste experimento, foram utilizadas soluções salina hiper-osmótica (para brânquias de animais aclimatados a salinidade 2) ou hipo-osmótica (para brânquias de animais aclimatados a salinidade 30). A concentração de cobre intracelular foi determinada pela técnica da fluorescência. O sinal de fluorescência (FS) foi verificado através de um espectrofluorímetro (Victor^2 TM 1420, Perkin-Elmer, USA) em condições controle (FS_0) e após 1 h de exposição ao cobre (FS_1). O "quenching" de fluorescência (ΔF) induzido pelo cobre foi calculado com base nos valores de FS_0 e FS_1. Os resultados foram expressos como ΔF /10^5 células.

2.3. Preparação e cultura primária das células branquiais e hepatopancreáticas

Os procedimentos para a obtenção da cultura primária das células branquiais (Fig. 4) foram realizados de acordo com a metodologia descrita por Kelly *et al*. (2000), com modificações. Já a obtenção das células do hepatopâncreas (Fig. 5), foi realizada seguindo a metodologia desenvolvida por Faucet *et al*. (2003), também com modificações. Para cada dissociação celular realizada foram utilizadas brânquias posteriores e hepatopâncreas de, no mínimo, dois siris. É importante salientar que todas as soluções utilizadas para a obtenção da cultura primária, bem como o meio de cultura, tiveram suas osmolalidades ajustadas à salinidade experimental (2 ou 30). O monitoramento das culturas celulares foi realizado por meio da visualização em microscópio invertido (XDS-IB.BIOVAL).

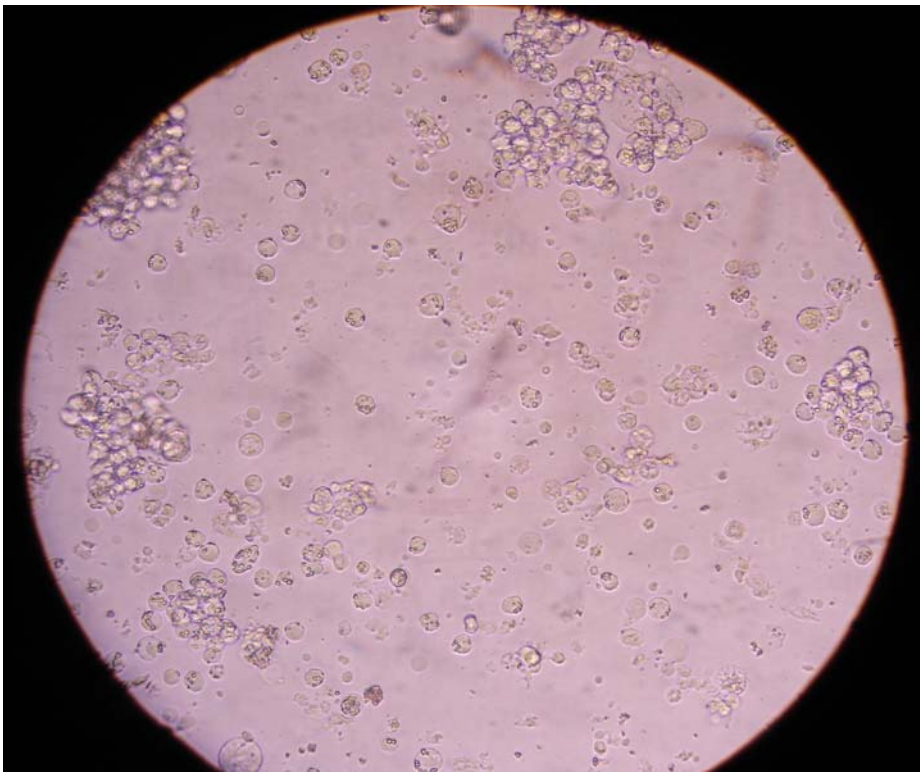

Figura 4: Cultura primária de células de brânquias posteriores de *Callinectes sapidus* (400x)

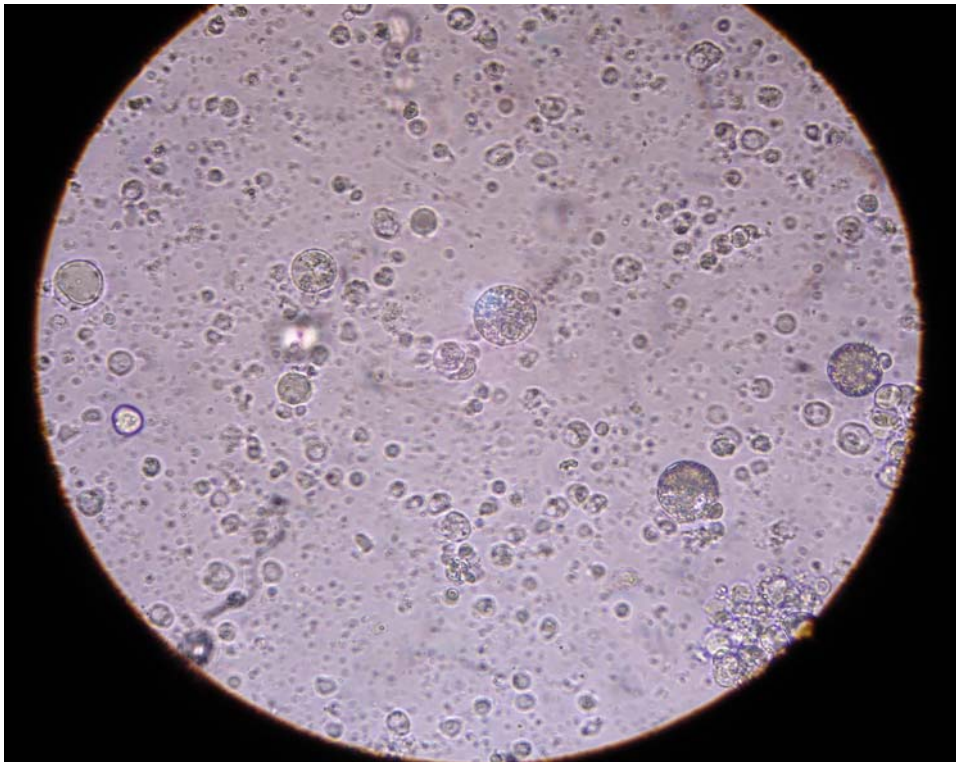

Figura 5: Cultura primária de células de hepatopâncreas de *Callinectes sapidus* (400x)

2.4. Determinação da toxicidade e acumulação de cobre

Experimentos foram realizados para determinar a toxicidade e a acumulação de cobre nas células branquiais e do hepatopâncreas em cultivo. Para tal, as células foram mantidas sob condições controle (sem adição do cobre) ou expostas a diferentes concentrações de cobre (1, 10 e 100 μM Cu). A viabilidade celular e a concentração de cobre nas células foram medidas em diferentes tempos experimentais (0, 1, 3 e 6 h). A viabilidade celular foi determinada pelo método de exclusão do Azul de Trypan, sendo que a contagem das células viáveis foi realizada em alíquotas do meio de cultivo após o tempo experimental desejado, utilizando-se câmara de Neubawer. A viabilidade celular foi expressa em percentagem, considerando o número de células presentes no início e no final do experimento. Por sua vez, a concentração celular de cobre foi determinada após lavagem das células em solução salina (sem cobre) e digestão do precipitado contendo as células com HNO_3 (Merck Suprapur). A determinação da concentração de cobre nas amostras digeridas foi realizada em espectrofotômetro de absorção atômica (AAS 932 Plus - GBC, IL, USA). A acumulação do cobre nas células foi expressa em ng Cu/10^5 células e avaliada comparando-se os diferentes tratamentos (concentrações de cobre e tempos experimentais).

Com base nos resultados obtidos nos experimentos descritos acima, foi escolhida uma concentração de cobre (100 μM) e um tempo (1 h) de exposição onde a viabilidade das células foi em torno de 50% e a acumulação de cobre alcançado um nível suficientemente elevado para permitir a detecção de possíveis variações causadas pela adição de inibidores do transporte iônico celular, para que fosse possível então identificar os mecanismos de transporte iônico celular envolvidos no transporte e acumulação do cobre nas células branquiais do siri azul, conforme será descrito a seguir.

2.5. Análise da participação dos mecanismos de transporte iônico na acumulação do cobre

A análise da participação das principais moléculas (transportadores ou enzimas) envolvidas no transporte de Na^+ e acumulação do cobre nas células branquiais de siri-azul *C. sapidus* foi realizada através de técnica de fluorescência, já descrita anteriormente. Esta metodologia além de ser muito sensível, permite quantificar a presença do cobre em células intactas, onde todos os mecanismos estão trabalhando em conjunto para internalizar o metal na célula. Neste caso, evita-se também a contaminação das leituras pelo cobre que possivelmente fique apenas adsorvido às membranas celulares.

As culturas primárias de siris aclimatados a salinidade 30 (780 mOsmoles.l^{-1}) foram pré-equilibradas por 1 h com o marcador fluorescente (1,5 μM) em meio de cultivo sem cobre. Posteriormente, as células foram lavadas e o precipitado ressuspendido em solução salina sem o marcador. Após este período de pré-equilíbrio das células com o marcador fluorescente, as células foram adicionadas no meio de cultivo contendo 100 μM de cobre, concentração escolhida conforme descrito anteriormente. As alterações na fluorescência ao longo do tempo de exposição (1 h), também escolhido conforme descrito acima, foram então quantificadas por espectrofluorimetria (Victor^2 TM 1420 - Perkin-Elmer).

Para avaliar a possível participação de uma determinada molécula (transportador ou enzima) envolvida no transporte de Na^+ sobre o transporte e acumulação do cobre nas células branquiais, os procedimentos descritos acima foram repetidos, porém associados com o uso de diferentes ferramentas farmacológicas. Os mecanismos de transporte iônico analisados foram: canais de Na^+, Cl^- e K^+, os transportadores Na^+/H^+, Na^+/Ca^{2+},e Cl^-/HCO_3^-, e os co-transportadores Na^+-K^+-$2Cl^-$ e Na^+-Cl^- (Fig. 5). Para tal, os seguintes inibidores/bloqueadores foram utilizados: fenamil (1 μM), 5-nitro 2- (3-phenilpropilamino) benzoato (NPPB, 100 μM), sulfato de quinina (500 μM), 5-etil-*N*-isopropil-amilorida (EIPA, 500 μM), amilorida

(1 mM), SITS (500 μM), furosemida (500 μM), and benztiazida (100 μM), respectivamente. A implicação das enzimas Na^+/K^+-ATPase, H^+-ATPase e anidrase carbônica intracelular na acumulação de cobre nas células branquiais foi analisada através da inibição da atividade destas enzimas utilizando ouabaína (100 μM), bafilomicina A_1 (1 μM), e acetazolamida (1 mM), respectivamente.

O grau de participação de cada molécula (transportador ou enzima) envolvida no transporte iônico sobre a acumulação do cobre nas células branquiais foi expresso em percentagem de "quenching" de fluorescência, considerando-se os dados obtidos na ausência e na presença do bloqueador/inibidor da molécula em questão.

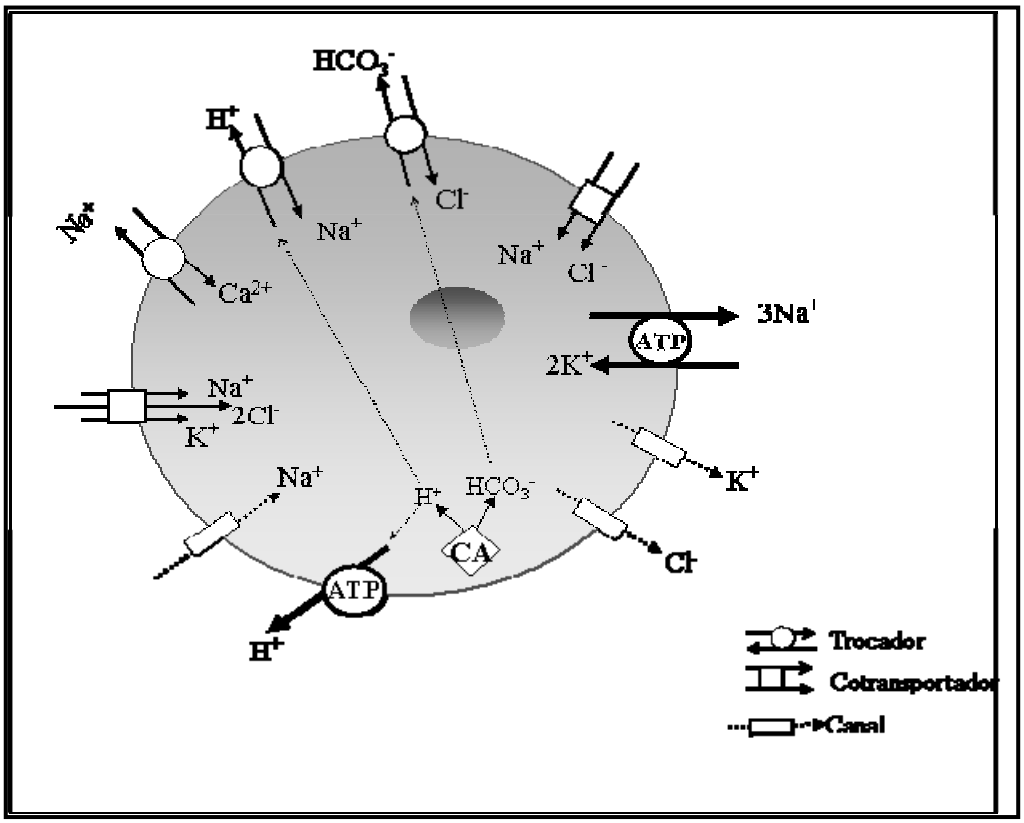

Figura 5: Ilustração esquemática dos mecanismos de transporte iônico investigados em célula branquial isolada de *Callinectes sapidus*

2.6. Análise estatística

Os dados foram expressos como média ± erro padrão. As diferenças entre os valores médios foram determinadas por análise de variância (ANOVA), seguido do teste *a posteriori* de Tukey (α=0,05). Os pressupostos da ANOVA (normalidade dos dados e homogeneidade de variâncias) foram previamente verificados. A relação entre a concentração do metal nas culturas celulares dos tecidos e a fluorescência, bem como as análises de cinética de transporte e acumulação foram realizadas através de análises de regressão linear e não linear (α=0,05).

3. SÍNTESE DOS RESULTADOS

3.1. Acumulação de cobre em células branquiais de *Callinectes sapidus* de diferentes áreas do estuário da Lagoa dos Patos (Anexo 6.1)

3.1.1. Características químicas da água

Os resultados referentes aos parâmetros químicos da água nos pontos de coleta dos exemplares de *C. sapidus* estão apresentados na tabela 1.

3.1.2. Concentração de cobre

Os resultados obtidos para a concentração de cobre total na água e nas células branquiais de *C. sapidus* coletados em diferentes pontos do estuário da Lagoa dos Patos estão listados na tabela 2.

Tabela 1: Características químicas da água dos pontos de coleta. Resultados expressos como média ± erro padrão.

Parâmetros	Período da coleta	Período de 6 meses que antecedeu à coleta
Temperatura (ºC)	24,2 ± 0,3	20,1 ± 0,7
Salinidade (ppt)	1,8 ± 0,5	2,7 ± 0,5

Tabela 2: Concentração de cobre total na água (μg Cu l^{-1}) dos diferentes pontos de coleta na porção sul do estuário da Lagoa dos Patos e o conteúdo total de cobre em células branquiais (ng Cu.10^5células^{-1}) de *C. sapidus in situ* e *in vitro* (após exposição a 100 μM de cobre por 1 h). Resultados expressos como média ± erro padrão. Letras diferentes representam médias significativamente diferentes entre si ($p < 0,05$).

Concentração de cobre	Ponto 1	Ponto 2	Ponto 3	Ponto 4
Água	14,7 ± 1,3 [a]	29,2 ± 4,0 [b]	11,2 ± 3,5 [a]	12,0 ± 4,0 [a]
Células - *in situ*	19,8 ± 0,9 [a]	32,6 ± 4,7 [b]	27,0 ± 3,1 [ab]	26,7 ± 2,6 [ab]
Células - *in vitro*	103,3 ± 37,5 [a]	343,9 ± 6,6 [b]	131,5 ± 35,6 [a]	99,8 ± 13,2 [a]

3.1.3. Efeito da salinidade de aclimatação e do choque osmótico na acumulação *in vitro*

Após 1 h de exposição ao cobre (100 μM) houve um aumento significativo do "novo cobre" acumulado nas células branquiais dos siris coletados no ponto 1 (Ilha dos Marinheiros) e aclimatados nas salinidades experimentais (2 ou 30). Este aumento foi significativamente maior (1,9 vezes) nos animais aclimatados à salinidade 2.

Nenhum efeito significativo de um choque osmótico agudo na acumulação *in vitro* de "novo cobre" após 1 h de exposição a 100 μM de cobre foi observado. Esta ausência de efeito foi verificada tanto em células de siris aclimatados à salinidade 2 e expostos ao choque hiperosmótico, quanto nos siris aclimatados à salinidade 30 e expostos ao cobre em situação de choque hiposmótico.

3.2. Acumulação e toxicidade do cobre em células de *Callinectes sapidus* (Anexo 6.2)

3.2.1. Acumulação de cobre

A acumulação de cobre foi determinada em células isoladas branquiais e hepatopancreáticas expostas a diferentes concentrações de cobre (1, 10 e 100 μM) por 1, 3 e 6 h. A concentração de cobre intracelular de ambos os tipos celulares foi significativamente maior que o controle (tempo zero), somente na concentração de 100 μM de cobre. Entretanto, apesar do significativo aumento da concentração de cobre nos diferentes tipos celulares durante 6 h, a cinética de acumulação foi completamente diferente. As células branquiais apresentaram um aumento linear da concentração do cobre ao longo do tempo enquanto as células do hepatopâncreas saturaram rapidamente (1 h) com a exposição ao cobre, mantendo os níveis acumulados do metal por até 6 h. Tanto as células branquiais quanto as hepatopancreáticas mostraram uma acumulação linear em função da concentração de cobre no meio de incubação.

3.2.2. Toxicidade do cobre

Quanto à toxicidade, a exposição ao cobre alterou a viabilidade das células branquiais somente nas maiores concentrações testadas (10 e 100 μM Cu). Em 10 μM Cu, foi observado um decréscimo na viabilidade celular de ~20% após 1 e 3 de exposição ao cobre, e de 30% após 6 h. Após 1 e 3 h de exposição a 100 μM Cu, um decréscimo de ~30% foi observado na viabilidade das células, enquanto uma redução de ~50% foi verificado depois de 6 h de exposição. Em relação às células hepatopancreáticas, uma mortalidade significativa de ~15% foi observada nas células expostas a 100 μM Cu, independente do tempo de exposição.

3.3. Participação dos mecanismos de transporte iônico na acumulação do cobre em células branquiais de *Callinectes sapidus* (Anexo 6.3)

A inibição dos transportadores de Na^+ e Cl^- ou da atividade das enzimas associadas induziram alterações significativas no "quenching" de fluorescência, indicando que o cobre

compete com o Na^+ para entrar ou sair da célula branquial. Os resultados obtidos para o acúmulo de cobre intracelular relacionados com os mecanismos transportadores estão listados na tabela 3.

Tabela 3: Percentagem do "quenching" de fluorescência (ΔF, media ± erro-padrão) em células isoladas de brânquias de *Callinectes sapidus* na presença de 100 μM de cobre junto com diferentes drogas. (-) menor acúmulo de cobre intracelular; (+) maior acúmulo de cobre intracelular.

