Universidade Estadual de Londrina

LUCIANA FERNANDES DE OLIVEIRA

MISTURA DE METAIS ESSENCIAIS (Zn, Mn, Fe) EM CONCENTRAÇÕES AMBIENTALMENTE RELEVANTES:

BIOACUMULAÇÃO E EFEITOS EM BIVALVE E TELEÓSTEO NEOTROPICAIS

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas da Universidade Estadual de Londrina, como parte dos requisitos para obtenção do título de Doutora em Ciências Biológicas (Biodiversidade e Conservação de Habitat Fragmentados).

Orientadora: Prof. Dra. Claudia Bueno dos Reis Martinez

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Discente: **Luciana Fernandes de Oliveira**

Título: "**Mistura de metais essenciais (Zn, Mn, Fe) em concentrações ambientalmente relevantes: bioacumulação e efeitos em bivalve e teleósteo neotropicais**".

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Ao meu afilhado Henrique,
DEDICO

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RESUMO

Pouco se conhece sobre a toxicidade de misturas metálicas em organismos aquáticos biomonitores, apesar dos efeitos isolados destes contaminantes ter sido amplamente investigados nas últimas décadas. No ambiente aquático e em efluentes líquidos diversos os contaminantes ocorrem combinados e, portanto, as interações desses elementos e consequentes efeitos nos organismos devem ser esclarecidos. A mineração de carvão é uma atividade que exemplifica essa problemática, pois seus resíduos contêm diversos metais componentes de rochas associadas ao carvão. Com isso, o presente estudo se preocupou em investigar os efeitos de misturas metálicas em duas espécies Neotropicais, um bivalve *Anodontites trapesialis* e um teleósteo *Prochilodus lineatus*, utilizando diferentes abordagens experimentais. Foi realizado um teste *in situ* de 96 horas com a espécie de bivalve em área de influência de mineração de carvão e testes em laboratório, também de 96 horas, nos quais os bivalves e os peixes foram expostos aos metais Zn, Mn e Fe, isolados ou em misturas, já que estes foram os metais considerados mais relevantes no teste realizado em campo. Nos testes, foi investigada a bioacumulação de metais em diferentes tecidos dos animais e foram considerados biomarcadores de estresse oxidativo, osmorregulação e danos no DNA. A atividade da acetilcolinesterase, concentração de glicogênio e variação da expressão de proteínas pela intensidade de *spots* em gel de eletroforese de duas dimensões também foram considerados nos testes em laboratório com bivalves. Entre os dois modelos biológicos estudados não é possível apontar aquele mais sensível aos efeitos dos metais, já que tanto peixes quanto bivalves apresentaram alterações nos *endpoints* avaliados, principalmente após exposição as concentrações mais altas testadas dos metais isolados (5 mg L^{-1} de Zn ou Mn). Quando em mistura, houve uma tendência de aumento de bioacumulação dos metais, tanto em bivalves quanto em peixes, demonstrando que as misturas testadas podem afetar a toxicocinética dos metais. Considerando-se a ocorrência de danos oxidativos e no DNA, percebe-se que o Mn é mais tóxico em comparação ao Zn e Fe para ambas as espécies, porém mesmo para esse metal os efeitos são mais evidentes após exposição à concentração mais elevada de 5 mg L^{-1}. Em bivalves e peixes, o Zn promove o aumento de metalotioneínas e tióis não proteicos, mostrando que as concentrações testadas deste metal promoveram respostas que protegem os animais contra a ação tóxica do Zn. No teste em laboratório com *A. trapesialis*, sugeriu-se que o Mn promove supressão metabólica, devido a uma série de alterações observadas nos *endpoints* analisados, esse comportamento também parece ter ocorrido após exposição *in situ*. Os biomarcadores relacionados à osmorregulação também sofreram alterações após exposição aos metais, tanto isolados como em mistura, porém não necessariamente causaram hipocalcemia, como esperado, já que o Mn promoveu aumento de Ca^{2+} tanto na hemolinfa dos bivalves, como no plasma dos peixes. Quando em mistura, os biomarcadores também se alteram, porém é difícil apontar os tipos de interações que estão ocorrendo ou atribuí-las a um metal. Com

isso, conclui-se que o estudo dos efeitos de misturas metálicas deve considerar as diversas variáveis atuantes, como o comportamento e defesas dos animais, bem como a particularidade de cada metal que compõe a mistura, concentrações e proporção testadas entre os metais componentes.

Palavras-chave: *Anodontites trapesialis*. Biomarcadores. Mineração de carvão. Mistura metálica. *Prochilodus lineatus*.

OLIVEIRA, Luciana Fernandes de. **Mixture of essential metals (Zn, Mn, Fe) in environmentally relevant concentrations:** bioaccumulation and effects on neotropical bivalve and teleost. 2017. 144f. PhD Thesis – Universidade Estadual de Londrina, Londrina.

ABSTRACT

Little is known about the toxicity of metal mixtures in aquatic biomonitor organisms, although the isolated effects of these contaminants have been widely investigated in recent decades. In the aquatic environment and in various liquid effluents the contaminants occur in combination and therefore the interactions of these elements and consequent effects on the organisms must be clarified. Coal mining is an activity that exemplifies this problem, since its residues contain several metal components in coal associated rocks. Thus, the present study investigated the effects of metallic mixtures on two Neotropical species, a bivalve *Anodontites trapesialis* and a teleost *Prochilodus lineatus*, using different experimental approaches. A 96 hour *in situ* test was carried out with the bivalve species in a coal mining area of influence and laboratory tests, also 96-h test, in which the bivalves and the fish were submitted to single or mixed exposures of the metals Zn, Mn and Fe, since these were the metals considered most relevant in the field test. In the tests, the metals bioaccumulation in different tissues of the animals was investigated and biomarkers of oxidative stress, osmoregulation and DNA damage were considered. The activity of acetylcholinesterase, glycogen concentration and variation of protein expression by the *spots* intensity on two-dimensional electrophoresis gel were also considered in laboratory tests with bivalves. Among the two studied biological models, it was not possible to point out the one most sensitive to the effects of the metals, since both fish and bivalves showed changes evaluated endpoints, especially after exposure to the highest tested concentrations of the isolated metals (5 mg L^{-1} of Zn or Mn). When mixed, there was a tendency for metals bioaccumulation increase in both bivalves and fish, demonstrating that the mixtures tested may affect the toxicokinetics of single metals. Considering the observed oxidative and DNA damages, it was noticed that Mn is more toxic in comparison to Zn and Fe for both species, but even for this metal the effects appear more after exposure to the highest concentration of 5 mg L^{-1}. In bivalves and fish, Zn promotes the increase of metallothioneins and non-protein thiols, showing that the tested concentrations of this metal promoted responses that protect the animals against the toxic action of Zn. In the laboratory test with *A. trapesialis*, it was suggested that Mn promotes metabolic suppression, due to a series of alterations observed in the endpoints, result that also appears to have occurred after *in situ* exposure. The biomarkers related to osmoregulation also underwent changes after exposure to the isolated and combined metals, but did not necessarily cause hypocalcemia as expected, since Mn promoted Ca^{2+} increase in both bivalve hemolymph and fish plasma. When mixed, biomarkers also change, but it is difficult to determine the types of interactions that are occurring or to assign them to one metal. Therefore, it is concluded that the study of the effects of metallic mixtures should consider the various variables, such as the behavior and defense mechanisms of the animals, as well as the particularities of each metal that composes the mixture, their concentrations and proportion.

Key words: *Anodontites trapesialis*. Biomarkers. Coal mining. Metal mix. *Prochilodus lineatus*.

ORGANIZAÇÃO DA TESE

A tese foi organizada de modo a atender às exigências do Programa de Pós-graduação em Ciências Biológicas. Primeiramente, é apresentada uma introdução geral (Capítulo I) que contempla a contextualização do trabalho desenvolvido, sua justificativa, hipóteses e objetivos. Então, são apresentados os cinco capítulos que correspondem a cada manuscrito gerado na presente tese. O Capítulo II refere-se ao artigo já publicado intitulado "Metals bioaccumulation and biomarkers responses in the Neotropical freshwater clam *Anodontites trapesialis*: Implications for monitoring coal mining areas". O terceiro e quarto capítulos abordam também estudos realizados com o bivalve *A. trapesialis*, porém com abordagem diferente já que se trata da bioacumulação e efeitos dos metais em animais expostos em testes de laboratório. O Capítulo III traz, portanto, o manuscrito "Single and combined effects of Zn, Mn and Fe on the Neotropical freshwater bivalve *Anodontites trapesialis*: bioaccumulation and biochemical biomarkers" a ser submetido para publicação no periódico *Aquatic Toxicology* (FI = 3,557). O Capítulo IV apresenta o manuscrito "Manganese promotes metabolic suppression in freshwater bivalve *Anodontites trapesialis*" a ser submetido como *short-communication* no periódico *Environmental Toxicology and Chemistry* (FI = 2,763). Os Capítulos V e VI trazem os resultados obtidos nos estudos de laboratório com o teleósteo *Prochilodus lineatus*, porém analisam biomarcadores distintos. O Capítulo V trata de biomarcadores no fígado e células sanguíneas relacionados ao estresse oxidativo e danos genotóxicos, além de apresentar os resultados de bioacumulação de metais. Esse manuscrito intitulado "Individual and combined effects of Zn, Mn and Fe on the Neotropical teleost *Prochilodus lineatus*: bioaccumulation and oxidative stress biomarkers", será também submetido para avaliação no periódico *Aquatic Toxicology*. O Capítulo VI intitulado "Efeitos do Zn, Mn e Fe isolados e em mistura na osmorregulação do teleósteo dulcícola *Prochilodus lineatus*" apresenta os resultados e discute, de modo ainda preliminar, os efeitos relacionados à biomarcadores de osmorregulação (enzimas e íons) nas brânquias e plasma dos peixes. Pretende-se submeter esse manuscrito para publicação no periódico *Comparative Biochemistry and Physiology, Part C* (FI = 2,546). A tese é, então, finalizada com as considerações finais (Capítulo VII), nas quais as hipóteses são verificadas e discussões comparativas entre os manuscritos são realizadas.

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INTRODUÇÃO GERAL

1.1 Contextualização

1.1.1 Metais como contaminantes em ecossistemas aquáticos

As atividades antrópicas promovem a entrada de substâncias químicas de diversas naturezas no ambiente. Os ecossistemas aquáticos são o receptor final destes compostos, seja por lançamento direto de efluentes, infiltração no solo e contaminação de águas subterrâneas e/ou superficiais, escoamento superficial e precipitação e carreamento de poluentes atmosféricos. Os metais são considerados contaminantes ambientais importantes, sendo que alguns podem ser altamente tóxicos. Por outro lado, muitos metais são essenciais para os organismos, ou seja, são elementos que apresentam funções nos sistemas biológicos e cuja deficiência pode trazer consequências. No caso de metais essenciais a toxicidade ocorre quando as concentrações ótimas são excedidas (Fig.1), já que sua regulação não é mais suficiente para manter os níveis internos do metal controlados (NORDBERG et al., 2007).

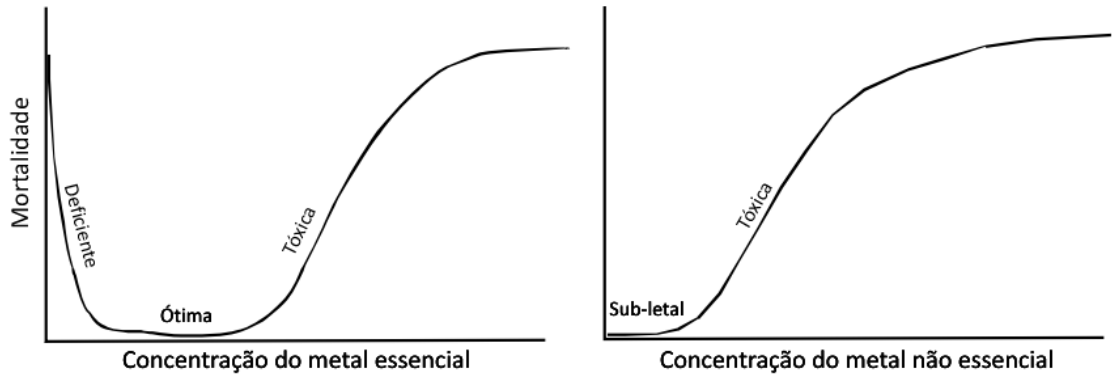

Figura 1 Mortalidade versus concentração para os metais essenciais (à esquerda) e não essenciais (à direita). Um metal essencial estará concentrações quando não for observada mortalidade. Porém em concentrações acima ou abaixo desse limite a mortalidade ocorre. Concentrações crescentes dos metais não essenciais aumentarão o nível de mortalidade experimentado em um grupo de indivíduos expostos (adaptado de NEWMAN, CLEMENTS, 2008).

Apesar dos processos naturais, geoquímicos e intemperismo, representarem importantes fontes de metais no ambiente, as fontes antropogênicas também contribuem com a dispersão destes elementos na atmosfera e na água, em destaque pelas atividades mineradoras, domésticas e industriais, já que esses elementos têm aplicações em diversos segmentos, como na construção civil com a produção de ligas metálicas, na área da saúde, na produção de pigmentos, além de comporem produtos como fungicidas, fertilizantes, combustíveis e produtos de limpeza (NORDBERG et al., 2007). A consciência social da presença de metais tóxicos no ambiente começou a ser mais evidente no início da década de 1950 após o caso da "Doença de Minamata" no Japão, no qual o acúmulo de metil-mercúrio em peixes e moluscos utilizados como fonte de alimentos, causaram sérias consequências para a saúde humana (MCALPINE, ARAKI, 1958). Desde então, foi despertado o interesse de pesquisadores acerca do tema e muitos estudos foram realizados visando o entendimento da distribuição e biodisponibilidade dos metais no ambiente, sua interação e bioacumulação em organismos aquáticos, além da toxicidade desses elementos (NORDBERG et al., 2007, WOOD, 2012).

1.1.2 A interação dos metais com a biota aquática

Uma vez no ambiente aquático os elementos metálicos sofrem especiação e sua forma e biodisponibilidade são dependentes das características químicas e físicas do local, tais como o pH, o teor de matéria orgânica, alcalinidade, temperatura, salinidade e dureza da água (PAQUIN, 2000). Os metais se distribuirão nos compartimentos ambientais, sejam esses abióticos ou bióticos, como consequência dos processos químicos e físicos favorecidos, tais como interações iônicas, complexação com ligantes, associação com partículas, aglomeração, reações de redução e oxidação, adsorção e absorção e o grau de interação entre os íons metálicos e a biota aquática será, portanto, consequência de um conjunto de fatores ambientais (ALLEN, HANSEN, 1996).

A assimilação de compostos exógenos pela biota, seguida de sua retenção, leva à bioacumulação desses nos organismos (NEWMAN,

CLEMENTS, 2008). A bioacumulação em tecidos alvo é resultante da toxicocinética (Fig.2) de um agente tóxico, ou seja, seu tempo de captação, translocação e da capacidade de transformação e eliminação nos organismos (ASHAUER, ESCHER, 2010). A entrada de metais nos animais aquáticos ocorre, principalmente, através do epitélio branquial e pela superfície de todo o corpo, além do alimento e água absorvida pelo epitélio digestivo. Para atravessar membranas biológicas por difusão os metais devem estar complexados a moléculas orgânicas, ou sua assimilação ocorrerá por meio de endocitose, proteínas carreadoras e/ou canais iônicos, que podem ser específicos ou não para cada íon metálico. Após assimilação, ocorrerá a translocação e distribuição desses elementos nos diferentes tecidos e órgãos, cujo padrão será específico para cada metal e dependente do tempo e concentrações de exposição. De modo geral, os órgãos que participam da assimilação, detoxificação e eliminação do excesso de metal são aqueles que apresentam maior acúmulo (WOOD, 2012).

A eliminação dos metais de organismos aquáticos é um processo lento que leva entre semanas e meses. Em peixes, a excreção desses elementos ocorre principalmente através das brânquias, apesar dos rins, intestino, fígado e bile também estarem envolvidos (WOOD, 2012). Em bivalves, algumas estratégias são apontadas como mecanismos de lidar com o excesso de metais, podendo esses ser incorporados em concreções de carbonato de cálcio e fosfato de cálcio em brânquias e manto e até mesmo na concha (HINZMANN et al., 2015), podem ser compartimentalizados em lisossomos após ligação de MT (VIARENGO, NOTT, 1993) ou ainda serem eliminados em pseudofezes (KLERKS, FRALEIGH, 1997) e muco (HIETANEN, SUNILA, KRISTOFFERSSON, 1988, PILLAY, 2013). Há muito ainda a ser esclarecido quanto às vias de eliminação dos metais de organismos aquáticos (WOOD, 2012).

O potencial tóxico de um metal não necessariamente tem uma relação direta com sua bioacumulação, já que os organismos aquáticos têm capacidade de regular e evitar a bioacumulação ou interação desses em locais ou com moléculas mais sensíveis (WOOD, 2012). Vários subsistemas celulares estão envolvidos na proteção contra a intoxicação por metais, incluindo o sequestro desses elementos por moléculas pequenas tais como as

metalotioneínas (MT) e a glutationa reduzida (GSH) e a formação de lisossomos e grânulos. A GSH é reconhecida como o tiol não proteico mais abundante nas células que tem capacidade quelante, ligando-se aos metais assim que eles entram nas células (WOOD, 2012). As MTs, por sua vez, são proteínas de baixo peso molecular, ricas em cisteína, que têm a propriedade de se ligar a vários metais devido à presença de grupos tiol (-SH). Essa característica permite que estas proteínas controlem a quantidade intracelular de metais essenciais, além de proteger contra a ação tóxica de metais não essenciais (AMIARD et al. 2006).

O mecanismo de ação de um agente tóxico varia, portanto, dependendo das suas características físicas e químicas, além das particularidades dos organismos afetados. A toxicodinâmica descreve o tempo de ação de um composto tóxico em seus sítios alvo, bem como subsequentes efeitos e respostas dos organismos à presença do contaminante, chegando aos efeitos em nível de organismos, como a morte (ASHAUER, ESCHER, 2010). A toxicocinética e toxicodinâmica de um metal determinarão o mecanismo de ação e o grau de toxicidade deste para determinada espécie animal (Fig.2).

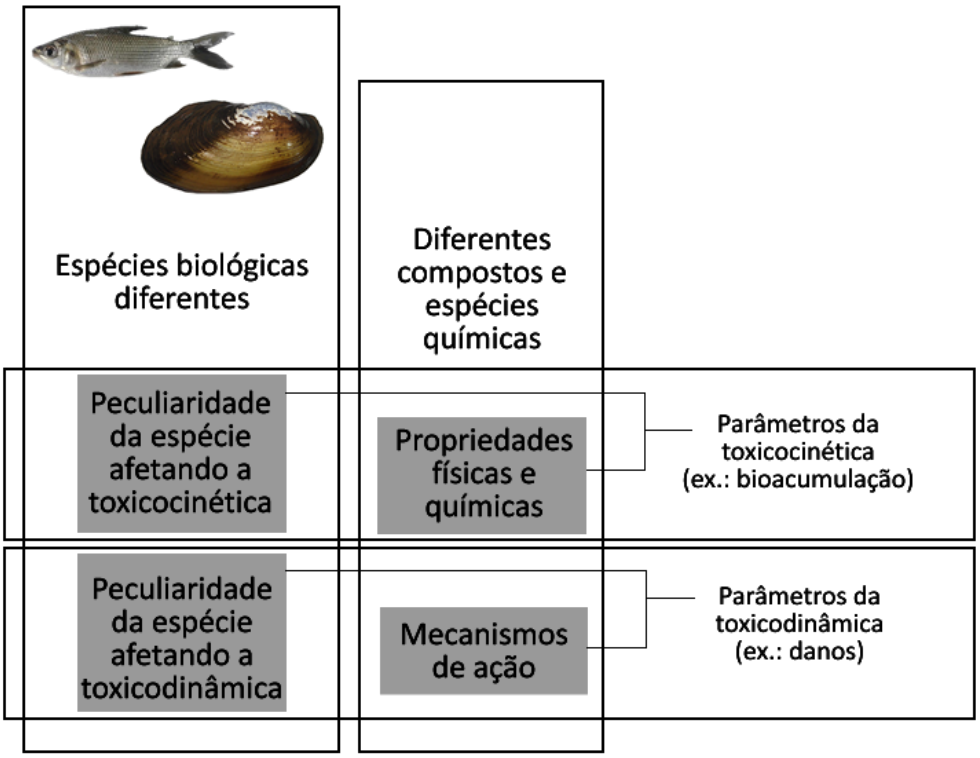

Figura 2 A conexão entre os parâmetros de toxicocinética e toxicodinâmica às características químicas e físicas dos compostos e peculiaridades das espécies afetadas (Ashauer e Escher, 2010, com modificações).

1.1.3 A problemática das misturas metálicas: o exemplo da atividade de mineração de carvão

Os organismos aquáticos são geralmente expostos a misturas de contaminantes, por vezes em altas concentrações, devido às ações antrópicas. Atualmente, os metais são considerados uma classe bem conhecida e estudada de contaminantes, porém, trabalhos que consideram os efeitos desses compostos em organismos aquáticos e discutem seus modos de ação conjuntos vêm sendo realizados mais recentemente (GAO et al., 2016, VAN GENDEREN et al., 2015). Muitos estudos ainda têm por objetivo o estudo da toxicidade de um composto individualmente e as regulamentações acerca das concentrações de metais permitidas em ambientes aquáticos são ainda fortemente baseadas nos efeitos de metais isolados (GAO et al., 2016). A legislação brasileira, representada pela Resolução CONAMA 357/2005, estabelece limites da concentração de compostos isoladamente em efluentes e água, sem que se conheçam de fato os efeitos que a interação deles pode causar aos organismos. Avaliar a toxicidade e os riscos associados à contaminação por misturas em ecossistemas ou aos seres humanos ainda é um dos maiores desafios de cientistas, avaliadores de risco e tomadores de decisão (SPURGEON et al., 2010). Os compostos presentes em uma mistura podem sofrer interações associadas a diferentes processos. As interações podem ocorrer no meio externo, de forma que a presença de um composto pode aumentar ou diminuir a disponibilidade de outro no meio, no processo de toxicocinética (absorção, distribuição, metabolismo e excreção) ou ainda no processo de toxicodinâmica (interações relacionadas aos sítios receptores e modo de ação) (Fig.2). As diferentes interações dos compostos presentes em uma mistura promovem nos organismos expostos a ela efeitos diferenciados, que podem ser aditivos, agonistas ou antagonistas (NIKINMAA, 2014, SPURGEON et al., 2010).

A mineração de carvão é uma das indústrias com maior impacto no meio ambiente e pode causar muitas mudanças ambientais, como a acidificação das águas superficiais, o aumento do total de sólidos suspensos e dissolvidos e o aumento dos níveis de metais, além de alterar as características dos sedimentos dos rios (TIWARY, 2001). Quando os minerais associados ao carvão são expostos à água e oxigênio na presença de bactérias *Thiobacillus*,

ácido sulfúrico e hidróxido ou sulfato de ferro são produzidos, o que provoca a dissociação de metais e outros constituintes, liberando-os na água e formando a drenagem ácida de mina (DAM). A composição desse efluente é variável e depende do tipo de depósito mineral de origem (SHEORAN, SHEORAN, 2006). Os metais ferro, alumínio, zinco e manganês são frequentemente encontrados em altas concentrações em corpos d'água próximos as mineradoras de carvão (BHARTI, BANERJEE, 2013, CAMPANER, LUIZ-SILVA, 2009, MACCAUSLAND, MCTAMMANY, 2007, ZOCCHE et al., 2014) e apresentam-se, portanto, como um problema recorrente deste tipo de atividade. Um *screening* preliminar realizado na região de mineração de carvão do município de Figueira, PR, Brasil, revelou que estes são os metais que ocorrem em concentrações mais elevadas, inclusive superando alguns limites estabelecidos pela legislação brasileira (CONAMA 357, 2005), apresentando maiores concentrações na água coletada à jusante em comparação a montante da mineradora (Tabela 1).

Tabela 1 Concentração de metal total (T) e dissolvido (D) em amostras de água coletadas a montante e jusante de mineradora de carvão.

	Montante		Jusante			**CONAMA 357**
(mg.L^{-1})	**T**	**D**	**T**	**D**		Classes 1 e 2 – Classes 3 e 4
Mn	0,254	0,236	0,671	0,965	**T**	0,1 – 0,5
Al	0,13	0,11	0,45	0,1	**D**	0,1 – 0,2
Fe	1,95	1,72	18,42	18,14	**D**	0,3 - 5
Zn	0,12	0,09	1,04	0,95	**T**	0,18 - 5
Ni	0,002	0,002	0,009	0,008	**T**	0,025
Ag	0,002	0,001	0,003	0,001	**T**	0,01 – 0,05
Cd	<0,001	<0,001	<0,001	<0,001	**T**	0,001 – 0,01
Pb	0,003	<0,001	0,001	<LD	**T**	0,01 – 0,033
Cr	0,005	0,005	0,007	0,006	**T**	0,05
Cu	<LD	<LD	<LD	<LD	**D**	0,009 – 0,013

<LD: abaixo do limite de detecção. Fonte: do autor.

Ferro, alumínio, zinco e manganês são metais abundantes na crosta terrestre e, com exceção do alumínio, são elementos traços considerados essenciais para os organismos. A toxicidade do zinco, manganês e ferro ocorre, portanto, quando a biota é exposta a concentrações elevadas e não naturais, seguindo o padrão demonstrado na Figura 1. No ambiente, as concentrações desses metais dissolvidos na água são bastante variáveis, pois

além de dependerem dos fatores físicos e químicos, como já discutido, também variam de acordo com as características geológicas locais. As concentrações naturais totais de Zn em água doce variam de 0,0001 – 0,05 mg L^{-1} e superam 4 mg L^{-1} em áreas impactadas (WHO, 2001). Os níveis de manganês dissolvido na em água doce superficial são geralmente mais baixas que 0,2 mg L^{-1}, dificilmente ultrapassando 1 mg L^{-1} em águas livres de fontes antropogênicas, porém valores acima de 4 mg L^{-1} também já foram observados em águas que recebem efluentes de DAM (WHO, 2004). Já as concentrações de ferro dissolvido em água doce são geralmente muito baixas (< 5 µg L^{-1}), pois em condições aeróbicas ocorre a formação de íons férricos insolúveis (PHIPPEN et al, 2008), entretanto concentrações preocupantes de ferro dissolvido podem ser encontradas em ambientes dulcícolas como em um rio no Colorado onde estas superaram 2,6 mg L^{-1} (FEY et al. 2002).

1.1.4 Estudos de campo x estudos em laboratório

Diferentes abordagens podem ser utilizadas em Ecotoxicologia a fim de avaliar a toxicidade de contaminantes para a biota aquática. A realização de testes *in situ*, que consistem na manutenção de organismos em recipientes-teste no ambiente, pode ser uma boa ferramenta para o estudo da qualidade de ambientes aquáticos, pois permitem o estudo de uma área específica abrangendo todas as variações físicas e químicas durante o período de experimento (VIARENGO et al., 2007). Entretanto, devido à falta de estabilidade destas variáveis ambientais estes experimentos de difícil repetibilidade e as relações de causa e efeito são difíceis de serem estabelecidas (RAND et al. 1995) o que dificulta ainda mais o estudo da toxicidade de misturas. Por outro lado, experimentos realizados em laboratório permitem o estabelecimento das relações de causa e efeito, assim como de comparações entre a toxicidade de compostos isolados ou em misturas mais simples. Apesar disso, os resultados obtidos nessa modalidade de teste muitas vezes superestimam os efeitos adversos de contaminantes, pois não consideram a habilidade adaptativa de populações naturais, as interações entre espécies, as mudanças que ocorrem no ambiente e a biodisponibilidade dos

contaminantes pode ser maior, devido ao uso de águas que não contém matéria orgânica como no ambiente natural (RAND et al. 1995).

1.1.5 Biomonitores

Animais aquáticos estão expostos diretamente a muitos compostos dissolvidos ou suspensos na água ou ainda depositados em algum compartimento do ecossistema, e podem, portanto, ser utilizados como biomonitores do ambiente. Segundo Rand (1995), biomonitores são organismos que sobrevivem em ambientes contaminados, mas são sensíveis às alterações ambientais, apresentando alterações subletais em nível individual e provendo sinais precoces de alerta da qualidade ambiental.

A toxicidade de contaminantes pode variar entre diferentes espécies aquáticas biomonitoras devido a fatores como o nível trófico, hábito alimentar e comportamento. A rota de entrada principal de compostos dissolvidos na água, tanto para peixes quanto moluscos, é a superfície branquial (WOOD, 2012), porém a captação de compostos por meio do alimento também é uma fonte importante de entrada de compostos tóxicos nos organismos aquáticos. As vias pelas quais um organismo aquático absorve um contaminante podem interferir nos seus efeitos bem como nos órgãos/tecidos-alvo nos quais esses efeitos serão observados. Um organismo que ocupa nível trófico alto, por exemplo, absorverá por via trófica maior quantidade de compostos em comparação a organismos de níveis mais baixos, devido ao processo de biomagnificação (NEWMAN, CLEMENTS, 2008).

Os hábitos alimentares também são fatores importantes na determinação da distribuição e vias de toxicidade de contaminantes em organismos aquáticos. Peixes detritívoros, por exemplo, ingerem diretamente partículas depositadas no fundo dos ecossistemas aquáticos, compartimento no qual são verificadas altas concentrações de contaminantes, enquanto peixes carnívoros têm como principal fonte de entrada de contaminantes seu alimento e a água filtrada nas brânquias (NEWMAN, CLEMENT, 2008). No caso de bivalves dulcícolas que se enterram em sedimentos lodosos, ocorre o íntimo contato com o sedimento, além do elevado volume de água filtrado

diariamente, em torno de 700 a 1000 mL/indivíduo/h para a espécie *Anodontites trapesialis*, por exemplo (LOAYZA-MURO, ELÍAS-LETTS, 2007).

Considerando os fatores interespecíficos que diversificam a ação de contaminantes em organismos aquáticos, é indicado que na avaliação e monitoramento da integridade de ecossistemas aquáticos sejam incluídas diversas espécies. A escolha dos biomonitores “ideais” precisa seguir alguns critérios, citados já na década de 90 por Johnson, Wiederholm, Rosemberg (1993), e, portanto, os organismos escolhidos devem atender o máximo de características possível desta lista: ser taxonomicamente bem definidos e facilmente reconhecíveis por não especialistas; apresentar distribuição geográfica ampla; ser abundantes ou de fácil coleta; ter baixa variabilidade genética e ecológica; preferencialmente possuir tamanho grande; apresentar baixa mobilidade e longo ciclo de vida; dispor de características ecológicas bem conhecidas; ter possibilidade de uso em estudos em laboratório.

No ambiente aquático, bivalves e teleósteos são frequentemente selecionados como biomonitores (MARKERT, BREURE, ZECHMEISTER, 2003), porém ainda existe uma carência de estudos com espécies neotropicais, especialmente no ambiente dulcícola. O teleósteo *Prochilodus lineatus* (Valenciennes, 1836) (Fig.3) da ordem Characiformes, família Prochilodontidae, conhecido popularmente no Brasil como curimba ou curimbatá, é nativo da região Neotropical. Esta espécie já se mostrou sensível a metais, tais como o alumínio (CAMARGO et al., 2009), chumbo (MONTEIRO et al., 2011, RIBEIRO et al., 2014), cobre (NASCIMENTO et al., 2012) e níquel (PALERMO et al., 2015) e é, portanto, um potencial biomonitor para os ambientes Neotropicais. É incluída como uma das espécies mais abundantes e importantes da região Sul e Sudeste do Brasil, com maior frequência na porção superior da bacia do rio Paraná, principalmente nos rios Grande, Pardo e Mogi-Guaçu (LEONHARDT et al., 2002), onde é muito utilizada na alimentação humana. É considerada vulnerável a alterações ambientais devido ao comportamento migratório e hábito alimentar detritívoro (SHIBATTA et al., 2007), ficando expostos a substâncias químicas, que eventualmente, tenham ficado retidas no sedimento (MARTINEZ, CÓLUS, 2002). Por outro lado, o bivalve *Anodontites trapesialis* (Lamarck, 1819) (Fig.3) da ordem Unionidae, pertencente ao gênero de maior representatividade da família Mycetopodidae que é endêmico da região

Neotropical, vem sendo considerada como um potencial biomonitor e seus aspectos biológicos e ecológicos passaram a ser mais bem estudados (CALLIL, JUNK, 1999). Essa espécie é exclusiva da América do Sul e frequentemente representa um problema em pisciculturas devido ao fato de sua larva do tipo lasídio ser parasita temporária de peixes (FELIPI, SILVA-SOUZA, 2008). Os bivalves apresentam uma série de características que seguem os critérios expostos anteriormente para definição de biomonitores adequados, dentre as quais se podem citar: a ocorrência em altas densidades; fácil coleta e identificação; tamanho suficiente para realização de análises em tecidos separadamente; são bentônicos ou sésseis e filtram grande volume de água diariamente; tem elevada capacidade de acumular contaminantes sem chegar à morte; e apresentam respostas a contaminantes presentes no meio (BOENING, 1999).

Figura 3 *Prochilodus lineatus* (à esquerda) e *Anodontites trapesialis* (à direita).

1.1.6 Biomarcadores como ferramentas de monitoramento

Biomarcadores são definidos como variações induzidas por agentes tóxicos em componentes moleculares ou celulares, processos, estruturas ou funções, determináveis em sistemas biológicos ou amostras (DEPLEDGE et al. 1995), cuja principal característica é o potencial de antecipar prejuízos em níveis de organização biológica superiores, podendo, portanto, serem usados de maneira preventiva (CAJARAVILLE et al. 2000). Devem ser selecionados biomarcadores capazes de indicar se o organismo foi exposto a poluentes (biomarcadores de exposição) e/ou se a magnitude da resposta ao poluente afeta o bem estar do organismo (biomarcadores de efeito) (CAJARAVILLE et

al. 2000; MONSERRAT et al. 2007). Os biomarcadores podem indicar, muitas vezes, as vias afetadas por determinado composto e o modo de ação que confere sua toxicidade.

A toxicidade de metais pode estar relacionada com a interação desses elementos com transportadores de membrana e atividade de enzimas envolvidas nos processos osmorregulatórios causando seu desajuste (KENNEDY, 2011, RIBEIRO et al., 2014). Em animais de água doce, cujo ambiente é hiposmótico em relação aos fluidos internos, há necessidade de captação ativa de íons através das brânquias. Alguns metais apresentam características que ocasionam o mimetismo iônico, ou seja, por sua similaridade utilizam as rotas de entrada de macronutrientes como Na^+, K^+, Cl^- e Ca^{2+} provocando alterações na captação desses íons. Íons metálicos divalentes como o Zn^{2+}, Mn^{2+}, Cd^{2+} e Pb^{2+} mimetizam o cálcio, enquanto os cátions monovalentes utilizam os transportadores e canais de Na^+ (WOOD, 2012). Para espécies cuja tomada de cálcio é elevada, como no caso de bivalves que utilizam esse elemento na formação das conchas, esse processo torna-se ainda mais relevante (NORDBERG et al., 2007). Além disso, certos metais podem interferir diretamente na atividade de ATPases transportadoras de macronutrientes (Na^+, K^+, Cl^-) e na atividade da anidrase carbônica, enzima que atua na manutenção do equilíbrio ácido-base e indiretamente na captação de íons sódio e cloreto (KENNEDY, 2011).

