UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

BIOACUMULAÇÃO E PARÂMETROS DE ESTRESSE OXIDATIVO EM TAMBAQUI (*Colossoma macropomum*) EXPOSTO A MnCl$_2$ EM DIFERENTES NÍVEIS DE OXIGÊNIO DISSOLVIDO

TESE DE DOUTORADO

Diogo Gabriel

Santa Maria, RS, Brasil

2013

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Diogo Gabriel

Tese apresentada ao Programa de Pós-Graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de

Doutor em Farmacologia

Orientadora: Profª. Drª. Susana Francisca Llesuy

Co-orientadora: Profª. Drª. Maria Amália Pavanato

Santa Maria, RS, Brasil

2013

Universidade Federal de Santa Maria
Centro de Ciências da Saúde
Programa de Pós-Graduação em Farmacologia

A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

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Elaborada por
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Como requisito parcial para a obtenção do grau de
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Dedico este trabalho aos meus pais, pois toda conquista de um filho é igualmente dos pais. Também dedico este trabalho a minha noiva Aline, meu amorzão, que sempre está ao meu lado.

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RESUMO

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BIOACUMULAÇÃO E PARÂMETROS DE ESTRESSE OXIDATIVO EM TAMBAQUI
(*Colossoma macropomum*) EXPOSTO A MnCl$_2$ EM DIFERENTES NÍVEIS DE
OXIGÊNIO DISSOLVIDO
AUTOR: DIOGO GABRIEL
ORIENTADORA: SUSANA FRANCISCA LLESUY
CO-ORIENTADORA: MARIA AMÁLIA PAVANATO
Local e data da defesa: Santa Maria, 18 de abril de 2013

Diversas atividades, incluindo a exploração petrolífera liberam manganês (Mn^{2+}) na água da bacia amazônica. O Mn^{2+} tem a capacidade de produzir estresse oxidativo em peixes. Logo, é necessário que se faça um controle do metal na água, mas também nos organismos aquáticos. Assim, o objetivo do nosso trabalho foi expor o tambaqui (*Colossoma macropomum*) ao manganês por 96h para encontrar a CL$_{50-96h}$ para o tambaqui e posteriormente traçar o perfil redox do peixe exposto ao metal em dose subletal em normóxia e hipóxia. Na primeira série de experimentos os animais foram expostos a normóxia (6 mg/L), hipóxia (0,25 mg/L) e hiperóxia (10 mg/L). Houve mortalidade somente em hipóxia, sendo o CL$_{50-96h}$ 4.03 mg/L de Mn^{2+}. A ordem da bioacumulação foi brânquias > fígado > músculo. Na segunda série de experimentos o tambaqui foi exposto a 3.88 mg/L de Mn^{2+} por 96 horas em normóxia (6 mg/L) e posteriormente foram mortos por secção medular, sendo os tecidos alvo do estudo retirados. Foram analisados vários biomarcadores de estresse oxidativo, como espécies reativas ao ácido tiobarbitúrico (TBARS), superóxido dismutase (SOD), catalase (CAT), glutationa-S-transferase (GST) e o conteúdo de tióis não protéicos (GSH). Em brânquias houve um aumento significativo dos níveis de TBARS e da atividade da SOD e uma redução do conteúdo de tióis não protéicos. Em fígado houve uma diminuição significativa dos níveis de TBARS e um aumento significativo nas atividades da SOD e GST, além do conteúdo de tióis não protéicos. No cérebro houve somente uma diminuição significativa da atividade da SOD e CAT. No rim houve uma aumento significativo dos níveis de TBARS e uma diminuição

significativa da atividade da SOD. Nesta segunda série de experimentos ficou claro a presença de estresse oxidativo em diferentes órgãos. Isso mostra que o Mn^{2+} possui toxicidade diferenciada em cada órgão. A ordem da bioacumulação foi brânquias > rim > cérebro > fígado. Na terceira série de experimentos o tambaqui foi exposto a 3.88 mg/L de Mn^{2+} por 96 horas em hipóxia (0,25 mg/L) e posteriormente foram mortos por secção medular, sendo os tecidos alvo do estudo retirados. Os biomarcadores de estresse oxidativo foram os mesmos da série anterior. Nas brânquias houve um aumento significativo dos níveis de TBARS e uma redução significativa nas atividades da SOD e GST. No fígado ocorreu um aumento significativo nos níveis de TBARS e uma diminuição significativas na SOD e GST. No cérebro e no rim a exposição não produziu alterações nos níveis de TBARS, mas houve um aumento significativo na atividade da SOD em ambos os tecidos. A ordem da bioacumulação foi rim > brânquias > fígado > cérebro. Da mesma maneira que na série anterior, a exposição ao metal provocou estresse oxidativo em alguns órgãos, entretanto, cada órgão obteve um perfil redox diferente. Assim, com os experimentos foi possível obter o CL_{50-96h} para o Mn^{2+} em tambaqui, além disso os valores de bioacumulação em cada órgão não se correlacionam com os resultados encontrados para os biomarcadores de estresse oxidativo e a SOD possuiu um papel chave nos experimentos. Por fim, o perfil redox encontrado tanto em normóxia como em hipóxia podem levar a novos experimentos que posteriormente podem proporcionar que o peixe possa ser utilizado como uma sentinela para o Mn^{2+} nas águas.

ABSTRACT

Thesis of Doctor's Degree
Programa de Pós-Graduação em Farmacologia
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**BIOACUMULAÇÃO E PARÂMETROS DE ESTRESSE OXIDATIVO EM TAMBAQUI
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AUTHOR: DIOGO GABRIEL
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Data and place of the defense: April, 18, 2013, Santa Maria

The aquatic organisms are susceptible to frequent interferences in their habitat, such as oxygen fluctuations and metal contamination. Industrial production and oil exploration, among other activities, release manganese (Mn^{2+}) into the water of the Amazon basin. Manganese may induce oxidative stress (OS) in fish, so a control of the metal in the water as well as in the aquatic organisms is necessary. In this way, the present work aimed at exposing tambaqui (*Colossoma macropomum*) to Mn^{2+} for 96 h to assess the LC_{50-96h} in the species and subsequently outline the redox profile of the fish subjected to a sublethal concentration of the metal in normoxia and hypoxia. In the first series of experiments the fish were exposed to normoxia (6 mg/L), hypoxia (0.25 mg/L) and hyperoxia (10 mg/L). Mortality was only observed in hypoxia, with a Mn^{2+} LC_{50-96h} of 4.03 mg/L. Bioaccumulation occurred in the following order: gills>liver>muscle. During the second series of experiments the fish were subjected to 3.88 mg/L Mn^{2+} for 96 h in normoxia (6 mg/L). After exposure the fish were euthanized by sectioning the spinal cord and the target tissues, brain, gills, liver and kidney, were excised. Biomarkers of OS were analysed: thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and the content of non-protein thiol groups (GSH). In gills there was a significant increase in TBARS levels and in SOD activity, and a reduction in GSH. Decreased levels of TBARS, increased SOD and GST activities, as well as increased GSH, were observed in the hepatic tissue. In brain SOD and CAT activities reduced significantly. An increase in TBARS levels and a decrease in SOD activity were found in the kidney. The induction of OS was clearly observed in this second series of trials, with a different pattern of Mn^{2+} toxicity in each of the evaluated organs. Bioaccumulation was as follows: gills>kidney>brain>liver. In the third series of experiments tambaqui was subjected to 3.88 mg/L Mn^{2+} for 96 h under hypoxia (0.25 mg/L). The procedures after exposure, the dissected tissues and the analysed biomarkers of OS were similar to the ones described for the second series, only GSH was not measured this time. In gills and liver there was a rise in the levels of TBARS and a decrease in the activities of SOD and GST. Exposure to Mn^{2+} did not trigger changes in the levels of TBARS in brain and kidney, but induced a significant increase in SOD activity. A reduction in the levels of GST was also observed in tambaqui kidney. The sequence of bioaccumulation was: kidney>gills>liver>brain. As it was observed in the second series, exposure to Mn^{2+} caused OS in certain organs, though with a different redox profile in each tissue. Therefore, the experiments allowed attaining Mn^{2+} LC_{50-96h} in tambaqui. The bioaccumulation values for each organ were not correlated with the findings on OS biomarkers. Furthermore, SOD displayed a key role in the tests. The redox profile

obtained in normoxia as well as in hypoxia may lead to further studies which will possibly indicate the tambaqui as a sentinel fish for Mn^{2+} in the water.

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LISTA DE ABREVIATURAS

$^1O_2$	Oxigênio singlet
AS	Sistema de defesa antioxidante
AOE	Espécies Ativas de Oxigênio
Ca^{2+}	Cálcio
$CaCO_3$	Carbonato de cálcio
CAT	Catalase
Cd^{2+}	Cádmio
CDNB	1-cloro-2,4-dinitrobenzeno
Cl^-	Cloreto
CL_{50-96h}	Concentração letal média em 96h de exposição
CONAMA	Conselho Nacional do Meio Ambiente
Cu/Zn SOD	Cobre/zinco superóxido dismutase
Cu^{2+}	Cobre
DNA	Ácido desoxiribonucleico
DOM	Matéria orgânica dissolvida
EDTA	Etileno diaminotetracético
EO	Estresse oxidativo
EROs	Espécies reativas de oxigênio
Fe SOD	Ferro superóxido dismutase
Fe^{2+}	Ferro
F_OF_1 ATPase	Bomba de prótons - fosforilação oxidativa
GPx	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
GST	Glutationa-S-transferase
H^+	Hidrogênio
H_2O_2	Peróxido de hidrogênio
H_2SO_4	Ácido sulfúrico
HNO_3	Ácido nítrico
LC_{50}	Concentração letal média
LDH	Lactato desidrogenase
LPO	Lipoperoxidação
MDA	Malondialdeído
Mg^{2+}	Magnésio
NH_4^+	Amônio
Mn (VII)	Manganês 7
Mn SOD	Manganês superóxido dismutase
Mn(III)	Manganês 3
Mn(IV)	Manganês 4

Mn^{2+}	Manganês
$MnCl_2$	Cloreto de manganês
N_2	Nitrogênio
Na^+	Sódio
NAOH	Hidróxido de Sódio
NF-κB	Fator nuclear Kappa B
NH_3	Amônia
O_2	Oxigênio
$O_2^{\bullet-}$	Ânion superóxido
OD	Oxigênio dissolvido
$OH^{\bullet}$	Radical hidroxil
$ONOO^{\bullet-}$	Radical peroxinitrito
PMSF	Fluoreto de fenil-metil-sulfonil
ROS	Espécies reativas de oxigênio
-SH	Tiol
SOD	Superóxido dismutase
TBA	Ácido tiobarbitúrico
TBARS	Espécies reativas ao ácido tiobarbitúrico
TCA	Ácido tricloroacético
USOD	Unidade de SOD
Zn^{2+}	Zinco

SUMÁRIO

1 Introdução

1.1 Características gerais da água da Bacia Amazônica

A Bacia Amazônica é constituída por três tipos de água, onde ocorrem alterações no pH e matéria orgânica dissolvida (DOM), a qual é constituída por substâncias húmicas as quais têm como principal constituinte o ácido húmico. A água branca possui pH próximo à neutralidade, com muito pouca DOM. A água clara apresenta baixa concentração de DOM e possui pH entre 5,5-7,5. A água preta se caracteriza por uma elevada concentração de DOM, baixa concentração iônica e pH ácido (5,0 -6,0). A temperatura da água varia em torno de 24-28 °C, podendo excepcionalmente alcançar a mínima de 17 °C e a máxima de 40°C (ARAUJO-LIMA e OLIVEIRA, 1998; MATSUO et al., 2005).

Além disso, a água da Bacia Amazônica é considerada mole, isto é, com baixa concentração de cálcio. Esta característica torna as espécies aquáticas mais susceptíveis à intoxicação por metais tóxicos, pois em água dura o cálcio compete com os metais tóxicos pelos sítios biológicos no peixe (o que confere proteção), fato que não ocorre nas águas moles. (MATSUO et al., 2005; BALDISSEROTTO et al., 2011).

Nos ambientes tropicais aquáticos, vários fatores contribuem com uma significante redução da quantidade de oxigênio dissolvido. Entre eles destacam-se a morfologia do sistema, as taxas de consumo e produção de oxigênio, o aumento da pressão parcial de outros gases e a temperatura do ambiente (Beadle, 1981). Assim, a região alagada apresenta níveis hipóxicos de oxigênio dissolvido, enquanto o leito principal do rio possui níveis normóxicos. A hiperóxia pode surgir em regiões com elevadas taxas de fotossíntese associadas a uma adequada renovação da água e menor temperatura (ARAUJO LIMA E OLIVERIA, 1998).

1.2 Tambaqui

1.2.1 Classificação

O tambaqui *Colossoma macropomum* (Cuvier, 1818), pertence à classe Actinopterygii, ordem Characiformes, família Characidae, Subfamília Serrasalmidae (Géry, 1977). Ele pode medir até 1 m de comprimento e pesar até 30 Kg (FIGURA 1) (ARAUJO-LIMA e GOMES, 2005).

1.2.2 Características Gerais

O tambaqui é um peixe de água doce, teleósteo (**FIGURA 1**). Em estado silvestre ele é encontrado somente nas bacias do Solimões/Amazônas e Orinoco. A espécie habita o Brasil, Bolívia, Venezuela, Colômbia e Peru (ARAUJO LIMA E GOULDING, 1998).

Ele é encontrado nas regiões de várzea na bacia Amazônica. Estes locais, logo após o pico de inundação anual, possuem estagnação por falta de circulação, muita matéria orgânica dissolvida. Logo, em quase todos os habitats da várzea, durante o ano todo, existe pouco oxigênio em uma profundidade abaixo de 3 m. Assim, o tambaqui é naturalmente exposto à hipóxia e até a anóxia. Talvez por isso seja extremamente resistente (ARAUJO LIMA e GOULDING, 1998; MATSUO et al., 2005). Além disso, o tambaqui também tem longevidade relativamente grande, podendo viver até 15 anos (MARCON e WILHELM, 1999; AFFONSO et al., 2002; CHAGAS e VAL, 2003).
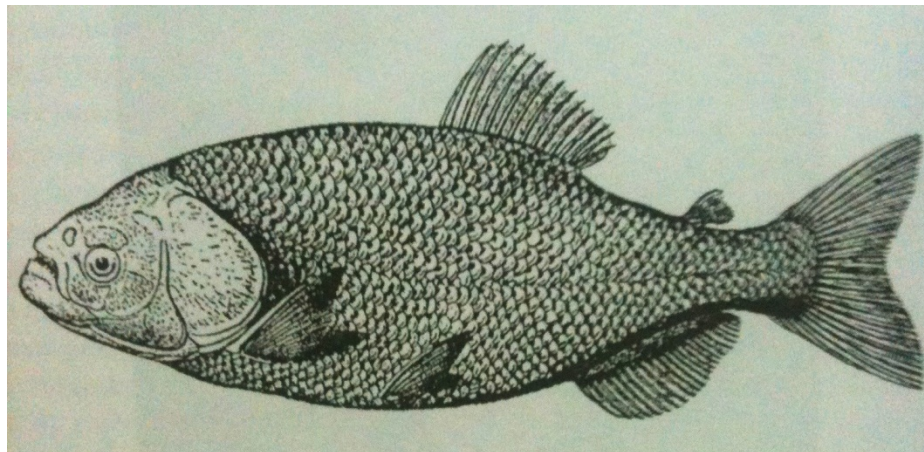
Figura 1 – O desenho de Cuvier do espécime tipo do tambaqui (CUVIER, 1818).

