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MARCADORES DE ESTRESSE OXIDATIVO EM PEIXES (*Astyanax sp*. e *Danio rerio*) EXPOSTOS A EFLUENTES URBANOS E AGRÍCOLAS NO BIOMA PAMPA

DISSERTAÇÃO DE MESTRADO

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RESUMO

Os ecossistemas aquáticos estão sob constante risco devido às atividades industriais, agrícolas e urbanos, comprometendo a qualidade da água e a preservação da biota aquática. A avaliação dos impactos toxicológicos causados por poluentes ao ambiente aquático utilizando medidas de biomarcadores em peixes pode fornecer dados confiáveis para estimar os efeitos sub-letais provocados por produtos químicos em áreas contaminadas. Neste estudo, os peixes (*Astyanax sp*. e *Danio rerio*, Hamilton, 1822) expostos aos efluentes agrícolas e urbanas no rio Vacacaí, no Brasil, foram testados quanto à potenciais sinais de contaminação aquática. Este rio compreende um dos principais cursos de água do Pampa brasileiro, um bioma com uma grande biodiversidade que vem sendo negligenciada em termos de desenvolvimento ambiental e sócio-econômico. Os locais S1 e S2 foram escolhidos por sua proximidade às monoculturas e aos pontos de descarga de águas residuais, enquanto local de referência estava localizada a montante de S1 e S2, em uma área aparentemente sem degradação. Tecidos musculares e cerebrais dos peixes foram processadas para determinação da acetilcolinesterase, bem como biomarcadores relacionados ao estresse oxidativo. Os resultados mostraram sinais de contaminação ambiental, caracterizada por alterações significativas na atividade da colinesterase, expressão de metalotioneína, enzimas antioxidantes, níveis de glutationa e ativação da resposta antioxidante / estresse por vias de sinalização celular em peixes expostos a locais contaminados quando comparados ao local de referência. Com base nesses resultados, evidencia-se que as atividades urbanas e agrícolas estão colocando em risco a qualidade ambiental dos recursos hídricos na área estudada. Também é demonstrado que os biomarcadores de estresse celular podem servir como ferramentas importantes para o biomonitoramento e desenvolvimento de protocolos de avaliação de risco no bioma Pampa.

Palavras chave: biomarcadores, estresse oxidativo, poluição, águas residuais, agricultura, peixe.

Abstract

Aquatic ecosystems are under constant risk due to industrial, agricultural and urban activities, compromising water quality and preservation of aquatic biota. The assessment of toxicological impacts caused by pollutants to aquatic environment using biomarker measurements in fish can provide reliable data to estimate sub-lethal effects posed by chemicals in contaminated areas. In this study, fish (*Astyanax sp.* and *Danio rerio* Hamilton, 1822) exposed to agricultural and urban effluents at the Vacacaí River, Brazil, were tested for potential signs of aquatic contamination. This river comprehends one of the main watercourses of the Brazilian Pampa, a biome with a large biodiversity that has being neglected in terms of environmental and social-economic development. Sites S1 and S2 were chosen by their proximity to crops and wastewater discharge points, while reference site was located upstream of S1 and S2, in an apparently non-degraded area. Fish muscle and brain tissues were processed for determination of acetylcholinesterase as well as oxidative stress related biomarkers. The results showed signs of environmental contamination, hallmarked by significant changes in cholinesterase activity, expression of metallothionein, antioxidant enzymes, glutathione levels and activation of antioxidant/cell stress response signaling pathways in fish exposed to contaminated sites when compared to reference. Based on these results, it is evidenced that urban and agricultural activities are posing risk to the environmental quality of water resources at the studied area. It is also demonstrated that cell stress biomarkers may serve as important tools for biomonitoring and development of risk assessment protocols in the Pampa biome.

Key words: biomarkers, oxidative stress, pollution, wastewater, agriculture, fish.

SUMÁRIO

1 APRESENTAÇÃO

Esta dissertação segue as normas do Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal do Pampa e compõe-se de uma introdução sumária, apresentação do estudo (objetivos e justificativa), artigo submetido a periódico especializado indexado (Qualis Capes e com fator de impacto JCR), conclusão breve e referências bibliográficas.

2 INTRODUÇÃO

2.1 Contaminação Ambiental

Os ecossistemas são frequentemente expostos a contaminantes ambientais, através de efluentes industriais, agrícolas e urbanos. O destino final da maioria desses contaminantes é o ambiente aquático, por meio de descargas diretas, ciclo hidrológico ou da atmosfera (STEGEMAN; LECH, 1991). A contaminação aquática compromete diretamente a saúde ambiental, através da incorporação de xenobióticos aos recursos hídricos, atingindo organismos unicelulares, peixes e organismos que estão no topo nas cadeias alimentares, causando por fim a contaminação humana pelo consumo de peixes e outros integrantes da biota aquática (CLARKSON, 1995 & KATAGI, 2010).

2.2 Biomarcadores

Dentre as abordagens metodológicas na avaliação ecotoxicológica nos últimos anos, vem se desenvolvendo o emprego de biomarcadores de contaminação ambiental, sendo estes excelentes ferramentas de respostas adaptativas e preventivas no biomonitoramento ambiental. Nesse contexto, biomarcadores são definidos como respostas biológicas adaptativas a estressores, evidenciadas através de alterações bioquímicas, celulares, histológicas, fisiológicas ou comportamentais (DEPLEDGE, 1993). Deste modo, a mensuração dos biomarcadores nos níveis celulares e moleculares, como primeiras respostas a poluição química, tornam se uma ferramenta sensível e confiável na avaliação da qualidade ambiental (VAN DER OOST et al., 2003).

Neste sentido, os parâmetros de estresse oxidativo constituem um grupo importante de biomarcadores, respondendo a diversos compostos tóxicos existentes no ambiente ou seu metabolitos que podem exercer efeitos tóxicos neste domínio (WINSTON; DI GIULIO, 1991). Entre as principais defesas não enzimáticas da célula esta o tripeptídeo glutationa (GSH). A GSH é composta por gamma-glutamil-citeinil-glicina, atuando contra a formação de radicais livres, na homeostase tiólica, na manutenção do balanço redox da célula e na defesa contra agentes eletrofílicos. Essa capacidade antioxidante se dá pelo grupamento tiol

(SH) reativo de sua cisteína, o qual também pode ser encontrado em proteínas (PSH) ou em tióis de baixo peso molecular (NPSH), como a cisteína (REISCHL et al., 2007).

Os sistemas de defesa que tendem a inibir a formação de oxiradicais incluem as enzimas antioxidantes, tal como superóxido-dismutase, catalase, glutationa-peroxidase e a glutationa-redutase, cuja medição enzimática é utilizada frequentemente em estudos de ecotoxicologia. A superoxido dismutase (SOD) é uma mataloenzima que age sobre o radical O^{2-}. dismutando a H_2O_2, que na sequência de eventos bioquímicos irá sofrer dismutação pelas enzimas catalase ou por outra peroxidase, originando água e oxigênio molecular (BERG et al. 2004), sendo a catalase (Cat) a função de dismutar H_2O_2 em H_2O e O_2 (AEBI, 1984).

O sistema glutationa, inclui glutationa reduzida, enzimas como glutationa peroxidase e glutationa redutase (DRINGEN et al., 2000). A glutationa peroxidase (GPx) está relacionada á função antioxidante da GSH com atividade peroxidásica contra peróxido de hidrogênio e peróxidos orgânicos (HALLIWLL & GUTTERIDGE, 2007). Para evitar a depleção da GSH e aumento da glutationa oxidada (GSSG), a glutationa redutase (GR) reduz a GSSG à custa de NADPH, regenerando a GSH e mantendo desta forma o estado redox intracelular (BERG et al., 2004) , servindo como biomarcador indireto de contaminação ambiental.