A toxicidade dos metais está frequentemente relacionada à capacidade desses elementos em causar estresse oxidativo (GHASEMI, ROSTAMPOUR, RANJBAR, 2014, SEVCIKOVA et al., 2011) por promoverem o aumento na geração de espécies reativas de oxigênio (ERO) e/ou enfraquecerem as defesas antioxidantes. Por outro lado, alguns metais, como o zinco e o manganês têm papel relacionado às defesas antioxidantes (COASSIN, URSINI, BINDOLI, 1992, POWELL, 2000), uma vez que estão associadas à atividade de superóxido dismutase (SOD). O zinco apresenta também relação íntima com a regulação da síntese de MTs que, por sua vez, regula as concentrações de íons metálicos essenciais ou não essenciais no citoplasma e participa da remoção de espécies reativas de oxigênio (ERO) (LAITY, ANDREWS, 2007). Nos bivalves Unionoida, altas concentrações de manganês em tecidos moles foram evidenciadas e relacionadas com a capacidade antioxidante desses

bivalves (CAMPANELLA, GATTA, RAVERA, 2005). Como já discutido anteriormente, o limiar entre a essencialidade e toxicidade desses metais é determinado por concentrações limites que atendem o potencial regulatório dos animais (Fig.1).

A indução do estresse oxidativo, em geral, pode ocorrer por mecanismos distintos e, por isso, os metais são divididos de acordo com suas propriedades químicas em dois grupos (LUSHCHAK, 2016). Metais redox ativos ou com "estado valência mutável" como ferro, manganês, cobre, cromo e vanádio geram ERO através de ciclo redox, podendo estar envolvidos na reação de Fenton e Haber–Weiss. Em organismos aeróbicos, o oxigênio molecular é essencial, principalmente por ser o receptor final na cadeia respiratória da mitocôndria. A redução de oxigênio à água, consequência deste processo de respiração celular, requer quatro elétrons transferidos em reações sequenciais, produzindo moléculas intermediárias, como o ânion radical superóxido ($O2^{\bullet-}$), peróxido de hidrogênio (H_2O_2) e radical hidroxil (•OH), que são as ERO. Metais redox ativos podem interferir nessas reações causando aumento da produção de ERO. Por outro lado, os metais sem potencial redox ou com "estado de valência constante" como mercúrio, níquel, chumbo e cádmio, prejudicam as defesas antioxidantes, especialmente as que envolvem antioxidantes que contém grupos -tiol e enzimas (LUSHCHAK, 2016, STOHS, BAGCHI, 1995). As defesas antioxidantes estão presentes normalmente nas células com papel de eliminar essas moléculas reativas e evitar sua toxicidade. Dão-se por ação conjunta de enzimas, como a SOD, catalase (CAT) e glutationa peroxidase (GPx) e moléculas de menor peso molecular como a GSH e as MTs (HERMES-LIMA, 2004).

Lushchak (2016) define o estresse oxidativo como "um aumento transitório ou crônico dos níveis basais de ERO, que perturba processos celulares essenciais e vias de sinalização, incluindo aquelas que envolvem as ERO, levando à modificação oxidativa de constituintes celulares que podem culminar na morte celular por necrose ou apoptose". Ele pode se estabelecer devido ao aumento da produção de ERO, redução das defesas antioxidantes ou, ainda, pela combinação desses dois fatores. As ERO são extremamente reativas, podendo se ligar a DNA, lipídios e proteínas causando danos deletérios (MACKENZIE, 2008). O radical hidroxil (•OH) é a principal ERO que

reage com ácidos graxos poliinsaturados (PUFAs), ácidos nucléicos e proteínas, causando danos. Quando uma espécie oxidante retira um átomo de hidrogênio de um grupo metileno de um PUFA inicia-se uma cadeia de reações chamada de lipoperoxidação. Forma-se um radical lipídico que em sequência reage com oxigênio molecular, formando um radical peroxil. Estes, por sua vez, alimentam a cadeia de reações da lipoperoxidação agindo como oxidantes de PUFAs ainda não oxidados (HERMES-LIMAS, 2004). Como consequências deste dano podem-se observar a redução na fluidez de membranas biológicas, inativação de receptores e enzimas de membrana e o aumento da permeabilidade da mesma (MANDUZIO et al. 2005). Danos oxidativos no DNA são reconhecidos como a principal causa de morte celular e mutações em organismos aeróbicos e são induzidos principalmente pelos radicais hidroxil que resultam da reação de Fenton (BJELLAND, SEEBERG, 2003). Outro efeito que o estresse oxidativo pode ter sobre o organismo é a carbonilação de proteínas, através da qual as cadeias laterais de aminoácidos são modificadas através da formação de grupamentos aldeídicos e cetônicos (LEVINE et al., 1994).

1.2 Justificativa

Os metais constituem uma classe de contaminantes amplamente estudada, porém ainda há uma falta de conhecimento de suas interações e efeitos nos ecossistemas quando presentes em misturas. É necessário considerar que os organismos aquáticos são expostos a misturas polimetálicas, que podem ocorrem no ambiente por ação antrópica, como em regiões de mineração de carvão. Em um estudo preliminar, realizado no município de Figueira (região central do estado do Paraná), verificamos o incremento na concentração de alguns metais na água de um córrego, provavelmente associado à atividade de mineração de carvão. Nessa área as concentrações de zinco, manganês, ferro e alumínio, aumentaram a jusante da mineradora e foram detectadas em concentrações acima da permitida pela resolução CONAMA (357/2005). A investigação dessa área de mineração e desses metais em particular foi considerada um estudo de caso de relevância ambiental que permitiria discutir a problemática de misturas metálicas e seus efeitos em animais aquáticos.

As diferentes modalidades de testes, *in situ* e em laboratório, quando usadas em conjunto, se complementam. Em campo avalia-se a situação real contendo, porém, variáveis não controladas, enquanto em laboratório é possível realizar testes controlados e verificar os efeitos dos metais isoladamente ou em misturas mais simples. No presente trabalho, foi realizado um teste *in situ* com o bivalve *A. trapesialis* e, posteriormente, os efeitos dos metais zinco e manganês foram avaliados individualmente em três diferentes concentrações, em mistura binária e na presença ou ausência de ferro, tanto na mesma espécie de bivalve quanto no teleósteo *P. lineatus*. Dois critérios foram utilizados para a escolha das concentrações de zinco, manganês e ferro utilizadas nos estudos realizados em laboratório, que foram as concentrações limite estabelecidas na legislação (CONAMA 357/2005) e as concentrações encontradas a jusante do córrego perto de uma área de mineração de carvão.

A toxicidade dos metais pode variar de acordo com as características do meio aquático bem como dos animais que interagem com estes compostos. As características de ecossistemas aquáticos da região Neotropical diferem daquelas de ambientes temperados e frios, tanto quanto às variáveis físicas e químicas, quanto às biológicas, porém a quantidade de estudos com espécies

de água doce nativas desta região ainda é bastante reduzida. As duas espécies escolhidas como alvo de estudo do presente trabalho são nativas da região Neotropical e alguns estudos mostram que elas são potenciais biomonitores para ambientes dulcícolas. Bivalves e peixes teleósteos diferem em diversos aspectos que podem interferir na toxicocinética de metais, como por exemplo, os diferentes tipos de circulação (aberta e fechada), as distintas vias de captação e obtenção de alimento, os padrões de comportamento, dentre outros. O uso desses dois modelos biológicos é importante, tanto para a determinação dos efeitos que são intrínsecos dos compostos químicos avaliados bem como daqueles mais relacionados à particularidade dos modelos utilizados.

A bioacumulação e os efeitos de um metal nos diversos órgãos dos animais podem ser alterados devido à presença de outro metal no meio. Isso pode ocorrer devido a interações entre as moléculas ainda fora do organismo, ou em nível toxicocinético e/ou toxicodinâmico. Assim, a quantificação do conteúdo de metais em diferentes órgãos e a análise de biomarcadores relacionados ao modo de ação dos mesmos devem contribuir para elucidar em que nível essas interações ocorrem, se elas são antagônicas ou agonistas, bem como o risco que elas representam para essas espécies quando encontradas em conjunto no ambiente.

1.3 Hipóteses

1. As diferentes abordagens experimentais, testes em campo e laboratório, utilizando *Anodontites trapesialis* devem produzir resultados que se sobrepõem.
2. Após testes, ocorre aumento da concentração de metais em *Anodontites trapesialis* e *Prochilodus lineatus*, mas varia entre os diferentes órgãos e concentrações testadas de Zn e Mn isoladamente.
3. Bivalves *Anodontites trapesialis* e peixes *Prochilodus lineatus* diferem quanto às respostas e efeitos em decorrência da exposição aos metais, sendo os peixes mais susceptíveis a ação tóxica dos mesmos após um período de 96 horas.
4. Os metais zinco, manganês e ferro têm propriedade pró-oxidante e causam estresse oxidativo.
5. Os metais zinco, manganês e ferro causam desajuste na osmorregulação desses animais, principalmente hipocalcemia.
6. A mistura dos metais promove interação antagônica já que os mesmos competem por vias de influxo, promovendo diminuição de bioacumulação e alteração em biomarcadores quando comparado aos efeitos do metal isolado.

1.4 Objetivos

Objetivo geral

Estudar os efeitos os metais essenciais zinco, manganês e ferro e suas possíveis interações por meio da análise da bioacumulação e de biomarcadores em diferentes órgãos de espécies neotropicais de bivalve e de teleósteo, após 96 horas de exposição aos metais, isoladamente e em mistura, tanto em campo quanto em laboratório.

Objetivos específicos

- Apontar os principais metais envolvidos com a problemática ambiental relacionada à atividade de mineração de carvão da região central do estado do Paraná.
- Confrontar os resultados obtidos em campo e laboratório a fim de caracterizar as respostas e os efeitos de metais, associados ou não, para o bivalve dulcícola Neotropical *Anodontites trapesialis*.
- Definir as concentrações basais dos metais Zn, Mn e Fe em vários tecidos de *A. trapesialis* e *P. lineatus*, bem como a alteração dessas concentrações em consequência da exposição a esses metais.
- Indicar diferenças e semelhanças entre as espécies neotropicais de bivalve e teleósteo quanto às variáveis estudadas.
- Evidenciar os efeitos do zinco, manganês e ferro em parâmetros relacionados ao estresse oxidativo em bivalve e teleósteo neotropicais de água doce.
- Verificar se biomarcadores de estresse oxidativo são adequados para examinar a toxicidade de metais essenciais.
- Verificar se zinco, manganês e ferro causam desajustes na osmorregulação de *A. trapesialis* e *P. lineatus*.
- Apontar a ocorrência de interações entre os metais estudados quanto à bioacumulação e efeitos nas duas espécies estudadas.

- Definir *endpoints* adequados para o monitoramento de áreas contaminadas por mistura dos metais zinco, manganês e ferro.

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Capítulo II

Metals bioaccumulation and biomarkers responses in the Neotropical freshwater clam *Anodontites trapesialis*: Implications for monitoring coal mining areas

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O referido artigo (Apêndice I) trata de um estudo realizado em campo, no qual bivalves *A. trapesialis* foram expostos ao longo de um córrego próximo à área de mineração de carvão. Essa problemática foi escolhida, devido a frequente contaminação por mistura metálica nessas áreas. Exposições *in situ* foram feitas por período de 96 horas em quatro pontos, dois a montante e dois a jusante da mineradora, para avaliar a bioacumulação de metais e efeitos subletais de metais nessa espécie. Com base nesse estudo foram estabelecidas concentrações de Zn, Mn e Fe para realização de testes em laboratório (Capítulos IV, V, VI), que investigaram, em condições controladas, a bioacumulação e efeitos desses metais, isolados ou em misturas ambientalmente relevantes.

Capítulo III

Single and combined effects of Zn, Mn and Fe on the Neotropical freshwater bivalve *Anodontites trapesialis*: bioaccumulation and oxidative stress biomarkers

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Single and combined effects of Zn, Mn and Fe on the Neotropical freshwater bivalve *Anodontites trapesialis*: bioaccumulation and biochemical biomarkers

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Abstract

In order to understand the effects of single or mixed Zn, Mn and Fe exposures on the Neotropical bivalves *Anodontites trapesialis*, we run short-term laboratory experiments. After 96 h-exposure, organs (gills, mantle, digestive gland, muscle, hemolymph) were removed for metal bioaccumulation analysis, oxidative stress biomarkers (reactive oxygen species (ROS), total antioxidant capacity, lipoperoxidation (LPO), proteins carbonylation (PC), metallothionein (MT), activity of superoxide dismutase and glutathione S-transferase and hemocytes DNA damage) and cholinesterase (ChE) activity evaluation. We run three independent tests. In Zn test clams were exposed to three concentrations of Zn (0.18 mg L^{-1}, 1.0 mg L^{-1}, 5.0 mg L^{-1}); in Mn test, clams were exposed to three concentrations of Mn (0.1 mg L^{-1}, 0.5 mg L^{-1}, 5.0 mg L^{-1}) and in Mix test, clams were exposed to the mixture Zn (1 mg L^{-1}) + Mn (0.5 mg L^{-1}), with and without Fe (5.0 mg L^{-1}). After single exposure to 5.0 mg L^{-1}, Zn bioaccumulated in all tissues, but only in mantle and hemolymph after exposure to 1.0 mg L^{-1}. The increased MT in gills of *A. trapesialis* exposed to Zn appears to be sufficient to avoid more severe damage, since LPO occurred only in digestive glands from animals exposed to 5.0 mg L^{-1}. We suggested that *A. trapesialis* showed metabolic suppression in consequence of Mn presence, based on the following results: the decrease of ROS in gills, the decrease of the Zn and Mn level in tissues and the decrease of ChE in muscle. Despite this physiological response, animals exposed to Mn showed oxidative damages (LPO and PC) in the mantle and digestive gland and MT increased in the mantle. These results indicated that *A. trapesialis* responded differently to each metal and Mn caused more damage. When exposed to Fe, gills level of ROS was increased, despite no changes in metal accumulation occurred. On the other hand, after exposure to the mixtures, tissues bioaccumulated Zn and previously observed damages caused by Mn and Fe disappeared. Consequently, biomarkers were less affected under mixture treatments, demonstrating mixtures effects or responses were not simply a combination of single exposures to Zn, Mn and Fe, but depend on metals toxicokinetics.

Keywords: essential metals, metabolic suppression, metallothionein, metal mixture, oxidative damage, valve closure behavior.

1 Introduction

Industrial and domestic wastes as well as many other contaminants ultimately reach a freshwater environment, which means that they contain a wide range of contaminants. Metals are a class of contaminants that have been extensively studied. However, the interactions of metals and effects of metal mixtures on aquatic ecosystems are not fully understood (Nordberg et al., 2007). If we are to elucidate how metals affect aquatic organisms, it should be borne in mind that these organisms are exposed to polymetallic mixtures in their natural environment. Metals can have additive, agonistic and antagonistic effects, depending on how they interact in the environment and within the organism (Nikinmaa, 2014, Spurgeon et al., 2010). Several interactions may occur at different levels. For example, one metal can affect the way in which another is absorbed, binds to proteins and is detoxified and excreted (Rand, 1995).

Some human activities produce liquid discharges containing particular metals at high concentrations. We recently detected elevated concentrations of Zn, Mn, Fe and Al in a stream contaminated by coal mining activities (Oliveira et al., 2016). The freshwater clam, *Anodontites trapesialis*, confined along this stream, bioaccumulated metals and showed several interesting biomarker alterations after only 96 h of exposure, making it a potentially useful biological model for monitoring polymetallic-contaminated environments. To better understand the interactions and possible combined effects of these elements in the field, it is essential to recognize individual modes of action in the species under laboratory controlled conditions.

Zn, Mn and Fe are essential elements involved in important cell functions, such as enzyme activities, membrane stability and protein and nucleic acid metabolization (Wood, 2012). These metals are frequently related to antioxidant defenses, since they are associated with the activity of superoxide dismutase (Cu/Zn-SOD or Fe/Mn-SOD), an important antioxidant enzyme that catalyzes the dismutation of the superoxide radical (O^{2-}) into molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) (Miller, 2004). It is worth noting that Zn plays a specific role in regulating metallothionein (MT) which, in turn, is involved in regulating concentrations of essential or non-essential metal ions in the cytoplasm, and is also involved in scavenging reactive oxygen

species (ROS) (Laity and Andrews, 2007). In unionoid bivalves, high concentrations of Mn have been found in soft tissues and are related to the antioxidant capacity of these bivalves (Campanella et al., 2005).

These essential metals can be toxic to aquatic organisms in unnatural concentrations associated with anthropogenic enrichment. Acute toxicity to freshwater bivalves can vary. The 96-h LC_{50} value for Zn ranges from 0.268 mg L^{-1} for *Anodonta imbecilis* (Keller, 1991) to 1.676 mg L^{-1} for *Lamellidens consobrinus* (Bhamre et al., 2010). In the marine species, *Ruditapes philippinarum,* the 96-h LC_{50} value for Zn can reach 16.4 mg L^{-1} (Dai et al., 1998). However, less information is available concerning acute Mn and Fe toxicity based on the 96-h LC_{50} value for marine or freshwater bivalves. For the marine clam, *Mya arenaria,* the 168-h LC_{50} value for Mn was determined at 300 mg L^{-1} (Eisler, 1977). For other freshwater invertebrates, the 96-h LC_{50} value for Mn ranges from 275.7 mg L^{-1} for the sludge worm (*Tubifex tubifex*) to 3.0 mg L^{-1} for the amphipod (*Hyallela azteca*), according to the WHO (2004). The 96-h LC_{50} value for Fe has been determined for invertebrates such as the crustaceans *Daphnia* (11.5 mg L^{-1}) and *Cyclops viridis* (11.5 – 36.0 mg L^{-1}) (Phippen et al., 2008).

Several sublethal effects have been observed in aquatic organisms, including bivalves, after exposure to unnatural concentrations. The toxicity of metals is often related to their ability to cause oxidative stress (Sevcikova et al., 2011, Trevisan et al., 2014) by increasing the generation of reactive oxygen species (ROS) and/or impairing antioxidant defenses. Zn can impair glutathione metabolization in the gills and alters antioxidant defense biomarkers in the marine bivalve, *Perna perna* (Trevisan et al., 2014). Moreover, the exposure of the freshwater bivalve, *Hyridella australis,* to Zn-contaminated sediments resulted in decreased antioxidant capacity and increased lipid peroxidation in the hepatopancreas (Wadige et al., 2014). In contrast to Zn, there is less information on Mn and Fe sublethal effects on freshwater organisms. Some studies have shown that 96-h exposure to Mn causes changes in the oxidative stress biomarkers of the freshwater fishes, *Colossoma macropomum* (Gabriel et al., 2013) and *Carassius auratus* (Vieira et al., 2012). Furthermore, Mn was found to cause a neurotoxic effect on the dopaminergic innervation of the gill in the marine bivalve, *Crassostrea virginica* (Martin et al., 2008). González et al.

(2010, 2015) demonstrated that Fe causes lipid peroxidation and impairment of the antioxidant enzyme (catalase) in tissues of the marine bivalve, *M. arenaria*.

In this study, under laboratory conditions we subjected the same bivalve species exposed in the field to coal mining pollutants (Capítulo II, Oliveira et al., 2016) to Zn, Mn and Fe with the aim of elucidating the responses to and effects of Zn, Mn and Fe under controlled conditions after a single exposure, binary exposure (Zn+Mn) or exposure to complex mixture (Zn+Mn+Fe), focusing on bioaccumulation and changes in oxidative stress biomarkers. Three different concentrations of Zn and Mn were tested over a 96-h period. A mixture at intermediate concentration (based on field observations) was also tested, with and without Fe. The aim was to investigate the possible effects of one metal on the effects of the others.

2 Material and methods

2.1 Collection and acclimation of clams

Individuals of *A. trapesialis* (n = 96; length: 126.1 ± 6.6 mm; height: 59.4 ± 3.6 mm; weight: 166.6 ± 27.4 g) were collected manually from the bottom of a dam in a fish farming facility in the Brazilian city of Londrina (23°41'26.41"S; 51°14'29.53"W) and immediately transported to the laboratory inside plastic bags containing dam water. They were acclimated for 10 days in tanks containing carbon filtered water (renewed every 24 h) under a 12/12 h photoperiod and at constant temperature (~ 21°C), pH (6.5 – 7.5) and oxygenation level (>85% saturation). During acclimation, the clams were fed on *Pseudokirchneriella subcapitata* algae (~10,000 cells mL^{-1}) every two days.

2.2 Experimental tests

Acute 96-h semi-static tests (water renewal every 24 h) were conducted by exposing clams to Zn (Zn test), Mn (Mn test) or a Zn/Mn mixture, with and without Fe (Mix test). In the Zn test, clams were exposed to the following nominal Zn concentrations: 0.18 mg L^{-1}, 1.0 mg L^{-1}, 5.0 mg L^{-1}. In the Mn test, clams were exposed to concentrations of 0.1 mg L^{-1}, 0.5 mg L^{-1} and 5.0 mg L^{-1}. In the Mix test, clams were exposed to intermediate concentrations of Zn+Mn (Zn: 1.0 mg L^{-1}; Mn: 0.5 mg L^{-1}), Zn+Mn+Fe (Zn: 1.0 mg L^{-1}; Mn: 0.5 mg L^{-1}; Fe:

5 mg L^{-1}) or Fe alone (5 mg L^{-1}). The tests were performed independently, each with its own control (CTR - clams exposed to water only). Metals were added to the water as sulfate salts $ZnSO_4.7H_2O$, $MnSO_4.H_2O$ and $FeSO_4.7H_2O$. Metal concentrations were chosen based on the environmental concentrations found in a stream contaminated by coal mining activities (Oliveira et al., 2016) and on the limits established in the Brazilian legislation for freshwater (CONAMA 357/2005), depending on the use of the water (Zn: 0.18 and 5.0 mg L^{-1}; Mn: 0.1 and 0.5 mg L^{-1}; Fe: 5.0 mg L^{-1}).

At the beginning of each test, the clams (n = 32) were transferred to beakers (1.5 L) where they were kept individually for 96 h and divided into four treatments groups. Food was provided daily 1 h before water renewal (*P. subcapitata*: ~20,000 cells mL^{-1}). Every day, before and after water renewal, non-filtered and filtered (Millipore Millex HV/PVDF 0.45-µm mesh filter) water samples were collected for analysis of total and dissolved Zn, Mn and Fe. During the tests, water temperature (21-23°C) and oxygenation (85% saturation) were kept constant.

After exposure for 96 h, the clams were removed from the beakers and samples of hemolymph, gills, mantle, muscle (foot) and digestive gland were collected and analyzed as described below. The same individuals were used to perform all analyses. All tissues except hemolymph were washed with a physiological solution (Sigma PBS), immediately frozen in liquid nitrogen and stored at -72°C.

2.3 Quantification of total and dissolved metals in exposure water

After collection, water samples were immediately fixed under acid conditions (Nitric acid 1%) and kept at -20°C before analysis. Metals were quantified against standard reference solutions (Specsol, Brazil) by flame (Zn) or electrothermic (Mn and Fe) ionization in a graphite tube attached in an atomic absorption spectrophotometer (AAnalyst 700, Perkin Elmer, USA). Metal speciation was simulated by PHREEQC Interactive Version 3.3.9.11951, based on alkalinity (ABNT, 1996), pH, temperature, sulfate (Tabatabai, 1974), dissolved Na, K (determined by flame photometry), Mg and Ca (determined by electrothermic ionization in a graphite tube attached in an atomic absorption spectrophotometer), and Mn, Fe and Zn concentrations.

2.4 Metal bioaccumulation

Concentrations of Zn, Mn and Fe in clam tissues (mantle, gills, digestive gland, muscle and hemolymph) were determined. Except for hemolymph, the tissues were completely dried at 60°C and subjected to acid digestion for 48 h at 60°C in suprapure nitric acid, according to Alves and Wood (2006). The digested tissues were subjected to flame analysis using an atomic absorption spectrophotometer (AAnalyst 700, Perkin Elmer, USA) and compared to standard reference solutions (Specsol, Brazil). Results were expressed in mg g dry weight^{-1} (mantle, gills, digestive gland and muscle) or mg L^{-1} (hemolymph).

2.5 Biomarkers

The methods and procedures used to analyze the applied biomarkers are described in detail in Oliveira et al. (2016). Below is a brief description of each method and some minor modifications, as required.

Reactive oxygen species (ROS) and total antioxidant capacity against peroxyl radicals (TAC) were measured in the gills (ROS and TAC) and mantle (TAC) using the method proposed by Amado et al. (2009). The fluorescence of the supernatants obtained (diluted to 0.5 mg mL^{-1} total proteins) were monitored (ex/em: 485/520 nm) over a period of 30 min in the presence and absence of 2,2′-azobis (2 methylpropionamidine) dihydrochloride (ABAP). The quantity of ROS was expressed in terms of the area calculated by computing the integral of fluorescence units over time in wells without ABAP, and TAC estimated in terms of the difference between graph areas (with and without ABAP).

In gills (n = 8), mantle (n = 8) and digestive gland (n = 8) homogenates, lipid peroxidation (LPO) and protein carbonylation (PC) were determined as oxidative damage endpoints, and CuZn-SOD activity and GST activity as antioxidant enzymes. Superoxide dismutase (CuZn-SOD) activity was determined in microplates by inhibiting the reduction rate of cytochrome c by the superoxide radical at 550 nm and 25°C, according to McCord and Fridovich (1969). GST activity was determined using the method described by Keen et al. (1976), in which GST conjugates reduce glutathione with 1-chloro-2,4-dinitrobenzene, monitored for 1 min in a spectrophotometer at 340 nm.

The content of metallothionein-like proteins was determined in gills (n = 8) and mantle (n = 8), as described by Viarengo et al. (1997) with some modifications. Partially purified metalloprotein fractions were obtained from the supernatant after ethanol/acid chloroform fractionation, and sulfhydryl groups (-SH) were quantified at 412 nm.

LPO was determined by the TBARS (thiobarbituric acid reactive substances) fluorescence assay (ex/em: 535/590nm), according to Camejo et al. (1998), after incubating supernatants at 60°C for 1 hour with thiobarbituric acid (1.3%). To determine the PC content, we used the method described by Levine et al. (1994), which is based on the reaction with 2,4-dinitrophenylhydrazine (DNPH 10 mM, prepared in HCl 2 M) and consequent formation of dinitrophenyl hydrazones quantified at 360 nm.

In order to evaluate hemocyte DNA damage, the Comet assay was performed (n = 8) as described in Oliveira et al. (2016b). DNA damage was expressed in terms of a score calculated using the following formula: (0xA) + (1xB) + (2xC) + (3xD), where A, B, C and D correspond to the number of cells out of 100, classified according to the damage exhibited: class 0 = absence of comet tail; class 1 = comet tail smaller than nucleoid diameter; class 2 = comet tail larger than nucleoid diameter; class 3 = comet tail more than twice nucleoid diameter.

ChE activity was determined in mantle (n = 8) and muscle (foot) (n = 8) as described by Ellman et al. (1961), with the modifications in Mora et al. (1999), in which absorbance was monitored for 30 min at 415 nm. All biochemical biomarkers were expressed in relation to total protein content determined at 595 nm by the method in Bradford (1976).

2.6 Statistical analysis

Data were first tested for normality and homogeneity of variance. Parametric (ANOVA) or non-parametric (Kruskal-Wallis) analysis of variance were performed, followed by a multiple comparison test (Newman-Keuls or Dunn's), where appropriate. Bioaccumulation and biomarker results for each tissue were compared for the different treatments against each specific control group. Differences were considered significant where $P < 0.05$.

3 Results

Three independent semi-static tests (Zn test, Mn test and Mix test) were successfully performed and no mortality was observed after 96-h exposure, confirming that the concentrations were sublethal to *A. trapesialis*. Some individuals in the Zn test at the higher Zn concentration (5 mg L^{-1}) exhibited an evident abundance of mucus (not quantified).

The metal concentrations determined in the water samples showed that the measured concentrations were always below the nominal figures and confirmed a gradient among treatment groups in the Zn test and Mn test (Table 1). Based on speciation simulations, it was determined that Zn, Mn and Fe divalent species were predominant under all conditions, representing over 90% of the dissolved metals. After the 24 h-period without renewal, dissolved metal levels dropped in the water. At intermediate concentrations in the Zn test and Mn test, there was a 48% drop in dissolved Zn and a 55% drop in dissolved Mn after 24 h, while in the Mix test groups (Zn+Mn and Zn+Mn+Fe), Zn dropped by 49% and 65% respectively, and Mn showed smaller respective drops of 29% and 23%.

Interesting results were obtained for metal bioaccumulation (Table 2). In the Zn test, clams from group Zn[1.0] exhibited an increase in mantle and hemolymph Zn levels, while group Zn[5.0] exhibited an increase in Zn in all tissues compared to the control (Zn[CTR]) and Zn[0.18]. Mn levels did not vary among groups in the Zn test. In contrast, Mn test clams from groups Mn[0.5] and Mn[5.0] showed a drop in Mn in the gills compared to the control (Mn[CTR]) and Mn[0.1], while clams from group Mn[5.0] exhibited an increase in hemolymph Mn compared to the other groups. Zn levels in muscle, mantle and gill tissue were significantly lower in clams from group Mn[5.0] and in hemolymph tissue from group Mn[0.1]. Mix test results were similar in the Zn+Mn and Zn+Mn+Fe groups. Zn was found in clams from both groups in muscle, digestive gland, mantle and hemolymph tissue, with significantly higher levels in mantle and hemolymph tissue compared to the control and Fe groups. Fe levels did not vary from one group to another in any of the tests.

Table 1 Total and dissolved metals concentrations (mean ± SD; mg L^{-1}) in all tested conditions at initial (0h) and final (24h) measurements in the 24 h-interval of water renovation.

		Zn initial		Zn final		Mn initial		Mn final		Fe initial		Fe final	
		Total	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**
Zn test	Zn[ctr]	< DL	< DL	< DL	< DL	< 0.01	< 0.01	< 0.01	< 0.01	0.02 ± 0.02	0.02 ± 0.01	0.04 ± 0.01	0.04 ± 0.00
	Zn[0.18]	0.03 ± 0.02	0.03 ± 0.01	< DL	< DL	< 0.01	< 0.01	< 0.01	< 0.01	0.03 ± 0.02	0.03 ± 0.02	0.09 ± 0.02	0.05 ± 0.00
	Zn[1.0]	0.80 ± 0.04	0.77 ± 0.09	0.41 ± 0.12	0.40 ± 0.12	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.05 ± 0.01	0.03 ± 0.00	0.08 ± 0.02	0.06 ± 0.01
	Zn[5.0]	4.38 ± 0.11	4.32 ± 0.16	2.81 ± 0.39	2.75 ± 0.38	0.03 ± 0.00	0.03 ± 0.00	0.13 ± 0.03	0.13 ± 0.03	0,03 ± 0.02	0.02 ± 0.02	0.07 ± 0.00	0.06 ± 0.02
Mn test	Mn[ctr]	< DL	< DL	< DL	< DL	< 0.01	< 0.01	< 0.01	< 0.01	0.20 ± 0.06	0.26 ± 0.03	0.12 ± 0.02	0.15 ± 0.06
	Mn[0.1]	< DL	< DL	< DL	< DL	0.05 ± 0.02	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.26 ± 0.04	0.23 ± 0.06	0.11 ± 0.06	0.20 ± 0.05
	Mn[0.5]	< DL	< DL	< DL	< DL	0.32 ± 0.15	0.27 ± 0.13	0.14 ± 0.08	0.12 ± 0.09	0.21 ± 0.01	0.21 ± 0.04	0.08 ± 0.01	0.15 ± 0.06
	Mn[5.0]	< DL	< DL	< DL	< DL	4.31 ± 0.95	4.09 ± 0.89	3.26 ± 0.90	3.08 ± 0.73	0.22 ± 0.04	0.25 ± 0.14	0.17 ± 0.04	0.11 ± 0.07
Mix test	Mix[ctr]	< DL	< DL	< DL	< DL	< 0.01	< 0.01	0.01 ± 0.01	0.01 ± 0.01	0.15 ± 0.13	0.16 ± 0.14	0.22 ± 0.20	0.22 ± 0.18
	Mix[Fe]	< DL	< DL	< DL	< DL	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	3.35 ± 0.19	1.86 ± 0.26	0.71 ± 0.70	0.37 ± 0.17
	Mix[Zn+Mn]	0.70 ± 0.03	0.71 ± 0.03	0.35 ± 0.12	0.36 ± 0.13	0.39 ± 0.01	0.37 ± 0.02	0.27 ± 0.02	0.27 ± 0.03	0.19 ± 0.17	0.21 ± 0.17	0.28 ± 0.19	0.22 ± 0.18
	Mix[Mix]	0.74 ± 0.04	0.68 ± 0.05	0.25 ± 0.11	0.23 ± 0.10	0.39 ± 0.02	0.39 ± 0.02	0.30 ± 0.02	0.30 ± 0.02	3.38 ± 0.60	2.64 ± 0.26	0.49 ± 0.26	0.40 ± 0.18

DL means *detection limit*. DL for each metal was the following - Al: 45 µg L-1; Mn: 1.5 µg L-1; Fe: 5 µg L-1; Zn: 1.5 µg L-1.

Table 2 Concentrations (mg Kg^{-1} or mg L^{-1} only for hemolymph) of Zn, Mn and Fe (mean ± SD) in different tissues of *Anodontites trapesialis* after Zn test, Mn test and Mix test. In each test, groups were compared with one respective control (statistical analyzes were applied separately for each mentioned Test).

		Zn					Mn					Fe				
		Muscle	**DG**	**Mantle**	**Gills**	**Hem**	**Muscle**	**DG**	**Mantle**	**Gills**	**Hem**	**Muscle**	**DG**	**Mantle**	**Gills**	**Hem**
Zn test	Zn[CTR]	138.5 ^{a} ± 21.5	819.9 ^{a} ± 246.6	443.3 ^{a} ± 108.8	600.6 ^{a} ± 155.5	0.39 ^{a} ± 0.20	1580.0 ^{a} ± 462.1	20354.3 ^{a} ± 6062.0	21890.3 ^{a} ± 6283.8	18598.4 ^{a} ± 2244.5	628.8 ^{a} ± 404.8	875.9 ^{a} ± 208.8	6462.8 ^{a} ± 1472.8	4877.3 ^{a} ± 1623.4	3862.0 ^{a} ± 716.1	8.7 ^{a} ± 2.3
	Zn[0.18]	141.7 ^{a} ± 11.5	767.4 ^{a} ± 142.8	485.0 ^{a} ± 124.9	482.7 ^{a} ± 112.3	0.67 ^{ab} ± 0.29	1287.0 ^{a} ± 626.9	21912.0 ^{a} ± 8369.9	21210.3 ^{a} ± 8300.4	15162.6 ^{a} ± 3691.4	765.1 ^{a} ± 210.8	795.2 ^{a} ± 172.8	5980.6 ^{a} ± 1165.6	5117.1 ^{a} ± 1520.2	3566.6 ^{a} ± 173.5	8.0 ^{a} ± 1.9
	Zn[1.0]	147.1 ^{a} ± 19.4	889.3 ^{a} ± 178.8	829.6 ^{b} ± 108.5	610.9 ^{a} ± 112.0	0.96 ^{b} ± 0.38	1430.7 ^{a} ± 1016.1	26014.0 ^{a} ± 7909.0	25415.7 ^{a} ± 9290.2	17101.0 ^{a} ± 2311.5	1077.1 ^{a} ± 175.9	759.7 ^{a} ± 271.0	7612.1 ^{a} ± 2461.8	4672.6 ^{a} ± 1950.9	3833.8 ^{a} ± 822.9	8.9 ^{a} ± 2.1
	Zn[5.0]	278.2 ^{b} ± 67.8	1212.8 ^{b} ± 232.4	1321.8 ^{c} ± 174.1	1320.9 ^{b} ± 296.4	0.80 ^{b} ± 0.19	2174.7 ^{a} ± 1372.2	20039.7 ^{a} ± 2556.9	23295.8 ^{a} ± 6555.5	15950.4 ^{a} ± 4334.6	1011.3 ^{a} ± 653.4	849.5 ^{a} ± 364.2	6807.2 ^{a} ± 2473.1	4942.2 ^{a} ± 1748.1	3788.9 ^{a} ± 1076.7	6.8 ^{a} ± 2.0
Mn test	Mn[CTR]	94.4 ^{a} ± 11.5	303.5 ^{a} ± 70.9	231.7 ^{a} ± 59.2	277.1 ^{a} ± 58.8	0.36 ^{a} ± 0.09	1110.2 ^{a} ± 669.3	22555.3 ^{a} ±2965.8	22695.2 ^{a} ± 5353.3	21687.7 ^{a} ± 1077.2	785.6 ^{a} ± 427.4	750.7 ^{a} ± 212.8	6302.0 ^{a} ± 1145.8	4337.8 ^{a} ± 988.9	3246.0 ^{a} ± 551.7	10.2 ^{ab} ± 2.2
	Mn[0.1]	89.9 ^{a} ± 8.8	321.9 ^{a} ± 132.0	238.9 ^{a} ± 102.8	315.8 ^{a} ± 25.4	0.12 ^{b} ± 0.05	1156.3 ^{a} ± 475.9	25304.6 ^{a} ± 7975.3	27499.8 ^{a} ± 9666.0	23552.1 ^{a} ± 3044.6	959.7 ^{a} ± 512.1	762.8 ^{a} ± 227.7	6205.1 ^{a} ± 1108.3	4790.0 ^{a} ± 1103.7	3752.1 ^{a} ± 218.3	12.8 ^{a} ± 2.4
	Mn[0.5]	85.0 ^{ab} ± 7.9	263.1 ^{a} ± 95.2	269.9 ^{a} ± 92.7	230.7 ^{ab} ± 71.9	0.39 ^{a} ± 0.17	1241.9 ^{a} ± 949.0	24318.4 ^{a} ± 8766.6	25755.8 ^{a} ± 11333.6	19090.0 ^{b} ± 2258.9	1224.4 ^{a} ± 381.9	775.2 ^{a} ± 288.6	6299.7 ^{a} ± 2245.0	4916.1 ^{a} ± 1810.0	3457.1 ^{a} ± 154.5	8.1 ^{b} ± 1.8
	Mn[5.0]	78.3 ^{b} ± 5.5	241.4 ^{a} ± 79.1	127.2 ^{b} ± 44.1	171.4 ^{b} ± 79.0	0.33 ^{a} ± 0.12	603.7 ^{a} ± 244.0	20319.1 ^{a} ± 4203.1	18666.3 ^{a} ± 3669.5	17485.2 ^{b} ± 2356.3	1973.1 ^{b} ± 395.0	797.2 ^{a} ± 260.7	4861.7 ^{a} ± 1051.1	4988.2 ^{a} ± 1714.0	3589.3 ^{a} ± 326.5	9.4 ^{ab} ± 2.2
Mix test	Mix[CTR]	97.8 ^{a} ± 7.3	293.9 ^{a} ± 71.6	348.9 ^{a} ± 181.1	413.5 ^{a} ± 192.2	0.09 ^{a} ± 0.05	1450.2 ^{a} ± 643.2	21155.0 ^{a} ± 3180.6	23880.1 ^{a} ± 9178.4	25942.8 ^{a} ± 11743.2	576.7 ^{a} ± 147.4	748.6 ^{a} ± 216.9	5462.4 ^{a} ± 1071.1	4604.4 ^{a} ± 810.0	4440.3 ^{a} ± 1571.9	9.8 ^{a} ± 2.1
	Fe	107.4 ^{a} ± 11.3	248.0 ^{a} ± 80.2	358.4 ^{a} ± 127.4	336.2 ^{a} ± 205.0	0.20 ^{a} ± 0.08	1407.4 ^{a} ± 740.9	17896.5 ^{a} ± 7912.0	23652.5 ^{a} ± 9583.6	21168.6 ^{a} ± 11079.3	602.2 ^{a} ± 315.7	783.1 ^{a} ± 480.0	4968.5 ^{a} ± 2523.7	3748.2 ^{a} ± 1659.7	3247.4 ^{a} ± 1617.0	8.0 ^{a} ± 2.2
	Zn+Mn	146.3 ^{b} ± 13.8	467.6 ^{b} ± 92.2	687.5 ^{b} ± 204.4	596.6 ^{a} ± 163.6	0.56 ^{b} ± 0.26	1934.0 ^{a} ± 890.5	22075.8 ^{a} ± 5449.0	27717.5 ^{a} ± 7471.2	21700.3 ^{a} ± 5837.8	1080.9 ^{b} ± 416.0	620.1 ^{a} ± 197.0	5472.1 ^{a} ± 1476.9	4888.8 ^{a} ± 1315.2	3988.3 ^{a} ± 1807.6	8.4 ^{a} ± 2.5
	Zn+Mn+Fe	143.0 ^{b} ± 8.9	386.9 ^{c} ± 48.6	639.1 ^{b} ± 80.3	565.9 ^{a} ± 276.1	0.78 ^{b} ± 0.30	1437.6 ^{a} ± 452.8	18244.1 ^{a} ± 3817.3	27213.0 ^{a} ± 7676.7	21875.3 ^{a} ± 9904.6	1151.2 ^{b} ± 441.5	729.8 ^{a} ± 526.1	4479.9 ^{a} ± 1171.0	3888.9 ^{a} ± 1438.7	3375.2 ^{a} ± 1451.5	9.7 ^{a} ± 3.7

D*etection limit* for each metal was the following - Al: 45 µg L-1; Mn: 1.5 µg L-1; Fe: 5 µg L-1; Zn: 1.5 µg L-1.

Different letters represent significant differences;

DG: Digestive gland; Hem: Hemolymph

The endpoints measured in gills (Fig.1), mantle (Fig. 2) and digestive gland tissues (Fig. 3) are shown in separate figures so that they are easier to compare. The same applies to ChE activity in mantle and muscle tissues (Fig. 4) and the comet assay (Table 3). In mantle tissue, ROS were not determined since this assay has to be performed on fresh samples and we prioritized gill tissue analysis on the day of sampling. There were too few digestive gland samples to perform all analysis procedures, so priority was given to those that could be carried out on the same homogenate (LPO, PC, GST, SOD). Comparisons were always performed on groups from the same test (Zn, Mn and Mix tests), since they were run independently and each test had its own control group.

In the gills (Fig.1), clams from groups Zn[1.0] and Zn[5.0] exhibited significantly higher MT-like protein levels compared the control (Zn[CTR]) ($H = 11.158$; $p = 0.011$), but no other endpoint showed gill alterations in clams subjected to the Zn test. However, the gills of clams exposed to Mn did not show alterations in MT-like protein levels or other endpoints, except for a significant decrease in ROS levels at the highest Mn concentration (Mn[5.0]) compared to other Mn test groups ($F = 9.486$; $p < 0.001$). In the Mix test, the Zn+Mn group exhibited a significant drop in ROS levels ($F = 9.806$; $p < 0.001$), but this was not observed in Zn+Mn+Fe group, probably because Fe induced an increase in this biomarker ($F = 9.806$; $p < 0.001$). When Fe was mixed with Zn and Mn, there was an antagonistic effect preventing a rise in ROS. No other effects were observed in the gill tissue of clams subjected to the Mix test.

In the mantle tissue (Fig.2), no effects were found in clams subjected to the Zn test. However, in the Mn test, the highest Mn concentration (Mn[5.0]) induced a significant increase in LPO ($F = 5.311$; $p = 0.006$) compared to all other groups, and in MT-like protein levels ($F = 4.265$; $p = 0.014$) in comparison to the control. In the Mix test, as in the Mn test, a significant increase in MT-like protein levels ($F = 5.042$; $p = 0.007$) was observed in the mantle of clams from the Zn+Mn group compared to all other groups, but no effect was evidenced when Fe was added to the mixture (Mix[Mix]).

The digestive glands (Fig.3) of clams exposed to Zn[5.0] showed a significant increase in LPO ($F = 4.598$; $p = 0.011$) compared to all groups in the Zn test. Similarly, clams exposed to Mn[5.0] exhibited significantly higher levels of LPO ($H =$

11.599; p = 0.009) in comparison to Mn[CTR] and Mn[0.1]. Moreover, exposure to Mn[0.1] and Mn[0.5] promoted a significant increase in PC (F = 5.089; p = 0.008) compared to the control. Mn also caused a significant decrease in GST activity in Mn[0.5] group (F = 2.973; p = 0.049) when compared to Mn[CTR]. In the digestive gland, the effects observed after Zn and Mn tests, in which metals were isolated, was not observed in clams subjected to the Mix test.

ChE activity (Fig. 4) was measured in mantle and muscle tissue and there were some statistical differences. In the mantle, there was one difference in the Mn test between Mn[0.1] and Mn[0.5] (F = 3.089; p = 0.046) but not in comparison to the control group. In the muscle, clams exposed to Zn[5.0] showed an increase in ChE activity (F = 5.476; p = 0.005) compared to all the other groups subjected to the Zn test. However, clams exposed to Mn[0.5] and Mn[5.0] showed a drop in ChE activity (F = 6.181; p = 0.003) compared to Mn[CTR] and Mn[0.1], respective drops of 43% and 32% compared to the control. In the Mix test, no differences were observed in muscle ChE activity. Finally, there were no differences in hemocyte DNA damage monitored by the comet assay in any of the tests (Table 3).

Table 3 DNA damage score (mean ± SD) in hemocytes from *Anodontites trapesialis* submitted to Zn test, Mn test and Mix test.

Zinc		**Manganese**		**Mixture**	
CTR	75.1 ± 19.2	CTR	79.3 ± 23.0	CTR	82.1 ± 18.7
[0.18]	82.4 ± 23.5	[0.1]	55.6 ± 19.7	Fe[5]	77.6 ± 19.1
[1.0]	72.0 ± 25.0	[0.5]	56.6 ± 21.0	[Zn+Mn]	85.6 ± 48.7
[5.0]	50.8 ± 22.7	[5.0]	67.8 ± 24.5	[Zn+Mn+Fe]	68.4 ± 14.4

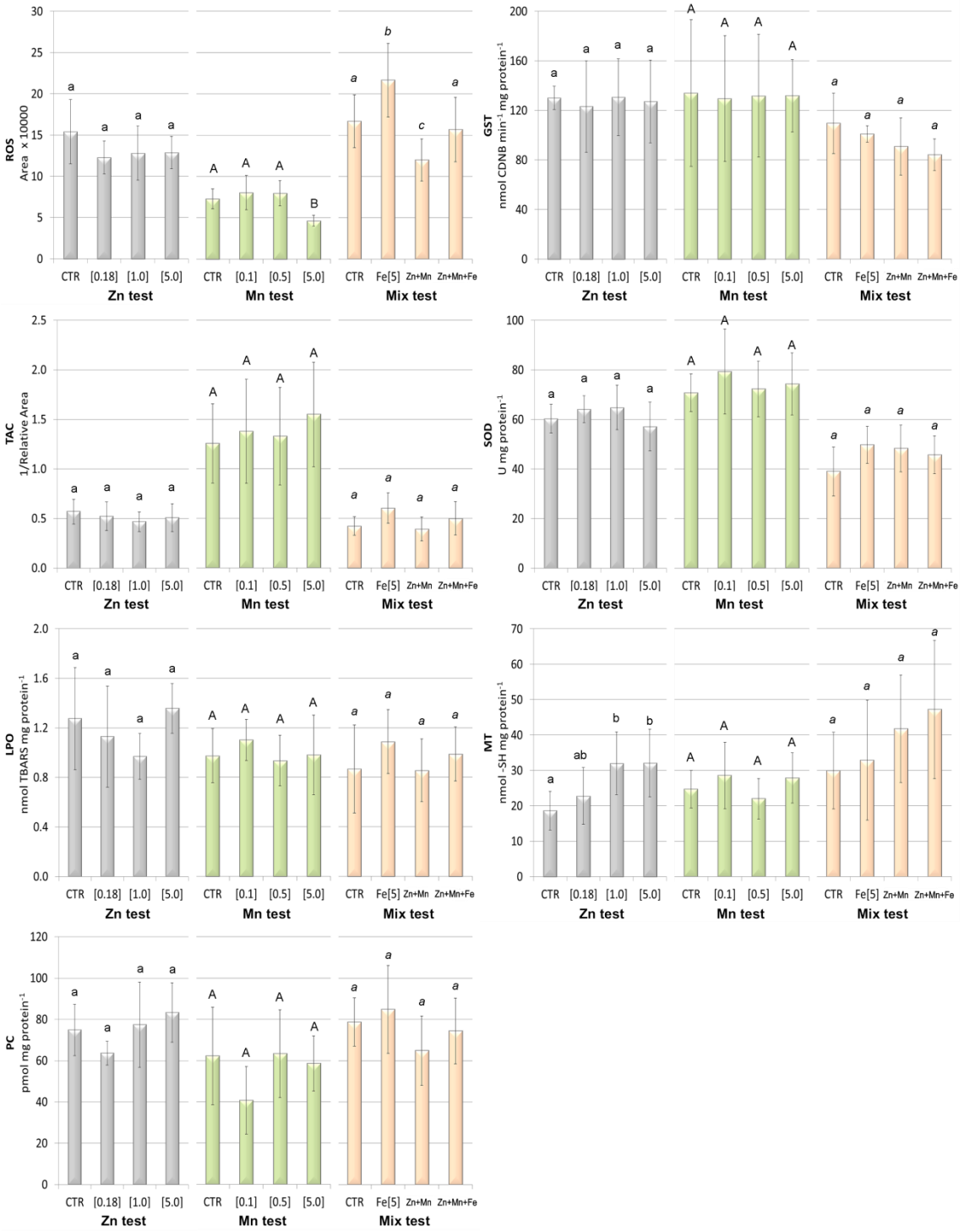

Figure 1 Biomarkers (mean ± SD) in gills from *Anodontites trapesialis* submitted to Zn, Mn and Mix tests. Statistical analyses were performed independently among tests and different patterns of letters were used for each test: Zn test – lowercase letters; Mn test – uppercase letters; Mix test – lower case italic letters. Significant differences were indicated by different letters.

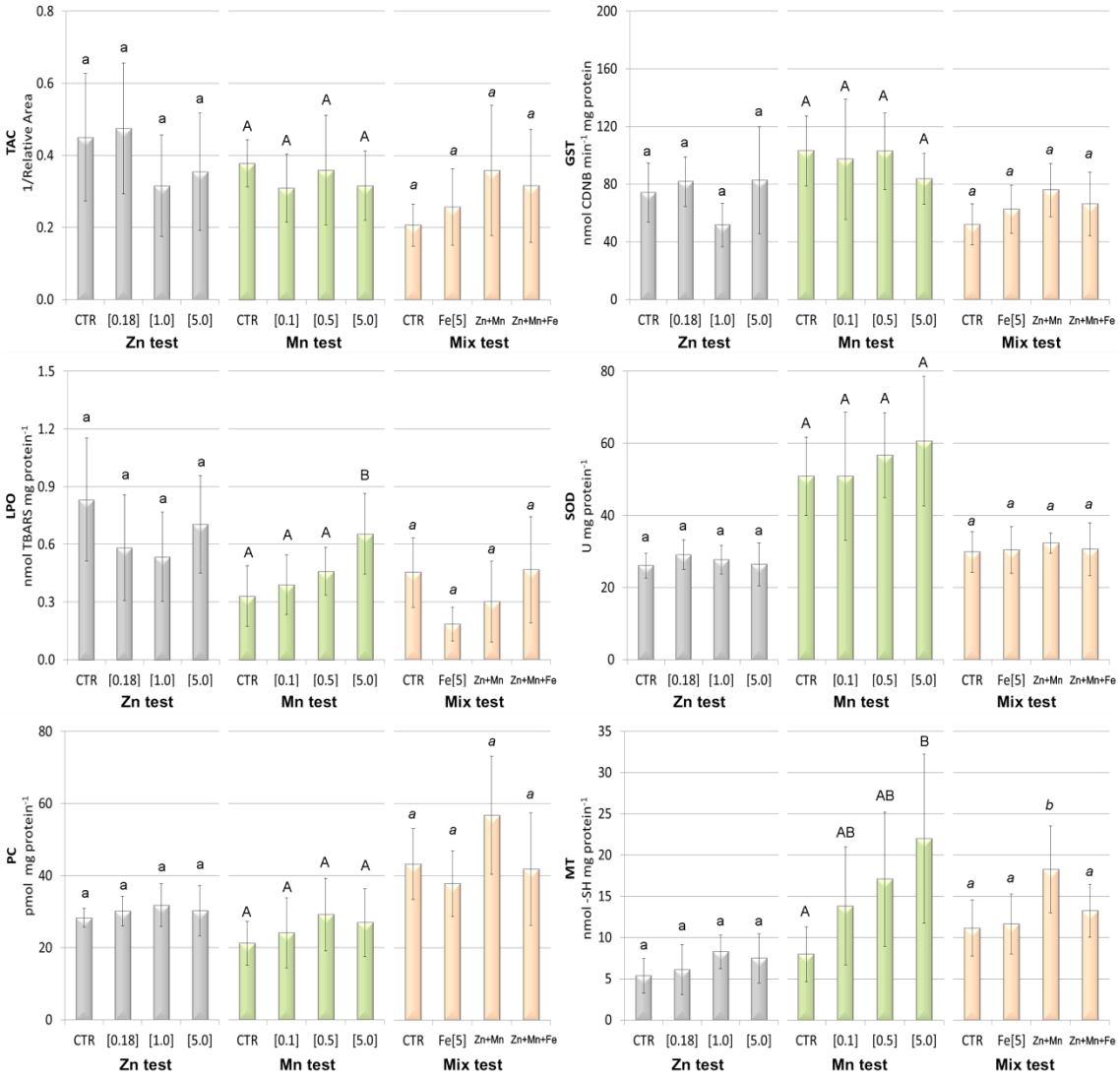

Figure 2 Biomarkers (mean ± SD) in mantle from *Anodontites trapesialis* submitted to Zn, Mn and Mix tests. Statistical analyses were performed independently among tests and different patterns of letters were used for each test: Zn test – lowercase letters; Mn test – uppercase letters; Mix test – lower case italic letters. Significant differences were indicated by different letters.

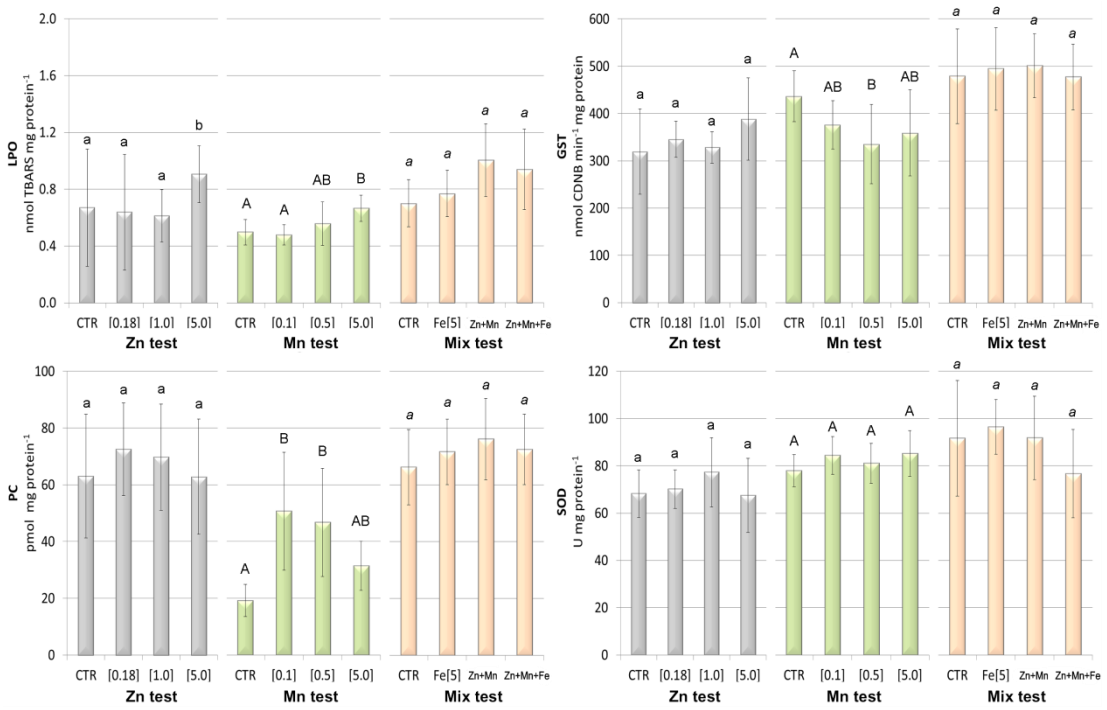

Figure 3 Biomarkers (mean ± SD) in digestive gland from *Anodontites trapesialis* submitted to Zn, Mn and Mix tests. Statistical analyses were performed independently among tests and different patterns of letters were used for each test: Zn test – lowercase letters; Mn test – uppercase letters; Mix test – lower case italic letters. Significant differences were indicated by different letters.

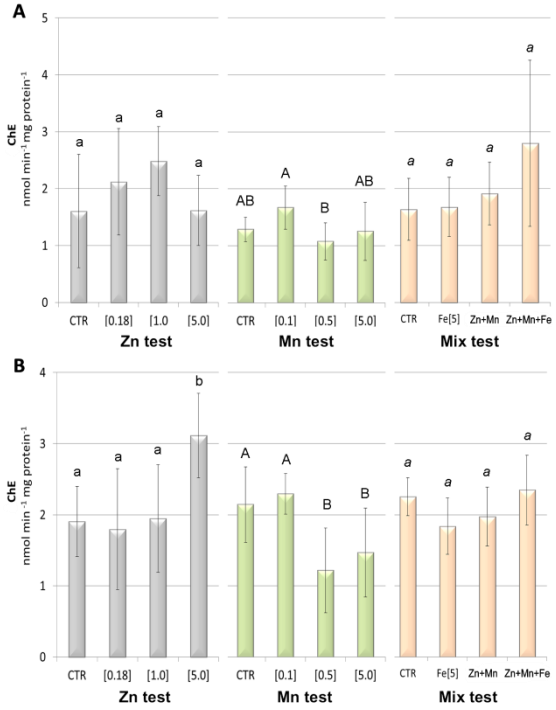

Figure 4 Colinesterase (ChE) activities (mean ± SD) in mantle (A) and muscle (B) from *Anodontites trapesialis* submitted to Zn, Mn and Mix tests. Statistical analyses were performed independently among tests and different patterns of letters were used for each test: Zn test – lowercase letters; Mn test – uppercase letters; Mix test – lower case italic letters. Significant differences were indicated by different letters.

4 Discussion

There are several anthropogenic metal sources, both punctual and diffuse, which contaminate the aquatic environment with complex metallic mixtures. In a previous study (Oliveira et al., 2016), we characterized metal contamination in a stream affected by coal mining activities and detected elevated concentrations of Zn, Mn and Fe downstream of the mine compared to upstream sites. In order to better understand the effects and bioaccumulation of these metals, we performed the tests described herein. Zn, Mn and Fe are essential to organisms. They play a part in a number of cell functions and are important for growth and survival. As essential metals, they are controlled by regulation mechanisms, especially in freshwater organisms living in an environment generally containing low concentrations. In clams, uptake and elimination of these metals is controlled to some extent, depending on the fitness of the organism and the environmental conditions (Bjerregaard and Andersen, 2007).

Zn cellular regulation is closely related to MTs and our results showed that exposure to Zn[1.0] and Zn[5.0] increased MTs in *A. trapesialis* gill cells. When intracellular Zn (Zn^{2+}) increases, the metal tends to bind to MTF-1, a Zn-dependent transcriptional activator of MT gene expression and a major regulatory component in higher eukaryotic Zn homeostasis (Laity and Andrews, 2007). Increased Zn concentration in the gills was found only in clams exposed to the highest Zn concentration (Zn[5.0]), in comparison to other Zn test groups, but an intermediate concentration of Zn (Zn[1.0]) was enough to boost MTs. It seems that the increase in MTs was enough to maintain normal cellular status since no other biomarker alteration was observed in the gills.

In addition to the gills, Zn concentrations in *A. trapesialis* were higher in all the other tissues analyzed (muscle, digestive gland, mantle and hemolymph) after exposure to Zn[5.0], and in the mantle and hemolymph after exposure to Zn[1.0]. These results suggest that in *A. trapesialis* exposed to Zn[1.0] and Zn[5.0], Zn uptake is overcoming efflux and elimination. Bivalves can employ several strategies and mechanisms to deal with excess metals in tissues and prevent toxicity. Metals can be incorporated in calcium carbonate and calcium phosphate concretions in the gills and mantle (Hinzmann et al., 2015) and even into the shell. They can be compartmentalized in lysosomes after MT binding (Viarengo and Nott, 1993) or

eliminated in pseudofeces (Klerks and Fraleigh, 1997) and mucus (Hietanen et al., 1988, Pillay, 2013). In fact, we did observe excess mucus production in clams exposed to Zn[5.0], which could indicate an attempt to remove excess Zn. Bioaccumulation does not necessarily imply toxicity, since bivalves are able to regulate metals internally and store them in inert forms, as mentioned above (Wood, 2012). Information concerning subcellular partitioning could clarify whether metals are metabolic active or not, improving our capability to predict metals toxicity (Campana et al., 2015).

Conversely, *A. trapesialis* seems to respond differently when exposed to Mn. We hypothesized that these clams had increased their valve closure frequency as a possible strategy to deal with Mn in the water and, consequently, after 96 h a possible state of metabolic suppression was established. This Mn-induced valve closure behavior has already been demonstrated in the freshwater bivalve, *Velesunio angasi* (Markich et al., 2000). The following observations support this argument. In the Mn test, with the exception of hemolymph tissue, the clams did not bioaccumulate Mn. Indeed, Mn concentration dropped in gill tissue sampled after Mn[0.5] and Mn[5.0]. Furthermore, Zn concentrations in muscle, mantle and gill tissue after Mn[5.0] were also lower than in other groups. These are essential metals obtained by feeding and water filtration, activities that were impaired after exposure to Mn leading to a decrease in metal uptake. Clayton et al. (2015) observed the same phenomenon in the freshwater mussel, *Strophitus undulatus*, finding that whole-body bioaccumulation of Mn, Zn and other metals decreased after *in situ* exposure in an area impacted by acid mine drainage, despite the higher concentrations of Mn in the water. Further evidence to support our hypothesis regarding valve closure and suppressed metabolism lies in the reduced amount of ROS in gill tissues after Mn[5.0]. When the valve is closed, gills become less active with reduced feeding, filtration rates and energy demand. ROS generation fell because it is dependent on oxygen availability (Lushchak, 2016). Decreased AChE activity in *A. trapesialis* muscle after Mn[0.5] and Mn[5.0] exposure is the third point that supports our hypothesis, and is probably related to low foot activity.

In Oliveira et al. (2016) we suggested that *A. trapesialis* also exhibited valve closure behavior after confinement downstream of a coal mine for 96 h since, as in the present study, Mn bioaccumulation in tissues tended to decrease in the confined

clams and AChE activity also decreased in muscle tissue, showing consistency between field and laboratory results. That particular stream contained dissolved Zn (0.27 ± 0.09 mg L^{-1}), Mn (0.88 ± 0.33 mg L^{-1}), Fe (3.59 ± 2.12 mg L^{-1}) and Al (0.31 ± 0.10 mg L^{-1}) as important contaminants. Our hypothesis regarding Mn potential to cause metabolic suppression in *A. trapesialis* is more consistently supported by the clams exposed to Mn[5.0]. In this treatment group, dissolved Mn concentration in the water was approximately 4 mg L^{-1}, but clams exposed to lower dissolved Mn concentrations (Mn[0.5]: 0.27 mg L^{-1}) also started to exhibit the first signs. The Mn effects on the digestive glands from the Mn[0.5] group were not observed in the Mn[5.0] group, which seems to suggest that Mn[5.0] clams used protective valve closure more intensely. Digestive gland tissue revealed significant sensitivity to Mn, since protein carbonylation increased after Mn[0.1] and Mn[0.5] exposures, and GST activity was inhibited in the Mn[0.5] group.

Short-term toxicity tests on bivalves have come under criticism and are not considered advantageous for evaluating sublethal effects in clams based on valve closure behavior, which is frequently observed as a general response to stress and introduces a source of variability (Hassal and Farris, 2007). Nevertheless, we believe that short-term toxicity tests on bivalves should be considered for monitoring issues as the sublethal effects we observed in consequence of this behavior could serve as an early signal of contamination. Moreover, elucidation of the regulatory mechanisms involved in the control of valve closure behavior could open up new approaches to bivalve ecotoxicology, since different conditions could interfere with particular pathways. For example, Mn can disrupt dopaminergic innervation of the gill, affecting the cilio-inhibitory system and leading to a drop in dopamine levels in the gills and cerebral and visceral ganglia of *Crassostrea virginica* clams (Martin et al., 2008). Therefore, since dopamine is involved in other neuronal control mechanisms, such as adductor muscle contraction (Almeida et al., 2003) and foot movements (Aiello et al., 1981), the effects of Mn on bivalves could be related to this pathway.

No evidence of oxidative stress was observed in the gills of *A. trapesialis* subjected to the Mn test, probably because of metabolic suppression and consequent lower ROS production in this tissue. Furthermore, we did not detect any increase in hemocyte DNA damage, despite the accumulation of Mn in the hemolymph. Conversely, LPO increased in mantle and digestive gland tissue of

clams subjected to Mn[5.0], and protein carbonylation was higher in digestive glands exposed to Mn[0.1] and Mn[0.5]. It has already been demonstrated that, under hypoxia, mitochondrial ROS production increases, causing oxidative damage (Hermes-Lima et al., 2015). Unfortunately, we did not measure ROS levels in these tissues to support this idea. Under hypoxia, ROS generation plays an important signaling role for the biosynthesis of antioxidants by activation of several transcriptional factors, such as hypoxia-inducible factor (HIF) and nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) (Hermes-Lima et al., 2015), both already detected in *Crassostrea gigas* bivalve tissue (Choi et al., 2013, Danielle et al., 2017).

In mantle tissue, we observed an increase in MT levels under the same conditions that led to elevated LPO (Mn[5.0]), lending weight to the idea that the mantle plays important role in the oxidative balance of *A. trapesialis*, mainly through MT (Oliveira et al., 2016). Le et al. (2016) discussed the multiple function of MT in different bivalve tissues and the inconsistency of results related to tissue-specific induction of the mantle. Mantle tissue has been found to contain high MT because of its high concentration of free radicals and intense metabolism, but digestive gland and gill tissue have also been suggested as target tissues. Our findings indicate that the mantle plays an important role in the MT function to protect against oxidative stress and should be considered in future studies.

Thus, Zn and Mn showed quite different effects on *A. trapesialis* after 96-h exposure and when we exposed the clams to Fe and mixtures in the Mix test, the effects on tissues were even more diverse. First, differences were observed in metal bioaccumulation. For example, in the Mix test, Mn levels did not fall in gill tissue as they did in the Mn test, but there was an increase in the hemolymph tissue. Regarding Zn, an increase in muscle, digestive gland and gill tissue levels in the Zn[1.0] group was only observed in the Mix test when Zn was mixed with Mn and Fe, but not after exposing the clams to Zn alone. These results suggest that Zn uptake and distribution are facilitated by the presence of the other metals. Combining these three cations in the experimental mixture elevated the positive charge outside the cells increasing the electrochemical gradient, which could have made it easier for Zn to enter the soft tissues of the clams. Since Zn, Mn and Fe are divalent transient metals, they have common uptake routes and can take advantage of the same transporters, such as calcium channels (epithelial Ca^{2+} channel - ECaC) or pumps

(Ca^{2+} -ATPase), divalent metal transporter-1 (DMT1) and ZIP family transporters (Garrick et al., 2006, Guerinot, 2000, Ivanina et al., 2013, Toyohara et al., 2005, Wood, 2012). Therefore, we would suggest that under the experimental conditions, Zn influx was enhanced. Moreover, as shown herein, natural Mn levels in *A. trapesialis* tissues are already high, as in other Unionidae (Campanella et al., 2005, Merlini et al., 1965, Sohail et al., 2016), and test concentrations in the mixtures were not sufficient to cause a significant, measurable increase of this metal in soft tissues.

In the Mix test, Fe alone caused an increase in ROS levels in *A. trapesialis* gill tissue. Under physiological conditions, soluble Fe^{2+} tends to be oxidized, in particular by molecular oxygen acting as pro-oxidant and catalyzing free radical reactions, as in the Fenton and Haber Weiss reactions (Lushchak, 2016). Nevertheless, neither LPO nor PC levels increased. On the other hand, a drop in ROS levels occurred after the clams were exposed to a mixture of Zn and Mn, probably due to the antioxidant role of Zn (Powell, 2000). This antioxidant Zn effect could also have counteracted the Fe pro-oxidant effect in Zn+Mn+Fe group, acting as an antagonist, since ROS levels did not differ from the control. We measured MT-like protein levels and SOD activity (two biomarkers related to the antioxidant role of Zn), but neither were significantly different from control group. MT-like protein levels were higher only in the mantle tissue of the Zn+Mn group. Once again, the mantle seems to be the tissue responsible for MT production.

No other effects were detected after exposing *A. trapesialis* to mixtures. Combined test concentrations of 1.0 mg L^{-1} Zn and 0.5 mg L^{-1} Mn were not sufficient to cause oxidative stress, nor metabolic suppression, in spite of Zn accumulation. It is estimated that 90% of intracellular Zn^{2+} is normally bound to organelles and molecules (Vallee and Falchuk, 1993) and it seems that after 96-h exposure to Zn, the tissues of *A. trapesialis* did not overshoot the essentiality threshold.

5 Conclusions

After short-term exposure (96 h) *A. trapesialis* used different strategies against Zn and Mn, the first metal stimulating MTs in gills and the second one probably promoting metabolic suppression after valve closure behavior. Thereby, using these strategies animals avoided damages in gills since LPO and PC did not occurred. Mantle and digestive gland seemed to be more sensitive organs as it was observed

LPO and PC even with increased MT in mantle. Since alterations on biomarkers occurred after short-term exposure we reinforce this species as a useful biomonitor which can provide early warning signals of metal contamination.

Our results showed that biomarkers were less affected under mixtures exposures when Zn bioaccumulation was enhanced by the presence of Mn and Fe (Zn+Mn and Zn+Mn+Fe groups). The evidences of Mn promoting metabolic suppression, oxidative damages in mantle and digestive glands or Fe effect on ROS generation did not appear in bivalves exposed to the mixtures. Thus, we can conclude, mixtures effects or responses were not simply a combination of single exposures to Zn, Mn and Fe, probably influenced by metals toxicokinetics.

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Capítulo IV

Manganese promotes metabolic suppression in freshwater bivalve *Anodontites trapesialis*

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Capítulo IV

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Capítulo IV

Manganese promotes metabolic suppression in gills of freshwater bivalve *Anodontites trapesialis*

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Capítulo IV

Abstract

A previous study indicated that Mn might stimulate valve closure behavior in *Anodontites trapesialis* leading to metabolic suppression. To support this hypothesis another endpoints were evaluated: glycogen levels dropped in gills after Mn exposure; elevated calcium and sodium levels and lower chloride in the hemolymph was observed; spot intensity of eleven gills proteins was suppressed in 2-DE gel. These endpoints reinforced that Mn might suppress gills metabolic rate *A. trapesialis*.

Keywords: acidification**,** ion regulation, glycogen, protein profile, valve closure behavior.

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Introduction

Bivalves present defense mechanisms against environmental stress agents such as the closure valve behavior which prevents the influx of contaminants [1]. Although a good strategy for preventing toxic effects, this response has consequences indicated by a set of biomarkers after short-term exposure to contaminants. Recently, we proposed that the freshwater clam, *Anodontites trapesialis*, possibly experienced metabolic suppression following an increase in valve closure frequency brought on by 96-h exposure to manganese [2]. Several biomarker alterations and a reduction in bioaccumulation led us to this hypothesis. In the gills of these clams, we observed a reduction in Mn and Zn levels and a lower concentration of reactive oxygen species (ROS) compared to other groups. Moreover, acetylcholinesterase (AChE) activity in muscle was decreased indicating that the bivalves were less active.

