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EFEITOS BIOQUÍMICOS E FISIOLÓGICOS DA EXPOSIÇÃO AO NÍQUEL NO CARANGUEJO EURIALINO *Neohelice granulata*

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ÍNDICE

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RESUMO

Em invertebrados eurialinos, a exposição a metais pode induzir distúrbios respiratórios, iônicos e osmóticos, bem como estresse oxidativo. Diversos estudos sobre o efeito combinado da salinidade da água e a exposição a metais em invertebrados estuarinos estão relatados na literatura, porém a maioria destes estudos estão focados em apenas alguns metais como Cd, Cu, Pb e Zn. Entretanto, poucos estudos avaliaram as respostas bioquímicas e fisiológicas de invertebrados eurialinos à exposição ao Ni em diferentes salinidades. No presente estudo, o caranguejo estuarino *Neohelice granulata* foi mantido sob condições controle (sem adição de Ni na água) ou exposto (96 h) a concentrações subletais de Ni (100 e 1000 µg/L) em duas salinidades (2 e 30). Após exposição, o consumo de oxigênio corporal foi medido e amostras de tecidos (hemolinfa, hepatopâncreas, músculo, e brânquias anteriores e posteriores) foram coletadas para análises posteriores. A concentração osmótica e a composição iônica (Na^+, Cl^-, Ca^{2+}, Mg^{2+} e K^+) foram determinadas nas amostras de hemolinfa. A atividade da lactato desidrogenase (LDH) foi medida na hemolinfa, hepatopâncreas e músculo, enquanto a peroxidação lipídica (LPO) foi analisada no hepatopâncreas, músculo e brânquias (anteriores e posteriores). Os caranguejos controle não apresentaram diferença na concentração osmótica em função da salinidade, porém aqueles aclimatados à salinidade 2 apresentaram menores concentrações hemolinfáticas de Na^+, K^+ e Mg^{2+}, bem como maiores níveis de LPO nas brânquias (anteriores e posteriores) e hepatopâncreas do que aqueles aclimatados à salinidade 30. O consumo de oxigênio corporal e a atividade tecidual da LDH foram semelhantes nos caranguejos controles aclimatados a 2 e 30. Estes resultados indicam que, após duas semanas de manutenção em laboratório, *N. granulata* apresenta ajustes fisiológicos da concentração osmótica (2‰: hiper-regulação; 30‰: hipo-regulação), composição iônica hemolinfática e taxas

metabólicas (aeróbica e anaeróbica) em função da salinidade, com conseqüente maior dano oxidativo em lipídios durante a hiper-regulação em baixa salinidade. Quanto à exposição ao Ni, houve aumento do consumo de oxigênio corporal, da atividade da LDH hemolinfática e da concentração hemolinfática de K^+ na salinidade 2. Na salinidade 30 foi observado um aumento da atividade da LDH hemolinfática, da concentração osmótica e de Cl^- hemolinfática, bem como uma diminuição das concentrações hemolinfáticas de K^+ e Mg^{2+}. Nos caranguejos aclimatados à salinidade 2, os efeitos do Ni parecem estar associados a distúrbios metabólicos (aeróbico e anaeróbico), enquanto distúrbios osmóticos e ionoregulatórios foram mais evidentes nos caranguejos aclimatados e expostos ao Ni na salinidade 30.

Palavras-chave: caranguejo, metabolismo, níquel, osmorregulação, salinidade

INTRODUÇÃO GERAL

Em termos oceanográficos e ecológicos, os estuários são definidos como ecossistemas costeiros semi-fechados que possuem ligação livre com o mar e onde a água marinha mistura-se com água doce proveniente das áreas terrestres. Os estuários, juntamente com as suas marismas adjacentes, estão entre os ecossistemas mais produtivos do planeta e são cruciais para a história de vida de muitas espécies aquáticas, servindo de berçário, bem como áreas de criação, alimentação e migração (Chapman & Wang, 2001). Além disso, alguns estuários, como o da Lagoa dos Patos (Rio Grande, RS, Brasil), apresentam uma importante função social e econômica para as comunidades que vivem em seu entorno, onde são desenvolvidas diversas atividades como pesca artesanal, agricultura e atividades portuárias e industriais.

Em decorrência das atividades antrópicas, os ambientes aquáticos estão sendo cada vez mais contaminados com diferentes tipos de poluentes orgânicos e inorgânicos. Um dos principais grupos de poluentes presentes no estuário da Lagoa dos Patos são os metais, tais como Cd, Pb, Zn, Cu e Cr, que podem ser poluentes comuns e preocupantes para a vida aquática. Suas principais fontes para a água, para o sedimento e até mesmo para os organismos são os esgotos industriais e domésticos, as águas de drenagem urbana, o lixo e as atividades relacionadas ao setor portuário, como as dragagens, os estaleiros e até mesmo as sucatas abandonadas no porto (Clark, 2001).

Os metais, assim como outros contaminantes, podem se acumular nos tecidos de animais aquáticos, incluindo crustáceos, em concentrações muito maiores do que aquelas encontradas na coluna da água ou no sedimento (Rainbow, 2007). Este fato pode ser relevante considerando que tanto os metais essenciais, aqueles que possuem uma participação no metabolismo, quanto os não essenciais, aqueles que não participam dos processos metabólicos, podem ser tóxicos aos organismos aquáticos, uma vez que

as concentrações destes contaminantes nos tecidos podem ser de dezenas e até mesmo milhares de vezes maiores do que suas concentrações no ambiente (Perevoznikov & Bogdanov, 1999; Moiseenko, 2003; Podgurskaya et al., 2004; Gremyachikh et al., 2006).

Os crustáceos assimilam os metais de forma proporcional à concentração dissolvida destes contaminantes na água (Jennings & Rainbow, 1979; White & Rainbow, 1984; Rainbow, 1985; Rainbow & White, 1989, 1990; Weeks & Rainbow, 1991; Chan & Rainbow, 1993). Assim, um aumento da taxa de captação do metal ou uma diminuição da sua taxa excreção refletirá um aumento da concentração do metal acumulado nos tecidos do animal, como ocorre, por exemplo, para o metal não-essencial Cd em crustáceos decápodes (Jennings & Rainbow, 1979; Rainbow, 1985; Rainbow & White, 1989), anfípodes (Rainbow & White, 1989) e cracas (Rainbow, 1985; Rainbow & White, 1989). No entanto, alguns crustáceos não mostram aumentos na concentração corporal de um metal, especialmente os metais essenciais, mesmo após uma série de exposições ao metal dissolvido no ambiente, embora as taxas de assimilação ainda se mostrem aumentadas com a concentração elevada de metais dissolvidos na água. Por exemplo, o crustáceo decápode *Palaemon elegans* mantém sua concentração corporal total de Zn em um nível constante quando exposto ao aumento da concentração deste metal em determinadas condições físico-químicas da água (White & Rainbow, 1984; Nugegoda & Rainbow, 1988, 1989a,b; Rainbow & White, 1989). Além disso, a maioria dos crustáceos eurialinos pode variar sua permeabilidade aparente à água em diferentes graus e, assim, alterar as taxas de captação absoluta do metal com a variação de salinidade (Rainbow, 1995).

O grau de sensibilidade dos tecidos/órgãos de animais aquáticos à toxicidade dos metais pode diferir se a exposição for aguda ou crônica (Brown et al., 1990), sendo que

a acumulação destes contaminantes pode ocorrer em diversos órgãos vitais (Perevoznikov & Bogdanov, 1999; Popov et al., 2002; Komov et al., 2004). Nos invertebrados, os metais associados aos alimentos se acumulam em quantidades consideráveis no intestino, estômago e apêndice pilórico (Chowdhury et al., 2005; Farag et al., 1995; Sobolev, 2005), enquanto aqueles dissolvidos na água se acumulam principalmente em outros órgãos, tais como estruturas de sustentação e brânquias (Cain et al., 1992; Craig et al., 1999). No camarão do gênero *Penaeus*, as maiores concentrações de Cu, Zn, Cd, Hg, Mn, Ni, Fe, Pb e Ag foram encontradas no hepatopâncreas, onde o Cu, o Zn e o Fe desempenham um papel importante no metabolismo celular (Pourang et al., 2004).

Normalmente, a toxicidade é resultante da ligação não específica de cátions metálicos reativos a macromoléculas biologicamente importantes, causando alterações em seu funcionamento. De forma geral, os mecanismos de ação tóxica do Cu, Zn, Hg e Cd são inespecíficos. No entanto, o modo geral de ação tóxica dos metais inclui a inibição de uma série de reações bioquímicas devido à ligação destes contaminantes com grupamentos sulfidrila (-SH) de proteínas funcionais ou a saída forçada dos elementos-traço dos centros ativos da enzima. Isto resulta em mau funcionamento do metabolismo celular, aumento da peroxidação lipídica (LPO), inibição da fosforilação oxidativa, desequilíbrio na homeostasia do Ca^{2+}, bem como alterações na estrutura e permeabilidade das membranas celulares (Gerhard, 1993).

Nas últimas décadas, as enzimas têm sido amplamente utilizadas como biomarcadores de contaminantes ambientais. Por exemplo, a lactato desidrogenase (LDH) desempenha um papel importante nas funções fisiológicas determinantes para a sobrevivência e o desempenho dos organismos, como produção de energia e desintoxicação. É uma enzima citoplasmática, que apresenta diferentes formas e catalisa

a interconversão de piruvato em lactato na glicólise. Neste contexto, alterações na sua atividade têm sido utilizadas como indicativo de potenciais efeitos do estresse químico sobre os mecanismos de produção de energia (Guilhermino et al., 1994; De Coen et al., 2001; Frasco & Guilhermino, 2002).

Por não participarem do metabolismo normal dos organismos, os metais não essenciais apresentam um potencial para causar dano ecotoxicológico em baixas concentrações. Porém, concentrações excessivas de metais essenciais também induzem efeitos negativos em animais aquáticos. Assim, diversos efeitos de metais, tanto essenciais quanto não essenciais, têm sido observados em crustáceos. Por exemplo, o Cd inibe a muda no caranguejo *Neohelice granulata* (Rodríguez Moreno et al., 2003) e produz injúria histopatológica no camarão-branco *Litopenaeus vannamei* (Wu et al., 2008). Por sua vez, o Cr induz desequilíbrio endócrino e da regulação da glicemia no caranguejo *Ucides cordatus* (Dias Corrêa et al., 2005), bem como diminui o consumo de oxigênio no mexilhão *Perna viridis* (Vijayavel et al., 2007). Quanto ao Cu, este causa várias anormalidades morfológicas em larvas eclodidas do caranguejo *N. granulata* (Lavolpe et al., 2004), bem como um aumento na resposta ao estresse oxidativo (Sabatini et al., 2009).

O estresse oxidativo é classicamente definido como uma perturbação no equilíbrio entre os pró-oxidantes e os antioxidantes em favor dos primeiros, levando a potenciais danos moleculares (Halliwell & Gutteridge, 2007). No entanto, a produção intracelular de espécies reativas de oxigênio (ROS), tais como o radical superóxido, peróxido de hidrogênio e radical hidroxila, não implica necessariamente em toxicidade celular. Porém, o estresse oxidativo ocorrerá quando a formação de ROS exceder a capacidade de defesa antioxidante celular ou interromper a sinalização e o controle redox, afetando assim a funcionalidade celular (Jones, 2006).

