Universidade Federal do Rio Grande – FURG
Programa de Pós-Graduação em Ciências Fisiológicas
Fisiologia Animal Comparada

ISOLAMENTO E IDENTIFICAÇÃO DE CÉLULAS BRANQUIAIS RICAS EM MITOCÔNDRIAS DE BIVALVES E SUA UTILIZAÇÃO EM ENSAIOS TOXICOLÓGICOS

Lygia Sega Nogueira

Rio Grande, maio de 2013

ISOLAMENTO E IDENTIFICAÇÃO DE CÉLULAS BRANQUIAIS RICAS EM MITOCÔNDRIAS DE BIVALVES E SUA UTILIZAÇÃO EM ENSAIOS TOXICOLÓGICOS

Lygia Sega Nogueira

Tese de doutorado apresentada no âmbito do Programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada, como requisito parcial para obtenção do título de Doutor.

Orientador: Dr. Adalto Bianchini
Co-orientador: Dr. Chris M. Wood

Banca examinadora:

Professora Dra. Marta Marques de Souza (ICB-FURG)

Professora Dra. Juliana Zomer Sandrini (ICB-FURG)

Professora Dra. Samantha Eslava Gonçalves Martins (ICB-FURG)

Professora Dra. Viviane Prodócimo (UFPR)

Rio Grande, maio de 2013

AGRADECIMENTOS

Ao meu orientador, Adalto Bianchini, pessoa que continuo admirando muito. Obrigada por todas as oportunidades que me foram cedidas durante o período de Doutorado (não tenho palavras para agradecer!), por toda confiança e ensinamentos durante todos esses anos.

À prof[a]. Dra. Marrrrrrrrta Marrrrrrrques de Souza, por novamente aceitar participar da minha banca examinadora. Além disso, obrigada por todas nossas conversas de laboratório e na sua sala, conselhos experimentais e pessoais e também por todo incentivo! (que foi fundamental para o desenvolvimento da técnica do "Percoll").

Às prof[as]. Dra. Juliana Zomer, Dra. Samantha Martins e Dra. Viviane Prodocimo por aceitarem o convite em participar da minha banca examinadora e também por todas as contribuições neste trabalho.

Ao Dr. Chris Wood, pesquisador que sempre admirei e que passei admirar ainda mais depois de conhecer pessoalmente. Obrigada por todos ensinamentos (que não são poucos!) durante (e após) minha estadia no Canadá. Obrigada também pela excelente receptividade e preocupação com meu bem-estar pessoal, além do empréstimo do "wood-móvel" !!! hehe...

À pesquisadora Dra. Patty Gillis, pela oportunidade em trabalhar na Environment Canada e por toda ajuda e ensinamentos. A experiência foi ótima. Também gostaria de agradecer aos técnicos do laboratório Rodney e Tina pela paciência que tiveram com a "invasão das brasileiras" no lab, e também por todo carinho enquanto estive lá.

À Indianara Barcarolli, Ana Kalb e prof. Dr. Robert Boyle por toda ajuda durante alguns experimentos desenvolvidos na tese.

À minha família: mãe, pai, irmão e irmã!!!! Obrigada por todo amor e toda torcida durante todos esses anos !! Mesmo distantes fisicamente sempre participam de todas as angústias e alegrias da vida pessoal e profissional!! Viva o mundo virtual !! Obrigada por TUDO !!!!! Amo vocês !!!

À família rio-grandina que escolhi: Robs, Flávio, "aitê", Nana e Samy. Feliz por continuar agradecendo à vocês por todos os momentos rio-grandinos! Nos momentos tristes ou felizes, sempre estão presente para ajudar, me aguentar ou incomodar!! hehe...

Também gostaria de agradecer à Nana pelo empréstimo de toda sua família durante todos esses anos! Obrigada por todos os almoços de domingo, festas de família, festas de Natal e Ano

Novo, Páscoa, além dos primos, tias e tios que “ganhei” !!!! A “bagunça de família” é fundamental!! hehe...

À duas grandes amigas que fiz durante minha estadia no Canadá: Emily e Margaret. Eu não tenho palavras para agradecer tudo que vocês fizeram por mim. Vocês foram amigas fundamentais durante um período muito feliz e ao mesmo tempo difícil da minha vida. Sem vocês, com certeza eu teria voltado para o Brasil um pouco mais cedo. Muuuuuuuuito obrigada! E Maggie, obrigada por todos os abraços !!! ;)

Às minhas amigas Lívia, Babi, Thaisa, Pri e Vanessa que ainda distante fazem parte da minha vida diária. Nossos contínuos e-mails coletivos (que ainda vão virar um livro!) são extremamente importantes nos momentos de desabafo, de conquistas e fofocas (hehe!).

Aos meus amigos desde a época de facul: Vitor, Kamilla, Aline e Rose. Sempre estiveram presente durante todos esses anos!! Muuuuuito obrigada pela amizade de vocês!!!

À Galera (atual ou não) da Sala 3: Robs Klein, Vanessa, Alê, Roberta Socoowski, Marcinha, Eve, Josencler (sempre sala 3), Ana Kalb, Indi (eterna rainha), Cinthia, Ana Laura, Fran, André “Caçapava”, Alzira e etc !! Sempre prontos a dar risadas, dar e receber abraços (importatíssimos em alguns momentos do desenvolvimento da tese!), passear no CC, tomar chimarrão, além de mandar ficar quieto e ir trabalhar!!! hehehe...

Aos amigos e colegas de laboratório por toda a boa convivência, ajuda e risadas durante esses anos: Abel, Roberta, Laura, Joseane, Marina, Laís, Vinícius, Iuri, Bruna, Cinthia, Arthur, Marianna Jorge, Mariana Lauer, Indi, Daiane.

E por fim, mas não menos importante, a todos os professores, amigos, professores/amigos e técnicos do ICB: Cami Dal, Camila(ão), Jú do Nino e o Nino, Biba, Kiti, “meninas do lab de Cultura Celular”, Sandrinha, Glauce, Thaís, Bernardo, Josencler e tantos outros ....

SUMÁRIO

RESUMO GERAL

No presente estudo, células branquiais do mexilhão de água doce *Lasmigona costata* e do marisco de água salgada *Mesodesma mactroides* foram isoladas, fracionadas, caracterizadas e utilizadas em ensaios toxicológicos com cobre (Cu). As células brânquiais do mexilhão e do marisco foram isoladas utilizando-se dissociação enzimática e mecânica do tecido, respectivamente. Para ambas as espécies, gradiente de densidade (Percoll) foi utilizado para separar as células isoladas em duas Frações (I e II). A presença de células ricas em mitocôndrias foi caracterizada na Fração II através de marcadores mitocondriais e da medida da atividade da Na^+,K^+-ATPase. A possibilidade de aplicação deste modelo biológico em estudos toxicológicos *in vitro* foi avaliada através da exposição das células da Fração II a concentrações ambientalmente relevantes de Cu (5, 9 e 20 µg/L) em condição isosmótica (840 mOsmol/Kg H_2O). Para ambas as espécies, o Cu não induziu toxicidade letal, mas causou acumulação celular do metal e diminuição do conteúdo de Na^+. De fato, uma correlação negativa entre estes parâmetros foi observada, sugerindo a competição do Cu com o Na^+ por sítios de ligação na membrana plasmática. Posteriormente, foi realizada a exposição de células ricas em mitocôndrias de *M. mactroides* seguida da análise da viabilidade celular, do conteúdo iônico (Na^+, K^+, Ca^{2+}, Mg^{2+} e Cl^-) e da atividade de enzimas envolvidas na regulação iônica (Na^+,K^+-ATPase e anidrase carbônica), nas condições descritas acima. Foi observada redução no conteúdo de Na^+, K^+ e Mg^{2+} e inibição da atividade da anidrase carbônica após exposição a 20 µg Cu/L, sem efeito na viabilidade celular, nos conteúdos Ca^{2+} e Cl^- e na atividade da Na^+,K^+-ATPase. Visto que a salinidade da água influencia a toxicidade do Cu, células ricas em mitocôndria de *M. mactroides* foram mantidas em condição isosmótica ou transferidas para meios hiposmóticos (730 e 670 mOsmol/Kg H_2O) na ausência e na presença de Cu. Neste caso, foram avaliadas a viabilidade celular, o conteúdo de Cu e de Na^+, bem como as atividades da Na^+,K^+-ATPase e anidrase carbônica. Na ausência de Cu, o choque hiposmótico reduziu a viabilidade celular e o conteúdo de Cu, sem alterar o conteúdo de Na^+ e a atividade das enzimas. Na presença de Cu, houve acúmulo de Cu, redução do conteúdo de Na^+ e inibição da atividade da anidrase carbônica em células expostas a 20 µg Cu/L em meio isosmótico. Na exposição em meio hiposmótico, houve acúmulo de Cu e inibição da atividade da anidrase carbônica nas células expostas a 9 µg Cu/L e redução do conteúdo de Na^+ e inibição das atividades da Na^+,K^+-ATPase e anidrase carbônica nas células expostas a 20 µg Cu/L. Estes resultados indicam a viabilidade das técnicas empregadas para isolamento e fracionamento de células ricas em mitocôndria de brânquias de *L. costata* e *M. mactroides*, bem como o uso destas células em testes toxicológicos visando a compreensão do mecanismo de toxicidade do Cu em ambientes dulcícolas e marinhos. Além disso, indicam que o Cu é um tóxico ionorregulatório em bivalves de água doce e marinha e que não somente a química da água, mas também a fisiologia do animal é importante para previsão da toxicidade do Cu. Neste contexto, os resultados do presente estudo apontam a atividade da anidrase carbônica de células branquiais ricas em mitocôndrias de bivalves como um potencial biomarcador da exposição ao Cu em ambientes aquáticos.

Palavras-chave: brânquia, cobre, célula isolada, ionorregulação, marisco, mexilhão, salinidade

ABSTRACT

In the present study, gills cells from the freshwater mussel *Lasmigona costata* and the seawater clam *Mesodesma mactroides* were isolated, fractionated, characterized and employed in toxicological assays with copper (Cu). Mussel and clam gill cells were isolated employing enzymatic and mechanical dissociation, respectively. For both species, density gradient (Percoll) was employed to separate isolated cells into two Fractions (I e II). The presence of cells rich in mitochondria was characterized in the Fraction II through mitochondrial dyes and Na^+,K^+-ATPase activity measurement. The possible application of this biological model in *in vitro* toxicological studies was evaluated exposing cells of Fraction II to environmental relevant Cu concentrations (5, 9 and 20 µg/L) in isosmotic medium (840 mOsmol/Kg H_2O). For both species, Cu exposure did not induce lethal toxicity, but caused cellular accumulation of Cu and reduction in Na^+ content. A negative correlation between these parameters was observed, suggesting a competition between Cu a Na^+ for binding sites on the plasma membrane. Following this experiment, cells rich in mitochondria isolated from gills of *M. mactroides* were exposed to Cu under the experimental conditions described above and cellular viability, ionic content (Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-), and the activity of enzymes involved in ionic regulation (Na^+,K^+-ATPase and carbonic anhydrase) were analyzed. Reduced Na^+, K^+ and Mg^{2+} content and inhibition of carbonic anhydrase activity was observed in cells exposed to 20 µg Cu/L, without changes in cell viability, Ca^{2+} and Cl^- contents, and Na^+,K^+-ATPase activity. Because water salinity can influence Cu toxicity, cells rich in mitochondria of *M. mactroides* were maintained in isosmotic medium or transferred to hypoosmotic media (730 e 670 mOsmol/Kg H_2O) for 3 h, in the absence or the presence of Cu. In this case, cell viability, Cu and Na^+ contents, and enzyme activity (Na^+,K^+-ATPase and carbonic anhydrase) were analyzed. In the absence of Cu, the hypoosmotic shock reduced cell viability and Cu content, without effects on Na^+ content and enzymes activities. In the presence of Cu, there was Cu accumulation, reduction in Na^+ content and inhibition of the carbonic anhydrase activity in cells exposed to 20 µg Cu/L in isosmotic medium. In the hypoosmotic media, there were Cu accumulation and inhibition of the carbonic anhydrase activity in cells exposed to 9 µg Cu/L and decrease in Na^+ content and inhibition of the activity of both Na^+,K^+-ATPase and carbonic anhydrase in cells exposed to 20 µg Cu/L. These findings indicate the viability of techniques employed to isolate and fractionate cells rich in mitochondria from gills of *L. costata* and *M. mactroides*, as well as their use in toxicological assays aiming to understand the mechanism of Cu toxicity in freshwater and seawater environments. Also, they indicate that Cu is an ionoregulatory toxicant in freshwater and seawater bivalves and suggest that not only the water chemistry, but animals physiology is important in predicting Cu toxicity. In this context, results from the present study point out the carbonic anhydrase activity of gill cells rich in mitochondria from bivalves as a potential biomarker of Cu exposure in aquatic environments.

Keywords: clam, copper, gills, isolated cell, ionoregulation, mussel, salinity

INTRODUÇÃO

Bivalves e seu uso em ecotoxicologia

Pertencente ao filo Mollusca, os bivalves são um grupo de animais que têm como característica exclusiva a presença de duas conchas carbonadas. Essas conchas são formadas por duas valvas que protegem os tecidos moles destes animais. Por filtração, os bivalves alimentam-se de material em suspensão na água através de estruturas chamadas sifões inalantes e exalantes. Exclusivamente aquáticos, esses animais são bentônicos e podem ser encontrados em ambientes de água salgada, doce ou salobra (Rupert e Barnes, 1996).

O sucesso evolutivo destes animais nos diferentes ambientes aquáticos se deve ao fato da presença de diferentes padrões osmorregulatórios. Os bivalves marinhos são osmoconformadores, ou seja, alteram a concentração osmótica de seus fluídos corpóreos e tornam-se isosmóticos em relação ao meio (Gilles, 1979; Zanotto e Wheatly, 2006), apresentando alto grau de tolerância osmótica celular (Randall et al., 2000). No entanto, osmoconformadores com tolerância baixa a variação de salinidade externa são chamados estenoalinos, e espécies com uma alto grau de tolerância à salinidade são chamados eurialinos (Evans, 2008). Eurialinidade portanto, é uma característica necessária a animais estuarinos ou àqueles que pertecem a a zona costeira onde as flutuações de salinidade ocorrem de forma natural. Já animais de água doce possuem a capacidade de manter a concentração osmótica de seus fluídos corpóreos estáveis até níveis semelhantes ao do ambiente, a partir disso, estes animais passam a se comportar de forma osmoconformadora.

Apesar dos diferentes padrões osmorregulatórios observados nestes animais, suas células branquiais usam estratégias osmorregulatórias que preservam seu volume e a concentração de eletrólitos durante essas possíveis variações da salinidade. Estas respostam ocorrem através da

regulação adaptativa de permeabilidade à água e aos íons, bem como regulação de osmólitos orgânicos (Kültz, 2001; Marshall e Grosell, 2005). Células expostas a um aumento da concentração osmótica do meio diminuiem seu volume celular e, para que o volume celular seja restaurado ocorre um aumento da concentração de osmólitos citoplasmáticos. No entanto, quando estas mesmas células são expostas a ambientes de menor osmolaridade ocorre um aumento do seu volume celular devido ao influxo de água. Nesse caso, o mecanimos para redução osmótica intracelular é a liberação de osmólitos para o fluído extracelular (Smith e Pierce, 1987; Quinn e Pierce, 1992). A ausência destas estratégias poderia resultar em problemas no funcionamento celular através das alterações no gradiente de difução de água e íons na membrana celular em função das mudanças osmóticas do fluído extracelular.

Além de afetar a regulação iônica e osmótica em animais aquáticos, a variação na osmolaridade externa pode alterar também a sensibilidade dos organismos à exposição a metais. Isto se dá em função da influência da quantidade de íons na água nos fenômenos de complexação com o metal, bem como especiação. Assim, considerando somente o efeito da química da água, é esperado que metais sejam menos tóxicos aos organismos em água salgada. Isto se dá pelo aumento da complexação do metal com os ânions presentes em grande quantidade na água, especialmente o Cl^-, bem como a maior competição entre o metal e os cátions presentes em maiores concentrações na água pelos sítios de ligação na membrana das células branquiais (Paquin et. al., 2002).

Efeitos do cobre sobre a regulação iônica celular

Os metais são constituintes naturais no ecossistema marinho e de água doce, sendo introduzidos no ambiente através de processos geoquímicos (Santore et al., 2000) . Entretanto, as

atividades humanas tem alterado de forma significativa os ciclos biogeoquímicos naturais destes elementos, que são estáveis e persistentes. O cobre, por exemplo, tem sua ocorrência considerada como um fenômeno natural, pois grande porcentagem do total anual lançado nos oceanos é resultado de processos de intemperismo (Pedroso e Lima, 2001). Nos ambientes aquáticos, este metal pode se encontrar na forma solúvel, associado a partículas sedimentares ou ainda em suspensão na água. A captação, distribuição e disposição desses metais nos organismos são tipicamente governadas por processos bioquímicos específicos (Nordberg et al., 2007).

Os efeitos biológicos relacionados à exposição ao cobre estão associados às propriedades químicas dos metais, tais como sua alta afinidade por grupos sulfidrilas (-SH) (Webb, 1979) e grupos imidazólicos, provenientes de ácidos nucléicos, substratos e metabólicos (afinidade ao S > N > O) (Freeman, 1973). Este metal é o único elemento que passa do estado oxidado (Cu^{2+}) a um estado reduzido (Cu^{+}) em ambiente celular, mudando significativamente sua afinidade pelos grupos –SH, sendo esta menor apenas que a do mercúrio ($Hg^{2+} > Cu^{+} > Cd^{2+} > Cu^{2+} > Zn^{2+}$) (Viarengo et al., 2002).

Nos bivalves, a captação de cobre pode ocorrer de várias maneiras, através das membranas celulares por meio da camada externa epitelial das brânquias, bem como de outros órgãos localizados na cavidade paleal, tais como o manto, as gônadas e a glândula digestiva (Le Pennec e Le Pennec, 2001). Durante a interação do cobre com quaisquer destes tecidos, ocorre a sua redução para Cu^{+}, mesmo que no ambiente aquático ele esteja predominantemente sob a forma de íon bivalente. A capacidade do cobre em ser transferido através da membrana celular é claramente um dos fatores principais na sua toxicidade, uma vez que este metal pode afetar a regulação iônica através da competição com cátions presentes na água, tais como Na^{+}, K^{+} e Ca^{2+}, por sítios de ligação na membrana plasmática das células branquiais (Bianchini e Wood, 2002;

Grosell et al. 2002; Paquin et al., 2002; Jorge et al., 2012). Consequentemente, qualquer distúrbio na regulação da concentração desses cátions interfere no metabolismo e função celular (Kültz, 2001). De fato, o metabolismo celular, durante o curso da evolução, foi adaptado para a manutenção das concentrações intracelulares de íons, particularmente K^+, Na^+, Mg^{2+}, Ca^{2+} e Zn^{2+}, bem como de outros eletrólitos que definem a força iônica intracelular, dentro de limites adequados.

Além do efeito gerado pela competição iônica por ligantes da membrana celular, o cobre pode afetar a atividade de enzimas relacionadas ao transporte iônico, tais como Na^+,K^+-ATPase e anidrase carbônica, devido sua afinidade por grupos tióis. Em alguns estudos, o mecanismo de toxicidade do metal tem sido relatado estar relacionado à uma insuficiência osmorregulatória associada a inibição de algumas ATPases branquiais (McGeer e Wood, 1998). Assim, Stagg e colaboradores (1992) sugeriram a avaliação da atividade de ATPases como um sinal de alerta de danos osmorregulatórios e do sistema ácido-base celular causados por poluentes, como os metais.

Estudos com análise da atividade da Na^+,K^+-ATPase em brânquias de peixes de água doce sugerem que o cobre pode afetar a atividade enzimática por ligar-se aos grupos tióis encontrados nas subunidades protéicas, bem como pela inibição estequiométrica dos sítios de ligação do Mg^{2+}, através da substituição do íon pelo metal (Li et al. 1998; Handy et al. 2002), sendo relacionado a um efeito indireto no distúrbrio na regulação de Na^+ (Blanchard e Grosell, 2006). No entanto, este mesmo efeito não foi observado em peixes de água salgada, ocorrendo uma alteração na captação de Na^+ porém sem alteração na atividade da Na^+,K^+-ATPase (Grosell et al., 2003, 2004; Blanchard e Grosell, 2006). Em virtude dessa alteração não estar relacionada à mudanças na atividade da Na^+,K^+-ATPase, alguns estudos relacionam a atividade da anidrase

carbônica. Tem sido relatado que vários metais pesados inibem a atividade da anidrase carbônica *in vitro* em uma grande diversidade de organismos, tais como peixes, caranguejos, anêmonas, corais e também os bivalves (Gilbert e Guzman, 2001; Lopes et al., 2011a; Jorge, 2012).

A inibição da atividade da anidrase carbônica se mostra tempo-dependente, sendo necessário pelo menos 10 min de exposição para a isoforma citosólica e 30 min para a isoforma presente na membrana plasmática. A isoforma de membrana possui sua atividade inibida através do sequestro do metal via componentes lipídicos localizados na membrana celular, porém é necessária uma alta concentração de cobre para que a inibição seja observada (Lioneto et al., 2012).

Nas brânquias de animais aquáticos, o efeito da inibição desta enzima pode interferir em diversas funções fisiológicas, tais como nas trocas gasosas, no balanço ácido-base, na osmoregulação e na excreção de amônia (Grosell et al., 2003, 2004, Zimmer et al., 2012).

A captação de Na^+ em alguns animais aquáticos ocorre através da membrana apical branquial pela troca direta de H^+ via trocador Na^+/H^+. O fato de a anidrase carbônica promover a hidratação do CO_2 e liberar substrato tanto para o trocador Na^+/H^+ quanto para o trocador Cl^-/HCO^{3-}, faz com que a mesma seja relacionada à captação de Na^+. Na truta arco-íris exposta *in vivo* ao cobre houve uma redução da captação tanto de Na^+ quanto de Cl^- em paralelo à uma inibição da atividade da anidrase carbônica branquial (Morgan et al., 2004). Nos caranguejos eurialinos *Neohelice granulata*, *Callinectes sapidus* e *Carcinus maenas*, a atividade da anidrase carbônica branquial também foi inibida após exposição *in vitro*. A exposição a diversos metais demonstrou que o cobre é mais potente inibidor da atividade desta enzima, seguido pelo zinco e cádmio (Vitale et al., 1999; Skaggs et al., 2002).