Em condições de hipóxia extrema, o tambaqui apresenta uma adaptação morfológica, o aumento do lábio inferior, para captar água junto à superfície, a qual apresenta maior concentração de oxigênio (ARAUJO-LIMA e GOMES, 2005).

1.2.3 Importância como modelo de estudo científico

A grande capacidade do tambaqui em se adaptar a condições adversas faz dele um bom modelo de estudo do estado redox em situações que geram estresse oxidativo, como na exposição à metais tóxicos e hipóxia (MARCON e WILHELM, 1999).

1.3 Espécies reativas de oxigênio

Os seres aeróbios necessitam de oxigênio (O_2) para seu metabolismo. Isto é especialmente verdade quando se aborda a produção de energia e o funcionamento enzimático (CADENAS, 1989). Grande parte da energia dos animais é produzida na mitocôndria, sendo que o oxigênio é importante no processo de transferência de elétrons, como aceptor final de elétrons (**FIGURA 2**).
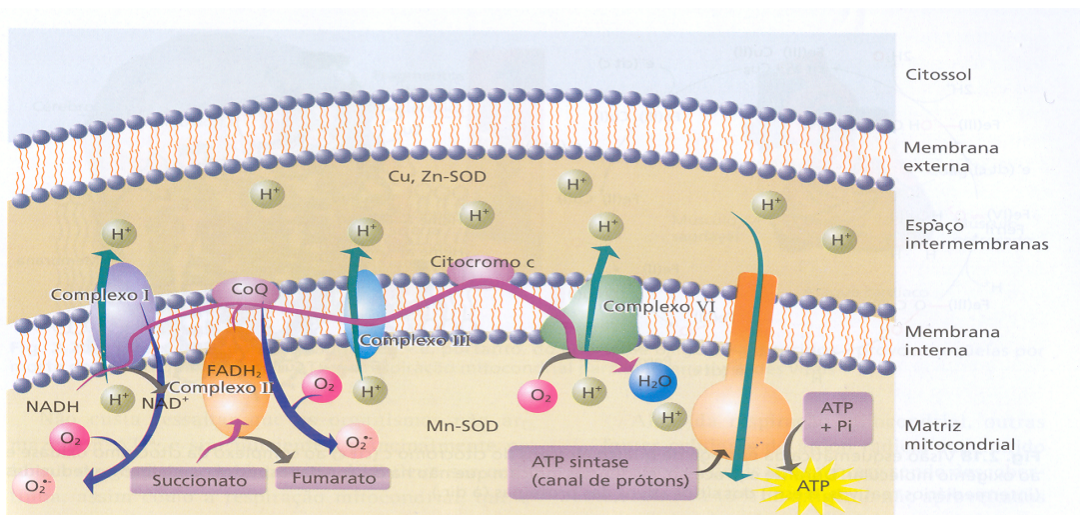
Figura 2 – Metabolização do oxigênio na mitocôndria (OHARA, 2006).

Cerca de 4% do oxigênio utilizado na cadeia respiratória sofre redução parcial, gerando o ânion superóxido ($O_2^{\bullet-}$), peróxido de hidrogênio (H_2O_2), "oxigênio

singlet" ($^1O_2$) e radical hidroxil (OH$^•$). Todas essas espécies são denominadas espécies reativas de oxigênio (EROs), por possuírem a capacidade de existir de forma independente (FRIDOVICH, 1995). Assim sendo, estas espécies são produzidas no organismo devido ao seu metabolismo normal.

Existe então o paradoxo do oxigênio, o qual é vital enquanto indispensável à vida e tóxico quando os seres vivos, dentre eles os peixes, são expostos a concentrações maiores às naturais. Quanto isso ocorre, surgem danos que podem ser reversíveis ou irreversíveis, chegando até à morte celular (BOVERIS e CHANCE, 1973; BRAUN et al., 2006; PAVANATO e LLESUY, 2008).

1.3.1 Estresse oxidativo e suas consequências

Em determinadas situações nas quais existe um aumento sustentado na concentração das espécies reativas de oxigênio pode ocorrer um desequilíbrio entre esta gênese e a capacidade do sistema de defesa antioxidante, o que leva ao estresse oxidativo (EO). Esta situação pode levar ao dano tecidual (PAVANATO e LLESUY, 2008).

Existem indicadores da presença do estresse oxidativo, como a lipoperoxidação, o dano ao DNA e a inativação enzimática. Da mesma forma são exemplos de mecanismos tóxicos mediados por EROs devido à agentes contaminantes do meio ambiente. Também estão envolvidos em processos patológicos e na etiologia de muitas doenças de peixes (MARTINEZ-ALVAREZ et al., 2005).

As pressões parciais de oxigênio na água podem diminuir abaixo da normalidade (hipóxia), permanecer em níveis normais (normóxia) ou ainda aumentar anormalmente (hiperóxia). Em meios de água doce, como na Bacia Amazônica, podem ocorrer momentos de hipóxia e até anóxia. Alguns autores demonstraram uma redução nos níveis de EROs em peixes nesta situação (LUSHCHAK e BAGNYUKOVA, 2006b), entretanto existem trabalhos que relatam um aumento das

EROs e presença de estresse oxidativo em hipóxia (LUSHCHAK et al., 2005a; LUSHCHAK et al., 2005b; BRAUN et al., 2008).

Existem diversos fatores que promovem uma variação na quantidade de OD. Quanto maior for a quantidade de matéria orgânica dissolvida (DOM) maior será a presença de microorganismos consumindo o oxigênio, isso leva a uma diminuição no OD. Da mesma forma, quanto maior for à quantidade de animais presentes no meio, maior será a diminuição do OD. Além disso, o aumento da temperatura também aumenta a perda do oxigênio da água devido ao aumento no metabolismo dos organismos aquáticos e da utilização do O_2. Também a taxa de fotossíntese aumenta durante o dia e diminui durante a noite, promovendo alterações nos níveis de OD (BALDISSEROTTO et al., 2009).

1.4   Sistema de defesa antioxidante

Os antioxidantes são definidos como qualquer substância que, quando está presente em baixas concentrações – comparadas com aquelas de um substrato oxidável – retarda significativamente ou impede a oxidação daquele substrato (o termo substrato oxidável inclui macromoléculas, tais como proteínas, lipídios, hidratos de carbono e DNA). Os antioxidantes podem atuar em distintos níveis como a prevenção da formação e interceptação das EAO ou de seus precursores, reparação das macromoléculas danificadas e regulação da produção de defesas antioxidantes endógenas. A ação protetora ou o mecanismo de ação dos antioxidantes depende das particularidades de geração da espécie ativa (HALLIWELL e GUTTERIDGE, 1989).

Os peixes, como organismos aeróbicos, também produzem EROs em seu metabolismo e por isso realizam processos envolvendo o sistema de defesa antioxidante com o intuito de manter as espécies reativas de oxigênio em equilíbrio. Além disso, como já referido, os peixes são expostos à diferentes situações envolvendo mudanças nos níveis de OD. Estas situações podem aumentar ou reduzir a produção de agentes pró-oxidantes e consequentemente modificar os

níveis das enzimas antioxidantes (MARTINEZ-ALVAREZ et al., 2005; SAMPAIO et al., 2008).

1.4.1   Sistema de defesa enzimático

O sistema de antioxidante enzimático é composto por diversas enzimas como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR) e glutationa-S-transferase (GST) (FIGURA 3).

A SOD participa da reação de dismutação do ânion superóxido ($O_2^{\bullet-}$) a H_2O_2. Existem três grandes grupos, segundo o metal que se encontra no sítio ativo: SOD que contém cobre (Cu^{2+}) e zinco (Zn^{2+}), localizada no citosol (Cu/Zn SOD); SOD que contém manganês (Mn^{2+}), localizada na mitocôndria (Mn SOD) e SOD que contém ferro (Fe^{2+}), encontrada nos procariontes (Fe SOD). A maior atividade da SOD se deve á isoforma que contém Cu^{2+} e Zn^{2+} (FRIDOVICH, 1974).

A CAT degrada o peróxido de hidrogênio (H_2O_2) em oxigênio e água. Ela está localizada nos peroxissomas, eritrócitos e em menor quantidade, no plasma (BOVERIS e CHANCE, 1973).

A GPx produz a decomposição de peróxido inorgânico e orgânicos (FLOHE et al., 1973). Ela utiliza glutationa reduzida (GSH) como co-substrato e produz glutationa oxidada (GSSG). A GSSG, por sua vez é reciclada para GSH pela GR, utilizando NADPH como cofator.

As enzimas antioxidantes atuam em conjunto (Figura 3), sendo que a atividade de uma pode influenciar na atividade da outra. Além disso, o nível de atividade destas enzimas pode ajudar a inferir sobre uma presença ou ausência de estresse oxidativo em peixes (STAICU et al., 2005).

A GST é uma enzima importante na detoxificação de xenobióticos e EROs. Ela é expressa em peixes, sendo utilizada para analisar a presença de estresse oxidativo em organismos aquáticos (MARTINEZ-ALVAREZ et al., 2005; OLIVEIRA et al., 2005; STAICU et al., 2005).
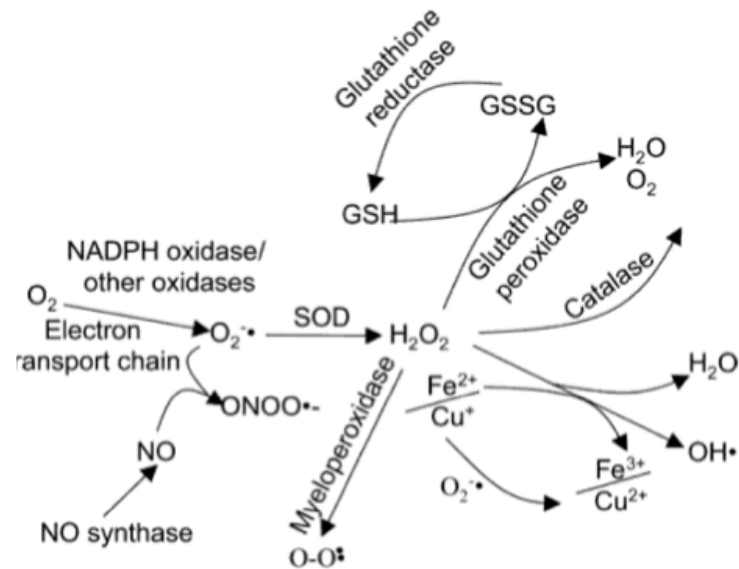
Figura 3 – Espécies reativas de oxigênio, sua produção e remoção nas células por diversas rotas (VINCENT et al., 2004).

Através da atividade da glutationa-s-transferase, componentes reativos bastante eletrofílicos podem ser removidos e conjugados com a glutationa antes de se ligarem covalentemente aos compostos teciduais nucleofílicos, ligação que produziria efeitos tóxicos (STAICU et al., 2005).

1.4.2 Antioxidantes não enzimáticos

Os antioxidantes não enzimáticos são moléculas que podem bloquear a produção ou neutralizar diretamente os radicais livres. De fundamental importância se destacam a glutationa (GSH), a vitamina E e o ácido ascórbico.

A glutationa é um tripeptídeo com resíduos de ácido γ-glutâmico, cisteína e glicina que atua como co-substrato da glutationa peroxidase na redução de peróxidos orgânicos. Além disso, ela também desempenha funções no metabolismo do ácido ascórbico, preservando e reduzindo grupos –SH de moléculas endógenas,

impedindo a toxicidade de metais (como substrato da GST) e protegendo o organismo do dano oxidativo (GIBSON et al., 1993).

A vitamina C (ácido ascórbico) protege o organismo contra o dano oxidativo devido suas propriedades redox e atua também em conjunto com a vitamina E (HILL et al., 2009).

A vitamina E (tocoferóis) é responsável pelo bloqueio direto de espécies reativas de oxigênio e também atua em outras rotas, como a inibição do NF-κB, o qual participa da resposta inflamatória. A vitamina E se localiza nas regiões hidrofóbicas da membrana celular (MEDLING et al., 2009).

Existem vários outros antioxidantes não enzimáticos, como alguns quelantes (que seqüestram metais impedindo reações de oxi-redução envolvidas na produção de radicais livres), os carotenóides (como o beta-caroteno), o selênio e compostos derivados (o qual é essencial para atividade de algumas enzimas antioxidantes como a tiorredoxina redutase e uma das isoformas da glutationa peroxidase).

1.5 Manganês

1.5.1 Características gerais

O manganês (Mn^{2+}) é um dos mais abundantes elementos na natureza. Em solução aquosa o estado mais estável de Mn^{2+} e as espécies com maior numero de oxidação como Mn(III), Mn(IV) e Mn(VII) também existem (HALLIWELL e GUTTERIDGE, 2007). O Mn^{2+} é encontrado em todos os tecidos, sendo essencial para o metabolismo de amino ácidos, lipídios, proteínas e carboidratos (ERIKSON et al., 2004), podendo interagir com o $O_2^{\bullet-}$ (BIELSKI e CABELLI, 1995). O Mn^{2+} é empregado na indústria (GERBER et al., 2002), em pesticidas (BELPOGGI et al., 2002), em vidro, cerâmicas e em baterias (SRIVASTAVA et al., 1991; MERGLER et al., 1994; BADER et al., 1999; ZHANG et al., 2008).

1.5.2 Presença de manganês na bacia amazônica

Iniciativas diversas para a exploração de petróleo em regiões como a Amazônia têm aumentado as chances de contaminação em ambientes aquáticos incluindo metais como o manganês. A extração de petróleo gera um volume significativo de água de captação, também chamada de água de formação. Apesar de receber tratamento, em um eventual acidente o risco de contaminação é presente. Estima-se que para cada barril de óleo cru sejam produzidos até nove barris de água de captação. Sua composição inclui, em geral, alto conteúdo de sulfatos, bicarbonatos, fenóis, cloretos, sódio, cálcio, magnésio, mercúrio, cádmio, cromo, chumbo, cianeto, arsênico e manganês (MATSUO et al., 2005; BALDISSEROTTO et al., 2011).