Neste contexto, o paradoxo do oxigênio surge do fato de que esse gás é essencial para a produção de energia nos organismos aeróbicos, mas ao mesmo tempo, o metabolismo celular está continuamente gerando ROS, que em altas concentrações podem ser extremamente prejudiciais aos constituintes celulares. Evidências recentes sugerem que os danos causados pelos radicais livres podem ser uma fonte significativa de toxicidade para os organismos aquáticos que vivem em ambientes que apresentam contaminantes dissolvidos na água (Livingstone, 2001). Os metais estão comumente associados à formação de ROS, que por sua vez levam ao estresse oxidativo, causando danos celulares (Leonard et al., 2004).

Assim, a presença de metais na água pode afetar a fisiologia dos crustáceos, sobretudo os eurialinos, uma vez que estes dependem de mecanismos de regulação iônica e osmótica para responder adequadamente às variações ambientais de salinidade (Crespo, 1984), que por sua vez afeta a biodisponibilidade e conseqüente toxicidade dos metais (Bianchini et al., 2003; Pedroso et al., 2007). Nos decápodes braquiúros, as brânquias anteriores estão envolvidas principalmente nas trocas gasosas, enquanto as brânquias posteriores estão envolvidas na regulação iônica e osmótica (Péqueux, 1995). Assim, a contaminação da água com metais pode causar modificações estruturais nas brânquias anteriores mudando a capacidade do animal em realizar as trocas gasosas. Nessa situação, espera-se, portanto, que o metabolismo aeróbico seja modificado, como de fato foi demonstrado em pós-larvas do camarão *Farfantepenaeus paulensis* expostas ao Zn e/ou Cu (Santos et al., 2000). Por outro lado, a atividade da Na^+,K^+-ATPase das brânquias posteriores é considerada a principal mediadora do transporte de NaCl ao longo do epitélio branquial, desempenhando um papel importante na regulação iônica e osmótica hemolinfática (Péqueux, 1995; Bianchini et al., 2008). Assim, alterações na atividade desta enzima induzida pela exposição a metais podem levar ao desequilíbrio

na regulação iônica e/ou osmótica nos animais osmorreguladores. De fato, tem sido demonstrado que a atividade da Na^+, K^+-ATPase de crustáceos é sensível a diversos metais, como Zn, Cu, Hg e Ag (Haya et al., 1983; Hansen et al., 1992; Péqueux et al., 1996; Bianchini & Castilho, 1999; Bianchini et al., 2004; Pedroso et al., 2007).

Neste contexto, Schmidt-Nielsen (1974) propõe que os efeitos tóxicos de compostos de Hg em organismos aquáticos são devido à interferência destes na habilidade osmorregulatória dos animais, enquanto Sprague (1984) sugeriu que os organismos osmorreguladores eurialinos são menos sensíveis aos tóxicos quando os animais estão perto de seu ponto isosmótico. Por sua vez, Jones (1975a,b) estudou os efeitos do Cd, Cu, Hg, Zn e Pb na mortalidade e osmorregulação em várias espécies marinhas e isópodes estuarinos, tendo relatado um efeito significativo dos metais na osmorregulação em algumas das espécies estudadas, especialmente em *Jaera albifrons*. Já Bjerregaard & Visle (1985a,b; 1986) estudaram os efeitos do Cd, Cu e Hg nas concentrações iônicas e na osmorregulação hemolinfática no caranguejo *Carcinus maenas*, tendo relatado efeitos significativos de todos os metais testados na osmolalidade e nos níveis iônicos hemolinfáticos.

Com base no exposto acima, verifica-se a existência de um conjunto de estudos significativos sobre o efeito de metais em organismos estuarinos e marinhos. No entanto, percebe-se também que estes estudos estão focadas em apenas um grupo restrito de metais, como a Ag, Cd, Cr, Cu, Hg, Pb e Zn. No entanto, importantes metais de uso humano, como o Ni, ainda carecem de estudos sobre sua biodisponibilidade em ambientes estuarinos e marinhos, bem como sobre sua bioacumulação e toxicidade nos organismos que habitam estes ambientes.

O níquel (Ni) é um elemento metálico que está naturalmente presente na crosta terrestre. Devido as suas propriedades físicas e químicas únicas, o Ni metálico e seus

compostos são importantes na indústria moderna, sendo utilizados na galvanoplastia, bem como na produção de baterias de Ni-Cd e equipamentos eletrônicos. Por sua vez, as ligas de Ni, como o aço inoxidável, são usadas na produção de ferramentas, maquinaria, armamentos e aparelhos eletrônicos. Também são usadas para forjar moedas, produzir jóias e próteses médicas. O alto consumo de produtos contendo Ni gera, inevitavelmente, contaminação e poluição ambiental por este metal e seus subprodutos em todos os estágios, desde a produção, passando pela reciclagem, até a sua disposição final (Denkhaus & Salnikow, 2002). De fato, efeitos tóxicos do Ni são relatados em organismos aquáticos que vivem perto da cidade de Sudbury (Província de Ontario, Canadá), um dos maiores locais de fundição do metal no mundo (Gunn, 1995), como conseqüência das elevadas concentrações deste metal no ambiente aquático (Borgmann et al., 2001).

Os compostos de Ni são encontrados no solo e estão presentes nas formas insolúveis, como sulfitos e silicatos, e em diversas formas solúveis (Garrett, 2000). Este metal está presente também na atmosfera e suas espécies dependem da fonte de contaminação. A partir de fontes antrópicas, o Ni é emitido como óxidos, sulfitos, silicatos, compostos solúveis e, em menor quantidade, como Ni metálico, sendo que a queima de combustíveis fósseis produz a maior parte dos compostos de Ni presentes no ar (Merian, 1984). A concentração atmosférica de Ni nas áreas de subúrbio em regiões industrializadas tem sido estimada entre 120 e 170 ng/m^3 (Norseth & Piscator, 1979).

Uma vez lixiviado diretamente das rochas e sedimentos, o Ni pode ser encontrado em concentrações significativas na água, onde está presente nas formas dissolvidas e também em partículas insolúveis suspensas. A descarga antrópica de Ni em águas estuarinas e marinhas pode ocorrer através de vários meios, incluindo efluentes costeiros de mineração, disposição de esgotos, deposição atmosférica,

mineração e perfuração marinha (Bryan, 1984). A concentração de Ni na água oceânica de profundidade varia geralmente entre 0,1 e 0,5 mg/L, enquanto a água superficial contém de 15 a 20 mg/L (Norseth & Piscator, 1979). No entanto, os níveis de Ni podem chegar a 82,2 mg/L em ambientes costeiros e estuarinos, onde o espaço confinado favorece um maior acúmulo do metal (Boyden, 1975). O Ni bivalente é a forma predominante nas fontes aquáticas (Förstner & Wittmann, 1983), enquanto a existência de outros compostos do metal depende do pH e de agentes ligantes orgânicos e inorgânicos.

Conforme mencionado acima, comparativamente a outros metais bivalentes, o Ni não tem sido muito estudado em termos de (eco)toxicidade, modo de ação e biodisponibilidade (Keithly et al., 2004). Enzimas que contêm Ni são bem conhecidas em bactérias (Ankel-Fuchs & Thauer, 1988; Hausinger, 1997). Atualmente, sete enzimas microbianas que contêm Ni já foram identificadas, incluindo urease, hidrogenase, CO-desidrogenase, metil-coenzima M redutase, Ni-superóxido dismutase, glioxilase I e cis-trans isomerase. Bactérias com enzimas que contêm Ni representam um bom modelo para estudar o biometabolismo do Ni, sistemas de transporte e proteínas ligantes do metal (Watt & Ludden, 1999).

Várias espécies animais necessitam da ingestão de Ni na dieta, embora elas não contenham urease ou qualquer outra das outras enzimas que contêm Ni e que estão presentes nas bactérias (Uthus & Seaborn, 1996; Anke et al., 1984). Contudo, o Ni é conhecido por ser um bloqueador dos canais de Ca^{2+} (Lee et al., 1999, Zamponi et al., 1996), levando a alteração na concentração intracelular deste íon, a qual geralmente é seguida por mudanças na sinalização da expressão gênica associada ao crescimento celular, diferenciação e apoptose (Valko et al., 2005). O Ni é também referido como um indutor de estresse oxidativo, aumentando os níveis de radicais livres nas células (Bal &

Kasprzak, 2002; Chen et al., 2003), causando depleção de glutationa (Rodriguez et al., 1996), induzindo LPO (Chen et al., 2002) e gerando danos no DNA associado à produção de ROS (Lynn et al., 1997).

No que se refere à biodisponibilidade dos metais, sabe-se que a composição química da água pode alterar de forma significativa este parâmetro e a conseqüentemente toxicidade destes contaminantes aquáticos (Di Toro et al., 2001). Em geral, as concentrações de íons metálicos livres são reduzidas em ambientes de alta salinidade em comparação a baixas salinidades ou água doce, devido ao aumento da presença de ânions complexantes. Para o Ni, os dois complexantes mais importantes em água marinha são o SO_4^{2-} e o Cl^- (Sadiq, 1989). Além disso, em altas salinidades deve ocorrer um aumento da competição entre íons metálicos e cátions de proteção, como o Na^+, Mg^{2+} e Ca^{2+}, pelos sítios de ligação no ligante biótico (Paquin et al., 2000; Janssen et al., 2003). Portanto, o aumento da salinidade da água atua como um fator de proteção contra a toxicidade de muitos metais, incluindo o Ni (Eisler, 1998).

Um número restrito de invertebrados tem sido testado como biomonitores da contaminação por Ni em sistemas estuarinos e marinhos (Widdows, 1985; Gibb et al., 1996; Yeh et al., 2009) e de água doce (Tochimoto et al., 2003). Espécies eurialinas são organismos potencialmente modelos para avaliar a influência da salinidade na toxicidade do Ni, já que este parâmetro químico da água pode afetar tanto a biodisponibilidade do metal quanto a fisiologia dos organismos. McLusky e Hagerman (1987) estudaram a evolução temporal do efeito dos metais na osmorregulação e mostraram que, para o Cr e o Zn, a concentração osmótica do sangue dos animais diminuiu progressivamente em função do tempo de exposição aos metais. No entanto, esta redução progressiva não foi observada para o Ni.

O caranguejo *N. granulata* habita áreas estuarinas e costeiras do Sudoeste do Brasil até a Argentina. Esta espécie é um importante elo da cadeia alimentar estuarina, devido à sua alta abundância e seus múltiplos papéis ecológicos, atuando tanto como predador quanto presa. Como espécie chave desses ecossistemas estuarinos, *N. granulata* desempenha um papel importante na transferência de contaminantes para níveis tróficos superiores. Devido ao importante papel ecológico de *N. granulata* e o extenso conhecimento sobre sua biologia e as condições para sua manutenção em cativeiro, este caranguejo tem se tornado, há décadas, alvo de estudos ecotoxicológicos (para revisão: Bianchini et al., 2008). No estuário da Lagoa dos Patos, *N. granulata* está freqüentemente exposto a grandes e freqüentes variações da salinidade da água, bem como sujeito à exposição a uma diversidade de contaminantes químicos nas marismas do estuário da Lagoa dos Patos, incluindo metais (para revisão: Bianchini et al., 2008).