In this paper, we present further evidence pointing to the potential of Mn cause metabolic suppression in *A. trapesialis*. In bivalves, anoxia-tolerant species exhibit several adaptive strategies to deal with this condition, including: large glycogen tissue stores; production of alternative end products other than lactic acid, like succinate, to minimize internal acidity during long-term anaerobiosis; and use of shell bicarbonate for buffering [3]. Even so, the internal bivalve environment may become acid because of anaerobic respiration. Ionic balance in unionids is closely linked to the acid-base balance, and low pH could be responsible for disrupting ion regulation [4]. Moreover, in consequence of some of these strategies, internal ATP production decreases, requiring a reduction in energy demand and leading to a drop of over 90% in metabolic rate in tolerant species [5], especially in terms of the reduction in macromolecule biosynthesis (protein, RNA and DNA) [6].

Changes in protein levels, glycogen concentration and hemolymph ions could provide useful indicators to support the hypothesis that Mn has the potential to suppress gill metabolism in *A. trapesialis*. This study investigated these endpoints, aiming to provide evidence to back up the hypothesis. Moreover, for the first time, the protein profile of *A.*

trapesialis gill tissue was determined, paving the way for future work using a proteomic approach.

Material and methods

Exposure

Individuals of *A. trapesialis* were collected manually from the bottom of a dam at a fish farming facility in Londrina (Brazil) and immediately transferred to the laboratory in bags containing dam water. They were acclimated for 10 days in tanks containing carbon filtered water (renewed every 24 h) under a 12/12 h photoperiod and at constant temperature (19-21°C), pH (6.5–7.5) and oxygenation level (>85% saturation). During this period, the clams were fed with *Pseudokirchneriella subcaptata* algae (~10.000 cells mL^{-1}) every two days.

Clams (n = 48) were exposed to Zn (1.0 mg L^{-1}), to Mn (0.5 mg L^{-1}) or a mixture of both metals (Zn+Mn) for 96 h under semi-static conditions. A control group was tested in parallel for each experimental treatment, as the exposure treatments were carried out independently. Eight individuals per group were exposed individually in 1.5 L of exposure medium. Food was offered daily 1 h before water renewal. After 96 h, hemolymph samples were collected from the adductor muscle and gills removed, washed with a physiological solution (Sigma PBS), immediately frozen in liquid nitrogen and stored at -72°C.

Osmolality and ion content in the hemolymph

Hemolymph osmolality was determined in a freezing point osmometer (Gonotec, Osmomat 030). Sodium and potassium were analyzed using a flame photometer (Digimed DM-62). Calcium and magnesium ions were analyzed by flame atomic absorption spectrophotometry (AAnalyst 700, Perkin Elmer, USA) in hemolymph samples diluted in lanthanum oxide (La_2O_3 0.1%). Chloride levels were determined by the mercury thiocyanate method at 470 nm using a commercial kit (Labtest, Brazil).

Glycogen in the gills

Gills samples were subjected to the method described by Bidinotto et al. [7] in order to dissolve reduced sugars and samples were subsequently analyzed using the hydrolytic acid method described by DuBois et al. [8] to determine glycogen content in this tissue. Samples were incubated with 6N KOH at 100°C for 5 min and after dissolution, ethanol and K_2SO_4 (10%) were added. The samples were centrifuged and the pellet was re-suspended in distilled water. Then 4.1% phenol and concentrated sulfuric acid were added and absorbance determined at 480 nm.

Statistical analysis

The results for osmolality, ions and glycogen levels were tested for significant alterations between groups (CTR vs Experimental group), and the parametric Student t-test or nonparametric Mann-Whitney test performed. Differences were considered significant where $P < 0.05$.

Gill protein profiles - differences in spot intensity in 2-D electrophoresis gels

Total protein extraction and quantification

Gills were homogenized in ice-cold buffer [20 mM HEPES (pH 7.5), 320 mM sucrose, 1 mM ethylene diamine tetra acetic acid (EDTA), 5 mM ethylene glycol tetra acetic acid (EGTA), 5 mM dithiothreitol (DTT), 1 mM phenyl-methylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4), 10 mM sodium fluoride (NaF), 10 mM sodium pyrophosphate ($Na_4P_2O_7$), and 10 mM β-glycerophosphate, supplemented with complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)], centrifuged at 800 *x g* for 10 min (4°C) and the collected supernatant was again centrifuged at 100,000 *x g* for 1 h (4°C) to obtain the cytosolic soluble fraction. Total protein content was quantified using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, USA). The homogenates were stored at -80°C after adding glycerol (13%).

Two-dimensional electrophoresis (2-DE)

Proteins (900 µg per sample) were precipitated using methanol (1:4) and chloroform (1:1) and re-solubilized in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1 mM DTT, 1% IPG buffer 3–10 NL, 1.2% DeStreak solution (GE Healthcare, UK) and traces of bromophenol blue. The resulting supernatant was used for two-dimensional electrophoresis (2-DE). For first dimensional electrophoresis, samples were added to immobilized pH gradient (IPG) strips (18 cm) with a non-linear gradient pH range of 3–10 (GE Healthcare), which were passively rehydrated for 1 h (20 °C, no current) and then focused at 20°C using the Ettan IPGphor II six-step system, as follows: 1) 12 h at constant 30 V; 2) 1 h of voltage increase to 500V; 3) 3 h of voltage increase to 3500V; 4) 3 h at constant 3500V; 5) 3 h of voltage increase to 8000V; 6) 6 h at constant 8000V. The IPG strips were then subjected to equilibration steps using a buffer (6 M urea, 2% SDS, 30% glycerol and 50 mM Tris/HCl, pH 8.8) containing 1% DTT to reduce proteins, and 2.5% iodoacetamide for alkalization. They were then immediately loaded onto a 12.5% 24-cm long, 1-mm-thick acrylamide gel overlaid with 1% agarose in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and traces of bromophenol blue). Electrophoreses were performed in an Ettan DALTsix unit (GE Healthcare) at 25 °C (1 h pre-run step of 2 W/gel; running step of 15 W/gel). Gels were fixed in methanol, acetic acid and ultrapure water solution (50:10:40), washed in ultrapure water and stained with colloidal Coomassie Blue, as suggested by the supplier (Gel Code Blu, Pierce).

Image analysis

Gel images were obtained using an ImageScanner II and analyzed with ImageMaster 2D Platinum software (Amersham Biosciences). Spot detection was performed automatically (minimal area = 50 pixels; smooth factor = 5; saliency = 300) and after spot splitting and noise removal, the results were revised and edited manually. For each separate experimental treatment (Zn, Mn or Zn+Mn), gels (n = 8; 4 control and 4 treated) were matched with a reference gel (the one with the higher number of protein spots) by placing landmarks, and spots found in all eight 2-DE gels were considered for further analysis. The

relative spot volume (%Vol. = 100 × Vol. single spot / Vol. all spots) was used in quantitative analysis to minimize experimental error in protein loading and staining. In order to identify statistical differences between differentially expressed proteins, the Student t-test for unpaired samples was used with a significance level of 5%. A minimum 50% change was used as a further criterion for differential expression [9] to eliminate false positives.

Results

Three independent non-lethal tests were performed. The physical and chemical properties of the water are given in Oliveira et al. [2].

Osmolality and ion levels in the hemolymph

Differences in Na^+, Cl^- and Ca^{2+} levels and osmolality were observed in experimental groups and compared to the respective controls (Fig.1). Mn promoted an increase in Na^+ ($P = 0.034$) and Ca^{2+} ($P = 0.009$) and a decrease in Cl^- levels ($P = 0.011$), while Zn exposure led to an increase in Ca^{2+} ($P = 0.012$). Hemolymph exposed to the mixture contained lower Na^+ levels ($P = 0.025$) compared to the control group. Osmolality dropped in bivalves from the Zn ($P = 0.009$) and Mix ($P = 0.002$) groups. Mg^{2+} and K^+ levels did not vary in any of the experimental groups.

Glycogen in the gills

Decreases in glycogen levels were observed in gills from the Mn and Mix groups compared to the respective controls (Fig.2). Mn ($P = 0.014$) promoted a reduction of 41% in gill glycogen while bivalves from the Mix ($P = 0.018$) group showed a 29% decrease.

Anodontites trapesialis gill protein profile

To our knowledge, this is the first description of the *A. trapesialis* protein profile (Fig.3). We set up the proper conditions to obtain a satisfactory 2-D electrophoresis gel for this species. Using Coomassie blue stain, 900 µg of protein was sufficient to ensure

adequate intensity and separation of spots. The number of spots counted for each gel ranged from 707 to 1249, but around 400 spots were common to the gels compared (i.e. Zn[CTR] x Zn or Mn[CTR] x Mn). The number of spots of different intensity on comparing the experimental and control groups are given in Table 1. Gills from *A. trapesialis* exposed to Zn showed only one protein that was 2.27-fold less intense than the control, while Mn promoted significant alterations in 12 proteins, one up-regulated and 11 down-regulated. However, gills from individuals exposed to the mixture showed 2 up-regulated and 2 down-regulated proteins, totaling 4 altered spots. There was no coincidence in altered spots among the different treatments.

Discussion

A range of endpoints is required to understand a contaminant's mode-of-action and/or its effects on a given species, always bearing in mind that environmental factors and species behavior alter bioavailability and contaminant uptake. If biomarker alterations are analyzed individually, misinterpretations and false conclusions can occur. A set of endpoints examined in Oliveira et al. [2] indicated that Mn might cause metabolic suppression in the gills. In this study, we added another three endpoints that corroborate this idea. Valve closure is not exclusively stimulated by Mn, but is commonly employed as a strategy of defense against environmental stressors. This led short-term bivalve exposure to be questioned as a useful approach in environmental monitoring [1]. However, since biomarker alterations occur in consequence of valve closure, they could be used to represent the overall state of the organism and be treated as stress endpoints rather than the direct effect of a specific contaminant.

One significant consequence of valve closure behavior is the decrease in food uptake, followed by a reduction in energy availability. During metabolic suppression, bivalves utilize glycogen, their primary stored energy source, to satisfy basal metabolic demands [10]. Glycogen is distributed throughout bivalve soft tissue [11], including the gills, and is considered a potential endpoint to assess the health status of bivalves [10]. In this study, we

demonstrated that Mn promotes a 41% drop in glycogen, further evidence of the way Mn can affect metabolism. Moreover, when Mn was mixed with Zn, the same effect occurred but was more moderate (29% drop), possibly indicating an antagonistic interaction between these metals.

Other than feeding, gaseous exchanges are also impaired during valve closure, causing a drop in oxygen influx. Anaerobic respiration is required to maintain the basal metabolism in a hypoxic condition. Although bivalves can produce succinate or other fermentation products instead of lactic acid, changes in pH occur in hemolymph under hypoxic conditions [3] and calcium carbonate is used as a buffer since this ion can be derived from the shell and calcium phosphate granules [12]. Ca^{2+} levels increased in the hemolymph after bivalve exposure to Mn but also after exposure to Zn, thus this ion alone cannot confirm the differences between the effects of Mn and Zn. The regulatory function of other ions, such as sodium and chloride, can be impaired by acidification. Uptake of these ions in the gill cells of unionid bivalves occurs independently [4], but they are closely related to the acid-base balance, since Na^+ uptake involves H^+ exchange and Na^+K^+ ATPase activity, while Cl^- uptake is associated with HCO^-_3 exchange. Once the pH drops as a result of anaerobic metabolization, the uptake and regulation of these ions could be affected. Taken together, the results for Ca^{2+}, Na^+ and Cl^- levels in bivalve hemolymph support our hypothesis that Mn has the potential to stimulate valve closure behavior, since the levels of these three ions change, indicating an internal acidification state.

During metabolic suppression, many mechanisms work together in order to decrease energy and ATP demand, including behavioral, physiological and biochemical processes and a reduction in protein synthesis [13]. Corroborating this idea, our results show that Mn promoted the suppression of eleven proteins in the gills of *A. trapesialis*. Mn also stimulated a 3.88-fold increase in one protein, which could be associated with mechanism regulation. On the other hand, as shown by Oliveira et al. [2], Zn promotes different responses in *A. trapesialis* and when mixed with Mn in the experimental media, it prevented the Mn effect on metabolism. Zn promoted alteration in only one isolated spot, and the mixture in another four

spots. These results indicate that Zn and Mn act differently in the gills of *A. trapesialis* and a mixture of the two did not cause any obvious antagonistic or agonistic effect.

Recently the number of ecotoxicology studies on aquatic organisms, including *omics* approaches, has increased. In ecotoxicology, these approaches are used mainly to understand the modes of action of a compound or mixture and bring to light new pathways and molecules that could serve as potential biomarkers [14]. Proteomics in particular could indicate the physiological or pathological status of an individual, cell or tissue in diverse environmental conditions, because it allows protein expression to be assessed after posttranslational modifications [15]. Once the methodology to determine the protein profile in gills of *A. trapesialis* has been standardized, this species could be easily used as a biological model in proteomics studies.

In conclusion, our investigation lends weight to the hypothesis that Mn promotes metabolic suppression in *A. trapesialis.* Future studies should be undertaken to understand how Mn acts and determine the threshold level for this species.

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Figure captions

Fig. 1 Ion levels (Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}) and osmolality (mean ± SD) in hemolymph from *Anodontites trapesialis* exposed to 1.0 mg L^{-1} zinc (Zn), 0.5 mg L^{-1} manganese (Mn) and a mixture of these metals (Mix). Statistical analysis (Student t-test) was performed independently on the test data sets and different font styles used to distinguish them: Zn – lowercase; Mn – uppercase; Mix – uppercase italic. Significant differences are indicated by different letters.

Fig. 2 Glycogen levels (mean ± SD) in gills of *Anodontites trapesialis* exposed to 1.0 mg L^{-1} zinc (Zn), 0.5 mg L^{-1} manganese (Mn) and a mixture of these metals (Mix). Statistical analysis (Student t-test) was performed independently on the test data sets and different font styles used to distinguish them: Zn – lowercase; Mn – uppercase; Mix – uppercase italic. Significant differences are indicated by different letters.

Fig.3 Two dimensional electrophoresis gel from a *control* bivalve showing the protein pattern in *Anodontites trapesialis* gills. Numbers indicate spots of statistically different comparative intensities between control and experimental groups, with a minimum of 50% change (see Table 1). *Green: Zinc; Red: Manganese; Blue: Mixture.*

Fig.1

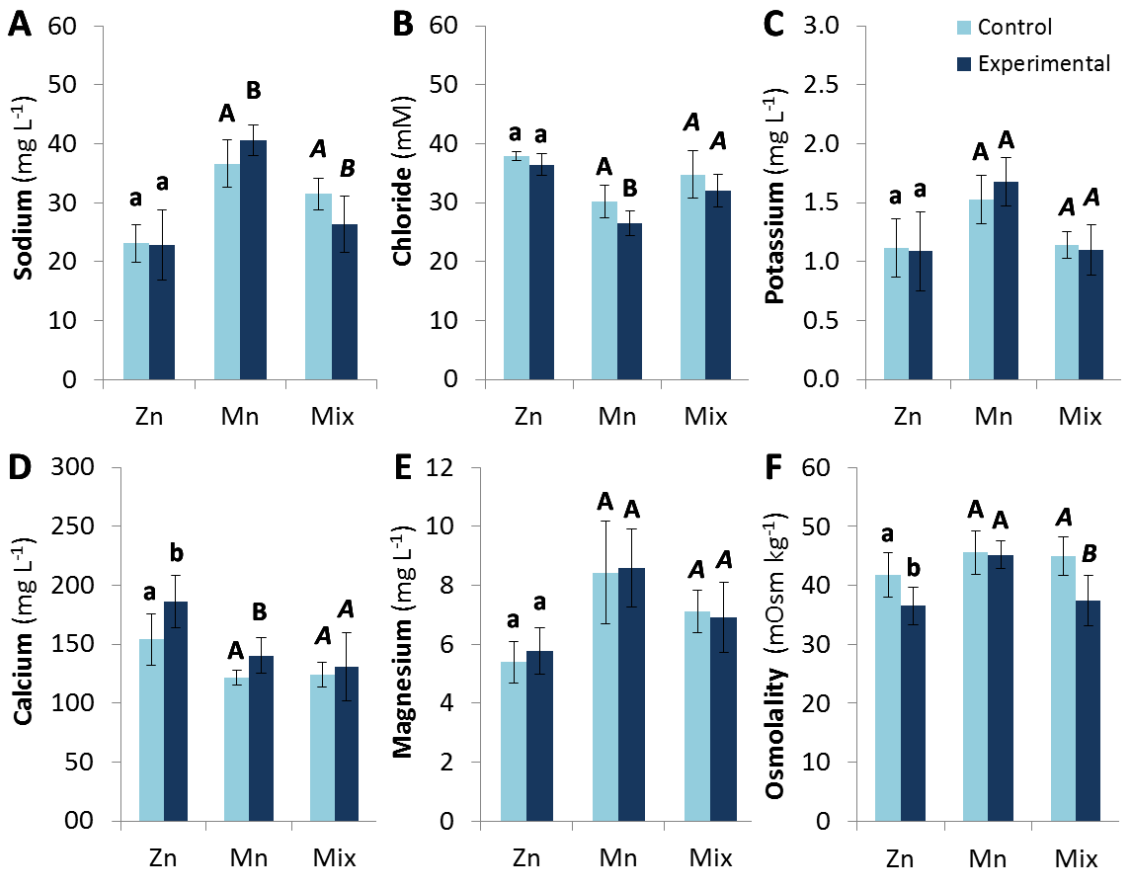
A
Sodium (mg L-1)
B
Chloride (mM)
C
Potassium (mg L-1)
Control
Experimental
D
Calcium (mg L-1)
E
Magnesium (mg L-1)
F
Osmolality (mOsm kg-1)
Zn
Mn
Mix

Fig.2

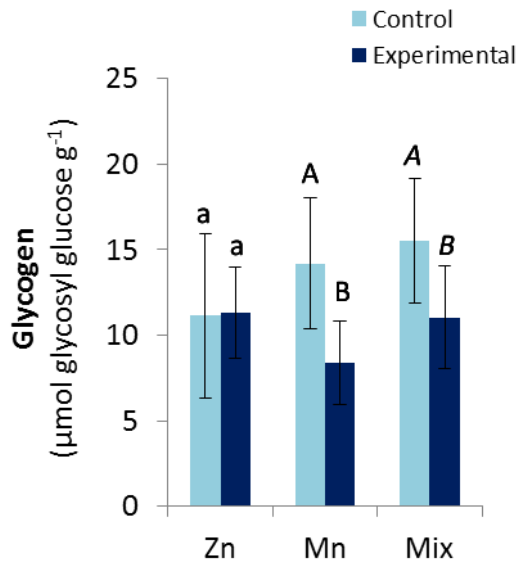
Control
Experimental
Glycogen
(µmol glycosyl glucose g-1)
Zn
Mn
Mix

Fig.3

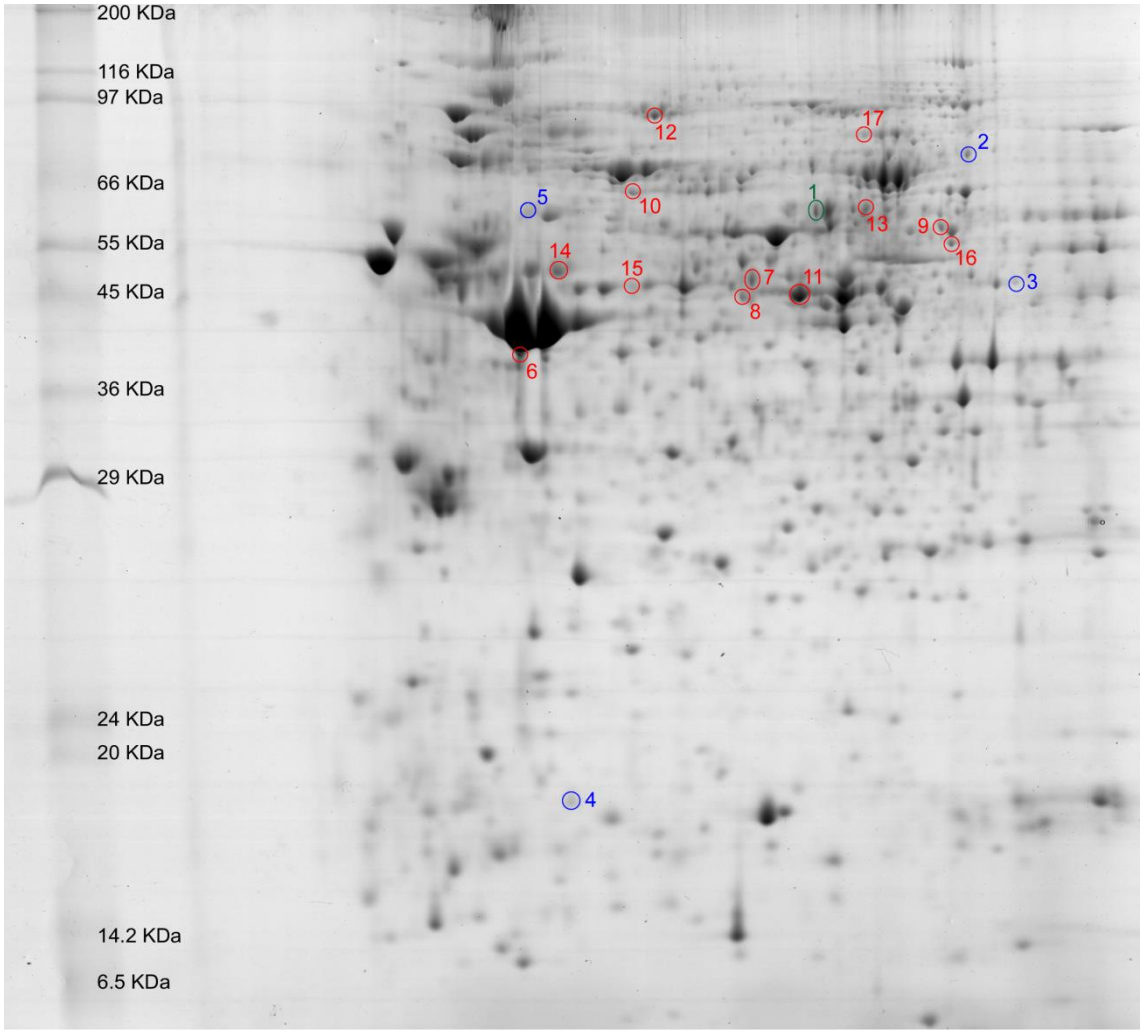
200 KDa
116 KDa
97 KDa
66 KDa
55 KDa
45 KDa
36 KDa
29 KDa
24 KDa
20 KDa
14.2 KDa
6.5 KDa
12
17
2
5
10
1
13
9
16
14
15
7
11
3
8
6
4

Table 1. Seventeen spots from all three tests (Zn, Mn and Mix) of statistically different comparative intensities between control and experimental groups (gl = 6; p = 0.01 → t > 3.707 **; p = 0.05 → t > 2.447), with a minimum of 50% change (at least 1.5-fold difference). For each spot *t-value* and *n*-fold *difference* the arrows indicate up- (↑) or down- (↓) regulation.

Spot	Test	Student t-value	*difference	↑↓	
1	Zn	2.46478	2.27	↓	
2	Mix	2.92114	1.82	↑	
3	Mix	2.60408	1.50	↑	
4	Mix	2.58080	1.50	↓	
5	Mix	2.55184	1.72	↓	
6	Mn	7.10552	3.88	↑	**
7	Mn	4.16115	1.65	↓	**
8	Mn	3.97414	1.55	↓	**
9	Mn	3.55323	2.26	↓	
10	Mn	3.42360	1.99	↓	
11	Mn	3.37866	1.73	↓	
12	Mn	2.83809	1.67	↓	
13	Mn	2.81859	1.57	↓	
14	Mn	2.68884	1.91	↓	
15	Mn	2.59720	1.55	↓	
16	Mn	2.58254	1.72	↓	
17	Mn	2.51878	1.54	↓	

Capítulo V

Individual and combined effects of Zn, Mn and Fe on the Neotropical teleost *Prochilodus lineatus*: bioaccumulation and oxidative stress biomarkers

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Manuscrito a ser submetido para avaliação no periódico *Aquatic Toxicology*

Individual and combined effects of Zn, Mn and Fe on the Neotropical teleost, *Prochilodus lineatus*: bioaccumulation and oxidative stress biomarkers

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Abstract

Metal bioaccumulation and oxidative stress biomarkers were determined in the Neotropical teleost, *Prochilodus lineatus,* in order to understand the effects of short-term exposure to essential metals Zn, Mn and Fe, both individually and combined in a mixture. Three independent tests were carried out. In the Zn test, fish were exposed to three concentrations (0.18, 1.0 and 5.0 mg L^{-1}) or to water only (Zn CTR); in the Mn test, fish were exposed to three concentrations (0.1, 0.5 and 5.0 mg L^{-1}) or to water only (Mn CTR); and in the Mix test fish were exposed to Fe (5.0 mg L^{-1}) and a mixture of Zn (1.0 mg L^{-1}) + Mn (0.5 mg L^{-1}), with and without Fe, or to water only (Mix CTR). After exposure for 96 h, tissues (muscle, blood cells, brain, bile, gills, liver, kidney and head kidney) were removed for metal bioaccumulation analysis. Oxidative stress biomarkers, such as lipid peroxidation (LPO), protein carbonylation (PCO), reduced glutathione (GSH), metallothionein (MT) and the activity of superoxide dismutase (SOD) and glutathione S-transferase (GST) were determined in liver tissue. DNA damage was analyzed in blood cells using the comet assay. In control groups, metal distribution varied from one tissue to another, depending on the metal analyzed, as follows: for Zn: *muscle* < *blood cells* < *brain* = *gills* < *bile* < *liver* < *kidney* < *head kidney*; for Mn: *bile* < *blood cells* < *head kidney* < *brain* < *muscle* < *gills* < *kidney* < *liver*; and for Fe: *muscle* < *brain* < *bile* < *gills* < *kidney* = *head kidney* < *blood cells* < *liver*. Our results revealed that Zn and Mn were bioaccumulated in fish tissues after 96 hours, mainly after exposure to a concentration of 5.0 mg L^{-1}, while Fe bioaccumulated only in muscle and gills after exposure to the mixture. In the Mix test, results indicated that one metal interfered with the toxicokinetics of the other, altering metal bioaccumulation and distribution among the organs of *P. lineatus* compared to exposure to one metal only. In *P. lineatus*, 5 mg L^{-1} levels of both Mn and Fe were toxic, since damage was observed (LPO in liver and DNA damage in blood cells). This same level of Zn induced liver responses (MT and GSH increases) to prevent damage. In terms of bioaccumulation and alterations of oxidative stress biomarkers, we showed that Zn, Mn and Fe interact agonistically, since fish exposed to the mixtures exhibited alterations not observed for individual metal exposure at the same concentrations. Since metals occur simultaneously in the environment and in industrial effluents, it is more appropriate to investigate the toxicity of mixtures rather than individual metals.

Keywords: DNA damage, metallothionein, reduced glutathione, toxicokinetic, lipid peroxidation.

1 Introduction

Fish are widely used as biomonitors of aquatic environments and are sensitive to a variety of contaminants, including metals. These organisms accumulate metals in their tissues, whether essential elements such as Zn, Cu, Mn and Fe, or non-essential elements such as Pb, Cd and Mg (Kennedy, 2011). In Neotropical regions, *Prochilodus lineatus* (Characiformes, Prochilodontidae), popularly known in Brazil as the *curimba*, is frequently used as a biomonitor. This species matches some requirements of an “ideal” biomonitor described by Johnson et al (1993), since it is one of the most abundant and widespread species in the South and Southeast of Brazil (Leonhardt et al., 2002) and is considered vulnerable to environmental changes due to its detritus feeding habits and migratory behavior (Shibatta et al., 2007). It has also been shown to be sensitive to metals such as aluminum (Camargo et al., 2009), lead (Monteiro et al., 2011, Ribeiro et al., 2014), copper (Nascimento et al., 2012) and nickel (Palermo et al., 2015), which affect different toxicity pathways.

Metals can cause oxidative stress in fish, increasing the production of reactive oxygen species (ROS) and impairing antioxidant defenses (Lushchak, 2016). Metals interact with other molecules and can affect the normal functioning of some cellular processes, such as cellular respiration, generating reactive oxygen species (ROS). The cells mobilize antioxidant defenses that neutralize ROS, but in situations where ROS production exceeds antioxidant capacity, oxidative damage occurs in biomolecules, such as membrane lipids, proteins and nucleic acids, characterizing oxidative stress. Antioxidant defenses involve some enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), as well as other molecules, such as reduced glutathione (GSH) and metallothioneins (MT). Several tissues were examined for investigating metal toxicity, but the liver is generally considered the main organ for the evaluation of oxidative stress biomarkers, since it plays an important role in the xenobiotic metabolism (Vieira, 2012) and because it contains higher concentrations of antioxidants compared to the gills, for example (Palermo et al., 2015). Several oxidative stress biomarkers previously evaluated in *P. lineatus* tissues were altered in the presence of metals such as nickel (Palermo et al., 2015) and copper (Carvalho et al., 2015), demonstrating their effectiveness as tools for evaluating the effects of metals on this fish.

Essential metals such as Zn, Mn and Fe are required for various functions in the organism but become toxic at levels exceeding a threshold above which the

organism can no longer regulate them (Newman and Clements, 2008). In consequence of the discharge of industrial or mining effluents, these metals can occur simultaneously in the aquatic environment at high concentrations (Jayaprakash et al., 2015, Oliveira et al., 2016). Zn, Mn and Fe play a part in regulating the production of antioxidants, such as MTs, and can be components of the antioxidant metalloenzyme SOD (Cu/Zn-SOD or Fe/Mn-SOD), acting as scavengers (Powell, 2000, Aguirre and Culotta, 2012). However, in spite of their antioxidant role, Zn, Mn and Fe ions can cause oxidative stress in fish (Gabriel et al., 2013, Mcrae et al., 2016). The use of biomarkers associated with oxidative stress is therefore of interest for evaluating the toxicity of these metals and verifying excessive concentrations at which they can be toxic. Where these metals are found together in the environment, interactions ranging from agonistic to antagonistic could occur (Spurgeon et al., 2010). For this reason, studies to evaluate the toxicity of mixtures can provide valuable information.

The aim of this study was to establish the distribution pattern of Zn, Mn and Fe in the tissues of *P. lineatus,* and to verify changes in the levels of these metals after acute exposure to both individual metals and metal mixtures. We also evaluated whether and how oxidative stress biomarkers change in animals exposed to environmentally relevant concentrations of these metals by comparing their effects, and whether interactions between them modifies their toxicity.

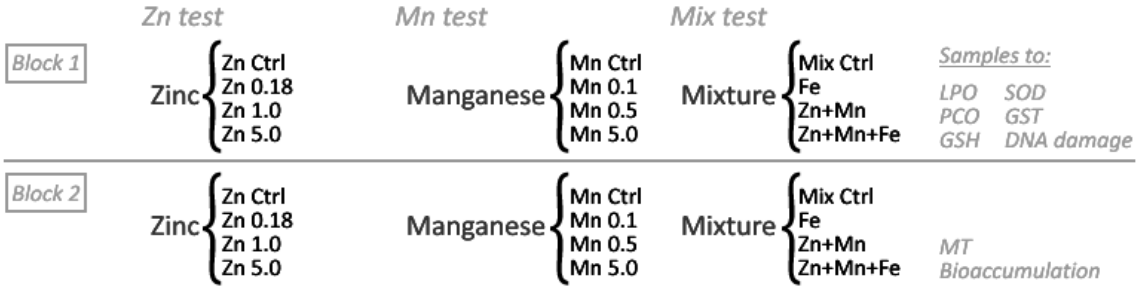
2 Material and methods

2.1 Experimental design

Juveniles of *P. lineatus* (n = 192; total length: 116.6 ± 9.8 mm; weight: 13.8 ± 2.9 g) were subjected to acute 96-h semi-static tests (water renewal every 24 h). Three tests were conducted, exposing fish to Zn (Zn test), Mn (Mn test) or a Zn/Mn mixture, with and without Fe (Mix test). In the Zn test, fish were exposed to the following nominal Zn concentrations: 0.18 mg L^{-1}, 1.0 mg L^{-1}, 5.0 mg L^{-1}. In the Mn test, fish were exposed to Mn at the nominal concentrations of 0.1 mg L^{-1}, 0.5 mg L^{-1} and 5.0 mg L^{-1}. In the Mix test, fish were exposed to intermediate nominal concentrations of Zn+Mn (Zn: 1.0 mg L^{-1}; Mn: 0.5 mg L^{-1}), Zn+Mn+Fe (Zn: 1.0 mg L^{-1}; Mn: 0.5 mg L^{-1}; Fe: 5 mg L^{-1}) and Fe alone (5 mg L^{-1}). The tests were performed independently, each test with its own control (CTR - fish exposed to water only). Metals were added to the water as sulfate salts ($ZnSO_4.7H_2O$; $MnSO_4.H_2O$ and

$FeSO_4.7H_2O$). Test concentrations of Zn, Mn and Fe were chosen based on two criteria: environmentally relevant concentrations observed in a stream near a coal mining area (Oliveira et al., 2016) and limit concentrations established in the specific Brazilian legislation (CONAMA 357/2005), either for class 1 and 2 (Zn: 0.18 mg L^{-1}; Mn: 0.1 mg L^{-1}), as for classes 3 and 4 (Zn: 5.0 mg L^{-1}; Mn: 0.5 mg L^{-1}; Fe: 5.0 mg L^{-1}).

In order to obtain samples in sufficient volume to analyze all the proposed biomarkers, six semi-static tests (24 h renewal period) were performed independently (one control group for each test), split into two blocks, as shown below.



Fish were obtained from the fish farm facility at the State University of Londrina and immediately transported to the laboratory. Prior to testing, the fish were acclimated for 5 days under a 12/12 h photoperiod in 300 L tanks containing carbon filtered water at constant temperature (~22.5 °C), pH (~7-8) and oxygenation level (>85% saturation). During acclimation, the fish were fed twice (2-day intervals) on commercial fish food (Guabi®, 36% protein). Feeding was suspended before beginning the experiments and during the tests.

2.2 Quantification of total and dissolved metals at mean exposure

Every day, before and after water renewal, non-filtered and filtered (Millipore Millex HV/PVDF 0.45-µm mesh filter) water samples were collected for analysis of total and dissolved Zn, Mn and Fe. These samples were immediately fixed with nitric acid (1%) and stored in decontaminated (10% nitric acid for 24 h) polypropylene plastic tubes. Zn was quantified by flame and Mn and Fe by electrothermic ionization in a graphite furnace in an atomic absorption spectrophotometer (AAnalyst 700, Perkin Elmer, USA) against standard reference solutions (Specsol, Brazil).

2.3 Fish sampling

Fish tissues were sampled according to the protocol approved by the Animal Ethics Committee of the State University of Londrina (Process 20032.2013.65). Prior to sampling, the fish were anesthetized with benzocaine (0.1 g L^{-1}) and blood samples collected from the caudal vein. One aliquot (10 µL) from each animal was preserved in fetal bovine serum (Gibco®) for subsequent use in the comet assay and another aliquot (~100 µL) was separated for bioaccumulation analyses. The fish were then killed by medullar section and samples taken from the gills, liver, bile, kidney, head kidney, axial muscle and brain. The sample tissues were washed (except blood) with physiological solution (Sigma PBS), immediately frozen and stored at -72°C.

2.4 Metal bioaccumulation

Metal concentrations were determined by flame (Zn and Fe) and electrothermic ionization in a graphite furnace (Mn) in an atomic absorption spectrophotometer (AAnalyst 700, Perkin Elmer, USA) against standard reference solutions (Specsol, Brazil) after acid digestion. Tissues (gills, liver, bile, kidney, head kidney, axial muscle and brain) and blood cells were completely dried at 60°C and digested in suprapure 5 N nitric acid for 48 h at 60°C, according to Alves and Wood (2006). Results are expressed in mg Kg dry $weight^{-1}$.

