UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG
PÓS-GRADUAÇÃO EM OCEANOGRAFIA BIOLÓGICA

BIOMARCADORES DE CONTAMINAÇÃO AQUÁTICA EM *Poecilia vivipara*

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Dissertação apresentada ao Programa de Pós-graduação em Oceanografia Biológica da Universidade Federal do Rio Grande, como requisito parcial à obtenção do título de Mestre.

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RIO GRANDE, Março 2012

Esta dissertação é dedicada à minha família,
que sempre esteve ao meu lado
apoiando e suportando
Pai, Mãe e Irmãos
Obrigado

Quand je parlerais toutes les langues des hommes, et même des Anges...et que j'aurais toute sorte de science; et quand j'aurais toute la foi qu'on puisse avoir, en sorte que je transportasse les montagnes, si je n'ai pas l'amour, je ne suis rien.

La Saint Bible

AGRADECIMENTOS

São poucas as conquistas em que há apenas um ganhador. Na maioria delas existem ganhadores, uma equipe que se esforça e trabalha junto por um objetivo, ou melhor por cada etapa para alcançar o objetivo. Como num corpo, cada parte da equipe desempenha um papel, alguns um papel mais aparente, se mostrando sempre presente. Outros, um papel mais específico, menos notado, mas não menos importante. Para quem trabalhou num laboratório de fisiologia, nada mais adequado que a analogia ao "corpo" que conviveu, se confrontou e confraternizou durante estes últimos 24 meses. E é esta equipe que, dividindo funções em união e unidade, vence. É por isso que aproveito este espaço pra lembrar alguns dos membros desta equipe que foram fundamentais para a conclusão deste trabalho.

Primeiramente quero agradecer à Mariana Hoff, que como um coração deste corpo-equipe, trabalhou todo o tempo, do planejamento à execução dos experimentos. Sei que não foi fácil me acompanhar como um projeto secundário que na verdade consumia mais que todo o seu tempo. Mesmo assim ela foi sempre sensível àquilo que lhe era proposto e, na ausência de estrogênio de minha parte, era ela quem se estressava, perdia cabelos e mesmo derrubava lágrimas quando as coisas davam certo ou errado demais.

Agradeço à Roberta Klein, que foi como um órgão de sustentação interna, que nos acompanha e permite que realizemos as coisas que fazemos. Foi ela quem, suportando minha falta de experiência em muitas das técnicas, perdeu sábados, domingos comigo no laboratório, além de suprir minha ausência no projeto durante o período no exterior.

Agradeço aos membros deste corpo: aqueles que foram os braços e pernas, colocando a mão na massa e levando o projeto adiante. Dentre estes incipientes heróis

do trabalho laboratorial merecem destaque Eduardo “o Guerreiro”, Janaína “a Índia”, Gilson “o Coletor”, Janine “Deusú” e Lucas “o Bianchini jr”.

Nos momentos de trabalho intenso o corpo aumentava e como simbiontes obrigatórios surgiam muitos que possibilitavam a execução de enorme trabalho em curto espaço de tempo. Por isso agradeço às técnicas de laboratório, em especial Patty e Sandrinha, às colegas Marina Giacomin e Cyntia Hayarashik, à Drª IndianaraBarcaroli, à professora Drª Marta Souza, à MSc Martina Prazeres ao Dr Flávio e sua companheira MSc Nana.

E como todo corpo precisa de um sistema nervoso, de onde surgem ideias e respostas, agradeço aos mentores intelectuais que me auxiliaram na compreensão de resolução dos problemas que apareciam. Para elaboração e execução deste projeto, tive o prazer de contar com a experiência e colaboração direta dos professores Dr Adalto Bianchini, Dr Elton Colares, Dr Chris Wood e Drª Grasiela Pinho. E também com contribuição dos professores Dr Juliano Zanette, Dr Juliane Ventura-Lima e Dr José Monserrat.

Também nenhum organismo vive só ou isolado nas suas ambições, vive sim, em geral, rodeado por outros da mesma espécie com quem aprende e estabelece os diversos tipos de relações. Então agradeço aos colegas de laboratório, amigos da oceano e biologia, os mais que amigos da Comunidade Cristã de Rio Grande, companheiros do CTG Farroupilha, “the canadian friends”, e especialmente à minha família. Estes proporcionaram apoio e suporte durante os melhores e não bons momentos destes anos.

Por fim, reconheço o apoio financeiro recebido do CNPq, CAPES, International Copper Association (EUA) e International Development Research Centre (Canadá).

PREFÁCIO

Este trabalho foi apresentado à comissão do curso de Pós-graduação em Oceanografia Biológica como requisito parcial à obtenção do título de mestre. Ele foi desenvolvido no âmbito do Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática (INCT-TA), num esforço de entender melhor as relações entre biomarcadores de contaminação aquática e exposição a contaminantes. Durante o decorrer do projeto foi realizada em laboratório a exposição do modelo biológico a três contaminantes aquáticos e a análise em campo de alguns dos principais biomarcadores. Por isso, o presente documento está separado em 4 partes: uma primeira parte em português e as três seguintes em inglês. Na primeira parte são rapidamente apresentados a problemática central do trabalho e um breve sumário dos resultados obtidos. Por sua vez, cada uma das três partes redigidas em língua inglesa constitui um manuscrito de artigo científico retratando a resposta dos biomarcadores a um dos contaminantes testados.

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RESUMO

A poluição aquática em zonas costeiras é crescente, exigindo cada vez mais o uso de ferramentas alternativas para seu monitoramento. Dentre estas, as de maior potencial são os biomarcadores, que são medidas biológicas capazes de expressar exposição ou efeito deletério de contaminantes. Porém, a falta de informação sobre respostas de biomarcadores a contaminantes em concentrações ambientalmente relevantes constitui empecilho ao emprego destas ferramentas. Neste trabalho foram analisados os efeitos de três contaminantes em diversos biomarcadores usualmente utilizados em estudos de avaliação ambiental. Os contaminantes testados foram cobre (metal), atrazina (herbicida) e fenantreno (hidrocarboneto policíclico aromático). Os biomarcadores avaliados foram atividades enzimáticas (superóxido dismutase, catalase, glutationa redutase e glutationa s-transferase), concentração de metalotioneínas, espécies reativas de oxigênio, capacidade antioxidante, peroxidação lipídica, quebras na fita de DNA, freqüência de células micronucleadas e de anomalias nucleares, e composição iônica corporal. Os três contaminantes demonstraram potencial para afetar os biomarcadores avaliados, porém cada um afetou distintamente os grupos de biomarcadores. O efeito do cobre foi caracterizado por um elevado potencial de geração de estresse oxidativo em brânquias, fígado e músculo, além de causar dano genético através da quebra da fita de DNA e clastogenicidade. A atrazina aumentou a atividade da glutationa s-transferase, causou estresse oxidativo branquial e danos genéticos não detectáveis no ensaio cometa. Por fim, o fenantreno gerou estresse oxidativo muscular, sendo a maioria dos danos possivelmente relacionada à geração de metabólitos reativos durante sua degradação, resultando em elevada genotoxicidade e citotoxicidade, bem como perturbações de sistemas enzimáticos e da fisiologia do animal.

PALAVRAS-CHAVE: Atrazina, Cobre, Desequilíbrio iônico, Estresse oxidativo, Fenantreno, Genotoxicidade.

ABSTRACT

Aquatic pollution in coastal areas is increasing, demanding the use of alternative tools for its adequate monitoring. Among them, biomarkers are those showing higher potential for practical use. They are biological measurements capable of detecting the exposure and effects of contaminants. However, the lack of information on the response of biomarkers to contaminants at environmentally relevant levels is a constraint for the employment of these tools in monitoring programs. In the present study, the effects of three contaminants on several biomarkers commonly employed in environmental analyses were evaluated. Contaminants tested were copper (metal), atrazine (herbicide) and phenanthrene (polycyclic aromatic hydrocarbon). Biomarkers evaluated were enzyme activities (superoxide dismutase, catalase, glutathione reductase and glutathione s-transferase), metallothioneins concentration, reactive oxygen species, antioxidant capacity, lipid peroxidation, DNA strand-breaks, micronucleated cells and nuclear anomalies frequencies, and whole-body ion composition. The three contaminants showed potential to affect the biomarkers evaluated. However, each contaminant affected distinctly the groups of biomarkers. Copper effect was characterized by a elevated potential for oxidative stress generation in gills, liver and muscle. It also induced genetic damage (DNA strand-breaks) and clastogenicity. Atrazine increased glutathione s-transferase activity, caused oxidative stress in gills and genetic damage non detectable by the Comet assay. Finally, phenanthrene induced oxidative stress in muscle, being most of the damage likely associated with the generation of reactive metabolites during the contaminant degradation process. Exposure to phenanthrene resulted in elevated genotoxicity and cytotoxicity, as well as perturbations of the enzymatic systems and the whole-animal physiology.

KEY WORDS: Atrazine, Copper, Genotoxicity, Ionic disturbance, Phenanthrene, Oxidative stress

1. INTRODUÇÃO

Há várias décadas que a sociedade humana apresenta crescimento, densidade e tecnologia suficientes para provocar massivas e profundas alterações ambientais (Phillips 1980). Atualmente, cerca de 70% da população mundial encontra-se situada no ambiente costeiro, e à medida que esta aumenta, as pressões sobre os recursos aquáticos se multiplicam (Bortone 2005). Isto implica que corpos aquáticos presentes nestes ecossistemas sejam utilizados para diversos fins, como turismo, lazer, extração de fontes alimentares, transporte marítimo e instalações de atividades industriais (Neto *et al.* 2008).

Tais atividades e usos geram poluentes característicos, com uma diversidade enorme de produtos químicos potencialmente tóxicos e em quantidade significativamente prejudicial ao ambiente (Zagatto 2006), tendo conseqüências diretas na qualidade do corpo receptor. Cenário este que se torna preocupante se for considerado que os estuários e ambientes marinhos costeiros têm sido utilizados como depósitos de diversas substâncias e resíduos antropogênicos durante as últimas décadas (Kennish 1991). Logo, os corpos aquáticos continentais e marinhos costeiros podem ser considerados como ecossistemas sensíveis e susceptíveis à degradação e à poluição (Nemerow 1991), pois atuam como receptáculos finais de inúmeros contaminantes. De fato, diversos efeitos deletérios para estes ambientes e suas respectivas comunidades biológicas têm sido relatados nas últimas décadas (Phillips 1980, Seeliger *et al.* 1988, Kennish 1991, Nemerow 1991, Heath 1995, Langston & Bebianno 1998, Neto *et al.* 2008).

Dentre os principais poluentes oriundos das atividades humanas estão os metais, pesticidas e hidrocarbonetos (Phillips 1980, Seeliger *et al.* 1988, Kennish 1991, Nemerow 1991, Heath 1995, Langston & Bebianno 1998, Neto *et al.* 2008). As maiores fontes de aporte antrópico destes contaminantes são o esgoto doméstico (Neto *et al.* 2008), atividades industriais (Kennish 1991), mineração (Phillips 1980), agricultura e erosão do solo (Heath

1995) e queima de combustíveis fósseis (Paytan *et al.* 2009). Estes contaminantes são considerados críticos devido à sua elevada toxicidade em altas concentrações, potencial para causar disfunção endócrina e, no caso de metais e hidrocarbonetos, tendência em acumular na biota e persistência ambiental (Kennish 1991). Logo, a contaminação por estes elementos pode produzir efeitos sub-letais e até mesmo letais nos organismos (Santos *et al*. 2000, Pinho *et al*. 2007), modificando significativamente a estrutura e as populações dos ecossistemas envolvidos (Bielmyer *et al.* 1995, Langston & Bebianno 1998, Paytan *et al.* 2009).

Com o intuito de detectar precocemente a poluição ambiental, prevenir seus efeitos nos organismos e fornecer informação para as atividades de manejo, inúmeros estudos têm sido realizados e diversas estratégias de monitoramento têm sido empregadas (Zagatto 2006). Para tanto, são realizadas medidas de qualidade ambiental que envolvem aspectos físicos, químicos e biológicos, onde os compartimentos monitorados são geralmente a água, o sedimento e a biota (Seeliger *et al*. 1988). Cada um destes compartimentos do ecossistema apresenta vantagens e desvantagens analíticas, porém a análise da biota é certamente a que oferece maiores informações de relevância biológica, em termos de biodisponibilidade e potencial toxicidade (Neto *et al.* 2008).

Uma vez que tais estudos objetivam manter a integridade e qualidade dos ecossistemas, se torna fundamental a compreensão das interações entre os contaminantes e organismos (Bortone 2005). Diferentes grupos de organismos são apontados como potenciais modelos ecotoxicológicos (Zagatto 2006), biomonitores ou bioindicadores (Seeliger *et al*. 1988), entre os quais se destacam os peixes, que são utilizados especialmente devido à sua importância econômica e também ecológica (Phillips 1980). Dentre os parâmetros avaliados nestes organismos estão a concentração química do contaminante em determinado tecido (Seeliger *et al*. 1988) e os efeitos biológicos do contaminante (Neto *et al.* 2008). Estes efeitos biológicos são chamados de biomarcadores, que são definidos como respostas mensuráveis nos fluídos

corporais, células ou tecidos ou organismo que indicam, em nível bioquímico, celular ou comportamental, a presença de contaminantes (para revisão: Monserrat *et al.* 2007).

De fato, os biomarcadores constituem uma ferramenta com grande potencial para o monitoramento ambiental, sendo recorrente o seu emprego em estudos de biomonitoramento (Rose *et al.* 2006), bem como crescente o consenso de que informações obtidas a partir deles são de grande importância para inferência em níveis biológicos superiores (Monserrat *et al.* 2007). Entretanto, estas práticas não estão incorporadas nas normativas ambientais, tampouco são comuns em monitoramentos ambientais para fins de fiscalização, em virtude da pequena quantidade de informação e controvérsia sobre como cada contaminante interage com determinado biomarcador e como suas repostas podem ser extrapoladas a níveis superiores da hierarquia biológica (Brown *et al.* 2004). Por isso, são necessários estudos no sentido de padronizar organismos e níveis de respostas para os distintos biomarcadores, bem como definir um espectro de respostas que possam ser consideradas "normais".

Diversos trabalhos têm sido desenvolvidos no sentido de compreender holisticamente a informação obtida a partir da análise de biomarcadores medidos em campo ou em exposições laboratoriais (Monserrat *et al.* 2007). Neste contexto, se insere o Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática (INCT-TA), cujos objetivos são padronizar espécies e biomarcadores para avaliação de ambientes aquáticos dulcícolas, salobros ou estuarinos e marinhos, bem como difundir informação sobre o seu uso dentre a população em geral. Dentre as diversas ações do INCT-TA, se insere o presente trabalho, cuja maior contribuição é apresentar ineditamente, em um potencial biomonitor, as respostas de diversos biomarcadores à exposição a substâncias típicas das três classes de contaminantes mais problemáticas no Brasil, a saber, metais, herbicidas e hidrocarbonetos policíclicos aromáticos (HPA).

O modelo biológico escolhido para o presente estudo foi o peixe teleósteo *Poecilia vivipara* (Bloch & Schneider 1801 *apud* Lucinda & Reis 2005), espécie abundante na maioria dos ambientes aquáticos continentais brasileiros, lênticos e hemi-lênticos, dulcícolas e estuarinos (Gomes & Monteiro 2008). Este organismo apresenta um comportamento alimentar oportunista, habitando preferencialmente águas rasas e calmas, com fundos ricos em argilas e macrófitas aquáticas (Mazzoni & Iglesias-Rios 2002), o que o torna potencialmente exposto no ambiente às três classes de contaminantes que foram avaliadas no presente trabalho. Esta espécie de peixe também apresenta abundância na natureza e fácil manutenção em laboratório, de modo que tem sido utilizado como modelo ecotoxicológico de espécie dulcícola e estuarina (Corrêa *et al.* 2010). Adicionalmente, estudos de sensibilidade com *P. vivipara* apontam para uma elevada tolerância desta espécie a metais (Chung 1983, Araújo *et al.* 2001). Tal tolerância sugere a existência de mecanismos de detoxificação que permitem a sua sobrevivência em ambientes contaminados, sendo que tais mecanismos são potenciais biomarcadores para avaliação da contaminação aquática. Além disso, por estarem presentes em vários gradientes de parâmetros ambientais, as populações de *P. vivipara* são expostas a variadas condições, inclusive de qualidade ambiental e exposição a poluentes (Gomes & Monteiro 2008). Logo, *P. vivipara* pode ser considerado um potencial biomonitor para as águas doces e estuarinas, de maneira que se torna evidente a necessidade de conhecimento sobre as respostas biológicas deste organismo a contaminantes ambientais.

Quanto aos modelos de contaminantes, para o presente estudo foram escolhidos o cobre, a atrazina e o fenantreno, como representantes das classes de metais, pesticidas e hidrocarbonetos, respectivamente. Estes contaminantes foram selecionados principalmente devido às diversas características comuns que compartilham. Primeiramente, são amplamente utilizados e economicamente importantes no Brasil, o que significa dizer que apresentam presença ambiental em níveis relevantes (Zagatto 2006, Neto *et al.* 2008). Além disso,

existem lacunas na legislação ambiental ou esta é inapropriada no tocante aos limites de concentração para estes compostos. Por exemplo, os limites estabelecidos para o cobre são baseados em legislações de outros países e não em estudos realizados com espécies nativas do Brasil. O limite para atrazina existe apenas para águas doces, enquanto o fenantreno sequer tem sua concentração limitada para águas nacionais (CONAMA, 2005). E por fim, estes contaminantes apresentam o comportamento ambiental e/ou mecanismos de toxicidade relativamente bem identificados, o que simplifica consideravelmente a interpretação e extrapolação das respostas dos biomarcadores.

Quanto aos biomarcadores avaliados no presente estudo, foram selecionados aqueles mais difundidos em programas de biomonitoramento de cunho científico e que apresentassem capacidade de integração de efeitos em distintas vias metabólicas e diferentes níveis da organização biológica. Assim, foram analisados brânquias e fígado, como exemplo órgãos-alvo da toxicidade dos contaminantes, bem como sangue e músculo como órgãos não-alvo. Nas brânquias, fígado e músculo foram avaliados biomarcadores de estresse oxidativo e/ou detoxificação, a saber, geração de espécies reativas de oxigênio (EROS), capacidade antioxidante (ACAP), atividades das enzimas superóxido dismutase (SOD), catalase (CAT), glutationa reductase (GR) e glutationa S-transferase (GST), lipoperoxidação (LPO) e concentração de metalotioneínas (MT). Nos eritrócitos foram analisados biomarcadores de dano de DNA relacionados com potencial mutagênico e carcinogênico, a saber, as anomalias nucleares e o ensaio cometa. Também foi avaliada a composição corporal dos íons maiores (Ca, K, Na, Mg e Cl), bem como a acumulação de cobre como respostas do organismo aos contaminantes.

2. MATERIAL E MÉTODOS

Os métodos utilizados no presente estudo estão detalhadamente descritos nos apêndices 1, 2 e 3. Estes descrevem os experimentos de exposição e procedimentos para análise dos biomarcadores nos experimentos de exposição ao cobre, atrazina e fenantreno, respectivamente. Assim, aqui serão apenas delineados os aspectos gerais do trabalho experimental.

2.1. Coleta dos animais e exposição aos contaminantes

Exemplares machos de *P. vivipara* foram coletados no Arroio do Gelo (Praia do Cassino, Rio Grande, RS, Brasil) entre maio de 2010 e março de 2011. Estes organismos foram mantidos sob fotoperíodo (12C:12E), temperatura (20˚C) e salinidade (24) constantes e alimentados com ração comercial (Alcon Basic) por duas semanas.

Após aclimatação, os peixes foram expostos ao cobre, atrazina ou fenantreno. Foram também realizados controles dos solventes utilizados para a atrazina (metanol) e fenantreno (DMSO). O meio experimental consistiu em água do mar natural diluída à salinidade 24, filtrada (0.45 μm) e contaminada em laboratório com um dos contaminantes testados. A exposição dos peixes aos contaminantes ocorreu apenas via água do mar contaminada, e os demais parâmetros experimentais foram mantidos conforme descrito acima para o período de aclimatação. Para a exposição aos contaminantes, a densidade de peixes foi fixada em 1 $g.L^{-1}$, sendo que o meio experimental foi completamente renovado diariamente. Para cada contaminante foram escolhidas três concentrações de exposição, que abrangem as concentrações mais típicas e ambientalmente relevantes. Assim, para o cobre foram testadas as concentrações de 5, 9 e 20 $\mu g.L^{-1}$. Para a atrazina, estas foram de 4, 10 e 100 $\mu g.L^{-1}$. No caso do fenantreno, os organismos foram expostos a 10, 20 e 200 $\mu g.L^{-1}$. Concomitantemente às exposições, foram avaliados grupos controles, que receberam o mesmo tratamento dos indivíduos expostos, à exceção da contaminação do meio com cobre, atrazina ou fenantreno. Para atrazina e fenantreno, também foram realizados controles específicos para avaliar possíveis efeitos do solvente utilizado para

dissolver o contaminante na água salgada. Neste caso, somente o solvente utilizado para cada contaminante foi adicionado ao meio experimental.

Após exposição aos contaminantes, os peixes foram anestesiados com benzocaína (0,1 $g.L^{-1}$), pesados (peso úmido) e medidos (comprimento total) e tiveram os seus tecidos (sangue, fígado, brânquias e músculo) dissecados. O sangue foi imediatamente usado no ensaio cometa e na avaliação de anomalias nucleares. Algumas amostras de fígado, brânquias e músculo foram imediatamente usadas para análise de produção de EROS, enquanto a maioria das amostras foi imediatamente congelada em ultra-freezer (-80^{o}C) para serem posteriormente analisadas quanto às atividades enzimáticas.

2.2. Análise dos contaminantes

A concentração de cada contaminante foi analisada no início e após 24 h de exposição, quando o meio experimental foi completamente renovado. A concentração de cobre foi analisada por espectrofotometria de absorção atômica no modo chama. As concentrações de atrazina e fenantreno foram avaliadas por cromatografia gasosa com sistema de detecção de massas acoplado. Estas últimas análises foram realizadas no Laboratório de Contaminantes Orgânicos e Ecotoxicologia da FURG.

2.3. Análise dos biomarcadores

Para avaliar os efeitos dos contaminantes em nível molecular e clastogênico, os biomarcadores de dano de DNA foram analisados em eritrócitos periféricos de *P. vivipara*. Foram considerados dois tipos de danos: (1) reversíveis, analisados com metodologia adaptada de Tice *et al.* (2000); e (2) irreversíveis, que foram avaliados conforme metodologia adaptada de Barsiene *et al.* (2006).

Para avaliar os efeitos em nível bioquímico e de danos em tecidos alvos e não alvos da toxicidade, biomarcadores de estresse oxidativo foram medidos em amostras de brânquias, fígado e músculo. A determinação da concentração de proteínas foi realizada utilizando-se um kit comercial de reagentes (Microprote, Doles, Brasil) baseado no método de Bradford. A produção de EROS e capacidade antioxidante contra radicais peroxil (ACAP) foram avaliadas conforme descrito por Amado *et al.* (2009). A lipoperoxidação (LPO) foi medida pelo método descrito por Oakes e Van Der Kraak (2003). A atividade da catalase (CAT) foi avaliada pela degradação do H_2O_2, conforme descrito por Beutler (1975). Por sua vez, a medida da atividade da superóxido dismutase (SOD) foi realizada através da análise da redução do citocromo C, de acordo com McCord e Fridovich (1969). A atividade da glutationa redutase (GR) foi avaliada pelo método do consumo de NADPH na presença de glutationa oxidada (Carlberg & Mannervik 1975), enquanto a atividade da glutationa S-transferase (GST) foi medida através da conjugação do CDNB com glutationa reduzida (Keen *et al.* 1976). O conteúdo de metalotioneínas foi analisado conforme o método descrito por Viarengo *et al.* (1997).

Como respostas em nível organísmico foram avaliadas a acumulação de cobre e a composição iônica corporal. Para tanto, organismos expostos aos contaminantes foram rapidamente (30 s) lavados em água tipo MilliQ, pesados e crio-sacrificados. Após 96 h de secagem em estufa (~70^{o}C), foi determinado o peso seco e o material digerido em acido nítrico 65% (SupraPur, Merck). Após completa digestão, as amostras foram apropriadamente diluídas para a análise da composição iônica (Ca, K, Mg e Na) e concentração de cobre corporal, as quais foram determinadas por espectrofotometria de absorção atômica no modo chama. A concentração de cloretos foi analisada pelo método de formação de cianeto férrico de enxofre utilizando-se um kit comercial de reagentes (Doles, Brasil).

2.4. Análises estatísticas

Todas as análises estatísticas foram realizadas utilizando-se uma abordagem bayesiana, de acordo com as soluções analíticas propostas por Kinas e Andrade (2010). É valido lembrar que no presente trabalho foi avaliado um grande número de biomarcadores e tratamentos, de maneira que o aumento de um organismo em cada biomarcador significaria inclusão de cerca de 140 animais adicionais. Assim restrição no tamanho amostral foi uma constante durante o desenvolvimento de todo o trabalho. Também os dados de dano de DNA eram altamente não normais, de modo que o enfoque bayesiano com a família Poisson-Gama melhor representou a distribuição destes parâmetros. Adicionalmente, o presente trabalho foi desenvolvido visando a promoção do emprego de biomarcadores em programas biomonitoramentos e manejo ambiental. Neste contexto, cabe ressaltar que o enfoque bayesiano fornece relevante ferramenta para o estabelecimento de modelos que visem fornecer base científica sólida para avaliação ambiental. Assim, devido à natureza dos dados e questões a serem respondidas, a abordagem bayesiana proporcionou uma interpretação mais realista e aprofundada dos dados, além de possibilitar a elaboração de modelos *a posteriori* que descrevem as relações de efeitos entre cada contaminante e biomarcador. Pelos motivos expostos acima, a abordagem estatística adotada constitui um diferencial do presente estudo, mais sensível às peculiaridades dos dados, e cujo muitos dos frutos poderão ser também observados nos futuros trabalhos com abordagens ambientais. Assim, médias e variâncias foram estimadas a partir da distribuição *a posteriori* dos dados. Para cada biomarcador, uma distribuição específica de probabilidade foi elaborada, sendo ela da família Poisson-Gama para os dados de contagens de dano de DNA e da família Normal-Normal para os demais biomarcadores. Testes de hipóteses foram realizados de acordo com a decisão de Bayes, baseados no fator de Bayes e nas chances *a priori* e *a posteriori*.

3. SÍNTESE DOS RESULTADOS

A maioria dos biomarcadores se mostrou sensível aos contaminantes nas concentrações testadas. Entretanto, poucos apresentaram uma evidente curva de resposta dependente da concentração nas condições experimentais utilizadas. O conjunto de biomarcadores que apresentou uma evidente resposta dependente da concentração do contaminante foi diferente para cada contaminante testado, conforme apresentado abaixo.

3.1. Cobre e biomarcadores

O efeito do cobre foi caracterizado por uma evidente indução de estresse oxidativo associado com genotoxicidade, clastogenicidade e desequilíbrio iônico. Entre os efeitos referentes ao status oxidativo se destacam a diminuição da atividade da SOD e aumento da atividade da CAT, da produção de EROS e da ACAP no fígado. A interação destas respostas culminou numa situação de estresse oxidativo, com ocorrência de peroxidação lipídica no fígado e brânquias. Danos oxidativos correlacionados com desequilíbrios iônicos evidenciaram a função do cobre, enquanto agente oxidativo, no seu mecanismo clássico de toxicidade: disfunção iônica. As quebras simples e duplas na estrutura do DNA de eritrócitos foram aumentadas pela exposição ao cobre, atingindo um máximo no grupo de peixes expostos a 9µg $Cu.L^{-1}$. A genotoxicidade também foi aumentada, sendo que anomalias nucleares como brotamentos e fragmentos apoptóticos seguiram o mesmo padrão de respostas das quebras de DNA. A clastogenicidade, verificada através da medida da frequência de células micronucleadas, foi significativamente aumentada com uma aparente resposta dependente da concentração de cobre. Quanto à composição iônica corporal, a exposição ao cobre aumentou significativamente as concentrações de Na, K e Mg, sendo que este último apresentou uma evidente resposta dependente da concentração (r^2=0,97).

3.2. Atrazina e biomarcadores

O efeito da atrazina foi marcado pelo aumento das atividades da GST e CAT no fígado, redução da CAT no músculo e aumento da geração de ROS em todos os tecidos analisados, o que culminou com um incremento da LPO nas brânquias após exposição dos peixes a 100 µg atrazina.L^{-1}. A medida de quebras da fita de DNA pelo ensaio cometa indicou uma diminuição deste tipo de dano, sugerindo que o efeito deletério associado à exposição à atrazina ocorra principalmente pela formação de adutos. A genotoxicidade foi observada após exposição dos peixes a qualquer uma das concentrações de atrazina testadas. Entretanto, a frequência de células micronucleadas foi significativamente aumentada somente na concentração de 100 µg atrazina.L^{-1}. Perturbações da composição iônica foram observadas nos conteúdos corporais de Cl, Mg, Na e K, além de ter sido observado um acúmulo corporal de cobre.

3.3. Fenantreno e biomarcadores

O hidrocarboneto fenantreno também causou diversos efeitos nos biomarcadores avaliados. O grupo de biomarcadores mais afetados nas condições testadas foram os de dano de DNA. O fenantreno demonstrou um elevado potencial para genotoxicidade. Além disso, foram observadas clastogenicidade e citotoxicidade. Em biomarcadores de estresse oxidativo, podem ser destacadas a inibição da atividade da SOD no fígado, incremento da concentração de metalotioneínas no fígado, grande aumento da geração de ROS em todos os tecidos e aumento da ACAP em fígado e brânquias. Estes efeitos induziram uma significativa LPO nos músculos. O fenantreno também afetou a composição iônica corporal, notadamente no que se refere aos conteúdos de Mg, K e Na.

4. CONCLUSÕES

Embora os três contaminantes testados (cobre, atrazina e fenantreno) sejam capazes de afetar todos os biomarcadores avaliados, cada um destes contaminantes afetou classes de

biomarcadores de forma diferente e característica. Assim, os resultados apresentados neste estudo evidenciam aspectos característicos sobre mecanismos de toxicidade de cada um dos contaminantes testados, bem como identificam biomarcadores de maior potencial para biomonitoramento em cada situação em particular.

Neste sentido, os danos oxidativos em lipídios e no DNA induzidos pelo cobre parecem estar associados a uma situação de estresse oxidativo. Assim, os biomarcadores que melhor evidenciam uma resposta dependente da concentração do contaminante são: lipoperoxidação, frequência de micronúcleos e conteúdo corporal de Mg. Já para a atrazina, os danos parecem estar relacionados a um dano oxidativo no DNA com formação de adutos e clastogenicidade. Deste modo, a elevada atividade de enzimas de detoxificação da fase II, como GST no fígado, associada a lipoperoxidação em brânquias e atividade genotóxica não associada à quebras na fita de DNA detectáveis no ensaio cometa, e perturbações iônicas generalizadas, potencialmente distinguem o efeito da atrazina. Por fim, o fenantreno parece alterar significativamente a formação de ROS, de tal forma que uma situação de estresse oxidativo é gerada no músculo. Neste caso, a maior parte dos efeitos tóxicos provavelmente está associada ao processo de detoxificação e geração de metabólitos altamente reativos capazes de danificar o DNA. Assim, os índices de dano de DNA são biomarcadores fundamentais que, associados a alterações generalizadas nas atividades de enzimas associadas ao estresse oxidativo e na composição iônica corporal, descrevem os efeitos do fenantreno.

Em resumo, os resultados do presente trabalho confirmam o potencial dos biomarcadores em estudos de diagnóstico da contaminação ambiental por compostos químicos. Futuros estudos modelando a interação entre cada classe de contaminante e os respectivos biomarcadores serão fundamentais para a adequada aplicação destas ferramentas em programas de biomonitoramento. Assim, os biomarcadores, além de constituírem ferramenta de avaliação da saúde ambiental com elevado significado biológico, poderão ser também utilizados com vistas a acessar e diferenciar

os níveis dos contaminantes aos quais os organismos foram expostos. Por fim, uma abordagem multi-biomarcadores, como a realizada no presente trabalho, será elementar para a correta compreensão do cenário ambiental.

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APÊNDICE 1

Biochemical, physiological and genetic responses to waterborne copper exposure in the guppy *Poecilia vivipara* acclimated to saltwater

Biochemical, physiological and genetic responses to waterborne copper exposure in the guppy *Poecilia vivipara* acclimated to saltwater

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ABSTRACT

The response of a large suite of biochemical, physiological and genetical biomarkers were evaluated in tissues (liver, gills, muscle and erythrocytes) or in the whole-body of the estuarine guppy *Poecilia vivipara* exposed to waterborne copper in salt water (salinity 24 ppt). Activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase, and glutathione S-transferase), concentration of metallothionein-like proteins, reactive oxygen species (ROS) production, total antioxidant capacity against peroxyl radicals (ACAP), and lipid peroxidation (LPO) were evaluated in liver, gills and muscle. Comet assay score and frequencies of nuclear abnormalities and micronucleated cells were analyzed in peripheral erythrocytes, while copper and ions (Na, K, Ca, Mg and Cl) concentrations were determined in the fish whole-body. The responses of these biomarkers were evaluated in fish exposed for 96 h to environmentally relevant concentrations of copper (5, 9 and 20 µg $Cu.L^{-1}$). Guppies were tolerant to copper and able to regulate the whole-body copper concentration at low copper concentrations. However, they accumulated copper in their body when exposed to 20 µg $Cu.L^{-1}$. Data from whole-body ionic composition clearly indicated that waterborne copper exposure induced ionoregulatory disturbances (Na, K, and Mg) in *P. vivipara*, suggesting that regulatory mechanisms taking place at gills and kidney were likely affected by copper exposure. Also, they showed that these disturbances were significantly correlated with the level of oxidative damage in gills, suggesting an indirect effect of copper on the ion regulatory mechanisms. Regarding the biomarkers of oxidative status, almost all of them proved to be affected by waterborne copper exposure. However, catalase activity in liver, ROS, ACAP and LPO in muscle, gills and liver, DNA damages in erythrocytes, and whole-body ion (Na, K, and Mg) composition showed to be the most promising biomarkers to assess the exposure to waterborne copper in *P. vivipara.* These biomarkers showed a response clearly dependent on copper concentration and can be considered as biologically relevant.

Keywords: biomarkers, ionic regulation, DNA damage, oxidative stress

INTRODUCTION

The constant effort to prevent or recover impacted environments has been proved to be not enough to stop or reduce the increasing concentration of many chemical contaminants in aquatic ecosystems. In this context, metals are anthropogenic pollutants of great concern (Phillips, 1980; Nemerow, 1991; Heath, 1995; Langston and Bebianno, 1998). They are naturally ubiquitous in aquatic environments, but their concentrations are increasing as a result of the growing discharges into the environment from domestic and industrial effluents, as well as fossil fuels burning (Neto et al, 2008; Paytan, 2009). Mining, groundwater use, and soil waterproofing and erosion are also considered as secondary sources of metals input into aquatic environments (Heath, 1995).

Metals can be considered as harmful contaminants to aquatic animals, especially if one considers their environmental persistency, bioavailability in water, potential for bioaccumulation, and toxicity (Kennish, 1991). Thus, water contamination with these chemicals may result in both sublethal and lethal effects (Santos et al., 2000; Pinho et al., 2007), modifying populations and the structure of the impacted ecosystems (Bielmyer et al., 1995; Langston and Bebianno, 1998; Paytan, 2009).

Copper is a transition metal essential to the metabolism (Heath, 1995), becoming toxic to estuarine and marine invertebrate and fish when at elevated concentrations (Santos et al., 2000; Pinho et al., 2007; Martins and Bianchini, 2008; Lopes et al., 2011a). It is well accepted that the key mechanism of acute copper toxicity in fish is associated with ionic and osmotic disturbances due to impairments especially on Na^+ and Cl^- regulation linked to the inhibition of the gill Na^+-K^+-ATPase activity. Furthermore, it is reported that these disturbances can lead to death in both freshwater and seawater fish (Grosell et al., 2002, 2007; Alsop and Wood, 2011). However, it is not understood if these disorders are resulting from a direct

inhibitory effect on the enzyme activity or are related to damages associated with a copper-induced oxidative stress. Therefore, the evaluation of the ionic disturbances induced by exposure to sublethal concentrations of waterborne copper paralleled by the response of oxidative stress parameters (biomarkers) would help to better understand the biochemical basis of these disturbances in fish. In addition, it would also help to explain some detrimental biological effects observed after fish exposure to waterborne copper. For example, it has been reported that copper can induce endocrine disruption and changes in metabolic rates (Handy, 2003), behavior (Phillips, 1980), immunological function, swimming performance (Heath, 1995), enzyme activities, and liver histology (Langston and Bebianno, 1998). As far as we know, studies evaluating concomitantly the responses of a large suite of biochemical, physiological and genetic damages biomarkers to characterize the sublethal effects of copper other than those associated with the ionic and osmotic regulation in a single marine fish species are lacking in the literature.

Biomarkers are considered useful alternative tools in biomonitoring programs and have been usually employed in environmental health assessment studies (Rose et al., 2006). They are being pointed out as potential tools for an early detection of environmental pollution, as well as to prevent its biological effects and provide information for management. In this context, biomarkers can be considered as measurable responses on body fluid, cells, tissues or organism indicating the presence of contaminants (for review: Monserrat et al., 2007). However, they are not being used to derive water quality criteria yet. Also, there is not a general agreement on the level of the biomarker response characterizing an environment as chemically polluted or biologically impacted. This likely arises from the fact that there is controversial information on how the biomarker response is specific to the contaminant exposure. Therefore, studies involving a large suite of biomarkers at different levels of the biological organization are necessary to identify the more adequate ones for biomonitoring

purposes, as well as to establish the relationship between the degree of the biomarker response and the environmental health condition.

With this background in mind, the present study was performed to describe, for the first time, the comparative response of a large set of biochemical, physiological and genetic biomarkers to sublethal concentrations of waterborne copper in the guppy *Poecilia vivipara* (Bloch and Schneider, 1801). This euryhaline teleost fish is native from Brazil and commonly found in coastal water bodies along the South Atlantic Ocean (Gomes and Monteiro, 2008). It has been pointed as a promising fish species to monitor the health conditions of tropical and sub-temperate coastal waters (INCT-TA, 2012). Endpoints evaluated in the present study included whole-body copper accumulation, whole-body ion (Na, K, Ca, Mg and Cl) composition, oxidative status parameters (reactive oxygen species production, total antioxidant capacity, enzymatic and non-enzymatic antioxidants), and oxidative damage to biomolecules (lipid peroxidation and DNA damage) in several tissues (liver, gills, and muscle or peripheral erythrocytes). The responses of these biomarkers were evaluated considering their suitability to detect biological effects associated with exposure to low and high environmental copper concentrations.

MATERIAL AND METHODS

Fish collection and copper exposure

Male fish (*P. vivipara*) were collected from May 2010 to December 2011 at the 'Arroio do Gelo', a small creek running into the Cassino Beach (Rio Grande, RS, Southern Brazil). Fish were transferred to the laboratory and kept under controlled conditions (12 L: 12

D photoperiod, temperature 20 ^{o}C, and salinity 24 ppt) for two weeks. Fish were daily fed until satiation with commercial fish food (Alcon Basic, Camboriú, SC, Brazil).

Seawater was diluted with dechlorinated tap water to prepare salt water at the desired salinity (24 ± 1 ppt). Salt water was then filtered (0.45 μm mesh filter) and contaminated with copper ($CuCl_2$ [Vetec Química Fina, São Paulo, Brazil]) from a stock solution (1 mg $Cu.L^{-1}$). All glassware and exposure chambers were previously acid washed and thoroughly rinsed with distilled water. Contamination of the exposure medium was performed 24 h before fish's introduction into the test chambers to allow copper equilibration with salt water. Three copper concentrations (nominal concentrations: 5, 9 and 20 μg $Cu.L^{-1}$) were tested along with a control (no copper addition into the experimental medium). The concentrations of 5 and 9 μg $Cu.L^{-1}$ actually represent the Brazilian water quality criteria for seawater and freshwater, respectively. In turn, 20 μg $Cu.L^{-1}$ was selected as a non-conforming copper concentration according to the Brazilian regulation (CONAMA, 2005). It is important to note that these concentrations can also be considered as representative of a non-polluted, moderately contaminated and highly contaminated environment, respectively.

Total and dissolved copper concentrations were measured immediately before fish's introduction into the exposure medium and 24 h after exposure. Copper concentrations were measured in non-filtered (total copper) and filtered (0.45 μm mesh filter; dissolved copper) water samples by atomic absorption spectrophotometry (AAS 932 Plus, GBC, IL, USA) following procedures previously described (Pinho et al., 2007; Martins and Bianchini, 2008; Lopes et al., 2011b). Results obtained are shown in Table 1, but for practical reasons, copper concentrations will be referred hereafter considering the nominal values.

Dissolved oxygen was kept close to the saturation level by continuously and gently bubbling air into the experimental medium, which was totally renewed every 24 h to maintain

pH (~7.8) and other water physicochemical parameters constant. Adult males of *P. vivipara* were exposed to copper in 30-L glass aquaria for 96 h. Fish stocking density was 1 g fish.L^{-1}.

After copper exposure, fish were anesthetized with benzocaine (0.1 g.L^{-1}), weighed (wet body weight), and measured (total body length). Blood was collected by puncture of the caudal vein and immediately used for comet and nuclear abnormalities assays. Liver, muscle and gills samples were then dissected and immediately used for ROS and ACAP measurements or frozen (-80^{o}C) for further enzyme activities analyses. For whole-body copper accumulation and ion composition, anesthetized fish were quickly rinsed in distilled water and immediately frozen (-80^{o}C) for further analyses. For practical reasons, the exposure procedure was repeated three times. Fish from the first exposure (wet body weight: 0.18 ± 0.07 g; total body length: 24.95 ± 4.57 mm) were used for whole-body copper concentration and ion composition analyses. Tissue samples of fish from the second exposure (wet body weight: 1.07 ± 0.31 g; total body length: 41.6 ± 4.4 mm) were used for enzymes activities, lipid peroxidation (LPO) and nuclear abnormalities analyses. Tissue samples of fish from the third exposure (wet body weight: 1.01 ± 0.31 g; total body length: 43.13 ± 4.32mm) were used for comet assay, production of reactive oxygen species (ROS), total antioxidant capacity against peroxyl radicals (ACAP) and metallothionein-like proteins (MT) assays.

All experiments were performed in duplicate. The number of fish exposed in each experiment was dependent on the variability of the biomarker to be analyzed, as previously determined, and fish availability in the laboratory, as described below.

Biomarkers analyses

Whole-body copper accumulation and ionic composition

Copper and major ions contents were determined in whole body of *P. vivipara* (n = 5). After the 96 h exposure period, fish were quickly rinsed in ultrapure water, dried at 68 ± 10ºC for ~ 96 h and weighted (dry body weight). Samples were completely digested in HNO_3 65% (Suprapur, Merck, USA) for 72 h, and properly diluted for measurements of Cu, Ca, K, Mg and Na concentrations by atomic absorption spectrophotometry (AAS 932 Plus, GBC, IL, USA) following procedures previously described (Pinho et al., 2007; Martins and Bianchini, 2008; Lopes et al., 2011a,b). Chloride concentration was measured using a commercial reagent kit based on the formation of sulfur cyanide ferric (Cloretos, Doles, Goiânia, GO, Brazil) with minor modifications.

Oxidative stress parameters

Biomarkers of oxidative status were measured in gills (target organ), liver (detoxification organ) and muscle (non-target organ) samples. ROS production and ACAP were determined (n = 4-6 fish for each experimental group) according to Amado et al. (2009). MT concentration was measured (n = 7-9 fish for each experimental group) based on the DTNB reaction with sulfidryl groups following the procedures described by Viarengo et al. (1997).

For analyses of antioxidant enzymes activities, protein concentration in the tissue homogenate was determined using a commercial reagent kit based on the Bradford reagent method (Sigma, São Paulo, SP, Brazil). Catalase (CAT) activity was evaluated (n = 7-9 fish for each experimental group) based on the H_2O_2 degradation according to Beutler (1975). Superoxide dismutase (SOD) activity was evaluated (n = 4-6 fish for each experimental group) following the cytochrome C reduction according to the procedures described by McCord and Fridovich (1969). Glutathione reductase (GR) activity was measured (n =5 fish for each experimental group) following the NADPH consumption in the presence of oxidized

glutathione (GSSH) according to Carlberg and Mannervik (1975). Glutathione *S*-transferase (GST) activity was assessed (n = 5-7 fish for each experimental group) through the conjugation of the reduced glutathione (GSH) with CDNB according to Keen et al. (1976).

Oxidative damage parameters

LPO was measured (n = 6-10 fish for each experimental group) using the thiobarbituric acid reactive substances method (TBARS), following the procedures described by to Oakes and Van Der Kraak (2003).

Erythrocytes freshly obtained were used to analyze oxidative DNA damages (reversible and non-reversible ones). Single or double reversible DNA strand breaks were scored (n = 4-6) using the comet assay following the procedures described by Tice et al. (2000), with some modifications. In this case, analysis was performed using red gel as fluorescent marker. The tail of each comet was classified into 4 types of damage, with indexes varying from 0 up to 3 (class 0, class 1, class 2 and class 3). Increasing class index corresponded to augmented DNA damage. Approximately 100 nucleoids were observed per fish. Measurements were performed in duplicate. Comet score was calculated multiplying the number of nucleoids belonging to each class by the respective class index. Therefore, higher comet scores corresponded to higher DNA strand breaks.

Non-reversible DNA damages were accessed in 10^3 cells per fish (n = 16) by counting the frequency of micronucleated cells, nuclear buds, binucleated cells and cells with nucleus presenting apoptotic fragments. These parameters were evaluated following the procedures described by Barsiene et al (2006). The frequency of cells with bilobed nucleus was also considered. It is known that hematopoietic organs can release immature cells into the fish bloodstream (Heath, 1995). These immature erythrocytes may show a nucleus still in division, which has the appearance of a cell with bilobed nuclei. Therefore, the frequency of

erythrocytes with bilobed nucleus was considered as an index of immature cells release into the blood stream.

Statistical analyses

Statistical analyses were performed using Bayesian methods according to Gelman et al. (2004). Briefly, all data of each biomarker generated at laboratory experimental work was conjugated with priori distribution of probability to build a posteriori probabilistic model that better describes the uncertainty on the response of the biomarker analyzed. Thus, for each biomarker, the assumed as most appropriate model of probability distribution for expectancies and variances was created, which was not limited to the normal model. Moreover, this method statistically evaluates the likelihood of each possible average associated to each standard deviation for each biomarker, allowing more deep and adjusted inference. It is worth to note that the Bayesian approach came up as the most adequate method to statistically analyze the probability of alternative hypothesis given the ecotoxicological context in which the present work was developed, and the data effectively available from the present work. Additionally, this approach allows for a permanent reevaluation of the strength (i.e. probability) of competing hypotheses, each time new data are incorporated into the analysis. Therefore, data shown in the present work, and most importantly their models, were used to obtain the present Bayesian a posteriori distributions, which allow the incorporation of new data from future laboratory and even environmental studies. This potentiates and better reflects the increment of the scientific knowledge produced, aiming to a more adequate and solid environmental management. The mathematical basis for all statistical procedures used in the present work is explained in details by Kinas and Andrade (2010). Methods used in the present study are briefly described below.

Conjugated families of probability were used to calculate posteriori distributions. In terms of variables the biomarkers analyzed were two kinds: the concentrations and/or rates, and the counting. As described by literature (Gelman et al., 2004) data of concentrations and rates trend to have Normal distributions, while counting are likely described by Poisson distribution. Thus, the Normal-Gamma posterior distributions conjugated family with non-informative priori were used to estimate averages and standard deviations for most of biomarkers analyzed and assumed to have a Normal distribution (Table 2). For nuclear abnormalities and comet assay data, the conjugated family Gama was used since these data were counting and assumed to have a Poisson distribution. This Gamma distributions have two prior hyper-parameter, which are α and β. They may be found because their strict relationship with prior averages and standard deviation (Gelman et al., 2004). In this case, α is the square of average divided by the square of standard deviation, and β is described as average divided by the square of standard deviation. Thus, prior averages and deviations used for DNA damage biomarkers were obtained from Barsiene et al. (2006), Negreiros et al. (2011) and Amado et al. (2006). Values reported in these studies for control animals were compiled and used to adjust priori parameters for one gamma probability distribution for each DNA damage biomarker, where the priori expectancies were the means found in those studies, while prior standard deviations were the values found in the studies multiplied by 3, to increase prior open-mindedness. The respective hyper-parameters (α and β) for each DNA damage biomarker are also shown in table 2.

To access the strength of evidence for differences among treatments frequentist hypothesis testing was replaced with Bayes's decision, based on the posteriori odds ratio and Bayes Factor (Jeffreys, 1961). The posterior distributions of differences of the most likely averages for each treatment and biomarker were obtained from simulations and evaluated using unilateral tests. Posterior distributions of differences concentrated around zero favor

hypothesis zero (H_0), wich negates the difference between averages; while posterior distributions of differences away from zero favor hypothesis 1 (H_1), which accept it. For these tests, priori odds ratio = 1 (i.e. giving the same prior probability to both H_0 and H_1), and loss w_0= 5 and w_1=1 for erroneously rejecting H_0 and H_1, respectively. Bayes Factor (BF) is shown when pointed significant difference at results sections. It may be worth to mention that BF express how many times H_1 is more probable than H_0, and BF bigger than 3.14 is considered substantial evidence against H_0 (Jeffreys, 1961). In this context, the present work assumed a conservative posture, rejecting H_0 only when BF was bigger than 5.

Relationships among the different parameters analyzed were determined using the Pearson linear correlation coefficient. Finally, in the graphs, data were shown as mean ± standard deviation for all biomarkers.

All data processing was performed using Excel and R software while graphs were plotted using the SigmaPlot software.

RESULTS

Whole-body copper and ion content

No mortality was observed in control and copper-exposed fish. An increase in whole-body copper content was observed only in fish exposed to 20 µg $Cu.L^{-1}$ in respect to those exposed to 9 µg $Cu.L^{-1}$ (BF > 5) (Fig. 1). Fish exposed to 5 µg $Cu.L^{-1}$ showed significantly increased whole-body concentrations of K (BF > 21) (Fig. 2), while those exposed to 9 µg $Cu.L^{-1}$ had increased whole-body levels of Na (BF > 5) (Fig. 2) and Mg (BF > 52) (Fig. 2). In turn, fish exposure to 20 µg $Cu.L^{-1}$ showed significant increased whole-body Na (BF > 5), K (BF > 6) (Fig. 2), and Mg (BF > 105) (Fig. 2) contents. In addition, whole-body Mg content

showed a strong linear and positive correlation with the increasing copper concentration in the experimental medium ($r^2 = 0.97$). Furthermore, a strong negative correlation ($r^2 = -0.97$) was observed between Mg and Ca content. On the other hand, whole-body Cl (Fig. 2) and Ca (Fig. 2) contents were not affected by waterborne copper exposure.

Oxidative status biomarkers

Almost all biomarkers evaluated were significantly affected by waterborne copper exposure. However, not all of them showed a clear concentration-dependent response in the range of concentrations tested.

ROS production was increased in liver of fish exposed to 20 µg $Cu.L^{-1}$ compared to control (BF > 22), and in gills of those exposed to 9 (BF > 8) and 20 µg $Cu.L^{-1}$ (BF > 7) compared to 5 µg $Cu.L^{-1}$. On the other hand, it was slightly reduced in muscle of fish exposed to 5 µg $Cu.L^{-1}$ (BF > 5) (Fig. 3A). ACAP was significantly increased in liver of fish exposed to 20 µg $Cu.L^{-1}$ (BF > 54) and slightly reduced in muscle of those exposed to 5µg $Cu.L^{-1}$ (BF > 77) (Fig. 3B). MT concentration was reduced in liver of fish exposed to 5 (BF > 5) and 20 µg $Cu.L^{-1}$ (BF > 7) and in gills of those exposed to 5 µg $Cu.L^{-1}$ (BF > 7). However, a significant increase in MT concentration was observed in gills of fish exposed to 9 (BF > 8) and 20µg $Cu.L^{-1}$ (BF > 6) (Fig. 3C).

Regarding the key-enzymes involved in the antioxidant defense system (Fig. 4), SOD activity was reduced in liver of fish exposed to 20 µg $Cu.L^{-1}$ (BF > 6) and in gills of those exposed to 5 (BF > 6) or 9 µg $Cu.L^{-1}$ (BF > 14). In muscle, no significant change was observed (Fig. 4A). A linear increase in CAT activity was observed in liver of fish exposed to copper ($r^2 = 0.89$), with enzyme activity being significantly different from control values in those exposed to 5 (BF > 14) and 20 µg $Cu.L^{-1}$ (BF > 833). Despite no significant effect was

observed in muscle CAT activity, a linear increment in enzyme activity was observed as a function of increasing copper concentrations (r^2 = 0.95) (Fig. 4B). GR activity was increased in liver of fish exposed to 5 (BF > 13) and 9 µg $Cu.L^{-1}$ (BF > 10) (Fig. 4C). A linear increase in GST activity was observed as a function of copper concentration in liver (r^2= 0.72). However, a significant increased enzyme activity was only observed in liver of fish exposed to 9 µg $Cu.L^{-1}$ (BF > 5). In muscle and liver, an increased GST activity was observed in fish exposed to 9 µg $Cu.L^{-1}$ (BF > 21), while a decreased enzyme activity was observed in muscle of those exposed to 20 µg $Cu.L^{-1}$ (BF > 9) (Fig. 4D).

In liver, all copper concentrations tested significantly increased LPO (BF > 1387). In gills, significant increases in LPO values were observed in fish exposed to 9 (BF > 123) and 20 µg $Cu.L^{-1}$ (BF > 138) (Fig. 5). In fact, a linear increase was observed in LPO values in liver (r^2= 0.84) and gills (r^2= 0.76) as a function of copper concentration in the water. A very strong positive correlation was observed between ROS production and LPO levels in gills (r^2 = 0.97) and between LPO levels in gills and liver (r^2 = 0.99). Furthermore, a marked correlation was observed between gill LPO values and whole-body Ca (r^2= -0.97) or Mg (r^2= 0.94) content. In muscle, only fish exposed to 9µg $Cu.L^{-1}$ showed significantly increased LPO values (BF > 21) (Fig.5).

DNA damage biomarkers

Regarding recoverable DNA strand breakage, class distribution and score values from the comet assay pointed to a significant increase of DNA single and double breaks in peripheral erythrocytes of fish exposed to any copper concentration tested (BF > 919) (Fig. 6). It is worth to note that fish exposed to 20 µg $Cu.L^{-1}$ showed a significant reduction in this kind of DNA damage when compared to those exposed to 9µg $Cu.L^{-1}$ (BF > 10), presenting

less nucleoids in classes 2 and 3, and a consequently lower comet score. This pattern is similar to that observed for nuclear buds and apoptotic fragment frequencies (Fig. 7A), which were significantly higher in fish exposed to 9 μg $Cu.L^{-1}$ (BF ~ ∞). However, micronucleated cells frequency was increased in fish exposed to 9 (BF > 45) and 20 μg $Cu.L^{-1}$ (BF > 171) (Fig. 7B), showing a highly significant and positive linear correlation with copper concentration in the water (r^2= 0.90). The frequency of erythrocytes with bilobed nuclei decreased in fish exposed to 5 μg $Cu.L^{-1}$ (BF > 3400), while it increased in those exposed to 20 μg $Cu.L^{-1}$ (BF > 28). The frequency of binucleated cells did not change after exposure to the copper concentrations tested (Fig. 7A).

DISCUSSION

Copper concentrations tested in the present study (0-20 μg $Cu.L^{-1}$) can be considered as being acutely sublethal to the guppy *P. vivipara* acclimated to salt water (salinity 24 ppt). In fact, no fish mortality was observed at any experimental condition tested. This finding indicates that the actual Brazilian environmental regulation (CONAMA, 2005) is effectively protecting the guppy *P. vivipara* in salt water if we consider only acute exposure (96 h) and lethal effects. However, as further discussed acute exposure to copper concentrations as low as 5 μg $Cu.L^{-1}$ can induce several relevant biochemical, physiological and genetic effects in *P. vivipara* acclimated to salt water.

Copper accumulation data reported in the present study indicate that fish was able to regulate the whole-body copper burden for at least 96 h when exposed to 5 and 9 μg $Cu.L^{-1}$. However, it seems that mechanisms involved in copper regulation began to be overwhelmed when guppies were exposed to 20 μg $Cu.L^{-1}$, since an enhanced whole-body copper accumulation was observed respect to those exposed to 9 μg $Cu.L^{-1}$ (Fig. 1). In saltwater fish,

dissolved copper is shown to be accumulated mainly through the gills and the gut (Grosell et al., 2007; Martins and Bianchini, 2008). Considering that these organs are the major responsible for the ionic and osmotic regulation in saltwater fish, it would be expected that copper exposure could impair the physiological mechanisms involved in the ionic balance in the osmoregulating *P. vivipara* acclimated to salinity 24 ppt. This aspect, as well as the suitability of using whole-body ionic composition as biomarkers of copper exposure is discussed below.

Data on whole-body ion composition reported in the present study show a significant increase in whole-body Na content in copper-exposed fish, especially at 9 and 20 μg $Cu.L^{-1}$ (Fig. 2). This finding indicates that the well-described mechanism of acute copper toxicity in fish, especially in freshwater ones, i.e., a Na regulation disturbance (Heath, 1995; McGeer et al., 2000; Grosell et al., 2002; Handy, 2003; Grosell et al., 2007; Alsop and Wood, 2011) was also observed in the guppy *P. vivipara* acclimated to salt water (salinity 24 ppt). Additionally, significant disturbances on whole-body K and Mg regulations were also observed. Considering all ions analyzed, the most marked and significant effect of waterborne copper exposure was on the whole-body Mg content. In this case, a significant linear and positive correlation was observed between the whole-body Mg content and the copper concentration in the water (Fig. 2). These findings clearly indicate that copper is disrupting not only the mechanisms involved in Na regulation in the guppy *P. vivipara* acclimated to salt water, but also those associated with K and Mg regulations. In addition, they point out that copper exposure is disturbing not only the mechanisms involved in ion regulation taking place at the gills, but also those at the kidney level. These facts ratify the idea that several osmoregulatory disturbances in fish exposed to copper may be due to alterations in gills transports and kidney effects McGeer et al. (2000). In fact, a strong negative correlation between whole-body Ca and Mg was observed, and it must be noticed that the concentrations of these divalent cations

are mainly regulated by the fish kidney (Grosell et al., 2007; Nordlie, 2009). Unfortunately, as discussed below, we evaluated the oxidative status parameters only in gills and liver, but not in kidney. Therefore, future studies are needed to characterize the possible toxic effects of copper in the kidney of the guppy *P. vivipara*.

It is worth to note that the ionic impairments observed in *P. vivipara* after exposure to waterborne copper were paralleled by significant changes in the response of several biochemical and physiological parameters related to the oxidative status in gills. An increased ROS production (Fig. 3A) paralleled by augmented LPO values (Fig. 5) was observed in gills of fish exposed to 9 and 20 μg $Cu.L^{-1}$. These facts clearly indicates that gill membranes would be suffering oxidative damage resulted from oxidative stress associated with a higher production of ROS induced by copper exposure. In fact, it is well known that copper can induce oxidative stress in estuarine and marine animals (for review: Monserrat et al., 2007). Therefore, indirect effects of copper through oxidative stress on ion-transporting mechanisms at the gills cannot be ruled out when explaining the whole-body ionic disturbances observed in the guppy *P. vivipara* in salt water. This hypothesis is supported by the strong and significant correlation observed between the gill LPO levels and the whole-body Ca (negative correlation) or Mg (positive correlation) content.

Since the key-mechanism of acute toxicity of waterborne copper is reported to be related to disturbances in fish ionic regulation, parameters associated with this physiological process have been proposed as biomarkers of metal contamination (Handy and Depledge, 1999). In general, studies are referred to the plasma ion content (Nordlie, 2009), which in fact has a high physiological relevance. However, due to the relative small size of *P. vivipara*, analyses reported in the present study were performed in the fish whole-body. In this context, results from the present study increases the knowledge on fish whole-body composition, as well as provide new information on the response of this parameter in fish exposed to low

concentrations of waterborne copper in salt water. Changes observed in whole-body ion composition can adequately reflect fish exposure to copper concentrations as low as 5 µg $Cu.L^{-1}$, especially when we consider the whole-body K and Mg contents. Regarding the relevance of these parameters in biomonitoring programs, it is important to stress that whole-body ionic composition is a biologically important parameter, since it does not report only a tissue specific response, but integrates the response of all fish tissues. Also, it is worth to note that the fish capability of ionic and osmotic regulation has been described as an important factor affecting fish growth and reproduction (Handy and Depledge, 1999), being also considered as a limiting factor to distribution and biogeography of fishes (Nordlie, 2009). Taking into account that the Poeciliidea is amongst the fish families showing the largest osmoregulation capability (Gomes and Monteiro, 2008), the observed effects induced by copper on whole-body ion composition in *P.vivipara* suggest that other fish species less adapted to cope with salinity changes would be even more impacted by exposure to copper waterborne in salt water.

Despite copper exposure induced whole-body ionic disturbances in the guppy *P. vivipara*, the observed impairments were not strong enough to induce fish mortality, even at the higher copper concentration tested (20 µg $Cu.L^{-1}$). Indeed, similar disruption of Na and K in plasma and non-mortality were reported by Mazon et al (2002) in *Prochilodus scrofa* at the same range of copper concentrations. In addition to the significant effects of copper on whole-body cations (Na, K, and Mg) regulation, copper exposure to the sublethal concentrations tested also induced the response of several other biochemical and physiological parameters in both gills and liver, as well as on genetic parameters in erythrocytes.

Over the last decades, the interest for biochemical, physiological and genetic parameters as biomarkers of exposure to pollutants has markedly increased (Handy and Depledge, 1999; Rose et al., 2006). It is well accepted that they integrate several molecular

and cell processes, providing a general picture on the organism's health. On the other hand, it is also recognized that they suffer interferences from distinct physiological pathways, which may mask their response at low concentrations of the contaminant, hampering their use as suitable tools in biomonitoring programs (for review: Monserrat et al., 2007).

All biomarkers analyzed in the present study are reported to respond to copper or other metals present in the aquatic environment (Heath, 1995; Handy, 2003; Amado et al., 2006; Monserrat et al., 2007; Nordlie, 2009; Alsop and Wood, 2011; Wang et al., 2011). However, findings from the present study show that not all of the analyzed biomarkers are able to respond at environmentally relevant concentrations of copper. Furthermore, only few of them showed a clear relationship between the degree of response and the concentration of copper in the experimental medium. After integrating the consistency of the response of each parameter evaluated, their physiological relevance, sensitivity to copper, as well as our previous knowledge on their response to high copper concentrations, it was possible to classify the potential of each biomarker response as a suitable biomarker to monitor the salt water contamination with low and high copper concentrations using *P. vivipara* (Table 3). Arguments supporting the selection of some biomarkers as potential tools for biomonitoring purposes are discussed below.

Let us first consider our findings on tissue MT concentration in the guppy *P. vivipara* exposed to waterborne copper in salt water, since this biomarker is generally used to identify exposure of aquatic animals to metals, including copper (Langston and Bibianno, 1998). MT is a family of cysteine-rich proteins, showing low molecular weight and high capacity for chelating metals, including copper. Also, it is reported their role as antioxidant agent, since it is able to bind free radicals (for review: Monserrat et al, 2007). Therefore, these cytosolic proteins can contribute to decrease the deleterious effects of metals and ROS (Viarengo et al., 1997; Martins and Bianchini, 2009). In fact, an increased ROS production (Fig. 3A)

associated with an augmented MT level (Fig. 3C) was observed in gills of fish exposed to 9 and 20 μg $Cu.L^{-1}$. In turn, this increased level of MT would be related to the observed higher levels of whole-body copper, especially at 20 μg $Cu.L^{-1}$ (Fig. 1). In fact, copper is shown to induce MT synthesis in aquatic animals (for review: Monserrat et al., 2007). As opposed to gills, liver showed an increased level of ROS production (Fig. 3A) paralleled by a decreased MT content (Fig. 3C). Taken altogether, these findings suggest that MT would be 'mobilized' at some extent from the liver to support in some way the gills to keep increased MT levels in order to avoid or reduce the direct impact of the waterborne copper on this multifunctional organ.

ROS are generated naturally during the aerobic metabolism through the Haber-Weiss reaction in the presence of the superoxide anion. Therefore, all aerobic animals show mechanisms to scavenge ROS (Geracitano et al., 2002). Such mechanisms are crucial in the whole-organism homeostasis, since ROS can react with biomolecules causing damages to DNA, proteins and lipids (Stohs and Bagchi, 1995). Genotoxicity and disturbances in membrane fluidity are some consequences from these injuries, respectively (Heath, 1995). However, several environmental natural and anthropogenic stressors, such as salinity changes and chemical pollution, can increase the natural effects of ROS. Under a physiological point of view, these stressors can induce an excessive ROS production and toxicity acting directly on the balance between ROS production and antioxidant (enzymatic and non-enzymatic) defenses (for review: Monserrat et al., 2007). These stressors can also indirectly affect ROS production and toxicity by increasing metabolic rates (Martins and Bianchini, 2009). In this context, some organisms may show a depressed metabolism in certain tissues as a strategy to reduce ROS production and deal with the contaminant exposure. This could explain the reduced ROS production observed in muscle of *P. vivipara* exposed to 5 μg $Cu.L^{-1}$ (Fig. 3A).

It is important to note that alterations in ROS production are associated with parallel changes in the activity of several enzymes and the concentration of antioxidant molecules to adequately scavenge the distinct types of ROS and avoid the subsequent deleterious oxidative damages in tissues. Some of these enzymes, such as SOD and CAT, are usually regulated by the concentration of their substrate, showing an increased activity when productions of superoxide and peroxide anions are increased, respectively (McCord and Fridovich, 1969; Beutler, 1975). The reactions catalyzed by these two enzymes are likely to occur one followed by the other. However, in the present study SOD activity was slightly reduced in liver of *P. vivipara* exposed to copper (Fig. 4A) while CAT activity was strongly increased (Fig. 4B), suggesting the existence of uncoupled reactions for superoxide and peroxide radicals. Some authors suggest that some contaminants may cause peroxissomal proliferation as a process related to a CAT activity increase after contaminant exposure (Amado et al., 2006). In fact, CAT activity has been shown also to be more sensitive to other water contaminants (atrazine and phenanthrene) than SOD activity in the guppy *P. vivipara* acclimated to salt water (Machado et al. 2012a, 2012b). Therefore, CAT activity seems to be a more suitable tool as a biomarker in water quality monitoring programs than SOD activity, especially because it is cheaper and easier to measure.

GR and GST are also among the main enzymatic antioxidants (Roche and Bogé, 1993). While GR recycles the GSSH to GSH, GST conjugates the GSH with toxicants or ROS, allowing their metabolism with the P-450 complex (for review: Monserrat et al., 2007). In the present study, the activities of these enzymes in gills and muscle of the guppy *P. vivipara* were not clearly responsive to copper exposure (Fig. 4). However, GR activity was increased in liver of fish exposed to 5 and 9 μg $Cu.L^{-1}$ (Fig. 4C), while GST activity appears to start to respond only at 9 μg $Cu.L^{-1}$ (Fig. 4D), when GR activity starts to decrease (Fig. 4C). These findings suggest that fish was firstly recycling GSSH at lower copper

concentrations, but starts to expend GSH through the more expensive GST route at higher copper concentrations. The increased GST activity observed in the liver as a function of copper concentration in the exposure medium can also be related to the higher levels of DNA breaks recorded in erythrocytes of fish exposed to copper. In fact, GST has been pointed to be involved in the defense mechanism against the DNA peroxidative products (Amado et al., 2006).

Despite the activities of the enzymes discussed above may provide important information on which mechanism of detoxification are turned on, they were not sensitive or did not show a clear copper concentration-dependent response, except in the case of the liver CAT activity. It may be possible that the activities of these enzymes are not monotonically responsive to low copper concentrations, mainly due to the integrated and complex way on how these enzymes are activated or deactivated within the whole chain of reactions. Furthermore, their responses are not easy to extrapolate at higher levels of biological organization, putting in check the need for measurement of the activities of all the antioxidant enzymes in biomonitoring programs. Therefore, we suggest that the measurement of a single exposure biomarker, such as liver catalase activity, may provide enough information on the exposure of the guppy *P. vivipara* to waterborne copper in salt water.

Regarding the most integrative parameters of the tissue oxidative status, it is important to notice that ACAP integrates the response of most enzymatic and non-enzymatic antioxidants, while LPO is an indicative of the ROS/ACAP balance. Moreover, some contaminants such as copper may interact with biomolecules, thus causing direct oxidative damages to these molecules, which in turn are reflected in part by increased LPO values (Stohs and Bagchi, 1995). Therefore, LPO and ACAP responses have been pointed as useful biomarkers of effect and exposure, respectively (Rose et al., 2006). In fact, metal-induced oxidative stress is well reported in estuarine and marine vertebrates and invertebrates (Roche

and Bogé, 1993; Heath, 1995; Geracitano et al., 2002; Ferreira-Cravo et al., 2009). In general, copper is considered as having a high oxidative potential (Stohs and Bagchi, 1995), a characteristic that mainly explains the toxic effects of this metal in estuarine animals (Ferreira-Cravo et al., 2009).

In the present study, ACAP was increased in liver of fish exposed to 20 µg $Cu.L^{-1}$ (Fig. 3B), while LPO showed a clear tendency of increase as copper concentration augmented in salt water (Fig. 5). The observed increase in ACAP is likely a result from the response of the biochemical and physiological mechanisms in attempt to avoid or reduce the tissue damage induced by the increased ROS production. It can be explained, at least in part, by the observed increase in liver CAT activity (Fig. 4B). However, it is important to note that the observed increase in ACAP was not enough to completely avoid the oxidative damage in the liver, since higher LPO levels were concomitantly observed (Fig. 5). These results are in agreement with those reported for seabirds exposed to metals (Kamiński et al., 2009). Considering the clear copper concentration-dependent response shown by liver LPO and its integrative relationship with ACAP, this biologically relevant biomarker of effect can be thus considered as a promising tool to biomonitor the water contamination with copper.

Let us now consider the oxidative damage induced by copper in the guppy *P. vivipara*. Waterborne copper exposure significantly induced DNA strand breaks (Fig. 6) and nuclear abnormalities (Fig. 7) in the fish erythrocytes. These oxidative damages are consistent with the increased ROS production and LPO values observed in gills and liver of copper-exposed fish discussed above. These findings have at least two implications, one related to the physiology of the guppy *P. vivipara*, and another linked to the use of these parameters as biomarkers in biomonitoring programs.

At the physiological point of view, genetic damage observed indicate that exposure to even low concentrations of waterborne copper can induce DNA damage in *P. vivipara*. This

statement is based on the consistent response of the large suite of parameters analyzed, i.e., comet score (Fig. 6) and frequency of nuclear buds, apoptotic fragments (Fig. 7A) and micronucleated cells (Fig. 7B). Despite comet scores were similar in 5 and 20 μg $Cu.L^{-1}$, erythrocytes from fish exposed to 20 μg $Cu.L^{-1}$ are likely to show significantly higher mutation levels, indicated by the increased frequency of micronucleated cells (Fig. 7B). The improvement observed in comet score (Fig. 6), as well as in the frequency of nuclear buds and apoptotic fragments (Fig. 7A) in erythrocytes of fish exposed to 20 μg $Cu.L^{-1}$ could be explained considering an increased release of young cells in the fish bloodstream. This statement is supported by the significant increase in bilobed cells observed in fish exposed to this copper concentration (Fig. 7A). Indeed, the release of young cell into the fish blood stream appears to be reduced in fish exposed at 5 μg $Cu.L^{-1}$, being stimulated in those exposed to 9 μg $Cu.L^{-1}$. An integrative analysis of the release of young cells rate and the efficiency of processes involved in DNA breaks recovery is reflected by the micronucleated cells frequency. Data reported in the present study suggest that even providing more cells into the bloodstream, there is an inefficiency of fish erythrocytes in reversing the single or double DNA breaks induced by copper exposure. This statement is supported by the significant increase in micronucleated cells frequency observed in erythrocytes of fish exposed to 9 and 20 μg $Cu.L^{-1}$ (Fig. 7B), as well as by the highly significant and positive linear correlation found between the micronucleated cells frequency and the copper concentration in water.

Considering the biomonitoring aspect, DNA breaks and clastogenicity parameters are considered as being closely related to genotoxicity and carcinogenesis (Mitchelmore and Chipman, 1998), thus positioning them among the most meaningful biological parameters. Therefore, findings reported and discussed above regarding these parameters just confirm the good perspective of their use as useful biomarkers to monitor the water contamination with

copper, especially because of their sensitivity and dependence on waterborne copper concentration.

In the sections above we discussed the responses of the various biomarkers in terms of sensitivity, consistency, dose-response and biological relevance. However, there is another important point to be considered regarding the use of biomarkers in environmental assessment: toxicant specificity. Despite many of biomarkers used in the present work were once considered specific to certain kinds of contaminants (Heath, 1995; Langston and Bebianno, 1998), the most accepted current thought is that none of them are really toxicant-specific. Taking the present study together to the similar ones performed with other contaminants (Machado et al., 2012a, 2012b) whole-body Mg, liver catalase activity, LPO and DNA damages appear to respond more clearly to copper, specially when considered jointly. However, they somehow respond to other contaminants. Additionally, it is known that responses to metal waterborne may change according to the period of exposure (McGeer et al., 2000), which implies that the biomarkers specificity reported in the present work is valid in the present experimental conditions. Nevertheless, the authors are confident that it is possible to infer which is the major contaminant or environmental stressors through biomarkers measurement, but more information and statistical analysis are needed. In fact, the effort to stocking information in a posteriori distribution by Bayesian methodology reflects these believe. Further works with more copper concentrations, varying water chemistry parameters and deeper modeling and exploratory approach would be able to clarify questions such as if there is a biomarker, or a combination of them, that diagnostic Cu or metal as major stressors.

In summary, the response of a large suite of biochemical, physiological and genetic biomarkers after acute exposure to environmentally relevant concentrations of copper were

evaluated in the salt water-acclimated guppy *P. vivipara*, an estuarine fish from Brazil. This fish species occurs all along the Brazilian South Atlantic coast (8,698 km long) and many inland water bodies. Findings reported clearly indicate that waterborne copper can significantly disturb biologically relevant parameters in *P. vivipara* exposed to metal concentrations as low as 5 μg $Cu.L^{-1}$ in salt water. Considering that *P. vivipara* shows wide distribution, high capability to face a wide range of salinities (0-30 ppt), high tolerance to inorganic and organic water contaminants, easy maintenance and cultivation in laboratory, small size, and response of several biomarkers to environmentally relevant concentrations of waterborne copper, it can be considered as a potential biomonitor of copper contamination in salt water. It is important to note that many of the biomarkers analyzed in the present study show different levels of response to copper exposure in each tissue analyzed. Indeed, some biomarkers seem to respond not monotonically to the copper concentrations tested. According to the physiological and biomonitoring weight considered in the present study (Table 3), catalase activity in liver, ROS, ACAP and lipid peroxidation in muscle, gills and liver, DNA damages in erythrocytes, and whole-body ion (Na, K, and Mg) composition are likely the most promising biomarkers to assess the exposure to waterborne copper in *P. vivipara.* These biomarkers showed a response clearly dependent on copper concentration and biological relevance. Further future studies would be important to analyze the response of these biomarkers to other contaminants and other environmental stressors for a better understanding of the relationship between each environment factor and the biomarker response.

ACKNOWLEDGMENTS

Financial support is acknowledged to the International Development Research Centre (IDRC, Ottawa, Canada), 'Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior' (CAPES – Programa Ciências do Mar, Brasília, DF, Brazil) and 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq – Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática,

Brasília, DF, Brazil). A. Bianchini is a research fellow from the Brazilian CNPq (Proc. # 304430/2009-9) and supported by the International Canada Research Chair Program from IDRC. We also thank Dr Marta Marques de Souza, Dr Ana Luiza M. Baisch (Universidade Federal do Rio Grande, Rio Grande, RS, Brazil) and Dr Carine Dahl Corcini (Universidade Federal de Pelotas, Pelotas, RS, Brazil) for their help during experiments and analyses.

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Table 1. Nominal and measured (total and dissolved) copper concentrations employed in experiments with the guppy *Poecilia vivipara* in salt water (24 ppt). Relevance according to the Brazilian environmental regulation is also presented. WQC: water quality control. BDL: below detection limit (1 μg Cu. L^{-1}). Data are mean ± standard deviation.

Copper concentration (μg Cu. L^{-1})			
Nominal	Total	Dissolved	Environmental relevance
0	BDL	BDL	Control
5	4.25 ± 1.25	3.45 ± 0.75	WQC - salt water
9	10.25 ± 1.75	6.70 ± 1.00	WQC - fresh water
20	21.50 ± 0.05	20.75 ± 0.25	Non-conforming

Table 2. Conjugated families of 'probability distribution' and 'priori parameters' used to obtain the 'posteriori distributions' for each biomarker analyzed in the guppy *Poecilia vivipara* acclimated to salt water (salinity 24 ppt) exposed (96 h) to copper. *According to solution presented by Kinas and Andrade (2010).

Biomarker	Conjugated Family	Priori Parameters	
Oxidative Status	Normal-Gamma & Normal	Jeffreys's non informative*	
Ions content	Normal-Gamma & Normal	Jeffreys's non informative*	
Score Comet	Normal-Gamma & Normal	Jeffreys's non informative*	
Comet Class 0	Gama & Poisson	α=13.0	β= 0.18
Comet Class 1	Gama & Poisson	α= 0.28	β= 0.03
Comet Class 2	Gama & Poisson	α= 3.57	β= 0.59
Comet Class 3	Gama & Poisson	α= 2.17	β= 2.17
Micronucleus	Gama & Poisson	α= 1.79	β= 5.97
Nuclear buds	Gama & Poisson	α= 0.41	β= 2.72
Apoptotic fragments	Gama & Poisson	α= 2.45	β= 24.49
Binuclear cells	Gama & Poisson	α= 3.61	β= 6.23
Bilobed nucleus	Gama & Poisson	α= 1.00	β= 0.01

Table 3. Biomarkers responsive to copper exposure in the guppy *Poecilia vivipara* in salt water. Sensitivity level, physiological relevance and potential as a useful tool to monitor low and high levels of copper in aquatic environments are presented. * indicates the lower copper concentration inducing a significant biomarker response. SOD: superoxide dismutase; CAT: catalase; GR: glutathione reductase; GST: glutathione S-transferase; MT: metallothionein-like proteins; ROS: reactive oxygen species; ACAP: antioxidant capacity against peroxyl radicals; LPO: lipid peroxidation; CA: comet assay; NB: nuclear buds; BN: bilobednucleus; AF: apoptotic fragments; MCF: micronucleated cells; WBIC: whole-body ion content.

Biomarker	Sensitivity* ($\mu g\ Cu.L^{-1}$)	Physiological relevance	Concentration response	Potential as biomonitor	
				Low levels	High levels
SOD	5	Exposure biomarker	No	Low	Medium
CAT	5	Exposure biomarker	Yes	High	High
GR	5	Exposure biomarker	No	Low	Low
GST	9	Exposure biomarker	No	Low	Medium
MT	5	Exposure biomarker	No	Low	High
ROS	9	Exposure biomarker	No	Medium	High
ACAP	5	Health condition	No	Medium	High
LPO	5	Effect biomarker	Yes	High	High
CA	5	Effect biomarker	Yes	High	High
NB	5	Effect biomarker	Yes	Medium	Medium
BN	5	Effect biomarker	No	Low	Low
AF	9	Effect biomarker	No	Low	Medium
MC	9	Effect biomarker	Yes	High	High
WBIC	9	Health condition	Yes	High	High

LEGEND TO FIGURES

Figure 1. Whole-body copper accumulation in the guppy *Poecilia vivipara* in control (open bar) and exposed (96 h) to 5 (diagonally hatched bars), 9 (diagonally inverted hatched bars) and 20 (diagonally hatched and diagonally inverted hatched bars) µg $Cu.L^{-1}$ in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among copper concentrations, and means sharing the same letter are not significantly different.

Figure 2. Whole-body ion in controls (open bars), 5 (diagonally hatched bars), 9 (diagonally inverted hatched bars) and 20 µg $Cu.L^{-1}$ (diagonally hatched and diagonally inverted hatched bars) of 96 h exposed guppy *Poecilia vivipara* in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among copper concentrations for each parameter, and means sharing the same letter are not significantly different.

Figure 3. (A) Reactive oxygen species (ROS) production, (B) total antioxidant capacity against peroxyl radicals (ACAP), and (C) metallothionein-like proteins (MT) concentration in liver, gills, and muscle of the guppy *Poecilia vivipara* exposed (96 h) to control (open bars), 5 (diagonally hatched bars), 9 (diagonally inverted hatched bars) and 20 µg $Cu.L^{-1}$ (diagonally hatched and diagonally inverted hatched bars) in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among copper concentrations for each tissue within the same parameter, and means sharing the same letter are not significantly different.

Figure 4. (A) Superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione reductase (GR) and (D) glutathione S-transferase (GST) activity in liver, gills, and muscle of the guppy *Poecilia vivipara* exposed (96 h) to control (open bars), 5 (diagonally hatched bars), 9 (diagonally inverted hatched bars) and 20 µg $Cu.L^{-1}$ (diagonally hatched and diagonally inverted hatched bars) in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among copper concentrations for each tissue within the same parameter, and means sharing the same letter are not significantly different.

Figure 5. Lipid peroxidation (LPO) in liver, gills, and muscle of the guppy *Poecilia vivipara* exposed (96 h) to control (open bars), 5 (diagonally hatched bars), 9 (diagonally inverted hatched bars) and 20 µg $Cu.L^{-1}$ (diagonally hatched and diagonally inverted hatched bars) in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among copper concentrations for each tissue, and means sharing the same letter are not significantly different.

Figure 6. DNA damage measured through comet assay in erythrocytes of the guppy *Poecilia vivipara* exposed (96 h) to different concentrations of waterborne copper in salt water (salinity 24 ppt). For comet class (left y-axis) (class 0: open bars; class 1: diagonally hatched bars; class 2: diagonally inverted hatched bars; class 3: double hatched bars), bars represent means and vertical lines indicate standard deviation. For comet score (right y-axis), data are expressed as mean (closed circles) ± standard deviation (vertical lines). Different letters indicate significant difference among copper concentrations for each parameter, and means sharing the same letter are not significantly different.

Figure 7. DNA damage measured through nuclear abnormalities in erythrocytes of the guppy *Poecilia vivipara* exposed (96 h) to control (open bars), 5 (diagonally hatched bars), 9 (diagonally inverted hatched bars) and 20 µg $Cu.L^{-1}$ (diagonally hatched and diagonally inverted hatched bars) in salt water (salinity 24 ppt). (A) Frequency of nuclear abnormalities: nuclear buds, binucleated cells, cells with nucleus presenting apoptotic fragments, cells with bilobed nucleus. (B) Frequency of micronucleated cells. Bars indicate means and vertical lines represent standard deviation. Different letters indicate significant difference among copper concentrations for each parameter, and means sharing the same letter are not significantly different.

Figure 1

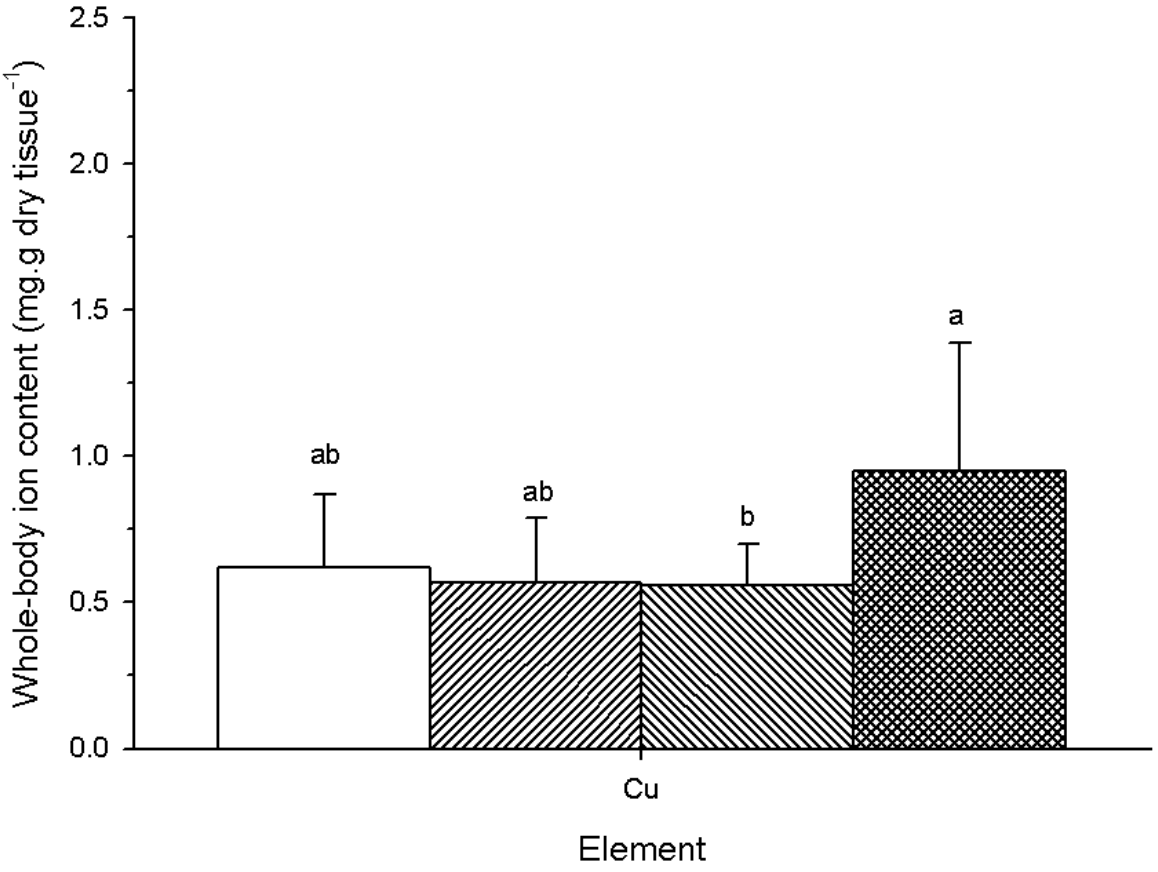

2.5
2.0
1.5
1.0
0.5
0.0
Whole-body ion content (mg.g dry tissue-1)
ab
ab
b
a
Cu
Element

Figure 2

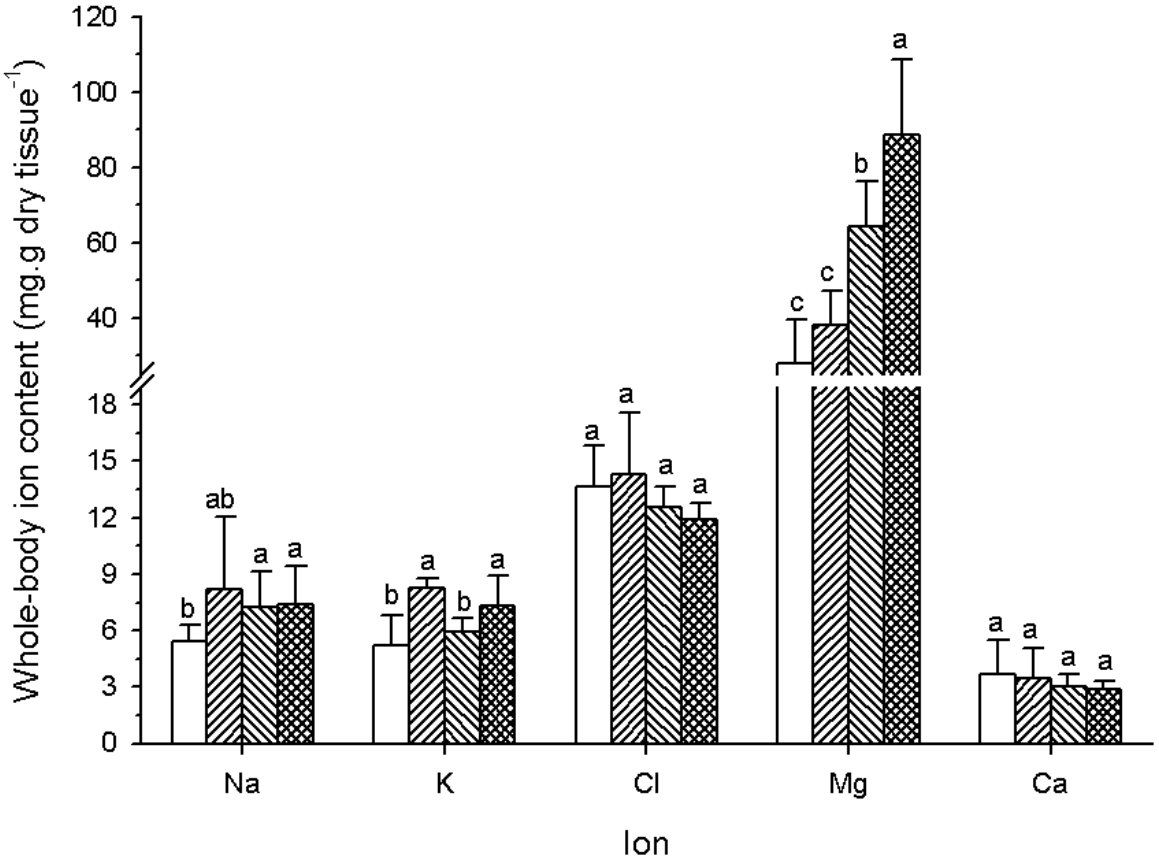

Whole-body ion content (mg.g dry tissue^{-1})
120
100
80
60
40
18
15
12
9
6
3
0
ab
b
a
a
b
a
b
a
a
a
a
a
c
c
b
a
a
a
a
Na
K
Cl
Mg
Ca
Ion

Figure 3

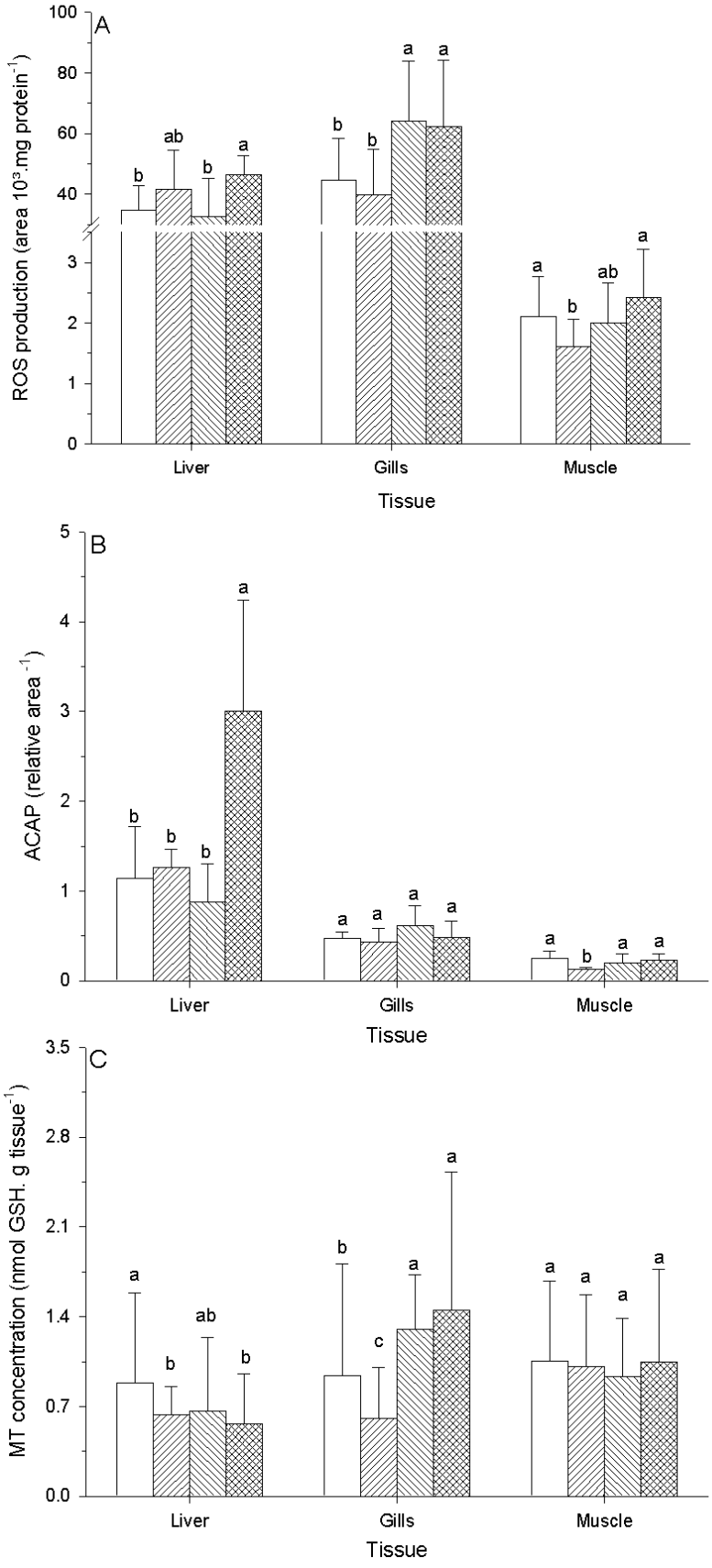

A
ROS production (area 10^3.mg protein^{-1})
100
80
60
40
3
2
1
0
b
ab
b
a
b
b
a
a
a
b
ab
a
Liver
Gills
Muscle
Tissue
B
ACAP (relative area^{-1})
5
4
3
2
1
0
b
b
b
a
a
a
a
a
a
b
a
a
Liver
Gills
Muscle
Tissue
C
MT concentration (nmol GSH. g tissue^{-1})
3.5
2.8
2.1
1.4
0.7
0.0
a
b
ab
b
b
c
a
a
a
a
a
a
a
Liver
Gills
Muscle
Tissue

Figure 4

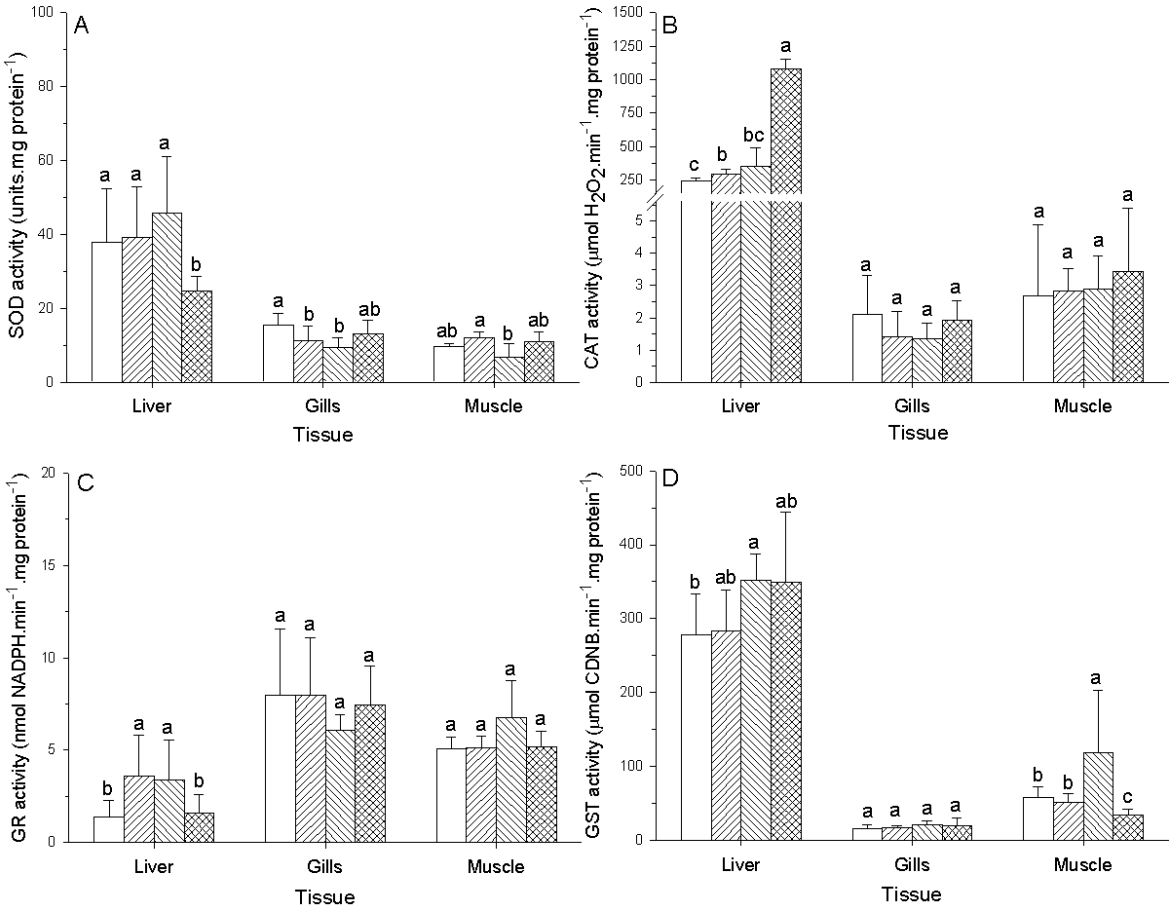

A
SOD activity (units.mg protein^{-1})
Liver
Gills
Muscle
Tissue
B
CAT activity (µmol H_2O_2.min^{-1}.mg protein^{-1})
C
GR activity (nmol NADPH.min^{-1}.mg protein^{-1})
D
GST activity (µmol CDNB.min^{-1}.mg protein^{-1})

Figure 5

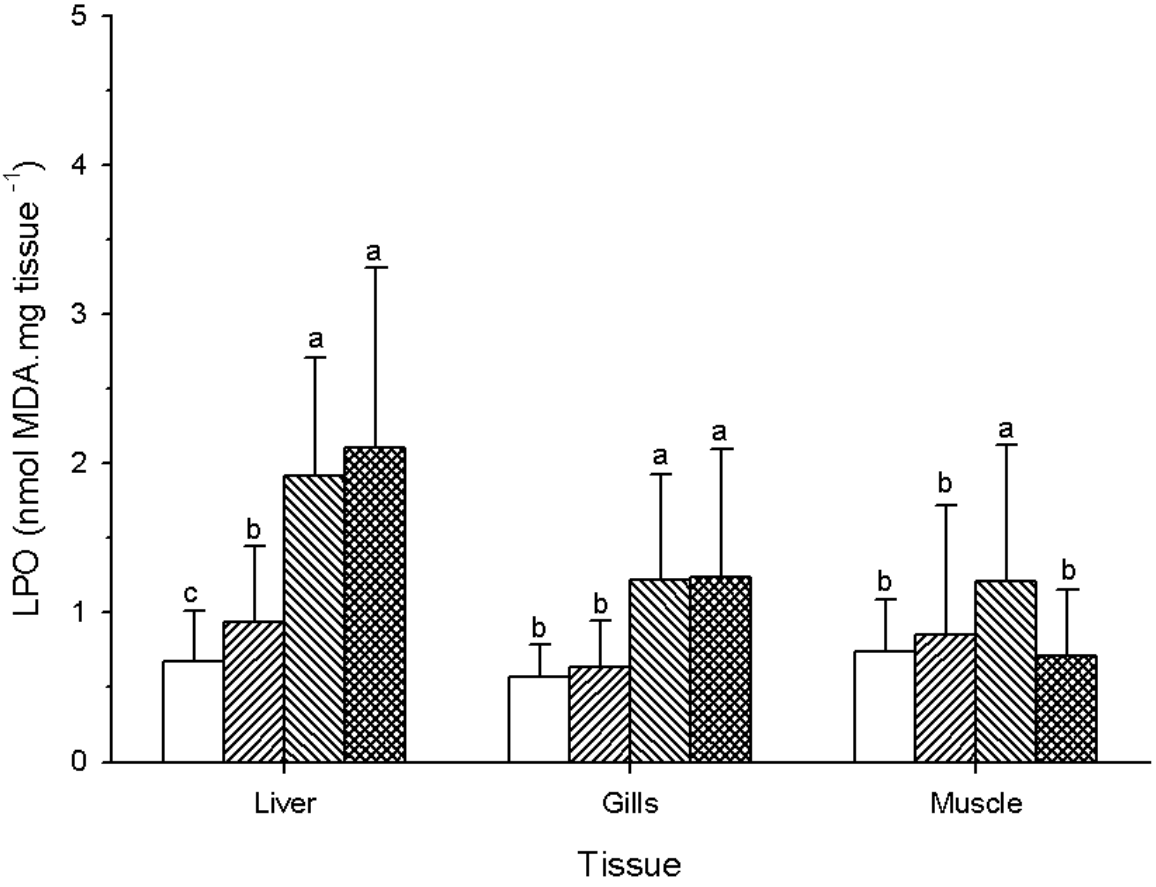

LPO (nmol MDA.mg tissue -1)
0
1
2
3
4
5
c
b
a
a
b
b
a
a
b
b
a
b
Liver
Gills
Muscle
Tissue

Figure 6

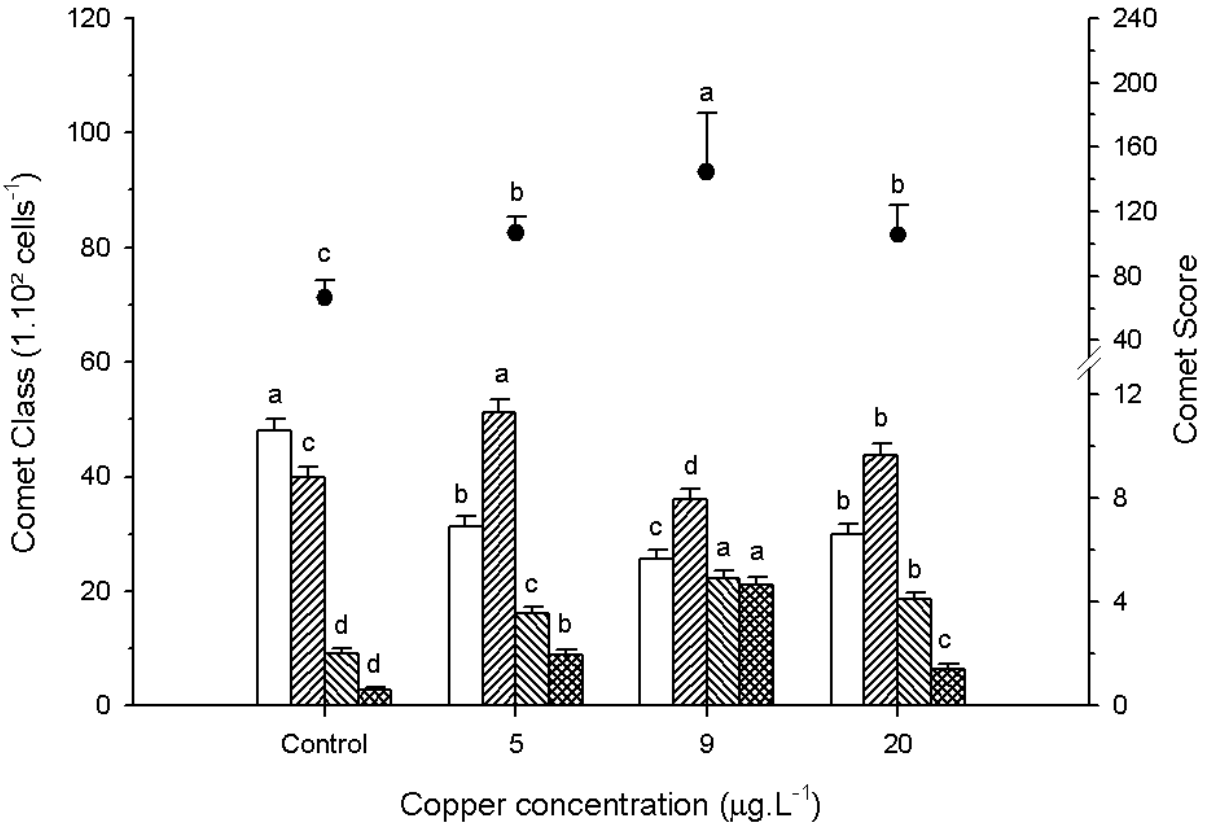
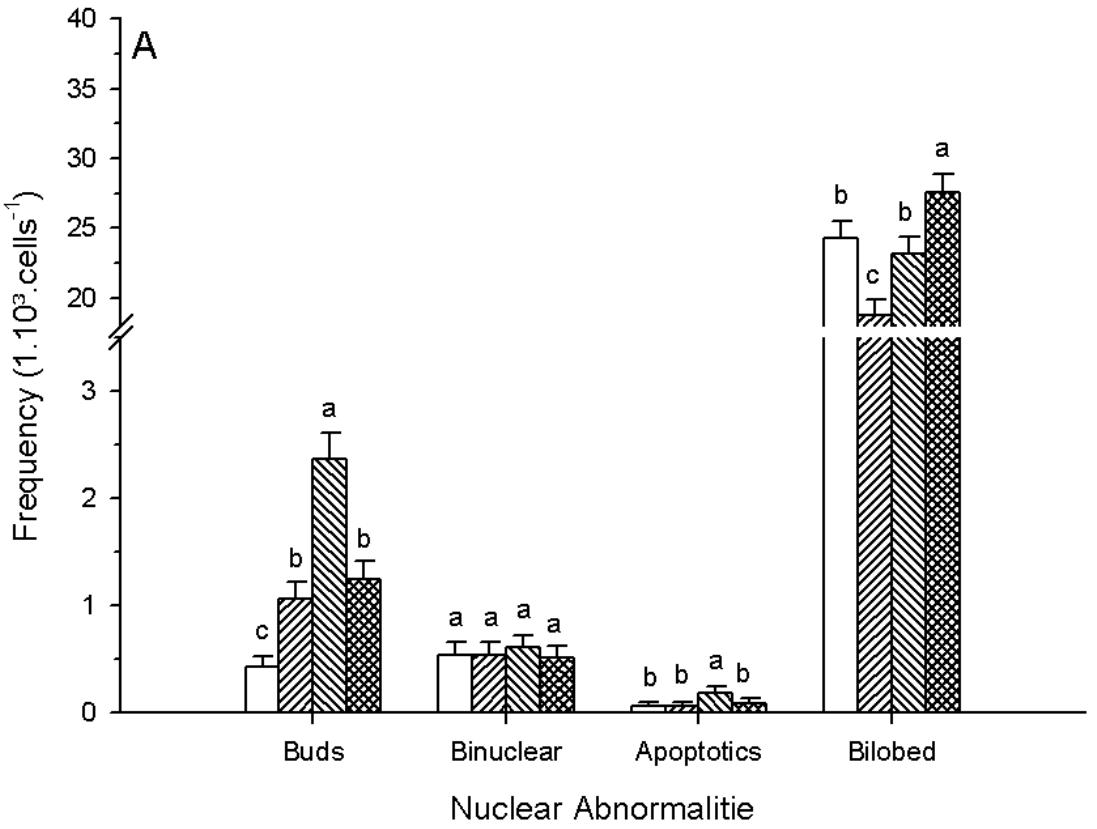
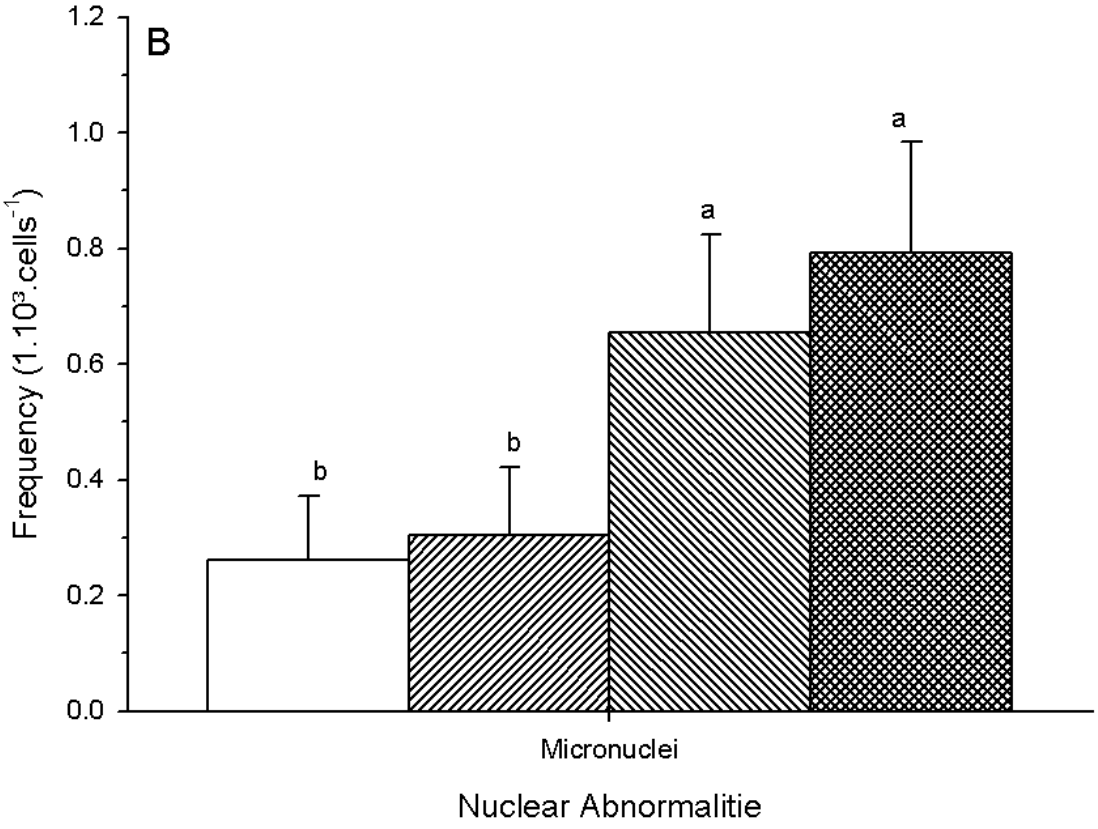

Comet Class (1.10² cells⁻¹)
Comet Score
Copper concentration (µg.L⁻¹)
Control
5
9
20
0
20
40
60
80
100
120
0
4
8
12
40
80
120
160
200
240
a
c
d
d
b
a
c
b
c
d
a
a
b
b
b
a
b
c
b
b
b

Figure 7


A
Frequency (1.10³.cells⁻¹)
40
35
30
25
20
3
2
1
0
c
b
a
b
a a a a
b b a b
b
c
b
a
Buds
Binuclear
Apoptotics
Bilobed
Nuclear Abnormalitie


B
Frequency (1.10³.cells⁻¹)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
b
a
a
Micronuclei
Nuclear Abnormalitie

APÊNDICE 2

Oxidative status, DNA damage and whole-body ions responses to atrazine exposure in the estuarine guppy *Poecilia vivipara*

Running Head: Biomarkers of atrazine exposure in estuarine guppy

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Word Count: 9,331

References Count: 40

Tables: 1

Figures: 6

OXIDATIVE STATUS, DNA DAMAGE AND WHOLE-BODY IONS RESPONSES TO ATRAZINE EXPOSURE IN THE ESTUARINE GUPPY *Poecilia vivipara*

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Abstract

Atrazine is one of most worldwide employed herbicide and it is commonly found as an agrichemical contaminant in surface waters. It causes several effects on aquatic animals, which may be used as biomarkers to monitor environmental contamination with this pesticide. The response of large suite of genetic biomarkers, biochemical and physiological in response to atrazine exposure using methanol as solvent was evaluated in the guppy *Poecilia vivipara*. Biomarkers were analyzed in erythrocytes (DNA damages), liver, gills and muscle (oxidative status parameters), as well as in fish whole-body (ion composition). Fish were kept in control conditions (no atrazine addition to the water) or exposed (96 h) to environmentally relevant concentrations of atrazine (4, 10 and 100 µg/L) in salt water (salinity 24 ppt). In atrazine-exposed fish, glutathione S-transferase activity was increased in liver and gills. In turn, glutathione reductase activity was increased in muscle. In a broad view, catalase activity was increased in liver and reduced in muscle. No significant changes in superoxide dismutase activity and metallothionein-like proteins were observed. Reactive oxygen species production was increased in muscle, while antioxidant capacity against peroxyl radicals was increased in muscle, but reduced in liver and gills. An increased lipid peroxidation was observed in gills. Despite no change was observed in the DNA strand breaks measured through comet assay in erythrocytes, atrazine exposure increased the frequencies of nuclear abnormalities and micronucleated cells. Significant disturbances in whole-body ion (Na, K, and Mg) composition were also observed.

Keywords: atrazine, biomarkers, fish, methanol solvent.

Introduction

Herbicides are a diverse assemblage of compounds with distinct mechanisms of action that shares the same purpose, i.e., control the undesired growth of plants. They are mostly employed in agriculture to improve production and yield. However, their indiscriminate or careless use allows them to enter into aquatic systems through surface runoff and leaching, contaminating surface and ground water [1]. Indeed, water pollution by agrichemicals is a current international concern, and constitutes one of the main inputs to water quality problems [2]. These chemicals were not meant to be toxic to animals. However, several effects of herbicides have been described in a large diversity of animal taxa, clearly indicating that these pollutants are often not entirely specific for their target organisms [3]. In fact, ecological effects on non-target organisms, especially the aquatic life forms have been recorded [4].

Atrazine (2-chloro-4-ethyl- amino-6-isopropylamino-s-triazine) is an effective and inexpensive inhibitor of photosynthesis, being reported as the most commonly used herbicide in the world [4, 5, 6, 7, 8]. This contaminant is considered as showing moderate or low toxicity to aquatic species. However, its environmental mobility, relative high water solubility (in the order of mg/L) and long environmental half-life (in the order of months) [1] place it among the most detected pesticides in streams, rivers, ponds, reservoirs and ground waters [2, 8, 9]. Additionally, triazine compounds are the third generations of new anti-fouling composts used in submerse structures. For these reasons, the acute and chronic toxic effects of atrazine have been studied in several species, including fishes [3, 10]. Endocrine disruption [7], feminization and carcinogenesis [6], ion regulation disturbances [11], behavioral impairments [5], oxidative stress [9], immunity deficiencies [12], DNA damaging, and cell apoptosis [10] are among the recently reported effects. Nevertheless, most of data described in the literature were obtained at very high concentrations of atrazine [7], thus some inconsistencies are

observed. Therefore, some authors states that atrazine at environmentally relevant concentrations does not impose biological risks [1,13].

Considering the unsolved question on the existence of atrazine effects at environmental levels, it is important to consider the fast growing in Brazilian economy, with agriculture playing a crucial role. In fact, farming area and water use in agriculture, as well as the use of herbicides keep increasing [14]. For non-drinking water, the actual Brazilian water quality criterion for atrazine in surface waters corresponds to 2 µg/L [15]. Despite that atrazine levels eventually reach peak values as high as 100 µg/L or even higher in plantation periods [12]. Therefore, biological measurements have been intensively searched in native aquatic organisms to assess the possible effect of short-term exposure to high concentrations of atrazine [16]. Among the parameters considered for biomonitoring, biomarkers are a promising tool for early warning of environmental deleterious effects [17, 18]. However, due to the complex interactive response of physiological and biochemical parameters [19], selecting adequate biomarkers and biomonitors is essential for an efficient assessment of environmental health. In this context, oxidative stress, DNA damage and ionic disturbances could be considered as meaningful biomarkers in aquatic animals. In turn, the guppy *Poecilia vivipara* (Bloch and Schneider, 1801) has been considered as potential biomonitor in coastal aquatic systems.

The guppy *P. vivipara* is a tiny viviparous teleost fish, which was described by Bloch and Schneider (1801) as the first Poeciliinae [20]. This species is endemic of South America and shows a wide distribution in hemi-lentic aquatic ecosystems all along the Brazilian South Atlantic coast. It can be found in salinities ranging from freshwater to seawater [21]. This guppy fish shows an opportunist feeding behavior, eating mainly algae and detritus. It occasionally feeds on bug larvae and lives in calm and shallow waters with clay and plant rich bottoms [22]. These environments are usually among the most likely to accumulate many

kinds of contaminants such as metals, herbicides or other organic chemicals. *Poecilia vivipara* is euryhaline and abundant in nature, showing fast reproduction. Also, it has a small size, being easily collected in the field and handled in the laboratory. These particular characteristics have led to its use as a model species in ecotoxicological studies in both fresh and salt water. Previous studies indicate that *P. vivipara* is highly tolerant to metallic and organic contaminants [23, 24], suggesting the existence of detoxification mechanisms. Therefore, it can provide potential biomarkers for monitoring of water contamination with chemical compounds. Moreover, this fish species is continuously subjected to marked and frequent changes in environmental conditions, thus requiring biochemical and physiological adaptive mechanisms to cope with the variations in physicochemical parameters, including some contaminants [21]. It is also important to note that *P. vivipara* populations did not display large movements, thus serving as an ideal bioindicator of local environmental stressors. All these characteristics point this fish as a potential biomonitor and model species for laboratory testing. However, biochemical, physiological and toxicological information is scarce for this species.

Considering this background, the present study was performed to evaluate the response of a large suite of biochemical, physiological and genetic biomarkers in the Brazilian guppy *P. vivipara* exposed to atrazine. Experiments were designed and data generated were evaluated focusing on the selection of those biomarkers more suitable for use as alternative tools for detection of environmental exposure to atrazine. Furthermore, our findings can contribute for a better understanding of mechanisms involved in acute toxicity of atrazine in fish exposed to environmentally relevant concentrations of this herbicide. Biomarkers evaluated in the present study included oxidative status and oxidative damage parameters in different tissues, as well as whole-body ionoregulatory disturbances.

Material and methods

Fish collection and exposure to atrazine

Male *P. vivipara* were collected at the 'Arroio do Gelo' creek (Cassino Beach, Rio Grande, Southern Brazil). Fish collected in October 2010 were used for enzyme activity, lipid peroxidation (LPO) level, metallothionein-like proteins (MT) concentration, and nuclear abnormalities analyses. Fish collected in January 2011 were used for the determination of reactive oxygen species (ROS) production, antioxidant capacity against peroxyl radicals (ACAP), DNA damage (through comet assay) and whole-body ionic composition. Both fish groups were kept at fixed photoperiod (12 L: 12 D), temperature (20^{o}C), and water salinity (24 ppt). Fish were fed until satiation with commercial fish food (Alcon Basic, Camboriú, SC, Brazil). They were acclimated to the laboratory conditions for at least 14 days before exposure to atrazine.

All glassware and exposure chambers were washed several times with nitric acid (3%), followed by methanol (10%), and thoroughly rinsed with distilled water. Fish were exposed to atrazine in salt water (salinity 24 ± 1 ppt) prepared by diluting natural seawater with overnight-aerated tap water. This mixture was filtered (0.45 μm mesh filter) and contaminated with atrazine immediately before the tests. Atrazine was purchased from Sigma-Aldrich (purity > 98%) and dissolved in methanol (J.T. Baker®)to prepare a stock solution (10 mg atrazine.mL^{-1}). Three concentrations of atrazine were made from the stock solution and tested (4, 10 and 100 μg atrazine/L). These atrazine concentrations were selected considering three different scenarios of contamination of environmental relevance. The first concentration tested (4 μg atrazine/L) is usually found in agricultural areas with atrazine spreading [1]. It is important to note that this concentration is close to the actual Brazilian water quality criterion for atrazine in fresh water [15]. The second concentration tested (10 μg atrazine/L) is usually

found during plantation periods, being reported to be the lower concentration showing significant deleterious effects in fish [11]. The highest concentration tested (100 µg atrazine/L) corresponds to the maximum concentration reported in agricultural areas in Brazil [12]. Also, this concentration is usually found in water bodies in countries that have make use of atrazine for long time, such as the United States, as well as in some streams in Canada [1] and Belgium [3]. Despite no data on *P. vivipara* sensitivity to atrazine are available, concentrations tested were expected to be far below the concentration causing 10% mortality after 96 h of exposure (96 h LC_{10}). For other fish species, 96 h LC_{10} values are actually estimated to be in the order of few mg atrazine/L [5, 10, 12]. Furthermore, the concentration of atrazine inducing 50% mortality of the guppy *P. reticulata* after 96 h of exposure (96 h LC_{50}) to the herbicide was estimated as being 4,300 µg/L [1].

Since methanol was used as solvent for atrazine, two controls were also run: (1) no atrazine or methanol addition into the salt water (referred hereafter as "control" group); and (2) methanol addition into the salt water (referred hereafter as "methanol" group). The final concentrations of the solvent (methanol) in the experimental concentrations of atrazine (4, 10 and 100 µg atrazine/L) corresponded to 0.4, 1 and 10 μL methanol/L of salt water. The final concentration of solvent in the methanol control group was 10 μL methanol/L of salt water. This volume corresponded to the maximum used for the highest concentration of atrazine tested (100 μg atrazine/L).

Adult male *P. vivipara* were exposed (96 h) in 30-L glass aquariums. Fishes were stocked in each aquarium at a density of 1 g fish/L of exposure media. Each aquarium received continuous and gentle air bubbling to keep the dissolved oxygen content close to the saturation level. In addition, the exposure medium was 100% renewed every 24 h to keep water pH (~7.8) and other physicochemical parameters constant, as well as low levels of nitrogenous compounds. Experiments were performed in duplicate. The number of fish

employed for the determination of each biomarker was adjusted according to fish availability in the laboratory, as well as our previous knowledge on the typical deviation of data distribution for the respective biomarker, as described below.

After exposure to atrazine, fish were anesthetized with benzocaine (0.1 g/L) and weighed (wet body mass). Blood was collected and immediately used in comet and nuclear abnormalities assays. Liver, muscle and gills were then dissected and frozen (-80 ^{o}C) for further analyses as described below.

Atrazine concentration measurements

Water samples were collected before fish introduction in the test chamber and after 24 h of exposure. They were pre-concentrated in a C18 (IST Isolute) activated solid-phase system following procedures described by Demoliner et al. [25]. All material used in this procedure, such as amber glass containers, silica gel (Merck, 60-200 mesh ASTM), neutral alumina (Al_2O_3) (Merck, 70-230 mesh ASTM) and granular sodium sulfate anhydrous, was previously calcinated at 450 ^{o}C for 6 h. Adsorbent compounds activation was performed at 160^{o}C for 4 h, and further deactivated with 5 % ultrapure water (MilliQ®) and extracted with *n*-hexane. All other reagents used were high purity reagents (J.T. Baker®). Samples were further eluted and atrazine concentrations were determined by gas chromatography with mass spectrometry detection system (GC-MS). The detection limit was 0.1 μg atrazine/L.

Analyses of oxidative stress biomarkers

Oxidative stress biomarkers were assessed in different tissues (gills, liver and muscle) of fish exposed to atrazine. Reactive oxygen species (ROS) production and antioxidant capacity against peroxyl radicals (ACAP) assays were performed according to Amado et al. (N = 4-6) [26]. Additionally, since the experiments reported in present work were performed

concomitantly to others closely related, control data for ROS and ACAP were earlier presented in Machado et al [23]. Lipid peroxidation (LPO) was determined as described by Oakes and Van Der Kraak [27]. For each experimental group, tissues of 8-10 fish were analyzed. Metallothionein-like proteins (MT) concentration was determined based on the DTNB reaction with sulphydryl groups [28]. Tissues of 4-5 fish were employed for measurements. Catalase (CAT) activity was determined by following the H_2O_2 degradation over time, following procedures described by Beutler [29]. Tissues of 5-6 fish were employed for determinations. Superoxide dismutase (SOD) activity was evaluated using a method based on the reduction of cytochrome C [30]. Tissues of 4 fish were employed. Glutathione reductase (GR) activity was determined indirectly by following the reduction of NADPH in the presence of oxidized glutathione [31]. Tissues of 4-5 fish were used for determinations. Glutathione *S*-transferase (GST) activity was measured based on the conjugation of CDNB, following procedures described by Keen et al. [32]. Tissues of 4-5 fish were used for the analysis. Fishes used in these assays had an average total body length and wet body mass of 40.06 ± 5.50 mm and 0.823 ± 0.341 g, respectively.

DNA damage analyses

Peripheral blood erythrocytes freshly collected from fish measuring (total body length) 37.71 ± 5.21 mm and weighing (wet body mass) 0.825 ± 0.355 g were employed in DNA damage assays. Reversible and non-reversible DNA damages were evaluated through comet and nuclear abnormalities assays, respectively.

Single or double DNA strand breaks can be repaired, thus being considered an index of reversible DNA damaging. Samples from 5-6 fish were employed to determine the index of damage, which was scored through the comet assay [33]. Procedures were adapted to use red gel as a fluorescent marker. Comet score was calculated classifying the tail of each comet into

4 classes of damage, with indexes varying from 0 up to 3. Higher class index corresponded to higher level of DNA damage. Approximately 100 nucleoids were analyzed per fish, with measurements being performed in duplicate. Comet score was calculated multiplying the number of nucleoids from each class by the respective class index, with higher comet scores corresponding to higher levels of DNA damage. Also for comet assay, control data were previous reported in Machado et al [23].

Non-reversible DNA damages are usually reported as biomarkers of effects, representing clastogenicity. In the present study, they were assessed through nuclear abnormalities analyses. Endpoints evaluated were the frequencies of micronucleated cells (MN), nuclear buds, bi-nuclear cells, apoptotic fragments and bilobed cells. They were determined following procedures previously described [34]. Samples from 11 ± 4 fishes were employed in the analysis.

Whole-body ionic composition

Concentrations of major ions (Na, K, Ca, Mg, and Cl) were assessed in fish whole-body. Briefly, whole-body of atrazine-exposed fish was dried (60 ^{o}C) for 72 h and digested in nitric acid (SupraPur, Merck) for 96 h. After properly dilution, ion (Na, K, Ca, and Mg) concentrations were measured by atomic absorption spectrophotometry (Avanta Plus, GBC, Australia). Chloride was measured by commercial kit through formation of sulfur cyanide ferric in acid medium (Cloretos, Doles, Goiânia, GO, Brazil). Despite in this method the reactions occur in acid medium, the excessive nitric acid used in tissue digestion inhibits its reactions. Thus it was necessary partially neutralize adding 100 µL of solution 10 N of NaOH (Vetec Química Fina, São Paulo, Brazil) to each 200 µL of digested sample. It is possible that this procedure slightly change the accuracy of the method, however the same procedure was

adopted for all samples, causing similar effect among all treatments. Besides that, this procedure was previous tested in our lab and did not caused significant effect on precision.

For all ion biomarkers, measurements were performed in 5 fish (total length: 24.53 ± 3.97 mm; wet body mass: 0.204 ± 0.09 g). Also for whole-body ion, control data presented in here were previously reported in Machado et al [23]

Statistical analyses

Data analyses were performed using a Bayesian approach [35]. Conjugated families of probability were used to calculate posteriori distributions according to mathematical solutions presented by Kinas and Andrade [36]. The method basically consists in extracting the information from data of each biomarker generated at laboratory experimental work to construct properly a distribution of probability that better describes the uncertainty about the parameter. Thus, the averages and standard deviation for data showing lack of normality or with normal distributions can be estimated from posteriori distributions. Consequently, this method statistically calculates the likelihood of the association of each possible average to each possible standard deviation for each specific biomarker, allowing deeper and fine-tuned inference. For this modeling, the biomarkers used in the present work were classified in two kinds. Firstly, biomarkers measured as rate or concentration, variables which are described to have normal distributions [35], were modeled by the conjugated family Normal-Gamma. Secondly, for biomarkers of DNA damages measured as counting, variables which are likely to have Poisson distributions [35], the conjugated family Poisson-Gamma was used in the modeling procedure. The mathematical basis for statistical procedures used in the present work is explained in details by Kinas and Andrade [36]. Moreover, all procedures and prior parameters are previously and detailed described in Machado et al [23].

Significant differences among treatments were accessed by hypothesis testing with Bayes's decision, which was based on posteriori odds ratio and Bayes Factor [36]. The posterior distributions of differences of the averages for each treatment and biomarker were obtained from simulations using unilateral tests. In the structure used, hypothesis zero (H_0) negated difference and alternative hypothesis (H_1) accepted it. Posterior distributions of differences concentrated around zero favor H_0, while posterior distributions of differences away from zero favor H_1. For these tests, priori odds ratio = 1 (i.e. giving the same prior probability to both H_0 and H_1), and loss w_0= 5 and w_1=1 for erroneously rejecting H_0 and H_1, respectively. In the section of results, Bayes Factor (BF) is shown to point the strength of the significant difference. It may be worth to mention that BF express how many times H_1 is more probable than H_0, and BF bigger than 3.14 is considered substantial evidence against H_0 [35]. In this context, the present work assumed a conservative posture, rejecting H_0 only when BF was bigger than 5. All data processing was performed using Excel and R software while graphs were plotted with the SigmaPlot software.

Considering that several parameters analyzed were affected by the solvent (methanol) employed to dissolve the herbicide atrazine (see Results section), comparisons to identify possible effects of atrazine were made between fish exposed to atrazine, which was previously dissolved in methanol, and those exposed only to methanol. In turn, a possible effect of solvent on the parameter analyzed was verified by comparing results from fish exposed to methanol (methanol group) and those kept under control conditions (control group). It is important to note that results showing a solvent effect on the parameters analyzed constitute important information, since many of the studies mentioned above (see Introduction section) have not considered the possible influence of the solvent employed. In addition, many of them have used much higher concentrations of methanol or acetone than

the one employed in the present study. Therefore, an interactive effect of methanol and atrazine cannot be ruled out for the parameters analyzed.

Results

Atrazine concentrations

Measured atrazine concentrations in the experimental media were quite similar to the desired nominal concentrations, ranging from 103 to 110 % at the beginning of the experiment and from 99 to 107.5 % after 24 h of exposure. Therefore, atrazine degradation over the 24 h period of exposure was low, varying from 2.2 to 4.5 % (Table 1).

Oxidative stress parameters

ROS production was higher in liver (BF > 35), gills (BF > 14) and muscle (BF > 11) of fish exposed to methanol than in those kept under control conditions. In liver and gills, no significant difference was observed in ROS production between fish exposed to methanol and those exposed to atrazine, except in liver of fish exposed to 100 µg atrazine/L which showed a lower ROS production (BF > 19). On the other hand, fish exposed to any atrazine concentration showed a significant higher muscle ROS production than those from control and methanol groups (BF > 435) (Fig. 1A).

ACAP was higher in muscle (BF > 5), gills (BF > 11) and liver (BF > 23) of fish exposed to methanol than in those kept under control conditions. When compared to the methanol group, ACAP was decreased in liver of fish exposed to 10 (BF > 9) and 100 µg atrazine/L (BF > 999). In gills, a significantly lower ACAP (BF > 13) was observed in fish exposed to any atrazine concentration. In muscle, ACAP was increased in fish exposed to 4 (BF > 6) and 100 µg atrazine/L (BF > 12) (Fig. 1B).

While changes in ROS production and ACAP were observed in all tissues analyzed of fish exposed to methanol or atrazine, significant changes were observed only in MT concentration of gills in those exposed to 100 µg atrazine/L (Fig. 1C).

GST activity was not affected by fish exposure to methanol. However, it was markedly increased in liver of fish exposed to 10 (BF > 31) and 100 µg atrazine/L (BF > 6) when compared to control or methanol-exposed fish. Also, a significant higher enzyme activity was observed in gills of fish exposed to 10 µg atrazine/L (BF > 8) respect to fish exposed to methanol (Fig. 2A).

SOD activity was lower in liver (BF > 6) and gills (BF > 31) of fish exposed to methanol than in those kept under control conditions, while in muscle no significant change was observed. In a broad view, no significant consistent effect of atrazine was also observed when enzyme activity in herbicide-exposed fish was compared with those exposed to methanol (Fig. 2B)

GR activity in liver of fish exposed to methanol was higher than in the liver of those kept under control conditions (BF > 22). However, no significant difference was observed in gills and muscle of fish from the two experimental groups. In general, there was no significant difference in GR activity in liver and gills of fish exposed to methanol and atrazine. However, a significant increase in the enzyme activity was observed in muscle of fish exposed to atrazine respect to that found in fish exposed to methanol (BF > 14) (Fig. 2C).

CAT activity in liver, gills and muscle was not affected by methanol. A slightly reduced enzyme activity was observed in liver of fish exposed to 4 µg atrazine/L (BF > 13) while an increased CAT activity was detected in liver of those exposed to 100 µg atrazine/L (BF > 10). In gills, a slightly reduced CAT activity was observed in fish exposed to 10 µg atrazine/L (BF > 12), while exposure to any concentration of atrazine significantly reduced the enzyme activity in muscle of fish exposed to the herbicide (BF >110) (Fig. 2D).

LPO was increased in gills (BF > 6) and reduced in muscle (BF > 30) of fish exposed to methanol respect to values found in control fish. There was no significant effect of atrazine in liver LPO, while a higher value was observed in gills of fish exposed to 100 μg/L (BF > 158). In muscle, there was no clear pattern of response in fish exposed to atrazine (Fig. 3).

DNA oxidative damages

DNA damage assessed by comet assay was surprisingly lower in erythrocytes of fish exposed to methanol than in those kept under control conditions (BF > 16). There was a reduction in all classes of high index, resulting in a lower comet score. However, exposure to atrazine did not change the degree of DNA damage in fish erythrocytes (Fig. 4).

Despite no significant effect of atrazine was observed in the index of recoverable DNA damage, the clastogenicity indexes were actually increased in erythrocytes of fish exposed to the herbicide (Fig. 5A), resulting in a markedly higher frequency of micronucleated cells in erythrocytes of fish exposed to 100 μg atrazine/L (BF > 22)(Fig. 5B). Nuclear buds were significantly higher in fish exposed to any atrazine concentration (BF > 48), with no significant effect of methanol. In general, the frequency of binucleated erythrocytes and cells with apoptotic fragments were not different among treatments. The frequency of bilobed cells was higher in methanol-exposed fish, while a no clear pattern of response was observed in fish exposed to atrazine (Fig. 5B).

Whole-body ion content

Whole-body sodium (BF > 13) and chloride (BF > 7) contents were reduced in methanol-exposed fish, while no significant change was observed in whole-body potassium content. Exposure to any concentration of atrazine induced a significant increase in whole-body sodium content (BF > 135), while a significant change (reduction) in whole-body

potassium content was observed in fish exposed to 10 (BF > 435) and 100 µg atrazine/L (BF >11) when compared to methanol-exposed fish. In turn, a reduction in whole-body chloride was detected only in fish exposed to 4 µg atrazine/L (BF > 5) (Fig. 6). No significant effect of methanol or atrazine on whole-body calcium was observed, while a significant increase in whole-body magnesium concentration was detected after exposure to methanol (BF > 28) or any atrazine concentration when compared to control (BF > 69). A significant change (increase) of magnesium was only observed in fish exposed to 10 µg atrazine/L when compared to those exposed to methanol (Fig. 6).

Discussion

As mentioned above, several studies have reported that atrazine induces several effects in a diversity of animal groups. However, it is clear the lack of consensus on the potential of atrazine to induce biological effects at environmental relevant concentrations [1, 13]. In fact, many of these studies were performed using very high concentrations of atrazine [7] or did not have measured the atrazine concentrations in the exposure media. In this context, results reported in the present study provide strong evidence of the atrazine potential to affect exposure biomarkers and even cause deleterious effects in fish at environmentally relevant levels. Also, findings reported here provide important information on the response of a large suite of biomarkers from different biological levels of organization, from molecules up to whole body. Therefore, they can contribute for a better understanding of the mechanisms involved in atrazine toxicity, as well as help us to select the most sensitive and reliable biomarkers to monitor the environmental exposure of the guppy *P. vivipara* to atrazine-based herbicides.

The present work was designed in accordance to guidelines of organization for economic cooperation and development (OECD) for aquatic toxicity testing of substances with difficult solubility [37], which limits the solvent concentration up to 100 μL/L. In this sense, we used a low solvent concentration (up to 10 μL/L) compared to the testing guidelines and some others atrazine works. However, many responses of biomarkers were affected by single vehicle exposure. This unexpected effects might be due to the fact that safety limits for solvent exposure in these guide lines were estimated based on historical data and endpoints such as mortality and growth, which are less sensitive than biochemical and genetic biomarkers used in the present work. In fact, interaction of methanol and biomarkers in fish remains very unknown, its mechanisms of toxicity are still unclear and most of studies in methanol toxicity were performed regarding only cryopreservation. The few works available suggest that methanol is a solvent potent in disrupting, crossing and extracting lipids from all membranes and compartments of fish bodies exposed during few minutes to 1 or 2 M methanol solutions [37]. Such sort of damages causes differential membranes permeability, which could explain the changes in ions contents of methanol-exposed fishes. Hutchinson et al [37] provide relevant review on solvent effects on aquatic toxicity testing. According to these authors, methanol is described to induce acidosis in cells of several vertebrates as well as increase peroxidative pathways during its metabolism. Cortisol rising, inhibition of P450 enzymes, endocrine disruption and increment of the toxicity of contaminant are also recently reported to the use of methanol as solvent in aquatic toxicity tests [37]. In this context, the present work provides important information in simultaneously showing control, methanol control and fish exposed to atrazine carried by methanol, contributing to understanding of both methanol and atrazine toxic responses. Despite it does not simplify the interpretation of single atrazine effects, this approach allows comparisons to most of atrazine works, which have used solvents in similar concentrations. Furthermore, this approach supplies relevant

information to environmental biomonitoring on the level of each biomarker that can be affected by toxicants when compared to a truly control.

Regarding effects related to atrazine, antioxidant enzymes evaluated in the present study responded to atrazine exposure. For example, GST activity was markedly increased in liver of the guppy *P. vivipara* exposed to 10 and 100 µg atrazine/L. An increased GST activity was also observed in liver of *Channa punctatus* exposed to high a concentration of atrazine (a few mg/L) [5] and liver of female zebrafish *Danio rerio* exposed to 100 µg atrazine/L for 14 days [9]. GST is a phase II detoxifying enzyme that catalyzes conjugation of GSH with a variety of compounds [16], allowing their metabolism by the P450 complex [19]. The observed increase in liver GST activity clearly points to the presence of a biochemical mechanism involved in atrazine metabolism in the liver of the guppy *P. vivipara*. Although previous studies report kidney as the organ involved in atrazine metabolism [4], our findings point to an important function of liver in atrazine detoxification. Indeed, it is known that atrazine is quickly metabolized, thus not being accumulated or concentrated in aquatic organisms [1].

It is interesting to note that a reduction in ACAP and no change in MT content were observed in liver of *P. vivipara* exposed to atrazine. Furthermore, an increased CAT activity was observed in the liver of fish exposed to 100 μg atrazine/L. Increases in CAT activity were also observed in the spleen of gold fish exposed to 50 μg atrazine/L [3], liver of *Channa punctatus* exposed to a high concentration of atrazine (a few mg/L) [5], and whole-body of earthworms [8]. Therefore, our findings clearly suggest that the reduced ACAP observed in liver of *P. vivipara* would be related to a reduction in liver GSH stock due to a higher consumption of this non-enzymatic antioxidant during atrazine detoxification and a lack of increase in GSH recycling. In fact, liver GR activity did not change in *P. vivipara* exposed to increasing concentrations of atrazine. Taking altogether, these findings strongly suggest the

involvement of GSH in the detoxification and elimination pathway for atrazine, as well as point the liver as an organ of detoxification for this herbicide.

SOD is one of first antioxidant enzyme to respond against the oxygen radical production. It converts O_2^- into peroxide and oxygen [30], providing substrate for CAT [19]. Interestingly, atrazine exposure did not change SOD activity in all tissues analyzed in the guppy *P. vivipara*. Also, no marked response in CAT activity was observed in gills and even an increased enzyme activity was observed in liver of fish exposed to 100 μg atrazine/L. In fact, these findings are in agreement with the observed lack of change in ROS production in both liver and gills of atrazine-exposed fish. Despite no changes in ROS production were observed, ACAP was reduced in both tissues of fish exposed to atrazine. As discussed above, this reduced ACAP would be related to an increased use of GSH in the atrazine detoxification metabolism. Therefore, oxidative damage to biomolecules would be expected in both liver and gills of atrazine-exposed fish. In fact, higher LPO values were observed in the liver of *Channa punctatus* exposed to high concentration of atrazine (a few mg. L^{-1}) [5] and in liver of female zebrafish *D. rerio* [9]. Unexpectedly, no change in LPO was observed in the liver of *P. vivipara* exposed to atrazine. However, a higher oxidative damage to lipids (LPO) was observed in gills of this fish.

Additionally, it has been reported that exposure to atrazine induces impairment in fish respiratory behavior, likely associated with histological gill damages induced by the herbicide [5, 9, 11]. These damages can be explained, at least in part, to an increased gill LPO, as observed in the present study in the guppy *P. vivipara*. Moreover, atrazine-induced oxidative damages are related to a depletion of GSH stocks in fish tissues [9, 10], as suggested in the present work. It is important to note that gills are a multifunctional organ, being involved not only in respiration, but also in ionic and osmotic regulation, acid-base balance and

nitrogenous compounds excretion. Therefore, oxidative-induced damages to this organ can lead also to disturbances in these physiological parameters.

Data from the present study clearly show that atrazine exposure induced marked increases in whole-body Na and Mg contents, as well as a significant decrease in whole-body K content. Only few studies have addressed the effects of atrazine on ionic regulation in fish. Increased whole-body Na content was also reported in fish exposed to endosulfan and others chlorinated hydrocarbons. Regarding atrazine, most studies on ion regulation are related to salmon smoltification. Plasma ionic disturbances after exposure to atrazine concentrations as high as 100 µg/L were previously reported. Decreased and increased plasma ions concentrations were observed in *Salmo salar* smolts in fresh and sea water, respectively [38]. Furthermore, a reduced Na, K-ATPase activity was observed in gills of the rare minnow exposed to atrazine concentrations higher than 3 µg/L for 28 days [11]. These findings are clear evidences that atrazine is able to disrupt the plasma and whole-body ionic regulation in fish. These effects are biologically relevant since homeostasis of fluids is crucial for the adaptive success of euryhaline fish like the guppy *P. vivipara* to the frequent and marked environmental changes occurring in estuarine and coastal waters. Like other vertebrates, fish must keep the whole-body hydromineral balance to ensure the physiological integrity of all the major biological systems [18]. Therefore, the whole-body ion disturbances observed in the guppy *P. vivipara* not only help us to better understand the mechanism involved in atrazine toxicity in fish, but also characterize the whole-body ion content as a potential biomarker of exposure to environmentally relevant concentrations of atrazine in salt water.

Despite muscle is not a target of atrazine toxicity, differently from liver and gills, this tissue showed a higher ROS production in fish exposed to 100 µg atrazine/L. An increased ROS production was also reported in the spleen of gold fish exposed to 50 µg atrazine/L in a mixture of herbicides [3]. Therefore, a higher ACAP would be expected to occur in muscle of

P. vivipara exposed to 100 µg atrazine/L in order to scavenge the observed surplus in ROS production. In fact, an increased ACAP was observed in muscle of fish exposed to 4 and 100 µg atrazine/L. This increased ACAP in muscle is likely due to an augmented rate of GSH recycling, which was observed in muscle GR of fish exposed to 4 and 100 µg atrazina/L. Moreover, this statement is based on the fact that no change in SOD activity, a reduced CAT activity, and no change in MT content were observed in muscle of fish exposed to atrazine. Furthermore, this increased ACAP was enough to scavenge the observed higher ROS production since no change in LPO was found in muscle of the atrazine-exposed fish. Therefore, this biochemical response is an important mechanism of defense against the deleterious effects of an excessive ROS production in muscle. In fact, oxidative damage induced by atrazine associated with an excessive ROS production lead to several behavioral disturbances in fish. The most usual effect of atrazine in fish behavior is faster swimming and greater jumping [1, 5]. It is important to remark that these behavioral disturbances were visually not observed in *P. vivipara* exposed to atrazine in the range of concentrations tested.

In addition to the oxidative damage to membrane lipids as discussed above, atrazine exposure has also shown to be related to cancer oncogenesis and loss of DNA integrity [6]. Despite oxidative stress parameters were not analyzed in erythrocytes of the guppy *P. vivipara* in the present study, these cells also showed evidences of damages, such as observed in gills of fish exposed to atrazine. The assessment of single or double DNA strand breaks through comet assay did not show oxidative damage in fish erythrocytes, however, clastogenicity indexes measured in the present study indicate an augmented DNA damage with increasing concentrations of atrazine. Similarly to what we show, a lack of comet assay change paralleled by higher levels of nuclear abnormalities and DNA damage have been also found in environmental monitoring studies using salt water fish [17]. Thus, it is likely that DNA strand breakage induced by atrazine exposure occurs through adduct incision and/or

dysfunction on DNA-related enzymes. Notwithstanding comet assay has been considered as one of most sensitive biomarkers for assessment of DNA damage, it does not distinguish the DNA damages associated with adduct incision into the DNA strands, dysfunction in endonucleases and topoisomerases or increases in intracellular calcium. In fact, DNA repair is slow in fish compared to mammals, decreasing considerably the sensitivity of this assay to strand breaks by adduct incision or transcriptional events [39].

In the present study, atrazine-induced clastogenicity was well characterized by a marked increase in the frequency of micronucleated cells in erythrocytes of *P. vivipara* after exposure to 100 μg atrazine/L. This nuclear abnormality is one of most well established index of clastogenicity and DNA damage. Indeed, micronuclei are produced from chromosome fragments or whole chromosomes that lag at cell division due to lack of centromere, damage in centromere or defect in cytokinesis. High frequency of micronucleated cells may indicate high level of mutagenicity. Since an increment in apoptotic cells is a way to eliminate micronucleated mutating cells, the observed lack of increase in the frequency of cells with apoptotic fragments in erythrocytes of *P. vivipara* exposed to atrazine indicates that these cells are likely subjected to higher mutating rates. Regarding the frequency nuclear buds, it was increased in fish exposed to any atrazine concentration tested. Although this nuclear abnormality has its mechanism of formation and consequences still not understood, it is reported that increments in the frequency of nuclear buds are related to genotoxic damages [40]. In turn, the increased frequency of cells with bilobed nucleus observed in *P. vivipara* exposed to 100 μg atrazine/L can be explained by an augmented release of young erythrocytes into the fish bloodstream from the hematopoietic organs.

Several authors point that oxidative stress is considered as a DNA damaging factor [5, 9, 10, 12, 39]. In fact, findings reported in the present study suggest that the DNA damages observed in the guppy *P. vivipara* are also related to an oxidative stress induced by atrazine

exposure. Since a high detoxification activity was observed in liver of fish exposed to atrazine, oxidative damages observed could be also a result from exposure to hepatic oxidative metabolites. Indeed, it has been shown that some extra-hepatic tissues, such as blood cells, may play an important role in the metabolism of xenobiotics [40]. In any case, DNA damage observed in erythrocytes of *P. vivipara* after exposure to atrazine is a clear evidence of the likely clastogenic effect of the herbicide.

In this context, more studies involving genetic and physiological parameters associated with the oxidative status in fish is needed for a better understanding of the atrazine effects on the complex and interactive metabolic processes in fish exposed to the herbicide. This approach also showed that the bulk of information reported in the present study allowed us to identify the potential of several oxidative stress-related parameters analyzed as sensitive tools to assess environmental exposure to atrazine.

The implication of the present results for biomonitoring herbicides in environment, in terms of biomarkers specificity, is that none of biomarkers analyzed showed to be specific to atrazine. Actually, as discussed above, most of then proved to be affect also by single methanol exposure. Additionally, many of then are reported to respond also to other chemicals, such as metals or hydrocarbons [23]. However, it is clear from our findings that evaluation in parallel of several biochemical, physiological and/or genetic damages is a potential tool for diagnostic atrazine exposure. In fact, when the response of the assembly of biomarkers is observed some very particular association of responses related to the presence of herbicide. The non-responding comet assay linked to higher clastogenicity and elevated GSH metabolism might be a strong indicative of atrazine exposure, for example. It is important to stress that these parameters could also be used to evaluate other agrichemicals that may be applied in combination with the herbicide atrazine, such as methyl parathion- and glyphosate-based compounds, as well as the fungicide tebuconazole, which have similar

biological effects [16]. Finally, it is important to mention that atrazine effects are reported to be initially reversible, tending to be more permanent with prolonged exposures [3]. Therefore, further studies using longer periods of exposure than the one employed in the present study (96 h) would be important to reinforce the findings reported here. This would allow a more confident application of the oxidative stress-related biomarkers in biomonitoring studies focused on the contamination of aquatic environments with atrazine.

Conclusions

In light of the discussed above, it is clear that exposure to environmentally relevant concentrations of atrazine can induce significant changes in the oxidative status of different tissues of *P. vivipara*, culminating in oxidative stress and oxidative damage in gills and erythrocytes. Also, our findings indicate that GSH is involved in the mechanism of atrazine detoxification in the liver. In terms of applicability in environmental biomonitoring, oxidative stress-related biomarkers such as ROS production, ACAP, GST activity, LPO, FMN and whole-body ionic disturbances appear to be those showing the most consistent response to atrazine exposure in the guppy *P. vivipara* acclimated to salt water.

Acknowledgements

This work was supported by the National Council for Scientific and Technological Development (CNPq, Brasília, DF, Brazil) in the scope of the National Institute of Science and Technology of Aquatic Toxicology, the Coordination for Training of Superior Studies Personnel (CAPES, Brasília, DF, Brazil), and the International Development Research Centre (IDRC, Ottawa, Canada). A. Bianchini is a research fellow from the Brazilian CNPq (Proc. #

304430/2009-9) and supported by the International Canada Research Chair Program from IDRC. Authors thank, Dr Marta M. Souza for help in tissues collection, and Dr Ana L.M. Baisch and Dr Carine Dahl Corcini for providing the laboratory conditions to perform the comet assay.

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Table 1. Environmental relevance [1], nominal and measured (mean ± standard deviation) atrazine concentrations at the beginning (Time 0) and after 24 h of exposure (Time 24), and atrazine degradation after 24 of exposure in experiments performed with the guppy *Poecilia vivipara* in salt water (salinity 24 ppt). No atrazine was detected in both control and methanol treatments.

Environmental relevance	Atrazine concentration (µg/L)			Degradation (%)
	Nominal	Measured		
		Time 0	Time 24	
Likely to occur	4	4.40 ± 0.56	4.30 ± 0.59	2.2 ± 0.92
Commonly detected	10	10.40 ± 1.03	9.90 ± 0.93	4.5 ± 0.55
Contaminated	100	103.00 ± 4.41	99.60 ± 3.76	3.2 ± 1.03

Figure Legends

Figure 1. (A) Reactive oxygen species (ROS) production, (B) total antioxidant capacity against peroxyl radicals (ACAP), and (C) metallothionein-like proteins (MT) concentration on liver, gills and muscle of guppy *Poecilia vivipara* after 96 h in control conditions (open bars) or exposed to methanol (diagonally hatched bars), 4 (diagonally inverted hatched bars), 10 (double hatched bar) and 100 µg/L (horizontally marked bars) of waterborne atrazine in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among atrazine concentrations for each tissue within the same parameter, and means sharing the same letter are not significantly different.

Figure 2. (A) Glutathione S-transferase (GST), (B) superoxide dismutase (SOD), (C) glutathione reductase (GR) and (D) catalase (CAT) on liver, gills and muscle of guppy *Poecilia vivipara* after 96 h in control conditions (open bars) or exposed to methanol (diagonally hatched bars), 4 (diagonally inverted hatched bars), 10 (double hatched bar) and 100 µg/L (horizontally marked bars) of waterborne atrazine in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among atrazine concentrations for each tissue within the same parameter, and means sharing the same letter are not significantly different.

Figure 3. Lipid peroxidation (LPO) on liver, gills and muscle of guppy *Poecilia vivipara* after 96 h in control conditions (open bars) or exposed to methanol (diagonally hatched bars), 4 (diagonally inverted hatched bars), 10 (double hatched bar) and 100 µg/L (horizontally marked bars) of waterborne atrazine in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among atrazine

concentrations for each tissue within the same parameter, and means sharing the same letter are not significantly different.

Figure 4. DNA damage measured through comet assay in erythrocytes of the guppy *Poecilia vivipara* exposed (96 h) to different concentrations of waterborne atrazine in salt water (salinity 24 ppt). For comet class (left y-axis) (class 0: open bars; class 1: diagonally hatched bars; class 2: diagonally inverted hatched bars; class 3: double hatched bars), bars represent average and lines indicate standard deviation. For comet score (right y-axis) data are expressed as mean (closed circles) ± standard deviation (vertical lines). Different letters indicate significant difference among atrazine concentrations for each parameter, and means sharing the same letter are not significantly different.

Figure 5. DNA damage measured through nuclear abnormalities in erythrocytes of the guppy *Poecilia vivipara* 96 h in control conditions (open bars) or exposed to methanol (diagonally hatched bars), 4 (diagonally inverted hatched bars), 10 (double hatched bar) and 100 µg/L (horizontally marked bars) of waterborne atrazine in salt water (salinity 24 ppt). (A) Frequency of nuclear abnormalities: nuclear buds, binucleated cells, cells with nucleus presenting apoptotic fragments, and cells with bilobed nucleus. (B) Frequency of micronucleated cells. Data are expressed as mean ± standard deviation. Different letters indicate significant difference among atrazine concentrations for each tissue within the same parameter, and means sharing the same letter are not significantly different. Data are expressed as mean ± standard deviation. Different letters indicate significant difference among atrazine concentrations for each parameter, and means sharing the same letter are not significantly different.

Figure 6. Whole-body sodium, potassium, chloride, calcium and magnesium in the guppy *Poecilia vivipara* 96 h in control conditions (open bars) or exposed to methanol (diagonally hatched bars), 4 (diagonally inverted hatched bars), 10 (double hatched bar) and 100 μg/L (horizontally marked bars) of waterborne atrazine in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among atrazine concentrations for each parameter, and means sharing the same letter are not significantly different.

Figure 1

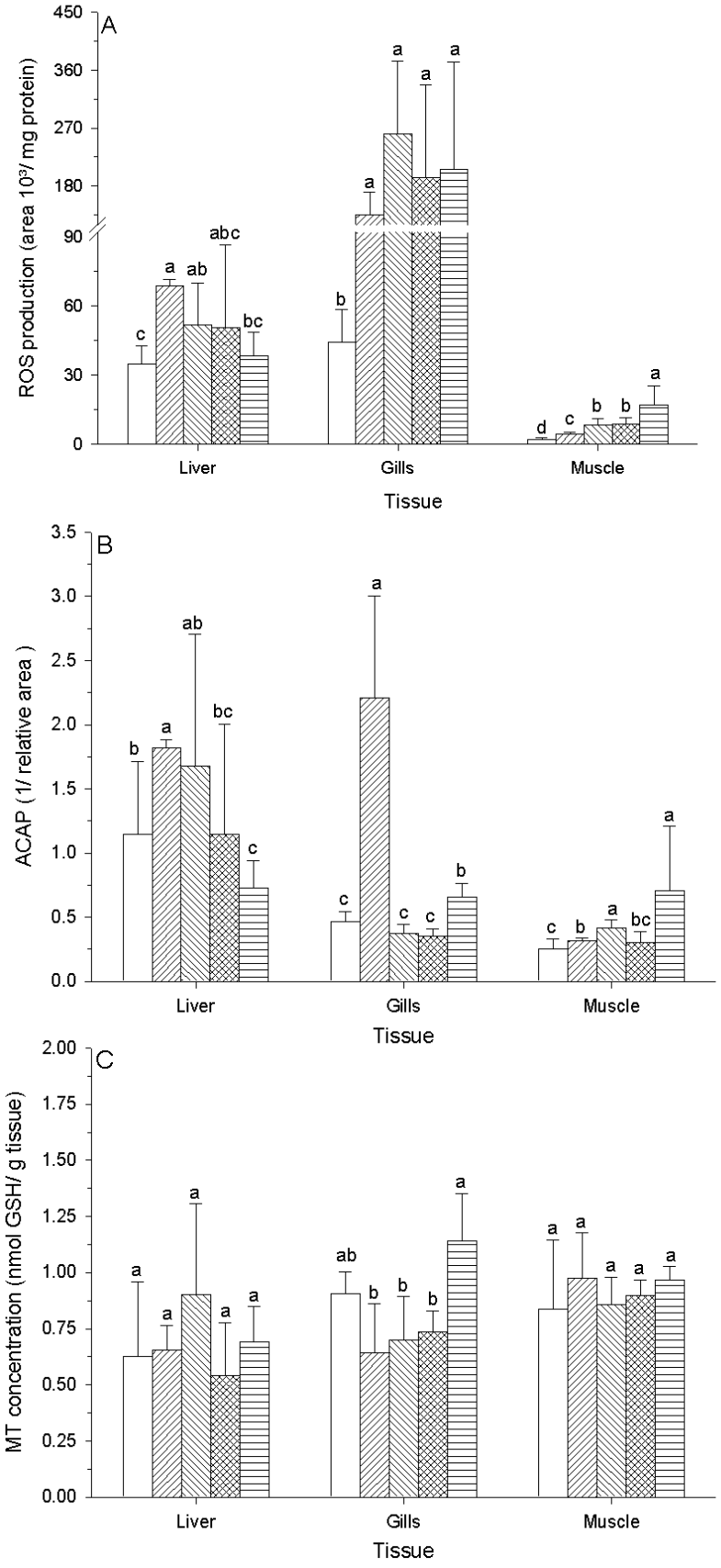

A
ROS production (area 10³/ mg protein)
450
360
270
180
90
60
30
0
c
a
ab
abc
bc
b
a
a
a
a
d
c
b
b
a
Liver
Gills
Muscle
Tissue
B
ACAP (1/ relative area)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b
a
ab
bc
c
c
a
c
c
b
c
b
a
bc
a
Liver
Gills
Muscle
Tissue
C
MT concentration (nmol GSH/ g tissue)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
a
a
a
a
ab
b
b
b
a
a
a
a
a
a
Liver
Gills
Muscle
Tissue

Figure 2

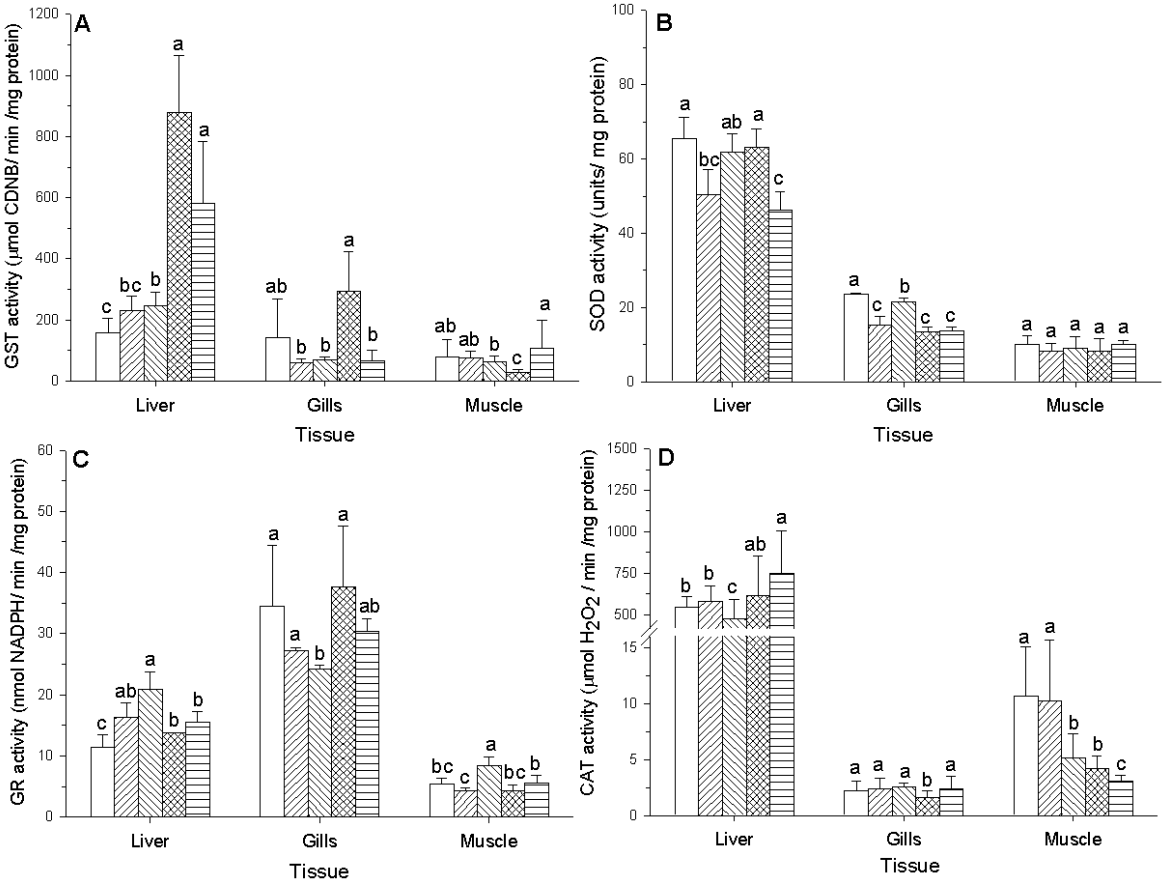

A
GST activity (µmol CDNB/ min /mg protein)
Liver
Gills
Muscle
Tissue
B
SOD activity (units/ mg protein)
C
GR activity (nmol NADPH/ min /mg protein)
D
CAT activity (µmol H_2O_2 / min /mg protein)

Figure 3

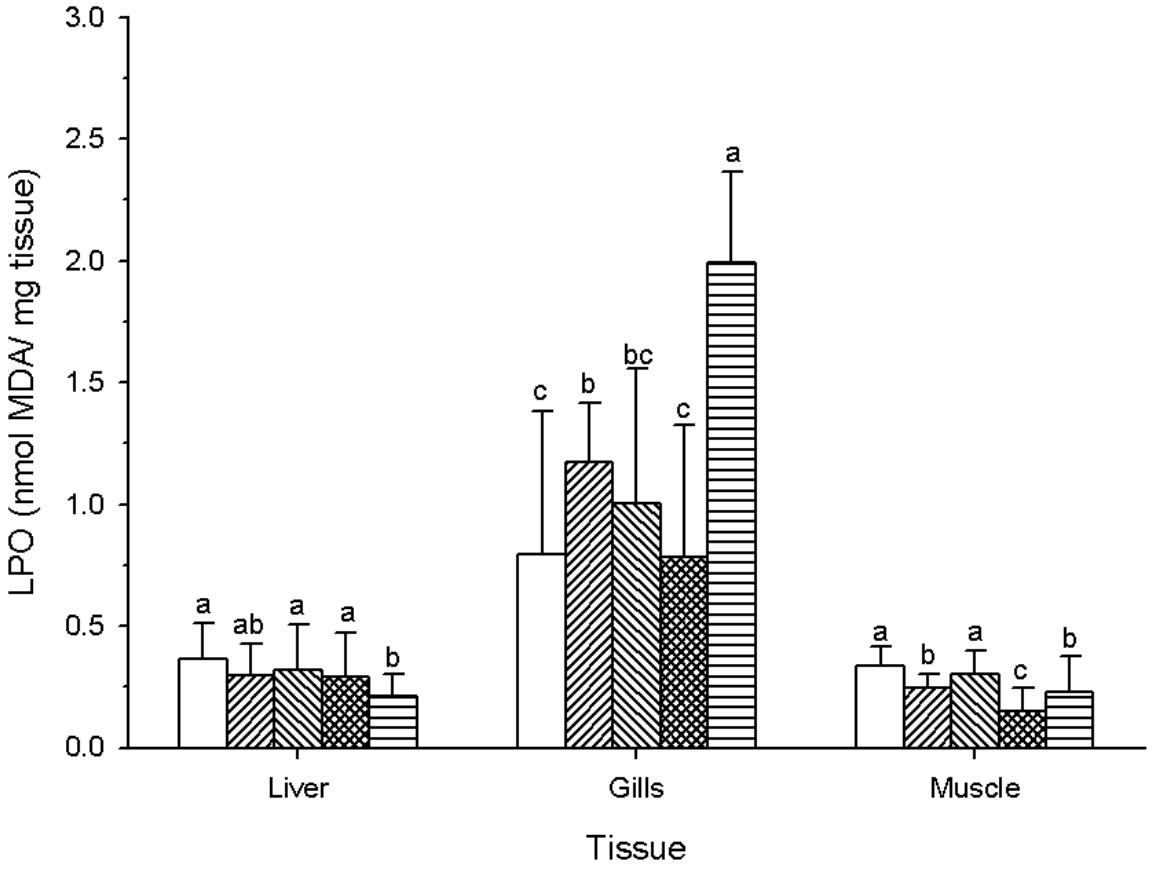

3.0
2.5
2.0
1.5
1.0
0.5
0.0
LPO (nmol MDA/ mg tissue)
a
ab
a
a
b
c
b
bc
c
a
a
b
a
c
b
Liver
Gills
Muscle
Tissue

Figure 4

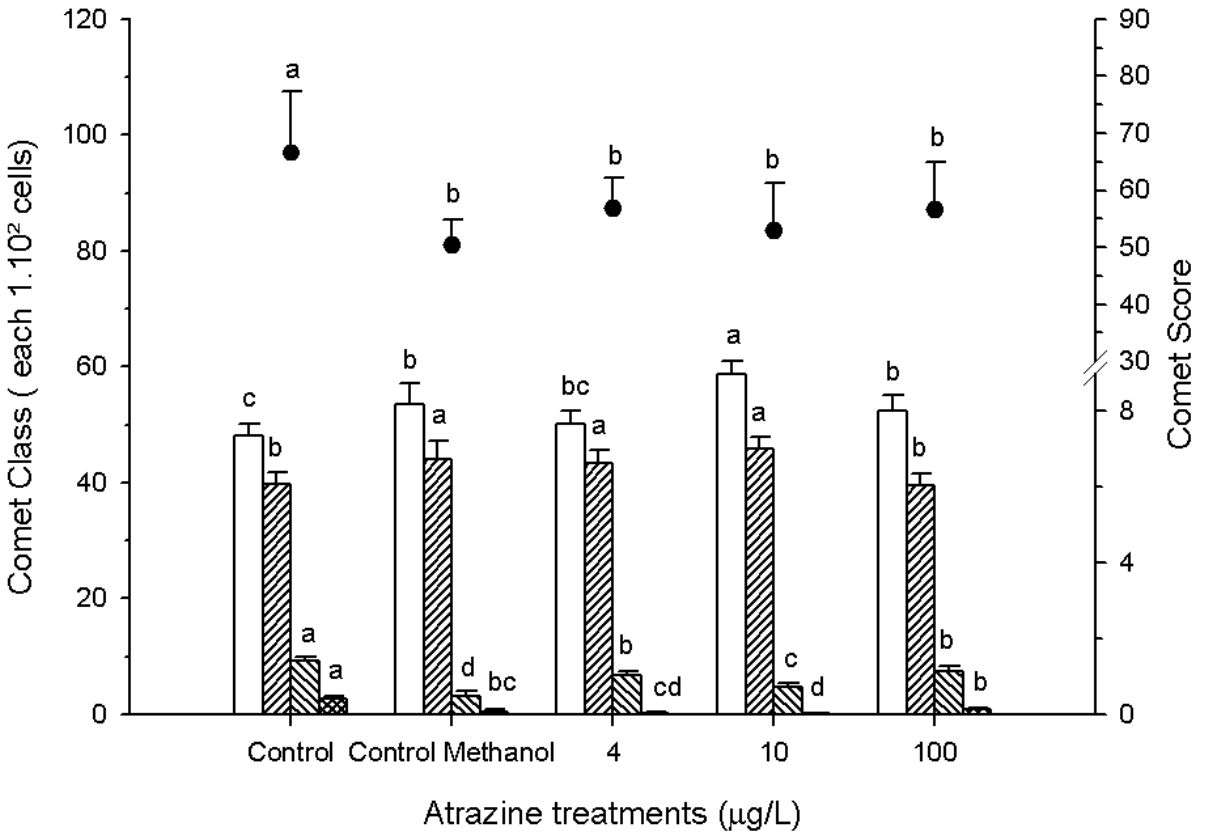
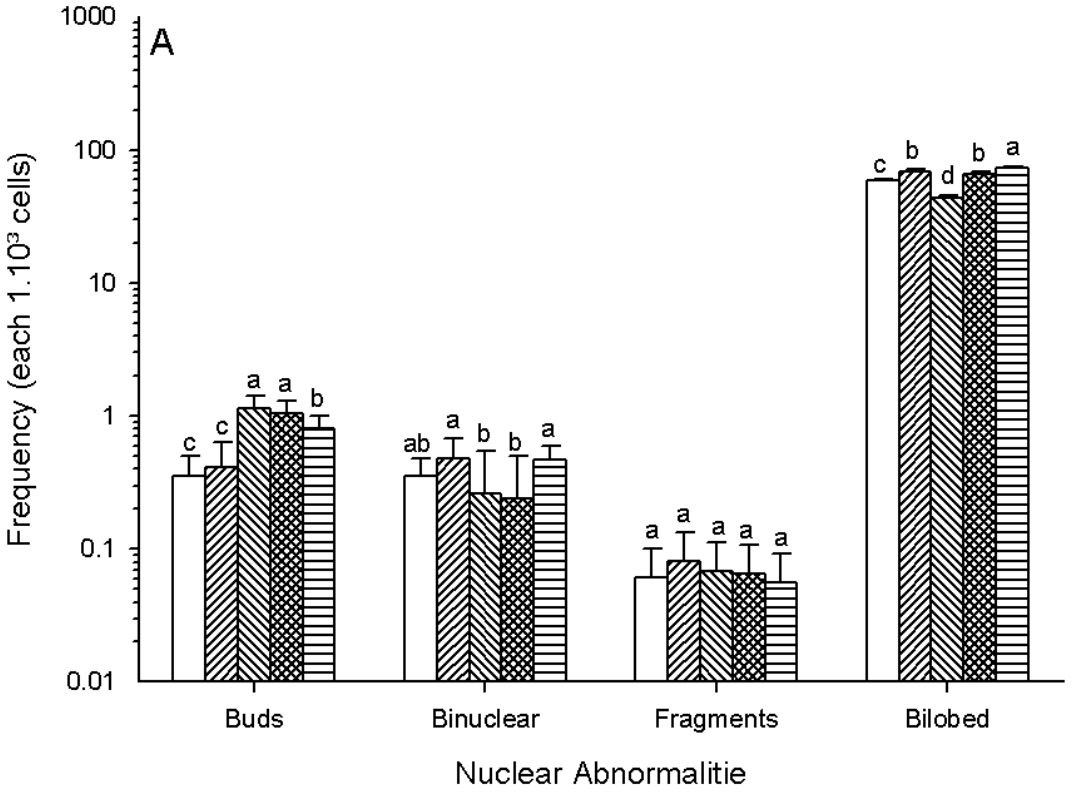

Comet Class (each 1.10² cells)
Comet Score
Atrazine treatments (µg/L)
Control
Control
Methanol
4
10
100
120
100
80
60
40
20
0
90
80
70
60
50
40
30
8
4
0
a
b
c
b
a
a
b
a
d
bc
b
a
b
bc
a
b
cd
a
a
c
d
b
b
b
b
b

Figure 5


A
1000
100
10
1
0.1
0.01
Frequency (each 1.10³ cells)
c c a a b
ab a b b a
a a a a a
c b d b a
Buds
Binuclear
Fragments
Bilobed
Nuclear Abnormalitie

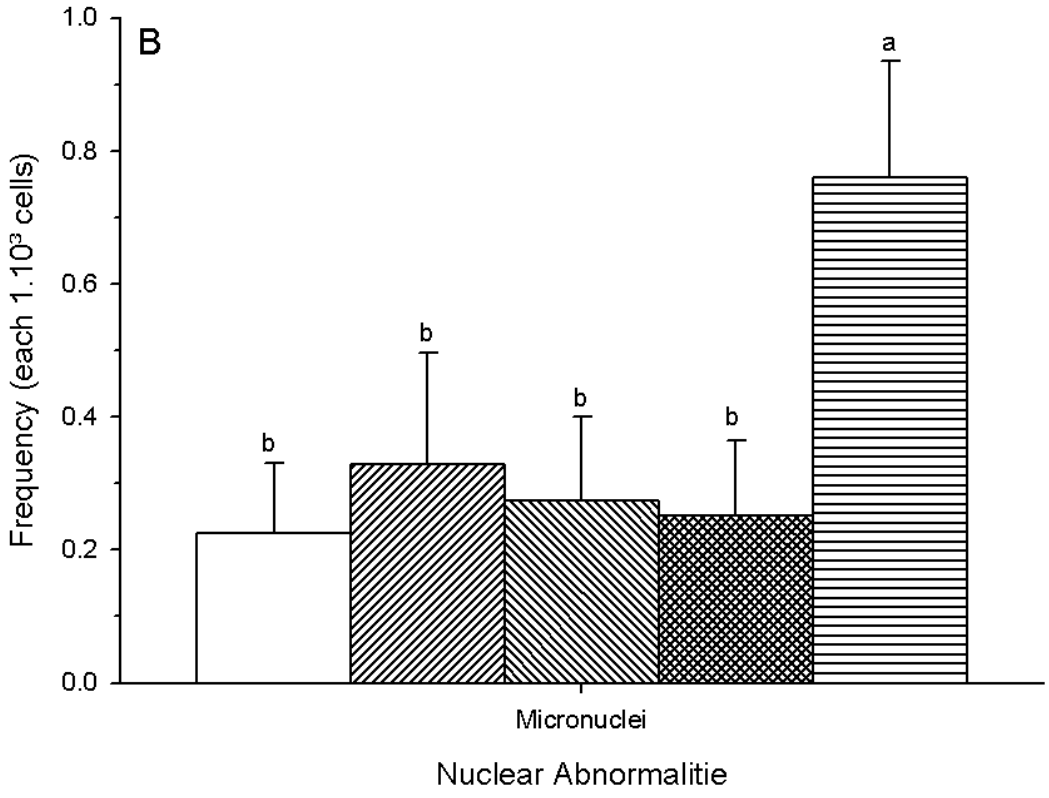
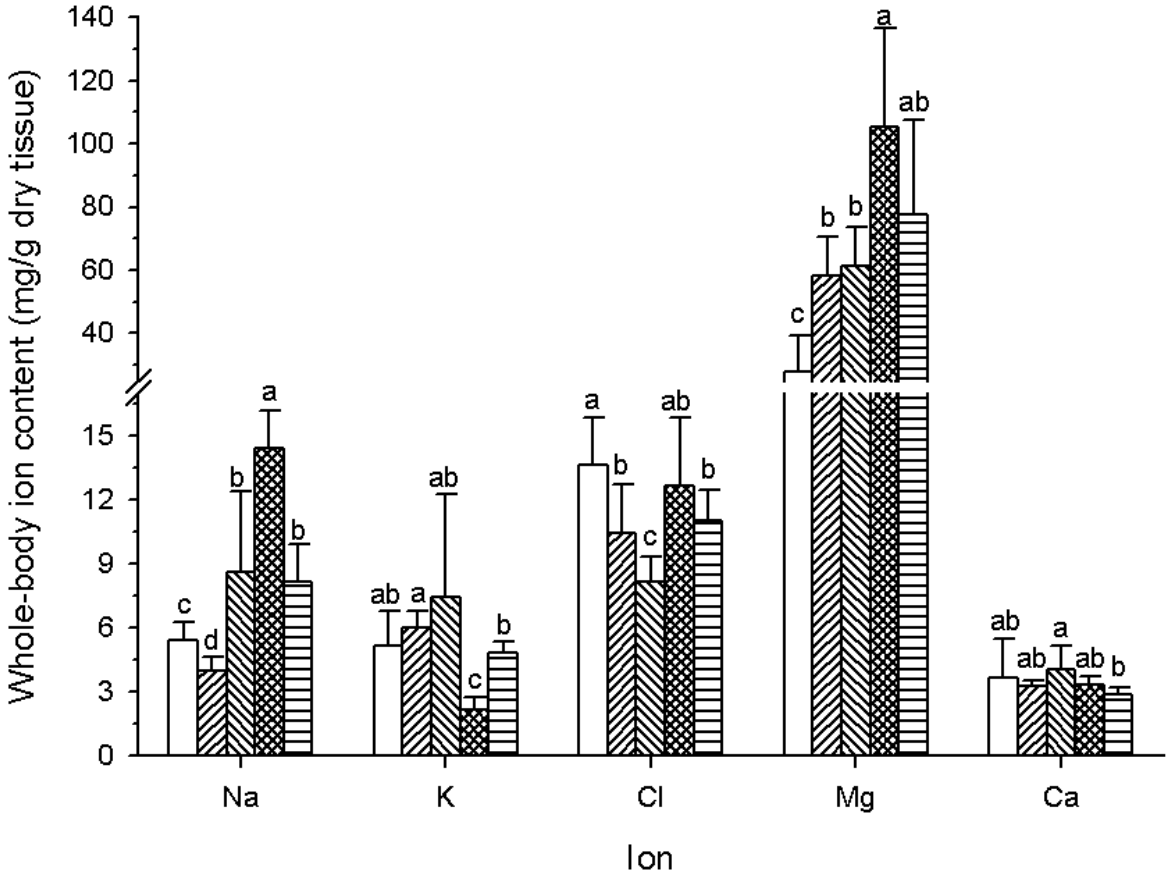

B
1.0
0.8
0.6
0.4
0.2
0.0
Frequency (each 1.10³ cells)
b b b b a
Micronuclei
Nuclear Abnormalitie

Figure 6


Whole-body ion content (mg/g dry tissue)
140
120
100
80
60
40
15
12
9
6
3
0
Na
K
Cl
Mg
Ca
Ion
a
ab
b
b
c
ab
a
b
c
ab
a
b
c
d
b
a
ab
b
c
ab
b
ab
ab
a
ab
b

APÊNDICE 3

Biomarkers response to phenanthrene and DMSO exposure in the estuarine guppy *Poecilia vivipara*

Biomarkers response to phenanthrene and DMSO exposure in the estuarine guppy *Poecilia vivipara*

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Abstract

Phenanthrene is one of most ubiquitous and aggressive contaminants. Unfortunately, little information on its effect on tropical estuarine fishes is available. We evaluated the response of a large suite of biomarkers (oxidative stress-related parameters, oxidative damage to biomolecules, and whole-body ionic composition) to phenanthrene exposure using DMSO as solvent (10, 20 and 200 $\mu g.L^{-1}$) was evaluated in the guppy *Poecilia vivipara* in salt water (salinity 24 ppt). Phenanthrene exposure affected the response of some oxidative stress-related parameters in liver, gills and muscle, with effects being dependent on tissue and phenanthrene concentration. Also, no evident DNA damage was observed in erythrocytes. However, marked and consistent disturbances in whole-body ionic content were observed. These effects were clearly not associated with the oxidative stress-related parameters. Our findings indicate that whole-body Na, Cl and Mg content as the most sensitive and practical biomarkers to monitor the exposure of the guppy *P. vivipara* to phenanthrene in salt water.

Keywords: biomarkers, DNA damage, ion regulation, oxidative stress, phenanthrene, *Poecilia vivipara*, salt water

1. Introduction

In the last decade, about 70% of world's population was already living in coastal environments, and as it increases, pressures on aquatic resources in these areas are multiplied (Bortone, 2005). In fact, water bodies of coastal ecosystems have been used for multiple purposes, such as tourism, fisheries, transport and industrial activities (Neto et al., 2008). These activities generate a miscellaneous of chemical substances potentially toxic to aquatic organisms, and in amounts enough to compromise the environmental health (Zagatto, 2006). Indeed, estuaries, fresh water and marine coastal environments have been used as natural deposits of chemical contaminants (Kennish, 1991), with many of them showing deleterious effects on ecosystems. Therefore, to monitor the effects of aquatic contaminants is crucial for a proper preservation of natural resources. In this context, the use of biomarkers in fish is considered as a cost effective strategy to obtain information on the state of the aquatic environment and the effect of pollutants on living resources (Pathiratne and Hemachandra, 2010). However the knowledge on the responses of fish to environmental changes and how they are affected by chemical pollutants is essential to identify suitable biomarkers with potential for use in biomonitoring programs.

Polycyclic aromatic hydrocarbons (PAHs) are among the most problematic contaminants. They are organic pollutants widespread in aquatic ecosystems (Lazartigues et al., 2010) that have their natural environmental concentrations raised up by anthropogenic combustion of fossil fuel, oil spilling and burning of organic matter (Kreitsberg et al., 2010). Once in the environment they can be trapped in sediments forming long-term contaminant reservoirs, thus increasing the risks of contamination and toxicity to biota (Jee et al., 2004). Although it is generally well accepted that PAHs does not markedly biomagnify along the food web, their cellular effects can lead to changes in organisms and their ecosystems (Kreitsberg et al., 2010). Phenanthrene is one typical example of these challenging compounds, which has three aromatic rings and is reported as the most abundant and ubiquitous PAHs in fresh, salt or brackish water, as well as seafood and organisms (Jee et al., 2004; Lazartigues et al., 2010; Oliveira et al., 2008; Yin et al., 2007; Wenju et al., 2009).

In fish, phenanthrene is shown to cause neurotoxicity (Barron et al., 2004), endocrine and reproductive disruptions (Han et al., 2010), cytotoxicity (Schirmer et al., 1998), genotoxicity (Oliveira et al., 2007), oxidative damage (Yin et al., 2007), impairment of growth (Jee et al., 2004), as well as carcinogenic and mutagenic effects (Pathiratne et al., 2010; Wenju et al., 2009). Its potential toxicity places phenanthrene among the most aggressive contaminants, being considered as a priority pollutant in several countries. Unfortunately, there is still little information on its effects on tropical aquatic organisms (Oliveira et al., 2008). Furthermore, the mechanism of its toxicity in fish remains still unclear (Sun et al., 2006; Pathiratne and Hemachandra, 2010). In addition, data on phenanthrene effects in estuarine tropical fishes are really scarce, and only few studies were performed focusing the analysis of biomarkers of exposure and effects in these organisms (Oliveira et al., 2007). This kind of information is so rare that Brazilian environmental regulators have actually no solid scientific information to establish adequate water quality criteria for phenanthrene in national surface waters or sediments.

In light of the above, the present study was designed to screen for the first time the effects of phenanthrene on a large suite of biological, physiological and genetic parameters in the tropical estuarine guppy *Poecilia vivipara* in salt water (salinity 24 ppt). Therefore, the aim of the present study was to generate information for a better understanding of the mechanisms involved in phenanthrene acute toxicity, as well as to select the more reliable and practical parameters to be applied as biomarkers of coastal contamination with this hydrocarbon. Endpoints analyzed included oxidative stress-related and oxidative damage parameters in tissues, as well as whole-body ion composition. The biotic model used was the guppy fish *P. vivipara*, one of the few species present along of all over the Brazilian coast, being found in fresh, brackish and salt waters (Gomes et al., 2008). Additionally, it has been already used in ecotoxicological studies (Araújo et al., 2001), and very recent studies show that *P. vivipara* is highly tolerant to chemical pollutants, displaying several biomarkers very sensitive to aquatic contaminants at environmentally relevant concentrations (Machado et al. 2012a,b). Therefore, this coastal fish species has been strongly recommended as one of most promising fish model for water quality monitoring in Brazilian coastal environments.

2. Material and Methods

2.1 Fish collection and exposure

Males *P. vivipara* were collected in the 'Arroio do Gelo' Stream (Cassino Beach, Rio Grande, Southern Brazil) between November 2010 and January 2011. Three exposures to phenanthrene were performed to obtain enough biological material for the analyses of all biomarkers selected. Fish resulting from the first exposure (29.48 ± 5.27 mm total length; 0.649 ± 0.348 g wet body mass) were used for the analyses of antioxidant enzymes activities. Fish from the second exposure (38.19 ± 6.85 mm total length; 0.818 ± 0.433 g wet body mass) were used for comet assay, reactive oxygen species (ROS) and total antioxidant capacity against peroxyl radicals (ACAP). Finally, fish from the third exposure (24.80 ± 3.74 mm total length; 0.204 ± 0.070 g wet body weight) were used to analyze the whole-body ions composition. All fish used were kept at fixed photoperiod (12 L: 12 D), temperature (20^{o} C), salinity (24ppt), and fed at satiation with commercial fish food (Alcon Basic, Camboriú, SC, Brazil) for at least 10 days prior to experiments.

Before exposure to phenanthrene, all glassware was washed several times with nitric acid (3%), methanol (10%) and thoroughly rinsed with distilled water. The exposure medium was prepared with seawater diluted to salinity 24 ± 1 ppt with overnight aerated tap water. This mixture was filtered (0.45-µm mesh filter) and contaminated with phenanthrene immediately before fish's introduction in the test chamber. Phenanthrene (purity >98%; Sigma-Aldrich, USA) was diluted in dimethylsulfoxide (DMSO) to obtain a stock solution of 2 mg phenanthrene.mL^{-1}, which was diluted into salt water prepared as described above to prepare the desired exposure concentrations (nominal concentrations: 10, 20 and 200 µg phenanthrene.L^{-1}). Two controls were also run in parallel: (1) fish exposed to salt water without addition of either DMSO or phenanthrene (referred hereafter as 'control' group), and (2) fish exposed to DMSO (100 µL DMSO.L^{-1}) at the same final concentration of that employed for the highest concentration of phenanthrene tested (200 µg.L^{-1}) (referred hereafter

as 'DMSO' group). Despite the phenanthrene concentration in the exposure medium was measured (see the Results section), treatments will be referred in the present work considering the nominal concentration of the hydrocarbon. Phenanthrene concentrations were selected taking into account three different scenarios of contamination with environmentally relevant concentrations of the hydrocarbon. In fact, environmental concentrations of phenanthrene in estuarine and coastal urban areas are reported to range from 14 to more than 1,000 $\mu g.L^{-1}$ (Oliveira et al., 2007; Wenju et al., 2009).

Adult males *P. vivipara* were exposed to phenanthrene for 96 h in 30-L glass aquariums under the acclimation conditions, as described above. Fish density at the beginning of the exposure corresponded to 1 g $fish.L^{-1}$. Exposure media received continuously and gentle air bubbling to keep the dissolved oxygen condition close to the saturation level. In addition, the exposure media were 100% renewed every 24 h to keep the water pH (~7.8) constant and low levels of nitrogenous compounds. All experiments were performed in duplicate. The number of fish tested for each biomarker measurement was planned taking into account the fish availability in the laboratory, as well as our previous knowledge on the typical deviation showed by each specific biomarker analyzed.

After exposure, fish were anesthetized with benzocaine (0.1 $g.L^{-1}$), weighed (wet body mass), and blood was collect by puncture of the caudal vein and immediately used in comet and nuclear abnormalities assays. Liver, gills and muscle were then dissected and frozen (-80^{o}C) for further analyses.

2.2 Phenanthrene concentration measurements

Water samples were collected immediately before fish's introduction in the test chamber and after 24 h of exposure. They were pre-concentrated in a C18 (IST Isolute) activated solid-phase system according to Lançcas (2004). Briefly, the method consists in filtering the water in a system able to bind to the PAHs, allowing samples storage for posterior elution and analysis. For this, all material used such as glass amber containers, silica gel (Merck, 60-200 mesh ASTM), neutral alumina (Al_2O_3) (Merck, 70-230 mesh ASTM) and granular sodium sulfate anhydrous were previously calcined at 450^{o}C for 6 h. Absorbent compounds were activated at 160^{o}C for 4 h, and further deactivated with 5% ultrapure water (MilliQ®) extracted with *n*-hexane. The extraction process occured when, after C18 conditioning in methanol, water samples were slowly aspirated through the column, where the analyte remained trapped. The filtering cartridge was dried and PAHs were eluted with ethyl acetate and *n*-hexane, being further concentrated by gentle N_2 bubbling and kept at 10^{o}C until the fractionation step. This step was performed by adsorption liquid chromatography in open column composed by alumina and silica. Phenanthrene identification was based on the retention time and mass spectra. Analyte quantification was performed comparing sample results with standard curves built with deuteron phenanthrene D10. Both processes were performed at the Laboratory of Organic Contaminants and Ecotoxicology of the Universidade Federal do Rio Grande (Rio Grande, RS, Brazil) using a gas chromatography (Perkin Elmer® Clarus 500) equipped with mass spectrophotometry detector (CG-EM). All chemicals used were HPLC grade and the quantification limit was 0.125 $ng.L^{-1}$.

2.3 Biomarkers measurements

2.3.1 Tissue oxidative stress-related parameters

Oxidative stress-related parameters were assessed in gills, liver, and muscle as examples of uptake, detoxification and non-target organs, respectively. For all analyses, protein concentration in tissue homogenates was determined using a commercial reagent kit (Microprote, Doles, Goiânia, GO, Brazil). Reactive oxygen species (ROS) production and antioxidant capacity against peroxyl radicals (ACAP) assays were performed according to Amado et al. (2009) using tissue samples from 4-6 fish for each treatment. Additionally, since the experiments reported in present work were performed concomitantly to others closely related, control data for ROS and ACAP were earlier presented in Machado et al. (2012a, b). Metallothionein-like proteins concentration was measured using the DTNB reaction with sulfhydryl groups (Viarengo et al., 1997) using tissue samples from 4 fish. Lipid peroxidation (LPO) was estimated following procedures described by Oakes and Van Der Kraak (2003) using tissue samples from 9-15 fish for each treatment. Catalase (CAT) activity was evaluated according to the method described by Beutler (1975) using tissue samples from 5-11 fish for each treatment. Superoxide dismutase (SOD) activity was evaluated following procedures described by McCords and Fridovich (1969) using tissue samples from 5-9 fish for each treatment. Glutathione reductase (GR) activity was determined according to Carlberg and Mannervik (1975) using tissue sample from 4 fish. Glutathione S-transferase (GST) activity was measured following procedures described by Keen et al. (1976) using tissue samples from 4 fish.

2.3.2 Erythrocytes DNA damage

Two kinds of DNA damages were evaluated in the present study. Reversible damage (single or double DNA strand breaks) was scored by the comet assay (Tice et al., 2000) using erythrocytes from 4-6 fish. This procedure was adapted for red gel use and to calculate the comet score according to Machado et al. (2012a,b). Also for comet assay, control data were previous reported in Machado et al. (2012a,b).

This assay provides a comparative analysis, where increasing comet classes and scores is related to higher level of DNA single or double strand breaks. Non-reversible DNA damages (clastogenicity) were accessed through nuclear abnormalities analysis (frequencies of micronucleated cells (MN), nuclear buds, bi-nuclear cells, apoptotic fragments and bilobed cells) following the procedure described by Barsiene et al (2006) and adapted by Machado et al. (2012a,b). Analysis was performed using erythrocytes from 9-15 fish.

2.3.3 Whole-body ion composition

Whole-body fish was dried (60^{o}C) for 72 h, digested in ultra-pure nitric acid (Sigma-Aldrich, Brazil) for 96 h, and properly diluted with MilliQ water. Atomic Absorption Spectrophotometry (AAS, Avanta Plus, Australia) in the flame mode was used to determine cation (Na, K, Ca and Mg) concentrations. Chloride was measured using a commercial reagent kit based on the formation of sulfur cyanide ferric (Doles, Goiânia, GO, Brazil). Analyses were performed using 5 fish per treatment. Also for whole-body ion, control data presented in here were previous reported in Machado et al. (2012a,b).

2.4 Statistical analysis

A Bayesian approach was used data analysis (Gelman et al., 2004). Conjugated families of probability were used to calculate posteriori distributions according to solutions presented by Kinas and Andrade (2010). The priori values and conjugate family of probability for each biomarker analyzed and all procedures adopted in the present work are described in details in Machado et al. (2012a,b). We Bayesian methods because the present study is a result from a toxicology network devoted to aquatic toxicology (INCT-TA, 2012) that aims to compare and provide models of the effect of different chemicals on biomarkers in *P. vivipara* for environmental monitoring (Machado et al., 2012a,b). Thus, the Bayesian approach came out as a powerful statistical tool to extract data information and store it in models of posterior distribution of probability, which can be used in future environmental works. Therefore, the posterior distributions can be analyzed by comparing many other parameters than only average, allowing a more deep and fine-tuned probabilistic understanding on how each chemical affects each biomarker.

Significant differences were accessed by hypothesis testing with Bayes's decision, based on posteriori odds ratio and Bayes Factor (Jeffreys, 1961). We considered two hypothesis hypothesis zero (H_0), wich negates the difference between averages, and hypothesis 1 (H_1), which accept it. For test which hypothesis is more likely we obtained the posterior distributions of differences of averages for each treatment and biomarker were estimated from simulations. Posterior distributions of differences concentrated around zero favor H_0, while posterior distributions of differences away from zero favor H_1. For these tests, priori odds ratio = 1, and loss w_0= 5 and w_1=1 for erroneously rejecting H_0 and H_1, respectively. Bayes Factor (BF) is shown in the section of results only when detected significant difference. It may be worth to mention that BF express how many times H_1 is more likely than H_0, and BF higher than 3.14 is considered substantial evidence against H_0 (Jeffreys, 1961). In this context, the present work assumed a conservative posture, rejecting H_0 only when BF was greater than 5. Data were expressed as average ± standard deviation for all biomarkers.

The potential effect of the solvent (DMSO) used to dissolve phenanthrene in salt water was verified by comparing responses of fish from the 'DMSO' group with those from the 'control' group. Since some of the parameters analyzed were affected by the single exposure to DMSO, the potential effects of phenanthrene were then identified by comparing the responses from fish exposed to any concentration of phenanthrene with those found for fish from the 'DMSO' group.

3. Results

3.1 Phenanthrene concentrations and toxicity

Measured concentrations of phenanthrene in the exposure media and respective 24-h degradation rates for the different experimental treatments are shown in Table 1. Measured concentrations of phenanthrene varied from 97.3 to 104.5% of the desired nominal concentrations. In turn, phenanthrene degradation rate ranged from 1.15 to 4.51% over the 24-h period of exposure (Table 1). No fish mortality was observed over the 96-h experimental period in any phenanthrene concentration tested.

3.2 Oxidative stress biomarkers

In fish singly exposed to DMSO, no significant change in ROS production was observed in liver, while increased mean values were found in gills (BF > 597) and muscle (BF > 5) (Fig. 1A). ACAP was also increased after DMSO exposure in the gills (BF > 38) (Fig. 1B). MT concentration decreased in liver (BF > 14) and increased in gills (BF > 5) and muscle (BF > 10) of fish exposed to DMSO for 96 h (Fig. 1C).

Regarding antioxidant enzymes activities, GST activity in liver, gills and muscle was not affected by single exposure to DMSO (Fig. 2A), while SOD activity was reduced in muscle (BF > 7) (Fig. 2B), GR activity was decreased in liver (BF > 31) and gills (BF > 5) (Fig. 2C), and CAT was not disturbed by DMSO exposure (Fig. 2D).

Exposure to DMSO induced a slightly reduced LPO in gills (BF > 13) (Fig. 3) and increased DNA damage [comet score (BF > 13) (Fig. 4) and frequency of micronucleated cells (BF > 5) (Fig. 5b)] in erythrocytes. Increases in whole-body Na (BF > 13) and K (BF > 11) concentrations (Fig. 6) and decreases in whole-body Cl (BF > 5) (Fig. 6) and Mg (BF > 7) concentrations (Fig. 6) were observed after fish exposure to DMSO.

ROS generation (Fig. 1) and ACAP (Fig. 2) were not affected in liver of fish exposed to any concentration of phenanthrene. However, a concentration-dependent reduction in both ROS generation (BF > 383) (Fig. 1A) and ACAP (BF > 38) (Fig. 1B) was observed in gills of fish exposed to the hydrocarbon. In muscle, a reduced ROS generation was observed only in fish exposed to 200 μg phenanthrene.L^{-1} (BF > 5) (Fig. 1A), while no significant change was observed in ACAP (Fig. 1B). MT concentration was increased in liver of fish exposed to 20 (BF > 79) and 200 μg phenanthrene.L^{-1} (BF > 63), while decreased mean values were observed in gills of these fish (BF > 14). In muscle, a significant reduction in MT concentration was observed in fish exposed to 10 (BF > 5) and 200 μg phenanthrene.L^{-1} (BF > 7) (Fig. 1C).

Regarding the activity of antioxidant enzymes, phenanthrene exposure only slightly reduced the GST activity in liver of fish exposed to 20 μg phenanthrene.L^{-1} (BF > 6), while no significant change was observed in gills of fish exposed to any concentration of phenanthrene. In muscle, increased GST activity was observed in fish exposed to 20 μg phenanthrene.L^{-1} (BF > 5) (Fig. 1A). SOD activity was reduced in liver of fish exposed to any concentration of phenanthrene (BF > 143), while no significant change was observed in gills. In muscle, exposure to any concentration of phenanthrene significantly increased SOD activity (BF > 34) (Fig. 1B). GR activity in both liver and gills was not altered by phenanthrene exposure, while enzyme activity was observed increased in muscle of fish exposed to 20 (BF > 6) and decreased in those exposed to 200 μg phenanthrene.L^{-1} (BF > 26) (Fig. 1C). CAT activity was reduced in liver of fish exposed to 20 μg phenanthrene.L^{-1} (BF > 7) and in gills of fish exposed to any concentration of phenanthrene tested (BF > 33). In turn, no significant change in CAT activity was observed in muscle of fish exposed to any concentration of phenanthrene (Fig. 1D).

LPO values were decreased in liver of fish exposed to 10 (BF > 6) and 20 μg phenanthrene.L^{-1} (BF > 7), while increased values were observed in gills of fish exposed to 10 μg phenanthrene.L^{-1} (BF > 341) and in muscle of those exposed to 200 μg phenanthrene.L^{-1} (BF > 24) (Fig. 3).

A higher comet score was observed in erythrocytes of fish exposed to 10 µg phenanthrene.L^{-1} (BF > 5) that was due to higher frequencies of cells showing comets in the class 3 after fish exposure to the hydrocarbon (Fig. 4). Nuclear buds and bilobed cells followed similar pattern, where higher frequencies of these nuclear abnormalities were observed in fish exposed to 10 and 20 µg phenanthrene.L^{-1} (BF ~ ∞) (Fig. 5A). Additionally, a higher frequency of micronucleated cells was observed in erythrocytes of fish exposed to 20 µg phenanthrene.L^{-1} (BF > 6) (Fig. 5B).

Fish exposure to 10 µg phenanthrene.L^{-1} did not significantly change the whole-body ion (Na, K, Cl, Ca and Mg) content (Fig. 6). However, exposure to 20 µg phenanthrene.L^{-1} significantly reduced whole-body Na (BF > 10) and Cl (BF > 12) concentrations (Fig. 6) and increased the whole-body Mg content (BF > 11) (Fig. 6). In turn, fish exposure to 200 µg phenanthrene.L^{-1} altered the whole-body concentration of all ions analyzed. Decreased whole-body Na (BF > 9), K (BF > 12), and Cl (BF > 8) contents (Fig. 6) and increased whole-body Ca (BF > 12) and Mg (BF > 36) concentrations (Fig. 6) were observed.

4. Discussion

Degradation rates of phenanthrene in salt water (salinity 24 ppt) over a 24-h period of exposure were very low (< 4.51 %). In fact, measured concentrations of phenanthrene in the exposure media were maximally 2.7% lower and 4.5% higher than the desired (nominal) concentrations. Hereafter, we will thus consider for practical reasons the nominal concentrations of phenanthrene tested in the present study (10, 20 and 200 µg.L^{-1}). The exposure to these phenanthrene concentrations was acutely sublethal to the guppy *P. vivipara* in salt water (salinity 24 ppt). In fact, no mortality was observed after 96 h of exposure to any concentration of phenanthrene tested. However, several biochemical, physiological and genetic parameters analyzed in the present study were responsive to phenanthrene exposure. As discussed below, the response of these parameters were used to evaluate the possible mechanism(s) of phenanthrene action in the guppy *P. vivipara* in salt water, as well as to select the most responsive parameters and point their potential as useful and practical biomarkers to be used in water quality biomonitoring programs in salt water.

Since it is lipophilic, phenanthrene can be easily taken up from water by fish. However, its mechanism(s) of toxicity remains unclear (Yin et al., 2007). The best knowledge of the phenanthrene toxicity is given by Alkyl Phenanthrene Model (Barron et al., 2004). In this model, interactions of phenanthrene and its metabolites with biomolecules is described to cause both sublethal and lethal toxicity. However, the mechanistic aspect involved in phenanthrene toxicity is widely open in this model. In fact, it is reported in the literature that phenanthrene is rapidly absorbed from water and metabolized by fish (Sun et al., 2006), eliciting its cytotoxicity by multiple modes of action (Schirmer et al., 1998), which are usually reflected in impairments of general health (Jee et al., 2004).

Among the parameters analyzed in the present study, whole-body ions content is the most integrative one since its response to water chemical contamination would depend on the exposure time and concentration, as well as the response of several other biochemical and physiological parameters. In a broad view, the most marked effect of phenanthrene exposure on the guppy *P. vivipara* acclimated to salt water (salinity 24 ppt) was whole-body

ionoregulatory disturbances. It is important to note that the present study is the first one to report phenanthrene effects on whole-body ions in fish. In fact, all ions analyzed (Na, K, Cl, Ca, and Mg) showed their whole-body values altered in fish exposed to the higher phenanthrene concentration tested (200 $\mu g.L^{-1}$), with significant effects on Na, Cl and Mg already occurring at a very low concentration of phenanthrene (20 $\mu g.L^{-1}$). These effects may be explained by considering possible phenanthrene-induced dysfunction of ion-transporting proteins and changes in cell membrane permeability. It is known that phenanthrene can cause a direct cytotoxic effect by interacting with biomolecules and disturbing cell membranes (Schirmer et al., 1998). In fact, effects of phenanthrene on the activity of enzymes involved in ions homeostasis have been recently reported (Sanchez et al., 2009). Also, it is well known for long time that phenanthrene can cause loss of membrane permeability (Schirmer et al., 1998; Viarengo and Moore, 1985). However, it seems that the whole-body ionoregulatory disturbances would be more associated with the phenanthrene effect on the ion-transporting proteins at gills level. This statement is based on the fact that phrenanthrene exposure did not induced consistent and significant effect on gill LPO, as further discussed. Therefore, singly or in combination, these potential effects of phenanthrene occurring in fish gills would lead to disturbances in whole-body ionic regulation. This statement is based on the fact that gills are the major surface of fish contact and ion exchange with the external environment, as well as the major route of entry for waterborne contaminants. Therefore, it is not surprising that phenanthrene toxicity may arise from perturbations in osmoregulatory processes taking place at the gill level (Handy and Depledge, 1999).

Despite phenanthrene can cause a cytotoxic effect by direct interaction with biomolecules (Schirmer et al., 1998), it is also known that living organisms exposed to PAHs may generate metabolites by breaking down these chemicals by adding hydroxyl radicals in the phase I of the biotransformation process. This may produce reactive compounds, which can yield ROS (redox cycling routes) and/or interact with DNA forming adducts, inducing several injuries in several biological endpoints (Oliveira et al., 2007). PAHs are biotransformated mainly by the liver through oxidation and conjugation reactions to hydrophilic metabolites that are excreted by bile (Pathiratne et al., 2010). In fact, liver of teleost fish is known to have high capacity for PAHs biotransformation, leading to the excretion of metabolites via gallbladder (Lazartigues et al., 2010). In this context, GST plays important function on detoxifying and bioactivating PAHs (Pathiratne and Hemachandra, 2010).Therefore, exposure of the guppy *P. vivipara* would be expect to cause an increased ROS generation and a possible oxidative stress condition not only in liver, but also in other fish tissues, culminating with oxidative damage to biomolecules. In the present study, ROS generation, ACAP, MT, and activity of several antioxidant enzymes, including GST, were evaluated in tissues (liver, gills and muscle) of the guppy *P. vivipara* in salt water (salinity 24 ppt). In addition, the response of biomarkers of oxidative damage to lipids (LPO) and DNA (comet score and nuclear abnormalities) were analyzed in erythrocytes of fish exposed to environmentally relevant concentrations of phenanthrene.

Regarding the response of enzymatic and non-enzymatic antioxidants agents, results from the present study indicate a differential response of each tissue analyzed to phenanthrene exposure. In liver, no significant damage to lipids (LPO) was observed in *P. vivipara* exposed to the higher concentration of phenanthrene tested (200 $\mu g.L^{-1}$), and even reduced LPO values

were observed in those fish exposed to lower concentrations of the hydrocarbon (10 and 20 μg phenanthrene.L^{-1}). These findings are in complete agreement with the general lack of changes in ROS generation, ACAP and CAT activity, as well as the clear reduction in SOD activity observed in liver of *P. vivipara* exposed to phenanthrene. Previous studies in fish report a variety of response of the antioxidant system to phenanthrene exposure. For example, an increased ROS production, notably OH^-, was observed in the freshwater goldfish *Carassius auratus* associated to phenanthrene bioaccumulation in the liver (Sun et al., 2006; Yin et al., 2007). Changes in CAT activity and LPO were observed in the golden grey mullet *Liza aurata* (Oliveira et al., 2008) and *C. auratus* (Yin et al., 2007) exposed to high concentrations of phenanthrene (~1 mg. L^{-1}). However, opposed to results reported for *L. aurata* and *C. auratus*, different findings were found in tilapia (female *Oreochromis niloticus* and male *O. aureus*) and flounder (*Paralichthys olivaceus*), where there was an increase in CAT, SOD, GR and glutathione peroxidase activity after exposure to phenanthrene (Wenju et al., 2009; Jee and Kang, 2005). Also, it is worth to note that variations in the response of oxidative stress-related enzymes were reported to occur as a function of the time and concentration of exposure in the mullet *L. aurata* and the goldfish *C. auratus* (Oliveira et al., 2008; Sun et al., 2006). Therefore, the differential responses observed in the saltwater guppy *P. vivipara* and other fish species, especially the freshwater ones, could be associated to differences in exposure time and concentrations of phenanthrene, as well as in the physiology of fish, since *P. vivipara* was shortly exposed to environmentally relevant concentrations of phenanthrene and is hyporegulating in salt water (salinity 24 ppt), while other fish were exposed to higher concentrations of phenanthrene. In the case of freshwater fish, it is important to consider that they are hyperegulating in fresh water and likely demanding a higher oxygen and energy consumption to deal with the environmental salinity. Higher oxygen consumption rates would lead to higher levels of ROS production. The low concentrations of phenanthrene used to expose *P. vivipara* probably also have resulted in lower levels of accumulation of the hydrocarbon in the liver, thus leading to a low rate of phenanthrene metabolism. This statement is based on the fact that no increase in GST activity was also observed in liver of *P. vivipara* exposed to 10 and 200 μg phenanthrene.L^{-1}, and even a reduced enzyme activity was found in those fish exposed to 20 μg phenanthrene.L^{-1}. Furthermore, no change in GR activity was observed in fish exposed to any concentration of phenanthrene. As discussed above, GSH metabolism has been pointed as a major route to deal with the stress related to phenanthrene exposure. In this context, the observed increase in MT concentration in the liver of *P. vivipara* after phenanthrene exposure would be an evidence of changes (likely increase) in the metabolism of –SH groups.

It is also important to stress that the variety of responses of oxidative stress-related parameters to phenanthrene exposure discussed above could also be associated with the possible and single effect of the solvent used to dissolve phenanthrene and prepare the exposure media. In the present study, the solvent (DMSO) used was unexpectedly able to significant affect several biomarkers under the experimental conditions employed in the present study. These effects were more clearly seem in gills than in liver and muscle.The authors recognize that this does not simplify the interpretation if the observed responses of the parameters analyzed in the present study are due or not to the single exposure to phenanthrene or to a combined effect of the hydrocarbon with the solvent. However it constitutes important

information since most of studies reported in the literature have been performed using only a positive control and have even used a higher DMSO concentration than that tested in the present study. In the physiological point of view, performing only a positive control mandatorily implies that the solvent does not interfere in the responses or there is a single additive effect of compounds. However, DMSO is also a lipophilic xenobiotic like phenanthrene, and data suggest that many effects of the DMSO and phenanthrene mixture may not be additive. Therefore, presence of a negative control is highly recommended when considering the possible application of results in biomonitoring studies.

Differently from the liver, gills of the guppy *P. vivipara* showed reduced ROS generation after exposure to any concentration of phenanthrene tested. This finding could be explained considering a possible lack of change in the rate of phenanthrene metabolism associated with phenanthrene-induced dysfunctions in mitochondrial respiratory function at the gill cells. The suggested lack of change in metabolism of the hydrocarbon in gill cells is based on the observed lack of significant effect of phenanthrene exposure on GST activity. As discussed above, this enzyme plays a major role in detoxification and bioactivation of PAHs (Pathiratne and Hemachandra, 2010). In turn, lower mitochondrial respiratory rates would lead to consequently reduced rates of ROS production. The reduced ACAP and the almost lack of changes in LPO observed in gills of fish exposed to phenanthrene are in complete agreement with the lowered ROS production discussed above. Based on the results reported in the present study, the reduced ACAP is clearly due at least in part to reduced contributions of both non-enzymatic (MT) and enzymatic (CAT) antioxidants.

Considering the lack of clear effects of oxidative stress in gills of the guppy *P. vivipara* and the marked disturbances observed in whole-body ionic regulation discussed above, further studies on the possible direct interaction of phenanthrene with gill ion-transport proteins and membrane permeability are needed to completely understand the mechanism involved in the acute toxicity of this hydrocarbon.

In muscle, as observed in gills, a lower ROS production was also found after *P. vivipara* exposure to phenanthrene, especially at the higher concentration tested. However, as opposed to gills, no significant change in ACAP was observed. Since a lower level of non-enzymatic antioxidants (MT and GSH) was observed associated with a decreased MT content and a lower GR activity, it is suggested the lack of change in ACAP was compensated by the contribution of the enzymatic antioxidants, notably by a consistent increase in SOD activity. Based on these findings, a lack of oxidative damage would be expected to occur in muscle of *P. vivipara* exposed to phenanthrene. In fact, a slight increase in LPO was observed only in fish exposed to the highest concentration of phenanthrene tested (200 $\mu g.L^{-1}$). As far as we know, there are no other studies reporting the effects of phenanthrene on oxidative responses in fish muscle, since most of studies regarding this tissue are focused in measurements of tissue PAHs concentrations. However, findings reported in the present study suggest that muscle may be also an important target organ of phenanthrene toxicity, like already reported for gills and liver. This statement is based on the fact phenanthrene seems to be accumulated and metabolized in this tissue, since an increased GST activity was observed in fish exposed to 10 and 200 µg phenanthrene.L^{-1}. This increased GST activity is in agreement with data reported for the flounder *P. olivaceus* (Jee and Kang, 2005), the Nile tilapia *O. niloticus*

(Pathiratne and Hemachandra, 2010), and the goldfish *C. auratus* (Yin et al., 2007) exposed to phenanthrene.

In light of the findings discussed above, no clear evidence of phenanthrene-induced oxidative stress in liver, gills and muscle of *P. vivipara* were observed in the present study. Despite this apparent lack of direct or ROS-mediated effect of phenanthrene on membrane lipids, measured through LPO, a possible effect of this hydrocarbon and/or its metabolites on other biomolecules such as proteins and DNA cannot be ruled out. In this context, it is worth to mention that several indirect effects of phenanthrene exposure were reported in salmon, such as enzymatic and proteomic dysfunctions concerning the antioxidant scavenging capacity, energy metabolism, cell communication, cell reproduction, homeostasis maintenance, among others (Sanchez et al., 2009). Such large spectrum of effects seems to imply a more precursor and general cause, like DNA damaging. In fact, a strong reduction in the activity of nuclear RNA polymerases I and II was described in mussel digestive gland after phenanthrene exposure (Viarengo and Moore, 1982). Although these authors did not measure the enzyme activity, they suggest that the observed reduction in the activity of the nuclear RNA polymerases could imply effects on the response of several enzymes in PAHs-exposed animals. In fact, Woo et al. (2006) highlighted that bioaccumulation of PAHs in intracellular medium cause modifications in DNA, inducing incomplete transcription from DNA to mRNA, which culminate in metabolic dysfunctions such as impairment of enzymatic reactions.

In the present study, possible DNA damage induced by phenanthrene was measured through comet assay and frequency of micronucleated cells. Increases in the frequency of micronucleated cells are associated with higher mutation rates and clastogenicity. In turn, DNA strand breaks measured through comet score, as well as the frequency of nuclear abnormalities are highly related to xenobiotic-initiated carcinogenesis and mutagenesis rate (Mitchelmore and Chipman, 1998; Barsiene et al., 2006). While comet assay offer information at a molecular level, micronucleus and nuclear abnormalities provide relevant integrative information on the dynamic interaction of chromosome breakage (observed in comet assay) and chromosome loss. Besides, DNA strands breakage as nuclear abnormalities have been reported as being an important effect of PAHs in both laboratory and field studies (Santos et al., 2010). Therefore, an integrative analysis of data generated by comet assay and nuclear abnormalities evaluation seems to be the best way to interpret DNA damages caused by xenobiotics. In this context, the detection of DNA damage resulting from contaminant exposure may be a key tactic in assessing the general health of marine organisms, either identifying the importance of genotoxicity or predicting the effects on populations or communities in the marine environment (Woo et al., 2006).

In any case, it is worth to note that any damage to DNA could lead to disturbances in the proper transcription of DNA into proteins. Therefore, the marked effects on whole-body ionic content observed in *P. vivipara* could be due not only to direct effects of phenanthrene and its metabolites on the activity of gill ion-transporting proteins, but also related to indirect effects on DNA transcription into these proteins, thus reducing the fish capability to maintain a proper whole-body ionic regulation. Unfortunately, the DNA expression was not evaluated in the present study. However, a consistent and significant increase in the frequencies of erythrocytes in class 3 (higher damage) of the comet assay was observed in *P. vivipara*

exposed to any concentration of phenanthrene. It is interesting to note that this increase was paralleled by a decrease in the frequencies of erythrocytes in class 1 in fish exposed to 10 and 20 µg phenanthrene.L^{-1} and of erythrocytes in class 2 in fish exposed to 200 µg phenanthrene.L^{-1}, resulting in an increased comet score only in *P. vivipara* exposed to 10 µg phenanthrene.L^{-1}. These findings suggest that in spite of a more general effect was not detected through comet score, especially at the higher concentrations of phenanthrene tested (20 and 200 µg.L^{-1}), fish erythrocytes showing already some degree of DNA damage had this damage enhanced by exposure to the hydrocarbon.

As observed for the comet assay data, a consistent effect of phenanthrene on the frequency of micronucleated cells was not observed, except in erythrocytes of fish exposed to 20 µg phenanthrene.L^{-1}. However, a consistent effect of phenanthrene exposure was observed in either the frequency of nuclear buds and cells with bilobed nucleus. Increased frequency of nuclear buds was also reported in the golden grey mullet *L. aurata* following short-term exposure to phenanthrene (Oliveira et al., 2007). There is no certainty about the biological implications of other nuclear abnormalities than micronucleated cells. However, they are also considered to be indicators of genotoxic damage (Çavas and Ergene-Gozukara, 2005). In turn, the increased frequency of cells with bilobed nucleus suggests a reduced release of young erythrocytes into peripheral circulation in *P. vivipara* exposed to phenanthrene. Since most of erythrocyte division occur in hematopoietic organs, where clastogenicity occurs, it is likely that a phenanthrene-induced inhibition of erythrocyte release into the blood stream may reflect changes in the frequency of micronucleated cells and other nuclear abnormalities indexes. It is interesting to mention that during the microscopic evaluation and counting of nuclear abnormalities, erythrocytes from *P. vivipara* exposed to the higher phenanthrene concentrations showed several membrane lesions and nuclear deformities that were not integrated in the indexes employed to measure DNA damage (data not shown). Similar findings were reported in erythrocytes of the flounder *P. olivaceus* exposed to phenanthrene (Jee et al., 2004). These authors also found several other hematological responses in flounders exposed to phenanthrene, including a significant decrease in erythrocytes counting at high concentrations of phenanthrene. In fact, they suggested a disruptive action of the PAH on the erythropoietic tissue, resulting in lower values of cell viability, thus being in alignment with data reported in the present study. In addition, the responses observed in blood cells after exposure to phenanthrene may also be due to the potential effect of the reactive metabolites from metabolism of phenanthrene in the liver. Nevertheless, it has been demonstrated that extra-hepatic tissues, such as blood, may be important components of the metabolism of xenobiotics, such as PAHs (Mitchelmore and Chipman, 1998).

Taken altogether, data from comet classes and the frequency of some nuclear abnormalities such as nuclear buds indicate that phrenanthrene exposure can be inducing DNA damage in erythrocytes of the guppy *P. vivipara* in salt water. In fact, highly reactive intermediate metabolites from PAHs degradation may bind to DNA inducing cancer and mutation (Pathiratne et al., 2010). However, comet score and frequency of micronucleated cells seems to be not sensitive enough to detect DNA damage induced by exposure to phenanthrene at environmentally relevant concentrations, as tested in the present study. These finding is in agreement with the fact that PAHs metabolites may interact with DNA and other biomolecules forming adducts, and comet score is not sensitive to such kind of damage

(Mitchelmore and Chipman, 1998). Consequently, it is suggested that a more deep and detailed analysis of data generated by comet assay (e.g. frequency of cells in each comet class), as well as the evaluation of other DNA damage than strand breaks should be considered in future studies on the genetic effects of phenanthrene in fish.

In summary, some oxidative stress-related parameters in the guppy *P. vivipara* were affected by exposure to phenanthrene at environmentally relevant concentrations. These effects were seem in all tissues analyzed (liver, gills and muscle), being the responses dependent on tissue and phenanthrene concentration. In addition, no clear DNA damage was observed in erythrocytes when measured through comet assay and frequency of micronucleated cells. However, marked and consistent disturbances in whole-body ionic content were observed. Therefore, our findings point that whole-body Na, Cl and Mg content as the most sensitive and practical biomarkers to monitor the exposure of the guppy *P. vivipara* to phenanthrene in salt water. Despite whole-body ion content does not clarify much about the mechanism involved in phenanthrene toxicity it clearly indicates that the contaminant has caused a more general effect on fish. Like other vertebrates, fishes must keep their hydromineral balance to ensure the physiological integrity of all the major body systems (Handy and Depledge, 1999). Therefore, our findings suggest that whole-body ion imbalance is the most consistent, reliable and practical biomarker of great biological relevance for biomonitoring *P. vivipara* exposure to environmentally relevant concentrations of phenanthrene in salt water. Notwithstanding the complexity of processes integrated by oxidative stress biomarkers, they are still meaningful in biomonitoring if careful interpretation is provided. In this context, findings reported in the present study corroborate the idea of Han et al. (2010), who suggest that a more realist and holistic scenario of environmental pollution is obtained by measuring oxidative biomarkers of exposure paralleled by the evaluation of biomarkers able to point disturbances in the general health of the organism.

5. Conclusions

Findings here reported in the present study provide evidence that phenanthrene effects on oxidative stress-related parameters are dependent on the concentration tested, tissue analyzed and the endpoint evaluate. Also, DNA damages could be seen in fish exposed to environmentally relevant concentrations of phenanthrene. However, the comet score and frequency of micronucleated cells were not sensitive enough to detect the phenanthrene-induced damage to DNA. In the other hand, the set of biomarkers more responsive and sensitive to phenanthrene exposure were the whole-body Na, Cl and Mg contents, thus being indicated as reliable, practical and biologically relevant biomarkers to detect exposure of the guppy *P. vivipara* to environmentally relevant concentrations of phenanthrene in salt water.

6. Acknowledgements

This work was supported by the “Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq” in the scope of the “Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática - INCT-TA (Brazilian Ministry of Science and Technology, Brasília, DF, Brazil), the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES” in the scope of the Marine Sciences Program (Brazilian Ministry of Education, Brasília, DF, Brazil), and the International Development Research Centre – IDRC (Ottawa, Canada). A.

Bianchini is a research fellow from the Brazilian CNPq (Proc. # 304430/2009-9) and supported by the International Canada Research Chair Program from IDRC. Authors thank the assistance provided by Dr Elton P. Colares, Dr Marta M. Souza and Dr Grasiela L.L. Pinho during experiments. Thanks also to Dr Ana Baisch and Dr Carine Dahl Corcini for allowing us the use of laboratory infrastructure for comet assays.

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TABLE

Table 1. Nominal and measured concentrations (mean ± standard deviation) and degradation rate of phenanthrene during exposure of *Poecilia vivipara* in salt water (salinity 24 ppt).

Phenanthrene concentration ($\mu g.L^{-1}$)			
Nominal	Measured		Degradation (%)
	Before exposure	After 24 h of exposure	
10	9.85 ± 0.85	9.73 ± 0.83	1.15 ± 0.21
20	20.90 ± 1.44	20.26 ± 1.46	3.09 ± 0.39
200	206.65 ± 6.71	197.35 ± 7.20	4.51 ± 0.58

FIGURE LEGENDS

Figure 1. (A) Reactive oxygen species (ROS) production, (B) total antioxidant capacity against peroxyl radicals (ACAP), and (C) metallothionein-like proteins (MT) concentration in liver, gills and muscle of *Poecilia* kept 96 h in control conditions (open bars) or exposed to DMSO (diagonally hatched bars), 10 (diagonally inverted hatched bars), 20 (double hatched bar) and 200 $\mu g.L^{-1}$. Data are expressed as mean ± standard deviation. Different letters indicate significant difference among treatments for each tissue within the same parameter, and means sharing the same letter are not significantly different.

Figure 2. (A) Glutathione S-transferase (GST), (B) superoxide dismutase (SOD), (C) glutathione reductase (GR) and (D) catalase (CAT) activity in liver, gills and muscle of *Poecilia vivipara* kept 96 h in control conditions (open bars) or exposed to DMSO (diagonally hatched bars), 10 (diagonally inverted hatched bars), 20 (double hatched bar) and 200 $\mu g.L^{-1}$. Data are expressed as mean ± standard deviation. Different letters indicate significant difference among treatments for each tissue within the same parameter, and means sharing the same letter are not significantly different.

Figure 3. Lipid peroxidation (LPO) on liver, gills and muscle of guppy *Poecilia vivipara* after 96 h in control conditions (open bars) or exposed to DMSO (diagonally hatched bars), 10 (diagonally inverted hatched bars), 20 (double hatched bar) and 200 $\mu g.L^{-1}$ (horizontally marked bars) of waterborne phenanthrene in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference among atrazine concentrations for each tissue within the same parameter, and means sharing the same letter are not significantly different.

Figure 4. DNA damage measured through comet assay in erythrocytes of *Poecilia vivipara* exposed to phenanthrene for 96 h in salt water (salinity 24 ppt). For comet class (left y-axis) (class 0: open bars; class 1: diagonally hatched bars; class 2: diagonally inverted hatched bars; class 3: double hatched bars), bars represent mean and lines indicate standard deviation. For comet score (right y-axis) data are expressed as mean (closed circles) ± standard deviation (vertical lines). Different letters indicate significant difference among treatments for each parameter, and means sharing the same letter are not significantly different.

Figure 5. DNA damage measured through frequency of nuclear abnormalities (A), and micronucleated cells (B) in erythrocytes of *Poecilia vivipara.* The organisms were maintained 96 h in control conditions (open bars) or exposed to DMSO (diagonally hatched bars), 10 (diagonally inverted hatched bars), 20 (double hatched bar) and 200 $\mu g.L^{-1}$ (horizontally marked bars) of waterborne phenanthrene in salt water (salinity 24 ppt). Bars indicate mean and vertical lines represent standard deviation. Different letters indicate significant difference among treatments for each parameter, and means sharing the same letter are not significantly different.

Figure 6. Whole-body sodium, potassium, chloride, calcium and magnesium in the guppy *Poecilia vivipara* 96 h in control conditions (open bars) or exposed to DMSO (diagonally hatched bars), 10 (diagonally inverted hatched bars), 20 (double hatched bar) and 200 $\mu g.L^{-1}$ (horizontally marked bars) of waterborne phenanthrene in salt water (salinity 24 ppt). Data are expressed as mean ± standard deviation. Different letters indicate significant difference

among atrazine concentrations for each parameter, and means sharing the same letter are not significantly different.

Figure 1

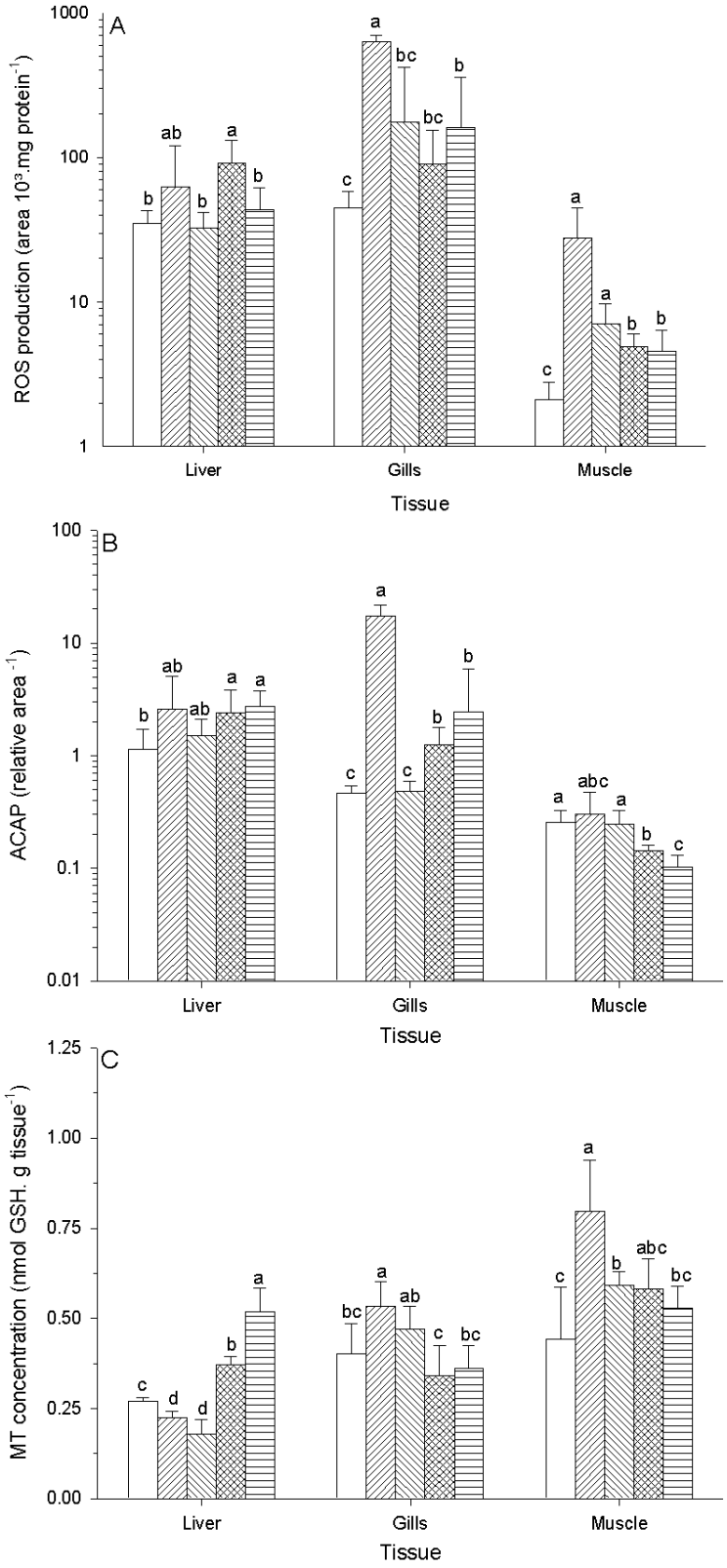

A
ROS production (area 10³.mg protein⁻¹)
1000
100
10
1
b
ab
b
a
b
c
a
bc
bc
b
c
a
a
b
b
Liver
Gills
Muscle
Tissue
B
ACAP (relative area⁻¹)
100
10
1
0.1
0.01
b
ab
ab
a
a
c
a
c
b
b
a
abc
a
b
c
Liver
Gills
Muscle
Tissue
C
MT concentration (nmol GSH. g tissue⁻¹)
1.25
1.00
0.75
0.50
0.25
0.00
c
d
d
b
a
bc
a
ab
c
bc
c
a
b
abc
bc
Liver
Gills
Muscle
Tissue

Figure 2

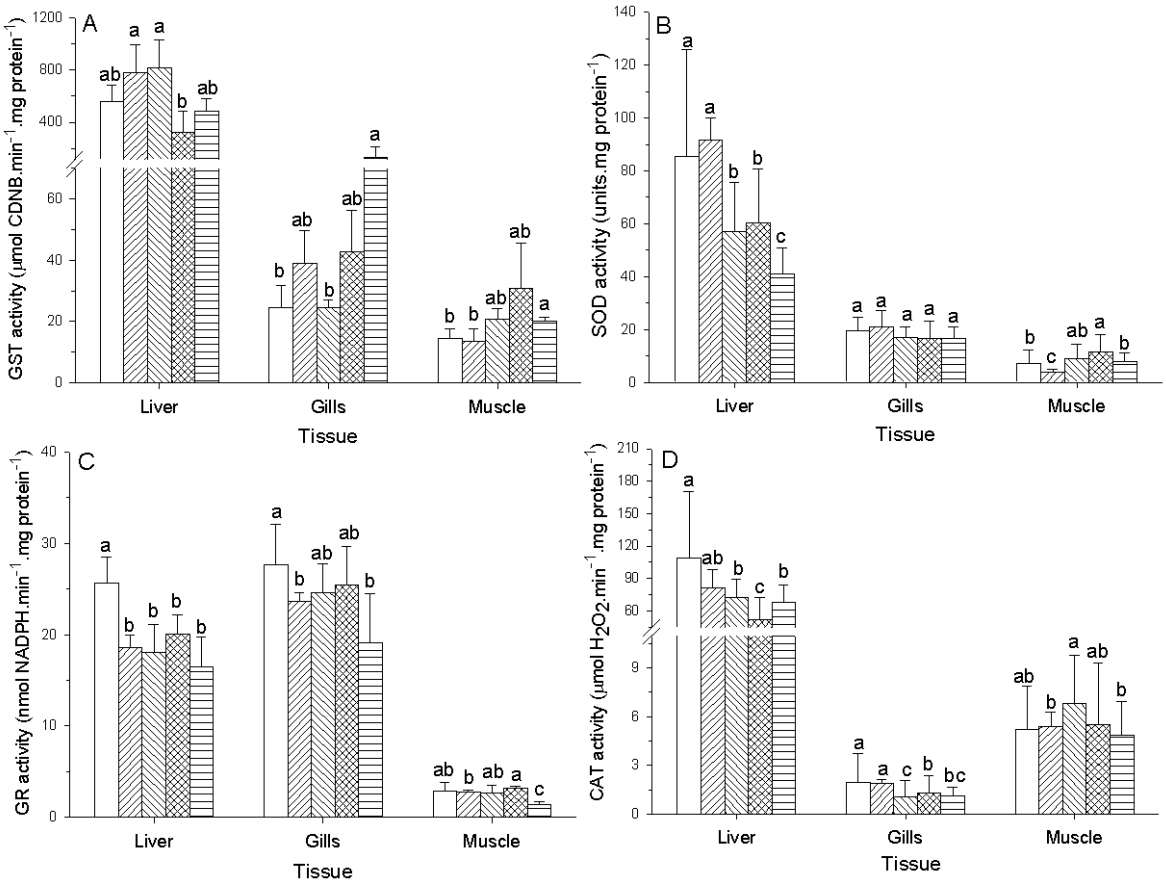

A
GST activity (µmol CDNB.min-1.mg protein-1)
Liver
Gills
Muscle
Tissue
B
SOD activity (units.mg protein-1)
C
GR activity (nmol NADPH.min-1.mg protein-1)
D
CAT activity (µmol H2O2.min-1.mg protein-1)

Figure 3

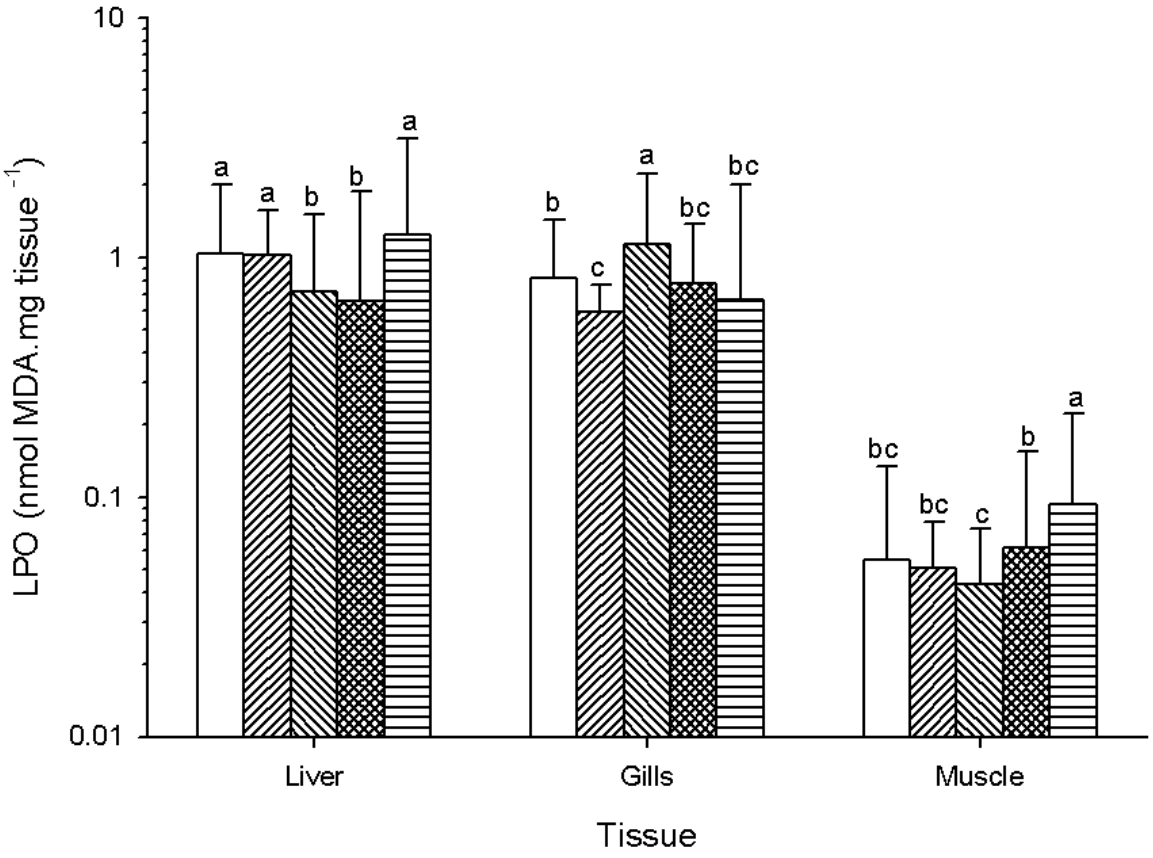

10
1
0.1
0.01
LPO (nmol MDA.mg tissue -1)
a
a
b
b
a
b
c
a
bc
bc
bc
c
b
a
Liver
Gills
Muscle
Tissue

Figure 4

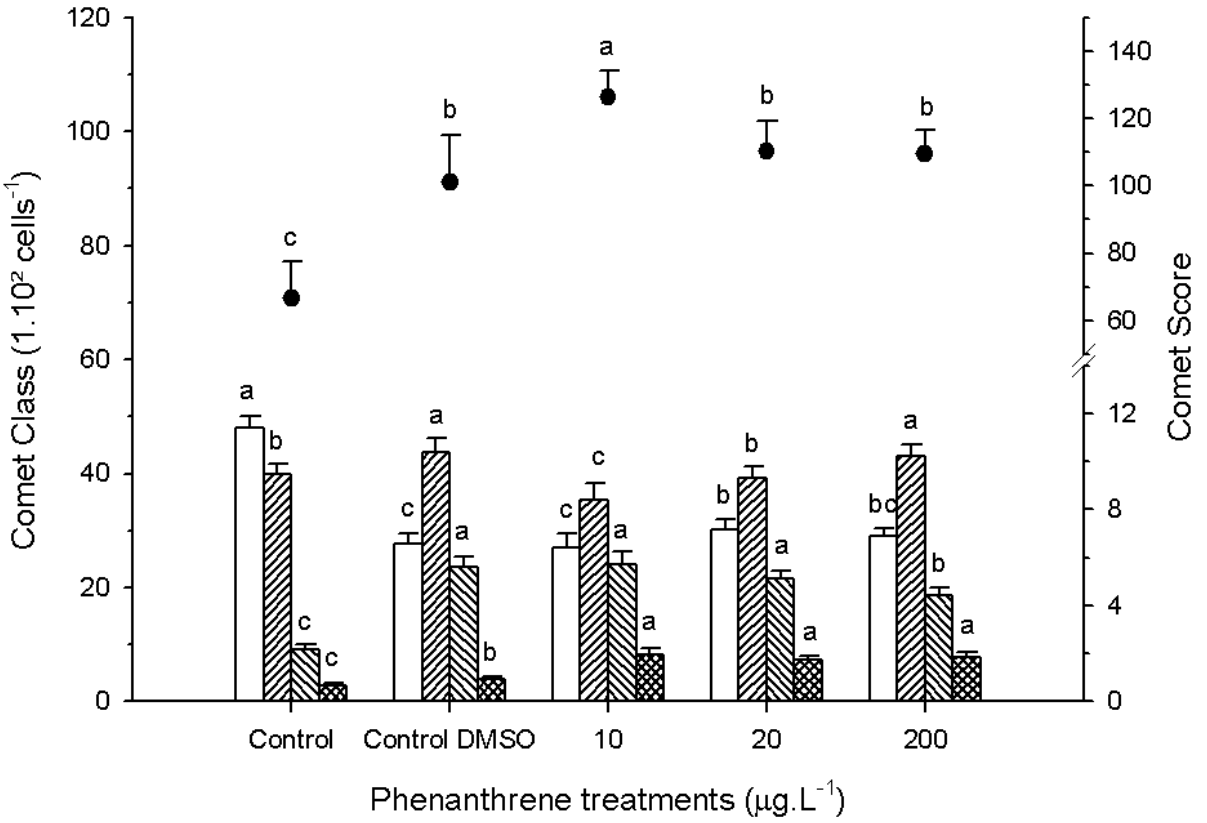
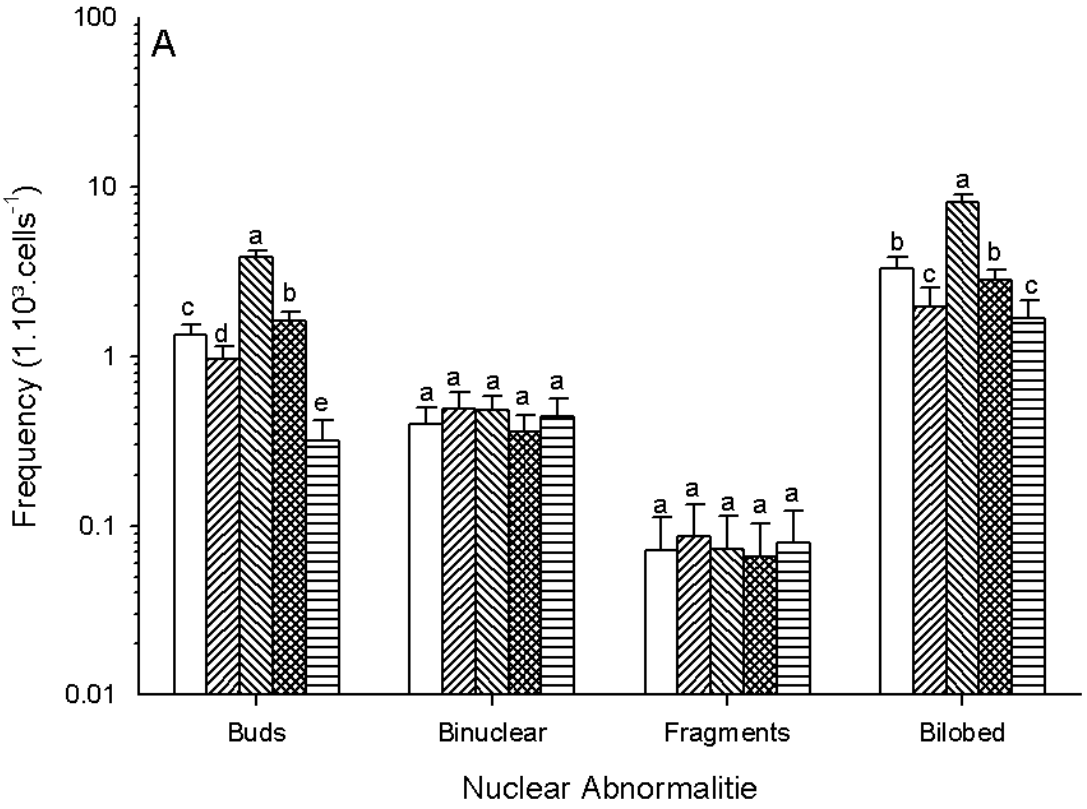

Comet Class (1.10² cells⁻¹)
Comet Score
Control
Control DMSO
10
20
200
Phenanthrene treatments (µg.L⁻¹)

Figure 5


A
100
10
1
0.1
0.01
Frequency (1.10³.cells⁻¹)
c d a b e
a a a a a
a a a a a
b c a b c
Buds
Binuclear
Fragments
Bilobed
Nuclear Abnormalitie

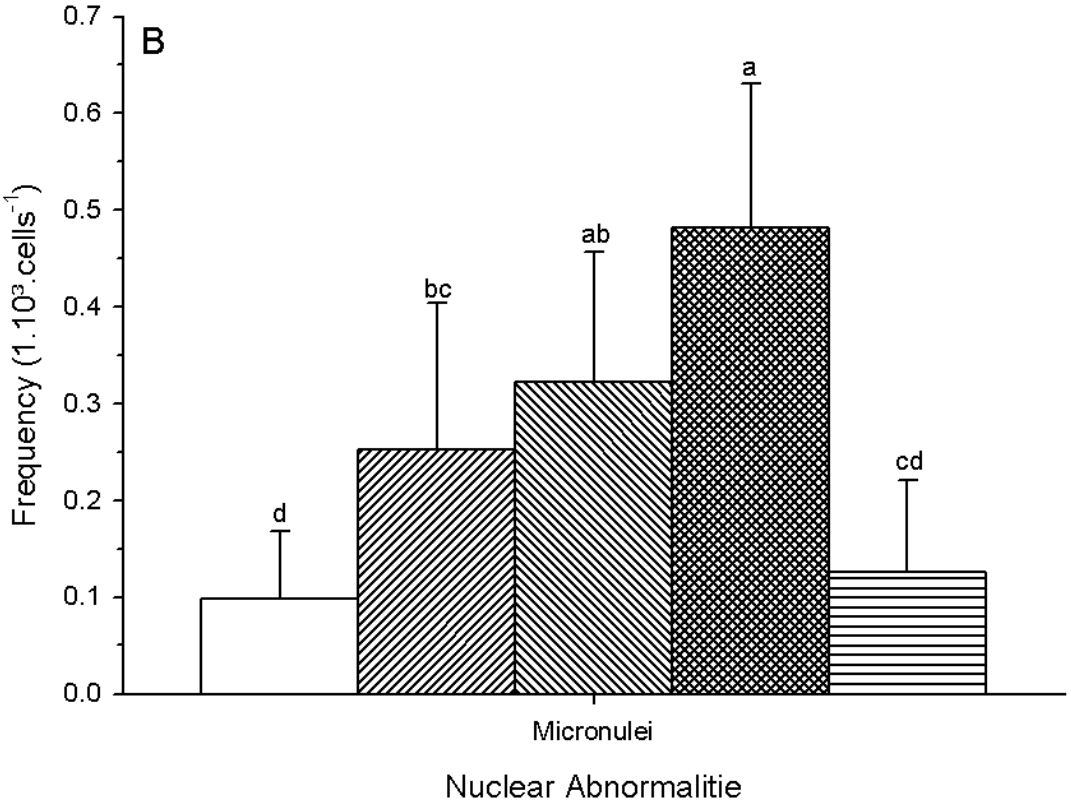
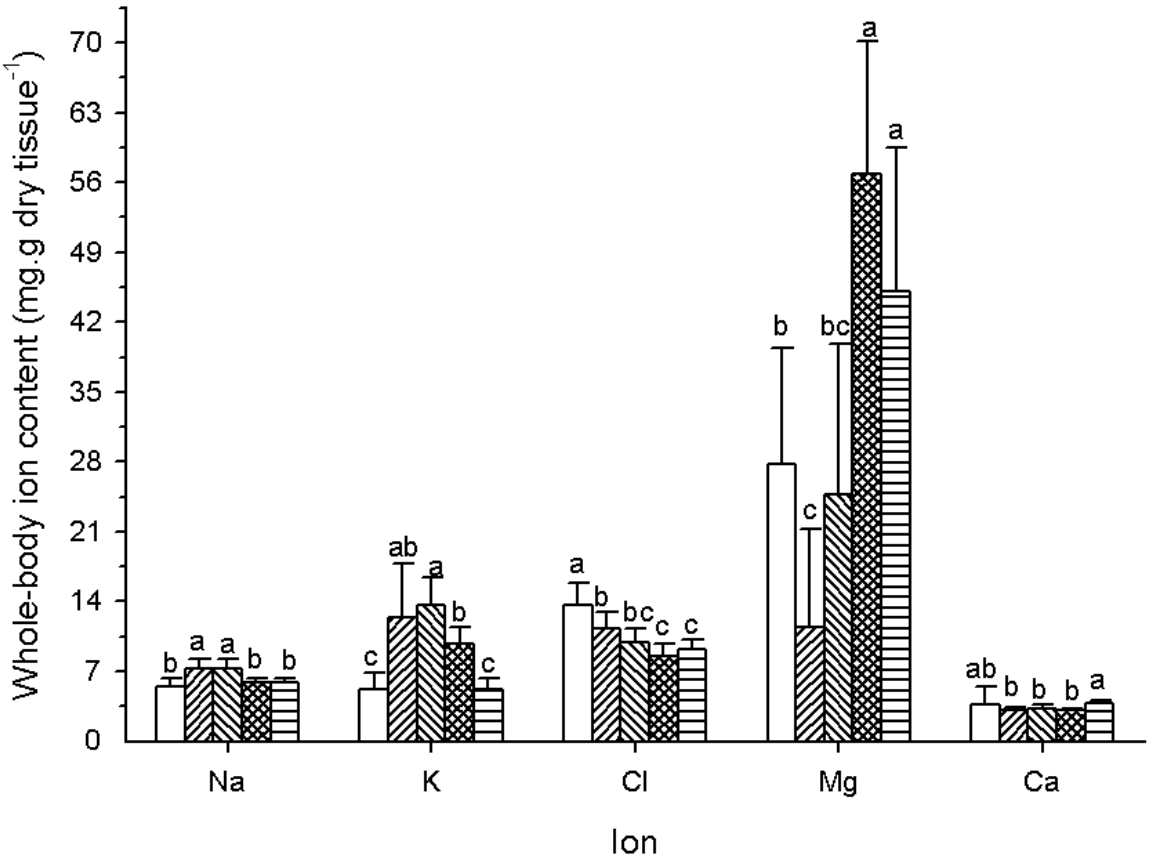

B
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Frequency (1.10³.cells⁻¹)
d
bc
ab
a
cd
Micronulei
Nuclear Abnormalitie

Figure 6


Whole-body ion content (mg.g dry tissue^{-1})
70
63
56
49
42
35
28
21
14
7
0
b a a b b
c ab a b c
a b bc c c
b c bc a a
ab b b b a
Na
K
Cl
Mg
Ca
Ion