Mecanismo de transporte	Inibidor/Bloqueador	Δ F (%)
Canal de Na^+	Fenamil	(-) 47 ± 4
Canal de K^+	Sulfato de Quinina	(-) 113 ± 8
Canal de Cl^-	NPPB	(+) 85 ± 1
Na^+ / Ca^{2+}	Amilorida	(+) 72 ± 6
Na^+ / H^+	EIPA	(-) 62 ± 23
Cl^- / HCO_3^-	SITS	(-) 88 ± 3
Na^+- K^+- $2Cl^-$	Furosamida	(-) 44 ± 2
Na^+- Cl^-	Benztiazida	(-) 82 ± 1
Na^+/ K^+- ATPase	Ouabaína	(+) 54 ± 2
H^+- ATPase	Bafilomicina A_1	(-) 158± 8
Anidrase Carbônica	Acetazolamida	(-) 80 ± 3

4. CONCLUSÕES

a) Células branquiais de *Callinectes sapidus* acumulam cobre de acordo com o nível do metal presente no meio.

b) As diferenças observadas no padrão de acumulação de cobre nas células branquiais de *C. sapidus* aclimatados à mesma salinidade em condições natural e experimental estão relacionadas com as respostas celulares desencadeadas devido à pré-exposição *in situ* ao metal.

c) A acumulação *in vitro* de cobre nas células branquiais de *C. sapidus* depende da salinidade de aclimatação, estando associada às mudanças bioquímicas e fisiológicas que ocorrem nas brânquias a longo-prazo durante o processo de aclimatação à salinidade.

d) Células branquiais de *C. sapidus* acumulam mais cobre e são mais sensíveis ao metal do que as células hepatopancreáticas.

e) A cinética de acumulação do cobre em função do tempo de exposição é diferente em células branquiais e hepatopancreáticas, provavelmente devido às funções desempenhadas por cada tipo celular.

f) Ambos os tipos celulares apresentaram uma cinética linear de acumulação de cobre em função da concentração do metal no meio de incubação, indicando que ambos tipos celulares podem ser utilizados para monitorar *in vitro* à contaminação ambiental pelo cobre.

g) A relação observada entre acumulação de cobre e a viabilidade das células branquiais indica que estas células são órgãos-alvo da toxicidade do cobre e podem servir como modelo biológico no desenvolvimento de uma versão *in vitro* do BLM para condições marinhas e estuarinas.

h) O bloqueio dos transportadores de Na^+ e Cl^- ou a inibição da atividade das enzimas a eles associadas caracteriza a competição entre cobre e o Na^+ pelos mesmos sítios de ligação em ambos os lados da membrana da célula branquial. Esta situação indica que a toxicidade

do cobre em células branquiais de *C. sapidus* pode estar relacionada ao desequilíbrio iônico e osmótico, com um possível impacto sobre a regulação osmótica e iônica intracelular.

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6. ANEXOS

6.1. Copper accumulation in gill cells of the blue crab *Callinectes sapidus*: influence of copper pre-exposure, acclimation salinity and acute osmotic shock

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Trabalho a ser enviado para: Comparative Biochemistry and Physiology – part C: Toxicology & Pharmacology

COPPER ACCUMULATION IN GILL CELLS OF THE BLUE CRAB *CALLINECTES SAPIDUS*: INFLUENCE OF COPPER PRE-EXPOSURE, ACCLIMATION SALINITY AND ACUTE OSMOTIC SHOCK

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Abstract

Copper accumulation in gills seems to control the toxicity of this metal for aquatic animals, since it is considered as an osmoregulatory toxicant. In the present study, *in vivo* and *in vitro* copper accumulation was measured in cells isolated from posterior gills of the blue crab *Callinectes sapidus* collected at four different sites of the Patos Lagoon estuary (Rio Grande, RS, Southern Brazil). Results obtained showed that *in situ* copper accumulation in gill cells of freshly collected crabs was different between sites of collection, being positively dependent on the water copper concentration. *In vitro* copper accumulation after exposure (1 h) to copper (100 μM) was higher in gill cells of crabs collected at the most polluted site when compared to those from crabs collected at the other sites. In gill cells of crabs collected at site 1, which showed the lowest level of copper burden, *in vitro* copper accumulation was dependent on the acclimation salinity. It was higher in gill cells isolated from crabs acclimated to low salinity (2 ppt) than to seawater (30 ppt). However, it was not significantly affected by cell exposure to an acute osmotic shock. Taken together, these findings indicate that cells isolated from the posterior gills of the blue crab *C. sapidus* are good indicators of the copper level in the surrounding medium. Furthermore, they show that copper accumulation in gill cells is influenced by the level of copper pre-exposure in the field and the acclimation salinity in the laboratory. The influence of this last parameter is likely associated with the long-term biochemical and physiological changes occurring in gills during the processes of crab acclimation to salinity.

Keywords: *Callinectes sapidus*, copper accumulation, gill cells, osmotic shock, metal contamination, salinity

1. Introduction

Pollution in estuaries is considered a critical environmental issue because of the high variation in several abiotic factors that impose severe restrictions to species living in these areas (Mathiensen and Law, 2002). Furthermore, estuarine environments are characterized by intense and frequent variations of water physico-chemical parameters, such as salinity, which can alter the biovailability and toxicity of metals (Witters, 1998). Generally, decreases in salinity are associated with higher metal bioavailability and toxicity to euryhaline animals (Blanchard and Grosell, 2006; Pedroso et al., 2007a; Pinho et al., 2007; Bianchini et al., 2008). In addition to the salinity effect on copper speciation and availability, changes in salinity can also affect the physiology of euryhaline animals through the energy demands for ionic and osmotic regulation (Grosell et al., 2007; Pedroso et al., 2007b; Pinho et al., 2007).

The Patos Lagoon (10360 km^2) is located in Southern Brazil and is part of the largest lagoon system in South America. Like many coastal lagoons, it receives untreated domestic, agricultural and industrial wastes associated with the populations living in several cities along its border. Local inputs from urban, industrial and harbor activities, especially those from the Rio Grande City, have produced an estuarine enrichment with many metals, including copper (Niencheski et al., 2006). In turn, the estuarine biota from the Patos Lagoon has shown to be sensitive to metals exposure (Bianchini et al., 2007; Pedroso et al., 2007a; Pinho et al., 2007). Either individually or in combination with other aquatic contaminants, metals can induce subletal effects at cellular level, causing injury in estuarine animals (Monserrat et al., 2007).

In coping with the new approach of regulatory tests for risk assessment, cultured cells have being applied as an important tool in mechanistic and toxic identification studies. Both fish and invertebrate cells are being considered as the key level of organization to evaluate

toxicity of several aquatic contaminants (Le Bihan et al., 2004; Schirmer, 2006; Zhou et al., 2006). In this context, isolated cells from fish and invertebrates have been used as biomonitors to assess the biological effects of metals, including copper (Le Bihan et al., 2004; Schirmer, 2006). However, studies with cultured cells from estuarine invertebrates are not available.

Gill epithelium is among the first organs to be in contact with waterborne contaminants. In turn, gills present a relatively large permeable surface for the exchange of these contaminants. In fact, waterborne copper exposure results in metal uptake and the binding of copper ions to molecular sites within the organism (Rainbow, 1995; Wright, 1995). In turn, copper accumulation in gills seems to control the toxicity of this metal, since it is considered as an osmoregulatory toxicant in aquatic animals, including estuarine species (Grosell et al., 2002; Pinho et al., 2007).

In the present study, the influence of copper pre-exposure and acclimation salinity on copper accumulation was investigated *in vitro* using isolated cells from the posterior gills of the blue crab *Callinectes sapidus* from the Patos Lagoon estuary. Posterior gills were selected considering their role in ionic and osmotic regulation in estuarine crabs (for review: Freire et al., 2007; Bianchini et al., 2007) and the fact that copper is considered as an iono- and osmoregulatory toxicant in aquatic invertebrates (Grosell et al., 2002; Pinho et al., 2007).

2. Material and methods

2.1. Collection sites

Juveniles of the blue crab *Callinectes sapidus* were collected in four different sites along the Southern portion of the Patos Lagoon estuary in November 2007 (Fig. 1). Site 1 is located at the Marinheiros Island and considered as a relatively clean area. Sites 2, 3 and 4

are located along the urban and industrial areas of Rio Grande City. Likely sources for metal contamination associated with the industrial complex include fertilizer and refinery plants. Additional information concerning the Patos Lagoon and collection sites selected for this study is available from other studies (Baumgarten and Niencheski, 1998; Niencheski and Baumgarten, 2000; Niencheski et al., 2006).

Water salinity, temperature and total copper concentration at the different collection sites were measured at the moment of crab collection and along the six months preceding it. Total copper concentration was measured in samples (10 ml) of surface water acidified with 1% HNO_3 (Suprapur, Merck, St. Louis, MO, USA), using atomic absorption spectrophotometry (AAS; GBC, AAS 932 Avanta Plus, IL, USA).

2.2. In situ and in vitro copper accumulation in gill cells

Twenty crabs were collected from each sampling site and immediately used to evaluate the *in situ* copper accumulation in gill cells and the influence of this pre-contamination on the *in vitro* copper accumulation. Immediately after collection, crabs were crioanesthetized, killed by removal of the exoskeleton, and had their posterior gills dissected. Isolated gill cells from crabs of each sampling site were obtained and had their viability checked, as described below. Cells obtained were divided into two aliquots. One aliquot was immediately used to measure *in situ* cell copper concentration. The other aliquot was exposed to copper (100 μM Cu as $CuCl_2$) for 1 h. Cell copper burden in gill cells non-exposed and exposed to copper was then measured by AAS. Procedures for gill cell isolation, copper exposure, and copper measurement are described below.

2.3. Influence of salinity and acute osmotic shock on in vitro copper accumulation

Considering that gill cells from crabs collected at site 1 showed the lower levels of copper contamination (see Results section), forty crabs were collected at this site and randomly divided into two groups (n = 20 for each group). Each group was acclimated to one of the experimental salinity (2 or 30 ppt) to evaluate the influence of acclimation salinity on copper accumulation in isolated gill cells. Crabs were acclimated to the experimental salinity for at least 15 days. During the acclimation period, they were fed three times a week with fish slices. Temperature and photoperiod were fixed at 20^{o}C and 12L:12D, respectively.

After acclimation, crabs were crioanesthetized, killed by removal of the exoskeleton, and had their posterior gills dissected. Gill cells were then isolated and had their viability checked. Each gill cell preparation was divided into three aliquots. To verify the possible influence of the measurement technique on results obtained, the first and second aliquots were exposed (1 h) to copper (100 μM Cu as $CuCl_2$) using an isosmotic saline solution. After copper exposure, cell copper burden in gill cells from the first and second aliquot was measured by AAS and a fluorescent technique, respectively. To verify the influence of an acute osmotic shock on the *in vitro* copper accumulation, gill cells from the third aliquot were exposed (1 h) to copper (100 μM Cu as $CuCl_2$) using hyperosmotic (gills from crabs acclimated to 2 ppt) or hyposmotic (gills from crabs acclimated to 30 ppt) saline solutions. Copper measurements in these cells were performed using the fluorescent technique. Procedures for gill cell isolation, copper exposure, and copper measurements are described below.

2.4. Gill cell isolation

General procedures to obtain isolated cells from posterior gill were performed according to the method described by Kelly et al. (2000), with modifications. Briefly, posterior gills of freshly collected crabs (salinity 1.8 ppt; see Results section) and of those

acclimated to low salinity (salinity 2 ppt) were excised and washed in a sterile Ca-free phosphate buffer solution (PBS: 250 mM NaCl, 20 mM Na_2HPO_4, 1.3 mM K_2HPO_4, and 12 mM KCl; pH 7.6) having an osmolality (580 mOsmoles kg^{-1}) similar to that of blue crabs acclimated to salinity 2 ppt (Henry, 2001). For crabs acclimated to seawater (salinity 30 ppt), posterior gills were excised and washed in a PBS (340 mM NaCl, 20 mM Na_2HPO_4, 1.7 mM K_2HPO_4, and 16 mM KCl; pH 7.6) having an osmolality (780 mosmol kg^{-1}) similar to that of blue crabs acclimated to salinity 30 ppt (Henry, 2001).

Hemolymph cells were flushed out by perfusing gills with the corresponding PBS. Before cell dissociation, each gill was perfused with PBS containing 0.05% trypsin (Gibco, UK). Tissues were minced into small pieces, placed in a beaker containing 20 ml of the corresponding PBS containing 0.035% trypsin and 0.04% chitinase (Sigma-Aldrich, St. Louis, MO, USA), and incubated in a shaking water bath (30^{o}C) for 30 min. The resulting cell suspension was filtered (30 μm-mesh nylon filter) to remove non-dissociated tissue and large debris, and mixed with fresh PBS containing 10% fetal bovine serum (Gibco, UK) to stop trypsin activity. The filtrate was centrifuged (Model 204-N, Fanem, São Paulo, SP, Brazil) at 180 x *g* for 10 min. The cell pellet was resuspended in fresh PBS containing 2.5% fetal bovine serum (Gibco, UK) and centrifuged (Model 204-N, Fanem, São Paulo, SP, Brazil) at 1,200 x *g* for 2 min. The cell pellet was resuspended in an adequate saline solution. For gill cells of freshly collected crabs (salinity 1.8 ppt) and of those acclimated to low salinity (2 ppt), a "low-salinity saline" solution was prepared (580 mOsmoles kg^{-1}; 220 mM NaCl, 7 mM KCl, 9 mM $CaCl_2$, 30 mM $MgCl_2$, 2 m M $NaHCO_3$; pH 7.6). For gill cells of crabs acclimated to seawater (salinity 30 ppt), a "seawater saline solution" was prepared (780 mOsmol Kg^{-1}, 370 mM NaCl, 9 mM KCl, 9 mM $CaCl_2$, 30 mM $MgCl_2$, 2 mM $NaHCO_3$, pH 7.6).

For each experimental condition, four different cell preparations were made using a pool of posterior gills from five crabs. Total number and viability of gill cells in each preparation was determined by the Trypan Blue exclusion method (0.08% trypan blue). In all preparations, cell viability was usually >80%.

2.5. Copper exposure and copper accumulation measurements

Each cell preparation from crabs freshly collected at the four different sites was incubated (1 h) in the "low-salinity saline" solution in the absence or the presence of copper (100 µM Cu as $CuCl_2$; Merck, St. Louis, MO, USA). One aliquot (80 µl) of "low-salinity saline" solution containing gill cells ($2x10^6$ cells ml^{-1}) was transferred into one well of a 96-wells plate using a micropipette. Each well of the plate already contained 320 µl of a fresh "low-salinity saline" solution without or with copper. Copper was added 3 h prior to the beginning of the experiment to allow copper to equilibrate with the saline solution. Final copper concentration in the exposure medium (100 µM) was checked by AAS (GBC, AAS 932 Avanta Plus, IL, USA).

Each one of the four cell preparations (n = 4) was tested in five replicates for each treatment (without and with copper). After cell exposure to copper, centrifugation and washing procedures were then repeated, as described above, with the exception that EDTA (12.5 mM) was added to the "low-salinity saline" solution. After the second washing, cell samples were dried (60ºC, 24 h) and digested with HNO_3 (Suprapur, Merck, St. Louis, MO, USA). Copper concentration in gill cells non-exposed or exposed to copper was determined by AAS (GBC, AAS 932 Avanta Plus, IL, USA), after sample dilution with Milli-Q water. Copper concentration was expressed as ng Cu 10^5 $cells^{-1}$.

Gill cells from crabs collected at site 1 and acclimated to low salinity (2 ppt) or seawater (30 ppt) were isolated as described above. Each cell preparation was divided into

three aliquots. One aliquot was exposed to copper in the corresponding saline solution and had their copper concentration measured by AAS, as described above. The other two aliquots were incubated (1 h) in the corresponding saline solution containing the copper-sensitive fluorescent dye Phen Green (1.5 μM; Molecular Probes, OR, USA). Experiments were performed according to the methodology described by Chavez-Crooker et al. (2001). After loading with the dye, cells were washed with a dye-free saline solution, centrifuged, and resuspended in the saline solution to be tested. One aliquot of gill cells from crabs acclimated to low salinity (2 ppt) was resuspended and tested in the "low-salinity saline" solution, while the other was resuspended and tested in the "seawater-saline" solution (hyperosmotic shock). On the other hand, one aliquot of gill cells from crabs acclimated to seawater (30 ppt) was resuspended and tested in the "seawater-saline" solution, while the other was resuspended and tested in the "low-salinity saline" solution (hyposmotic shock).

For each cell preparation, gill cells loaded with the fluorescent dye were acutely exposed (1 h) to copper (100 μM Cu as $CuCl_2$), as described above. For each well of the microplate, 30 μl of the tested saline solution containing 10^5 cells were added to 150 μl of the same saline solution containing copper. For each cell preparation, gill cells loaded with the fluorescent dye were also maintained under control conditions (no copper addition to the saline solution).

For each well of the microplate, fluorescence signal (FS) was measured (excitation 490 nm; emission 520 nm) using a spectrofluorometer (Victor^2 TM 1420, Perkin-Elmer, USA) at the beginning (FS_0) and after 1 h of copper exposure (FS_1). Fluorescence quenching (ΔF) induced by copper was calculated based on FS_0 and FS_1 values. All experiments were performed at room temperature (20°C). Results were expressed as ΔF 10^5 cells^{-1}.

To compare results obtained with the AAS and fluorescent techniques in gill cells of crabs acclimated to low salinity (2 ppt) or seawater (30 ppt), the accumulation of "new

copper" was calculated for each treatment. This was achieved by subtracting the mean copper concentration already present in gill cells before the *in vitro* exposure to copper, measured by AAS, from that obtained after copper exposure. This was performed because of measurements with the fluorescent dye Phen Green only consider the quenching of fluorescence induced by the "new copper" accumulated into the gill cell.

2.7. Statistical analyses

All data are expressed as mean ± standard error (n = 4 different cell preparations). Differences between treatments were assessed by one way analysis of variance (ANOVA) followed by the Tukey's test. ANOVA assumptions (data normality and homogeneity of variances) were previously checked. Significance level adopted was 95% ($\alpha = 0.05$).

3. Results

3.1. Water chemistry parameters

Mean (± SE) water temperature and salinity were not significantly different between sites at the moment of crab's collection (general means: 24.2 ± 0.3^{o}C and 1.8 ± 0.5 ppt, respectively) and along the six months preceding the crab's collection (general means: 20.1 ± 0.7^{o}C and 2.7 ± 0.5 ppt, respectively).

Total copper concentration at the moment of crab's collection was not significantly different between sites (general mean: 10.8 ± 0.5 μg Cu l^{-1}). However, a significant difference between the mean values along the six months preceding the crab's collection was observed. It was significantly higher in the site 2 (29.2 ± 4.0 μg Cu l^{-1}) than in the other sites. Furthermore, no significant difference was observed between the site 1 (14.7 ± 1.3 μg Cu l^{-1}), site 3 (11.2 ± 3.5 μg Cu l^{-1}), and site 4 (12.0 ± 4.0 μg Cu l^{-1}).

3.2. Influence of collection site on in situ and in vitro copper accumulation

The mean *in situ* copper accumulation was significantly higher (1.6-fold) in gill cells of freshly blue crabs collected from site 2 than in those from crabs freshly collected at site 1. In turn, gill cells from crabs freshly collected at sites 3 and 4 showed intermediary values (Fig. 2A).

