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Eduardo Stringini Severo

PEIXES COMO MODELO BIOLÓGICO PARA O MONITORAMENTO AMBIENTAL EM ÁREAS AGRÍCOLAS

Santa Maria, RS
2021

Eduardo Stringini Severo

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Tese 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 **Doutor em Biodiversidade Animal**.

Orientadora: Profª. Drª. Vania Lucia Loro

Santa Maria, RS, Brasil
2021

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Santa Maria, RS
2021

DEDICATÓRIA

Gostaria de dedicar esta tese de doutorado a todos os cientistas brasileiros que, assim como eu, passam por uma difícil situação, a qual com os frequentes cortes nos orçamentos dos programas de pesquisa, a ciência brasileira é cada vez mais desestimulada.

Quando entrei no curso de Ciências Biológicas no ano de 2011, presenciei um grande investimento na educação brasileira. Muitas oportunidades de viajar para congressos pelo país e até mesmo fora do Brasil, além da possibilidade de se estudar em outras universidades ao redor do mundo. Naquele tempo, a ciência brasileira parecia próspera, assim, logo após terminar a graduação, vi como uma oportunidade para crescer na vida através do programa de pós-graduação oferecido pela própria Universidade.

Durante o mestrado, eu já começava a notar cortes no orçamento. Percebi que talvez para realizar a minha própria pesquisa, eu tivesse que futuramente arcar com os custos de forma particular, como alguém que paga com seu próprio “salário” para poder trabalhar.

Nesse mesmo tempo, além dos cortes, cresceu no país uma onda de críticas aos investimentos em programas estudantis e parte da nossa sociedade começou a vê-los com "maus olhos". Não demorou muito para começar a faltar verbas nas universidades.

No doutorado, passei por frequentes momentos de incerteza se no mês seguinte teria bolsa de estudo, assim como vários outros colegas do curso. Isso trouxe muita insegurança para os estudantes de pós-graduação, que acabam se desmotivando em continuar dando o melhor de si. Afinal, talvez, existisse a possibilidade de ter que adequar todo o planejamento na pós-graduação e ainda procurar emprego repentinamente.

Somado a tudo isso, trabalhar com meio ambiente e com poluição ambiental no momento em que ambos são totalmente negligenciados acaba sendo bastante frustrante. Por isso, dedico este documento a todos que continuam na luta, que acreditam em um futuro melhor onde a educação é prioridade para o desenvolvimento de uma nação.

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Em primeiro lugar, quero agradecer a quem sempre esteve ao meu lado desde o início e presenciou minhas frustrações e momentos de alegria. Tiessa, muito obrigado por tudo nesses últimos anos, sem teu apoio nada disso seria possível. Obrigado pela companhia nas saídas de campo, nas idas ao laboratório nos finais de semana, muitas vezes tarde da noite para colocar os peixes-zebra para reprodução. Foram momentos cansativos que se tornaram mais leves graças a tua ajuda e parceria.

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A todos os peixe-zebras que participaram desse projeto.

A todos, o meu mais sincero agradecimento!

“O universo não tem obrigação de fazer sentido para você. ”

(Neil deGrasse Tyson)

RESUMO

PEIXES COMO MODELO BIOLÓGICO PARA O MONITORAMENTO AMBIENTAL EM ÁREAS AGRÍCOLAS

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ORIENTADORA: Vania Lucia Loro

A alta demanda por alimentos tem levado a um maior número de pesticidas utilizados nas lavouras, causando impacto ambiental nos ambientes aquáticos próximos a essas localidades. Para avaliar os impactos nos ambientes aquáticos são necessários estudos de biomonitoramento. Os peixes são comumente usados como modelo animal para a realização deste tipo de estudo. O estado do Rio Grande do Sul está entre os maiores produtores agrícolas do Brasil, porém a região carece de estudos acerca da presença de contaminantes em seus rios e riachos. No Artigo 1, objetivou-se realizar uma revisão sobre as principais metodologias de biomonitoramento realizadas em países da América Latina. Assim, foram analisados 127 artigos científicos publicados entre 2010 e 2020 que relatam a realização de metodologias de biomonitoramento em ambientes aquáticos utilizando peixes como bioindicadores de poluição ambiental. Desse modo, foram avaliadas as principais vantagens e desvantagens de cada metodologia, bem como as principais análises realizadas durantes e após os estudos de biomonitoramento. No artigo 2, embriões de *Danio rerio* foram utilizados como bioindicadores para avaliar a qualidade da água no rio Vacacaí. Realizaram 8 coletas de água em três pontos do rio durante o ano de 2018. As águas coletadas foram utilizadas para quantificar pesticidas e realizar a exposição dos embriões durante 96 horas. Vinte e quatro pesticidas foram encontrados em amostras de água do rio. Os mais encontrados foram atrazina, quinclorac e clomazone. Durante a exposição avaliou-se o movimento espontâneo, a frequência cardíaca e a taxa de eclosão. Após o final da exposição, os embriões foram eutanasiados para a realização de ensaios bioquímicos. Foram analisados biomarcadores como substância reativa ao ácido tiobarbitúrico (TBARS), acetilcolinesterase (AChE), glutationa S-transferase (GST) e catalase (CAT). Observou-se aumentos em GST e TBARS, especialmente durante os períodos de maior contaminação da água, como janeiro, fevereiro, outubro e novembro. No Artigo 3, foi realizada a exposição de embriões de peixe-zebra ao herbicida Basagran® nas concentrações do seu princípio ativo (bentazona) encontradas no estudo de biomonitoramento realizado no Artigo 2. O objetivo do estudo foi o de avaliar o desenvolvimento de embriões de *D. rerio* expostos a concentrações ambientais deste herbicida (3 $\mu g.L^{-1}$, 6 $\mu g.L^{-1}$, 12 $\mu g.L^{-1}$), bem como o seu limite estabelecido seguro para água potável (300 $\mu g.L^{-1}$). Realizaram-se análises comportamentais e de desenvolvimento durante o período de exposição. Os resultados mostraram alterações apenas na frequência cardíaca avaliada em 48 horas pós fertilização, mostrando que o herbicida Basagran® nas concentrações testadas ocasionou poucos efeitos adversos no desenvolvimento e comportamento dos embriões avaliados. De maneira geral, constatou-se a possibilidade do uso de embriões de peixe-zebra para realizar estudos de biomonitoramento.

Palavras-chave: Biomonitoramento. Ecotoxicologia. Poluição Aquática. Biomarcador. *Danio rerio*

ABSTRACT

FISH AS A BIOLOGICAL MODEL FOR ENVIRONMENTAL MONITORING IN AGRICULTURAL AREAS

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ADVISOR: Vania Lucia Loro

The high demand for food has led to a greater number of pesticides used in crops, causing an environmental impact on aquatic environments near these locations. To assess the impacts on aquatic environments, biomonitoring studies are needed. Fish are commonly used as an animal model to carry out this type of study. The state of Rio Grande do Sul is among the largest agricultural producers in Brazil, but the region lacks studies on the presence of contaminants in its rivers and streams. In Article 1, the objective was to carry out a review of the main biomonitoring methodologies carried out in Latin American countries. Thus, 127 scientific articles published between 2010 and 2020 reporting the implementation of biomonitoring methodologies in aquatic environments using fish as bioindicators of environmental pollution were analyzed. Thus, the main advantages and disadvantages of each methodology were evaluated, as well as the main analyzes carried out during and after the biomonitoring studies. In article 2, *Danio rerio* embryos were used as bioindicators to assess water quality in the Vacacaí River. They carried out 8 water collections at three points in the river during the year 2018. The collected waters were used to quantify pesticides and expose the embryos for 96 hours. Twenty-four pesticides were found in river water samples. The most common were atrazine, quinclorac and clomazone. During the exposure, spontaneous movement, heart rate and hatching rate were evaluated. After the end of exposure, the embryos were euthanized for biochemical assays. Biomarkers such as thiobarbituric acid reactive substance (TBARS), acetylcholinesterase (AChE), glutathione S-transferase (GST) and catalase (CAT) were analyzed. Increases in GST and TBARS were observed, especially during periods of greater water contamination, such as January, February, October and November. In Article 3, zebrafish embryos were exposed to the herbicide Basagran® at the concentrations of its active principle (bentazone) found in the biomonitoring study carried out in Article 2. The aim of the study was to evaluate the development of embryos of *D. rerio* exposed to environmental concentrations of this herbicide (3 µg.L-1, 6 µg.L-1, 12 µg.L-1), as well as its established safe limit for drinking water (300 µg.L-1) . Behavioral and developmental analyzes were performed during the exposure period. The results showed alterations only in the heart rate evaluated in 48 hours after fertilization, showing that the herbicide Basagran® at the tested concentrations caused few adverse effects on the development and behavior of the evaluated embryos. In general, the possibility of using zebrafish embryos to carry out biomonitoring studies was found.

Keywords: Biomonitoring. Ecotoxicology. Water Pollution. Biomarker. *Danio rerio*.

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1 INTRODUÇÃO

1.1 CONTAMINAÇÃO DE ECOSSISTEMAS AQUÁTICOS

Anualmente são despejados grandes volumes de compostos químicos em ecossistemas aquáticos decorrentes da produção agrícola, indústrias e resíduos domésticos. O meio aquático é afetado principalmente por dois tipos de fontes de contaminação, as chamadas fontes pontuais (que podem ser localizadas no espaço e no tempo) e as não pontuais (em que a localização espacial e temporal não podem ser identificadas). As fontes pontuais são os esgotos, por exemplo, provenientes das indústrias e dos grandes centros urbanos, ambos lançados diretamente nos cursos d'água. Quando misturados acabam por se tornar muito mais perigosos e de difícil tratamento (MERTEN E MINELLA, 2002).

As fontes dispersas são fontes de contaminação de difícil identificação do ponto de lançamento de origem. Os tipos mais comuns são oriundos de atividades agrícolas, como os fertilizantes e pesticidas. Em grandes centros urbanos, devido à falta de saneamento básico, a contaminação por esgoto doméstico também pode ser definida como não pontual (ZAGATTO E BERTOLETTI, 2008) (Figura 1).

Figura 1 - Principais fontes de poluição pontuais e difusas.
Fonte: http://7fbiolugar.blogspot.pt/2012/06/fontes-de-poluicao-aquatica.htmlt

A contaminação difusa causada por pesticidas ocorre através de uma série de fatores ambientais como o escoamento superficial causado pelo excesso de chuvas e a deriva de pulverização ocasionada pelo vento (Figura 2). O desmatamento também é responsável por facilitar a contaminação de ambientes aquáticos, uma vez que a mata ciliar é considerada eficaz na interceptação e controle de cargas químicas de fontes agrícolas difusas (WEISSTEINER et al., 2014).

Os processos envolvidos no destino ambiental de pesticidas também são afetados devido às suas propriedades físico-químicas e forma de aplicação. Nesses processos, os compostos, sofrem alterações na concentração, a qual pode diminuir (diluição), aumentar (reconcentração) ou sofrer transferência de fase. Além disso, os compostos são molecularmente alterados em função de processos como a degradação (bio e fotodegradação) e das múltiplas reações químicas de que os mesmos podem participar, que podem aumentar, diminuir ou mesmo inativar seu poder tóxico (ZAGATTO E BERTOLETTI, 2008; SPADOTTO et al., 2010; PETERS et al., 2013).

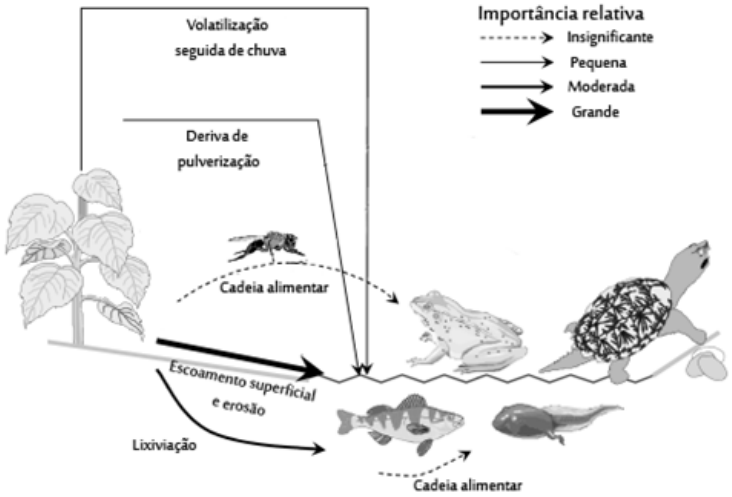

Figura 2 – Processos relacionados à contaminação ambiental.
Fonte: adaptado de SOLOMON et al., 2008, p.725.

1.2 USO DE AGROTÓXICOS NO BRASIL E NO RIO GRANDE DO SUL

Entre as principais tecnologias utilizadas para o crescimento da agricultura brasileira estão os pesticidas. No ano de 2017, conforme o Censo Agropecuário divulgado pelo Instituto Brasileiro de Geografia e Estatística (IBGE), cerca de 36% do total de 5.073.324 unidades agropecuárias recenseadas naquele ano, alegaram utilizar algum tipo de pesticida. Além disso, segundo dados do IBAMA (2021) no ano de 2019 foi comercializado o total de 620.537,98 toneladas de ingredientes ativos, o que representa um aumento de 12,97% nas vendas internas em relação ao ano de 2018 (Figura 3).

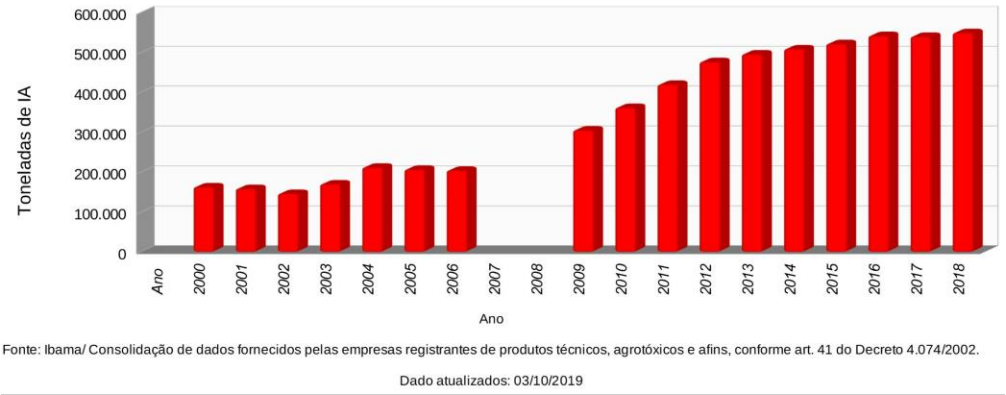

Figura 3: Consumo de pesticidas e afins (2000- 2018).
Fonte: adaptado de http://www.ibama.gov.br/phocadownload/qualidadeambiental/relatorios/2018/grafico%20-%20Consumo%20agrotoxicos%202000-2018.pdf.

Segundo dados do IBGE (2018) o estado do Rio Grande do Sul produziu 17.538.725 toneladas de soja (em grão) seguida por 8.401.787 toneladas de arroz (em casca), sendo o estado de maior produção de arroz no país em 2018. Relatórios do IBAMA apontam o estado do Rio Grande do Sul como o terceiro maior consumidor de pesticidas no Brasil no ano de 2019 (Figura 7).

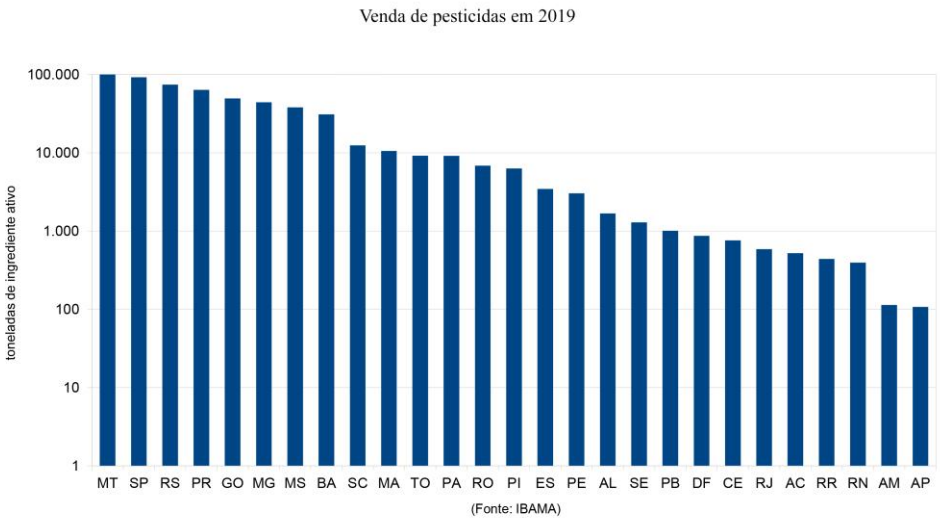

Figura 4: Venda de pesticidas no ano de 2019.
Adaptado de http://www.ibama.gov.br/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos.

1.3 BIOMONITORAMENTO EM ECOSSISTEMAS AQUÁTICOS

A avaliação da presença de pesticidas em ambientes aquáticos é possível através de análises que detectam a presença desses compostos como a Cromatografia Líquida de Alta Eficiência (CLAE), porém essas análises realizadas de forma isolada, não possuem a capacidade de mostrar o real impacto desses compostos na fauna aquática.

A interação entre contaminantes e organismos aquáticos pode levar a uma série de alterações como mudanças fisiológicas, bioquímicas, genéticas e comportamentais, as quais podem ser avaliadas através da análise de bioindicadores que consistem em organismos ou partes de organismos capazes de conter informações sobre a qualidade de um determinado ambiente (MARKET, 1994). Desde o ano 2005, o Conselho Nacional do Meio Ambiente (CONAMA) recomenda a realização de estudos toxicológicos para avaliar efeitos agudos e crônicos de contaminantes em organismos aquáticos de forma a avaliar possíveis impactos.

Entre as formas de se realizar estas avaliações, alguns estudos executam ensaios utilizando os contaminantes de interesse em condições laboratoriais, avaliando diferentes níveis de concentração desses compostos (MURUSSI et al., 2015; MARINS et al., 2021). Também é possível avaliar efeitos de contaminantes através de biomonitoramentos ambientais, que consistem em uma série de análises

integradas realizadas em locais possivelmente contaminados pelos mais diversos tipos de contaminantes (DO AMARAL et al. 2018, GONÇALVES et al., 2020).

A forma mais usual de se realizar estudos de biomonitoramento em ambientes aquáticos consiste na captura de espécimes de animais dos mais diversos níveis tróficos e de diferente táxons, acompanhado de análises de físico-químicas da água (temperatura, pH, condutividade, entre outros) e coletas de amostras de água e sedimento para avaliar a presença de eventuais contaminantes, como metais, pesticidas, fármacos, entre outros. As coletas são realizadas por um longo período, geralmente procurando fechar o ciclo de um ano, realizando coleta em todas as estações do ano ou em períodos de condições climáticas opostas (LORO et al., 2015; CEREZER et al., 2020; MARINS et al., 2020).

Uma possível alternativa para a realização de estudos de biomonitoramento consiste na coleta de amostras de água ou sedimento nos locais de interesse de estudo para posterior exposição dessas amostras em animais criados e mantidos em condições controladas de laboratório (COSTA-SILVA et al., 2011; RIBEIRO et al., 2020).