A concentração de Mn^{2+} na Bacia Amazônica varia de 0,06 à 0,09 mg/L (RODRIGUES, 2003; AZEVEDO, 2006), já a concentração do metal na água de captação da Província Petrolífera de Urucu é de 6,44 mg/L, segundo ROSSONI, (2005). Estes dados indicam que a atividade petrolífera pode liberar através da água de captação uma quantidade significativa de manganês na Bacia Amazônica, mesmo que diluída na água.

1.5.3 Toxicidade do manganês

Existem poucos trabalhos com exposição em Mn^{2+} em peixes. Já em humanos a intoxicação por manganês é bem estudada. A intoxicação do metal pode levar à distúrbios neuropsiquiátricos como disfunções extrapiramidais (hipocinesia, rigidez e tremores) lembrando a doença de Parkinson (ROBISON et al., 2012). O nível de manganês no plasma, em humanos, deve estar entre 8-25 µg/dl. Alguns estudos sugerem que a toxicidade do manganês é resultado de uma interferência na fosforilação oxidativa por ligação à F_OF_1 ATPase. Outra hipótese é um possível deslocamento do Fe^{2+} dos citocrômos da cadeia respiratória produzindo disfunção mitocondrial (ZHENG et al., 1999). A capacidade do manganês em passar da valência dupla Mn^{2+} para a tripla Mn^{3+} aumenta a capacidade pró-oxidante, podendo levar ao estresse oxidativo, sendo então o Mn^{3+}, mais danoso (ONO et al., 2002; ZHANG et al., 2008). Segundo alguns estudos, a presença de Mn^{2+} gera uma elevação do Fe^{2+} no organismo, levando ao aumento da peroxidação lipídica

(ZHENG et al., 1999; ZHENG e ZHAO, 2001). Outros estudos mostram inibição da atividade da aconitase como causa de morte celular (ZHENG et al., 1998; CROOKS et al., 2007). A ação pró-oxidante direta do Mn^{2+} também é considerada como mecanismo de dano tecidual (CHTOUROU et al., 2010).

STAICU et al., (2005), verificaram que o douradinho (*Carassius auratus*) exposto ao manganês (0,5 mg/L) por até sete dias, apresentou uma alteração nas enzimas CAT, GPx, GR e GST, bem como um aumento da lactato desidrogenase (LDH), em especial no segundo dia de exposição. Da mesma forma, a análise histológica mostrou danos celulares no rim e fígado, sendo que o último foi o órgão mais atingido. A exposição ao manganês também está relacionada a hiperglicemia e diminuição de glicogênio hepático e alterações hematológicas em peixes (NATH e KUMAR, 1987; PARTRIDGE e LYMBERY, 2009; DOLCI et al., 2013).

2 Objetivos

2.1 Geral

Analisar o perfil redox e a bioacumulação em tambaqui exposto a diferentes níveis de oxigênio dissolvido em presença e ausência de manganês.

2.2 Específicos

- Verificar a concentração letal média em 96h para o manganês em tambaqui exposto a hiperóxia, normóxia e hipóxia;

- Verificar o efeito da exposição aguda ao manganês sobre a bioacumulação em brânquias, cérebro, fígado, rim e músculo de tambaqui mantido em normóxia e hipóxia;

- Verificar o efeito da exposição aguda ao manganês sobre o perfil redox de tambaqui expostos ao manganês.

3   Desenvolvimento

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo científico e manuscritos. Os itens Resumo, Introdução, Materiais e métodos, Resultados, Discussão e Referências encontram-se no próprio artigo e manuscritos. O Artigo está disposto da mesma forma que foi publicado e os manuscritos conforme as normas das respectivas revistas científicas às quais foram submetidos.

3.1 Manuscrito 1 – Bulletin of environmental contamination and toxicology.

Manganese Accumulation in Tissues of *Colossoma macropomum C.,* under Different Dissolved Oxygen Levels

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ABSTRACT. This study evaluated the lethal concentration (LC_{50}) and the bioaccumulation of manganese (Mn) in tambaqui (*Colossoma macropomum*) exposed to normoxia (6 mg L^{-1}), hypoxia (0.25 mg L^{-1}) or hyperoxia (10 mg L^{-1}) for 96 hours. Mortality was observed only in tambaqui subjected to hypoxia, and the $LC_{50-96 h}$ was 4.03 mg Mn L^{-1}. Mn accumulation occurred in the following order: gills > liver > muscle. Hypoxia and hyperoxia altered Mn bioaccumulation in the examined tissues.

Manganese (Mn) is a metal required for biological processes in all organisms, including fish, which obtain it from the water or diet (Watanabe et al. 1997). Nonetheless, an excess of Mn is toxic to fish, triggering the production of free radicals and the consequent development of oxidative stress. Mn can also cause hyperglycemia and decrease the levels of liver glycogen in fish (Nath and Kumar 1987). There are reports on Mn bioaccumulation in fish (Adam et al. 1997; Burger et al. 2002), but none relates Mn toxicity or bioaccumulation to dissolved oxygen levels. A significant amount of Mn is released into the water due to oil exploration in the Amazon basin. The water of such basin is very soft, thus providing a high bioavailability of the metal and increasing its toxicity (Baldisserotto et al. 2011). Another important feature is the seasonal variation in dissolved oxygen levels in the Amazon region, which affects the physiology of the aquatic organisms (Val 1996).

Tambaqui (*Colossoma macropomum*) inhabits floodplain lakes in the Amazon basin and is continuously exposed to variations in dissolved oxygen level. The species has great longevity, is tolerant of hypoxia and pH changes and is very important to the local riverside economy (Affonso et al. 2002; Gomes et al. 2006). These characteristics make of tambaqui a good model for the study of metal bioaccumulation at different oxygen dissolved levels. Therefore, the purpose of this study was to evaluate the lethal concentration (LC_{50}) and the bioaccumulation of Mn in tambaqui exposed to different dissolved oxygen levels.

Materials and Methods. Juvenile tambaqui (7 – 10 g) were obtained from a local fish farm (Fazenda Santo Antônio, Rio Preto da Eva, Amazonas, Brazil) and kept in aerated well water in the Instituto Nacional de Pesquisas da Amazônia. Composition (in µmol) and characteristics of the water were as follows: Ca^{2+} = 11; Na^+ = 34; Cl^- = 28; Mg^{2+} = 0.8; K^+ = 15; pH 6.3; dissolved organic matter = 0.9 mg C; Cu= 1.7 µg L^{-1}; Cd = 0.3 µg L^{-1}; and temperature = 28 ^{o}C. The fish were acclimated for at least three weeks prior to experimentation and were fed commercial dry food pellets once a day (Nutripisces).

Feeding was suspended 48 h before the tests. The tambaqui were acutely exposed to 0.13, 3.45, 8.48, 15.39 and 18.12 mg Mn^{2+} (measured values) under normoxic (6 mg L^{-1}), hypoxic (0.25 mg L^{-1}) or hyperoxic (10 mg L^{-1}) conditions for 96 h (10 L aquaria; 10 fish per aquarium). Dissolved oxygen levels were adjusted by bubbling the water with N_2 or O_2. The experiments were performed in triplicate.

After the experimental period, the fish were sacrificed by section of the spinal cord. Gills, liver and muscle were removed, weighed and immediately frozen in liquid nitrogen. The same procedure was performed with the fish that died during the experiment. The tissues were stored at -20 $^{\circ}$C and later digested with nitric acid (HNO$_3$, 1 N, Merck) for 48 h at 60 $^{\circ}$C. The Mn concentrations in the digested tissues and water samples were analyzed using graphite furnace atomic absorption spectrophotometry (ICP-MS, Elan DRCII Perkin Elmer SCIEX – Canada). The certified standards provided by the manufacturer were used throughout this study.

Estimates of the LC$_{50}$ and the associated 95% confidence limits were determined by the Trimmed Spearman–Karber method using the measured concentrations of Mn (Hamilton et al. 1977). Fish mortality was recorded every 12 h, and the LC$_{50}$ values were determined after 96 h.

Data are reported as the means ± SEM. Homogeneity of variances was assessed by a Levene test, and homogeneous variances were obtained after log transformation. Comparisons between the different groups were performed using a two-way ANOVA and Tukey's test. The minimal significance level was $P<0.05$. All of the analyses were made with the Statistica software 7.0.

Results and Discussion. Mortality was observed only in the tambaqui exposed to hypoxia and was proportional to the waterborne Mn level (up to 86.67% in the fish exposed to 18.12 mg Mn L^{-1}). It can be expressed as $y = 37.82 + 2.506x$, where y = mortality (%) and x = mg Mn L^{-1}. The lethal concentration at 96 h was 4.03 mg Mn L^{-1} (confidence interval: 3.38 – 6.52 mg Mn L^{-1}).

There are a few reports describing the LC$_{50}$ of Mn in fish. A study observed a LC$_{50\text{-}96\,h}$ of 1176.5 mg Mn L^{-1} in *Colisa fasciatus* (165.33 mg CaCO$_3$ L^{-1} water hardness) (Nath and Kumar 1987). Mn LC$_{50\text{-}96\,h}$ in tambaqui was much lower probably because the water used in the present study was very soft (1.12 mg CaCO$_3$ L^{-1} hardness). This situation could have increased Mn toxicity in tambaqui, since Mn availability is higher in waters with low hardness (Baldisserotto et al. 2011).

The increase in waterborne Mn concentration corresponded to a progressive increase in Mn levels in gills, liver and muscle, irrespective of the dissolved oxygen level (**figure 1**). The maximum Mn accumulation in gills and liver of the tambaqui exposed to normoxia was approximately 3.5 and 8.5 mg Mn L^{-1}, respectively, whereas the maximum accumulation in liver and muscle of the fish subjected to hyperoxia was about 8.5 mg Mn L^{-1}. Apparently, a maximum Mn accumulation in the tambaqui exposed to hypoxia was not reached at the highest waterborne Mn level tested. In the tambaqui

subjected to hypoxia or normoxia, bioaccumulation was similar in gills and liver but lower in muscle. Conversely, under hyperoxic conditions, Mn accumulation occurred in the following order: gills > liver > muscle. Liver and muscle showed higher Mn bioaccumulation in the fish exposed to hypoxia than in those exposed to normoxia or hyperoxia, while higher Mn levels were observed in gills of the fish maintained in hypoxia or hyperoxia.

Exposure to hypoxia increased the ventilatory frequency and volume and, consequently, the water flow through the gills of tambaqui (Val 1996). Furthermore, hypoxia changed ion fluxes in silver catfish *Rhamdia quelen* (Rosso et al. 2006), and, as Mn uptake in the gills is related to ion transporters, any change in the gill transporters as a result of hypoxia could also alter Mn bioaccumulation. Hypoxia reduced the expression of some antioxidant enzymes, thus increasing the toxicity of pro-oxidant agents, which would most likely be a source of the damage observed at this level of dissolved oxygen (Pierron et al. 2007). Hypoxia was reported to have no influence on the toxicokinetics of cadmium in the common carp *Cyprinus carpio*, and the increased toxicity of this metal under this condition is related to other mechanisms, such as the internal anoxia due to gill damage (Hattink et al. 2005). Thus, the observed mortality of the tambaqui exposed to hypoxia and Mn may have a multifactorial explanation, as suggested by the studies abovementioned.

In the present investigation, a higher Mn bioaccumulation was observed in the gills and liver. Similar results were obtained for the African catfish *Clarias gariepinus* (Crafford and Avenant-Oldewage 2011). According to these authors, the liver is highly active in the uptake and storage of pollutants, whereas the large surface area of the gills and the large volume of water that passes over them further facilitates metal uptake. Two long-term exposure studies demonstrated that Mn was found in the bony and cartilaginous tissues, and that the gills, which are also composed of cartilage, may be a long-term fixation site for Mn (Adam et al. 1997; Baudin et al. 2000).

In conclusion, the present study showed that Mn bioaccumulation in tambaqui is proportional to its waterborne levels and that hypoxia increases its bioaccumulation. Moreover, Mn bioaccumulation in the tissues occurred in the following order: gills > liver > muscle.
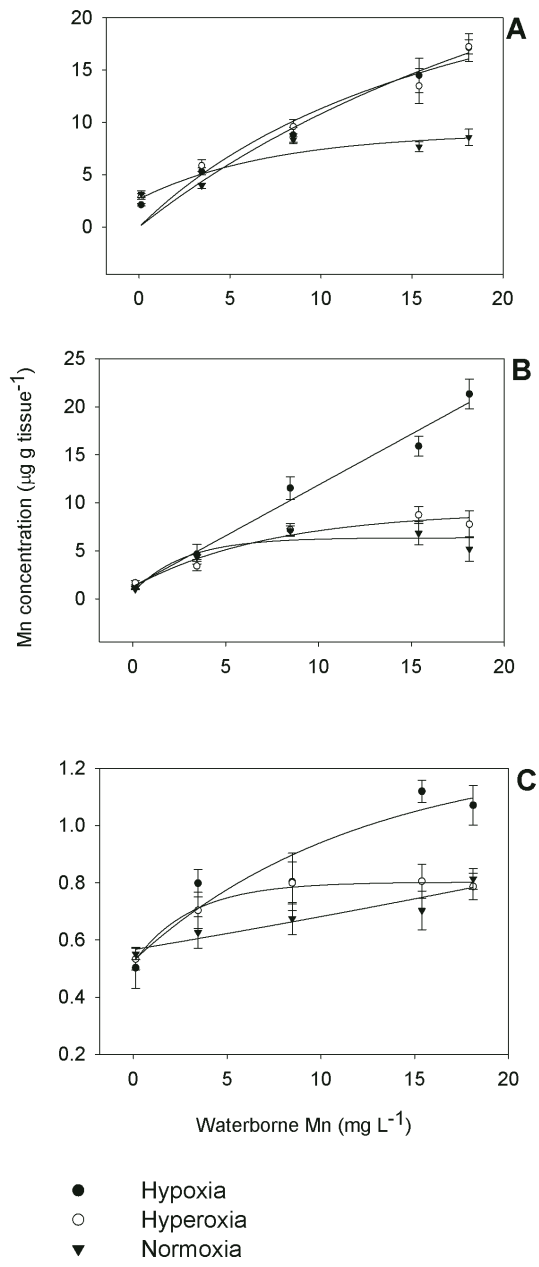
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FIGURE LEGEND

Fig. 1 Manganese levels in gills (A), liver (B) and muscle (C) of *Colossoma macropomum* exposed to different waterborne Mn and dissolved oxygen levels for 96 h. (A) normoxia: $y = 2.67 + 6.37(1 - e^{-0.14x})$ hypoxia: $y = \frac{1.38x}{(1+0.03x)}$ hyperoxia: $y = \frac{1.71x}{(1+0.05x)}$. (B) normoxia: $y = \frac{6.35 - 5.68}{(1+2.14^{-0060x})^{\frac{1}{5.83^{-0060}}}}$, hypoxia: $y = 1.27 + 1.06x$, hyperoxia: $y = \frac{9.15 - 7.92}{(1+6.96^{-0060x})^{\frac{1}{5.18^{-0060}}}}$. (C) normoxia: $y = 0.56 + 0.01x + (5.70^{-0.05}x)^2$, hypoxia: $y = 0.52 + 0.28(1 - e^{-0.08x})$, hyperoxia: $y = 0.52 + 0.28(1 - e^{-0.32x})$. Where y = Mn levels (µg.g tissue^{-1}) and x = waterborne Mn (mg.L^{-1}).