The level of *in vitro* copper uptake by isolated gill cells markedly changed according to the site of collection. In all cases, a significant copper accumulation was observed after 1 h of exposure to 100 μM copper. No significant difference was observed between cells from crabs collected at sites 1, 3, and 4. However, copper accumulation was in average 2.3-fold higher in gill cells of crabs from site 2 than in those from crabs collected at the other sites. A 10.6-fold increase in copper burden was observed in cells from crabs collected at site 2 while a 4.6-fold increase was observed in gills from crabs collected at the other sites. (Fig. 2B).

3.2. Influence of acclimation salinity on in vitro copper accumulation

After 1 h of exposure to copper (100 μM), a significant accumulation of "new copper" was observed in gill cells of crabs collected at site 1 and acclimated to either low (2 ppt) or high (30 ppt) salinity. However, it was significantly higher (1.9-fold) in crabs acclimated to low salinity (2 ppt). It is important to note that a similar difference (1.6-fold) was observed when the fluorescent technique was used to measure gill copper burden (Fig. 3).

3.3. Influence of osmotic shock on in vitro copper accumulation

No significant effect of an acute osmotic shock was observed on the *in vitro* accumulation of "new copper" after 1 h of exposure to 100 μM copper. This lack of effect was observed for isolated cells from crabs acclimated to low salinity (2 ppt) and exposed to

copper in a hyperosmotic saline solution or those acclimated to seawater (30 ppt) and exposed to copper in a hyposmotic saline solution (Fig. 4).

4. Discussion

Gill is in direct contact with waterborne toxicants, being considered as the main route of copper uptake from the dissolved phase. Consequently, it is also considered as the first target for copper toxicity (Paquin et al., 2002; Di Toro et al., 2001). Accordingly, previous studies demonstrated that this organ rapidly accumulates copper following the onset of waterborne exposure, resulting in disturbance of multiple physiological processes (Grosell et al., 2007).

Data obtained in the present study clearly indicate that gill cells of the blue crab *Callinectes sapidus* accumulate copper according to the level of this metal in the surrounding medium. In fact, a higher copper accumulation was observed in isolated gill cells of crabs freshly collected at site 2, which showed a higher long-term copper concentration in the dissolved phase. On the other hand, a lower copper accumulation was observed in isolated gills of crabs freshly collected at site 1, which showed a lower long-term copper concentration in the dissolved phase. These results are in agreement with the fact that a straight linear relationship between copper concentration in the incubation medium and in isolated gill cells was observed *in vitro* for *C. sapidus* (Paganini and Bianchini, 2008).

The lower long-term level of copper in the dissolved phase at site 1 is in agreement with the fact that this area has been considered as a relatively clean area (Baumgarten and Niencheski, 1998). On the other hand, the elevated level of copper and other metals have been reported to be associated with intense harbor activities and discharge of untreated sewage. This area is also characterized by a slow water turnover rate, resulting in high

residence time of total suspend matter. In fact, surface water and suspended sediments appears to be significantly enriched with copper (Niencheski and Baumgarten, 2000; Niencheski et al., 2006). In turn, sites 3 and 4 are located near urban and industrial areas and are also affected by harbor activities. However, they are located at semi-enclosed bays and are dominated by smaller sized particles of lower density and higher organic matter content, which are in permanent suspension (Niencheski et al., 1994). Therefore, the higher rate of water turnover and concentration of copper complexing agents (organic matter) are probably reducing copper bioavailability at sites 3 and 4. Furthermore, it has been demonstrated that sediments contaminated by local sources of copper associated with urban, industrial and harbor activities are diluted by the natural sediments transported through the main channel of the Patos Lagoon (Niencheski et al., 2006). Taken together, these findings can explain the intermediary levels of copper registered in isolated gills of blue crabs freshly collected at sites 3 and 4.

Regarding the influence of *in vivo* pre-exposure to copper on the isolated gill cell response to *in vitro* copper exposure, a markedly higher copper accumulation was observed in cells of blue crabs freshly collected at site 2 than in those of crabs freshly collected at the other sites. This finding clearly indicates that long-term exposure to higher levels of copper under field conditions had led to a differential response of gill cells to copper exposure. The higher ability of these cells to accumulate copper could be associated with the presence of a higher content of copper-binding ligands such as metallothioneins and glutathione, as observed in other aquatic invertebrates exposed for long term to copper (Amiard et al., 2006; Ross et al., 2002).

Based on the evidences presented above, it is suggested that gill cells of blue crabs *C. sapidus* are a good biomarker of exposure to environmental copper contamination. Furthermore, they indicate that studies involving experimental exposure to copper should

consider the background level of copper already present in gill cells of blue crabs. Based on this fact, only crabs collected at site 1 were used for experiments testing the influences of acclimation salinity and acute osmotic shock on the *in vitro* copper accumulation in isolated gill cells.

Regarding the influence of acclimation salinity, a markedly higher "new copper" accumulation was observed in isolated gill cells of crabs acclimated to low salinity (2 ppt) than in those of crabs acclimated to seawater (30 ppt). This result is in complete agreement with other reported in the literature for aquatic invertebrates (Bianchini et al., 2007; Monserrat et al., 2007; Pinho et al., 2007). It is important to note that similar results were observed when two different techniques were employed for copper measurements, *i.e.*, AAS and fluorescence using Phen Green as a copper-sensitive dye. Considering that the fluorescence quenching is induced only by intracellular copper, results described above indicate that the cell washing procedure used before copper determination by AAS was adequate to avoid the possible influence of the copper loosely bound onto the cell surface on copper accumulation measurements. Despite the fact that the fluorescence technique allows to measure only the accumulation of "new copper", its use can be recommended considering its practicality, low cost and effectiveness. In fact, this technique has been employed for other cell types isolated from aquatic invertebrates (Ahearn et al., 2004; Chavez-Crooker et al., 2001)

In the present study, the fluorescent technique was employed to evaluate the possible influence of an acute osmotic shock on the "new copper" accumulation in gill cells of the blue crab. Data obtained showed a lack of influence of an acute osmotic shock on the accumulation of "new copper" in isolated gill cells of crabs either acclimated to low salinity (2 ppt) and subjected to a hyperosmotic shock or acclimated to seawater (30 ppt) and subjected to a hyposmotic shock. These findings indicate that the differential copper

accumulation observed in gill cells of crabs acclimated to low salinity (2 ppt) and seawater (30 ppt) when incubated in an isosmotic saline solution is likely associated with the different adaptive mechanisms associated with the intracellular copper homeostasis, which are expressed after long-term acclimation to salinity (15 days). For example, these mechanisms can involve those related to intracellular Na^+ regulation. This suggestion is based on the following facts: (1) mechanisms of gill Na^+ uptake and extrusion markedly changes according to the acclimation salinity in crabs (Bianchini et al., 2007; Freire et al., 2007); (2) copper competes for Na^+ binding sites at the gills of fish and invertebrates (Di Toro et al., 2001, Grosell et al., 2004; Handy et al., 2002), and (3) copper is an iono- and osmoregulatory toxicant in aquatic animals (Blanchard and Grosell, 2006; Grosell et al., 2002; Grosell et al., 2007; Pinho et al., 2007).

In summary, results from the present study show that gill cells from the blue crab *C. sapidus* can be used as an interesting model to monitor the environmental exposure to copper. Furthermore, they point to the need to consider the influence of the background levels of copper exposure in the field on the cell response to experimental exposure to the metal. Finally, our data indicate that acute changes in the hemolymph osmolality are not able to induce significant changes in gill copper accumulation, but that long-term acclimation to salinity has an important influence on the *in vitro* copper accumulation in gill cells.

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Legends to figures

Figure 1. Map showing the collection sites in the Southern portion of the Patos Lagoon (Southern Brazil).

Figure 2. Copper accumulation in isolated cells from posterior gills of the blue crab *Callinectes sapidus* collected at four different sites along the Southern portion of the Patos Lagoon (Southern Brazil). (A) *In situ* copper accumulation. (B) *In vitro* copper accumulation after 1 h exposure to 100 μM copper ($CuCl_2$). Measurements were done using atomic absorption spectrophotometry. Data are expressed as mean ± SE (n = 4). Different letters represent significant different means ($p < 0.05$).

Figure 3. Copper accumulation in isolated cells from posterior gills of the blue crab *Callinectes sapidus* collected at site 1 in the Patos Lagoon (Southern Brazil), acclimated to two different salinities and exposed to 100 μM copper ($CuCl_2$) for 1 h. Copper measurements were done using two different techniques: atomic absorption spectrophotometry (ASS) and fluorescence (PG). Data are expressed as mean ± SE (n = 4). * Indicates means significantly different for the same technique ($p<0.05$).

Figure 4. Effect of an *in vitro* acute osmotic shock on copper accumulation in isolated cells from posterior gills of the blue crab *Callinectes sapidus* collected at site 1 in the Patos Lagoon (Southern Brazil) acclimated to two different salinities and exposed to 100 μM copper ($CuCl_2$) for 1 h. Copper measurements were done using fluorescence. Data are expressed as mean ± SE (n = 4). Different letters indicate means significantly different ($p<0.05$).

Figure 1

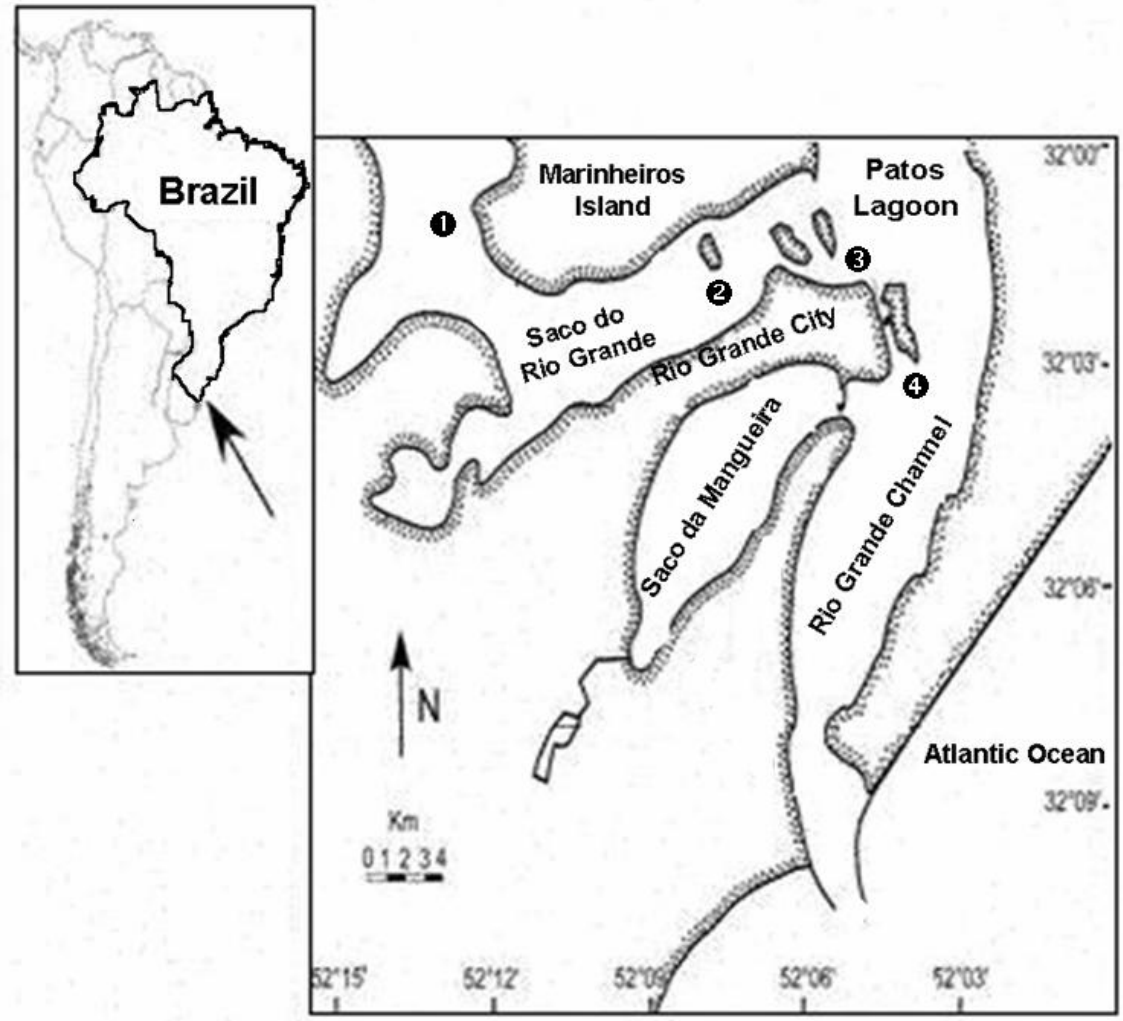
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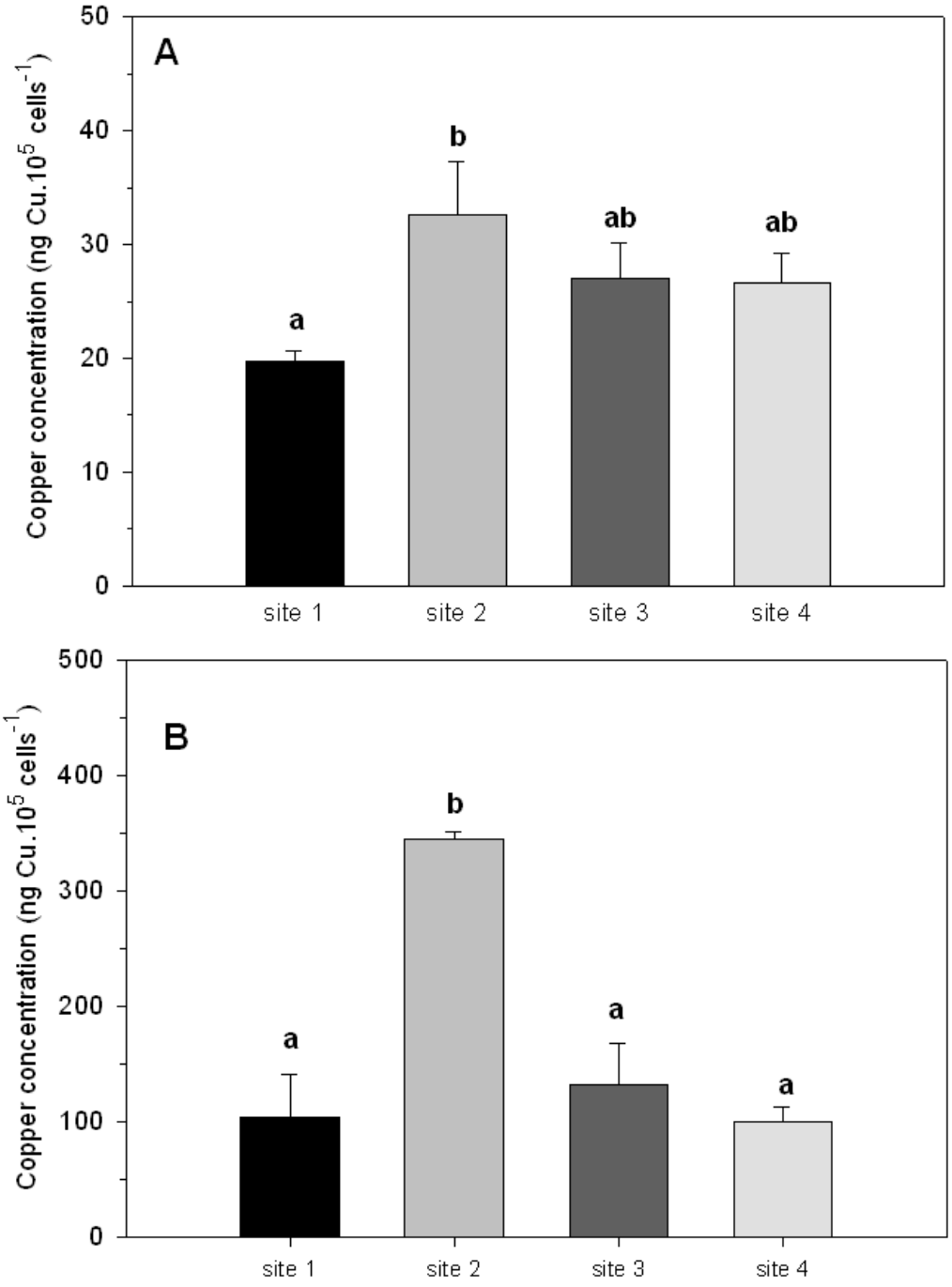
Figure 2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Copper concentration (ng Cu.10^5 cells^{-1})
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Copper concentration (ng Cu.10^5 cells^{-1})
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site 4

Figure 3

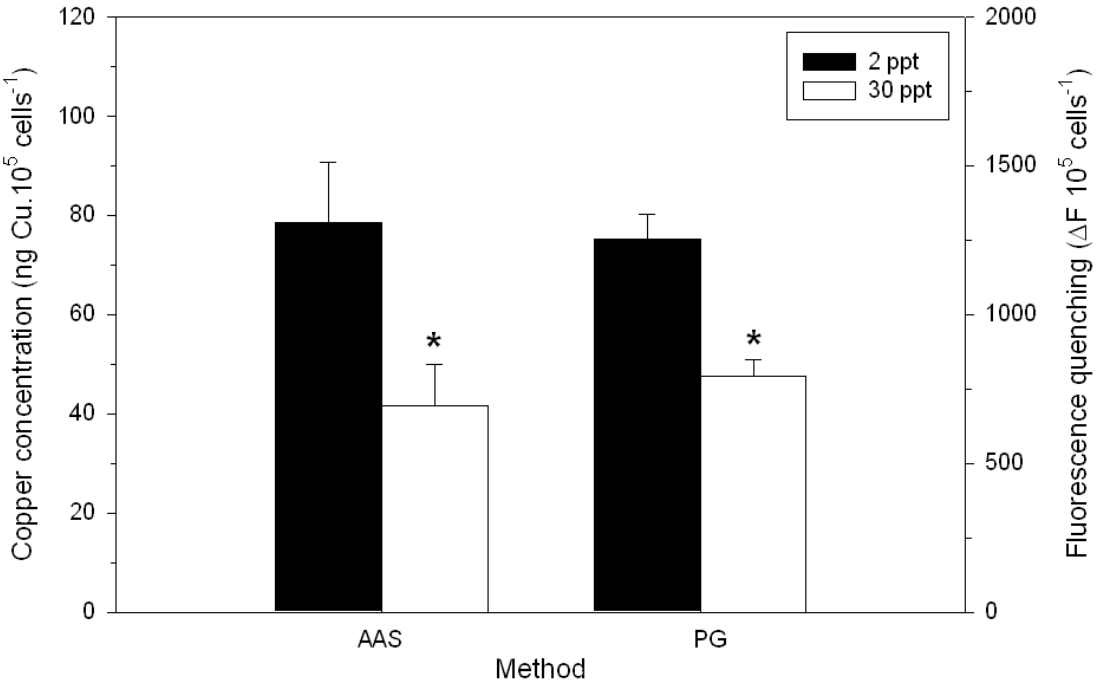
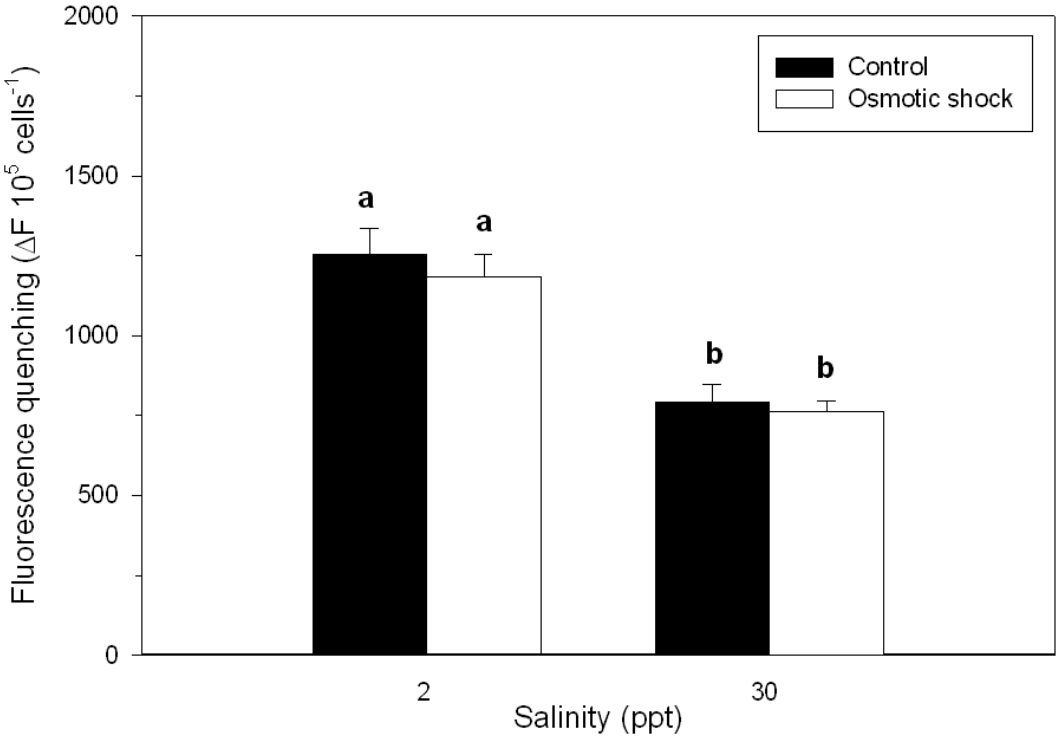

