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EUGÊNIA CARLA KUHN

AVALIAÇÃO ECOTOXICOLÓGICA DO MÉDIO RIO URUGUAI USANDO O *Caenorhabditis elegans* PARA BIOMONITORAMENTO

Uruguaiana

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica, da Universidade Federal do Pampa, como requisito parcial para a obtenção do título de Mestra em Bioquímica.

Orientadora: Daiana Silva de Ávila
Coorientadora: Caroline Brandão Quines

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1. RESUMO

O Rio Uruguai faz parte de uma relevante bacia hidrográfica brasileira que se estende desde o Rio Grande do Sul e Santa Catarina até países como Uruguai e Argentina, demarcando fronteiras. A maioria das regiões banhadas pelo rio é caracterizada por intensa atividade agroindustrial. O intenso uso de agrotóxicos relacionados às grandes plantações de soja e arroz no sul do país, associado a outros fatores como a falta de proteção ambiental adequada, expõem o Rio Uruguai a grandes chances de poluição, comprometendo todo o ecossistema, colocando em potencial risco não só o habitat, mas toda a população da região. Pensando nisso, este estudo teve como objetivo analisar e correlacionar as alterações nas águas do Rio Uruguai no município de Uruguaiana – Rio Grande do Sul, cidade conhecida pela alta produção de arroz, inicialmente com analises de amostras pré e pós a aplicação de pesticidas nas lavouras e depois de nossas descobertas estendemos nosso estudo para amostras mensais em um período de 12 meses. Foram avaliados inicialmente para ambos os estudos os parâmetros físico-químicos, pH, oxigênio dissolvido, condutividade, salinidade e temperatura da água. Para o estudo de biomonitoramento, utilizamos o nematoide de vida livre *Caenorhabditis elegans* como modelo de bioindicador, expondo-os as amostras coletadas do rio. Os vermes foram expostos por 24 horas a amostras coletadas sob agitação constante e depois transferidos para meio sólido em placas NGM para testes de sobrevivência, longevidade, reprodução e comprimento corporal. Para complementar nossos estudos foram quantificados metais e pesticidas nas amostras e no segundo projeto também utilizamos a cepa CF1553, além do tipo selvagem N2, com o intuito de observar uma possível expressão da *Sod-3*. Algumas amostras de ambos os trabalhos demostraram a presença residual de agrotóxicos (Tebuconazol, Imazetapir, Clomazone, sulfentrazone e 2,4-D) e metais (As, Hg, Fe e Mn) e foi possível correlacionar efeitos fisiológicos do *C. elegans* com a presença dos contaminantes. Essas amostras causaram redução na reprodução, comprimento, sobrevivência e na vida útil dos vermes expostos. Esta pesquisa teve como objetivo reforçar a importância de estudos frequentes na área de ecotoxicologia, devido à constante poluição e dos impactos nos organismos vivos.

Palavras-Chave: Biomarcadores; Contaminação Ambiental; Pesticidas; Metais Pesados; Contaminação Ambiental**.**

2. ABSTRACT

The Uruguay River is part of a relevant Brazilian hydrographic basin that extends from Rio Grande do Sul and Santa Catarina to countries such as Uruguay and Argentina, demarcating borders. Most of the regions bathed by the river are characterized by intense agro-industrial activity. The intense use of pesticides related to the large soy and rice plantations in the south of the country, associated with other factors such as the lack of adequate environmental protection, exposes the Uruguay River to great chances of pollution, compromising the entire ecosystem, putting in potential risk not only the habitat, but the entire population of the region. This study aimed to analyze and correlate the changes in the waters of the Uruguay River in the municipality of Uruguaiana - Rio Grande do Sul, a city known for high rice production, initially with analysis of samples before and after the application of pesticides in crops, extending our study to monthly samples over a period of 12 months. The physicochemical parameters, pH, dissolved oxygen, conductivity, salinity and temperature of the water were initially evaluated for both studies. For the biomonitoring study, we used the free-living nematode *Caenorhabditis elegans* as a bioindicator model, exposing them to samples collected from the river. The worms were exposed for 24 hours to samples collected under constant agitation and then transferred to solid medium on NGM plates for survival, longevity, reproduction and body length tests. To complement our studies, metals and pesticides were quantified in the samples and in the second project we also used the CF1553 strain, in addition to the wild type N2, in order to observe a possible expression of Sod-3. Some samples from both studies showed the residual presence of pesticides (Tebuconazole, Imazetapir, Clomazone, sulfentrazone and 2,4-D) and metals (As, Hg, Fe and Mn) and was possible to correlate with physiological effects in *C. elegans*. Several samples, in different periods and locations of the body of the Uruguay River caused a reduction in reproduction, length, survival and lifespan of exposed worms. This research aimed to reinforce the importance of frequent studies in the area of ecotoxicology, due to the constant pollution and impacts on living organisms.

Key Words: Biomarkers; Environmental Contamination; Pesticides; Heavy Metals; Environmental Contamination

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3. REVISÃO BIBLIOGRÁFICA

3.1. Rio Uruguai

O rio Uruguai é uma grande bacia hidrográfica do sul da América Latina que começa desde as nascentes do rio Pelotas e do rio Canoas e sua área estende-se até o estuário do rio da Prata. A área total drenada pela Bacia é de cerca de 385.000 km², conforme a resolução CNRH n.º 30/2003. (Uruguai. 2006) Aproximadamente 49,8% desta área de drenagem são pertencentes ao território nacional, sendo que 73% estão no Rio Grande do Sul e 27% em Santa Catarina. Seus principais tributários estão situados no setor leste da bacia. A vazão média anual é de 3600 m³/s e seu volume médio anual são de 114 km³. Além disso, o rio faz divisa entre o Uruguai e a Argentina, sendo este último o país com menor porcentagem do rio em seu território (Por volta de 17%). (Marcuzzo 2017)

Uma característica relevante sobre deste corpo hídrico são as frequentes inundações e secas ao longo do rio, acompanhando os impactos climáticos das estações do ano. (Righi 2011) Os períodos mais frios do ano apresentaram maior registro de decretos de inundação, enquanto nas épocas de calor há mais registros para as enxurradas, principalmente na porção Média do rio Uruguai, onde temos, por exemplo, os municípios de São Borja, Itaqui e Uruguaiana, visto que a planície apresenta um acúmulo fluvial elevado relativo ao escoamento da bacia hidrográfica ao longo do seu curso. (Sausen and Reis 2013, Agência Nacional das Águas 2014)

Usando como base o Caderno da Região Hidrográfica do Uruguai, a maior parte da região que circunda o rio Uruguai, é classificada como clima Temperado, com chuvas distribuídas em todas as estações do ano, proporcionado por sua posição subtropical, mas em maior concentração nos meses mais frios, como maio até setembro. (INMET , Sartori 1993, Sartori 1993) A precipitação média da bacia hidrográfica é de 1623 mm^3. (Uruguai. 2006) No entanto, a decorrência das precipitações pode variar, devido a existência de fenômenos de oscilação como “El Niño”, resultando em longos períodos de estiagens ou de inundações, que podem acontecer em qualquer época do ano, o que pode levar a diferentes níveis de água nas margens mesmo em épocas do ano não condizentes. (Rossato 2011, Guimarães, Fan et al. 2018)

Além de ser fonte de água para as populações, o Rio Uruguai também proporciona outros benefícios como, por exemplo, a pesca, agricultura, meio de

transporte e influencia de forma direta a ocupação do solo a sua volta. (Almeida, Koefender et al. 2016) A bacia hidrográfica do rio Uruguai tem grande importância não só para a região Sul do Brasil, como também Argentina e Uruguai devido a toda a rede de atividades agroindustriais que se desenvolveu a sua volta e seus canais, além do seu potencial hidrelétrico. A bacia do rio Uruguai ainda proporciona muitos outros benefícios ecológicos e sociais, sendo um sistema fundamental para a conservação de populações de peixes desta região, uma vez que é o principal canal de migração de diversas espécies. (Silva 2018)

Entre todas as diversas regiões abastecidas pelo rio Uruguai dentro do território nacional, a cidade de Uruguaiana – Rio Grande do Sul tem sua economia baseada na pecuária e a agricultura, destacando-se como forte produtora de arroz. O rio Uruguai, não só garante o fornecimento de água para o abastecimento regional e agrícola da fronteira oeste como também oferece o mesmo recurso à cidade Argentina fronteira a Uruguaiana, além de ser abrigo de vasta diversidade ictiológica, considerada uma das principais riquezas desta região. Logo, é de extrema importância que as águas do rio Uruguai sejam preservadas. (Pessano 2008, Silva 2018)

3.2. Contaminação por pesticidas

Segundo a UN-WATER, fatores globais como a mudança de padrões de consumo das últimas décadas, desenvolvimento econômico e o aumento da população mundial são partes responsáveis pela crescente demanda de água. Estima-se que ocorre um aumento de cerca de 1% ao ano neste consumo, principalmente quando tratamos de países em desenvolvimento. Segundo a própria Organização das Nações Unidas para a Alimentação e Agricultura (FAO. 2019.), as atividades agrícolas são as principais consumidoras da água doce disponível. De acordo com a entidade, 70% de toda a água consumida no mundo é utilizada na irrigação das lavouras, número que se eleva para 72% no caso do Brasil. Ainda é apontado que o inevitável crescimento da demanda de recursos vem atrelado à deterioração da qualidade da água. Descrevem também que essa perda da qualidade da água relacionada ao crescimento pode ser encontrada na maior parte dos cursos de água da Ásia, África e América Latina desde a década de 1990. (UN-WATER 2018)

Além do grande consumo de água, as atividades agrícolas atuais baseiam-se em um alto uso de pesticidas para evitar perdas e aumentar a produtividade. Os pesticidas comerciais se tornaram cada vez mais usuais nas plantações ao decorrer dos anos, o que contribuiu com o aumento da produção em larga escala, devido à demanda crescente de alimentos ao longo do tempo. (Sanches 2003) Existem muitos relatos do uso incorreto destas fórmulas, consequentemente provocando um aumento na utilização de pesticidas nas produções agrícolas. Este supracitado crescimento do uso de pesticidas deu início a um dos maiores problema ambientais com relação às fontes naturais de água e à segurança das populações, gerando diversos resíduos tóxicos que passaram a ser encontrados não só em alimentos como no ambiente, causando toxicidade em diversas espécies e acarretando danos deliberados. Relata-se que esses contaminantes chegam às fontes de águas costuma ser pelo desague das enxurradas, levando água da chuva que passa pelas lavouras, condições climáticas que levam a cheia ou secas dos rios ou ainda a percolação desses resíduos no solo, atingindo os lençóis freáticos e águas subterrâneas. (Arias, Buss et al. 2007, Berti, Düsman et al. 2009)Sendo assim todo o ser que consuma destas fontes está constantemente exposto a uma quantidade considerável de substâncias tóxicas, como metais pesados, agrotóxicos, compostos orgânicos, entre outros. E não só a falta de controle na dosagem de pesticidas que gera impacto nos corpos hídricos, mas também a mineração, construção de barragens, o desvio de cursos naturais, lançamento de efluentes e a exploração dos recursos pesqueiros sem controle dos órgãos reguladores têm provocado danos extremamente relevantes quando falamos em queda da qualidade da água e alteração dos ecossistemas aquáticos. (Rojo, Álvarez-Muñoz et al. 2019, Rojo, Cristos et al. 2021)

3.3. Importância do monitoramento ambiental e da qualidade da água

Tendo em mente que a água é um recurso natural renovável e finito e que a qualidade da água é influenciada pelo processo de crescimento industrial, populacional e atividades agrícolas e agropecuárias, temos então neste contexto que o monitoramento da qualidade desse recurso é de suma importância. (Von Sperling 2005, Zamberlan, Robaina et al. 2013) Este constante monitoramento

possibilita a criação de estratégias para garantir a continuidade do mesmo e assegurar uma boa gestão dos recursos hídricos. As atividades humanas dependem de forma extensiva da água seja para o abastecimento urbano, desenvolvimento de indústrias, geração de energia e para a irrigação destinada à agricultura. (Gloria, Horn et al. 2017, Santos, Lima et al. 2017) A qualidade da água pode ser representada por diversas variáveis que traduzem as suas principais características físico-químicas e biológicas. (Souza, Cecconello et al. 2021)

Existem no território nacional órgãos e agências reguladoras responsáveis pelo manejo e monitoramento dos recursos hídricos de forma adequada. Podemos citar a *Agência Nacional de Águas* (ANA), *o Programa Nacional de Avaliação da Qualidade das Águas (*PNQA), *a Rede Nacional de Monitoramento da Qualidade de Água (*RNQA), além do programa destinado à Divulgação de Dados de Qualidade de Água (Qualiágua). Ainda possuímos uma resolução própria do CONAMA n° 357, de 17 de março de 2005, que estabelece a classificação e enquadramento dos corpos d'água no Brasil. Esta resolução propõe organizar e estabelecer os parâmetros e variáveis físico-químicas e biológicas dos corpos hídricos dentro do território brasileiro.