2.5 Biomarkers

Liver tissues were homogenized in potassium phosphate buffer (0.1 M, pH 7), centrifuged (14,000 ×g; 20 min, 4 °C) and the supernatants used to determine antioxidant and oxidative stress end-points. The biomarker methods used herein were as defined previously (Oliveira et al., 2016, Vieira et al., 2016) with some minor modifications. Lipid peroxidation (LPO) was determined by the TBARS fluorescence assay (ex/em: 535/590nm), according to Camejo et al. (1998), after incubating the supernatants for 1 hour with thiobarbituric acid (1.3%) at 60°C. To determine protein carbonylation (PCO) levels, we used the method described by Levine et al. (1994), based on the reaction with 2,4-dinitrophenylhydrazine (DNPH 10 mM, prepared in HCl 2 M) and consequent formation of dinitrophenyl hydrazones quantified at 360 nm. Non-protein thiol (NPSH) levels were determined according to the method in Beutler et al. (1963), measured at 412 nm. Superoxide dismutase (Cu/Zn-SOD)

activity was determined in microplates by the method that involves inhibiting the reduction rate of cytochrome c by the superoxide radical, at 550 nm and 25°C, according to McCord and Fridovich (1969). GST activity was determined using the method described by Keen et al. (1976), in which GST conjugates reduced glutathione with 1-chloro-2,4-dinitrobenzene, monitored for 1 min in a spectrophotometer at 340 nm. Levels of metallothionein-like proteins were determined using the method described by Viarengo et al. (1997) with modifications. Partially purified metalloprotein fractions were obtained from the supernatant after ethanol/acid chloroform fractionation, and sulfhydryl groups (-SH) quantified at 412 nm. All biochemical biomarkers are expressed in relation to the total protein content, determined at 595 nm by the method in Bradford (1976).

DNA damage was quantified by the length of DNA migration (comet tail length), visually determined in 100 randomly selected non-overlapping cells from each fish (n = 8 for each experimental group), as previously described in Vieira et al. (2016). DNA damage was expressed as a score calculated by the following formula (0xA) + (1xB) + (2xC) + (3xD), where A, B, C and D correspond to the number of cells in each comet class: class 0 = absence of comet tail; class 1 = comet tail shorter than the diameter of the nucleoid, class 2 = comet tail longer than the diameter of the nucleoid; class 3 = comet tail more than twice the diameter of the nucleoid.

2.6 Statistical analysis

Data were first tested for normality and homogeneity of variance to check the statistical requirements. Parametric analysis of variance (ANOVA) or non-parametric (Kruskal-Wallis) tests were run, followed by a multiple comparison test (Student Newman-Keuls or Dunn's), where recommended. Bioaccumulation and biomarker results for each tissue were compared separately for each test (Zn, Mn and Mix tests). Pearson correlation coefficients were calculated for metal accumulation and biomarkers in the liver. Differences were considered significant for $P < 0.05$.

3 Results

Six independent semi-static tests were successfully performed, despite some mortality after 96 h. In the first block of tests, the highest Zn concentration (5.0 mg L^{-1}) caused the death of 3 fish (total n = 8). In the Mix test control, only one fish died and in the Fe group two fish died. In the second block, mortality occurred only in the

Mix test, with two fish dying after Zn+Mn exposure and one after Zn+Mn+Fe exposure. Mn concentrations were confirmed as sublethal to *P. lineatus*.

In the water, metal concentrations were generally slightly lower than nominal, but the intended gradient was produced (Table 1).

Zn, Mn and Fe concentrations in *P. lineatus* tissues are shown in Figure 1. Metal concentrations in control fish (background concentrations) vary from one tissue to another, with Zn appearing at increasing clevels as follows: *muscle*< *blood cells*< *brain = gills* < *bile* < *liver* < *kidney* < *head kidney*. For Mn, levels found in tissues were as follows: *bile*< *blood cells* < *head kidney* < *brain* < *muscle* < *gills* < *kidney* < *liver*; and for Fe: *muscle* < *brain* < *bile* < *gills* < *kidney = head kidney* < *blood cells* < *liver*.

In the Zn test, after exposure for 96 h, Zn levels increased in gills, bile, liver and kidney after the Zn[5.0] treatment compared to fish in other groups, but only in muscle an increase was observed in both Zn[1.0] and Zn[5.0] treatments. The lowest Zn test concentrations did not alter Zn levels in any tissue compared to the control. Mn increased in the kidney after the Zn[5.0] treatment and Fe levels dropped in gill tissue after the Zn[5.0] treatment.

In the Mn test, fish blood, gills, bile, liver and kidney from the Mn[5.0] group all accumulated Mn after 96-h exposure. Mn levels in the gills increased for all three concentrations tested compared to control group, and there was a drop in gill tissue Zn. For the Zn+Mn+Fe mixture, there was an increase in Fe levels in muscle and gill tissue, an increase in Zn in the gills and liver and Mn in blood cells and kidney. When Fe was not present in the mixture (Zn+Mn group), Fe levels dropped in the brain tissue, but gill and liver Zn was still elevated, and blood, kidney and head kidney Mn levels remained high. A drop in Zn was observed in head kidney after exposure to Zn+Mn. Figure 2 summarizes the results of metal bioaccumulation in organs from fish exposed to mixtures (Zn+Mn and Zn+Mn+Fe) and to Zn[1.0], Mn[0.5] and Fe[5.0] (same metal concentrations as in the mixtures).

Table 1. Total and dissolved metal concentrations (mean ± SD; mg L^{-1}) for all treatments at the beginning (0h) and end (24h) over 24-water renewal period.

	Zn (0h)		Zn (24h)		Mn (0h)		Mn (24h)		Fe (0h)		Fe (24h)	
	Total	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**
Zn[Ctrl]	< DL	< DL	< DL	< DL	< 0.01	< 0.01	< 0.01	< 0.01	< 0.1	< 0.1	< 0.1	< 0.1
Zn[0.18]	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.02	< 0.01	< 0.01	< 0.01	< 0.01	< 0.1	< 0.1	< 0.1	< 0.1
Zn[1.0]	0.79 ± 0.04	0.77 ± 0.02	0.80 ± 0.05	0.77 ± 0.04	< 0.05	< 0.05	< 0.05	< 0.05	< 0.1	< 0.1	< 0.1	< 0.1
Zn[5.0]	4.31 ± 0.23	4.22 ± 0.17	4.45 ± 0.31	4.26 ± 0.21	< 0.05	< 0.05	< 0.05	< 0.05	< 0.1	< 0.1	< 0.1	< 0.1
Mn[Ctrl]	< DL	< DL	< DL	< DL	< 0.01	< 0.01	< 0.01	< 0.01	< 0.1	< 0.1	< 0.1	< 0.1
Mn[0.1]	< DL	< DL	< DL	< DL	0.15 ± 0.05	0.12 ± 0.03	0.08 ± 0.02	0.08 ± 0.03	< 0.1	< 0.1	< 0.1	< 0.1
Mn[0.5]	< DL	< DL	< DL	< DL	0.38 ± 0.03	0.37 ± 0.02	0.32 ± 0.07	0.27 ± 0.11	< 0.1	< 0.1	< 0.1	< 0.1
Mn[5.0]	< DL	< DL	< DL	< DL	4.46 ± 0.60	4.26 ± 0.65	4.51 ± 0.34	4.24 ± 0.55	< 0.1	< 0.1	< 0.1	< 0.1
Mix[Ctrl]	< 0.05	< 0.05	< 0.05	< 0.05	< 0.01	< 0.01	< 0.01	< 0.01	< 0.1	< 0.1	< 0.1	< 0.1
Mix[Fe]	< 0.05	< 0.05	< 0.05	< 0.05	< 0.02	< 0.02	< 0.02	< 0.02	4.59 ± 0.55	1.68 ± 0.66	2.34 ± 2.34	0.25 ± 0.31
Mix[Zn+Mn]	0.89 ± 0.04	0.89 ± 0.03	0.88 ± 0.03	0.86 ± 0.03	0.38 ± 0.08	0.34 ± 0.06	0.29 ± 0.12	0.25 ± 0.09	< 0.1	< 0.1	< 0.1	< 0.1
Mix[Zn+Mn+Fe]	0.89 ± 0.03	0.75 ± 0.07	0.86 ± 0.09	0.71 ± 0.06	0.34 ± 0.07	0.32 ± 0.06	0.32 ± 0.04	0.33 ± 0.04	4.86 ± 0.60	0.82 ± 0.20	3.49 ± 1.15	0.74 ± 0.63

DL = detection limit. DL for each metal was as follows - Al: 45 µg L-1; Mn: 1.5 µg L-1; Fe: 5 µg L-1; Zn: 1.5 µg L-1.

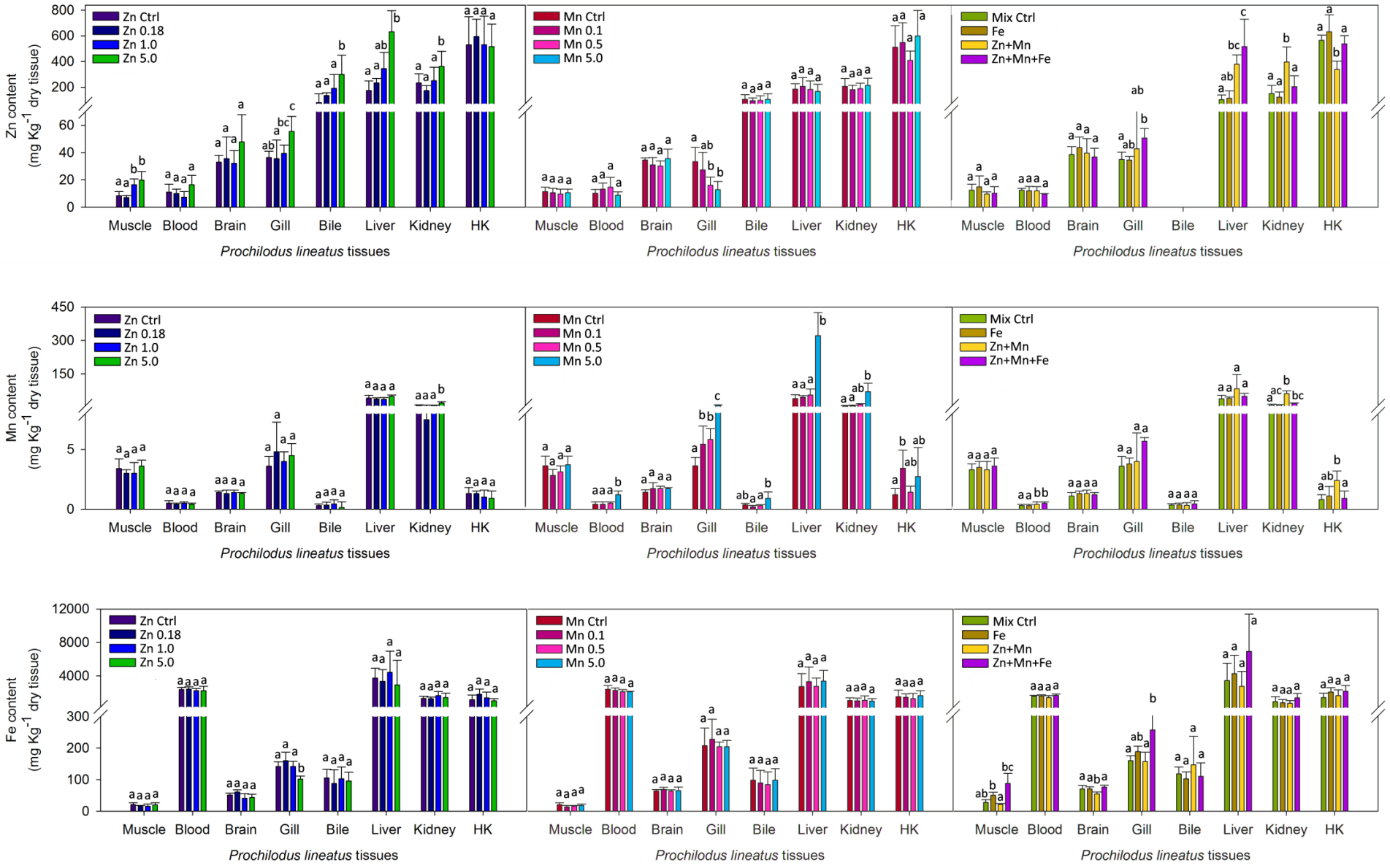


Fig.1 Zn (A), Mn (B) and Fe (C) levels (mean ± SD) in *P. lineatus* tissues (muscle, blood cells, brain, gill, bile, liver, kidney and head kidney-HK) after the Zn , Mn and Mix tests.

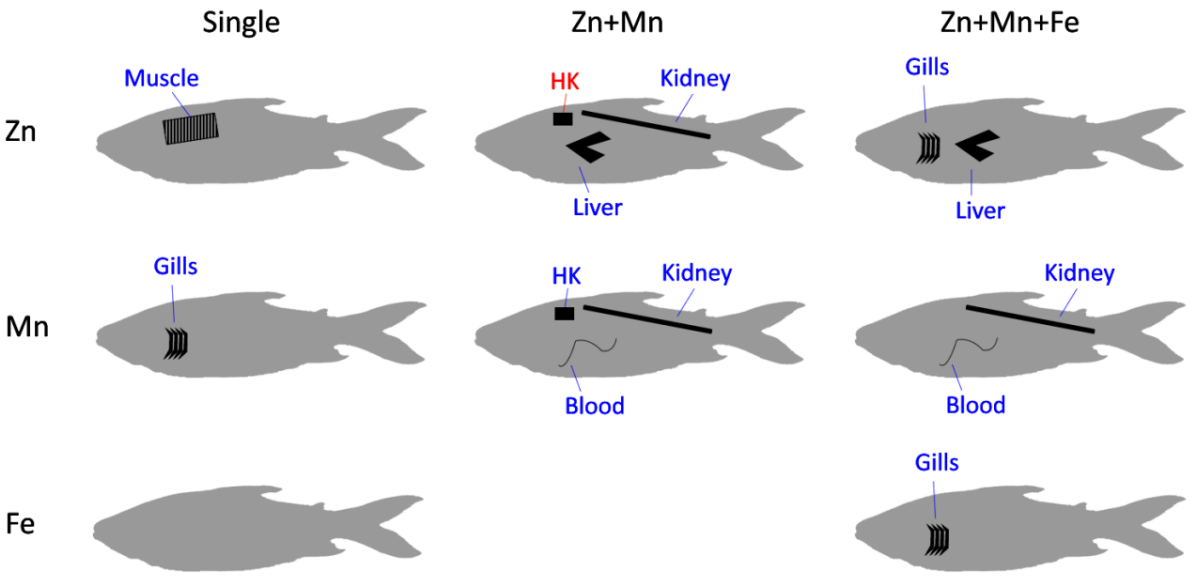


Fig. 2 Organs in which metal bioaccumulation showed significant increases (blue) or decreases (red) after 96-h exposure to Zn[1.0], Mn[0.5] and Fe[5.0] (single metal exposures at the same concentrations as in the mixtures) compared to exposure to mixtures (Zn+Mn and Zn+Mn+Fe).

Biomarkers were always compared for fish from the same test (Zn, Mn or Mix tests) and not between different tests since they were run independently, each having its own control group. Zn and Mn induced significant changes in biomarkers only at the highest concentrations. Liver from the Zn[5.0] group showed higher NPSH ($P < 0.001$, $F = 9.42$) and MT ($P < 0.001$, $H = 24.63$) levels than the control and Zn[0.18] groups (Fig.3), together with a decrease in SOD activity ($P = 0.008$, $F = 4.90$) (Fig.5). The Mn[5.0] group showed signs of oxidative stress, with higher LPO ($P = 0.036$, $H = 8.54$) (Fig.4) and increased DNA damage ($P = 0.008$, $F = 5.11$) (Fig.6). Although no differences were observed in biomarkers from fish exposed to intermediate concentrations of Zn and Mn, fish from Zn+Mn+Fe group showed some significant alterations, including increases in liver NPSH ($P = 0.005$, $F = 5.45$) and LPO ($P = 0.043$, $F = 3.19$) compared to the control group (Fig.3 and 4) and reduced GST activity (Fig.5) compared to the Zn+Mn group ($P = 0.021$, $F = 3.89$). Finally, exposure to Fe alone induced DNA damage, with a damage score significantly higher than that of all other groups ($P = 0.004$, $F = 6.19$) (Fig.6).

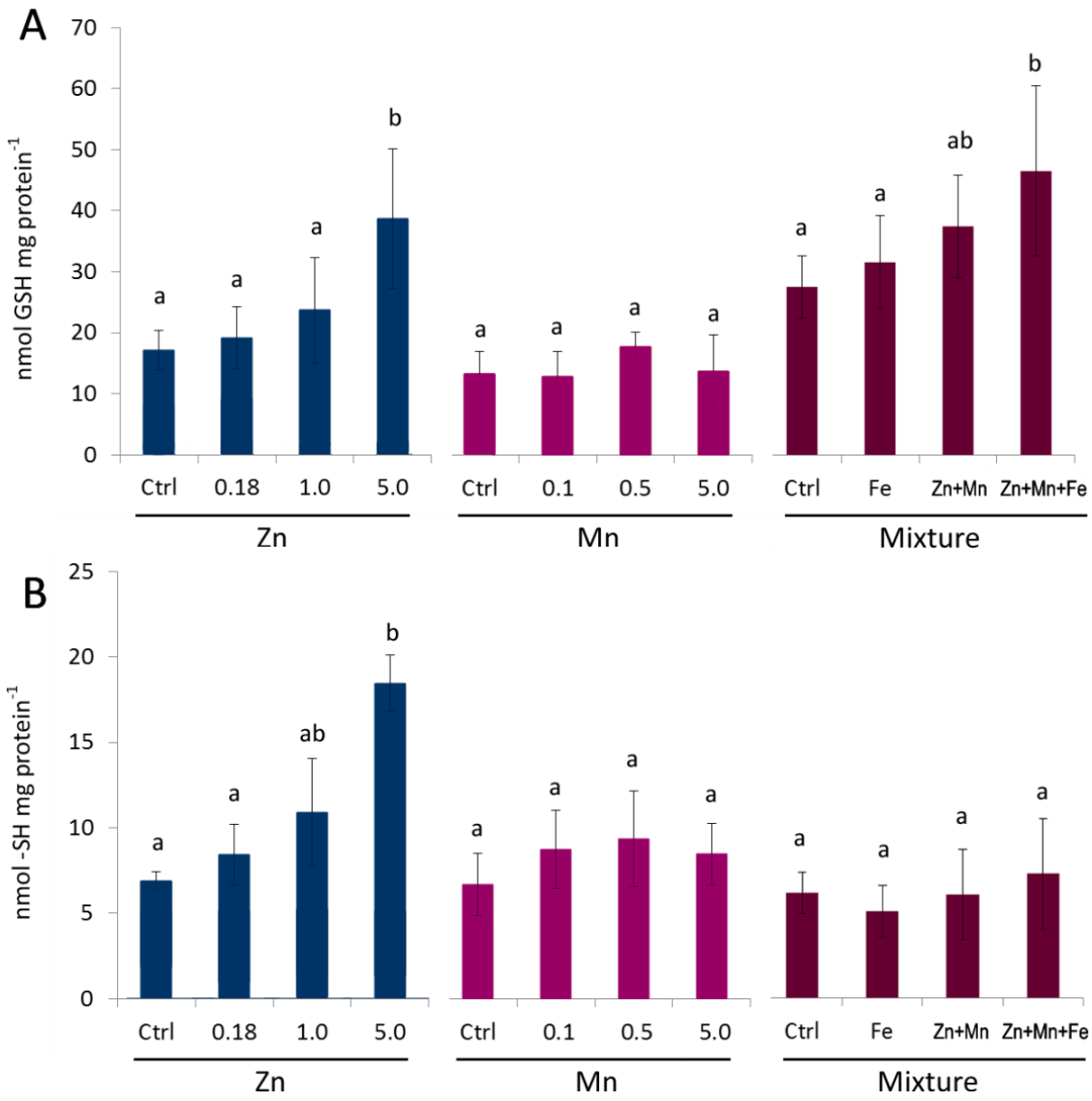


Fig.3 A: Non-protein thiols and B: metallothionein-like proteins (mean ± SD) in liver from *P. lineatus* subjected to the Zn, Mn and Mix tests.

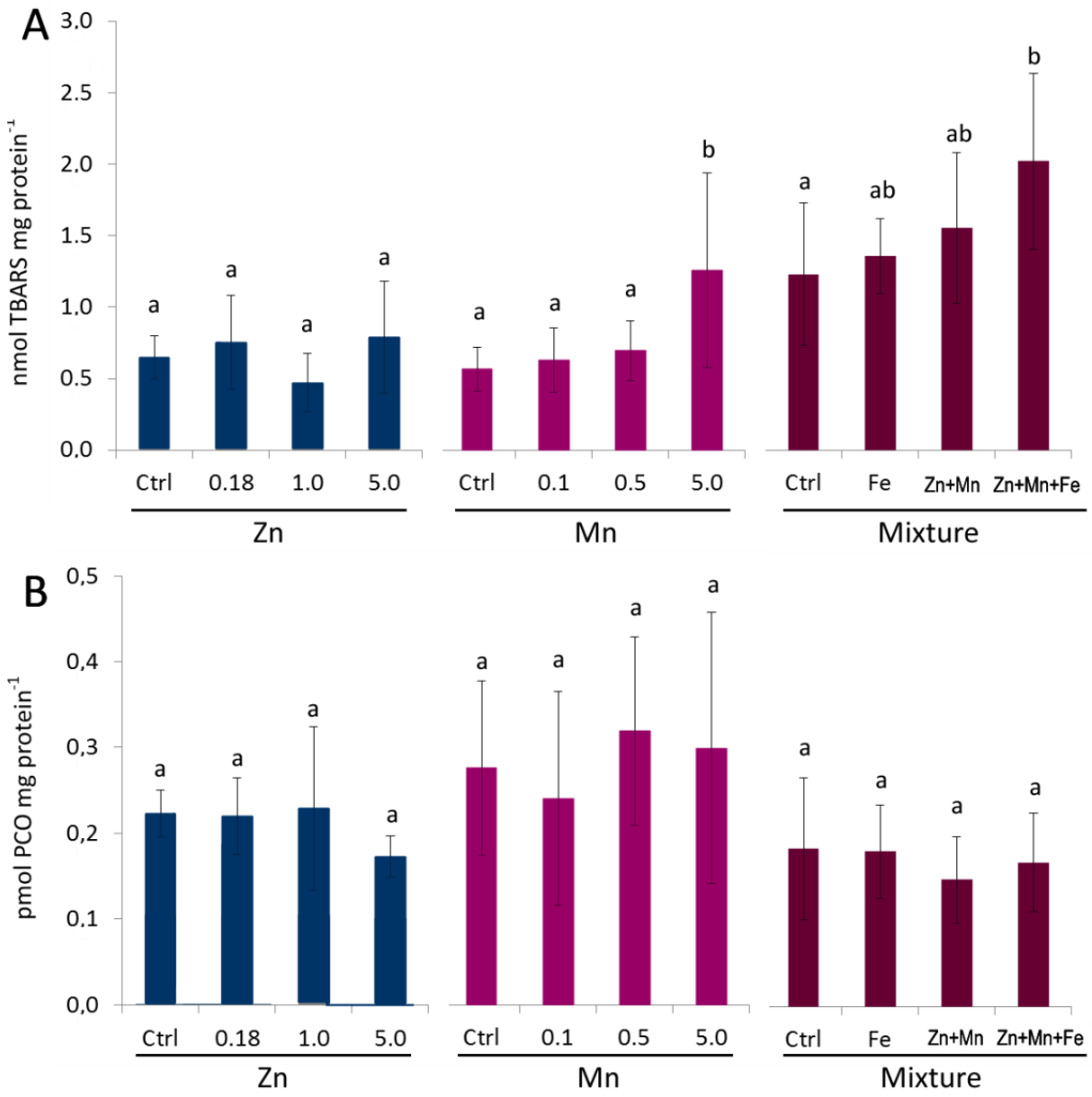


Fig.4 Oxidative damage in liver from *P. lineatus* subjected to the Zn, Mn and Mix tests. A: Lipid peroxidation (LPO); B: Protein carbonylation (PCO) (mean ± SD).

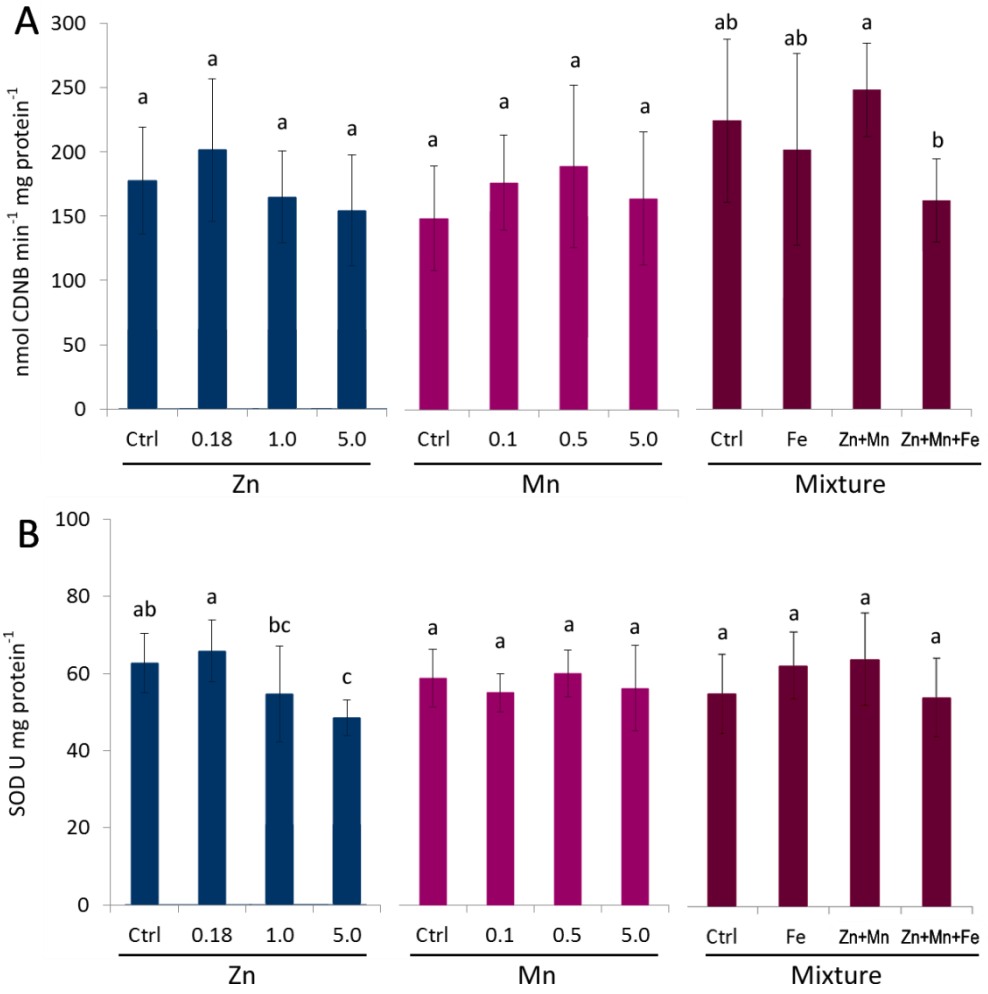


Fig.5 A: glutathione S-transferase (GST) and B: superoxide dismutase (SOD) activity (mean ± SD) in liver from *P. lineatus* subjected to the Zn, Mn and Mix tests.

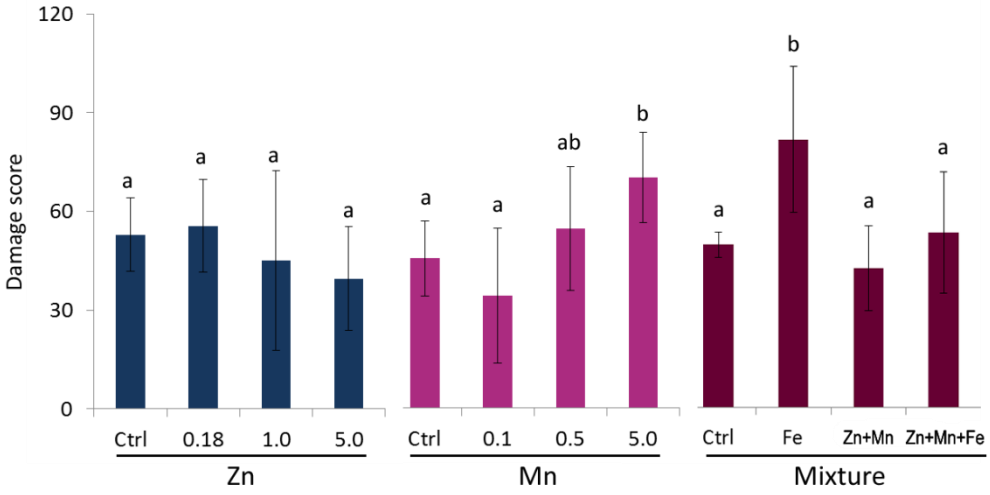


Fig.6 Erythrocyte damage score (mean ± SD) of *P. lineatus* subjected to Zn, Mn and Mix tests.

The Pearson's correlation matrix (Table 2) indicates that liver Zn levels showed a significant positive correlation with NPSH and MT, while liver Fe accumulation was correlated with NPSH and LPO levels.

Table 2 Pearson's correlation coefficients for metal bioaccumulation in the liver and hepatic biomarkers.

	Mn	**Fe**	**NPSH**	**MT**	**LPO**	**PC**	**GST**	**SOD**
Zn	-0.176	0.250	0.620 *	0.713 *	0.201	-0.470	-0.292	-0.521
	P = 0.584	P = 0.433	P = 0.032	P = 0.009	P = 0.532	P = 0.123	P = 0.358	P = 0.082
Mn		-0.149	-0.298	-0.020	0.195	0.461	-0.166	-0.114
		P = 0.644	P = 0.347	P = 0.951	P = 0.545	P = 0.131	P = 0.606	P = 0.723
Fe			0.680 *	-0.265	0.701 *	-0.451	-0.089	-0.111
			P = 0.015	P = 0.406	P = 0.011	P = 0.141	P = 0.782	P = 0.731

Asterisks indicate significant differences (P≤0.05).

4 Discussion

This study makes some important contributions to the literature by revealing the background levels of three essential metals in eight different tissues of a Neotropical freshwater fish species (*P. lineatus*) and highlighting liver and blood cell biomarker responses to short-term exposure to environmentally relevant concentrations of Zn, Mn, Fe, both individually and combined.

4.1 Metal distribution and bioaccumulation

The levels and distribution of Zn, Mn and Fe found in *P. lineatus* provide valuable data for future studies, mainly serving as basal values in field approaches. In general, Fe levels were the highest, followed by Zn, and then Mn. This seems to be a pattern in liver from freshwater fish (Table 3). Fe plays an essential role as an oxygen carrier in vertebrates, forming the heme group of hemoglobin and acting as an electron acceptor or donor in cytochrome *c* oxidases in the respiratory chain (Bury et al., 2012). Elevated Fe levels in fish tissues/organs were associated with the blood. In fact, in addition to the blood cells, liver, kidney and head kidney, as well-perfused organs, exhibited the highest Fe background concentrations. In contrast, blood levels of Zn and Mn were lower, but liver and kidney tissues showed higher levels of Zn and Mn.

Table 3 Zn, Mn and Fe levels in freshwater fish tissues.

	Freshwater fish	Liver	Gill	Muscle	Brain	Kidney	
Zn	***Prochilodus lineatus***	**100.9 – 184.8 mg Kg^{-1} dw**	**33.3 – 36.4 mg Kg^{-1} dw**	**8.4 – 12.3 mg Kg^{-1} dw**			**This study**
	Salmo trutta		~290 mg Kg^{-1}				Hansen et al. (2007)
	Oreochromis niloticus	~ 140 mg Kg^{-1} dw	~120 mg Kg^{-1} dw				Firat et al. (2008)
	Carassius auratus	~120 mg Kg^{-1} dw					Qu et al. (2014)
	Ctenopharyngodon idellus	~42 mg Kg^{-1} ww	~21 mg Kg^{-1} ww	~4.9 mg Kg^{-1} ww			Liu et al. (2012)
	Carassius auratus	~33 mg Kg^{-1}	~87 mg Kg^{-1}	~4.7 mg Kg^{-1}			Jia et al. (2017)
	Pelteobagrus fulvidraco	~17 mg Kg^{-1}	~17 mg Kg^{-1}	~3.7 mg Kg^{-1}			Jia et al. (2017)
	Squaliobarbus curriculus	~23 mg Kg^{-1}	~25 mg Kg^{-1}	~4.1 mg Kg^{-1}			Jia et al. (2017)
	Pseudoplatystoma corruscans	~60 mg Kg^{-1} ww		~20 mg Kg^{-1} ww			Arantes et al. (2016)
	Barbus grypus	~78 mg Kg^{-1}	~103 mg Kg^{-1}	~23 mg Kg^{-1}			Alhashemi et al. (2012)
	Barbus luteus	~119 mg Kg^{-1}	~59 mg Kg^{-1}	~45 mg Kg^{-1}			Alhashemi et al. (2012)
	Barbus sharpeyi	~92 mg Kg^{-1}	~89 mg Kg^{-1}	~45 mg Kg^{-1}			Alhashemi et al. (2012)
	Cyprinus carpio	~528 mg Kg^{-1}	~611 mg Kg^{-1}	~28 mg Kg^{-1}			Alhashemi et al. (2012)
	Silurius trisostegus	~49 mg Kg^{-1}	~65 mg Kg^{-1}	~24 mg Kg^{-1}			Alhashemi et al. (2012)
Mn	***Prochilodus lineatus***	**38.8 – 53.6 mg Kg^{-1} dw**	**~3.6 mg Kg^{-1} dw**	**3.3 – 3.6 mg Kg^{-1} dw**	**1.1 – 1.4 mg Kg^{-1} dw**	**9.0 – 9.8 mg Kg^{-1} dw**	**This study**
	Colossoma macropomum	> 1 mg Kg^{-1}	~ 3 mg Kg^{-1}		> 1 mg Kg^{-1}	> 1 mg Kg^{-1}	Gabriel et al. (2013)
	Rhamdia quelen	~ 2 mg Kg^{-1}	> 1 mg Kg^{-1}		> 1 mg Kg^{-1}	> 1 mg Kg^{-1}	Dolci et al. (2014)
	Ctenopharyngodon idellus	~2.5 mg Kg^{-1} ww	~6.8 mg Kg^{-1} ww	<1 mg Kg^{-1} ww			Liu et al. (2012)
	Carassius auratus	~1.6 mg Kg^{-1}	~7.2 mg Kg^{-1}	<1 mg Kg^{-1}			Jia et al. (2017)
	Pelteobagrus fulvidraco	~1.7 mg Kg^{-1}	~4.9 mg Kg^{-1}	<1 mg Kg^{-1}			Jia et al. (2017)
	Squaliobarbus curriculus	~2.0 mg Kg^{-1}	~14 mg Kg^{-1}	<1 mg Kg^{-1}			Jia et al. (2017)
	Barbus grypus	~4.4 mg Kg^{-1}	~4.5 mg Kg^{-1}	~1.7 mg Kg^{-1}			Alhashemi et al. (2012)
	Barbus luteus	~5.8 mg Kg^{-1}	~17 mg Kg^{-1}	~4.2 mg Kg^{-1}			Alhashemi et al. (2012)
	Barbus sharpeyi	~4.3 mg Kg^{-1}	~13 mg Kg^{-1}	~2.2 mg Kg^{-1}			Alhashemi et al. (2012)
	Cyprinus carpio	~5.6 mg Kg^{-1}	~6.7 mg Kg^{-1}	~1.0 mg Kg^{-1}			Alhashemi et al. (2012)
	Silurius trisostegus	~3.8 mg Kg^{-1}	~13 mg Kg^{-1}	<1 mg Kg^{-1}			Alhashemi et al. (2012)
Fe	***Prochilodus lineatus***	**2810 – 4123 mg Kg^{-1} dw**	**141.8 – 205.7 mg Kg^{-1} dw**	**20.1 – 26.9 mg Kg^{-1} dw**			**This study**
	Carassius auratus	~ 1900 mg Kg^{-1} dw					Qu et al. (2014)
	Carassius auratus	~193 mg Kg^{-1}	~90 mg Kg^{-1}	~3.1 mg Kg^{-1}			Jia et al. (2017)
	Pelteobagrus fulvidraco	~127 mg Kg^{-1}	~46 mg Kg^{-1}	~3.7 mg Kg^{-1}			Jia et al. (2017)
	Squaliobarbus curriculus	~129 mg Kg^{-1}	~48 mg Kg^{-1}	~3.7 mg Kg^{-1}			Jia et al. (2017)
	Pelmatochromis guentheri		~5.1 mg Kg^{-1} ww	~4.6 mg Kg^{-1} ww			Ajima et al. (2015)
	Pelmatochromis pulcher		~6.0 mg Kg^{-1} ww	~4.4 mg Kg^{-1} ww			Ajima et al. (2015)

Zn and Mn play a part in determining antioxidant status (Powell, 2000, Coassin et al., 1992), and levels in fish liver may in part be related to the importance of this tissue in preserving the oxidative balance, since detoxification processes are usually triggered in the liver once multiple oxidative reactions and high free radical generation occur (Martínez-Álvarez et al., 2005). Metals are stored and/or detoxified in fish by molecules like GSH and MT. Increases in these molecules may cause the metals to accumulate and be retained in an inert form inside lysosomes and granules (Wood, 2012). In *P. lineatus*, hepatic GSH and MT increases were positively correlated (Table 2) with Zn bioaccumulation in the liver after exposure to 5.0 mg L^{-1}, but not with Mn bioaccumulation. Thus, one possible hypothesis is that excess Mn in the liver tissue of fish exposed to Mn[5.0] was bound to other ligands or free.

The kidney in freshwater fish is essential for eliminating large volumes of diluted urine and reabsorbing ions (Martinez, 2017), but it also excretes excess trace elements. Elevated concentrations of Zn and Mn in the water are probably indicative of Zn and Mn concentrations in the ultrafiltrate as fish attempt to eliminate excess metal in urine, and this may stimulate metal reabsorption and accumulation in the kidney, since divalent ions Zn^{+2} and Mn^{2+} can compete with Ca^{2+} and be reabsorbed through passive (epithelial Ca^{2+} channel - ECaC) or active (Ca^{2+} -ATPase) calcium transport pathways. There is a gap in our knowledge of excretion routes in fish (Wood, 2012) for Zn and other metals, such as Mn. Most Zn is thought to be excreted by the gills, but the intestine may also play an important role, as in mammals, while the kidney accounts for less than 1% of total Zn losses (Hardy et al., 1987). In our experimental design, the fish food supply was suspended 48 h prior beginning the tests and no food was offered during testing, which decreased metal excretion in the faeces and increased urine output. Metals can also be excreted in the bile in fish (Hauser-Davis et al., 2012), and our results lend weight to the idea that the bile should be considered a potential biomarker for environmental metal contamination, since Zn and Mn were found to have bioaccumulated in the bile after exposure to the highest metal concentrations.

Although the gills were not among the organs with the highest background concentrations of the analyzed metals, an increase in gill metal bioaccumulation was observed after 96 h, even in fish exposed to intermediate concentrations. Because the gills are in intimate contact with the environment in waterborne contamination, they are often shown to be bioaccumulation target tissues in fish (Wood, 2012). In

freshwater fish, ion uptake occurs primarily in the gills through metal-specific carriers, i.e. apical ZIP (Zn importer) (Guerinot, 2000) and divalent metal transporter-1 (DMT1) (Garrick et al., 2006). Alternatively metals can use other ions on active transport pathways. As mentioned above, divalent ions mimic Ca^{2+} and can enter the gill cells through channels and pumps (Ivanina et al., 2013).

One important finding of this study was that increases in metal bioaccumulation occurred in several organs after exposure to mixtures, although no accumulation was observed after individual metal exposure at the same concentration. These results indicate that one metal affected the toxicokinetics of another, probably altering its uptake and distribution in *P. lineatus* organ tissues. As discussed in Oliveira et al. (Capítulo III), Zn, Mn and Fe are divalent cations which elevate the positive charge outside the organism, inducing an increase in the electrochemical gradient that could favor the influx of these elements. Figure 2 is a simple illustration of how metals were distributed in the organs after the fish were exposed to individual and mixed metals at the same concentrations. Our results revealed that Zn accumulation was more closely associated with the liver, while Mn accumulation was associated with the blood and kidney, each probably following its own metal detoxification and elimination pathway. Moreover, Fe and Zn bioaccumulation in the gills of fish exposed to the Zn+Mn+Fe mixture seems to override the Mn bioaccumulation observed in exposure to the individual metals, and the presence of Fe in the mixture favored Zn bioaccumulation in the gills rather than the kidney.

4.2 Biomarkers in liver and blood cells

Both Zn and Mn caused hepatic and/or blood alterations only after exposure to a concentration of 5.0 mg L^{-1}, with metal bioaccumulation in the liver and blood cells only in fish from the Zn[5.0] and Mn[5.0] groups. On the other hand, the concentrations found in a stream near coal mining activities (Oliveira et al., 2016), equivalent herein to intermediate concentrations of Zn (1.0 mg L^{-1}) and Mn (0.5 mg L^{-1}), when tested individually did not induce alterations or bioaccumulation in the liver and blood cells of *P. lineatus*, indicating that the fish were able to regulate the homeostasis of these trace metals at these concentrations. Nevertheless, Zn and Mn were found at increased levels in other organs like muscle (Zn), and gills and head kidney (Mn), which could be used as target organs in future studies. Moreover, metal

toxicity is often associated with impairment of the calcium metabolism, osmo-ionic regulation and the acid-base balance (Kennedy, 2011), which involves some biomarkers not investigated in this study. Further investigation is therefore required to confirm that these intermediate concentrations are not toxic to *P. lineatus*.

Fish exposed in the Zn[5.0] group did not exhibit any evidence of oxidative stress in the liver. On the contrary, the increase in NPSH and MT-like protein levels in fish exposed to the highest Zn concentration showed that they were able to respond to the presence of Zn. GSH and MTs are the primarily cytosol ligands of Zn in cells (Convin et al., 2008) because of the high affinity of this metal to cysteine. Trace metals like Zn and Cu are associated with MT regulation and can induce MT synthesis (Olsson, 1993), making the liver cells more resistant to metal toxicity, since this tripeptide, as well as GSH, bind to free Zn ions removing them from the cytosol. Thus, in consequence of increases in NPSH and MT-like proteins, no other alterations were observed in *P. lineatus* biomarkers in the liver, nor in LPO or PC activity. However, Cu/Zn-SOD activity decreased in the liver of these same fish, indicating that the antioxidant role of Zn is more related to non-enzymatic defenses than to antioxidant enzymes. A drop in SOD activity could be ascribed to Zn scavenging by GSH and MT, in turn reducing the availability of free metal ions to the enzyme.

Our results show that although Zn was bioaccumulated in different tissues, the toxicity threshold for this species was not exceeded in liver. Zn toxicity varies from one fish species to another and, based on oxidative stress biomarkers, there are no typical responses and/or effects. Several studies have shown that Zn causes oxidative damage to lipids and proteins (Loro et al., 2012; McRae et al., 2016) and can impair antioxidant defenses (Qu et al., 2014). LPO increased in the liver of *Galaxias maculatus* exposed to only 1.0 mg L^{-1} Zn for 96 h (McRae et al., 2016) and reductions in enzymatic and non-enzymatic antioxidants were observed in the liver of *Carassius auratus* exposed to 0.1 and 1.0 mg L^{-1} Zn for acute and sub-chronic periods (Qu et al., 2014). Variations in metal toxicity can be caused by differences in species sensitivity and experimental media properties, such as water hardness, temperature and pH.

The idea, based on the liver biomarkers, that the fish were able to respond to the presence of Zn runs counter to the evidence, i.e. mortality of 40% of the fish in the Zn[5.0] group. These conflicting results may indicate an intra-specific variation in

biomarker responses, or that liver and blood were not the tissues most affected by Zn exposure. Once again, further biomarker analysis of other tissues is required before we can make any conclusive statement regarding Zn water concentrations that are safe for *P. lineatus.*

In contrast to the results obtained for Zn exposure, on the liver and blood cells of individuals exposed to Mn (Mn[5.0]) effects (damages) were observed rather than responses. Mn accumulates primarily in cell mitochondria, and because of its high pro-oxidant capacity, Mn affects oxidative respiration, increasing ROS production and leading to mitochondrial dysfunction (Farina, 2013, Dolci, 2013). In the liver tissue of *P. lineatus*, neither NPSH and MT-like protein concentrations nor Cu/Zn-SOD activity responded to Mn exposure, leading to oxidative damage as indicated by the increase in hepatic LPO. In goldfish, *C. auratus,* exposed for 96 h to 0.1mM (~5.5 mg L^{-1}) Mn, LPO also increased in the liver and at the same time, hepatic catalase (CAT) dropped and there was no impact on SOD activity. Only glutathione peroxidase (GPx) activity increased under these conditions (Vieira, 2012). This particular study corroborates our idea that concentrations of around 5 mg L^{-1} Mn cause oxidative damage in freshwater fish after exposure for 96 h, even though the Mn concentrations may be not sufficient to induce significant alterations in antioxidant mechanisms.

Like Mn, Fe is a pro-oxidant that easily changes its oxidation state, alternating between 2+ to 3+, endowing this metal with important functions in living organisms (Bury et al., 2012). Because of this characteristic, Fe is involved in the Fenton reaction which produces the hydroxyl radical ($HO^{\bullet}$), the most reactive oxygen species radical, initiating a cycle of cellular damage, including DNA damage. In this study, the erythrocytes of fish exposed to Fe suffered more DNA damage than control or mixture groups, in spite of the fact that the dissolved Fe concentration in the water was lower (1.68 ± 0.66 mg L^{-1}) than the total concentration (4.59 ± 0.55 mg L^{-1}). This effect disappeared in fish from Zn+Mn+Fe group, in which the level of dissolved Fe was even lower (0.82 ± 0.20 mg L^{-1}) and may not have been enough to cause DNA damage.

As already discussed, the intermediate concentrations of Zn and Mn did not affect or induce any responses in liver and blood cells of *P. lineatus.* However, when mixed with Fe the results were different. Biomarker changes were observed only when Fe was present in the mixture, pointing to an agonism among these three

metals. The way the combined effects of contaminants are interpreted requires further investigation in ecotoxicology research (Spurgeon et al., 2010). Contaminant interactions could occur at a chemical level or affect toxicokinetic or toxicodynamic parameters (Sexton and Hattis, 2007). Our results indicate that changes in toxicodynamics probably occurred as a consequence of metal toxicokinetics and biomarkers were altered once internal Zn levels in the target organ (liver) overshot a threshold (Ashauer and Escher, 2010). In fish exposed to the mixture (Zn+Mn+Fe), Zn levels in the liver (492.9 ± 202.2 mg Kg^{-1}) were sufficient to boost NPSH, suggesting that this Zn level exceeds the threshold for this biomarker. On the other hand, the same Zn level did not significantly boost hepatic concentration of MT-like proteins. The initial Zn is thought to bind to NPSH (GSH, for instance) and when they become scarce, the Zn starts to bind to MTs (Hogstrand and Wood, 1996), but in our study only NPSH increased and liver integrity was not guaranteed since LPO occurred. Great care is required in interpreting and predicting the effects of metal mixtures, since they depend on a number of issues, such as the endpoint considered, exposure time, toxicant concentrations (Ashauer and Escher, 2010), and especially the toxicodynamics. Our results suggest that mixture toxicity could alter biomarkers dynamically and not necessarily in a linear or unique pattern.

5 Conclusions

Essential metals become toxic when they overshoot essential requirements and regulation thresholds, which vary from one fish species to another and from one metal to another. In *P. lineatus*, levels of 5 mg L^{-1} of Mn and Fe seem to be toxic since at this concentration metals caused oxidative damage (liver LPO) and DNA damage in blood cells. In contrast, Zn at the same level stimulates liver responses to regulate and prevent metal toxicity (MT and GSH increase). Metal toxicity can affect different functional mechanisms (not just inducing oxidative stress) and also target organs/tissues other than liver and blood cells, and especially the gills. Complementary approaches including other biomarkers are important for elucidating the toxicity of these metals. In regard to bioaccumulation and oxidative stress biomarkers, we have shown that Zn, Mn and Fe acted as agonists, since fish exposed to a mixture of these metals exhibited alterations that were not observed after exposure to only one of these metals at the same concentration. Although high metal levels were necessary to cause any effect after exposure to a single metal,

relevant environmental concentrations were sufficient to cause bioaccumulation and damage when the metals were mixed. Since metals occur simultaneously in the environment, it would be more appropriate to consider the toxicity of mixtures rather than focusing on a single metal.

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Capítulo VI

Efeitos do Zn, Mn e Fe isolados e em mistura na osmorregulação do teleósteo neotropical *Prochilodus lineatus*

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Efeitos do Zn, Mn e Fe isolados e em mistura na osmorregulação do teleósteo dulcícola *Prochilodus lineatus*

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Resumo

Juvenis do teleósteo dulcícola *Prochilodus lineatus* foram utilizados para avaliar os efeitos osmorregulatórios dos metais essenciais Zn, Mn e Fe, isolados e em mistura. Os peixes foram submetidos, por 96 h, a três testes independentes, cada qual com o seu respectivo grupo CTR (peixes expostos apenas à água): teste do Zn e teste do Mn, com peixes expostos ao Zn (0,18; 1,0; 5,0 mg L^{-1}) ou ao Mn (0,1; 0,5; 5,0 mg L^{-1}) e teste da mistura, com peixes expostos ao Fe (5 mg L^{-1}), à mistura Zn (1,0 mg L^{-1}) + Mn (0,5 mg L^{-1}) e à mistura Zn+Mn+Fe, nessas mesmas concentrações. Nas brânquias foram analisadas as atividades das enzimas anidrase carbônica (AC), H^+ATPase (HATP), Ca^{2+}ATPase (CaATP), Na^+K^+ATPase (NKA) e Mg^{2+}ATPase (MgATP) e no plasma as concentrações de Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^- e osmolalidade. Nos peixes expostos ao Zn observou-se uma diminuição do K^+, Na^+ e Ca^{2+} plasmáticos, porém nenhuma alteração foi observada na atividade das enzimas branquiais. Já a exposição ao Mn causou alterações apenas do Ca^{2+} plasmático, com a menor concentração promovendo redução do Ca^{2+}, enquanto as maiores concentrações causaram aumento do Ca^{2+}. A maior concentração de Mn também promoveu aumento de HATP e AC indicando acidose. Nos animais expostos ao Zn+Mn verificou-se redução de Ca^{2+} plasmático e aumento da CaATP, enquanto nos peixes expostos a Zn+Mn+Fe verificou-se aumento da CaATP e redução da HATP. Portanto, esses biomarcadores mostraram-se úteis para o monitoramento de locais contaminados por Zn, Mn e Fe em mistura, mas também Zn e Mn isoladamente, podendo ser aplicados em estudos de regiões Neotropicais no teleósteo *P. lineatus*.

Palavras-chave: acidose, anidrase carbônica, Ca^{2+}ATPase, equilíbrio ácido-base, H^+ATPase, hipercalcemia, osmorregulação, mistura metálica.

Introdução

A toxicidade dos metais em peixes tem sido extensivamente estudada já que estes contaminantes são bioacumulados nos tecidos desses animais e causam efeitos letais e subletais. No entanto, as interações e os efeitos das misturas de metais ainda não são totalmente compreendidos (Nordberg et al., 2007; Wood, 2012). Os compostos presentes em uma mistura podem interferir na biodisponibilidade ou toxicidade de outro, seja por meio das interações químicas que reduzem sua biodisponibilidade ou causando alterações na toxicocinética e/ou toxicodinâmica do metal para determinada espécie (Ashauer e Escher, 2010). As consequências dessas interações geram efeitos diferenciados, que podem ser aditivos, agonistas ou antagonistas (Spurgeon et al., 2010).

Previamente (Oliveira, 2017 - Capítulo V), os efeitos dos metais Zn, Mn e Fe, individualmente e em conjunto, foram verificados na espécie de peixe Neotropical *Prochilodus lineatus* em biomarcadores genotóxicos e de estresse oxidativo. Estes são metais essenciais que se tornam tóxicos ao superar um limiar, que varia entre diferentes espécies e metais (Newman e Clements, 2008). Em *P. lineatus*, 5 mg L^{-1} de Mn e Fe parecem representar concentrações tóxicas devido aos danos oxidativos causados no fígado. Por outro lado, esta mesma concentração de Zn estimulou as respostas hepáticas para regular e evitar a toxicidade do metal. As alterações nos biomarcadores de estresse oxidativo, mostraram que a mistura de Zn, Mn e Fe promovem interações agonistas, uma vez que os peixes expostos à mistura apresentaram alterações não observadas após exposições aos metais isolados, nas mesmas concentrações.

A toxicidade dos metais pode ocorrer por meio de diferentes vias e atingir vários tecidos e órgãos. As brânquias, por exemplo, são reconhecidas como órgãos-alvo da toxicidade de metais dissolvidos na água, já que estão em contato direto com o meio. Esses elementos interagem com transportadores de membrana branquiais e alteram a atividade de enzimas envolvidas na osmorregulação e balanço ácido-base, causando seu desajuste (Kennedy, 2011). Em animais de água doce ocorre a captação ativa de íons através das brânquias, por meio direto da atividade das enzimas Na^+ /K^+-ATPase (NKA), Ca^{2+}-ATPase (CaATP), Mg^{2+}-ATPase (MgATP), H^+-ATPase (HATP) e indireto da anidrase carbônica (AC). A AC participa do balanço ácido-base do organismo, pois catalisa a reação reversível de hidratação/desidratação do CO_2, proveniente da respiração, formando ácido

carbônico (H_2CO_3), que rapidamente se dissocia em HCO_3^- e H^+ por ser um ácido de baixa estabilidade. Os íons HCO_3^- e H^+, por sua vez, participam do contratransporte de Na^+ e Cl^-, respectivamente (Gilmour e Perry, 2009). Alguns metais apresentam características que ocasionam o mimetismo iônico, ou seja, por sua similaridade utilizam as rotas de entrada de macronutrientes como Na^+, K^+, Cl^- e Ca^{2+} provocando alterações na captação desses íons, como é o caso dos íons metálicos divalentes como o Zn^{2+}, Mn^{2+}, Cd^{2+} e Pb^{2+}, que mimetizam o cálcio (Wood et al., 2012).

Com isso, o presente trabalho propôs avaliar as alterações agudas em teleósteos da espécie *P. lineatus* provocadas pelos metais Zn, Mn e Fe na osmolalidade e concentrações iônicas plasmáticas, além da atividade branquial das enzimas envolvidas no transporte de íons. Ainda, objetivou-se verificar como essas variáveis se alteram após exposição às misturas destes metais.

Material e métodos

Exposição e amostragem

Juvenis do teleósteo *P. lineatus* (n = 96; comprimento total: 12,1 ± 1,1 cm; peso: 14.1 ± 2.2 g) foram expostos por 96 h ao Zn, Mn e a combinação desses dois metais, com ou sem a adição de Fe, como já descrito no Capítulo V. Foram realizados três testes independentes (Teste do Zn, teste do Mn e teste da Mistura), cada qual com seu controle. Todos os experimentos foram realizados de acordo com o guia OECD 171 (2012) e aprovados pelo comitê de ética em experimental animal da Universidade Estadual de Londrina (Processo CEUA/UEL 20032.2013.65). Após o período de exposição os animais foram anestesiados com benzocaína (0,1 g L^{-1}) e uma amostra de sangue total foi retirada da veia caudal. Em seguida os animais foram mortos por secção medular, os quatro arcos branquiais do lado esquerdo dos animais foram retirados, lavadas em solução fisiológica (PBS Sigma) e as amostras foram armazenadas em tubos plásticos contendo tampão SEI (Sacarose 150 mM, EDTA 10 mM, Imidazol 50 mM, pH 7,5) a -72°C.

Parâmetros iônicos

As amostras de sangue total foram centrifugadas (1870 x *g*; 10 min) para obtenção do plasma que foi utilizado para as análises das concentrações de sódio (Na^+), potássio (K^+), cloreto (Cl^-), cálcio (Ca^{2+}) e magnésio (Mg^{2+}) e osmolalidade. A dosagem de Na^+ e K^+ foi determinada em plasma diluído em água deionizada

(1:100) usando fotômetro de chama (DM-62, Digimed). A análise de Cl^- foi realizada com auxílio de kit comercial (Labtest Diagnostica e BioClin, Brasil) pelo método do tiocianato de mercúrio, com leitura em espectrofotômetro a 490 nm. A dosagem de Ca^{2+} e Mg^{2+} foi realizada com amostras de plasma diluídas (1:50) em óxido de lantânio (La_2O_3 0,1%) em espectrofotômetro de absorção atômica (AAnalyst 700, Perkin Elmer). A osmolalidade foi determinada por osmômetro de pressão a vapor (Vapro 5600, Wescor, Logan, UT, USA).

Análises enzimáticas

Os filamentos branquiais (1:5 p/v) foram homogeneizados em tampão SEID 0,5% (sacarose 150 mM, EDTA 10 mM, imidazol 50 mM, deoxicolato de sódio 2,4 mM, pH 7,5), centrifugadas (7500 x *g*, 15 min, 4 °C) e o sobrenadante utilizado nas seguintes análises. No sobrenadante foi determinada a concentração de proteínas totais conforme Bradford (1976) a 595 nm, para normalização dos resultados.

Na^+/K^+-ATPase (NKA) e H^+-ATPase (HATP)

A análise da atividade das enzimas NKA e HATP foi feita segundo descrito por Gibbs e Somero (1989) e adaptado para microplacas (Silva e Martinez, 2014). O sobrenadante contendo 1 mg L^{-1} de proteína foi utilizado para determinação da atividade das enzimas. A atividade foi mensurada de modo indireto pelo declínio da absorbância, em virtude da hidrólise do ATP e consequente produção de ADP, nas amostras incubadas em meio de reação (ATP 1 mM, NADH 0,2 mM, piruvato quinase 3 U mL^{-1}, lactato desidrogenase 2 U mL^{-1}, frutose-1,6-difosfato 0,1 mM, fosfoenolpiruvato 2 mM, pH 9,0), contendo N-etilmaleimida (NEM – 2 mM, inibidor da HATP) ou ouabaína (2 mM, inibidor da NKA). A absorbância foi lida a cada minuto, durante 15 min, em espectrofotômetro de microplaca (Victor^3, Perkin Elmer) a 340 nm. A atividade da NKA foi calculada pela diferença da atividade entre os meios contendo ou não ouabaína, e a atividade da HATP, pela diferença da atividade entre os meios contendo ou não NEM, sendo os valores expressos em µmol ADP mg proteína^{-1} h^{-1}.

Ca^{2+}-ATPase (CaATP) e Mg^{2+}-ATPase (MgATP)

A atividade das enzimas CaATP e MgATP foi mensurada segundo método descrito por Vijayavel et al. (2007) com modificações. Primeiramente, as amostras

foram incubadas em uma solução reativa (NaCl 189 mM, $MgCl_2$ 5 mM, Tris 20 mM, $CaCl_2$ 5 mM, ouabaína 2 mM, pH 7,6) sem ATP para determinação da concentração basal de fosfato inorgânico (Pi) na amostra. Em seguida, as amostras foram incubadas em solução reativa (CaATP: NaCl 189 mM, $MgCl_2$ 5 mM, Tris 20 mM, $CaCl_2$ 5 mM, ouabaína 2 mM, pH 7,6; MgATP: NaCl 189 mM, $MgCl_2$ 5 mM, Tris 20 mM, KCl 14 mM, EDTA 0,2 mM, ouabaína 2 mM, pH 7,6) contendo ATP (3 mM). A atividade da CaATP e MgATP foi determinada pela diferença de absorbância entra as amostras com ATP e as amostras sem ATP, pela quantificação de Pi liberado na amostra usando-se uma solução de coloração segundo Ames (1966). As leituras foram realizadas em leitora de microplacas (ELX 800, Bio-Tek Instruments) a 620 nm, sendo os valores expressos em µmol Pi mg proteína^{-1} min^{-1}.

Anidrase carbônica (AC)

A atividade da AC branquial foi determinada de acordo com Vitale et al. (1999) com base na catálise de uma solução saturada de CO_2 com liberação de H^+ e consequente diminuição do pH. Ao sobrenadante foi adicionado o tampão de homogeneização utilizado e água saturada com CO_2. A atividade catalítica foi quantificada pelo decaimento do pH a cada 4 s durante 20 s. A inclinação da reta resultante do decaimento do pH da amostra forneceu a taxa de reação catalisada (TRC) e da leitura do pH na ausência de amostra forneceu a taxa de reação não-catalisada (TRNC). A atividade específica da anidrase carbônica (UAC) foi calculada como [(TRC/TRNC)$^{-1}$]/[proteína] da amostra^{-1} e os resultados foram expressos em UAC mg proteína^{-1} min^{-1}.

Análise estatística

Os resultados obtidos foram primeiro testados quanto à normalidade e homogeneidade da variância. Em seguida, utilizou-se a análise paramétrica de variância (ANOVA) ou não paramétrica (Kruskal-Wallis), seguida de um teste de comparação múltipla (teste de Newman-Keuls ou Dunn), quando indicado. Os testes, Zn, Mn e Mistura, foram feitos independentemente e, por isso, os resultados foram comparados apenas entre os grupos de cada teste, sendo consideradas significativas diferenças cujo $P < 0,05$.

Resultados

As concentrações dos íons determinadas no plasma de *P. lineatus* após 96 h de exposição às diferentes condições testes estão apresentadas na Tabela 1. Em relação aos respectivos grupos controle, a exposição ao Zn promoveu diminuição significativa de K^+ (Zn[1,0] e Zn[5,0]), Na^+ (Zn[5,0]) e Ca^{2+} (Zn[0,18] e Zn[5,0]), enquanto o Mn causou diminuição significativa de Ca^{2+} no grupo Mn[0.1] e aumento significativo desse íon nas duas maiores concentrações (Mn[0.5] e Mn[5.0]). Os peixes expostos à mistura Zn+Mn apresentaram redução significativa de Ca^{2+} As concentrações de Cl^- e Mg^{2+} não variaram significativamente em nenhum dos testes realizados.

Tabela 1 Concentrações iônicas (mM) e osmolalidade (mOsm.kg H_2O^{-1}) do plasma de *Prochilodus lineatus* submetidos aos testes do Zn, Mn e Mistura.

		Osmolalidade	**K^+**	**Na^+**	**Cl^-**	**Ca^{2+}**	**Mg^{2+}**
Zn	Ctrl	325±8 a	5,8±0,8 a	147±10 a	140±12 a	1,8±0,1 a	0,5±0,02 a
	0,18	333±12 a	5,1±0,6 a	150±6 a	127±28 a	1,6±0,2 b	0,6±0,03 a
	1,0	332±13 a	4,9±0,6 b	138±10 ab	130±22 a	1,8±0,1 ab	0,6±0,03 a
	5,0	326±19 a	4,2±0,4 b	127±14 b	121±15 a	1,5±0,2 b	0,5±0,02 a
Mn	Ctrl	325±6 a	5,3±0,8 a	145±17 a	138±5 a	1,8±0,2 a	0,5±0,02 a
	0,1	326±17 a	5,1±0,6 a	147±11 a	140±8 a	1,5±0,3 b	0,6±0,02 a
	0,5	322±20 a	5,0±0,7 a	151±15 a	148±30 a	2,4±0,1 c	0,6±0,03 a
	5,0	327±9 a	4,5±0,3 a	145±23 a	129±23 a	2,3±0,2 c	0,6±0,03 a
Mistura	Ctrl	361±11 a	5,9±1,1 ab	155±14 a	147±7 a	2,2±0,2 a	0,6±0,03 a
	Fe	334±15 a	5,4±0,9 ab	160±7 a	151±7 a	2,1±0,2 a	0,6±0,03 a
	Zn+Mn	323±14 a	10,4±5,9 a	146±18 a	144±15 a	1,1±0,2 b	0,5±0,02 a
	Zn+Mn+Fe	337±21 a	4,9±0,4 b	150±8 a	150±10 a	2,1±0,2 a	0,6±0,03 a

Diferenças significativas entre os grupos de cada teste estão indicadas por diferentes letras.

Quanto à atividade branquial das enzimas envolvidas no transporte de íons, não foram observadas alterações significativas nos peixes expostos ao Zn em relação ao respectivo controle. Por outro lado, a exposição ao Mn na concentração de 5,0 mg L^{-1} promoveu aumento significativo da atividade das enzimas AC e HATP (Fig.1). A atividade da enzima CaATP aumentou significativamente após exposição às misturas Zn+Mn e Zn+Mn+Fe (Fig.2), enquanto a atividade da HATP diminuiu significativamente nas brânquias dos peixes expostos à mistura Zn+Mn+Fe (Fig.1).

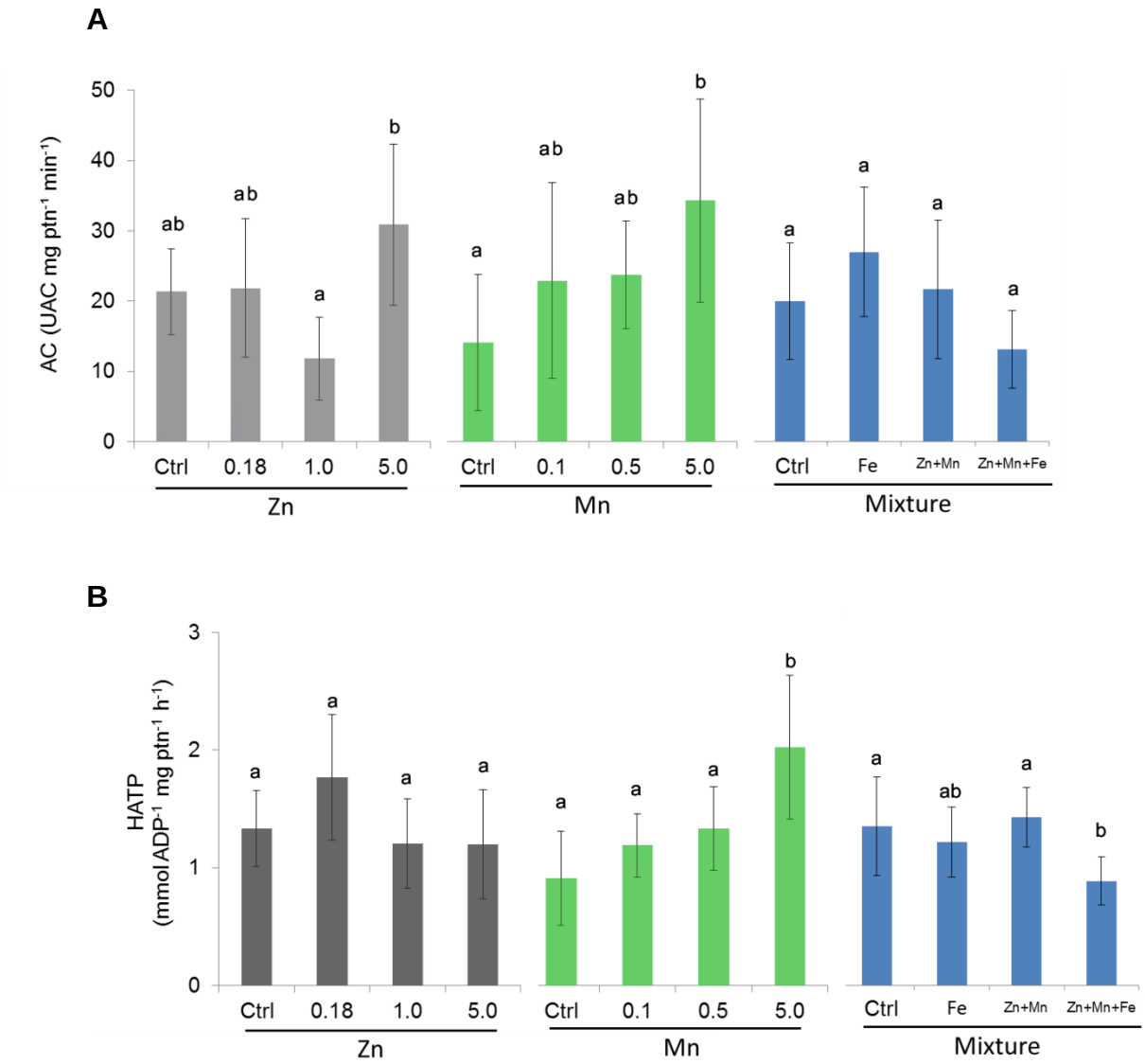

Figura 1 Atividade das enzimas anidrase carbônica **(A)** e H^+ATPase **(B)** em brânquias do teleósteo *Prochilodus lineatus* submetidos aos testes Zn, Mn e Mistura. Diferenças significativas entre os grupos de cada teste estão indicadas por diferentes letras.

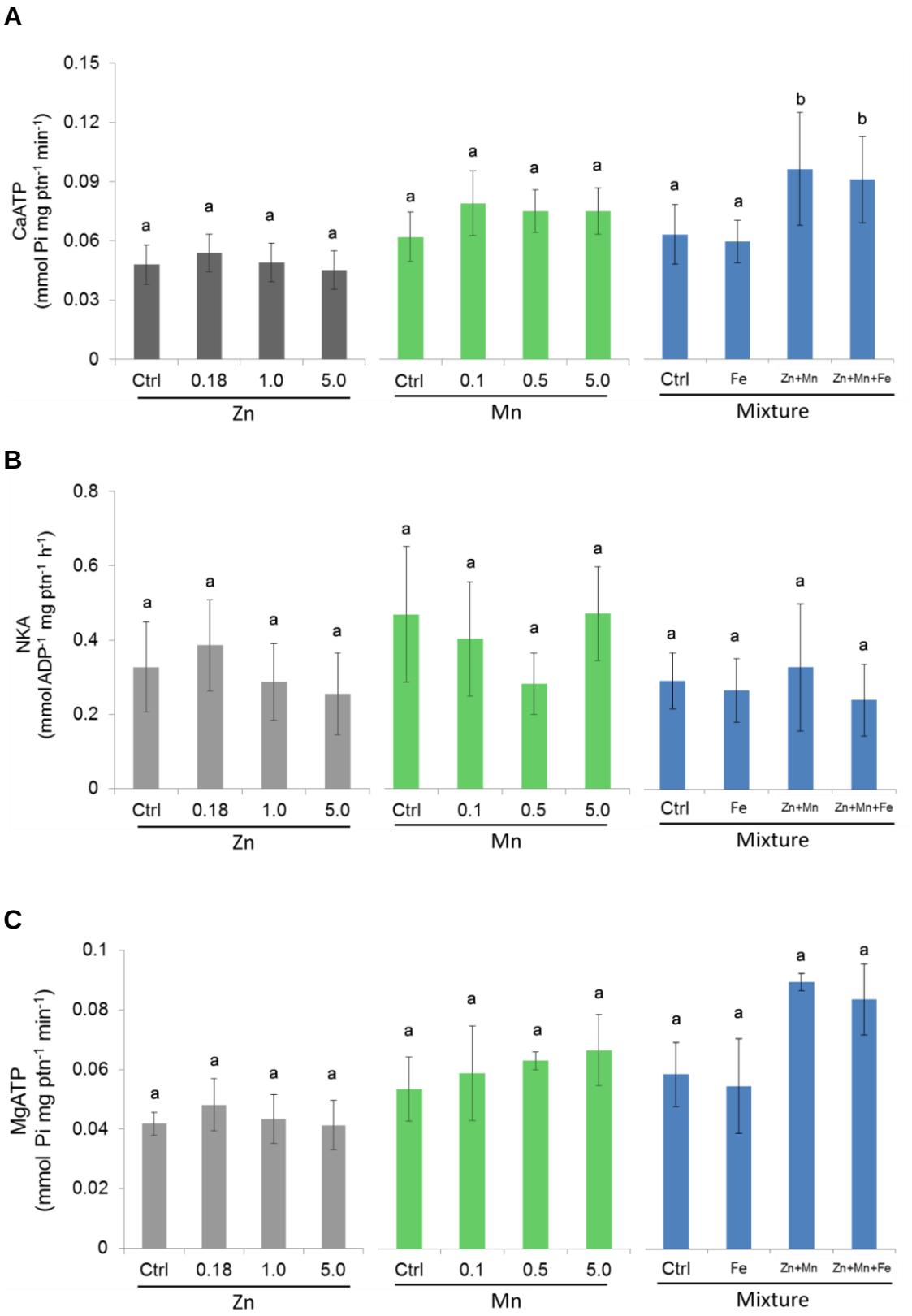

Figura 2 Atividade das enzimas Ca^{2+}ATPase **(A)**, $Na^{+}K^{+}$ATPase **(B)** e Mg^{2+}ATPase **(C)** em brânquias de *Prochilodus lineatus* submetidos aos testes do Zn, Mn e Mistura. Diferenças significativas entre os grupos de cada teste estão indicadas por diferentes letras.

Discussão

A osmorregulação de *P. lineatus* foi afetada após exposição aos metais Zn e Mn isoladamente, assim como após exposição a mistura desses metais com ou sem Fe. Assim como observado para os biomarcadores de estresse oxidativo (Oliveira, 2017 - Capítulo V), a concentração de 5,0 mg L^{-1} de Zn e Mn foi aquela que promoveu mais alterações, porém no caso dos biomarcadores relacionados à osmorregulação, alterações foram já observadas após exposição às menores concentrações (Zn: 0,18 e 1,0 mg L^{-1}; Mn: 0,1 e 0,5 mg L^{-1}). Devido ao fato das brânquias serem os órgãos de contato íntimo com o meio externo e local de influxo desses metais acabaram sofrendo efeitos mesmo em concentrações menores, ou ainda, possivelmente efeitos mais precoces que aqueles de estresse oxidativo observados no fígado.

A exposição ao Zn promoveu a diminuição de íons K^+, Na^+ e Ca^{2+} plasmáticos em *P. lineatus*, apesar da ausência de alterações nas atividades das enzimas branquiais envolvidas no transporte de íons. Assim, a redução do Ca^{2+} plasmático deve ter ocorrido por consequência da competição com Zn pelos transportadores como o canal epitelial de cálcio (ECaC) e a Ca^{2+}ATPase, já que este metal bivalente mimetiza o íon Ca^{2+} (Wood, 2012). Já a diminuição de Na^+ e K^+ pode indicar um efeito do Zn em outros canais e trocadores branquiais específicos para estes íons, os quais não foram avaliados, ou ainda, poderia estar relacionada a problemas na reabsorção desses íons nos rins, apontando a necessidade de investigações mais pontuais neste órgão.

Os peixes expostos ao Mn (5,0 mg L^{-1}) apresentaram aumento da atividade branquial das enzimas AC e HATP indicando um provável estado de acidose dos animais. As brânquias constituem o principal órgão em peixes de água doce responsáveis pela manutenção do equilíbrio ácido-base por meio da atividade dessas enzimas, as quais regulam as concentrações intra e extracelular dos íons H^+ e HCO_3^-. A HATP, localizada na membrana apical das células, está intimamente relacionada a este mecanismo, pois secreta o H^+ proveniente da atividade da AC intracelular, para o meio externo, controlando o pH do organismo (Gilmour e Perry, 2009). A acidose pode ocorrer devido à diminuição da ventilação branquial e trocas gasosas, com consequente aumento da concentração de CO_2, bem como devido ao metabolismo anaeróbico (Perry e Gilmour, 2006).

Além disso, o Mn também promoveu alterações na concentração plasmática de Ca^{+2-}, sendo que nos peixes do grupo Mn[0,1] houve redução significativa deste íon, enquanto nos peixes dos grupos Mn[0,5] e Mn[5,0] houve aumento significativo do cálcio plasmático. A regulação do Ca^{2+} ainda não é totalmente esclarecida em peixes teleósteos, entretanto recentemente estudos mostraram o envolvimento de hormônios nessa regulação. Hormônios, como o cortisol, calcitonina e hormônio da paratireoide (PHT), entre outros, modulam a captação da Ca^{2+} em *Danio rerio* por meio da regulação de seus transportadores (Lin e Hwang, 2016). Outro hormônio também citado como participante na regulação do Ca^{2+} em peixes teleósteos é a prolactina (Hoseini et al., 2014), atuando inclusive como inibidora da atividade osteoclástica em *Carassius auratus* (Takahashi et al., 2008). A secreção de prolactina é controlada pelo sistema dopaminérgico que, por sua vez, é afetado pela ação do Mn (Santos et al., 2011). Assim, pode-se sugerir que o Mn, indiretamente, estaria causando alteração nas concentrações do Ca^{2+} devido a sua ação neurotóxica sobre o sistema dopaminérgico e esse efeito seria concentração dependente.

Após a exposição à mistura Zn+Mn, ocorreu uma diminuição do Ca^{2+} plasmático, possivelmente como já comentado, devido à capacidade de metais bivalentes mimetizarem esse íon (Wood, 2012). O aumento da atividade da CaATP nas brânquias desses animais deve ter atuado de modo compensatório à diminuição de Ca^{2+} plasmático. A CaATP localiza-se na membrana basolateral das células branquiais de peixes e atua, em conjunto com um trocador Na^{+}/Ca^{2+}, na absorção de Ca^{2+} da água (Perry et al., 2003). Esses transportadores criam um gradiente eletroquímico favorável a entrada de Ca^{2+} via canais epiteliais ECaC presentes na membrana apical (Zheng et al., 2014). O aumento da atividade desta enzima também ocorreu após exposição à mistura Zn+Mn+Fe e foi suficiente para evitar a hipocalcemia. A atividade da CaATP foi um biomarcador que respondeu apenas após a exposição às misturas (Zn+Mn e Zn+Mn+Fe), demonstrando uma interação agonista entre os metais Zn e Mn.

Os peixes expostos à mistura dos três metais (Zn+Mn+Fe) apresentaram diminuição na atividade branquial da HATP. Essa bomba está associada à captação de Na^{+} na membrana apical das brânquias, pois a extrusão de prótons H^{+} promove um gradiente elétrico favorável a entrada de cátions (Evans, 2008). Todavia, a

concentração de Na^+ plasmático não sofreu alteração, indicando que a captação desse metal não foi comprometida.

Conclusões

As concentrações iônicas no plasma de *P. lineatus* foram sensíveis à presença de Zn e Mn, principalmente o Ca^{2+}, cujas concentrações se alteraram no plasma de peixes expostos aos três tratamentos. No teste do Mn, a menor concentração promove diminuição Ca^{2+}, a mesma resposta observada no teste do Zn. Entretanto, as concentrações de 0,5 e 5,0 mg L^{-1} de Mn causaram um efeito inverso, provocando um aumento deste íon. Nossos resultados revelam que esses metais causam hipo- ou hipercalcemia, agindo por diferentes modos de ação. A hipocalcemia está possivelmente relacionada ao fato de que os cátions divalentes mimetizam o Ca^{2+}, competindo pelas vias de entrada, enquanto a hipercalcemia causada pelo Mn poderia estar relacionada com sua ação neurotóxica, que interfere na regulação hormonal deste íon. Quando expostos em mistura (Zn+Mn) provocaram hipocalcemia, prevalecendo, portanto, o efeito desses metais sobre o influxo de Ca^{2+}.

As enzimas branquiais estudadas, por sua vez, não apresentaram alterações após tratamentos com Zn isoladamente, mas a maior concentração de Mn (5 mg L^{-1}) promoveu aumento da AC e HATP. Assim, em comparação às concentrações iônicas plasmáticas, as atividades das enzimas de transporte iônico são biomarcadores menos sensíveis a esses metais isolados. Entretanto, podemos observar que, quando submetidos aos tratamentos das misturas, alguns biomarcadores mostraram-se mais sensíveis. A CaATP aumentou após tratamento com as misturas Zn+Mn e Zn+Mn+Fe e a HATP diminuiu após exposição à mistura com os três metais (Zn+Mn+Fe).

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Capítulo VII

CONSIDERAÇÕES FINAIS

O presente trabalho utilizou diferentes abordagens experimentais, bem como dois modelos biológicos, para verificar alterações na bioacumulação e biomarcadores por consequência da exposição a misturas metálicas. Esse trabalho gerou uma publicação (Capítulo II) e outros quatro manuscritos (Capítulos III, IV, V, VI) a serem submetidos à avaliação e possível publicação em revistas científicas. As discussões referentes a cada um dos manuscritos já foram abordadas individualmente, porém considerações finais foram feitas com intuito de discutir aspectos comparativos entre as abordagens experimentais e os modelos biológicos, além de apontamentos gerais sobre as conclusões da tese, referentes aos objetivos e hipóteses levantadas no início do trabalho.

No Capítulo II, por meio de estudo de caso em área de influência de mineração de carvão, foi possível identificar os principais metais relacionados à problemática ambiental desse local. Foram apontados os metais Zn, Mn, Fe e Al como aqueles mais importantes, metais que são recorrentes em efluentes desse tipo de atividade. Baseando-se nesses resultados, optou-se por trabalhar nos testes em laboratório com Zn, Mn e Fe, já que a disponibilidade de Al na água tem relação íntima com o pH e seria necessário acrescer mais uma variável ao estudo. Essas outras combinações entre os metais, incluindo o Al e Fe em misturas binárias ou complexas podem ser consideradas em estudos futuros. Além disso, é importante considerar que quando se trata desse tipo de mineração a composição de metais é variável, ocorrendo também metais como selênio, chumbo e cobre.

Algumas das concentrações nominais de Zn, Mn e Fe testadas nos testes em laboratório com *A. trapesialis* e *P. lineatus* são as concentrações limites para os corpos de água doce no Brasil, dependendo do uso preponderante da água (Classes 1 e 2, 3 e 4) regulados pela Resolução CONAMA 357 (2005). A concentração de 0,18 mg L^{-1} de Zn total é o limite estabelecido para as classes 1 e 2. A exposição a esta concentração não promoveu alterações de bioacumulação em nenhum dos modelos biológicos estudados, e a única alteração significativa observada foi a diminuição do Ca^{2+} plasmático nos peixes. Já a concentração de 5 mg L^{-1} de Zn total, que é o limite estabelecido para classes 3 e 4, promoveu alterações nas duas

espécies estudadas. As concentrações limites de Mn, 0,1 mg L^{-1} (Classes 1 e 2) e 0,5 mg L^{-1} (Classes 3 e 4), e de Fe 5,0 mg L^{-1} (Classes 3 e 4) também promoveram alterações apontadas no Quadro abaixo.

	Zn		Mn		Fe
CONAMA 357 (2005)	Classe 1 e 2 0,18 mg L^{-1}	Classe 3 e 4 5,0 mg L^{-1}	Classe 1 e 2 0,1 mg L^{-1}	Classe 3 e 4 0,5 mg L^{-1}	Classe 3 e 4 5,0 mg L^{-1}
		- Bioacumulação: músculo, manto, glândula digestiva, brânquias e hemolinfa; - Aumento de MT nas brânquias; - LPO na glândula digestiva; - Aumento de ChE no músculo.	- Aumento de PCO na glândula digestiva;	- Diminuição de Mn nas brânquias; - Aumento de PCO na glândula digestiva; - Diminuição da ChE no músculo; - Diminuição de glicogênio nas brânquias; - Hipercalcemia; - Diminuição de proteínas.	- Aumento de ERO nas brânquias;
	- Hipocalcemia plasmática.	- Bioacumulação: músculo, brânquias, bile, fígado e rim; - Aumento de MT; - Aumento de NPSH; - Diminuição de sódio, potássio e cálcio plasmáticos;	- Bioacumulação nas brânquias; -Hipocalcemia plasmática;	- Bioacumulação nas brânquias; - Hipercalcemia plasmática;	- Aumento de danos no DNA de eritrócitos.

Como demonstrado na Tabela 1, as duas abordagens experimentais, teste *in situ* e teste em laboratório realizados com o bivalves *A. trapesialis*, apresentaram sobreposição de certos resultados, confirmando a hipótese 1.

A diminuição do acúmulo de Mn nas brânquias e a diminuição da AChE no músculo, dois *endpoints* apontados no presente trabalho como consequências do fechamento das valvas, ocorreram tanto em campo, quanto em laboratório. A princípio, o Al foi indicado como o responsável por causar o fechamento das valvas de *A. trapesialis* expostos em campo, já que apresentou maior acúmulo, porém a utilização da abordagem de teste em laboratório esclareceu que o Mn tem papel importante nesse comportamento, apesar da diminuição de sua concentração nas brânquias. Outro *endpoint* cujo resultado se repete em ambas as abordagens experimentais é o aumento da MT no manto que, como já discutido, reforça a importância da análise deste biomarcador neste órgão de *A. trapesialis*.

Tabela 1 Comparação entre os resultados obtidos nos experimentos realizados em campo (*in situ*) (Capítulo II) e em laboratório (Capítulo III) com os bivalves *Anodontites trapesialis*. São considerados apenas os *endpoints* comuns utilizados nos dois experimentos. As flechas indicam se houve aumento (↑) ou diminuição (↓).

	Órgãos		Zn	Mn	Fe	TAC	LPO	PC	AChE	MT	GST
IN SITU	Brânquias	Up2									↓
		Dw1		↓	↑		↑				
		Dw2		↓	↑		↑				
	Manto	Dw1		↓	↓		↑				
		Dw2				↓				↑	
	Glândula digestiva	Dw1	↓	↓							
	Músculo	Dw1							↓		
		Dw2			↑				↓		
	Hemolinfa	Up2		↑							
		Dw1		↑	↑						
		Dw2		↑	↑						
LABORATÓRIO	Brânquias	Zn[1.0]								↑	
		Zn[5.0]	↑							↑	
		Mn[0.5]		↓							
		Mn[5.0]	↓	↓							
	Manto	Zn[1.0]	↑								
		Zn[5.0]	↑								
		Mn[0.5]								↑	
		Mn[5.0]	↓				↑			↑	
		Zn+Mn	↑								
		Zn+Mn+Fe	↑								
	Glândula digestiva	Zn[5.0]	↑				↑				
		Mn[0.1]						↑			
		Mn[0.5]						↑			↓
		Mn[5.0]	↑				↑				
		Zn+Mn	↑								
		Zn+Mn+Fe									
	Músculo	Zn[5.0]	↑								
		Mn[0.5]	↓						↓		
		Mn[5.0]							↓		
		Zn+Mn	↑								
		Zn+Mn+Fe	↑								
	Hemolinfa	Zn[1.0]	↑								
		Zn[5.0]	↑								
		Mn[5.0]	↓	↑							
		Zn+Mn	↑								
		Zn+Mn+Fe	↑								

A hipótese 2 foi aceita, pois ocorreu aumento de bioacumulação de Zn e Mn, tanto no bivalve quanto no peixe. De modo geral, esse aumento aconteceu apenas após exposição às concentrações maiores de Zn e Mn (5,0 mg L^{-1}). A bioacumulação de Zn, Mn e Fe após exposição às misturas ocorreu de modo diferenciado nos dois modelos biológicos estudados. Em ambos os casos, ocorreu maior bioacumulação de metais quando em mistura quando comparado aos

tratamentos de mesma concentração dos metais isolados, já que esses metais são cátions bivalentes e geram maior gradiente eletroquímico que favorece a entrada desses elementos nos animais. Entretanto, no caso dos bivalves houve bioacumulação apenas de Zn, enquanto nos peixes a bioacumulação de Mn e Fe também aumentou. A concentração de Mn nos tecidos de bivalves Unionídeos é naturalmente alta, e possivelmente por isso, o aumento da bioacumulação desse metal nesses bivalves não ocorreu.

Ao contrário do que era esperado e apontado na hipótese 3, os peixes não apresentaram maior susceptibilidade a exposição a Zn, Mn e Fe. Como é possível observar na Tabela 2, tanto em *P. lineatus* quanto em *A. trapesialis* a maior parte dos efeitos ocorreu após exposição à concentração de 5,0 mg L^{-1}.

Tabela 2 Comparação entre os resultados obtidos nos experimentos com os bivalves *Anodontites trapesialis*. São considerados apenas os *endpoints* comuns utilizados nos dois experimentos. As flechas indicam se houve aumento (↑) ou diminuição (↓).

	Órgãos		**LPO**	**PC**	**MT**	**GST**	**SOD**	**Danos no DNA**
A. trapesialis	Brânquias	Zn[1.0] Zn[5.0]			↑ ↑			
	Manto	Mn[0.5] Mn[5.0] Zn+Mn	↑		↑ ↑			
	Glândula digestiva	Zn[5.0] Mn[0.1] Mn[0.5] Mn[5.0]	↑ ↑	↑ ↑		↓		
P. lineatus	Fígado	Zn[5.0] Mn[5.0] Zn+Mn+Fe	 ↑ ↑		↑		↓	
	Sangue	Mn[5.0] Fe						↑ ↑

Ambos os animais apresentaram efeitos (↑LPO e ↓GST) após exposição ao Mn, mas responderam ao Zn com o aumento de MT. Nos bivalves ainda ocorreu aumento de PC na glândula digestiva de animais expostos a 0,1 e 0,5 mg L^{-1} de Mn e aumento de MT em brânquias de animais expostos a 1,0 mg L^{-1} de Zn, revelando que quando considerados esses biomarcadores *A. trapesialis* pode ser mais sensível. Entretanto, não é possível afirmar qual das espécies seria mais indicada como biomonitora para estudos dessa natureza.

Os metais estudados têm propriedade pró-oxidante, porém também desempenham papel nas defesas antioxidantes. O presente trabalho mostrou que a

concentração de 5 mg L^{-1} de Mn causou danos oxidativos em manto e glândula digestiva dos bivalves, assim como no fígado dos teleósteos. Ainda, a exposição ao Mn promoveu o aumento de PC na glândula digestiva dos bivalves nas concentrações de 0,1 e 0,5 mg L^{-1}. A exposição ao Zn, por sua vez, promoveu danos oxidativos apenas na glândula digestiva dos bivalves após exposição a 5 mg L^{-1}, enquanto o Fe na mesma concentração promoveu danos no DNA de eritrócitos em *P. lineatus*. Comparativamente, o Mn foi o metal mais tóxico, pois promoveu danos em diversos órgãos, tanto em bivalves quanto em peixes. Já quando expostos ao Zn, os animais foram capazes de responder à presença do metal por meio do aumento de MT e GSH, evitando a ocorrência de danos mais severos. Com isso, se aceita parcialmente a hipótese 4, que supõe que os três metais têm propriedade pró-oxidante e causariam estresse oxidativo. Entretanto, evidencia-se que biomarcadores relacionados ao estresse oxidativo são uma escolha interessante para avaliação da toxicidade mesmo de metais essenciais.

Ambos os modelos biológicos apresentaram alterações em biomarcadores relacionados à osmorregulação, porém não necessariamente ocorreu a hipocalcemia, como era esperado. Portanto, a hipótese 5 foi aceita apenas parcialmente. No caso dos bivalves, as concentrações de íons na hemolinfa foram resultados que contribuíram para o entendimento do efeito do Mn sobre o comportamento de fechamento das valvas nesses animais, mas que contrariaram a expectativa de diminuição do cálcio na hemolinfa, já que o armazenamento deste íon é abundante nas conchas e grânulos destes animais e em condição de acidose é liberado para exercer função de tamponamento do pH. No caso dos peixes, o Mn também provocou alteração nas concentrações plasmáticas de Ca^{2+}, revelando que uma possível ação neurotóxica deste metal pode alterar indiretamente biomarcadores relacionados à osmorregulação. Esses resultados mostram que alguns metais podem apresentar efeitos específicos sobre esses biomarcadores, sendo, portanto, necessária a investigação e a determinação desses mecanismos de ação.

A hipótese 6 também foi rejeitada já que a mistura de metais favoreceu a bioacumulação, sendo a toxicocinética dos metais afetada devido a presença dos outros metais. Por consequência do aumento do conteúdo de metais em alguns órgãos também foram observadas alterações nos biomarcadores de animais expostos às misturas, as quais não foram observadas nas exposições individuais. O

aumento do influxo dos metais deve estar relacionado à elevação do gradiente eletroquímico entre os meios extra e intracelular. Esses metais apresentam vias comuns de entrada nas células, mas isso não refletiu em diminuição da bioacumulação. Em bivalves ocorreu favorecimento da entrada de Zn em detrimento do Mn e Fe, possivelmente pela maior afinidade do Zn com transportadores comuns, além de que a concentração de Mn já é naturalmente elevada nos órgãos desses animais. No caso dos teleósteos, todos os metais tiveram aumento do acúmulo após exposição às misturas, porém distribuíram-se em órgãos distintos.

Refletimos que a definição das interações entre os metais como aditivas, agonistas ou antagonistas é uma tarefa complicada quando avaliados os biomarcadores, já que as alterações nesses *endpoints* podem ocorrer como consequência da ação dos metais em diferentes vias, uso de estratégias de defesa distintas, tanto comportamentais como moleculares, ou do quanto os metais entram e estão disponíveis nos organismos. Esses fatores serão possivelmente influenciados pela particularidade de cada metal que compõe a mistura e pelas concentrações e proporção testadas entre os metais componentes. Com isso, conclui-se a necessidade de considerar tais variáveis na avaliação e estudo da toxicidade de misturas.

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Metals bioaccumulation and biomarkers responses in the Neotropical freshwater clam *Anodontites trapesialis*: Implications for monitoring coal mining areas

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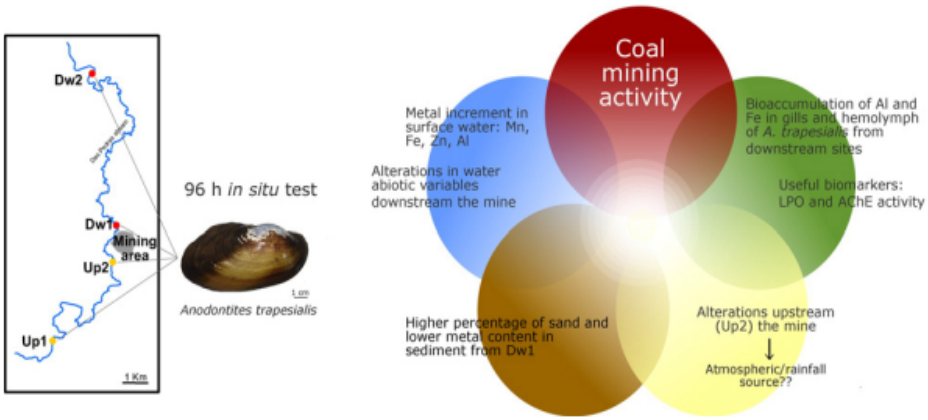
HIGHLIGHTS

- Neotropical bivalve *Anodontites trapesialis* was caged in a stream near a coal mine.
- Clams were caged, for 96 h, at sites upstream and downstream (Dw) from the mine.
- Clams caged at Dw sites showed higher levels of Al and Fe in different tissues.
- Increased lipoperoxidation was detected in gills and mantle of clams from Dw sites.
- Reduced acetylcholinesterase activity was observed in clams muscle from Dw sites.

GRAPHICAL ABSTRACT



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ABSTRACT

As one of the most impactful industries, coal mining can promote several alterations at surrounding environment. In surface water, elevated concentrations of metals like Mn, Zn, Fe and Al are often observed. Thus, the aim of this study was to investigate the bioaccumulation and the sub-lethal effects of these metals on various organs of the Neotropical bivalve *Anodontites trapesialis* confined along a stream located near a coal mine, in order to assess a set of biomarkers that could be used for effectively monitoring coal mining areas. Clams were caged, for 96 h, at two sites located upstream (Up1 and Up2) and two sites downstream (Dw1 and Dw2) from the mine. Metals bioaccumulation was determined in gills, mantle, digestive gland, muscle and hemolymph and the following biomarkers were measured in *A. trapesialis* tissues: total antioxidant capacity against peroxyl radicals, metallothionein content, lipid peroxidation (LPO), proteins carbonylation, glutathione S-transferase activity, superoxide dismutase activity and acetylcholinesterase (AChE) activity. The results showed that Al and Fe bioaccumulation in the gills and hemolymph, Al bioaccumulation in the mantle and muscle, increased LPO in the gills (Dw1 and Dw2) and mantle (Dw1), as well as reduced AChE activity in the muscle (Dw1 and Dw2) should be considered effective biomarkers for monitoring coal mining areas. *A. trapesialis* proved to be an efficient biological model, considering that biomarkers responses were observed in the clams after only 96 h of confinement at Dw sites, accordingly this species could be a good candidate for monitoring Neotropical freshwaters.

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1. Introduction

Coal mining is one of the industries most likely to heavily impact the environment and can cause many environmental changes, such as acidifying surface water, increasing total suspended and dissolved solids, and boosting metal levels, as well as altering the features of streams beds (Tiwary, 2001). When the pyretic minerals associated with coal are exposed to water and oxygen in the presence of *Thiobacillus* bacteria, this produces sulfuric acid and iron hydroxide or sulfate. This acid fluid causes the dissociation of metals and other constituents, releasing them into the water, forming acid mine drainage (AMD) containing metals whose composition depends on the type of originating mineral deposit (Sheoran and Sheoran, 2006). Coal mining is a good example of human activity that can release metals into the environment.

Metals are a well-known class of pollutant that can accumulate in organic tissues and cause alterations in the biomarkers of aquatic organisms (Nordberg et al., 2007; Viarengo, 1985). Some are trace elements, *i.e.* essentials for the survival and growth of organisms, and are abundant in the earth's crust, which could become toxic to aquatic biota when present in the environment at elevated concentrations (Nordberg et al., 2007). While others are non-essentials and low concentrations are sufficient to cause toxicity and sub-lethal effects in aquatic organisms. In recent years, there has been increased interest in understanding metal interactions and their combined effects on organisms, often occurring simultaneously in environment. Anthropogenic activities can be responsible for very high concentrations which, depending on how they interact in the environment and within the organism, can have additive, synergetic or antagonistic effects (Spurgeon et al., 2010). In field studies, these interactions have been found to be even more complex, and several tools are required in order to understand them.

Active biomonitoring is a useful approach to diagnose and monitor water quality in which several endpoints can be quantified in translocated organisms. This method permits the control of many variables such as time of exposure, age and sex as opposed to traditional passive monitoring in which resident and sometimes resistant organisms are collected (Wepener, 2013). Biomarkers have been used as such endpoints (Hook et al., 2014), and can provide valuable information on the toxicity mechanisms enhanced by contaminants, as well as the deleterious effects that may impair the performance of the organism (Moreira et al., 2004). As such, alongside chemical analysis, biomarkers should be included in environmental monitoring programs as a fast method of screening for the biological effects of contaminants, prior to the implementation of preventive bioremediation strategies (Livingstone, 2001).

Classical biomarkers, such as those involved in biotransformation processes (Glutathione S-transferase), oxidative stress (total antioxidant capacity, reduced glutathione and metallothionein), oxidative damage (lipid peroxidation, protein carbonylation), metals regulation (metallothionein) and acetylcholinesterase activity (AChE) have been extensively studied in various aquatic organisms (Oliveira et al., 2014; Vieira et al., 2016). Bivalves are often used as biological models in ecotoxicology studies (Farris and Van Hassel, 2007), since they live in close contact with sediment and filter large volumes of water. However, so far few studies have been conducted on Neotropical freshwater bivalves and therefore there is no established biological model for this region. It is important to use native species for environmental monitoring since they are less tolerant to contaminants than invasive species (Bielen et al., 2016).

In the central region of Paraná state (Southern Brazil), coal has been mined since the 1940s, resulting in a long history of effluent discharge and water contamination with metals. Against this backdrop, the aim of this study was to investigate the metals bioaccumulation and the sub-lethal effects of these metals on the Neotropical bivalve species, *Anodontites trapesialis*, after 96 h of confinement along a stream located near a coal mine in order to assess a set of biomarkers that could be used for effectively monitoring coal mining areas.

2. Material and methods

2.1. Study area

In situ tests were performed at Das Pedras stream (DP stream), located in a coal mining area of Paraná state (Southern Brazil). Currently, in this particular mine, coal production is achieved in an underground mine in which AMD does not occur. However, DP stream is near the waste disposal area active since 1940s and has been submitted to leaching and effluent discharges. Four sites were chosen to perform the tests, two upstream (Up1 and Up2) and two downstream (Dw1 and Dw2) from the mine as represented in Fig.1. Up1 (23°51′139″ S; 50°26′779″ W) and Up2 (23°49′708″ S; 50°25′571″ W) are located, respectively, approximately 7 km and 400 m upstream from the mine. Dw1 (23°49′026″ S; 50°25′506″ W) is located just after the mine and Dw2 (23°46′265″ S; 50°26′068″ W) around 7 km downstream the mine.

2.2. Animals – Anodontites trapesialis (LAMARCK, 1819)

The freshwater clam *A. trapesialis* was chosen as biological model because is easily obtained and as a Neotropical native species becomes a potentially biomonitor for this region. To perform the experiments 40 individuals of *A. trapesialis* (length: 131.75 ± 0.96 mm; weight: 178.9 ± 36.4 g) were collected manually at a fish farming facility in Londrina, Paraná, Brazil. Then, the bivalves were maintained for one week in tanks containing dechlorinated water, renewed every 24 h, with temperature 20 °C, pH 7.5 and conductivity 100 μS cm^{-1}, approximately. Animals were fed with algae *Pseudokirchneriella subcapitata* (~~ 10.000 cells mL^{-1}) every two days.

2.3. Experimental design

After the depuration period, animals were transported in plastic bags filled with dechlorinated water and constant aeration to the study area and were distributed in the four sites of interest (n = 10 in each site). The tests at the four sites were performed simultaneously.

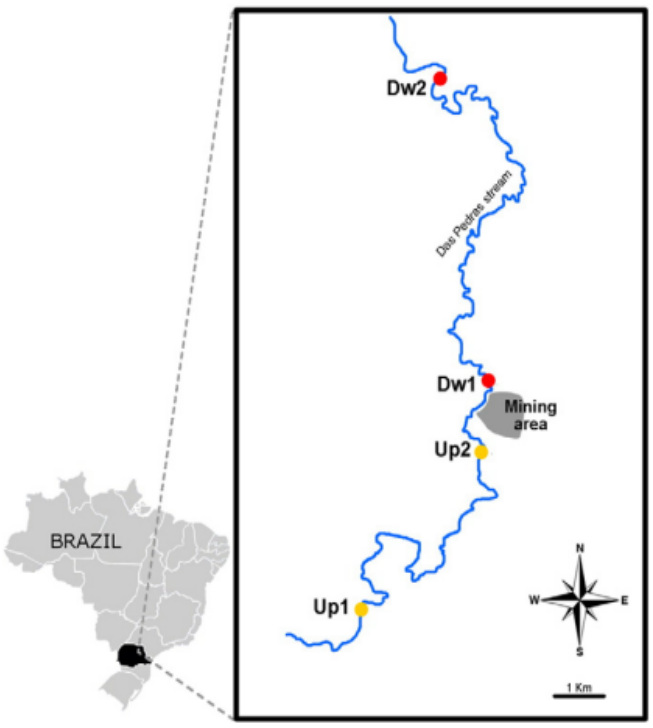

Fig. 1. Map of Brazil highlighting Paraná State (left). In detail the location of four sites where *in situ* tests were carried out: two sites upstream (Up1 and Up2) and two sites downstream (Dw1 and Dw2) from the coal mine.

Individuals were maintained for 96 h into cages (50 × 60 × 40 cm – 120 L) that allowed water circulation and free access to sediment (5-mm mash screen). Animals were weighted before and after the confinement. After the caging period, the bivalves were removed from the cages and transported to a laboratory located in the mine facilities (maximum 1 h of transport time) in plastic bags containing water collected from each site. Samples of hemolymph, gills, mantle, muscle (foot) and digestive glands were collected and immediately frozen on liquid nitrogen. Samples from the same 40 individuals were used to perform all analysis.

2.4. Physical and chemical variables

At each *in situ* experimental site, at the first and last days of exposure, measures of temperature, pH, turbidity and water conductivity were done with a multiparameter reader (Horiba U-52) and water samples were collected to determine hardness by EDTA titrimetric method and dissolved organic carbon (DOC) (Rodrigues and Bianchini, 2007).

Metal concentrations were analyzed in sediment and water samples collected from each caging site. After a more complete screening of metals in water (Ag, Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn) (data not shown) and based on the monitoring records done by the mining company, Al, Fe, Mn and Zn were chosen as target metals. Thus, for each caging site, water samples were collected once a day during the test and superficial sediment samples were collected two times, at the beginning and end of the test. Water samples were stored in plastic tubes in order to analyze total and dissolved metals (filtered at 0.45 μm pore size). After sampling, they were immediately acidified (1% HNO_3) and stored refrigerated for metal (Al, Fe, Mn and Zn) analysis, by flame atomic absorption spectrophotometry, using an atomic absorption spectrophotometer (AAnalyst 700, Perkin Elmer, USA) against references standard solutions (Specsol, Brazil).

To perform metal (Al, Fe, Mn and Zn) analyses in sediment samples, hydrochloric acid (HCl) 0.1 M was added in 1 g of sediment sample, previously dried at 60 °C, then submitted to 2 h of horizontal shaking and filtered (8 μm pore size) (Mozeto, 2004). Granulometric characterization was done using a 53 μm mesh size sieve to quantify the percentage of sand against silt and clay.

2.5. Bioaccumulation of metals

Metals were measured in different bivalve tissues (n = 7–10 per tissue): mantle, gills, digestive gland, muscle and hemolymph. These (except hemolymph samples) were completely dried at 60 °C and, then, submitted to acid digestion for 48 h at 60 °C in nitric acid suprapure 5 N, according to Alves and Wood (2006). The digested tissues were analyzed by flame or electrothermal atomic absorption spectrophotometry, using an atomic absorption spectrophotometer equipped with a graphite furnace atomizer (AAnalyst 700, Perkin Elmer, USA) against references standard solutions (Specsol, Brazil). Results were expressed mg g dry tissue^{-1} (mantle, gills, digestive gland and muscle) or mg L^{-1} (hemolymph).

2.6. Biomarkers

2.6.1. Total antioxidant capacity against peroxyl radicals (TAC)

TAC was measured according to the method proposed by Amado et al. (2009). Gills and mantle (n = 8–10) were homogenized (1:4 – m/v) in a Tris-HCl buffer (100 mM; pH 7.75) containing 2 mM EDTA and 5 mM $MgCl_2$ and then centrifuged (14,000 ×*g*; 20 min; 4 °C). The samples (0.5 mg mL^{-1} of protein per well) were subjected to the presence and absence of 2,2′-azobis (2 methylpropionamidine) dihydrochloride (ABAP) at 35 °C and the fluorescence (ex/em: 485/520 nm) was monitored over a period of 30 min employing 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) as substrate. TAC was estimated through the difference between graphs' areas (presence and absence of ABAP), obtained by computing the integral of fluorescence units over time and expressed by the relative area.

2.6.2. Superoxide dismutase (CuZn-SOD), glutathione S-transferase (GST) and oxidative damage

Gills (n = 8–10), mantles (n = 8–10) and digestive glands (n = 8–10) were individually homogenized in potassium phosphate buffer (0.1 M, pH 7) and then centrifuged (14,000 ×*g*; 20 min, 4 °C). The supernatant was used for the determination of CuZn-SOD activity, GST activity, lipid peroxidation (LPO) and protein carbonylation (PC).

Copper–zinc Superoxide dismutase (CuZn-SOD) activity was determined based on the measurement of the inhibition of the reduction rate of cytochrome *c* by the superoxide radical at 550 nm and 25 °C, according to McCord and Fridovich (1969), adapted to microplate. SOD activity was expressed in SOD U mg protein^{-1}, with one unit of SOD corresponding to the quantity of enzyme that promoted the inhibition of 50% of the reduction rate of cytochrome c.

The GST activity was determined using the method described by Keen et al. (1976). This method is based on the GST catalyzed conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB), monitored for 1 min in a spectrophotometer at 340 nm. The enzyme activity was expressed in nmol CDNB conjugate min^{-1} mg of protein^{-1}.

The TBARS (thiobarbituric acid reactive substances) assay was performed according to Camejo et al. (1998) as a measure of lipid peroxidation. Butylated hydroxytoluene (BHT 1 M), phosphate buffered saline (2 mM KCl; 1.4 mM NaH_2PO_4; 357 mM NaCl; 10 mM Na_2HPO_4; pH 7.4), trichloroacetic acid (TCA 50%) and thiobarbituric acid (TBA 1.3%) dissolved in 0.3% NaOH were added to the supernatant and the mixture was kept in an incubator at 60 °C for 1 h. For digestive gland, samples were first treated with TCA, centrifugated at 2400 ×*g* for 5 min at 4 °C and, than, BHT and TBA were added. A fluorescence reading was made (ex/em: 535/590 nm) and the TBARS concentration was expressed in nmol TBARS mg of protein^{-1} using a malondialdehyde (MDA) standard curve.

The carbonylated proteins were quantified based on their reaction with 2,4-dinitrophenylhydrazine (DNPH) and consequently formation of dinitrophenyl hydrazones, as described by Levine et al. (1994). Briefly, samples were added (v:v/1:5) to DNPH (10 mM, prepared in HCl 2 M), vigorously mixed for 3 min and incubated for 90 min at 30 °C. A blank without DNPH was performed simultaneously with each sample. After, proteins were precipitated with thiobarturic acid (28%), centrifuged (9.000 ×*g* for 10 min) and the pellet was washed (ethanol:ethil acetate/1:1) three times. Guanidine hydrochloride (6 M) was used to protein solubilization. The carbonyls content was determined in spectrophotometer at 360 nm and expressed in nmol carbonyls mg of protein^{-1}.

2.6.3. Metallothionein-like proteins (MT-like)

The MT-like content was determined in gills (n = 9–10) and mantle (n = 8–10), following the methodology described by Viarengo et al. (1997) with modifications. Tissues were homogenized (1:4 - m/v), centrifuged for 45 min (18,650 ×*g*; 4 °C) and the supernatant was subjected to ethanol/acid chloroform fractionation to obtain a partially purified metalloprotein fraction. In this fraction sulfhydryl groups (−SH) were quantified in a spectrophotometer at 412 nm, using Ellman's reagent. Reduced glutathione (GSH) was used as standard and the metallothionein content was expressed in nmol GSH mg of protein^{-1}.

2.6.4. Acetylcholinesterase (AChE) activity

Mantle (n = 8–9) and muscle (foot) (n = 9–10) samples were individually homogenized (w:v/1:4) in potassium phosphate buffer (0.1 M, pH 8) and then centrifuged (14,000 ×*g*; 20 min, 4 °C) for the determination of AChE. The assay was performed as described by Ellman et al. (1961), with Mora et al. (1999) modifications, using acetylcholine iodide (75 mM) as substrate and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (5 mM) as color reagent. The absorbance was monitored for

30 min at 415 nm. Two blanks were done in order to evaluate the substrate spontaneous hydrolysis rate. AChE activity was expressed in nmol min^{-1} mg $protein^{-1}$.

Biochemical biomarkers were expressed in relation to the total protein concentration which was determined by the Bradford (1976) method, against a bovine serum albumin (BSA) calibration curve at 595 nm.

2.7. Statistical analysis

Data were first tested for normality and homogeneity of variance to check statistical demands. The parametric analysis of variance (ANOVA) or non-parametric (Kruskal-Wallis) were used, followed by a multiple comparison test (Newman-Keuls test or Dunn's), when indicated. Bioaccumulation and biomarkers results, for each tissue, were compared among bivalves caged at different sites (Up1 × Up2 × Dw1 × Dw2). Still, metal bioaccumulation was compared between organs (gills x mantle × digestive gland × muscle) from animals of a single site. Animals weights taken at the beginning (0 h) and at the end (96 h) of the caging period at each site were compared by using paired *t*-test. Differences were considered significant for $P < 0.05$.

In order to illustrate the data synthetically and to evaluate the contributions of metals in tissues, sediments and water in the explanation of the variations between caging sites, principal components analysis (PCA) were carried out using the software CANOCO for Windows 4.25.

3. Results

Several physical and chemical variables showed alterations in sites downstream the mine (Dw1 and Dw2) when confronted with upstream sites (Up1 and Up2) (Fig. 2). It was observed an elevation in electric conductivity, total dissolved solids and hardness and a reduction in pH values. Some of these changes were more pronounced at the end of the caging period (96 h).

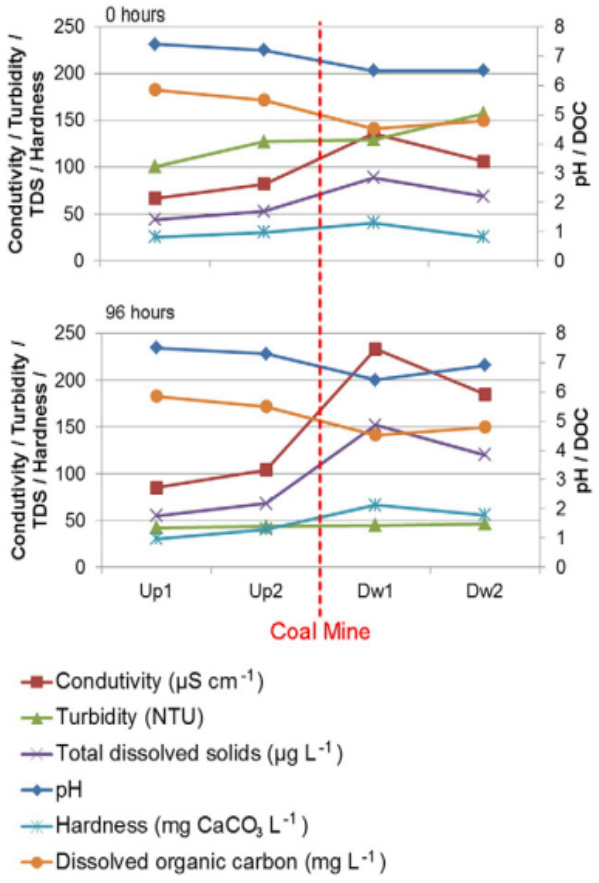

Fig. 2. Physical and chemical variables from water measured in the beginning (0 h) and end (96 h) of the *in situ* test at each studied site (Up1, Up2, Dw1 and Dw2). TDS: Total dissolved solids; DOC: Dissolved organic carbon.

The concentrations of Al, Mn, Fe and Zn in water (total and dissolved) and sediment are shown in Table 1. All these metals appear to increase in the water samples from downstream and significant increases were evidenced in total and dissolved Mn concentrations in Dw1 and Dw2 in comparison to Up1 and in total Fe from Dw1 and Dw2 in relation to Up2. Zn was only detected in water samples from downstream sites. On the other hand, in sediment samples a different pattern was observed (Table 1) as Dw1 site seems to contain lower amounts of metals, but in Dw2 samples higher contents of Al, Fe and Zn were detected. Granulometric analyses showed that Dw1 sediment presents 99.4% of sand while in the other three sites sand content was between 80.6 and 86.0%.

No clam mortality was observed during the experiment and animals caged at Dw1 presented a statistical gain of 7% in total weight. Metal concentrations in different tissues of *A. trapesialis* are shown in Table 2. The distribution of metals varied among different tissues and higher concentrations for each metal were highlighted in Table 2, excluding hemolymph results because these present a different unit (mg L^{-1} instead of mg g^{-1}). Gills and mantle were the organs with higher content of Zn and Mn, while gills and digestive gland presented higher amounts of Al and gills, mantle and digestive gland of Fe.

Gills of *A. trapesialis* caged at Dw1 and Dw2 showed significant increases in Al and Fe concentrations, while a significantly lower content of Mn was observed in this same tissue in comparison to animals confined in upstream sites. Animals caged at Dw1 also presented statistically higher amounts of Al in mantle, muscle and hemolymph when compared to bivalves from Up1. Iron concentrations were statistically higher in hemolymph of animals caged at Dw1 when compared to clams caged at upstream sites (Up1 and Up2) and in animals from Dw2 only compared to Up1. In muscle, Fe content was significantly higher in animals from Dw1 in comparison to Up1. Mn concentrations were significantly lower in mantle and digestive glands of animals caged at Dw1 in relation to bivalves from Up2 and Dw2. This same pattern was observed in Zn concentration in the digestive gland. Animals confined at Dw1 showed lower concentrations of Al in digestive gland and of Fe in mantle when compared to Up2. A significant increase in Zn content was only observed in muscle of animals caged at Up2, Dw1 and Dw2 in comparison to Up1 together with a significantly higher content of Mn in the hemolymph of animals caged in these same sites.

The principal component analysis (PCA) was applied to the metal accumulation in clam tissues and metals in sediment and water from the different sites and the results are presented in a biplot (Fig. 3). The first two axes (PC1 and PC2) accounted for 44.5% of total variance. Therefore, the other axes were neglected because they did not provide significant additional information. The first PCA axis (horizontal) accounted for 33.4% of total variability and was responsible for a clear separation of upstream (Up1 and Up2) from the downstream (Dw1 and Dw2) sites. The factors that most contributed for this separation were metal concentrations in water, Fe and Al bioaccumulation in gills and Fe in hemolymph. The second PCA axis (vertical) accounted for 11.2% of the total variability of the data and was responsible for the clear separation of Dw1 from Dw2. Important factors that determinate this separation were higher concentrations of Al and Fe in sediment of Dw2, lower concentrations of Zn in gills and mantle, Mn in muscle and Al in digestive gland of animals caged at Dw1 compared to all the other sites and higher concentrations of Al in hemolymph of bivalves from Dw1. Fe and Al bioaccumulation were still important to separate Dw1 from Dw2 because their concentrations were higher at Dw1.

Concerning biomarker results, *A. trapesialis* showed differences among tissues. The gills showed a total antioxidant capacity (Fig. 4A) slightly lower than the mantle and seemed to be the organ with a high natural occurrence of oxidative damage (Fig. 5A-B). On the other

Table 1
Concentrations of Al, Mn, Fe and Zn in water (total and dissolved) (mean ± SD) and sediment samples (beginning: 0 h; end: 96 h) collected from sites located upstream (Up1 and Up2) and downstream (Dw1 and Dw2) from a coal mine.

	Water (total)	Water (dissolved)	Sediment 0 h	Sediment 96 h
	mg L^{-1}	mg L^{-1}	mg g^{-1}	mg g^{-1}
Aluminum				
Upstream1	0.77 ± 0.59 a	0.11 ± 0.07 a	0.44	0.47
Upstream2	0.91 ± 0.48 a	0.13 ± 0.07 a	0.56	0.59
Downstream1	1.55 ± 0.84 a	0.31 ± 0.10 a	0.40	0.55
Downstream2	1.64 ± 0.38 a	0.25 ± 0.18 a	0.78	0.85
Manganese				
Upstream1	0.23 ± 0.03 a	0.14 ± 0.04 a	0.27	0.41
Upstream2	0.69 ± 0.35 ab	0.40 ± 0.05 ab	0.17	0.29
Downstream1	1.98 ± 2.25 b	0.88 ± 0.33 b	0.01	0.01
Downstream2	1.08 ± 0.31 b	0.79 ± 0.20 b	0.14	0.18
Iron				
Upstream1	1.25 ± 0.17 ab	0.15 ± 0.10 a	2.83	2.98
Upstream2	0.79 ± 0.18 a	0.25 ± 0.25 a	2.72	3.18
Downstream1	5.01 ± 1.88 b	3.59 ± 2.12 a	1.13	1.53
Downstream2	5.35 ± 1.59 b	3.37 ± 2.87 a	4.32	4.49
Zinc				
Upstream1	<dl	<dl	<0.01	<0.01
Upstream2	<dl	<dl	0.01	0.02
Downstream1	0.33 ± 0.12 a	0.27 ± 0.09 a	0.01	0.02
Downstream2	0.22 ± 0.06 a	0.19 ± 0.08 a	0.02	0.08

dl (detection limit) for Al: 45 μg L^{-1}; Mn: 1.5 μg L^{-1}; Fe: 5 μg L^{-1}; Zn: 1.5 μg L^{-1}. Different letters represent significant differences among sites for a given metal. Differences for Al, Mn and Fe were tested by Kruskal-Wallis and for Zn were tested by Student *t*-test.

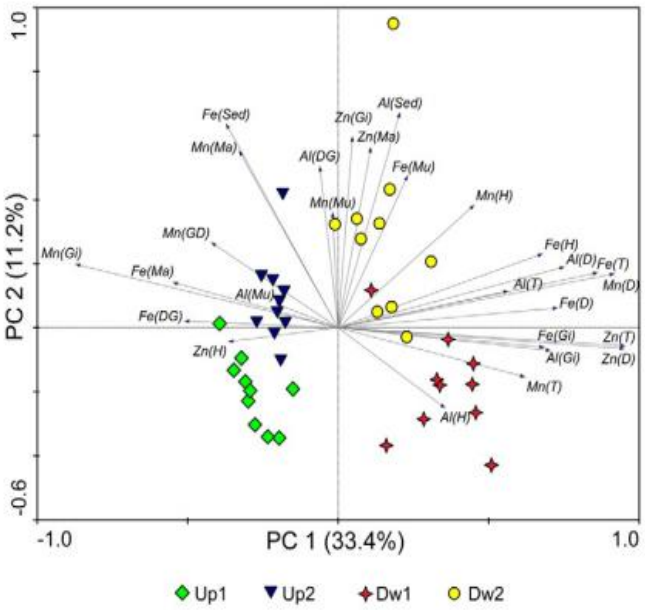

Fig. 3. Principal components analyses (PCA) performed using metal concentrations in samples of sediment (Sed) and water (T: total metals; D: dissolved metals) from each site (Up1, Up2, Dw1 and Dw2), and in digestive gland (DG), gills (Gi), muscle (Mu), mantle (Ma) and hemolymph (H) of *Anotontites trapesialis* confined for 96 h upstream (Up1 and Up2) and downstream (Dw1 and Dw2) from a coal mine.

hand, digestive gland was the tissue with the highest GST activity (Fig. 5C).

Biomarkers results (Mean ± SD) varied among caging sites in gills and mantle, while no alterations were observed in digestive gland. In the mantle of animals caged at Dw2 a significant ($H = 8.079$; $P = 0.044$) lower TAC was detected in comparison to Up1 (Fig. 4A) and a significant ($H = 14.988$; $P = 0.002$) higher MT content in relation to upstream sites (Fig. 4B). An increase in oxidative damage occurred in this same tissue only in clams confined at Dw1, as indicated by a significant ($H = 11.418$; $P = 0.010$) higher LPO when compared to Up2 (Fig. 5A). On the other hand, a significant ($H = 11.637$; $P = 0.009$) increase in LPO was verified in gills of animals confined at Dw1 and Dw2 in comparison to Up2 (Fig. 5A). PC in the gills was significantly ($F = 3.497$; $P = 0.026$) higher in animals caged at Dw2 in comparison to Up 2 (Fig. 5B). GST activity in the gills was significantly ($F = 3.982$; $P = 0.015$) lower in bivalves caged at Up2 in comparison to all other sites (Fig. 5C) while SOD activity did not vary significantly among sites (Fig. 5D). It was also observed a significant ($H = 25.121$; $P < 0.001$) decrease in AChE activity in the muscle of *A. trapesialis* caged downstream from the mine (Dw1 and Dw2) in comparison to both upstream sites (Fig. 6).

Table 2
Bioaccumulation of Al, Mn, Fe and Zn in different tissues of *Anodontites trapesialis* caged for 96 h at upstream (Up1 and Up2) and downstream (Dw1 and Dw2) sites from a coal mine. Organs with higher concentrations of a specific metal were highlighted in italic, excluding hemolymph as the results are presented using a different unit (mg L^{-1} instead of mg g^{-1}).

	Gills	Mantle	Digestive gland	Muscle	Hemolymph
	mg g^{-1}	mg g^{-1}	mg g^{-1}	mg g^{-1}	mg L^{-1}
Aluminum					
Upstreaml	*0.26 ± 0.19 a AC*	0.03 ± 0.02 a B	*0.35 ± 0.16 a C*	0.05 ± 0.03 a AB	0.14 ± 0.12 a
Upstream2	*0.48 ± 0.35 a A*	0.09 ± 0.06 ab B	*0.21 ± 0.06 ab A*	*0.31 ± 0.14 b A*	0.20 ± 0.10 ab
Downstreaml	*2.96 ± 2.09 b A*	0.12 ± 0.06 b B	0.15 ± 0.08 b B	0.22 ± 0.08 b B	0.31 ± 0.16 b
Downstream2	*1.78 ± 0.88 b A*	0.08 ± 0.03 ab B	*0.50 ± 0.33 a AC*	0.13 ± 0.07 ab BC	0.23 ± 0.11 ab
Manganese					
Upstreaml	*32.05 ± 2.91 a A*	*30.10 ± 5.27 ab A*	*20.04 ± 7.05 ab A*	1.10 ± 0.41 a B	0.10 ± 0.03 a
Upstream2	*31.42 ± 3.19 a AB*	*34.27 ± 9.01 a A*	23.61 ± 5.83 a B	1.61 ± 0.72 a C	0.83 ± 0.29 b
Downstream1	*19.43 ± 5.08 b A*	*24.88 ± 8.44 b A*	*15.80 ± 3.65 b AB*	1.15 ± 0.48 a B	0.88 ± 0.40 b
Downstream2	*26.03 ± 3.45 c AB*	*34.54 ± 6.45 a B*	19.85 ± 4.76 a AC	1.51 ± 0.85 a C	0.68 ± 0.25 b
Iron					
Upstream1	2.96 ± 0.48 a AC	*4.34 ± 1.50 a AB*	*6.01 ± 2.02 a B*	0.50 ± 0.15 a C	36.2 ± 21.3 a
Upstream2	*2.94 ± 0.46 a A*	*3.57 ± 0.79 ab A*	*5.11 ± 1.96 a A*	0.85 ± 0.37 ab C	74.3 ± 23.1 ac
Downstream1	*6.02 ± 1.92 b A*	2.63 ± 1.16 b B	*3.83 ± 1.70 a AB*	1.03 ± 0.27 b C	144.1 ± 46.5 b
Downstream2	*5.07 ± 1.47 b A*	3.41 ± 1.29 a B	*4.21 ± 1.56 a AB*	1.15 ± 0.80 ab C	119.8 ± 59.8 bc
Zinc					
Upstream1	*0.27 ± 0.12 a A*	*0.28 ± 0.07 a A*	0.12 ± 0.05 ab B	0.06 ± 0.01 a B	0.30 ± 0.24 a
Upstream2	*0.35 ± 0.15 a A*	*0.34 ± 0.10 a A*	*0.19 ± 0.06 a AB*	0.08 ± 0.03 b B	0.47 ± 0.10 a
Downstream1	*0.25 ± 0.06 a A*	*0.21 ± 0.13 a AB*	0.09 ± 0.04 b BC	0.09 ± 0.02 b C	0.30 ± 0.11 a
Downstream2	*0.40 ± 0.13 a A*	*0.32 ± 0.13 a AB*	0.18 ± 0.10 a BC	0.10 ± 0.01 b C	0.31 ± 0.09 a

Different letters represent significant differences: lowercase letters indicate differences among sites considering each metal and organ separately; uppercase letters indicate differences among organs (gills, mantle, digestive gland and muscle) of animals from the same site.

4. Discussion

In situ tests were carried out in the DP stream, located near a coal mining area, in which clams were confined for 96 h and chemical and biological variables evaluated. Our results showed that this particular coal mining activity has clearly affected the stream, as evidenced by variations in metals bioaccumulation and biomarkers in *A. trapesialis*. In fact, several alterations in physical and chemical variables such as electrical conductivity, total dissolved solids (Fig. 2) and water metals content (Table 1), were more marked at the two downstream sites than at the upstream sites in the mining area, indicating that the mine has indeed affected water quality. The PCA results (Fig. 3) show that upstream (Up) and downstream (Dw) sites differed mainly in terms of total and dissolved metals concentration and bioaccumulation of Fe and Al. Anthropogenic sources of surface water pollution vary enormously and understanding the effects of contaminants on aquatic ecosystems requires a full analysis of both the physical and chemical variables, and the biological variables. In general, aquatic organisms are exposed to numerous stressors and establishing causal links between the levels of a specific contaminant and biomarker responses is a rather complex task. Our findings help identify a number of potential tools that could be used to monitor aquatic ecosystems near coal mining areas.

Fe, Al, Zn and Mn have been found to present problems at this particular mine (Campaner and Luiz-Silva, 2009). High concentrations of these metals have already been monitored in water bodies near coal mining operations (Bharti and Banerjee, 2013; MacCausland and McTammany, 2007; Zocche et al., 2014). Our findings show evidence of significantly higher concentrations of total Fe, Zn and Mn in water from sites downstream of the mine. However, there was no evidence of this pattern at site Dw1, which showed lower metal concentrations in sediment samples. The environmental impacts of coal mining activities are not restricted to contamination by AMD. A significant impact occurs after rainfall, when runoff causes erosion and silting, resulting in an increase in particulates in nearby bodies of water (Tiwary, 2001). This probably has occurred at Dw1 over the years of coal mining. At this site, a higher percentage of sand was observed and because of this particular physical impact, the capacity of the sediment to retain contaminants has been altered. The PCA results (Fig. 3) reflect this alteration, showing that sediment metals concentrations at downstream sites are determinant factors separating Dw1 from Dw2.

The bioaccumulation data show a different pattern of metals distribution among tissues (Table 2). For essential metals, such as Mn, Zn and Fe, partitioning to specific organs is related to the minimum background concentration required for regular metabolic activity. When essential or non-essential metals exceed a certain threshold, some organs act as temporary or permanent "dump sites", and can show high concentrations (Rainbow, 1993). In our results, the gills could have acted as dump sites and accumulated Al and Fe exclusively at downstream sites (statistically different from Up1 and Up2). Indeed, gills are considered the target organ for metals bioaccumulation in bivalves, since a large volume of water is filtered by this organ, reaching rates of 700 mL h^{-1} in *A. trapesialis* (Loayza-Muro and Elías-Letts, 2007).

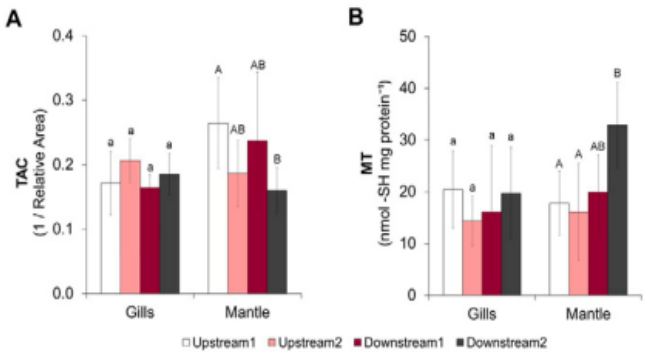

Fig. 4. A) Total antioxidant capacity against peroxyl radicals (TAC) and B) metallothionein content (MT) in gills and mantle of *Anodontites trapesialis* confined for 96 h upstream (Up1 and Up2) and downstream (Dw1 and Dw2) from a coal mine. Values are mean ± SD (n = 8–10) and different letters represent significant differences (*One-way ANOVA*: *TAC-gills*; *Kruskal-Wallis*: *TAC-mantle*, *MT-both tissues*, $P < 0.05$) between sites, for each tissue separately (Gills: lowercase letters; Mantle: uppercase letters).

A clear elevation in Al and Fe levels was evidenced in the gills of clams from Dw1 and Dw2, and statistical differences were observed in hemolymph levels of these two metals for clams sampled at Up1 and Dw1 (Table 2). It is important to highlight that there was no statistical difference in total and dissolved Al and dissolved Fe between the downstream and upstream sites, but the bioaccumulation of these two metals at downstream sites was notable. Bivalve mollusks have been used as biomonitors in environmental monitoring programs (Goldberg and Bertine, 2000) because they are filter-feeders with a very low metabolic rate that results in the bioaccumulation of many chemicals. *A. trapesialis* showed a significant increase in metals bioaccumulation after only 96 h of confinement in the field and seems to be a good biological model for monitoring metal-contaminated environments. Al and Fe bioaccumulation in *A. trapesialis* tissues after 96 h seems to provide a good indication of contamination from this particular coal mine, and these clams should be considered as effective biomonitors. In contrast to the other three metals (Mn, Fe and Zn), Al is the only non-essential metal, and is therefore very important in the case in point, since it can cause toxicity at lower concentrations (Nikinmaa, 2014).

Zinc and manganese levels were elevated in muscle and hemolymph tissues, respectively, in individuals from both Dw sites and, surprisingly, Up2. Moreover, the Al concentration was elevated in the muscle tissue of *A. trapesialis* clams confined at Up2. Water analysis also showed a slightly higher but not statistically different concentration of Mn and Al at Up2 when compared to Up1, possibly indicating that the effects of mining at this site are influenced by atmospheric or rainfall events. This data is an indication of how necessary it is to monitoring upstream of the contamination source and implement control measures for other sources of contamination in coal mining activities. Even so, biomarkers results indicate that *A. trapesialis* did not suffer significantly from contamination problems at Up2.

Bioavailability and metal-tissue interactions depend on several factors, such as the physical and chemical characteristics of the water (pH, hardness, dissolved organic matter) which are determinants for metal speciation (Niyogi and Wood, 2004), interactions with other atoms and molecules (including other contaminants), the entrance routes of contaminants (filtration, feeding, *etc.*) and animal behavior (Belzunce-Segarra et al., 2015; Rainbow, 2007). Thus, simply determining total metals concentration in the water is not enough to characterize its toxicity. For example, Al speciation is highly influenced by low pH, high conductivity and sulfate concentrations, common characteristics of AMD effluents. Consequently, the distribution of Al species varies depending on the water chemistry, and will therefore determine the level of toxicity (Chamier et al., 2015).

Several classical biomarkers measured in *A. trapesialis* showed alterations after 96 h of confinement downstream of the coal mine. Oxidative stress occurred in clams caged at both downstream sites (Dw1 and Dw2) compared to Up2, evidenced by LPO in the gills. However, in the mantle tissues, different alterations were observed in downstream animals. LPO alteration was observed only in clams from Dw1, yet TAC and MT-like content were modified in clams from Dw2. Bivalves use different strategies and mechanisms to deal with excess metals in the environment, depending on the situation (Thorsen et al., 2007). These strategies include avoidance (valve closure or a drop in the filtration rate), induction of MT, incorporation of metals into lysosomal granules, histidine-rich glycoproteins and calcium-phosphate granules (Thorsen et al., 2007). Despite several results indicating metal concentrations in the water and Al and Fe accumulation in gill tissue, there were no significant differences between the conditions at Dw1 and Dw2, although these sites seemed to provoke different responses in *A. trapesialis*, evidenced by biomarker alterations.

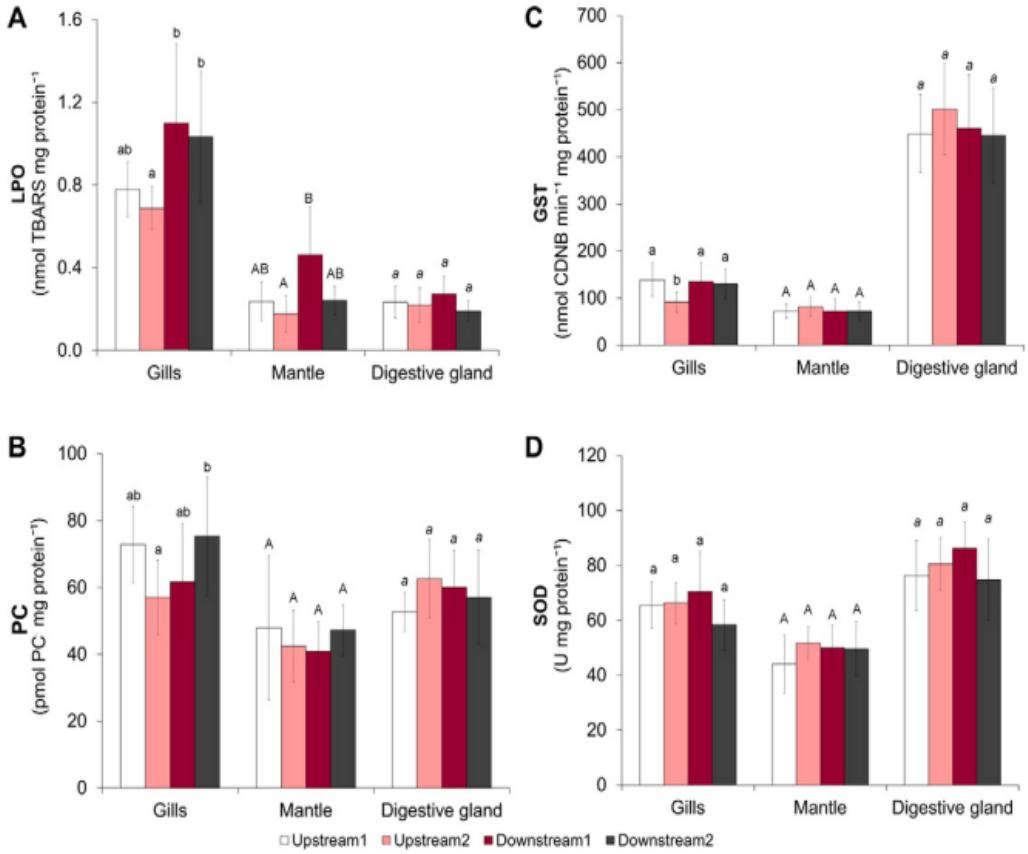

Fig. 5. A) Lipid peroxidation (LPO), B) protein carbonylation (PC), C) Glutathione S transferase activity (GST) and D) Superoxide dismutase activity (SOD) in gills, mantle and digestive gland of *Anodontites trapesialis* confined for 96 h upstream (Up1 and Up2) and downstream (Dw1 and Dw2) from a coal mine. Values are mean ± SD (n = 8–10) and different letters represent significant differences (*One-way ANOVA*: *LPO-Digestive gland*, *PC-gills and mantle*, *GST-all tissues*, *SOD-all tissues*; *Kruskal-Wallis*: *LPO-gills and mantle*, *PC-Digestive gland*, $P < 0.05$) between sites, for each tissue separately (Gills: lowercase letters; Mantle: uppercase letters; Digestive gland: italic letters).

In bivalves, valve closure and reduction of filtration rate are common behaviors under variable and poor environmental conditions resulting from the presence of contaminants (Farris and Van Hassel, 2007). Valve closure causes a reduction in gas and ion exchange through the gills and increases anaerobic respiration. These factors can promote a decrease in internal pH, which can be associated with increased Ca^{2+} in the hemolymph (released by the shell and calcium-phosphate granules), probably because the buffering role of $CaCO_3$ (Burton, 1983). *A. trapesialis* confined at Dw1 showed osmo-ionic alterations in the hemolymph (elevated Ca^{2+} concentration, low osmolarity, and concentrations of Na^{2+}, Cl^{-}, Mg^{2+}) (data not shown). These results, together with a 7% gain in total weight, support the hypothesis that, at Dw1, environmental conditions stimulated an increase in valve closure frequency and the increase in total weight was probably associated with water retention after a drop in urine production (Robertson, 1964). Clams from Dw1 showed Al bioaccumulation mainly in the gills, but also in the mantle, muscle and hemolymph, and this could have caused valve closure, as already shown in *Anodonta cygnea* when exposed to 500 μg L^{-1} of aluminum for 15 days (Kádár et al., 2011). *Anodonta cygnea* showed a 50% reduction in shell gape after the exposure period and increased aluminum concentration in the gills, digestive gland and kidney.

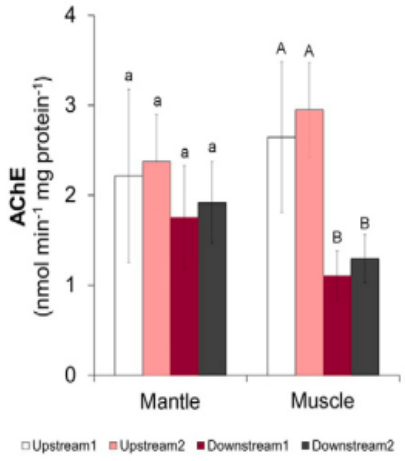

Fig. 6. Acetylcholinesterase activity in mantle and muscle of *Anodontites trapesialis* confined for 96 h upstream (Up1 and Up2) and downstream (Dw1 and Dw2) from a coal mine. Values are mean ± SD (n = 8–10) and different letters represent significant differences (*One-way ANOVA*: *mantle*; *Kruskal- Wallis*: *muscle*, $P < 0.05$) between sites, for each tissue separately (Mantle: lowercase letters; Muscle: uppercase letters).

Our results show that the gills and mantle of *A. trapesialis* exhibited similar MT-like content, but this varied from one site to another only in the mantle (Fig. 4B). In this tissue, MT-content was higher in individuals confined at Dw2 compared to those at upstream sites. Higher concentrations of metals were not observed in the mantle tissue of individuals confined at Dw2 and it was not possible to establish a direct relationship between MT-content and metals bioaccumulation, although we cannot rule out the possible accumulation of metals not evaluated. MTs also have an antioxidant role (Viarengo et al., 2000) and higher levels of these peptides could justify the absence of oxidative damage (LPO and PC levels) in the mantle tissue of clams from Dw2 (Fig. 5). Despite the higher MT-content, a decrease was observed in TAC against peroxyl radicals. The antioxidant capacity of a tissue to withstand these reactive oxygen species (ROS) is 70% promoted by non-enzymatic low-molecular-weight scavengers (Amado et al., 2009), including reduced glutathione (GSH), MTs, ascorbic acids and vitamin E. Thus, the drop in TAC is probably related to the depletion of these other antioxidants and the fact that

the increase in MT-content was insufficient to maintain normal TAC levels. On the other hand, the clams confined at Dw1 showed elevated LPO levels in the mantle compared to those at Up2, indicating oxidative stress.

The gills of the clams caged at downstream sites (Dw1 and Dw2) also showed evidence of oxidative stress, with LPO levels higher than those found in specimens at Up2 (Fig. 5). This is directly associated with the higher amounts of aluminum and iron observed in the tissues of these animal. Although Al is considered a non-redox-active metal, it can enhance the production of ROS and cause oxidative damages. Hypothetically, Al can interact in biological samples to form an Al-superoxide complex that has pro-oxidant activity (Exley, 2004) by catalyzing the Fenton reaction cycle (Ruipérez et al., 2012), such as Fe.

AChE activity in bivalves appears also to be involved in burying behavior, especially in those species that live on soft sediments, like *Anodonta* (Corsi et al., 2007, Perić and Ribarić, 2013), and the inhibition of AChE could impair the burying capability. Currently, it is known that AChE activity can be decreased by many molecules, and not just organophosphates and carbamates. Several studies have demonstrated that some metals (*e.g.* Cd, Cu, Zn, Al and Fe) cause alterations in the activity of this enzyme, probably by direct interaction with anionic sites causing protein denaturating (De Lima et al., 2013; Pohanka, 2014). In the muscle tissue of clams confined at the two downstream sites, lower AChE activity (Fig. 6) was associated with higher Al and Fe levels in specimens from Dw1, and Zn in clams confined at Dw1 and Dw2. However, we cannot confirm that this is a direct result of the presence of metals, since no other chemical analyses were carried out. This might be a consequence of contamination by mine effluents, since a drop in AChE activity was detected only at downstream sites.

5. Conclusions

Coal mining adversely affects freshwater environments in many ways, and the effects can persist for a long time. Some metals, like Al, Mn, Zn and Fe, are often found in high concentrations in AMD and water bodies near mining activities. *A. trapesialis* proved to be an efficient biological model, since alterations were observed in the animals after only 96 h of confinement at downstream sites, and this invertebrate species could be a good candidate for monitoring Neotropical freshwaters. Al and Fe bioaccumulation in the gills and hemolymph, Al bioaccumulation in the mantle and muscle, LPO in the gills (Dw1 and Dw2) and mantle (Dw1), as well as AChE activity in the muscle should be considered effective biomarkers for monitoring coal mining areas. Longer *in situ* exposures should be performed in future works to detect other sub-lethal effects, considering that along 96 h exposure *A. trapesialis* probably used valve closure behavior as a protection mechanism.

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