Copper concentration (ng Cu.10^5 cells^{-1})
Fluorescence quenching (ΔF 10^5 cells^{-1})
2 ppt
30 ppt
120
100
80
60
40
20
0
2000
1500
1000
500
0
*
*
AAS
PG
Method

Figure 4


2000
1500
1000
500
0
Fluorescence quenching (ΔF 10^5 cells^-1)
Control
Osmotic shock
a
a
b
b
2
30
Salinity (ppt)

6.2. Copper accumulation and toxicity in isolated gill and hepatopancreas cells of the blue crab *Callinectes sapidus*.

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Trabalho submetido para: Environmental Toxicology & Chemistry

Running Head: Copper accumulation and toxicity in isolated cells of the blue crab

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COPPER ACCUMULATION AND TOXICITY IN ISOLATED CELLS FROM GILLS AND HEPATOPANCREAS OF THE BLUE CRAB *CALLINECTES SAPIDUS*

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Abstract

In the present study, we used fresh preparations of mixed-cell populations to evaluate accumulation and toxicity of dissolved copper (1, 10 and 100 μM) in isolated cells from posterior gills and hepatopancreas of the blue crab *Callinectes sapidus*. For both gill and hepatopancreatic cells, significant increases in copper accumulation were only observed after exposure to 100 μM copper. In gill cells, a linear increase in copper accumulation was observed over time. In hepatopancreatic cells, a maximum level of copper accumulation was achieved after 1 h of exposure, remaining unchanged up to 6 h. After 6 h of exposure, copper content in gill cells was 6.6-fold higher than in hepatopancreatic cells. In both cell types, copper accumulation always followed a linear relationship with copper concentration in the incubation medium. Significant decreases in cell viability were observed after exposure to either 10 (gill cells) or 100 (gill and hepatopancreatic cells) μM copper. Furthermore, an exponential rise to maximum relationship between copper accumulation and toxicity was observed in gill cells. Altogether, these findings indicate that isolated cells from posterior gills of the blue crab *C. sapidus* fulfill the premise behind the BLM approach, *i.e.*, toxicity is driven by copper accumulation in the biotic ligand, the gill cell. Therefore, these cells can be used as a model for the development of an *in vitro* BLM version for marine conditions. However, isolated cells from the hepatopancreas could be used as a model to better understand the mechanism of copper tolerance at a cellular level in crustaceans.

Keywords: blue crab, *Callinectes sapidus*, copper accumulation, copper toxicity, isolated cells, gills, hepatopancreas.

Introduction

Copper is an essential micronutrient and acts as co-factor in multiple enzymatic processes, but it is potentially toxic to aquatic organism when in excess in the water. Actually, this metal is brought into the coastal and marine environments by human activities, resulting in elevated concentrations. This increases the exposure and potential toxicity of this metal to coastal and marine organisms, including crustaceans. These contrasting considerations of essentiality, bioavailability, and toxicity of copper are recognized as being important factors to address the consequences of this metal in aquatic ecosystems.

Previous studies have shown that copper accumulation and toxicity are highly variable depending on crustacean species and life stage examined [1-4]. The cellular events underlying these effects are not yet fully understood, but studies reported in the literature indicate adverse effects of copper on active and passive ion transports. Therefore, copper has been recognized as an osmoregulatory toxicant that induces ionoregulatory disturbances in aquatic organisms [5-11]. However, information on routes of copper accumulation and the mechanism of copper toxicity in brackish or marine invertebrates is still scarce.

Long-term copper exposure results in uptake and binding of copper ions to molecular sites of aquatic organisms [12,13]. Since gills are in contact with waterborne copper, these organs are the first target for the metal toxicity [14,15]. In turn, hepatopancreas is a central organ involved in sequestration and detoxification of heavy metals [16]. Considering that cell is the main site of accumulation, metabolism and toxicity of metals like copper, isolated and cultured cells from gills and hepatopancreas could be a powerful tool for *in vitro* studies for a better understanding of these process. In fact, it has been shown that *in vivo* exposure conditions to aquatic contaminants can be reproduced under *in vitro* conditions using both fish and mollusks gill cells, as well as fish hepatocytes, and crustacean and mollusks

hepatopancreatic cells [17-21]. Although the relevance of the systematic effects on toxicity and toxicant metabolism cannot be reflected from the *in vitro* results, cell culture allows rapid and rather cheap testing. Furthermore, it often brings relevant information on the mechanism of toxicity.

In light of the above, the main goal of the present study was to use fresh preparations of mixed-cell populations to evaluate accumulation and toxicity of dissolved copper to isolated cells from the gills and hepatopancreas of the blue crab *Callinectes sapidus*.

Material and methods

Animals

Male crabs (*Callinectes sapidus*) were captured at the Patos Lagoon estuary (Rio Grande, RS, Southern Brazil), transferred to the laboratory, and acclimated to continuously aerated seawater at salinity 30 ppt (800 mosmol kg^{-1}), for at least one week. During acclimation and tests, temperature and photoperiod were fixed at 20°C and 12L:12D, respectively. Crabs were fed twice a week with sliced fish. After acclimation, crabs were crioanesthetized, killed by removal of the exoskeleton, and had their posterior gills and hepatopancreas dissected.

Primary culture and cell preparation

General procedures to obtain isolated cells from posterior gill cells of *C. sapidus* were performed according to the method described by Kelly *et al.* [22], with modifications. Briefly, posterior gills were excised and washed in sterile Ca-free phosphate buffer saline (PBS: 342 mM NaCl, 20 mM Na_2HPO_4, 1.7 mM K_2HPO_4, and 16 mM KCl; pH 7.6; 780

mosmol kg^{-1}). Hemolymph cells were flushed out by perfusing the gills with PBS. Before cell dissociation, each gill was perfused with PBS containing 0.05% trypsin (Gibco, UK). Tissues were minced into small pieces, placed in a beaker containing 20 mL of PBS containing 0.035% trypsin and 0.04% chitinase (Sigma-Aldrich, St. Louis, MO, USA), and incubated in a shaking water bath (30°C) for 30 min. The resulting cell suspension was filtered (50 μm-mesh nylon filter) to remove non-dissociated tissue and large debris, and mixed with a fresh PBS solution containing 10% fetal bovine serum (Gibco, UK) to stop trypsin activity. The filtrate was centrifuged (Model 204-N, Fanem, São Paulo, SP, Brazil) at 180 x *g* for 10 min. The cell pellet was resuspended in culture medium (pH 7.6; 726 mOsmol Kg^{-1}) prepared adding $NaHCO_3$ (4.9 mM), KCl (8.1 mM), and NaCl (205 mM) and fetal bovine serum (5%) to the 199 medium (Sigma-Aldrich, St. Louis, MO, USA).

Procedures to obtain isolated cells from hepatopancreas were carried out following the method described by Faucet *et al.* [23] with modifications. Hepatopancreas from 3 crabs was dissected, pooled, washed, and incubated (20 min) in PBS containing 10 mM Hepes (PBS-Hepes; pH 7.6; 790 mOsmol Kg^{-1}), antibiotics (4% penicillin-streptomycin; Gibco, UK) and fungicide (1% fungizone; Gibco, UK). After incubation, the hepatopancreas was transferred to a new flask and rinsed twice with a freshly prepared PBS-Hepes solution without antibiotics and fungicide. It was sliced into small pieces with a razor blade and shacked at 80 rpm (Certomat-MO-II; Sartorius Stedim Biotech GmbH, Göttingen, Germany) for 30 min at room temperature (20°C). Dissociated cells were filtered (100-μm mesh filter). The filtrate was collected in a 15-mL plastic tube and centrifuged at 90 x *g*, for 3 min (Model 204-N, Fanem, São Paulo, SP, Brazil). The pellet containing cells was resuspended in PBS-Hepes. These procedures were repeated three times to wash cells. After the last wash, the pellet was resuspended in the same culture medium used for the gill cells, as described above.

Cell viability in gill and hepatopancreas preparations was determined by the Trypan Blue exclusion method (0.08% trypan blue).

Copper accumulation

One aliquot (80 µL) of culture containing gill or hepatopancreatic cells was transferred into one well of a 96-wells plate using a micropipette. Each well of the plate already contained 320 µL of a fresh culture medium prepared as described above, but containing copper, as $CuCl_2$ (Merck, St. Louis, MO, USA). Therefore, cells were tested at a final density of 10^6 cells mL^{-1}. As fetal bovine serum seems to increase copper toxicity by binding copper and increasing its uptake by the cell [24], it was not added to the incubation medium. Copper was added 3 h prior to the beginning of the experiment to allow copper to equilibrate with the culture medium. The final copper concentrations tested were 1, 10, and 100 µM. Copper concentration in the exposure medium was checked by atomic absorption spectrophotometry (GBC, AAS 932 Avanta Plus, IL, USA). Each copper concentration was tested in replicates (n = 5) for each cell culture preparation. Control tests (no copper addition to the exposure medium) were also run. All procedures were performed in triplicate, i.e., in three different cell culture preparations.

At the beginning and after 1, 3, and 6 h of test, the exposure medium was homogenized, transferred to 1.5-mL plastic tube, and centrifuged at 1,200 x *g*, for 10 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA). For cell washing, the supernatant was discarded and the pellet was resuspended with PBS (gill cells) or PBS-Hepes (hepatopancreas cells). Centrifugation and washing procedures were repeated. After the second washing, cell samples were dried (60ºC, 24 h) and digested with 50 µL of HNO_3 (Suprapur, Merck, St. Louis, MO, USA). Copper concentration in the

samples was determined by atomic absorption spectrophotometry (GBC, AAS 932 Avanta Plus, IL, USA) after sample dilution with Milli-Q water. Copper concentration was expressed as μg Cu $.10^6$ $cells^{-1}$.

Copper toxicity

Culture medium was pre-equilibrated for 3 h with different copper concentrations (1, 10, and 100 μM Cu). Copper was added to the culture medium as $CuCl_2$ (Merck, St. Louis, MO, USA). Gill and hepatopancreas cells were then added to the culture medium without (control) or with copper at a final density of 10^4 mL^{-1}. Test media containing cells were placed in a 96-wells plate, which was kept in an incubator (20°C) over the exposure time. At the beginning and after 1, 3 and 6 h of exposure, three samples from each well were collected and cell viability was determined using the Trypan Blue exclusion method. Total and viable cells were counted using a haemocytometer (Neubauer chamber, 0.1 mm depth)**.** Cell viability was expressed in percentage considering the total number of cells in the respective control as 100%. Control (no copper addition) and different copper concentrations were tested in triplicate for the same primary cell culture preparation. Also, all procedures were replicated (n = 6), *i.e.*, different primary cell cultures for each organ were prepared and analyzed.

Statistical analyses

All data were expressed as mean ± standard error. Copper accumulation and cell viability data were subjected to analysis of variance (ANOVA) followed by the Tukey's test. ANOVA assumptions (data normality and homogeneity of variances) were previously

checked. Mathematical data transformations were performed before statistical analysis of copper accumulation (log-transformation) and cell viability (√(arcsine)-transformation) data. In all cases, the significance level adopted was 95%.

The relationship between copper accumulation and copper concentration in the incubation medium was determined by linear regression analysis. The relationship between copper accumulation and gill cell mortality was determined by non-linear regression analysis (exponential rise to maximum).

Results

Copper accumulation

In control cells, no significant change in copper concentration was observed over the exposure time for both gill and hepatopancreatic cells. Copper concentration in gill cells (11.75 ± 2.27 ng Cu $.10^6$ cells^{-1}) was 3.0-fold higher than in hepatopancreatic cells (3.91 ± 0.69 ng Cu $.10^6$ cells^{-1}).

Copper accumulation was measured in gill and hepatopancreas cells over 6 h of exposure to different copper concentrations (1, 10 and 100 μM). For both cells types, copper content did not significantly change after exposure to the lower copper concentration tested (1 and 10 μM). However, a significant increase was observed after exposure to the highest copper concentration (100 μM). After 6 h of exposure, the increase in cell copper content was higher in gill (9.5-fold; Fig. 1A) than in hepatopancreas (4.3-fold; Fig. 1B). Furthermore, copper accumulation kinetics was completely different between the two cell types. Gill cells showed a significant linear increase in copper accumulation over time (Fig. 1A) while hepatopancreatic cells showed a saturation-type kinetics. These cells were saturated with

copper after 1 h of exposure and remained saturated for up 6 h of exposure (Fig. 1B). Disregarding the time of exposure (1 or 6 h), both gill (Fig. 2A) and hepatopancreatic (Fig. 2B) cells showed a linear increase in copper accumulation as a function of copper concentration in the exposure medium.

At the beginning of the exposure period, mean (± S.E.) cell viability corresponded to 78.8 ± 2.5 and 87.5 ± 0.8% in gill and hepatopancreatic cells, respectively. Viability of both gill and hepatopancreatic cells did not significantly change over the exposure period under control conditions. The mean (± S.E.) cell viability observed was 79.5 ± 1.3 and 87.6 ± 0.7%, respectively. It is important to note that no significant changes were observed in the total number of cells at the end of the exposure period (6 h) when compared to that registered at the beginning of the exposure period (data not shown).

Copper exposure to the lowest copper concentration (1 μM) did not induce significant decrease in gill cell viability. However, incubation with higher copper concentrations (10 or 100 μM) induced a significant decrease in cell viability. At 10 μM copper, 21.6, 28.5 and 31.0% decrease in cell viability was observed after 1, 3 and 6 h of exposure, respectively. At 100 μM copper, 36.9, 38.8 and 44.1% decrease in cell viability was observed after 1, 3 and 6 h of exposure, respectively (Fig. 3A). Mortality of gill cells was clearly dependent on the cell copper content. Cell mortality markedly increased up to ~40% as copper accumulation augmented up to ~40 ng $Cu.10^6$ $cells^{-1}$. Higher copper levels in the gill cells did not induce any further significant cell mortality (Fig. 4).

In hepatopancreatic cells, incubation with 1 or 10 μM copper did not induce any significant change in cell viability over the exposure period. Significant reduction in cell viability was only observed after exposure to 100 μM copper. In this case, maximum mean reduction in cell viability was only 14.6%. Furthermore, mortality was not dependent on the

time of exposure (Fig. 3B) and did not show any significant relationship with cell copper accumulation in the range of the copper concentrations tested (Fig. 4).

Discussion

In the present study, isolated cells from posterior gills and hepatopancreas of the blue crab *C. sapidus* were used to evaluate the potential accumulation and toxicity of dissolved copper. Data reported in the present study clearly indicate that procedures adopted for tissue dissection, washing, and incubation were quite adequate to obtain fresh preparations of isolated cells from gill and hepatopancreas of the blue crab *C. sapidus*. Therefore, procedures described in the present study could be adopted to obtain preparations of fresh isolated cells from gills and hepatopancreas of other crustaceans acclimated to seawater for many purposes, including short-term toxicological studies to access the possible different mechanisms involved in copper accumulation and toxicity at a cellular level in different tissues. This statement is based on the following facts: (1) isolated gill and hepatopancreatic cells always showed viability values higher than 75% for up to 6 h under control conditions (no copper addition to the experimental medium); (2) it was already possible to detect a significant copper accumulation in both gill and hepatopancreatic cells after 1 h of exposure to 100 μM copper; (3) it was possible to detect a significant copper toxicity in gill and hepatopancreatic cells after only 1 h of exposure to 10 or 100 μM copper; and (4) different kinetics of copper accumulation and sensitivity to metal exposure were observed in gill and hepatopancreatic cells.

Marine invertebrates can take up toxic metals like copper across the integument via the gills when in contact with dissolved copper or through the gut after food ingestion. In turn, metal excretion may occur across the same tissues [7, 16, 25]. Since the gill epithelium is in

direct contact with copper dissolved in the surrounding water, it is considered as being the main route of copper uptake from the dissolved phase. Consequently, it is also considered as being the first target for copper toxicity [14, 15].

Data obtained in the present study clearly indicate that gill cells are able to regulate their copper levels in the presence of relatively low levels of copper (1 μM) in the surrounding medium. However, at very high levels of copper (100 μM) the mechanisms of copper extrusion from the cell are completely overwhelmed by those of copper uptake. At this point, it is interesting to note that once the cell capacity to regulate copper concentration is overwhelmed, as observed at 100 μM Cu, copper toxicity is significantly expressed. In fact, a time-dependent copper accumulation and toxicity was observed in isolated gill cells. It is also worth noting that a very significant correlation was observed between copper accumulation and toxicity in isolated cells from posterior gills of the blue crab *C. sapidus*. This finding is in complete agreement with the premise behind the Biotic Ligand Model (BLM) approach [14, 15]. According to this model, copper toxicity is a direct function of the amount of copper accumulated on or at the sites of toxicity at the gills of aquatic animals. Therefore, we suggest that isolated gill cells from the posterior gills of the blue crab *C. sapidus* can be used to better understand the mechanisms involved in copper accumulation and toxicity in marine crustaceans, as well as to develop an *in vitro* BLM version for copper to be applied in marine conditions. This last suggestion is based on the following facts: (1) gills are the main route for copper entry and the first target for copper toxicity in aquatic animals [14, 15]; (2) copper is considered as an osmoregulatory toxicant [7, 8, 11]; (3) there is a straight linear relationship between copper concentration in the cell incubation medium and cell copper concentration; (4) there is an exponential relationship between cell copper burden and copper toxicity in isolated gill cells of the blue crab; and (5) gill cells were markedly more sensitive to copper exposure than the hepatopancreatic ones.