Diferentes padrões de osmorregulação são observados entre os animais estuarinos, sendo que os crustáceos são geralmente osmorreguladores em baixas salinidades e osmoconformadores em altas salinidades (Péqueux, 1995; Kirschner, 2004; Bianchini et al., 2008). No entanto, adultos de *N. granulata* são claramente caracterizados como sendo hiper-hipo-osmorreguladores (Bromberg, 1992; Miranda, 1994; Novo et al., 2005; Bianchini et al., 2008). Estudos realizados em adultos de *N. granulata* mostraram que os machos e as fêmeas são capazes de hiper-osmorregular em baixas salinidades (2-5) e hipo-osmorregular em alta salinidade (40), apresentando um ponto isosmótico correspondente à concentração osmótica da água em torno de 29‰ (Bromberg, 1992; Miranda, 1994; Novo et al., 2005; Bianchini et al., 2008). Como resultado de todos os ajustes metabólicos necessários para aclimatação à salinidade, maiores taxas de consumo de oxigênio são observadas em caranguejos jovens e adultos de *N. granulata* após aclimatação a salinidades extremas do ambiente (Santos et al.,

1987; Bianchini & Castilho, 1999). De acordo com Gimenez (2003), essa plasticidade pode contribuir para manter certo grau de conectividade da população e persistência, independentemente da heterogeneidade do habitat. Dentre as respostas dos caranguejos às mudanças da salinidade ambiental, aquelas diretamente relacionadas à capacidade de *N. granulata* em manter o equilíbrio iônico e osmótico hemolinfático adequado são certamente imperativas para sua tolerância frente às mudanças ambientais de salinidade.

Em relação à interação entre atividades antrópicas e fatores ambientais, fica evidente que a presença de metais em águas estuarinas pode afetar a regulação iônica e osmótica em animais eurialinos, tais como *N. granulata*, modificando assim a capacidade destes em se ajustarem fisiologicamente às mudanças de salinidade da água. De fato, demonstrou-se que a exposição a diferentes metais, tais como Ag, Cd, Cu e Zn afetou os mecanismos envolvidos na regulação iônica e osmótica hemolinfática em *N. granulata* quando aclimatado a baixas salinidades (para revisão: Bianchini et al., 2008). Também é importante ressaltar que a salinidade da água afeta de forma significativa a toxicidade de poluentes inorgânicos em *N. granulata*. Por exemplo, o aumento da salinidade da água diminuiu a toxicidade do Cd, Cu e Ag em caranguejos adultos (Vitale et al., 1999; Rodríguez et al., 2001; Bianchini et al., 2003; Lauer et al., 2005). Como relatado anteriormente, este efeito protetor da salinidade contra a toxicidade aguda dos metais está associado a uma menor biodisponibilidade dos metais devido a uma maior complexação destes com ânions, especialmente o Cl^-, presentes em altas concentrações na água do mar. Além disso, ocorre uma maior competição entre os metais e outros cátions, como Na^+, Ca^{2+} e Mg^{2+} presentes em concentrações elevadas na água do mar, pelos sítios de ligação nas brânquias (Bianchini et al., 2003; 2004; Lauer et al., 2005; Pedroso et al., 2007).

Tendo em vista que mudanças na salinidade alteram a biodisponibilidade e conseqüentemente a absorção e toxicidade dos metais em invertebrados eurialinos, e sabendo-se que, de maneira geral, a exposição de crustáceos aos metais pode afetar o metabolismo aeróbico e a capacidade osmorregulatória, esta dissertação de Mestrado foi desenvolvida com vistas ao estudo dos efeitos da exposição aguda do caranguejo eurialino *N. granulata* a concentrações subletais de Ni em diferentes salinidades.

OBJETIVOS

Objetivo geral:

O objetivo geral deste estudo é analisar efeitos bioquímicos e fisiológicos da exposição aguda a concentrações subletais de Ni no caranguejo eurialino *Neohelice granulata* aclimatado a diferentes salinidades.

Objetivos específicos:

Ao longo deste estudo foram avaliados os efeitos da exposição a concentrações subletais de Ni sobre o consumo corporal de oxigênio, os níveis de peroxidação lipídica (LPO) em diferentes tecidos (brânquias anteriores e posteriores, músculo e hepatopâncreas), a atividade da lactato desidrogenase (LDH) em diferentes tecidos (hemolinfa, hepatopâncreas e músculo), bem como sobre as concentrações osmóticas e iônicas (Na^+, Cl^-, Ca^{2+}, Mg^{2+} e K^+) hemolinfáticas em caranguejos *N. granulata* aclimatados às salinidades 2 e 30.

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Biochemical and physiological toxic effects of nickel in the euryhaline crab
***Neohelice granulata* acclimated to different salinities**

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Abstract

In euryahaline invertebrates, metal exposure can induce respiratory, osmotic and ionic disturbances, as well as oxidative stress. Despite several studies on the combined effect of water salinity and metal exposure have been reported for estuarine invertebrates, most of them are focused only on few metals like Cd, Cu, Pb and Zn. Actually, a limited number of studies were performed to evaluate the biochemical and physiological responses of euryhaline invertebrates to Ni exposure at different water salinities. In the present study, the estuarine crab *Neohelice granulata* was kept under control condition (no Ni addition in the water) or exposed (96 h) to sublethal concentrations of Ni (100 and 1000 µg/L) in two different water salinities (2 and 30 ppt). After exposure, whole-body oxygen consumption was measured and tissue samples (hemolymph, hepatopâncreas, muscle, and anterior and posterior gills) were collected for further analyses. Osmotic and ionic (Na^+, Cl^-, Ca^{2+}, Mg^{2+} and K^+) concentrations were determined in hemolymph. Lactate dehydrogenase (LDH) activity was measured in hemolymph, hepatopancreas and muscle while lipid peroxidation (LPO) was analyzed in hepatopancreas, muscle and gills (anterior and posterior). Control crabs acclimated to salinity 2 ppt showed lower hemolymph concentrations of Na^+, K^+ and Mg^{2+} and higher LPO levels in gills (anterior and posterior) and hepatopancreas than those acclimated to salinity 30 ppt. Ni exposure induced higher whole-body oxygen consumption, as well as higher hemolymph LDH activity and K^+ concentration in crabs acclimated to salinity 2 ppt. In crabs acclimated to salinity 30 ppt, increased hemolymph LDH activity, osmolality and Cl^- concentration, as well as reduced hemolymph K^+ and Mg^{2+} concentrations were observed after Ni exposure. Taken altogether, these findings indicate that *N. granulata* is osmoregulating in salinities 2 (hyper-regulation) and 30 ppt (hypo-regulation), showing adequate

adjustments of the hemolymph ionic composition, aerobic and anaerobic metabolic rates, with consequent higher oxidative damage to lipids in low salinity (2 ppt). Regarding Ni effects, they are associated with metabolic (aerobic and anaerobic) disturbances in crabs acclimated to salinity 2 ppt, while osmotic and ionoregulatory disturbances were more evident in crabs acclimated and exposed to Ni in salinity 30 ppt.

Introduction

Nickel (Ni) is an essential trace metal naturally occurring in the whole environment worldwide. However, domestic sewage, industrial effluents, incineration of wastes, mining and marine drilling activities have significantly increase Ni concentrations in both terrestrial and aquatic systems (Bryan, 1984). In estuaries, Ni concentration generally varies between 1 and 75 µg/L (Eisler, 1998). In impacted sites, its concentration in underground waters can be as high as 2,500 µg/L.

Like other essential metals, such as Cu and Zn, the bivalent Ni is the predominant form in aquatic environments (Förstner and Wittmann, 1983). However, in contrast to other essential metals, Ni bioavailability, toxicity and mode of action in invertebrates have being analyzed only in a few studies (Keithly et al., 2004).

In vertebrates, Ni is reported as a Ca^{2+} channel blocker, inducing changes in intracellular Ca^{2+} concentration (Zamponi et al., 1996; Lee et al., 1999). Ni is also referred as an oxidative stress inducer, causing increased cellular levels of reactive oxygen species (Bal and Kasprzak, 2002; Chen et al., 2003), with consequent damage to lipids (Chen et al., 2002) and DNA (Lynn et al., 1997). However, as far as we know, no similar reports are available for euryhaline animals, especially invertebrates. Acute respiratory effects were observed in the rainbow trout *Oncorhynchus mykiss* after waterborne Ni exposure. These effects were followed by increased blood lactate levels and hematocrite values (Pane et al., 2003a). In the freshwater flea *Daphnia magna*, significant reduced oxygen consumption and impaired whole-body Mg^{2+} content was observed after acute and chronic exposure to waterborne Ni (Pane et al., 2003b). Based on these findings, it is expected that Ni exposure would induce respiratory impairments, changes in energy metabolism, and oxidative stress followed by damage to molecules

such as lipids and DNA in euryhaline invertebrates. A disturbance in the regulation of divalent cations, especially Ca^{2+}, would be also expected. However, these effects would depend on the water salinity, since it is well recognized that this water parameter largely influences metal bioavailability and the consequent bioaccumulation and toxicity of metals, including Ni, in estuarine invertebrates (Bianchini et al., 2004; Pedroso et al., 2007; Pinho et al., 2007; Lauer and Bianchini, 2010; Pinho and Bianchini, 2010; Leonard et al., 2011). Also, it is known that water salinity changes markedly influences the biochemistry and physiology of estuarine invertebrates, including crabs (for review: Bianchini et al., 2008).

The euryhaline crab *Neohelice granulata* inhabits estuarine and coastal areas of South America, being distributed from Southeastern Brazil to Argentina. In the Patos Lagoon estuary (Rio Grande, RS, Southern Brazil), it is subjected to frequent and marked changes in water salinity. To cope with the environmental changes in water salinity, *N. granulata* shows a suite of biochemical and physiological mechanisms of adaptation involved in iono- and osmotic regulation. In fact, this euryhaline crab can survive in a wide range of salinities (from freshwater to hypersaline water), being considered as a hyper-hypo-osmoregulator. Anterior gills are mainly involved in gas exchange while posterior gills are implicated in iono- and osmoregulation (for review: Bianchini et al., 2008). In this context, it is important to consider that water contamination with metals can induce structural changes in anterior gills altering their ability to exchange gases, with consequent changes in the aerobic metabolism and energy production. In turn, the Na^+,K^+-ATPase activity present in posterior gills is considered the major mediator of the NaCl uptake across the gills, playing an extremely important role in both ionic and osmotic regulation. Exposure to elevated concentrations

of essential metals such as Cu was shown to inhibit gill Na^+,K^+-ATPase activity in *N. granulata* (for review: Bianchini et al., 2008).

With this background in mind, the aim of the present study was to identify the most likely mechanism of Ni toxic action in estuarine crustaceans. Therefore, the response of some biochemical and physiological parameters associated with metabolism, oxidative status, and hydro mineral homeostasis were evaluated in the estuarine crab *N. granulata* after acute exposure to sub-lethal concentrations of waterborne Ni. Experiments were performed using crabs acclimated to two different salinities (2 and 30 ppt) to evaluate the influence of salinity on the biochemical and physiological responses analyzed.

2. Material and methods

2.1. Crab collection and acclimation

Adult male crabs *N. granulata* (13.2 ± 1.41 g wet body mass) were collected in salt marshes from the Patos Lagoon estuary (Rio Grande, RS, Southern Brazil), transferred to the laboratory, and acclimated for two weeks under control conditions (temperature: 20ºC and photoperiod 12 h light:12h dark). Crabs were acclimated to two different water salinities (2 and 30 ppt), which were prepared from natural sea water filtered (0.45-µm mesh filter; PVDF membrane, Durapore, Millipore, São Paulo, SP, Brazil) and diluted with distilled water to obtain the desired salinities. Crabs were fed ground beef three times a week.