Os parâmetros encontrados na água de um rio nos dizem muito sobre suas influências, incluindo a formação do solo, condições atmosféricas e tipo de vegetação. Neste contexto, o monitoramento frequente da água possibilita avaliar sua qualidade, com o intuito de garantir a continuidade e assegurar uma boa gestão dos recursos hídricos. (Bartram and Ballance 1996, Von Sperling 2005) Há diversos motivos para a realização do monitoramento, e em algumas circunstâncias as informações coletadas com um propósito podem sobrepor-se e serem aproveitadas para um objetivo distinto. Um programa de monitoramento inclui coletas frequentes nos pontos pré-definidos de amostragem com coordenadas e análise em laboratório com o maior número de variáveis possíveis, resultando em hipóteses em dimensões maiores e interpretações mais complexas. (Andrade, Araújo et al. 2007, Olsen, Chappell et al. 2012, Zamberlan, Robaina et al. 2013) Ainda, para entender a situação dos recursos hídricos, as mudanças ocorridas no tempo e no espaço, a interação entre variáveis físicas e ambientais, estudos para avaliação da qualidade da água de uma bacia hidrográfica em geral incluem também a análise de uma série de constituintes (variáveis químicas, físicas e biológicas), por um período de tempo

significativo, o que gera uma complexa e extensa quantidade de dados onde os resultados muitas vezes não são facilmente interpretados. (Calazans, Pinto et al. 2018, Martins 2020, de Souza, Cecconello et al. 2021)

3.4. Caenorhabditis elegans como modelo de biomonitoramento

Graças ao avanço do campo da toxicologia aquática e a maior frequência de artigos voltada a essa ideia, temos agora uma maior compreensão dos efeitos dos poluentes sobre os organismos e é dada bastante ênfase à importância de avaliar respostas fisiológicas dos animais e o uso de espécies como meio de estudo. Deve-se notar que uma abordagem que faz uso de biomarcadores não substitui as estratégias de monitoramento químico-físico convencionais, mas os integra fornecendo grande contribuição na padronização de resultados e na avaliação de poluentes nos meios aquáticos, mesmo em baixas concentrações. Fazendo assim com que tenhamos uma ideia micro de um efeito em maior escala no ambiente ou que possa se acumular em mais espécies da cadeia. (Viarengo, Lowe et al. 2007)

A toxicidade na água causada por contaminantes tem sido descrita usando diferentes modelos biológicos como bioindicadores, incluindo pequenos peixes como o *Danio rerio* ou micro crustáceo, como por exemplo, a *Daphnia magna*, mas o nematoide de vida livre *Caenorhabditis elegans*, já amplamente utilizado em estudos de toxicidade, vem sendo bastante usado como um modelo viável de biomonitoramento para água, solo e sedimentos fluviais. (Bluhm, Otte et al. 2014, Sancho, Villarroel et al. 2016, Stevanovic, Gasic et al. 2017, Lei, Wu et al. 2018)

Seu corpo pequeno e translucido ciclo de vida curto, reprodução rápida e facilidade de cultivo são vantagens que tornam *C. elegans* ideal para uso como modelo em estudos de monitoramento ambiental. (Du and Wang 2009) Como algumas de suas vias bioquímicas são semelhantes às dos seres humanos, ele tem sido usado em pesquisas em vários campos. Seu uso como modelo biológico em avaliações ambientais tóxicas permite a determinação de diversos desfechos incluindo sobrevivência, crescimento, reprodução, fertilidade, locomoção, sobrevivência, expressão proteica, expressão gênica, danos ao DNA, entre outros. (Anbalagan, Lafayette et al. 2012, Salgueiro, Xavier et al. 2014) *C. elegans* transgênicos portadores do gene GFP também são usados em estudos de vias

bioquímicas, incluindo resposta a metais pesados, estresse celular e oxidativo. (Bianchi, Stockert et al. 2015, Tejeda-Benitez and Olivero-Verbel 2016)

O *C. elegans* também é sensível a metais, expressando metalotioneínas *mtl-1* e *mtl-2*, pequenas proteínas ricas em cisteína com forte afinidade a metais, envolvidas no sequestro de metais, transporte, desintoxicação e proteção contra agentes oxidantes, (Wang, Wang et al. 2010, Monteiro, Brinke et al. 2014) possibilitando ser utilizado para indicar alterações no ambiente.

Neste sentido, já foi possível determinar diversos achados científicos recentes e ambientalmente relevantes usando o *C. elegans*, como por exemplo, (Karengera, Bao et al. 2021) descrevendo a difícil detecção de contaminantes ambientais em baixas concentrações e a necessidade de desenvolvimento de ferramentas bioanalíticas, expondo o modelo *C. elegans* em meio líquido, mostrando que o perfil de expressão gênica de nematoides pode ser usado para identificar os mecanismos potenciais subjacentes à toxicidade de compostos químicos. Estes achados reforçam o *C. elegans* como um organismo de teste promissor como ferramenta bioanalítica para a quantificação de tóxicos em uma ampla gama de contaminantes hidrofílicos. (Brucker, Menezes et al. 2021) Também utilizaram *C. elegans* em amostras de água coletadas em uma área rural brasileira, com o objetivo determinar a presença de agrotóxicos e metais. Os metais detectados em maior concentração foram Cu, Cr, Mg, Fe e Mn além de alterações fisiológicas no verme. Ainda sobre estes estudos, podemos citar (Abbas, Valek et al. 2018, Lei, Wu et al. 2018, Tejeda-Benítez, Noguera-Oviedo et al. 2018, Jacques, Bornhorst et al. 2019, Rai, Sjöberg et al. 2019, Han, Ji et al. 2020, Hu, Xu et al. 2021, Huang, Yen et al. 2021, Martín, Fajardo et al. 2021, Li, Yang et al. 2022), trazendo os micropoluentes antropogênicos e como afetam negativamente os ecossistemas aquáticos e os recursos hídricos, destacando a necessidade de uma avaliação integrada do tratamento de águas residuais, abrangendo parâmetros biológicos e químicos. Por fim, trazemos (Haegerbaeumer, Höss et al. 2018) em seu estudo *“Caenorhabditis elegans é representativo de espécies de nematóides de água doce em testes de toxicidade?”*, onde o estudo justifica o uso de *C. elegans* como modelo representativo para espécies de nematoides de água doce em testes de toxicidade.

4. JUSTIFICATIVA

Considerando a importância do rio Uruguai, um importante pilar para a vida da região sul do nosso país, e o uso de pesticidas nas lavouras, o que pode levar a contaminação do rio Uruguai, é necessário que seja conhecida a real situação deste importante recurso hídrico. A contaminação deste rio não só trás risco a todas as espécies nativas, como também à saúde da população local e regiões próximas pertencentes a outros países. Usando o *C. elegans* em associação com outros parâmetros, poderemos analisar os efeitos dos contaminantes e correlacioná-los para determinar o nível de contaminação, uma vez que o nematoide já é um modelo bastante usado em pesquisas de monitoramento ambiental. Este biomonitoramento é importante para o embasamento de políticas públicas de proteção ambiental que podem ser criadas e implementadas a fim de preservar este recurso hídrico e o bioma que dele depende.

5. OBJETIVO GERAL

Avaliar a qualidade da água do rio Uruguai, em sua porção média, em diversos períodos através de análises físico-químicas, quantificação de metais e pesticidas, utilizando *C. elegans* como modelo de biomonitoramento.

5.1. OBJETIVOS ESPECÍFICOS

- Avaliar parâmetros fisiológicos em *C. elegans* expostos às amostras de água coletadas;
- Verificar se as amostras de água coletadas contem contaminantes como pesticidas ou metais tóxicos;
- Relacionar os dados obtidos das amostras mensais com a atividade de plantio do arroz;
- Correlacionar os resultados das amostras com as alterações fisiológicas em *C. elegans*.
- Avaliar a qualidade e segurança da água proveniente do rio Uruguai, em sua porção mediana.

6. MATERIAIS, MÉTODOS, RESULTADOS E DISCUSSÃO.

As seções Materiais e Métodos, Resultados e Discussão serão apresentadas na forma de artigos científicos. A primeira parte foi publicada em 2021 na revista “Environmental Science and Pollution Research”. A segunda parte está apresentada na forma de manuscrito que não está formatado, pois a revista para a qual será submetido será definida após as considerações da banca.

7. ARTIGO I

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RESEARCH ARTICLE

Ecotoxicological assessment of Uruguay River and affluents pre- and post-pesticides' application using *Caenorhabditis elegans* for biomonitoring

Eugênia Carla Kuhn [1] · Maurício Tavares Jacques [1] · Daniela Teixeira [2] · Sören Meyer [3] · Thiago Gralha [4] · Rafael Roehrs [2] · Sandro Camargo [5] · Tanja Schwerdtle [3] · Julia Bornhorst [6] · Daiana Silva Ávila [1]

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Abstract
Uruguay River is the most important river in western Rio Grande do Sul, separating Brazil from Argentina and Uruguay. However, its pollution is of great concern due to agricultural activities in the region and the extensive use of pesticides. In a long term, this practice leads to environmental pollution, especially to the aquatic system. The objective of this study was to analyze the physicochemical characteristics, metals and pesticides levels in water samples obtained before and after the planting and pesticides' application season from three sites: Uruguay River and two minor affluents, Mezomo Dam and Salso Stream. For biomonitoring, the free-living nematode *Caenorhabditis elegans* was used, which were exposed for 24 h. We did not find any significant alteration in physicochemical parameters. In the pre- and post-pesticides' samples we observed a residual presence of three pesticides (tebuconazole, imazethapyr, and clomazone) and metals which levels were above the recommended (As, Hg, Fe, and Mn). Exposure to both pre- and post-pesticides' samples impaired *C. elegans* reproduction and post-pesticides samples reduced worms' survival rate and lifespan. PCA analysis indicated that the presence of metals and pesticides are important variables that impacted *C. elegans* biological endpoints. Our data demonstrates that Uruguay River and two affluents are contaminated independent whether before or after pesticides' application season. In addition, it reinforces the usefulness of biological indicators, since simple physicochemical analyses are not sufficient to attest water quality and ecological safety.

Keywords Heavy metals · Pesticides · Contamination · Arsenic · Environmental pollution · Uruguay River

Responsible Editor: Chris Lowe

✉ Daiana Silva Ávila
avilads1@gmail.com

[1] Laboratório de Bioquímica e Toxicologia em Caenorhabditis elegans, Programa de Pós-Graduação em Bioquímica, Universidade Federal do Pampa- UNIPAMPA, Campus Uruguaiana, BR 472 – Km 592 – Caixa Postal 118, Uruguaiana, RS CEP 97500-970, Brazil

[2] Laboratório de Estudos Físico-químicos e Produtos Naturais, Universidade Federal do Pampa, Campus Uruguaiana, Uruguaiana, Brazil

[3] Institute of Nutritional Science, University of Potsdam, Nuthetal, Germany

[4] Núcleo de Pesquisa em Ictiologia, Limnologia e aquicultura da Bacia do Rio Uruguai, Universidade Federal do Pampa, Campus Uruguaiana, Uruguaiana, Brazil

[5] Programa de Pós-Graduação em Computação Aplicada (PPGCAP), Universidade Federal do Pampa, Campus Bagé, Bagé, Brazil

[6] Food Chemistry, Faculty of Mathematics and Natural Sciences, University of Wuppertal, Wuppertal, Germany

Introduction

The aquatic environments are dynamic ecosystems, exhibiting great variability in the water quality. This variability can be partially attributed to the intrinsic characteristics of the system and to extrinsic variations such as climatic changes, droughts, and rainy seasons. However, most of these variations have been originated from pollution. Anthropogenic activities (i.e., industrial wastewater, transport, agriculture) release a wide variety of pollutants that are important threats to the conservation of the water resources (Ferreira et al. 2016).

The presence of pesticides in hydrological systems due to agricultural activities is the most common type of contamination. This is of particular concern because commercial formulations contain many different ingredients, such as potentially toxic metals and surfactants. They have potentially toxic properties to the living organisms, and due to their persistence in the environment, they are of high ecological risk (Gonzalez-Macias et al. 2006). In particular, Brazil has been one of the

largest pesticide consumers since the 1970s. Notably, 30% of the pesticides applied in Brazil have been banned in Europe, which brings issues for commodities' exportation. Among the most important agricultural commodities produced in Brazil and, particularly, in Rio Grande do Sul state (RS) is rice (Carneiro et al. 2015; Fao et al. 2019).