On the other hand, hepatopancreatic cells of the blue crab *C. sapidus* do not seem to be the adequate "biotic ligand" for the development of an *in vitro* BLM version for marine conditions, as suggested for the gill cells. Despite the fact that these cells also showed a linear increase in copper concentration as a function of the copper concentration in the incubation medium, no relationship was observed between cell copper burden and copper toxicity. It is also interesting to note that the maximum copper accumulation observed in the hepatopancreatic cells was achieved after only 1 h of exposure. This finding suggests that copper homeostasis after copper exposure is quickly reached in hepatopancreatic cells while no copper homeostasis was observed in gill cells even after 6 h of exposure to 100 μM. It also indicates that different mechanisms of copper transport and accumulation are operating in membranes of gill and hepatopancreatic cells. Regarding copper toxicity, it was very low in hepatopancreatic cells, even after 6 h of exposure to 100 μM copper. Altogether these data suggest that these cells could be used as an interesting model to investigate the mechanisms of copper uptake and extrusion, leading to a copper homeostasis at the cellular level, as well as the role of the hepatopancreas in tissue copper distribution in crustaceans.

Regarding copper sensitivity, a marked linear increase in copper toxicity as a function of copper concentration in the incubation medium was observed in gill cells in the range of 12 to 40 ng $Cu.10^6$ $cells^{-1}$. After this level, no further toxicity was observed with increased cell copper burdens. This finding suggests that only one population of gill cells are being affected by copper exposure, the remaining showing tolerant to the metal exposure. In fact, the epithelium of posterior gills in brachyuran crabs is composed by a monolayer of cells sat on the cuticle in each side of the ion-transporting gill lamella, where aggregates of ionocytes can be found. On the other hand, the pillar cells are present in the gill epithelium forming columns regularly disposed in the hemolymphatic space. This feature creates a series of lacunas that allows the hemolymph flow through the lamella [26, 27]. Therefore, ionocytes

are directly responsible for the ion transport across the gills and seems to be the preferred target for copper toxicity, since copper is recognized as an osmoregulatory toxicant that induces ionoregulatory disturbances in aquatic organisms [5-11]. Further studies are needed to confirm this hypothesis.

As previously discussed, copper cytotoxicity is clearly controlled by the level of copper accumulation in the gill cell. Therefore, the higher percentages of mortality observed in gill cells than in hepatopancreas cells at the same time of exposure and copper concentration can be explained based on the higher copper accumulation in gill cells likely associated with their low ability to metabolize copper when compared to hepatopancreas cells. In fact, it has been reported that the capacity to metabolize toxicants is much lower in fish gill cells than in fish hepatocytes [21, 28]. Furthermore, copper appeared to has no effect on isolated digestive gland cells of the mollusk *Sepia officinalis* [19].

In hepatopancreas cells, copper can be associated with metallothioneins or glutathione, transported into mitocondria, accumulated by lisossomes, transferred into the endoplasmic reticulum, or undergo efflux across the basolateral cell membrane [16, 17, 29]. In fact, hepatopancreas has been recognized as a major organ of heavy metal homeostasis in crustaceans, sequestering and detoxifying dietary or waterborne metals. However, the hepatopancreas involvement in copper sequestration and accumulation seems to more apparent at long-term exposures to the metal. This statement is based on the following facts (1) gill cells of the blue crab *C. sapidus* accumulated 6.6-fold more copper than the hepatopancreatic cells after 6 h of exposure to 100 μM Cu; (2) a significant copper accumulation in the hepatopancreatic cells was observed only after short exposure to a high copper concentration (100 μM); and (3) the maximum capacity of copper accumulation by hepatopancreatic cells was achieved after only 1 h of exposure to 100 μM Cu and did not change up to 6 h of exposure. Otherwise, we need to consider that copper is continuously

being sequestered, detoxified, and extruded from the hepatopancreas cells. Unfortunately, our data do not allow us to verify this hypothesis. Further studies are needed for a better understanding of the processes undergoing at the cellular level. On the other hand, gill cells seem to play a minor role in detoxification. Despite that, they are in direct contact with waterborne toxicants, are considered a major route of uptake for virtually all of them, and a key organ for lethal damage for many of them, including metals [21, 30]. As reported in the present study, gills rapidly accumulate copper following the onset of waterborne exposure, resulting in disturbance of multiple physiological processes [25].

Regarding the mechanisms involved in toxicity in gill cells, copper has been recognized as an osmoregulatory toxicant that induces ionoregulatory disturbances in aquatic animals, including crustaceans [7, 8, 11]. Also, copper can affect the cell acid-base balance in aquatic animals. It seems that acidification, in addition to other effects of copper, contributes to copper toxicity [11]. In hepatopancreatic cells, oxidative stress could be the physiological basis of copper toxicity. In fact, copper is known to cause lipid peroxidation at the cell membrane due to enhanced production of reactive oxygen species, inducing cytoxicity [31]. Further studies are needed to identify and characterize the mechanisms involved.

To sum up, data reported in the present study clearly indicate that isolated cells from the posterior gills of the blue crab *C. sapidus* fulfill the premise behind the BLM approach, becoming an excellent model for the development of an *in vitro* BLM version for marine conditions. Furthermore, they showed that isolated cells from the hepatopancreas could be used as a model to better understand the mechanism of uptake, detoxification, and extrusion of copper at a cellular level in crustaceans.

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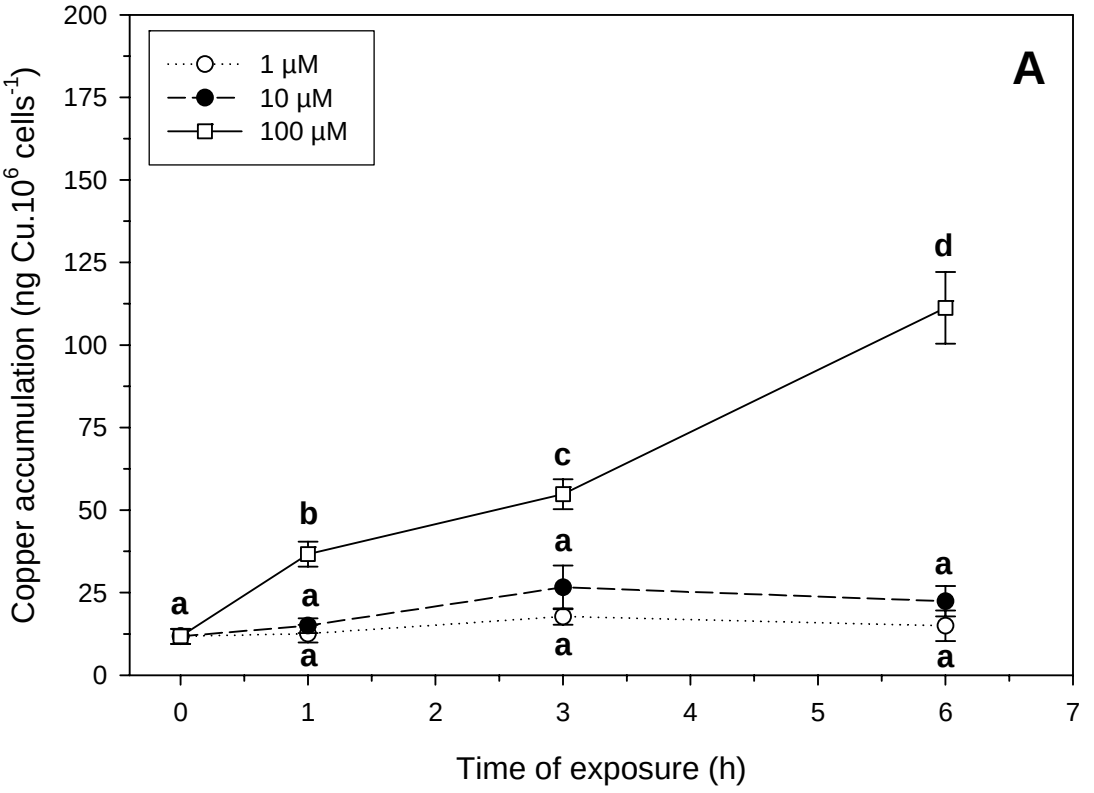
Legend for figures

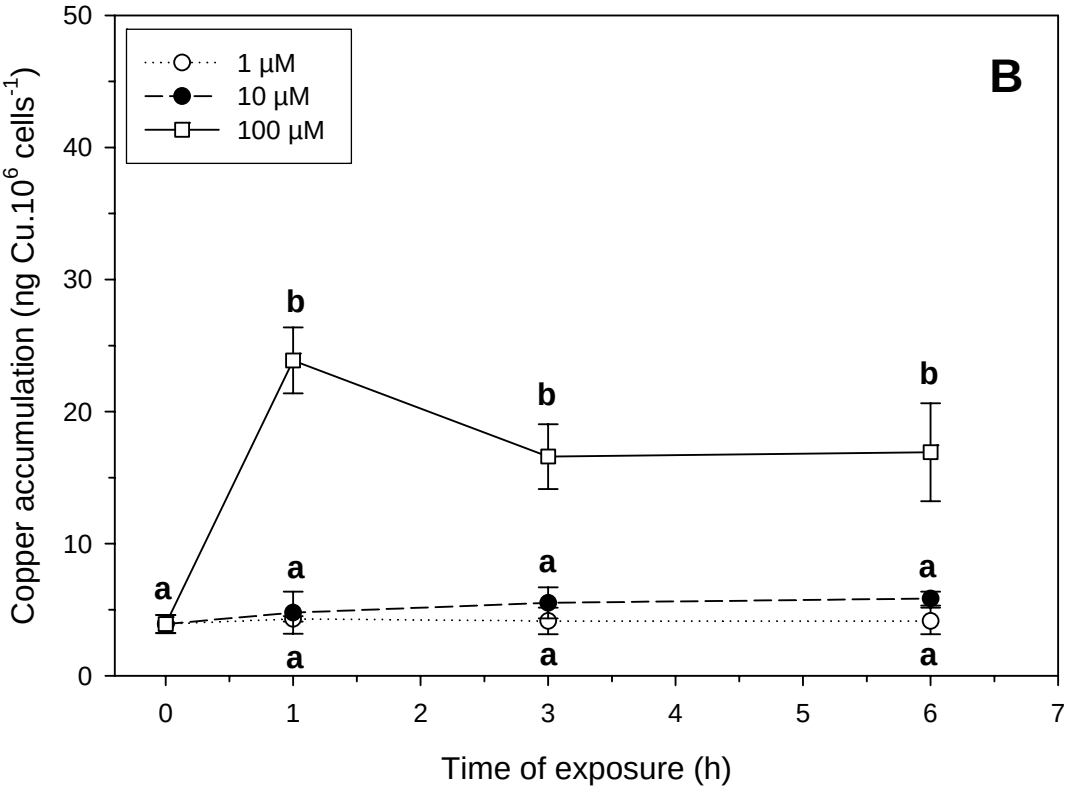
Figure 1. Copper accumulation in isolated cells from posterior gills (A) and hepatopancreas (B) of the blue crab *Callinectes sapidus* exposed to 1, 10 or 100 μM copper ($CuCl_2$) for up to 6 h. Data are expressed as means ± SE of three different cell preparations. Different letters indicate means significantly different for cells exposed to the same copper ($p<0.05$).

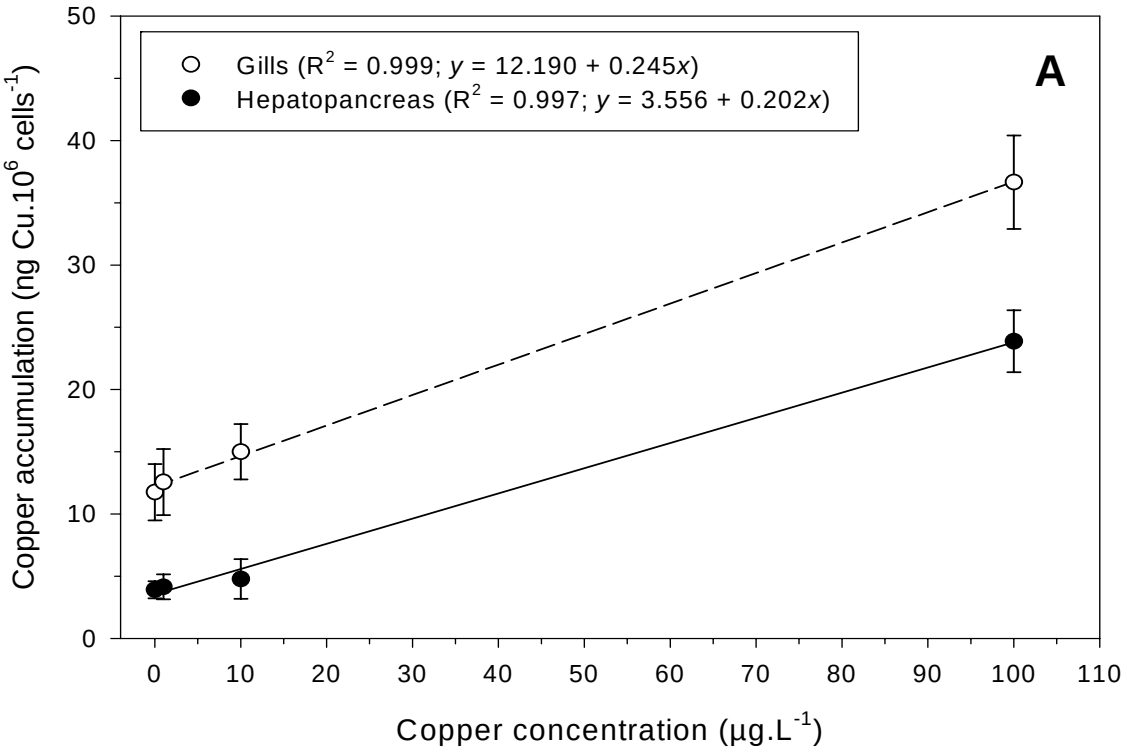
Figure 2. Copper accumulation in isolated cells from gill and hepatopancreas of the blue crab *Callinectes sapidus* exposed to 1 (A) or 6 (B) h to different copper ($CuCl_2$) concentrations. Data are expressed as means ± SE of three different cell preparations.

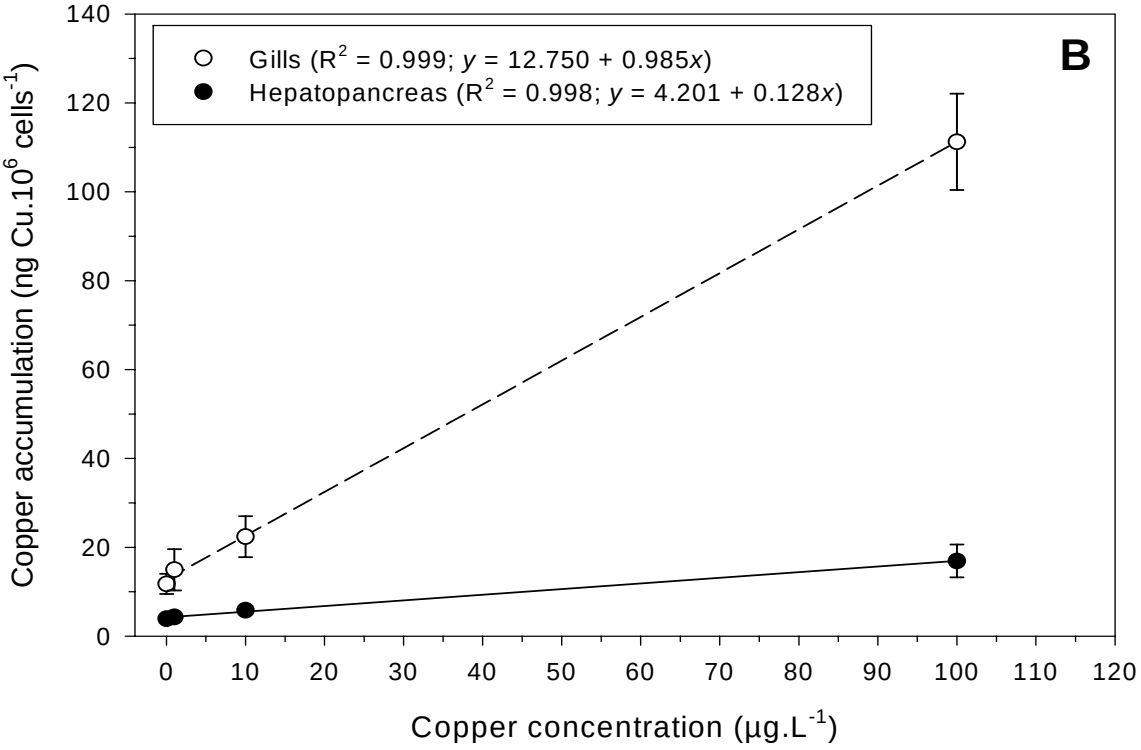
Figure 3. Viability of isolated cells from posterior gills (A) and hepatopancreas (B) of the blue crab *Callinectes sapidus* exposed to different copper ($CuCl_2$) concentrations (1, 10, and 100 μM) for up to 6 h of test. Data are expressed as mean ± SE of six different cell preparations. * Indicates significantly different means ($p<0.05$) from the control condition.

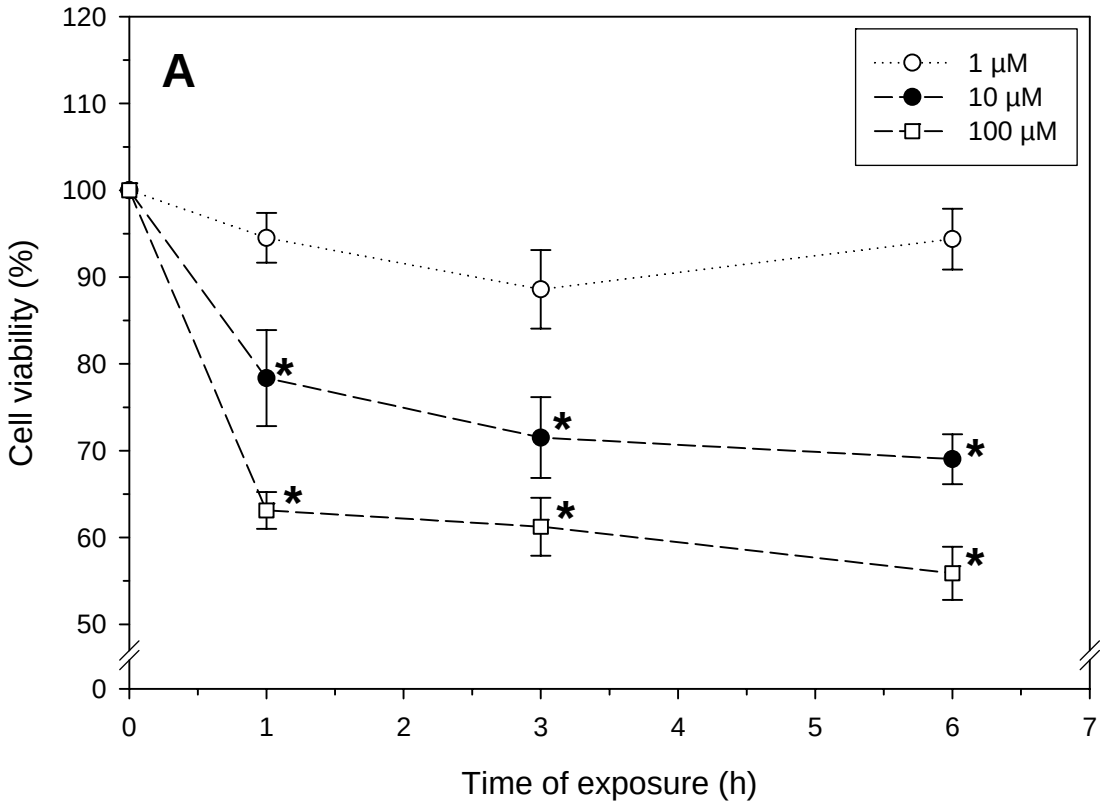
Figure 4. Copper toxicity as a function of copper accumulation in gill and hepatopancreatic cells of the blue crab *Callinectes sapidus*. Data are expressed as mean ± SE of three (copper accumulation) or six (copper toxicity) different cell preparations.