2.2. Ni exposure

All glassware employed were previously washed in 10% HNO_3 and thoroughly rinsed with distilled water before use. Exposure media were prepared using natural sea water adjusted to the desired experimental salinities (2 and 30 ppt) as described above. Nominal concentrations of Ni (0, 100 and 1000 µg Ni/L) were obtained from a stock solution prepared with $NiCl_2.6H_2O$ (Merck, Haar, Germany). Experimental media were prepared 24 h before crab's introduction in the test chamber to allow Ni to completely equilibrate with salt water. Crabs were exposed to Ni in 5-L glass aquaria in the absence of food for 96 h. Every 24 h, the experimental media were completely renewed and crab survival was monitored.

After Ni exposure, whole-body oxygen consumption was measured as described below. Crabs were then cryoanesthetized and hemolymph samples were collected by puncture of the hemolymph sinus at the basis of the 3rd or 4th pair of pereiopods. Crabs were then killed by removal of the exoskeleton and had their gills (anterior and posterior), muscle and hepatopancreas dissected and frozen (-75ºC) for further analyses, as described below.

Filtered (0.45-µM mesh filter) and non-filtered samples (10 mL) from the experimental media were daily collected for measurements of dissolved and total Ni concentrations, respectively. All samples were acidified with 50 µL of 65% HNO_3 (Suprapur, Merck). Ni concentration was determined by atomic absorption spectrophotometry (AAS, Avanta 932 Plus, GBC, Hampshire, IL, USA) using a standard curve built with standard solutions prepared from a certified standard solution (Tritisol-Merck).

2.3. Oxygen consumption

After 96 h of Ni exposure, whole-body oxygen consumption of crabs was measured using a 140-mL static respirometer mounted over a magnetic agitator and connected to an oximeter (Digimed, São Paulo, SP, Brazil). Every 5 min, dissolved O_2 content was measured. After 30 min, crabs were weighed (wet body mass) and the oxygen consumption calculated based on changes in oxygen content in the experimental medium, respirometer volume, time elapsed and crab wet body mass. Therefore, results were expressed as mg O_2/ g wet body mass/ h.

2.4. Hemolymph osmolality and ionic concentration

Hemolymph osmolality was measured using a osmometer based on vapor pressure (Wescor, Model VAPRO, USA) and was expressed as mOsmoles/kg H_2O. Na^+ and K^+ concentrations were measured by atomic absorption spectrophotometry (AAS, Avanta, 932 Plus, GBC, Hampshire, IL, USA) using certified standard solutions (Tritisol, Merck, USA). Cl^-, Ca^{2+} and Mg^+ concentrations were measured using commercial reagent kits (Reference 49, Reference 95-2/50 and Reference 50; Labtest Diagnóstica, Lagoa Santa, MG, Brazil). Hemolymph ion concentrations were expressed in mM.

2.5. Lipid peroxidation (LPO)

For LPO measurements, gills (anterior and posterior), muscle and hepatopancreas were weighed and homogenized (1:9 w/v) with a solution containing KCl (1.15%) and BHT (35 μM). The supernatant obtained was used for analysis. LPO measurements were performed following the procedures described by Ohkawa et al. (1979). Briefly, supernatant samples (10 μl) were added to the reaction mixture

containing (BHT 20 µL), 20% acetic acid (pH 3.5; 150 µL), TBA (150 µL), Milli-Q water (50 µL) and SDS (20 µL). Reaction mixture was kept in water bath (95ºC) for 30 min. After cooling, Milli-Q water (100 µL), n-butilic alcohol (500 µL) were added to the reaction mixture, which was homogenized using a Vortex. After centrifugation (3,000 ×g) for 10 min at 10ºC, the organic phase was removed and the fluorescence (excitation: 515 nm; emission: 553 nm) of the reaction mixture was determined using a microplate reader (Victor 2, Perkin, USA). LPO values were expressed as nmol MDA/mg wet tissue, using TMP as an external standard.

2.6. Lactate dehydrogenase (LDH) activity

LDH activity was measured in hemolymph, hepatopancreas and muscle samples. They were weighed, homogenized (1:5 w/v) using sodium citrate (100 mM), and centrifuged (7,000 ×g) for 10 min at 4ºC. The supernatant obtained was used for analysis. LDH activity was measured using a commercial reagent kit (TOX7, Sigma-Aldrich, EUA). The assay is based on the NAD reduction by LDH. The resulting reduced NAD (NADH) is employed in the stoichiometric conversion of the tetrazolium dye. The concentration of the resulting compound was analyzed by spectrophometry in a microplate reader (Biotek, Model EL 808, USA) at two different wavelengths (490 nm and 550 nm). Protein concentration in the homogenized was measured using a commercial kit (Doles Ltda, Belo Horizonte, MG, Brazil) based on the Biuret reagent's method (Gornall et al., 1949). Specific enzyme activity was expressed as enzyme units/mg proteins/h.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation. Comparisons among treatments were made by two-way (salinity and Ni concentration) analysis of variance (ANOVA) followed by the Duncan's test. ANOVA assumptions (data normality and homogeneity of variances) were previously verified. Mathematical (log) transformations were performed when necessary (Zar, 1984). In all cases, the significance level adopted was 95% ($p<0.05$).

3. Results

3.1. Ni concentrations and crab survival

Nominal, total and dissolved Ni concentrations in experimental media used to expose crabs are shown in Table 1. In all cases, including the control media (no Ni addition to the water), Ni concentration was always lower in dilute media (salinity 2 ppt) than in sea water (salinity 30 ppt). This difference was associated with the different Ni backgrounds in the two experimental salinities, which in turn was related to the dilution of sea water with distilled water to prepare the water at salinity 2 ppt. No significant difference was observed between total and dissolved Ni concentration. Nominal values are thus used to refer Ni concentrations thereafter. All crabs survived after Ni exposure.

3.2. Oxygen consumption

In control crabs, no significant difference in oxygen consumption was observed between crabs acclimated to 2 and 30 ppt salinity. Exposure to 1000 µg Ni/L significantly increased the oxygen consumption of crabs acclimated to 2 ppt salinity. In

fact, Ni effect was dependent on metal concentration in the experimental medium. In the other hand, no significant Ni effect was observed in seawater-acclimated crabs (Fig. 1).

3.3. Hemolymph osmolality and ionic composition

In control crabs, hemolymph osmolality (Fig. 2A), as well as Cl^- (Fig. 2C) and Ca^{2+} (Fig. 2D) concentrations were similar between crabs acclimated to 2 and 30 ppt salinity. However, hemolymph Na^+ (Fig. 2B), Mg^{2+} (Fig. 2E) and K^+ (Fig. 2F) concentrations were significantly higher in crabs acclimated to 30 ppt salinity than in those acclimated to 2 ppt salinity. In crabs acclimated to 2 ppt salinity, exposure to 1000 µg Ni/L reduced the hemolymph Cl^- concentration (Fig. 2C), while exposure to 100 µg Ni/L increased the hemolymph K^+ concentration (Fig. 2F). In seawater-acclimated crabs, exposure to 100 and 1000 µg Ni/L increased hemolymph osmolality (Fig. 2A) and Cl^- concentration (Fig. 2C) while exposure to 100 µg Ni/L increased Ca^{2+} concentration (Fig. 2D). Also, exposure to 1000 µg Ni/L reduced the hemolymph Mg^{2+} (Fig. 2E) and K^+ (Fig. 2F) concentration.

3.4. Lipid peroxidation

In control crabs, LPO was higher in anterior (Fig. 3A) and posterior (Fig. 3B) gills, as well as hepatopancreas (Fig. 3C) of crabs acclimated to salinity 2 ppt than in those acclimated to sea water. However, it was similar in muscle of the two groups of crabs (Fig. 3D). Ni exposure did not increase LPO in anterior (Fig. 3A) and posterior (Fig. 3B), as well as muscle (Fig. 3D) of crabs acclimated to salinities 2 and 30 ppt. LPO was even reduced in hepatopancreas of crabs acclimated to sea water and exposed to 1000 µg Ni/L (Fig. 3C).

3.5. LDH activity

In control crabs, LDH activity was similar in tissues (hemolymph, hepatopancreas and muscle) of crabs acclimated to salinity 2 and 30 ppt (Fig. 4). Increased LDH activity was observed in the hemolymph of crabs exposed to 1000 µg Ni/L in both salinities 2 and 30 ppt (Fig. 4A). Enzyme activity was also higher in hepatopancreas of crabs acclimated to salinity 2 ppt and exposed to 100 and 1000 µg Ni/L, as well as of those acclimated to salinity 30 ppt and exposed to 1000 µg Ni/L (Fig. 4B). Also, a higher LDH activity was observed in muscle of crabs acclimated to salinity 30 ppt and exposed to 100 and 1000 µg Ni/L. However, a reduced LDH activity was observed in muscle of crabs acclimated to salinity 2 ppt and exposed to 1000 µg Ni/L (Fig. 4C).

4. Discussion

Oxygen consumption rate is considered an indirect measurement of the physiological status of the organism and is often employed as an indicator of the stress imposed to aquatic organisms by changes in environmental factors. It can vary with changes in respiration rate, internal transport and utilization of oxygen by tissues (Grobler et al., 1989). Therefore, oxygen consumption rate can provide useful information to understand the effect of environmental stressors (Watenpaugh and Beitinger, 1985).

Contrary to previous data reported for other euryhaline crab species where an increased (Engel and Eggert, 1974; Engel et al., 1975; Sabourin, 1983) or reduced (Findley et al., 1978; Chen and Chia, 1996b; Robles et al., 2002) oxygen consumption rate was observed after acclimation to low salinity, no significant difference in oxygen

consumption rate was observed between control *N. granulata* acclimated to 2 and 30 ppt. This finding associated with the lack of difference in hemolymph osmolality and tissue LDH activity between control crabs acclimated to the different water salinities indicate that *N. granulata* is fully acclimated to the experimental salinities. In fact, hemolymph osmolality data are in agreement with the fact that *N. granulata* is a good osmoregulator in the range of salinities tested, hyper-regulating in low salinities and hypo-regulating in low salinities (for review: Bianchini et al., 2008). Also, as previously shown (Bianchini et al., 2008), different hemolymph ionoregulatory patterns were observed for some of the ions analyzed (Na^+, K^+ and Mg^{2+}). Regarding LDH, this enzyme plays an important role in physiological functions associated with survival and performance of organisms, having a key role in energy production and detoxification. LDH is a citosolic enzyme showing different isoforms which interconvert piruvate in lactate during glycolisis, playing an essential role during hypoxic conditions. Therefore, the observed lack of change in LDH activity in tissues of control crabs is also evidence that *N. granulata* were fully acclimated to the two experimental salinities. Taken altogether these findings suggest that both aerobic and anaerobic metabolism are in a "steady state" in crabs acclimated to the two experimental salinities (2 and 30 ppt).

Oxygen consumption rate is also considered a good indicator of the general metabolic status of aquatic animals exposed to sublethal concentrations of metals (Vosloo et al., 2002). In fact, exposure to chemical substances can cause changes in the respiratory rate of organisms, indicating possible disturbances or adaptive responses of physiological processes involved in metabolic rate regulation (Watenpaugh and Beitinger, 1985). Considering that no significant difference was observed in the oxygen consumption rate as a function of water salinity in *N. granulata*, changes observed in this parameter after exposure to Ni could be thus associated with the metal exposure. A

possible combined effect between water salinity and metal exposure cannot be also ruled out.

In mollusks and crustaceans, oxygen consumption rate is generally reduced after exposure to different metals (Spicer and Weber, 1991). However, increased oxygen consumption was observed in the estuarine crab *N. granulata* acutely exposed (96 h) to 1,000 μg Ni L^{-1} in salinity 2 ppt. It is important to note that this response was dependent on Ni concentration in the experimental medium. Also, it is worth to mention that the same effect was not observed in crabs exposed to the metal in salinity 30 ppt. Bianchini and Castilho (1999) also observed an increased oxygen consumption rate in *N. granulata* acutely exposed to zinc in low water salinities. However, there is not a unique pattern of response of the oxygen consumption rate to metal exposure. Actually, metabolic rate was shown to decrease (Spicer & Weber, 1991; Varghese et al., 1992; Bambang et al., 1995; Barbieri, 2007), increase (Calow, 1989) or vary only over the exposure period (Vosloo et al., 2002).

In previous studies with different crustacean species, including *N. granulata*, toxicity of metals like Cd, Cu and Ni was reduced as water salinity increased (Zaunders and Rojas, 1996; Rodríguez et al., 2001; Bianchini et al., 2003, 2004; Leonard et al., 2011). In fact, salinity increase will interfere with metal speciation, reducing metal bioavailability and its consequent bioaccumulation (Verslycke et al., 2003). Therefore, the increased oxygen consumption rate observed after *N. granulata* exposure to Ni in low salinity (2 ppt) could be explained considering a higher energy consumption associated with a higher demand of processes involved in Ni detoxification, mobilization and excretion, as suggested for the crab *Carcinus maenas* exposed to Hg (Depledge, 1984). Unfortunately, tissue Ni accumulation was not evaluated in the present study to confirm this hypothesis.

It is also possible that the increased oxygen consumption rate observed after *N. granulata* exposure to Ni is reflecting a better ability of *N. granulata* to cope with the stress induced by Ni exposure at low salinities than in high salinities. This statement is based on the fact that Ni-induced disturbances in hemolymph osmotic and ionic regulation was much more evident in crabs exposed to the metal in salinity 30 ppt than in those exposed to Ni in 2 ppt. Significant disturbances in hemolymph osmolality and concentrations of all ions analyzed, except Na^+, were observed in crabs exposed to Ni in salinity 30 ppt, while in those exposed to the metal in salinity 2 ppt disturbances were observed only for Cl^- and K^+. Disturbances of whole-body Na^+ homeostasis were not also observed in rainbow trout and water flea exposed to Ni in freshwater (Pane et al., 2003a,b).

Osmotic disturbances found in crabs exposed to Ni in salinity 30 ppt can be explained by the observed increase in hemolymph Cl^- concentration. In turn, the lack of change in hemolymph osmotic concentration of crabs exposed to Ni in low salinity (2 ppt) seems to be a combined result of the significant decrease observed in Cl^- concentration and the observed tendency of increase in Na^+ concentration.

It is reported that changes in whole-body Ca^{2+} content are typical in aquatic animals exposed to Ni. It is well accepted that there is an antagonism between Ni and Ca^{2+}. It is also known that Ni is an efficient blocker of several types of Ca^{2+} channels (McFarlane and Gilly, 1998; Todorovic and Lingle, 1998; Lee et al., 1999). In addition, it has been shown that Ca^{2+} protects against Ni toxicity in rainbow trout *O. mykiss*, fathead minnow *Pimephales promelas* (Meyer et al., 1999; Deleebeeck et al., 2007), and water flea *Daphnia pulex* (Kozlova et al., 2009). However, an antagonism between Ni and Ca^{2+} was not observed in the crab *N. granulata* in the present study, since no decreases in hemolymph Ca^{2+} concentration was observed with increasing Ni

concentration in the experimental medium. In contrast, an even increased hemolymph Ca^{2+} concentration was observed in crabs exposed to 100 µg Ni L^{-1} in high salinity (30 ppt). This lack of negative effect of Ni on hemolymph Ca^{2+} concentration could be explained by the high active Ca^{2+} metabolism associated with molting and the consequent growth observed in crustaceans (Greenaway, 1985). This high ability of crustaceans to deal with $Ca2^{+}$ homeostasis could thus explain the lack of Ni effect on hemolymph Ca^{2+} concentration in *N. granulata* (Huner et al., 1979).

As described for Ca^{2+}, Ni is also recognized as being an Mg^{2+} antagonist (Pane et al., 2003a, b). This fact has been described for different animal groups, including mammals, birds, bacteria, yeast (Eisler, 1998) and also in the rainbow trout, where Ni uptake is facilitated by Mg^{2+} transporters (Pane et al., 2003a). In euryhaline crustaceans (shrimp and isopod), we have recently demonstrated that significant decreases in hemolymph Mg^{2+} concentration are well correlated with significant increases in whole-body Ni concentration (Leonard et al., 2011). Therefore, the significant decrease observed in the hemolymph Mg^{2+} concentration in the crab *N. granulata* exposed to Ni in high salinity is in complete agreement with the idea that Ni is a Mg^{2+} antagonist not only in freshwater fish and crustaceans (Pane et al., 2003a,b), but also in euryhaline crustaceans. Taken all findings together, it seems that disturbances in Mg^{2+} homeostasis might be the mechanism of Ni toxic action in freshwater, brackish and marine crustaceans.

Ni exposure also disturbed the hemolymph K^+ homeostasis in crabs acclimated to 2 and 30 ppt. It is well known that K^+ is the main intracellular electrolyte and the ratio between its concentrations in the intracellular and the extracellular media is the major factor determining the transmembrane electric potential. Therefore, any change in the extracellular concentration of K^+ can induce deleterious effects not only on the

metabolic function but also in the nervous conduction of the action potential with important consequences in muscle function, especially in the cardiac muscle (Barbosa and Sztajnbok, 1999). It is also reported that exposure to metals can affect the cellular membrane properties with consequences on K^+ permeability. As the K^+ concentration is higher in the cell than in the extracellular medium, increases in membrane permeability induced by metal exposure would favor the efflux of K^+ from the cell, thus increasing the concentration of this ion in the extracellular medium (Davson and Danielli, 1938). This situation can help to explain the increased hemolymph K^+ concentration observed in *N. granulata* exposed to Ni in low salinity. However, this hypothesis thus not explain the decreased hemolymph K^+ concentration observed in crabs exposed to Ni in high salinity. In this case, a possible explanation would be to consider an increased membrane permeability to both K^+ and Na^+ in crabs exposed to Ni in high salinitiy. In this case, a higher Na^+-K^+-ATPase activity would be necessary to maintain the intracellular Na^+ homeostasis observed in crabs exposed to Ni in salinity 30 ppt, thus increasing the intracellular K^+ concentration with a consequent reduction in the concentration of this ion in the hemolymph. The increment in summary, findings reported in the present study clearly indicate that Ni can be considered as an osmo- and ionoregulatory toxicant for the estuarine crab *N. granulata* acclimated to a wide range of salinities, especially during the hypo-regulation in high salinities.

Oxygen consumption due to the variety of biochemical reactions occurring in aerobic organisms produces oxygen species that are only partially reduced, which are known as reactive oxygen species (ROS). The superoxide and hydroxil radicals, as well as hydrogen peroxide are some examples of ROS. These oxygen species are shown to be able to induce lipid peroxidation (LPO), resulting in damage to biological membranes. Actually, LPO has been considered as a good index of oxidative stress

damage in tissues for almost all animals analyzed up to date, including estuarine invertebrates (Monserrat et al., 2007). As gills are the primary site of the crab contact with the surrounding medium, it is expected that this organ would be more impacted by the stress induced by changes in environmental factors. Considering that changes in environmental factors such as temperature, pH and salinity can induce ROS formation with consequent increase in LPO levels (for review: Monserrat et al., 2007), and the fact that gills are the main organ involved in respiration and ionic and osmotic regulation in crabs (Henry and Cameron, 1983; Henry, 1984; Lucu, 1990) including *N. granulata* (for review: Bianchini et al., 2008), higher LPO levels would be expected in gills than in other tissues of control crabs. Actually, LPO levels were ~2- to 3-fold higher in gills (anterior and posterior) than in hepatopancreas and muscle of *N. granulata* kept under control conditions. Also, the higher levels of LPO observed in gills (anterior and posterior) and hepatopancreas of control *N. granulata* acclimated to low salinity than in those acclimated to salinity 30 ppt are in complete agreement with the fact that biochemical and physiological changes observed over the salinity acclimation process are more pronounced when crabs are acclimated to low salinity (Piller et al., 1995; Bianchini et al., 2008).

Regarding metal exposure, it is reported that transition metals like Cd, Co, Cu, Hg, Pb, Fe, Sn, V, and Ni can induce peroxidation of membrane lipids (Knight and Voorhees, 1990). However, no significant increase in LPO was observed after *N. granulata* exposure to Ni. This general lack of Ni increase in LPO would be explained considering one of the following possibilities: (1) a lack of increase in ROS production after Ni exposure; (2) increased tissue total antioxidant capacity after Ni exposure; or increased ROS production associated with a parallel a similar increase in the total antioxidant capacity. Actually, the decreased LPO value observed in muscle of *N.*

granulata exposed to Ni in high salinity (30 ppt) suggest that an increased level of antioxidant agents would be occurring after Ni exposure. However, a better understanding of the crab oxidative response to Ni exposure would be only possible with data from future studies evaluating the response of ROS production and total antioxidant capacity in tissues, especially gills, of crabs exposed to Ni under the same conditions employed in the present study.

In light of the discussed above, we report evidences that different mechanisms of Ni toxicity are occurring as a function of the water salinity where crabs were acclimated and exposed to Ni. In high salinity (30 ppt), disturbance in ionic/osmotic regulation without oxidative damage in lipids was observed, while disturbance in oxygen consumption without oxidative damage in lipids was observed after Ni exposure in low salinity (2 ppt). It is worth to note that the increased oxygen consumption rate induced by Ni exposure was paralleled by an increased LDH activity in tissues (hemolymph, hepatopancreas and muscle), except in muscle of crabs acclimated and exposed to Ni in 2 ppt. This increased LDH activity is in agreement with data reported for the water flea *D. magna* exposed to Hg (De Coen et al., 2001) and the shore crab *C. maenas* exposed to Zn and Hg (Elumalai et al., 2007). These findings suggest that an increased anaerobic production of energy would be occurring under metal exposure, including Ni. They are also in agreement with the use of tissue LDH activity in muscle or whole-body homogenates as indicative of potential effects of chemical stress on energy production in aquatic animals (Guilhermino et al., 1994; De Coen et al., 2001; Frasco and Guilhermino, 2002).

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Table 1. Ni concentrations (μg/L) in experimental media used to expose the euryhaline crab *Neohelice granulata*. Data are expressed as mean ± standard deviation (n = 4).

Water salinity	Nominal Ni concentration	Total Ni concentration	Dissolved Ni concentration
2 ppt	0	44.8 ± 10.3	45.5 ± 13.7
	100	179.8 ± 20.2	181.5 ± 11.0
	1000	1019.6 ± 21.8	1026.2 ± 73.5
30 ppt	0	353.3 ± 38.1	351.0 ± 27.4
	100	441.0 ± 23.9	441.8 ± 26.6
	1000	1459.8 ± 26.7	1459.8 ± 34.4

Figure captions

Figure 1. Oxygen consumption rate in the estuarine crab *Neohelice granulata* kept under control conditions (no nickel addition to the water) or exposed (96 h) to nickel (100 and 1000 µg/L) in salinities 2 and 30 ppt. Data are expressed as mean ± standard deviation (n = 5). No significant difference was observed between mean values for control crabs acclimated to 2 and 30 ppt. Different letters indicate significant different mean values among experimental treatments for each salinity ($p < 0.05$).

Figure 2. Hemolymph osmotic (A) and ionic (Na^+: B; Cl^-: C; Ca^{2+}: D; Mg^{2+}: E; and K^+: F) concentrations in the estuarine crab *Neohelice granulata* kept under control conditions (no nickel addition to the water) or exposed (96 h) to nickel (100 and 1000 µg/L) in salinities 2 and 30 ppt. Data are expressed as mean ± standard deviation (n = 5). * indicates significant different mean values between control crabs acclimated to 2 and 30 ppt. Different letters indicate significant different mean values among experimental treatments for each salinity ($p < 0.05$).

Figure 3. Lipid peroxidation in anterior gills (A), posterior gills (B), hepatopancreas (C) and muscle (D) of the estuarine crab *Neohelice granulata* kept under control conditions (no nickel addition to the water) or exposed (96 h) to nickel (100 and 1000 µg/L) in salinities 2 and 30 ppt. Data are expressed as mean ± standard deviation (n = 5). * indicates significant different mean values between control crabs acclimated to 2 and 30 ppt. Different letters indicate significant different mean values among experimental treatments for each salinity ($p < 0.05$).