1.4 BIOMARCADORES

Após a captura dos animais, alguns tecidos de interesse são removidos para posterior análise de determinados biomarcadores. Os biomarcadores são classificados como biomarcadores de exposição, efeito e suscetibilidade.

Os biomarcadores de exposição são análises realizadas em organismos bioindicadores aptos a indicar a biodisponibilidade de um xenobiótico, possuem como principal característica, o fado de serem marcadores bem caracterizados de sua exposição a determinado compostos (VAN DER OOST et al., 2003). Como exemplo, podemos citar a atividade da enzima acetilcolinesterase (AChE), que consiste em um excelente biomarcador de exposição para avaliar alterações causadas por contaminantes como organofosforado e organoclorados, além de alguns metais (MARINS et al., 2021).)

Os biomarcadores de efeito são medidas que indicam alterações bioquímicas, comportamentais e fisiológicas associadas à exposição a um xenobiótico, podendo indicar possíveis danos em um organismo. (VAN DER OOST et al., 2003). Entre os biomarcadores utilizados para avaliar os possíveis danos causados nos organismos, os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) que indicam danos em lipídios pela peroxidação lipídica. A lipoperoxidação pode ser definida como uma série de eventos bioquímicos decorrentes da ação de espécies reativas sobre os lipídios insaturados das membranas celulares, que em casos mais extremos pode levar à morte celular (BENZIE, 1996).

Biomarcadores de suscetibilidade auxiliam a entender diferenças nas respostas entre indivíduos de uma mesma população. As diferenças podem ser provenientes de qualidades inerentes do organismo, como sexo e idade, entre outros, os quais são responsáveis por gerar uma recuperação ou vulnerabilidade no organismo a determinados xenobióticos (VAN DER OOST et al., 2003; ZAGATTO e BERTOLETTI, 2008).

1.5 *DANIO RERIO* COMO MODELO DE ESTUDO

O *Danio rerio*, também conhecido com peixe zebra ou paulistinha, vem ganhando destaque por possuir uma série de vantagens como o seu pequeno tamanho, fácil manutenção e baixo custo para manter essa espécie em laboratório (Figura 4). Além disso, a espécie possui uma vasta bibliografia de artigos publicados nas mais diversas áreas do conhecimento, como toxicologia, neurociência, farmacologia e biologia do comportamento (COSTA-SILVA et al., 2018; NUNES et al., 2019)

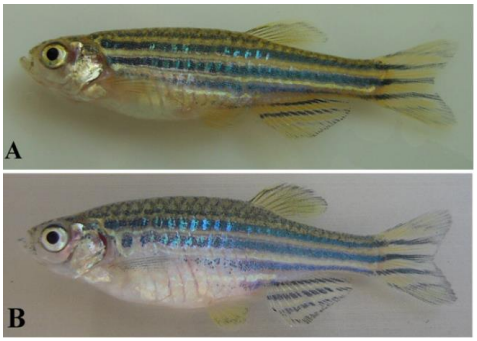

Figura 5: *Danio reri*o macho (A) e fêmea (B).
Fonte: AVDESH (2012)

Outro fator que contribui para a popularidade do peixe zebra em estudos científicos é a possibilidade de se realizar a sua reprodução em laboratório de forma relativamente simples. Um casal de peixe zebra pode facilmente produzir cerca de 200 embriões, e sendo a fecundação externa, torna-se viável a coleta desses embriões para a realização de estudos experimentais. O ciclo completo de desenvolvimento do embrião ocorre em até 96 horas (Figura 5), e por possuir o seu ovo transparente, é possível acompanhar o seu pleno desenvolvimento, possibilitando observar eventuais alterações em diversos órgãos como coração e olhos. Outras vantagens no uso de embriões de *D. rerio* estão relacionadas ao pouco espaço necessário para a realização do experimento, uma vez que os grupos amostrais podem ser organizados em placas de cultura de célula, resultando em um baixo custo de manutenção durante os experimentos realizados (OECD, 2013).

Uma das principais referências para a realização de testes com embriões de peixe-zebra é o guia Fish Embryo Acute Toxicity (FET) Test (OECD, 2013) o qual detalha todas as etapas experimentais dos testes com embriões, inclusive as alterações possíveis de se observar durante o seu desenvolvimento.

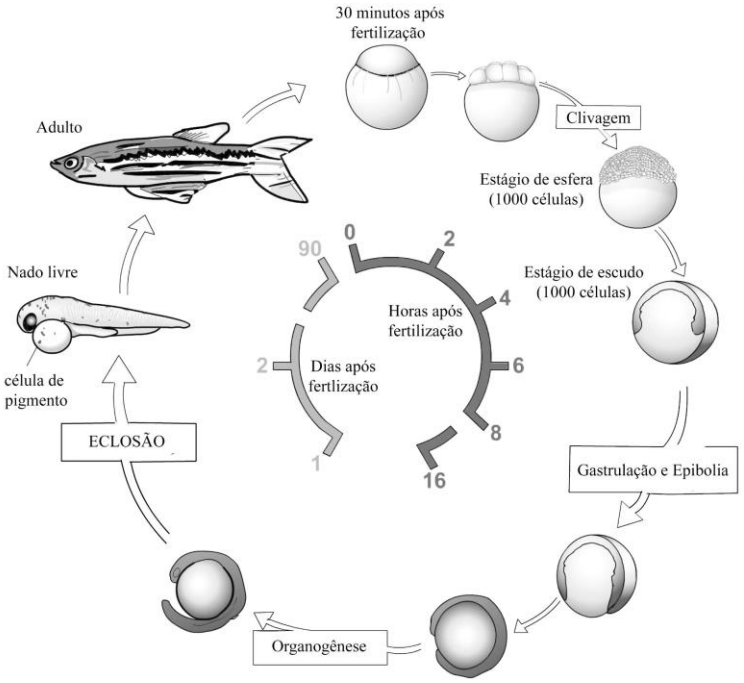

Figura 6: Desenvolvimento dos embriões de peixe-zebra, começando logo após a fertilização até sua forma adulta.
Fonte: adaptado de D'COSTA, A.; SHEPHERD, I. T. (2009)

1.6 CONTAMINAÇÃO DE ECOSSISTEMAS AQUÁTICOS NO RIO GRANDE DO SUL

Diversos estudos realizados no estado do Rio Grande do Sul relatam a presença de pesticidas em seus rios e riachos, como herbicidas, fungicidas e inseticidas encotrados em amostras de águas e sedimentos coletadas em ecossistemas aquáticas (Figura 7). Na região central do Rio Grande do Sul estão presentes algumas das maiores cidades do estado, como Santa Maria, a qual possui como uma de suas matrizes econômicas o cultivo de arroz, soja, milho, fumo e feijão preto (EMATER, 2021). Um dos principais recursos hídricos da cidade de Santa Maria é o rio Vacacaí, situado na Bacia Hidrográfica do Vacacaí-Vacacaí Mirim, a qual possui área de 11.177 km² e população estimada de 415.094 habitantes (2020), sendo 373.264 habitantes em áreas urbanas e 41.830 habitantes em áreas rurais (SEMA, 2021). O rio Vacacaí é hábitat de espécies endêmicas, como os peixes anuais *Cynopoecilus intimus* e *Austrolebias paucisquama*, ambas consideradas vulneráveis, além de espécies de peixes migratórios, como o *Salminus brasilienses* (dourado) (REIS et al., 2003; ICMBio, 2018).

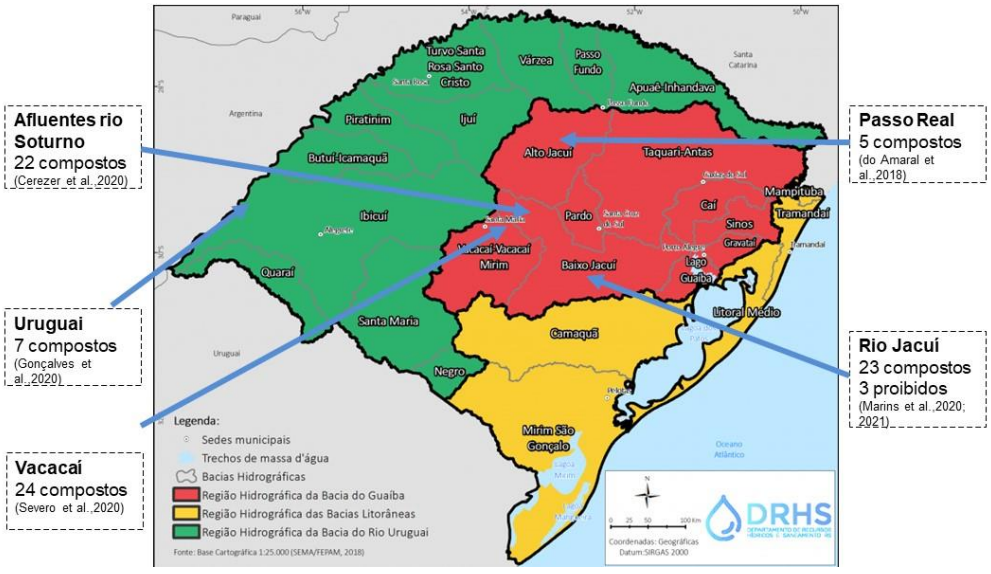

Figura 7: Artigos publicados com dados sobre a presença de pesticidas em rios e riachos no Estado do Rio Grande do Sul, Brasil.
Fonte: Adaptado de https://www.sema.rs.gov.br/bacias-hidrograficas.

2 HIPÓTESES

Diante do exposto, a presente tese propõe investigar três hipóteses:

a) coleta de peixes é a metodologia mais empregada em estudos de biomonitoramento em ecossistemas aquáticos continentais em países latino-americanos;
b) as amostras de água coletadas no rio Vacacaí são capazes de induzir alterações bioquímicas e comportamentais em embriões de peixe-zebra;
c) o herbicida Basagran® é capaz de induzir alterações comportamentais e de desenvolvimento em embriões de peixe-zebra, em concentrações ambientalmente relevantes;

3 OBJETIVOS

3.1 OBJETIVO GERAL

Identificar e avaliar as vantagens e desvantagens do uso de peixes em estudos de biomonitoramento, assim como, as principais análises a serem realizadas utilizando estes modelos.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar as principais metodologias de biomonitoramento realizadas em ecossistemas aquáticos dulcícolas de países latino-americanos.
- Avaliar parâmetros comportamentais e de desenvolvimento, bem como biomarcadores bioquímicos em embriões de peixe-zebra expostos à água superficial do rio Vacacaí, localizado na cidade de Santa Maria, RS.
- Avaliar os efeitos do herbicida Basagran®, em condições de laboratório, no comportamento e desenvolvimento de embriões de peixe-zebra, usando as concentrações encontradas em rios impactados por pesticidas, bem como o limite estabelecido seguro para água potável.

3.3 ESTRUTURA DO DOCUMENTO

O restante desta tese está organizado da seguinte forma:

O item 4 apresenta o artigo de revisão intitulado “Fish as biological model for environmental biomonitoring in Latin American countries: a systematic review” ; o item 5 apresenta o artigo já publicado “Ecological risk of pesticide contamination in a Brazilian river located near a rural area: A study of biomarkers using zebrafish embryos”; o item 6 apresenta o artigo intitulado “A preliminary study of the effects of Basagran® herbicide in *Danio rerio* embryonic development”; o item 8 apresenta a discussão geral da tese e o item 9 são feitas as considerações finais sobre o trabalho.

4 ARTIGO 1

Fish as biological model for environmental biomonitoring in Latin American countries: a systematic review

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ABSTRACT

To assess impacts on aquatic environments, biomonitoring studies are needed. Fish are commonly used as an animal model to carry out this type of study. Thus, the most common biomonitoring methodologies are field collections, in situ exposure and laboratory tests. We analyzed 127 scientific articles published by Latin American countries between 2010 and 2020 that report the use of one of these three biomonitoring methodologies. Thereby, the main advantages and disadvantages of each methodology were evaluated, as well as the analyzes carried out during and after the biomonitoring study. The objective of this study was to evaluate the main biomonitoring studies carried out in Latin American countries, in this way, this review can be used as a guide for researchers interested in conducting biomonitoring studies, as well as pointing out suggestions for improving and innovating in this area of knowledge. We have identified three biomonitoring methodologies using fish: field collection, in situ exposure and laboratory tests. Brazil was the country with the highest number of publications in the three evaluated methodologies. We also check the biomarkers used and the main analyzes performed in water and sediment. This study provides an overview of the main articles on biomonitoring carried out in freshwater ecosystems in Latin America. At the end of the article, we suggest approaches to techniques that have not yet been explored in biomonitoring studies carried out in latin america.

Keywords: aquatic pollution, bioindicator, ecotoxicology, biomarker,

1 INTRODUCTION

Latin American countries are historically characterized by their rapid and disorganized development. This led to a series of socio-environmental problems, such as the precariousness of sewage and water treatment systems (PAHO 2012). They are also countries with a strong presence of agribusiness, which is responsible for transforming natural biomes into monocultures (Lapola et al., 2014; Bonanomi et al., 2019).

At the same time, the countries of this region have a rich biodiversity, which is mainly threatened by illegal exploratory practices, such as mining, logging and land grabbing. These factors lead to deforestation, advances in agriculture, livestock frontiers and, consecutively, an increase in the degradation of water resources (DeFries et al., 2010; Richards et al., 2012; Borras et al., 2012), resulting in the contamination of aquatic environments, which is reported from all regions of Latin America (Echeverría-Sáenz et al., 2016; Vieira et al., 2019; Starling et al., 2019)

Pollution that affects aquatic environments are called point and non-point or diffuse pollution. Point pollution is characterized by the type of impact which is known exactly where it occurs, such as sewage thrown directly into a river. Diffuse pollution is characterized by the difficulty of controlling its final destination as the pollution caused by pesticides in crops near aquatic environments (Geissen et al. 2015; Tran et al., 2019)

The physical-chemical characteristics of each contaminant can modify their interaction with environmental matrices as the environmental characteristics (such as the physical, chemical and biological properties, soil textural class and, mineralogy, organic matter content, pH, cation exchange capacity, microbial activity, temperature and intensity of sunlight) can alter the mobility of chemical compounds (Mosher et al., 2006; Lei et al., 2017; Du et al., 2017). So the environmental interactions with chemical structure can lead to some transformations, as decomposition that, lead to an increase in chemical diversity of pollutants, which can enhance the toxic effects through additive and sinergic processes (Juhel et al., 2017; Salomão et al, 2020; Marins et al, 2021).

It is possible to assess the occurrence of water contamination through quantitative analysis such as high-performance chromatography (Donato, 2015)

for pesticides, as well as metering of metals through atomic absorption spectrometry with a coupled graphite oven, according to the methodology described by Nadella et al. (2009). Although these techniques are very accurate in quantifying the presence of pollutants in aquatic environments, they do not have the capacity to demonstrate any impacts that these compounds could have on local fauna (Soininen et al., 2004; Dos Santos et al., 2016)

One way to assess possible impacts on aquatic environments is through biomonitoring techniques. According to Zhou (2008), biomonitoring is a scientific technique to assess environments exposed to synthetic or natural contamination, based on analyzes of an organism tissue or fluids samples. Still, this technique considers that contaminants once in contact with an organism, leave marks that can characterize the exposure of the organisms to the contaminant, which can be analyzed through the analysis of biomarkers (Van der Oost et al., 2003).

Biomonitoring can be carried out with different groups of animals, plants and even microorganisms, the choice of the organism varies according to the research objective (Cerezer et al., 2020; Dében et al., 2016). Fish are popularly used in biomonitoring studies, since they are animals that have ecological importance, due to their wide distribution, being present in more than one trophic level and being consumed by humans (Lushchak, 2015). These animals can go through a series of changes in their organism, which can help to assess possible environmental impacts at the study site. Genotoxicity techniques such as micronucleus or biochemical biomarkers of oxidative stress are just a few of the many analyzes that can be performed to assess any changes caused by water pollutants (Vieira et al., 2019; Marins et al., 2020; Severo et al., 2020)

Taking this issue into consideration, the aim of this study was to evaluate the main biomonitoring methodologies carried out in Latin American countries. Pointing out the main advantages and disadvantages of each methodology and main points to be considered when planning an environmental impact study. This systematic review can be used as a guide for researchers who live in similar socioeconomic realities, in order to be encouraged to carry out biomonitoring studies in their localities.

2 MATERIAL AND METHODS

2.1 SEARCH STRATEGY

The Pubmed (https://pubmed.ncbi.nlm.nih.gov), ScienceDirect (https://www.sciencedirect.com), Scopus (https://www.scopus.com) and Scielo (https://www.scielo.org) platform were used to search for research articles for this review. We used the following search terms: “fish” and “biomonitoring”. The filters “results by year” and “research article” were selected in all research platforms. Additionally, the country filter was used for the Scopus platform. The search was carried out in the last ten years of publication (2010 to 2020), and the keywords were searched in English language. We initially screened studies based on general titles and abstracts. Then, we read the full text to extract all the necessary information.

The criteria adopted in this search period were: only research articles carried out in Latin American countries or using water samples collected in Latin America. During the search, articles written in English, Portuguese and Spanish were selected. The chosen research articles should involve research related to environmental impact in aquatic environments and use fish as a bioindicator animal. As well, biomonitoring studies must have been carried out in continental aquatic ecosystems.

In the search for research articles, we focused on studies that used the most common techniques in biomonitoring using fish: collection of animals in a potentially contaminated place (field collection), exposure of animals in contaminated environments (in situ exposure), and also studies that performed the collection of water in potentially contaminated places and performed the exposure of fish to these waters under controlled conditions (laboratory tests). Studies carried out in the coastal region or offshore were discarded.

When we found research articles that met those criteria, we also evaluated related articles suggested by the research platforms in “similar articles”. Furthermore, during the research, when we found referenced articles that fit the criteria adopted in our search, these articles were also selected. We

also selected articles with different techniques than those used in Latin American countries, to suggest approaches that can be taken by researchers from these countries.

2.2 DATA EXTRACTION

As the research articles were selected, they were cataloged in a program (Libreoffice calc, version 7.1.5.) to later facilitate writing about the types of research carried out. Tables were created focusing on the similarities between the selected articles such as: fish species, number of river sites, techniques used, types of environmental impact, among others. The cataloged data were used to create the figures and tables present in this study.

We grouped the biomarkers used in the studies and standardized their names to better organize the data in the assembled tables. They are organized into biochemical, genotoxicity, histological and molecular biomarkers. In addition to analysis of bioaccumulation, physiological indexes, endocrine disruption, hematological parameters and lethal and morphological endpoints.

3 RESULTS AND DISCUSSION

Database searches resulted in an initial total of 4445 documents (n=894 from Pubmed, n=3.318 from Science Direct, n=196 from Scopus and n=37 from Scielo) obtained through systematic search. After deleting redundant documents, a total of 127 research articles that fit all the chosen criteria were found. According to our survey, the most used methodology was “field collection” (n=99), followed by “in situ exposure” (n=17) and “laboratory tests” (n=11). Among the main types of environmental impacts found in our research, we can highlight the impact caused by pesticides, metals, domestic sewage and industrial effluents.