Rice (*Oryza sativa* L.) is a grain widely consumed in the basic diet of more than half of the world population, becoming the second largest crop after wheat. Rice crops are cultivated in more than 100 countries with 90% of the global production located in the Asians countries (Fukagawa and Ziska 2019; Kaur et al. 2016; Sun et al. 2017). Studies indicate that the consumption of rice, mainly those grown in flooded areas, are the main source of dietary exposure to arsenic (As) and residual pesticides (Fao et al. 2019; Khammanee et al. 2020; Kumarathilaka et al. 2018; Meharg et al. 2009; Zhao et al. 2013; Zhu et al. 2008).

In the southern Brazilian region, rice accounts for 10% of the crops, and according to local data, rice cultivation uses, on average, 10 L of pesticides per planted hectare, which is very high compared to other cultures (Primel et al. 2005). Notably, the municipality of Uruguaiana, located in the extreme west in the state Rio Grande do Sul, is well known as one of the top producers of irrigated rice in Brazil. The ideal climatic conditions, the vast lands, and the presence of a great river like the Uruguay River are the main reasons for this success. However, the amount of pesticides applied and the trafficking of illegal pesticides such as paraquat, which enters Brazil from the Uruguay and Argentina borders, are great concerns. There are many small dams and streams that were created to allow the water flow to and from rice crops. Therefore, pesticides may be dragged into the Uruguay River. The Uruguay River is very important for agriculture, fishing, and also for recreational activities in Brazil, Argentina, and Uruguay along its 1838 km. However, water quality has been neglected by regulatory agencies of these three countries. Erosion and silting of the banks and near extinction of some plant species are consequences of the pollution in this river (Arocena et al. 2018; Goncalves et al. 2020; Rojo et al. 2019; Speranza et al. 2020).

It is known that continuous monitoring is one of the most reliable practices for obtaining information on the quality of natural water resources. Different analyses have been used to detect variations in addition to the standardized physicochemical monitoring. However, most of these techniques cannot identify all the pollutants present in the aquatic environment. For this reason, ecotoxicological biomonitoring practices have increased in the last years and are have been added to the monitoring plans (Clavijo et al. 2016; Pignati et al. 2017; Ruan et al. 2009; Salem et al. 2016; Viarengo et al. 2007).

Some criteria must be met for an organism to be adopted in biomonitoring: for example, must be sensitive to the toxic agent, easy to handle, and available throughout the year (Wah Chu and Chow 2002). Many authors have already demonstrated that the free-living nematode *Caenorhabditis elegans* (*C. elegans*) is a viable model as a bioindicator in ecological risk assessments as it meets the requirements (Clavijo et al. 2016). In addition, *C. elegans* is a globally accepted model for environmental impact analysis (Wah Chu and Chow 2002). Remarkably, this model is very attractive to assess aquatic toxicity because it has a short life cycle, a small body size, is easy to handle, and has low cost for maintenance. *C. elegans* has a high tolerance to pH variations, salinity, and water hardness and offers a wide range of ecological and toxicologically relevant parameters such as mortality, growth, and reproduction (Tejeda-Benitez et al. 2016).

Therefore, the objective of the present study was to evaluate the water quality from Uruguay River samples obtained before and after pesticides application in the rice crops, using *C. elegans* as bioindicator. In addition, we sought to correlate biological outcomes and limnological water analysis for future regulatory purposes.

Materials and methods

Sample collection

Samplings were carried out in two periods, before the planting and pesticides application period and after pesticides application in the crops. The sampling sites were selected based on the different proximities to the rice crops (Fig. 1): (A) the margin of the Uruguay River basin (U), which provides water for drinking and for crops irrigation, but also receives all kinds of wastes from the affluents (coordinates 29° 44′ 50.3″ S, 57° 05′ 19.6″ W); (B) Salso Stream (S) located at BR 472 (coordinates 29° 47′ 54.2″ S, 57° 05′ 31.6″ W), which receives water from the sewage treatment plant, which then drains back to the Uruguay River; (C) Mezomo Dam (M), chosen for receiving water from some irrigated rice plantation properties, draining this water back to the Uruguay River (coordinates 29° 59′ 14.5″ S, 57° 07′ 19.3″ W). Pre-pesticide water samples were collected in triplicates on August 19th, 20th, and 22nd/2016 (pre-D1, pre-D2, and pre-D3, respectively), and post-pesticide application samples on February 28th, March 2nd, and March 3rd/2017 (post-D1, post-D2, and post-D3, respectively) at same period of the day, between 11:00 and 12:00 a.m. using sterilized flasks, from the top layer to the water flow. The pesticide application occurred between December and February. Following limnologic analysis, samples were stored at − 20 °C.

Physicochemical analysis

In order to determine the characteristics of the water samples, we have subjected them to physical and chemical analyses. Based on that, we evaluated dissolved oxygen, temperature,

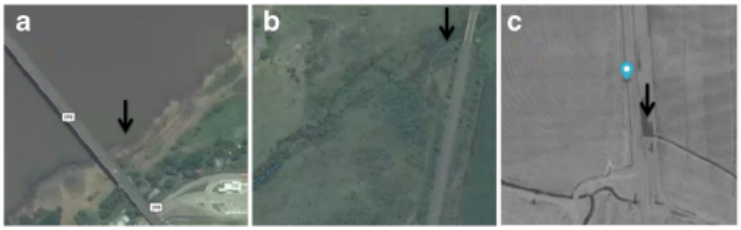

Fig. 1 Geographic locations from samples collection. Arrows indicate the exact point of water collections. **a** The margin of the Uruguay River basin, which receives tillage water and wastes from the region and also supplies water for drinking and irrigation (coordinates 29° 44′ 50.3″ S, 57° 05′ 19.6″ W). **b** Salso Stream located at BR 472, known for receiving the treated sewage material (coordinates 29° 47′ 54.2″ S, 57° 05′ 31.6″ W). **c** Mezomo Dam, located next to a rice farms (coordinates 29° 59′ 14.5″ S, 57° 07′ 19.3″ W). Source: Google Earth, scale 100 m

pH, and conductivity using a portable multiparameter; turbidity was analyzed with a turbidimeter and ammonia and nitrite by colorimetric assays.

Worms' maintenance and synchronization

Wild-type N2 worms were used in all the experiments and were obtained from the *Caenorhabditis* Genetics Center (CGC). Animals were kept in 21–22 °C and grown on nematode growth medium (NGM) seeded with *Escherichia coli*/OP50 as food source. To obtain animals at the same larval stage, the population was synchronized by exposing gravid adult worms to a lysing solution (0.45 N NaOH, 2% HOCl). After 14 h, the eggs hatched, releasing L1 larvae that were used for the treatments (Brenner 1974).

Exposure to water samples

One thousand worms at L1 stage were placed in a 100-mL Erlenmeyer, containing 5 mL of the water samples and *E. coli*/OP50 as food source and left on constant agitation for 24 h (on orbital shaker at 70 RPM, in an incubator at 21 °C). K-medium (composed of 3 g of sodium chloride, 2.4 g of potassium chloride, and 1 L of H_2O distilled) was used as control. After 24-h exposure, worms were pelleted and transferred to NGM plates seeded with *E. coli*/OP50 for recovery.

Survival and longevity assays

When worms reached L4 stage, the live ones were scored with the aid of a grid. This assay was performed only with animals exposed to post-pesticide samples. Afterwards, 20 treated animals were transferred to NGM plates seeded with *E. coli*/OP50, in duplicates. Survival was assessed daily until all the worms were dead. The worms were transferred daily during the reproductive period to avoid contamination of the progeny and then were transferred every 2 days. Each experiment was repeated at least three times.

Brood size

One worm from each treatment was individually transferred daily to NGM plates with *E. coli*/OP50 and reproduction was evaluated by counting the brood size until the end of the reproduction period. Each experiment was performed in triplicates and three independent experiments were performed.

Pesticides determination

The extraction was carried out using Strata X cartridges (500 mg/3 mL), 6 mL of methanol, 6 mL of ultrapure water, and 6 mL of acidified ultrapure water of pH 3 were used for the conditioning. Two liters of sample were percolated maintaining the flow rate of 1 mL/min, and subsequently, 3 mL of acidified pH 3 water was added to affect the cleaning of the cartridge. The analytes were eluted with 9 mL of methanol and under vacuum for another 10 min. The solvent was evaporated to dryness and the eluate was resuspended in 1.5 mL of methanol. Subsequently, the samples were filtered through a 0.22-μm syringe filter and analyzed by HPLC-DAD. The chromatographic system used was YL9100 (Young Lin, South Korea) equipped with a YL90 vacuum degasser YL9110, YL9150 autosampler, YL9131 column oven, and YL9160 diode array detector. The control of the equipment and the data acquisition were made through the YL-Clarity software. The acetonitrile and methanol used in the chromatographic analyses were HPLC grade (J.T. Baker, The Netherlands); ultrapure water was purchased from a Milli-Q system (Millipore, USA). Analyses were performed using Synergi 4 μ Fusion-RP 80 Å (250 × 4.6 mm) and pre-column Fusion-RP (4 × 3.0 mm) chromatography column (Phenomenex, USA). The method initially consists of acidified ultrapure water up to pH = 3, methanol, and acetonitrile (46/38/16, v/v) at the flow rate of 0.9 mL/min; after 10 min, the mobile phase, it became 40% of water pH 3 and 22% acetonitrile, maintaining the initial amount of methanol and with the flow rate of 1.0 mL/

min, which is maintained up to 15 min. At 15 min, the mobile phase, it became 36% water pH 3 and 32% acetonitrile and methanol. After 30 min, the mobile phase went to 40% methanol, 36% acetonitrile, and 24% water pH 3 to 35 min. At 35 min, the mobile phase passed on a 44% methanol, 36% acetonitrile, and 20% water pH 3 and flow of 1.2 mL/min. The method was finished at 40 min with mobile phase in 48% methanol and 16% water pH 3. The wavelengths used were 220 nm for tebuconazole and clomazone and 248 nm for imazethapyr; the injection volume was 20 μL of sample. We have also analyzed the presence of diuron, 2,4D, bentazone, quinclorac, propanil, sulfentrazone, 3,4 DCA, and 3,5 DCA.

Metal analysis

Metals as arsenic, copper, iron, manganese, cadmium, zinc, aluminum, mercury, and lead were quantified through inductively coupled plasma mass spectrometry (ICP-MS/MS (Agilent 8800 ICP-QQQ)) as described before (Lohren et al. 2015; Meyer et al. 2018).

Statistical analysis

All assays were performed at least three individual times and GraphPad Prism 6 software was used to generate charts and statistical analysis. One or two-way ANOVA were used and $p < 0.05$ was considered statistically significant. Repeated measures one-way ANOVA was used for lifespan analysis. Post hoc tests were performed using Tukey post hoc test. Correlation and multiple linear regression were also performed.

Results

Physicochemical analyses

The data obtained from water samples collected pre- and post-pesticide application seasons in the crops are shown in Table 1. It can be observed that dissolved O2, turbidity, nitrite, ammonia, and conductivity were within the desired levels.