A glutationa S-transferase (GST) é responsável pela conjugação de xenobióticos eletrofílicos ao tripeptídeo GSH, reduzindo sua toxicidade. A GST e o citocromo P450, por serem enzimas sensíveis a compostos exógenos, têm sido largamente utilizados como biomarcadores (STEGEMAN et al., 1990; BUCHELI & FENT, 1995). Alterações nestas defesas podem estar relacionadas a diferentes classes de poluentes, diferenças de sensibilidade de espécies e a fatores ambientais e biológicos (WINSTON; DI GIULIO, 1991).

A acetilcolinesterase (AChE) é a enzima responsável pela hidrólise do neurotransmissor acetilcolina presente nas fendas sinápticas durante a transmissão colinérgica. Esta enzima é inibida por dois grupos de pesticidas os organofosforados e os carbamatos, os quais se combinam covalentemente à resíduos de aminoácidos específicos para inativar a enzima (JUNG et al., 2007). A análise de determinados parâmetros neurotóxicos tem assumido uma particularidade clássica como biomarcadores de exposição a agroquímicos, sendo a atividade da enzima colinesterase um marcador confiável frente a exposição a agroquímicos organofosforados e carbamatos (GALLOWAY et al., 2004).

Na avaliação de risco ecotoxicológico, estudos relacionados à modulação de vias de sinalização celular vem ganhando destaque, pois evidências recentes apontam para este tipo de abordagem como ferramentas úteis para o desenvolvimento de novos protocolos de biomonitoramento, incluindo a análise das mais diversas vias de sinalização celular, como potencias biomarcadores de contaminação aquática (LEAL et al., 2006).

Apenas recentemente, o estudo da modulação de membros da família de proteínas quinases ativadas por mitógeno (MAPKs) em organismos submetidos a estresse ambiental tem sido empregado na ecotoxicologia. MAPKs representam uma família de proteínas altamente conservadas entre diferentes espécies e desempenham importantes funções regulatórias na fisiologia das células, sendo envolvidas em processos como morte celular, proliferação, diferenciação e resposta adaptativa (CHANG; KARIN, 2001 & LEAL et al., 2006).

Dentre os membros desta família inclui-se a proteína quinase regulada por estresse extracelular (ERK1/2), c-Jun N-terminal quinase (JNK) e a p38. Quando duplamente fosforilados em resíduos de treonina e tirosina estas proteínas são ativadas, podendo então fosforilar e ativar outros componentes em sua via de transdução de sinal (CHANG; KARIN, 2001), nesse contexto a ativação de MAPKs tem sido observadas em resposta a estresse osmótico, estresse oxidativo, exposição à citocinas e injúria tóxica (COWAN; STOREY, 2003).

A análise de proteínas de choque térmico (HSPs) tem sido aplicadas como biomarcadores de exposição a contaminantes ambientais. Estas proteínas tem papel importante na manutenção da conformação de proteínas celulares atuando como chaperonas (MACARIO; CONWAY DE MACARIO, 2007). Além disso, diversos contaminantes demonstraram modular a expressão destas proteínas em organismos aquáticos (KILEMADE; MOTHERSILL, 2001).

Dentre outras respostas biológicas ao estresse ambiental, destaca-se a atividade de expressão de fatores de transcrição nuclear (Nrf2), que no núcleo das células, se liga a elementos de resposta a antioxidantes (ARE) na região promotora dos genes que transcrevem varias enzimas antioxidantes endógenas como GST, GPX, GR, SOD, CAT e sistema tioredoxina (HUR; GRAY, 2011 e TANITO et al., 2007). Glutationa peroxidase 4 (GPX-4) é expressa em uma variedade de tecidos, no entanto a sua localização subcelular nos tecidos se

encontra diferenciada (CONRAD et al., 2007). Os principais substratos para GPX4 são hidroperoxidos fosfolipídios, podendo indicar um papel crucial na peroxidação lipídica causada por xenobióticos (BRIGELIUS-FLOHÉ, 2006).

A flavoproteina homodimérica NADPH quinona oxiredutase (NQO-1), catalisa a redução de dois elétrons de quinonas inibindo assim o ciclo redox de xenobióticos, estresse oxidativo e lesão neoplásica (DINKOVA-KOSTOVA; TALALAY, 2010). Com isso, reforça a hipótese de que sua expressão pode ser um indicativo de contaminação ambiental frente à geração de dano oxidativo por xenobióticos.

As metalotioneínas (MTs) são proteínas primordialmente citosólicas de baixo peso molecular, caracterizadas por possuírem altos níveis de cisteína e ausência de aminoácidos aromáticos e histidina. A presença de tióis (SH) permite a estas proteínas se ligarem ao excesso de metais essenciais e a metais poluentes, protegendo assim organismos aquáticos a exposição de compostos inorgânicos. A produção de metalotioneínas é induzida pelo aumento de entrada de metais na célula, o que torna estas proteínas biomarcadores específicos de exposição à contaminação por metais (VIARENGO et al., 2007).

O reparo do DNA em resposta ao estresse ambiental, através de PARP, uma poli (ADP-ribose) polimerase nucelar de 116KDa, um dos principais alvos de caspase-3, sendo a clivagem de PARP em fragmento de 89 KDa, um biomarcador de células em processo apoptótico (SATOH; LINDAHL, 1992).

O uso da atividade de enzimas de estresse oxidativo e alterações nas vias de sinalização celular podem representar uma ferramenta preventiva nas análises da qualidade ambiental em ecossistemas impactados. Portanto, este tipo de abordagem tende a elucidar o nível de dano biológico no sítio de estudo, podendo ser correlacionado a parâmetros analíticos como dosagem de concentração de poluentes, análise físico-química do corpo receptor, gerando assim um protocolo eficiente para o estudo ecotoxicológico de áreas contaminadas.

2.3 Peixes como bioindicadores

As espécies sentinelas que são utilizadas como primeiros indicadores de danos ao ambiente são definidas como bioindicadores (ADAMS, 2002). A utilização de peixes para o monitoramento ecotoxicológico se justifica pelo seu íntimo contato com o habitat, muitas espécies com seu ciclo de vida bem documentado, posição chave na cadeia trófica e importância no consumo humano (VIARENGO et al., 2007).

2.3.1 Astyanax sp

A ordem Characiformes é o grupo dominante dentre os peixes de água doce da América do Sul, sendo a família Characidae a maior e mais complexa desta ordem (FOWLER, 1999). O gênero *Astyanax sp*, tem importância para o consumo humano e possuem um grande valor ecológico como espécie forrageira (GODOY, 1975), além disso, tem papel chave na ciclagem de nutrientes em ambientes aquáticos e servem de alimento para aves e peixes pertencentes a níveis tróficos superiores.

2.3.2 Danio rerio

Danio rerio (Hamilton, 1822*)*, também conhecido como Zebrafish, é uma espécie bentopelágica de água doce pertencente ordem de Cypriniformes, família Cyprinidae, com distribuição no continente Asiático (Westerfield, 2000). O Zebrafish se tornou um modelo biológico versátil nas pesquisas, sendo um vertebrado diploide com um bom equilíbrio entre a complexidade e a simplicidade (STREISINGER et al., 1981). O modelo alternativo Zebrafish possui vantagens em bioensaios, por possuir pequeno tamanho, fácil manutenção e crescente número de publicações nas diferentes áreas da ciência, como a ecotoxicologia. Portanto, Zebrafish é largamente usado como espécie modelo em protocolos ecotoxicológicos (OECD, 1992).

2.4 Área de Estudo

O bioma pampa possui uma grande biodiversidade e uma vasta gama de espécies endêmicas (BEHLING & PILLAR, 2007). O bioma pampa possui uma grande riqueza em recursos hídricos de água doce, inseridas nas bacias hidrográficas, sendo vitais para o sustento de atividades agrícolas, industriais, abastecimento público e preservação da biodiversidade aquática (INSTITUTO IBAMA).

A bacia hidrográfica do Vacacaí-Vacacaí Mirim, pertencente à Região Hidrográfica do Guaíba está localizada na porção centro-ocidental do RS, possuindo uma área de 11.077,34 km². Os principais usos da água se destinam a irrigação, pecuária e abastecimento público, sendo que o rio Vacacaí e seus respectivos afluentes abrangem 40% da população da bacia incluindo a zona urbana e agrícola dos municípios de São Gabriel, Caçapava do Sul e São Sepé (SEMA RS).

O rio Vacacaí nasce em São Gabriel, passando por Santa Maria até desembocar no rio Jacuí. O solo é ocupado por latifúndios, com pecuária extensiva e agricultura. (FEPAM RS). O município de São Gabriel-RS tem como principais atividades econômicas a pecuária extensiva e o cultivo de monoculturas de arroz irrigado e soja, sendo o rio Vacacaí o principal manancial para estas atividades. Os pontos amostrados para avaliação de biomarcadores de contaminação ambiental S1 e S2, demonstram visível degradação ambiental, apresentando fontes difusas de contaminação como atividades agrícolas e descargas de efluentes urbanos.

3 OBJETIVOS

Geral:

Utilização de biomarcadores bioquímicos em *Astyanax sp.* e *Danio rerio* como ferramenta para avaliação da qualidade ambiental do rio Vacacaí São Gabriel – RS.

Objetivos específicos:

a) Avaliar as alterações bioquímicas em *Astyanax sp*. em estudo de campo nos pontos de coleta do rio Vacacaí.

b) Avaliar respostas biológicas (biomarcadores) no modelo *Danio rerio* exposto às amostras ambientais coletadas no rio Vacacaí.

4 JUSTIFICATIVA

A bacia hidrográfica Vacacaí-Vacacaí Mirim drena parte do Estado do Rio Grande do Sul, sustentando atividades econômicas importantes para a região como pecuária extensiva e agricultura (FEPAM RS). No entanto, essas atividades aliadas à ausência de planejamento e gestão ambiental dos recursos naturais, vêm comprometendo o equilíbrio de ecossistemas que compõe a bacia, dentre eles o bioma pampa.

No rio Vacacaí, região alvo do nosso estudo, tem-se observado problemas de erosão, uso inadequado do solo através das monoculturas, descargas diretas de efluentes urbanos e esgotos domésticos *in natura*. Desta forma, esses impactos ambientais acabam por comprometer a saúde ambiental dos ecossistemas aquáticos, tornando o biomonitoramento ambiental da bacia importante não só como instrumento de diagnóstico ecológico, mas também como uma ferramenta para fins de sustentabilidade no desenvolvimento da região do rio Vacacaí do ponto de vista social e econômico.

A avaliação de áreas impactadas necessita de técnicas rápidas e preditivas para a análise de risco ecotoxicológico. Entre essas técnicas estão os biomarcadores, indicados como possíveis sensores de impacto local, diagnóstico, controle e prevenção em programas de biomonitoramento ambiental (BERTOLETTI & ZAGATTO, 2006). Portanto a investigação de biomarcadores de contaminação aquática em peixes com espécies naturais (*Astyanax sp*) e bioensaios padronizados com o modelo (*Danio rerio*), se tornam relevantes na prevenção de danos ambientais e diagnóstico preventivo em programas biomonitoramento ambiental na bacia hidrográfica do Vacacaí-Vacacaí Mirim.

5 CONFIRMAÇÃO DE ACEITE DO ARTIGO

De: "Cinta Porte" <em@editorialmanager.com>
Data: 15/05/2015 05:47
Assunto: ESPR: Your manuscript ESPR-D-15-00324R4 - [EMID:cb6785138b87bef0]
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Ref.: Ms. No. ESPR-D-15-00324R4
Oxidative stress markers in fish (Astyanax sp. and Danio rerio) exposed to urban and agricultural effluents in the Brazilian Pampa biome
Environmental Science and Pollution Research

Dear Dr. Franco,

I am pleased to tell you that your work has now been accepted for publication in Environmental Science and Pollution Research.

Thank you for submitting your work to this journal.

With kind regards,

Dr Cinta Porte
Editor
Environmental Science and Pollution Research

6 ARTIGO

Oxidative stress markers in fish (*Astyanax sp.* and *Danio rerio*) exposed to urban and agricultural effluents in the Brazilian Pampa biome

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Abstract

Aquatic ecosystems are under constant risk due to industrial, agricultural and urban activities, compromising water quality and preservation of aquatic biota. The assessment of toxicological impacts caused by pollutants to aquatic environment using biomarker measurements in fish can provide reliable data to estimate sub-lethal effects posed by chemicals in contaminated areas. In this study, fish (*Astyanax sp.* and *Danio rerio*) exposed to agricultural and urban effluents at the Vacacaí River, Brazil, were tested for potential signs of aquatic contamination. This river comprehends one of the main watercourses of the Brazilian Pampa, a biome with a large biodiversity that has being neglected in terms of environmental and social-economic development. Sites S1 and S2 were chosen by their proximity to crops and wastewater discharge points, while reference site was located upstream of S1 and S2, in an apparently non-degraded area. Fish muscle and brain tissues were processed for determination of acetylcholinesterase as well as oxidative stress related biomarkers. The results showed signs of environmental contamination, hallmarked by significant changes in cholinesterase activity, expression of metallothionein, antioxidant enzymes, glutathione levels and activation of antioxidant/cell stress response signaling pathways in fish exposed to contaminated sites when compared to reference. Based on these results, it is evidenced that urban and agricultural activities are posing risk to the environmental quality of water resources at the studied area. It is also demonstrated that cell stress biomarkers may serve as important tools for biomonitoring and development of risk assessment protocols in the Pampa biome.

Key words: biomarkers, oxidative stress, pollution, wastewater, agriculture, fish.

Introduction

The Brazilian Pampa biome comprehends one of six terrestrial biomes in Brazil. It is located between latitudes 28°00' S and 34°00' S and longitudes 49°30' W and 58°00' W (IBGE – Brazilian Institute of Geography and Statistics 2014) occupying an area of 176,496 km^2. It represents about 63% of the Rio Grande do Sul State and 2.07% of the national territory (Roesch et al. 2009). It also extends to another two South American countries, Argentina and Uruguay. The improper management of urban waste and widespread use of pesticides in monocultures, especially soybeans and rice, are the main causes of environmental degradation of the Pampa biome (Behling and Pillar, 2007). The Vacacaí-Vacacaí Mirim basin is a major watershed located at the central-western region of the State, supplying potable water for approximately 385,000 inhabitants. To date, no studies assessed aquatic environmental pollution and hence no monitoring of its effects on the animal species has been performed within the Pampa biome borders. Therefore, efforts focusing at the monitoring of this fragile biome waters are welcome and necessary.

The use of biomarkers in aquatic organisms, especially in fish, have been pointed out as an effective approach to obtain information about the aquatic environmental quality and the potential threats caused by pollutants to the local ecosystem (Viarengo et al. 2007). Biomarkers, by definition, consist in a range of biological responses related to exposure to contaminants and may include molecular, cellular, physiological and behavioral responses (Montserrat 2003). The measurement of biomarkers at the molecular and cellular levels have been proposed as early hallmarks of chemical pollution exposures, thus being reliable and sensitive tool in the assessment of environmental quality (van der Oost et al. 2003). Classical biomarkers include acetylcholinesterase (AChE) inhibition and cytochrome P450 (CYP) induction, whereas oxidative stress-related parameters, such as antioxidant enzymes, glutathione status and oxidative damage to lipids are complementary biomarkers (Franco et al. 2010). Among phase II enzymes, the glutathione S-transferase (GST) induction is an important factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals and/or environmental conditions (Gadagbui and James, 2000). Metallothioneins (MT) expression also can be used as a biomarker since those molecules play a role in the intracellular metal homeostasis. The thiol component of the MT protein is responsible for metal binding, and intracellular storage of both essential and no-essential metal ions (Eaton et

al. 1980; Schlenk 1998). Metallothioneins are induced by exposure to metals, therefore such effect is a well-established marker of metal contamination (Roesijadi 1994).

Oxidative stress is defined as an unbalance between pro-oxidants, including reactive species and free radicals derived from numerous toxicological processes, that can generate biomolecules damage. The cellular antioxidant apparatus, which include both protein and non-protein antioxidants, is the prime barrier against oxidative damage to biomolecules (Halliwell 2007). Metal, organic compounds and a range of environmental contaminants are demonstrated to induce oxidative stress by a myriad of mechanisms (Storey 1996). Aquatic organisms become targets of oxidative effects from environmental pollutants, when they ingest such molecules increasing the production of ROS, such as transition metals (Viarengo 1989).

Astyanax is a widespread genus composed of nearly 100 species, occurring in almost all Brazilian territory (Neto 2009). As a forage species, it plays a central role in the aquatic ecosystems and despite the small size it is frequently utilized for human consumption (Godoy 1975). Some characteristics make such group of species a model organism to study the effect of pollutants in water basins as elevated reproductive potential, trophic opportunism and because they have short migration only during the higher precipitation periods (Filho and Braga 1996; Suzuki and Agostinho 1997). Recent studies have demonstrated the effectiveness of aquatic biomonitoring using *Astyanax* genus as model organism (Erbe et al. 2011; Uieda and Barreto 1999; Orsi et al. 2004).

Considering the complete lack of studies on the aquatic pollution risk assessment and the absence of pollution effects monitoring strategies in the Brazilian Pampa biome, the present study aimed to search for potential threats to local water supplies by using oxidative stress based biomarkers and evaluate a suite of biomarker for biomonitoring the Brazilian Pampa biome.

Material and methods

Chemicals

Glutathione reductase (GR), reduced glutathione (GSH), oxidized glutathione (GSSG), tert-butylhydroperoxide (t-BOOH), 5,5-dithio-bis(2-nitrobenzoic)acid (DTNB), 1-chloro-2,4-dinitrobenzene(CDNB), acetylthiocholine iodide, nicotinamide adenine dinucleotide phosphate—reduced (NADPH), quercetin, *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine **(**TEMED), anti-rabbit-HRP secondary antibodies, beta-actin HRP conjugated antibody was purchased from Sigma-Aldrich, (São Paulo, Brazil). Other chemicals were obtained from highest commercial grade available.

Animals and area of study

The reference site (30°38’32.93”S/54°40'29.63”W) was chosen due to its apparent conservation aspect, with the presence of riparian vegetation and no proximity to urban areas neither agricultural activity. The S1 site (30°22’35.45”S/54°21’24.82”W) was located nearby rice crops and S2 (30°20’27.28”S/54°1819.96”W) was located in an area presenting both urban and agricultural activities nearby. Reference, S1 and S2 sites were at Vacacaí River, São Gabriel, RS, Brazil. The distances from each site are as follows: Ref/S1: 42.3 Km; Ref/S2: 48.7 Km; S1/S2: 6.4 Km. Satellite view of each site can be seen in Supplementary figure 1. Analysis of the physical-chemical parameters of the water was evaluated *in–situ* with the aid of a multi-probe instrument (Manta 2, Eureka, USA).

Lambari fish (*Astyanax sp.*)

A total of thirty (30) adult Lambari fish (*Astyanax sp.*), both male and females, were captured using hand-made plastic traps (1.0 x 1.0 cm mesh) at studied sites (n=10 animals per site). We limited our analyses to adult specimens (body weight 5-8g; length 50-60mm) obtained from each site. After being captured, animals were gently transferred to tanks containing water from the capture site and rapidly transported to the laboratory with constant aeration. No signs of stress or potential injuries were observed in fish after capturing procedures. Animals were allowed to adapt to the laboratory conditions for 3 hours before sample preparation. Fish were removed from the tanks and anesthetized on ice-cold water. Then, animals were killed by medullar section and samples of muscle and brain were taken and kept in ultra-freezer (-80°C) for further biochemical analyses. Muscle tissue from

Astyanax specimens were used for determination of thiol status and enzyme activities while brain tissue was used for western blot analysis.

The genus *Astyanax* has hundreds of morphologically similar species forming a complex of species from the taxonomic point of view (Fernandes and Martins-Santos 2004, 2005). Therefore, there is the possibility that different *Astyanax* species might be sampled in this study. However, no influence in the biomarkers measurements were observed as suggested by the low degrees of standard error among the biological replicates (see Results section).

Zebrafish (*Danio rerio*)

A total of 30 adult zebrafish (wild-type; both genders) (n=10 individuals per treatment) from our own breeding colony were kept in tanks, under controlled temperature and light–dark (12-12h) photoperiod. Feeding and maintenance of fish were done according to Westerfield (2000). For the experiments, fish were transferred to 3 liter tanks containing water collected from reference, S1 and S2 sites. After 48h exposure, animals were anesthetized in ice-cold water and then killed by medullar section and samples of brain tissue were taken for biomarker evaluation by western blotting techniques. All experimental procedures utilized in this study involving animals were approved by the university's ethical committee for the use of experimental animals (CEUA Unipampa protocol 043/2013).

Thiol status

Muscle tissue was collected, weighed and homogenized in 0.5 M perchloric acid (PCA) and centrifuged at 15,000 g for 2 min, 4°C and the supernatant was assayed for glutathione levels in the form of non-protein thiols (NPSH). The *pellet* was washed 3 times in 0.5 M PCA and re-suspended in 1 ml 0.1 M TRIS/HCl pH 8.0 for determination of protein thiols (PSH). Both NPSH and PSH were measured spectrophotometrically (Agilent Cary 60 UV-VIS) at 412 nm (Ellman 1959). Data were expressed as µmol NPSH or PSH/g wet tissue.

Enzyme activity

For enzymatic analysis, fish muscle was homogenized in 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), pH 7.4 and centrifuged at 20,000 g for 30 min at 4°C.

Glutathione reductase (GR) was determined according to the method described by Carlberg and Mannervik (1985). Briefly, GR reduces GSSG to GSH, expending NADPH and the disappearance of which can be measured spectrophotometrically at 340 nm. One unit will cause the oxidation of 1.0 μmole of NADPH to NADP+ per minute at 25 °C at pH 7.0.

Glutathione peroxidase (GPx) activity was measured as originally described by Wendel (1981). The method uses an indirect determination based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP+ is indicative of GPx activity, since GPx is the rate limiting factor of the coupled reactions. One unit of GPx will produce 1.0 μmole of NADP+ from NADPH per minute at pH 7.0 at 25 °C in a coupled reaction in the presence of reduced glutathione, glutathione reductase, and tert-butyl hydroperoxide.

The *glutathione S-transferase* (GST) activity was done as described by Habig and Jakoby 1981 using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, which is suitable for the broadest range of GST isozymes. Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is a linear increase in the absorbance at 340 nm. One unit of GST activity is defined as the amount of enzyme producing 1.0 μmol of GS-DNB conjugate/min under the conditions of the assay.

Activity of catalase (CAT) was measured according to Aebi 1984, in which the disappearance of H_2O_2 is continuously monitored at 240 nm. One unit of CAT is defined as the amount of enzyme degrading 1.0 nmol of H_2O_2 per minute under the conditions of the assay.

Superoxide dismutase (SOD) was measured following the procedures established by Kostyuk and Potapovich (1989). In this assay, quercetin is used as a superoxide radical sensor in the presence of N,N,N′,N′-Tetramethylethane-1,2-diamine (TEMED) at pH 10. Tissue SOD activity is expressed in units SOD/mg of total protein, where 1 unit is the amount of SOD required to give 50% maximal inhibition of the initial rate of quercetin reduction.

Acetylcholinesterase (AChE) activity was assayed by measuring the hydrolysis ratio of acetylthiocholine in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and formation of thionitrobenzoic acid (Ellman et al. 1961), monitored at 412 nm. In this assay, thiocholine,

produced by AChE, reacts with DTNB to form a colorimetric (412 nm) product, proportional to the AChE activity present in the sample. One unit of AChE is the amount of enzyme that catalyzes the production of 1.0 μmole of thiocholine per minute at 25 °C at pH 7.5.

Total protein was determined according to Bradford (1976).

Western blotting

Western blotting was performed according to Franco et al., 2010a,b with minor modifications. The whole fish brains were homogenized at 4 °C in a buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride and protease inhibitor cocktail (Sigma, MO). The homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C and the supernatants are collected. After total protein determination (Bradford 1976) using bovine serum albumin as standard, β-mercaptoethanol was added to samples to a final concentration of 8%. Then samples were frozen at −80 °C for further analysis. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Then, membranes were incubated with specific primary antibodies for the determination of specific protein targets.

Primary antibodies for MAPK family (total and phosphorylated forms) were purchased from Cell Signaling Technologies (Danvers, USA). Primary antibodies anti-rabbit Nrf2, NQO-1, GPx-1 GPx-4, MT-1, HSP70, PARP, Phospho-ERK, total ERK B-Actin and enhanced chemiluminescent western blot substrate were purchased from Santa Cruz Biotechnology (Dallas, USA). Phospho-Nrf2 primary antibody was purchased from Abcam (Cambridge, UK) and nitrocellulose membranes were provided by GE Healthcare (São Paulo, Brazil). Anti-Rabbit IgG, produced in goat, used as secondary antibody was purchased from Sigma-Aldrich, (São Paulo, Brazil). Densitometric analysis of immunoreactive bands was performed using Scion Image® software.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's *post-hoc* test when needed. Results were considered statistically significant when $p<0.05$.

Results

Astyanax sp. biomarkers

Water physical-chemical parameters from reference, S1 and S2 sites are depicted in Table 1. According to the obtained data, no differences were observed when comparing the reference with S1 and S2 sites.

Table 1

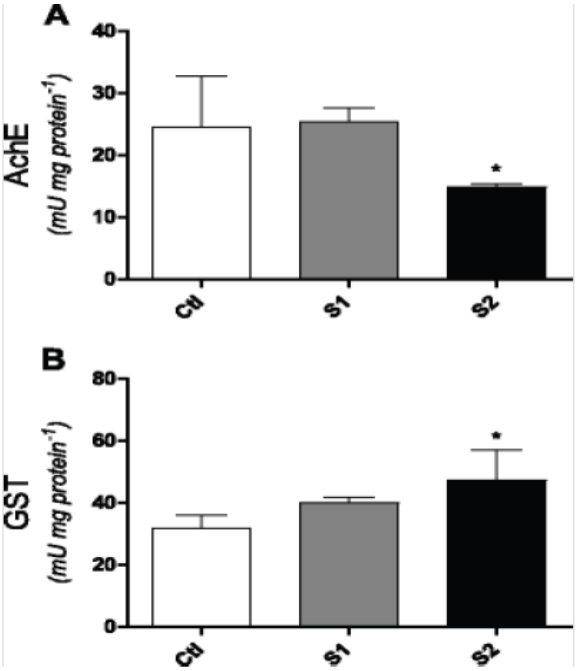
Physical-chemical parameter of Vacacaí water at each indicated site

Parameter	Reference	Site 1 (S1)	Site 2 (S2)
Temperature (°C)	25	24	25.4
pH	6.65	6.77	7.44
Conductivity (mS/cm)	0.095	0.110	0.090
Dissolved oxygen (mg/L)	7.27	8.48	7.4

The activity of acetylcholinesterase (AChE) in fish muscle tissue was significantly inhibited ($p<0.05$) in S2, when compared to S1 and reference sites (Fig. 1a). The glutathione S-transferase activity was substantially ($p<0.05$) increased in the muscle of fish collected at S2 site, when compared to both S1 and reference (Fig. 1b).

Fig. 1

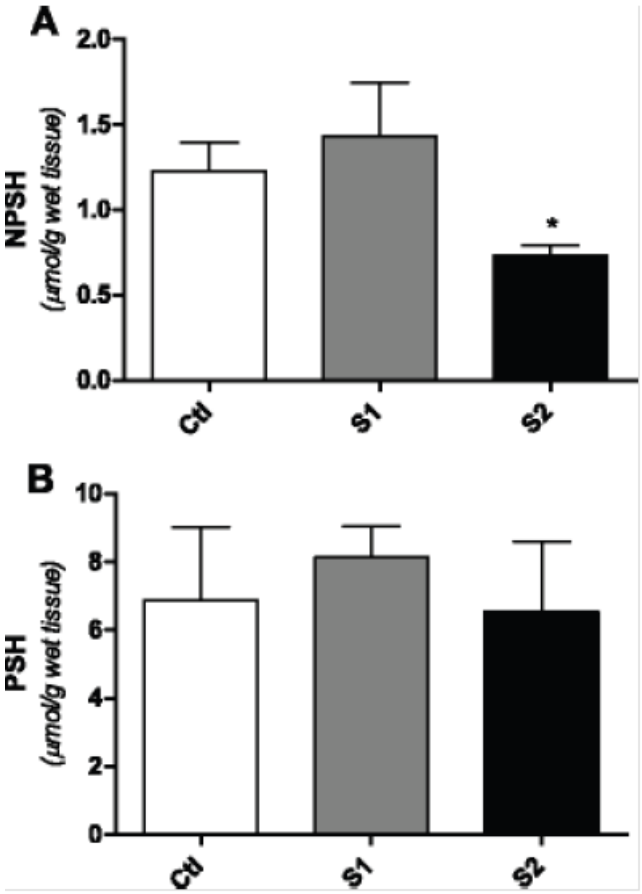
a AChE activity and **b** GST activity from muscle tissue of Lambari fish (*Astyanax sp.*) captured in Vacacaí River sites. Data are expressed as Mean±SD of enzyme activity ($mU\ mg\ protein^{-1}$). * $p<0.05$ when compared to reference site.



We measured thiol status, as non-protein (NPSH) and protein (PSH) thiols in order to evaluate a potential oxidative stress condition in fish muscle (Fig. 2). According to the data, a significant ($p<0.05$) decrease in NPSH (Fig. 2a) was observed in S2 site while PSH (Fig. 2b) was not changed at all evaluated sites.

Fig. 2

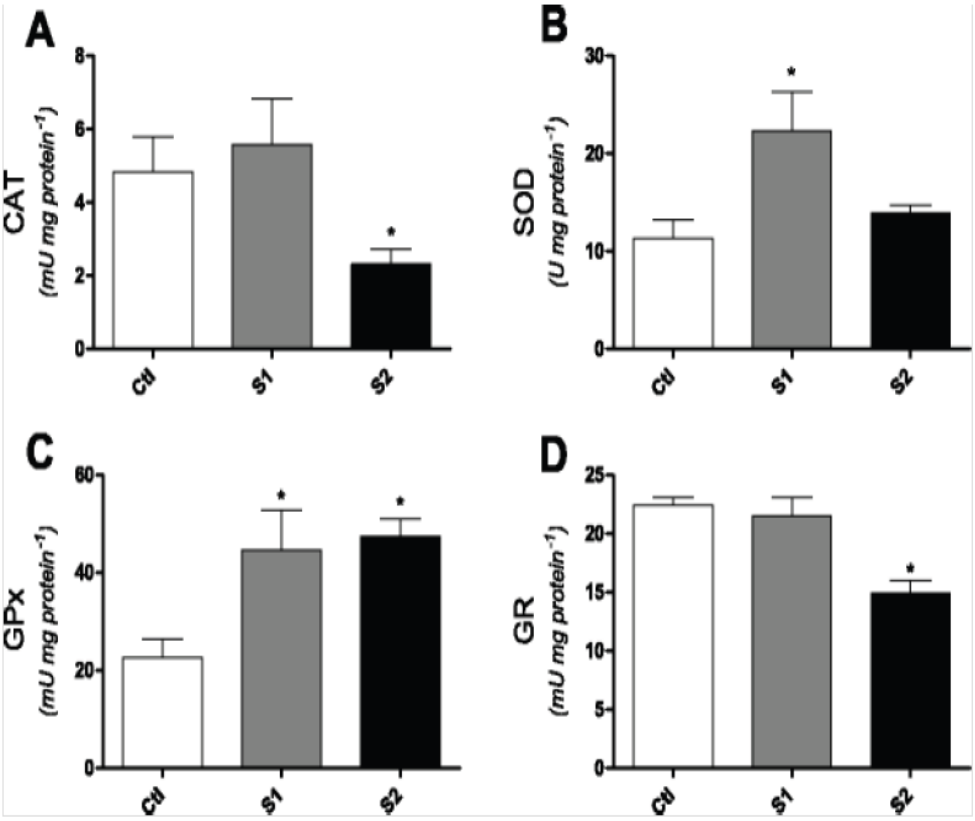
a Non-protein thiols and **b** protein thiols content in Lambari fish (*Astyanax sp.*) muscle collected in different Vacacaí River locations. Data are expressed as Mean±SD of tiol content (μmol/g of wet tissue). * $p<0.05$ when compared to reference site (control).



Regarding the activity of antioxidant enzymes, a significant ($p<0.05$) decrease of catalase (CAT) activity was observed at S2 (Fig. 3a), while superoxide dismutase (SOD) activity was enhanced ($p<0.05$) at S1 (Fig. 3b). The glutathione peroxidase (GPx) activity was significantly ($p<0.05$) increased in both S1 and S2 sites, when compared to reference site (Fig. 3c). The activity of glutathione reductase (GR) was significantly decreased ($p<0.05$) at S2 when compared to reference and S1 sites (Fig. 3d).

Fig. 3

Enzymatic activity of **a** CAT, **b** SOD, **c** GPx and **d** GR in muscle of Lambari fish (*Astyanax sp.*) captured in Vacacaí River sites. Data are expressed as Mean±SD of enzyme activity. CAT, GPx and GR activities are expressed as *mU mg protein*$^{-1}$. SOD activity is expressed as *U mg protein*$^{-1}$. * $p<0.05$ when compared to reference site (control).



It was also determined whether antioxidant responsive markers would present changes in the Lambari fish brains at the investigated sites at the Vacacaí River. As shown on Figure 4, the expression of total (Fig. 4a) and phosphorylated (Fig. 4b) forms of Nrf2 was substantially ($p<0.05$) up-regulated in the brain of animals collected in both S1 and S2 sites when compared to the reference site. A significant ($p<0.05$) increase in protein expression levels was also observed for NQO-1 (Fig. 4c), indicating an activation of the Nrf2 pathway in the S1 and S2 sites, when comparing to the reference site. The expression of glutathione peroxidase isoform 4 (GPx-4) and metallothionein 1 (MT-1) was also significantly ($p<0.05$) up-regulated in S1 and S2 (Fig. 5a,b respectively).

Fig. 4

Expression of **a** Nrf2, **b** pNrf2 and **c** NQO-1 in Lambari fish (*Astyanax sp.*) brains. Data are expressed as Mean±SD (percentage (%) of control). Densitometric analysis of immunoreactive bands was performed using Scion Image® software. Beta-actin was used as loading control. * $p<0.05$ when compared to reference site (control).

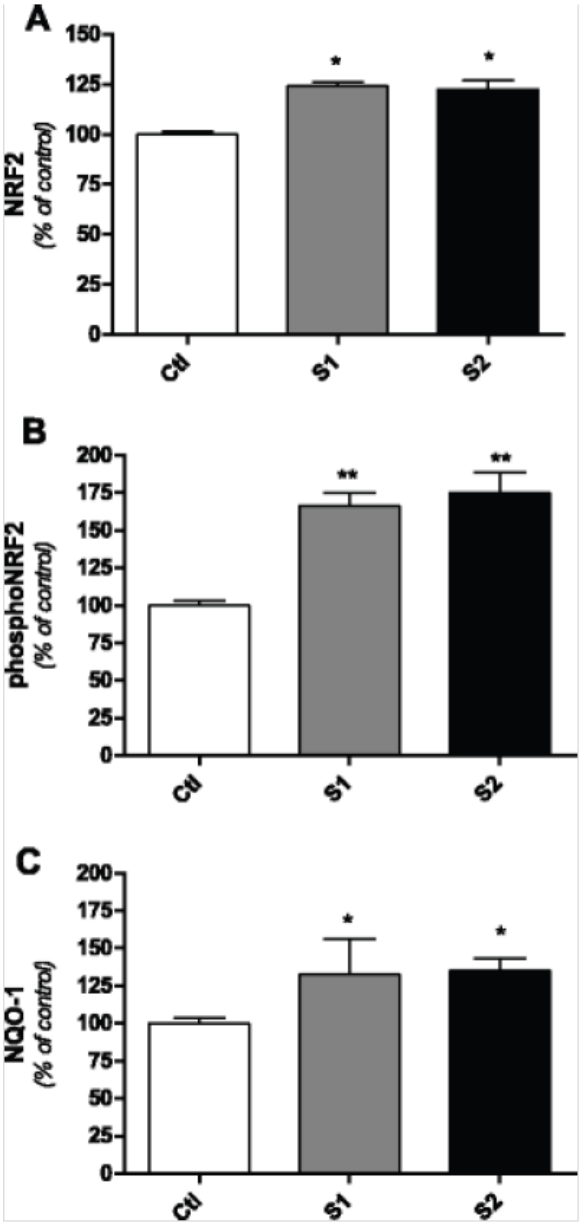
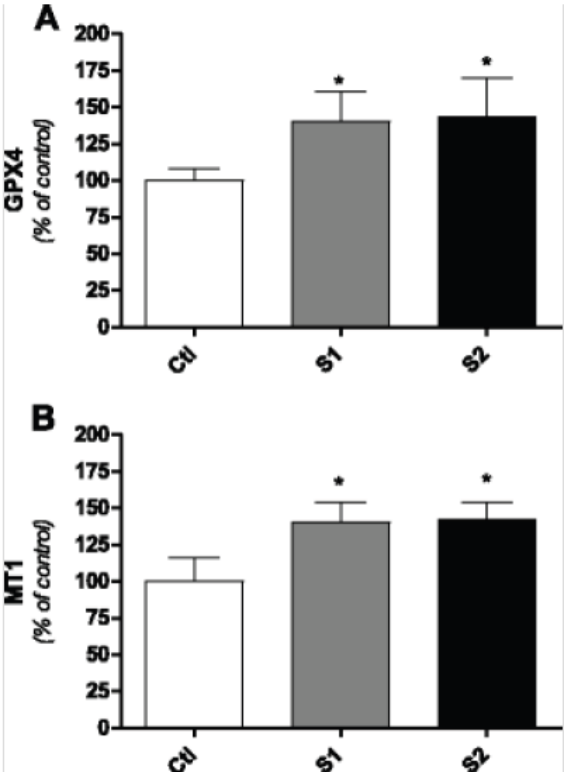


Fig. 5

Optical densitometry of immunoreactive bands of **a** GPx4 and **b** MT1 in lambari fish (*Astyanax sp.*) brain tissues. Data are expressed as Mean±SD (percentage (%) of control). Densitometric analysis of immunoreactive bands was performed using Scion Image® software. Beta-actin was used as loading control. * $p<0.05$ and ** $p<0.01$ when compared to reference site (control).



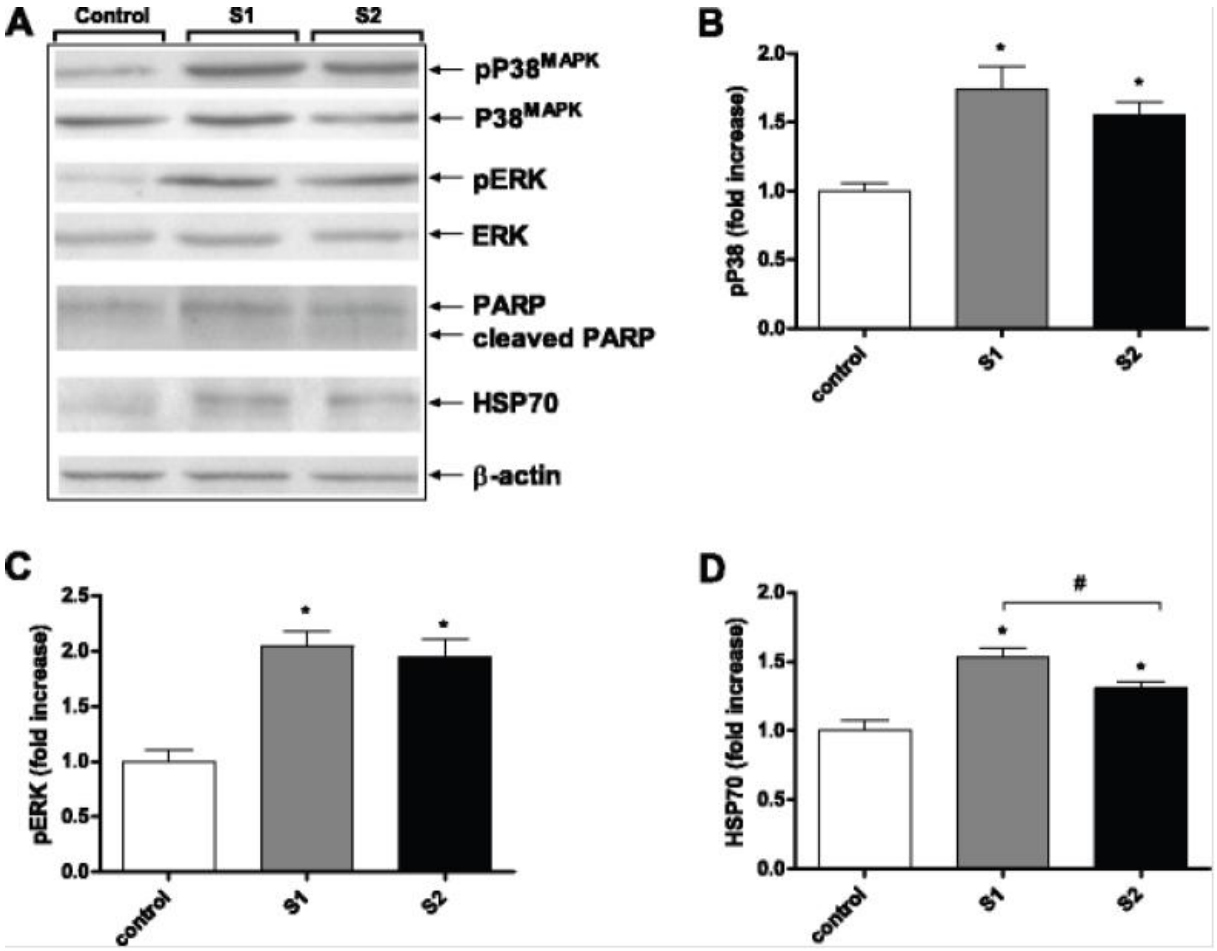
Danio rerio biomarkers

We also tested a protocol in which zebrafish, a widely used model for toxicity tests (Scholz et al., 2008), was exposed to the water from reference, S1 and S2 sites during 48h, in order to explore potential changes in stress-related proteins in zebrafish brains. As observed in Fig. 6, a significant ($p<0.05$) increase in p38 (Fig. 6a,c) and ERK (Fig. 6a,c) phosphorylation was evident after exposure to S1 and S2 waters when compared with the fish kept in the water from the reference site. It was also observed a significant increase in HSP70 levels in the brains of S1 and S2 exposed fish when compared to the reference site (Fig. 6d). At the analyzed time point (48h), it was not possible to observe significant changes in PARP cleavage (Fig. 6a).

Fig. 6

Analysis of stress responsive proteins in brain tissues of Zebrafish (*Danio rerio*) exposed to Vacacaí River water collected in S1 and S2 sites. After 48hs of fish exposure, samples were collected and processed for western blot evaluation of each protein target. **a**

Representative immunoblots for protein targets of zebrafish. **b** Optical densitometry of immunoreactive bands of pP38 **c** pERK and **d** HSP70. Results are expressed as arbitrary units (mean±SD). *p<0.05 compared to control.



Discussion

Based on the results presented here substantial alterations in biochemical markers of cell stress responses of fish exposed to Vacacaí River water, a major watercourse in the Brazilian Pampa, is demonstrated. At least in our knowledge, this is the first report on the assessment of environmental quality using toxicological biomarkers in fish within the Brazilian Pampa biome borders.

As mentioned before, the Brazilian Pampa is one of the six Brazilian biomes located at the so called “southern half” of the Rio Grande do Sul State (Overbeck 2007; Roesch et al. 2009). In the last years, some studies reported a vast biodiversity, of both animals and plants,

in this biome. However, it still remains neglected in terms of economical and environmental aspects. Regarding ecotoxicological risk assessment programs, there is a complete absence of studies. In the last decades, the remarkable expansion of agricultural activities in this region (Overbeck 2007; Roesch et al. 2009) has led to a dependence on conventional agricultural methods, which by definition, depends on the use of harmful chemical fertilizers and pesticides (Santos 2009). As a result, there is an urgent need for standardized surveys focusing on the assessment of toxicological impacts caused by urban and agricultural activities in this biome. In addition, the development of such surveys can be relevant for the management of the Pampa biome in countries other than Brazil, as Argentina and Uruguay.

Stress markers in Lambari fish

Acetylcholinesterase (AChE) is a classic biomarker for the presence of sublethal concentrations of organophosphorous compounds, as well as carbamates, which are widely used for pest control and can reach water streams through agricultural and urban releases (Viarengo et al. 2007). Several studies using fish species have shown inhibition of cholinesterase activity in the presence of organophosphate compounds in water (Sancho et al. 1997; Dutta and Arends 2003). In addition to organophosphate and carbamates, other agricultural compounds such as glysophate are shown to inhibit AChE in fish (Moraes et al. 2007). We found a significant decrease in the activity of this enzyme in muscle of fish captured at Vacacaí River, a fact that probably reflects the presence of cholinesterase inhibitors. This is in agreement with the extensive crop activities in the region such as soybeans and rice (Roesch et al. 2009). In line with this hypothesis, Table 2 shows the most utilized agrochemicals (in descending order) used in the Rio Grande do Sul state, according to Brazilian regulatory agencies (Barreto and Garibotti 2012). Among them, four compounds can potentially inhibit AChE activity as organophosphates (acephate and methamidophos), glycine analogs (glyphosate) and carbamates such as carbofuran, suggesting that one or more of such compounds can be inhibiting AChE in Lambari fish exposed to Vacacaí river water.

Table 2. Most common agrochemicals used in Rio Grande do Sul State, Brazil.

Agrochemical	*Chemical Group*
Glyphosate	Glycine analogue
Acephate	Organophosphate
Difenoconazole	Triazole
Methamidophos	Organophosphate
Metalaxyl	Phenylamide
Cypermethrin	Pyrethroid
Diflubenzuron	Benzamide
Carbofuran	Carbamate

Adapted from Barreto et al., 2012.

Under oxidative stress conditions, the cellular adaptive response is mediated by the transcription factor Nrf2 through the antioxidant response element (ARE). Once oxidative stress signals are generated, Nrf2 triggers the transcription of endogenous antioxidant enzymes as glutathione S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), NAD(P)H oxidase, NAD(P)H dehydrogenase (quinone) 1 (NQO-1), glutamate-cysteine ligase (GCL) and thioredoxin system through its binding to the ARE (Tanito 2007; S. Schulke 2012). We observed a significant increased Nfr2 and phosphoNrf2 in both S1 and S2 sites (Figure 4A and B). Moreover, we also observed a significant increase in NQO-1 at both sites. This activation of the Nrf2-ARE pathway coupled with increased expression of the NQO-1 evidences an oxidative stress condition, probably generated by exposure of fish to pollutants and agrochemicals.

The GST enzyme is widely used as a biomarker for environmental exposures to exogenous compounds, since its activity is related to detoxification of xenobiotic molecules (Stegeman and Lech 1991; Buchelli and Fent 1995). Lambari fish collected at S2 site, in close proximity with crops and urban area, showed a significant increase of GST activity (Fig. 1B). The antioxidant activity of several enzymes and the amount of thiol groups also can be used for monitoring the presence of pro-oxidant compounds in aquatic environments. The amount of thiols, both in proteins (PSH) or not (NPSH), is an indicative of the antioxidant capacity of the organism (Reischl et al. 2007). We observed a significant decrease in NPSH at the S2 site (Figure. 2A). Literature reports are in line with our findings. For instance, the exposure of the silver catfish to clomazone, one of the most used herbicides in the region, also reduced the

levels of NPSH (Crestani et al. 2007). NPSH depletion in S2 site may be related to the possible presence of herbicides/pesticides, highly used in crop production in the Pampa Biome, or heavy metals from wastewater discharges, since the S2 site is in close proximity of both crops and urban area.

Another marker known to be over expressed in organisms under heavy metal stress conditions are the metallothioneins (MT). Metal metabolism involves formation of GSH–metal complexes, subsequently transferring the metal to MT apoproteins (Atli et al. 2006). Our results showed an increase in the expression of MT1 in both S1 and S2 sites (Figure 5B) suggesting that lambari fishes are in contact with metal compounds. The presence of such metallic compounds also may be a result of the use of agrochemicals applied in the rice and soybean crops near to the Vacacaí River as well as from urban effluents from the São Gabriel municipality. An important component of metal environmental contamination came from organic and mineral fertilizers, soil correctives and pesticides used in this region (Amaral Sobrinho 1992). Another study also reported a watershed metal contamination by Cadmiun, Plumbum and Manganese provident from agrochemicals (Ramalho 2000). On the other hand, MT may also be up-regulated as an adaptive response to oxidative stress induced by a myriad of xenobiotics (Oliveira 2010; Viarengo 1999). Therefore, the presence of toxic levels of metal and its role in the observed MT response in fish should be further addressed.

CAT and GR activity showed a significant inhibition at the S2 site (Fig. 3A) suggesting that some pollutants present in the Vacacaí River waters may be blocking their activity. Accordingly, Bainy and colaborators 1996, showed an inhibition of the CAT activity in *Oreochromis niloticus* from polluted areas. SOD and GPx activities also acts as detoxifying enzymes removing free radicals and hence protecting macromolecules against damage (Ballesteros et al. 2009). Here, both enzyme activities were significantly increased when compared with the reference site (Fig. 3B, 3C). However, SOD activity only increased in the S1 site, while GPx increased in both S1 and S2 sites (Figure 3B and C). In agreement with our findings the activity of SOD increased in the liver carp exposed to Carbofuran under rice field and laboratory conditions (Clasen et al. 2011). Regarding different GPx isoforms, we could observe an increase in the expression of GPx4 in both S1 and S2 sites (Figure 5A), while GPx1 was unchanged (data not shown). This result indicates that GPx4 increased expression plays major role in the observed increase of total GPx activity in fish, demonstrating the potential use of this isoform as a responsive biomarker in biomonitoring studies.

Stress markers in Zebrafish

Zebrafish is a model for the investigation of several aspects of human diseases and represent a valuable model for environmental risk assessment of pollutants (Scholz et al. 2008). Here, we exposed adult zebrafish to the Vacacaí River water and after 48h, examined the expression of oxidative stress biomarkers. After exposure experimental animals presented a significant increase in pP38 MAPK, pERK and HSP70 in the evaluated sites in comparison with the fish exposed to the reference site. Mitogen-activated protein kinases (MAPKs) are a family of proteins that participate in transduction pathways affected by various environmental pollutants, including heavy metals and organic compounds (Peti and Page 2013). Usually, the extracellular signal-related kinase - ERK is activated by mitogenic stimuli and regulates mainly the growth and differentiation. The 38 kilodantons protein kinase - p38MAPK is activated by cellular stress and inflammatory cytokines, and is involved in the apoptotic process (Harper and LoGrasso 2001). These proteins regulate the transmission of signals from membrane to the nucleus and are well conserved from unicellular to more complexes organisms (Harper and LoGrasso 2001; Peti and Page 2013). It's well known that MAPK pathways are susceptible to oxidative stress. Despite the apparent antagonic actions of ERK and P38, both enzymes have been demonstrated to be modulated by several cell stressors (Chu et al. 2004).

Heat shock proteins (HSPs) are widely used biomarkers of environmental stress (Nadeau et al. 2001; Iwama et al. 2004). The main function of these chaperones is to support the proper folding of proteins, thus preventing damage under stress conditions. Disturbances in the cellular redox balance are shown to evoke HSP70 overexpression (Padmini 2010), a chaperone widely used in biomonitoring studies (Iwama et al. 2004).

According to our results, PARP cleavage, a marker of apoptotic cell death was not evident in zebrafish exposed to Vacacaí River water, however, the increased phosphorylation MAPK proteins and the up-regulation of the chaperone HSP70 are indicative of environmental stress, since all these three proteins are targets involved in stress response and antioxidant signaling (reviewed in Lushchak 2011), suggesting that the Vacacaí river water have pollutants and agrochemicals that could affect a broad range of fish species.

When considering biomarkers of pollution in fish species, liver and gills are the most utilized tissues reported in literature, even though recent studies have been using muscle and

brain tissues in biomarker analysis (Ghazala et al., 2014; Aich et al., 2015). Here, we have shown that both fish species studied showed visible alterations in stress related biomarkers in muscle and brain. These results reinforce the idea that muscle may be as responsive as other organs used for evaluation of biomarkers of aquatic pollution (as liver, gills or brain). The advantages of using muscle tissues from fish relies on its abundance and the possibility of collecting samples without the necessity of killing specimens, which may be of interest in terms of species conservation.

Conclusion

As far as we know, this is the first report evaluating the impact of wastewater and agrochemical pollutants on the naturally living and controlled laboratory fish in the Pampa biome, a threatened Brazilian biome. Our study highlights that role of highly used organophosphate agrochemical compounds on the blockage of the activity of acetylcholinesterase, expression of oxidative stress markers and toxicity in fish. As suggested by our results AChE, GST, CAT, SOD, GPx, GR activity and the expression of MT1, GPX4, p38, pERK and HSP70 are good markers for stress related biomonitoring studies. It also showed that both naturally captured Lambari fishes and laboratory breed zebrafish are complementary models for biomonitoring studies. Additional experiments are needed to clarify which compounds are responsible for such effects and evaluate their impact in other species.

Conflict of Interest

Authors declare no conflict of interest.

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Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary Figure 1

(A) Map of Brazil detailing Rio Grande do Sul state. (B) Map of Rio Grande do Sul state highlighting in green the Vacacaí River Basin. (C) Reference site, locate within riparian forest. (D) Site 1 – S1, located nearby intense agricultural activity. (E) Site 2 – S2, located nearby crops and urban localities (PPTX 2474 kb)

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7 CONSIDERAÇÕES FINAIS

As alterações bioquímicas entre os organismos usados neste estudo, como biomarcadores clássicos, acetilcolinesterase, atividade de enzimas de estresse oxidativo (catalase, glutationa peroxidase), enzimas de biotransformação de xenobióticos (glutationa S- transferase), alterações nos níveis de expressão de Nrf2 ,GPX4 e NQO-1,pERK, p38 e HSP70, reforçam a hipótese de que estes peixes se encontram em ambientes sob estresse oxidativo, como também uma possível exposição a compostos organofosforados, sugerindo a descarga destes compostos por atividades agrícolas inadequadas na região de estudo.

Com base nos dados apresentados neste trabalho, é possível observar uma correlação entre os resultados de testes de ecotoxicidade em laboratório na espécie *Danio rerio*, com o estudo em campo no gênero *Astyanax sp*, através da análise de respostas biológicas (biomarcadores) propostas neste estudo. Portanto, os resultados apresentados nesta investigação, permitem sugerir que estes organismos servem como potenciais bioindicadores da qualidade ambiental em regiões impactadas.

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