Já em mexilhões, acredita-se que a sensibilidade da anidrase carbônica é tecido-específica (Jernelov et al., 1996; Soto et al., 2000; Caricato et al., 2010; Jorge, 2012). Devido à grande aplicação de bivalves no monitoramente da qualidade ambiental, a determinação da sensibilidade da anidrase carbônica a metais pesados representa um importante passo na aplicação futura de variações na atividade desta enzima como biomarcador de exposição a metais pesados no organismo sentinela (Lionetto et al., 2012).

O estudo toxicológico *in vitro*

Para a avaliação dos impactos da poluição sobre os animais aquáticos que vivem em ecossistemas costeiros, estuarinos e de água doce existe um considerável interesse no desenvolvimento de modelos *in vitr*o. Células oriundas de vários tipos de cultura, bem como aquelas obtidas a partir da dissociação de tecidos de bivalves adultos, tem sido utilizadas em estudos ecotoxicológicos e podem ser obtidas a a partir da glândula digestiva (Lowe e Pipe, 1994; Birmelin e Pipe, 1999; Le Pennec e Le Pennec, 2001) ou da hemolinfa (Cajaraville et al., 1996; Venier et al., 1997; Olabarrieta et al., 2001; Gómez- Mendikute et al., 2002; Gómez-Mendikute e Cajaraville, 2003).

De forma semelhante, as células branquiais de bivalves são considerados bons modelos para estudos ecotoxicológicos, já que as brânquias são o primeiro alvo e local de captação para muitos tóxicos no ambiente aquático e, assim, suas células são frequentemente afetadas pela exposição a poluentes (Bigas et al., 2001). Este tecido possui diversas funções fisiológicas, incluindo respiração, excreção, balanço ácido-base, regulação iônica e transferência de solutos, sendo considerado como um órgão fundamental para manutenção da homeostase do meio extracelular (Péqueux, 1995; Goss et al., 2001).

Por exemplo, experimentos *in vivo* e *in vitro* com brânquias do mexilhão marinho *Mytilus galloprovincialis* demonstraram que estes animais possuem 58% de células epiteliais ciliadas, sendo o restante composto por células não ciliadas. Neste caso, os mucócitos corresponderam a 2,3% e os hemócitos 4,3% do total de células isoladas (Gómez-Mendikute et al., 2005). Por sua vez, os bivalves de água doce *Pyganodon cataracta*, *Utterbackia imbecillis* e *Ligumia substrata* (Família Unionidae) também foram analisados morfologicamente, tendo sido observada uma grande diversidade nos tipos celulares branquiais, incluindo àquelas ricas em mitocôndrias (Kays et al., 1990; Schwartz e Dimock, 2001). Nestes trabalhos, as células ricas em mitocôndrias foram designadas por participarem ativamente do transporte de soluto e da captação iônica.

Além da identificação destas células em alguns bivalves de água doce, sabe-se que em crustáceos decápodas estas células estão presente independente do padrão osmorregulatório osmoconformador (ambiente marinho) ou hiper-reguladores (ambiente de água doce ou salobra). Nestes animais, estas células possuem invaginações basais com grandes quantidades de mitocôndria, além de vários canais iônicos, transportadores, trocadores e bombas, sugerindo que estes estão inseridos diferencialmente entre as membranas apical e basolateral da célula branquial, agindo assim em série e dirigindo o transporte de Na^+ e Cl^- para a hemolinfa (Martelo e Zanders, 1986; Taylor e Taylor, 1992; Kirschner, 2004; Freire et al., 2008).

No entanto, as células ricas em mitocôndrias foram primeiramente descritas em brânquias de peixes há mais de 30 anos (Hwang e Hirano, 1985). Desde esta época, as células são conhecidas por participarem ativamente dos processos de osmorregulação e então foram formalmente conhecidas como células de cloreto ou ionócitos. Por serem amplamente estudadas neste grupo de animais, as células ricas em mitocôndrias tem reconhecido papel na absorção

(animais de água doce) ou secreção (animais de água salgada) ativa de íons, bem como na regulação ácido-base e de excreção de amônia (Hwang e Lee, 2007; Evans, 2008; Hwang, 2009).

Atualmente, somente nos diferentes grupos de animais, é reconhecida uma heterogeneidade de subtipos de células branquiais ricas em mitocôndrias com distintas funções na ionorregulação. Em peixes teleósteos de água doce *Oryzias dancena*, por exemplo, tiveram seu epitélio branquial descrito e dois diferentes tipos de células ricas em mitocôndria (MRC) foram identificadas. O primeiro tipo de MRC foi descrito como sendo responsável pela captação de Na^+, devido a presença do trocador Na^+/H^+ na membrana apical, e o segundo tipo celular por possuir em sua membrana apical o trocador $Na^+/2Cl^-$, responsável pela captação de Na^+ e Cl^- (Kang et al., 2013). Já na truta arco-íris *Oncorhynchus mykiss*, as células do epitélio branquial foram isoladas e diferenciadas em células pavimentosas e células ricas em mitocôndrias, sendo estas últimas caracterizadas como sendo positivas à reação com "lectin agglutinin" (PNA+MRC) ou negativas à reação com "lectin agglutinin" (PNA-MRC). Diferentes componentes associados ao transporte iônico estão presentes nestes diferentes subtipos celulares. Experimentos *in vitro* demonstraram que as células PNA-MRC são responsáveis pela captação de Na^+, enquanto que as PNA+MRC estão envolvidas na captação de Ca^{2+} (Reid et al., 2003; Galvez et al., 2006; Goss et al., 2011).

Dessa forma, a participação ativa das células ricas em mitocôndrias na regulação iônica em nível do epitélio branquial nos diferentes grupos animais faz com que essas células sejam foco de interesse de estudos, tanto em animais de água doce quanto de água salgada (Lee et al., 1996; Chang et al., 2001; Goss et al., 2001; Galvez et al., 2002; Reid et al., 2003; Galvez et al., 2006; Hiroi e McCormick, 2007). No entanto, quando relaciona-se metal e exposição *in vitro* de células ricas em mitocôndrias isoladas, somente reporta-se um trabalho com a truta arco-íris

Oncorhynchus mykiss. Goss et al. (2011) demonstrou o efeito da exposição ao cobre, cádmio e prata na captação de Na^+ em células branquiais ricas em mitôndrias isoladas (PNA+ e PNA-), bem como sobre a atividade da Na^+,K^+-ATPase.

Bivalves em estudo

1. Mexilhão de água doce *Lasmigona costata*

O mexilhão de água doce *Lasmigona costata* (Rafinesque, 1820) é pertencente a família Unionidae. Os animais adultos desta família podem medir entre 4 e 30 cm, no entanto a espécie em estudo possui um tamanho médio de 10,4 cm. Estes animais são encontrados em áreas de fluxo lento a moderado em rios de grande e médio porte que apresentam sedimento arenoso, lamoso ou composto de cascalho fino. Estes mexilhões são planctívoros e também podem servir de alimento para peixes e outros predadores (Willians et al., 1993).

A maior diversidade desses animais da família Unionidae está localizada nos Estados Unidos e Canadá, no entanto esses os mexilhões de água doce veêm sofrendo um grande declínio com relação ao número de indivíduos e também espécies. Esse declínio é em função da alta sensibilidade destes animais, principalmente suas larvas, a alguns contaminantes ambientais advindos das atividades antropogênicas (Cope et al., 2008; Gillis, 2011).

O desenvolvimento larval destes mexilhões pertencentes à família Unionidae ocorre pela fertilização dos ovos que são mantidos dentro das brânquias das fêmeas, chamadas de marsupio. Após alguns dias, as larvas, denominadas *glochidias*, são liberadas na água e podem permanecer semanas no ambiente aquático até infestarem as brânquias de peixes específicos e desenvolver seu estágio parasitário até se tornar juvenil (Cope et al., 2008). Em fêmeas grávidas as células

branquiais ricas em mitocôndrias foram identificadas microscopicamente e, atribuiu-se a função no transporte ativo de membrana para dentro e fora do marsúpio (Schwartz e Dimock, 2001).

2. Marisco de água salgada *Mesodesma mactroides*

Pertencente a família Mesodesmatidae, o marisco branco ou sernambi, *Mesodesma mactroides*, está distribuído desde o Rio de Janeiro, no Brasil, até a Bahia Blanca, na Argentina (Rios, 1994). Nesta espécie, o indivíduo adulto é considerado aquele com mais de 4,3 cm de comprimento. As praias favoráveis ao desenvolvimento desta espécie de marisco não apresentam substrato lodoso ou com alta porcentagem de fragmentos de rocha, sendo encontrados na zona de varredura das praias. Esta espécie possui, na maior parte do seu tempo, um hábito alimentar suspensívoro. Entretanto, quando há escassez de alimento em suspensão na coluna da água, este passa a se alimentar de detritos contidos no sedimento (Defeo e Scarabino, 1990).

A presença de cursos de água doce é um dos fatores que influenciam na distribuição destes animais, pois mesmos eurialianos esse animais têm baixa tolerância a salidade (entre 15 e 35). Além deste fator, a morfologia da praia, a composição do substrato e superexploração são fatores que também afetam sua presença na praia (Olivier et al., 1971; Bastida et al., 1991; Defeo et al., 1992).

Assim como todos bivalves, estes animais são considerados bons bioindicadores biológicos pois metais pesados e outras substâncias nocivas aos seres vivos ficam incorporados aos seus tecidos (Mansur et al., 1994; Gil e Thomé, 1998).

OBJETIVOS

Objetivo geral

O presente estudo teve como objetivo geral a obtenção de células branquiais ricas em mitocôndrias do mexilhão de água doce *Lasmigona costata* e do marisco de água salgada *Mesodesma mactroides* e avaliação da viabilidade do uso destas células em estudos toxicológicos *in vitro* com o cobre em diferentes condições osmóticas.

Objetivos específicos

- Isolar as células branquiais do marisco de água salgada *Mesodesma mactroides* e mexilhão de água salgada *Lasmigona costata* e separar o *pool* total de células em duas distintas frações;
- Identificar as distintas frações celulares obtidas do marisco de água salgada *M. mactroides* e do mexilhão de água doce *L. costata* através da aplicação da identificação de mitocôndrias e análise da atividade da Na^+,K^+-ATPase;
- Avaliar a toxicidade e o conteúdo de cobre em células branquiais ricas em mitocôndria isoladas do *M. mactroides* e do *L. costata* após exposição a 5, 9 e 20 µg Cu/L;
- Avaliar o efeito do cobre sobre o conteúdo de Na^+, Ca^{2+}, K^+ ,Mg^{2+} e Cl^- em células isoladas ricas em mitocôndrias do *M. mactroides*;
- Avaliar o efeito do cobre sobre a atividade da Na^+,K^+-ATPase e anidrase carbônica em células isoladas ricas em mitocôndrias do *M. mactroides*;
- Avaliar o efeito do cobre associado ao choque hiposmótico sobre o conteúdo de Na^+ e cobre, bem como a atividade enzimática da Na^+,K^+-ATPase e anidrase carbônica em células ricas em mitocôndrias isoladas do *M. mactroides*.

I. ARTIGO CIENTÍFICO

O primeiro manuscrito desta tese foi submetido para a revista *Cytotechnology*. As normas da revista para submissão encontra-se no ANEXO 1.

Isolation and fractionation of gill cells from freshwater (*Lasmigona costata*) and seawater (*Mesodesma mactroides*) bivalves for use in toxicological studies with copper

Lygia S. Nogueira[a], Chris M. Wood[b], Patricia L. Gillis[c], Adalto Bianchini[a]

[a] Instituto de Ciências Biológicas, Universidade Federal do Rio Grande-FURG, Av. Itália, km 8, 96203-900, Rio Grande, RS, Brazil

[b] Department of Biology, McMaster University, Hamilton, ON, Canada L8S 4K1

[c] Aquatic Contaminants Research Division, National Water Research Institute, Environment Canada, P.O. Box 5050, Burlington, ON, Canada L7R 4A6

AUTHOR EMAIL ADDRESS: nogueiralygia@gmail.com

CORRESPONDING AUTHOR:

M.Sc. Lygia S Nogueira, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Av. Itália km 8, 96201-900, Rio Grande-RS, Brasil. E-mail: nogueiralygia@gmail.com. Phone: +55 53 3233-5198. FAX: +55 53 3233-6848

ABSTRACT

Gills cells of the freshwater mussel *Lasmigona costata* and the seawater clam *Mesodesma mactroides* were isolated (mussel: chemical dissociation; clam: mechanical dissociation) and fractionated (Percoll gradient) into Fractions I and II. Mitochondrial dyes (DASPEI: mussel; MitoTracker^{R}: clam) and Na^{+},K^{+}-ATPase activity measurement were used to distinguish between cells of Fractions I and II. For mussel and clam, 80.5 ± 1.5% and 48.3 ± 3.2% of cells were in Fraction II, respectively. For both species, cells of Fraction II had higher fluorescence emission and higher enzyme activity than those of Fraction I, being characterized as 'cells rich in mitochondria'. Cells of Fraction II were kept under control condition (no Cu addition) or exposed (3 h) to copper (Cu: 5, 10 and 20 µg Cu/L). Cell viability and Cu and Na^{+} content were measured. For both species, Cu content was higher and Na^{+} content was lower in cells exposed to 20 µg Cu/L. Furthermore, a strong negative correlation was observed between cell Na^{+} and Cu content in the two bivalve species, indicating a possible competition between Cu and Na^{+} for ion-transporting mechanisms or binding sites at gill cells of Fraction II. Considering that Cu is an ionoregulatory toxicant in aquatic invertebrates, our data support the idea of using isolated gill cells rich in mitochondria to study the mechanisms underlying the acute toxicity of waterborne Cu in freshwater and marine bivalves.

Keywords: bioaccumulation, clam, copper, gills, isolated cells, mussel

INTRODUCTION

In vitro studies in bivalves have been largely performed with digestive glands, mantle, haemocytes and gills (Le Pennec and Le Penec 2001; Chelomin et al. 2005; Lopes et al. 2011a,b). However, studies involving the identification of cells present in these tissues are generally scarce. Scarce studies examined the gill tissue and analysed the cells of freshwater mussels, *Pyganodon cataracta*, *Utterbackia imbecillis* and *Ligumia substrata* (Unionid mussels), and reported different cellular types, including the 'cells rich in mitochondria', which play a significant role in active solute transport into and out of the gill cells and uptake of ions (Kays et al. 1990; Schwartz and Dimock, 2001). *In vivo* and *in vitro* gill epithelium analyses were also performed in the seawater mussel *Mytilus galloprovincialis* (Gómez-Mendikute et al. 2005). Findings reported the presence of ciliated (58%) and non-ciliated (42%) cells, including epithelial cells and haemocytes. Mucocytes and haemocytes corresponded to 2.3 and 4.3%, respectively.

Although morphological studies in bivalves describe the gill epithelium as being heterogeneous, the specific types of cells and their physiological functions are still unknown. Moreover, there is considerable interest in the development of *in vitro* models due to the action of aquatic pollutants in cell membranes via the outer epithelial layer of the gills, as well as other organs located in the paleal cavity, and the consequent toxicity to cells (Bigas et al. 2001; Le Pennec and Le Pennec 2001; Lopes et al., 2011a,b). Therefore, the first goal of the present study was to isolate and fractionate cells from the gills of the freshwater mussel *Lasmigona costata* and seawater clam *Mesodesma mactroides*. Fractionation was performed using discontinuous Percoll gradient and confirmed by fluorescence microscopy using specific mitochondrial dyes and Na^+,K^+-ATPase activity measurement, as earlier applied for teleost fish (Fletcher et al. 2000; Kelly et al. 2000; Wong and Chan 2001; Tse et al. 2006). Two distinct fractions were identified, one of which (Fraction II) was rich in mitochondria and Na^+,K^+-ATPase activity.

In teleost fish, separation of different isolated cell types has been useful for biochemical and cytological analyses, e.g. functional characterization of cell membrane transporters related to Na^+ uptake (Goss et al., 2001; Galvez et al., 2002; Reid et al., 2003), and particularly in analyzing the ionoregulatory disrupting effects of metals such as Cu (Goss et al., 2011). Cu is now generally recognized to be an ionoregulatory toxicant in aquatic invertebrates (Pinho et al.,

2007; Lopes et al., 2011a,b) as well as in fish. Therefore, we exposed gill cells of Fraction II isolated from the freshwater mussel *L. costata* and the seawater clam *M. mactroides* to three environmentally relevant concentrations of copper (Cu) (5, 9 and 20 µg Cu/L) for 3 h and analysed the cellular content of both Cu and Na^+. Results obtained were compared between species and the methodology used to obtain gill cells of bivalves for *in vitro* toxicological studies was evaluated.

MATERIAL AND METHODS

Experimental animals

Wild adults of the fluted-shell mussel *L. costata* (Rafinesque, 1820) were collected from a reference site in the Grand River (southern Ontario, Canada). In turn, adults of the yellow clam *M. mactroides* (Deshayes, 1854) were collected from a reference site at the Mar Grosso beach (southern Rio Grande do Sul, Brazil). Both species were acclimated to laboratory conditions for at least 1 week prior to experimentation. Mussels were held in aerated reconstituted moderately-hard water (composition in mM: 2.28 $NaHCO_3$, 0.78 $CaSO_4.H_2O$, 1 $MgSO_4$, 0.1 KCl; pH 7.8-8.0; ASTM 2006) under a 16h:8h light:dark cycle at 14°C. The water was renewed once a week when the mussels were fed with a commercial shellfish diet (Instant Algae Shellfish Diet 1800®, Richmond Hill, Ontario, Canada) at the rate of approximately $1.2x10^{10}$ algal cells per mussel. Clams were held in natural filtered (45 µm-mesh filter) sea water (salinity 30 ppt) continuously aerated and in the absence of sediment. Room temperature (20ºC) and photoperiod (12L:12D) were fixed. The acclimation medium was renewed three times a week, when clams were fed with the diatom *Thalassiosira weissflogii* approximately $2x10^2$ cells per clam.

Cell isolation

Initially, cell isolation from the gill epithelium of the mussel *L. costata* was performed using mechanical dissociation as this approach was thought to be less aggressive. However, the maintenance of small pieces of gill tissue in freshwater phosphate buffer solution (PBS) for 40 min caused massive cell death (data not shown). Therefore, we subsequently employed

enzymatic dissociation as described by Quinn et al. (2009) with modifications. Briefly, gill tissue from two mussels were dissected, pooled, and held in calcium-free phosphate buffer solution (freshwater PBS; composition in mM: 9 NaCl, 5 $NaHCO_3$, 0.5 KCl, 5 Na_2PO_4; pH 7.6; 20°C) for 10 min to remove the excess mucus. Tissues were sliced into small pieces and incubated (120 rotations per minute) with pronase from *Streptomyces griseus* (0.025% in freshwater PBS) (Sigma-Aldrich, Oakville, Ontario, Canada), for 15 min at room temperature (21^{o}C). After enzymatic digestion, isolated cells were filtered using a 30 µm-mesh nylon filter into stop buffer (1:10 fetal bovine serum/FBS; Sigma-Aldrich, Oakville, Ontario, Canada), centrifuged (600 x*g* for 4 min; Sorvall Legend X1, Thermo Scientific, Walthan, Massachusetts, USA) and resuspended in freshwater PBS.

Isolation of gill cells from the seawater clam *M. mactroides* was performed following the procedures previously described by Lopes et al. (2011a,b) with modifications. Briefly, gill tissue from three clams was dissected, pooled, sliced into small pieces, incubated in calcium-free phosphate buffer solution (seawater PBS; composition in mM: 342 NaCl, 20 Na_2HPO_4, 1.7 K_2HPO_4, 16 KCl, 5.5 EDTA; pH 7.6; 20ºC), and shaken (120 rotations per minute; Certomat-MO-II; Sartorius Stedim Biotech GmbH, Göttingen, Germany) for 30 min. After incubation, dissociated cells were filtered (30 µm-mesh nylon filter) to remove the non-dissociated tissue and large debris. The filtered solution containing isolated cells was transferred to plastic tubes, centrifuged (360 x*g*) for 3 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, Minnesota, USA). The pellet obtained was resuspended in seawater PBS.

Fractionation of isolated gill cells

Isolated gill cells from both species were layered onto a discontinuous Percoll gradient (Sigma-Aldrich, St. Louis, Missouri, USA). The gradient for *L. costata* was obtained by diluting the Percoll stock solution to obtain the following densities: 1.03 g/mL (18.6% Percoll stock solution, 71.4% bi-distilled H_2O, 10% 1.5 M NaCl), 1.05 g/mL (34.0% Percoll stock solution, 56.0% bi-distilled H_2O, 10% 1.5 M NaCl); 1.09 g/mL (64.8% Percoll stock solution, 25.2% bi-distilled H_2O, 10% 1.5 M NaCl). The gradient for *M. mesodesma* was obtained as described for *L. costata*, but replacing bi-distilled H_2O with seawater PBS.

Each layer of the Percoll gradient was placed into a plastic falcon-type tube (15 mL) in the following sequence: 1.09; 1.05; and 1.03 g/mL. Finally, the pool of isolated gill cells was added and centrifuged at 2000 x*g* for 10 min as described by Galvez et al. (2002), with modifications. Briefly, gill cells from both species were separated into Fraction I (1.03-1.05 g/mL Percoll interface) and Fraction II (1.05-1.09 g/mL Percoll interface). These Fractions were washed with 10 mL of the respective PBS for each species and centrifuged at 600 x*g* for 4 min. Finally, the new pellet obtained for each species was resuspended in 1 mL of saline solution of similar composition to the freshwater mussel hemolymph (composition in mM: 18 NaCl, 1.6 $CaCl_2$, 0.9 KCl, 0.4 $MgCl_2$, 2 $NaHCO_3$, and 1.4 glucose; pH 7.6; 20°C; Dietz 1979) and to the seawater clam hemolymph (composition in mM: 350 NaCl, 9 KCl, 30 $MgSO_4$, 9 $CaCl_2$, 2 $NaHCO_3$; pH 7.6; 20ºC; Lopes et al. 2011a,b).

Cell distinction

For both species, a specific mitochondrial dye was used to distinguish between cells from Fractions I and II (Lin and Hwang 2004; Lin et al. 2006; Buhariwalla et al. 2012). The fluorescent dye DASPEI (2-(4-dimethylaminostyryl)-1-methylpyridinium iodide; Sigma-Aldrich, St. Louis, Missouri, USA) was used in isolated gill cells of *L. costata*. Cells from Fractions I and II were incubated with DASPEI (0.2 µM) for 20 min, washed with freshwater PBS, and held in the corresponding saline solution for image processing. Images were analyzed using a fluorescent microscope (Leica DMR microscope; Leica Microsystems, Wetzlar, Germany) equipped with a Qimaging FAST 1394 digital camera. In turn, fractionated gill cells of *M. mactroides* were incubated with the fluorescent dye MitoTracker Green^R (1µM) for 10 min, washed with seawater PBS, and held in the corresponding saline solution for image processing. Images were analyzed using a fluorescent microscope (Olympus IX 81, Markham, Ontario, Canada) equipped with a DP72 digital camera. For both species, gills cells from Fractions I and II were evaluated for the presence or the absence of fluorescence emission.

Gill cells present in Fractions I and II were also distinguished through measurements of the cell Na^+,K^+-ATPase activity. Following cell isolation and Percoll gradient fractionation, gill cells of *L. costata* were homogenized in buffer solution (composition in mM: 150 sucrose; 5 EDTA; 50 imidazole; pH 7.5). Na^+,K^+-ATPase activity was measured based on the difference in

the amount of inorganic phosphate (Pi) released from ATP in the presence of K^+ (medium A) and in the absence of K^+ (medium B) and the presence of ouabain as described by Bianchini and Castilho (1999). For each cell fraction, 10 µL of the cell homogenate was incubated in medium A (composition in mM: 100 NaCl; 6 $MgCl_2$; 50 imidazole; 20 KCl; 3 ATP; pH 7.5) and in medium B (composition in mM: 100 NaCl; 6 $MgCl_2$; 50 imidazole; 3 ATP; 2 ouabain; pH 7.5) at 30ºC for 30 min. The reaction was stopped by adding 0.2 mL trichloroacetic acid (50%) to the reaction medium. Phosphate concentration was determined using a colorimetric method (Fiske and Subbarow, 1925). Protein concentration in the cell homogenate was determined using the method described by Bradford (1976). The specific enzyme activity was expressed as µmol Pi/mg protein/h.

Isolated gill cells of *M. mactroides* were homogenized in buffer solution (composition in mM: 300 sucrose; 20 EDTA; 100 imidazole; pH: 7.4). Na^+,K^+-ATPase activity was measured based on the ADP production in the presence of K^+ (medium A) and in the absence of K^+ and the presence of ouabain (medium B) as described by McCormick (1993). An aliquot (10 µL) of the cell homogenate was added in medium A (composition in mM: 100 NaCl; 10.5 $MgCl_2.H_2O$; 30 KCl; 50 imidazole) and in medium B (composition in mM: 100 NaCl; 10.5 $MgCl_2.H_2O$; 50 imidazole; 1 ouabain). The reaction solution (composition in mM: 2.8 phosphoenolpyruvate; 3.5 ATP; 0.22 NADH; 50 mM imidazole; composition in µL/ml: 4 lactate dehydrogenase; 5 pyruvate kinase) was then added to the mixture, which was incubated for 30 min. Protein concentration in the cell homogenate was determined using the method described by Bradford (1976). The specific enzyme activity was expressed as µmol ADP/mg protein/h.

Copper accumulation and Na^+ content after Cu exposure

For both species, isolated cells (10^6 cells) present in Fraction II (cells rich in mitochondria, see Results section) were kept in their respective saline solution without Cu addition (control) or exposed (3 h) to Cu (5, 9 and 20 µg/L), as previously described for mantle cells of the clam *M. mactroides* (Lopes et al. 2011a,b) with some modification. The 5 and 9 µg Cu /L concentrations used in our study are within toxicological endpoint in the environmental protection regulation in Brazil (Conselho Nacional do Meio Ambiente, 2005) and Canada (Canadian Water Quality Guideline, 2005). In turn, the 20 µg Cu/L exposure is an extrapolation

of the environmental legislation concentrations. Briefly, Cu as $CuCl_2$ (Merck, St. Louis, Missouri, USA) was added to the respective saline solution for *L. costata* and *M. mactroides*. After exposure, cells rich in mitochondria were centrifuged 360x*g*, 3 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, Minnesota, USA). The supernatant was discarded and the pellet was quickly washed with the respective saline solution containing EDTA (12 mM) to remove the loosely Cu bound on the cell surface. The washing and centrifugation procedures were repeated. The pellet was resuspended in the respective (freshwater or seawater) PBS solution. An aliquot of the suspension was collected to determine cell viability using the Trypan Blue exclusion method. Remaining cells were then split into two aliquots. The first aliquot was dried (50ºC; 24 h), completely digested with HNO_3 (Suprapur, Merck, St. Louis, Missouri, USA), diluted with Milli-Q water, and analyzed for Cu concentration. The second aliquot was sonicated (Sonozap, Ultrasonic Processor, New York, USA) and diluted with Milli-Q water for Na^+ concentration analysis.

For *L. costata* samples, Cu and Na^+ concentration was analyzed by graphite furnace atomic absorption spectrometry (GFAAS, Varian Spectra AA-20 equipped with graphite tube atomizer [GTA-110], Mulgrave, Australia) and flame atomic absorption spectrometry (SpectrAA 220FS Atomic Absorption Spectrometer), respectively. For *M. mactroides* samples, Cu and Na^+ concentration was analyzed by atomic absorption spectrophotometry (AAS 932 Avanta Plus, GBC, Hampshire, Illinois, USA), as previously described (Pinho et al. 2007; Lopes et al. 2011b). Cell Cu or Na^+ content was expressed as µmol Cu or $Na^+/10^6$ cells, considering the respective cell viability in each experimental condition.

Cell viability

Cell viability was determined using the Trypan Blue exclusion assay (0.08% Trypan Blue). Cells from each sample were counted under a light microscope. Cell viability was determined by dividing the number of non-stained (living) cells by the number of total cells counted. Measurements were performed immediately after cell isolation, after 5 h of maintenance in the corresponding saline solution, after cell fractionation (Percoll gradient), and the 3-h period of Cu exposure (Fraction II cells only).

Statistical analyses

Data are expressed as mean ± standard error (n = 5), where each measurement represents a pool of cells (Fraction I or II) prepared with gills from two (*L. costata*) and three (*M. mactroides*) animals. Each pool of cells was analyzed at least in triplicate and a mean value was calculated for each pool of cells. Na^+,K^+-ATPase activity, as well as Na^+ and Cu cell content data were compared using one-way analysis of variance (ANOVA) followed by the Fisher least significant difference (LSD) test. Correlations between cell Cu content and Cu concentration in the incubation medium or between cell Cu content and Na^+ content were evaluated through the Spearman correlation index. Statistical analyses were performed using the software Statistica 7.0 (Stat Soft, Tulsa, Oklahoma, USA). The significance level adopted was 95% ($\alpha = 0.05$).

RESULTS

Cell dissociation, fractionation and distinction

The enzymatic cellular dissociation employed for the freshwater mussel *L. costata* showed satisfactory results when cell viability data were considered. After isolation, cell viability was 87.4 ± 2.7% and no significant change was observed after 5 h of cell maintenance in saline solution of similar composition to the mussel hemolymph. Also, the mechanical dissociation of gill cells applied to the gill epithelium of the seawater clam *M. mactroides* proved to be satisfactory with respect to the number of isolated cells acquired and the cell viability observed (82.4 ± 1.0%).

For both species, the discontinuous Percoll gradient technique separated gill cells into two different groups of cells (Fraction I: cells retained in the 1.03-1.05 g/mL Percoll interface; Fraction II: cells retained in the 1.05-1.09 g/mL Percoll interface). For both fractions, cell viability did not differ from that observed for the total pool of cells. Cell separation was confirmed through two different approaches (mitochondrial dyes and Na^+,K^+-ATPase activity measurement), as described below.

For *L. costata*, cells collected from the 1.03-1.05 (Fraction I) and the 1.05-1.09 g/mL (Fraction II) Percoll interfaces were incubated separately in the mitochondrial fluorescent dye

DASPEI. Cells present in Fraction I (Fig. 1A) virtually showed no fluorescence emission (Fig. 1B) while those from Fraction II (Fig. 1C) emitted higher levels of fluorescence (Fig. 1D). For *M. mactroides*, each cell fraction was incubated separately in the mitochondrial fluorescent dye MitoTracker Green^{R}. Cells from Fraction I (Fig. 2A) showed lower fluorescence emission (Fig. 2B) than those present in Fraction II (Fig. 2C,D).

For both the freshwater mussel (Fig. 3A) and seawater clam (Fig. 3B), Na^+,K^+-ATPase activity was significantly higher in cells from Fraction II than in those from Fraction I. Cell counting data indicated that 80.5 ± 1.5% and 48.3 ± 3.2% of the isolated cells were actually cells showing high fluorescence emission and Na^+,K^+-ATPase activity in *L. costata* and *M. mactroides*, respectively.

Copper accumulation and Na^+ content after Cu exposure

For both species, Cu exposure (3 h) did not significantly affect viability of gill cells present in Fraction II. For *L. costata*, control cells showed 87.35 ± 2.69% viability while those exposed to 5, 9 and 20 µg Cu/L had 89.86 ± 2.18 %, 88.02 ± 2.46%, and 87.99 ± 4.48% viability, respectively. Cell Cu content in control cells was 1.02 ± 0.06 µmol/10^6 cells, and significantly increased in those exposed to 20 µg Cu/L (1.53 ± 0.21 µmol/10^6 cells). Notably, a significant positive correlation between Cu content in gill cells and Cu concentration in the incubation medium was observed (R = 0.72; p <0.05). Notably, the Na^+ content was significantly lower in cells exposed to 20 µg Cu/L (3.44 ± 0.16 µmol/10^6 cells) than in control cells (5.02 ± 0.43 µmol/10^6 cells). In addition, a significant negative correlation between cell Cu content and cell Na^+ content was observed (R = -0.57; p < 0.05) (Fig. 4A).

For *M. mactroides*, viability in control cells was 81.28 ± 2.75% while it was 78.81 ± 3.22%, 75.81 ± 3.05% and 76.42 ± 2.87% in those exposed to 5, 9 and 20 µg Cu/L, respectively. As for *L. costata*, a significant increase in Cu content was also observed in cells exposed to 20 µg Cu/L (0.048 ± 0.004 µmol/10^6 cells) when compared to that observed in control cells (0.037 ± 0.001 µmol/10^6 cells). Also, there was a positive correlation between Cu content in gill cells and Cu concentration in the incubation medium (R = 0.44; p<0.05). The Na^+ content was also significantly lower in cells exposed to 20 µg Cu/L (5.77 ± 0.88 µmol/10^6 cells) than in cells

(12.31 ± 1.22 µmol/10^6 cells). Finally, a significant negative correlation between cell Cu content and cell Na^+ content was also observed (R = -0.59; $p < 0.05$) (Fig. 4B).

DISCUSSION

In the two bivalve species analyzed in the present study, the gill epithelium is a thin tissue playing essential role in respiration, excretion, acid-base regulation, ionic regulation, and solute transfer. However, the freshwater mussel *L. costata* hold embryos in their internal cavity (marsupium) until the larval (glochidia) stage is fully developed and released into the water column in order to parasitize fish and metamorphose into juveniles (Schwartz and Dimock, 2001).

Two different techniques were employed to dissociate and isolate gill cells from the freshwater mussel *L. costata* (enzymatic technique) and the seawater clam *M. mactroides* (mechanical technique). Although the mechanical dissociation approach is considered to be less aggressive for cells during the isolation process, it proved to be inappropriate for dissociating gill cells of the freshwater mussel *L. costata*. The massive cell mortality observed using this technique (during preliminary trials) may have resulted from the high sensitivity of freshwater bivalves to the low K^+ concentration in the external medium (Fisher et al., 1991). In contrast, our data indicate that the enzymatic method was an excellent option to obtain isolated cells from gills of *L. costata*. Pronase, the enzyme used in the present study, has been described as the most satisfactory in cell dissociation in the freshwater mussel *Dreissena polymorpha* (Quinn et al., 2009). However, mechanical shaking was included and the time of tissue incubation with the enzyme was improved (reduced from 16 h to 20 min) in the protocol applied for *L. costata* in the present study, thus resulting in a satisfactory number of isolated cells at a high percentage of viability in a shorter time. On the other hand, the mechanical cell isolation technique applied to the seawater clam *M. mactroides* proved very effective, liberating a satisfactory number of isolated cells with a higher percent viability. In addition, gill cells showed a high viability even 5 h after being isolated. According to Lilius et al. (1995), cells are able to maintain their membrane polarity and normal functions up to 5 h after being isolated and then they likely lose this ability after that period.

Following the successful application of the different techniques for isolation of gill cells in the two bivalve species, a three-step Percoll gradient was applied to separate the pool of gill cells obtained into two major fractions. The gill cells of *L. costata* and *M. mactroides* present in Fraction II (1.05 –1.09 g/L) of the Percoll gradient showed a markedly higher fluorescence emission in the presence of the DASPEI and Mito tracker Green^{R}, respectively. To confirm our finding in the freshwater mussel, Mito tracker Green^{R} was also used and similar results were obtained (data not shown). According to Galvez et al. (2002), isolated gill cells from fish retained in the first interface of the Percoll gradient (1.03 –1.05 g/L) were pavement cells (PVCs), while those retained in the second interface of the Percoll gradient (1.05 –1.09 g/L) were mitochondria-rich cells. Therefore, we could assume that these gill cells of bivalves would correspond also to cells rich in mitochondria.

Cells rich in mitochondria are known to have a high metabolic potential due to their high ability for energy production, thus usually exhibiting high Na^{+},K^{+}-ATPase activity. Therefore, enzyme activity analysis was the second approach used to differentiate the two pools of cells isolated from gills of the freshwater mussel *L. costata* and seawater clam *M. mactroides.* As in other animal species (Wong and Chan, 1999; Galvez et al. 2008) , both methodologies used in our studies were also satisfactory and cells collected from the Fraction II (1.05-1.09 g/L Percoll gradient interface) showed a markedly higher Na^{+},K^{+}-ATPase activity than those collected from the Fraction I (1.03-1.05 g/L Percoll gradient interface). In the freshwater mussel, the Na^{+},K^{+}-ATPase activity was 2-fold higher in Fraction II than Fraction I while seawater clam had 6-fold. This finding was in accord with Na^{+},K^{+}-ATPase activity in gill cells isolated from Japanese eels where a greater relative difference between Fractions was observed in the seawater animal (Wong and Chan, 1999).

In these bivalves, the ratio of gill cells rich in mitochondria to the other cell types present after Percoll gradient separation was very different from that observed for other animals. For example, around 85% of gills cells in rainbow trout (*Oncorhynchus mykiss*) were characterized as pavement cells and only 15% as mitochondria-rich cells after using the Percoll gradient technique to fractionate isolated gill cells (Galvez et al. 2008). In the present study, $80.5 \pm 1.5\%$ of cells isolated from gills of the freshwater mussel *L. costata* were characterized as showing high mitochondrial density and Na^{+},K^{+}-ATPase activity and 48.3 ± 3.26 % of gill cells isolated from the seawater clam *M. mactroides* show to be the same properties.

As previously mentioned, Unionid mussels have a high amount of cells rich in mitochondria in their gill epithelium, but pavement cells are present in a greater proportion as in fish (Schwartz and Dimock 2001). However, the higher percentage of cells rich in mitochondria found in the present study could be associated with the cellular dissociation technique employed. Although it is regarded as the best method for cell isolation in the freshwater bivalve *Dreissena polymorpha* (Quinn et al. 2009), enzymatic digestion is considered to be an "aggressive" method to perform cell isolation. Therefore, we suspect that a large proportion of the debris and dead cells found after applying the enzymatic method in *L. costata*, i.e., tissue digestion with pronase, may have been derived from cells showing low mitochondrial density and low Na^+,K^+-ATPase activity. The two cell fractions were more equal in *M. mactroides* (Fraction I: 51.7 ± 3.2%; Fraction II: 48.3 ± 3.2%) but still not comparable with fish results (Reid et al., 2003; Galvez et al. 2008). Probably, this is a consequence of the less aggressive mechanical method applied for cell dissociation. Regardless, the methods employed in both bivalve species in the current study produced an adequate abundance of viable cells (>80%) for *in vitro* experiments.

Following separation and fractionation, gill cells of Fraction II from both species of bivalves were exposed to different Cu concentrations, and analysis of cellular Cu and Na^+ content were performed. Cell Cu content in freshwater mussel *L. costata* as well as seawater clam *M. mactroides* increased linearly with the increasing concentration of Cu in the incubation medium. Also, a significant decrease in cell Na^+ content was observed after exposure to the highest concentration of Cu tested (20 µg Cu/L). As a cation, Cu would outcompete with Na^+ present in the incubation medium for active sites of ion transport at the membrane cell surface. Therefore, an increase in Cu concentration in the incubation medium could interfere with cell metabolism and function through disturbance of essential ion regulation, especially Na^+ (Kültz, 2001; Grosell et al. 2002; Blanchard and Grosell 2006). In the present study, the rate of cell survival was not significantly affected by exposure to any Cu concentration tested. Therefore, the ionic (Na^+) disturbance observed after Cu exposure was not enough to induce cell death. However, a possible lethal effect after exposure to higher concentrations of Cu (>20 µg Cu/L) or for a longer period of time (>3 h) to those tested (5, 9, 20 µg Cu/L) in the present study cannot be ruled out.

Our data indicate that cells isolated from gills of bivalves are a suitable model for *in vitro* toxicological studies in both freshwater and seawater bivalves. This statement is based on two

facts: (1) the significant and positive correlation observed between Cu content in isolated gill cells and the Cu concentration in the incubation medium; (2) the significant and negative correlation observed between the Na^+ content and the Cu content in isolated cells present in Fraction II. It is interesting to note that the negative correlation in the freshwater mussel *L. costata* (R = -0.57) and the seawater clam *M. mactroides* (R = -0.59) is very similar. This finding is likely associated with the high concentration of Na^+ employed in the freshwater saline solution (similar to that in freshwater mussel hemolymph - 18 mM NaCl), when compared to that generally found in fresh water (~1 mM). Presumably, 18 mM NaCl was sufficient to induce the maximum cellular Na^+ influx in isolated gills cells of both *L. costata* and *M. mactroides*, thus limiting the competitive effect of Cu on cellular Na^+ uptake and its consequent toxicity to isolated cells. Although we did not observe any lethal toxicity of Cu to isolated gill cells, studies at the cellular level like the one described here, allow the investigation and a better understanding of the mechanisms involved in Cu toxicity because they simulate *in vitro* the exposure of aquatic animals to contaminants (Castaño et al. 2003).

CONCLUSION

The methodologies employed for isolation and fractionation of cells from the gills of the freshwater bivalve *L. costata* and the seawater clam *M. mactroides* were found to be effective, as previously reported for fish (Goss et al. 2001; Galvez et al. 2002; Tse e al. 2006). Also, the application of two different approaches (mitochondria density and Na^+,K^+-ATPase activity) proved to be reliable in characterizing different sub-types of gill cells in both bivalve species analyzed. Finally, our findings support the idea that Cu outcompetes with Na^+ for sites of ion transport on the plasma membrane, as previously observed in the gills of intact crustaceans and fish. Taken altogether, these findings indicate that cells isolated from gills of freshwater and seawater bivalves are a useful tool for studying the cellular mechanisms of metal uptake and toxicity.

ACKNOWLEDGEMENTS

We thank Rodney McInnis, Tina Hooey, Jim Bennett, Mark McMaster (Environment Canada) and Robert Boyle and Ana Cristina Kalb (Universidade Federal do Rio Grande, Brazil).

This research was supported by awards from the International Development Research Centre (IDRC, Ottawa, ON, Canada) to A. Bianchini and C.M. Wood, the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília, DF, Brazil). A. Bianchini is a research fellow from the Brazilian CNPq (Proc. #304430/2009-9) and is supported by the International Research Chair Program from IDRC. C.M. Wood is supported by the Canada Research Chair Program.

REFERENCES

ASTM (2006) Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (E2455-05). ASTM International, West Conshohocken, PA

Bianchini A, Castilho PC (1999) Effects of zinc exposure on oxygen consumption and gill Na^+,K^+-ATPase of the estuarine crab *Chasmagnathus granulata* Dana, 1851 (Decapoda, Grapsidae). Bull Environ Contam Toxicol **62,** 63-69

Bigas M, Durfort M, Poquet M (2001) Cytological effects of experimental exposure to Hg on the gill epithelium of the European flat oyster *Ostrea edulis*: ultrastructural and quantitative changes related to bioaccumulation. Tissue Cell 33:178–188

Blanchard J, Grosell M (2006) Copper toxicity across salinities from freshwater to seawater in the euryhaline fish *Fundulus heteroclitus*: Is copper an ionoregulatory toxicant in high salinities? Aquat Toxicol 80, 131-139

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254

Buhariwalla HEC, Osmond EM, Barnes KR, Cozzi RRF, Robertson GN, Marshall WS (2012) Control of ion transport by mitochondrion-rich chloride cells of eurythermic teleost fish: Cold shock vs. cold acclimation. Comp Biochem Physiol 162A, 234-244

Castaño A, Bols N, Braunbeck T, Dierickx P, Halder M, Isomaa B, Kawahara K, Lee LEJ, Mothersill C, Pärt P, Repetto G, Sintes JR, Rufli H, Smith R, Wood C, Segner H (2003) The use of fish cells in ecotoxicology: The report and recommendations of ECVAM Workshop 47. ATLA 31, 317-351

CCME, 2005. Canadian Water Quality Guidelines. Canadian Council of Ministers of the Environment, Environment Canada, Ottawa, ON.

Chelomin VP, Zakhartsev MV, Kurilenko AV, Belcheva NN (2005) An in vitro study of the effect of reactive oxygen species on subcellular distribution of deposited cadmium in digestive gland of mussel *Crenomytilus grayanus*. Aquat Toxicol 73:181–189

CONAMA, 2005. Conselho Nacional do Meio Ambiente. Resolução nº 357, de 17 de março de 2005

Dietz TH (1979) Uptake of sodium and chloride by freshwater mussels. Can J Zool 57, 156-160

Fisher SW, Stromberg P, Bruner KA, Boulet LD (1991) Molluscicidal activity of potassium to the zebra mussel, *Dreissena polymorpha*: Toxicity and mode of action. Aquat Toxicol 20, 219-234

Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorus. J Biol Chem 66, 375-400

Fletcher M, Kelly SP, Part P, O'Donnell MJ, Wood CM (2000) Transport properties of cultured branchial epithelia from freshwater rainbow trout: a novel preparation with mitochondria-rich cells. J Exp Biol 203, 1523-1537

Galvez F, Tsui T, Wood CM (2008) Cultured trout gill epithelia enriched in pavement cells or in mitochondria-rich cells provides insights into Na^+ and Ca^{2+} transport. In Vitro Cell Dev Biol Anim 44, 415-425

Galvez F, Wong D, Wood CM (2006) Cadmium and calcium uptake in isolated mitochondria-rich cell populations from the gills of the freshwater rainbow trout. Am J Physiol 291, R170-R176

Galvez F, Reid SD, Hawkings G, Goss GG (2002) Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. Am J Physiol 282, R658-R668

Gómez-Mendikute A, Elizondo M, Venier P, Cajaraville MP (2005) Characterization of mussel gill cells *in vivo* and *in vitro*. Cell Tissue Res **321,** 131-140

Goss GG, Adamia S, Galvez F (2001) Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium Am J Physiol 281, R1718-R1725

Goss G, Gilmour K, Hawkings G, Brumbach JH, Huynh M, Galvez F (2011) Mechanism of sodium uptake in PNA negative MR cells from rainbow trout, *Oncorhynchus mykiss* as revealed by silver and copper inhibition. Comp Biochem Physiol 159A, 234-241

Grosell M, Nielsen C, Bianchini A (2002) Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comp Biochem Physiol 133C, 287-303

Horng JL, Lin LY, Huang CJ, Katoh F, Kaneko T, Hwang PP (2007) Knockdown of V-ATPase subunit A (atp6v1a) impairs acid secretion and ion balance in zebrafish (*Danio rerio*). Am J Physiol 292, R2068-R2076

Kang CK, Yang WK, Lin ST, Liu CC, Lin HM, Chen HH, Cheng CW, Lee TH, Hwang PP (2013) The acute and regulatory phases of time-course changes in gill mitochondrion-rich cells of seawater-acclimated medaka (*Oryzias dancena*) when exposed to hypoosmotic environments. Comp Biochem Physiol 164A, 181-191

Katoh F, Hasegawa S, Kita J, Takagi Y, Kaneko T (2001) Distinct seawater and freshwater types of chloride cells in killifish, *Fundulus heteroclitus*. Can J Zool 79, 822-829

Kays WT, Silverman H, Dietz TH (1990) Water channels and water canals in the gill of the freshwater mussel, *Ligumia subrostrata*: ultrastructure and histochemistry. J Exp Zool 254, 256–269

Kelly SP, Fletcher M, Part P, Wood CM (2000) Procedures for the preparation and culture of 'reconstructed' rainbow trout branchial epithelia. Methods Cell Sci 22, 153-163

Kültz D (2001) Cellular osmoregulation: beyond ion transport and cell volume. Zoology 104, 198-208

Le Pennec G, Le Pennec M (2001) Acinar primary cell culture from the digestive gland of *Pecten maximus* (L): an original model for ecotoxicological purposes. J Exp Mar Biol Ecol 259, 171-187

Lilius H, Sandbacka M, Isomaa B (1995) The use of freshly isolated gill epithelial cells in toxicity testing. Toxicol In Vitro 9, 299-305

Lin LY, Hwang PP (2004) Mitochondria-rich cell activity in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) larvae acclimatized to different ambient chloride levels. J Exp Biol 207, 1335-1344

Lin LY, Horng JL, Kunkel JG, Hwang PP (2006) Proton pump-rich cell secretes acid in skin of zebrafish larvae. Am J Physiol Cell Physiol C371-C378

Lopes TM, Barcarolli IF, Oliveira CB, Souza MM, Bianchini A (2011a) Effect of copper on ion content in isolated mantle cells of the marine clam *Mesodesma mactroides*. Environ Toxicol Chem 30, 1582-1585

Lopes TM, Barcarolli IF, Oliveira CB, Souza MM, Bianchini A (2011b) Mechanism of copper accumulation in isolated mantle cells of the marine clam *Mesodesma mactroides*. Environ Toxicol Chem 30, 1586-1592

McCormick SD (1993) Method for non-lethal gill biopsy and measurement of Na^+,K^+-ATPase activity. Can J Fish Aquat Sci 50, 656-658

Pinho GLL, Pedroso MS, Rodrigues SC, Souza SS, Bianchini A (2007) Physiological effects of copper in the euryhaline copepod *Acartia tonsa*: waterborne versus waterborne plus dietborne exposure. Aquat Toxicol 84, 62-70

Quinn B, Costello MJ, Dorange G, Wilson JG, Mothersill C (2009) Development of an *in vitro* culture method for cells and tissues from the zebra-mussel (*Dreissena polymorpha*). Cytotechnol 59,121-134

Reid SP, Hawkings GS, Galvez F, Goss GG (2003) Localization and characterization of phenamil-sensitive Na^+ influx in isolated rainbow trout gill epithelial cells. J Exp Biol 206, 551-559

Schwartz ML, Dimock RV Jr (2001) Ultrastructural evidence for nutritional exchange between brooding unionid mussels and their glochidia larvae. Invertebr Biol 120, 227-236

Tse WKF, Au DWT, Wong CKC (2006) Characterization of ion channel and transporter mRNA expressions in isolated gill chloride and pavement cells of seawater acclimating eels. Biochem Biophys Res Commun 346, 1181-1190

Wong CKC, Chan DKO (1999) Isolation of viable cell types from the gill epithelium of Japanese eel *Anguilla japonica*. Am J Physiol 276, R363-R372

Wong CKC, Chan DKO (2001) Effects of cortisol on chloride cells in the gill epithelium of Japanese eel, *Anguilla japonica*. J Endocrinol 168, 185-192

FIGURE CAPTIONS

Figure 1. Fluorescent images of isolated gill cells from the freshwater mussel *Lasmigona costata* after layering over a discontinuous Percoll gradient (1.03-1.05-1.09 g/mL) and separation by centrifugation. Cells from each interface were collected, incubated with a specific mitochondrial dye (DASPEI) and examined under a fluorescence microscope (200x magnification). A and C = phase contrast image; B and D = fluorescent images of the same fields from A and C. A and B = 1.03-1.05 g/L Percoll density interface (Fraction I); C and D = 1.05-1.09 g/L Percoll density interface (Fraction II).

Figure 2. Fluorescent images of isolated gill cells from the seawater clam *Mesodesma mactroides* after layering over a discontinuous Percoll gradient (1.03-1.05-1.09 g/mL) and separation by centrifugation. Cells from each interface were collected, washed, incubated with a mitochondrial dye (MitoTracker^{R}) and examined under a fluorescence microscope (400x magnification). A and C = phase contrast images; B and D = fluorescent images of the same fields from A and C. A and B = 1.03-1.05 g/L Percoll density interface (Fraction I); C and D = 1.05-1.09 g/L Percoll density interface (Fraction II).

Figure 3. Na^{+},K^{+}-ATPase activity in cells of Fraction I (1.03-1.05 g/L Percoll density interface) and Fraction II (1.05-1.09 g/L Percoll density interface) isolated from gills of the freshwater mussel *Lasmigona costata* (A) and seawater clam *Mesodesma mactroides* (B). Data are expressed as mean ± standard error (n = 5). Different letters indicate significant different mean values among treatments ($p<0.05$).

Figure 4. Copper and Na^{+} content in isolated gill cells from Fraction II of the freshwater mussel *Lasmigona costata* (A) and the seawater clam *Mesodesma mactroides* (B) after exposure to copper (5, 9 and 20 µg Cu/L). Data are expressed as mean ± standard error (n=5). Different letters indicate significant different mean values among treatments ($p<0.05$). Closed circle: Cu content; open circle = Na^{+} content.

Figure 1

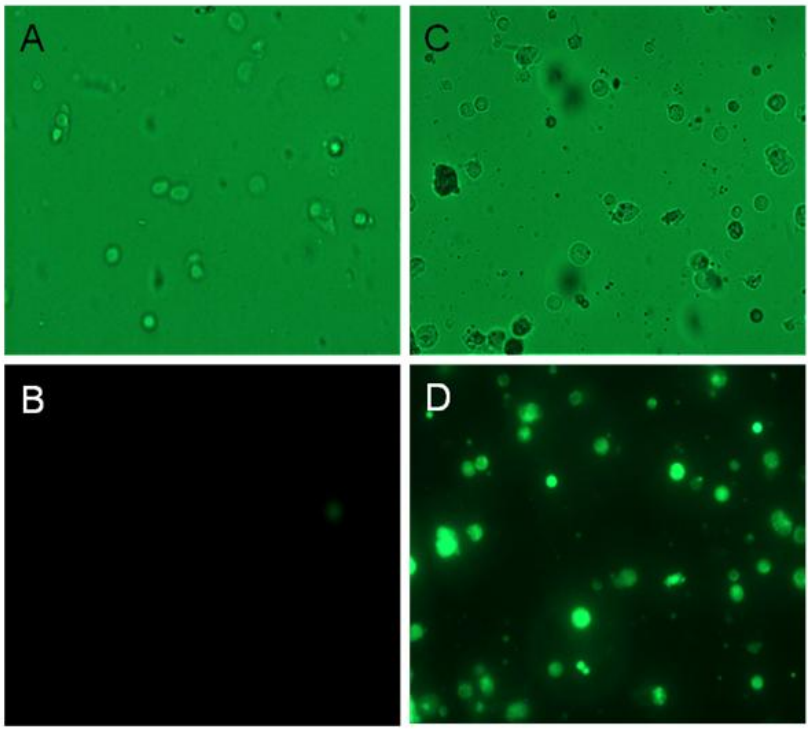


Figure 2

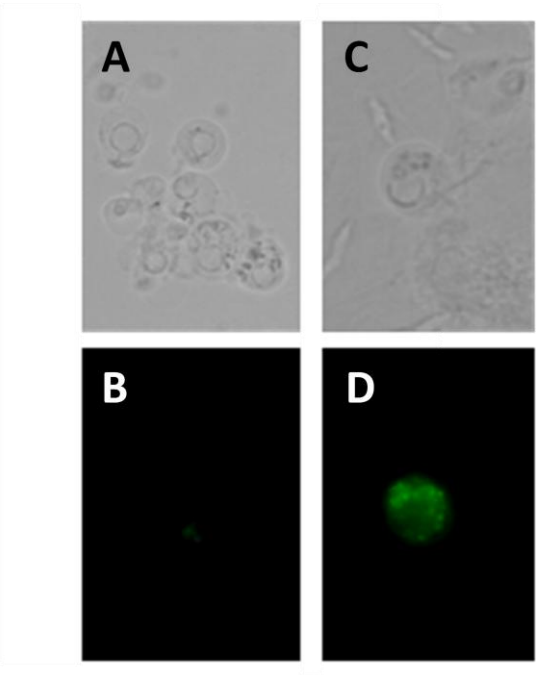


Figure 3

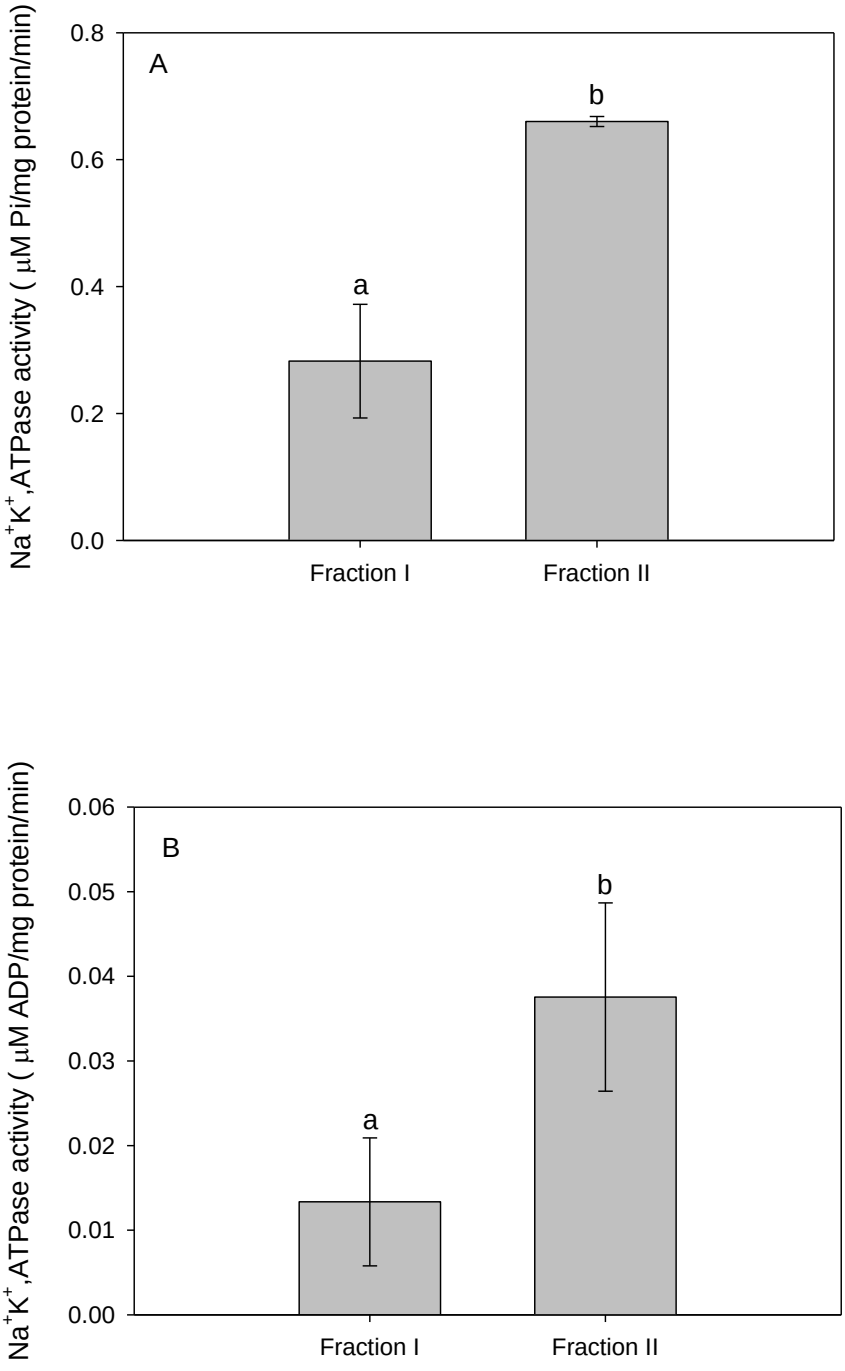
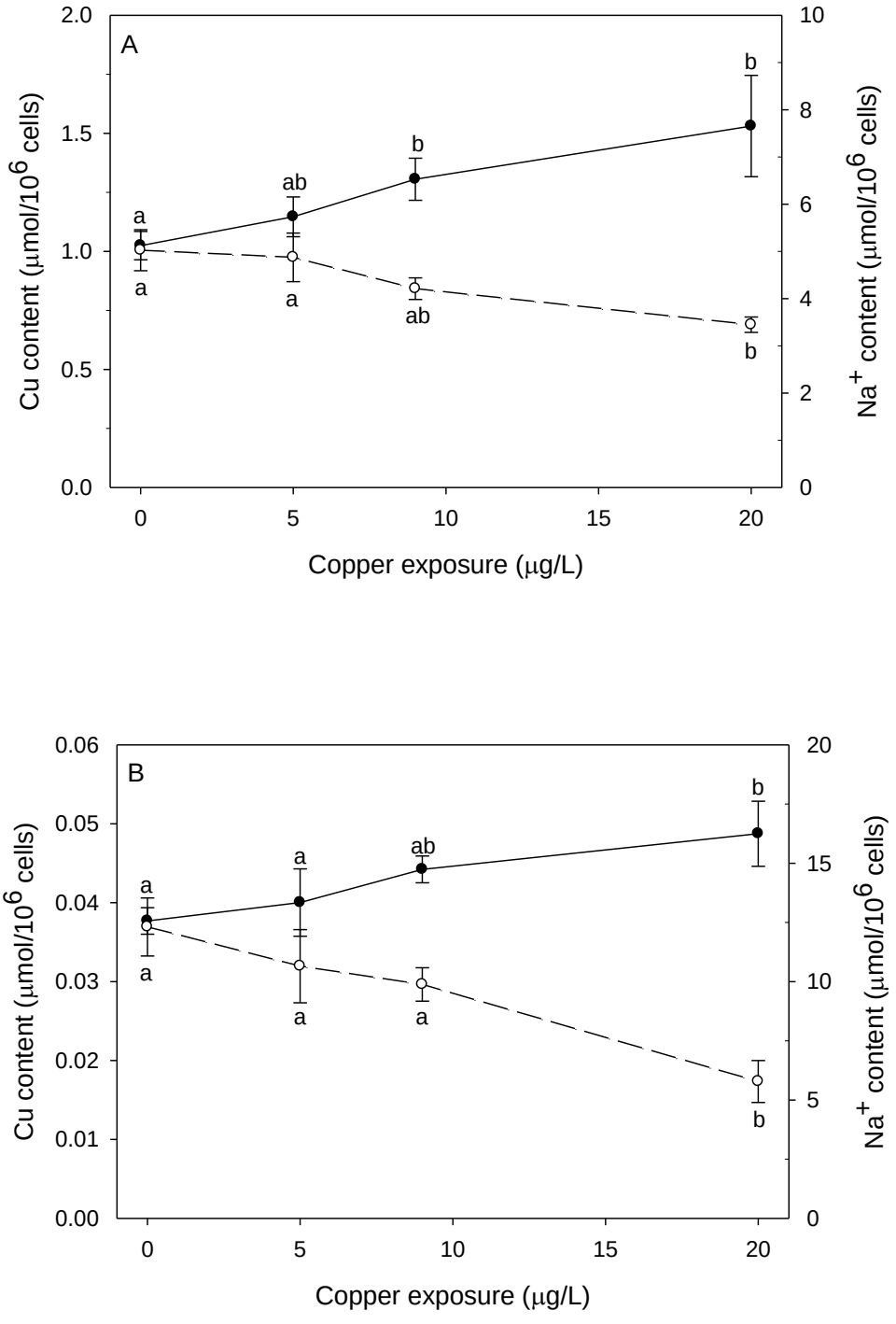

A
B
a
b
a
b
0.8
0.6
0.4
0.2
0.0
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Fraction I
Fraction II
Fraction I
Fraction II
Na+K+,ATPase activity (µM Pi/mg protein/min)
Na+K+,ATPase activity (µM ADP/mg protein/min)

Figure 4


A
B
Cu content (μmol/10^6 cells)
Na+ content (μmol/10^6 cells)
Copper exposure (μg/L)
0.0
0.5
1.0
1.5
2.0
0
2
4
6
8
10
0.00
0.01
0.02
0.03
0.04
0.05
0.06
5
10
15
20
a
ab
b

II. ARTIGO CIENTÍFICO

O segundo manuscrito desta tese foi submetido para a revista *Aquatic Toxicology*. As normas da revista para submissão encontra-se no ANEXO 2.

Ionic disturbances in mitochondria-rich cells isolated from gills of the yellow clam *Mesodesma mactroides* exposed to copper under different osmotic conditions

Lygia Sega Nogueira and Adalto Bianchini*

Universidade Federal do Rio Grande, Instituto de Ciências Biológicas, Programa de Pós-Graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, Av. Itália km 8, 96203-900, Rio Grande, Rio Grande do Sul, Brazil

*Corresponding author: Adalto Bianchini

Universidade Federal do Rio Grande

Instituto de Ciências Biológicas

Av. Itália km 8, Campus Carreiros

96203-900, Rio Grande, RS, Brazil

e-mail: adaltobianchini@furg.br

Phone: +55 53 3293-5193

FAX: +55 53 3233-6633

ABSTRACT

Gill cells rich in mitochondria are involved in ion regulation in fish, crustaceans and bivalves. Copper (Cu) toxicity to these animals is generally associated with disturbances in ionic regulation. Therefore, cells rich in mitochondria were isolated from gills of the seawater clam *Mesodesma mactroides*, incubated in isosmotic saline solution (840 mOsmol/Kg H_2O), and exposed (3 h) to environmentally realistic Cu concentrations (5, 9 and 20 µg/L). In cells exposed to 20 µg Cu/L, there were a decrease in Na^+, K^+ and Mg^{2+} content and an inhibition of carbonic anhydrase activity, without changes in cell viability, Ca^{2+} and Cl^- contents, and Na^+,K^+,ATPase activity. Considering that changes in environmental salinity may influence Cu toxicity, isolated cells were maintained (3 h) under isosmotic or transferred to hyposmotic conditions (730 and 670 mOsmol/Kg H_2O) in either the absence or the presence of Cu. In the absence of Cu, cell viability and Cu content were reduced in the hypoosmotic media, without changes in Na^+ content and enzymes activities. In the presence of Cu, cells exposed to 20 µg Cu/L in isosmotic medium showed increased Cu content, reduced Na^+ content and inhibited carbonic anhydrase activity. In hypoosmotic media, Cu content increased in cells exposed to 5 and 9 µg Cu/L while Na^+ content and Na^+,K^+,ATPase activity were reduced in cells exposed to 20 µg Cu/L. In turn, carbonic anhydrase activity was dependent on Cu concentration in the incubation medium, being reduced in cells exposed to 9 and 20 µg Cu/L. These findings indicate that Cu is an ionoregulatory toxicant to gill cells of *M. mactroides* and that metal toxicity increases under hypoosmotic conditions. They also suggest that physiology rather than water chemistry is more important in predicting Cu toxicity in environments with varying salinities, pointing out carbonic anhydrase activity of isolated gill cells of *M. mactroides* as a potential biomarker of Cu exposure.

Keywords: carbonic anhydrase, copper, gill, ion regulation, Na^+,K^+,ATPase, seawater clam

INTRODUCTION

Mitochondria-rich cells (MRC) are present in the gill epithelium of several groups of aquatic animals. These cells are formally known as chloride cells or ionocytes and they are involved in osmoregulation (active ion absorption in fresh water and ion secretion in sea water), acid-base regulation, and ammonia excretion (Hwang and Lee, 2007; Evans, 2008; Gilmour and Perry, 2009; Hwang, 2009). Their active participation in the ionic regulation at the gill epithelium makes these cells well studied in both freshwater and seawater animals (Lee et al., 1996; Chang et al., 2001; Chang et al., 2003; Galvez et al., 2006; Hiroi and McCormick, 2007).

Although MRC can differ between fish species, Cl^- channel and Na^+/H^+ exchanger are present in the apical membrane while K^+ channel, Na^+/K^+-ATPase, and $Na^+/K^+/2Cl^-$ co-transporter are present in the basolateral membrane of epithelial gill cells in freshwater teleosts (Hwang et al., 2011). In some invertebrates, such as marine crabs, cells with large amounts of mitochondria have been also identified in the gill epithelium and are shown to be specialized in cellular ion transport (Taylor and Taylor, 1992). These cells were also found in both freshwater and seawater bivalves. In the freshwater mussels *Pyganodon cataract*, *Utterbackia imbecillis* and *Ligumia substrata,* these cells were microscopically identified and were shown to provide energy to the glochidia larvae and uptake of ions (Kays et al. 1990; Schwartz and Dimock, 2001). In the freshwater mussel *Lasmigona costata* and the seawater clam *Mesodesma mactroides,* these cells were isolated from the gill epithelium, fractionated and recognized as good models for *in vitro* toxicological studies (Nogueira et al., 2013).