Table 1 Physicochemical analysis of water samples

Samples	Dissolved O_2 (mg/L)	Turbidity (NTU)	Ammonia (mg/L)	pH	Nitrite (mg/L)	Conductivity (μS/cm)
U1 (D1) pre-pesticides	9.2	7.49	0.25	6.14	<LOD	78.24
U2 (D2) pre-pesticides	9.2	6.3	0.25	6.7	<LOD	82.02
U3 (D3) pre-pesticides	9.2	6.36	0.25	6.67	<LOD	71.46
U4 (D1) post-pesticides	3.9	54.9	0.25	6.55	<LOD	66.27
U5 (D2) post-pesticides	7.8	8.21	<LOD	6.4	<LOD	52.12
U6 (D3) post-pesticides	7.6	27	0.1	6.5	<LOD	73.52
S1 (D1) pre-pesticides	9.5	4.36	1	6.23	0.3	443.8
S2 (D2) pre-pesticides	9.3	3.84	1	6.45	0.3	451.7
S3 (D3) pre-pesticides	9.3	4.28	1	6.48	0.3	449
S4 (D1) post-pesticides	7.4	18.7	0.25	6.73	<LOD	178.7
S5 (D2) post-pesticides	7.7	12.1	0.1	6.71	0.025	240.7
S6 (D3) post-pesticides	7.5	17.8	0.25	6.7	0.05	133
M1 (D1) pre-pesticides	9.8	81.2	0.1	6.42	<LOD	155.9
M2 (D2) pre-pesticides	9.6	8.61	0.1	6.57	<LOD	155.1
M3 (D3) pre-pesticides	9.5	5.34	0.1	6.63	<LOD	153.8
M4 (D1) post-pesticides	5.8	5.34	0.1	5.81	<LOD	56.22
M5 (D2) post-pesticides	6.6	81.2	<LOD	6.33	<LOD	122
M6 (D3) post-pesticides	7.3	8.61	0.1	5.46	<LOD	80.18
RV	> 5	Up to 100	Up to 3.7	6–9	1	NA

(U1) sample collected on the Uruguay River on August 19th; (U2) sample collected on the Uruguay River on August 20th; (U3) sample collected on the Uruguay River on August 22nd; (U4) sample collected on the Uruguay River on February 28th; (U5) sample collected on the Uruguay River on March 2nd; (U6) sample collected on the Uruguay River on March 3rd; (S1) sample collected at Salso Stream on August 19th; (S2) sample collected at Salso Stream on August 20th; (S3) sample collected at Salso Stream on August 22nd; (S4) sample collected at Salso Stream on February 28th; (S5) sample collected at Salso Stream on March 2nd; (S6) sample collected at Salso Stream on March 3rd; (M1) sample collected at Mezomo Dam on August 19th; (M2) sample collected at the Mezomo Dam on August 20th; (M3) sample collected at the Mezomo Dam on August 22nd; (M4) sample collected at Mezomo Dam on February 28th; (M5) sample collected at Mezomo Dam on March 2nd; (M6) sample collected at the Mezomo Dam on March 3rd

<LOD, below the limit of detection; *RV*, reference values by CONAMA 357/05-Class II water; *NA*, not available

Pesticides and metal quantification

Table 2 depicts the amounts of pesticide residues of clomazone, imazethapyr, and tebuconazole that were found in all samples. There were no differences between pre- and post-samples, not even among locations. We have also detected metals as As and Hg as well as Cu, Fe, and Mn in all samples. Overall, pre-pesticide samples presented higher amounts of Mn, Cu, As, and Hg in relation to post-pesticides. No significant differences were found among locations, since they all presented similar metal levels. Most of the samples presented higher levels than the recommended for class II waters, according to the Brazilian regulatory agency CONAMA.

Brood size

In Fig. 2a and b, it can be observed that *C. elegans* reproductive capacity was impaired by exposure to all pre- and post-pesticide samples. When separately analyzing these data, we observed that some samples strongly contributed to these results (Supplementary Figure S1). For pre-pesticide samples, D1 Mezomo Dam and D2 Salso Stream and Uruguay River depicted significant results (S1 A and B, $p < 0.05$), whereas D1 post-pesticide samples from Salso and Mezomo had higher statistical power (S1 D, $p < 0.05$). These different effects can be attributed to the different metal, pesticides, and other contaminant composition in these samples that could be changed by factors such as flow dynamics, the possibility of residues addition in those particular collection days, or that the bottom of the River or the affluents could have been moved. Of note, samples were collected from the upper water column.

Lifespan assay

Next, we investigated the long-term effects of sample exposure to worms by analyzing the whole lifespan. We have found that only post-pesticides significantly reduced *C. elegans* longevity compared to the control group (Fig. 3a–c). The most powerful statistical difference was detected

Table 2 Pesticide levels in water samples

Samples	Clomazone (mg/L)	Imazethapyr (mg/L)	Tebuconazole (mg/L)
U1 (D1) pre-pesticides	0.003	0.00175	0.00338
U2 (D2) pre-pesticides	<LOD	<LOD	<LOD
U3 (D3) pre-pesticides	0.01176	0.00158	0.00226
U4 (D1) post-pesticides	0.01176	0.00158	0.00226
U5 (D2) post-pesticides	0.003	0.00175	0.00338
U6 (D3) post-pesticides	<LOD	<LOD	<LOD
S1 (D1) pre-pesticides	0.00318	0.00152	0.00343
S2 (D2) pre-pesticides	<LOD	<LOD	<LOD
S3 (D3) pre-pesticides	0.00356	0.00146	0.00199
S4 (D1) post-pesticides	0.00356	0.00146	0.00199
S5 (D2) post-pesticides	0.00318	0.00152	0.00343
S6 (D3) post-pesticides	<LOD	<LOD	<LOD
M1 (D1) pre-pesticides	0.00868	0.00248	0.00366
M2 (D2) pre-pesticides	<LOD	<LOD	<LOD
M3 (D3) pre-pesticides	<LOD	<LOD	<LOD
M4 (D1) post-pesticides	<LOD	<LOD	<LOD
M5 (D2) post-pesticides	0.00868	0.00248	0.00366
M6 (D3) post-pesticides	<LOD	<LOD	<LOD
RV	NA	NA	NA

(U1) sample collected on the Uruguay River on August 19th; (U2) sample collected on the Uruguay River on August 20th; (U3) sample collected on the Uruguay River on August 22nd; (U4) sample collected on the Uruguay River on February 28th; (U5) sample collected on the Uruguay River on March 2nd; (U6) sample collected on the Uruguay River on March 3rd; (S1) sample collected at Salso Stream on August 19th; (S2) sample collected at Salso Stream on August 20th; (S3) sample collected at Salso Stream on August 22nd; (S4) sample collected at Salso Stream on February 28th; (S5) sample collected at Salso Stream on March 2nd; (S6) sample collected at Salso Stream on March 3rd; (M1) sample collected at Mezomo Dam on August 19th; (M2) sample collected at the Mezomo Dam on August 20th; (M3) sample collected at the Mezomo Dam on August 22nd; (M4) sample collected at Mezomo Dam on February 28th; (M5) sample collected at Mezomo Dam on March 2nd; (M6) sample collected at the Mezomo Dam on March 3rd

<*LOD*, below the limit of detection; *RV*, reference values by CONAMA 357/05-class II water; *NA*, not available

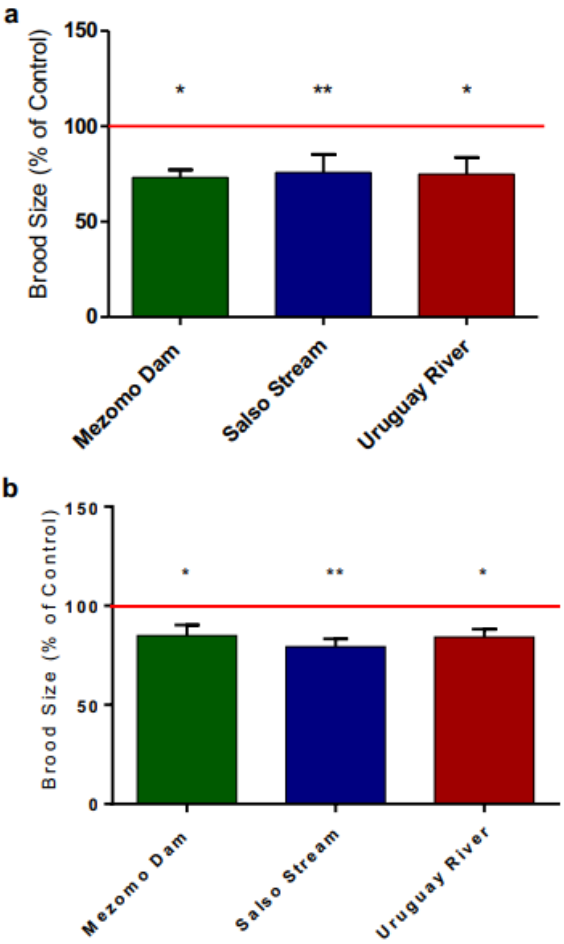

Fig. 2 Reproduction rate in *C. elegans* exposed to water samples. **a** Pre-pesticide application and **b** post-pesticide application. * indicates statistically significant differences in comparison to control (red line) following one-way ANOVA ($p < 0.05$, Tukey post hoc test)

in Mezomo Dam data, in which worms exposed to post-pesticide samples had also a significant shorter lifespan in relation to pre-pesticide-exposed animals. The same was evidenced when analyzing separately these samples (Supplementary Figures 2 and 3).

Survival

In order to detect short-term effects of sample exposure and considering that we previously hypothesized that post-pesticide samples would have been more contaminated and therefore would cause higher toxicity, we analyzed the survival rate 48 h after the end of exposure. We observed that worms exposed to all the water samples collected on D1 and D2 after the pesticides application season caused a reduced survival rate (Fig. 4, $p < 0.05$). However, worms exposed to D3 samples did not present reduced survival. This finding might be associated to the pesticides and metal levels, since all post-D3 samples depicted lower levels or below the detection levels of these contaminants in relation to D1 and D2 (Tables 2 and 3).

Correlation analyses

In order to determine whether physicochemical parameter or pesticides would be causing alterations in the biological endpoints, we have performed a correlation analysis (Fig. 5). We have observed that reproduction may be impaired by conductivity, nitrite, tebuconazole, and imazethapyr levels. Lifespan can be shortened by dissolved oxygen, conductivity, and ammonia in the samples. We have found positive correlations between turbidity and pesticide levels and a negative correlation between dissolved oxygen and pesticides, which indicate that pesticide presence impairs the water quality by reducing available O_2 and changing the limpidity of the samples, possibly by stimulating anaerobic microorganism growth. The normality test demonstrated a great variation of the parameters in the samples, even those collected from the same sites in the same period, which may have impaired our analysis. As previously mentioned, these variations were expected, and in order to avoid inaccurate conclusions from a single sampling, we performed triplicate collections.

The principal component analysis (PCA) demonstrated a clearly separation between the data obtained from pre- and post-pesticide samples. Considering PC1 and PC2 composition, pre-pesticide samples are mostly influenced by metals, conductivity, ammonia, and dissolved O_2, whereas post-pesticide samples are mostly influenced by turbidity, tebuconazole, imazethapyr, and clomazone levels (Fig. 6). This indicates that, despite the great variability among the samples, the presence of metals and pesticides are important variables that modify the biological outcomes in worms exposed to pre- and post-pesticide samples.

Discussion

The assessment of water quality is critical to water management policies. In the present study, the *C. elegans* nematode was used as a bioindicator to evaluate the quality of the Uruguay River and two affluents before and after pesticide application on rice crops. We have confirmed the presence of pesticide residues and toxic metals in both pre- and post-application samples. In addition, we have found that independent on whether samples were collected before or after pesticides applications, they caused physiological alterations in the worms, which indicate that samples had poor quality. One of the most important observations is that *C. elegans* reproduction and lifespan were impaired, even when the

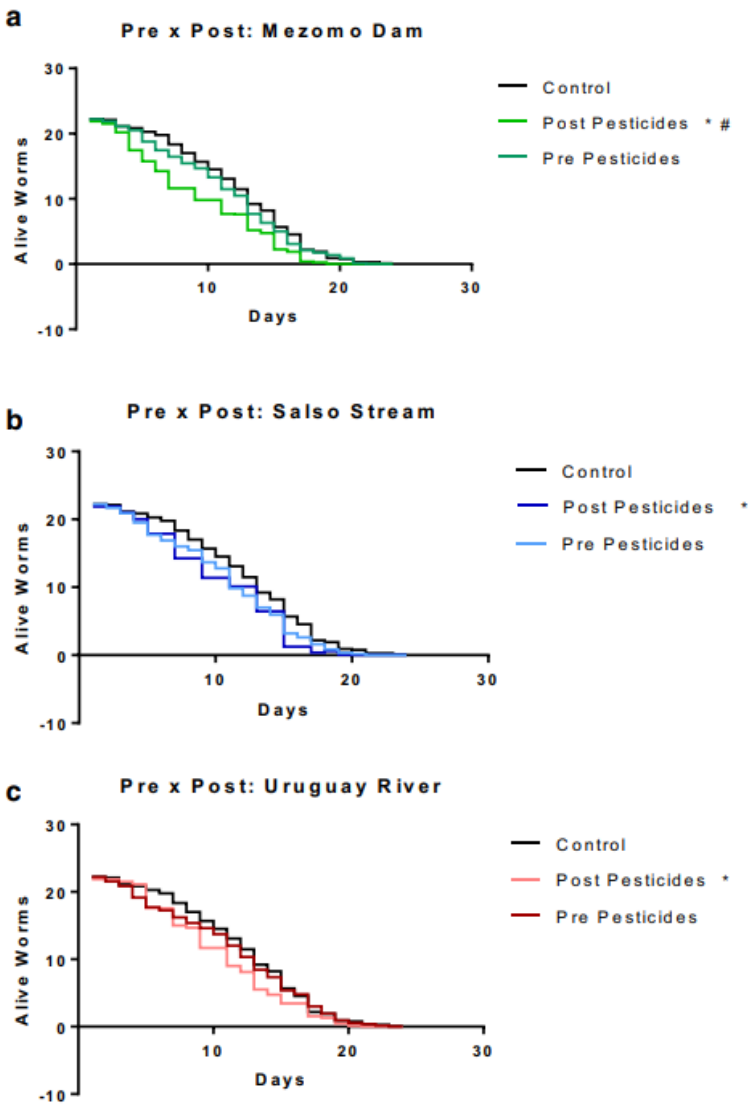

Fig. 3 Lifespan of worms exposed to samples pre- and post-pesticide application. **a** Mezomo Dam. **b** Salso Stream. **c** Uruguay River. * indicates significant differences in comparison to control and # indicate significant difference between pre- and post-samples. Data were analyzed by repeated measures one-way ANOVA followed by Tukey post hoc test ($p < 0.05$)

physicochemical data were within the acceptable levels. This aspect reinforces the concept that physicochemical data are not sufficient to attest water quality and that the use of bioindicators is a powerful tool. We hypothesize that these samples are constantly contaminated due to the repeated anthropogenic activities, especially agricultural activities.