Figure 1.

Waterborne Mn (mg L^{-1})

Mn concentration (μg g tissue^{-1})

● Hypoxia
○ Hyperoxia
▼ Normoxia

3.2 Artigo 1 – Archives of environmental contamination and toxicology

Effects of Subchronic Manganese Chloride Exposure on Tambaqui (*Colossoma macropomum*) Tissues: Oxidative Stress and Antioxidant Defenses

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Abstract This study aimed to evaluate oxidative stress parameters in juvenile tambaqui (*Colossoma macropomum*) exposed to 3.88 mg l^{-1} Mn^{2+} for 96 hours. Biomarkers of oxidative stress, such as thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) activities, as well as content of reduced glutathione (GSH), were analyzed in gill, liver, brain, and kidney. The presence of Mn^{2+} in the water corresponded to increased levels of Mn^{2+} accumulation according to the following sequence: gill > kidney > brain > liver. There was a significant increase in TBARS levels (40 %) and SOD activity (80 %) in addition to a significant decrease in GSH content (41 %) in gills of fish exposed to waterborne Mn^{2+}. In hepatic tissue of the exposed animals, TBARS levels decreased significantly (35 %), whereas SOD (82 %) and GST activities (51 %) as well as GSH content (43 %) increased significantly. In brain of exposed juvenile fish, only significant decreases in SOD (32 %) and CAT activities (65 %) were observed. Moreover, the kidney of exposed fish showed a significant increase in TBARS levels (53 %) and a significant decrease in SOD activity (41 %) compared with the control. Thus, the changes in biomarkers of oxidative stress were different in the tissues, showing a specific toxicity of this metal to each organ.

Manganese (Mn^{2+}), an essential trace metal, is found in all tissues of bacteria, plants, humans, and fish because it is required for normal amino acid, lipid, protein, and carbohydrate metabolism in vivo (Erikson et al. 2004). This metal is one of the most abundant elements and is widely used in industry (Gerber et al. 2002), pesticide formulations (Belpoggi et al. 2002), glass and ceramic production, and manufacture of dry cell (Srivastava et al. 1991; Mergler et al. 1994; Bader et al. 1999). It is also present at very high concentrations in formation water (produced water or oil field brine) from oil and gas extraction (Baldisserotto et al. 2012). Whereas Mn^{2+} deficiency is extremely rare, toxicity due to Mn^{2+} overexposure is more prevalent (Crossgrove and Zheng 2004). Mn^{2+} undergoes oxireduction reactions and may have negative physiological effects owing to oxidative stress induction (Huang et al. 2011).

Oxidative stress occurs due to either the overproduction of reactive oxygen species (ROS) or a decrease in cellular antioxidant levels. As a metal ion, Mn^{2+} is toxic because it enhances ROS formation and catecholamine oxidation by products (Prabhakaran et al. 2008; Falfushynska et al. 2011). ROS generated in tissues and subcellular compartments are efficiently scavenged by the antioxidant defense system, which is composed of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and nonenzymatic antioxidants, such as

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reduced glutathione (GSH). These antioxidant defenses can protect cells from lipid peroxidation (LPO), protein oxidation, and DNA damage (Halliwell and Gutteridge 1999).

There are several studies on exposure to Mn^{2+} and other metals in different aquatic species. In general, these studies aimed to analyze mortality and metal bioaccumulation in tissues (Nath and Kumar 1987; Seymore et al. 2006; Crafford and Avenant-Oldewage 2011). Only a few investigations have evaluated possible oxidative damage involved in aquatic animals exposed to Mn^{2+} (Jena et al. 1998; Falfushynska et al. 2011).

Tambaqui (*C. macropomum*) is an abundant species in the Amazon basin and is very important to the local economy (Affonso et al. 2002). This species has great longevity and high tolerance to changes in dissolved oxygen levels and pH (Marcon and Wilhelm 1999; Milsom et al. 2002; Florindo et al. 2004). Such characteristics make of tambaqui a good model for the study of metals.

Experiments with metals and native fish have become essential to assess the risk of environmental contamination. Thus, the purpose of this study was to evaluate oxidative stress generated in several organs of tambaqui exposed to high waterborne Mn^{2+} levels for 96 hours.

Materials and Methods

Chemicals

All reagent-grade chemicals were purchased from Sigma (St. Louis, MO).

Fish

Juvenile tambaqui (100–300 g) were obtained from Fazenda Santo Antônio in Rio Preto da Eva, Amazonas, Brazil. Fish were transported to the Laboratory of Ecophysiology and Molecular Evolution, National Institute of Amazon, and maintained in aerated well water for at least 21 days and were fed commercial dry food pellets once a day. Water parameters were as follows: temperature 28 °C, pH 6.3, Ca^{2+} 11 μmol l^{-1}, Na^+ 34 μmol l^{-1}, Cl^- 28 μmol l^{-1}, Mg^{2+} 0.8 μmol l^{-1}, K^+ 15 μmol l^{-1}, dissolved organic matter 0.9 mg C l^{-1}, background Cu^{2+} 1.7 μg l^{-1}, and background Cd^{2+} 0.3 μg l^{-1}. The experimental protocol was approved by the Animal Health Committee of Federal University of Santa Maria, Rio Grande do Sul, Brazil.

Exposure to Mn

Stock solutions were prepared by dissolving manganese chloride ($MnCl_2$) in water and added it to the experimental aquarium after the acclimation period. Juvenile fish were randomly separated into two 72-l aquaria ($n = 10$ each aquarium), one exposed to 3.88 ± 0.239 mg l^{-1} Mn^{+2} (measured level) and another nonexposed to the metal (control) for 96 hours. The water was not renewed during the experimental period. Then the fish were placed in containers filled with water and ice for 5 minutes for anaesthetization, after which blood was sampled from the caudal vein with heparinized syringes; the fish were killed by section of the spinal cord. The gills, liver, brain, and kidneys were removed and immediately frozen in liquid nitrogen. The tissues were stored at −70 °C for measurement of oxidative stress parameters or at −20 °C for posterior digestion with concentrated nitric acid (HNO_3, 1 N; Merck). Mn concentrations in digested tissues and water samples were analyzed using graphite furnace–atomic absorption spectrophotometry (inductively coupled plasma–mass spectrometry (Elan DRCII Perkin Elmer SCIEX–Canada). Certified standards provided by the manufacturer were used throughout this study. Mn activity was calculated using the speciation program Visual MINTEQ version 3.0 (Gustafsson 2012).

Water Parameters

Water samples were collected from each aquarium to determine water-quality parameters at the beginning and at the end of the experiment. Water alkalinity (10.83 ± 0.48 mg l^{-1} $CaCO_3$) was determined by the sulfuric acid method (Eaton et al. 2005). Measurements of dissolved oxygen (YSI model Y5512 oxygen meter) and water pH (7.1 ± 0.04) (Quimix 400A pH meter) were performed daily. Water hardness (13.22 ± 0.66 mg l^{-1} $CaCO_3$) was determined by the ethylene diamine tetraacetic acid titrimetric method, and total ammonia ($NH_3 + NH_4^+$, final value 1.23 ± 0.05 mg l^{-1}) was determined by the direct nesslerization method (Eaton et al. 2005).

Oxidative Stress Parameters

The tissues were homogenized as described previously by Azambuja et al. (2011). The homogenates were centrifuged at $1000 \times g$ for 10 minutes at 4 °C to discard nuclei and cell debris, and the supernatant fraction obtained was frozen at −70 °C for analyses of oxidative stress parameters.

Lipid peroxidation (LPO) was measured by TBARS assay (Buege and Aust 1978). Results were expressed as nmol mg protein^{-1}. Commercially available malonaldehyde was used as a standard. Protein content was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Total SOD activity was based on the inhibition rate of autocatalytic adenocrome generation at 480 nm in a reaction medium containing epinephrine and glycine/NaOH

(pH 10.2). The enzyme activity was expressed as USOD mg protein^{-1}. One SOD unit was defined as the amount of enzyme needed for 50 % inhibition of adenochrome formation as described by Misra and Fridovich (1972). CAT activity was evaluated according to the decrease in the 240 nm absorption in a reaction medium consisting of phosphate buffer (pH 7.4) and hydrogen peroxide (H_2O_2), thereby determining the pseudo–first order reaction constant ($k^.$) of the decrease in H_2O_2 absorption. This was reported as nmol mg protein^{-1} (Boveris and Chance 1973). GST activity, expressed as µmol min^{-1} mg protein^{-1}, was determined according to Habig et al. (1974). The assay was performed using potassium phosphate buffer (pH 6.5) with reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene. Activity was calculated from the changes in absorbance at 340 nm (ε_{340} nm $= 9.6$ mM^{-1} cm^{-1}). One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of CDNB with GSH/min at 25 °C. Tissue sulfhydryl groups, an indirect measure of GSH, were evaluated at 412 nm after reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). Proteins were eliminated through the addition of perchloric acid. The final product formed is the yellow 2-nitro-5-mercapto-benzoic acid. The results were reported as nmol protein^{-1} using ε_{412} nm $= 13.6$ mM^{-1} cm^{-1} (Ellman 1959).

Statistical Analysis

The results are expressed as the means $\pm$ SEs. Levene's test was performed to evaluate the homogeneity of variances. Unpaired Student t test was used for comparison of means. All analyses were executed by using GraphPad Instat software (San Diego, CA). Differences were considered significant at $p < 0.05$.

Results

The presence of Mn^{2+} in the water corresponded to increased levels of Mn^{2+} in the gill, brain, and kidney. The percentage of accumulation in liver was not significant. Mn^{2+} accumulation in the tissues occurred in the following sequence: gill > kidney > brain > liver (Fig. 1). Gills of tambaqui exposed to waterborne Mn^{2+} exhibited a significant increase in thiobarbituric acid reactive substances (TBARS) levels (40 %) in addition to a significant increase in SOD activity (80 %) and a significant decrease in GSH content (40 %). GST activity was unaffected, whereas CAT activity could not be detected (Fig. 2). Hepatic TBARS levels of the fish exposed to waterborne Mn^{2+} was decreased (35 %) compared with the control. This tissue also showed a significant increase in SOD (82 %) and GST

activities (51 %), as well as GSH content (43 %), whereas no change in CAT activity was observed in animals exposed to this metal (Fig. 3). In brain, SOD and CAT activities were significantly decreased (32 and 65 %, respectively) in the group exposed to Mn^{2+} compared with control fish. Nonetheless, GST activity and TBARS levels were unaffected (Fig. 4). Moreover, TBARS levels increased significantly (53 %) in kidney of tambaqui exposed to waterborne Mn^{2+}. SOD activity was significantly decreased (41 %) in renal tissue of these animals, whereas no change in CAT activity was observed (Fig. 5).

Discussion

Because fish constitute an important link in the food chain, their contamination by toxic metals causes a direct threat not only to the entire aquatic environment but also to humans (Obasohan 2008). Toxicity of Mn^{2+} in fish, despite its highly variable levels in water (Linnik 2000) and dependence on complexation (Liccione and Maines 1988), has scarcely been studied (Falfushynska et al. 2011). In the present study, the calculated Mn speciation by Visual Minteq 3.0 showed that 97 % of total Mn existed mainly as the free ionic species, Mn^{2+}.

The maximum allowed concentration of Mn^{2+} in Brazilian waters is 0.1 mg L^{-1} (Conselho Nacional do Meio Ambiente-CONAMA 2005), whereas this metal is present at 6.44 mg L^{-1} in the formation water from Urucu Reserve, Amazon (Baldisserotto et al. 2012). Therefore, the concentration used in the study is in between the maximum acceptable concentration and the concentration present in oilfield process water. Manganese is not distributed homogeneously throughout the organs in tambaqui. Most of the assessments on Mn^{2+} bioaccumulation are in accord with the measurement of trace metals in
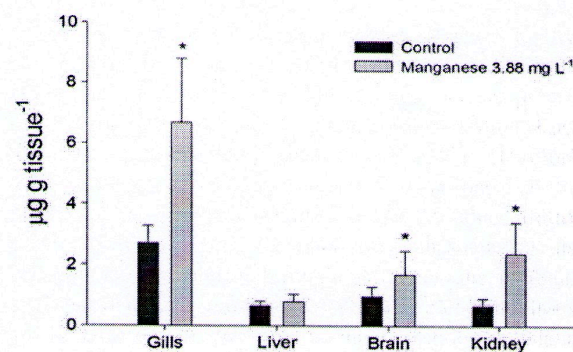
Fig. 1 Mn^{2+} levels in gill, liver, brain, and kidney of *C. macropomum* exposed to 3.88 mg L^{-1} waterborne Mn for 96 hours. *Significantly different from control by unpaired Student t test ($p < 0.05$)

Fig. 2 TBARS levels (**a**), SOD (**b**), GST activities (**c**), and GSH content (**d**) in gill of tambaqui exposed to 3.88 mg l^{-1} Mn^{2+} for 96 hours. Data are reported as means $\pm$ SEs ($n = 10$). *Significantly different from control by unpaired Student t test ($p < 0.05$)
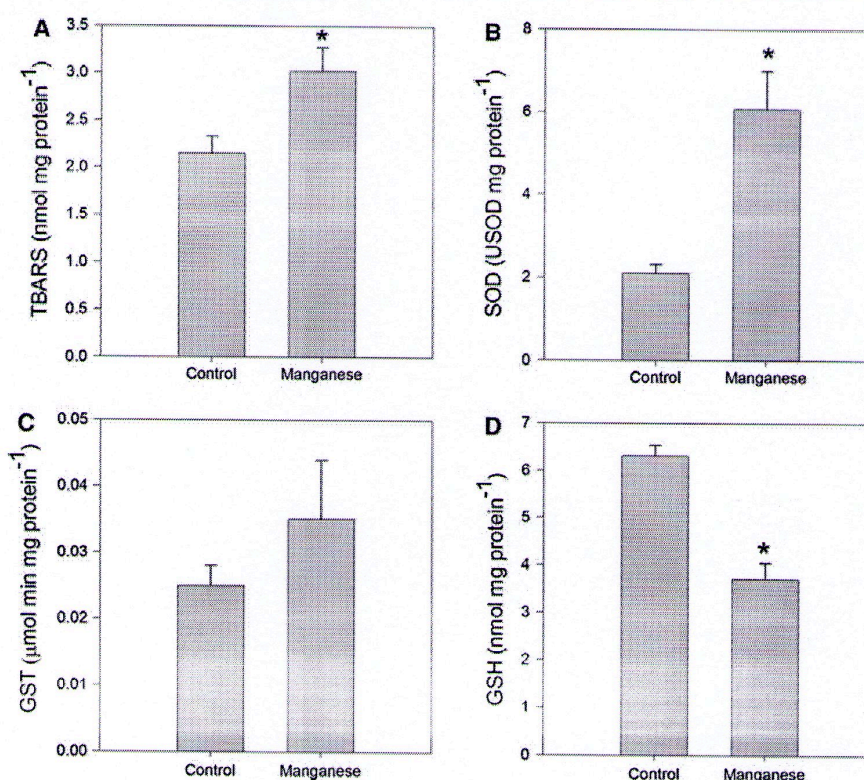
certain habitats rather than in a controlled exposure setting (Bharti and Banerjee, 2011; Alhashemi et al. 2012). Despite the lack of correlation between the data on bioaccumulation and oxidative stress parameters, the greater accumulation of the metal in tambaqui gills is in accordance with a preceding work with *Esox lucius* and *Abramis brama* (Rajkowska and Protasowicki 2012).

