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EDUARDO STRINGINI SEVERO

ANÁLISE DE BIOMARCADORES EM JUNDIÁS (*Rhamdia quelen*) EXPOSTOS *IN SITU* COMO UMA FERRAMENTA DE AVALIAÇÃO AMBIENTAL

Santa Maria, RS
2017

Eduardo Stringini Severo

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Dissertação apresentada ao Curso de Pós-Graduação em Biodiversidade Animal, Área de Concentração em Bioecologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Biodiversidade Animal**.

Orientadora: Profª. Drª. Vania Lucia Loro

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Aprovado em 24 de março de 2017:

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RESUMO

ANÁLISE DE BIOMARCADORES EM JUNDIÁS (*Rhamdia quelen*) EXPOSTOS *IN SITU* COMO UMA FERRAMENTA DE AVALIAÇÃO AMBIENTAL

AUTOR: Eduardo Stringini Severo
ORIENTADORA: Vania Lucia Loro

Os ecossistemas aquáticos são afetados pelo uso de agroquímicos em áreas agrícolas próximas a rios. Para avaliar possíveis impactos, são necessários estudos com animais que habitam esses ecossistemas. A exposição *in situ* é uma das formas de avaliar possíveis danos em ecossistemas. O objetivo deste estudo foi investigar o uso da espécie *Rhamdia quelen* como bioindicador, através de sua exposição *in situ*. Grupo de 10 peixes foram transportados e colocados em gaiolas em três locais (S1, S2 e S3) do rio Vacacaí na cidade de São Gabriel, Rio Grande do Sul. Após o período de exposição de 96 horas, os peixes foram eutanizados e cérebro, brânquia, fígado e músculo foram removidos para ensaios bioquímicos. Foram analisados biomarcadores de danos oxidativos como substâncias reativas ao ácido tiobarbitúrico (TBARS) e carbonilação de proteínas (PC), biomarcador de neurotoxicidade como a enzima acetilcolinesterase (AChE) e parâmetros antioxidantes como glutationa s-transferase (GST), superóxido dismutase (SOD), catalase (CAT), capacidade total contra peróxidos (ACAP) e tióis não protéicos (NPSH). O cálculo do Índice Integrado de Respostas de Biomarcadores (IBR) foi utilizado para auxiliar na interpretação dos resultados. Houve uma diminuição da AChE no cérebro dos peixes expostos nos três locais estudados (S1, S2 e S3). Além disso, diminuiu os níveis de TBARS no cérebro e aumentou a PC no cérebro, fígado e brânquias em S2 e S3. A atividade da SOD diminuiu em todos os pontos nas brânquias e CAT aumentou em S3. O aumento de GST foi observado principalmente no fígado nos três pontos. ACAP aumentou em S3. O cálculo do IBR mostrou um valor maior em S2, seguido por S3 e S1. *Rhamdia quelen* mostrou ser um excelente bioindicador para ser usado em experimentos *in situ*. O uso dos biomarcadores com o auxílio do IBR, mostraram-se bastante úteis para o resultado do trabalho.

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Palavras-chave: Peixes. Estresse oxidativo. Ecotoxicologia, Biomonitoramento

ABSTRACT

ANALYSIS OF BIOMARKERS IN JUNDIÁS (*Rhamdia quelen*) EXPOSED *IN-SITU* AS AN ENVIRONMENTAL ASSESSMENT TOOL

AUTHOR: Eduardo Stringini Severo
ADVISOR: Vania Lucia Loro

Aquatic ecosystems are affected by the use of agrochemicals in agricultural areas near rivers. To evaluate possible impacts, studies with animals that inhabit these ecosystems are needed. *In situ* exposure is one way of assessing possible damage to ecosystems. The aim of this study was to investigate the use of *Rhamdia quelen* as a bioindicator through its *in situ* exposure. Group of 10 fishes were transported and placed in cages at three sites (S1, S2 and S3) of the Vacacaí river in the city of São Gabriel, Rio Grande do Sul. After the exposure period of 96 hours, fish were euthanized and brain, gill, liver and muscle were removed for biochemical assays. Biomarkers of oxidative damage as thiobarbituric acid reactive substances (TBARS) and protein carbonylation (PC), biomarker of neurotoxicity as the acetylcholinesterase enzyme (AChE) and antioxidant parameters such as glutathione s-transferase (GST), superoxide dismutase (SOD), catalase (CAT), antioxidant capacity against peroxyl radicals (ACAP) and non-protein thiols (NPSH) were evaluated. The calculation of the Integrated Biomark Response Index (IBR) was used to aid in the interpretation of the results. There was a decrease in AChE in brain of exposed fishes in the three studied sites (S1, S2 and S3). In addition, it decreased levels of TBARS in the brain and increased PC in the brain, liver and gills in S2 and S3. SOD activity decreased at all site in the gills and CAT increased at S3. The increase in GST was mainly observed in the liver at all three sites. ACAP increased in S3. The IBR calculation showed a higher value in S2, followed by S3 and S1. *Rhamdia quelen* has been shown to be an excellent bioindicator for use in *in situ* experiments. The use of biomarkers with the help of the IBR proved to be very useful for the results of the study.

Keywords: Fish. Oxidative stress. Ecotoxicology. Biomononitoring

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LISTA DE ABREVIATURAS E SIGLAS

INTRODUÇÃO

ACAP	Capacidade total contra peróxidos
AChE	Acetilcolinesterase
CAT	Catalase
EROS	Espécies reativas de oxigênio
GSH	Glutationa reduzida
GST	Glutationa S-transferase
HAPs	Hidrocarbonetos aromáticos policíclicos
IBR	Índice Integrado de Respostas de Biomarcadores
NPSH	Tióis não protéicos
PC	Carbonilação de proteínas
SOD	Superóxido dismutase
TBARS	Substâncias reativas ao ácido tiobarbitúrico

MANUSCRITO

ACAP	Antioxidant capacity against peroxyl radicals
AChE	Acetilcolinesterase
CAT	Catalase
DO	Dissolved oxygen
GST	Glutathione S-transferase
IBR	Integrated biomarker response
NPSH	Non-protein thiols
PC	Carbonylated protein
ROS	Reactive oxygen species
SOD	Superoxide dismutase
S1	Site 1
S2	Site 2
S3	Site 3
TBARS	Thiobarbituric acid reactive substances

SUMÁRIO

1 INTRODUÇÃO

1.1 EXPOSIÇÃO *IN SITU*

Os estudos de ecotoxicologia integram os conceitos da ecologia e da toxicologia. Essa ciência compõe a diversidade e importância dos organismos nos ecossistemas com os efeitos adversos dos poluentes sobre as comunidades biológicas (PLAA, 1982)

Testes ecotoxicológicos são frequentemente realizados através de experimentos controlados em laboratório, através de relações concentrações/resposta. Embora haja correlação muito próxima entre os dados obtidos em laboratório para avaliação de impacto ambiental, existem casos em que essa extrapolação não se aplica (ZAGATTO E BERTOLETTI, 2008).

A exposição *in situ* surgiu como uma alternativa para avaliação de locais potencialmente contaminados. Esta técnica possui algumas vantagens em relação a coleta de bioindicadores nos locais de interesse como o conhecimento preciso do local e a duração precisa da exposição. A migração de muitas espécies de peixes para alimentação e reprodução cria incerteza sobre o quão bem reflete a qualidade da água no local ou ao redor do local de captura (OIKARI, 2006).

Além disso, todo os animais utilizados na exposição *in situ* vem de um mesmo local, evitando assim grandes variedades de tamanho e peso. Diversas espécies de peixes já foram testadas para a utilização dessa metodologia como *Prochilodus lineatus, Cyprinus carpio, Oncorhynchus tshawytscha* (KELLEY et al., 2011; SCARCIA et al., 2012; VIERA et al., 2016). Moluscos e crustáceos também tem demonstrado serem bons organismos aquáticos para serem utilizados em estudos *in situ* (LACAZE et al., 2011; GILTRAP et al., 2013; LEBRUN et al., 2015; TURJA et al., 2015).

1.2 CONTAMINAÇÃO DE AMBIENTES AQUÁTICOS

Os agrotóxicos estão entre os principais instrumentos utilizados no crescimento da agricultura brasileira. Segundo o IBGE (2015), o uso de agrotóxicos saltou de 2,7 quilos por hectare para 6,9 quilos por hectare entre os anos de 2002 e

2012. Desses agrotóxicos utilizados em 2012, 64,1% foram considerados como perigosos e 27,7% muito perigosos.

Os processos envolvidos no destino ambiental dos agrotóxicos dependem de suas propriedades físico-químicas e forma de aplicação, características do solo e condições ambientais (Figura 1). Dentre os processos que favorecem a contaminação ambiental estão a lixiviação, escoamento superficial, sorção, degradação química e biológica e volatização (SPADOTTO et al., 2010; PETERS et al., 2013).

O meio aquático é afetado por dois tipos de fontes de contaminação, as chamadas fontes pontuais e as não pontuais ou difusas. As fontes pontuais são os esgotos domésticos lançados diretamente nos cursos d'água, provenientes dos grandes centros urbanos. Quando misturados com resíduos provenientes das grandes indústrias, acaba por se tornar muito mais perigoso e de difícil tratamento (MERTEN E MINELLA, 2002).

As fontes não pontuais ou difusas, tratam-se de fontes de contaminação que não são possíveis a identificação do ponto de lançamento do contaminante no ambiente aquático. Os tipos mais comuns são causados por fertilizantes (arraste de nutrientes) e pesticidas pelo escoamento superficial. Em grandes centros urbanos a contaminação por esgoto doméstico também pode ser definida como não pontual, isso ocorre em cidades sem planejamento na coleta de esgoto. Esse tipo de contaminação pode ocorrer pelo escoamento superficial das águas das chuvas (ZAGATTO E BERTOLETTI, 2008).

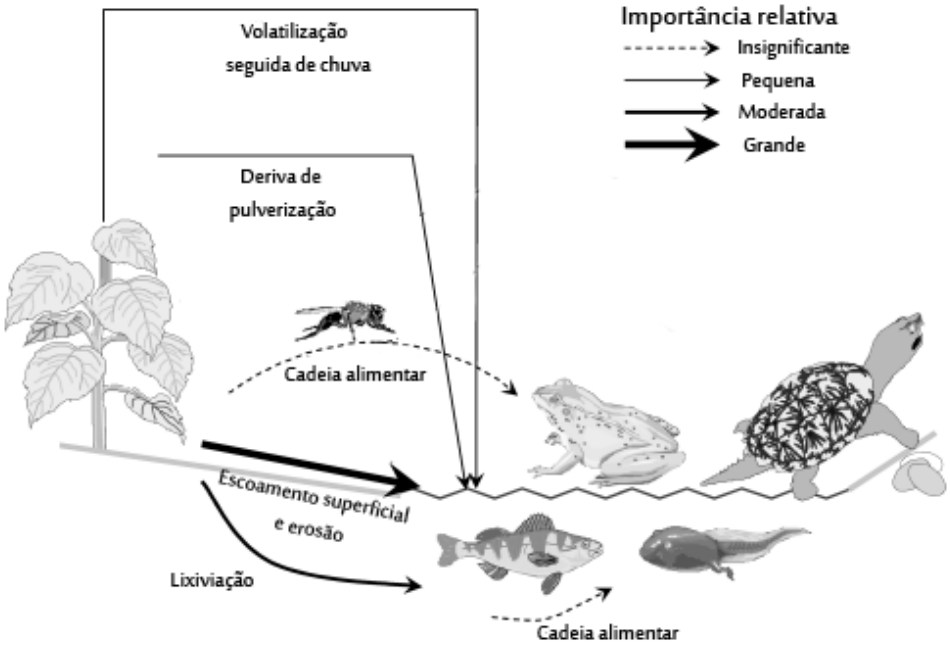

Figura 1 – Alguns processos que afetam o destino ambiental dos agrotóxicos, a largura da seta indica a importância relativa da rota de exposição.
Fonte: adaptado de SOLOMON et al., 2008, p.725.

1.3 BIOMARCADORES

O contato de organismos aquáticos a agentes tóxicos pode levar a mudanças bioquímicas, fisiológicas ou histológicas nestes organismos. Estas alterações podem ser avaliadas através do uso de biomarcadores que possuem o potencial de antecipar prejuízos em níveis de população, comunidade e ecossistemas.

Quando expostos a agentes tóxicos a produção de espécies reativas de oxigênio (EROS) pode ultrapassar a capacidade de defesa do organismo, caracterizando-se uma situação de estresse oxidativo (PISOSCHI, POP, 2015). Entre os biomarcadores utilizados para avaliar os possíveis danos causados nos organismos destaca-seos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), que indica danos em lipídios pela peroxidação lipídica. A lipoperoxidação pode ser definida como uma cascata de eventos bioquímicos resultante da ação de radicais livres sobre os lipídeos insaturados das membranas celulares, em casos mais extremos pode levar à morte celular (BENZIE, 1996).

Danos em proteínas podem ser avaliados através de proteínas carbonil (PC) formadas. A oxidação de proteínas pode ser reversível ou irreversível, dependendo

do alvo e da forma de dano oxidativo. Todos os resíduos de aminoácidos podem ser oxidados por EROS, podendo também sofrer agregação e fragmentação, formando grupos carbonilas (VALKO et al., 2006; TRACHOOTHAM et al., 2008).

É possível avaliar danos neurológicos através da atividade da enzima acetilcolinesterase (AChE). Alterações comportamentais podem estar relacionada com a inibição da enzima acetilcolinesterase (AChE), cuja ação é crucial na propagação do impulso nervoso e também na transmissão nervo-músculo. A AChE inativa a ação do neurotransmissor acetilcolina (AChE) hidrolisando-o em acetato e colina. Este processo de hidrólise evita a propagação contínua do impulso nervoso, evitando hiperatividade, asfixia e morte (ROEX, 2003).

São exemplos de marcadores de defesa a atividade de enzimas de biotransformação (GST), defesa antioxidante enzimática (SOD, CAT e ACAP) e não-enzimáticas (NPSH). A GST pertence a uma família de enzimas de fase II que conjugam compostos eletrofílicos (hidrocarbonetos aromáticos policíclicos - HAPs) com glutationa (GSH) e participam na proteção celular contra os efeitos tóxicos de uma variedade de xenobióticos e subprodutos metabólicos oxidados (HAYES et al., 2005; TONI et al., 2011; PEREIRA et al., 2013).

A enzima antioxidantes superóxido dismutase (SOD), catalisa a dismutação de $O_2^{\bullet -}$ em H_2O_2 enquanto que a enzima catalase (CAT), é responsável por catalisar a redução do H_2O_2 a H_2O e O_2. O principal tiol não protéico é a GSH, a sua função antioxidante é mediada pelo grupo tiol (-SH) reativo da cisteína, que confere a capacidade redutora da GSH.

A capacidade antioxidante contra peróxidos (ACAP) determina a capacidade antioxidante total, como uma alternativa para reduzir o número de análises de antioxidantes (AMADO et al., 2009).

1.4 ÍNDICE INTEGRADO DE RESPOSTAS DE BIOMARCADORES

Embora os biomarcadores possam fornecer informações valiosas e possam ser usados para medir uma ampla gama de respostas fisiológicas a agente tóxicos, seu uso é limitado se estes não puderem ser integrados em um esquema geral que facilite a análise e interpretação de dados. O “índice integrado de respostas de biomarcadores” (IBR) descrito por Beliaeff e Burgeot (2002) é uma metodologia que integra as respostas de diferentes biomarcadores em um único valor ou gráfico e

vem sendo utilizada em estudos de campo e laboratótio (MURUSSI et al., 2015; VIEIRA et al., 2016). O resultado de cada análise é primeiramente dívidido pelo valor do grupo controle (o grupo controle é dívidido por ele mesmo resultando em valor igual a 1) em seguida, cada resultado é logaritmizado para diminuir a variância. Do resultado da logaritimização (Y_i) é realizado a média (μ) e o desvio padrão (s) de todos os resultados. Então os valores de Y_i são padronizados pela seguinte fórmula — $Zi = (Yi - \mu) / s$ — e a diferença entre Z_i e Z_0 (controle) é calculada para determinar os valores de A. Os valores de A representam o resultado atribuído para cada biomarcador calculado. O IBR é estimado para cada grupo pela soma dos valores de A convertidos em valores absolutos (S). Os resultados são apresentados como gráficos de radar que indica o desvio de todos os biomarcadores em relação ao controle (0). Os valores acima da linha do zero representam a indução dos biomarcadores, enquanto os valores abaixo de zero representam inibição dos biomarcadores.

	A	B	C	D	E	F	G
1	GST Fígado						
2				Y_i	Z	A	S
3		Resultados					
4	Controle	10	B4/B4	LOG10(C4)	D4-D9/D10	E3-E4	ABS(F4)
5	Grupo 1	5	B5/B4	LOG10(C5)	D5-D9/D10	E5-E4	ABS(F5)
6	Grupo 2	15	B6/B4	LOG10(C6)	D6-D9/D10	E6-E4	ABS(F6)
7	Grupo 3	3	B7/B4	LOG10(C7)	D7-D9/D10	E7-E4	ABS(F7)
8							
9			μ	MÉDIA(D4:D7)			
10			s	DESVPAD(D4:D7)			

Figura 2 – O cálculo do IBR foi realizado no programa Microsoft Excel 2010.

	A	B	C	D	E	F	G
1	GST Fígado						
2				Y_i	Z	A	S
3		Resultados					
4	Controle	10	1	0	0.520799	0	0
5	Grupo 1	5	0.5	-0.301029996	0.219769	-0.30103	0.30103
6	Grupo 2	15	1.5	0.176091259	0.69689	0.17609	0.17609
7	Grupo 3	3	0.3	-0.522878745	-0.00208	-0.52288	0.52288
8							
9			μ	-0.16195437			
10			s	0.310972935			

Figura 3 – O resultado A de cada grupo foi utilizado para a geração do gráfico em radar. O valor de S de todos os biomarcadores de cada grupo foram somados e colocados acima do gráfico.

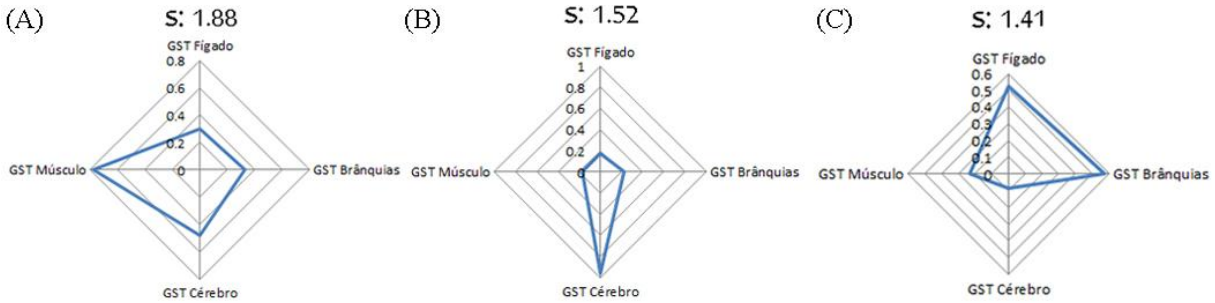

Figura 4 – A comparação entre o grupo 1 (A), grupo 2 (B) e grupo 3 (C) neste exemplo hipotético demonstra que o grupo 1 foi o que mais sofreu alteração nos biomarcadores estudados.

1.5 ORGANISMO TESTE

Rhamdia quelen (QUOY & GAIMARD, 1824), popularmente conhecido como jundiá, é um peixe teleósteo pertencente à família Heptapteridae (BOCKMANN, GUAZZELLI, 2003). Sua coloração varia de marrom-avermelhado claro a cinza ardósia, pode ser diferenciado de outras espécies pertencentes ao mesmo gênero através de algumas características como: espinho da nadadeira peitoral serrilhado em ambos os lados, barbilhões maxilares no mínimo 28,8% do comprimento padrão (SILFVERGRIP, 1996) (Figura 2). Os adultos são onívoros, possuem preferência por determinados alimentos de acordo com a sua abundância. Peixe nativo da região Sul do Brasil, vive em lagos e rios, com preferência a águas calmas com fundo de

areia e lama junto as margens e vegetações. Possuem hábito noturno, procurando alimentos principalmente após períodos de chuva. Estudos mostram que são generalistas em relação a escolha de alimento (GOMES et al., 2000; MEURER & ZANIBONI FILHO, 1997).

Figura 5 – Exemplar de jundiá, *Rhamdia quelen*.
Fonte: arquivo pessoal.

A maturidade sexual é atingida por volta de um ano de idade nos dois sexos. Os machos iniciam o processo de maturação gonadal com 13,4 cm, e as fêmeas com 16,5 cm. A partir de 16,5 cm e 17,5 cm todos os exemplares machos e fêmeas, respectivamente estão potencialmente aptos para reprodução (NARAHARA et al., 1985). Os alevinos de *Rhamdia* quelen possuem uma tolerância a mudanças de salinidade suportando até 9,0 g/L de sal comum (NaCl) por 96h. Alguns estudos relatam que esta espécie suporta variações de pH (4,0 a 9,5) além de suportarem temperaturas de 15 a 34ºC (MARCHIORO et al., 1999; CHIPPARI GOMES et al., 2000).

Os jundiás são amplamente utilizados em estudos de avaliação toxicológica de diversos compostos. Quando expostos a compostos químicos em condições laboratoriais, os jundiás apresentaram mudanças em seu perfil antioxidante, parâmetros de estresse oxidativo, parâmetros metabólicos e alterações histológicas. Devido a estas características, o jundiá é considerado um bom organismo

bioindicador (GLUSCZAK et al., 2007; MENEZES et al., 2011; MURUSSI et al., 2015).

Segundo Akaishi (2004) algumas características devem ser levadas em consideração ao escolher um determinado organismo bioindicador, como sobreviver em ambientes saudáveis e possuir resistência a ambientes contaminados, assim como abundância da espécie escolhida no ambiente e facilidade em adaptar-se aos ensaios laboratoriais.

1.6 OBJETIVOS

1.6.1 Objetivo Geral

Investigar o potencial uso de *Rhamdia quelen* exposto *in situ* como organismo bioindicador de ambientes contaminados.

1.6.2 Objetivos Específicos

- Avaliar a presença de agroquímicos em água e sedimento do rio Vacacaí, no município de São Gabriel.
- Verificar neurotoxicidade através de atividade da enzima acetilcolinesterase.
- Avaliar danos oxidativos através dos biomarcadores TBARS e carbonilação de proteínas.
- Determinar o perfil antioxidante enzimático e não enzimático em diferentes tecidos de jundiás, medindo-se as atividades de CAT e SOD, níveis de NPSH e capacidade antioxidante contra peróxidos (ACAP).
- Analisar a atividade da enzima de detoxificação GST.
- Avaliar a eficácia da abordagem do Índice Integrado de Resposta de Biomarcadores (IBR) em três localidades do rio Vacacaí.

2 MANUSCRITO:

BIOMARKERS RESPONSES IN *RHAMDIA QUELEN* EXPOSED *IN SITU* ON A BRAZILIAN RIVER LOCATED NEAR AGRICULTURAL AREAS

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PERIÓDICO: ENVIRONMENTAL TOXICOLOGY

ABSTRACT:

Aquatic environments are impacted by the use of agrochemicals in agricultural areas near rivers. To evaluate its impact on wildlife, *in situ* exposure has become an alternative to evaluate these environments. In this study the use of the *Rhamdia quelen* as a bioindicator was investigated through in situ exposition. Were placed 10 fishes per cage in each site of a total of three sites in Vacacaí river. After 96-hours of exposure, the fishes were euthanized and brain, gill, liver and muscle were removed for biochemical assay. Biomarkers of oxidative damage (thiobarbituric acid-reactive substance - TBARS, carbonylated protein - CP), neurotoxicity (acetylcholinesterase - AChE) and antioxidant parameters (glutathione S-transferase - GST, superoxide dismutase - SOD, catalase - CAT, antioxidant capacity against peroxyl radicals - ACAP and non-protein thiols - NPSH) were analyzed. The IBR calculation was used to aid in the interpretation of the results. Biomarker variations were observed at the three sites of *in situ* exposure. Decrease in AChE, TBARS, PC and SOD mainly in the most contaminated sites. In addition, CAT, GST and ACAP biomarkers presented increase in the results. IBR showed a higher value at the points of exposure considered to be the most contaminated. The *Rhamdia quelen* showed to be an excellent bioindicator to be used for in situ experiments. All biomarkers also contributed to the result of this work.

KEYWORDS: fish, bioindicator, oxidative stress, ecotoxicology

1 INTRODUCTION:

The aquatic ecosystem is considered the most susceptible to pollution and contamination. Commonly associated with the discharge of domestic, industrial or agricultural effluents [1]. The presence of pollutants in these environments can affect the health and survival of fishes and other aquatic animals. According to the data from IBGE [2], the use of chemicals in Brazilian agriculture more than doubled, in a period of 10 years, jumping from 2.7 to 6.9 kilograms per hectare. Quantitative chemical analyzes are used to evaluate the amount of these pollutants in the environment, but they are not able to demonstrate the possible effect on aquatic

fauna. In this way, biomonitoring has been proven to be a necessary way to complement traditional monitoring techniques [3].

There are several ways to perform biomonitoring studies, and one of the most usual ways is to collect animals directly at the places of interest [4,5]. Nevertheless, according to Oikari et al. [6], the migration of many species of fish for feeding and breeding creates uncertainty about how well an individual's tissue sample reflects the quality of the water at or around the site of capture.

An individual could recently have entered in a more or less contaminated area and therefore exhibit accumulated contaminants from earlier site. Thereby, an alternative for biomonitoring would be the use of *in situ* exposure. This method has been used because of its efficiency in demonstrating the effects of the mixture of contaminants on the environment [7,8]. The *in situ* exposure also offers numerous advantages such as the precise site knowledge and species selection at the development stage of interest. In addition, the use of animals from the same source also reduces inter-individual variability of exposed organisms, and minimizes the influence of adaptive mechanisms [6,9].

The fish species chosen for this study was used in previous toxicology studies [10–12]. The silver catfish (*Rhamdia quelen*) belongs to the family Heptapteridae and it is a native species from the southern region of Brazil, although the genus *Rhamdia* has a wide distribution in the South America continent. The silver catfish has an important role in the environment since its diet is based on animals belonging from diverse levels of the food chain, such as insects, mollusks and even other fish. Moreover, has a commercial relevance due to its high quality meat [13,14].

To check possible changes in the animals after exposure, it is necessary to evaluate biomarkers. Biomarkers are defined as biochemical, physiological or histological changes caused by contact with xenobiotics [15,16]. Exposure to different pollutants may cause oxidative damage resulting from the generation of free radicals and increased production of reactive oxygen species (ROS). Hence, the increase of ROS can cause changes in the antioxidant profile [17,18].

The place chosen for this study was the Vacacaí river at city of São Gabriel, that has a population of approximately 62,874 inhabitants [19]. The Vacacaí river belongs to the Vacacaí-Vacacaí Mirim basin, which is an important hydrographic basin located in the Center-West region of the Rio Grande do Sul State, providing drinking water for approximately 385,000 inhabitant [20]. A study conducted by Marchezan et

al. [21] showed that contamination of agrochemicals occurs in the Vacacaí River mainly due to rice cultivation. Although this study was carried out in another municipality, rice is one of the main sources of economy of the municipality of São Gabriel [22].

The aim of this study was test biomarkers in *Rhamdia quelen* exposed *in situ* and to evaluate those biomarkers during the 96-hour period, making possible the use of this species in similar studies in other environments with potential interest for research.

2 MATERIAL AND METHODS:

2.1. STUDY SITES

The sites chosen in this study are located in the Vacacaí river, located in rural areas and in an urbanized region of the municipality of São Gabriel. The first site (S1) was chosen because of this distance from the urban and rural area, in a region with a large riparian forest (30°27'14.7"S 54°22'25.5"W). The second site (S2) is located at a distance of approximately 10 km from the first point, this location is close to rice and soybean crops (30°22'40.7"S 54°20'54.8"W). The third site (S3) is an urbanized region (30°20'30.5"S 54°18'19.9"W) (Fig 1).

2.2. ANIMALS

Juvenile silver catifsh of both sexes (mean weight 22.3 ± 7.05 g, body length 13.55 ± 0.98 cm) were obtained from a fish farm (Rio Grande do Sul, Brazil) and were acclimatized at the aquatic toxicology laboratory at Universidade Federal de Santa Maria (UFSM).

The animals were acclimated for ten days to laboratory conditions, with natural photoperiod (14h light/10h dark). They were maintained in static system in 250 L boxes of fiberglass, with continuously aerated tap water and physical and biological filters. Water quality parameters were verified daily (temperature 24.05 ± 0.96°C, pH 7.15 ± 0.17, dissolved oxygen 7.90 ± 0.74 mg/L, non-ionized ammonia 0.15 ± 0.04 µg/mL, nitrite 1.11 ± 0.78 mg/mL). The fish were fed twice a day with commercial feed to satiety during the acclimation period. Feces and pellet residues were removed by suction. This study was authorized by the responsible environmental entity (ICMBio, protocol number: 52452-1).

2.3. EXPERIMENTAL DESIGN

A total of 50 silver catfish were transported in oxygenated plastic bags to the municipality of São Gabriel. Eugenol was used as anesthesia according to Cunha et al. [23]. After transport, 10 animals were anesthetized using eugenol solution (concentration of 50 µL/L) and then euthanized by spinal cord section. After dissection, brain, liver, gills and muscle were removed and stored at -80°C for later analysis. We named this group as “transport group” in order to evaluate changes in the biomarkers associated with animal transportation.

Another group of 10 silver catfish was kept in an aquarium (32 x 32 x 48 cm, 49 L) at Universidade Federal do Pampa campus São Gabriel, located at the city of São Gabriel – Rio Grande do Sul, with conditions suitable for survival over the 96 hours period (we named this group as “control group”). The other fish were transported to the site mentioned in item 2.1 and transferred to cages (32 x 32 x 48 cm, 49 L), where they remained for 96 h. The cages were completely submerged (depth ≤ 1 m), and their design allows full water circulation following the methodology used by Vieira et al. [8]. It were placed 10 fishes per cage and each group of fish received the name of the place where they were exposed (S1, S2 and S3).

At the end of 96 h of in situ exposure all fishes were transported to the Universidade Federal do Pampa, where they were anesthetized using eugenol solution (concentration of 50 µL/L) and euthanized by spinal cord section. After dissection, brain, liver, gills and muscle were removed and stored at -80°C for later analysis. We did the same with “laboratory control” after the end of the 96 h.

2.4. ANALYSIS OF WATER AND SEDIMENT

The samples of water and sediment were collected at the same sites mentioned in item 2.1, in the first and the last day of *in situ* exposure. We performed water and sediment collections in the months of november and december before *in situ* exposure which was held in late january and early february. The water was collected in 500 mL amber bottle and the sediment was collect in a sediment collector pot, both were placed immediately on ice. The water and sediment samples were evaluated qualitatively and quantitatively for agrochemicals, according to the methodology described by Donato et al. [24].

With the aid of multiparameter water quality equipment (Horiba) the following parameters were determined: temperature, pH, dissolved oxygen. Were also measured nitrite and ammonia in water sample.

2.5. BIOCHEMICAL BIOMARKERS

2.5.1. Tissue preparation

Samples of brain, liver, gills and muscle (1:20) were homogenized with Tris-HCl 50 mM buffer (pH 7.4) in a Potter-Elvejhem glass/Teflon homogenizer, centrifuged (3000 g for 10 min, - 4º C) and the supernatant was transferred to microtubes and kept at -80 ºC for posteriors assays.

2.5.2. Biomarkers of oxidative damage

Lipid peroxidation and carbonylated protein were determined in brain, liver, gills and muscle. Lipid peroxidation was estimated by thiobarbituric acid-reactive substance (TBARS) production, which is widely performed for measurement of lipid redox state [25]. Results were expressed as nmol MDA/mg of protein. Further details of the methodology can be found in Nunes et al. [26].

The levels of carbonylated protein (PC) were assayed based on the method described by Yan et al [27], adapted to 96 wells microplate reader. Further details of the methodology can be found in Müller et al. [28].

2.5.3. Antioxidant parameters

Catalase (CAT) and superoxide dismutase (SOD) activities were determined in liver and gills. CAT was expressed as µmol/min/mg protein, according to Nelson and Kiesow [29]. The SOD activity was measured in accordance with Misra and Fridovich [30] and it was expressed as U/mg protein. Further details of those methodologies can be found in Murussi et al. [10].

Glutathione S-transferase (GST) activity was analyzed according to Habig et al. [31] in brain, liver, gills and muscle. The activity was expressed as µmol GSDNB/ min/mg protein. Further details of the methodology can be found in Nunes et al. [26]

Non-protein thiols levels (NPSH) were determined in brain, liver, gills and muscle and were assayed based on the method described by Ellman [32] in a

microplate reader. Results were expressed as nmol SH/ mg protein. Further details of the methodology can be found in Nunes et al. [26].

The antioxidant capacity against peroxyl radicals (ACAP) determines total antioxidant capacity as an alternative to reduce the number of antioxidant analyzes.The peroxide radicals are generated by thermal decomposition at 35°C of 2,2'-azobis (2 methylpropionamidine) dihydrochloride (ABAP, 4 mM) following the procedures described by Amado et al. [33]

2.5.4. Biomarker of neurotoxicity

The AChE activity was measured by the rate of hydrolysis of acetylthiocholine iodide (0.88 mM) was determined in a final volume of 300 µL, with 33 µL of homogenized sample and 33 µL of 100 mM phosphate buffer, pH 7.5 mixed to 2.0 mM 5,5'-dithionitrobis 2-nitrobenzoic acid (DTNB). The hydrolysis of acetylthiocholine iodide was monitored in a microplate reader by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) [32]. Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. AChE activity was expressed as µmol thiocholine (SCh)/h/mg of protein.

2.5.5. Protein determination

Protein was determined by the Coomassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm as described by Bradford [34].

2.6. IBR

The results obtained in this study were applied to the "Integrated biomarker response" (IBR) to characterize the effects in each site that silver catfish was exposed *in situ*. Representative results are shown as star plot charts indicating the deviation of all biomarkers in relation to the control, the IBR was estimated for each site resulting in S values. The area above 0 reflects biomarker induction, and the area below 0 indicates a biomarker inhibition. IBR is based in a method described by Beliaeff and Burgeot [35] and modified by Sanchez et al. [36]. Further details of these methodology can be found in Vieira et al. [8].

2.7. STATISTICAL ANALYSIS

The results are presented as means ± standard error of mean (S.E.M.). The data were homogeneous, proven by the Shapiro-Wilk test. Comparisons between groups were by one-way ANOVA followed by Newman-Keuls multiple comparisons test. The significant level considered was $P < 0.05$. Analyzes were performed with the software GraphPad Prism version 6.01.

3 RESULTS:

The agrochemicals of the water and sediment from the *in situ* exposures and collections made in previous months are given in Table 1. The herbicide clomazone was found at all sites and the fungicide tebuconazole at S2. Physical and chemical parameters of the water from the *in situ* exposure and previous water collection are given in Table 2. The results of the transport group did not show significant differences in relation to the control group and the animals from the *in situ* exposure, so we did not put their data in the tables and graphs. The use of eugenol to transport silver catfish demonstrated to be useful, since it was not responsible for the changes of biomarkers.

The TBARS levels in brain decreased in S2 and S3 in relation to the control group, S3 show decrease in relation to S2. In muscle there was decrease in the TBARS levels in S1 and S2 and in S3 was an increased in relation to S1. An increase in liver was observed only in S1 in relation to all others groups. In gills there was no significant change in the TBARS levels between the sites and control group (Fig. 2)

The PC levels in brain increased in S2 and S3 in relation to S1 and control group. In liver it was observed that PC level increased in S3, but S1 and S2 shows no significant difference in relations to control group and S3. In gills it was observed an increase only in S3 in relation to all groups and in muscle there was no significant change between the sites and control group (Fig.3)

The results of GST show a decrease of this activity in brain in S1. GST increase in muscle in S3 in relation to control group and S1, S2 do not show difference in relation to the other groups. In liver it is observed an increase in S1 and

S2 in relation to the control group and in S3 in relation to all groups. In gills, an increase occurred only in S2 in comparison to other groups (Table 3).

NPSH results show no variation in liver and gills. In muscle there was an increase in S1 and S3 in relation to control group, but not in relation to S2. In brain was observed an increase in S2 in comparison to control group (Table 3).

CAT activity in liver increased in S1 in relation to the control group and S2, S3 increased only in relation to control group, but do not show significant differences in relation to S1 and S2. In gills it was an increase in S3 in relation to S2, and both control group and S1 do not show significant differences in relation to S2 and S3 (Table 4).

In SOD, there were no significant differences in liver. In gills was observed a decrease in SOD activity at the three sites studied in comparison to control group (Table 4).

There were no significant differences in ROS production ACAP increased at S3 in relation to S1 and S2, those result were no significant in relation to control group (Table 4).

AChE activity in the brain decreased at all sites studied, in comparison to control group. In muscle, AChE activity increased in S2 in relation to control group and S1 (Fig. 4).

Values of IBR for silver catfish at S1, S2 and S3 was 2.10, 3.12 and 3.06 respectively. The star plots indicate that CAT, GST and PC were the most representative biomarker in S1, showing an increase especially in liver tissue, together with a reduction in CAT and AChE in gills and brain respectively. In S2 and S3, GST, CAT, NPSH and PC levels were the most representative biomarker as they showed greater variations in comparison to the basal group, with a reduction in SOD at S2 and S3 in gills tissue, and AChE and TBARS in S3 in brain tissue (Fig. 5)

4 DISCUSSION:

When we chose the sites of this work, we decided that S1 would be the place used as a reference, since it presents ciliary forest and is far from crops, however, as seen in the last water collection (*in situ* exposure), it was found equal clomazone concentrations of S2 and S3 water samples. However, previous collections (november and december) show the increase in clomazone concentrations found.

The water quality analyzes did not show any changes above that allowed in Brazilian waters [37]. In addition, these concentrations are within the stipulated limits for the creation of this species under optimum conditions. According to Chiparri-Gomes et al. [38] Lethal temperatures for Rhamdia quelen are below 3ºC and above 32ºC. According to Braun et al. [39] Dissolved oxygen (OD) below 1.6mg / L occurs mortality. The ideal pH range for juveniles is between 4.0 and 9.0 according to Zaions et al. [40] And the levels of ammonia and nitrite are below the concentrations considered dangerous of 0.44mg / L and 1.2mg / L respectively [41,42].

Some results found in this study are in agreement with previous study performed by our research group. Coincidentally our group performed laboratory studies with clomazone and tebuconazole, the same agrochemicals found in water samples collected during the *in situ* exposure period and still using *Rhamdia quelen* as model organism [43–46].

The results obtained in this study show, in general, that *Rhamdia quelen* presented different results according to the analyzed organ. Lipid peroxidation is a common response observed after herbicide exposure, and is usually estimated by TBARS. However, TBARS levels in brain and muscle showed a decrease, thus indicating that lipid peroxidation did not occur in this organ probably due to the action of antioxidant system. This same result was observed in Murussi et al. [45] in liver tissue, who exposed silver catfish to clomazone in two different concentration.

Another parameter used to verify damage caused by agrochemicals is the estimation of protein carbonyl caused by oxidative damage. Protein carbonyl increased in almost all tissue and sites that silver catfish were exposed. The hypothesis for this result would be the inefficient protection of the antioxidant system. In our study, this explanation makes sense if we consider that GST increased in much the same way as carbonyl protein levels in the liver in S3 and brain in S2 and S3.

GST is a phase II enzyme that conjugates xenobiotics or their metabolites with glutathione, making them less toxic and more easily able to be excreted. This biomarker have been used in many biomonitoring studies [5,47]. Our study presented a very satisfactory result in relation to this enzyme. It is observed the increase of its activity in relation to the sites with the highest number of agrochemicals found in water and sediment collections, mainly in S2 and S3. Although the increase in its

activity is notorious, it is probably not enough to prevent oxidative damage as show in PC.

As seen in Vieira et al. [8] the non-increase in NPSH might have impaired the antioxidant capacity of the organisms, leading to oxidative damage. This may been responsible for the oxidative damage caused in PC in liver and gills at S3.

The SOD and CAT enzymes are an important group to protect organism against ROS. SOD convert superoxide anions O_2^- into H_2O_2 and CAT subsequently into H2O and O2. In our results, we observe a decrease in SOD at gills tissue in all sites. A similar result was observed in Xing et al. [48] exposing common carp to different agrochemicals, indicating that the decrease of this enzyme contributes to the elimination of ROS from the cell, after a period of recovery the increase of the activity of this enzyme was observed. According to Doyotte [49], depending on the intensity and duration of chemical stress, it may increase or inhibit the activity of antioxidant enzymes and may reflect an adaptation of the organism to the presence of xenobiotic. CAT increased is usually related as a mechanism against oxidative damage caused by chemical stress induction and inhibition were observed after the exposure of fish to environmental pollution [47,50].

ACAP is a fast and general method to determine the total antioxidant capacity against ROS. In our results it was possible to observe that ACAP increased at S3, this result indicates that at this site the fish antioxidant system was that most worked to reduce ROS. Although this biomarker is generalist, this result was in agreement with the results of CAT at S3 in liver and gills. A similar result was found by Beauvais et al. [51].

Inhibiton of AChE can result in excessive stimulation of cholinergic nerves, resulting in behavioral alterations such as tremors, convulsions, and erratic or lethargic swimming [52,53]. AChE is usually associated with exposure to organophosphates and carbamates however studies indicate that this enzyme may be inhibited by the presence of metals and other agrochemicals [54–56]. Our result corroborates with this hypothesis, since inhibition of this enzyme was observed in all sites in brain tissue. A similar result was observed by others field study on the most impacted site [8,57]. Some authors suggest that the increase in TBARS levels could be associated with AChE inhibition, but in our results was observed the inhibition in brain in both biomarkers. However, another hypothesis is the involvement of protein carbonyl to disrupt AChE activity [58], we observed that protein carbonyl increased in

S2 and S3, in S1 was not observed increased in this biomarker even though AChE it has decreased.

IBR results was a great tool in this work as it helped to summarize all the biomarkers in a single result. As expected, the value of IBR was higher in the sites with higher pollution presence. Although agrochemicals appeared at all sites during the exposure, previous collections indicate that S2 and S3 are more contaminated. The results of IBR are in agreement with the results seen in Vieira et al. [8] Where higher values of the sum of the biomarkers were also observed in the most contaminated sites.

It is evident in our study the differences of biomarkers evaluated at each site of exposure. As expected, the sites considered more contaminated resulted in significant alterations in relation to the control group and the animals exposed in S1. This result makes sense, since riparian forest is thought to be effective at intercepting and controlling chemical loads from diffuse agricultural sources to entering water bodies [59].

It was observed that none of the agrochemicals found in our water collections are described in the last report provided by CONAMA [37] responsible for determining maximum amounts of the chemicals found in the environment In waters of the Brazilian territory. The concentrations of clomazone and tebuconazole found in our study deserve attention and should be studied under controlled conditions, in an isolated and synergistic way once that effects of mixture toxicity are complex and poorly understood [60].

5 CONCLUSION:

The use of silver catfish in an *in situ* exposure for 96 hours proved to be quite efficient, since the animals showed good adaptation, surviving during the exposure period. The evaluation of all the biomarkers used proved to be quite adequate for the study, since all gave some kind of response. This study has the potential to serve as a reference for future studies in the field of ecotoxicology, making possible the use of *Rhamdia quelen* in *in situ* experiments in regions where environmental risks are assessed. Even, our study helps to corroborate the importance of the preservation of riparian forests and their role in the preservation of aquatic fauna.

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TABLES

Table 1. Agrochemicals found in water and sediment from *in situ* exposure sites and previous collections.

	Water		
	S1	**S2**	**S3**
November			
Clomazone	-	<LOQ	<LOQ
December			
Bentazon	-	1.8	0.77
Clomazone	-	0.33	0.31
Clorimuron	<LOQ	<LOQ	<LOQ
Quinclorac	-	0.45	0.4
***In situ* exp. 0h**			
Clomazone	0.4	0.4	0.4
In situ exp. 96h			
Clomazone	0.34	0.41	-
Tebuconazole	-	0.3	-
	Sediment		
	S1	**S2**	**S3**
December			
Carbofuran	<LOQ	0.029	-

Concentrations of agrochemicals are reported as µg L^{-1}, LOQ = limit of quantification, indicates concentration less than the limit of qualification of the method.

Table 2. Physical and chemical parameters of the water from the *in situ* exposure sites.

	S1	S2	S3
***In situ* exp. 0h**			
Temperature	25.4	26.19	27.28
pH	7.54	7.48	7.33
DO	9.07	9.27	9.47
Ammonia	0.013	0.022	0.019
Nitrite	0.034	0.077	0.070
***In situ* exp. 96h**			
Temperature	24.84	25.66	28.64
pH	7.36	7.17	7.2
DO	8.64	9.79	10.08
Ammonia	0.014	0.011	0.017
Nitrite	0.032	0.054	0.05

Values of temperature are reported as ºC, dissolved oxygen as mg/L, ammonia as mg/mL and nitrite as mg/mL.

Table 3. NPSH levels and GST activity in brain , gills, liver and muscle of silver catfish exposed *in situ*.

	Control	S1	S2	S3
NPSH				
Brain	0.42 ± 0.032^{a}	0.47 ± 0.023^{ab}	0.54 ± 0.006^{b}	0.52 ± 0.023^{ab}
Gills	0.51 ± 0.006^{a}	0.49 ± 0.02^{a}	0.53 ± 0.012^{a}	0.49 ± 0.017^{a}
Liver	0.59 ± 0.01^{a}	0.59 ± 0.013^{a}	0.57 ± 0.027^{a}	0.59 ± 0.02^{a}
Muscle	0.47 ± 0.019^{a}	0.56 ± 0.01^{b}	0.53 ± 0.016^{ab}	0.57 ± 0.016^{b}
GST				
Brain	0.12 ± 0.005^{a}	0.097 ± 0.002^{b}	0.105 ± 0.005^{ab}	0.12 ± 0.007^{ab}
Gills	0.09 ± 0.002^{a}	0.09 ± 0.003^{a}	0.15 ± 0.007^{b}	0.10 ± 0.008^{a}
Liver	0.44 ± 0.033^{a}	0.68 ± 0.038^{b}	0.70 ± 0.044^{b}	0.98 ± 0.025^{c}
Muscle	0.10 ± 0.009^{a}	0.10 ± 0.005^{a}	0.11 ± 0.006^{ab}	0.15 ± 0.013^{b}

NPSH levels was expressed as µmol NPSH/g tissue, and GST activity was expressed as µmol GS-DNB/min/mg. Data are reported as mean ± S.E.M (n = 10). Different letters indicate differences between groups ($p < 0.05$).

Table 4. CAT, SOD, ROS and ACAP activities in liver and gills of silver catfish exposed *in situ*.

	Control	S1	S2	S3
CAT				
Gills	0.101 ± 0.01^{ab}	0.112 ± 0.01^{ab}	0.071 ± 0.005^{a}	0.13 ± 0.012^{b}
Liver	1.18 ± 0.075^{a}	2.54 ± 0.107^{b}	1.60 ± 0.123^{ac}	2.10 ± 0.169^{bc}
SOD				
Gills	6.65 ± 0.45^{a}	3.81 ± 0.06^{b}	1.92 ± 0.29^{b}	2.75 ± 0.33^{b}
Liver	7.47 ± 0.74^{a}	6.21 ± 0.41^{a}	5.96 ± 0.43^{a}	6.60 ± 0.060^{a}
ROS				
Liver	4.49 ± 0.55^{a}	3.47 ± 0.23^{a}	5.61 ± 0.41^{a}	5.54 ± 0.54^{a}
ACAP				
Liver	1.28 ± 0.055^{ab}	1.17 ± 0.10^{a}	1.09 ± 0.025^{a}	1.58 ± 0.1^{b}

CAT activity was expressed as µmol/min/mg protein, SOD activity was expressed as U/mg protein, ROS production was expressed as area 10^{3} /mg protein, and ACAP was expressed as relative area^{-1}. Data are reported as mean ± S.E.M (n = 10). Different letters indicate differences between groups ($p < 0.05$).

FIGURES

Figure 1. Map of Rio Grande do Sul State and the location of the municipality of São Gabriel. The three sites where in situ tests were carried out are marked with a red dot on the map.

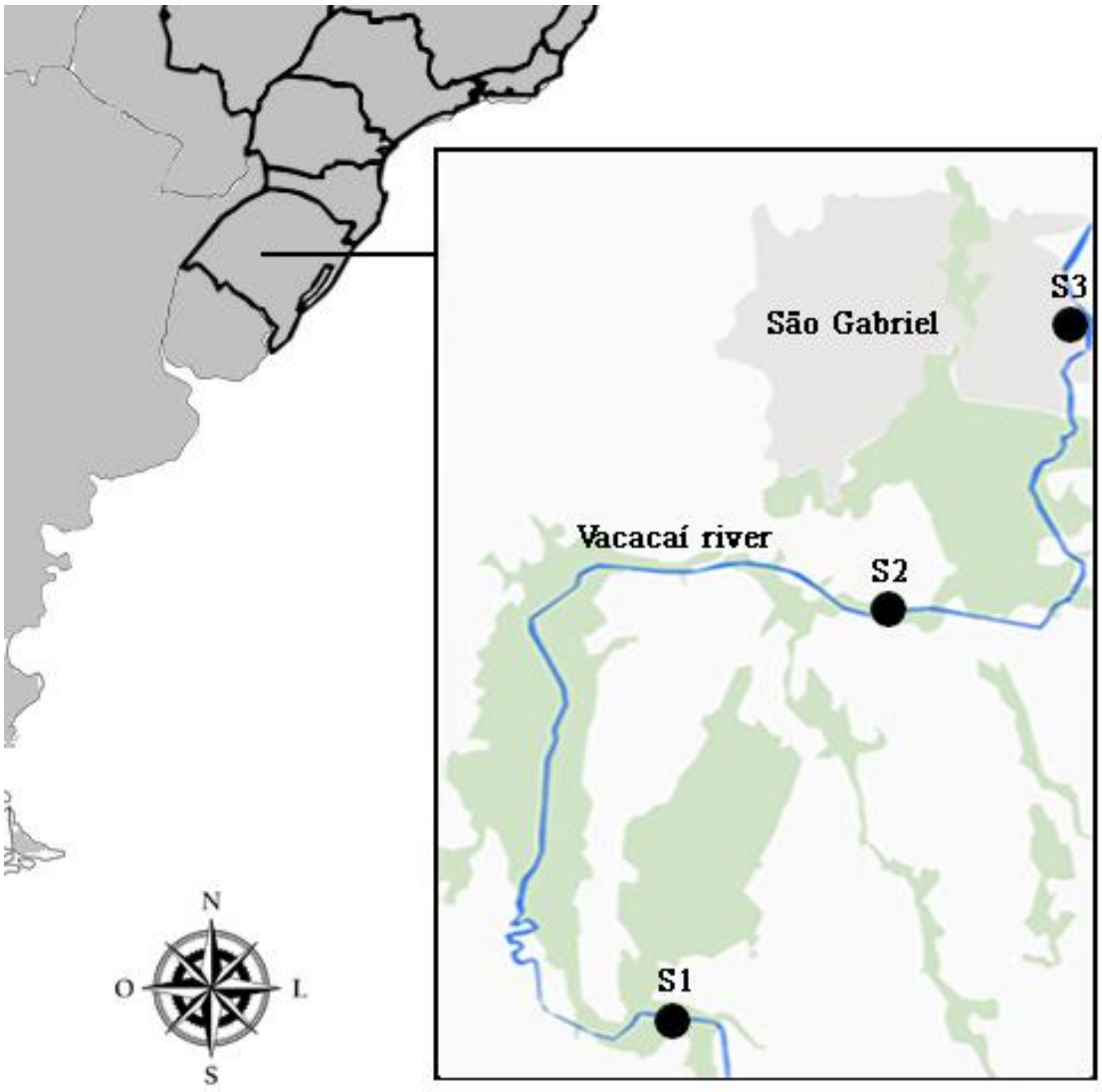


Figure 2. TBARS levels in brain (A), gills (B), liver (C) and muscle (D) from silver catfish exposed *in situ*. Data are reported as mean ± S.E.M. (n = 10). Different letters indicate differences between groups ($p < 0.05$).

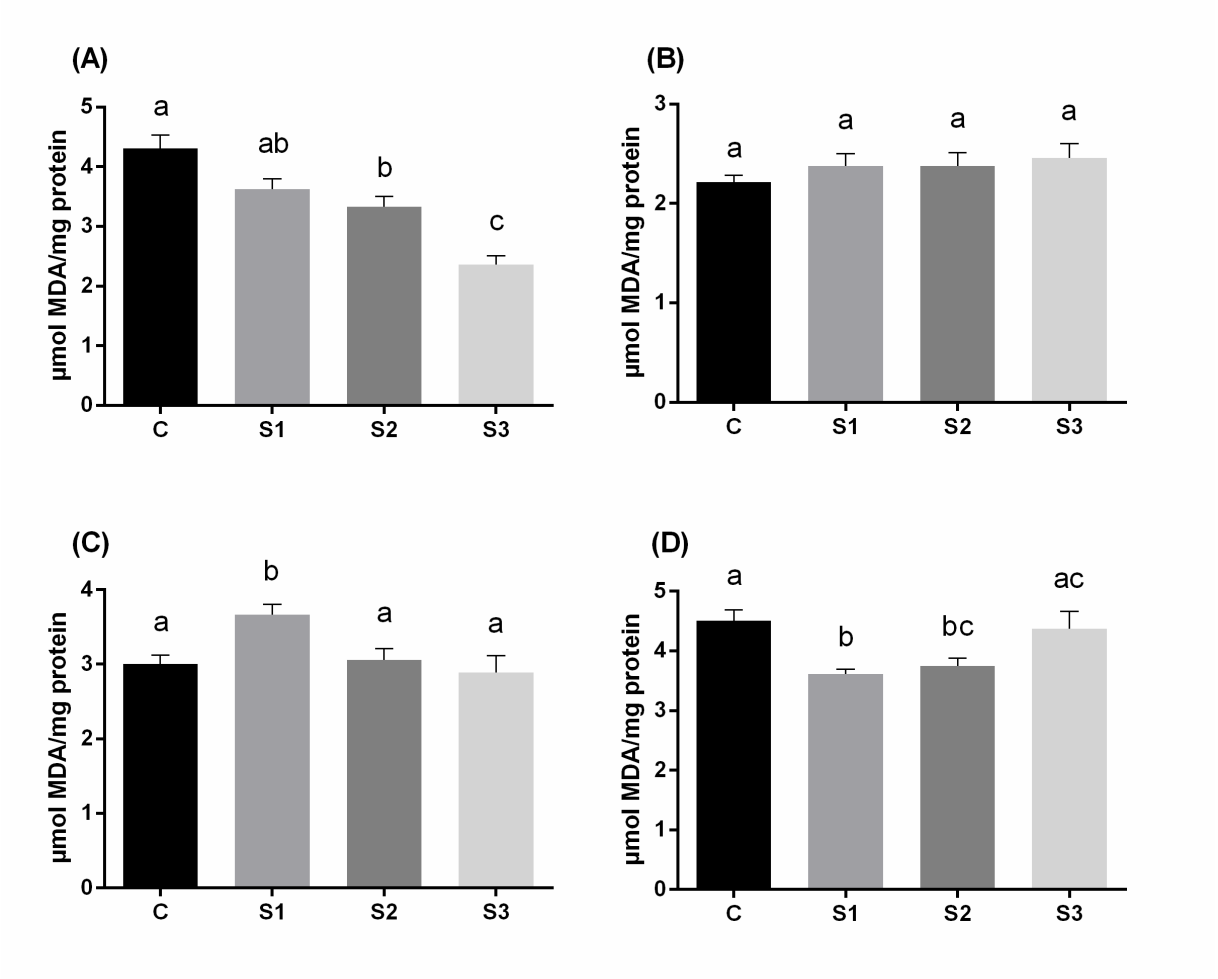


Figure 3. PC levels in brain (A), gills (B), liver (C) and muscle (D) from silver catfish exposed *in situ*. Data are reported as mean ± S.E.M. (n = 10). Different letters indicate differences between groups ($p < 0.05$).

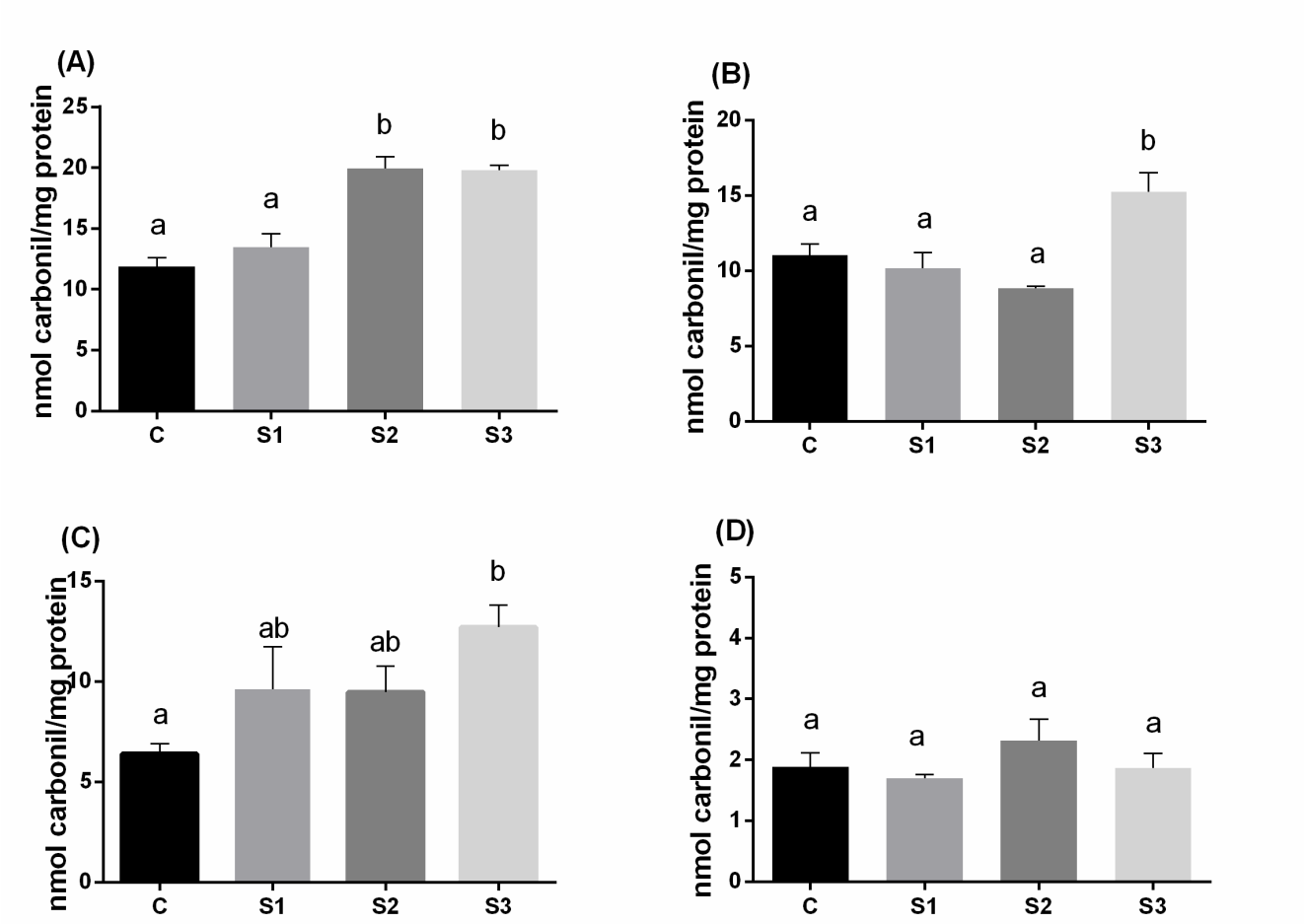


Figure 4. AChE activity in brain (A) and muscle (B) from silver catfish exposed *in situ*. Data are reported as mean ± S.E.M. (n = 10). Different letters indicate differences between groups ($p < 0.05$).

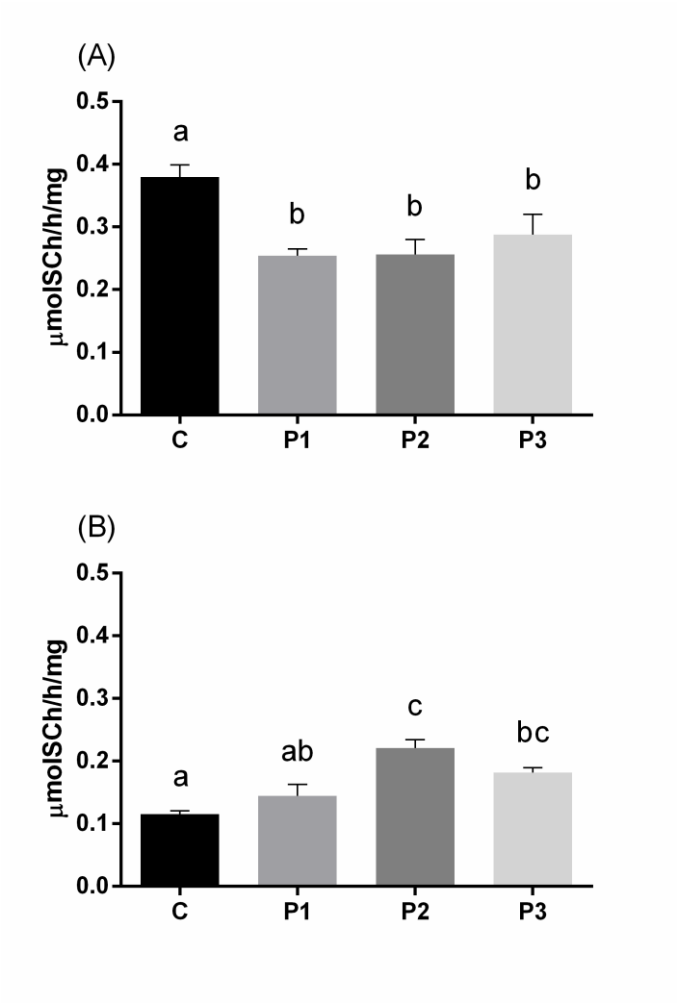
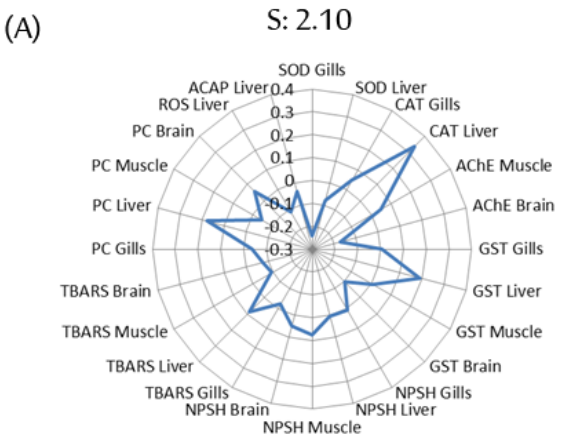
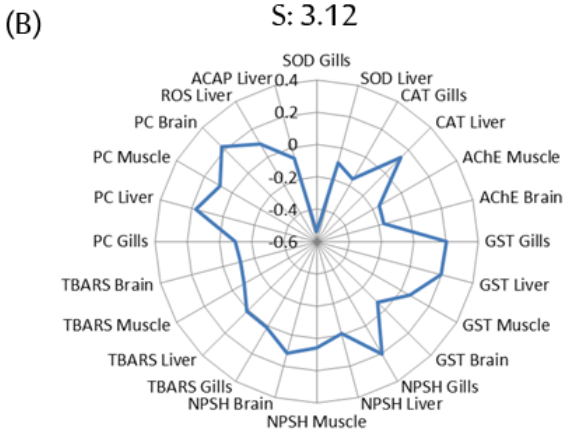


Figure 5. IBR values of S1 (A), S2 (B) and S3 (C) from *in situ* exposure.

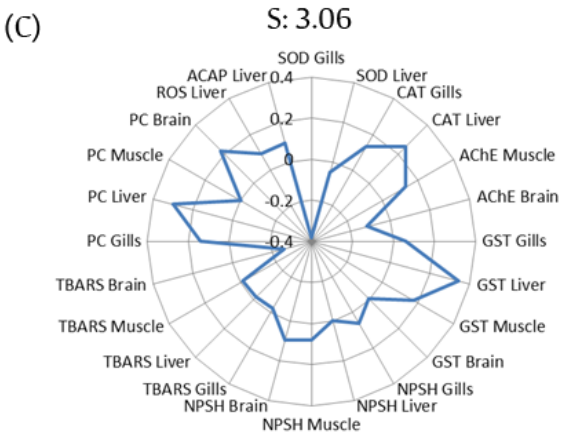
(A)



(B)



(C)



3 CONCLUSÃO

No presente estudo evidenciou-se que:

- O uso de jundiás em exposição *in situ* é viável no período de 96 horas, uma vez que os animais mostraram boa resistência a vários fatores estressores.
- Os biomarcadores utilizados neste trabalho se mostraram bastante úteis e responderam de forma variada em cada local onde os peixes foram expostos.
- O uso de IBR foi bastante útil para a interpretação geral dos resultados, uma vez que esse cálculo transforma todos os resultados dos biomarcadores em um único valor.

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