Remarkably, we detected high levels of As, much higher than those recommended by CONAMA (Brazilian regulatory agency) and by the World Health Organization. Arsenic forms are easily absorbed, particularly in rice grains, which has been associated to the increased incidence of cardiovascular diseases in populations that are major rice consumers (Chanpiwat and Kim 2019; Fao et al. 2019; Majumder et al. 2020). Besides, As, Fe, Mn, and Hg were also found at higher then recommended levels. Remarkably, the damaging effects of the bioaccumulation of these metals to the aquatic ecosystems are of concern, considering their long-term effects (He et al. 2019; Vijver et al. 2004). In addition, some samples depicted conductivity levels above the recommended. There are no reference values in Brazilian legislation for conductivity; however, according to Von Sperling (2007), natural waters should have 10–100 µS/cm, whereas polluted environments may have up to 1000 µS/cm (Von Sperling 2005).

The presence of commonly used pesticides in rice crops as clomazone, imazethapyr, and tebuconazole (Glinski et al. 2018) was detected in the samples obtained prior and after pesticide application, mainly in the Uruguay River, which is the recipient of the crop affluents. We expected significantly

Table 3 Metal concentrations in water samples (μg/L)

Pre-pesticides samples	Mn	Fe	Cu	As	Hg
U1 (D1) pre-pesticides	2.49	50.52	1.14	0.38	28.6
U2 (D2) pre-pesticides	31.41	67.78	0.75	0.43	65.34
U3 (D3) pre-pesticides	26.04	21.64	<LOD	1.37	56.8
U4 (D1) post-pesticides	0.17	29	<LOD	<LOD	<LOD
U5 (D2) post-pesticides	7.1	245.3	<LOD	<LOD	<LOD
U6 (D3) post-pesticides	0.78	143.5	<LOD	<LOD	<LOD
S1 (D1) pre-pesticides	3.37	58	1.41	0.4	32.14
S2 (D2) pre-pesticides	10.43	27.26	<LOD	0.44	96.34
S3 (D3) pre-pesticides	60.7	35.55	<LOD	1.41	86.77
S4 (D1) post-pesticides	0.11	3.5	<LOD	1.4	<LOD
S5 (D2) post-pesticides	<LOD	307.9	<LOD	0.4	<LOD
S6 (D3) post-pesticides	18.6	<LOD	<LOD	1.1	<LOD
M1 (D1) pre-pesticides	1.86	104.1	0.98	0.21	41.77
M2 (D2) pre-pesticides	18.57	67.34	1.03	0.44	89.9
M3 (D3) pre-pesticides	69.56	51.65	<LOD	1.44	103.02
M4 (D1) post-pesticides	0.05	8.2	<LOD	<LOD	<LOD
M5 (D2) post-pesticides	0.11	<LOD	<LOD	<LOD	<LOD
M6 (D3) post-pesticides	<LOD	2.5	<LOD	<LOD	<LOD
MAV	0.1	0.3	0.009	0.14	0.2

(U1) sample collected on the Uruguay River on August 19th; (U2) sample collected on the Uruguay River on August 20th; (U3) sample collected on the Uruguay River on August 22nd; (U4) sample collected on the Uruguay River on February 28th; (U5) sample collected on the Uruguay River on March 2nd; (U6) sample collected on the Uruguay River on March 3rd; (S1) sample collected at Salso Stream on August 19th; (S2) sample collected at Salso Stream on August 20th; (S3) sample collected at Salso Stream on August 22nd; (S4) sample collected at Salso Stream on February 28th; (S5) sample collected at Salso Stream on March 2nd; (S6) sample collected at Salso Stream on March 3rd; (M1) sample collected at Mezomo Dam on August 19th; (M2) sample collected at the Mezomo Dam on August 20th; (M3) sample collected at the Mezomo Dam on August 22nd; (M4) sample collected at Mezomo Dam on February 28th; (M5) sample collected at Mezomo Dam on March 2nd; (M6) sample collected at the Mezomo Dam on March 3rd

<LOD, below the limit of detection; *RV*, reference values by CONAMA 357/05-class II water; *NA*, not available

higher levels of pesticides as well as higher toxicity in the post-application samples. However, there are 2 factors to consider: (i) we did not measure other pesticides used in the region because we did not have standards or standardized methods for quantification, and (ii) the persistence of the pesticides found and also those ones that could not be measured could be the cause of the toxicity found in pre-application samples. Recently, Gonçalves et al. demonstrated that another location of the Uruguay River presented other seven pesticides: atrazine, byspiribac-sodium, imidacloprid, malathion, propoxur, quinclorac, and simazine. Their presence were associated to alterations in several oxidative biomarkers in *Astyanax jacuhiensis*, a native species (Goncalves et al. 2020).

Remarkably, we observed that, collectively, exposure to pre- and post-pesticide samples impaired worms reproduction. In addition, post-pesticide exposure affected *C. elegans* survival in both short and long terms. Given all the factors that could modify worms' biological responses, correlation and PCA analyses were performed (Figs. 5 and 6). We have observed that lifespan is negatively modulated by the reduction of dissolved oxygen in the medium. It is known that *C. elegans* can enter into an alternative larval stage known as "dauer" in response to environments with low oxygen concentrations and can even die under highly anaerobic conditions due to energy deficit (Kitazume et al. 2018; Miller and Roth 2009). However, the levels of dissolved oxygen were not as low to cause worms' mortality; therefore, this parameter by itself cannot be used to explain reduced worms' lifespan. Notably, as dissolved oxygen levels decreased, the levels of clomazone were increased, and this factor has been negatively correlated with worms' longevity in our study. This herbicide has been widely used in various crops, including in rice. In 2009, clomazone was among the top ten most used herbicides in Brazil (Primel et al. 2005; Rebelo et al. 2010). Although very effective, it is known for contributing to environmental contamination due to its high solubility in water (1100 mg L^{-1}) (Zanella et al. 2002). Previous studies have already described that clomazone caused increased mortality of *Rhamdia quelen* (LC50 7.32 μL L^{-1}) (Crestani et al. 2007),

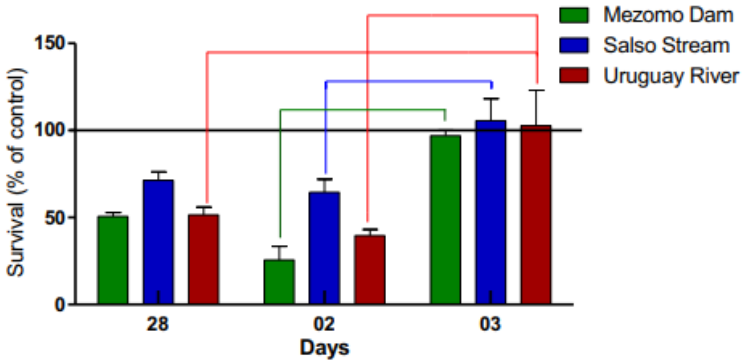

Fig. 4 Survival rate in *C. elegans* exposed to samples collected post-pesticide application. Lines indicate significant differences among groups by two-way ANOVA ($p < 0.05$, Tukey post hoc test)

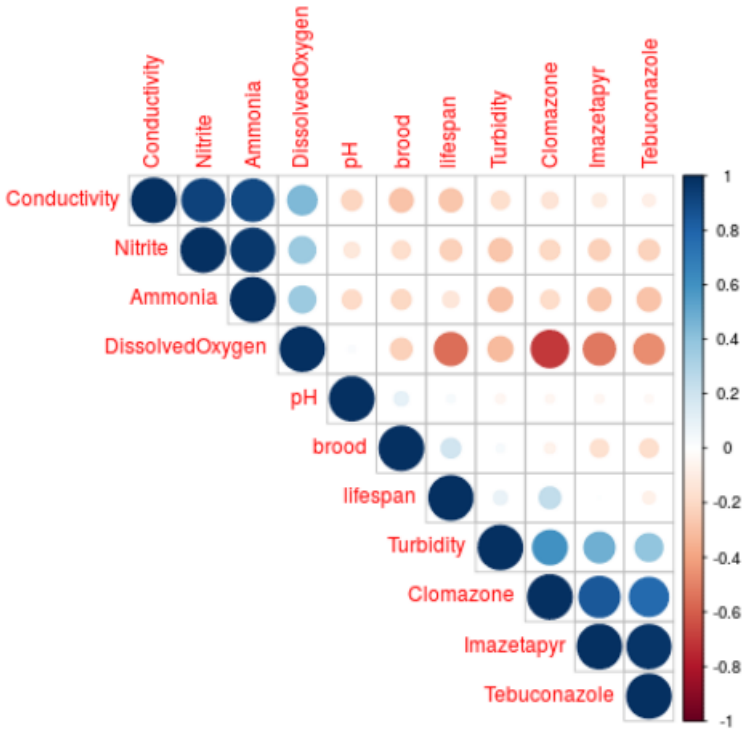

Fig. 5 Correlation analysis of all data, independent on samples collected pre- or post-pesticide application

and convulsions occurred after 30-min exposure to 20 and 50 mL/L (Miron et al. 2004). In addition, clomazone exposures have caused suppression of catalase antioxidant enzyme and increased lipid peroxidation in *Rhamdia quelen*, activation of glutathione transferase enzyme activity in *Prochilodus lineatus* (Pereira et al. 2013); and to cause high mortality, teratogenic effects, and underdevelopment in *Danio rerio* embryos, findings that evidence its toxicity (Stevanovic et al. 2017).

The correlation and PCA analysis have also indicated that tebuconazole presence may reduce longevity and egg viability in *C. elegans*. This fungicide toxicity in reproduction has already been reported in different animal models (Guimaraes et al. 2018; Li et al. 2020; Machado-Neves et al. 2018;

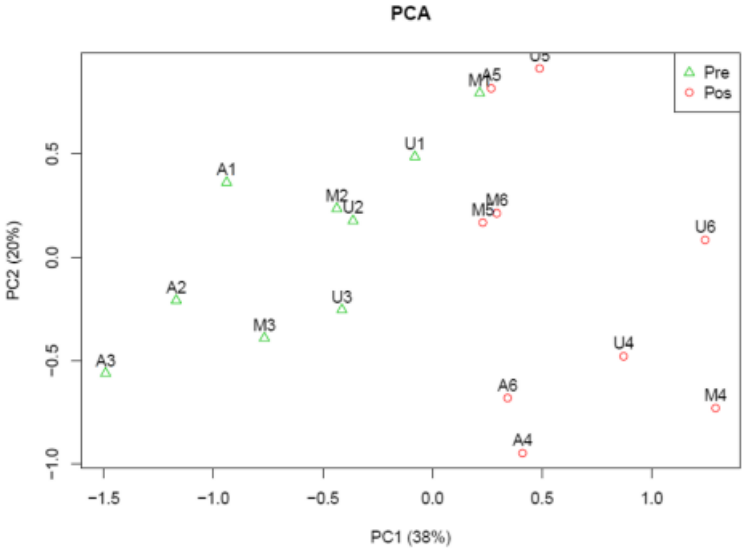

Fig. 6 PCA analysis of the data considering pre- (green triangles) and post-pesticide (red circles) samples

Zanella et al. 2002), but not in this nematode. Tebuconazole is a fungicide that has been frequently detected in agriculture systems at concentrations that affect endocrine function in organisms. For instance, it has been found to cause oxidative stress and endocrine disruption in rats (Yang et al. 2018) and developmental toxicity associated to thyroid dysfunction in zebrafish (Li et al. 2020). Finally, imazethapyr has also been reported by causing metabolic alterations in *Cyprinus carpio* (Moraes et al. 2011), genotoxicity in tadpoles of *Rhinella arenarum* and *Hypsiboas pulchellus* species (Carvalho et al. 2019; Perez-Iglesias et al. 2017; Perez-Iglesias et al. 2015), and also in mammalian cells (Soloneski et al. 2017); however, the toxicity of herbicide is poorly characterized and needs to be further investigated.