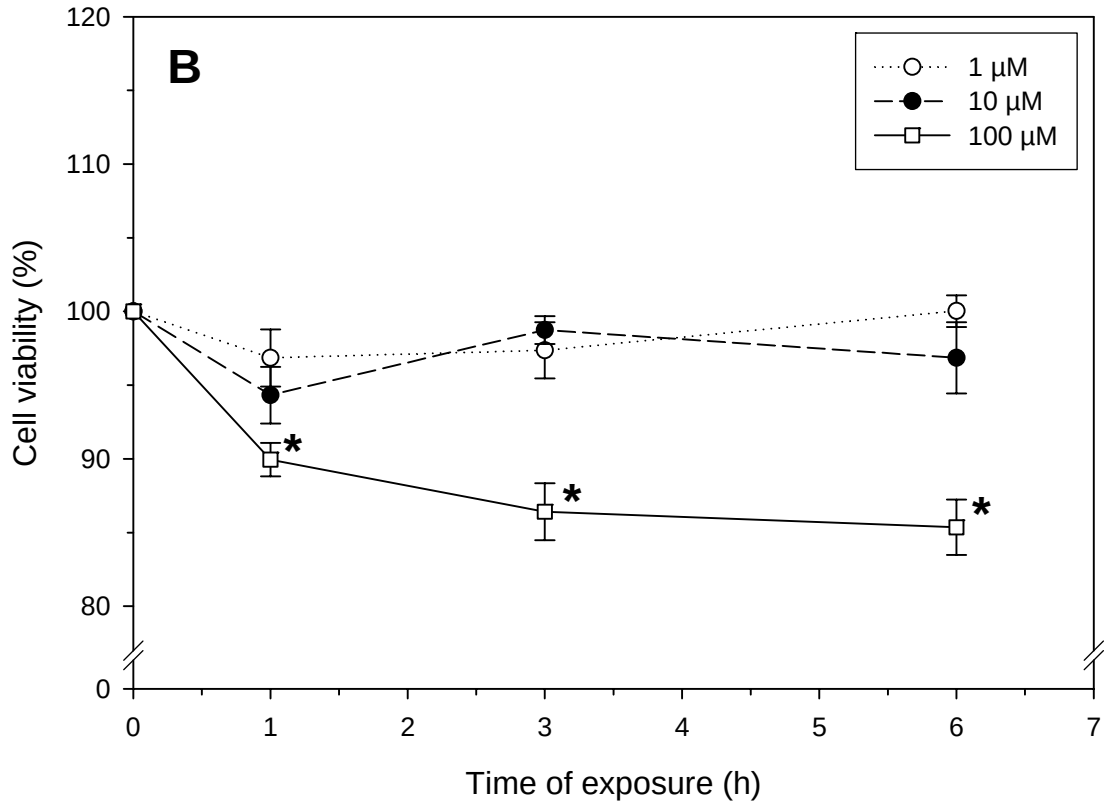

A
1 µM
10 µM
100 µM
Copper accumulation (ng Cu.10^6 cells^-1)
Time of exposure (h)
0
25
50
75
100
125
150
175
200
0
1
2
3
4
5
6
7
a
b
c
d
a
a
a
a
a
a

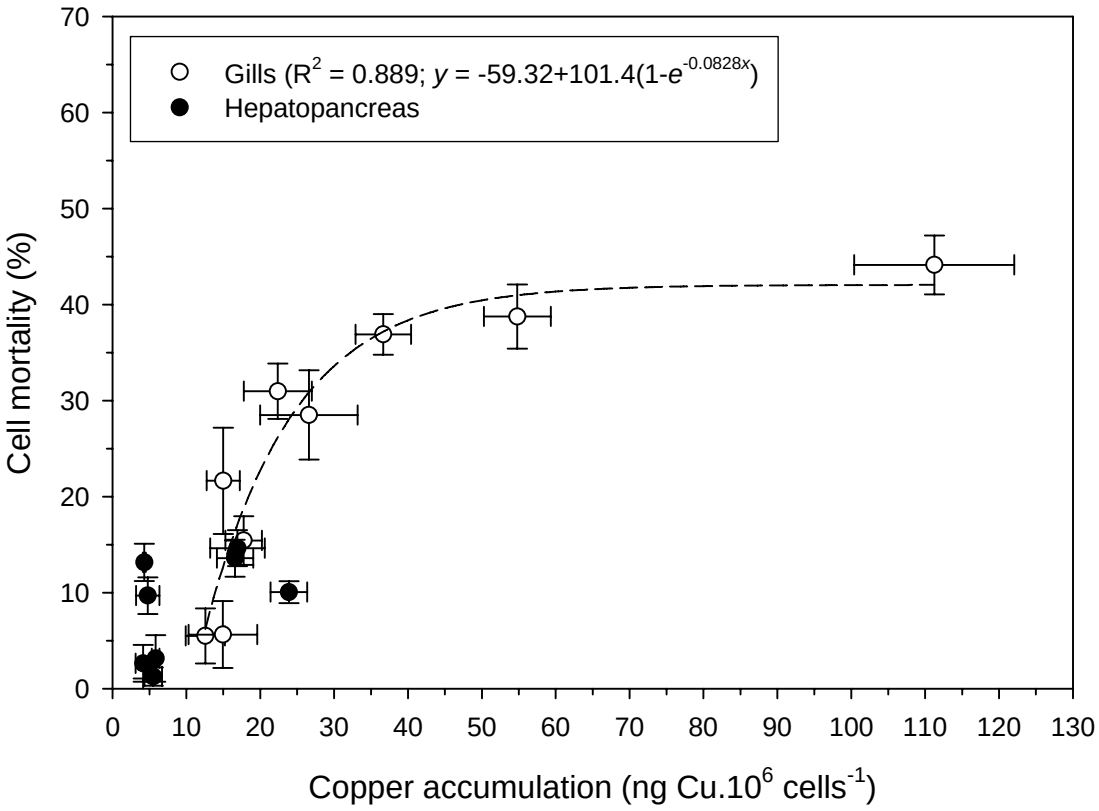

B
1 µM
10 µM
100 µM
Copper accumulation (ng Cu.10^6 cells^-1)
Time of exposure (h)
0
10
20
30
40
50
0
1
2
3
4
5
6
7
a
b
b
b
a
a
a
a
a
a


Gills (R^2 = 0.999; y = 12.190 + 0.245x)
Hepatopancreas (R^2 = 0.997; y = 3.556 + 0.202x)
A
Copper accumulation (ng Cu.10^6 cells^{-1})
Copper concentration (µg.L^{-1})
0
10
20
30
40
50
60
70
80
90
100
110


Gills (R^2 = 0.999; y = 12.750 + 0.985x)
Hepatopancreas (R^2 = 0.998; y = 4.201 + 0.128x)
B
Copper accumulation (ng Cu.10^6 cells^{-1})
Copper concentration (µg.L^{-1})
0
20
40
60
80
100
120
140


A
1 µM
10 µM
100 µM
Cell viability (%)
Time of exposure (h)
120
110
100
90
80
70
60
50
0
0
1
2
3
4
5
6
7
*


B
1 µM
10 µM
100 µM
Cell viability (%)
Time of exposure (h)
120
110
100
90
80
0
0
1
2
3
4
5
6
7
*


Gills (R^2 = 0.889; y = -59.32+101.4(1-$e^{-0.0828x}$)
Hepatopancreas
Cell mortality (%)
Copper accumulation (ng Cu.10^6 cells^{-1})
0
10
20
30
40
50
60
70
80
90
100
110
120
130

6.3. Mechanisms of copper accumulation in isolated gill cells of the blue crab *Callinectes sapidus*.

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Trabalho submetido para: Aquatic Toxicology

MECHANISMS OF COPPER ACCUMULATION IN ISOLATED GILL CELLS OF THE BLUE CRAB CALLINECTES SAPIDUS

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Abstract

Mechanisms involved in copper accumulation in isolated cells from posterior gills of the blue crab *Callinectes sapidus* acclimated to seawater were identified using a fluorescence technique combined with pharmacological tools. Isolated gill cells were loaded with a fluorescent die (Phen Green; 15 μM) and exposed (1 h) to dissolved copper only (100 μM) or together with different ion transporter blockers or enzyme inhibitors. In the last case, cells were pre-treated with one of the tested drugs before being exposed to copper. Drug effect was tested pre-treating cells with the drug for 1 h and exposing them to copper. Change in fluorescence quenching (ΔF) after copper exposure was used as a measurement of changes in the amount of copper accumulated in the isolated gill cells. ΔF significantly reduced after treatment with furosemide (100 μM), phenamil (1 μM), SITS (500 μM), benzthiazide (100 μM), acetazolamide (1 mM), quinine sulfate (500 μM), bafilomycin A1 (1 μM), and EIPA (500 μM). It was significantly increased after treatment with NPPB (100 μM), amiloride (1 mM) and ouabain (100 μM). Cells exposed to copper (100 μM) showed 28% mortality. These findings indicate that the Na^+/K^+ exchanger, the H^+-ATPase/Na^+ channel system, and the Na^+-K^+-$2Cl^-$ and Na^+-Cl^- cotransporters are involved in copper uptake by the gill cells while the Na^+/K^+-ATPase and Na^+/Ca^{2+} exchanger is involved in copper extrusion from the gill cells. Furthermore, they suggest that copper uptake or extrusion is also dependent on the intracellular electrical state. The influence of competitive interactions between copper and Na^+ for binding sites at the gill cells on the acute copper toxicity observed in gill cells of the blue crab *C. sapidus* is discussed.

Keywords: *Callinectes sapidus*, copper accumulation, copper toxicity, crab, gills, ion transport, ion regulation, isolated cells.

Introduction

Copper is ubiquitous in the environment and occurs both naturally and as a result of releases from anthropogenic sources (Santore et al., 2000). It is considered an essential element for most organisms, being part of proteins involved in several physiological functions. For example, copper acts as cofactor of a number of key enzymes and plays a pivotal role in cellular respiration (Dameron and Howe, 1998; Bury *et al.*, 2003), particularly in mollusks and crustaceans, which utilize copper-containing hemocyanin as their respiratory pigment (Rainbow, 1985; Viant et al., 2002).

Aquatic organisms can take up copper directly from water and elevated ambient copper concentrations can lead to excessive copper accumulation in several tissues, including gills (Skaggs and Henry, 2002; Grosell et al., 2002, 2004; Matsuo et al., 2005; Blanchard and Grossel, 2006). Copper has been recognized as an osmoregulatory toxicant inducing disturbances in the balance of the main ions involved in osmoregulation in aquatic animals, including crustaceans (Laurén and McDonald, 1985; Barnber and Depledge, 1997; Morgan et al., 1997; Grosell et al., 2002; Handy et al., 2002; Brooks and Mills, 2003; Bianchini et al., 2004; Blanchard and Grosell, 2006).

Copper toxicity is assumed to occur as the result of free metal ion interaction with the physiologically active binding sites at the site of action, the gills (Di Toro et al., 2001; Paquin et al., 2002). Therefore, both monovalent and divalent copper forms could be transported across the gill cells of aquatic animals through multiple pathways, including the mechanisms involved in membrane Na^+ and Cl^- transport. This implies that at least four ion transporters may contribute to gill copper uptake. In euryhaline crustaceans, Na^+ is believed to be taken up either in exchange for H^+ by action of the Na^+/H^+ exchanger or through apical Na^+-channels that are associated with an outwardly H^+-ATPase (Towle et al., 1997; Skaggs and Henry, 2002). In turn, Cl^- seems to cross the apical membrane through a Cl^-/HCO_3^- exchanger and/or a Na^+-K^+-$2Cl^-$ cotransporter (Riestenpatt et al., 1996; Kirschner, 2004).

Other components of the branchial Na^+ uptake include two transport-related enzymes: the Na^+/K^+-ATPase and the cytosolic carbonic anhydrase (CA). These enzymes play a central role in the general process of osmoregulation by supporting the ion transport processes involved (Genovese et al., 2005; Blanchard and Grosell, 2006). Therefore, the ability of euryhaline crustaceans to regulate hemolymph ionic and osmotic concentrations is believed to depend on the presence of a number of ion-transport proteins and transport-related enzymes in the gills (Towle, 1997; Henry, 2001).

Copper has been shown to disrupt sodium ion regulation in estuarine animals like the crab *Carcinus maenas* (Lawson et al., 1995), the shrimp *Peneaus japonicus* (Bamhang et al., 1995), the isopod *Excirolana armata* (Bianchini et al., 2003), and the copepod *Acartia tonsa* (Pinho et al., 2007). It also inhibited the branchial CA in the estuarine crabs *Chasmagnathus granulatus* (Vitale et al., 1999) and *Callinectes sapidus* (Skaggs and Henry, 2002).

Considering that depletion of extracellular sodium is the primary cause of copper toxicity, inhibition of or competition with any of the transporters mentioned above would influence sodium uptake across the gills and could lead to animal death (Bianchini and Wood, 2002; Bianchini et al., 2002; Grosell et al., 2002). Unfortunately, information regarding routes of copper accumulation and the mechanism of copper toxicity in brackish or marine invertebrates, like crustaceans, is still scarce. According to Bianchini et al. (2003), marine invertebrates are generally osmoconformers, but they can act as ionoregulators and this condition suggests that gill may also become a key site for copper toxicity.

Most studies on copper accumulation in invertebrates were focused on whole animals, which implies the influence of variable internal factors that may complicate the interpretation of results from *in vivo* experiments. Therefore, the development of techniques using isolated cells offers the possibility to mimic the copper exposure situation in aquatic environment in an *in vitro* system (Kelly et al., 2000; Le Bihan et al., 2004; Zhou et al., 2006). Although the

relevance of systematic effects on toxicity and toxicant metabolism cannot be reflected from the *in vitro* results, cell culture allow rapid and rather cheap testing and often bring relevant information on the mechanism of toxicity. It thus offer an alternative tool to investigate and better understand the processes of accumulation, metabolism and toxicity of metals like copper.

In the present study, isolated cells from the osmoregulating (posterior) gills of the blue crab *Callinectes sapidus* were used as an experimental model to characterize the involvement of membrane Na^+ and Cl^- transporters on the cellular accumulation of copper.

Material and methods

Crabs

Male crabs (*Callinectes sapidus*) were collected at the Patos Lagoon estuary (Rio Grande City, RS, Southern Brazil), immediately brought to the laboratory, and acclimated for at least 7 days in tanks containing continuously aerated water at 30 ppt (800 mosmol kg^{-1}). During acclimation and experimental periods, temperature and photoperiod were fixed at 20°C and 12L:12D, respectively. Crabs were fed twice a week. After acclimation, they were crioanesthetized, killed by removing the exoskeleton, and posterior gills were dissected.

Primary culture and cell preparation

General procedures to obtain isolated cells from posterior gills were performed according to the method described by Kelly et al. (2000) with modifications. Briefly, posterior gills were excised and washed in sterile Ca-free phosphate buffer saline (PBS: 342

mM NaCl, 20 mM Na_2HPO_4, 1.7 mM K_2HPO_4, 16 mM KCl; pH 7.6; 780 mosmol kg^{-1}). Hemolymph cells were rinsed out by gill perfusion with PBS. Before cell dissociation, each gill was perfused with PBS containing 0.05% trypsin (Gibco, UK). Gills were then minced into small pieces, placed in a beaker containing 20 ml of PBS containing 0.035% trypsin and 0.04% chitinase (Sigma-Aldrich, St. Louis, MO, USA), and incubated in a shaking water bath (30^{o}C) for 30 min. The resulting cell suspension was filtered through a 30-µm nylon mesh to remove non-dissociated tissue and large debris, and mixed with a fresh PBS solution containing 10% fetal bovine serum (Gibco, UK) to reduce trypsin activity. The filtrate obtained was centrifuged (Fanem-Model 204-N; São Paulo, SP, Brazil) at 180 x *g* for 10 min to spin down the cells. The pellet was resuspended in 199 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 4.9 mM $NaHCO_3$, 8.1 mM KCl, and 205 mM NaCl (pH 7.6; 726 mosmol kg^{-1}). As fetal bovine serum seems to influence copper toxicity by binding copper and increasing its uptake into the cell (Dowling and Mothersill, 2001), no serum was added to the culture medium. Cell viability was determined by the Trypan Blue exclusion method (0.08% trypan blue). In all preparations, cell viability was always >80%.

Copper toxicity tests

To test copper toxicity to isolated cells, copper was added to the culture medium 3 h prior to cell exposure to allow pre-equilibration. Copper concentrations tested were 1, 10, and 100 µM Cu, as $CuCl_2$ (Merck, St. Louis, MO, USA). Gill cells were then maintained under control conditions or exposed to copper at a final density of 10^4 cells mL^{-1}. Cells were exposed in a 96-wells plate, which was kept in an incubator (20°C) for 1 h. After exposure, aliquots of the cell suspensions were collected and cell viability was determined using the Trypan Blue exclusion method. Total and viable cells were counted using a haemocytometer

(Neubauer chamber, 0.1 mm depth)**.** Cell viability was expressed in percentage considering the total number of cells as 100%. Based on results from these experiments, a final concentration of 100 μM Cu was chosen for the gill copper accumulation studies described below.

Copper accumulation studies

Copper accumulation in isolated gill cells was evaluated using the copper-sensitive fluorescent dye Phen Green (Molecular Probes, OR, USA). Experiments were performed according to the methodology described by Chavez-Crooker et al. (2001). To calibrate the methodology employed, two series of tests were performed. Firstly, a calibration curve was built using different copper concentrations (25, 50, and 100 μM Cu as $CuCl_2$) in the presence of 1.5 μM Phen Green. Isolated gill cells were incubated for 1 h in saline solution (350 mM NaCl, 9 mM KCl, 9 mM $CaCl_2$, 30 mM $MgCl_2$, and 2 mM $NaHCO_3$) containing the fluorescent dye. After loading with the dye, cells were washed with a dye-free saline solution and lised by sonication (15 min) with an ultra sonic cleaner (USC 1450 – Unique, São Paulo, SP, Brazil). Copper was then added to the cell lisate until to reach the final desired concentration. Copper-induced quenching of the fluorescence (Phen Green: excitation 490 nm; emission 520 nm) was measured using a spectrofluorometer (Victor^2 TM 1420, Perkin-Elmer, USA). Secondly, the dye fluorescence quenching was analyzed over 2 h in intact cells pre-equilibrated with Phen Green (1.5 μM) and incubated with 100 μM Cu. All experiments were performed at room temperature (20°C).

After the calibration of the methodology, the involvement of the different mechanisms of Na^+ and Cl^- transport in gill copper accumulation was analyzed using the fluorescence technique described above. However, this technique was associated with the use of different

pharmacological tools. Briefly, intact gill cells were loaded with the fluorescent dye Phen Green for 1 h in an orbital shaker (Certomat-MO-II; Sartorius, Germany) at 80 rpm and 20°C. After incubation, cells were washed by centrifugation (180 x *g* for 10 min) and resuspended in saline solution. Suspended cells were plated and maintained under control condition or exposed to 100 µM Cu. To evaluate the possible involvement of a single mechanism of Na^+ or Cl^- transport on cellular accumulation of copper, the following ion transport blockers were used: phenamil (1 µM), NPPB (100 µM), quinine sulfate (500 µM), EIPA (500 µM), amiloride (1 mM), SITS (500 µM), furosemide (500 µM), and benzthiazide (100 µM). Therefore, the following mechanisms of ion transport were considered: Na^+, Cl^- and K^+ channels, Na^+/H^+, Na^+/Ca^{2+}, and Cl^-/HCO_3^- exchanger, Na^+-K^+-$2Cl^-$ and Na^+-Cl^- cotransporters, respectively. Furthermore, the implication of Na^+/K^+-ATPase, H^+-ATPase and intracellular carbonic anhydrase in cell copper accumulation was analyzed by inhibiting the activity of these enzymes using ouabain (100 µM), bafilomycin A_1 (1 µM), and acetazolamide (1 mM), respectively. SITS and ouabain were purchased from Fluka Chemie (Buchs, SG, Switzerland). The other compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were dissolved in 0.8% DMSO. The possible influence of DMSO alone on cell copper accumulation was also tested.

Changes in fluorescence quenching after exposure to the different blockers of Na^+ and Cl^- transporters or inhibitors of the related enzymes were monitored over 1 h either in the absence or the presence of 100 µM Cu using the spectrofluorometer previously mentioned. Changes in fluorescence quenching was expressed in percentage, considering the fluoresce quenching in the absence of blockers or inhibitors and the presence of 100 µM Cu as 100%.

Statistical analysis

All data are expressed as mean ± standard error of the number of independent experiments (n = 3-4) performed on different cell preparations. Each cell preparation was obtained from 2 to 6 crabs. Mean data were compared by analysis of variance (ANOVA) followed by the Tukey test. ANOVA assumptions were previously checked (Sokal and Rohlf, 1981). The level of significance adopted was 95% ($\alpha = 0.05$).

Results

Copper toxicity

Under control conditions, viability of cells non-exposed to copper was quite constant and around 100% over the 1-h period of experiment (Fig. 1). Cell exposure to 1 or 10 µM Cu did not significantly affect cell viability. However, cell incubation with 100 µM Cu significantly reduced (28%) cell viability.

Quantitative Phen Green fluorescence quenching induced by copper

Two series of experiments were performed. The first series was performed to built a calibration curve of the dye fluorescence quenching after addition of three different copper concentrations to the lisate of dye-equilibrated cells. Increasing copper concentration resulted in a reduction of the fluorescence signal (Fig. 2A). The magnitude of the fluorescence quenching ($\Delta F_{25\ \mu M} = 1663$, $\Delta F_{50\ \mu M} = 2213$, and $\Delta F_{100\ \mu M} = 2561$; inset to Fig. 2A). The second series of experiments evaluated the fluorescence signal in intact cells pre-equilibrated with Phen Green (1.5 µM) exposed to copper (100 µM) over 2 h. A typical fluorescence signal is shown in Figure 2B.

Copper accumulation data

Fluorescence quenching induced by copper was significantly reduced in the presence of the following drugs: furosemide (44 ± 2%), phenamil (47 ± 4%), benzthiazide (82 ± 1%), SITS (88 ± 3%), acetazolamide (80 ± 3%), quinine sulfate (113 ± 8%), bafilomycin A1 (158 ± 8%), and EIPA (262 ± 23%) (Fig. 3 A). On the other hand, it was significantly increased in the presence of the following drugs: NPPB (85 ± 1%), amiloride (72 ± 6%) and ouabain (54 ± 2%) (Fig. 3B). At this point, it is interesting to note that a reduced fluorescence quenching indicates a higher amount of copper accumulated inside the gill cell, while an increased fluorescence quenching indicates a lower amount of intracellular copper.

Discussion

Results from the present study clearly show that dissolved copper at high concentration (100 μM) is acutely toxic to gill cells, reducing their viability in a short period of time (1 h) (Fig. 1). In comparison to isolated cells from other organisms such as rat hepatocytes (Pourahmad and O'Brien, 2000), roman snail hepatopancreas (Manzl et al., 2004), and trout gill cells (Mazon et al., 2004), a rather lower concentration of copper was required, after the same time of exposure, to induce a significant decrease in cell viability in the crab gill cells. These findings suggest that one or more physiological roles of the gill cells (ionoregulation, osmoregulation, respiration, nitrogenous compounds excretion, and acid-base regulation) could be compromised by copper exposure. Although it is difficult to precise the primary cause of copper toxicity based on results from the present study, it is clear that dissolved copper could be disrupting the ionic (particularly Na^+) and osmotic balance in the blue crab. This statement is based on the following facts: (1) copper is very quickly accumulated by the gill cell, since an immediate fluorescence quenching was observed after exposure of intact

gill cells to 100 μM Cu (Fig. 2B); (2) blockade of Na^+ and Cl^- transporters or activity inhibition of the associated enzymes induced significant changes in the dye fluorescence quenching, indicating that copper is sharing some mechanisms of ion transport to enter or leave the gill cell (Fig. 3). This situation could characterize a competition between copper and Na^+ for the same binding sites both at the extracellular or intracellular sides of the gill cell membrane. This competition certainly could lead to a cellular ionic and osmotic imbalance, with a possible impact on extracellular and whole body ionic and osmotic regulation.

Previous studies with euryhaline crustaceans, such as the blue crab, demonstrated the presence of ionic transport pathways from ambient medium to the gill epithelial cells (Péqueux, 1995; Henry, 2001; Kirschner, 2004). There is conflicting evidence concerning the exact mechanism of branchial ion transport, but the current ideas can be summarized as follows. Na^+ uptake across the apical surface of the gill occurs via a combination of Na^+/H^+ and Na^+/NH_4^+ exchangers (Henry, 2001; Kirschner, 2004), and possibly also via Na^+ channel linked to a H^+ extrusion by an apical H^+- ATPase, as it is believed to occur in fish gills (Henry, 2001; Hwang and Lee, 2007). Results from the present study indicate that both Na^+/H^+ exchanger and apical Na^+ channels are present in the membrane of gill cells from the blue crab and that copper is sharing these mechanisms to enter these cells. These statements are based on the following facts: (1) cell exposure to copper together with EIPA, a specific blocker of the Na^+/H^+ exchanger, induced a significant and marked decrease in the fluorescence quenching, indicating a lower amount of copper accumulated in the gill cell (Fig. 3A); (2) the Na^+/H^+ exchanger has been previously reported for several euryhaline crabs, including the blue crab *C. sapidus* (Cameron, 1978; Henry, 2001) and identified in *Carcinus maenas* (Towle et al., 1997); (3) a similar response, but of a lower magnitude, was observed when cells were exposed to phenamil, a specific blocker of the apical Na^+ channel

(Fig. 3A); (4) the bafilomycin A1-induced inhibition of the apical H^+-ATPase, an enzyme associated with the generation of the apical electric gradient responsible for the Na^+ inward current (Grosell et al., 2002), also led to an important reduction of the fluorescence quenching, indicating a reduced copper uptake (Fig. 3A); (5) the H^+-ATPase/Na^+ channel system have been described not only in freshwater or low salinity acclimated crabs (Onken and Putzenlechner, 1995; Weihrauch et al., 2004; Genovese et al., 2005), but also in moderate strong regulators at lower salinity (Kirschner, 2004); (6) inhibition of the carbonic anhydrase by acetazolamide or blockade of the Cl^-/HCO_3^- exchanger by SITS led to a similar and marked decrease in the amount of copper accumulated in the gill cell, indicating that copper uptake is highly dependent on the H^+ provided by carbonic anhydrase from the CO_2 hydration. In both cases, lower H^+ production can be easily explained considering a direct inhibition of the enzyme activity by acetazolamide and an indirect reduction in HCO_3^- extrusion from the cell after exposure to SITS. Carbonic anhydrase has been described to be the enzyme responsible for the generation of the counterions (H^+ and HCO_3^-) for Na^+ and Cl^- uptake in gills of euryhaline crustaceans (Henry, 2001; Genovese et al., 2005).

The fact that a markedly higher decrease in fluorescence quenching, and by analogy in the amount of copper accumulated, was observed after cell exposure to the blocker of the Na^+/H^+ exchanger (phenamil) than to the H^+-ATPase/Na^+ channel system (bafilomycin A1/EIPA) perfectly agrees with the idea that the last system is more expressed in freshwater and low salinity acclimated crabs *Eriocheir sinensis*, *Dilocarcinus pagei* and *Chasmagnathus granulatus* (Onken and Putzenlechner, 1995; Weirauch et al., 2004; Genovese et al., 2005), while the Na^+/H^+ exchanger would be more important for strong or moderate strong regulators euryhaline crabs like *C. sapidus* and *C. maenas* (Cameron, 1978; Henry, 2001). Taken all these evidences together, it is clear that both transporters are constituting a membrane system involved in the coupled exchange of Na^+ with H^+, being an important

pathway for copper uptake in gill cells of the blue crab *C. sapidus* acclimated to seawater. At this point, it is important to note that this system is also involved in intracellular pH regulation and active ammonia excretion (Grosell et al., 2002; Weihrauch et al., 2002). Therefore, copper is potentially affecting the ionic balance in gill cells of the blue crab, but possible effects on the intracellular pH regulation and ammonia excretion must also be considered to understand the primary cause of gill cell mortality.

Evidences have been reported suggesting that the Na^+-K^+-$2Cl^-$ cotransporter is also involved in monovalent ion uptake by crustacean gills (Riestenpatt et al., 1996; Henry, 2001). More recently, Kirschner (2004) proposed that a Na^+-K^+-$2Cl^-$ -mediated Cl^- uptake pathway may exist in estuarine fish, as well as in brackish water crabs. This idea is in line with that reported for estuarine crabs like *C. granulatus* and *C. maenas*, where an electrogenic ion uptake across the gill epithelium would be taking place through an apical Na^+-K^+-$2Cl^-$ cotransporter in series with the Na^+/K^+-ATPase, and K^+ and Cl^- channels at the basolateral membrane (Riestenpatt et al., 1996; Onken et al., 2003). To access a possible involvement of the Na^+-K^+-$2Cl^-$ cotransporter in copper uptake, isolated gill cells from the blue crab were pre-exposed to furosemide, a potent inhibitor of this cotransporter, and then exposed to copper. Furosemide only partially reduced the amount of copper accumulated (Fig. 3A), suggesting that copper uptake in isolated gill cells could be involving other sodium-dependent mechanisms than the Na^+-K^+-$2Cl^-$ cotransporter. In tilapia gills, a heterologous antibody revealed apical Na^+-K^+-$2Cl^-$ cotransporter signals in mitochondria-rich (MR) cells, and this apical cotransporter was suggested to be associated with a Na^+-Cl^- cotransporter for Na^+ and Cl^- uptake (Hwang and Lee, 2007). Therefore, another mechanism of ion transport possibly involved in copper uptake in gill cells would be the Na^+-Cl^- cotransporter. In fact, our results clearly show that this cotransporter is playing an important role as via for copper uptake in the gill cells of the blue crab. This statement is supported by

the marked decrease in the amount of intracellular copper observed when cells were exposed to copper together with benzthiazide, a Na^+-Cl^- cotransporter blocker (Fig. 3A).

To sum up, copper uptake by the isolated gill cells of the blue crab *C. sapidus* seems to be dependent on the combined action of the following mechanisms of Na^+ uptake: Na^+/H^+ exchanger, Na^+ channel, and Na^+-K^+-$2Cl^-$ and Na^+-Cl^- cotransporters. Therefore, a possible physiological mechanism of copper toxicity in gill cells would be a decrease in intracellular Na^+ concentration caused by copper competition on the binding sites associated with Na^+ uptake. Once copper enters the gill cell, it can induce a further decrease in Na^+ uptake due to its inhibitory effect on the Na^+/K^+-ATPase and carbonic anhydrase activities. In fact, Na^+/K^+-ATPase provides the main driving force for active Na^+ uptake across the gill epithelium in euryhaline crabs (Péqueux, 1995; Towle et al., 2001; Lucu and Towle, 2003), and copper has been previously shown to inhibit both Na^+/K^+-ATPase (Bianchini et al., 2003; Pinho et al., 2007) and carbonic anhydrase activity (Vitale et al., 1999; Grosell et al., 2002; Skaggs and Henry, 2002; Grosell et al., 2007) in euryhaline crustaceans, including crabs.

Until now it had been assumed that the interactions between copper and the Na^+/K^+-ATPase appears to be of a competitive nature at the magnesium binding site, which is critical for phosphorylation of the enzyme. An interference of copper with sulphydryl groups has also been pointed to affect the three dimensional structure of the enzyme (Grossel et al., 2002; Brooks and Mills, 2003). However, our results provided *in vitro* pharmacological evidences for the involvement of the Na^+/K^+-ATPase in copper extrusion from the gill cells of the blue crab *C. sapidus*. In fact, a significant increase in the amount of copper accumulated was observed in cells exposed to copper together with ouabain, a specific inhibitor of the Na^+/K^+-ATPase activity (Fig. 3B). This finding suggests that copper is possibly competing also for the Na^+ binding sites of the enzyme.

Na^+/K^+- ATPase is not only involved in the Na^+ extrusion from the gill cell, but it also energizes secondary active transporters in both apical and basolateral membranes. These may include the Na^+/Ca^{2+} exchanger firstly identified in membrane vesicles of *C. maenas* gills (Flik et al., 1994). Although studies suggest that Na^+/K^+- ATPase plays a central role in driving Na^+/Ca^{2+} exchanger in the molting process (Ziegler, 1997), this transporter may also be a target of the ionoregulatory processes (Lucu and Towle, 2003). Our results showed that amiloride induced at elevated concentration (1mM) a marked increase in the amount of copper accumulated in the isolated cell of the blue crab. It has been shown that milimolar amiloride at concentration blocks the Na^+/Ca^{2+} exchanger (Masereel et al., 2003). Therefore, this findings suggest that the Na^+/Ca^{2+} exchanger, as Na^+/K^+- ATPase, is a possible via for copper extrusion from the gill cell.

In light of the results discussed above, we can state that copper accumulation in isolated gill cells of the blue crab is very dependent on the activity of several ion transporters involved in Na^+ uptake or extrusion from the cell. However, some of our data also clearly suggest that copper accumulation in the isolated gill cells is also dependent on the electrical state of the cytosol. This hypothesis is supported by the marked increase in copper accumulation after cell exposure to NPPB, a Cl^- channel blocker (Fig. 3B). Under this situation, the outwardly current of Cl^- through the Cl^- channels would be reduced, inducing to an increased intracellular concentration of Cl^- and a consequent intracellular electronegativity. In turn, this electronegativity could be driving more copper to inside the cell. On the other hand, blockade of the K^+ channel with quinine sulfate would induce a decrease in the outwardly current of K^+ through the K^+ channels, inducing to an increased intracellular concentration of K^+ and a consequent intracellular electropositivity. This new electrical state of the cytosol would difficult the copper entry to the cell.

Based on results from the present study, we propose a general model depicting possible mechanisms of ion transport involved in copper uptake and extrusion in isolated cells from the posterior gills of the blue crab *C. sapidus* (Fig. 4). According to this model, extracellular copper can enter gill cells competing with Na^+ for binding sites on the following ion transporters: Na^+/H^+ exchanger, Na^+ channel, and Na^+-K^+-$2Cl^-$ and Na^+-Cl^- cotransporters. Carbonic anhydrase and H^+-ATPase also play pivotal roles in copper accumulation in the gill cells, since they are indirectly or directly involved in H^+ extrusion from the cell coupled to a Na^+ uptake. On the other hand, copper extrusion from the cells seems to occur via the Na^+/K^+-ATPase and Na^+/Ca^{2+}. Also in this case, copper seems to compete with Na^+ by the binding sites on these molecules. Interferences of copper on the mechanisms of intracellular ionic and osmotic regulation are certainly part of the mechanism(s) causing the observed copper toxicity in the isolated gill cells of the blue crab *C. sapidus*.

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Legend for figures

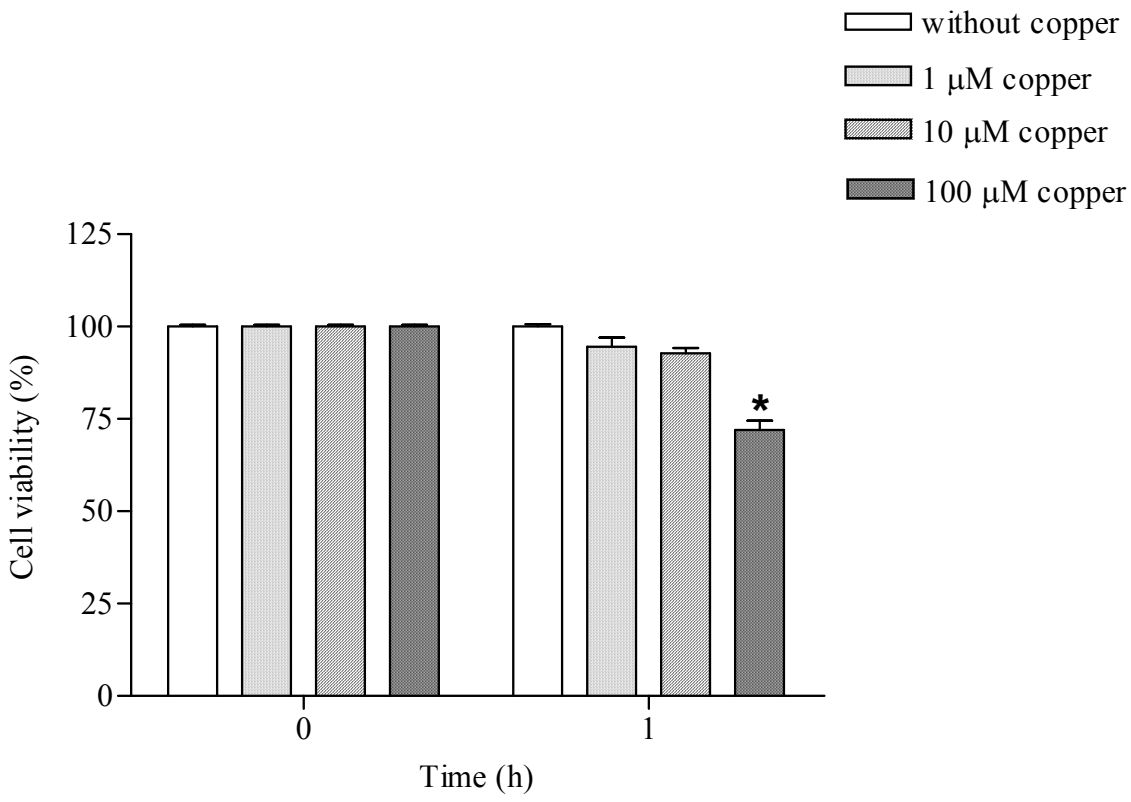
Figure 1. Viability of isolated cells from the posterior gill cells of the blue crab *Callinectes sapidus* exposed to different concentrations of copper ($CuCl_2$). Cells were tested at a final density of 10^4 cells mL^{-1}. * Indicates significantly different means ($p<0.05$) between copper-exposed cells and the respective control, for the same experimental time.

Figure 2. Fluorescence intensity of the dye Phen Green induced by increasing concentrations of copper ($CuCl_2$) in cell lisates (A) or by addition of 100 μM Cu in the saline solution used to incubate intact cells (B). In both cases, isolated cells (10^4 cells mL^{-1}) from posterior gills of the blue crab *Callinectes sapidus* were previously loaded with the fluorescent dye (1.5 μM) for 1 h. Inset: fluorescence quenching (ΔF) at different copper concentrations over time.

Figure 3. Percentages of fluorescence quenching (ΔF) in isolated cells from posterior gills of the blue crab *Callinectes sapidus* in the presence of copper (100 μM) or copper (100 μM) together with different drugs: (A) 100 μM furosemide (blocker of Na^+-K^+-$2Cl^-$ cotransporter), 1 μM phenamil (blocker of Na^+ channel), 500 μM SITS (inhibitor of Cl^- /HCO_3^- exchanger), 100 μM benzthiazide (blocker of Na^+-Cl^- cotransporter), 1 μM acetazolamide (blocker of carbonic anhydrase enzyme), 500 μM quinine sulfate (blocker of K^+ channel), 1 μM bafilomycin A_1 (specific inhibitor of the H^+-ATPase) and 500 μM EIPA (blocker of Na^+/H^+ exchanger); (B) 100 μM ouabain (blocker of Na^+/K^+-ATPase), 1 mM amiloride (inhibitor of Na^+/Ca^{2+} exchanger) and 100 μM NPPB (blocker of Cl^- channel). Fluorescence quenching (ΔF) in the copper only exposure was considered as 100%. * Indicates significantly different means ($p<0.05$) from the copper only exposure.

Figure 4. A schematic illustration of a general model indicating possible mechanisms involved in copper uptake and extrusion in isolated cells from posterior gills of the blue crab *Callinectes sapidus*. Dotted lines represent passive diffusive movements and solid lines describe all other events. Carriers marked with ATP perform ion active transport dependent on the dephosphorylation of adenosine triphosphate (ATP). CA = carbonic anhydrase.

Figure 1


without copper
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Cell viability (%)
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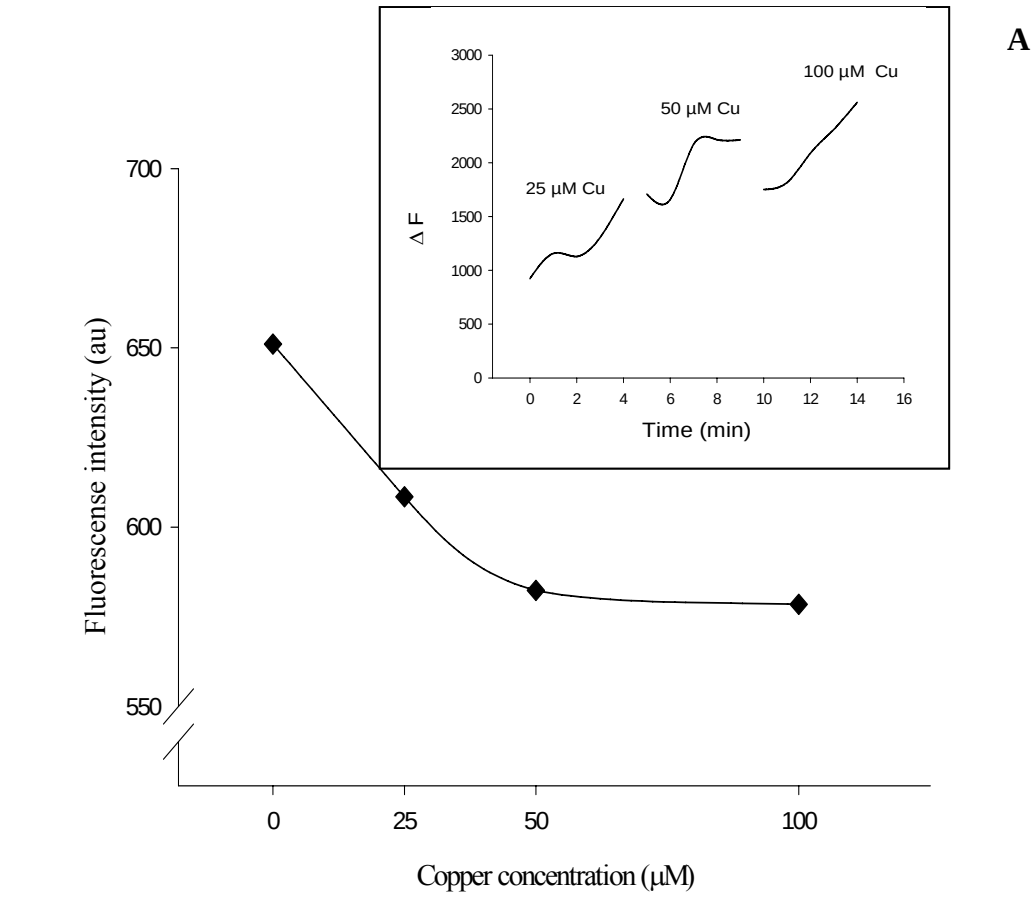
Time (h)

Figure 2



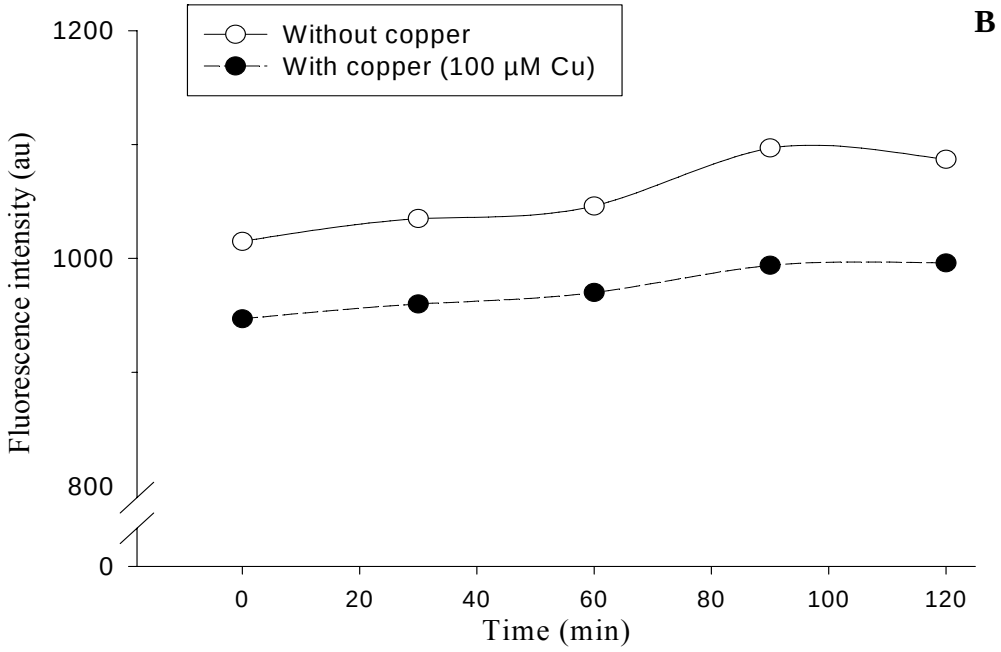
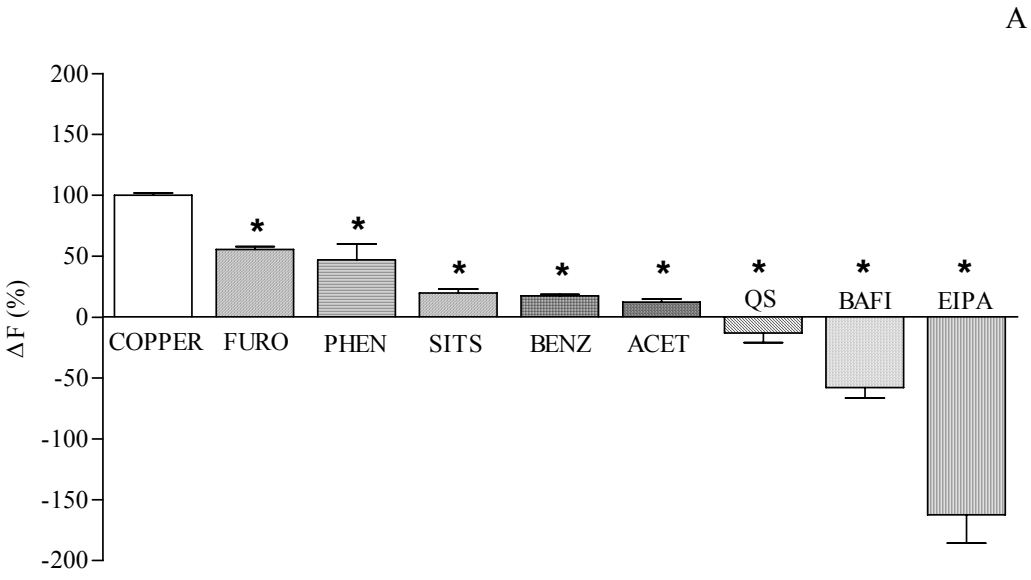


Figure 3

Figure 3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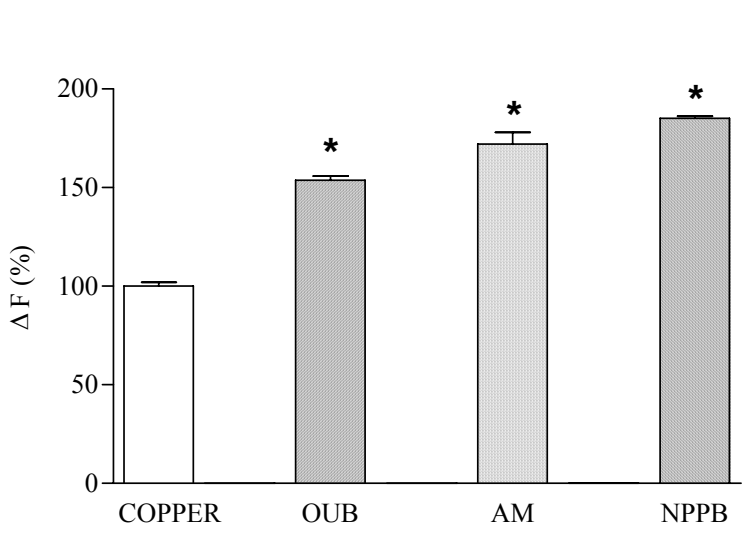
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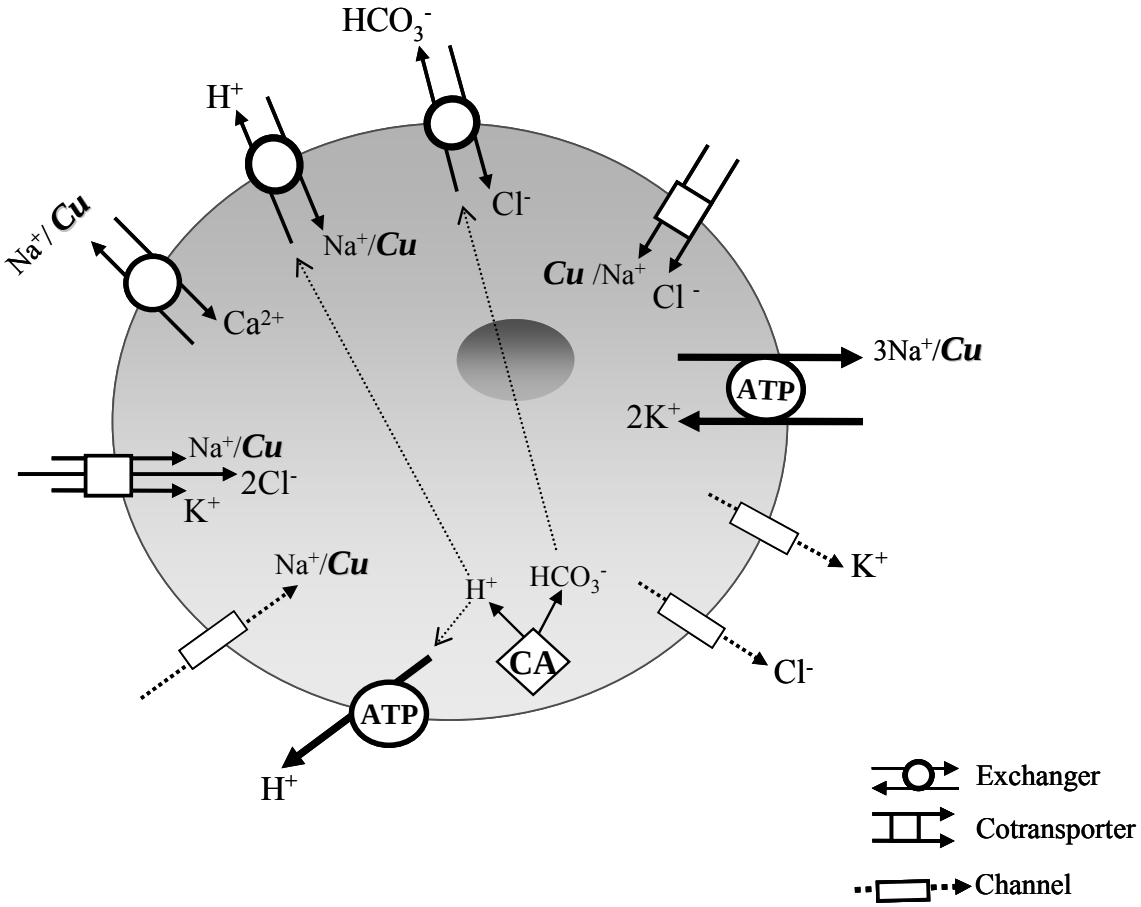
Figure 4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3Na+/Cu
ATP
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Na+/Cu
2Cl-
K+
K+
Na+/Cu
H+
HCO3-
CA
Cl-
ATP
H+
Exchanger
Cotransporter
Channel

Anexo 4. Sumário dos dados estatísticos

Análises estatísticas referentes ao ANEXO 6.1

➦ **Análise química da água:**

1) Fator: área amostral

Níveis: ponto 1, ponto 2, ponto 3 e ponto 4

- período de 6 meses que antecedeu à coleta:

2) Nº de réplicas: 3 amostras de 10 ml de água por ponto amostral coletadas sazonalmente.

3) Variável avaliada: concentração de cobre expressa em μg Cu/ml.

Foi realizada análise de variância – ANOVA: variável independente foi considerada os pontos amostrais e a variável dependente a concentração de cobre.

Quanto aos dados de temperatura e salinidade, estes foram apresentados como médias.

- período da coleta:

Foi realizada uma média dos dados de temperatura, salinidade e concentração de cobre.

➦ **Análise *in situ* da concentração de cobre em células branquiais:**

1) Fator: área amostral

Níveis: ponto 1, ponto 2, ponto 3 e ponto 4

2) Nº de réplicas: 4 diferentes preparações de dissociações celulares por ponto amostral – cada dissociação celular composta por brânquias de 5 animais.

3) Variável avaliada: concentração de cobre expressa em ng Cu/10^5 células.

Foi realizada análise de variância – ANOVA: variável independente foi considerada os pontos amostrais e a variável dependente a concentração de cobre.

➦ **Análise *in vitro* da concentração de cobre em células branquiais:**

1) Fator: área amostral

Níveis: ponto 1 + 100 μM Cu, ponto 2+ 100 μM Cu, ponto 3+ 100 μM Cu e ponto 4 + 100 μM Cu.

2) Nº de réplicas: 4 diferentes preparações de dissociações celulares por ponto amostral – cada dissociação celular composta por brânquias de 5 animais.

3) Variável avaliada: concentração de cobre expressa em ng $Cu/10^5$ células.

Foi realizada análise de variância – ANOVA: variável independente foi considerada os pontos amostrais e a variável dependente a concentração de cobre.

➦ **<u>Análise da influência da salinidade de aclimatação</u>: técnica EAA**

1) Fator: salinidade

Níveis: salinidade 2 e salinidade 30

2) Nº de réplicas: 4 diferentes preparações de dissociações celulares por salinidade experimental – cada dissociação celular composta por brânquias de 5 animais.

3) Variável avaliada: concentração de cobre expressa em ng $Cu/10^5$ células.

Foi realizado teste – t para comparar as duas salinidades experimentais.

➦ **<u>Análise da influência da salinidade de aclimatação</u>: técnica da Fluorescência**

1) Fator: salinidade

Níveis: salinidade 2 e salinidade 30

2) Nº de réplicas: 4 diferentes preparações de dissociações celulares por salinidade experimental – cada dissociação celular composta por brânquias de 5 animais.

3) Variável avaliada: variação do sinal de fluorescência expresso em $\Delta F/10^5$ células.

Foi realizado teste – t para comparar as duas salinidades experimentais.

➦ **<u>Análise da influência do choque osmótico</u>:**

1) Fator: salinidade de aclimatação e solução

Níveis: solução isosmótica para salinidade 2, solução hiper-osmótica para salinidade 2, solução isosmótica para salinidade 30 e solução hipo-osmótica para salinidade 30.

2) Nº de réplicas: 4 diferentes preparações de dissociações celulares por salinidade experimental – cada dissociação celular composta por brânquias de 5 animais.

3) Variável avaliada: variação do sinal de fluorescência expresso em $\Delta F/10^5$ células.

Foi realizada análise de variância – ANOVA: variável independente foi considerada a solução isosmótica para salinidade 2, solução hiper-osmótica para salinidade 2, solução isosmótica para salinidade 30 e solução hipo-osmótica para salinidade 30; e a variável dependente a concentração de cobre e a variável dependente o ΔF.

Análises estatísticas referentes ao ANEXO 6.2

➦ **Análise da acumulação de cobre:**

1) Fator: tempo e concentração de exposição

Níveis: tempo 0 (T0), tempo 1 (T1), tempo 3(T3) e tempo 6 (T6); controle (sem cobre), concentração de 1 μM Cu, concentração de 10 μM Cu e concentração de 100 μM Cu.

2) Nº de réplicas: 3 diferentes preparações de dissociações celulares por ponto amostral – cada dissociação celular composta por brânquias ou hepatopâncreas de 5-6 animais.

3) Variável avaliada: concentração de cobre expressa em ng $Cu/10^6$ células.

Foi realizada análise de variância – ANOVA ao longo do tempo para cada concentração experimental: variável independente foi considerada a concentração de cobre experimental e a variável dependente a concentração de cobre intracelular.

➦ **Análise da toxicidade de cobre:**

1) Fator: tempo e concentração de exposição

Níveis: tempo 0 (T0), tempo 1 (T1), tempo 3(T3) e tempo 6 (T6); controle (sem cobre), concentração de 1 μM Cu, concentração de 10 μM Cu e concentração de 100 μM Cu.

2) Nº de réplicas: 6 diferentes preparações de dissociações celulares– cada dissociação celular composta por brânquias ou hepatopâncreas de 5-6 animais.

3) Variável avaliada: viabilidade celular expressa em %.

Foi realizada análise de variância – ANOVA ao longo do tempo para cada concentração experimental: variável independente foi considerada a concentração de cobre experimental e a variável dependente foi considerada a viabilidade celular (transformação utilizada para % foi √arco-seno(Variável)).

Análises estatísticas referentes ao ANEXO 6.3

➦ **Análise da participação dos mecanismos de transporte:**

1) Fator: cobre e bloqueador/inibidor

Níveis: solução salina, solução de cobre, solução salina com bloqueador/inibidor e solução de cobre com bloqueador/inibidor.

2) Nº de réplicas: 3-4 diferentes preparações de dissociações celulares por salinidade experimental – cada dissociação celular composta por brânquias de 2-6 animais.

3) Variável avaliada: variação do sinal de fluorescência expresso em % ΔF.

Foi realizada análise de variância – ANOVA para cada droga utilizada, onde: a variável independente foi considerada os níveis (solução salina, solução de cobre, solução salina com bloqueador/inibidor e solução de cobre com bloqueador/inibidor) e a variável dependente, o ΔF.