Figure 4. Lactic dehydrogenase (LDH) activity in hemolymph (A), hepatopancreas (B) and muscle (C) of the estuarine crab *Neohelice granulata* kept under control conditions (no nickel addition to the water) or exposed (96 h) to nickel (100 and 1000 μg/L) in salinities 2 and 30 ppt. Data are expressed as mean ± standard deviation (n = 5). * indicates significant different mean values between control crabs acclimated to 2 and 30 ppt. Different letters indicate significant different mean values among experimental treatments for each salinity ($p < 0.05$).
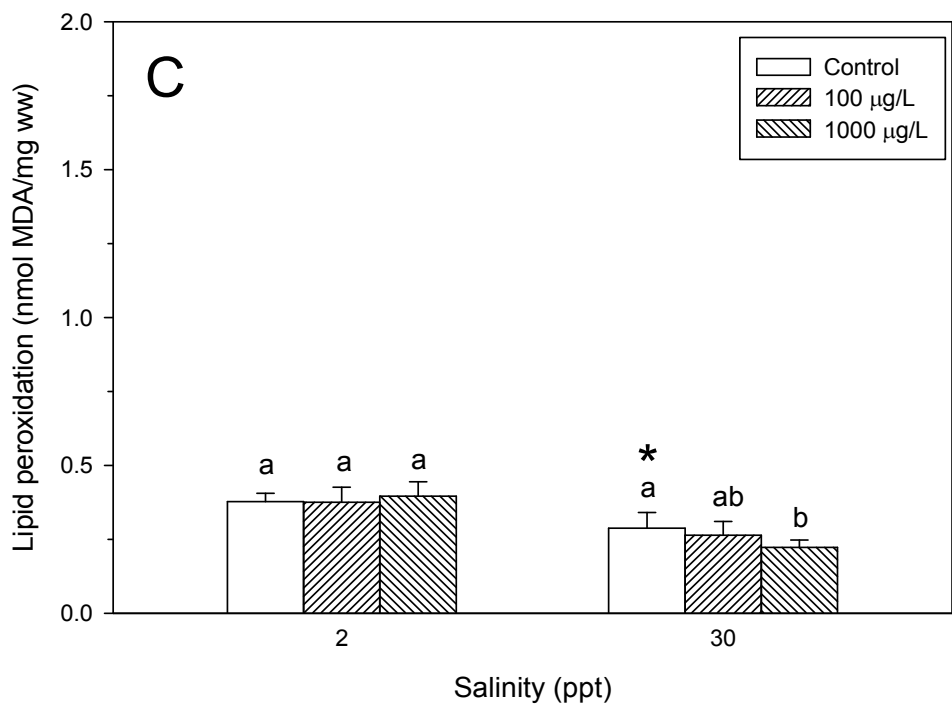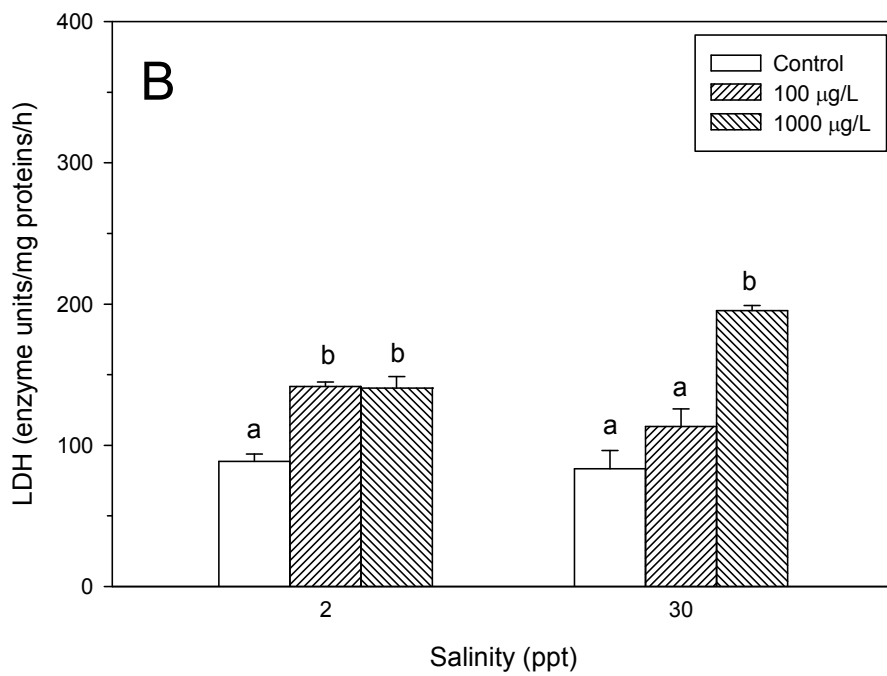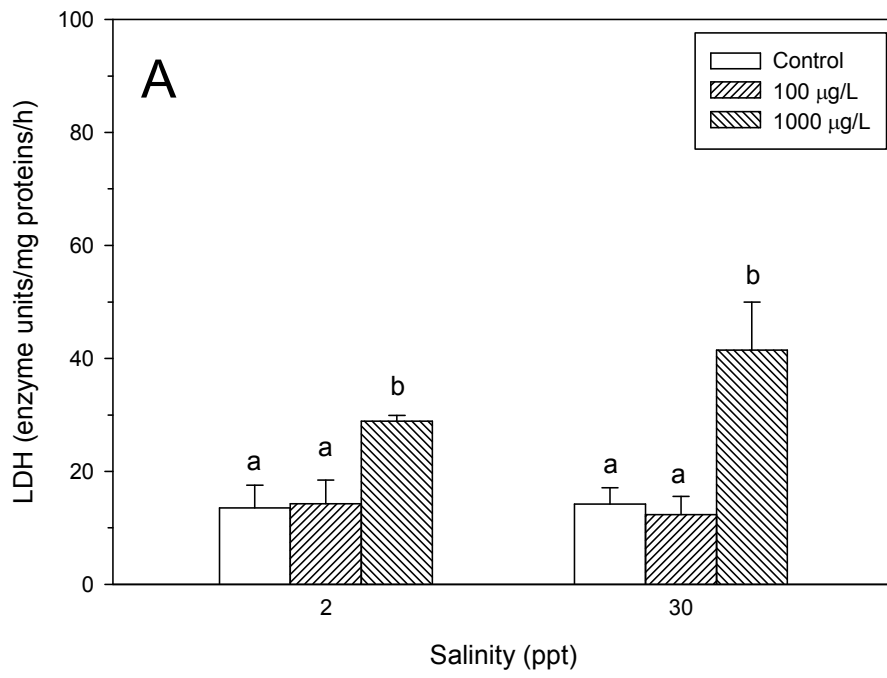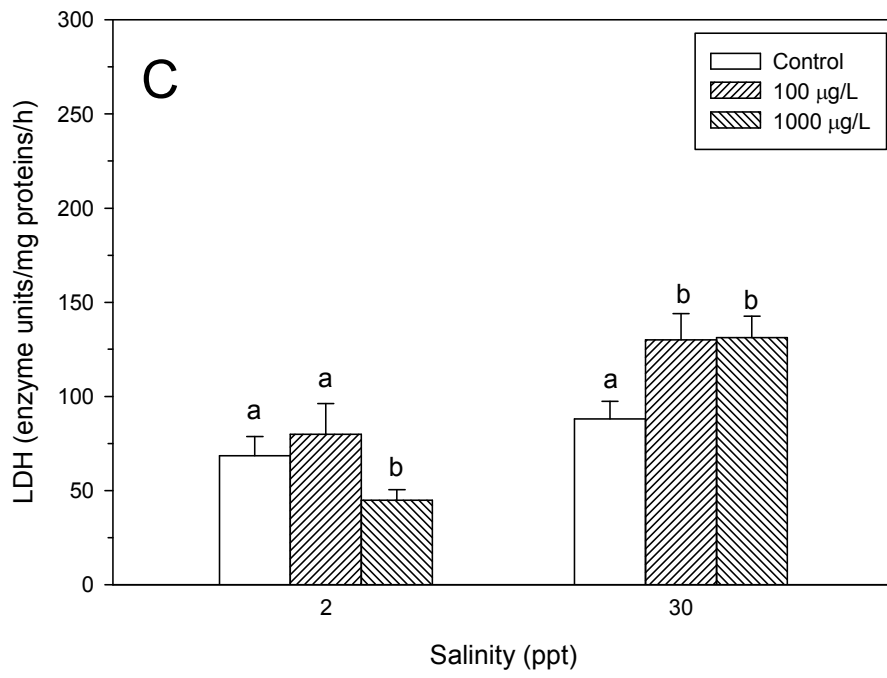
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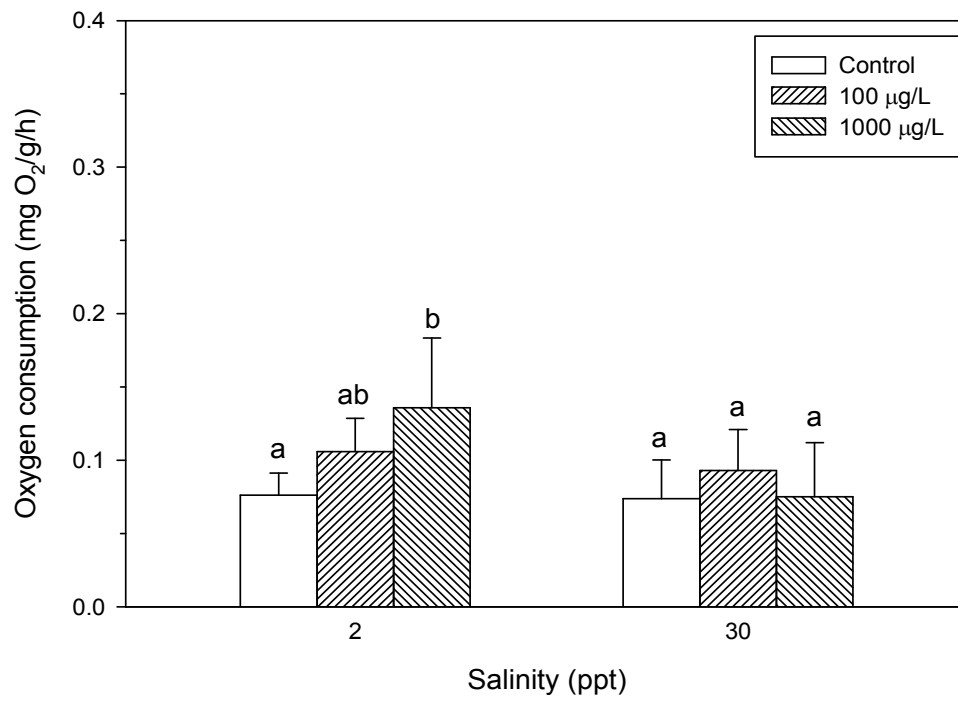
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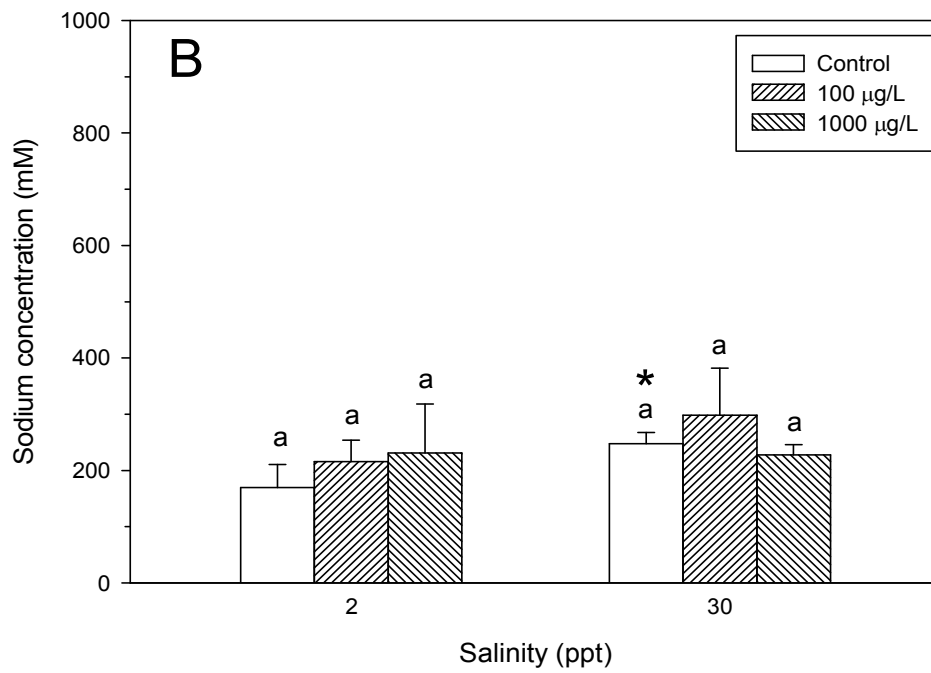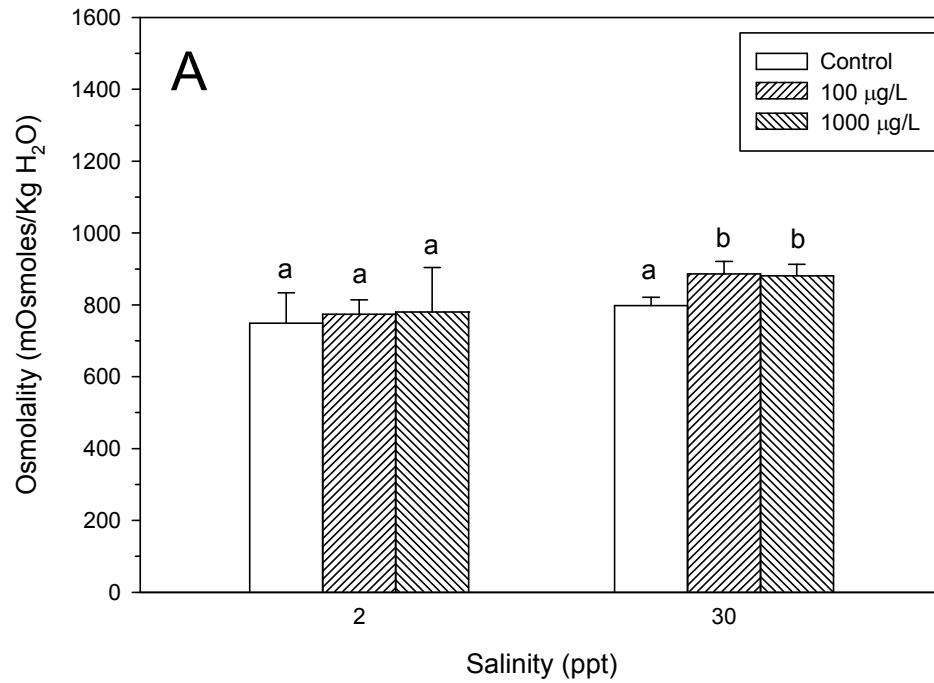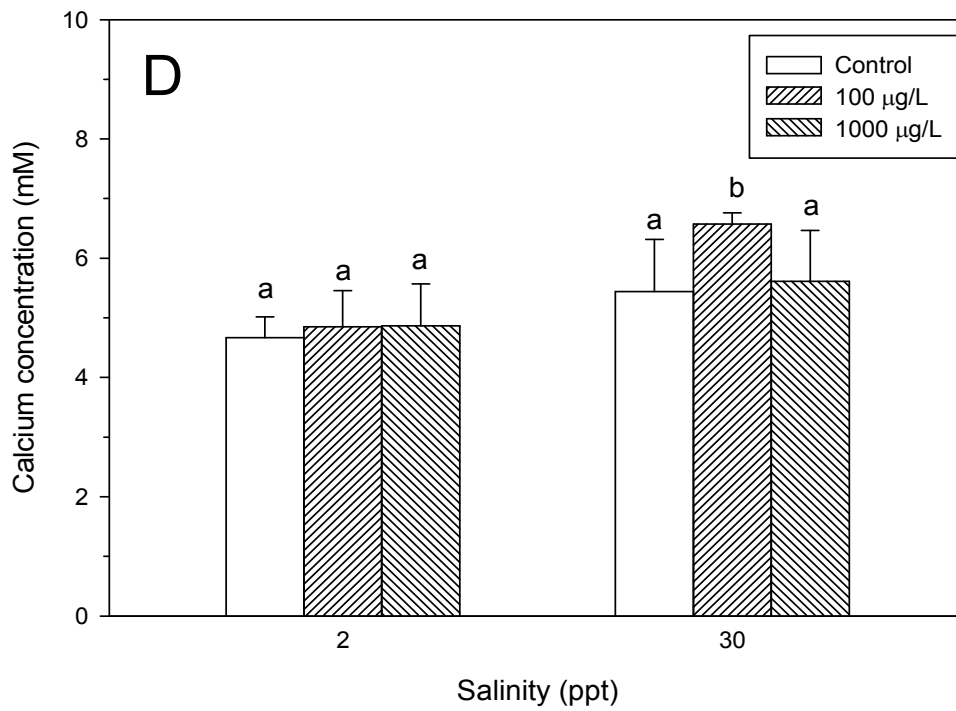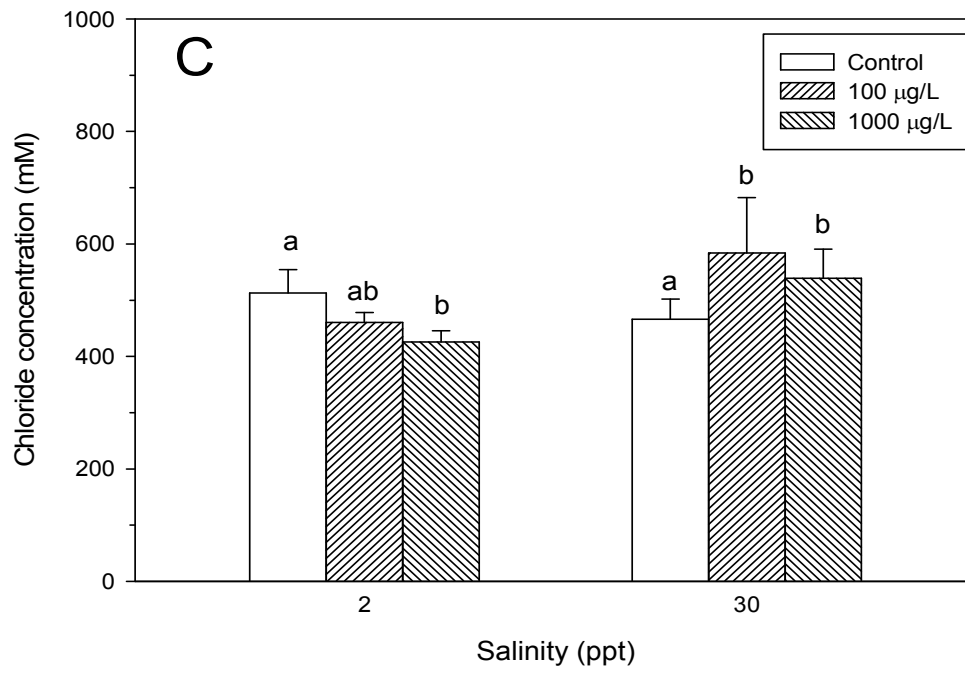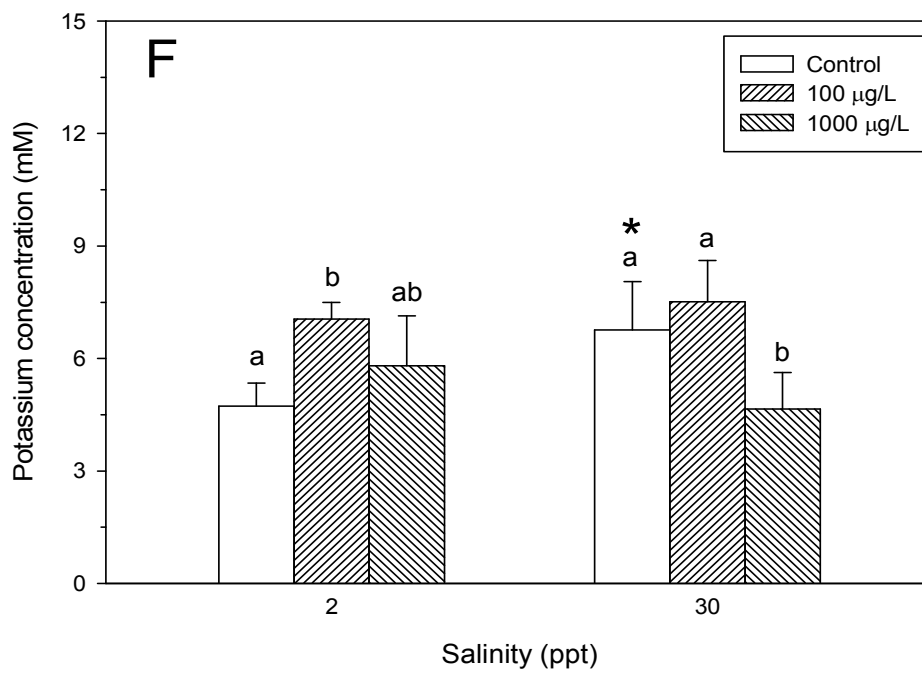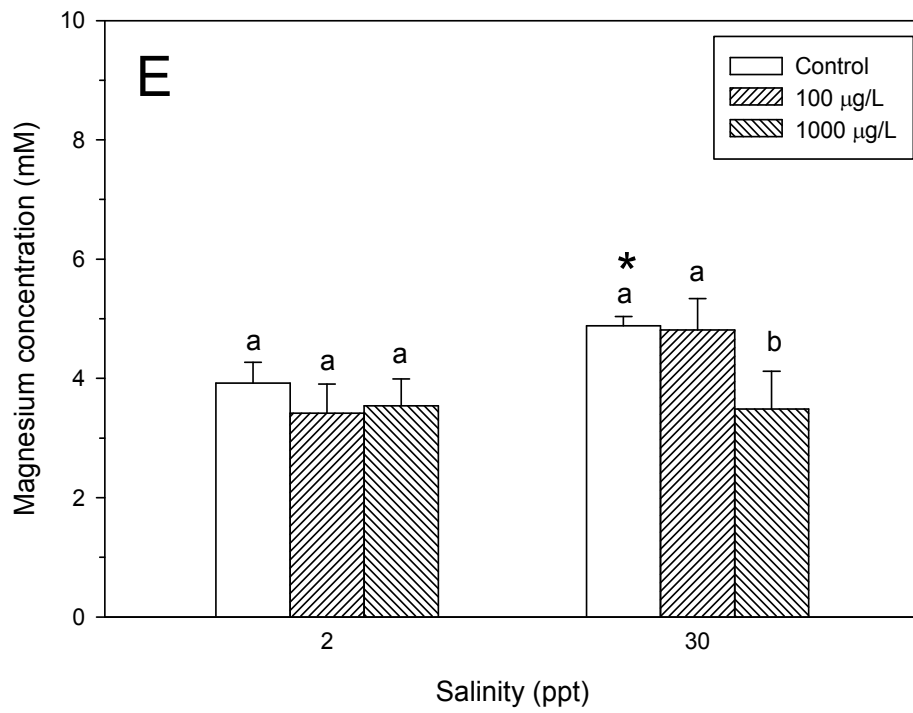
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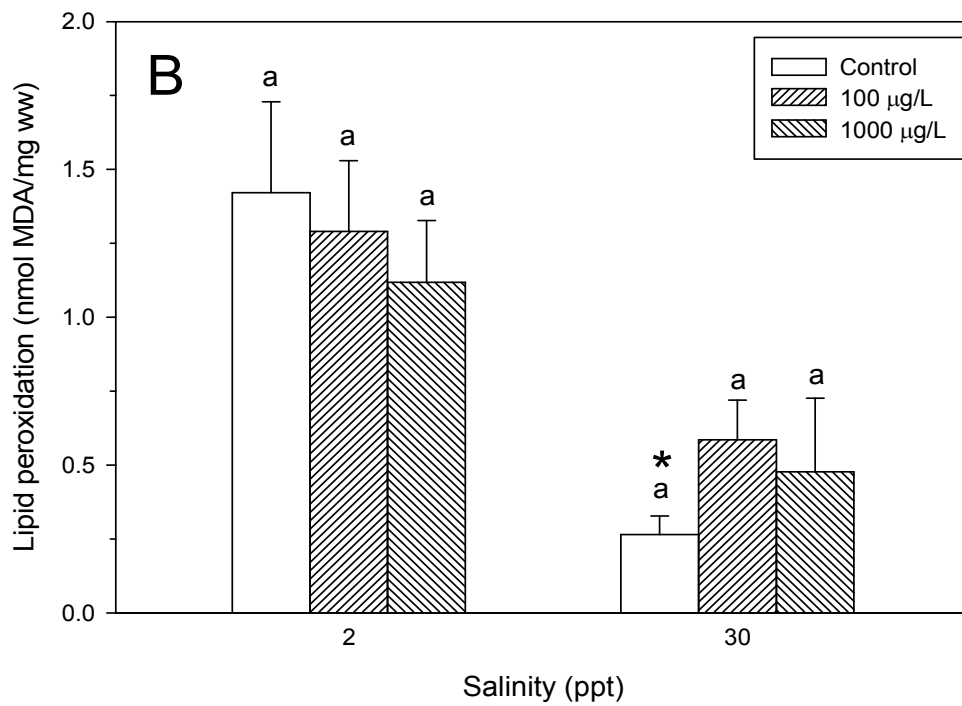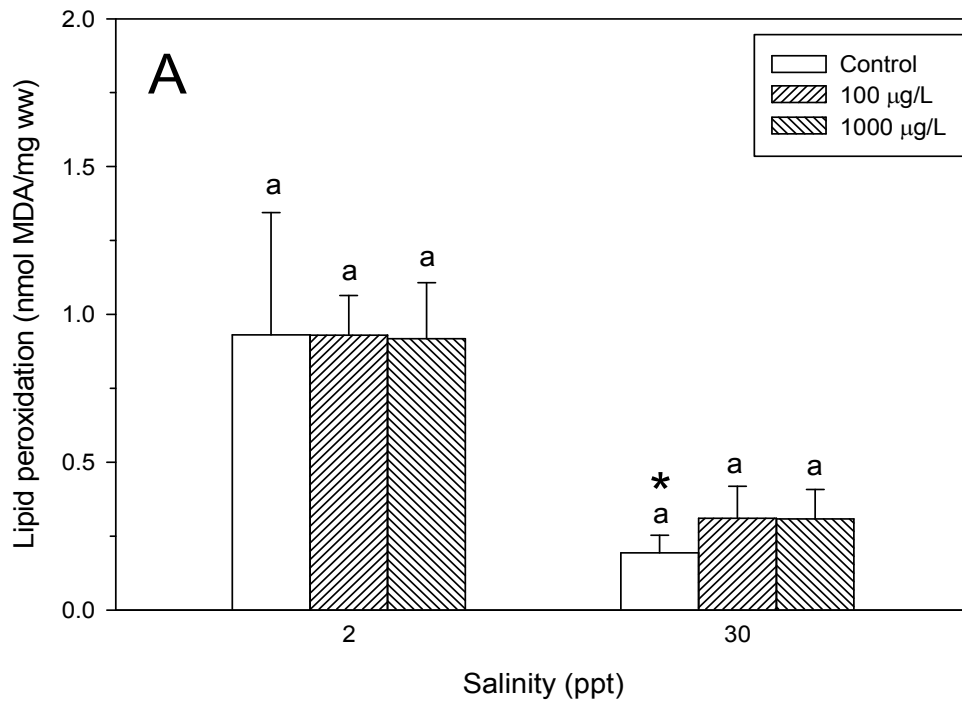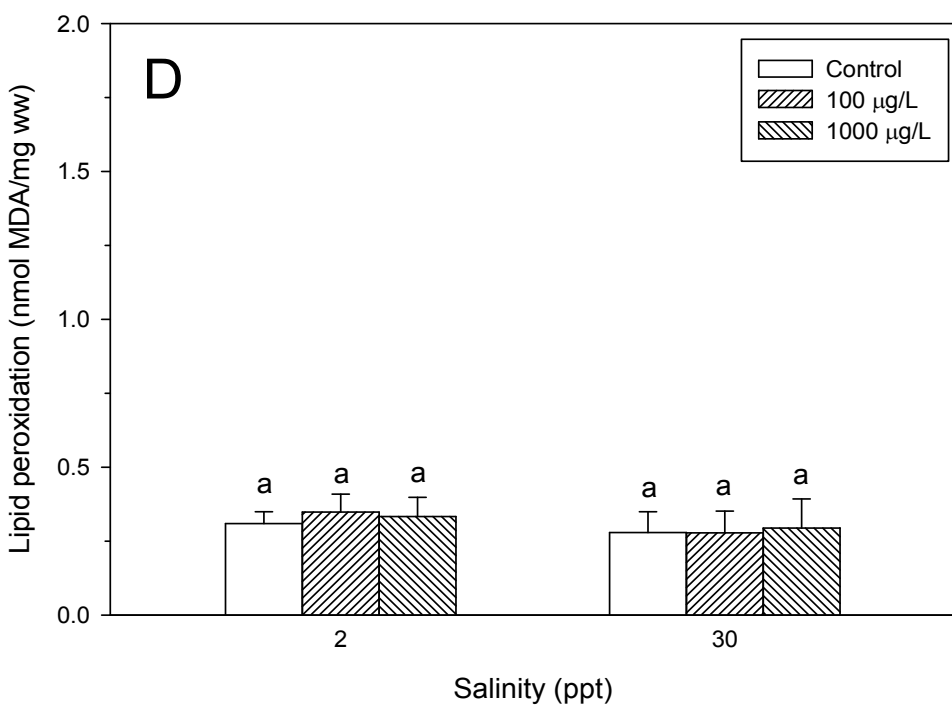
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CONCLUSÕES

Tem sido descrito que a exposição a metais pode induzir distúrbios respiratórios, iônicos e osmóticos, bem como estresse oxidativo em invertebrados eurialinos. Porém, um número muito limitado de estudos foi realizado para avaliar as respostas bioquímicas e fisiológicas de invertebrados eurialinos à exposição ao Ni em diferentes salinidades. No presente estudo, o caranguejo estuarino *Neohelice granulata* foi agudamente exposto a diferentes concentrações do metal em duas salinidades distintas, representando águas estuarinas (salinidade 2) e águas marinhas (salinidade 30).

De uma forma geral, os dados referentes à regulação osmótica e iônica hemolinfática relatados no presente estudo confirmam que *N. granulata* regula adequadamente sua concentração osmótica hemolinfática (hiper-regulação na salinidade 2 e hipo-regulação na salinidade 30) e apresenta ajustes fisiológicos na composição iônica (Na^+, Cl^-, Ca^{2+}, Mg^{2+} e K^+) hemolinfática nas duas salinidades experimentais. Os dados relatados no presente estudo indicam também que, após o período de duas semanas, os caranguejos estavam completamente aclimatados às salinidades experimentais, uma vez que as taxas do metabolismo aeróbico (consumo de oxigênio) e anaeróbico (atividade da LDH) foram semelhantes nas duas salinidades experimentais. No entanto, observou-se que o estresse oxidativo tecidual, especialmente nas brânquias, foi maior quando associado aos ajustes fisiológicos à hiper-regulação osmótica hemolinfática do que quando associado aos processos hipo-regulatórios.

Com relação às respostas dos caranguejos *N. granulata* expostos ao Ni, os dados do presente estudo indicam claramente que os efeitos deste metal estão associados a distúrbios metabólicos (aeróbico e anaeróbico) na salinidade 2, enquanto distúrbios osmóticos e ionoregulatórios são os efeitos mais evidentes do metal na salinidade 30.