Fish gills represent a thin and extensive surface (≤ 90 % of total body surface) in intimate contact with water. They carry out three main functions: gas exchange, ion regulation, and excretion of metabolic waste products. Due to constant contact with the external environment, gills are the first target of waterborne pollutants (Perry and Laurent 1993) and are susceptible to damage caused by heavy metals. Metals induce oxidative stress by the overproduction of ROS; thus, a strong antioxidant defense is essential to neutralize the impact of these species (Ahmad et al. 2000; Kochhann et al. 2009). The increase in SOD activity observed in gills of tambaqui exposed to Mn^{2+} could represent a tissue response to compensate for the increased LPO.

SOD is a key antioxidant enzyme in the metabolism of ROS because it removes superoxide anion (O$_2^{\bullet-}$) and prevents the formation of other ROS, such as hydroxyl radicals (OH$^{\bullet}$) (Enghild et al. 1999). O$_2^{\bullet-}$ is the first species in the cascade of univalent decrease of molecular oxygen and

therefore is the first indicator of increased generation of ROS. Steady-state concentrations of O$_2^{\bullet-}$ are directly proportional to its rate of production and inversely proportional to the activity of scavenging enzymes, such as SOD (Ferreira et al. 2004). If there is an increase in SOD activity, there will be a decrease in O$_2^{\bullet-}$ and an increase in H$_2$O$_2$ production. H$_2$O$_2$ is removed by two enzymes: CAT and glutathione peroxidase (GPx). The latter uses GSH as a cofactor to remove the H$_2$O$_2$. The present study data also showed a decrease in GSH levels. Mn^{2+} toxicity is related to the depletion of GSH in different animal phyla, including aquatic animals (Madejczyk et al. 2009). The depletion of GSH can enhance Mn^{2+} toxicity, albeit to a lesser extent than that registered for Cu^{2+} (Maracine and Segner 1998; Bozocaarmutlu and Arinc Bozcaarmutlu and Arinc 2004).

There is no pattern of antioxidant behavior in gills of fish exposed to metals. Chromium (Cr) exposure (10 mg l^{-1} Cr^{3+} or Cr^{6+}) for 96 hours did not change GSSG and total GSH ratio, GST and glutathione reductase (GR) activities, and LPO levels in gills of *C. auratus*. However, Cr^{6+} treatment resulted in decrease of carbonyl proteins levels, whereas exposure to both concentrations led to a decrease in CAT activity (Kubrak et al. 2010). In turn, gills of *C. auratus gibelio* exposed to 1.7 mg l^{-1} Mn^{2+} for 14 days showed increased SOD activity in

Fig. 3 TBARS levels (a), SOD (b), CAT (c), GST activities (d), and GSH content (e) in liver of tambaqui exposed to 3.88 mg l^{-1} Mn^{2+} for 96 hours. Data are reported as means $\pm$ SEs ($n = 10$). *Significantly different from control by unpaired Student t test ($p < 0.05$)
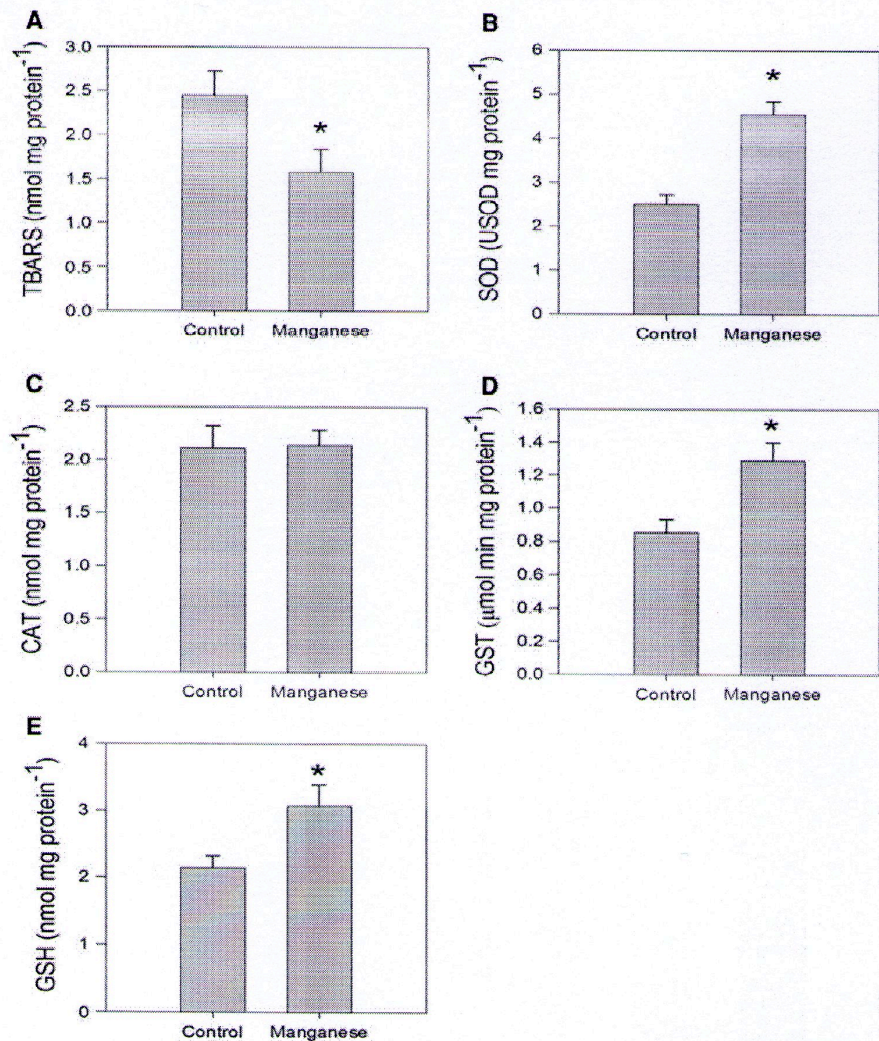
addition to decreases in LPO and GSH levels (Falfushynska et al. 2011). Moreover, exposure of *Channa punctatus* to different cadmium (Cd^{2+}) levels did not modify the amount of LPO and CAT activity in the gills, although Cd^{2+} induced a significant increase in the activity of the other enzymes, such as SOD, GPx, and GST as well as GSH content. Finally, Arabi and Alaeddini (2005) showed that supplementation of 5.5 mg l^{-1} Mn^{2+} reverted the deleterious effects of mercury (Hg^{2+}) and copper (Cu^{2+}) to *Oncorhynchus mykiss* exposed because its application inhibited LPO levels, decreased GST activity, and increased GSH content in the gill samples.

Liver is the main organ of various key metabolic pathways and the most frequently studied tissue regarding oxidative stress. Our data showed that liver LPO levels were decreased in tambaqui exposed to Mn^{2+}. In turn, the activity of antioxidant enzymes, such as SOD and GST,

and the content of nonenzymatic antioxidant GSH presented an opposite pattern, whereas CAT activity was unaffected. The increased formation of GSH in liver of tambaqui exposed to Mn^{2+} suggests a role in the defense of cells against oxidative stress. Furthermore, our study also showed that GST plays an important role in the detoxification of the end products of LPO.

Similar results were also shown in *C. auratus gibelio* exposed to 1.7 mg l^{-1} Mn^{2+} for 14 days. This species showed a decrease in levels of liver LPO associated with an increase in Mn-SOD activity compared with the respective control (Falfushynska et al. 2011). Moreover, Huang et al. (2011) also described a decrease in LPO levels in addition to an increase in GSH content in liver of rats exposed to Mn^{2+}. However, in opposition to our data, Casalino et al. (2004) reported an increase in LPO levels in liver of rats 24 hours after administration of 2.0 mg kg^{-1} Mn^{2+}.

Fig. 4 TBARS levels (**a**), SOD (**b**), CAT (**c**), and GST activities (**d**) in brain of tambaqui exposed to 3.88 mg l^{-1} Mn^{2+} for 96 hours. Data are reported as means $\pm$ SEs ($n = 10$). *Significantly different from control by unpaired Student t test ($p < 0.05$)
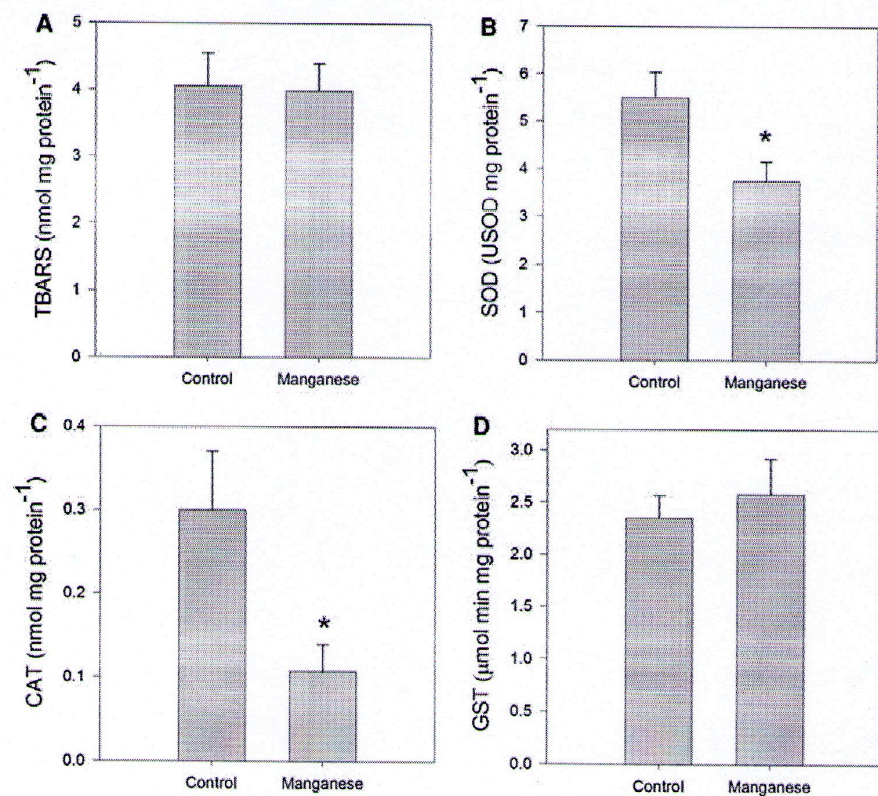
Nevertheless, these investigators found an increase in GST activity in this organ, thus corroborating our findings in tambaqui. Induction of GST activity depends on the type of tissue and nature of the inducer. In another experiment, *C. punctatus* exposed to sublethal concentrations of Cd^{2+} for 24, 48, 72, and 96 hours presented increased levels of liver LPO and modulated activities of SOD, CAT, GPx, GR, and GST as well as GSH content (Dabas et al. 2011). Thus, the current results suggest that the increase in both types of antioxidants (enzymatic and nonenzymatic) in liver of tambaqui exposed to Mn^{2+} is compensating for the decrease in LPO levels.

The brain is very susceptible to oxidative damage by ROS as it contains high amounts of unsaturated lipids and uses approximately 20 % of total the body's oxygen demand (Stella and Lajtha 1987). Our data showed that LPO levels and GST activity remained unchanged, whilst SOD and CAT activities decreased in brain tissue of tambaqui exposed to Mn^{2+} compared with the respective control. This decrease observed in SOD and CAT activities indicates oxidative damage to organs in the presence of Mn^{2+}.

Chtourou et al. (2010) described similar data because they verified a decrease in the antioxidant enzymes in cerebral cortex of rats that received Mn^{2+} in drinking water for 30 days. Mn^{2+} is an important cofactor for a variety of enzymes, including SOD (Hurley and Keen 1987). This metal scavenges $O_2^{\bullet-}$ and $OH^{\bullet}$ even when SOD activity is inhibited (Hussain and Ali 1999). However, the prooxidant effects of Mn^{2+} have been confirmed repeatedly in in vitro and in vivo studies (Ali et al. 1995; Zhang et al. 2004; Jiao et al. 2008). An in vitro analysis showed that 18.31 mg l^{-1} Mn^{2+} significantly inhibited CAT activity in brain of fish and lizards (Jena et al. 1998). Cr exposure (10 mg l^{-1} Cr^{3+} or Cr^{6+}) of *C. auratus* for 96 hours resulted in increased brain content of carbonyl protein and no changes in SOD, CAT, and GST activities in this tissue (Kubrak et al. 2010). The same investigators published another study in 2011, in which they evaluated the effects of various concentrations of cobalt (Co^{2+}) on brain of *C. auratus*. Exposure to 50 mg l^{-1} Co^{2+} for 96 hours did not affect LPO levels and GR activity; however, this induced a decrease in SOD, CAT, and glucose-6-phosphate dehydrogenase activities (Kubrak et al. 2011). These findings are in accordance with our data.

Finally, our results also showed that Mn^{+2} exposure of tambaqui may reflect the development of renal oxidative stress because it led to an increase in LPO levels associated with a decrease in SOD activity, although no change was observed in CAT activity. SOD, along with CAT, represents the first barrier against ROS and is essential to cell
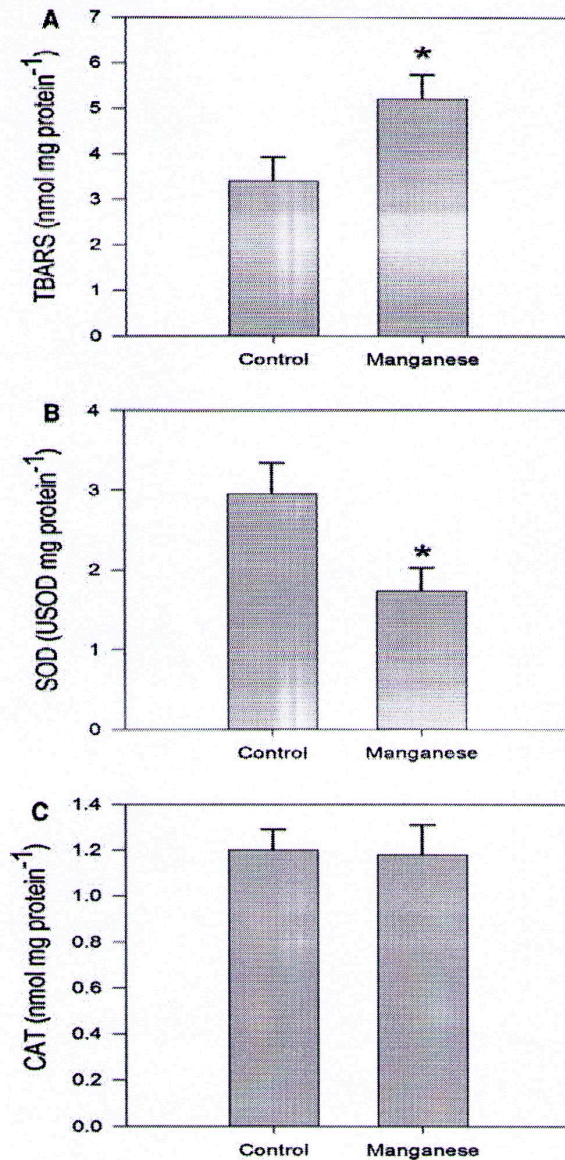
Fig. 5 TBARS levels (**a**), SOD (**b**), and CAT activities (**c**) in kidneys of tambaqui exposed to 3.88 mg.L^{-1} Mn^{2+} for 96 hours. Data are reported as mean ± SE ($n = 10$). *Significantly different from control by unpaired Student t test ($p < 0.05$)

survival (Remacle et al. 1992; Mates et al. 1999; Halliwell 2001). Travacio and Llesuy (1996) reported that different models of oxidative stress involve a biphasic response of antioxidant enzyme activities. At first, enzymatic activities are markedly decreased, but with time the activity levels increase, probably as a consequence of a new synthesis and/or enzymatic activation.

C. punctatus exposed to Cd^{2+} (6.7, 13.4, and 20.1 mg l^{-1}) for various time periods (24, 48, 72, and 96 hours) presented increased levels of LPO as well as SOD, GST, and GR activities, whereas CAT activity was decreased (Dabas et al. 2011). In turn, *C. auratus* exposed to various concentrations of Cr^{6+} for 96 hours showed increased renal hydroperoxide levels and SOD activity and no significant differences in CAT activity (Velma and Tchounwou 2010).

The results of the current research clearly show that there were changes in the balance of pro-oxidants and antioxidants in different organs of tambaqui. Such changes were more evident in liver and kidney. Furthermore, there was no correlation between the oxidative stress results and the bioaccumulation data. Present findings may contribute to the scarce literature regarding fish subchronic exposure to Mn^{2+}.

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3.3   Manuscrito 2 – Ecotoxicology and environmental safety

Redox profile and bioaccumulation in tambaqui (*Colossoma macropomum)* following subchronic manganese exposure under hypoxia

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This study aimed to delineate the redox profile and evaluate the bioaccumulation in juvenile tambaqui (*Colossoma macropomum*) exposed to 3.88 mg L^{-1} Manganese (Mn^{2+}) for 96 h in hypoxia (0.2 mg L^{-1}). In gills there was a rise in thiobarbituric acid reactive substances (TBARS) (37.74%) and a reduction in the activities of superoxide dismutase (SOD) (36.58%) and glutathione-S-transferase (GST) (96.52%). The level of TBARS increased (369.10%) in the hepatic tissue, while the antioxidant enzymes SOD and GST reduced (47.66 and 36.91%, respectively). Exposure to Mn^{2+} did not affect TBARS levels in brain, but induced elevation of SOD (28.90%) and GST (40.53%) activities. The presence of Mn^{2+} in the water corresponded to increased levels of Mn^{2+} bioaccumulation in the following sequence: kidney>gills>liver>brain. Thus, the redox profile and the bioaccumulation were distinct in each organ. A correlation between bioaccumulation and toxicity was observed in gills and liver.

Keywords: contamination; fish; metal toxicity.

1. Introduction

Aerobic animals depend on a proper oxygen supply in order to survive. When a partial reduction in the water levels of oxygen occurs, fish enter the so-called hypoxic condition. This may arise from fluctuation in variables like temperature, dissolved organic matter and partial pressure of gases, as well as metal pollution (Beadle, 1981). Contamination by metals such as manganese occurs in the Amazon basin as a result of the industrial activity and the heavy oil exploration, especially in the Urucu province. For the extraction of every nine barrels of petroleum, one barrel of influent liquid of raw petroleum is released, which emerges from the soil under high pressure containing high ion and metal concentrations (Baldisserotto et al., 2011; Matsuo et al., 2005). This compound is called formation water and, in case of leakage, it may add significant amounts of manganese to the medium (Holdway, 2002). Considering that the highest concentration of manganese that is allowed in Brazilian waters is 0.1 mg L^{-1} (Conselho Nacional do Meio Ambiente – CONAMA, 2005; Portaria do Ministério da Saúde n. 2914, 2011) and that the concentration that might be released through formation water is 6.44 mg L^{-1}, the danger of environmental pollution under the latter circumstance cannot be overlooked. The present study tested a manganese concentration of 3.88 mg L^{-1}; it approaches the concentration found in formation water, but it remains under the mean lethal concentration for tambaqui (4.03 mg L^{-1}, unpublished data).

Another feature of the Amazon basin water that adds to the toxicity of the metals is its low water hardness. Hard waters show a protective effect because they have large amounts of ions such as calcium and magnesium that compete with toxic metals for their binding site in aquatic organisms, what is not observed in the soft water of the Amazon basin (Baldisserotto et al., 2011; Matsuo et al., 2005).

Manganese (Mn^{2+}) is a trace element which is essential for the metabolism of lipids, proteins and carbohydrates, acting as a cofactor in many mammalian enzymes (Keen et al., 2000). This metal is one of the most abundant elements and is widely used in industry (Gerber et al., 2002), pesticide formulation (Belpoggi et al., 2002), glass and ceramic production, and manufacture of dry cell (Bader et al., 1999; Mergler et al., 1994; Srivastava et al., 1991). Manganese intoxication causes neurologic, hepatic and cardiac disorders in humans with a combination of symptoms that characterize manganism (Bowler et al., 2006; Lee, 2000). According to some studies, Mn^{2+} promotes an elevation in the levels of Fe^{2+} in the organism, what induces lipoperoxidation (LPO) (Zheng and Zhao, 2001; Zheng et al., 1999). Other reports suggest inhibition of aconitase activity as the cause of cellular death (Crooks et al., 2007; Zheng et al., 1998), or a direct pro-oxidant action of Mn^{2+} (Chtourou et al., 2010).

Several investigations correlate heavy metals with the generation of reactive oxygen species (ROS) (Romeo et al., 2000; Ruas et al., 2008; Sanchez et al., 2005), as superoxide radical, hydrogen peroxide and hydroxyl radical (Romeo et al., 2000; Ruas et al., 2008; Sampaio et al., 2008). In order to control ROS production, fish possess an antioxidant defense system (AS) which varies in expression according to the tissue (Lemaire et al., 1994). The AS is mainly consisted of enzymes as superoxide dismutase (SOD) and catalase (CAT), and glutathione-related enzymes, as glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) (Halliwell B, 1999; Halliwell and Gutteridge, 1989; Martinez-Alvarez et al., 2005). Superoxide dismutase and catalase catalyze the reduction of superoxide radical into hydrogen peroxide. Glutathione peroxidase also participates in the reduction of hydrogen peroxide. On the other hand, GST catalyzes the

conjugation of pollutants to eliminate them from the cellular system, and GR reduces the oxidized glutathione to GSSH (Halliwell and Gutteridge, 1989). Certain environmental conditions, as metal pollution and hypoxia, may lead to an imbalance between ROS (also termed pro-oxidants) generation and the antioxidants rates (Sies, 1991). In the case of overproduction of ROS or reduction in the antioxidants levels, a condition known as oxidative stress (OS) takes place. It can cause detrimental effects like oxidation of protein, DNA and steroid constituents, and peroxidation of unsaturated lipids in cell membranes (Martinez-Alvarez et al., 2005).

Tambaqui (*Colossoma macropomum*) (Cuvier, 1818) has great importance for the economy of the Amazon region, where it occurs in floodplain areas (Affonso et al., 2002; Chagas and Val, 2003; Marcon and Wilhelm, 1999). This habitat undergoes constant alterations in dissolved oxygen (DO) concentrations, exposing the fish to hypoxic and even anoxic conditions. In order to survive in such circumstances, tambaqui presents some general modifications as increased gill ventilation, altered cardiac dynamics, biochemical adjustments related to erythrocytes and reduction in the metabolism. These changes happen along with some more specific adaptations like surface swimming associated to swelling of the lower lip to absorb the more oxygenated surface water (Affonso et al., 2002; Aride et al., 2007; Bailey et al., 1999; Matsuo et al., 2005; Milsom et al., 2002). Due to its great resistance and local importance, tambaqui is an adequate model for studying the Mn^{2+} that is released into the water as a result of the oil extraction activity. Since tambaqui may be subjected to hypoxic conditions in its natural habitat, it is relevant to test the effect of the metal exposure in a similar environmental setting.

Fish have been largely used to evaluate variations in the quality of aquatic systems, while the biochemical and physiological changes may be used as biomarkers in the

presence of environmental pollution (Monferran et al., 2011). Only a few studies relate short time exposure to heavy metals and hypoxia (Mustafa et al., 2012). The present work aims to outline the redox profile and to evaluate the bioaccumulation in juvenile tambaqui exposed to 3.88 mg L^{-1} Mn^{2+} for 96 h in hypoxia.

Materials and methods

2.1 Reagents

The reagent-grade chemicals manganese chloride ($MnCl_2$), phenylmethylsulfonyl fluoride (PMSF), 1-chloro-2,4-dinitrobenzene (CDNB), L- reduced glutathione (GSH), epinephrine and glycine were purchased from Sigma Chemical Co. (USA). Hydrogen peroxide, trichloroacetic acid (TCA), thiobarbituric acid (TBA) and albumin were purchased from Sigma (St. Loius, MO). All of the other reagents were of analytical grade.

2.2 Fish

Tambaqui juveniles weighing 100-300 g were obtained from a local fish farm (Fazenda Santo Antônio, Rio Preto da Eva, Amazonas, Brazil). The specimens were kept for at last three weeks in aerated well water with an average composition of: Ca^{2+} = 11, Na^+ = 34, Cl^- = 28, Mg^{2+} = 0.8, K^+ = 15, all in µmol L^{-1}; pH 6.3; dissolved organic matter = 0.9 mg Cl^-; background Cu^{2+} = 1.7 µg L^{-1}; background Cd^{2+} = 0.3 µg L^{-1}; temperature = 27 ± 1°C. The fish were fed commercial dry food pellets once a day. The experimental protocol was approved by the Animal Health Committee of Federal University of Santa Maria, RS, Brazil.

2.3 Exposure to Mn^{2+} and hypoxia induction

After the acclimation period, the juveniles were randomly separated into 72 L aquaria (ten fish per aquarium) and assigned to two treatments. One group was exposed to 3.88 ± 0.24 mg L^{-1} Mn^{2+} (measured concentration) for 96 h in hypoxia (0.2 mg L^{-1} DO) and the other was subjected to similar conditions, only there was no addition of Mn^{2+} to the test water. The DO levels were maintained by nitrogen bubbling or turning off the bubbling air. Once the 96h had elapsed, the fish were placed in recipients containing water and ice during 5 min for anaesthetization. Gills, liver, brain and kidney were removed and immediately frozen in liquid nitrogen. The tissues were stored at -70°C for measurement of OS parameters or at -20°C for posterior digestion with concentrated nitric acid (HNO_3, 1N, Merck). The Mn^{2+} concentrations in the digested tissues and water samples were analyzed using graphite furnace atomic absorption spectrophotometry (ICP-MS, Elan DRCII Perkin Elmer SCIEX – Canada). The certified standards provided by the manufacturer were used throughout this study. Manganese activity was calculated using the speciation program Visual MINTEQ version 3.0 (Gustafson, 2012).

2.4 Water parameters

Water samples were collected from each aquarium to determine water quality parameters at the beginning and at the end of the experiment. Water alkalinity (10.66 ± 1.08 mg $CaCO_3$ L^{-1}) was determined by the sulfuric acid (H_2SO_4) method (Eaton et al., 2005). Measurements of DO (YSI model Y5512 oxygen meter) and water pH (7.1 ± 0.04) (Quimix 400A pH meter) were performed daily. Water hardness (14.66 ± 0.88 mg $CaCO_3$ L^{-1}) was determined by the EDTA titrimetric method and total ammonia (0.98 ± 0.08 mg L^{-1}) (NH_3 + NH_4^+) was determined by the direct nesslerization method (Eaton et al., 2005).

2.5 Oxidative stress parameters

The tissues were homogenized as previously described by Azambuja et al., (2011). The homogenates were centrifuged at 1000 x g for 10 min at 4°C to discard nuclei and cell debris, and the supernatant fraction obtained was frozen at -70°C for analyses of OS parameters.

Lipid peroxidation was measured by the TBARS assay (Buege and Aust, 1978). Results are expressed as nmol mg protein^{-1}. Commercially available malonaldehyde (MDA) was used as a standard. Protein content was measured by the method of Lowry et al., (1951) using bovine serum albumin as standard.

Total SOD activity was based on the inhibition rate of autocatalytic adenocrome generation at 480 nm in a reaction medium containing epinephrine and glycine/NaOH (pH 10.2). The enzyme activity is expressed as USOD mg protein^{-1}. One SOD unit was defined as the amount of enzyme needed for 50% inhibition of adenochrome formation, as described by Misra and Fridovich, (1972). The activity of CAT was evaluated by following the decrease in the 240 nm absorption in a reaction medium consisting of phosphate buffer (pH 7.4) and hydrogen peroxide (H_2O_2), thereby determining the pseudo-first-order reaction constant (k`) of the decrease in H_2O_2 absorption. It is reported as nmol mg protein^{-1} (Boveris and Chance, 1973). GST activity, expressed as µmol min^{-1} mg protein^{-1} of protein, was determined according to Habig et al., (1974). The assay was performed using potassium phosphate buffer (pH 6.5) with GSH and 1-chloro-2,4-dinitrobenzene (CDNB). Activity was calculated from the changes in absorbance at 340 nm (ε_{340} nm = 9.6 mM^{-1} cm^{-1}). One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of CDNB with GSH per minute at 25°C.

2.6 Statistical analysis

Data are reported as the means ± standard error of the mean (SEM). Homogeneity of variances among groups was tested with a Levene test. Unpaired Student's t-test was used for comparison of means. All analyses were executed by using GraphPad Instat software (San Diego, CA). Differences were considered significant at $p < 0.05$.

3. Results and discussion

Fish have been susceptible to great changes in the levels of oxygen in the Amazon basin, what imposed the development of certain strategies in order to survive. A strong relationship between the ventilatory frequency and the absorption of pollutants in aquatic environments has been suggested, resulting in a heavier toxicological impact under hypoxic conditions (Schiedek et al., 2007).

The fish exposed to 3.88 mg L^{-1} Mn^{2+} in hypoxia presented higher concentrations of this metal in the analysed tissues than their control counterparts (**Table 1**). Bioaccumulation of Mn^{2+} in the tissues occurred in the following order: kidney>gills>liver>brain. This sequence differs from the one previously found in normoxia: gills>kidney>brain>liver (Gabriel et al., 2013). Indeed, some works highlight the changes in metabolism, gill permeability and renal function under hypoxia (Iftikar et al., 2010; Matey et al., 2011; Wood et al., 2009). The observed higher bioaccumulation may be explained by a rise in ventilatory frequency increasing the water flow through the gills, thus intensifying Mn^{2+} absorption (Mustafa et al., 2012). The gills are naturally exposed to metals in the water. Manganese has binding sites on their cartilaginous structure (Adam et al., 1997), what may explain the high levels of the metal found in this tissue. In the present study there was an increase of 37.74% in the levels of TBARS and a decrease of 36.58 and 96.52% in the activities of SOD and GST, respectively (**Figure 1**). Besides a possible pro-oxidant role of Mn^{2+} (Ali et al., 1995), the increased blood supply to the gills due to hypoxia, as a result of the vasodilation promoted by catecholamines and nitric oxide (Florindo et al., 2004), may have potentiated ROS

production (Affonso and Rantin, 2005; Braun et al., 2006; Burleson et al., 2002; Kind et al., 2002). This was observed in the levels of LPO (**Figure 1**). A significant decline in the levels of the antioxidant enzymes occurs as a consequence of lipid oxidation, in an attempt to compensate for ROS generation.

A similar response was verified in *Clarias gariepinus* exposed to 0.2 and 0.4 mg L^{-1} Cd^{2+} in normoxia for 21 days; there was an increase in the levels of TBARS and a decrease in SOD activity (Asagba et al., 2008). There are only a few studies involving hypoxia and metals.

The liver is a key organ for the metabolism in aquatic animals. Exposure to Mn^{2+} produced results which are similar to the ones observed in gills: an increase of 369.10% in TBARS levels and a reduction of 47.66 and 36.81% in the activities of SOD and GST, respectively (**Figure 2**). Catalase was not affected by Mn^{2+} treatment. As well as the gills, the liver has binding sites for Mn^{2+}. The reduced activities of both SOD and GST are correlated with an attempt to protect the organism from the damage caused by the manganese-induced overproduction of ROS and indicate OS in the tissue. A similar enzymatic behaviour has been reported in liver of *Piaractus mesopotamicus* by Sampaio et al., (2010); the fish were subjected to 0.4 mg L^{-1} Cu^{2+} for 48 h in an acid medium. Another study using the same experimental conditions as the latter, only exposing the fish to hypoxia, described an increase in the levels of pro-oxidants determined by ferrous oxidation in xylenol orange which is similar to the one found in this investigation; nonetheless, SOD activity increased, in opposition to the present results (Sampaio et al., 2008).

No effect of Mn^{2+} and hypoxia exposure was observed in either TBARS or GST in brain of tambaqui. The activity of SOD, however, was significantly affected (28.90%). Some theories about the damage induced by Mn^{2+} indicate an elevation in ROS levels in brain of rats *in vivo* and *in vitro* (Ali et al., 1995), inhibition of aconitase in preparations of rat brain (Zheng et al., 1998), iron overload in cultures of rat neurons (Zheng and Zhao, 2001), and changes in Fe^{2+} homeostasis in rats (Zheng et al., 1999). Nevertheless, there is a paucity of information

regarding OS as a consequence of metal exposure and hypoxia in fish brain. Vieira et al., (2012) did not find changes in TBARS in *Carassius auratus* subjected to 0.1 mM (5.5 mg L^{-1}) and 1 mM (55 mg L^{-1}) Mn^{2+} in normoxia for 96 h. In accordance with the current study, Sithara et al., (2010) reported a rise in SOD in *Catla catla* exposed to 0.01 mg L^{-1} Cd^{2+} in normoxia for 96 h. Induction of SOD activity in this study probably resulted in a protective effect in the tissue, thus avoiding damage, what is demonstrated by the absence of difference in TBARS levels between the groups.

The kidney is responsible for detoxifying and eliminating toxic substances (Üner et al., 2005). The highest bioaccumulation was observed in this organ, what may have resulted from the decline in metabolism under hypoxia, since it was not noted in normoxia (Gabriel et al., 2013). Dolci et al., (2013) verified a higher concentration of Mn^{2+} in kidney of *Rhamdia quelen* exposed to 8.4 mg L^{-1} Mn^{2+} in moderate hypoxia rather than in normoxia, what is consistent with the present results. Literature is scarce with regard to reports on metals, OS parameters and hypoxia in kidney. There were no changes in LPO or CAT in tambaqui kidney (**Figure 4**). Prevention of oxidative damage in this organ may have happened because of the increase in SOD activity (40.53%) and the reduction in GST activity, for these antioxidant enzymes are modulated by the presence of ROS (Possamai et al., 2007). Superoxide dismutase is an essential enzyme of the AS (Fridovich, 1995), and was the one that altered the most in the different evaluated organs. Tambaqui exposed to Mn^{2+} and normoxia presented lower SOD levels in kidney than the unexposed fish (Gabriel et al., 2013). This result corroborates the importance of such enzyme, which seems to be extremely sensitive to the fluctuations in the levels of oxygen. The increased SOD levels in brain and kidney under hypoxia is in agreement with the theory of preparation to OS prior reoxygenation proposed by Buzadžić et al., (1997); Hermes-Lima et al., (1998); Lushchak et al., (2001). The same behaviour was not displayed by the enzyme in gills and liver, since there is a larger imbalance between the levels of pro-oxidants and antioxidants in these organs.

4. Conclusion

Manganese induced damage in gills and liver, what was correlated with the bioaccumulation. Brain and kidney, on the other hand, were protected by the increased activities of the antioxidant enzymes.

Exposure to sublethal concentrations of Mn^{2+} altered the redox profile in tambaqui, which may be used as a sentinel fish for Mn^{2+} in hypoxic regions.

Conflict of interest

The authors report no conflict of interest.

Tables

Table 1 – Manganese levels in brain, gills, liver and kidney of *Colossoma macropomum* exposed to 3.88 mg L^{-1} Mn^{2+} for 96 h in hypoxia.

Organs	Groups	
	Control	Manganese
Brain	1.104 ± 0.140	1.599 ± 0.253
Gills	2.858 ± 0.254	6.280 ± 0.661*
Liver	0.664 ± 0.086	2.243 ± 0.384*
Kidney	3.353 ± 0.663	7.894 ± 0.784*

Values are expressed as means ± SEM (n = 10). * indicates significant difference between exposed and control (unexposed groups) by Student's t-test (p<0.05).

Figures

Figure 1.
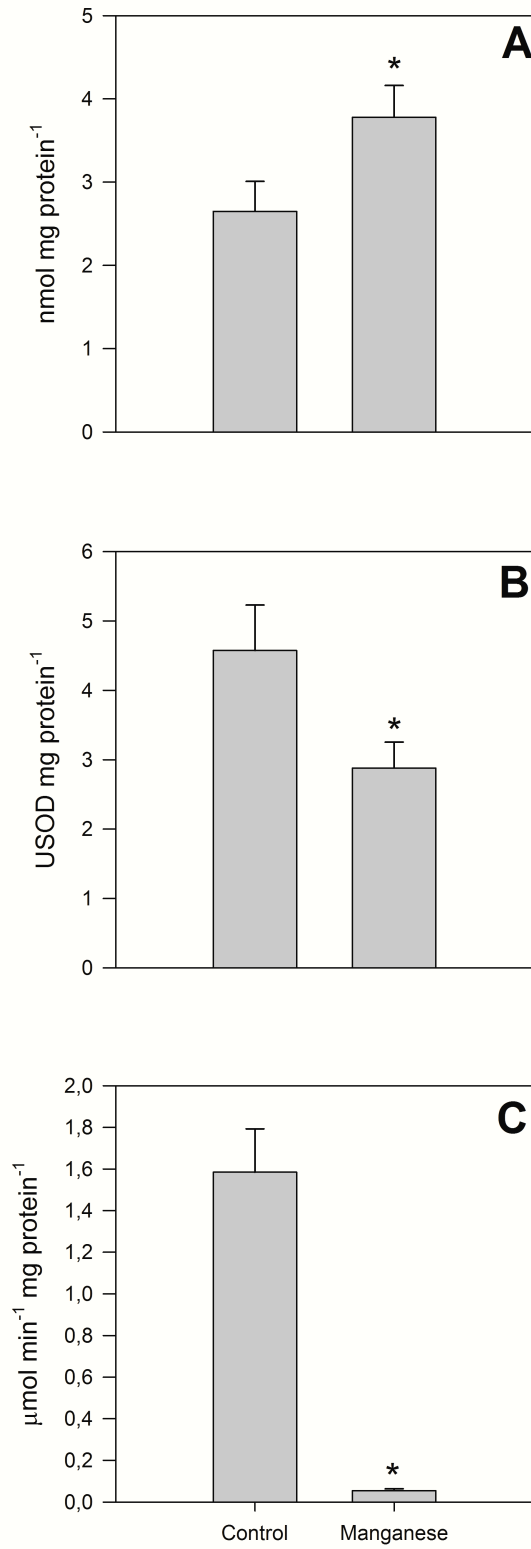
Figure 2.
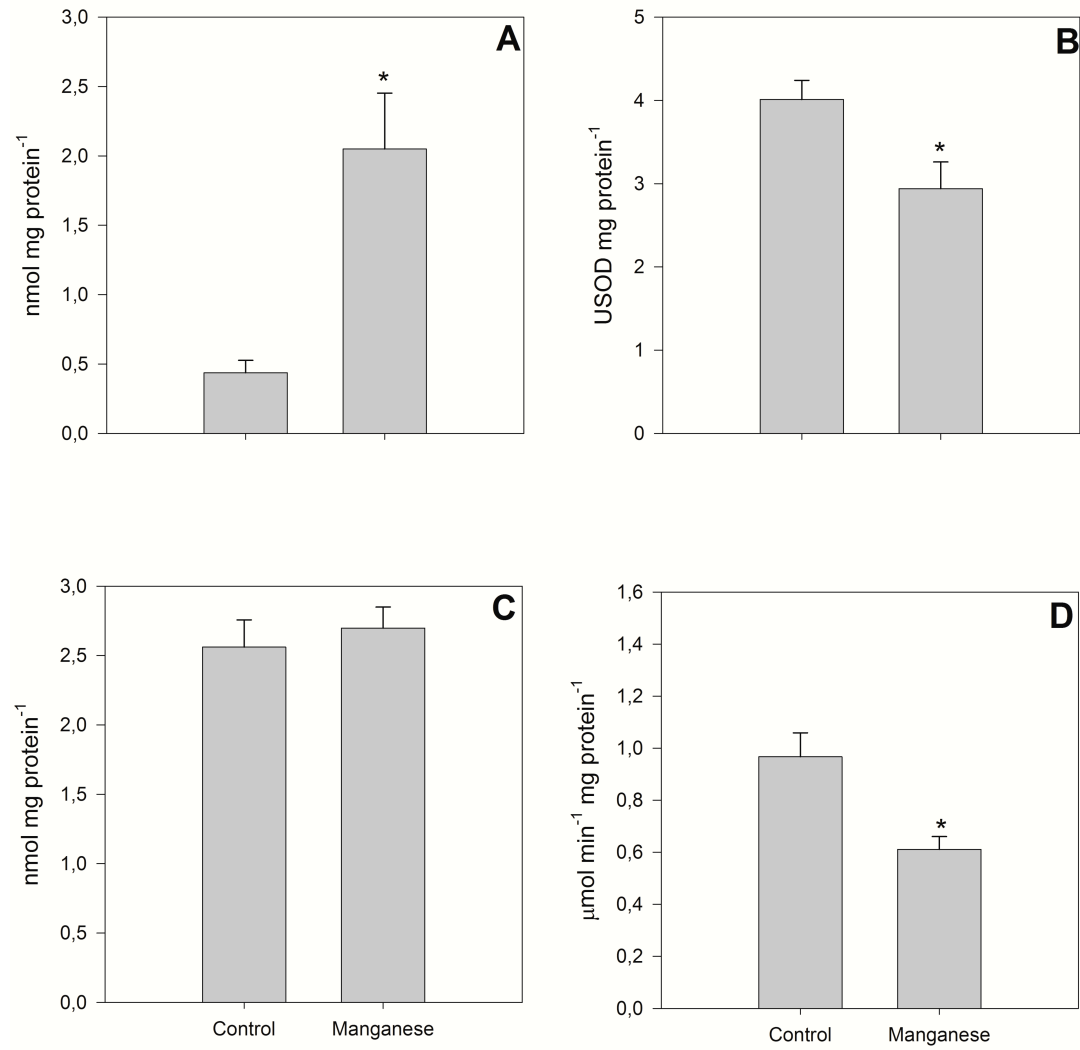
Figure 3.
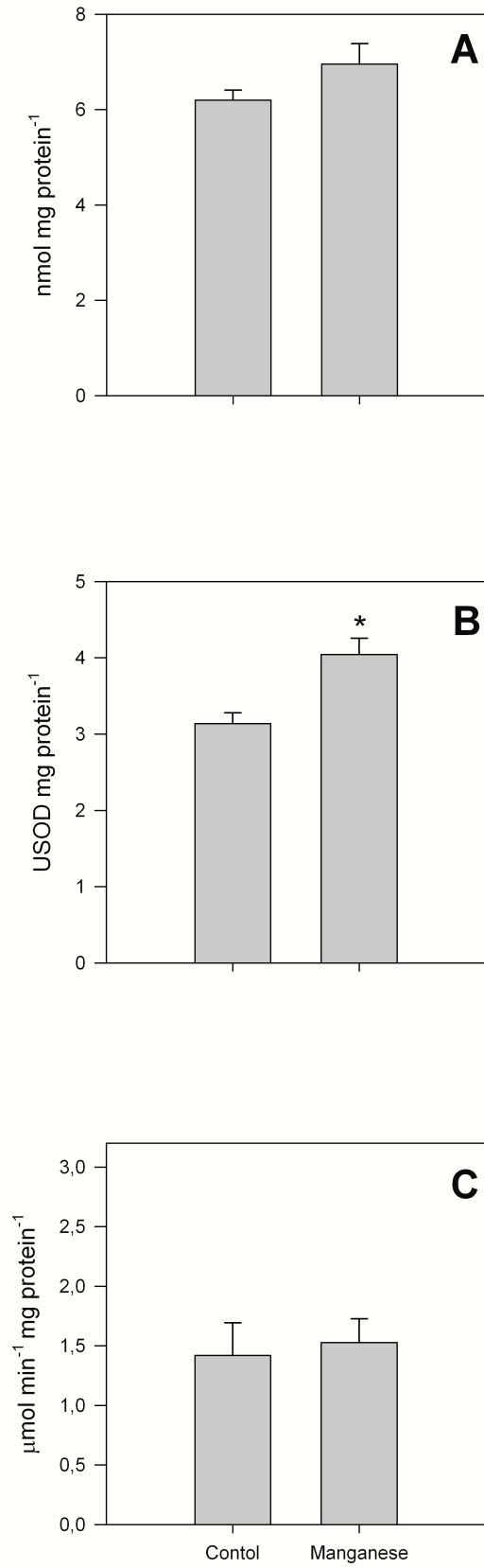
Figure 4.
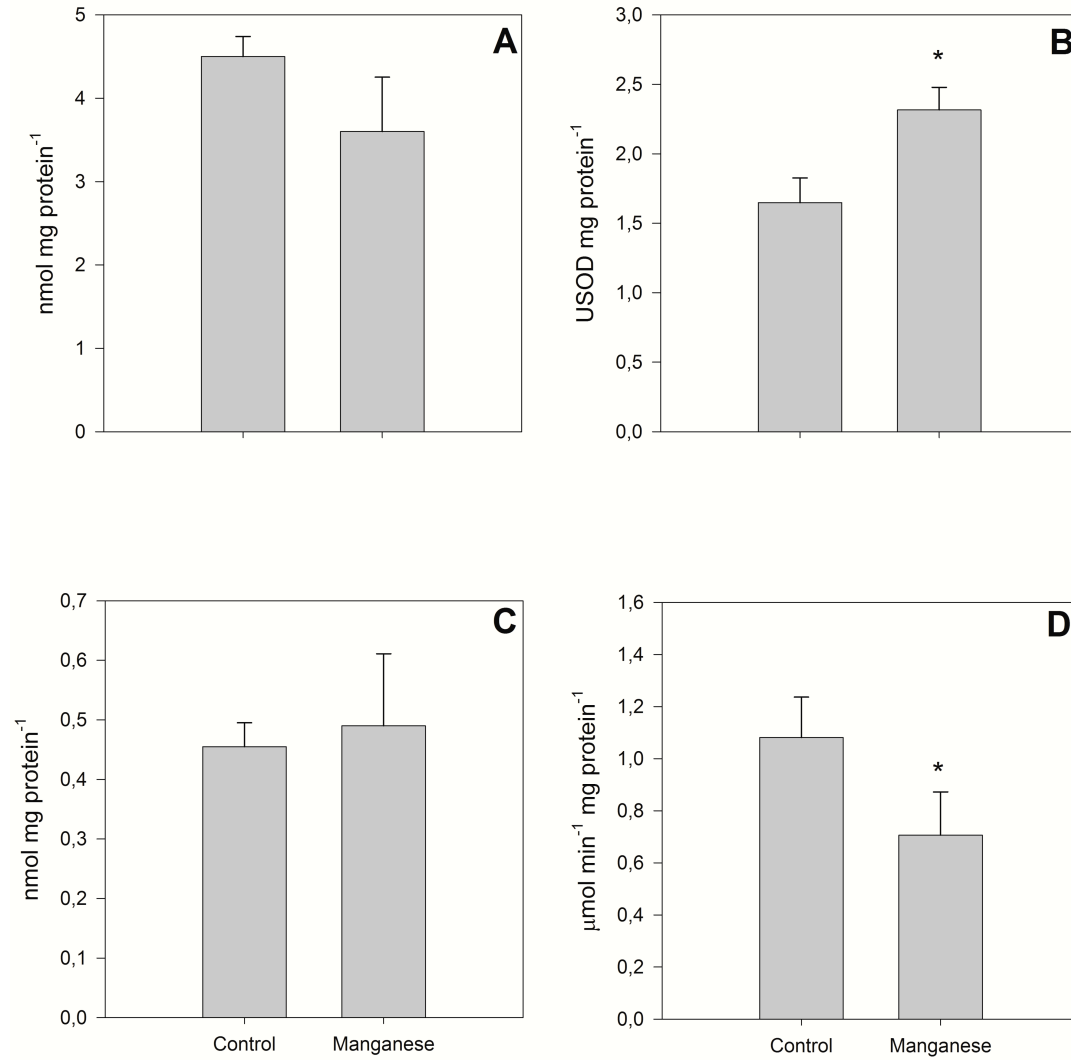
Figure captions

Figure 1. TBARS levels (A), SOD (B) and GST (C) activities in gills of tambaqui exposed to 3.88 mg L^{-1} Mn^{2+} for 96 h in hypoxia. Data are reported as mean ± S.E. (n=10). * Significantly different from control by unpaired Student's t-test ($p < 0.05$).

Figure 2. TBARS levels (A), SOD (B), CAT (C) and GST (D) activities in liver of tambaqui exposed to 3.88 mg L^{-1} Mn^{2+} for 96 h in hypoxia. Data are reported as mean ± S.E. (n=10). * Significantly different from control by unpaired Student's t-test ($p < 0.05$).

Figure 3. TBARS levels (A), SOD (B) and GST (C) activities in brain of tambaqui exposed to 3.88 mg L^{-1} Mn^{2+} for 96 h in hypoxia. Data are reported as mean ± S.E. (n=10). * Significantly different from control by unpaired Student's t-test ($p < 0.05$).

Figure 4. TBARS levels (A), SOD (B), CAT (C) and GST (D) activities in kidney of tambaqui exposed to 3.88 mg L^{-1} Mn^{2+} for 96 h in hypoxia. Data are reported as mean ± S.E. (n=10). * Significantly different from control by unpaired Student's t-test ($p < 0.05$).

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4 Discussão

O estudo da contaminação do ambiente aquático por metais é bem estudado. Estes são liberados no ambiente aquático através da atividade industrial, de mineração e pela atividade petrolífera. Assim a pesquisa envolvendo metais na água vem ganhando grande destaque. Existem muitos trabalhos com mercúrio e cadmio (ELIA, et al., 2003; HATTINK, et al., 2005), no entanto existem poucos estudos com o manganês, o qual pode ser liberado na água por ser empregado em diversas atividades, como na indústria (GERBER et al., 2002), em pesticidas (BELPOGGI et al., 2002), em vidro, cerâmicas e em baterias (SRIVASTAVA et al., 1991; MERGLER et al., 1994; BADER et al., 1999; ZHANG et al., 2008), como também a extração petrolífera (BALDISSEROTTO et al., 2012).

Em nosso trabalho utilizamos o tambaqui (*Colossoma macropomum*) para realizar os experimentos com o manganês. Este peixe foi utilizado por possuir grande resistência e habitar regiões de normóxia e de hipóxia, esta última por ele ser um peixe encontrado principalmente em regiões de várzea. Como o oxigênio dissolvido varia conforme o local analisado, seja pelo ciclo dia/noite, seja pela quantidade de matéria orgânica dissolvida ou pelo local onde o tambaqui se encontra, resolvemos realizar um experimento com hipóxia e normóxia (ARAUJO-LIMA e GOULDING, 1998). Neste, foi analisado o perfil redox dos animais expostos ao Mn^{2+} em normóxia e hipóxia.

O manuscrito 1 foi a linha de partida para os demais. Como não havia uma CL50 para o manganês em tambaqui em 96h era necessária esta determinação. O valor encontrado foi abaixo do liberado pela água de formação, isto é, o valor liberado por ocasião da atividade petrolífera possui importância significativa para o tambaqui. A bioacumulação também não encontrou um platô.

Assim, partindo dos resultados encontrados no **manuscrito 1** foi escolhida uma dose subletal para se realizar os demais experimentos. Esta concentração letal (3,88 mg/L) tem uma representatividade significativa, pois está no intervalo entre a concentração liberada pela água de captação (6,44 mg/L) e o valor máximo preconizado pela CONAMA (0,1 mg/L). Está concentração letal encontrada indica

que em hipóxia, valores menores que aqueles liberados na água de captação podem ser potencialmente tóxicos.

No **Artigo 1** e no **manuscrito 2** a exposição ao Mn^{2+} por 96h causou danos oxidativos em vários órgão e de maneira diferenciada. No **Artigo 1**, em normóxia, a bioacumulação foi diferente em cada órgão (brânquias > rim > cérebro > fígado). Já a bioacumulação em hipóxia, no **manuscrito 2** foi diferente em cada órgão, mas diferente dos animais expostos em normóxia (rim > brânquias > fígado > cérebro). Este resultado diverso da bioacumulação em normóxia e hipóxia também foi observado no trabalho de HATTINK et al., (2005) onde trabalharam com *Cyprinus carpio* expostos a 6,5 nmol/L de Cd^{2+} em nomóxia e várias saturações de oxigênio dissolvido até a hipóxia.

A produção de EROs não se correlacionaram com presença de manganês no órgão, tanto em normóxia, no **Artigo 1** como em hipóxia, no **manuscrito 2.** A explicação deste dado deve ser multifatorial, mas uma das elucidações sem dúvida reside no fato de que cada órgão possui a sua sensibilidade ao metal. Assim alguns órgãos sofreram maior alteração, mesmo com pouca concentração de manganês. O mesmo ocorreu com o estudo de ELIA et al., (2003), que expuseram *Ictalurus mela* à 35, 70 e 40 microg/L de Hg^{2+} por 10 dias.

Em brânquias de tambaqui exposto ao manganês em normóxia houve dano oxidativo, evidenciado pelo aumento da lipoperoxidação e observado no **Artigo 1**. O mesmo ocorreu nos animais em hipóxia, observado no **manuscrito 2**. Já as enzimas antioxidantes apresentaram resultado inverso. Isto pode estar relacionado ao grau de estresse oxidativo no órgão. Enquanto em normóxia se observou um aumento da SOD e um aumento na GST, em hipóxia estas enzimas provavelmente foram saturadas devido ao metal e as EROs, com a atividade reduzida. Em hipóxia ocorre um aumento da ventilação branquial (MATEY et al., 2008). O aumento da passagem de água aumenta a biodisponibilidade ao metal.

Não foi observada alterações nos níveis de lipoperoxidação no fígado em normóxia, no **Artigo 1**. Provavelmente o aumento da atividade da SOD e da GST protegeu o órgão de lesões por lipoperoxidação. Já no **manuscrito 2** os peixes em hipóxia apresentaram dano por lipoperoxidação no fígado e uma saturação da SOD

e GST, o inverso da normóxia. Isso indica que os peixes podem ser mais susceptíveis ao manganês, em fígado, quando em hipóxia.

Os autores divergem sobre o efeito da normóxia e hipóxia em relação a atividade das enzimas antioxidantes (LUSHCHAK e BAGNYUKOVA, 2006a; HENRIK HANSEN et al., 2007; CHEN et al., 2012; PÉREZ-JIMÉNEZ et al., 2012; STARA et al., 2012).

Existem muitos trabalhos com manganês em cérebros de ratos (ALI et al., 1995a; DESOLE et al., 1997; HAMAI et al., 2001; ERIKSON et al., 2004; NORMANDIN et al., 2004), mas o estudo deste órgão em peixes é raro. O cérebro é protegido por barreiras fisiológicas, que explica a pequena presença do metal no órgão, tanto em normóxia como em hipóxia nos peixes. Já a SOD uma apresentou resposta inversa, reduzindo a atividade em normóxia e aumentando a atividade em hipóxia. A redução da atividade da SOD e da CAT em normóxia demonstrou dano oxidativo sofrido pelo órgão. A atividade da GST em cérebro no **artigo 1** e no **manuscrito 2** não apresentou modificação em relação aos controles. Uma possível explicação para este dado é a concentração pequena de Mn^{2+} neste tecido. Como a GST é uma enzima detoxificadora (STAICU et al., 2005), e ocorreu pouca presença do metal no tecido, possivelmente não houve um aumento na atividade desta enzima devido a pouca concentração de substrato.

No rim houve maiores danos em normóxia, **artigo 1**. Ocorreu uma redução da atividade da SOD e um aumento dos níveis de lipoperoxidação. A catalase, assim como na hipóxia, **manuscrito 1,** permaneceu inalterada. Em hipóxia não houve alteração nos níveis de lipoperoxidação. Possivelmente ocorreu uma compensação pelo aumento na atividade da SOD e uma redução da atividade da GST. TRAVACIO e LLESUY, (1996) relataram que diferentes modelos de estresse oxidativo envolvem uma resposta bifásia da atividade antioxidante das enzimas. Em primeiro, a atividade enzimática é claramente diminuída, mas com o tempo, o nível de atividade aumenta, provavelmente por uma nova síntese e/ou ativação enzimática.

5 Conclusão

Em nosso estudo foi possível concluir diversos pontos:

- A $CL_{50\text{-}96h}$ para o Mn^{2+} em tambaqui mantido em hipóxia foi 4.03 mg Mn/L com valores de confiança 3.38-6.52 mg Mn/L;

- O Mn^{2+} na dose subletal é capaz de produzir estresse oxidativo, em normóxia e hipóxia;

- Os valores da bioacumulação em cada órgão geralmente não se correlacionaram com o estresse oxidativo em normóxia e hipóxia;

- A enzima antioxidante que apresentou um papel importante nos experimentos foi a superóxido dismutase;

- O perfil redox encontrado para o tambaqui frente ao Mn^{2+} em normóxia e hipóxia, pode fornecer suporte para novos estudos, os quais podem levar à utilização do peixe como biomarcador para o Mn^{2+} nas águas em normóxia e hipóxia.

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