We have found great variability of metals and pesticide levels among samples collected in the same site and same period. Of note, the samples were collected from the top layer of the water sources; therefore, the lower metal levels in post-pesticide samples could be attributed to sedimentation of these materials due to many days without rain (Ajima et al. 2015). Another explanation for this phenomenon is that the inert components present in pesticides and other contaminants may have decanted, sedimented, and accumulated, as reported by previous studies (Caballero-Gallardo et al. 2015; Kim et al. 2018; Tejeda-Benitez et al. 2016; Vallejo Toro et al. 2016). On the other hand, higher metal levels detected in 3rd day from all pre-pesticide samples could be attributed to movements in the bottom of the river or that some deposit was done on those sites in that day. We knew that these daily changes could occur since hydric systems are highly dynamic, and because of that, we have sampled in triplicates for each site, avoiding a unique collection and inaccurate conclusions.

In summary, this study demonstrated that pesticides and metals are present in the Uruguay River and two of its affluents, with particular attention to metals above the recommended levels. Furthermore, the biological data demonstrated that the worms suffered negative consequences from the exposure to contaminated samples, therefore indicating that aquatic organisms would be impaired as well. We did find correlation between these biological effects and the presence of the analyzed metals and pesticides; however, we did not find a correlation between pre- and post-pesticide application season, therefore indicating that Uruguay River and affluents are constantly contaminated. In addition, the presence of other contaminants and their association must be taking into consideration. The regulatory authorities from Brazil, Uruguay, and Argentina must be aware of the presence of these contaminants in this important River and take actions to reduce the ecotoxicological outcomes to all live forms that depend on it.

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Authors' contributions E.C.K conceptualization, investigation, methodology, data curation, formal analysis, writing- original draft; M.T.J conceptualization, supervision, methodology, formal analysis, writing - original draft; D.T methodology, data curation, formal analysis, writing; S.M methodology, data curation, formal analysis; T.G methodology, data curation, formal analysis, writing; R.R data curation, formal analysis, supervision, funding acquisition, writing; S. C data curation, formal analysis, writing- original draft; T. S methodology, data curation, formal analysis, resources, writing; J. B methodology, data curation, formal analysis, writing; D.S.A conceptualization, funding acquisition, investigation, methodology, project administration, data curation, formal analysis, resources, supervision, writing.

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Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Ethics approval and consent to participate Not applicable

Consent to publish Not applicable

Competing interests The authors declare that they have no competing interests.

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8. MANUSCRITO

Annual Ecotoxicological Assessment of medium Uruguay River using *Caenorhabditis elegans* as a biomonitor

Eugênia Carla Kuhn[1], Andressa Pedroso Belmonte[1], Ana Thalita Gonçalves Soares[1], Maria Eduarda Oliveira De Souza[1], Matheus Chimelo Bianchini[2], Murilo Ricardo Sigal Carriço[4], Rafael Roehrs[4], Márcia Foster Mesko[5], Caroline Brandão Quines[3], Daiana Silva Ávila[1]*

1. Laboratório de Bioquímica e Toxicologia em *Caenorhabditis elegans* (GBToxCe), Universidade Federal do Pampa, Campus Uruguaiana;
2. Laboratório de Bioquímica e Toxicologia de Produtos Naturais e Sintéticos, Universidade Federal do Pampa, Campus Uruguaiana;
3. Grupo de pesquisa em Bioquímica e Toxicologia em eucariontes (GPBTE), Universidade Federal do Pampa, Campus Uruguaiana;
4. Laboratório de Análises Químicas Ambientais e Toxicológicas (LAQAT) Universidade Federal do Pampa, Campus Uruguaiana;
5. Laboratório de Controle de Contaminantes em Biomateriais – LCCBio, Universidade Federal de Pelotas - UFPel

*Corresponding author:

Daiana Silva Ávila

Universidade Federal do Pampa - UNIPAMPA

Programa de Pós-Graduação em Bioquímica

BR 472 – Km 592 – Caixa Postal 118

CEP 97500-970 Uruguaiana /RS

Email: avilads1@gmail.com

9. INTRODUCTION

The Uruguay River is a hydrographic basin with frequent floods that covers an area of approximately 384,000 km² in length. This hydrographic basin occupies 3% of the Brazilian territory, specifically at the southern of Brazil, demarcating borders among Argentina and Uruguay, dewatering in the River Plate. Its course can be divided into three parts, Upper, Middle and Lower Uruguay River, where the regions of the middle and lower are widely used for irrigation of crops such as corn, soybeans and rice. (Marcuzzo 2017) Such intense and conventional use of the land results in excessive use of pesticides, which are occasionally drained to the river. This region has also inefficient environmental protection, beyond of some points of raw sewage release from municipalities and rural areas, contributing with the alteration of this important aquatic ecosystem. (Basso 2004, Cravo 2010) These stressors can result in impacts on the water body, which is fundamental for the economic and social development of the entire region, in addition to the degradation of habitat and various species of local fauna. (Soininen and Könönen 2004, Boehler, Strecker et al. 2017, Tran, Reinhard et al. 2019)

Pesticides are one of the largest groups of pollutants due to their intensive use. The Brazilian Special Secretariat for Family Agriculture and Agrarian Development (Sead) and the National Agency for Sanitary Vigilance have reported that Brazil has been the largest consumer of pesticides in the world since 2009. (MDA 2013) Majority environmental contamination is related to the application of pesticides in the crops. (Dellamatrice and Monteiro 2014) Currently, there is a large number of extremely toxic pesticides authorized in Brazil, without the obligation of toxicological evaluation. (Gilson, Rocha et al. 2020) Also, Brazil shows an ineffective control over the sale of pesticides and is common the lack of proper equipment in their application. (Carvalho Dores 1999, Coutinho, Tanimoto et al. 2006) Additionally, the increased frequency of application, their use in cultures which they are not recommended for, in addition to overdose, where the producer believes in greater efficiency by administering doses higher than those recommended by the manufacturer, are additional factors that contribute to environmental, and particularly, aquatic contamination. (Carvalho Dores 1999, Filho. 2019 , Eloisa Lovison Sasso 2020)

To detect the alterations of water quality, the traditional methods based only on physicochemical parameters have become obsolete over time, as they only assess the specific environment at a given time. It is much more appropriate to perform a greater number of analyzes along the time, associated with the use of biomonitoring. (Rodrigues 2010) Biomonitoring is based on the responses of organisms when exposed to a sample. For this, a combination of classical methods with biological analysis is necessary, considering that only the physicochemical method assesses the type and intensity of factors, while biomonitoring demonstrates the effects of stressors on the water body, indicating changes in the location through different parameters. To carry out this monitoring, choosing a model that is ideal is important.

The free-living nematode *Caenorhabditis elegans* is a model that has been used in *in vivo* assays for environmental assessments (Leung, Williams et al. 2008) due its sensitivity, versatility and well-characterized physiology, in addition to being abundantly found in soil and adaptable to aquatic environments. Other advantages that make it a good model for ecotoxicological assessments are its simple and fast maintenance and cultivation, short life cycle of 20 days on average, and fast reproductive cycle, making experiments faster and with lower costs.(Abbas, Valek et al. 2018, Lei, Wu et al. 2018, Xiao, Zhao et al. 2018, Xiao, Zhao et al. 2018, Tran, Reinhard et al. 2019)

Traditional water quality studies could not identify the toxic effects on living organisms. Analyzing the waters from Tunuyán River Basin was possible to identify that physicochemical and bacteriological analyzes explained less than 62% of the responses obtained in delayed growth in *C. elegans*, therefore reinforcing that this type of experimental design is important to obtain a realistic selection scenario for water quality. (Clavijo, Kronberg et al. 2016) Recently, we have been demonstrated the presence of pesticides such as Tebuconazole, Imazetapyr and Clomazone, as well as metals such as arsenic, in samples collected from the Uruguay River. Associated with this, both the survival, longevity, and reproductive viability of the worms exposed to the samples were altered in relation to the control. (Kuhn, Jacques et al. 2021) These results were observed in different collections points in two different periods of the year, based on the planting and harvesting calendar of rice crops in Uruguaiana. Based on this data, we aimed to monitor the water quality of the

Uruguay River along the entire year in order to determine the relationship between the presence of contaminants during the seasons and the physiological damages caused to the nematode *C. elegans* exposed to samples collected monthly from February 2019 to January 2020.

10.MATERIALS AND METHODS

10.1. Sample collection and physicochemical analysis

Monthly samples were made on the margin of the Uruguay River, municipality of Uruguaiana, Brazil (Fig. 1), coordinates 29°44'55 "S and 57°05'12"W, in the period of 12 months, from February 2019 to January 2020. For sampling, collections took place on the 10th day of each month, always in the morning between 10:00 am and 12:00 pm, using sterilized 50 ml Falcons. After analysis, the samples were stored at -20ºC.

Figure 1: Uruguay River site chosen for collecting water samples, on the banks of the Uruguay River. Source: Google Earth.

Most of analyzes for water quality parameters and physicochemical characteristics were carried out at the site. We evaluated dissolved oxygen (DO), salinity and conductivity right after the collection with portable multiparameter equipment and the pH was measured in the laboratory. Rainfall data the sample collection were also taken into account (Pluviometric data obtained from a local meteorological agency).

10.2. Maintenance and synchronization of *C. elegans*

The wild type N2 and CF1553 *{muIs84 [(pAD76) sod-3p::GFP+rol-6(su1006)]}* strain was used for all experiments and were obtained from the *Caenorhabditis* Genetics Center (CGC). The animals were kept at 21- 22°C and cultured in Nematode Growth Medium (NGM) seeded with *Escherichia coli* OP50 as a food source. To obtain animals at the same larval stage, the population was synchronized by exposing adult pregnant worms to a lysis solution (0.45 N NaOH,

2% HOCl). After 14 hours, the eggs hatched, releasing L1 larvae that were used for the following treatments. (Brenner)

10.3. Sample exposure

About one thousand (1,000) worms at the L1 larval stage were placed in a 50mL Erlenmeyer flasks with 5 ml of the samples or 5 ml of Medium K (3 g sodium chloride, 2.4 g potassium chloride and 1 L of autoclaved distilled H_2O) for the control group. Additionally, 50 µl of *E.coli* OP50 was used as a food source. The flasks were left under constant agitation for 24 hours in an orbital shaker at 70 RPM, in an incubator to maintain the temperature at 21 ° C. Shortly after 24 hours, the worms were collected into NGM plates seeded with *E. coli* OP50 for recovery, the tests followed 48 hours after the worms were collected for the plates. The CF1553 mutant worms were exposed to the water samples in the same way, however, they were removed and placed on the slides for photos soon after 24 hours. all experiments were repeated at least three (3) times.

10.4. Survival and Longevity

When the worms reached the L4 stage after exposure, the live animals were counted on a grid with marked quadrants with the aid of a microscope. Following survival, 25 animals (on duplicate plates) were transferred to new NGM plates seeded with *E. coli* OP50. Animal survival was assessed daily until all worms were dead. The worms were transferred daily during the reproductive period to avoid contamination of the progeny and were then transferred every two days. Each for the experiment was repeated at least three times.

10.5. Brood Size

One worm from each sample was transferred daily for 3 days to NGM plates with *E. coli* OP50 and reproduction was evaluated by counting the progeny daily, until the end of the reproduction period. Each experiment was repeated at least three times in duplicates.

10.6. Body length

To assess body length, worms were collected 48 hours after treatment, at the L4 stage, in 1.5 ml microtubes and washed with distilled water at least three times. Worms were transferred to slides containing a levamisole solution to paralyze them and to obtain photographs with the aid of the ECLIPSE 50I® (NIKON®) microscope. 10 animals were selected and imageJ® software was used to measure the body length of the worms.

10.7. Determination of the sod-3 expression

The transgenic strain CF1553 with GFP reporters was used to detect changes in sod-3 expression. The L1 stage transgenic worms were treated in the same way as N2, with constant agitation and the presence of *E.coli* OP50 as a food source. After the 24-h period of agitation, the transgenic worms were collected and kept on microscope slides and paralyzed with a levamisole solution. Fluorescence images were captured by a Floid Cell Imaging Station® microscope (Thermo Fisher Scientific), and expressions were verified by checking the GFP signals using ImageJ software.

10.8. Extraction of pesticides

A 100 mL aliquot of sample (Water 2018) was previously acidified to pH 1.2 and a sufficient amount of NaCl was added to reach the final concentration of 25 mM.

Pesticides were extracted by Solid Phase Extraction, using 500 mg / 6 mL Strata-X cartridges (Phenomenex) under the following conditions: cartridge conditioning with 6 mL of methanol, 6 mL of water and 6 mL of acidified water (pH 3 with H_3PO_4), followed by percolation of the sample with 500 mL and cleaning with 6 mL of water. Finally, pesticides were eluted with 10 ml of methanol.

The eluate was evaporated on a rotary evaporator (Buchi) to dryness and resuspended in 1 mL of methanol. The sample was filtered through a nylon syringe filter (22 µm), and stored at – 20 ºC until analysis by HPLC-DAD.