Considering the background described above, it is worth to mention that many chemicals present in the environment can adversely affect the functioning of cells with large amounts of

mitochondria. Among these compounds, copper (Cu) is an essential metal which affects the Na^+ content in mantle cells (Lopes et al., 2011a) and gill cells rich in mitochondria (Nogueira et al., 2013) isolated from the seawater clam *M. mactroides*. In fact, it has been reported that the key mechanism of the acute Cu toxicity in aquatic animals is associated with ionoregulatory disturbances. This effect is associated with a competition between Cu and other essential cations, especially Na^+, for binding sites at the gill cell membrane. Also, the activity of ion-transporting proteins, such as Na^+/K^+-ATPase, and other enzymes involved in osmoregulation, such as carbonic anhydrase, was shown to be affected by Cu exposure in both seawater and freshwater animals (Bury et al., 1999; Grosell et al., 2002; Bianchini et al., 2004; Pinho et al., 2007; Boyle et al., 2013), including bivalves (Boyle et al., 2013).

Changes in physicochemical parameters such as water salinity, pH and temperature can influence Cu bioavailability and its consequent toxicity. Metal complexation with major anions (e.g., Cl^-, OH^-, HCO_3^-, SO_4^{2-}) present in the water will increase with increasing salinities (Wright 1995). Also, the competition between Cu and major cations (e.g., Na^+, K^+, Mg^{2+} and Ca^{2+}) present in the water for specific transport ligands in the plasma membrane will increase (Nordberg et al., 2007) . It is important to note that animals living in intertidal zones and estuaries, such as bivalves, are directly affected by natural variations in water salinity (Grosell et al., 2007; Monserrat et al., 2007; Prevodnik et al., 2007). In this context, it is worth to mention that studies on the mechanisms involved in ionic regulation and Cu effects on this function are very scarce, especially in seawater animals (Wang, 2001; Grosell et al., 2007). In bivalves, the complexity and adaptive functionality of the gill epithelium provide an excellent model for studying the ion regulation processes because this organ is directly exposed to the surrounding water at the paleal cavity (Gómez-Mendikute et al., 2005; Tse et al., 2006).

In light of the above, we evaluated the effect of Cu on ionic regulation in cells rich in mitochondria isolated from gills of the seawater clam *Mesodesma mactroides* under isosmotic and hypoosmotic conditions. Endpoints analyzed were Cu and ion (Na^+, Cl^-, K^+, Mg^{2+} and Ca^{2+}) contents, as well as Na^+K^+-ATPase and carbonic anhydrase activities after the *in vitro* exposure of isolated cells to three environmentally realistic concentrations of Cu (5, 9 and 20 µg/L).

MATERIAL AND METHODS

Clam collection and acclimation

Clams (*M. mactroides*) were collected at Mar Grosso Beach (São José do Norte, RS, Southern Brazil), immediately transferred to the laboratory, and acclimated for at least 5 days in a plastic tank containing sea water (salinity: 30 ppt) continuously aerated and in the absence of sediment. Temperature (20°C) and photoperiod (12L: 12D) were fixed. The tank was covered with a black plastic to minimize stress. The acclimation medium was completely renewed three-times a week, when clams were fed with the diatomacea *Thalassiosira weissflogii* ($2x10^4$ cells/L). After acclimation, clams were crioanesthetized and had their gills dissected.

Gill cell isolation and fractionation

Gill cell isolation was performed following the procedures previously described by

Nogueira et al. (2013). Briefly, gill tissue of 2 or 3 clams was dissected, pooled, sliced in small pieces, and incubated in a calcium-free phosphate buffer solution (PBS; composition in mM: 342 NaCl; 20 Na_2HPO_4; 1.7 K_2HPO_4; 16 KCl; 780 mOsm; pH 7.5; 20ºC) and shaken (100 rpm; Certomat-MO-II; Sartorius Stedim Biotech GmbH, Göttingen, Germany) for 30 min. Dissociated cells were filtered (30 µm-mesh nylon filter) to remove the non-dissociated tissue and large debris. The filtered solution containing isolated cells was transferred to plastic tubes and centrifuged (360 x*g*) for 3 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA).

Isolated cells were fractionated using the density gradient technique as described by Nogueira et al. (2013). The density gradient was created by diluting a Percoll stock solution (Sigma-Aldrich, St. Louis, MO, USA) to achieve the desired densities: 1.03 g/mL (18.6% Percoll stock solution, 71.4% PBS, 10.0% 1.5 M NaCl), 1.05 g/mL (34.0% Percoll stock solution, 56.0% PBS, 10.0% 1.5 M NaCl); 1.09 g/mL (64.8% Percoll stock solution, 25.2% PBS, 10.0% 1.5 M NaCl). Each density solution was placed in a Falcon-type tube (15 mL) according to the following sequence: 1.09 g/mL, 1.05 g/mL, and 1.03 g/mL. The pool of isolated gill cells was then added to the tube and centrifuged (2.000 x*g*; 10 min). The Fraction II (1.05-1.09 g/mL Percoll interface), which contain the isolated cells rich in mitochondria were collected, washed in PBS (10 mL), and centrifuged (360 x*g*; 3 min). The pellet obtained was resuspended in 1 mL of saline solution isosmotic with the clam hemolymph (composition in mM: 350 NaCl; 9 KCl; 30 $MgCl_2$; 9 $CaCl_2$; 2 $NaHCO_3$; pH 7.5; 20ºC; 740 mOsmol/Kg H_2O) (Lopes et al., 2011a,b). Cell counting was performed under a light microscope (20x magnification) and cell viability (% of viable cells from the total number of cells) was determined using the Trypan Blue exclusion

assay (0.08% Trypan Blue). Only preparations showing more than 80% viable cells were used in the experiments.

Cellular Cu content

Cells rich in mitochondria (10^6 cells) were incubated in 1 mL of isomotic saline solution without Cu (control; 840 mOm/Kg H_2O) or with Cu (5, 9 and 20 µg/L) for 3h, following the procedures described by Lopes et al. (2011a,b) with modifications. Briefly, Cu was added to the saline solution as $CuCl_2$ (Merck, St. Louis, MO, USA). After exposure, cells were centrifuged (360 x*g*) for 1 min (Hettich Zentrifugen, Model Mikro 22 R, Global Medical Instrumentation, Ramsey, MN, USA). The supernatant was discarded and the pellet was quickly washed with an EDTA solution of osmolality similar to the clam hemolymph (composition in mM: 12 EDTA, 385 sucrose) to remove the loosely bound Cu on the cell surface. The washing and centrifugation procedures were repeated. The pellet containing the cells rich in mitochondria was resuspended in a fresh Cu-free saline solution. An aliquot was collected to determine cell viability using the Trypan Blue exclusion assay (0.08% Trypan Blue). The cell sample was then dried (50ºC; 24 h), digested in 50 µL of HNO_3^- suprapur (Merck, St. Louis, MO, USA), and diluted with 450 µL of Milli-Q water. Copper concentration in the digested sample was analyzed by atomic absorption spectrophotometry (AAS 932 Avanta Plus, GBC, Hampshire, IL, USA), as previously described (Pinho et al., 2007; Lopes et al., 2011a,b). Cu content in cells rich in mitochondria was expressed as µmol Cu/10^6 cells, considering the amount of Cu measured in the cells and the respective cell viability in each experimental condition. Four different and independent cell preparations were tested for each experimental condition (n = 4).

Cellular ion content

Cells rich in mitochondria (10^6 cells) were incubated, exposed to Cu, and prepared for cellular ion content measurement as described above for the cell Cu content analysis. An aliquot was collected to determine cell viability using the Trypan Blue exclusion method. The remaining cells were sonicated (Sonozap, Ultrasonic Processor, New York, USA) and adequately diluted for Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- concentration measurement. Ions concentrations were measured by atomic absorption spectrophotometry (AAS 932 Avanta Plus, GBC, Hampshire, IL, USA), except for Cl^-, which was measured using a colorimetric assay (Cloretos, Doles, Goiânia, GO, Brazil).

Ion content in cells rich in mitochondria was expressed as μmol Cu/10^6 cells, considering the amount of ion measured in the cells and the respective cell viability in each experimental condition. Four different and independent cell preparations were tested for each experimental condition (n = 4).

Cellular Na^+,K^+-ATPase activity

Cells rich in mitochondria (10^6 cells) were incubated, exposed to Cu, and prepared for Na^+,K^+-ATPase activity measurement as described above for the cell Cu and ion content analyses. The pellet containing cells rich in mitochondria was homogenized in a buffer solution (composition in mM: 150 sucrose; 5 EDTA; 50 imidazole; pH 7.5). Na^+,K^+-ATPase activity was measured through the difference between the amount of inorganic phosphate (Pi) released from

ATP in the presence of K^+ (medium A) and in the absence of K^+ (medium B) and presence of ouabain, as described by Bianchini and Castilho (1999). Cell homogenate (10 µL) was incubated in medium A (composition in mM: 100 NaCl; 6 $MgCl_2$; 50 imidazole; 20 KCl; 3 ATP; pH 7.5) and medium B (composition in mM: 100 NaCl; 6 $MgCl_2$; 50 imidazole; 3 ATP; 2 ouabain; pH 7.5). The assay was performed at room temperature (20^{o}C) for 1 h. Pi concentration both media (A and B) was determined using a commercial reagent kit (Fosfato; Doles, Goiânia, GO, Brazil). Protein concentration in the cell homogenate was determined using a commercial reagent kit based on the piragalol assay (Microprote; Doles, Goiânia, GO, Brazil). The specific enzyme activity was expressed as µmol Pi/mg protein/h. Five different and independent cell preparations were tested for each experimental condition (n = 5).

Carbonic anhydrase activity

Cells rich in mitochondria (10^6 cells) were incubated, exposed to Cu, and prepared for carbonic anhydrase activity measurement as described above for the Na^+,K^+-ATPase activity analysis. Carbonic anhydrase activity was measured using the method based on the catalysis of the hydration of the carbon dioxide present in a saturated CO_2 solution by the enzyme with subsequent release of H^+ and consequent reduction of the pH. Measurement as performed following the procedures described by Henry (1991). Cell homogenate was added into the reaction solution (composition in mM: 225 mannitol; 75 sucrose; 10 Tris Base; 10 Na_2HPO_4) and an aliquot of a solution saturated with CO_2 was added. The pH was measured every 15 s for 1 min. The enzymatic activity was calculated based on the slope of the linear regression for the pH change over time and the protein content in the cell homogenate. Protein concentration in the cell

homogenate was determined using a commercial reagent kit based on the piragalol assay (Microprote; Doles, Goiânia, GO, Brazil). The specific enzyme activity was expressed as arbitrary unit of carbonic anhydrase (slope of the linear regression)/mg protein/h. Five different and independent cell preparations were tested for each experimental condition (n = 5).

Cu effect under hypoosmotic condition

Based on the observed effects of Cu on the endpoints analyzed in the experiments with the isosmotic saline solution (840 mOsmol/Kg H_2O), additional experiments were performed under hypoosmotic conditions. Following cell isolation and fractionation, cells rich in mitochondria (10^6 cells) were kept under control condition (no Cu addition to the incubation medium) or exposed to Cu (5, 9 and 20 µg Cu/L) for 3 h, as described above for experiments under isosmotic condition. Experiments were then performed using two hypoosmotic saline solutions: 730 mOsmol/Kg H_2O (composition in mM: 318 NaCl; 8 KCl; 9.5 $MgCl_2$; 30 $NaHCO_3$; 1.35 $CaCl_2$; pH 7.6) and 670 mOsmol/Kg H_2O (composition in mM: 285 NaCl; 7.25 KCl; 8.5 $MgCl_2$; 26.6 $NaHCO_3$; 1.17 $CaCl_2$; pH: 7.6). The isosmotic solution (840 mOsm/Kg H_2O) kcorrespond 25 ppt salinity which is acceptable considering the variation of salinity in the coastal region. The hypoosmotic solutions 730 and 670 mOsm/Kg H_20 correspond 22.5 and 20 ppt, respectively, and they are within the tolerance of the animal (15 to 35 ppt; Seelinger et al., 1998). Cell homogenates were prepared and endpoints analyzed as described above for experiments with the isosmotic saline solution. Based on results obtained under isosmotic conditions, endpoints analyzed were cell viability, cellular Cu and Na^+ content and Na^+,K^+-

ATPase and carbonic anhydrase activity. The number of replicates analyzed and data expression were as described above for experiments with the isosmotic saline solution.

Data presentation and statistical analyses

Data were expressed as mean ± standard error, where each measurement represents a pool of cells present in Fraction II and isolated from the gill epithelium of 2 or 3 clams. For each treatment, the general mean value and standard error were calculated based on the mean value obtained for the pools of cells within the same treatment. For cellular Cu content (n = 4), ion content (n = 4), Na^+,K^+-ATPase (n = 5) and carbonic anhydrase activity (n = 5), comparisons among treatments were performed through one-way analysis of variance (ANOVA) followed by the Fisher LSD test. For experiments performed under isosmotic and hypoosmotic conditions, comparisons among treatments were performed through two-way ANOVA followed by the Fisher LSD test. In all cases, ANOVA assumptions (data normality and homogeneity of variances) were previously verified. Data were mathematically transformed (square root transformation) when ANOVA assumptions were not verified. The significance level adopted was 95% ($\alpha = 0.05$). Statistical analyses were performed using the software Statistica 7.0 (Stat Soft, USA).

RESULTS

Cu exposure under control osmotic condition

Viability of cells rich in mitochondria isolated from gills of the seawater clam *M. mactroides* kept under control osmotic condition (840 mOsmol/Kg H_2O) without Cu addition in the incubation medium (81.3 ± 2.7%) was not significantly different from that observed under the same osmotic condition but in the presence of 5 µg Cu/L (82.8 ± 2.5%), 9 µg Cu/L (78.8 ± 3.2%), or 20 µg Cu/L (78.8 ± 1.7%) for 3 h. Cellular Cu content increased with increasing Cu concentration in the incubation medium, being significantly higher in cells exposed to 20 µg Cu/L (Fig 1). However, a significant decrease in cellular Na^+ (Fig. 2A), K^+ and Mg^{2+} content (Fig. 2B) was observed in cells exposed to 20 µg Cu/L without changes in the cellular Cl^- (Fig. 2A) and Ca^{2+} content (Fig. 2B). A significant decrease in cellular K^+ content was also observed in cells exposed to 5 µg Cu/L (Fig. 2B). Regarding the activity of enzymes involved in ionic regulation, Na^+,K^+-ATPase activity was not affected by Cu exposure (Fig. 3A). However, carbonic anhydrase activity was significantly reduced in cells exposed to 9 and 20 µg Cu/L (Fig. 3B).

Cu exposure under hypoosmotic condition

The viability of cells rich in mitochondria isolated from gills of the seawater clam *M. mactroides* immediately after dissociation was 82.4 ± 1.0% and significantly reduced after 3 h of incubation in hypoosmotic saline solutions (730 and 670 mOsmol/Kg H_2O) in the absence of Cu addition to the incubation medium. No significant effect of Cu on cell viability was observed in any of the osmotic conditions tested (Table 1).

When control cells were compared, a significant reduction in cellular Cu content was observed in cells maintained in the hypoosmotic saline solution at 670 mOsmol/Kg H_2O. Also,

significant increases in cellular Cu content were observed in cells exposed to 9 µg Cu/L in the hypoosmotic saline solution at 730 mOsmol/Kg H_2O and to 5 or 9 µg Cu/L in the hypoosmotic saline solution at 670 mOsmol/Kg H_2O (Fig. 4A). On the other hand, cellular Na^+ content was significantly reduced in cells exposed to 20 µg Cu/L at 640 mOsmol/Kg H_2O as observed in the isosmotic saline solution. When control cells were compared, no significant difference in cellular Na^+ content was observed among the osmotic conditions tested (Fig. 4B).

The activity of enzymes involved in ionic regulation (Na^+,K^+-ATPase and carbonic anhydrase) did not change with the osmotic condition tested in control cells. Also, no Cu effect was observed in Na^+,K^+-ATPase activity of cells incubated in the isosmotic saline solution or the hypoosmotic saline solution at 730 mOsmol/Kg H_2O. However, a significant inhibition of the Na^+,K^+-ATPase activity was observed in cells exposed to 20 µg Cu/L in hypoosmotic saline solution at 670 mOsmol/Kg H_2O (Fig. 5A). Also, a significant inhibition of carbonic anhydrase activity was observed in cells exposed to 9 and 20 µg Cu/L in all osmotic conditions tested (Fig. 5B).

DISCUSSION

Cu effects under isosmotic condition

The separation of cell types have been consider useful and adequate for biochemical and cytological analyses, such as functional characterization of cell membrane transporters related to Na^+ uptake (Goss et al., 2001; Galvez et al., 2002; Reid et al., 2003; Nogueira et al., 2013). Therefore, the paucity of *in vitro* toxicological studies using cells isolated from bivalves led us to

obtain gill cells rich in mitochondria, exposed them to environmentally realistic Cu concentrations, and analyse a suite of parameters involved in ionic regulation.

Under isosmotic condition (840 mOsm/Kg H_2O), exposure to 20 µg Cu/L resulted in significant Cu accumulation in cells rich in mitochondria isolated from gills of the seawater clam *M. mactroides* but any evident lethal toxicity was observed. These findings are in agreement with a previous report from our laboratory (Nogueira et al., 2013). An increased cellular Cu content was also reported in cells isolated from the mantle of *M. mactroides* exposed *in vitro* to Cu. However, this Cu accumulation resulted in reduced cell viability, likely associated with the much higher (8-fold) concentrations of Cu tested than those used in the present study (Lopes et al., 2011a). In fact, results from our laboratory (data not published) suggest that isolated gills cells (total pool of cells) of *M. mactroides* accumulate Cu in concentrations of Cu ranging from 7 to 360 µg Cu/L without causing lethal toxicity. As previously suggested for whole bivalves (Salánki et al., 2003), these findings clearly suggest that cells isolated from gills and mantle of seawater bivalves are useful tools to monitor the water contamination with Cu, as well as adequate models to understand the mechanism involved in Cu toxicity in aquatic invertebrates. It is worth to mention that Cu has being characterized as a ionoregulatory toxicant in both freshwater and seawater fish and invertebrates (Bury et al., 1999; Grosell et al., 2002; Bianchini et al., 2004; Pinho et al., 2007; Boyle et al., 2013), including bivalves (Lopes et al., 2011a; Boyle et al., 2013; Nogueira et al., 2013).

In the present study, isolated gill cells rich in mitochondria had reduced Na^+, K^+ and Mg^{2+} content after the 3-h period of exposure to Cu (20 µg/L) when compared to that of cells maintained under control condition (no Cu addition in the water). This effect was not paralleled by significant changes in cellular Ca^{2+} and Cl^- contents. It is likely related to an extracellular

competition between Cu and Na^+ for the transporting sites available at the cell plasma membrane. Therefore, an extracellular Cu competition to entry into the cell the Na^+/H^+ exchange is expected, thus affecting the Na^+ entry (Grossel et al. 2002; Nordberg, 2007). The other possible explanation for the Cu-induced decreased in cellular Na^+ content would be the influence of Cu on activity of enzymes involved in ionic regulation, such as Na^+,K^+-ATPase and carbonic anhydrase (Grosell et al., 2002; Bianchini et al., 2004; Pinho et al., 2007; Lopes et al., 2011a; Boyle et al., 2013).Regarding K^+ disturbance, the exact mechanism involved in Cu-induced in cellular K^+ regulation is still not clear (Tellis et al., 2012; Lopes et al., 2011a). Although, no clear and significant change was observed in cellular Cl^- content, the observed reduction in cellular Na^+ content after exposure to 20µg Cu/L could help to explain the decreased cellular K^+ content at this condition. This statement is based on the fact that a Na^+ gradient is necessary for the K^+ transport through the $Na^+,K^+,2Cl^-$ co-transporter (Hiroi and McCormick, 2007). In turn, the reduced cellular content of Mg^{2+} would be associated with its participation in the Na^+,K^+-ATPase activity. It is reported that exposure to Cu induces a replacement of Mg^{2+}, the enzyme co-factor, with Cu thus leading Mg^{2+} available for extrusion from the cell (Handy et al. 2002). However, the *in vitro* methodology used for assessment of Na^+,K^+-ATPase not allow us to evaluate this replacement effect once we evaluated the "optimal operation" of this enzyme by supplying high amounts of Mg^{2+}. Further studies focused on the Cu influence on cellular Mg^{2+} regulation are needed for a better understanding of the observed effects.

In the present study, no significant Cu effect on Na^+,K^+-ATPase activity of isolated gill cells was observed. This enzyme requires the presence of Na^+, K^+ and Mg^{2+} to be active. The presence of Ca^{2+} facilitates the ATP hydrolysis, thus releasing the energy required for ion (Na^+ and K^+) transport against the concentration gradient (Marshall and Grosell, 2005). Despite the

observed reduction in cellular Na^+ content and the higher amount of Cu accumulated in the cell observed after exposure to 20 µg Cu/L, these effects were not enough to significantly affect the enzyme activity in the present study. In fact, expression of Na^+,K^+-ATPase in gills of the seawater clam *M. mactroides* was shown to increase after exposure to a sublethal concentration of Cu (300 µg Cu/L) for 96 h (Boyle et al., 2013). Authors suggested that this response would be a compensatory mechanism to ameliorate a possible Cu-induced inhibition of the gill enzyme activity. It was also reported that Na^+,K^+-ATPase activity in mitochondria-rich cells PNA- isolated from gill tissue of the freshwater fish *Oncorhynchus mykiss* was also not affected by exposure to 63.45 µg Cu/L for 1 and 30 min (Goss et al., 2011).

As observed for other metals (Vitale et al., 1999; Skaggs and Henry, 2002; Lionetto et al., 2006; Soyut et al., 2008; Goss et al., 2011), carbonic anhydrase activity of cells rich in mitochondria isolated from gills of the seawater clam *M. mactroides* was significantly inhibited after exposure (3 h) to Cu (20 µg Cu/L). Carbonic anhydrase is a key enzyme in the adaptation of marine organisms to changes in environmental conditions. Responsible for acid-base balance and ammonia excretion, it is reported to be inhibited in both fish (Zimmer et al., 2012) and invertebrates (Vitale et al., 1999; Skaggs and Henry, 2002) exposed *in vivo* to low concentrations of heavy metals, including Cu. In turn, carbonic anhydrase activity in mitochondria-rich cells PNA- isolated from gills of the freshwater fish *O. mykiss* did not change after *in vitro* exposure to 63.5 µg Cu/L for 1and 30 min (Goss et al., 2011). Inhibition of carbonic anhydrase activity induced by Cu occurs through a non-competitive manner, i.e., binding imidazole side and blocking proton transfer from the zinc-bound to buffer molecules located outside of the active site region (Tu et al., 1981; Lionetto et al., 2000). It is important to note the time-dependence as an important aspect to analyse the carbonic anhydrase inhibition. This statement is based on the

fact that Cu concentrations used in the present study is ~3-fold lower than that used to expose the mitochondria-rich cells PNA- isolated from gills of the freshwater fish *O. Mykiss* (Goss et al., 2011). However, the longer (3-fold) period of exposure to Cu adopted in the present study was adequate to reveal a Cu-induced enzyme inhibition.

Cu effects under hypoosmotic condition

Under control condition (no Cu addition in the water), viability of cells rich in mitochondria isolated from gills of the seawater clam *M. mactroides* was significantly reduced after incubation in hypoosmotic media (730 and 670 mOsmol/Kg H_2O) for 3h. During cell counting, it was observed a cell swelling after maintenance at 670 mOsm/Kg H_2O medium. It is known that cells exposed to environments osmotic variation causes changes in cellular volume (Neufeld and Wright, 1996; Lang et al., 1998; Leguen and Prunet, 1998; Neufeld and Wright, 1998). In hypoosmotic medium, the cells swell due to an influx of water, following a cellular volume regulation occurs by release constituents of the cytoplasmic pool of organic molecules into the extracellular medium and cytoplasmic inorganic ions which can induce the enzymes related to ion transport (Neufeld and Wright, 1996; Henry et al., 2003). Despite this phenomenon, no significant change was observed in cellular Na^+ content and also in the enzymes (Na^+,K^+-ATPase and carbonic anhydrase) activity among the different control osmotic conditions tested (840, 730 and 670 mOsmol/Kg H_2O).

Previously data reported for mitochondria-rich cells isolated from gills of the killifish *Fundulus heteroclitus* and the seawater-acclimated medaka *Oryzias dancena* (Marshall et al., 1999; Katoh et al., 2002; Kang et al., 2013). In turn, another study showed an increased enzyme

activity after incubating cells isolated from the sea bream under hypoosmotic condition (Kelly and Woo, 1999). In invertebrates, carbonic anhydrase activity was also shown to increase in the gill tissue of the bivalves *Rangia cuneata*, *Ligumia subtrata* and *Corbicula fluminea* (Henry and Saintsing, 1984) and the crab *Neohelice granulata* (Genovese et al., 2005) acclimated to hypoosmotic condition. However, it is important to stress that the animals tested in these studies were fully acclimated to a hypoosmotic medium, differently from the approach adopted in the present study where cells were abruptly exposed to the hypoosmotic medium. In the former case, the experimental approach would have allowed to the animals the time enough for adjusting the level of enzyme activity required for homeostasis maintenance (Kang et al., 2013). Therefore, the reduced viability and the lack of change in carbonic anhydrase activity observed in isolated cells of *M. mactroides* in the present study could be related to the experimental approach adopted. Further studies on fully acclimated clams to hypoosmotic condition would help to clarify this point.

Let us know consider the response of the endpoints analyzed when cells rich in mitochondria isolated from gills of the seawater clam *M. mactroides* were exposed to Cu under different osmotic conditions. The Cu content in isolated cells exposed to Cu under both the hypoosmotic conditions showed a similar pattern to that observed in cells maintained in isosmotic medium (840 mOsmol/Kg H_2O), except for cells exposed to 20 µg Cu/L. An increased Cu accumulation after decreasing the osmolality of the incubation medium would be reasonable considering a possible reduction in competition between Cu and major cations, especially Na^+, for binding sites on the cell membrane with a consequent protection against Cu toxicity (Grosell and Wood, 2002; Lauren and McDonald, 1985; Lauren and McDonald, 1986). However, an increased Cu content was only observed in cells exposed to 5 and 9 µg Cu/L in the hypoosmotic

media. These findings support the idea that physiology rather than Cu speciation and competition is determining the relative sensitivity to Cu in animals often subjected to changes in environmental salinity (Grossel et al., 2007). In fact, the physiological significance of regulating the Cu content in cells rich in mitochondria isolated from gills of the seawater clam *M. mactroides* is supported by the observed decrease in Cu content of control cells incubated in the more dilute media (670 mOsmol/Kg H_2O) without Cu addition, as well as those exposed to 20 µg Cu/L in both hypoosmotic media (730 and 670 mOsmol/Kg H_2O).

Regarding the Cu effect on cell ion regulation, exposure of isolated cells to 20 µg Cu/L in the isosmotic medium (840 mOsmol/Kg H_2O) and the more dilute medium (670 mOsmol/Kg H_2O) significantly reduced the cellular Na^+ content. At this point, it is important to note that both Na^+,K^+-ATPase and carbonic anhydrase activities are related to Na^+ uptake. However, the Na^+,K^+-ATPase activity is considered the major determinant of cytoplasm Na^+ concentration, exchanging intracellular Na^+ for extracellular K^+ at a 3:2 ratio (Marshall and Bryson, 1998; Handy et al., 2002; Marshall, 2002). In the present study, both enzymes were inhibited by Cu exposure.

A significant decrease in Na^+,K^+-ATPase activity was observed after exposure of cells rich in mitochondria isolated from gills of *M. mactroides* to Cu. This effect was observed in cells exposed to 20 µg Cu/L in the more dilute saline solution (670 mOsmol/Kg H_2O). This finding is in agreement with previous studies in seawater fish, where Na^+,K^+-ATPase inhibition is directly related to Cu exposure under hypoosmotic conditions (Grosell and Wood, 2002; Blanchard and Grosell, 2006). However, this is not clear in hyperosmotic conditions, since lethal toxicity is observed and related to Na^+ regulation in seawater fish, but without significant change in

Na^+,K^+-ATPase activity (Grosell et al., 2003, 2004; Blanchard and Grosell, 2006; Zimmer et al., 2012).

As for Na^+,K^+-ATPase, a significant inhibition of carbonic anhydrase activity was also observed in isolated cells exposed to Cu. However, this enzyme showed to be more sensitive to Cu exposure than the Na^+,K^+-ATPase. This statement is based on the fact that a significant inhibition of the carbonic anhydrase activity was observed in isolated cells exposed to 9 and 20 µg Cu/L in all osmotic conditions tested. As discussed above, this finding also support the idea that physiology, rather than water chemistry alone, may be more important in predicting Cu toxicity in environments with varying salinities (Grossel et al., 2002; Grossel et al., 2007; Zimmer et al., 2012). Taken altogether, these findings clearly suggest that an environmentally realistic Cu concentration (9 µg Cu/L) has the potential to inhibit the carbonic anhydrase activity in gill cells, especially when Cu exposure is combined with a reduction in environmental salinity.

Finally, it is clear from the present results the influence of the activities of enzymes associated with ionic regulation (Na^+,K^+,ATPase and carbonic anhydrase) on Cu accumulation in gill cells rich in mitochondria. This statement is based on the fact that the observed reduction in Cu content was paralleled by a decrease in Na^+ content and inhibition of the activity of both enzymes when isolated cells were incubated in the more dilute medium (640 mOsmol/Kg H_2O) and exposed to the higher Cu concentration tested (20 µg Cu/L). This finding strongly suggests that Cu is driven by the cytoplasm electronegativity generated mainly by Na^+,K^+-ATPase activity and the direct action of the carbonic anhydrase activity to drive Na^+ and/or Cu into the cell. Further studies on the influence of the activity of these enzymes on Cu accumulation in isolated cells of aquatic animals should help to confirm this hypothesis.

In summary, findings reported in the present study show that Cu is accumulated in cells rich in mitochondria isolated from gills of the seawater clam *M. mactroides* after *in vitro* exposure to environmentally realistic concentrations of Cu. Although no lethal toxicity of Cu was observed, metal exposure induced ionic (especially Na^+) disturbance paralleled by a marked inhibition of the carbonic anhydrase activity under both isosmotic and hypoosmotic conditions. As suggested for other marine osmoconforming animals (Lioneto et al., 2012), the acute Cu toxicity to cells rich in mitochondria isolated from gills of the seawater clam *M. mactroides* could be associated with the inhibition of the carbonic anhydrase activity. This statement is based on the fact that this effect was observed after exposure to environmentally realistic copper concentrations in a concentration-dependent manner. As previously suggested by Lionetto et al. (2005; 2006), the wide use of bivalves to monitor and assess the quality of aquatic environments together with the findings reported in the present study strongly support the idea of developing an *in vitro* bioassay based on the carbonic anhydrase activity to determine the toxicity of environmental water samples contaminated with metals, including Cu.

ACKNOWLEDGMENTS

We thank Indianara Fernanda Barcarolli (Universidade do Estado de Santa Catarina, Brazil). Financial support is acknowledged to the International Development Research Centre (IDRC, Ottawa, Canada), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES – Programa Ciências do Mar, Brasília, DF, Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática, Brasília, DF, Brazil). A. Bianchini is a research fellow from the

Brazilian CNPq (Proc. # 304430/2009-9) and supported by the International Canada Research Chair Program from IDRC.

REFERENCES

Bianchini, A., Castilho, P.C. 1999. Effects of zinc exposure on oxygen consumption and gill Na^+,K^+-ATPase of the estuarine crab *Chasmagnathus granulata* Dana, 1851 (Decapoda, Grapsidae). Bull. Environ. Contam. Toxicol. **62,** 63-69.

Bianchini, A., Martins, S.E.G., Barcarolli, I.F. 2004. Mechanism of acute copper toxicity in euryhaline crustaceans: implications for the Biotic Ligand Model. Int. Cong. Ser. 1275, 189-194.

Blanchard, J., Grosell, M. 2006. Copper toxicity across salinities from freshwater to seawater in euryhaline fish *Fundulus heteroclitus*: is copper an ionoregulatory toxicant in high salinities? Aquat. Toxicol. 80, 131-139.

Boyle, R.T., Oliveira, L.F., Bianchini, A., Souza, M.M. 2013. The effects of copper on Na^+/K^+-ATPase and aquaporin expression in two euryhaline invertebrates. Bull. Environ. Contam. Toxicol. 90, 387-390.

Bury, N.R., Grosell, M., Grover, A.K., Wood, C.M. 1999. ATP-dependent silver transport across the basolateral membrane of rainbow trout gills. Toxicol. Appl. Pharmacol. 159, 1-8.

Chang, I.C., Wei, Y.Y., Chou, F.I., Hwang, P.P. 2003. Stimulation of Cl^- uptake and morphological changes in gill mitochondria-rich cells in freshwater tilapia (*Oreochromis mossambicus*). Physiol. Biochem. Zool. 76, 544-552.

Chang, I.C., Lee, T.H., Yang, C.H., Wei, Y.W., Chou, F.I., Hwang, P.P. 2001. Morphology and function of gill mitochondria-rich cells in fish acclimated to different environments. Physiol. Biochem. Zool. 74, 111-119.

Evans, D.H. 2008. Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. Am. J. Physiol. Regul. Integr. Comp. Physiol. 704-713.

Galvez, F., Wong, D., Wood, C.M. 2006. Cadmium and calcium uptake in isolated mitochondria-rich cell populations from the gills of the freshwater rainbow trout. Amer J of Physiol 291: 170–176.

Galvez, F., Reid, S.D., Hawkings, G., Goss, G.G. 2002. Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. Am. J. Physiol. Regul. Integr. Comp. Physiol. 282, 658-668.

Genovese, G., Ortiz, N., Urcola, M.R., Luquet, C.M. 2005. Possible role of carbonic anhydrase, V-H^+-ATPase, and Cl^-/HCO^{3-} exchanger in electrogenic ion transport across the gills of the euryhaline crab *Chasmagnathus granulatus*. Comp. Biochem. Physiol. A 142, 362-369.

Gilmour, K.M., Perry, S.F. 2009. Carbonic anhydrase and acid-base regulation in fish. J. Exp. Biol. 212, 1647-1661.

Gómez-Mendikute, A., Elizondo, M., Venier, P., Cajaraville, M.P. 2005. Characterization of mussel gill cells *in vivo* and *in vitro*. Cell Tissue Res. 321, 131-140.

Goss, G., Gilmour, K., Hawkings, G., Brumbach, J.H., Huynh, M., Galvez, F. 2011. Mechanism of sodium uptake in PNA negative MR cells from rainbow trout, *Oncorhynchus mykiss* as revealed by silver and copper inhibition. Comp. Biochem. Physiol. A 159, 234-241.

Goss, G.G., Adamia, S., Galvez, F. 2001. Peanut lectin binds to a subpopulation of mitochondria- rich cells in the rainbow trout gill epithelium Am. J. Physiol. Regul. Integr. Comp. Physiol. 281, 1718-1725.

Grosell, M., Blanchard, J., Brix, K.V., Gerdes, R. 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquat. Toxicol. 84, 162-172.

Grosell, M., McDonald, M.D., Wood, C.M., Walsh, P.J. 2004. Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) I. Hydromineral balance and plasma nitrogenous waste products. Aquat. Toxicol. 68, 249-262.

Grosell, M.,Wood, C.M., Walsh, P.J. 2003. Copper homeostasis and toxicity in the elasmobranch *Raja erinacea* and the teleost *Myoxocephalus octodecem spinosus* during exposure to elevated waterborne copper. Comp. Biochem. Physiol. C 135, 179-190.

Grosell, M., Nielsen, C., Bianchini, A. 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comp. Biochem. Physiol. C 133, 287-303.

Grosell, M., Wood, C.M. 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179-1188.

Handy, R.D., Eddy, F.B., Baines, H. 2002. Sodium-dependent copper uptake across epithelia: a review of rationale with experimental evidence from gill and intestine. Bioch. Bioph. Acta 1566, 104-115.

Henry, R.P., Saintsing, D.G. 1983. Carbonic anhydrase activity and ion regulation in three species of osmoregulating bivalve molluscs. Physiol. Zool. 56, 274-280.

Henry, R.P. 1991. Techniques for measuring carbonic anhydrase in vitro: the electrometric delta pH and the pH stat methods. In: Dodgsom, S.J., Tashianm, R.E., Gros, G., Carter, N.D. (Eds.), The Carbonic Anhydrases. Plenum Press, pp. 119-125.

Henry, R.P., Gehnrichc, S., Weihrauch, D., Towle, D.W., 2003. Salinity-mediated carbonic anhydrase induction in the gills of the euryhaline green crab, *Carcinus maenas*. Comp. Biochem. Physiol. A 136, 243–258.

Hiroi, J., McCormick, S.D. 2007. Variation in salinity tolerance, gill Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. J. Exp. Biol. 210, 1015-1024.

Hwang, P.P., Lee, T.H., Lin, L.Y. 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, 28-47.

Hwang, P.P. 2009. Ion uptake and acid secretion in zebrafish (*Danio rerio*). J. Exp. Biol 212, 1745-1752.

Hwang, P.P., Lee, T.H. 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp. Biochem. Physiol. A 148, 479-497.

Kang, C.K., Yang, W.K., Lin, S.T., Liu, C.C., Lin, H.M., Chen, H.H., Cheng, C.W., Lee, T.H., Hwang, P.P. 2013. The acute and regulatory phases of time-course changes in gill mitochondrion-rich cells of seawater-acclimated medaka (*Oryzias dancena*) when exposed to hypoosmotic environments. Comp. Biochem. Physiol. A 164, 181-191.

Katoh, F., Shimizu, A., Uchida, K., Kaneko, T. 2002. Shift of chloride cell distribution during early life stages in seawater adapted killifish, *Fundulus heteroclitus*. Zool. Sci. 17, 11-18.

Kays, W.T., Silverman, H., Dietz, T.H. 1990. Water channels and water canals in the gill of the freshwater mussel, *Ligumia subrostrata*: ultrastructure and histochemistry. J. Exp. Zool. 254, 256–269.

Kelly, S.P., Woo, N.Y.S. 1999. The response of sea bream following abrupt hypoosmotic exposure. J. Fish Biol. 55, 732-750.

Lang, F., Busch, G.L. Ritter, M., Völkl, H., Waldegger, S., Gulbins, E., Üssinger, D., 1998. Functional significance of cell volume regulatory mechanisms. Physiol. Rev. 78 (1), 247-306.

Lauren, D.J., McDonald, D.G. 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* (Richardson). J. Comp. Physiol. B 155, 635-644.

Lauren, D.J., McDonald, D.G. 1986. Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri* . Can. J. Fish. Aquat. Sci. 43, 1488-1496.

Lee, T.H., Hwang, P.P., Lin, H.C., Huang, F.L. 1996.Mitochondria-rich cells in the branchial epithelium of the teleost, *Oreochromis mossambicus*, acclimated to various hypotonic environments. Fish Physiol. Biochem. 15, 513-523.

Leguen, I., Prunet, P. 2001. In vitro effect of various xenobiotics on trout gill cell volume regulation after hypotonic shock. Aquat. Toxicol. 53, 201–214.

Lionetto, M.G., Giordano, M.E., Vilella, S., Schettino, T., 2000. Inhibition of eel enzymatic activities by cadmium. Aquat. Toxicol. 48, 561-571.

Lionetto, M.G., Caricato, R., Erroi, E., Giordano, M.E., Schettino, T. 2005. Carbonic anhydrase based environmental bioassay. Intern. J. Environ. Anal. Chem. 85, 12-13, 895-903.

Lionetto, M.G., Caricato, R., Erroi, E., Giordano, M.E., Schettino, T. 2006. Potential application of carbonic anhydrase activity in bioassay and biomarker studies. Chem. Ecol. 22(S1), 119-125.

Lionetto, M.G., Caricato, R., Giordano, M.E., Erroi, E., Schettino, T. 2012. Carbonic anhydrase as pollution biomarker: An ancient enzyme with a new use. Int. J. Environ. Res. Public Health 9, 3965-3977.

Lopes, T.M., Barcarolli, I.F., Oliveira, C.B., Souza, M.M., Bianchini, A. 2011a. Effect of copper on ion content in isolated mantle cells of the marine clam *Mesodesma mactroides*. Environ. Toxicol. Chem. 30, 1582-1585.

Lopes, T.M., Barcarolli, I.F., Oliveira, C.B., Souza, M.M., Bianchini, A. 2011b. Mechanisms of copper accumulation in isolated mantle cells of the marine clam *Mesodesma mactroides*. Environ. Toxicol. Chem. 30, 1586-1592.

Marshall, W.S. 2002. Na^{+}, Cl^{-}, Ca^{+2} and Zn^{+2} transport by fish gills: retrospective review and prospective synthesis. J. Exp. Zool. 293, 264-283.

Marshall, W.S., Bryson, S.E. 1998. Transport mechanisms of seawater teleost chloride cells: an inclusive model of a multifunctional cell. Comp. Biochem. Physiol. A 119, 97-106.

Marshall, W.S., Emberley, T.R., Singer, T.D., Bryson, S.E., McCormick, S.D. 1999. Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. J. Exp. Biol. 202, 1535-1544.

Marshall, W.S., Grosell, M. 2005. Ion transport, osmoregulation, and acid-base balance. In: Evans, D.H., Clairborne, J.B. (Eds.), The Physiology of Fish, 3rd Edition, CRC Press, pp. 177-230.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M.G., Pinho, G.L.L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. Comp. Biochem. Physiol. C 146, 221-234.

Neufeld, D.S., Wright, S.H. 1996. Response of cell volume in *Mytilus* gill to acute salinity change. J. Exp. Biol. 199, 473–484.

Neufeld, D.S., Wright, S.H. 1998. Effect of cyclical salinity changes on cell volume and function in *Geukensia demissa* gills. J. Exp. Biol. 201, 1421–1431.

Nogueira, L.S., Wood, C.M., Gillis, P., Bianchini, A., 2013. Isolation and fractionation of gill cells from freshwater (*Lasmigona costata*) and seawater (*Mesodesma mactroides*) bivalves for use in toxicological studies with copper. Cytotechnol. (*In preparation*).

Nordberg, G.F., Fowler, B.A., Nordberg, M., Friberg, L. 2007. Handbook on the Toxicology of Metals. 3rd Edition. Academic Press, New York.

Pinho, G.L.L., Pedroso, M.S., Rodrigues, S.C., Souza, S.S., Bianchini, A. 2007. Physiological effects of copper in the euryhaline copepod *Acartia tonsa*: waterborne versus waterborne plus dietborne exposure. Aquat. Toxicol. 84, 62-70.

Prevodnik, A., Gardestrom, J., Lilja, K., Elfwing, T., McDonagh, B., Petrovic, N., Tedengren, M., Sheehan, D., Bollner, T. 2007. Oxidative stress in response to xenobiotics in the blue mussel *Mytilus edulis* L.: evidence for variation along a natural salinity gradient of the Baltic Sea. Aquat. Toxicol. 82, 63-71.

Reid, S.P., Hawkings, G.S., Galvez, F., Goss, G.G. 2003. Localization and characterization of phenamil-sensitive Na^+ influx in isolated rainbow trout gill epithelial cells. J. Exp. Biol. 206, 551-559.

Salánki, J., Farkas, A., Kamardina, T., Rózsa, K.S. 2003. Mollusc in biological monitoring of water quality. Toxicol. Lett. 140/141, 403-410.

Seeliger, U., Ode brec ht, C., Castello, J.P. 1998. Os ecossistemas costeiro e marinho do extremo sul do Brasil. Rio Grande: Ecoscientia, 341 p.

Schwartz, M.L., Dimock, R.V. Jr. 2001. Ultrastructural evidence for nutritional exchange between brooding unionid mussels and their glochidia larvae. Invert. Biol. 120, 227-236.

Skaggs, H.S., Henry, R.P. 2002. Inhibition of carbonic anhydrase in the gills of two euryhaline crabs, *Callinectes sapidus* and *Carcinus maenas*, by heavy metals. Comp. Biochem. Physiol. C 133, 605-612.

Soyut, H., Beydemir, Ş., Hisar, O. 2008. Effects of some metals on carbonic anhydrase from brains of rainbow trout. Biol. Trace Elem. Res. 123, 179-190.

Taylor, H.H., Taylor, E.W. 1992. Gills and lungs: the exchange of gases and ions. In: Harrison, F.W., Humes, A.G. (Eds.), Microscopic Anatomy of Invertebrates, Decapod Crustacea, vol. 10, Wiley-Liss, pp. 203-293.

Tellis, M.S., Alsop, D., Wood, C.M. 2012. Effects of copper on the acute cortisol response and associated physiology in rainbow trout. Comp. Biochem. Physiol. C, 281- 289.

Tse, W.K.F., Au, D.W.T., Wong, C.K.C. 2006. Characterization of ion channel and transporter mRNA expressions in isolated gill chloride and pavement cells of seawater acclimating eels. Biochem. Bioph. Res. Commun. 346, 1181-1190.

Tu, C., Wynns, G.C., Silverman, D.N. 1981. Inhibition by cupric ions of 18O exchange catalyzed by human carbonic anhydrase. II. Relation to the interaction between carbonic anhydrase and hemoglobin. J. Biol. Chem. 256, 9466-9470.

Vitale, A.M., Monserrat, J.M., Castilho, P., Rodriguez, E.M. 1999. Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). Comp. Biochem. Physiol. C 122, 121-129.

Wang, W.X. 2001. Comparison of metal uptake rate and absorption efficiency in marine bivalves. Environ. Toxicol. Chem. 20, 1367-1373.

Wright, D.A., 1995. Trace metal and major ion interactions in aquatic animals. Mar. Pollut. Bull. 31, 8-18.

Zimmer, A.M., Barcarolli, I.F., Wood, C.M., Bianchini, A. 2012. Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both freshwater and seawater. Aquat. Toxicol. 122-123, 172-180.

Table 1. Viability of the cells rich in mitochondria isolated from gills of the seawater clam *Mesodesma mactroides* kept under control condition (no Cu addition in the water) or exposed (3 h) to different Cu concentrations (5, 9 and 20 µg Cu/L) under different osmotic conditions (840, 730 and 670 mOsmol/Kg H_2O). Data are expressed as mean ± standard error (n = 4).

Osmotic condition	Treatment				
(mOsmol/Kg H_2O)	Control-0 h	Control-3 h	5 µg Cu/L	9 µg Cu/L	20 µg Cu/L
840	82.4 ± 1.0[a]	81.2 ± 2.7[a]	78.8 ± 3.2[a]	78.3 ± 1.8[a]	78.7 ± 1.7[a]
730	82.4 ± 1.0[a]	75.0 ± 1.0[b*]	74.1 ± 0.9[b]	74.0 ± 1.9[b]	74.9 ± 1.0[b]
670	82.4 ± 1.0[a]	71.0 ± 3.4[b*]	71.0 ± 3.6[b]	72.9 ± 3.2[b]	73.1 ± 2.2[b]

FIGURE CAPTIONS

Figure 1. Copper content in cells rich in mitochondria isolated from gills of the seawater clam *Mesodesma mactroides* kept under control condition (no Cu addition in the water) and exposed (3 h) to different copper concentrations (5, 9 and 20 µg Cu/L). Data are expressed as mean ± standard error (n = 4). Different letters indicate significantly different mean values among treatments (p<0.05).

Figure 2. Cellular Na^+, Cl^- (A) and Ca^{2+}, K^+ and Mg^{2+} (B) content in cells rich in mitochondria isolated from gills of the seawater clam *Mesodesma mactroides* kept under control condition (no Cu addition in the water) and exposed (3 h) to different Cu concentrations (5, 9 and 20 µg Cu/L). Data are expressed as mean ± standard error (n= 4). Different letters indicate significantly different mean values among treatments (p<0.05).

Figure 3. Na^+,K^+-ATPase (A) and carbonic anhydrase (B) activity in cells rich in mitochondria isolated from gills of the seawater *Mesodesma mactroides* kept under control condition (no Cu addition in the water) and exposed (3 h) to different Cu concentrations (5, 9 and 20 µg Cu/L). Data are expressed as mean ± standard error (n= 5). Different letters indicate significantly different mean values among treatments (p<0.05).

Figure 4. Cellular Cu (A) and Na^+ (B) content in cells rich in mitochondria isolated from gills of the seawater clam *Mesodesma mactroides* kept under control condition (no Cu addition in the water) and exposed (3 h) to different Cu concentrations (5, 9 and 20 µg Cu/L) under different

osmotic conditions (670, 730 and 840 mOsmol/Kg H_2O). Data are expressed as mean ± standard error (n = 5). Different capital letters indicate significant different mean values among osmotic conditions within the same treatment (control or Cu exposure). Different small case letters indicate significant different mean values among treatments (control or Cu exposure) within the same osmotic condition (p<0.05).

Figure 5. Na^+,K^+-ATPase (A) and carbonic anhydrase (B) activity in cells rich in mitochondria isolated from gills of the seawater clam *Mesodesma mactroides* kept under control condition (no Cu addition in the water) and exposed to different Cu concentrations (5, 9 and 20 µg Cu/L) under different osmotic conditions (670, 730 and 840 mOsmol/Kg H_2O). Data are expressed as mean ± standard error (n = 5). Different capital letters indicate significant different mean values among osmotic conditions within the same treatment (control or Cu exposure). Different small case letters indicate significant different mean values among treatments (control or Cu exposure) within the same osmotic condition (p<0.05).

Figure 1

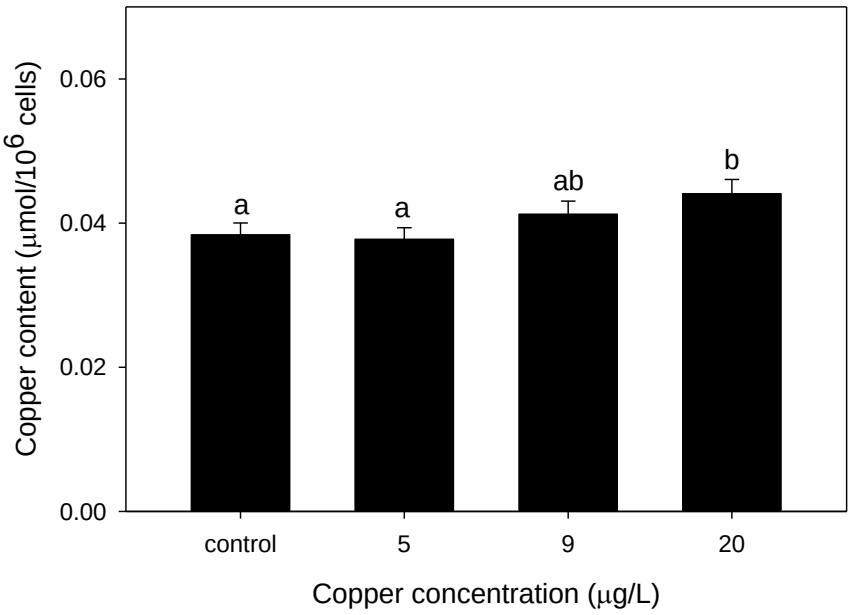
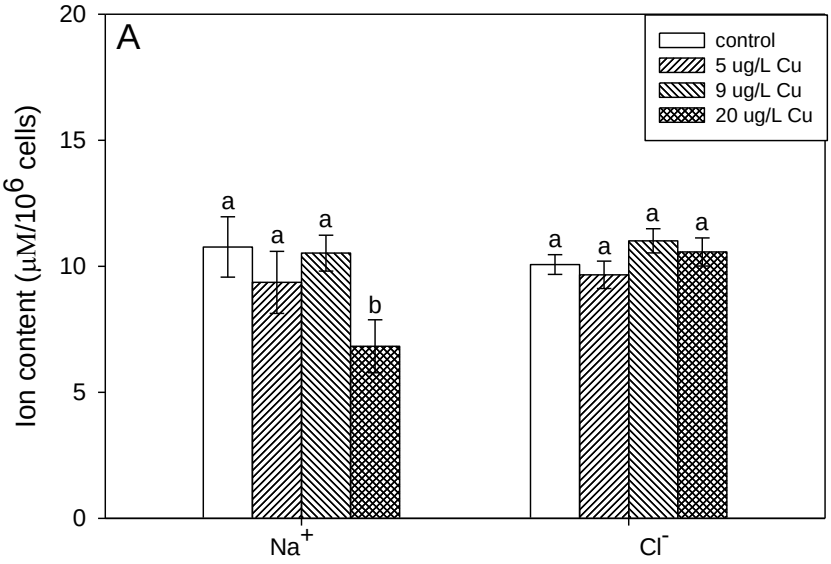

Copper content (μmol/10^6 cells)
0.06
0.04
0.02
0.00
a
a
ab
b
control
5
9
20
Copper concentration (μg/L)

Figure 2


A
control
5 ug/L Cu
9 ug/L Cu
20 ug/L Cu
Ion content (μM/10^6 cells)
20
15
10
5
0
a
a
a
b
a
a
a
a
Na+
Cl-

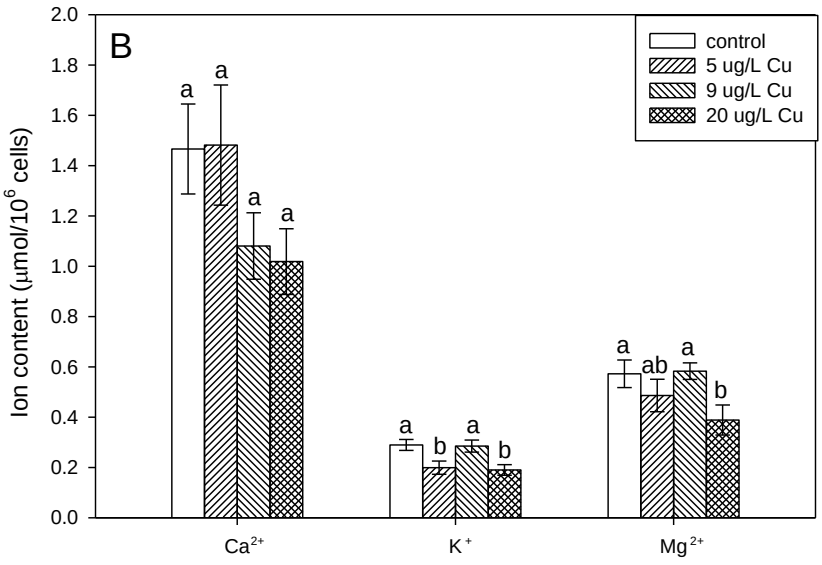

B
control
5 ug/L Cu
9 ug/L Cu
20 ug/L Cu
Ion content (μmol/10^6 cells)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
a
a
b
a
b
a
ab
a
b
Ca2+
K+
Mg2+

Figure 3

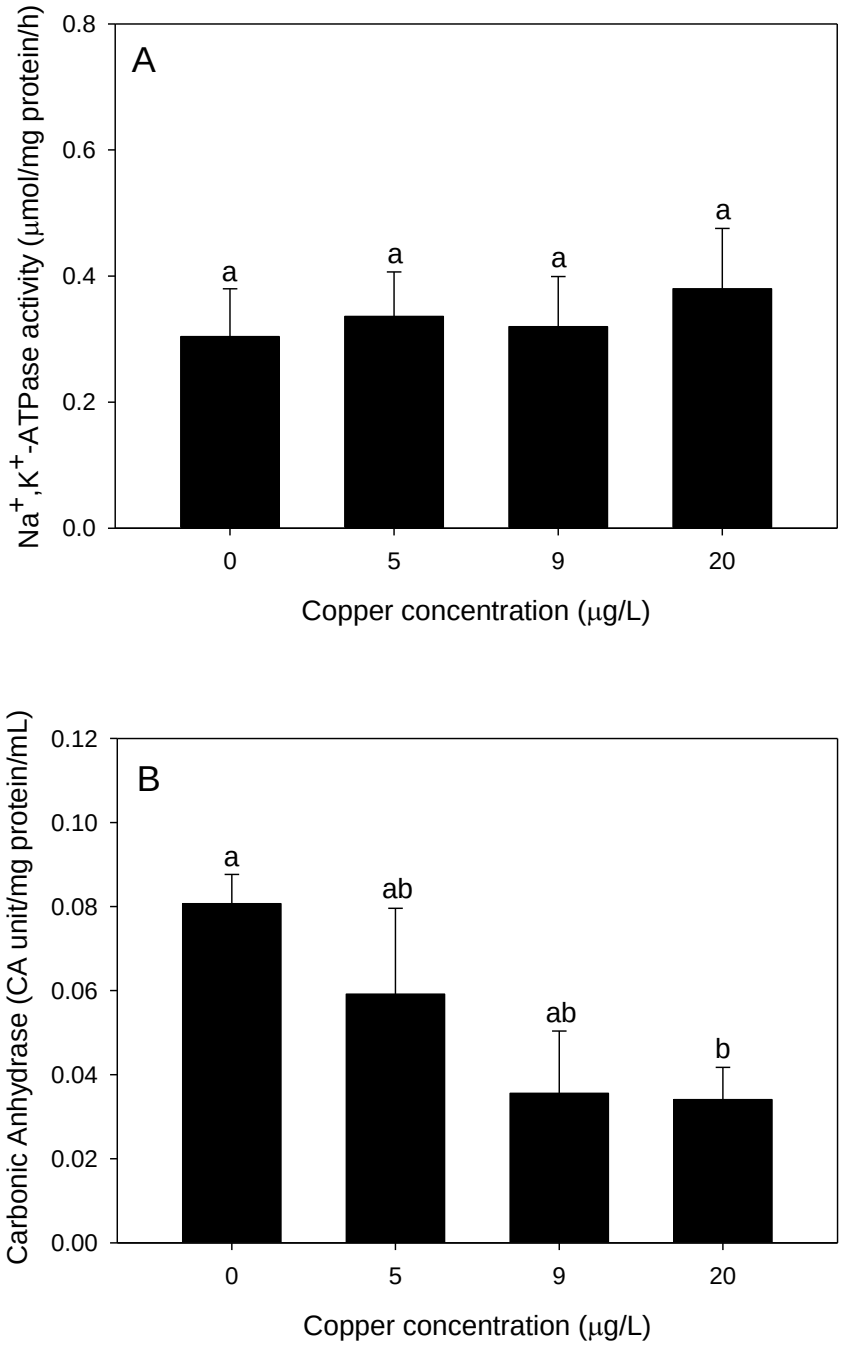
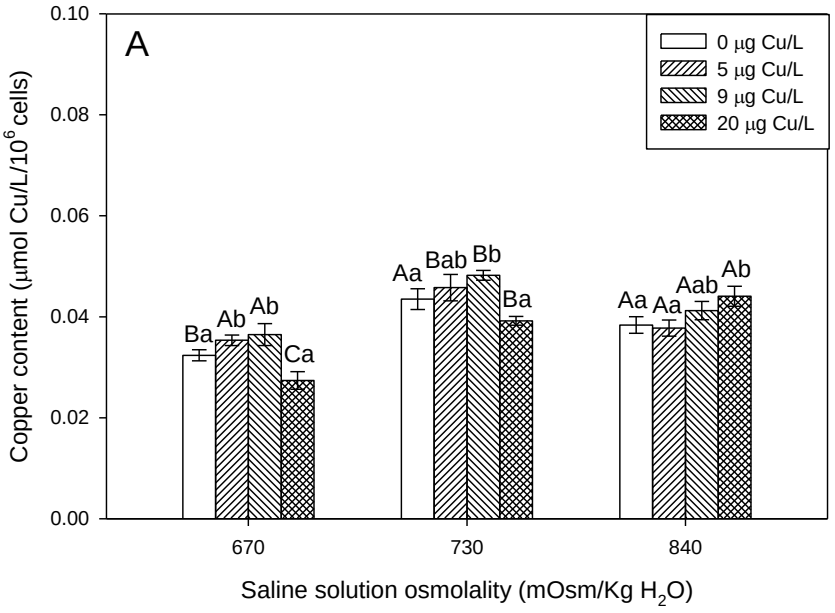

A
Na^+,K^+-ATPase activity (μmol/mg protein/h)
0.0
0.2
0.4
0.6
0.8
a
a
a
a
0
5
9
20
Copper concentration (μg/L)
B
Carbonic Anhydrase (CA unit/mg protein/mL)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
a
ab
ab
b
0
5
9
20
Copper concentration (μg/L)

Figure 4


A
0 μg Cu/L
5 μg Cu/L
9 μg Cu/L
20 μg Cu/L
Copper content (μmol Cu/L/10^6 cells)
0.10
0.08
0.06
0.04
0.02
0.00
Ba
Ab
Ab
Ca
Aa
Bab
Bb
Ba
Aa
Aa
Aab
Ab
670
730
840
Saline solution osmolality (mOsm/Kg H2O)

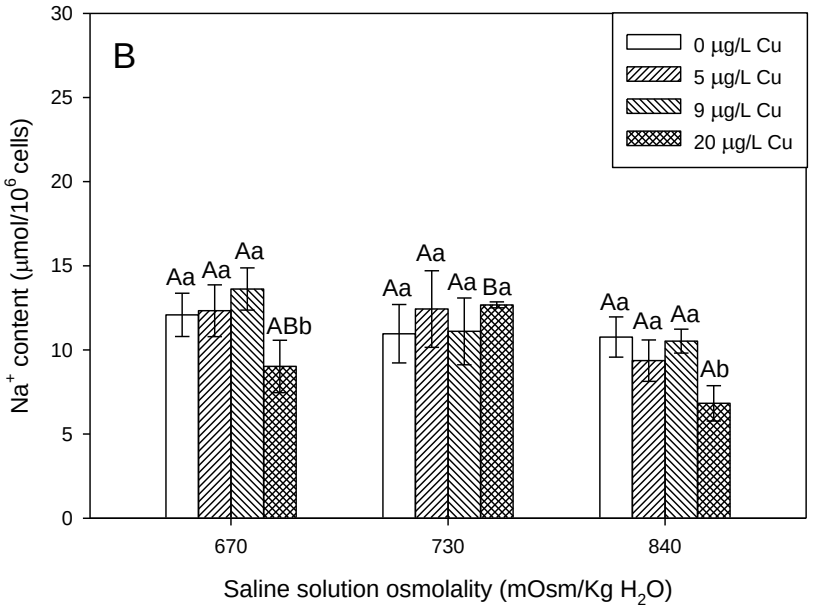
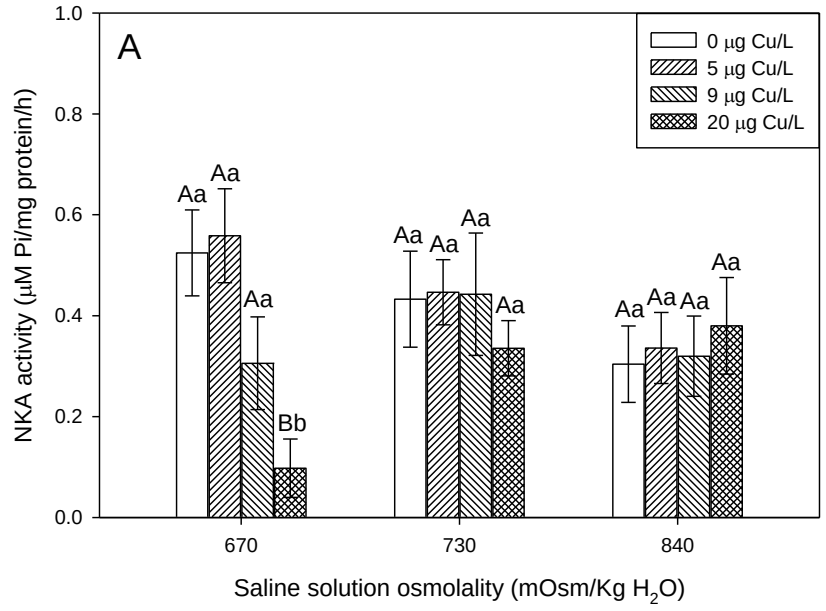
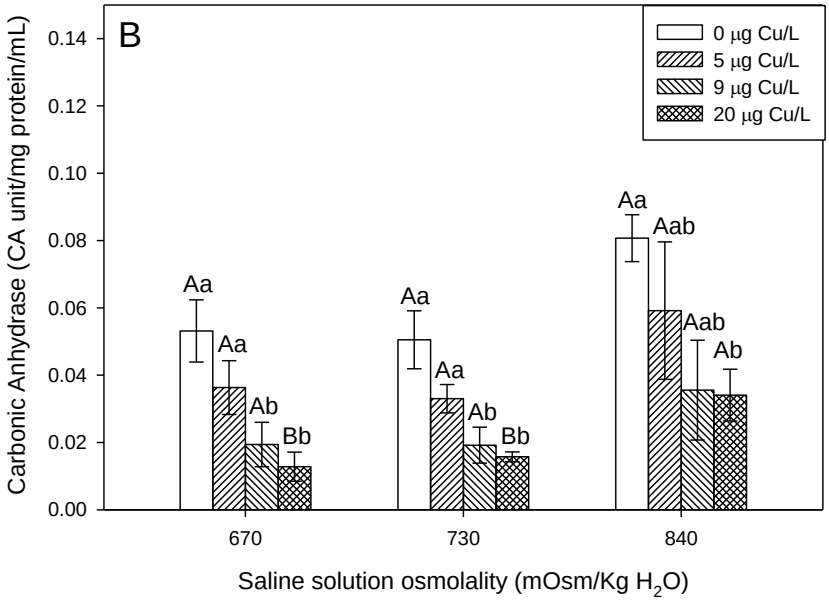

B
0 μg/L Cu
5 μg/L Cu
9 μg/L Cu
20 μg/L Cu
Na+ content (μmol/10^6 cells)
30
25
20
15
10
5
0
Aa
Aa
Aa
ABb
Aa
Aa
Aa
Ba
Aa
Aa
Aa
Ab
670
730
840
Saline solution osmolality (mOsm/Kg H2O)

Figure 5


A
0 μg Cu/L
5 μg Cu/L
9 μg Cu/L
20 μg Cu/L
NKA activity (μM Pi/mg protein/h)
1.0
0.8
0.6
0.4
0.2
0.0
Aa
Aa
Aa
Bb
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
670
730
840
Saline solution osmolality (mOsm/Kg H2O)


B
0 μg Cu/L
5 μg Cu/L
9 μg Cu/L
20 μg Cu/L
Carbonic Anhydrase (CA unit/mg protein/mL)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Aa
Aa
Ab
Bb
Aa
Aa
Ab
Bb
Aa
Aab
Aab
Ab
670
730
840
Saline solution osmolality (mOsm/Kg H2O)

CONCLUSÕES GERAIS

As técnicas empregadas para dissociação do tecido branquial do mexilhão de água doce *Lasmigona costata* e do marisco de água salgada *Mesodesma mactroides* se mostraram eficazes para obtenção de células isoladas e viáveis para futuros estudos *in vitro*. Os procedimentos empregados para fracionamento das células obtidas permitiram a separação das células branquiais em duas frações distintas. Além disso, a aplicação de duas abordagens (marcação mitocondrial e análise enzimática) para identificação da presença de células ricas em mitocôndrias nas frações celulares obtidas para as duas espécies de bivalves também se mostrou adequada. Por sua vez, as células ricas em mitocôndrias isoladas das brânquias das duas espécies de bivalves mostraram ser uma potencial ferramenta para o estudo dos mecanismos celulares envolvidos na acumulação e toxicidade do cobre (Cu) em bivalves de água doce e marinhos. De fato, os resultados obtidos após a exposição das células ricas em mitocôndria isoladas do mexilhão de água doce *L. costata* e do marisco marinho *M. mactroides* ao Cu indicam uma importante competição entre o Na^+ e Cu por sítios de ligação na membrana plasmática. Além disso, eles demonstram que o Cu é um tóxico ionorregulatório para ambas as espécies de bivalves, e que a toxicidade celular do metal aumenta em ambientes hiposmóticos. Por fim, o presente estudo fornece evidências claras de que não só a química da água, mas também a fisiologia do organismo em estudo é importante para a previsão da toxicidade do Cu em ambientes aquáticos. Neste contexto, os resultados apresentados indicam a atividade da anidrase carbônica de células branquiais ricas em mitocôndria do marisco de água salgada *M. mactroides* como um potencial biomarcador da exposição ao Cu em ambientes aquáticos.

REFERÊNCIAS BIBLIOGRÁFICAS

Bastida RA, Roux A, Bremec C, Gerpe M, Sorensen M (1991) Estructura poblacional de la almeja amarilla (Mesodesma mactroides) durante el verano de 1989, en la provincia deBuenos Aires, Argentina. Frente Marítimo 9A: 83-92.

Bianchini A, Wood C M (2002) Physiological effects of chronic silver exposure in *Daphnia magna*. Comparative Biochemistry Physiology 133C, 137-145.

Bigas M, Durfort M, Poquet M (2001) Cytological effects of experimental exposure to Hg on the gill epithelium of the European flat oyster *Ostrea edulis*: ultrastructural and quantitative changes related to bioaccumulation. Tissue Cell 33:178–188.

Birmelin C, Pipe RK (1999) Primary cell culture of the digestive gland of the marine mussel *Mytilus edulis*: a time-course study of antioxidant and biotransformation-enzyme activity and ultrastructural changes. Marine Biology 135:65–75.

Boyle RT, Bianchini A, Souza MM (2013) The effects of copper on Na^+/K^+-ATPase and aquaporin expression in two euryhaline invertebrates. Bulletin of Environmental Contamination and Toxicology (2013) 90: 387-390.

Blanchard J, Grosell M (2006) Copper toxicity across salinities from freshwater to seawater in the euryhaline fish Fundulus heteroclitus: Is copper an ionoregulatory toxicant in high salinities? Aquatic Toxicology 80: 131–139.

Cajaraville MP, Olabarrieta I, Marigómez I (1996) In vitro activities in mussel haemocytes as biomarkers of environmental quality: a case study in the Abra estuary (Biscay Bay). Ecotoxicology Environmental and Safety 35:253–260.

Caricato R, Lionetto MG, Dondero F, Viarengo A, Schettino T (2010) Carbonic anhydrase activity in *Mytilus galloprovincialis* digestive gland: sensitivity to heavy metal exposure. Comparative Biochemistry and Phyiology: Toxicology & Pharmacology, 3C: 241-247.

Chang IC, Lee TH, Yang CH, Wei YW, Chou FI, Hwang PP (2001) Morphology and function of gill mitochondria-rich cells in fish acclimated to different environments. Physiology and Biochemistry Zoology 74:111–119.

Cope WG, Bringolf RB, Buchwater DB, Newton TJ, Ingersoll CG, Wang N, Augsperger T, Dwyer FJ, Barnhart MC, Neves RJ, Hammer E (2008) Differential exposure, duration and sensitivity of unionidean bivalve life stages to environmental contaminants. Journal of the North American Benthological Society, 27(2):451–462

Dallinger R (1994) Invertebrate organisms as biological indicators of heavy metal pollution. Applied Biochemistry and Biotechnology 48: 27–31.

Defeo O, Ortiz E, Castilla JC (1992) Growth, mortality and recruitment of the yellow clam *Mesodesma mactroides* on Uruguayan beaches. Marine Biology Oldendorf/Luhe, n. 114: 429-437.

Defeo O, Scarabino V (1990). Ecological significance of a possible deposit-feeding strategy in Mesodesma mactroides Deshayes, 1854 (Mollusca: Pelecypoda). Atlântica 12: 55-65.

Evans DH (2008)a Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. American Journal of Physiology 295, 704–713.

Evans DH (2008)b Osmotic and ionic regulation: cells and animals. CRC Press 598 p.

Freeman HC (1973) Metal complexes of amino acid and peptides, in Inorganic Biochemistry (Eichorn, G. L., ed.), Elsevier, Amsterdam, pp. 121–127. In: Edward J. Massaro editor (2002) Handbook of Copper Pharmacology and Toxicology. HUMANA PRESS, 696p.

Freire CA, Amado EM, Souza LR, Veiga MPT, Vitule JRS, Souza MM, Prodocimo V (2008) Muscle water control in crustaceans and fishes as a function of habitat, osmoregulatory capacity, and degree of euryhalinity. Comp Biochem Physiol 149A:435–446

Galvez FS, Reid D, Hawkings G, Goss GG (2002) Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology 82, 658- 668.

Galvez F, Wong D, Wood CM (2006) Cadmium and calcium uptake in isolated mitochondria-rich cell populations from the gills of the freshwater rainbow trout. American Journal of Physiology 291: 170–176..

Gil GM, Thomé W (1998) *Donax hanleyanus* Philippi, 1847 como indicador ambiental. Biociências 6: 189-193.

Gilbert, AL**, Guzmán,** HM (2001) Bioindication potential of carbonic anhdrase activity in anemones and corals". Marine Pollution Bulletin 42: 742-744.

Gillis, PL (2011) Assessing the toxicity of sodium chloride to the glochidia of freshwater mussels: Implications for salinization of surface waters. Environmental Pollution 159, 1702-1708.

Gilles R, (1979) Intracellular organic osmotic effectors. In: Gilles R, editor, Mechanisms of osmoregulation in animals. Chichester:Wiley, 111–154pp.

Gómez-Mendikute A, Elizondo M, Venier P, Cajaraville MP (2005) Characterization of mussel gill cells in vivo and in vitro. Cell Tissue Research 321:131–140.

Gómez-Mendikute A, Cajaraville MP (2003) Comparative effects of cadmium, copper and benzo(a)pyrene on the actin cytoskeleton and production of reactive oxygen species (ROS) in mussel haemocytes. Toxicology In Vitro 17:539–546.

Gómez-Mendikute A, Etxeberria A, Olabarrieta I, Cajaraville MP (2002) Oxygen radicals production and actin filament disruption in bivalve haemocytes treated with benzo(a)pyrene. Marine Environmental Research 54:431–436.

Goss GG, Adamia S, Galvez F (2001) Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium. American Journal of Physiology 281: 1718–1725.

Goss G, Gilmour K, Hawkings G, Brumbach JH, Huynh M, Galvez F (2011) Mechanism of sodium uptake in PNA negative MR cells from rainbow trout, *Oncorhynchus mykiss* as revealed by silver and copper inhibition. Comparative Biochemistry and Physiology 159A: 234–241.

Grosell M, McDonald MD, Walsh PJ, Wood CM (2004) Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) II: copper accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues. Aquatic Toxicology 68 (3): 249-262.

Grosell M, Wood CM,Walsh PJ (2003) Copper homeostasis and toxicity in the elasmobranch Raja erinacea and the teleost *Myoxocephalus octodecem spinosus* during exposure to elevated water-borne copper. Comparative Biochemistry and Physiology 135C: 179-190.

Grosell M, Nielsen C, Bianchini A (2002) Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comparative Biochemistry and Physiology 133C: 287-303.

Hamed MA, Emara AM (2006) Marine molluscs as biomonitors for heavy metal levels in the Gulf of Suez, Red Sea. Journal of Marine Systems 60: 220–234.

Handy RD, Eddy FB, Baines H (2002) Sodium-dependent copper uptake across epithelia: a review of rationale with experimental evidence from gill and intestine. Biochemical et Biophysica Acta 1566, 104-115.

Hiroi J, McCormick SD (2007) Variation in salinity tolerance, gill Na+/K+- ATPase, $Na^+/K^+/2Cl^-$ cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. Journal of Experimental Biology 210, 1015–1024.

Hwang PP, Hirano R (1985) Effects of environmental salinity on intercellular organization and junctional structure of chloride cells in early stages of teleost development. Journal of Experimental Zoology. 236, 115–126.

Hwang PP, Lee TH (2007) New insights into fish ion regulation and mitochondrion-rich cells. Comparative Biochemistry and Physiology 148A: 479– 497.

Hwang, PP (2009) Ion uptake and acid secretion in zebrafish (*Danio rerio*). Journal of Experimental Biology 212, 1745–1752.

Jernelov A (1996) The international mussel watch: a global assessment of environmental levels of chemical contaminats. Science of the Total Environment, 188: 37-44.

Jorge, MB, Loro, VL, Bianchini, A, Wood, CM, Gillis, PL (2013) Mortality, bioaccumulation and physiological responses in juvenile freshwater mussels (Lampsilis siliquoidea) chronically exposed to copper. Aquatic Toxicology 126, 137– 147.

Kang CK, Yang WK, Lin ST, Liu CC, Lin HM, Chen HH, Cheng CW, Lee TH, Hwang PP (2013) The acute and regulatory phases of time-course changes in gill mitochondrion-rich cells of seawater-acclimated medaka (*Oryzias dancena*) when exposed to -hypoosmotic environments. Comparative Biochemistry and Physiology 164A: 181-191.

Katoh F, Hasegawa S, Kita J, Takagi Y, Kaneko Y (2001) Distinct SW and freshwater types of chloride cells in killifish, *Fundulus heteroclitus*. Canadian Journal of Physiology 79: 822–829.

Kirschner LB (2004) The mechanism of sodium chloride uptake in hyperregulating animals. Journal of Experimental Biology, 207, 1439–1452.

Kültz D (2001) Cellular osmoregulation: beyond ion transport and cell volume. Zoology 104: 198-208.

Lee TH, Hwang PP, Lin HC, Huang FL (1996) Mitochondria-rich cells in the branchial epithelium of the teleost, *Oreochoromis mossambicus,* acclimated to various hypotonic environments. Fish Physiology and Biochemistry 15, 513-523.

Li J, Quabius ES, Wendelaar BSE, Flik G, Lock RAC (1998) Effects of water-borne copper on branchial chloride cells and Na^+/K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). Aquatic Toxicology 43, 1–11.

Lowe DM, Pipe RK (1994) Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an *in vitro* study. Aquatic Toxicology 30, 357–365.

Le Pennec G, Le Pennec M (2001) Acinar primary cell culture from the digestive gland of Pecten maximus (L): an original model for ecotoxicological purposes. Journal of Experimental Marine Biology and Ecology 259: 171– 187.

Lionetto MG, Caricato R, Giordano ME, Erroi E, Schettino T (2012) Carbonic anhydrase and heavy metals, biochemistry, Prof. Deniz Ekinci (Ed.), ISBN: 978-953-51-0076-8.

Mansur MCD, Valer RM, Aires NCM (1994) Distribuição e preferência ambientais dos moluscos bivalves no açude do parque de proteção ambiental COPESUL, no município de Triunfo, Rio Grande do Sul, Brasil. Biociências 2(1): 27-45.

Martelo MJ, Zanders IP (1986) Modifications of gill ultrastructure and ionic composition in the crab *Goniopsis cruentata* acclimated to various salinities. Comparative Biochemistry and Physiology A, 383-389.

Marshall WS, Grosell M (2005) Ion Transport, osmoregulation, and acid–base balance. In: Evans, D.H., Clairborne, J.B. The Physiology of Fish. CRC PRESS. pp 177-230.

McGeer JC, Wood CM (1998) Effects of water chloride concentration on physiological response of rainbow trout to silver. Canadian Journal of Fisheries and Aquatic Sciences 55:2447–2454.

Morgan TP, Grosell M, Gilmour KM, Playle RC, Wood CM (2004). Time course analysis of the mechanism by which silver inhibits active Na^+ and Cl^- uptake in gills of rainbow trout. American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 287: R234-242.

Nordberg GF, Fowler BA, Nordberg M, Friberg L (2007) Handbook on the toxicology of metals. 3 rd ed. Academic Press, Inc. pp. 392.

Olabarrieta I, L'Azou B, Yuric S, Cambar J, Cajaraville MP (2001) *In vitro* effects of cadmium on two different animal cell models. Toxicology *In Vitro* 15:511–517.

Olivier, S., Penchaszadeh, P., (1971) Estructura de la Comunidad Dinámica de la Población y Biología de la Almeja Amarilla. Instituto de Biología Marina, Mar del Plata Proy. Desarr. Pesq. FAO, Ser. Inf. Tecn. Publ., Mar del Plata 27: 1-90.

Paquin PR, Gorsuch JW, Apte S, Batley GE, Bowles KC, Campbell PGC, Delos CG, Di Toro DM, Dwyer RL, Galvez F, Gensemer RW, Goss GG, Hogstrand C, Janssen CR, McGeer JM, Naddy RB, Playle RC, Santore RC, Schneider U, Stubblefield WA, Wood CM, Wu KB (2002) The biotic ligand model: a historical overview. Comparative Biochemistry and Physiology 133C: 3–35.

Pedroso MFM, Lima IV (2001). Ecotoxicologia do cobre e seus compostos. CRA: Salvador, 128p.

Péqueux A (1995) Osmotic regulation in crustaceans. Journal of Crustacean Biology 15:1– 60.

Quinn, RH, Pierce, SK (1992) The ionic basis of the hypoosmotic depolarization in neurons from the opisthobranch mollusc *Elysia chlorotica*. J. Exp. Biol ., 163, 169–186.

Randall D, Burggren W, Frenck K (2000) Fisiologia animal: mecanismos e adaptação. 4.ed. Rio de Janeiro: Editora Guanabara Koogan S.A.

Reid SD, Hawkings GS, Galvez F, Goss GG (2003) Localization and characterization of phenamil-sensitive Na^+ influx in isolated rainbow trout gill epithelial cells. Journal of Experimental Biology 206, 551-559.

Rios E C (1994). Seashell of Brazil. 2. ed. Rio Grande: Fundação Universidade Rio Grande. 368p.

Ruppert, EE, Barnes, RD (1996) Zoologia dos Invertebrados. 6 ed. São Paulo: Ed. Roca 1028p.

Santore RC, Di Toro DM, Paquin PR. (2000) A biotic Ligand Model of the acute toxicity of metals II. Application to acute copper toxicity in freshwater fish and daphnia. Technical reported, Environmental Protection Agency, Office of Water Regulations and Standards, Washington, DC.

Sancho E, Fernandez-Vega C, Ferrando MD, Andreu-Moliner E (2003) Eel ATPase activity as biomarker of thiobencarb exposure. Ecotoxicological Environmental and Safety 56:434–441.

Schwartz ML, Dimock RVJr. (2001) Ultrastructural evidence for nutritional exchange between brooding unionid mussels and their glochidia larvae. Invertebrate Biology 120: 227–236.

Skaggs HS, Henry RP (2002) Inhibition of carbonic anhydrase in the gills of two euryhaline crabs, *Callinectes sapidus* and *Carcinus maenas*, by heavy metals. Comparative Biochemistry and Physiology 133C, 605-612.

Smith, LH, Pierce, SK (1987) Cell volume regulation by molluscan erythrocytes during hypoosmotic stress: Ca^{2+} effects on ionic and organic osmolyte fluxes. Biol. Bull ., 173, 407–418.

Stagg R, Goksoyr A, Rodger G (1992) Changes in branchial Na^{+},K^{+}–ATPase, metallothionein and P450 1A1 in dab *Limanda limanda* in the German bight: indicators of sediment contamination? Marine Ecology Progress Series 91:105–115.

Soto M., Ireland MP, Marigomez I (2000) Changes in mussel biometry on exposure to metals: Implications in estimation of metal bioavailability in 'Mussel-Watch' programmes. Science of the Total Environment. 247: 175- 187.

Taylor HH, Taylor EW (1992) Gills and lungs: The exchange of gases and ions. In F.W. Harrison and A. G. Humes (eds.), Microscopic anatomy of invertebrates, Decapod Crustacea, Vol. 10, pp. 203–293. Wiley-Liss, New York.

Venier P, Maron S, Canova S (1997) Detection of micronuclei in gill cells and haemocytes of mussels exposed to benzo(a)pyrene. Mutat Res 390:33–44.

Viarengo A, Pertica M, Mancinelli G, Burlando B, Canesi JL, Orunesu M (1996) *In vivo* effects of copper on the calcium homeostasis mechanisms of mussel gill cell plasma membranes. Comparative Biochemistry Physiology 113C: 421-425.

Viarengo A, Burlando B, Bolognesi C (2002) Cellular Responses to Copper in Aquatic Organisms Importance of Oxidative Stress and Alteration of Signal Transduction. In: Edward J. Massaro editor (2002) Handbook of Copper Pharmacology and Toxicology. HUMANA PRESS, 696p

Vitale AM, Monserrat JM, Castilho P, Rodriguez EM (1999) Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). Comparative Biochemistry and Physiology 122C: 121-129.

Wang WX (2001) Comparison of metal uptake rate and absorption efficiency in marine bivalves. Environmental Toxicology and Chemistry 20, 1367–1373.

Webb M (1979) Interactions of cadmium with cellular components, in The Chemistry, Biochemistry and Biology of Cadmium, Elsevier Science, 285–340. In: Viarengo A, Burlando B, Bolognesi C. Cellular Responses to Copper in Aquatic Organisms Importance of Oxidative Stress and Alteration of Signal Transduction. In: Edward J. Massaro editor (2002) Handbook of Copper Pharmacology and Toxicology. HUMANA PRESS, 696p.

Wilson RW, Taylor EW (1993) Differential responses to copper in rainbow trout (*Oncorhynchus mykiss*) acclimated to seawater and brackish water. Journal of Comparative Physiology 163B, 239–246.

Williams JD, Warren ML, Cummings KS,Harris JL, Neves RJ (1993) Conservation status of the freshwater mussels of the United States and Canada. Fisheries 18:6-22.

Wong CK, Chan DK (1999) Chloride cell subtypes in the gill epithelium of japanese eel *Anguilla japonica*. American of Journal Physiology 277, 517- 522.

Zanotto FP, Wheatly MG (2006) Ion Regulation in Invertebrates: Molecular and Integrative Aspects. Physiological and Biochemical Zoology 79(2):357–362.

Zimmer AM, Barcarolli IF, Wood CM, Bianchini A (2012) Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both freshwater and seawater. Aquatic Toxicology 172-180.

ANEXO 1

Normas para o autor da revista **CYTOTECHNOLOGY.**

Manuscript submission

Title Page

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

- LaTeX macro package (zip, 182 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.
Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.
Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.
Reference list entries should be alphabetized by the last names of the first author of each work.

- Journal article
  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8
  Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:
  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI
  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086
- Book
  South J, Blass B (2001) The future of modern genomics. Blackwell, London
- Book chapter
  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257
- Online document
  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
- Dissertation
  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

- www.issn.org/2-22661-LTWA-online.php

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

- EndNote style (zip, 3 kB)

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork and Illustrations Guidelines

For the best quality final product, it is highly recommended that you submit all of your artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the quality of the artwork provided.

Electronic Figure Submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.

- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art

- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art

- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

Combination Art

- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color art is free of charge for online publication.
- If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.
- If the figures will be printed in black and white, do not refer to color in the captions.
- Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).

- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

Figure Numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

- When preparing your figures, size figures to fit in the column width.
- For most journals the figures should be 39 mm, 84 mm, 129 mm, or 174 mm wide and not higher than 234 mm.
- For books and book-sized journals, the figures should be 80 mm or 122 mm wide and not higher than 198 mm.

ANEXO 2

Normas para o autor da revista **AQUATIC TOXICOLOGY**

Types of paper

1. Original Research Papers (Regular Papers)
2. Review Articles
3. Short Communications
4. Letters to the Editor

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.

Review Articles can be divided into three types:
• Regular reviews covering subjects falling within the scope of the journal which are of active current interest. These should generally not exceed 12 printed pages (approx. 6000 words).
• Mini-reviews. These will be short reviews or overviews (not exceeding 2-3 printed pages, approx. 1000-1500 words) on topics of above-average emerging interest.
• Commentaries. This label will be given to mini-reviews which clearly contain the personal opinions of the author concerned. All types of review articles will be solicited by the Reviews Editor, Prof. M.N. Moore, Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth, PL1 3DH, UK. E-mail: mnm@pml.ac.uk.

Short Communications will be restricted to papers describing short, complete studies. They should not exceed 3 printed pages, including figures and tables (approx. 1500 words), and should be written in a continuous style, without subdivisions of introduction, materials and methods, results, discussion and acknowledgements; they should always begin with a summary. A short communication, although brief, should be a complete and final publication, and figures and tables from the communication should not occur in a later paper.

Letters to the Editor should either offer comment on a paper published in the journal, or comment on any general matter providing that this is relevant to the scope of the journal. In the case of letters commenting on published papers, the author(s) of the latter will be given the opportunity to react to the letter and the two items will subsequently be published together in the journal.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• **Author names and affiliations.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**
• **Present/permanent address.** If an author has moved since the work described in the article

was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required of no more than 400 words. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separate from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, they must be cited in full, without reference to the reference list. Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using

superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.
Table footnotes
Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the printed version.
• Submit each illustration as a separate file.
A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures

will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style
Text: All citations in the text should refer to:

1. Single author: the author's name (without initials, unless there is ambiguity) and the year of publication;
2. Two authors: both authors' names and the year of publication;
3. Three or more authors: first author's name followed by 'et al.' and the year of publication.
Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Journal abbreviations source
Journal names should be abbreviated according to:
List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php; NLM Catalog (Journals referenced in the NCBI Databases): http://www.ncbi.nlm.nih.gov/nlmcatalog/journals; CAS (Chemical Abstracts Service): via http://www.cas.org/content/references/corejournals.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

AudioSlides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.
Ensure that the following items are present:
One author has been designated as the corresponding author with contact details:
• E-mail address
• Full postal address
• Phone numbers
All necessary files have been uploaded, and contain:
• Keywords
• All figure captions
• All tables (including title, description, footnotes)
Further considerations
• Manuscript has been 'spell-checked' and 'grammar-checked'
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)
• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print
• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes