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EFEITOS DO COBRE NO METABOLISMO ENERGÉTICO DO BIVALVE MARINHO *Mesodesma mactroides*

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RESUMO

Apesar de ser um micronutriente essencial aos organismos, o cobre (Cu) é tóxico quando presente em elevadas concentrações na água. O mecanismo pelo qual este metal exerce sua toxicidade em invertebrados marinhos ainda não está bem estabelecido. Dentre os diversos efeitos relatados, observa-se uma redução do consumo de oxigênio corporal e tecidual no marisco *Mesodesma mactroides* exposto (96 h) ao Cu (150 µg L^{-1}) em água do mar (salinidade 30). Portanto, o objetivo do presente estudo foi avaliar os efeitos desta exposição ao Cu no metabolismo energético em teciduais do marisco *M. mactroides*. Os conteúdos de ATP e coenzimas (NAD^+ e NADH) nas brânquias, glândula digestiva e músculo pedal não foram alterados pela exposição ao Cu, indicando que estes tecidos mantiveram suas capacidades de produção aeróbica de energia. Porém, foi observada uma redução no conteúdo hemolinfático de ATP. Quanto ao conteúdo de proteínas, houve um aumento na glândula digestiva, que pode estar associado à maior oxidação de proteínas já relatada para esse tecido após exposição ao Cu. Os conteúdos de lipídios, glicogênio e glicose permaneceram inalterados em todos os tecidos analisados, exceto no músculo pedal, onde foi observada uma redução no conteúdo de glicose. Por isso, os conteúdos de piruvato e lactato também foram analisados no músculo pedal e na hemolinfa. Em ambos tecidos, foi observado um aumento do conteúdo de lactato, sem alteração no conteúdo de piruvato. Portanto, os resultados do presente estudo sugerem que os tecidos de *M. mactroides* utilizam a anaerobiose para obtenção de energia durante a exposição ao Cu, conforme demonstrado no músculo pedal e hemolinfa. Apesar disso, a hemolinfa não é capaz de manter o nível de ATP nas condições experimentais testadas.

Palavras-chave: anaerobiose, cobre, exposição aguda, marisco, metabolismo energético.

ABSTRACT

Copper (Cu) is an essential micronutrient to all living organisms, but it is toxic when at elevated concentrations in the water. Its mechanism of toxicity to marine invertebrates is still not well established. Among the several effects of Cu, a reduced whole-body and tissue oxygen consumption is reported in the marine clam *Mesodesma mactroides* exposed (96 h) to Cu (150 μg L^{-1}) in sea water (salinity 30 ppt). Therefore, the objective of the present study was to evaluate the effects of this Cu exposure on the energy metabolism in tissues of *M. mactroides*. ATP and dinucleotides (NAD^+ and NADH) contents in gills, digestive gland and adductor muscle were not altered by Cu exposure, indicating that these tissues were able to maintain the generation of energy through the aerobic metabolism. However, a decrease in the hemolymph ATP content was observed. An increase in protein content was observed in digestive gland, which could be related to the higher protein oxidation level previously observed in this tissue under the same experimental conditions. Lipids, glycogen and glucose contents were not affected by Cu exposure in all tissues analyzed, except in the adductor muscle, where a decrease in glucose content was observed. Therefore, pyruvate and lactate contents were also analyzed in the adductor muscle and hemolymph. In both tissues, an increase in the lactate content was observed, but without significant change in the pyruvate content. Taken altogether, these findings suggest that tissues of the marine clam *M. mactroides* use the anaerobic metabolism to generate energy during Cu exposure, as demonstrated in the adductor muscle and hemolymph. Despite this capacity, hemolymph was shown to be not able to maintain the ATP level under the experimental conditions tested in the present study.

Keywords: acute exposure, anaerobiosis, clam, copper, energy metabolism.

INTRODUÇÃO

As zonas costeiras são caracterizadas como a região de transição entre os ecossistemas continentais e marinhos, e consequentemente possuem uma dinâmica natural bastante intensa, as quais estão sujeitas a ação de fatores externos, como correntes marinhas, circulação atmosférica e descarga continental. Ainda, devido a sua localização privilegiada, são conhecidas como locais de elevada atividade antrópica. Essas características tornam esses ambientes altamente sujeitos a contaminação oriunda de diversas atividades humanas, tais como a exploração de petróleo e gás, mineração, pesca, turismo, agricultura, industrialização e transporte da produção (Vitousek et al., 1997). Dentre as classes de contaminantes que atingem os ecossistemas costeiros e marinhos encontram-se os metais, elementos naturais da biosfera, mas que quando presentes em quantidades acima das normais são potencialmente perigosos aos ecossistemas (Santore et al., 2001).

O cobre (Cu) é um metal de transição que apresenta alta condutibilidade elétrica e térmica e, portanto, é amplamente utilizado pela indústria como material condutor (fios e cabos), bem como na fabricação de tubos de encanamentos, motores elétricos, interruptores, entre outros (Lander e Reuther, 2004). As maiores fontes antrópicas de aporte deste elemento para o meio ambiente são as atividades industriais (Kennish, 1997), de mineração (Phillips, 1980), erosão do solo (Heath, 1995), queima de combustíveis fósseis e despejo de esgoto doméstico (Neto et al., 2008).

O Cu é um micronutriente essencial para todos os organismos, participando de várias funções fisiológicas (Solomon e Lowery, 1993). No caso dos invertebrados marinhos, este metal encontra-se em abundância no sistema circulatório, pois é constituinte do pigmento respiratório hemocianina (Taylor e Anstiss, 1999). O Cu também desempenha um papel importante como cofator de inúmeras enzimas, tais como a citocromo oxidase, superóxido

dismutase, álcool desidrogenase, dopamina hidroxilase, tirosinase e lisil oxidase (Suzuki et al., 2002; Ryu et al., 2003; Serafim e Bebianno, 2009). Porém, mesmo sendo essencial, o Cu pode se tornar tóxico quando presente em elevadas concentrações na água (D'Silva e Kureishy, 1978).

Para muitos organismos de água doce, o mecanismo de toxicidade do Cu está relacionado com distúrbios na regulação iônica e osmótica, especialmente devido a inibição da atividade da Na^+/K^+-ATPase (Laurén e McDonald, 1985). Esta enzima é responsável pela geração da diferença de potencial elétrico, que resulta na captação ativa de íons Na^+ da água para o meio intracelular (Grosell, 2012; Grosell et al., 2002). Organismos de água doce possuem a concentração dos seus fluídos corporais acima daquela do ambiente em seu entorno. Desta forma, a água tende a fluir por osmose para dentro do organismo, enquanto os solutos presentes nos fluídos corporais tendem a se difundir para o ambiente. Para contrabalancear essas perdas e manter a homeostase de seus fluídos corporais, esses organismos captam ativamente íons do meio externo através de um conjunto de mecanismos de regulação iônica e osmótica. Sabe-se que uma redução de 30% no sódio plasmático ou corporal é suficiente para gerar mortalidade em peixes teleósteos de água doce (Wood et al., 1996; Hogstrand e Wood, 1998). Desta forma, a redução na atividade da Na^+/K^+-ATPase diminui consequentemente a captação ativa desse elemento, levando o organismos à morte.

Além de ser conhecido por induzir um desequilíbrio no balanço iônico, o Cu é considerado um potente causador de estresse oxidativo em muitas espécies de animais aquáticos (Aust et al., 1985; Luschak et al., 2011). Além das alterações fisiológicas e bioquímicas já mencionadas, a exposição ao Cu também pode causar um aumento na concentração de metalotioneínas (Brouwer e Hoexum-Brouwer, 1998; Brown et al., 2004), inibição da excreção de amônia e da atividade de enzimas relacionadas à regulação iônica e osmótica (Laurén e McDonald, 1985; Wilson e Taylor, 1993; Blanchard e Grosell, 2006;

Zimmer et al., 2012; Jorge et al., 2013), alterações nas defesas antioxidantes enzimáticas (Regoly et al., 1998), ou ainda, redução da atividade de enzimas da via glicolítica (Hansen et al., 1992; Lauer et al., 2012).

Apesar das informações descritas acima, ainda não existe um consenso acerca do mecanismo de toxicidade do Cu em organismos marinhos e estuarinos, especialmente os invertebrados. No entanto, diversos trabalhos utilizando diferentes abordagens vem sendo realizados por nosso grupo de pesquisa, visando elucidar os efeitos fisiológicos da exposição Cu em invertebrados estuarinos e marinhos (Bianchini et al., 2004; Pinho et al., 2007; Lauer e Bianchini, 2010; Pinho e Bianchini, 2010; Lopes et al., 2011a,b; Martins et al., 2011; Jorge et al., 2013). Estudos com o siri-azul *Callinectes sapidus* aclimatado a baixas salinidades demonstraram que o mecanismo de toxicidade aguda do Cu pode estar relacionado a uma diminuição na expressão de genes que codificam para proteínas envolvidas na osmorregulação (Martins et al., 2011). No entanto, o mesmo resultado não foi encontrado quando o siri-azul é aclimatado a altas salinidades, indicando que o mecanismo de toxicidade do Cu pode variar conforme a condição fisiológica a qual o animal está submetido (Grosell et al., 2007; Martins et al., 2011). Este mesmo padrão de resposta foi encontrado para o copépode eurialino *Acartia tonsa* (Pinho et al., 2007).

Já no bivalve eurialino *Mesodesma mactroides*, que é um marisco osmoconformador, o Cu é capaz de afetar a regulação iônica a nível intracelular (Lopes et al., 2011a,b) e tecidual (Jorge et al., 2013). Jorge et al. (2013) e Nogueira et al. (2013), utilizando abordagens *in vivo* e *in vitro* respectivamente, demonstraram que o Cu também é capaz de diminuir a atividade da anidrase carbônica, uma enzima relacionada à respiração, excreção de amônia e manutenção do equilíbrio ácido-base. Além disso, Jorge et al. (2013) relataram uma diminuição no consumo de oxigênio corporal e tecidual (brânquias e glândula digestiva) em *M. mactroides* exposto agudamente (96 h) ao Cu. Estes autores também observaram uma

redução da succinato-desidrogenase (complexo II da cadeia transportadora de elétrons) quando tecidos (brânquias e glândula digestiva) de *M. mactroides* foram expostos *in vitro* a 100 µM de Cu, indicando a possibilidade de uma disfunção respiratória induzida pela exposição ao metal (Jorge et al., 2013).

Outros autores sugerem ainda que o mecanismo de toxicidade do Cu em invertebrados marinhos e estuarinos pode estar relacionado com a inibição da atividade de enzimas essenciais do metabolismo energético, resultando em uma diminuição da quantidade de energia produzida, necessária para combater alterações fisiológicas decorrentes da exposição ao metal (Lauer et al., 2012). Neste contexto, Yano (2010) relatou que a exposição ao Cu inibe a atividade da citrato sintase, a primeira enzima do ciclo de Krebs, na anêmona-do-mar *Actinia bermudensis*, um cnidário osmoconformador. Além disso, sabe-se que a exposição ao Cu causa uma diminuição na taxa de consumo de oxigênio corporal em diversos grupos de invertebrados, bem como no consumo de oxigênio tecidual em estudos *in vitro* (Brown e Newell, 1972; Spicer e Weber, 1991; De Boeck et al., 1995; Santos et al., 2000; Brown et al., 2004; Paila e Yallapragada, 2010; Jorge et al, 2013). Neste caso, a redução no consumo de oxigênio pode ser um resultado de vários fatores. Dentre estes, pode-se considerar uma diminuição na taxa ventilação do órgão respiratório na tentativa de reduzir o grau de exposição ao metal (Spicer e Weber, 1991); uma diminuição na eficiência das trocas gasosas em nível do órgão respiratório associada a um aumento na produção de muco para diminuir o contato do metal com a superfície respiratória e/ou a biodisponibilidade do metal (Sze e Lee, 1995); uma alteração na capacidade de transporte de oxigênio pela hemocianina associada ao efeito do metal sobre a afinidade deste pigmento respiratório pelo oxigênio (Taylor e Antiss, 1999); e uma redução da atividade mitocondrial induzida pela exposição ao Cu (Jorge et al., 2013; Lauer et al., 2013). Independente do fator envolvido na redução do consumo de oxigênio corporal ou tecidual, esta situação poderia estar levando o animal a produzir uma

menor quantidade de trifosfato de adenosina (ATP) e consequentemente uma deficiência no balanço energético desses organismos.

O metabolismo é o conjunto de processos pelos quais as células e os organismos adquirem, reorganizam e eliminam os nutrientes de modo a sustentar a vida. O catabolismo é o conjunto de processos pelos quais os compostos químicos complexos são degradados para liberar energia, produzindo moléculas menores. Por sua vez, o anabolismo consiste no conjunto de processos que sintetizam compostos químicos maiores a partir de moléculas menores, utilizando energia (Hill et al., 2008). A via principal do catabolismo aeróbico pode ser subdividida em quatro grandes conjuntos de reações: a glicólise, o ciclo de Krebs, a cadeia transportadora de elétrons e a fosforilação oxidativa. Cabe ressaltar aqui que o principal produto resultante do catabolismo aeróbico é o trifosfato de adenosina (ATP), um nucleotídeo que armazena energia nas ligações entre os seus grupos fosfato, não sendo transportado entre células e caracterizada por ser uma forma de energia para consumo imediato.

Na glicólise, uma molécula de glicose (6 carbonos) é degradada através de dez reações catalisadas enzimaticamente, para liberar duas moléculas de piruvato, cada uma contendo 3 carbonos (Nelson e Cox, 2008). Durante as reações sequenciais da glicólise, parte da energia livre liberada da glicose é conservada na forma de ATP e NADH. O piruvato formado pela glicólise pode seguir três rotas catabólicas alternativas. Nos tecidos sob condições aeróbicas, o piruvato é oxidado com perda do seu grupo carboxila na forma de CO_2 para liberar o grupo acetila da acetil-Coenzima A, a qual é totalmente oxidada pelo ciclo de Krebs (Nelson e Cox, 2008). A segunda rota para o metabolismo do piruvato é chamado de glicólise anaeróbica, e promove a sua redução a lactato por meio da lactato desidrogenase, que utiliza elétrons provenientes do NADH e consequentemente recicla o NAD^+, permitindo a continuação da via glicolítica (Campbell e Farrell, 2006). Em condições aeróbicas, essa reação é reversível, sendo que o piruvato pode ser regenerado a partir do lactato. A terceira e última rota do

metabolismo do piruvato consiste na conversão deste composto em etanol e CO_2, no processo chamado de fermentação alcoólica.

O ciclo de Krebs ou ciclo do ácido tricarboxílico é uma via catabólica, onde o citrato formado a partir de oxaloacetato e acetil-CoA é oxidado produzindo CO_2, sendo que a energia desta oxidação é armazenada na forma das coenzimas reduzidas NADH e $FADH_2$ (Nelson e Cox, 2008). Após uma volta completa do ciclo de Krebs, uma molécula de piruvato terá produzido três moléculas de NADH, um $FADH_2$ e dois ATPs (Berg et al., 2002).

A cadeia transportadora de elétrons é constituída por quatro grandes complexos protéicos localizados nas membranas internas das mitocôndrias formando uma cadeia. Nesta cadeia, os elétrons do NADH e $FADH_2$ são transferidos em sequencia, de um complexo para outro, em uma série de reações de oxidação-redução. O último complexo da cadeia (citocromo-oxidase) transfere os elétrons, juntamente com os prótons (íons H^+), para o oxigênio, reduzindo-o a água. Assim, o oxigênio atua como aceptor final de elétrons, sendo que o resultado final da cadeia transportadora é retirar os elétrons do NADH e do $FADH_2$, transferindo-os para o oxigênio, re-oxidando o NAD^+ e o FAD. Desta forma, é na cadeia transportadora de elétrons que ocorre a transferência de energia das ligações das moléculas dos alimentos ingeridos para o ATP (Hill et al., 2008). Por sua vez, o processo de síntese de ATP realizado pela ATP sintase, por meio da utilização da energia contida no gradiente eletroquímico originado do transporte dos elétrons pela cadeia transportadora de elétrons, é chamada de fosforilação oxidativa (Nelson e Cox, 2008).

Considerando-se que o metabolismo energético é dependente do conteúdo das reservas energéticas disponíveis para transformação e do fornecimento adequado de oxigênio, os efeitos da exposição ao Cu sobre o conteúdo das principais reservas energéticas e de produtos metabólicos intermediários e finais foram avaliados nos tecidos do marisco *M. mactroides*. A hipótese central deste estudo é de que a exposição ao Cu, em níveis subletais, causa uma

diminuição na produção de ATP, devido a uma diminuição do suprimento de oxigênio intracelular, resultando em uma disfunção metabólica.

O bivalve marinho *M. mactroides* (Deshayes 1854) é popularmente conhecido como marisco branco ou sernambi. Esse molusco bivalve pertence à infauna cavadora profunda (Olivier e Penchaszadeh, 1971) e habita o mesolitoral de praias arenosas dissipativas (baixa inclinação, areias finas e alta energia de ondas), sendo que a composição do substrato exerce forte influência na distribuição de *M. mactroides* (Bastida et al., 1991; Defeo et al., 1992). Sua distribuição geográfica (Fig. 1) vai desde o sudeste do Brasil (24ºS, Rio de Janeiro, Brasil) até a planície central da Argentina (40ºS, Província de Buenos Aires, Argentina) (Olivier and Penchaszadeh, 1968; Fiori e Morsan, 2004). Ele é abundante ao longo de todo o ano e considerado um dos principais representantes da biomassa bêntica da zona de varrido de praias arenosas do sul do Brasil (Gianuca, 1983, 1985). No inverno, *M. mactroides* é encontrado enterrado no sedimento a uma profundidade de 30 a 40 cm de profundidade, enquanto no verão este marisco permanece próximo à superfície do sedimento (Herrman, 2008). Este marisco apresenta prioritariamente um hábito alimentar suspensívoro fitoplanctófago. Quando há escassez de alimento em suspensão na coluna da água, ele passa a se alimentar de detritos presentes no sedimento (Defeo e Scarabino, 1990). O marisco branco é considerado eurialino, tolerando salinidades variando de 15 a 35 (Santos e Bianchini, 1998).

OBJETIVOS

Objetivo geral

O objetivo geral do presente estudo foi avaliar o efeito da exposição subletal aguda ao Cu sobre o estado metabólico de juvenis do marisco branco *Mesodesma mactroides*.

Objetivos específicos

- Avaliar o efeito da exposição ao Cu no conteúdo tecidual (brânquias, glândula digestiva, músculo pedal e hemolinfa) de coenzimas (NAD^+ e NADH) e de ATP.
- Determinar as alterações causadas pela exposição ao Cu no conteúdo tecidual (brânquias, glândula digestiva, músculo pedal e hemolinfa) de reservas energéticas (glicose, glicogênio, lipídios totais e proteínas totais).
- Analisar o conteúdo tecidual (músculo pedal e hemolinfa) de glicose e produtos metabólicos (piruvato e lactato) após exposição ao Cu.

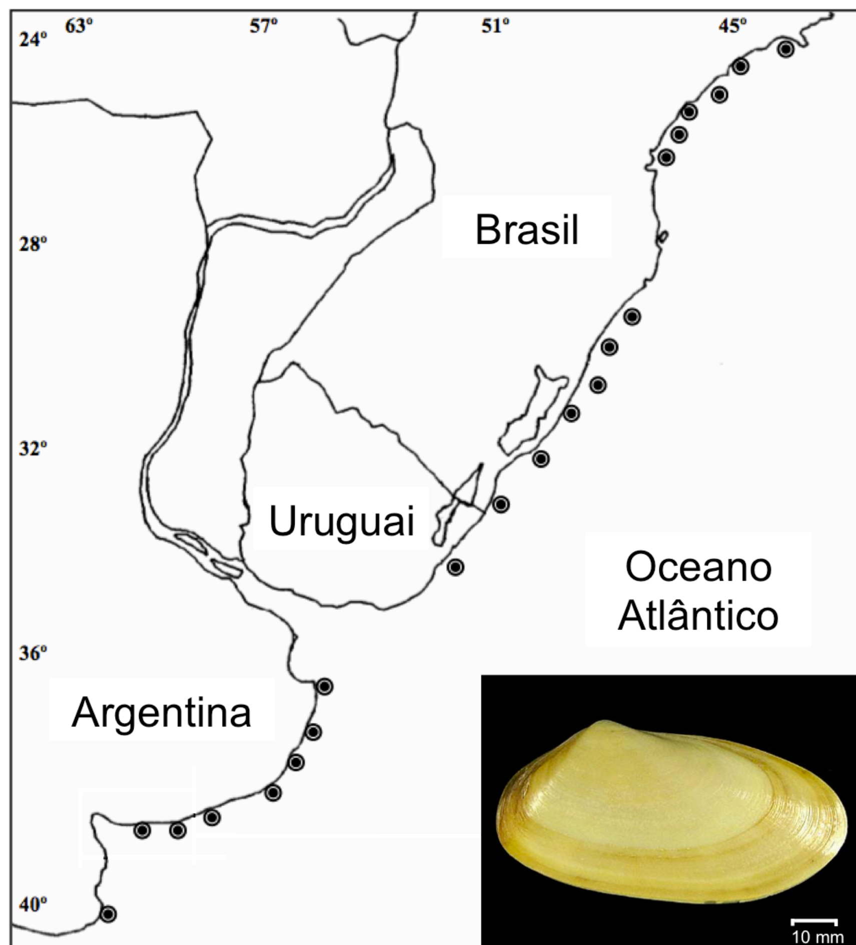

Figura 1. Distribuição geográfica do marisco branco *Mesodesma mactroides* (adaptado de Olivier e Penchaszadeh, 1971). No detalhe é mostrado um exemplar adulto da espécie.

ARTIGO CIENTÍFICO

Effects of copper exposure on the energy metabolism in juveniles of the marine clam *Mesodesma mactroides*

Artigo científico preparado para submissão ao periódico "Aquatic Toxicology" conforme o "Guia para Autores", que se encontra ao final desta dissertação.

Effects of copper exposure on the energy metabolism in juveniles of marine clam *Mesodesma mactroides*

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Abstract

In freshwater invertebrates, Cu is described as an ionoregulatory toxicant, inhibiting the activity of key enzymes involved in Na^+ uptake and consequently inducing ionic and osmotic disturbances. However, the mechanism involved in Cu toxicity in osmoconforming invertebrates remains still unclear. Recent findings from our laboratory have suggested that Cu toxicity in marine invertebrates is more related to a respiratory impairment than to ionic and osmotic disturbances. Therefore, metabolic changes induced by waterborne Cu exposure were evaluated in the osmoconforming clam *Mesodesma mactroides*, a bivalve species widely distributed along the South American sandy beaches. Juvenile clams were kept under control conditions (no Cu addition in the water) or acutely (96 h) exposed to Cu (96-h LC_{10} = 150 µg L^{-1}) in artificial sea water (30 ppt). ATP, proteins, lipids, glycogen and glucose content was analyzed in gills, digestive gland, adductor muscle and hemolymph. Dinucleotides (NAD^+ and NADH) content was also analyzed in gills, digestive gland, and adductor muscle while pyruvate and lactate content was determined in adductor muscle and hemolymph. In all tissues analyzed, Cu exposure did not affect ATP content and NAD^+/NADH ratio, except in the hemolymph, where a decrease in ATP content was observed. These findings indicate that clam cells, except those from hemolymph, were able to maintain constant the level of free energy. A significant increase in total protein content was observed in the digestive gland, which could be a compensatory mechanism to counteract the higher level of protein oxidation previously observed in *M. mactroides* exposed to Cu under the same experimental conditions. Finally, reduced glucose content in the adductor muscle paralleled by increased lactate content in the adductor muscle and hemolymph was observed in Cu-exposed clams. Overall, these findings indicate that Cu exposure is leading to an increased reliance upon the anaerobic energy production to maintain the overall cellular ATP production in the clam *M. mactroides*.

Keywords: acute toxicity, anaerobiosis, copper, marine clam, metabolism

1. Introduction

Copper (Cu) is a transition metal characterized by its high electrical and thermal conductibility, which explains the wide industrial use of this metal (Lander and Reuther, 2004). Although there are important natural sources of Cu in the environment, a variety of anthropogenic sources such as agricultural, industrial, and harboring activities have considerably increased Cu releasing into aquatic environments worldwide (Kennish, 1997).

Cu is an essential micronutrient required by all living organisms, playing an important role as cofactor of many enzymes and being part of other proteins (Solomon and Lowery, 1993; Taylor and Anstiss, 1999). However, it can be potentially toxic to aquatic organisms when available at elevated concentrations in the water. In freshwater animals, including invertebrates, Cu is described as an ionoregulatory toxicant. It is reported to inhibit the activity of ion-transporting proteins such as Na^+/K^+-ATPase and carbonic anhydrase, key enzymes involved in gill Na^+ uptake (Laurén and McDonald, 1985; Grosell and Wood, 2002; Zimmer et al., 2012). On the other hand, there is still no agreement on the mechanism involved in Cu toxicity in seawater invertebrates (Bianchini et al., 2004; Pinho et al., 2007; Lauer e Bianchini, 2010; Pinho et al., 2010; Lopes et al., 2011a,b; Martins et al., 2011a,b; Jorge et al., 2013; Monteiro et al., 2013).

In the euryhaline blue crab *Callinectes sapidus* acclimated to low salinities, the mechanism of Cu toxicity appears to be related to a down-regulation of genes encoding some key proteins involved in osmoregulation. However, the same effect was not seen in blue crabs acclimated to high salinities, indicating that different mechanisms could be involved in Cu toxicity under different osmoregulatory conditions (Martins et al., 2011a). In the marine clam *Mesodesma mactroides*, Lopes et al. (2011a,b) provided some evidence that Cu is able to impair the intracellular ion regulation without change in the hemolymph ion regulation. These

authors further concluded that Cu was entering and being accumulated in mantle cells of the marine clam mainly through the $Na^+/K^+/2Cl^-$ cotransporter (Lopes et al., 2011a,b). Also, exposure to Cu was shown to reduce the carbonic anhydrase activity, an important enzyme related to cellular acid-base regulation, respiration, and ammonia excretion. This effect was reported both *in vivo* (Jorge et al., 2013a) and *in vitro* (Nogueira et al., 2013). Also, Lauer et al. (2013) reported that the acute exposure to a sublethal concentration of Cu affect the activity of key enzymes involved in the energy metabolism in gills of the estuarine crab *Neohelice granulata*. Furthermore, these authors reported a decrease in mitochondrial membrane potential, which could lead to a reduced mitochondrial ATP production.

In the osmoconformer clam *M. mactroides*, Cu exposure was shown to decrease the whole-body and tissue (gill and digestive gland) oxygen consumption. Therefore, it was suggested that Cu toxicity would be associated with a respiratory impairment. However, no significant changes were observed in the activity of succinate-dehydrogenase (respiratory Complex II) when gills and digestive gland were exposed *in vitro* to 1 and 10 μM Cu (Jorge et al., 2013a). These findings suggest that energy metabolism could be affected by clam exposure to waterborne Cu. However, this effect seems not to be associated with changes in the ability of cells to generate energy through the aerobic metabolism. In general, energy metabolism is consisted of processes by which energy is acquired, transformed and channeled to useful functions. It relies on a set of biochemical reactions that are interconnected, forming the metabolic pathways necessary to provide enough energy for cell functioning (Hill et al., 2008).

In light of the above background, the present study aimed to examine the metabolic responses of the marine clam *M. mactroides* to acute waterborne Cu exposure. Juvenile clams were exposed to 150 μg Cu L^{-1} (96 h – LC_{10}) for 96 h. It is important to note that this Cu concentration was previously reported to induce 10% mortality of *M. mactroides* under the

same experimental conditions tested in the present study (Jorge et al., 2013a,b). We predict that acute exposure to this sublethal concentration of Cu would negatively affect the energy metabolism in the marine clam *M. mactroides*. According to findings from the recent studies with the osmoregulating crab *N. granulata* (Lauer et al., 2013) and the osmoconforming clam *M. mactroides* (Jorge et al., 2013a), this effect would be occurring through changes in ATP production and energy reserves. In the present study, this hypothesis was tested by assessing the possible changes induced by waterborne Cu on the level of free energy, content of energy reserves, and content of metabolic end products in tissues of juvenile clam *M. mactroides*.

2. Material and Methods

2.1. Clam collection and acclimation

Juvenile clams (*M. mactroides*) were collected in winter (July-August 2012) at Mar Grosso Beach (São José do Norte, southern Brazil). Clams (shell length: 39-54 mm; shell width: 22-32 mm) were transferred to the Animal Care Facility of the Federal University of Rio Grande, where they were separated in batches and maintained (1 clam $2L^{-1}$) in natural filtered (45-µm mesh filter) sea water (salinity 30 ppt). During the first 7 days of acclimation, water was partially renewed daily using natural filtered sea water. Afterwards, artificial sea water (30 ppt) prepared with sea salts (Coralife®, Central Aquatics, Franklin, WI, USA) dissolved in MilliQ water was used to gradually (20% per day) replace the natural sea water, until the acclimation media was consisted of only artificial sea water. Clams were maintained under this condition for at least 3 days. Water was partially renewed daily. During the whole acclimation period, clams were fed every 2 days with the microalgae *Thalassiosira weissflogii*

(~ $2x10^7$ cells L^{-1}) at a ratio of 2.5 mL of algae culture in 1L of acclimation sea water. Room temperature (20ºC) and photoperiod (12 h light:12 h dark) were fixed.

2.2. Acute Cu exposure and water and tissue sampling

Juvenile clams were exposed to Cu in artificial sea water (salinity 30 ppt), prepared as described above. Clams were individually exposed in 250-mL glass beakers containing 200 mL of control (no Cu addition in the artificial sea water) or Cu-contaminated medium. The latter was prepared by adding Cu (nominal concentration: 150 µg Cu L^{-1}) as $CuCl_2$ (Vetec®, Rio de Janeiro, RJ, Brazil) in artificial sea water and allowed to equilibrate for 24 h prior to clam's introduction into the test chamber (Bielmyer et al., 2004). All glassware was pre-washed with 1% HNO_3 and thoroughly rinsed with MilliQ water before use. Exposure was performed for 96 h using a static-renewal system. Exposure media were completely renewed every 24 h. Non-filtered and filtered (0.45-µm mesh filter; Ministart®, Sartorius AG, Goettingen, Germany) water samples were collected right before clam's introduction into the experimental media and at 24 h of exposure, i.e., prior to the daily renewal of the test media. All water samples were acidified with HNO_3 (Suprapur; Vetec®, Rio de Janeiro, RJ, Brazil) to a final concentration of 1% HNO_3.

Room temperature and photoperiod conditions were kept as described for the acclimation period. To ensure adequate levels of dissolved O_2 in the water, experimental media were continuously aerated. Feeding was stopped 24 h prior to the beginning of the test and no food was provided throughout the experimental period (96 h). Clam survival was monitored every 24 h and dead animals were discarded. At the end of the exposure period, hemolymph from each living clam was collected by puncture of the hemolymph sinus at the adductor muscle using a disposable syringe with needle (13x0.45 mm). Gills, digestive gland

and adductor muscle were dissected, immediately frozen in liquid N_2, and stored in an ultra-freezer (-80°C) until further analysis.

2.3. Cu concentration analysis

Total (non-filtered water samples) and dissolved (filtered water samples) Cu concentrations were measured by atomic absorption spectrophotometry (AAS 932 Plus - GBC, IL, USA). Measurements were performed as previously described (Lauer et al., 2012; Prazeres et al., 2012; Jorge et al., 2013a).

2.4. Tissue energy reserves and metabolic end products analyses

2.4.1. ATP content

ATP content was determined in deproteinized samples. Tissue (gills, digestive gland and muscle) were homogenized using a liquid N_2-cooled mortar and pestle until a fine powder was obtained. Powdered samples were weighed and 5 μL of perchloric acid (6%) was added per mg of tissue. Samples were neutralized with K_2CO_3 (3 M) and the resultant sample extract was used for subsequent analysis. The hemolymph samples were deproteinized by addition of perchloric acid (6%) at a ratio of 1:3 (v:v) and neutralized with K_2CO_3 (3 M). ATP content was measured in sample extracts using a commercial reagent kit (Molecular Probes®, Eugene, OR, USA) based on light production by luciferase, as measured by a luminometer with maximum emission at 560 nm (Victor 2, Perkin Elmer, Waltham, MA, USA). Results were expressed as μg ATP g wet weight^{-1} (gills, digestive gland and adductor muscle) or μg ATP mL^{-1} (hemolymph).

2.4.2. NAD^+ and NADH content

Content of dinucleotides (NAD^+ and NADH) was determined in gills, digestive gland and adductor muscle through the spectrophotometric (450 nm) cycling method, using a commercial reagent kit (Sigma-Aldrich, St. Louis, MO, USA). Samples were homogenized using the buffer solution provided in the reagent kit and was split into two aliquots. One aliquot was incubated at 60ºC (30 min) to decompose NAD^+ while the other one was left stand to preserve both NAD^+ and NADH present in samples. NAD^+ concentration was then calculated by subtracting the value obtained for the sample containing only NADH (decomposed NAD^+ sample) from that obtained for the intact sample containing NAD^+ and NADH. Results were then normalized by the protein content in the sample aliquot, which was measured as described below. Therefore, NAD^+ and NADH contents were expressed in pmol NADH ng protein^{-1}. Finally, data were expressed as the ratio between NAD^+ and NADH contents.

2.4.3. Total proteins and glucose contents

Gills, digestive gland and adductor muscle samples were homogenized (1:5; w:v) with a phosphate buffer solution (1 M K_2HPO_4; 1 M NaH_2PO_4; 1 mM phenylmethanesulfonyl fluoride; pH 7.4) and centrifuged (10,000 x *g*) at 4ºC for 2 min. Supernatant was collected for further analyses. Hemolymph samples were used intact.

Total protein content was determined in tissue homogenized using a commercial reagent kit (Doles, Goiania, GO, Brazil), and in hemolymph using the Bradford Reagent (Sigma-Aldrich, St. Louis, MO, USA). In both cases, protein concentration was calculated based on a standard curve built with bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as standard. Results were expressed as mg protein g wet weight^{-1} (gills, digestive gland and adductor muscle) or µg protein mL^{-1} (hemolymph).

Total glucose content was measured in tissue homogenized and hemolymph samples by using a commercial reagent kit (Doles, Goiania, GO, Brazil) based on the enzymatic (glucose oxidase) method. Glucose concentration was calculated based on a standard curve built according to the manufacturer's instructions. Results were expressed as µg glucose g wet weight^{-1} (gills, digestive gland and adductor muscle) or µg glucose mL^{-1} (hemolymph).

2.4.4. Glycogen content

Gills, digestive gland and adductor muscle samples were homogenized using a liquid N_2-cooled mortar and pestle. Powdered samples were weighed and an ice-cold sodium citrate buffer solution (0.1 M; pH 5.0) was added (1:5; w:v) while hemolymph samples were simply diluted (1:3; v:v) using the same buffer solution. Samples were incubated (5 min) at 100ºC and then centrifuged (2,790 x *g*) at 20ºC for 5 min. The supernatant was collected, split into 2 aliquots and treated as described by Carr and Neff (1984). One aliquot was hydrolyzed with amyloglucosidase (Sigma-Aldrich, A7095) and the other one was left intact. Both aliquots were incubated at 25ºC for 12 h, and the glucose concentration en each aliquot was determined using the commercial reagent kit used for glucose content analysis, as described above. Glycogen content in each sample was calculated based on the difference between the glucose content measured in the enzyme hydrolyzed aliquot and the non-hydrolyzed aliquot. A glycogen standard solution (1 mg mL^{-1}) was used throughout the analysis to verify the enzyme conversion rate, which is considered acceptable when between 86 and 130%. Results were then expressed as µg glycogen Kg wet weight^{-1} (gills, digestive gland and adductor muscle) or µg glycogen mL^{-1} (hemolymph).

2.4.5. Total lipids content

Total lipids were extracted from tissue samples using a mixture of chloroform and methanol (2:1), as described by Folch et al. (1956). Extracts were analyzed

spectrophotometrically (550 nm) using the sulfophosphovanilin method, as described by Cheng et al. (2011). Total lipids content was calculated based on a standard curve and expressed as mg lipids g wet weight^{-1} (gills, digestive gland and adductor muscle) or mg lipids mL^{-1} (hemolymph).

2.4.6. Pyruvate and lactate contents

Muscle and hemolymph samples were deproteinized as described above (Section 2.4.1.). Pyruvate and lactate contents were determined in deproteinized samples following procedures described by Bergmeyer (1983). For pyruvate determination, samples were incubated at 25 ºC in a reaction buffer solution (0.5 M triethanolamine, 5 mM EDTA, and 0.17 mM NADH; pH 7.6) for 90 min prior to the addition of lactate dehydrogenase (LDH) to a final concentration of 5.76 enzyme units mL^{-1}. Sample absorbance was read at 340 nm every 15 s for 2 min. The amount of pyruvate present in the sample is inversely proportional to NADH decrease reflected by a reduction in sample absorbance. Pyruvate concentration was calculated based on a standard curve built with sodium pyruvate as standard. Results were then expressed as nmol pyruvate g wet weight^{-1} (muscle) or pmol pyruvate L^{-1} (hemolymph).

For lactate measurement, deproteinized samples were mixed with a reaction buffer solution (0.5 M glycine, 0.4 M hydrazine; pH 9.0), NAD^+ (2 mM final concentration) and LDH (20 enzyme units mL^{-1}). The reaction mixture was incubated (25ºC) for at least 90 min or until the reaction had stabilized. Sample absorbance (340 nm; extinction coefficient = 6.22 L mmol^{-1} cm^{-1}) was read and lactate content was expressed as mmol lactate g wet weight^{-1} (muscle) and pmol lactate L^{-1} (hemolymph).

2.5. Data presentation and statistical analysis

Data were expressed as mean ± 1 standard deviation (n = 4-6 for gills, digestive gland and adductor muscle; n = 12 for hemolymph). For each parameter, mean values for the same tissue were compared through Student's t-test. In all cases, mean values were considered significantly different when $p<0.05$. Parametric assumptions (data normality and homogeneity of variances) were previously checked, and if not achieved, data were mathematically transformed using a square root transformation (Zar, 1984).

3. Results

3.1. Copper concentration in the experimental medium and clam survival

Total (145.32 ± 2.36 μg Cu L^{-1}) and dissolved (145.27 ± 8.73 μg Cu L^{-1}) Cu concentrations in the experimental medium at the beginning of the 24-h period of exposure were virtually the same and quite similar to the desired nominal concentration. In turn, no Cu was detected in the control medium (detection limit = 5 μg Cu L^{-1}). A notable reduction in Cu concentration was observed in experimental medium after 24 h of exposure (total Cu: 42.84 ± 13.48 μg Cu L^{-1}; dissolved Cu: 39.98 ± 11.27 μg Cu L^{-1}). This trend was consistent throughout the 96-h period of experiment. After 96 h of exposure, mortality rate corresponded to 5% in Cu-exposed clams while no mortality was observed in clams maintained under control conditions (no Cu addition into the artificial sea water).

3.2. Energy reserves and metabolic end products

Exposure to waterborne Cu did not affect the ATP content in gills, digestive gland, and adductor muscle of *M. mactroides* (Fig. 1). Also, no significant change in NAD^+ or

NADH content was observed in these tissues after Cu exposure (data not shown). Therefore, the NAD^+/NADH ratio was also similar in control and Cu-exposed clams (Fig. 2). On the other hand, a significant decrease (28%) in ATP concentration was observed in the hemolymph of Cu-exposed clams (Fig. 1).

Cu exposure only affected the total proteins content in the digestive gland. In this case, a significant increase (39%) was observed in Cu-exposed clams respect to the control ones (Fig. 3). In turn, total lipids content was not affected by Cu exposure in any of the tissues analyzed (Fig. 4). As expected, the adductor muscle showed the highest content of glycogen among the tissues analyzed. No significant difference was observed between control and Cu-exposed clams (Fig. 5). Also, no Cu effect on total glucose content was observed in gills, digestive gland and hemolymph. However, adductor muscle of Cu-exposed clams showed a significant decrease (23%) in the total glucose content (Fig. 6).

Cu exposure did not affect the pyruvate content in the adductor muscle and hemolymph (Fig 7A). However, a marked increase in the lactate content was observed in the adductor muscle (56%) and hemolymph (1,124%) of Cu-exposed clams (Fig. 7B).

4. Discussion

There was a negligible (5%) mortality of clams during the 96-h period of experiment in the Cu-exposed group and no mortality was seen in the control group. Therefore, all effects reported in the present study were considered to be sublethal. Throughout the experiment, a reduction in total (68.3%) and dissolved (70.2%) Cu concentration was observed between 0 and 24 h after the beginning of exposure period (24 h) using the same experimental medium. Although free Cu ions are readily adsorbed onto the walls of the exposure chamber and even the body surface of clams, a recent study from our research group showed that *M. mactroides*

tissues (gills, digestive gland and hemolymph) accumulate significant amounts of Cu under the same experimental conditions tested in the present study. It was shown that Cu is more accumulated in hemolymph (12-fold increase respect to the control value) followed by gills (5-fold increase) and digestive gland (4-fold increase) (Jorge et al., 2013).

The maintenance of oxygen supply is crucial for all aerobic living organisms. In fact, oxygen plays an important role as the final electron acceptor in the mitochondrial respiratory chain. It has been reported that metals, including Cu, are able to impair whole-body respiratory rate in fish, crustaceans and mollusks (Brown and Newell, 1972; Santos et al., 2000; Paila and Yallapragada, 2010; Das and Gupta, 2013). It is described that Cu at lethal and sublethal concentrations acts on the respiratory system of crustaceans and mollusks primarily by disrupting gill function (Spicer and Weber, 1991; Soegianto et al., 1999; Frias-Espericueta et al., 2008). Gill damage induced by Cu would result in a reduction in the surface area available for gas exchange (Nonnotte et al., 1993). In turn, Brown and Newell (1972) showed that high Cu burdens exert an inhibitory effect on gill cilia of the bivalve *Mytilus edulis*. In this context, it is important to stress that the oxygen consumption rate is considered as a useful indicator of the overall metabolic rate of animals (Schmidt-Nielsen, 1997). In fact, this physiological endpoint is commonly and widely used to assess the effects of chemical contaminants on animal physiology.

It is well established that exposure to Cu induces a decrease in both whole-body (*in vivo*) and tissue (*in vitro*) oxygen consumption rate in aquatic animals, including mollusks (Brown and Newell, 1972; Spicer and Weber, 1991; De Boeck et al., 1995; Santos et al., 2000; Brown et al., 2004; Paila and Yallapragada, 2010; Jorge et al, 2013). In fact, juveniles of the marine clam *M. mactroides* acutely (96 h) exposed to 150 µg Cu L^{-1} showed a 38% reduction in whole-body oxygen consumption rate (Jorge et al., 2013). This effect could be resulted from several factors. Among them, we could consider a decreased gill ventilation rate

as an attempt to reduce Cu uptake (Spicer and Weber, 1991), an increased distance for the diffusive gas exchange due to mucus secretion in response to waterborne Cu exposure (Sze and Lee, 1995), a change in haemocyanin affinity for oxygen (Taylor and Anstiss, 1999), and a reduced activity of mitochondrial enzymes (Jorge et al., 2013; Lauer et al., 2013). Though it is clear that Cu can display a variety of negative effects on gill function, the resulting oxygen availability to cells could lead to a decreased mitochondrial respiration rate and a consequently reduced ATP production, as described in the euryhaline crab *N. granulata* acutely exposed to Cu (Lauer et al., 2013).

Though a decrease in tissue ATP content after Cu exposure was predicted, this was only observed in the hemolymph of Cu-exposed clams. In contrast, the other tissues (gills, digestive gland and adductor muscle) of Cu-exposed clams were able to maintain their ATP content at levels very similar to those showed by clams maintained under control condition (Fig. 1). This finding combined with the reduced oxygen consumption rate previously reported in juvenile *M. mactroides* exposed to Cu under the same conditions tested in the present study (Jorge et al., 2013) suggest that clams are reducing their whole-body metabolic rate in order to minimize energy expenditure. Also, an increased utilization of other pathways than glycolysis to produce ATP, such as the catabolism of fats and/or proteins (Hansen et al., 1992), as well as a shift from aerobic to anaerobic metabolism cannot be ruled out. These possibilities will be further explored and discussed considering the findings reported in the present study.

In mollusks, it was shown that the Krebs cycle remains partially or totally functional under anaerobic situations (De Zwaan and Van Marrewijk, 1973; Hochachka et al*.,* 1973; Zs.-Nagy, 1974; De Zwaan et al*.,* 1975). This might happen because fumarate or specific non-substrate electron acceptors are replacing oxygen (De Zwaan and Wijsman, 1976). The lack of change in NAD^+/NADH ratio in gills, digestive gland and adductor muscle of Cu-exposed

clams supports the idea that energy transfer processes until the mitochondrial level are not affected by Cu exposure. However, it is important to note that NAD^+/NADH ratio values were consistently below one (Fig. 2), indicating a higher NADH availability. This finding could indicate that less NADH is being re-oxidized into NAD^+ by the electron transport chain during Cu exposure. In this context, it is important to note that during low oxygen stress, like the one observed after *M. mactroides* exposure to Cu, ATP utilization in bivalves may be as low as 5% of that seen during aerobic metabolism (De Zwaan, 1983). Taken altogether, these findings associated with those already reported in the literature suggest that juvenile clam *M. mactroides* could be shifting the energy source employed to produce a similar amount of ATP with lower oxygen consumption. At this point, the reduced ATP content observed in the hemolymph of juvenile *M. mactroides* exposed to Cu could be related to a possible incapacity of this tissue to shift metabolic pathways in order to keep constant the cellular levels of free energy (ATP).

In general, organisms can obtain energy from the aerobic oxidation of carbohydrates, lipids and proteins. Therefore, analysis of body composition can indicate which components are available and may be the primary fuel for metabolism (De Zwaan and Wijsman, 1976). In the present study, we evaluated the effects of Cu on the main energy reserves and substrates, such as carbohydrates (glycogen and glucose), total lipids and total proteins. When glucose is found in excess within the cell, it might be converted into storage polymeric compounds such as glycogen (Nelson and Cox, 2006). Under aerobic conditions, glycogen breakdown leads to glucose generation in a catabolic pathway called glycogenolysis. Although glycogen is an important energy reserve for most animal species, bivalves are reported to have their metabolism predominantly centered on glycogen metabolism (Giese, 1969; De Zwann and Zandee, 1972). Furthermore, it has been shown that tissue glycogen content decreases when mussels are exposed to high levels of organic compounds (Pellerin-Massicotte, 1994) and

metals (Hummel et al., 1996; Duquesne et al., 2004). However, no significant change in tissue (gills, digestive gland, adductor muscle and hemolymph) glycogen content was observed between control and Cu-exposed juvenile clams from the present study (Fig. 5). This might be due to a lack of a higher energy demand, since glucose content in all tissues analyzed was also found unaltered in Cu-exposed clams. However, we cannot rule out a possible negative effect of Cu on tissue ability to convert glycogen into glucose. Further studies are needed to clarify this point.

Although glycogen is generally the most suitable substrate for energy production under anaerobic condition, some authors have pointed out the role of lipids under this stressful condition in several mollusk species (Oudejans and Van der Horst, 1974; Zs.-Nagy and Galli, 1977). In fact, changes in lipid indices have been demonstrated in blue mussels in response to organic pollutants exposure (Capuzzo and Leavitt, 1988; Leavitt et al., 1990). However, the effects of metal exposure on bivalve lipid composition and distribution are quite variable, depending on species and metals tested (Abdullah and Ireland, 1986; Lowe and Clarke, 1989; Duquesne et al., 2004). The marine clam *M. mactroides* shows a typical lipidic composition characteristic of marine organisms. In this case, fatty acid composition is mainly regulated by diet and, to a lesser extent, by other mechanisms (De Moreno et al., 1976). The lack of change in total lipids (Fig. 4) in all tissues analyzed in the present study suggest that fats metabolism is likely not altered by Cu exposure in juvenile *M. mactroides*.

Measurements of total proteins content in tissues have been widely used as an index of animal health. Indeed, global protein synthesis rates have even been suggested as a reliable biomarker of stress in the mussel *M. galloprovicialis* following exposure to metals (Pytharopoulou et al., 2006). In fact, a decrease in total protein levels might be attributed to a decrease in general metabolic activity (Baudrimont et al., 1997; Géret et al., 2003). Despite the lower whole-body and tissue oxygen consumption observed in *M. mactroides* exposed to

Cu (Jorge et al., 2013), no significant change was observed in the total proteins content in gills, adductor muscle and hemolymph of Cu-exposed clams (Fig. 3). This finding suggests that the global protein metabolism in these tissues is also likely not affected by Cu exposure, as observed for lipids. On the other hand, there was a marked increase in total proteins content in the digestive gland of clams exposed to Cu when compared to those maintained under control condition. This effect could be explained considering a higher synthesis of metallothioneins to bind and detoxify the excess of Cu accumulated in the digestive gland of *M. mactroides* after exposure to Cu (Jorge et al., 2013). In fact, several metals including Cu have shown to induce metallothioneins synthesis in bivalve mollusks (Géret et al., 2002; Serafim and Bebiano, 2009). However, a previous study from our laboratory showed no significant change in metallothioneins content in the digestive gland of *M. mactroides* exposed to Cu under the same experimental conditions tested in the present study. However, an increased level of protein carbonylation was observed in the digestive gland of these clams (non published data). Therefore, the observed higher content of total proteins in digestive gland of Cu-exposed clams in the present study (Fig. 3) could represent an adaptive response to counteract the increased protein oxidation rate resulting from the oxidative stress induced by exposure to Cu.

Taken altogether, data on energy reserves (proteins, lipids, glycogen and glucose) reported in the present study for gills, digestive gland and hemolymph of *M. mactroides* exposed to Cu cannot explain the lower whole-body and tissue (gills and digestive gland) oxygen consumption previously reported under the same experimental condition (Jorge et al., 2013). In fact, Zwaan and Wijsman, (1976) stated that no simple explanation exists to account for how carbohydrates and other substances become degraded during functional hypoxia in bivalves and that several intermediate and end products (pyruvate, lactate, octopine, succinate, alanine, acetate, propionate and CO_2) would be involved in energy generation

under such conditions. At this point, it is interesting to note that Cu-exposed clams showed a significantly reduced glucose content in the adductor muscle (Fig. 6) paralleled by a lack of change in pyruvate content (Fig. 7A) and a marked increase in lactate content in both the adductor muscle and hemolymph (Fig. 7B).

Glycolysis is an anaerobic process that yields two molecules of ATP. As the first stage of carbohydrate metabolism, it ends 2 units of pyruvate, which under aerobic conditions, is oxidized to acetyl-CoA and enters the Krebs cycle pathway. When the oxygen is not available at adequate levels, pyruvate is reduced through LDH to lactate, thus recycling NAD^+ that can be again used by glycolysis (Campbell and Farell, 2006). In fact, the rate of conversion of pyruvate into lactate can be considered as an index of physiological stress under anaerobic conditions (Sivaramakhrishna and Radhakhrishnaiah, 2000). Taken altogether, these findings suggest that glucose is being consumed by Cu-exposed clams at a higher rate through glycolysis, with the resulting pyruvate being promptly converted to lactate in order to maintain the overall cell energy production. Indeed, increased LDH activity following Cu exposure has been reported in aquatic animals, including invertebrates (Carvalho and Fernandes, 2008; Lauer et al., 2012). Therefore, we suggest that lactate largely accumulated in the adductor muscle and hemolymph of Cu-exposed clams is being used as an energy source to maintain the general supply of free energy as ATP (Fig. 1).

In summary, the reduced glucose content in the adductor muscle paralleled by the increased lactate content in the adductor muscle and hemolymph of Cu-exposed clams strongly suggest that juveniles *M. mactroides* are facing the stress induced by Cu exposure by relaying more in the anaerobic metabolism in order to maintain constant levels of cellular ATP. Also, the observed lack of Cu effects on NAD^+ and NADH concentrations, and consequently on the ratio of these dinucleotides, can be an indicative that clams are keeping the Krebs cycle functional even after exposure to Cu. Further studies focused on the analysis

of metabolic end products more specific to mollusks could help to better elucidate the metabolic strategies adopted by *M. mactroides* to cope with the acute waterborne Cu exposure. Finally, based on the findings reported in the present study one can infer that the mechanism involved in lethal acute toxicity of Cu in the osmoconforming clam *M. mactroides* seems not to be related to impairment on energy metabolism.

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6. References

Abdullah, A.M., Ireland, M.P., 1986. Seasonal studies on cadmium concentration and toxicity, oxygen consumption, digestive gland glycogen, and lipid in dog whelks collected at Aberystwyth, Wales. Mar. Pol. Bull. 17, 562 - 566

Baudrimont, M., Metivaud, J., Maury-Brachet, R., Ribeyre, F., Boudou, A. 1997. Bioaccumulation and metallothionein response in the asiatic clam (*Corbicula fluminea*) after experimental exposure to cadmium and inorganic mercury. Environ. Toxicol. Chem. 16, 2096 – 2105.

Bergmeyer, H., 1983. Methods of enzymatic analysis. Academic Press, New York

Bianchini, A., Pedroso, M.S., Said, J.S., Spengler, A., Martins, S.E., 2003. Biotic Ligand Model in fresh and sea water in Brazil. In: Lagos, G.E., Warner, A.E.M., Sánchez, M. (Eds.), Copper 2003 - Health, Environment and Sustainable Development. MetSoc Publication. Quebec, Canada, pp. 543-552.

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. Congr. Ser. 1275, 189–194.

Bielmyer, G.K., Klaine, S.J., Tomasso, J.R., Arnold, R.W., 2004. Changes in water quality after addition of sea salts to fresh water: implications during toxicity testing. Chemosphere 57, 1707 – 1711.

Brown, B.E., Newell, R.C. 1972. The effect of copper and zinc on the metabolism of the mussel *Mytilus edulis*. Mar. Biol. 16, 108 – 118.

Brown, R.J., Galloway, T.S., Lowe, D., Browne, M.A., Dissanayake, A., Jones, M.B., Depledge, M.H., 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquat. Toxicol. 66, 267–278.

Campbell, M.K., Farrell, S.O., 2006. Biochemistry. 5th ed. Thomson Brooks/Cole, Belmont, CA, USA

Capuzzo, J. M., & Leavitt, D. F. (1988). Lipid compo- sition of the digestive glands of *Mytilus edulis* and *Carcinus maenas* in response to pollutant gradients. Marine Ecology Progress Series, 46, 139–145.

Carr, R.S., Neff, J.M., 1984. Quantitative semi-automated enzymatic assay for tissue glycogen. Comp. Biochem. Physiol. 77B, 447–449.

Carvalho, C.S., Fernandes, M.N., 2008. Effect of copper on liver key enzymes of anaerobic glucose metabolism from freshwater tropical fish *Prochilodus lineatus*. Comp. Biochem. Physiol. 151, 437 – 442.

Cheng, Y., Zheng, Y., VanderGheynst, J.S. 2011. Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. Lipids 46, 95 – 103.

Das, S., Gupta, A. 2013. Accumulation of copper in different tissues and changes in oxygen consumption rate in Indian flying barb, *Esomus danricus* (Hamilton-Buchanan) Exposed to sub-letal concentrations of copper. Jordan Journal of Biological Sciences. 6, 21-24.

De Boeck, G., Smet, H.D., Blust, R., 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio.* Aquat. Toxicol. 32, 127 – 141.

De Moreno, J.E.A., Moreno, V.J., Brenner, R.R., 1976. Lipid metabolism of the yellow clam, *Mesodesma mactroides*: I. Composition of lipids. Lipids, 4, 334 – 340.

De Zwaan, A., Zandee, D.I. 1972. The utilization od glycogen and accumulation of some intermediates during anaerobiosis in *Mytilus edulis* L. Comp. Biochem. Physiol. 43B, 47 – 54.

De Zwaan, A., Van Marrewijk, W.J.A., 1973. Anaerobic glucose degradation in the sea mussel *Mytilus edulis (L.).* Comp. Biochem. Physiol. 44B, 429 - 439.

De Zwaan, A., De Bont, A.M.T. Kluytmans, J. H. F. M., 1975. Metabolic adaptations on the aerobic-anaerobic transition in the sea mussel *Mytilus edulis (L.).* Proc. 9th Europ. Mar. Biol. Symp. 121-t38.

De Zwaan, A., Wijsman, T.C.M., 1976. Anaerobic metabolism in bivalvia (Mollusca): Characteristics of anaerobic metabolism. Comp. Biochem. Physiol. 54B, 313-324

De Zwaan, A., 1983. Carbohydrate catabolism in bivalves. In : Hochacka, P.W. (ed) The Mollusca, v.1. Metabolic Biochemistry and Molecular Biomechanics. Academic Press, New York, p 137

Duquesne, S., Liess, M., Bird, D.J. 2004. Sub-lethal effects of metal exposure: physiological and behavioural responses of the estuarine bivalve *Macoma balthica*. Mar. Environ. Res. 58, 254 – 250.

Folch, J., Lees, M., Stanley, G.H. 1956. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497 – 509.

Frias-Espericueta, M.G., Castro-Longoria, R., Barron-Gallardo, G.J., Osuna-Lopez, J.I., Abad-Rosales, S.M., Paez-Osuna, F., Voltolina, D., 2008. Histological changes and survival of *Litopenaeus vannamei* juveniles with different copper concentrations, Aquaculture 278, 97–100.

Géret, F., Jouan, A., Turpin, V., Bebianno, M.J., Cosson, R.P., 2002. Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalves mollusks: the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*). Aquatic Living Resources 15, 61-66.

Géret F., Serafim A., Bebianno M.J., 2003. Antioxidant enzyme activities, metallothioneins and lipid peroxidation as biomarkers in *Ruditapes decussatus*? Ecotoxicology, 12, 417-426.

Giese, A.C., 1969. A new approach to the biochemical composition of the molluscs body. Oceanoqr. Mar. Biol. Ann. Rev. 7, 175 229.

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

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.

Hansen, J.I., Mustafa, T., Depledge, M., 1992. Mechanisms of copper toxicity in the shore crab, *Carcinus maenas* II. Effects on key metabolic enzymes, metabolites and energy charge

potential. Mar. Biol. 114, 259–264.

Hill, R., Wyse, G.A., Anderson, M. 2008. Animal Physiology. Sinauer Associates, Sunderland, USA.

Hochachka, P.W., Fields, J., Mustafa, T., 1973. Animal life without oxygen: Basic biochemical mechanisms. Am. Zool. 13. 543-555.

Hummel, H., Amiard-Triquet, C., Bachelet, G., Desprez, M., Marchand, J., Sylvand, B., Amiard, J.C., Rybarckzyk, H., Bogaards, R.H., Sinke, J., De Wit, Y., De Wolf, L. 1996. Sensitivity to stress of the estuarine bivalve *Macoma balthica* from areas between the Netherlands and its southern limits (Gironde). J. Sea Res. 35, 315-321.

Jorge, M.B., Lauer, M.M., Martins, C.M.G., Bianchini, A., 2013a. Ionoregulatory and respiratory disturbances induced by copper in the osmoconforming clam *Mesodesma mactroides.* Aquat. Toxicol. (submitted).

Jorge, M.B., Rodrigues, S.C., Bianchini, A., 2013b. Effects of dissolved organic matter on tissue accumulation and acute toxicity of copper in the clam *Mesodesma mactroides*. Environ. Toxicol. Chem. (*In preparation*).

Kennish, M.J., 1997. Practical handbook of estuarine and marine pollution. *CRC Press*, Boca Raton, Florida, USA.

Lander, L., Reuther, R., 2004. Metals in society and in the environment: a critical review of current knowledge on fluxes, speciation, bioavailability and risk adverse effects of copper, chromium, nickel and zinc. *Kluwer Academic Publishers*, Boston, USA.

Lauer, M.M., Bianchini, A., 2010. Chronic copper toxicity in the estuarine copepod *Acartia tonsa* at different salinities. Environ. Toxicol. Chem. 29, 2297 – 2303.

Lauer, M.M., Oliveira, C. B., Yano, N.I., Bianchini, A., 2012. Copper effects on key metabolic enzymes and mitochondrial membrane potential in the gills of the estuarine crab *Neohelice granulata* at different salinities. Comp. Biochem, Physiol. 156C, 140-147.

Laurén, D.J., McDonald, D.G., 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson - modulation by water hardness and pH. J. Comp. Physiol. 155B, 635-644.

Leavitt, D. F., Lancaster, A. S., & Capuzzo, J. M. (1990). Changes in the biochemical composition of a sub-tropical bivalve, *Arca zebra*, in response to contaminant gradient in Bermuda. Journal of Experimental Marine Biology and Ecology, 138, 85–98.

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

Lowe, D.M., Clarke, K.R., 1989. Contaminant-induce changes in the structure of the digestive epithelium of *Mytilus edulis*. Aquat. Toxicol. 15, 345 – 358.

Martins, C.M.G., Barcarolli, I.F., Menezes, E.J., Giacomin, M.M., Wood, C.M., Bianchini, A., 2011a. Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectes sapidus* acclimated to different salinities: in vivo and in vitro studies. Aquat. Toxicol. 101, 88–99.

Martins, C.M.G., Almeida, D.V., Marins, L.F.F., Bianchini, A., 2011b. mRNA expres- sion and activity of ion-transporting proteins in gills of the blue crab *Callinectes sapidus*: effects of waterborne copper. Environ. Toxicol. Chem. 30, 206–211.

Monteiro, S.C.R., Pinho, G.L.L., Hoffmann, K., Barcarolli, I.F., Bianchini, A., 2013. Acute waterborne copper toxicity to the euryhaline copepod *Acartia tonsa* at different salinities: Influence of natural freshwater and marine dissolved organic matter. Environ. Toxicol. Chem. DOI: 10.1002/etc.2197.

Nonnotte, L., Boitel, F., Truchot, J.P., 1993. Waterborne copper causes gill damage and haemolymph hypoxia in the shore crab *Carcinus maenas*, Can. J. Zool. Rev. 71, 1569–1576.

Oudejans, R.C.H., Van der Horst, D.J., 1974. Aerobic – anaerobic biosynthesis of fatty acids and other lipids from glycolytic intermediates in the pulmonate land snail *Cepae nemoralis* (L.). Comp. Biochem. Physiol. 47B, 139 – 147.

Paila, R.V., Yallapragada, P.R., 2010. Bioaccumulation and toxic effects of copper on growth and oxygen consumption by the postlarvae of *Penaeus indicus*. Chemistry and Ecology, 26, 209 – 221.

Pellerin-Massicotte, J., Vincent, B., Pelletier, E., 1993. Evaluation ecotoxicologique de la baie des Anglais a Baie-Comeau (Quebec). Water Pollut. Res. J. Can. 28, 665–686.

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

Pinho, G.L.L., Bianchini, A., 2010. Acute copper toxicity in the euryhaline copepod *Acartia tonsa*: Implications for the development of an estuarine and marine biotic ligand model. Environ. Toxicol. Chem. 29, 1834– 1840.

Pytharopoulou, S., Kouvela, E.C., Sazaki, E., Leostsinidis, M., Kalpaxis, D.L. 2006. Evaluation of the global protein synthesis in *Mytilus galloprovincialis* in marine pollution monitoring: Seasonal variability and correlations with other biomarkers. Aquat. Toxicol. 80, 33-41.

Santos, M.H.S., Cunha, N.T., Bianchini, A. 2000. Effects of copper and zinc on growth, feeding and oxygen consumption of *Farfantepenaeus paulensis* postlarvae (Decapoda: Penaeidae). J. Exp. Mar. Biol. Eco. 247, 233-242.

Schmidt-Nielsen, K. 1997. Animal Physiology: adaptation and environment. Cambridge: Ed. Cambridge University, 607 p.

Serafim, A., Bebiano, M.J., 2009. Metallothionein role in the kinetic model of copper accumulation and elimination in the clam *Ruditapes decussates*. Environmental Research 109, 390-399.

Sivaramakhrishna, B., Radhakhrishnaiah, K., 2000. Mercury induced alterations in the energetics of hepatopancreas of two freshwater molluscs, *Pila globosa* and *Lamellidens marginalis*. In: Markert, B., Friese, K. (eds.) Trace elements - Their distribution and effects in the environment, Kidlington, UK.

Soegianto, A., Charmanitier-Daures, M., Trilles, J.P., Charmaniter, G., 1999. Impact of copper on the structure of gills and epipodites of the shrimp *Penaeus japonicus*. J. Crust. Biol. 19, 209–223.

Solomon, E. I., Lowery, M. D., 1993. Electronic structure contributions to function in bioinorganic chemistry. Science 259, 1575–1581.

Spicer, J.I., Weber, R.E., 1991. Respiratory impairment in crustaceans and molluscs due to exposure to heavy metals. Comp. Biochem. Physiol. 100C, 339 – 342.

Sze, P.W.C., Lee, S.Y., 1995. The potential role of mucus in the depuration of copper from the mussels *Perna viridis* (L.) and *Septifer virgatus*. Mar. Pollut. Bull. 31, 390–393.

Taylor, H.H., Anstiss J.M., 1999. Copper and haemocyanin dynamics in aquatic invertebrates. Mar. Freshwater. Res. 50, 907–931.

Zar, J.H., 1984. Biostatistical Analysis. Prentice Hall, New Jersey.

Zimmer, A., 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 fresh water and sea water. Aquat. Toxicol. 122-123, 172–180.

Zs.-Nagy, I., 1974. Some quantitative aspects of oxygen consumption and anaerobic metabolism of molluscan tissues. A review. Comp. Biochem. Physiol. 49A, 399-405.

Zs.-Nagy, I., Galli, C., 1977. On the possible role of unsaturated fatty acids in the aerobiosis of *Anodonta cygnea* L. (Mollusca, Pelecypoda). Acta. Biol. Acad. Sci. Hung. 28, 123 – 131.

Figure Captions

Figure 1. ATP content in gills, digestive gland, adductor muscle (n = 4-6) and hemolymph (n = 12) of juvenile clam *Mesodesma mactroides* maintained under control condition (no Cu addition into the water) or exposed to Cu (150 µg L^{-1}) in artificial sea water (salinity 30 ppt) for 96 h. Data are mean ± 1 standard deviation. * denotes significant different mean values between control and Cu-exposed clams within the same tissue.

Figure 2. NAD^{+}/NADH ratio in gills, digestive gland and adductor muscle (n = 4-6) of juvenile clam *Mesodesma mactroides* maintained under control condition (no Cu addition into the water) or exposed to Cu (150 µg L^{-1}) in artificial sea water (salinity 30 ppt) for 96 h. Data are mean ± 1 standard deviation. No significant difference was observed between control and Cu-exposed clams within the same tissue.

Figure 3. Total proteins content in gills, digestive gland, adductor muscle (n = 4-6) and hemolymph (n = 12) of juvenile clam *Mesodesma mactroides* maintained under control condition (no Cu addition into the water) or exposed to Cu (150 µg L^{-1}) in artificial sea water (salinity 30 ppt) for 96 h. Data are mean ± 1 standard deviation. No significant difference was observed between control and Cu-exposed clams within the same tissue.

Figure 4. Total lipids content in gills, digestive gland, adductor muscle (n = 4-6) and hemolymph (n = 12) of juvenile clam *Mesodesma mactroides* maintained under control condition (no Cu addition into the water) or exposed to Cu (150 µg L^{-1}) in artificial sea water (salinity 30 ppt) for 96 h. Data are mean ± 1 standard deviation. No significant difference was observed between control and Cu-exposed clams within the same tissue.

Figure 5. Glycogen content in gills, digestive gland, adductor muscle (n = 4-6) and hemolymph (n = 12) of juvenile clam *Mesodesma mactroides* maintained under control condition (no Cu addition into the water) or exposed to Cu (150 µg L^{-1}) in artificial sea water (salinity 30 ppt) for 96 h. Data are mean ± 1 standard deviation. No significant difference was observed between control and Cu-exposed clams within the same tissue.

Figure 6. Glucose content in tissues gills, digestive gland, adductor muscle (n = 4-6) and hemolymph (n = 12) of juvenile clam *Mesodesma mactroides* maintained under control condition (no Cu addition into the water) or exposed to Cu (150 µg L^{-1}) in artificial sea water (salinity 30 ppt) for 96 h. Data are mean ± 1 standard deviation. * denotes significant different mean values between control and Cu-exposed clams within the same tissue.

Figure 7. Pyruvate (A) and lactate (B) content in adductor muscle (n = 4-6) and hemolymph (n = 12) of juvenile clam *Mesodesma mactroides* maintained under control condition (no Cu addition into the water) or exposed to Cu (150 µg L^{-1}) in artificial sea water (salinity 30 ppt) for 96 h. Data are mean ± 1 standard deviation. * denotes significant different mean values between control and Cu-exposed clams within the same tissue.

Figures

Figure 1

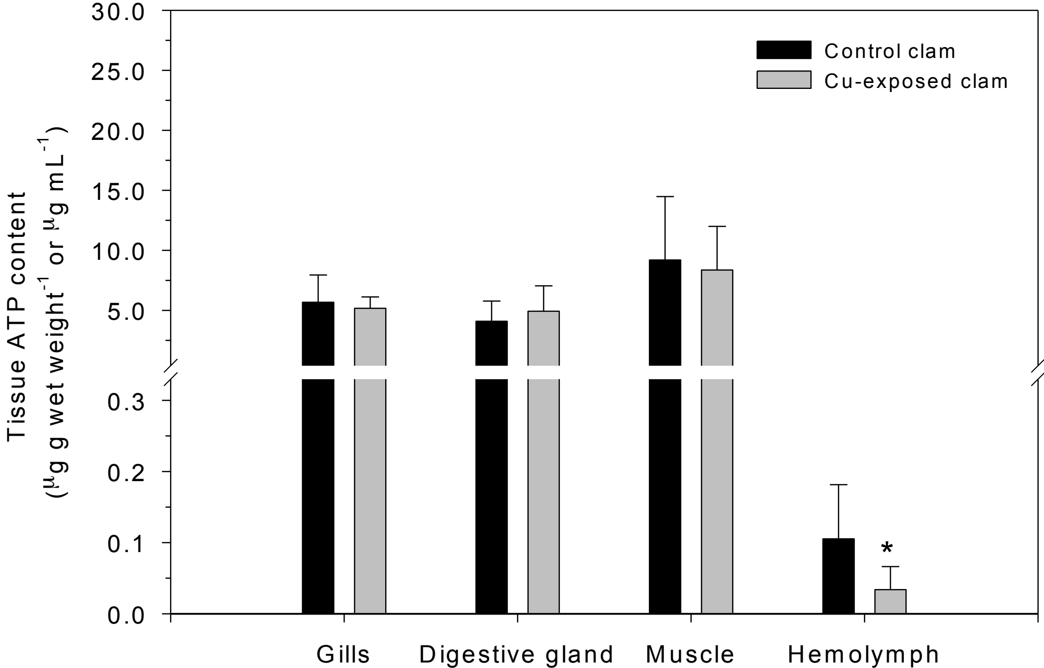


Figure 2

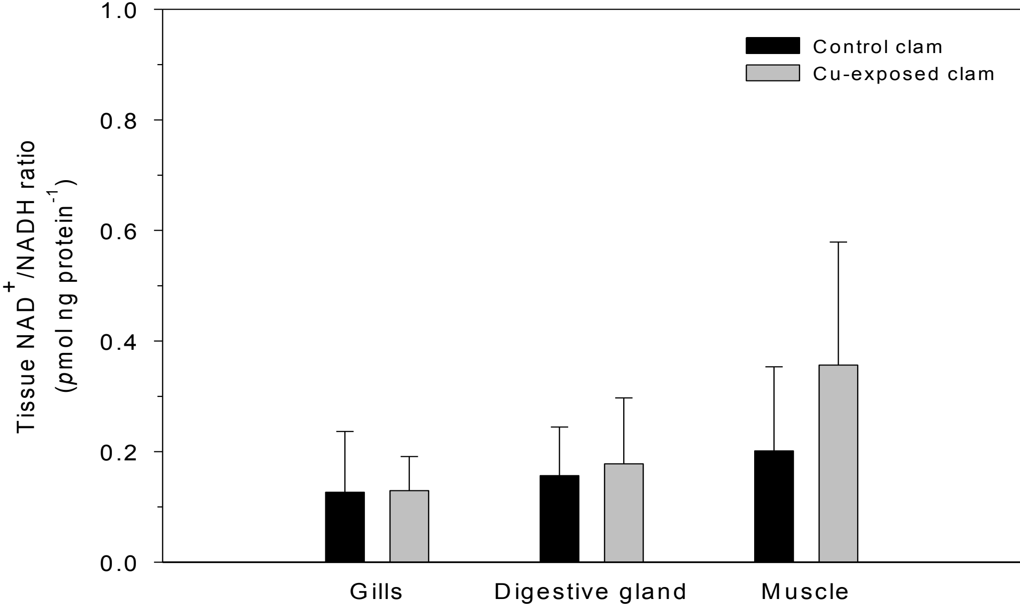


Figure 3

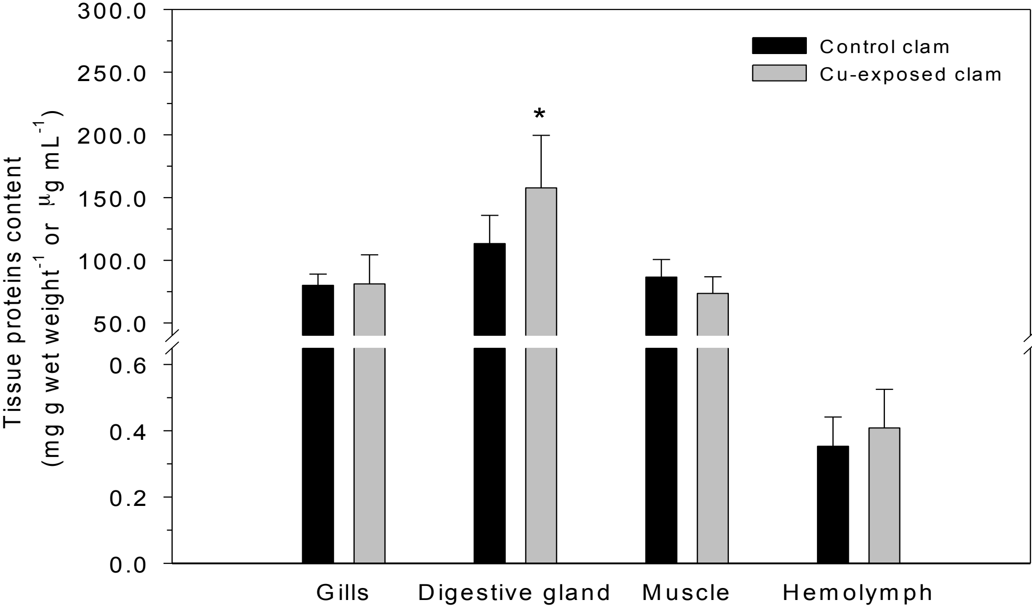


Figure 4

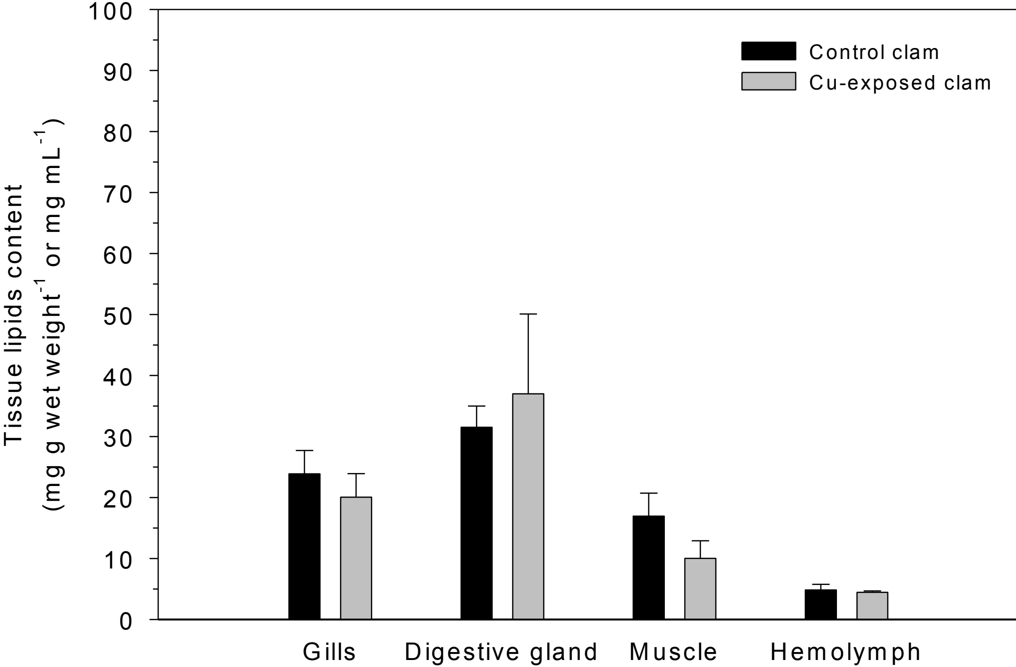


Figure 5

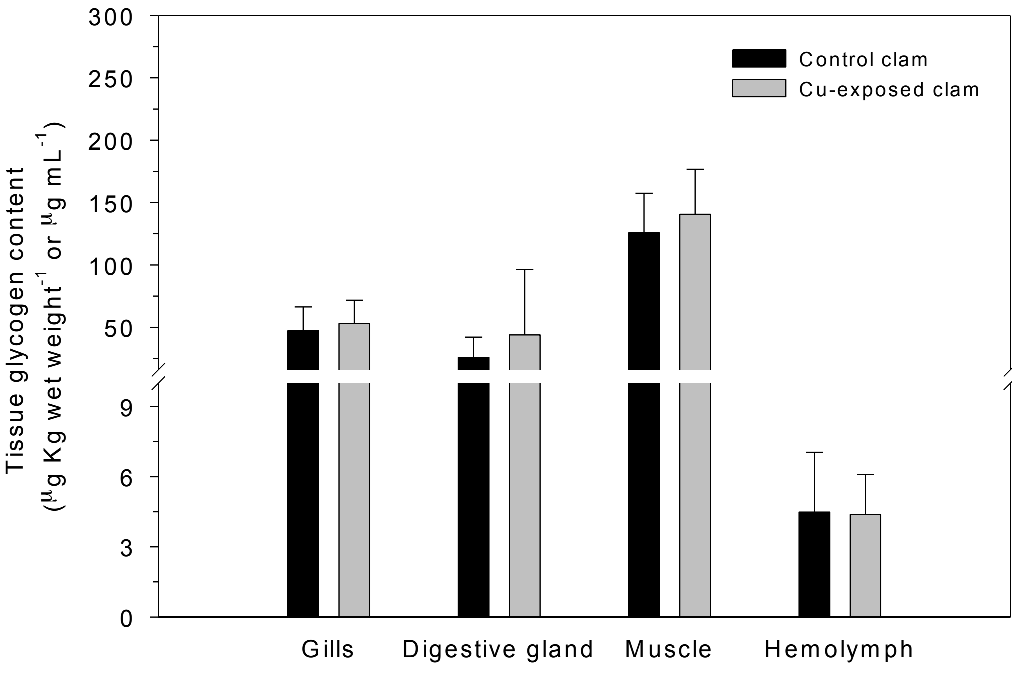


Figure 6

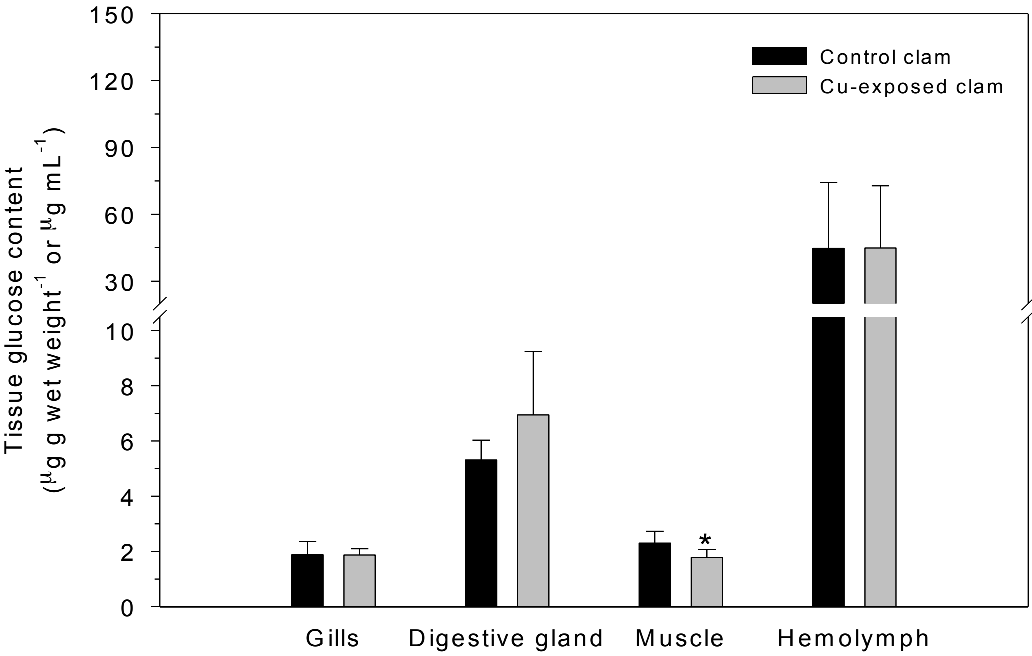
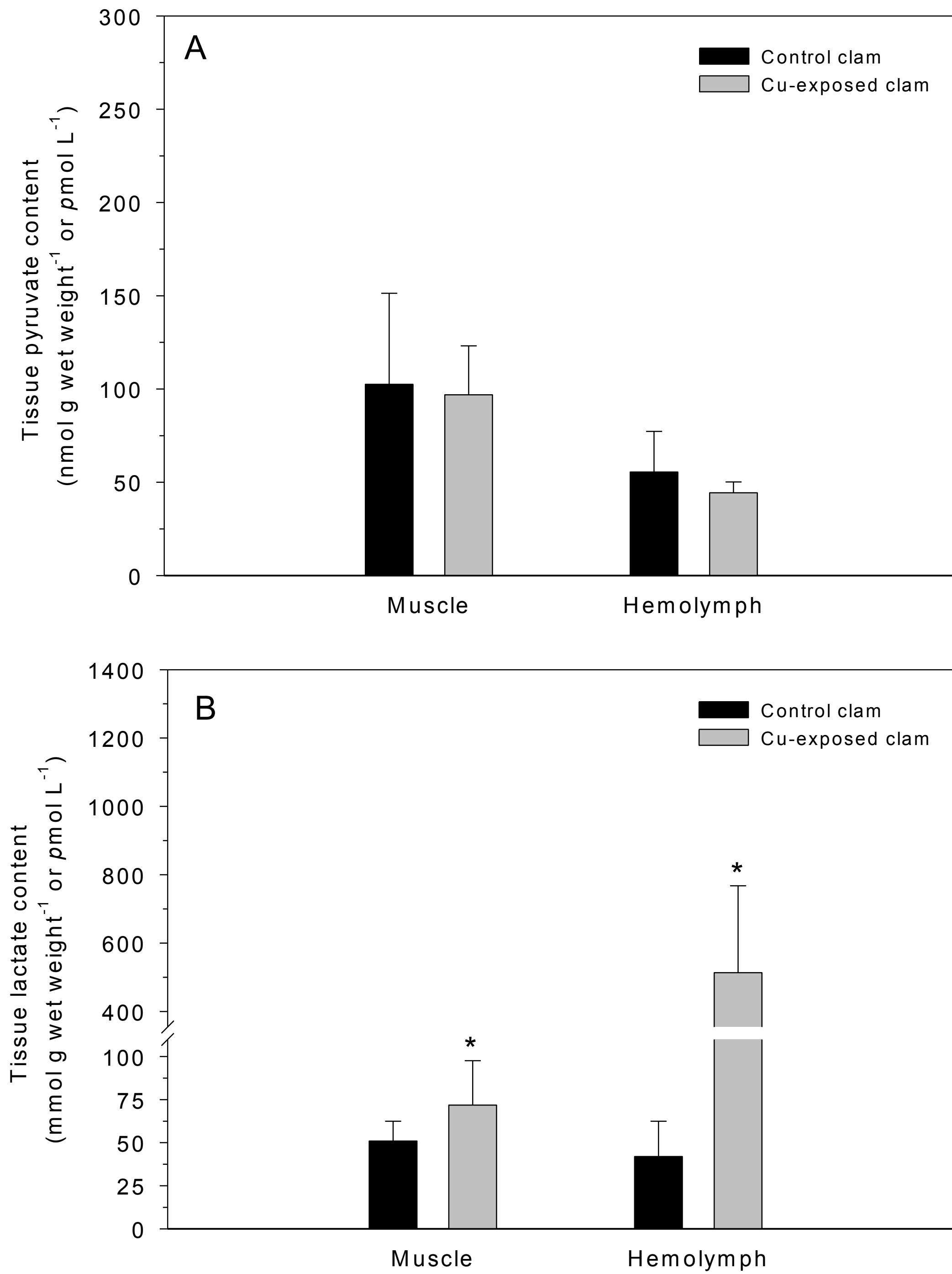


Figure 7



CONCLUSÕES GERAIS

Foi observado que o bivalve *Mesodesma mactroides* é capaz de manter os níveis intracelulares de ATP constantes após 96 h de exposição a uma concentração subletal de cobre. Através dos resultados das análises do conteúdo de glicose, piruvato e lactato, propõe-se que o mecanismo utilizado para manutenção dos níveis intracelulares de ATP é uma mudança de vias metabólicas. Ou seja, ao invés da oxidação do piruvato em acetil-CoA este estaria sendo reduzido a lactato, contribuindo para manutenção da concentração de NAD^+ e do funcionamento do ciclo de Krebs. Os dados obtidos no presente trabalho rejeitam a hipótese inicial proposta. Por fim, concluímos que o mecanismos de toxicidade aguda do cobre para *Mesodesma mactroides* não está relacionado com um desbalanço nos estoques energéticos e perturbações das vias metabólicas centrais.

REFERÊNCIAS BIBLIOGRÁFICAS DA INTRODUÇÃO

Aust, S.D., Morehouse, L.A., Thomas, C.E., 1985. Role of metals in oxygen radical reactions. J. Free Rad. Biol. Med. 1, 3-25

Bastida, R.A., 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 de Buenos Aires, Argentina. Frente Marítimo 9 A, 83-92.

Berg, J.M., Tymoczko, J.L., Stryer, L., 2002. Biochemistry. W. H. Freeman and Company. New York, NY, USA.

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. Congr. Ser. 1275, 189–194.

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. Tox. 80, 131–139.

Brouwer, M., Hoexum-Brouwer, T., 1998. Biochemical defense mechanisms against copper-induced oxidative damage in the blue crab, *Callinectes sapidus*. Arch. Biochem. Biophys. 3512, 257–264.

Brown, R.J., Galloway, T.S., Lowe D., Browne, M.A., Dissanayke, A., Jones, M.B., Depledge, M.H. 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquat. Toxicol. 66, 267 – 278.

Brown, B.E., Newell, R.C. 1972. The effect of copper and zinc on the metabolism of the mussel *Mytilus edulis*. Mar. Biol. 16, 108 – 118.

Campbell, M.K., Farrell, S.O., 2006. Biochemistry, Thomson Brooks/Cole, Belmont, CA, USA.

De Boeck, G., Smet, H.D., Blust, R., 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio.* Aquat. Toxicol. 32, 127 – 141.

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

Defeo, O., Ortiz, E., Castilla, J.C., 1992. Growth, mortality and recruitment of the yellow clam *Mesodesma mactroides* on Uruguayan beaches. Mar. Biol. Oldendorf/Luhe 114, 429-437.

D'Silva, C., Kureishy, T.W., 1978. Experimental studies on the accumulation of copper and zinc in the green mussel. Mar. Pollut. Bull. 9, 187–190.

Fiori, S.M., Morsán, E.M., 2004. Age and individual growth of *Mesodesma mactroides* (Bivalvia) in the southernmost range of its distribution. J. Mar. Sci. 61, 1253 – 1259.

Gianuca, N.M., 1983. A preliminary account of the ecology of sandy beaches in southern Brazil. In: McLachlan A., Erasmus T. (Eds.). Sandy beaches as ecosystems. Dr. W. Junk Publishers, Hague, NL, pp. 413-419.

Gianuca, N.M., 1985. The ecology of a sandy beach in southern Brazil. Tese de Doutorado, University of Southhampton, USA, 330 p.

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.

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., 2012. Copper, in: Wood, C.M., Farrell, T., Brauner, C. J. (Eds.), Homeostasis and Toxicology of Essential Metals, Fish Physiology, vol 31A. Academic Press, San Diego, pp. 53-133.

Hansen, J.I., Mustafa, T., Depledge, M., 1992. Mechanisms of copper toxicity in the shore crab, *Carcinus maenas* II. Effects on key metabolic enzymes, metabolites and energy charge potential. Mar. Biol. 114, 259-264.

Herrman, M., 2008. Population dynamics of the Argentinean surf clams *Donax hanleyanus* and *Mesodesma mactroides* from open-Atlantic beaches off Argentina. Tese de Doutorado. Universidade de Bremen, Bremen, Alemanha.

Heath, A.G., 1995. Water Pollution and Fish Physiology. Florida: *CRC Press*, Boca Raton, USA, 359 p.

Hill, R., Wyse, G.A., Anderson, M. 2008. Animal Physiology. Sinauer Associates, Sunderland, USA.

Hogstrand, C., Wood, C.M., 1998. Towards a better understanding of the bioavailability, physiology, and toxicity of silver in fish: implications for water quality criteria. Env. Toxicol. Chem. 17, 547–561.

Jorge, M.B., Lauer, M.M., Martins, C.M.G., Bianchini, A., 2013. Ionoregulatory and respiratory disturbances induced by copper in the osmoconforming clam *Mesodesma mactroides.* Aquat. Toxicol. (submitted).

Kennish, M.J., 1997. Practical handbook of estuarine and marine pollution. Florida: CRC Press, Boca Raton, USA.

Lander, L., Reuther, R., 2004. Metals in society and in the environment: a critical review of current knowledge on fluxes, speciation, bioavailability and risk adverse effects of copper, chromium, nickel and zinc. Boston: Kluwer Academic Publishers.

Lauer, M.M., Bianchini, A., 2010. Chronic copper toxicity in the estuarine copepod *Acartia tonsa* at different salinities. Environ. Toxicol. Chem. 29, 2297 – 2303.

Lauer, M.M., 2012. Efeito do cobre sobre a atividade de enzimas-chave do metabolismo energético do órgão respiratório de animais aquáticos. Tese de Doutorado. Universidade Federal do Rio Grande - FURG, Rio Grande, 117p.

Lauer, M.M., Oliveira, C. B., Yano, N.I., Bianchini, A., 2012. Copper effects on key metabolic enzymes and mitochondrial membrane potential in the gills of the estuarine crab *Neohelice granulata* at different salinities. Comp. Biochem. Physiol. 156C, 140-147.

Laurén, D.J., McDonald, D.G., 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson - modulation by water hardness and pH. J. Comp. Physiol. 155B, 635-644.

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

Lushchak, V. I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 100, 13-30.

Martins, C.M.G., Almeida, D.V., Marins, L.F.F., Bianchini, A., 2011. mRNA expres- sion and activity of ion-transporting proteins in gills of the blue crab *Callinectes sapidus*: effects of waterborne copper. Environ. Toxicol. Chem. 30, 206–211.

Nogueira, L.S., 2013. Isolamento, identificação e ultilização de células branquiais ricas em mitocôndrias de bivalves em ensaios ecotoxicológicos. Tese de Doutorado. Universidade Federal do Rio Grande - FURG, Rio Grande, 96p.

Nelson, D.L., Cox, M.M., 2008. Lehninger: Principles of biochemistry. W. H. Freeman and company. New York, NY.

Neto, J.A.B., Wallner-Kersanach, M., Patchineelam, S.M., 2008. Poluição Marinha. Rio de Janeiro: Interciência, 440 p.

Olivier, S. R., and Penchaszadeh, P. E. 1968. Evaluación de los efectivos de almeja amarilla (*Mesodesma mactroides* Deshayes 1854) en las costas de la provincia de Buenos Aires. Proyecto de Desarrollo Pesquero e FAO Serie Informes Tecnicos, 8: 1e10.

Olivier, S.R., Penchaszadeh, P.E., 1971. Ecologia. In: Olivier, S.R., Capezzani, D.A.A., Carreto, J.I., Christiansen, H.E., Moreno, V.J., Moreno, J.E.A., Penchaszadeh, P. 1971. Estructura de la comunidad, dinamica de la poblacion y biologia de la almeja amarilla (*Mesodesma mactroides* Deshayes 1854) en Mar Azul (Pdo. De Gral. Madariaga, Bs. As.,

Argentina). Instituto de Biologia Marina – Serie Contribuciones. Impresora Atlantica, Mar del Plata, n.122, Chap. 1: 13-35.

Paila, R.V., Yallapragada, P.R., 2010. Bioaccumulation and toxic effects of copper on growth and oxygen consumption by the postlarvae of *Penaeus indicus*. Chemistry and Ecology, 26, 209 – 221.

Phillips, D.J.H., 1980. Quantitative Aquatic Biological Indicators. London, Applied Science Publishers, 588 p.

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

Pinho, G.L.L., Bianchini, A., 2010. Acute copper toxicity in the euryhaline copepod *Acartia tonsa*: Implications for the development of an estuarine and marine biotic ligand model. Environ. Toxicol. Chem. 29, 1834– 1840.

Regoly, F., Hummel, H., Amiard-Triquet, C., Larroux, C., Sukhotin, A., 1998. Trace metals and variations of antioxidant enzymes in Arctic bivalve populations. Arch. Environ. Contam. Toxicol. 35, 249–256.

Ryu, S.K., Park, J.S., Lee, I.S., 2003. Purification and characterization of copper binding protein from Asian periwinkle *Littorina brevicula*. Comp. Biochem. Physiol. C 134, 101–107.

Santos, E.A., Bianchini, A. 1998. O ambiente e a biota do estuário da Lagoa dos Patos: Adaptações fisiológicas de invertebrados e peixes. In: Seeliger, U.C., Odebrecht, C., Castello, J.P. (Eds). Os ecossistemas costeiro e marinho do extreme sul do Brasil. Rio Grande: Ecoscientia. 341p.

Santos, M.H.S., Cunha, N.T., Bianchini, A. 2000. Effects of copper and zinco n growth, feeding and oxygen consumption of *Farfantepenaeus paulensis* postlarvae (Decapoda: Penaeidae). J. Exp. Mar. Biol. Eco. 247, 233-242.

Santore, R.C., Di Toro, D.M., Paquin, P.R., Allen, H.E., Meyer, J.S., 2001. A biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and daphnia. Environ. Toxicol. Chem. 20, 2397–2402.

Serafim, A., Bebianno, M. J., 2009. Metallothionein role in the kinetic model of copper accumulation and elimination in the clam *Ruditapes decussatus*. Environ. Res. 109, 390–399.

Solomon, E. I., Lowery, M. D., 1993. Electronic structure contributions to function in bioinorganic chemistry. Science 259, 1575–1581.

Spicer, J.I., Weber, R.E., 1991. Respiratory impairment in crustaceans and molluscs due to exposure to heavy metals. Comp. Biochem. Physiol. 100C, 339 – 342.

Susuki, K.T., Someya, A., Komada, Y., Ogra, Y., 2002. Roles of metallothionein in copper homeostasis: responses to Cu-deficient diets in mice. J. Inorg. Biochem. 88, 173–182.

Sze, P.W.C., Lee, S.Y., 1995. The potential role of mucus in the depuration of copper from the mussels *Perna viridis* (L.) and *Septifer virgatus*. Mar. Pollut. Bull. 31, 390–393.

Taylor, H.H., Anstiss J.M., 1999. Copper and haemocyanin dynamics in aquatic invertebrates. Mar. Freshwater. Res. 50, 907–931.

Vitousek, P.M., Mooney, H.A., Lubchenco, J., Melillo, J.M., 1997. Human domination of the Earth's ecosystems. Science 277, 494–499

Wilson, R.W., Taylor, E.W., 1993. The physiological responses of freshwater rain- bow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. J. Comp. Physiol. 163B, 38–47.

Wood, C.M., Hogstrand, C., Galvez, F., Munger, R.S., 1996. The physiology of water borne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) I: The effects of ionic Ag. Aquat. Toxicol. 35, 93–109.

Yano, N.L.I., 2010. Efeito do cobre no metabolismo energético da anêmona-do-mar *Actinia bermudensis*. Monografia de conclusão de curso. Universidade Federal do Rio Grande - FURG, Rio Grande, RS. 37 pp.

Zimmer, A., 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 fresh water and sea water. Aquat. Toxicol. 122-123, 172–180.

AQUATIC TOXICOLOGY

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Aquatic Toxicology publishes original scientific papers dealing with the mechanisms of **toxicity** and the responses to toxic agents in **aquatic environments** at the community, species, tissue, cellular, subcellular and molecular levels, including aspects of uptake, metabolism and excretion of **toxicants.**

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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.

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