10.9. Analysis of pesticides by HPLC-DAD

The chromatography system used was an HPLC-DAD, Young Lin (Andrade, Araújo et al. 2007) 9100 (Anyang, Korea) with a diode array detector (Trindade, Almeida et al. 2016) (YL9160). An Inertsil ODS-3 column (4.6 × 250 mm) was used, with an elution gradient composed of acetonitrile (A) and water acidified to pH 3 with

H3PO4 (B). The gradient starts with 30:70 (A:B) to 86:14 (A:B) in 42 minutes, the initial conditions are re-established until 44 minutes and continue until 50 minutes with a constant flow of 0.5 mL/min. The calibration curve for Imazetapir, Sulfentrazone, Diuron, 3,4-dichloroaniline (DCA), Propanil (220 nm), 3,5-dichloroaniline (3,5-DCA) (230 nm), tebuconazole, 2,4-dichlorophenoxyacetic acid (2,4-D) (202 nm) and quinchlorac (254 nm) was performed with the 5 constituent points of the curve (0.5, 1, 2, 4 and 8 mg.L-1) by linear regression.

10.10. Metals Determination

Instrumentation

An inductively coupled plasma mass spectrometer (Elan DCR II, Perkin Elmer, Canada) equipped with a concentric nebulizer (Meinhard, USA) and a cyclonic nebulization chamber (Glass Expansion, Australia), it was used for the determination of As, Cd , Hg, Mn and Pb. Inductively coupled plasma optical emission spectrometer (Optima 4300 DV, Perkin Elmer, USA), equipped with a concentric nebulizer (Meinhard , USA) and cyclonic nebulization chamber (Glass Expansion Inc., Australia), was used to determine Fe and Mn.

Reagents

5% v/v nitric acid (HNO_3) solution in the procedures was prepared by diluting double-distilled concentrated HNO_3 in ultrapure water. The calibration solutions for the ICP-MS (0.05 to 10 µg L^{-1} in HNO_3 5%) and for the ICP-OES (1 to 100 µg L^{-1} in HNO_3 5%) were prepared from the dilution of a 10 mg L^{-1} multielement stock solution (SCP33MS, SCP Science, Canada). The plasma used in the equipment was generated from argon gas with 99.998% purity (White Martins- Praxair, Brazil).

Sample packaging and pre-treatment

Samples in 50 mL Falcon tubes, kept refrigerated at -4 °C. Prior to the analyte determination step, the samples were thawed and filtered with a chromatographic syringe filter (PTFE, 0.22 µm). Samples were diluted (dilution factor: 2-fold) in 5% HNO3 for determination by both techniques.

10.11. Statistical analysis

All tests were performed at least three times and the GraphPad Prism 5 software was used to generate graphs and statistical analysis. One-way ANOVA was used for the analyzes and a $p < 0.05$ was considered statistically significant. Post hoc

tests were performed in these cases using Tukey's post hoc test. Values are normalized as a percentage, assuming control to be 100%.

11. RESULTS

11.1. Physicochemical analysis

The physicochemical data obtained at the time and place of collection is described at table 1, where a reduced level of dissolved oxygen in the months of October and November 2019 were observed. It is also important to highlight an increase in conductivity in September 19 through to January 20. We also reported that there was no rain on the collection days, but it rained in the early morning of the collection day in May.

Table 1

Physicochemical data obtained at the collection site.

Samples	Dissolved O_2 (mg/L)	pH	Salinity (ppt)	Conductivity (µS/cm)
February 19	7.2	5.12	0.03	50.3
March 19	5.0	6.24	0.05	87.6
April 19	9.6	6.9	0.03	53.5
May 19	8.3	5.96	0.03	54.0
June 19	8.6	6.7	0.06	58.0
July 19	7.9	5.2	0.04	64.21
August 19	8.9	5.4	0.03	95.51
September 19	6.6	6.1	0.05	178.97
October 19	4.7	6.9	0.05	143.2
November 19	4.3	6.7	0.03	75.16
December 19	7.2	8.4	0.03	159.87
January 20	6.5	7.2	0.03	112.63
RV	>5	6-9		NA

11.2. Longevity

Almost all samples showed a significant reduction in survival compared to the control, with the exception of February 19 and January 20 (Fig. 2).

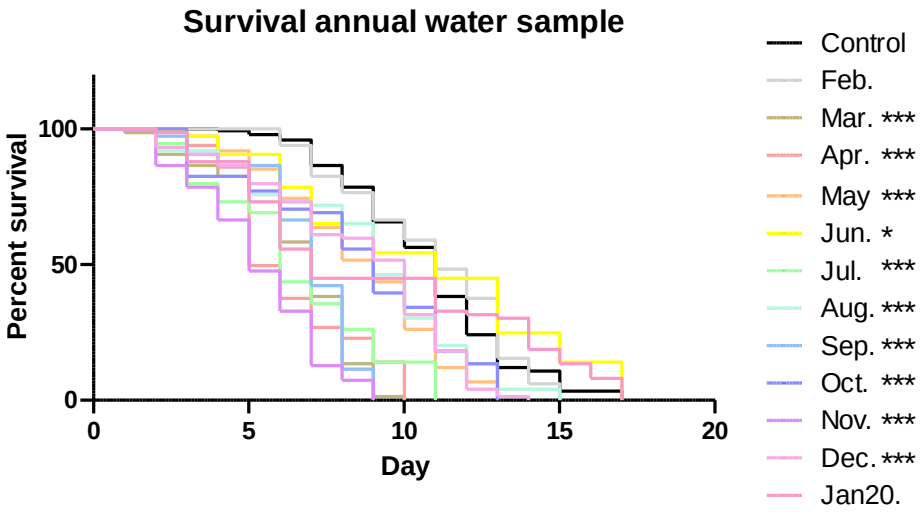

Fig 2. Reduced longevity in *C. elegans* exposed to river water samples sampled monthly during a period of 12 months. Asterisk indicates significant differences from control after one-way ANOVA ($p<0.05$, Tukey's post-hoc test. *<0.05, **<0,001 and ***<0,0001)

11.3. Brood size

Regarding the data obtained in relation to the size of the progeny in *C. elegans* exposed to river water samples, we found that only the sample from Jan 20 caused a significant difference in relation to the control (Figure 3). However, there is statistical difference between the samples, where August is different from April, October and November and September is different from November. The worms exposed to the sample collected in June also tended to present this increase in the reproductive rate.

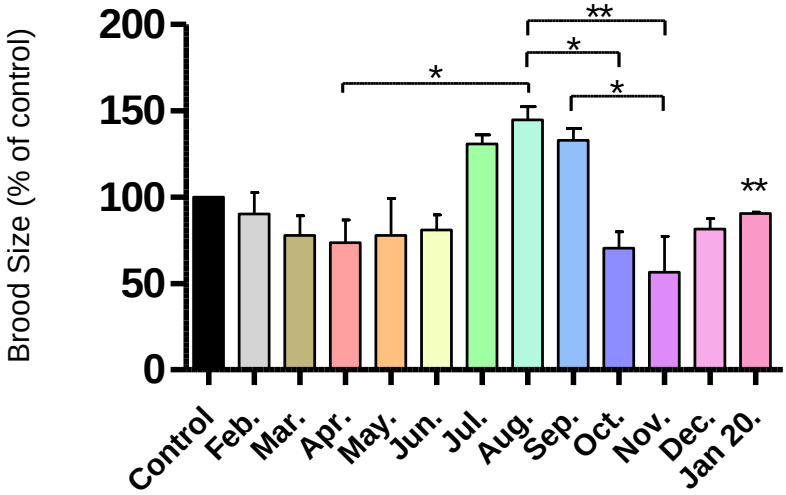

Fig 3. Altered reproduction in *C. elegans* exposed to monthly river water samples. * indicates significant differences with control following one-way ANOVA ($p < 0.05$, Tukey's post hoc test. *<0.05, **<0,001 and ***<0,0001)

11.4. Survival Rate

In Figure 4 we demonstrate the percentage of survival rate in *C. elegans* exposed to water samples. We observed a significant reduction compared to the control in the survival of animals exposed to February, March, April, June, July, August and November samples.

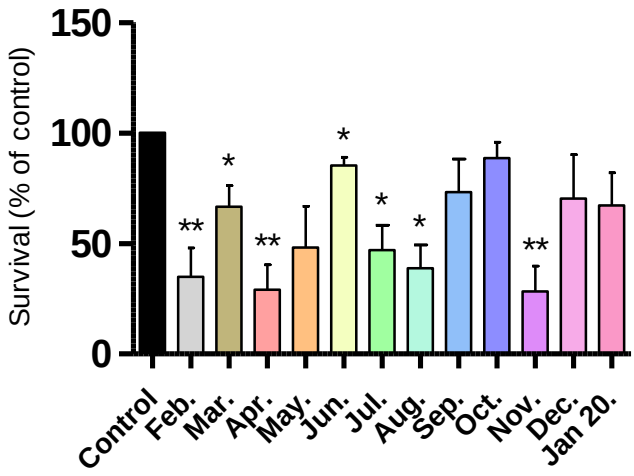

Fig 4. Reduced survival in *C. elegans* exposed to monthly river water samples. * indicates significant differences with the following control one-way ANOVA ($p < 0.05$, Tukey's post hoc test. *<0.05, **<0,001 and ***<0,0001).

11.5. Body Length

Regarding the body length of worms exposed to river water samples, even though there is an apparent decrease in body length in other months such as April, August and May, we observed a significant reduction in the size of *C. elegans* exposed only to the November 19 sample, in relation to control.

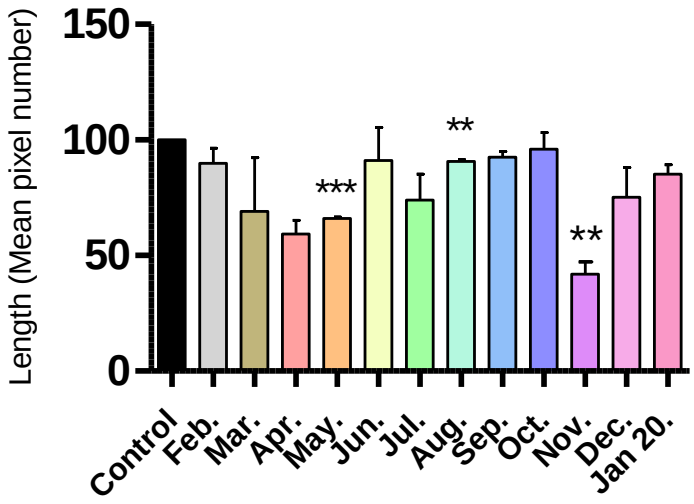

Fig 5. Body length in *C. elegans* exposed to monthly river water samples *indicates significant differences from control after one-way ANOVA ($p < 0.05$, Tukey's post hoc test. *<0.05, **<0,001 and ***<0,0001).

11.6. SOD-3 expression

The expression of sod-3 ::GFP fluorescence with CF1553 strain was not statistically different among the water samples and the control. However, there was an apparent increase in some months, such as May, September and January 20 (Fig. 6).

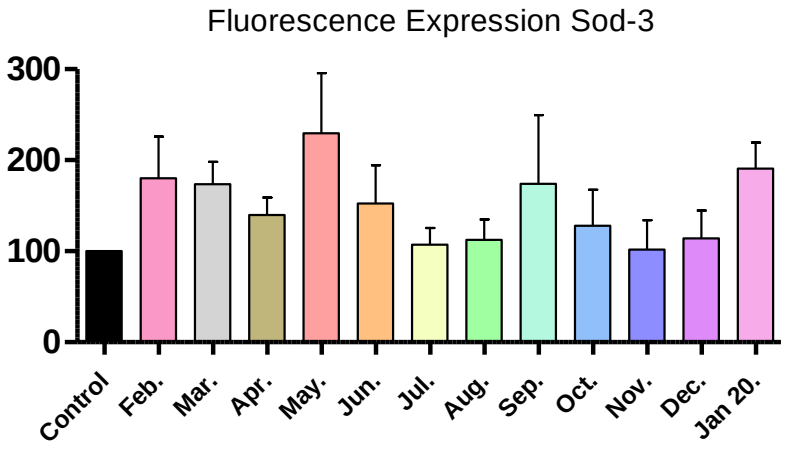

Fig 6. Measurement of sod-3 ::GFP gene expression in CF1553 *C. elegans* exposed to water samples. The results are expressed with three repeated experiments,*indicates significant differences from control after one-way ANOVA ($p<0.05$, Tukey's post-hoc test and test T. ($p < 0.05$, Tukey's post hoc test. *<0.05, **<0,001 and ***<0,0001)

11.7. Pesticides Levels

We observed the presence of imazethapyr (Feb and Mar samples), of tebuconazole (Sep sample), and 2,4-D (March sample), a pesticide that had not been identified in the samples from our previous study in this place (Kuhn et al., 2021).

Table 2

Concentration of pesticides detected in the different months (-) indicates not detected. Results are expressed as mean ± standard deviation.

	Pesticide		
Sample	**imazethapyr (µg/L)**	**sulfentrazone (µg/L)**	**2,4-D (µg/L)**
February	26.01 ± 23.49	-	
March	15.13 ± 4.16	-	47.29 ± 0.45
April	-	-	-
May	-	-	-
June	-	-	-
July	-	-	-
August	-	-	-
September	-	5.57 ± 0.23	-
October	-	-	-
November	-	-	-
December	-	-	-
January	-	-	-

(-) indicates below our detection limits.

11.8. Metals Levels

It was not possible to quantify the Cd and Hg elements in the evaluated samples. The As and Pb elements were quantified, together with Mn, only in the April sample. On the other hand, it was possible to quantify Mn in almost all samples, with the exception of the June sample.

Table 3

As, Cd , Hg, Mn and Pb concentrations determined by ICP-MS, and Fe and Mn concentrations determined by ICP-OES, µg L -1 , n = 3, mean ± SD (RSD).

Sample	**^{75}As**†	**^{112}Cd**†	**^{200,201,202}Hg**†	**^{55}Mn**†	**^{208}Pb**†	**Fe (239.562 nm)** ‡	**Mn (257.610 nm)** ‡
February	< 0,05*	< 0,05*	< 0,05*	0,164 ± 0,015 (9,1%)	< 0,05*	< 5,00*	< 1,00*
March	< 0,05*	< 0,05*	< 0,05*	0,104 ± 0,011 (11,0%)	< 0,05*	< 5,00*	< 1,00*
April	0,124 ± 0,007 (5,5%)	< 0,05*	< 0,05*	0,688 ± 0,013 (1,9%)	0,056 ± 0,003 (5,0%)	< 5,00*	< 1,00*
May	< 0,05*	< 0,05*	< 0,05*	0,586 ± 0,021 (3,7%)	< 0,05*	< 5,00*	< 1,00*
June	< 0,05*	< 0,05*	< 0,05*	< 0,05*	< 0,05*	< 5,00*	< 1,00*
July	< 0,05*	< 0,05*	< 0,05*	1,028 ± 0,054 (5,2%)	< 0,05*	< 5,00*	1,128 ± 0,051 (4,5%)

August	< 0,05*	< 0,05*	< 0,05*	0,116 ± 0,006 (5,5%)	< 0,05*	< 5,00*	< 1,00*
September	< 0,05*	< 0,05*	< 0,05*	1,254 ± 0,096 (7,7%)	< 0,05*	< 5,00*	1,470 ± 0,102 (6,9%)
October	< 0,05*	< 0,05*	< 0,05*	0,204 ± 0,015 (7,3%)	< 0,05*	< 5,00*	< 1,00*
November	< 0,05*	< 0,05*	< 0,05*	1,658 ± 0,104 (6,3%)	< 0,05*	< 5,00*	1,814 ± 0,160 (8,8%)
December	< 0,05*	< 0,05*	< 0,05*	0,892 ± 0,015 (1,6%)	< 0,05*	< 5,00*	0,904 ± 0,054 (6,0%)
January	< 0,05*	< 0,05*	< 0,05*	1,402 ± 0,060 (4,3%)	< 0,05*	< 5,00*	1,466 ± 0,035 (2,4%)

† Determination by ICP-MS.
‡ Determination by ICP-OES.
* Instrumental LOQ.

Regarding the results obtained for the concentration of Mn in the samples, it was observed that the values varied from about 0.104 to 1.658 µg L-1. Figure 7 and Table 5 illustrates the behavior of the variation in Mn concentration over time, according to the dates of sample collection.

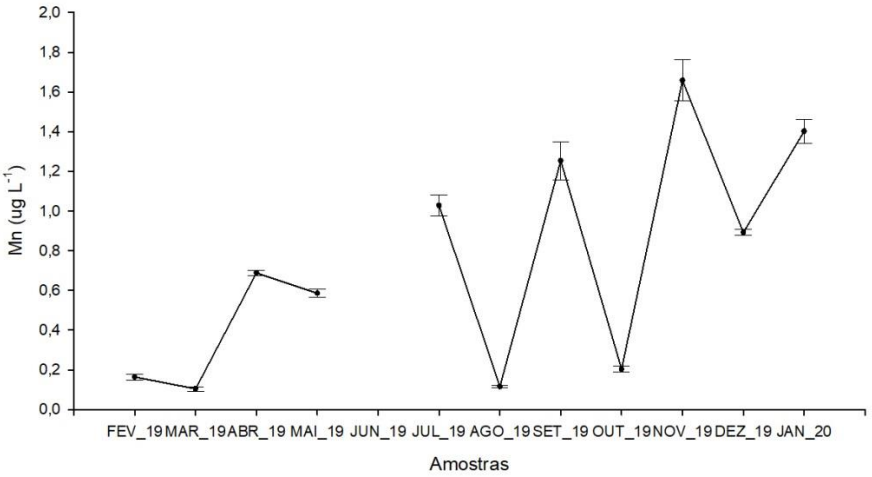

Fig 7. Fluctuation in Mn concentration (µg L -1) in samples evaluated after determination by ICP-MS.

11.9 Correlations

Finally, in order to identify putatively links between our findings, correlations analysis were made between some of the data. We have found negative correlations between Body Length and Brood, and between Survival and Brood in August samples. Brood size and SOD-3 in April samples and Survival and SOD-3 in February samples also showed negative correlations. Positive correlations were identified between Body Length and Brood (March, April and January), Survival and

Brood (October and December), and Body Length and Survival (February, April, July, August and November)

Table 4

Correlations analyses results.

Sample	Body Lenght x Brood	Survival x Brood	Body Lenght x Survival	Survival x SOD-3	Body Lenght x SOD-3	Brood size x SOD-3
February	NS	NS	0.75	-0.82	NS	NS
March	0.84	NS	NS	NS	NS	NS
April	0,74	NS	0.88	NS	NS	-0.95
May	NS	NS	NS	NS	NS	NS
June	NS	NS	NS	NS	NS	NS
July	NS	NS	0.83	NS	NS	NS
August	-0.98	-0.91	0.91	NS	NS	NS
September	NS	NS	NS	NS	NS	NS
October	NS	0.93	NS	NS	NS	NS
November	NS	NS	0.95	NS	NS	NS
December	NS	0.87	NS	NS	NS	NS
January	0.82	NS	NS	NS	NS	NS

NS- not significant

12. DISCUSSION

In the last decade, the nematode *C. elegans* has gained considerable notoriety in freshwater ecotoxicology studies and its use in toxicity tests. As mentioned before, its use is already well established for its multiple advantages such as its life cycle, sensitivity to metals, easy reproduction, and multiple occurrences in the environment, ecological relevance and applicability to varied ecotoxicological tools, which make nematodes excellent bioindicators. (Tejeda-Benitez and Olivero-Verbel 2016)

Our present study sought to investigate the environmental changes, probably from anthropic origin, for a period of one year. Given our previous published findings, (Kuhn, Jacques et al. 2021) we wondered if the presence of metals and pesticides would remain throughout the year or would it be present only in the times that precede or proceed the application of pesticide in rice crops. Previously, our findings already indicated a general contamination of the river and tributaries as the levels of contaminants were present in samples that preceded the application of pesticides, including the damage caused to *C. elegans* exposed to pre-application water samples. It is necessary to highlight that in this study we limited to only one collection point because of the number of samples.

Our first finding was the increasing level of conductivity of the samples throughout the year (Table 1), indicating the possible presence of metals in the water, metals that come from commercial pesticide formulas. (Gulati, Banerjee et al. 2010, Chen, Xu et al. 2015, Shao, Zang et al. 2021) The days of collection did not have the presence of rain, which may lead to the idea of a concentration of these metals, however, the samples, were collected from the upper water column, which rules out this possibility, since the accumulation would be most likely in the lower column of the river.

All monthly samples caused a significant lifetime reduction in relation to untreated worms, except for the months of February 2019 and January 2020. This could happen for several reasons, including isolated events in the water on the day of collection, however, when we observed the longevity from our previous study, we come across some similar data. The worms exposed to all samples pre-application of pesticides did not show a statistically significant reduction in the life expectancy of *C. elegans* in relation to the control. The collection period established in the first pre-

application samples of pesticides in crops extends until February. When we think about how these chemicals reach water sources, as mentioned earlier, the dynamics of the rains that carry the residues of pesticides from the crops to the river or even the water used in the plantations. Since during this period the crops were not irrigated in their entirety and the dry period characteristic of that time in the region, which leads to a drop in the water level, may indicate that all pesticides applied to the plantations have not yet reached the main body of the water, which shows us that these chemical residues carried by the water are dispersed in the main body of the river throughout the year.

But this hypothesis cannot lead us to the idea that in the first months of the year (January and February) the water in the region is probably free of contamination, because we observed a reduction in survival rate in February (Figure 4), and reduced brood size in worms exposed to January sample (Figure 3). We can assume that there are other types of contaminants present in the sample that we were not able to detect or even the same presence of imazethapyr detected in February, as indicated in table 3. Also in figure 4, given the large number of samples that offered a reduction in population to the worms, and the data referring to the body length of the animals (Figure 5) reinforce the idea that there are more interfering toxicants that it was not possible to identifies in our study, since we could only evaluate metals and pesticides.

It is also remarkable that when we observe the significant differences in Figure 3 we also observed differences among the months. This can be explained by a fluctuation in the amount and types of toxicants over the months, altered even by the natural flow of the river, dragging toxics even from other regions. The increase in the reproduction rate can also indicate toxic damage. In face of small damage, there is a stimulus to increase in progeny to maintain the specie. This idea is supported, for example, by the data found in September, where we identified the presence of Mn (table 3 and figure 7) and sulfentrazone (Table 2) and increased brood size. However, the increase in the profile in months, such as July, August and January, may indicate that there are other pesticides that were not possible to identify.

One of the pesticides recently found in annual water samples that was not identified in our previous work is 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D is a

phenoxyalkanoic acid herbicide and it is among the most widely distributed pollutants in the environment, according to. (Ju, Liu et al. 2019)

These authors reported the combined toxicities of 2,4-D and 2,4-DCP (major metabolite) to *Vibrio qinghaiensis* sp.-Q67 (Q67) and *C. elegans*, however depending on the concentrations they may have antagonistic effects in *C. elegans.*

When correlating the data, we observed that most of the important physiological correlations between the data were from April samples these correlations seem to be related to elevated As levels.

There are several studied that help us to associate the damage in *C. elegans* exposed to Uruguay river samples with the presence of pesticides and metals. We can mention the findings obtained in sediment studies of the Magdalena River, in Colombia, in which even if wastewater is treated, various pollutants, such as heavy metals from petroleum used in the refining process, flow into the river and are incorporated into the sediments. Exposure to these sediments caused high genetic response of stress, survival and inhibition of locomotion in animals. (Tejeda-Benitez, Flegal et al. 2016, Tejeda-Benitez and Olivero-Verbel 2016, Tejeda-Benítez, Noguera-Oviedo et al. 2018) Two studies carried out in the Reservoir of Three Gorges in the upper Yangtze River in China, one during the flood season and one during the normal period, indicated locomotor damage and reactive oxygen species (ROS) production in the nematode intestine. (Xiao, Zhao et al. 2018, Xiao, Zhao et al. 2018) Water and sediment samples collected from the Mud River in West Virginia, a river heavily impacted by mining, were responsible for inhibiting body development in *C. elegans* due to heavy metal contamination. (Turner, Kroeger et al. 2013) These studies also corroborate the importance of monitoring important springs and, like other articles, (Loro, Murussi et al. 2015) can help to reveal more clearly what happens in the Uruguay River.

13. CONSIDERAÇÕES FINAIS

Acreditamos que nosso estudo reforça a grande importância do controle da qualidade da água para diversas áreas de interesse, utilizando o modelo alternativo *C. elegans* como ferramenta útil de monitoramento em cursos d'água conhecidos, como o Rio Uruguai, principalmente em regiões com forte produção agrícola. E sua participação, juntamente com outros métodos, pode ser bastante significativa na avaliação do meio ambiente e da qualidade dos recursos naturais.

Em nosso estudo, descobrimos que:

- As amostras de água do Rio Uruguai contêm a presença de diversos contaminantes como clomazone (primeiro trabalho), imazetapir, tebuconazol e 2,4-D;
- As amostras de água contêm vários metais tóxicos, inclusive arsênio;
- As amostras, nos dois trabalhos, causaram alterações fisiológicas em *C. elegans*, indicando que os animais que vivem no Rio Uruguai podem estar sofrendo impactos.

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