3.1 BIOMONITORING METHODOLOGIES

3.1.1 Field collection

Studies involving the collection of fish directly from places impacted by the most diverse forms of pollution were called in our study "field collection". The Latin American countries with the highest number of scientific articles published using this biomonitoring technique were Brazil (79) followed by Argentina (11) and México (5). The total of published articles can be seen in Figure 1. This form of biomonitoring in aquatic environments is the most used, possibly due to its practicality and the need for few resources. In order to carry out the fish collection, low-cost materials are generally used, such as nets, hooks and homemade traps (de Jesus et al., 2013; Morais et al., 2016; Cardoso et al.,2019). In a few studies, more expensive equipment such as the electro-fisher backpack were used (Guyón et al., 2016; Assef et al., 2019).

In most cases, several points are chosen in the study site. The researcher generally chooses a site considered as a reference (a place with little or no environmental impact), another close to the impacted site and another ahead of the impacted site (Maggioni et al., 2012; Nime et al., 2018). Some studies used as a reference even another river or lake, as well as animals collected in fish cultures or even acclimated in a laboratory (da Silva Montes et al., 2020; da Silva et al 2018; Nimet et al., 2018). The types of environments vary among the studies, such as lentic environments as lakes and reservoirs (Hauser-Davis et al., 2015; Marcon et al.,2010) and lotic environments such as rivers estuaries and streams (Assef et al., 2019; Albergaria-Barbosa et al., 2016). Some of these studies perform collections in different aquatic environments in the same biomonitoring study (Ribeiro et al., 2015; Hinojosa-Garro et al., 2020). In some cases, finding a site considered as a reference becomes very difficult, since many regions are widely impacted by anthropogenic interference as reported by Marins et al, (2020).

The number of collection sites used in the studies varied from biomonitoring using only a single collection site to collect fish, as well as a few studies that collected from more than ten collection sites (Ruelas-Inzunza et al., 2015; Hinojosa-Garro et al., 2020). The average of collection sites in the total of analyzed studies

was around four collection sites (Silva et al 2015; Ernst et al., 2018). Some studies perform more than one biomonitoring per year. In these cases, we observe that the most used strategy is to catch fish in opposite seasons during the year, such as winter and summer or autumn and spring (warm and cold seasons) as seen in Freire et al. (2015) and Yamamoto et al. (2016). Other biomonitoring studies perform capture in all seasons of the year as seen in Gonçalves et al. (2020). One of the reasons for performing biomonitoring in opposite seasons is directly related to the different types of agricultural crops in each season of the year and their possible impacts on aquatic environments. In addition, it is possible to evaluate whether seasonality interferes with the results obtained in biomonitoring (do Amaral et al., 2018).

We observed that there is no pattern for the number of fish collected per collection site. Some studies collected fixed values per study site as seen in Ghisi et al. (2016) who collected 20 specimens of *Hypostomus ancistroides*, as well as Trujillo-Jiménez et al. (2014) which collected 60 *Astyanax aeneus* per study site. Other studies only described the total fish collected in the entire study, without distinguishing the number of fish collected per study site as seen in de Lima Cardoso el al. (2018) who reported having collected 55 specimens of *Prochilodus lacustris* in the dry season and 43 specimens in the rainy season at a total of three collection points per season. Other studies only described the total amount of fish collected in the entire biomonitoring study as seen in Leone et al. (2018), which collected a total of 319 specimens of *Odonthestes bonariensis* in a total of six collections carried out at two sites during the year. Thus, it was difficult to define a pattern of collected animals due to the great variation observed.

The species collected are described in Table 1. Most studies used native species, but in some studies exotic species were collected as seen in Carvalho et al. (2012) who collected *Oreochromis niloticus* and Assef et al. (2019) who collected *Salmo trutta*. In conjunction with fish collections, several studies carry out analyzes in the water and sediment of the studied location. The most common analyzes performed with water are physical-chemical analyses. These analyzes consist of a set of analyzed parameters, such as temperature, pH, conductivity, turbidity, dissolved oxygen, total dissolved solids, among others. As mentioned by Ruaro et al. (2015), several studies have used the physical-chemical parameters for the identification of reference sites, since the analysis of physical-chemical parameters

are recommended to verify water quality. Although they provided information about the effects of stressors on the physical and chemical processes of the ecosystem, they are not able to explain how they affect biological communities (Serpa et al., 2014).

In order to complement these analyses, water samples are collected to later verify and quantify the presence of compounds which are presumed to be present in the studied environment, such as metals, pesticides, among others. The list of analyzes performed can be seen in Table 1. Although evaluating the presence of such compounds is important for the performance of biomonitoring, several studies do not carry out such analyses. We assume that in many cases it is due to the lack of financial resources to carry out the analysis. In a few studies, sediment collections were also carried out from the studied site. In general, the analysis carried out in sediment are the same carried out in water (physical-chemical, metals, pesticides, among others). The list of analyzis carried out in sediment can be seen in Table 1.

In conjunction with the use of fish species, some studies performed biomonitoring using organisms from other taxa at the same time. This type of study can be seen in Bueno-Krawczyk et al. (2015) who used *Daphnia magna* and *Desmodesmus subspicatus* together with *Astyanax bifasciatus* to monitor a river impacted by urban and rural effluents. Biomonitoring studies performed by Seriani et al. (2015), Batista et al. (2016) and Beraldi et al. (2019) used *Daphnia similis*, *Allium cepa* and macrophytes (the study did not identify the species), respectively. Another approach was used by de Carvalho et al. (2020) which used periphyton sampling (biofilm) and Alcala-Orozco et al. (2020) used blood and human hair samples from the population of the studied region to assess the presence of mercury, cadmium and selenium.

After collection, the fish are usually taken to the laboratory for the removal of biological samples for later use in biomarker analysis. Practically all organs can be used for biomarker analysis, Ribeiro et al. (2013) extracted samples of bile, liver, muscle, kidney and blood from *Atherinella brasiliensis* to analyze biochemical, histological and genotoxic biomarkers as well as Paschoalini et al. (2019) extracted samples of liver, muscle, spleen and gonads from *Prochilodus argenteus* to analyze bioaccumulation of metals, histological and immunohistochemistry biomarkers. Some researchers carry out the collection of biological samples in the field to reduce the stress caused by transporting fish to the laboratory (Yamamoto et al., 2017).

The list of biomarkers found in our searches can be seen in Table 1 in the column "fish analysis". The analysis of biochemical biomarkers was the most used by researchers (representing a total of 24,74% of the analysis) (Figure 1). These biomarkers consist of a series of techniques that assess changes in antioxidant enzymes and non-enzymatic antioxidants and possible damage to lipids and proteins resulting from fish contact with pollutants, in addition to many other changes. Some biochemical biomarkers are often related to damage caused by specific pollutants, such as the enzyme acetylcholinesterase (AChE) since this enzyme is inhibited by some contaminants (carbamates, organophosphate, and organophosphorus pesticides) (Marins et al., 2021). Another widely used enzyme is glutathione S-transferase (GST), which is considered a phase II enzyme that conjugates xenobiotics or their metabolites with glutathione, making them less toxic and more easily excreted (Severo et al., 2020). The non-enzymatic antioxidant metallothionein (MT) is involved in the homeostasis of essential metals and features high scavenging activity against different free-radical species and heavy metals (Sakuragui et al., 2013).

The second most performed analysis was bioaccumulation (20,61%) (Figure 1). As defined by Streit (1998), bioaccumulation is the accumulation of a contaminant into an organism or a biological community, resulting either from direct uptake from the water (by bioconcentration) or from ingestion (biomagnification). The most performed bioaccumulation analysis was the one involving the presence of metals, especially mercury (Hg) (Ruelas-Inzunza et al, 2015; Hinojosa-Garro et al., 2020). Other studies show the bioaccumulation of polycyclic aromatic hydrocarbons (PAHs), pesticides, pharmaceuticals and persistent organic pollutants (POPs) (Osório et al., 2014; Paulino et al., 2014; Valdes et al., 2016; Pizzochero et al., 2019). In some of these studies, the analysis of these same compounds in water and sediment was performed (Araujo et al., 2018b; Gonçalves et al., 2020).

The analysis of DNA alterations in fish using genotoxicity biomarkers (18,55%) has been shown to be a suitable method to assess genotoxic contamination of aquatic environments, being able to detect exposure to contaminants in a wide range of species (Wachtel et al., 2019; Ghisi et al., 2020; Montes et al., 2020) (Figure 1). These studies evaluated genotoxicity biomarkers in *Astyanax bifasciatus*, *Psalidodon bifasciatus* and *Serrasalmus rhombeus* to assess the environmental impact caused by industrial, urban, and agricultural pollutants and metals, respectively. The

genotoxicity analyzes used in biomonitoring were micronucleus and comet assay. These techniques are well defined and are valuable tool for being simple, versatile, of rapid execution, visual and highly sensitive to detect DNA lesions (Dhawan et al., 2009).

Histological biomarkers (15.98%) are sensitive tools to detect the toxic effects of chemical compounds on target organs and are indicators of previous exposure to environmental stressors (Figure 1). The gills and liver are organs normally used for this type of analysis, the gill for being in direct contact with the external environment and having an important role in fish respiration and osmoregulation, and the liver for being related to metabolism and excretion of contaminants (Coutinho and Gokhale., 2000). Other organs such as the kidney and pancreas are frequently used for the evaluation of histological biomarkers. To assess the impact of Hg from gold mining, Rabitto et al. (2011), analyzed histological changes in gills and liver of *Cichla monoculus*, as well as the study by Vreys et al. (2019) which evaluated the impact of urban pollutants using *Heptapterus mustelinus* gill histology. The histopathology translates the lesion as a function of the duration and intensity of exposure to the toxic element and the adaptive capacity of a tissue (Van der Oost et al., 2003). Thus, toxic effects of pollutants may be evident in cells and tissues, before significant changes in behavior or outward appearance can be identified.

Condition factor (CF), hepatosomatic index (HSI) and gonadosomatic index (GSI) analyzes are the usual physiological analysis performed (11.34%). These biomarkers can be used to assess environmental contamination, since all these factors can be directly or indirectly affected (Guyón et al., 2016) (Figure 1). The CF is a quantitative indicator of individual well being, reflecting recent food availability conditions (Le Cren, 1951). CF can increase in polluted and rich organic matter areas due to increased feeding sources used by tolerant species that take advantage of these resources. This biomarker was used by Ghisi et al. (2016) to assess the impact of agricultural and industrial activity through CF analysis in *Hypostomus ancistroides* The HSI is used to assess liver size as a percentage of total body weight, contaminants can lead to an increase in liver size from hypertrophy and/or hyperplasia of hepatocytes. This biomarker was used by Araújo et al. (2018a) to assess the impact of urban and industrial pollutants in a river through HSI analysis in *Pimelodus maculatus*. The GSI is a bioindicator that provides information about the health and state of gonadal maturation and can be related to fish fecundity, there is

evidence that exposition to several environmental pollutants can result in gonad alterations like reduction of GSI, morphological changes, or both. This biomarker was used by de Albergaria-Barbosa et al. (2016) to assess the impact of urban pollutants in an estuary region through GSI analysis in *Mugil curema*.

3.1.2 In situ exposure

Studies involving the exposure of fish in possibly impacted locations through the use of cages were called "in situ exposure". The Latin American countries with the highest number of scientific articles published using this biomonitoring technique were Brazil (10) followed by Costa Rica (4) and Argentina, Chile and México (each with 1 article published). The total published articles can be seen in Figure 1.

This is the most used technique after "field collection". This method has been used due to its efficiency in demonstrating the effects of mixing contaminants in the environment (Rodríguez-Fuentes et al., 2012; Vieira et al., 2016; da Silva et al., 2020). According to Oikari et al. (2006), the migration of many fish species for breeding and feeding creates uncertainty about how well an individual tissue sample reflects the water quality at or around the capture site in biomonitoring using the "field collection" technique. An individual may have recently entered a more or less contaminated area and therefore exhibit accumulated contaminants from the previous location. In situ exposure offers numerous advantages, such as accurate site knowledge and selection of species at the developmental stage of interest. Most of the studies carried out chose to use juveniles (Table 2), probably to avoid changes related to sex and because of their smaller size, facilitating the handling of the technique.

In all studies, the animals used in the experiment were acquired from local fish cultures except for Echeverría-Sáenz et al. (2012) who used specimens of *Bryconamericus scleroparius* captured at a reference site and transported to the study site, the same methodology was used by Mena et al. (2014) using specimens from *Poecilia gillii*. Although most studies used native species, *Danio rerio*, *Oreochromis niloticus*, and *Oncorhynchus mykiss* were the exotic species used in biomonitoring studies, as seen in Table 2. However, we believe that this idea should be discarded, although the researcher assures the safety of his used cage model, eventual leaks can occur and cause ecological imbalances.

As explained by Oikari (2006), the use of animals from the same origin reduces the inter-individual variability of exposed organisms and minimizes the influence of adaptive mechanisms. The size of the cages and the amount of fish varies between studies, Vieira et al. (2016) used cages in the size of 0.125m³ with 8 specimens of *Astyanax altiparanae*, and Santana et al. (2018) used cages of 8m³ with 60 specimens of *Oreochromis niloticus*. Most studies used one cage per study site, but studies such as those of Chiang et al. (2015) and Lunardelli et al. (2018) used two cages per location, yet Rodríguez-Fuentes et al. (2012) used a total of 3 cages per study point. As described by Echeverría-Sáenz et al., (2016), one of the difficulties in using this technique is the possibility of losing the cages due to abrupt changes in the levels and currents at the sampling sites, which are frequent in tropical rivers. Depending on the place installed, the cages can be stolen, since it is practically impossible to carry out constant vigil during the period of exposure.

This methodology can be applied in several aquatic ecosystems, but in the analyzed studies we only found *in situ* exposures carried out in rivers and streams. The technique was mainly used to assess sites contaminated by urban and agricultural effluents. As in "field collection" biomonitoring it is usual to choose a location as a reference site. The number of sites chosen to carry out the exposure varies widely between studies, ranging from two sites (a reference site and a contaminated site) as seen in Vieira et al. (2019) and ten sites (two reference sites and eight contaminated) as seen in Souza-Bastos et al. (2017). The exposure time varies widely between the analyzed studies, ranging from 2 to 120 days, and up to 6 months, as shown in Table 2.

Some studies used organisms from other taxons in parallel with the *in situ* study. To monitor a river impacted by effluents from local pineapple plantations, Echeverría-Sáenz et al. (2012) used *Daphnia magna* and *Lactuca sativa* in addition to specimens of *Poecilia gillii* and *Bryconamericus scleroparius*, and Fournier et al. (2016) used macroinvertebrate communities in parallel with *Parachromis dovii* to monitor rivers impacted by agricultural effluents.

The steps of this methodology are very similar to the "field collection" except for the use of cages and animals previously selected for the exposure. Thus, the next steps are similar to those mentioned in item 3.1.1, during exposure, many studies usually assess the quality of water and sediment as can be seen in Table 2.

After the exposure period, the animals are collected for the removal of tissues of interest for further analysis of biomarkers. Biochemical biomarkers were the most performed analyzes in this type of biomonitoring, representing a total of 37.5% of the total biomarkers used, followed by genotoxic (17.5%) and histological (12.5%) biomarkers. The list of biomarkers used can be seen in Table 2 and Figure 1.

3.1.3 Laboratory tests

When researching biomonitoring, we observed a less common methodology, which consists of collecting water in places possibly impacted by human activities, transporting this water to a research laboratory and exposing to it a species of fish of interest. Here in this review we call these studies “laboratory tests" since we have not found a term that defines this methodology. This methodology was mostly explored by Brazilian research groups totaling a total of 10 studies, followed by a study carried out by a Colombian study group (Figure 1) In general, the steps of this type of biomonitoring are very similar to those in point 3.1.1 and 3.1.2, basically changing the fact that the exposure is carried out in the laboratory.

Control groups for laboratory exposures are carried out in some different ways. Some studies collect water in places considered to be a reference (without the occurrence of effluents) as seen in Costa Silva et al (2015) which performed 48 hours of exposure in *D. rerio* using water collected from a river impacted by urban and rural effluents and used as a control the water collected in a location far from the city and with a large presence of riparian forest. Other researchs use an artificial medium as a control, as observed in Ribeiro et al. (2020), which used a reconstituted water (ISO, 1996) as control in a study with *D. rerio* embryos to assess the effects of water collected from a river impacted by wastewater treatment plants. In addition, positive controls in toxicological tests are also used, as in Ossa-López et al (2017), which used $HgCl_2$ in *D. rerio* in a biomonitoring of a river impacted by mining effluents.

In general, there is no pattern in the number of sites chosen, as seen in field collection and *in situ* exposure biomonitoring studies. In the study carried out by Ferreira et al. (2016) three sites were chosen, two impacted sites and one site as a reference, as well as Francisco et al. (2019) which carried out water collection in eight sites, all presenting different levels of environmental impact.

The main impacts evaluated are related to rivers and streams close to cities and crops, basically the same types of impacts of greater concern in the other types of biomonitoring. Two of the studies carried out aimed to evaluate rivers impacts by mining leading to pollution caused mainly by metals used in mining, as can be seen in Ossa-López et al (2017) and Macedo et al. (2020) who used *D. rerio* exposed for 12 hours and *Astyanax lacustris* for 168 hours, respectively. Another approach to environmental impact was taken by Walter et al. (2018) who exposed D. *rerio* embryos for 96 and 120 hours to assess the contamination caused by cyanobacteria in dams.

In most of the studies carried out, the water collected from the impacted sites was the main material used for the exposure. Sediment samples were used to carry out the exposure in Rocha et al. (2011) who performed toxicity tests with *D. rerio* embryos exposed for 48 and 96 hours to sediments from selected locations in the Tietê River Basin (river impacted by urban pollutants). Ferreira et al. (2016) conducted studies, exposing *Phalloceros caudimaculatus* for 8 hours to sediment samples from a watercourse impacted by urban discharges.

In general, the collection and transport of water and sediment are carried out in such a way as to preserve their physical-chemical characteristics, so researchers usually transport and keep these materials at low temperatures as described by Walter et al (2018) and Ferreira et al. (2016). The methods used to assess and quantify the presence of pollutants in samples varies from study to study, Severo et al. (2020) analyzed water samples to identify pesticides in a river located near to soybean and rice fields, performing the analysis by high-performance chromatography in accordance with Donato et al. (2015). To assess the presence of pollutants from agriculture and urban areas in river sediment, Rocha et al. (2011) used the freeze–drying technique, more details of each step of this process can be seen in their studies.

The number of animals used to carry out the studies with collected samples varied widely among the analyzed studies, ranging from 10 specimens of *D. rerio* used per site (Costa Silva et al., 2015) to 180 *D. rerio* embryos used per site (Severo et al., 2020). The exposure time also varied widely between the analyzed studies ranging from 8 hours to 168 hours, with the most common occurrence of 48 and 96 hours, the usual period of toxicology tests, as can be seen in Table 3.

Among the species used in this methodology, we observed that *D. rerio* appeared in most published articles, possibly because this species is widely used in research and has a vast bibliography about this animal research model. There is also a greater interest in using this species in its embryo stage, it is possible to carry out a series of morphological analysis due to the transparency of the egg and the organism itself, allowing to assess the development of the heart, eyes, somites between others (OECD 2013). In addition, this experimental model offers the practicality of its size, which exposure can be performed using petri dishes or 24/96-well plates. There are two toxicological tests used on zebrafish embryos to assess the effects of a compound known as Fish Embryo Acute Toxicity (FET) Test and Test Guideline 236 (TG236), and are considered standard for assessing acute toxicity at 96 hours' post-fertilization (hpf) (OECD 2013). In these tests, some observations may indicate toxicity in zebrafish embryos at 24, 48, 72 and 96 hpf. The complete list of species used can be seen in Table 3.

This biomonitoring technique allows the performance of analyzes carried out during the exposure, since the experiment is carried out in a controlled environment. Thus, there is a large amount of analyzes regarding possible deaths that may occur during this period and developmental changes, added together, these analyzes correspond to a total of 55.56% of the analyzes performed after exposure, this is due to the the fact that all these studies involve the use of *D. rerio* embryos, which allow such analyses. After the end of the exposure period, the use of the same techniques performed in the aforementioned biomonitoring is observed, involving genotoxicity (16.67%), biochemical (11.11%), molecular (11.11%) and histological biomarkers (5.56%). These data can be seen in Table 3 and Figure 1. It is observed that many biomarkers were not explored by this methodology, such as bioaccumulation, physiological indexes, endocrine disruption and hematological parameters.

3.2 FINAL CONSIDERATIONS

The studies analyzed demonstrated a wide variety of ways to perform biomonitoring studies. It is noted that many biomonitoring studies did not carry out any kind of physicochemical analysis in water, nor collect water samples to analyze the presence of pollutants. Even more unusual are the chemical analyzes of the sediment. One of the strategies adopted in some of these studies is to cite reports or

articles that demonstrate the presence of pollutants at the biomonitoring site, without necessarily carrying out the analyzes during the biomonitoring (Costa-Silva et al., 2015; Freire et al., 2015).

In our research, we found only two studies using exposure to sediment samples (Rocha et al., 2011; Ferreira et al., 2016), since sediment samples often hold different types of contaminants (de Andrade et al., 2018). It is necessary to carry out more studies using this type of exposure, which is widely used in studies outside Latin America (Barjhoux et al., 2017; Boehler et al., 2017; Boulanger et al., 2018).

Another approach that has not yet been explored is to use fish scales as a non-lethal tool of the toxicity of wastewater. Scales are the external structures of the fish's body and are in direct contact with contaminants present in the water. Some types of pollutants such as metals, are known to alter the structure of scales, causing malformations, changing patterns and fragility (Lin-Sun et al., 2009; Shikha and Sushma 2011; Sultana et al., 2017).

Among the methodologies aimed at replacing and decreasing the use of animals is the use of transgenic fish cell lines with reporter genes to detect certain contaminants, as an example the use of intestinal cells (RTgutGC) and gill cells line (RTgill- W1) (Langan et al., 2017; Scot et al., 2021). In this context, it is possible to perform ecotoxicological predictions, such as bioaccumulation in fish through software such as quantitative structure-activity relationship (QSAR). Its use is encouraged to reduce the use of animals, as well as a complementary tool in the initial screning of new contaminants with unknown biological effect due to its relationship of structure and activity with similar compounds. It is possible to predict some relevant points in ecotoxicity in aquatic environments and consequently to fish (Kobayashi et al., 2021).

This review presented the perspectives of how it is possible to carry out biomonitoring studies using fish as a study model. It is evident that there is no definitive way to carry out a biomonitoring study. There are techniques that may be incorporated in future biomonitoring studies, and we encourage the use more than one of the three mentioned biomonitoring techniques presented in this review which can be used in a complementary way.

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Table 1. List of studies using the biomonitoring methodology "field collection", between 2010 and 2020 (to be continued)

Species	Fish analysis	Water analysis	Sediment analysis	Reference
Centropomus parallelus; Mugil cephalus; Ariopsis felis; Eugerres axillaris; Oreochromis sp	Bioaccumulation of persistent organic pollutants (POPs), genotoxic biomarkers		POPs	González-Mille et al., 2010
Prochilodus lineatus	Biochemical biomarkers and bioaccumulation of lead (Pb)			Lombardi et al., 2010
Oreochromis niloticus	Genotoxic biomarkers and bioaccumulation of metals	Total alpha and beta radiation, metals, cyanobacteria analysis		Marcon et al., 2010
Cichla monoculus	Bioaccumulation of mercury (Hg) and pesticide, biochemical and histological biomarkers			Rabitto et al., 2011
Astyanax fasciatus; Pimelodus maculatus	Biochemical biomarkers, hematological parameters and physiological indexes	Metals, pesticide residues, physical-chemical parameters		Sadauskas-Henrique et al., 2011
Atherinella brasiliensis	Biochemical biomarkers and hematological parameters	Petroleum products and physical-chemical parameters	Petroleum products	Souza-Bastos et al., 2011
Cathorops spixii; Genidens genidens	Bioaccumulation of metals			Azevedo et al., 2012
Oreochromis niloticus	Biochemical biomarkers	Metals and physical-chemical parameters		Carvalho et al., 2012
Hoplias malabaricus	Bioaccumulation of Hg, biochemical, histological and genotoxic biomarkers			da Silva et al., 2012
Geophagus brasiliensis; Astyanax bimaculatus	Histological biomarkers			Gomes et al., 2012
Jenynsia multidentata	Biochemical and histological biomarkers	Metals, pesticide residues, and physical-chemical parameters	Metals and pesticides residues	Maggioni et al., 2012
Oncorhynchus mykiss	Bioaccumulation of POPs, physiological indexes	POPs		Ondarza et al., 2012

Table 1. (continued)

Species	Fish analysis	Water analysis	Sediment analysis	Reference
Astyanax sp.	Genotoxic biomarkers			Ramsdorf et al., 2012
Sciades herzbergii	Biochemical biomarkers and physiological indexes	Physical-chemical parameters, Metals	Metals	Carvalho-Neta et al., 2013
Cathorops spixii	Biochemical and histological biomarkers			Azevedo et al., 2013
Astyanax fasciatus; Pimelodus maculatus	Biochemical and histological biomarkers, bioaccumulation of metals and pesticides	Metals, pesticides residues, and physical-chemical parameters		Fernandes et al., 2013
Gymnotiformes (eight species)	Genotoxic biomarkers			Melo et al., 2013
Atherinella brasiliensis	Physiological indexes , bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) , biochemical, histological and genotoxic biomarkers			Ribeiro et al., 2013
Atheriniformes (one species); *Characiformes* (five species); *Cichliformes* (one species); *Cyprinodontiformes* (one species); *Siluriformes* (two species)	Bioaccumulation of arsenic	Physical-chemical parameters, arsenic	Arsenic	Rosso et al., 2013
Astyanax fasciatus; Pimelodus maculatus	Bioaccumulation of metals and pesticides, biochemical biomarkers	Metals, pesticides, and physical-chemical parameters		Sakuragui et al., 2013
Plagioscion squamossissimus; Lithodoras dorsalis	Biochemical and histological biomarkers			Viana et al., 2013
Astyanax bimaculatus	Biochemical biomarkers	Physical-chemical parameters		Batista et al., 2014
Hyphessobrycon luetkenii	Genotoxic biomarkers			Bühler et al., 2014
Astyanax sp.	Biochemical, histological and genotoxic biomarkers			da Silva et al., 2014

Table 1. (continued)

Species	Fish analysis	Water analysis	Sediment analysis	Reference
Rhamdia quelen	Genotoxic biomarker and citogenetic analysis	Physical-chemical parameters, fecal coliform, metals		de Campos Júnior et al., 2014
Hoplias malabaricus; Serrasalmus brandtii	Bioaccumulation of metals			de Jesus et al., 2014
Characiformes (six species); *Perciformes* (one species); *Siluriformes* (two species)	Genotoxic biomarkers			Furnus et al., 2014
Geophagus brasiliensis	Biochemical, endocrine disruption, genotoxic, and histological biomarkers, bioaccumulation of PAH		Metals	Osório et al., 2014
Astyanax fasciatus; Pimelodus maculatus	Histological biomarkers and bioaccumulation of metals and pesticides	Physical-chemical parameters, metals, pesticides		Paulino et al., 2014
Astyanax paranae	Genotoxic biomarkers			Ribeiro et al., 2014
Plagioscion squamosissimus; Lithodoras dorsalis;Geophagus proximus; Curimata inornat; Anchoa spinifer; Cathorops agassizii; Brachyplatystoma vaillantii	Bioaccumulation of metals			Serrão et al., 2014
Astyanax aeneus	Biochemical biomarkers and physiological indexes	Water quality index (WQI), physical-chemical parameters, and total and fecal coliform counts.		Trujillo-Jiménez et al., 2014
Astyanax bifasciatus	Biochemical and genotoxic biomarkers, physiological indexes	Physical-chemical parameters , emergent contaminants		Bueno-Krawczyk et al., 2015
Astyanax sp.	Biochemical biomarkers			Costa-Silva et al., 2015

Table 1. (continued)

Species	Fish analysis	Water analysis	Sediment analysis	Reference
Astyanax sp.	Biochemical, histological and genotoxic biomarkers, bioaccumulation of PAH, physiological indexes			Freire et al., 2015
Oreochromis niloticus	Bioaccumulation of microcystin, biochemical biomarker			Hauser-Davis et al., 2015
Astyanax sp.; Corydoras paleatus	Bioaccumulation of Hg		Mercury	Kütter et al., 2015
Astyanax jacuhiensis	Biochemical and genotoxic biomarkers	Physical-chemical parameters and pesticide residues		Loro et al., 20015
Astyanax bimaculatus	Biochemical biomarkers	Physical-chemical parameters		Ribeiro et al., 2015
Oreochromis aureus	Bioaccumulation of Hg			Ruelas-Inzunza et al., 2015
Oreochromis niloticus	In vitro transportability of mucus, genotoxic biomarkers and hematological parameters	Physical-chemical parameters, metals		Seriani et al., 2015
Astyanax fasciatus; Astyanax altiparanae; Characidium fasciatum	Genotoxic biomarkers	Physical-chemical parameters, metals	Physical-chemical parameters, metals	Silva et al., 2015
Plagioscion squamosissimus	Biochemical biomarkers and physiological indexes			Wunderlich et al., 2015
Oreochromis niloticus	Hematological parameters	Physical-chemical parameters		Corrêa et al., 2016
Gambusia affinis; Jenynsia multidentata	Pharmaceuticals bioaccumulation			Valdés et al., 2016
Tilapia rendalli; Hoplias malabaricus	Genotoxic biomarkers	Physical-chemical parameters, total and thermotolerant coliforms, trace elements analysis		Batista et al., 2016
Jenynsia multidentata; Phalloceros caudimaculatus	Molecular biomarker	Physical-chemical parameters	PAHs	Chivittz et al., 2016

Table 1. (continued)

Species	Fish analysis	Water analysis	Sediment analysis	Reference
Mugil curema	Bioaccumulation of PAH, physiological indexes			de Albergaria-Barbosa et al., 2016
Hypostomus ancistroides	Biochemical, histological, and genotoxic biomarkers, physiological indexes	Physical-chemical parameters		Ghisi et al., 2016
Jenynsia multidentata	Molecular biomarker, physiological indexes	Physical-chemical parameters, total coliforms		Guyón et al., 2016
Geophagus brasiliensis	Genotoxic biomarkers	Physical-chemical parameters, metals		Morais et al., 2016
Plagioscion squamosissimus	Erythrocyte cell cycle, genotoxic biomarker	Physical-chemical parameters		Rocha et al., 2016
Genidens genidens	Biochemical biomarkers			Sardi et al., 2016
Astyanax bifasciatus; Chrenicicla iguassuensis; Geophagus brasiliensis	Biochemical, histological and genotoxic biomarkers, bioaccumulation of PAH, physiological indexes	Physical-chemical parameters, microcystin-LR, and metals		Yamamoto et al., 2016
Geophagus brasiliensis	Physiological indexes, biochemical and genotoxic biomarkers, Paralytic Shellfish Toxins (PSTs) bioaccumulation	Physical-chemical parameters, phytoplankton and PSTs analyses.		Calado et al., 2017
Oreochromis niloticus	In vitro mucociliary transport, genotoxic biomarker, hematological parameters	Physical-chemical parameters		da Silva et al., 2017
Geophagus brasiliensis	Biochemical, Endocrine disruption, and histological biomarkers			Doria et al., 2017
Astyanax aff. paranae	Biochemical, histological and genotoxic biomarkers	Physical-chemical parameters		Ghisi et al., 2017
Astyanax bifasciatus	Biochemical biomarkers and physiological index	Physical-chemical parameters	Pesticide residues	Nimet et al., 2017

Table 1. (continued)

Species	Fish analysis	Water analysis	Sediment analysis	Reference
Astyanax altiparanae	Biochemical and genotoxic biomarkers	Physical-chemical parameters and pesticide residues	Pesticide residues	Vieria et al., 2017
Astyanax bifasciatus; Chrenicicla iguassuensis; Geophagus brasiliensis	Endocrine disruption and histological biomarkers, physiological index			Yamamoto et al., 2017
Pimelodus maculatus	Biochemical and genotoxic biomarkers, physiological indexes			Araújo et al., 2018a
Gobionellus oceanicus	Bioaccumulation of metals, biochemical and histological biomarkers	Physical-chemical parameters, metals	Metals	Araujo et al., 2018b
Astyanax jacuhiensis	Histological biomarkers			Batista et al., 2018
Bryconamericus iheringii	Biochemical, histological and genotoxic biomarkers, bioaccumulation of metals, physiological index	Physical-chemical parameters, metals, and microbiological analysis		Dalzochio et al., 2018
Loricariichthys anus	Biochemical biomarkers	Physical-chemical parameters, pesticide residues		do Amaral et al., 2018
Prochilodus lacustris	Gonadal stage macroscopic analysis, genotoxic and histological biomarkers	Physical-chemical parameters, microbiological analysis, metals		Cardoso et al., 2018
Characiformes (four species); *Siluriformes* (four species)	Bioaccumulation of pesticides			Ernst et al., 2018
Geophagus brasiliensis	Bioaccumulation of Hg		Mercury	Furlan et al., 2018
Odonthestes bonariensis	Histological biomarkers	Physical-chemical parameters		Leone et al., 2018
Oreochromis niloticus	Bioaccumulation of metals, biochemical biomarker	Physical-chemical parameters, metals		Muñoz-Nájera et al., 2018
Ancistrus mullerae	Histological biomarkers	Physical-chemical parameters		Neves et al., 2018
Astyanax bifasciatus	Histological biomarkers	Physical-chemical parameters		Nimet et al., 2018

Table 1. (continued)

Species	Fish analysis	Water analysis	Sediment analysis	Reference
Hatcheria macraei; Salmo trutta; Oncorhynchus mykiss; Oncorhynchus tshawytscha	Molecular biomarker	Physical-chemical parameters		Assef et al., 2019
Oncorhynchus mykiss	Bioaccumulation of metals			Barrientos et al., 2019
Hoplias malabarius; Geophagus brasiliensis	Bioaccumulation of metals		Metals	Beraldi et al., 2019
Geophagus brasiliensis	Bioacumulação Paralytic shellfish Toxins (PSTs)	Physical-chemical parameters, PSTs, phytoplankton analysis		Calado et al., 2019
Centropomus undecimalis	Biochemical, immunohistochemistry, and genotoxic biomarkers			Cardoso et al., 2019
Hoplerythrinus unitaeniatus; Hoplias malabaricus; Pterygoplichthys pardalis	Bioaccumulation of Hg			da Silva et al., 2019
Rhamdia quelen; Hypostomus commersoni; Hoplias lacerdae; Prochilodus lineatus	Bioaccumulation of contaminants of emerging concern (CEC)	Physical-chemical parameters		Ondarza et al., 2019
Trichomycterus areolatus; Percilia irwini	Biochemical and histological biomarkers, physiological indexes	Passive sampling of contaminants with sex steroid receptors binding role		Orrego et al., 2019
Prochilodus argenteus.	Bioaccumulation of metals, histological and immunohistochemistry biomarkers, physiological indexes			Paschoalini et al., 2019
Colossoma macropomum	Genotoxic and histological biomarkers	Physical-chemical parameters, metals		Pinheiro-Sousa et al., 2019
Micropogonias furnieri	Bioaccumulation of POPs			Pizzochero et al., 2019

Table 1. (continued)

Species	Fish analysis	Water analysis	Sediment analysis	Reference
Heptapterus mustelinus	Biochemical and histological biomarkers, physiological indexes	WQI, pharmaceuticals and pesticide residues		Vreys et al., 2019
Astyanax bifasciatus	Genotoxic biomarkers			Wachtel et al., 2019
Characiformes (nine species); *Cichliformes* (three species); *Osteoglossiformes* (one species); *Perciformes* (one species); *Siluriformes* (ten species)	Bioaccumulation of Hg, Cd, and Se			Alcala-Orozco et al., 2020
Plagioscion squamosissimus; Colossoma macropomum	Biochemical and molecular biomarkers, bioaccumulation of Hg	Physical-chemical parameters		Bittarello et al., 2020
Geophagus brasiliensis	Biochemical, genotoxic, and osmoregulatory biomarkers			Calado et al., 2020
Mugil curema	Genotoxic biomarker, hematological parameters, physiological indexes	Physical-chemical parameters		Cicero et al., 2020
Characiformes (nine species); *Cichliformes* (two species); *Clupeiformes* (one species); *Perciformes* (one species*); Siluriformes* (four species)	Bioaccumulation of Hg			da Silva and Lima, 2020
Serrasalmus Rhombeus	Biochemical, histological and genotoxic biomarkers, bioaccumulation of metals	Physical-chemical parameters, metals	Metals	Montes et al., 2020
Hypostomus spp. (eight species)	Nitrogen stable isotopic analysis			de Carvalho et al., 2020
Loricariichthys anus; Geophagus brasiliensis	Biochemical biomarkers	Physical-chemical parameters, pesticide residues, metals	Pesticide residues	do Amaral et al., 2020

Table 1. (continued)

Species	Fish analysis	Water analysis	Sediment analysis	Reference
Psalidodon bifasciatus	Biochemical, histological and genotoxic biomarkers			Ghisi et al., 2020
Astyanax jacuhiensis	Biochemical biomarkers, bioaccumulation of pesticides	Pesticide residues		Gonçalves et al., 2020
Astyanax aeneus	Biochemical biomarkers and bioaccumulation of metals			Hinojosa-Garro et al., 2020
Hypostomus francisci	Histological and immunohistochemistry biomarkers			Macêdo et al., 2020
Astyanax fasciatus; Astyanax jacuhiensis	Biochemical biomarkers, physiological indexes	Physical-chemical parameters, pesticide residues	Pesticide residues	Marins et al., 2020

Table 2. List of studies using the biomonitoring methodology "*in situ* exposure", between 2010 and 2020 (to be continued)

Species	Life stage	Exposure time	Fish analysis	Water analysis	Sediment analysis	Reference
*Bryconamericus scleroparius, Poecilia gillii**	Adult and juvenile	48 hours	Biochemical biomarkers	Pesticide residues and physical-chemical parameters		Echeverría-Sáenz et al., 2012
*Danio rerio**	Juvenile	15 days	Molecular biomarkers	Physical-chemical parameters		Rodríguez-Fuentes et al., 2012
Prochilodus lineatus	Juvenile	96 hours	Biochemical biomarkers, hematological parameters, and physiological indexes	Bacteriological analyses and physical-chemical parameters		Cazenave et al., 2014
*Parachromis dovii, Poecilia gillii**	Adult and juvenile	48 hours	Biochemical biomarkers	Pesticide residues		Mena et al., 2014
Astyanax altiparanae		7 days	Biochemical and genotoxic biomarkers	Physical-chemical parameters		Vieira et al., 2014
*Oncorhynchus mykiss**	Juvenile	11, 21, and 31 days	Biochemical, endocrine disruptions, and histological biomarkers, physiological indexes	Physical-chemical parameters		Chiang et al., 2015
*Oreochromis niloticus**	Juvenile	6 months	Bioaccumulation of metals and PAH, biochemical, endocrine disruption, and histological biomarkers, physiological indexes			dos Santos et al., 2016
Parachromis dovii	Juvenile	48 hours	Biochemical biomarkers	Pesticide residues and physical-chemical parameters		Echeverría-Sáenz et al., 2016
Prochilodus lineatus	Juvenile	96 hours	Biochemical and genotoxic biomarkers	Metals, pesticide residues, and physical-chemical parameters	Metals	Vieira et al., 2016
Parachromis dovii	Juvenile	48 hours	Biochemical biomarkers	Pesticide residues and physical-chemical parameters		Fournier et al., 2017

Table 2. (continued)

Species	Life stage	Exposure time	Fish analysis	Water analysis	Sediment analysis	Reference
Rhamdia quelen	Juvenile	7 days	Biochemical biomarkers and hematological parameters	Physical-chemical parameters		Souza-Bastos et al., 2017
Astyanax altiparanae	Adult	7 days	Biochemical and genotoxic biomarkers	Pesticide residues and physical-chemical parameters	Pesticide residues	Vieira et al., 2017
Prochilodus lineatus	Juvenile	96 hours	Bioaccumulation of metals, biochemical and genotoxic biomarkers	Metals and physico-chemical parameters	Metals	Lunardelli et al., 2018
*Oreochromis niloticus**	Adult	60 days	Bioaccumulation of metals and PAHs, biochemical, genotoxic, and histological biomarkers	Metals		Santana et al., 2018
*Oreochromis niloticus**	Juvenile	60 days	Biochemical, endocrine disruption, histological, and molecular biomarkers	Persistent organic pollutans		Yamamoto et al., 2018
Prochilodus lineatus	Juvenile	5, 15, 30, 60, 90, and 120 days	Bioaccumulation of metals and pesticides, biochemical, genotoxic, and histological biomarkers	Metals, pesticide residues, and physical-chemical parameters	Metals and pesticide residues	Vieira et al., 2019
Astyanax lacustris	Adult	96 hours	Genotoxic biomarkers	Metals and physical-chemical parameters	Metals and physical-chemical parameters	da Silva et al., 2020

* Exotic fish species

Table 3. List of studies using the biomonitoring methodology "laboratory tests", between 2010 and 2020.

Species	Life stage	Exposure matrix	Exposure time	Fish analysis	Water analysis	Sediment analysis	Reference
Hyphessobrycon luetkenii		Water	48 hours	Genotoxic biomarkers	Metals		Scalon et al., 2010
*Danio rerio**	Embryo	Sediment	48 and 96 hours	Lethal and morphological endpoints			Rocha et al., 2011
*Danio rerio**	Adult	Water	48 hours	Biochemical biomarkers	Physical-chemical parameters		Costa Silva et al., 2015
Phalloceros caudimaculatus	Adult	Sediment	8 hours	Molecular biomarker			Ferreira et al., 2016
*Danio rerio**	Adult	Water	12 hours	Genotoxic and molecular biomarkers	Physical-chemical parameters		Ossa-López et al., 2017
Rhamdia quelen	Embryo	Water	24, 48, 72 and 96 hours	Lethal and morphological endpoints	Metals and PAH		Brito et al., 2018
*Danio rerio**	Embryo	Water	96 and 120 hours	Lethal and morphological endpoints	Physical-chemical parameters, metals, cyanobacteria abundance, cyanotoxin determination		Walter et al., 2018
Astyanax altiparanae	Immature	Water	168 hours	Genotoxic biomarkers	Physical-chemical parameters and metals	Metals	Francisco et al., 2019
Astyanax lacustris	Immature	Water	168 hours	Histological and immunohistochemistry biomarkers	Metals		Macêdo et al., 2020
*Danio rerio**	Embryo	Water	144 hours	Lethal and morphological endpoints	Physical-chemical parameters and metals		Ribeiro et al., 2020
*Danio rerio**	Embryo	Water	96 hours	Lethal and morphological endpoints, biochemical biomarkers	Physical-chemical parameters, pesticide residues		Severo et al. 2020

* Exotic fish species

FIGURES

Figure 1. Number of scientific articles published by Latin American countries between 2010 and 2020 and percentage of the main biomarkers used in each biomonitoring methodology.

5 ARTIGO 2

Ecological risk of pesticide contamination in a Brazilian river located near a rural area: A study of biomarkers using zebrafish embryos

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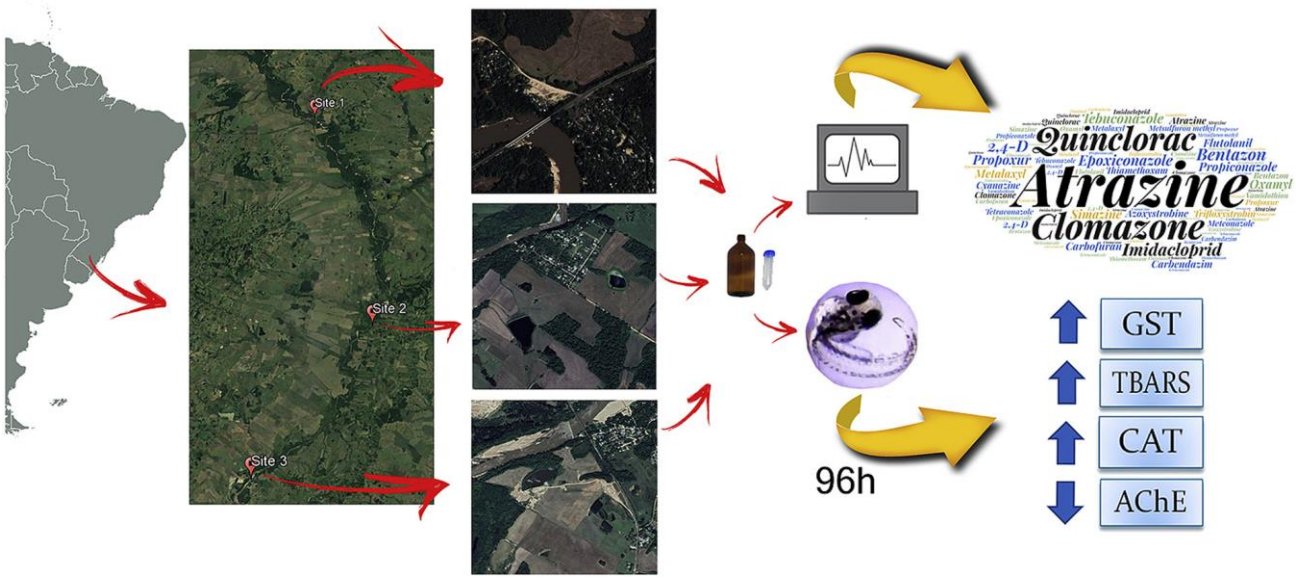
ABSTRACT

Aquatic environments are affected by the use of pesticides in agricultural areas near rivers. To assess the impact of pesticide residues on affected environments Danio rerio (zebrafish) embryos have become an alternative model for biomonitoring studies. In the present study, zebrafish embryos were used as bioindicator of water quality in the Vacacaí river, located in the city of Santa Maria, southern Brazil. We hypothesized that it would be possible to observe changes in the biomarkers tested in the embryos. Exposures were performed over a total of eight months during the year 2018 using water collected in a river located near agricultural areas. Twenty-four pesticides were found in river water samples. The most frequently found were atrazine, quinclorac and clomazone. During exposure (96 h) spontaneous movement, the heart rate and hatching rate were evaluated. After the exposure time the embryos were euthanized for biochemical assays. We analyzed biomarkers such as thiobarbituric acid reactive substance (TBARS), acetylcholinesterase (AChE), glutathione S-transferase (GST) and catalase (CAT). We observed increases in GST and TBARS, especially during periods of major water contamination such as January, February, October, and November. Pesticides can affect the development of native species that reproduce during periods of high agricultural production. These results demonstrate the potential use of biochemical parameters combined with developmental and behavioral analyses in zebrafish embryos for biomonitoring studies.

KEYWORDS: Biomonitoring. Pesticides. Toxicity. Bioindicators. Ecotoxicology

GRAPHICAL ABSTRACT



1 INTRODUCTION

Agricultural production in Brazil has grown rapidly in recent years. High production demand has led to an increase in the use of chemicals such as fertilizers and pesticides (Boccolini et al., 2013). The application of these products does not respect natural barriers like relief and riparian forest, since it is almost impossible to control their course in the field. This type of contamination is usually called diffuse or nonpoint, reaching sites distant to their introduction point through wind, superficial runoff, erosion and leaching (Boehler et al., 2017; Tran et al., 2019). The dispersion of these products can reach non-target organisms such as fish, which can suffer physiological changes and, in some cases, may even die. In order to evaluate the possible impact of these contaminants on the local fauna, studies of organisms caught at contaminated sites have been performed (Amado et al., 2005; Azevedo et al., 2013; Do Amaral et al., 2018). Quantitative chemical analysis is used to evaluate the pollutant amounts in the environment, but it cannot demonstrate the possible effect on aquatic fauna. In this way, the use of bioindicators in biomonitoring studies has been chosen as an important method to complement traditional water and sediment monitoring techniques (Soininen et al., 2004). There are several ways to perform a biomonitoring study. The most common is the capture of animals using gillnets, and fishing rods (Loro et al., 2015; Do Amaral et al., 2018). These methodologies, although classic, have disadvantages such as the variation in size, age, and weight of the fish caught, as well as stress caused in the animals. Another disadvantage is the uncertainty of weather the animal was in the affected site at the time of its collection because the fish may have migrated from another region where they may have been contaminated by unknown products or may not have been contaminated (Oikari, 2006).

A possible alternative for studies of biomonitoring of aquatic environments is the use of zebrafish embryos (Danio rerio). This model is well established in toxicology tests but has also been gaining relevance in biomonitoring studies (Rocha et al., 2011; Zhang et al., 2015). The use of zebrafish embryos has several advantages, such as the transparent chorion, which permits observation of its development. The ability to produce several thousand embryos daily under laboratory conditions and use them for parallel experimental treatments with a low maintenance cost is another vantage of this model. In addition, zebrafish embryos offer the possibility of carrying

out small-scale analysis and are an alternative to correlation with conventional in vivo tests with adult fish (Scholz et al., 2008; Lammer, 2009). According to Stelzer et al. (2018), the use of the Fish Embryo Acute Toxicity (FET) test has been spreading worldwide as a reliable method for effluent risk assessment. The FET is becoming a substitute for previous fish tests with larvae and juveniles, not only in the assessment of chemicals but also for environmental samples and wastewater.

The Brazilian Pampa Biome was only designated a biome in 2004 (IBGE, 2004). This biome is impacted by intensive farming of soybeans and rice. In the river where the study was performed there are species of fish considered vulnerable, in addition, many species of fish reproduce in the summer (ICMBIO, 2018). There are no studies evaluating possible the impact of pesticide use in this region. Considering the hypothesis that the use of fish embryos is important for evaluating impacted environments, the goal of this study was to evaluate behavioral and development parameters as well biochemical biomarkers in zebrafish embryos exposed to the water of a river located in a rural region of southern Brazil. There are few studies relating these data and the use of biochemical biomarkers in embryos is still relatively unexplored. In addition, this study aims to evaluate the potential risks of pesticides to the aquatic ecosystem of the river using a valid model for biomonitoring studies.

1 MATERIALS AND METHODS

2.1 STUDY SITE AND WATER ANALYSIS

The sites chosen for this study are located in the Vacacaí river. The river passes throught rural area of Santa Maria, Rio Grande do Sul, Brazil. The first study site (S1) is located near Passo do Verde (29° 56′11.6″S; 53°42′40.8″W), the surrounding region is heavily used for agriculture. Riparian forest is almost non-existent in this area. The second study site (S2) is located in the district of Arroio do Só (29°52′12.1″S; 53°32′19.9″W) and this region has riparian forest on the river bank. There are several monoculture areas of soybeans and rice a few meters from the river. Site 3 (S3) is located at Praia das Tunas (29°55′29.8″S; 53° 24′55.2″W).

This region is the most populous of the three sites, and a few meters from the river are several areas used by agriculture. The distance between the collection sites is around 15 km and the farthest sites (S1 and S3) is at distance of about 30 km.

Water was collected at the sampling sites in summer (January and February), autumn (May and June), winter (August and September), and spring (October and November), during the year of 2018. The water was collected in 500 mL amber bottles and placed immediately on ice.

The water samples were analyzed by high-performance chromatography for pesticides, in accordance with Donato et al. (2015). The following parameters such as temperature, pH, dissolved oxygen, turbidity, conductivity and total dissolved solids were determined with the aid of multiparameter water quality equipment (Horiba). At each collection point, water samples were collected in 50-mL falcon tubes (in triplicate) and were frozen for posterior use in zebrafish embryo exposure assays. The methodology used was according to Zhang et al. (2015).

2.2 ZEBRAFISH MAINTENANCE AND REPRODUCTION

Adult Danio rerio (wild-type) were kept in 25 L aquariums, each aquarium containing 6 females and 12 males. The matrices were fed twice daily with commercial feed, supplemented with Artemia sp. once daily. The fish were kept with a photoperiod of 14 h light/10 h dark and appropriate water conditions (pH 7.2, 28 °C). Adult zebrafish were obtained from a local store (Hobby Aquários) in Santa Maria, Rio Grande do Sul, Brazil.

The zebrafish embryos used in the present work were obtained from the reproduction of adult specimens and maintained in a room with a temperature of 28 °C and photoperiod of 14 h light/10 h dark following the protocol Fish Embryo Acute Toxicity (FET) test (OECD, 2013). After breeding the zebrafish embryos were collected, immersed in E3 medium (solution to cleaned fish embryos) and placed on a petri plate for random selection, according to Westerfield (2000). The experimental protocols used in this study were approved by the local Ethics committee (CEUA – UFSM: protocol 4735080419).

2.3 EXPOSURE OF ZEBRAFISH EMBRYOS TO COLLECTED WATER

Toxicological assays were adapted from Fish Embryo Acute Toxicity (FET) Test (OECD, 2013). The embryos were placed in 24-well plates with a total of 5

embryos per well. The wells were filled with 2 mL of the water collected in item 2.1 within 2 h post-fertilization (hpf). Sixty embryos were exposed per group (Control, S1, S2 and S3) for a total of 240 embryos in each experiment. A total of 30 embryos for each group (Control, S1, S2 and S3) were used to study spontaneous movement, and heart rate and other 30 were used for biochemical analysis.

2.4 MORTALITY AND HATCHING RATE

Following treatment, embryo mortality was evaluated at 24 hpf. Embryo viability was analyzed by observation of egg coagulation. The hatching rate was assessed at 96 hpf. All parameters described here were assessed according to the (FET) test (OECD, 2013). Images were obtained with the aid of a Vastar Mega Pixels 500X microscope and observed with Many-Cam.

2.5 SPONTANEOUS MOVEMENT

The spontaneous movement (bends) of zebrafish embryos was assessed under a microscope and recorded with ManyCam by counting the number of total body axis movements performed by embryos within their chorions (Xia et al., 2017). Following treatment, 30 embryos from each group were selected at random for the test. Embryos were counted

2.6 HEART RATE

The heart rate of embryos was measured at the 48 hpf time point. The heart beats were counted with the aid of a Vastar Mega Pixels 500X microscope and recorded with ManyCam. Following treatment, 30 embryos from each group were selected at random and adapted for 2 min in Petri dishes containing water and their heart rate was measured for 1 min, according to Xia et al. (2017).

2.7 BIOCHEMICAL BIOMARKERS

After 96 h of exposure, 30 zebrafish larvae were placed in a plastic micro-tube and euthanized under 60 min of hypothermia. A total of 6 micro-tubes were used for

each treatment. After water removal, 200 µl of Tris-HCl 50 mM buffer (pH 7.4) were added and the samples homogenized with a glass cane, centrifuged (3000 g for 10 min, - 4 °C). The supernatant was transferred to microtubes and kept at −80 °C for subsequent assays. Lipid peroxidation levels were estimated by thiobarbituric acid-reactive substance (TBARS) production. Results were expressed as nmol MDA/mg of protein, according to Draper and Hadley (1990). Catalase (CAT) activitiy was expressed as µmol/min/mg protein, according to Nelson and Kiesow (1972). Glutathione S-transferase (GST) activity was analyzed according to Habig (1974). The activity was expressed as µmol GS-DNB/min/mg protein. Acetylcholinesterase (AChE) activity was determined according to Ellman (1961), and expressed as µmol Sch/min/mg of protein. Further details of the biochemical analysis can be found in Nunes et al. (2016) and Leitemperger (2019).

2.8 STATISTICAL ANALYSIS

The data were tested for normality and homogeneity according to the methods of Kolmogorov-Smirnov and Levene, respectively. Statistical comparisons were performed with two-way ANOVA (factor 1 local, factor 2 month), and the Tukey post hoc-test. Correlation among biomarkers and pesticide water residues was performed by using Spearman correlation analysis. Results were considered significant at $P < 0.05$. Statistical analyses were performed with the software
GraphPad Prism version 6.01. Results are presented as means ± standard error of the mean (SEM).

3 RESULTS

3.1 ENVIRONMENTAL DATA

Twenty-four pesticides were detected in the water: 10 fungicides, 8 herbicides and 6 insecticides. The active ingredients found, in decreasing frequency order were atrazine, quinclorac, clomazone, imidacloprid, tebuconazole, epoxiconazole, tetraconazole, propiconazole, bentazon, propoxur, trifloxystrobin, metsulfuron methyl, azoxystrobin, thiamethoxam, carbofuran, metalaxyl, flutolanil, 2,4-D, carbendazim, simazine, metconazole, cyanazine, oxamyl, and vamidothion. The months with the

highest pesticide presence were, in descending order; October, February, November, January, May, June, September, and August. The S3 presented more pesticide residues than S1 and S2. All these data are present in Table 1. The physical and chemical parameters of the water were in agreement with the standards of animal health care (Supplementary material Table 1).

3.2 MORTALITY RATE, SPONTANEOUS MOVEMENT, HEART RATE AND HATCHING RATE

The mortality rate was below 10% at 24 hpf in all groups (Control, S1, S2 and S3) and there were no significant differences between exposure groups. Spontaneous movement was not significantly different between the control group and the studied sites in any month nor and among the control groups of each month. Spontaneous movement increased in S1 in September relative to January, February, May, June, August and November. There were no significant differences in any month for S2 and S3 (Fig. 1a). There were no significant differences in heart rate between the control group and the studied sites in any given month nor among the control groups of each month. Heart rate increased in January and February in S1 in comparison with other months, and in August there was an increase compared with October. In S2 there was an increase in January, relative to June, August, September, October and November, an increase in February relative to September and October, and an increase in May, October and August in relation to October. In S3, there was only an increase in January relative to the other months, as well as an increase in February, June, August and November relative to October (Fig. 1b).

The hatching rate was not significantly different between the control group and the studied sites in any given month nor among the control groups of each month. The hatching rate in S1 was higher in May relative to August, September and October. In S2 the hatching rate in May was higher than in February, August, September and October. In S3 May presented a higher hatching rate than August, September and October (Fig. 1c).

3.3 BIOCHEMICAL ANALYSIS

The TBARS levels in S1 were higher in February compared with the control group and with all other months. The months of January, May, June and August had higher levels of TBARS compared with September. In S2, there was an increase in TBARS levels in February relative to the control group and to all other months, and in January TBARS were increased relative to the control group and relative to the months of October and November. In S3 TBARS levels in February were higher compared with the control and relative to all other months. The month of January presented a significant increase in relation to its control and to September (Fig. 2a).

The GST activity was not significantly different among control groups within the studied sites in any given month nor control groups of each month. In S1, GST activity was the highest in January compared with February, August, September and October. In S2, GST activity in January was higher relative to the months of February, May, August, September and October. In S3, GST activity was not significantly different between the months (Fig. 2b).

The CAT activity in S1 was higher in October than in January. No significant differences were observed among control groups within the studied sites in all months nor among control groups within each month. In S2, CAT activity was higher in October compared with its control group in January, February, May, June, August, September, and November but was not significantly different between control groups in any given month. In S3, CAT activity was higher in February than in to August, and there was not significant difference between control groups in any given month (Fig. 3a).

There was no significant difference in AChE activity between the control groups of the studied sites in all months nor among the control groups of each month. In S1, AChE activity decreased in January, May, June, August, October and November relative to February. In S2, AChE activity decreased in May, June, August, September and November relative to October. In S3, AChE activity decreased in January, May, June, August, October and November relative to February and, January and May decreased in relation to September (Fig. 3b).

Only the data considered as significantly different were placed in Spearman correlation tables. The correlation coefficient, rs, ranged from −1 to +1, where from 0 to 1 means the two variables tend to increase or decrease together and from −1 to 0

means one variable increases and the other decreases. Spearman correlation results are presented in Table 2.

4 DISCUSSION

The presence of pesticide residues was observed during the eight months of water collection. Besides the great distance between points, the presence of pesticides followed a very similar distribution at the three collection sites. The southern region of Brazil is the fourth largest agricultural producer in the country. The cycles of preparation and harvesting of rice and soybeans are consistent with the highest concentrations of pesticides found in the river (spring and summer). This same period consists of the reproduction phase of fishes, demonstrating an alert to the harmful effects of pesticides on native species populations. Only two of the pesticides found in our water collections were described in the last report provided by the National Council of the Environment (CONAMA, 2005), the government agency responsible for determining the maximum allowable amounts of chemicals in waters of the Brazilian territory. Those substances, 2,4-D and atrazine, were found in amounts below that allowed by law which is 30 µg/L and 2 µg/ L, respectively.

The problem found with pesticides is the sum of many different chemicals in the water, that could affect aquatic organisms in different ways and also influence their reproductive patterns.

As expected the FET test of water from the chosen sampling sites showed alterations in fish embryo biomarkers that, could be related to pesticides found in the water. Pioneering studies suggest that chemicals can, in many cases have very similar toxicological and teratological effects in zebrafish embryos and humans (Yang et al., 2009). Our study focused in places where aquatic contamination is not evident, but organisms could be exposed to a sum of different chemicals. Probably because of the low levels of pesticides found, no abnormalities were observed in the embryos during the exposure period. The embryo mortality rate did not exceed 10% and did not show significant differences during exposure. No significant differences were shown between control groups. This supports the hypothesis that significant changes observed in other parameters between exposure sites occur due to differences between river water samples.

Spontaneous movement is the first movement performed by the zebrafish embryo at around 28 hpf and occurs due to the movement of the spinal cord triggered by the development of motor neurons that innervate the muscles. According to Xia et al. (2017), a decrease in spontaneous movement suggests some type of

neurotoxicity and may be a reason delayed hatching. In our study, we only observed an increase in the number of spontaneous movements in September. This could not be related to the level of pesticides recorded. An increase in the rate of spontaneous movement leading to acceleration of the hatching process of zebrafish embryos was reported after exposure to an insecticide (Jin et al., 2009). According to Wang et al. (2018), hatching is an important stage in the life cycle of fish, and a combination of biochemical and physical mechanisms regulates the process. We observed a delay in the hatching rate in February (S2), August, September and October. This reduction in the hatching rate was probably due to exposure to contaminants. Qian et al. (2018) observed a decrease in the hatching rate when zebrafish embryos were exposed to the herbicide boscalid and attributed alterations in hatching enzymes to this compound. A delay in hatching time also increases vulnerability to predation according to Todd and Leeuwen (2002). In the context of the present study, reduced hatching represented alterations in reproduction and increase the vulnerability of the organisms.

The heart is one of the first functional organs developed in zebrafish, and the heart rate is an important toxicology response in the fish embryonic test (Glickman and Yelon, 2002). Some studies involving fungicides and herbicides indicated a reduction in the heart rate. These injuries to the heart could lead to a reduction of blood transport in embryos and then affect energy transport, further influencing embryo development (Aksakal and Ciltas, 2018; Qian et al. (2018)). We noticed a decrease mainly in the month of October. This month showed amounts of pesticides that could explain, at least in part, the alterations observed in the heart rate.

Oxidative stress biomarkers are widely used in biomonitoring works, as well as in laboratory studies. The responses of these biomarkers are well established in several studies (Vieira et al., 2016; Marins et al., 2018). Lipid peroxidation is a common response observed after pesticide exposure and is usually estimated by TBARS. Contact with pesticides leads to increased free radicals and can lead to oxidative cell injury (Clasen et al., 2018). We observed an increase of TBARS mainly in the months of January and February, in which Clomazone and Propoxur showed positive correlation. These months also represent the highest concentrations of pesticides found during the analysis. Notably, Quinclorac was found in concentrations ten times greater than those found during winter. There are no studies showing the effects of clomazone on zebrafish embryos however, exposure of

Cyprinus carpio to this compound presented increased TBARS levels (Toni et al., 2013). We cannot say that this compound is responsible for the lipid damage observed, but it should be noted that the result is related to its high concentration, as well as the presence of other compounds that may have acted together to promote lipid peroxidation. It is possible that the antioxidant system was not able to avoid damage. This is concordant with the increase in GST levels observed in February.

GST is a phase II enzyme that conjugates xenobiotics or their metabolites with glutathione, making them less toxic and more easily excreted. This enzyme has been used as biomarker in biomonitoring studies (Loro et al., 2015; Scarcia et al., 2009) and in studies with zebrafish embryos (Costa-Silva et al., 2018). An increase in the activity of this enzyme was observed in zebrafish embryos only in January. We expected but did not observe a similar increase in February. On the other hand, in February a very significant increase in lipid damage was observed, perhaps due to enzymatic detoxification failure. Spearman analysis showed a positive correlation of GST with azoxystrobin, quinclorac and simazine in October and with bentazon and carbofuran in November. This result shows the role of this enzyme in protecting the zebrafish embryo from pesticide toxicity.

Contact with pesticides leads to an increase in reactive oxygen species (ROS), which are responsible for oxidative stress. The CAT enzyme is important for protecting the organism against ROS converting H2O2 into H2O and O2. Several studies have shown an increase in the activity of this enzyme in aquatic organisms exposed to toxicants (Nunes et al., 2016; Muller et al., 2017). The highest levels of this enzyme in zebrafish embryos were observed in October, and the activity of this enzyme remained high in the months when more pesticides were found in the water. Clomazone, Metalaxyl and Thiamethoxam showed positive correlations with this antioxidant enzyme, showing that a increase in these pesticides could increase the generation of ROS. In this way, CAT activity would be a means of reducing their effects in the zebrafish embryo. Clomazone is considered persistent in the environment and appeared at all three sites, Murussi et al. (2015) showed a relationship of the increase of CAT with this herbicide in livers of C. carpio.

Inhibition of AChE can result in excessive stimulation of cholinergic nerves, resulting in behavioral alterations such as tremors, convulsions, and erratic or lethargic swimming (Payne et al., 1996; Fernández-Vega et al., 2002; Miron, 2005). In May, the carbamate Propoxur was found; coincidentally, this month showed one of

the highest levels of AChE inhibition. This enzyme is usually inhibited by organophosphates and carbamates; however, studies indicated that it may also be inhibited by the presence of other pesticides such as clomazone and quinclorac (Miron, 2005; Moraes et al., 2007; Pretto et al., 2011). The enzyme AChE showed a negative correlation with propoxur in May but also with other pesticides such as Atrazine, Clomazone, Imidacloprid and Quinclorac. This means that the decrease in this enzyme may be related to the increase in pesticides in general.

Biomarkers such as GST and TBARS have been demonstrated to be strongly allied to FET use, since they showed significant responses in the month with the highest contaminant concentration. The results presented in the correlation help to corroborate the alteration in behavioral parameters and development analysis. All changes are related to biochemical alterations. The zebrafish embryo is a great tool that can be used in an integrated way with water quality parameters to verify the influence of pesticide residues in biological systems. Thus, it is possible to conduct toxicity studies with realistic concentrations occurring in the environment.

Authors Credit statements

Eduardo S. Severo - Writing- Original draft preparation, execution of experimental methods. Statistical analysis and graphical preparations., Aline Marins and Cristina Cerezer – biochemical analysis and specifically critical review, commentary or revision – including pre-publication stages., Dennis Costa and Mauro Nunes – Development of FET test in our experimental conditions and laboratory structure. Dennis and Mauro help Eduardo to beginning the experiment and also to analyze behavior of fish embryos., Osmar D. Prestes and Renato Zanella – Support all materials and methodologies needed to analyze water pesticides in samples of the present study., Vania Loro - Management and coordination responsibility for the research activity planning and execution; Funding acquisition and also acquisition of the financial support for the project leading to this publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.110071.

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TABLES

Table 1. Pesticide residues (µg/l) found in water samples in the Vacacaí river, RS, Brazil (*to be continued*)

Pesticide	*Class*	*LOD*	*LOQ*	*January*			*February*			*May*			*June*		
				S1	*S2*	*S3*	*S1*	*S2*	*S3*	*S1*	*S2*	*S3*	*S1*	*S2*	*S3*
2.4 D	H	0.006	0.02	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Atrazine	H	0.006	0.02	0.100	0.090	0.110	0.066	0.079	0.075	0.048	0.065	0.065	0.106	0.062	0.062
Azoxystrobine	F	0.006	0.02	<LOQ		<LOQ	0.083	0.076	0.075						
Bentazon	H	0.240	0.80	3.100	2.700	1.300									
Carbofuran	I	0.006	0.02				0.024	<LOQ	<LOQ						
Carbendazim	F	0.006	0.02	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Clomazone	H	0.006	0.02	0.490	0.570	0.590	0.180	0.207	0.215	0.209	0.240	0.237	0.351	0.291	0.359
Cyanazine	H	0.006	0.02											<LOQ	
Epoxiconazole	F	0.006	0.02	0.030	0.030	0.030	0.093	0.080	0.089	<LOQ	<LOQ				
Flutolanil	F	0.006	0.02												
Imidacloprid	I	0.006	0.02	0.250	0.170	0.120	0.821	0.652	0.651	0.037	0.042	0.036	0.043	0.055	0.051
Metalaxyl	F	0.006	0.02				<LOQ		<LOQ						
Metconazole	F	0.006	0.02												
Metsulfuron methyl	H	0.006	0.02											<LOQ	
Oxamyl	I	0.040	0.13						<LOQ						
Propiconazole	F	0.006	0.02				<LOQ	0.027	0.027	0.020	<LOQ				
Propoxur	I	0.012	0.04	0.010	0.010	0.020	0.058	0.060	0.061	0.029	0.033	0.026			
Quinclorac	H	0.006	0.02	4.290	4.280	4.720	2.714	1.437	2.104	0.418	0.515	0.486	0.278	0.269	0.274
Simazine	H	0.006	0.02												
Tebuconazole	F	0.012	0.04	0.230	0.120	0.100	1.016	0.881	0.730	0.032	0.022	0.028			<LOQ
Tetraconazole	F	0.006	0.02					<LOQ	<LOQ	0.028	<LOQ	<LOQ		0.043	
Thiamethoxam	I	0.006	0.02				0.125	0.077	0.089						
Trifloxystrobin	F	0.012	0.04	0.100		0.010				0.026	0.021	<LOQ			
Vamidothion	I	0.012	0.04						<LOQ						

H: herbicide; I: insecticide; F: fungicide; LOD: limit of detection; LOQ: limit of quantification; na: not analyzed.

Table 1. Pesticide residues (µg/L) found in water samples in the Vacacaí river, RS, Brazil (*conclusion*)

Pesticide	*Class*	*LOD*	*LOQ*	*August*			*September*			*October*			*November*		
				S1	*S2*	*S3*	*S1*	*S2*	*S3*	*S1*	*S2*	*S3*	*S1*	*S2*	*S3*
2.4 D	H	0.006	0.02	n.a	n.a	n.a	<LOQ	<LOQ	<LOQ				n.a	n.a	n.a
Atrazine	H	0.006	0.02	<LOQ	<LOQ	<LOQ	0.046	0.064	0.076	0.034	0.041	0.086	0.090	0.131	0.129
Azoxystrobine	F	0.006	0.02							<LOQ	<LOQ	<LOQ			
Bentazon	H	0.240	0.80								0.232	0.114	0.332	0.321	0.516
Carbofuran	I	0.006	0.02									<LOQ			<LOQ
Carbendazim	F	0.006	0.02	n.a	n.a	n.a	<LOQ	<LOQ	<LOQ	n.a	n.a	n.a			
Clomazone	H	0.006	0.02	0.328	0.302	0.230	0.175	0.220	0.235	0.314	0.440	0.176	0.547	0.591	0.587
Cyanazine	H	0.006	0.02												
Epoxiconazole	F	0.006	0.02									0.023	<LOQ	<LOQ	
Flutolanil	F	0.006	0.02										0.056	0.095	0.055
Imidacloprid	I	0.006	0.02	0.026	<LOQ	<LOQ	0.042	0.042	<LOQ				0.072	0.070	0.069
Metalaxyl	F	0.006	0.02							<LOQ	<LOQ				
Metconazole	F	0.006	0.02									0.020			
Metsulfuron methyl	H	0.006	0.02	0.031	0.026	0.023	0.021	0.020	0.026	<LOQ					
Oxamyl	I	0.04	0.13												
Propiconazole	F	0.006	0.02									0.043	<LOQ	<LOQ	
Propoxur	I	0.012	0.04												
Quinclorac	H	0.006	0.02	0.080	0.141	0.181	0.097	0.122	0.103	<LOQ	<LOQ	<LOQ	2.184	1.391	2.099
Simazine	H	0.006	0.02							<LOQ	<LOQ	<LOQ			
Tebuconazole	F	0.012	0.04	<LOQ	0.020	<LOQ				0.032		0.131	0.037	<LOQ	
Tetraconazole	F	0.006	0.02									0.045	0.042	0.049	<LOQ
Thiamethoxam	I	0.006	0.02								<LOQ		0.061	0.075	0.067
Trifloxystrobin	F	0.012	0.04									0.037	0.024	0.042	0.026
Vamidothion	I	0.012	0.04												

H: herbicide; I: insecticide; F: fungicide; LOD: limit of detection; LOQ: limit of quantification; na: not analyzed.

Table 2. Spearman correlation coefficient among pesticides and biomarkers of zebrafish embryos (*to be continued*)

	January				*February*				
	GST	CAT	TBARS	Spontaneous movement	CAT	AChE	TBARS	Spontaneous movement	Heart rate
AChE		0.493							
TBARS	0.497	0.615			0.460				
Spontaneous movement					-0.489		-0.481		
Atrazine				-0.493	0.489		0.721		
Azoxystrobin									0.843
Carbofuran						0.527	0.454		0.735
Clomazone			0.636	-0.509	0.714		0.745	-0.607	
Epoxiconazole			0.671			0.698			0.551
Imidacloprid									0.843
Metalaxyl					0.486	0.815			
Oxamyl					0.611	0.471		-0.553	-0.454
Propiconazole					0.634		0.773	-0.537	
Propoxur			0.552	-0.528	0.714		0.745	-0.607	
Quinclorac				-0.493		0.698			0.551
Tebuconazole									0.843
Tetraconazole					0.555		0.659	-0.522	
Thiamethoxam						0.698			0.551
Vamidothion					0.611	0.471		-0.553	-0.454

Only correlation coefficients with a statistically significant difference are shown ($p < 0.05$).

Table 2. Spearman correlation coefficient among pesticides and biomarkers of zebrafish embryos (*to be continued*)

	May				*June*				
	GST	AChE	TBARS	Heart rate	GST	CAT	AChE	TBARS	Heart rate
TBARS		-0.588			0.510		-0.571		
Heart rate		-0.576					-0.763	0.583	
Atrazine	-0.458	-0.822	0.593	0.702		-0.564			
Clomazone		-0.776	0.512	0.785			-0.481		0.497
Epoxiconazole				0.480					
Imidacloprid		-0.481	0.465	0.715			-0.589		0.603
Propoxur		-0.481	0.465	0.715					
Quinclorac		-0.776	0.512	0.785					
Tebuconazole			0.621				0.451		0.616
Tetraconazole			0.601						
Trifloxystrobin			0.520						

Only correlation coefficients with a statistically significant difference are shown ($p < 0.05$).

Table 2. Spearman correlation coefficient among pesticides and biomarkers of zebrafish embryos (*to be continued*)

	August			*September*			
	Spontaneous movement	Heart rate	Hatching rate	CAT	AChE	TBARS	Hatching rate
Hatching rate		-0.451					
Spontaneous movement						-0.462	
2,4-D							0.604
Atrazine		0.445					0.596
Azoxystrobin							
Carbendazim							0.604
Clomazone							0.596
Imidacloprid				0.450			
Metsulfuron methyl					0.628		0.654
Quinclorac		0.584	-0.645				
Tebuconazole	-0.519						

Only correlation coefficients with a statistically significant difference are shown ($p < 0.05$).

Table 2. Spearman correlation coefficient among pesticides and biomarkers of zebrafish embryos (*conclusion*)

	October				*November*			
	GST	CAT	Spontaneous movement	Hatching rate	GST	CAT	TBARS	Heart rate
CAT					0.576			
TBARS					0.743	0.561		
Heart rate						-0.473		
Hatching rate	0.631							
Atrazine			-0.700	0.485				
Azoxystrobin	0.491		-0.502	0.686				
Bentazon			-0.629		0.659	0.652	0.644	-0.660
Carbofuran			-0.492		0.731		0.571	
Clomazone		0.737		0.539				
Epoxiconazole			-0.492					
Imidacloprid								-0.652
Metalaxyl		0.746						
Metconazole			-0.492					
Propiconazole			-0.492					
Quinclorac	0.491		-0.502	0.686		0.667	0.458	-0.785
Simazine	0.491		-0.502	0.686				
Tebuconazole								-0.501
Tetraconazole			-0.492					
Thiamethoxam		0.611						
Trifloxystrobin			-0.492					

Only correlation coefficients with statistically significant difference are shown ($p < 0.05$).

FIGURE CAPTION

Figure 1. Spontaneous movement (A), heart rate (B) and hatching rate (C) of *Danio rerio* embryos exposed to water river samples. Data are reported as mean ± S.E.M. (n = 6). Different letters indicate significant differences among months at each site or in each control group ($p < 0.05$).

Figure 2. TBARS levels (A) and GST activity (B) of *Danio rerio* embryos exposed to water river samples. Different letters indicate significant difference among months at each site or in each control group. Asterisks represent significant differences between the control group and the sites in the same month ($p < 0.05$).

Figure 3. CAT (A) and AChE activities (B) of *Danio rerio* embryos exposed to water river samples. Different letters indicate significant differences among months at each site or in each control group. Asterisks represent significant difference between the control group and the sites in the same month ($p < 0.05$).

Figure 1

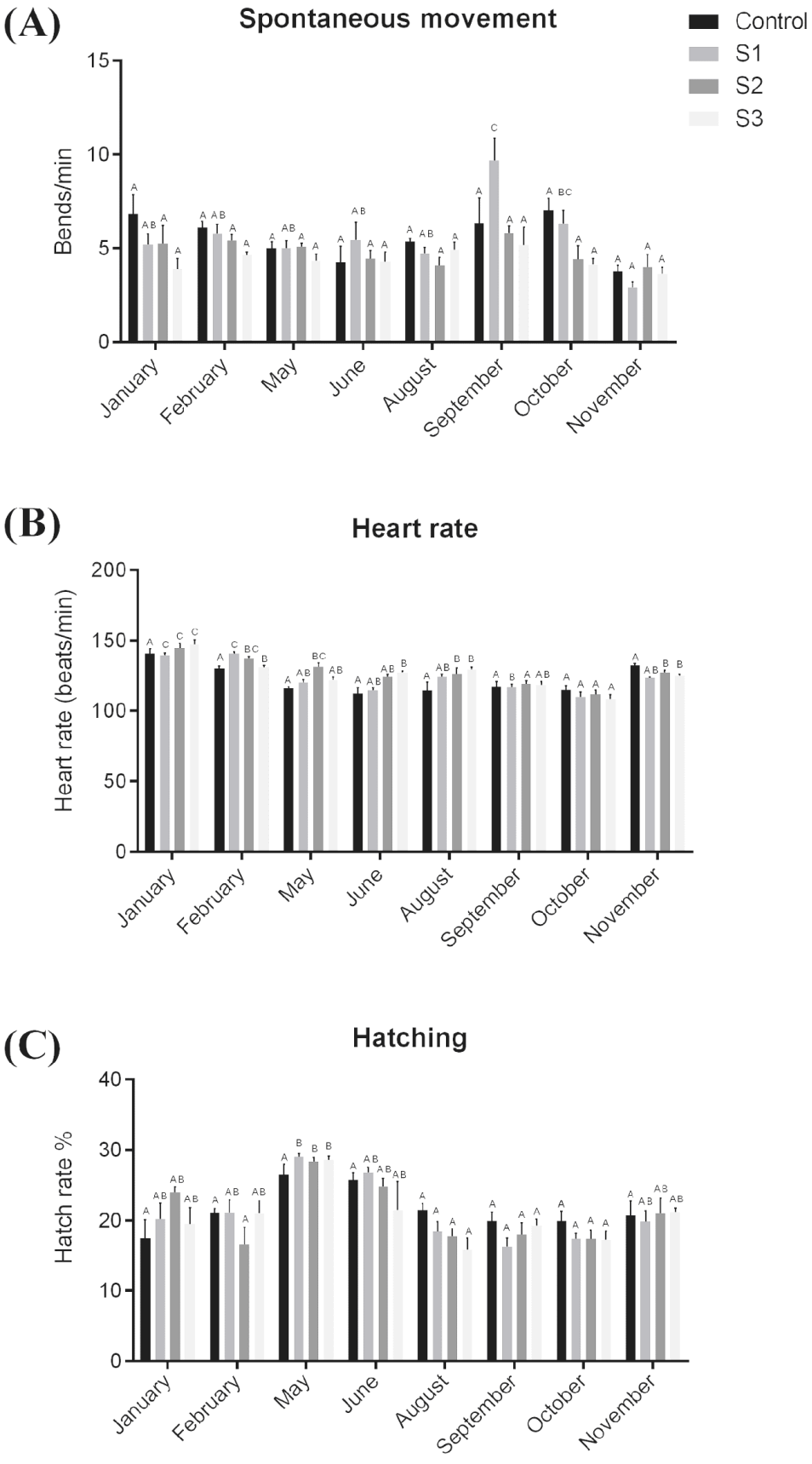

(A)
Spontaneous movement
Control
S1
S2
S3
Bends/min
15
10
5
0
January
February
May
June
August
September
October
November
(B)
Heart rate
Heart rate (beats/min)
200
150
100
50
0
January
February
May
June
August
September
October
November
(C)
Hatching
Hatch rate %
40
30
20
10
0
January
February
May
June
August
September
October
November

Figure 2

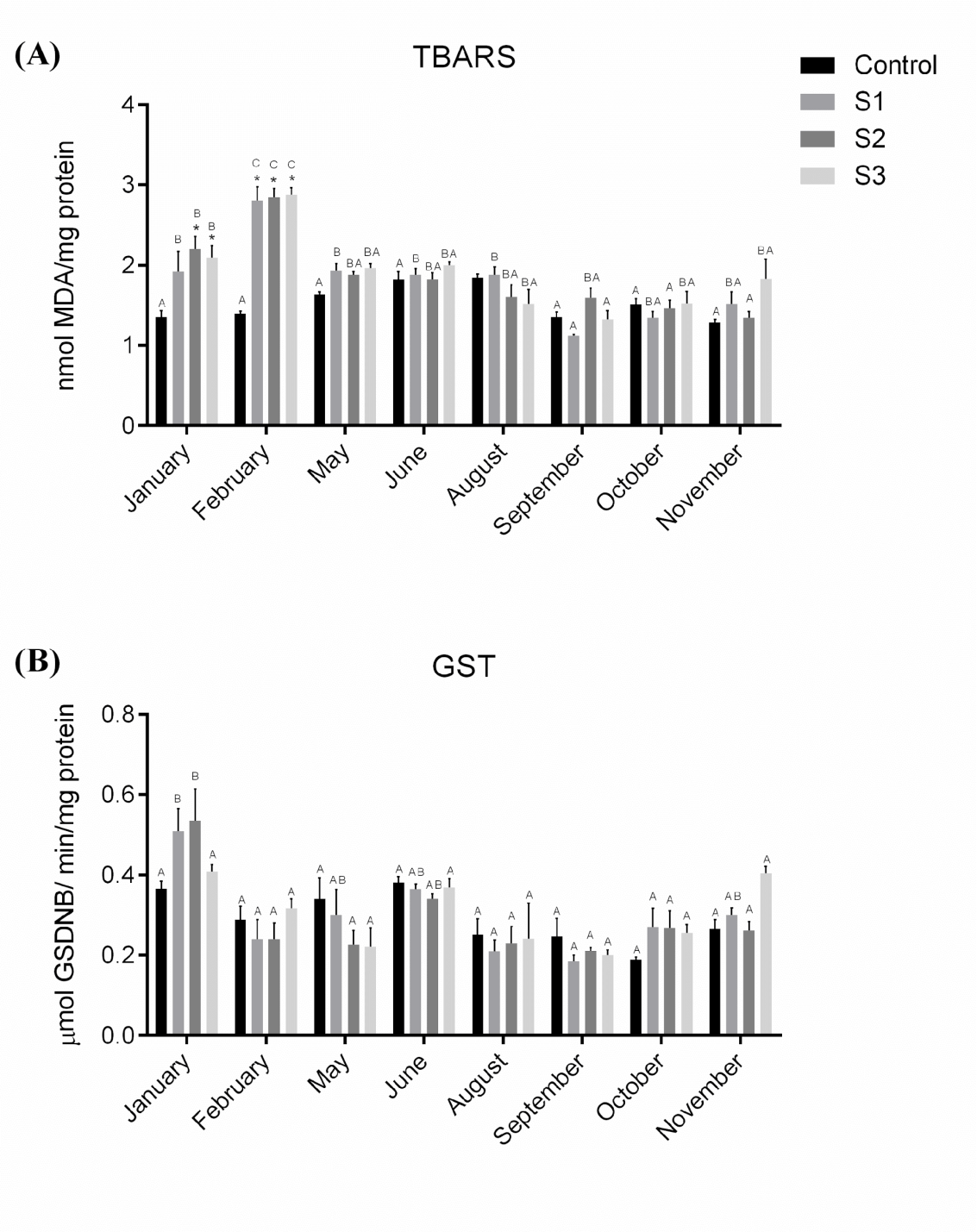
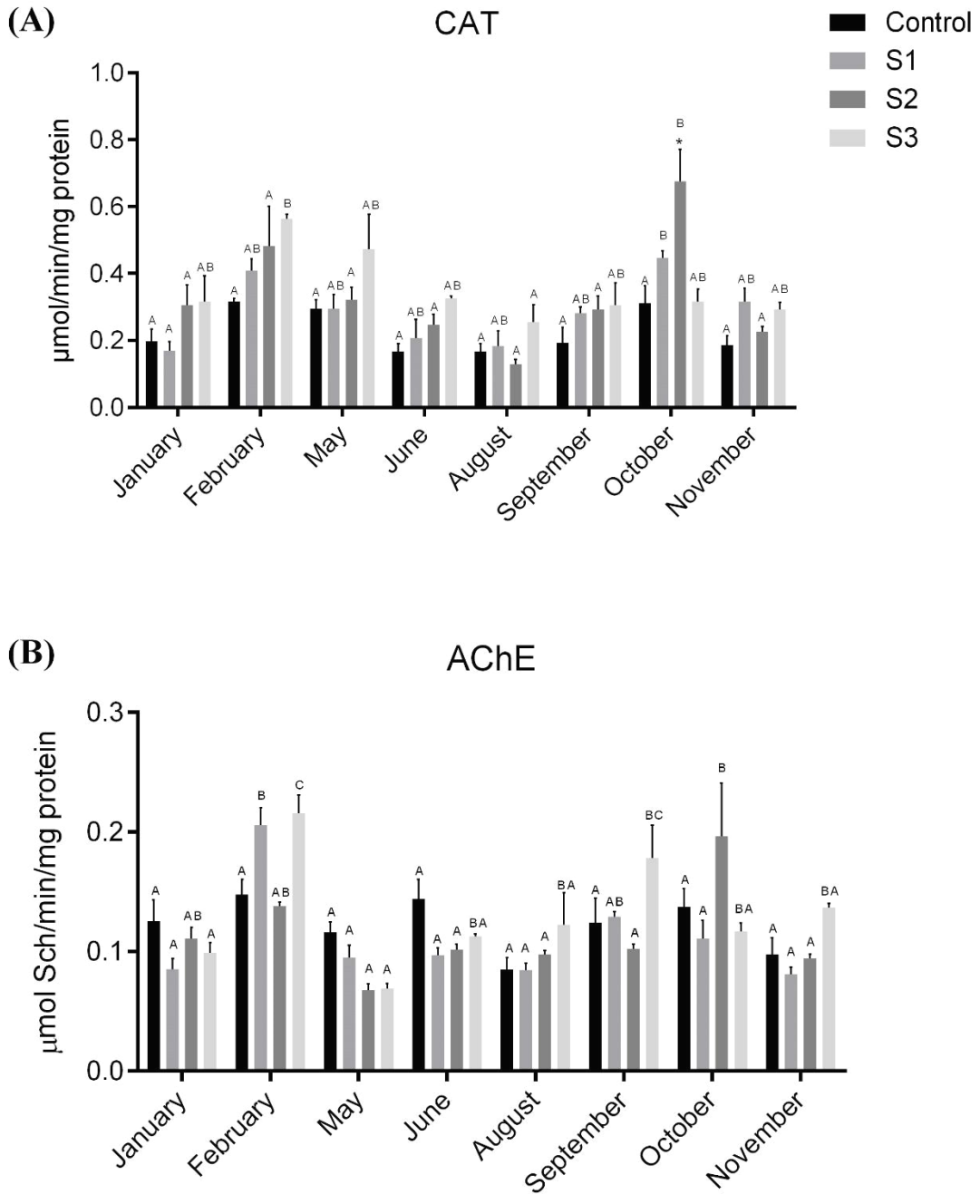

(A)
TBARS
Control
S1
S2
S3
nmol MDA/mg protein
0
1
2
3
4
January
February
May
June
August
September
October
November
(B)
GST
µmol GSDNB/ min/mg protein
0.0
0.2
0.4
0.6
0.8
January
February
May
June
August
September
October
November

Figure 3


(A)
CAT
Control
S1
S2
S3
µmol/min/mg protein
1.0
0.8
0.6
0.4
0.2
0.0
January
February
May
June
August
September
October
November
(B)
AChE
µmol Sch/min/mg protein
0.3
0.2
0.1
0.0
January
February
May
June
August
September
October
November

6 ARTIGO 3

A preliminary study of the effects of Basagran® herbicide in *Danio rerio* embryonic development

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ABSTRACT

The high demand for food has led to a greater number of pesticides used in crops, causing an environmental impact on aquatic environments close to these locations. There is a lack of knowledge about the effects of some of these pollutants on environmental concentrations. Thus, this study aimed to estimate the influence of herbicide Basagran® in *Danio rerio* embryos development exposed to environmental concentrations of this herbicide (3 $\mu g.L^{-1}$, 6 $\mu g.L^{-1}$, 12 $\mu g.L^{-1}$) as well as the limit established safe for drinking water (300 $\mu g.L^{-1}$). We performed behavioral and developmental analyzes during the exposure period. Our results showed changes only in heart rate evaluated at 48hpf, showing that the herbicide Basagran® at the concentrations tested had few adverse effects on the development and behavior of the *Danio rerio* embryos evaluated.

Keywords: fish, bioindicator, ecotoxicology, pesticides, environment pollution

1 INTRODUCTION

Brazil is among the largest producers of agricultural commodities in the world (Paumgartten 2020). To ensure large-scale production, the use of pesticide becomes indispensable. In the year 2019, 620,538 tons of active ingredients were marketed in Brazil, according to IBAMA (2021). Inevitably, during their application, these compounds reach non-target locations such as rivers and lakes surrounding agricultural fields (Tran et al., 2019).

The occurrence of environmental contaminants can be assessed through quantitative analysis such as high-performance liquid chromatography (HPLC) (Donato et al., 2015). This methodology is commonly used to assess the amount of pesticide residues present in the environment (Loro et al., 2015; do Amaral et al., 2018; Marins et al., 2020). Although highly accurate, these analyzes do not have the capacity to demonstrate any impacts that these compounds could induce in local fauna (dos Santos et al., 2016). Pesticides are well known to cause impact to non-target organisms, mainly aquatic organisms (Bashnin et al., 2019; Lima-Fernandes et al., 2019; He et al., 2019; Dievel et al., 2019).

Biomonitoring studies are needed to assess possible impacts of contaminants to the ecosystems. There are several ways to conduct a biomonitoring study, such as

by collecting animals in the field or exposing the organism model in the natural environment (*in situ*) (Amado et al., 2005; Vieira et al, 2016). These methods have some disadvantages, such as environmental variations that can not be controlled, as well as all the stress caused by handling, which can mask the physiological response to contaminant exposure (Oikari, 2006). In this context, exposure in laboratory conditions emerge as an alternative for biomonitoring study. Although it is difficult to reproduce all the characteristics of the natural environment, it is possible to infer some deleterious effects for wild animals (Murussi et al., 2015; Marins et al., 2021).

Among the aquatic animals most used in laboratory research, the zebrafish (*Danio rerio*) has been a great biological model due to its several advantages such as rapid development, low maintenance cost, and small space occupied in the laboratory, among others (OECD, 2013). Besides that, the transparency of its egg enables monitoring embryo development by observation of behavioral and morphological changes (Oliveira et al., 2017; Costa-Silva et al., 2018).

During the year of 2018, biomonitoring studies were carried out in the southern region of Brazil, on rivers surrounded by crop fields (Cerezer et al., 2020; Severo et al., 2020). Among the pesticides found, bentazon drew our attention because it was found in several water samples at high concentrations (with values up to 12 $\mu g.L^{-1}$). Besides that, other studies report the presence of the herbicide bentazon in aquatic environments (Lazartigues et al., 2012; Rodrigues et al., 2018; de Oliveira et al., 2019; Marins et al., 2020). Bentazon is the active ingredient of Basagran®, belongs to the chemical group of benzothiadiazinones, being a post-emergent herbicide selective to soy, rice, beans, corn and wheat crops. It acts by inhibiting weed photosynthesis and is considered extremely toxic and harmful if swallowed and may cause sensitization on skin contact in humans (Bessegato et al., 2012). In non-target organisms, different studies reported the effects ranging from endocrine disrupting, cytotoxic effect, genotoxicity, and developmental malformations (Orton et al., 2009; Pistl et al., 2003; Kaya et al., 2004, Oliveira et al., 2017). In a review carried out by Vieria et al. (2016), the presence of concentrations of bentazon ranging from 9 $\mu g.L^{-1}$ to 135 $\mu g.L^{-1}$ in the southern Brazilian coast is reported. Bentazone was found in Japan at concentrations up to 14 $\mu g.L^{-1}$ in rice growing areas and in drinking water it was found in concentrations of 1 $\mu g.L^{-1}$. In Germany, the maximum concentration of bentazone measured in drinking water was 0.185 $\mu g.L^{-1}$ (WHO, 2016)

Thereby, the goal of this study was evaluate the effects of the herbicide Basagran® on the behavior and development of zebrafish embryos, using concentrations found in rivers impacted by pesticides as well as the limit established safe for drinking water (Brasil, 2005).

2 MATERIALS AND METHODS

Bentazon (CAS number 25057-89-0; 3-isopropyl-1H-2,1,3-benzothiadiazin-4 (3H)-one 2,2-dioxide) was obtained from the commercial formulation Basagran®. The stock solution was prepared by diluting the herbicide in distilled water. For the exposures, the stock solution was diluted in E3 medium (Westerfield, 2000).

Adult zebrafish were obtained from a local store and maintained in a tank under appropriate water conditions (3 animals. L^{-1} density, pH 7:2±0:5, 400 ± 50 µS conductivity, 28 ± 1° C, and dissolved oxygen equal or above 95% saturation) with a 14 h / 10 h (light / dark) photoperiod. Ammonia, nitrite, and nitrate values were kept lower than 0.2 ppm. The matrices were fed twice daily with commercial feed, supplemented with *Artemia sp*. once daily. The embryos were obtained from the standard reproduction, described by Westerfield (2000). After breeding the zebrafish embryos were collected, immersed in E3 medium (solution to clean fish embryos) and placed on a petri plate for random selection, according to Westerfield (2000). The experimental protocols used in this study were approved by the local Ethics committee (CEUA – UFSM: protocol 4735080419).

Toxicological assays were adapted from OECD guidelines for the testing of chemicals 236, Fish, Early-life Stage Toxicity Test (FET) (OECD, 2013). The embryos with 2 hours post-fertilization (hpf), without abnormality in cell division or chorion, were placed in 24-well plates with five embryos per well (n = 120 by group). The experiment was composed of a control group (E3 medium without Basagran®) and four exposure groups (3 $\mu g.L^{-1}$, 6 $\mu g.L^{-1}$, 12 $\mu g.L^{-1}$ and 300 $\mu g.L^{-1}$ of Basagran®).

A total of 30 embryos for each group were used to study heart rate, spontaneous movement, and the touch response test. Following treatment, embryo mortality was evaluated during the first 24 hpf time point. Embryo viability was analyzed by observation of egg coagulation. The spontaneous movement (bends) was measured at 28 hpf and was assessed by counting the number of total body axis movements performed by embryos within their chorions according to Xia et al.

(2017). The heart rate of embryos was measured at the 48 hpf by counting the number of heart beats per minute, according to Xia et al. (2017). The hatching rate was assessed at 72 hpf with all surviving embryos. Images were obtained with the aid of a Vastar Mega Pixels 500X microscope and observed with Many-Cam. Behavioral touch response test was measured at the 72 hpf, counting the required number of stimuli needed for zebrafish larvae to escape. This technique was adapted from Saint-Amant and Drapeau (1998) and more details can be seen at Costa-Silva et al. (2018).

The data were tested for normality and homogeneity according to the methods of Kolmogorov-Smirnov and Shapiro-Wilk, respectively. Statistical comparisons of parametric data were performed with one-way ANOVA, and the Tukey post-hoc test. Non-parametric data were tested using Kruskal-Wallis. Results were considered significant at $P < 0.05$. Results are presented as means ± standard error of the mean (SEM).

3 RESULTS AND DISCUSSION

The mortality showed no statistically significant difference among groups, being in the range (around 10%) as expected by the FET test (OECD, 2013).

Among the morphological and behavioral tests performed during 96 hours of development, the only one that showed significant differences was the heart rate, which was significantly higher in groups exposed to all Basagran® concentrations compared to control groups. However, there was no significant difference among the exposed groups (Fig. 1a). The heart is one of the first functional organs developed in zebrafish, and the heart rate is an important toxicological response in the fish embryonic test (Glickman and Yelon, 2002). The persistent increase in heart rate triggers the death of cardiomyocytes in response to elevated inflammation and impaired calcium manipulation. These injuries to the heart could lead to a reduction of blood transport in embryos and then affect energy transport, further influencing embryo development (Kossack et al., 2017; Aksakal and Ciltas, 2018; Qian et al., 2018).

The first movement performed by the zebrafish embryo is spontaneous movement. It happens at around 28 hpf and occurs because of the movement of the spinal cord triggered by the development of motor neurons that innervate the

muscles (Saint-Amant and Drapeau, 1998). According to Xia et al. (2017), the decrease in spontaneous movement suggests some type of neurotoxicity and may be a reason for delayed hatching. In our study, no significant changes were observed in spontaneous movement among the groups analyzed (Fig 1b).

Touch stimulation test was performed to assess sensory-motor capacity at 72 hpf, when normally most embryos have already hatched (Fig 1c). Locomotor behaviors carry out an important role in activities during zebrafish development, such as scape behavior, feeding, social, and defensive activities (Colwill and Creton, 2011). We did not observe changes in the escape behavior performed by this test, perhaps because the Basagran® in the tested concentrations may not affect the locomotor development of the embryo, as observed by Costa Silva (2018) which observed changes in locomotion in embryos exposed to the fungicide Mancozeb (1.88; 2.81; 3.75 µM).

Hatching is one of the important stages of the fish life cycle, which directly affects the rate of embryonic development. The normal time for the embryos to hatch is between 48 to 72 (hpf), depending on enzyme activity and embryonic movements (Kimmel et al., 1995; Yumnamcha et al., 2015, Samaee et al., 2015). In our study, we did not observe changes in the hatching rate between groups exposed to the herbicide in relation to the control group and among Basagran® concentrations. The exposure of zebrafish embryos to much higher concentrations of Basagran® (120.0 to 480.6 mg / L), delayed the embryo development (Oliveira et al., 2017) alleged that the herbicide may have altered the physical structure of the chorion layer, consequently causing increased hatchability

Despite the few adverse effects that occurred in our study, Severo et al. (2020) observed a series of changes in embryos exposed to river water samples, which were detected the presence of bentazon. In that study, bentazon showed positive correlations with glutathione S-transferase, catalase, and lipid damage, as well as negative correlation with heart rate and spontaneous movement. Thereby, it is possible that only Basagran® at the concentrations tested was not enough to lead to several damage in the parameters observed. In addition, in that study, other pesticides were found together with bentazone

In conclusion, no damages on the morphology and behavioral parameters were observed in embryos exposed to environmental concentrations of Basagran®. In this way, there is a chance that bentazone acts together with other environmental

contaminants in an additive or synergistic way. On the other hand, it is possible that the analyzed parameters are not good effect biomarkers for this compound. However, more studies are necessary to determine which combinations with bentazon can be more or less harmful to aquatic organisms.

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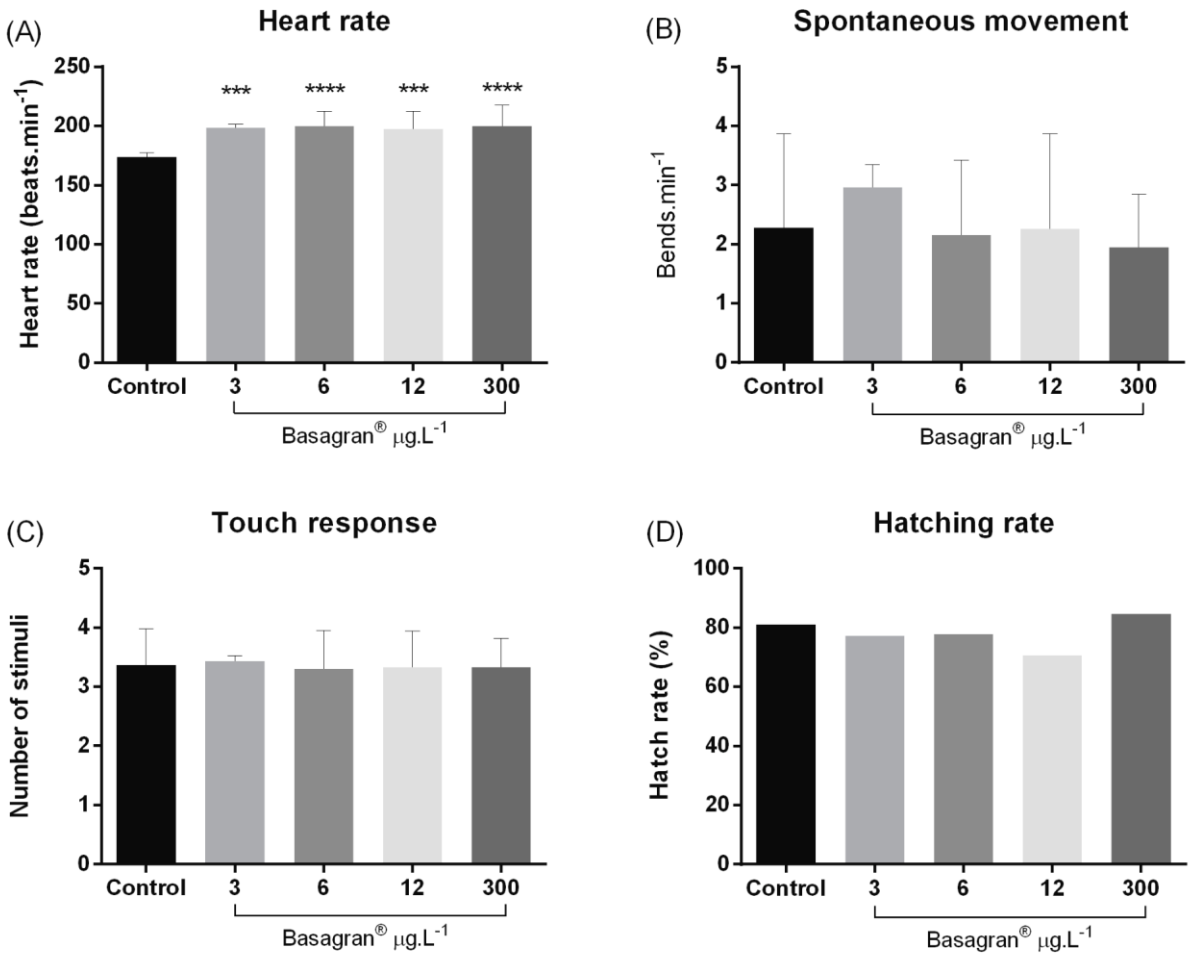
FIGURE CAPTION

Figure 1. Heart rate (A), spontaneous movement (B), touch response (C), and hatching rate (D) of *Danio rerio* embryos exposed to Basagran®. Data are reported as mean ± S.E.M. Asterisks represent statistical significant difference with the control (***p<0,001; ****p < 0,0001, n=30).



7 DISCUSSÃO

Existe uma ampla série de possibilidades para a realização de estudos de biomonitoramento em ambientes aquáticos, cada uma delas oferecendo suas devidas vantagens. Nesse sentido, se faz necessário a busca pelas metodologias para avaliação de impactos ambientais que melhor respondam sobre os reais danos causados em organismos que habitam esses locais. A possibilidade de realizar mais de uma metodologia de biomonitoramento em um mesmo experimento é algo ainda pouco explorado, sendo possivelmente uma forma de tornar os resultados do biomonitoramento mais elucidativos sobre a chance de determinados impactos ambientais estarem causando algum dano na biodiversidade local.

Em países onde os recursos financeiros para a ciência são cada vez mais escassos, alternativas que possibilitam fazer pesquisas com baixo custo tornam-se verdadeiros desafios. Dessa forma, a utilização de embriões de *Danio rerio*, demonstrou ser um aliado nas pesquisas de biomonitoramento pois o esforço para se realizar a captura de peixes em determinados locais exige uma série de recursos como barco e materiais profissionais de pesca, a construção de equipamentos para a exposição *in situ* também requer um investimento considerável (DO AMARAL et al., 2018; CARDOSO et al., 2019). Além disso, o modelo experimental utilizando embriões de peixe-zebra contornam algumas das dificuldades encontradas nas outras metodologias, como a grande variabilidade de tamanho e peso dos animais capturados, além de eventuais perdas de gaiolas em exposições *in situ* (ECHEVERRÍA-SÁENZ et al., 2016).

O local escolhido para a realização do estudo no artigo 2 apresentou contaminação causada por pesticidas durante todo o período do biomonitoramento, com maior presença desses contaminantes nos períodos de maior atividade agrícola (primavera e verão). Do total de 24 contaminantes encontrados, apenas dois desses compostos possuem determinação de valores máximos permitidos em águas superficiais no território brasileiro (CONAMA, 2005). Essas substâncias, 2,4-D e atrazina, foram encontradas em quantidades abaixo do permitido por lei que é 30 µg / L e 2 µg /L, respectivamente. Dessa forma, fica evidente a necessidade da atualização das normas locais a respeito dos níveis máximos permitidos de uma ampla classe de agrotóxicos, ainda mais que nos últimos anos, vem ocorrendo a liberação de novos compostos (Figura 9) (MAPA,2021).

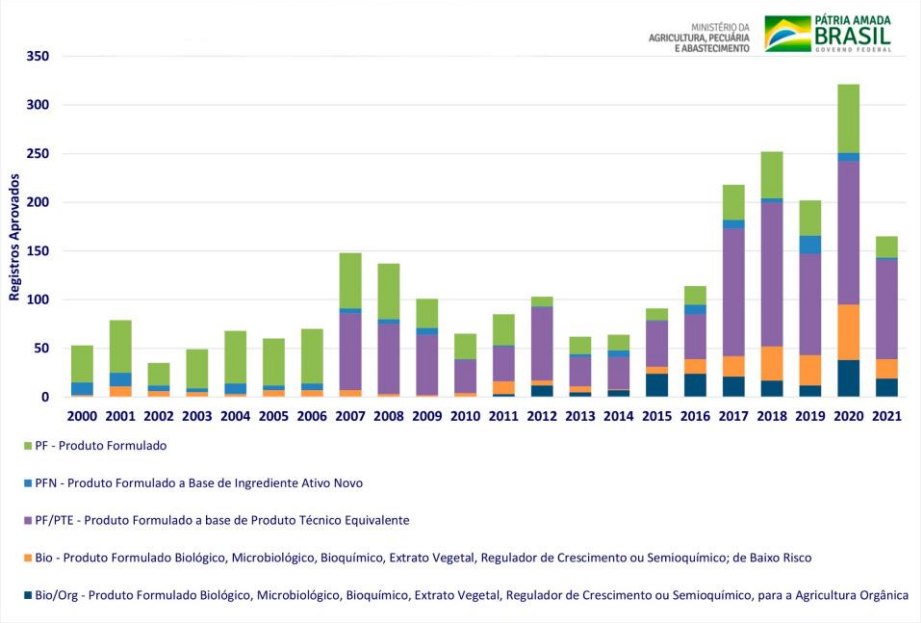


Figura 8 : Registro de agrotóxicos e Afins - Por Tipo.
Fonte: adaptado de https://www.gov.br/agricultura/pt-br/assuntos/insumos-agropecuarios/insumos-agricolas/agrotoxicos/informacoes-tecnicas

Os resultados obtidos no artigo 2 mostram um maior número de alterações nos biomarcadores analisados justamente nos meses de coleta em que as análises de água apontaram uma maior presença de pesticidas. A análise de correlação realizada neste estudo ajudou a corroborar os resultados obtidos, mostrando que o aumento de determinados biomarcadores ocorreu em função do aumento da presença de determinados compostos.

Entre os contaminantes encontrados no estudo de biomonitoramento realizado no rio Vacacaí, a Bentazona e o Quinclorac apresentaram a maior amplitude nas suas concentrações encontradas no decorrer do biomonitoramento. Uma das características da bentazona é seu baixo coeficiente de adsorção, característica que torna esse composto muito móvel no solo. Embora a fotodegradação ocorra tanto no solo como na água, o tempo de meia-vida da bentazona no solo é estimado entre 14 e 98 dias e devido à sua alta mobilidade, a bentazona é suscetível de lixiviar sob condições de chuvas extremas que ocorram após a sua aplicação, levando o composto atingir águas subterrâneas e próximas ao seu local de aplicação (WHO, 2016)

A bentazona ocorre frequentemente em águas superficiais. No Japão foi encontrada em concentrações de até 14 $\mu g.L^{-1}$ em áreas de cultivo de arroz e na água potável foi encontrada a concentração de 1 $\mu g.L^{-1}$. Na Alemanha, a

concentração máxima de bentazona medida na água potável foi de 0,185 $\mu g.L^{-1}$ (WHO, 2016). Em estudos realizados no Brasil, as concentrações encontradas em água superficial variaram entre 0,102 $\mu g.L^{-1}$ até 12,92 $\mu g.L^{-1}$ em um estudo de biomonitoramento realizado na região central do Rio Grande do Sul (CEREZER et al., 2020) e em um estudo realizado no estado de Santa Catarina, foram encontradas concentrações de bentazona variando entre 9 $\mu g.L^{-1}$ to 135 $\mu g.L^{-1}$ em lavouras de arroz irrigado (VIEIRA et al., 2016). Além disso, a portaria do Ministério da saúde 518/2004 permite a concentração máxima da bentazona de 300 $\mu g. L^{-1}$ em água potável (BRASIL, 2005).

Existe uma necessidade de se avaliar os efeitos de agrotóxicos em concentrações ambientalmente relevantes, uma vez que grande parte dos estudos de toxicologia publicados focam em avaliar concentrações comerciais ou acima delas. Desse modo, o artigo 3 focou em realizar um estudo prévio a respeito dos efeitos do herbicida Basagran, o qual o ingrediente ativo é a bentazona. Nesse estudo foram realizadas somente análises de mortalidade, desenvolvimento e comportamento em embriões de peixe-zebra. Inicialmente pretendíamos realizar as mesmas análises bioquímicas realizadas no artigo 2, porém, no experimento realizado, obtivemos uma quantidade insuficiente de embriões para a realização das análises bioquímicas. Das análises realizadas, somente a frequência cardíaca apresentou alterações significativas em relação ao grupo controle, segundo apontam estudos, o aumento da frequência cardíaca pode acarretar na morte de cardiomiócitos afetando o desenvolvimento do embrião (KOSSACK et al., 2017; AKSAKAL AND CILTAS, 2018; QIAN et al., 2018).

8 CONCLUSÃO

A partir da interpretação dos dados gerados por esta tese podemos concluir que:

- O Brasil destaca-se como um dos principais países Latino-americanos na publicação de estudos sobre biomonitoramento;
- A dispersão descontrolada de pesticidas atinge o ambiente aquático, podendo afetar o desenvolvimento de organismos aquáticos;
- Embriões de peixe-zebra possuem o potencial para serem utilizados como uma ferramenta em conjunto com análises de qualidade de água;
- O Basagran® em concentração ambientalmente relevante pode afetar o desenvolvimento de embriões de peixe-zebra, através do aumento da frequência cardíaca;
- Existe a necessidade de se realizar estudos que avaliem os efeitos de pesticidas em concentrações ambientais;

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ANEXO A - AUTORIZAÇÃO DA CEUA

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "EFEITOS DA ÁGUA DO RIO VACACAÍ UTILIZANDO-SE EMBRIÕES DE DANIO RERIO COMO BIOINDICADORES", protocolada sob o CEUA nº 4735080419 (ID 002591), sob a responsabilidade de **Vânia Lucia Loro** *e equipe; Aline Teixeira Marins; Cristina Cerezer; Eduardo Stringini Severo* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 16/07/2019.

We certify that the proposal "EFFECTS OF VACACAÍ RIVER WATER USING DANIO RERIO EMBRYOS AS BIOINDICATORS", utilizing 7260 Fishes (males and females), protocol number CEUA 4735080419 (ID 002591), under the responsibility of **Vânia Lucia Loro** *and team; Aline Teixeira Marins; Cristina Cerezer; Eduardo Stringini Severo* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 07/16/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 03/2019 a 03/2020 Área: Bioquímica E Biologia Molecular

Origem:	Não aplicável biotério						
Espécie:	Peixes	sexo:	Machos e Fêmeas	idade:	6 a 12 meses	N:	60
Linhagem:	Selvagem			Peso:	4 a 6 g		
Origem:	Não aplicável biotério						
Espécie:	Peixes	sexo:	Machos e Fêmeas	idade:	1 a 96 horas	N:	7200
Linhagem:	Selvagem			Peso:	1 a 3 g		

Local do experimento: Os peixes serão aclimatados e mantidos durante o período experimental no biotério de experimentação do laboratório de fisiologia de peixes (anexo sala 2226 prédio 18) sobre responsabilidade do Professor Bernardo Baldisserotto e da professora Vania Lucia Loro.

Santa Maria, 14 de julho de 2021

Profa. Dra. Patrícia Severo do Nascimento
Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria