



Universidade Federal do Rio Grande
Instituto de Ciências Biológicas
Pós-graduação em Biologia de
Ambientes Aquáticos Continentais



**Monitoramento ecotoxicológico da Lagoa
Mirim – RS, um dos maiores sistemas
lagunares do Brasil**

Josiane Araujo da Silva

Orientador: Dr^a. Camila de Martinez Gaspar Martins

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Aluno: Josiane Araujo da Silva

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*Dedico esta dissertação a todos os que me ajudaram
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RESUMO

A Bacia Hidrográfica da Lagoa Mirim vem sofrendo degradação ao longo dos anos, principalmente devido à intensa atividade agrícola que ocorre no seu entorno. Neste sentido, o presente estudo teve como objetivo avaliar a qualidade da água superficial da Lagoa Mirim do ponto de vista ecotoxicológico através de ensaios agudos e crônicos realizados com os seguintes organismos: microalgas *Pseudopediastrum boryanum*, microcrustáceos *Daphnia magna* e *Ceriodaphnia dubia* e peixe *Danio rerio*, expostos a amostras de água superficial coletadas em diferentes pontos da bacia hidrográfica e em três períodos distintos (Janeiro, Março e Junho de 2022). Além disso, para os organismos *D. magna* e *D. rerio* foram realizadas análises de biomarcadores comportamentais e bioquímicos. As microalgas foram os organismos mais sensíveis, com efeitos principalmente em março, enquanto para os demais organismos não foi observado efeitos letais, evidenciando a importância de avaliar organismos de diferentes níveis tróficos. Já em relação aos biomarcadores, os resultados mostraram maiores efeitos, principalmente através do índice de Resposta do Biomarcador Integrado (IBR), para *D. magna* e *D. rerio* no período de março, porém a *D. magna* se mostrou mais sensível. Ademais, as Análises de Redundância indicaram correlação entre os biomarcadores para ambos os organismos e os parâmetros abióticos, principalmente os nutrientes (fósforo total e nitrogênio total), coliformes termotolerantes, sólidos totais e turbidez. As análises multivariadas e o índice IBR se mostraram ferramentas eficientes para auxiliar no monitoramento dos corpos hídricos como a Lagoa Mirim, permitindo observar de forma espacial e temporal um panorama geral dos efeitos causados aos organismos pelas amostras coletadas.

Palavras-chave: ambiente aquático, testes de toxicidade, comportamento, biomarcadores.

ABSTRACT

The Mirim Lagoon watershed has been suffering from degradation over the years, mainly due to the intense agricultural activity that occurs in its surroundings. In this sense, the present study aimed to evaluate the quality of surface water in Mirim Lagoon from an ecotoxicological point of view through acute and chronic tests performed with the following organisms: microalgae *Pseudopediastrum boryanum*, microcrustaceans *Daphnia magna* and *Ceriodaphnia dubia*, and fish *Danio rerio*, exposed to samples of surface water collected at different sites in the watershed and in three distinct periods (January, March, and June 2022). Furthermore, behavioral and biochemical biomarker analyses were performed in *D. magna* and *D. rerio*. Microalgae were the most sensitive organisms, with effects mainly in March, while no lethal effects were observed for the other organisms, highlighting the importance of evaluating organisms from different trophic levels. Regarding biomarkers, the results showed greater effects, mainly through the Integrated Biomarker Response (IBR) index, for *D. magna* and *D. rerio* in March, but *D. magna* proved to be more sensitive. Furthermore, Redundancy Analyses indicated a correlation between the biomarkers for both organisms and the abiotic parameters, mainly nutrients (total phosphorus and total nitrogen), thermotolerant coliforms, total solids, and turbidity. Multivariate analyses and the IBR index proved to be useful tools to assist in the monitoring of water bodies such as Mirim Lagoon, allowing for spatial and temporal observation of a general overview of the effects caused to the organisms by the collected samples.

Keywords: aquatic environment, toxicity tests, behavior, biomarkers.

APRESENTAÇÃO

Esta dissertação contém uma introdução geral, seguida de objetivos e referências bibliográficas. Após, é apresentado o capítulo único em formato de artigo científico, redigido em inglês e de acordo com as normas da revista a qual pretende-se submeter o trabalho. O capítulo trata do monitoramento ecotoxicológico realizado na Lagoa Mirim-RS, intitulado “Uso de ferramentas integrativas e múltiplos modelos para avaliação da qualidade ambiental aquática: Lagoa Mirim, um exemplo de estudo no sul do Brasil” / “The use of integrative tools and multiple models for aquatic environmental quality assessment: Mirim Lagoon, an example of study in Southern Brazil”, e que será submetido para a revista *Environmental Monitoring and Assessment*. Por fim, são apresentadas as considerações finais e perspectivas do estudo.

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INTRODUÇÃO GERAL

Contaminação dos ambientes aquáticos

O crescente aumento populacional e avanço tecnológico levaram ao aumento na diversidade de produtos comercializados e, conseqüentemente, do seu aporte no ambiente, comprometendo assim a qualidade dos ecossistemas aquáticos em função do lançamento de uma variedade de produtos químicos originários de atividades antropogênicas (Freire et al., 2008). Os contaminantes atingem os corpos hídricos através de diversas fontes, podendo ser pontuais e difusas (Figura 1). As emissões de fontes pontuais são caracterizadas por descargas diretas dos contaminantes nos corpos d'água, sendo de fácil detecção e controle, como, por exemplo, efluentes industriais e domésticos advindos de estações de tratamento e derramamentos acidentais. Ao contrário, as emissões de fontes difusas são de difícil controle e identificação de sua origem, variam com o tempo e espaço e podem envolver rotas que resultem na deposição parcial dos contaminantes antes deles atingirem os corpos d'água, como a utilização de agrotóxicos no solo que, por meio da lixiviação e drenagem das águas utilizadas nas plantações de arroz irrigado, acabam contaminando os corpos hídricos (Holt, 2000).

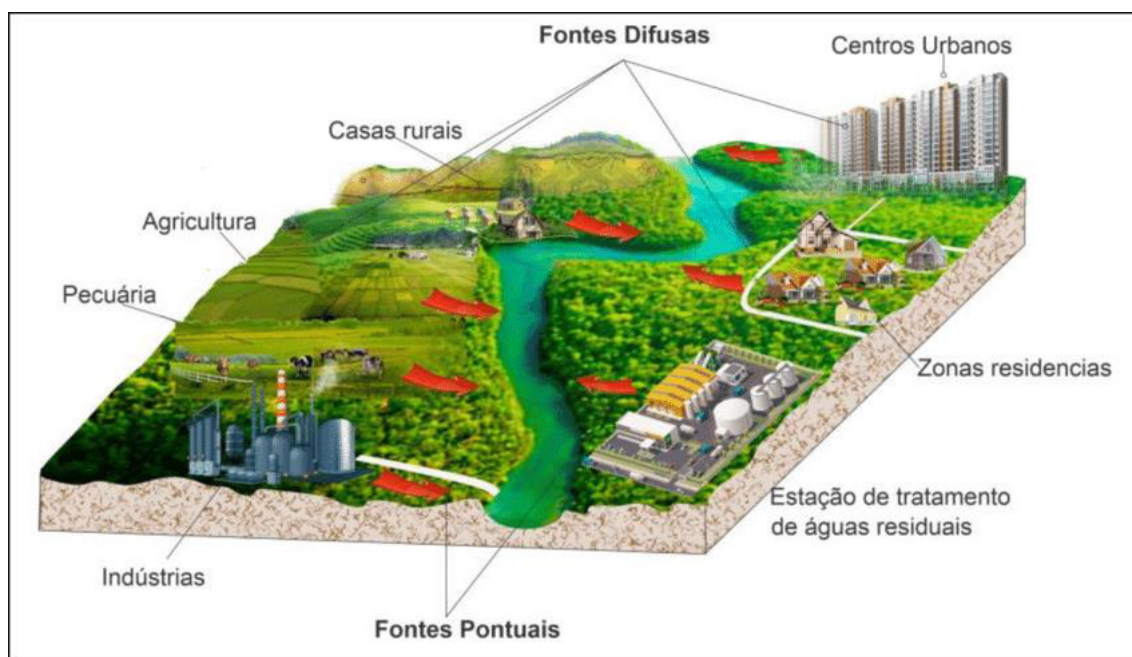


Figura 1: Principais vias de entrada de contaminantes em águas superficiais.
Fonte: Ribeiro et al., 2019.

Nos ambientes aquáticos, os contaminantes podem sofrer uma combinação de processos que possivelmente venham a afetar o seu comportamento e destino final (Holt,

2000). Os compostos podem ser degradados por processos abióticos como a fotólise, causada pela luz, principalmente pela radiação ultravioleta, ou bióticos como a biodegradação por ação de bactérias, fungos e outros microrganismos. No entanto, muitas substâncias resistem por certo tempo aos processos de degradação, desta forma os compostos podem persistir no ambiente por longos períodos de tempo, por possuírem uma meia-vida mais longa. O tempo de meia-vida de um composto significa o tempo necessário para que metade da concentração inicial leva para ser degradada, seja em horas, dias, meses ou até mesmo anos (Andrade et al., 2010). Os compostos químicos presentes na água podem sofrer processos de complexação. Por exemplo, metais se complexam com a matéria orgânica dissolvida, precipitação e ressuspensão em caso de remobilização do sedimento, adsorção a sólidos suspensos, entre outros (Figura 2) (Costa et al., 2008). Porém, o conceito de persistência não está apenas relacionado à meia-vida do contaminante, mas também ao seu descarte contínuo no ambiente aquático. De qualquer forma, a maior persistência pode levar ao maior contato e/ou acumulação dos contaminantes pela biota e, conseqüentemente, resultar em toxicidade aos organismos (Costa et al., 2008).

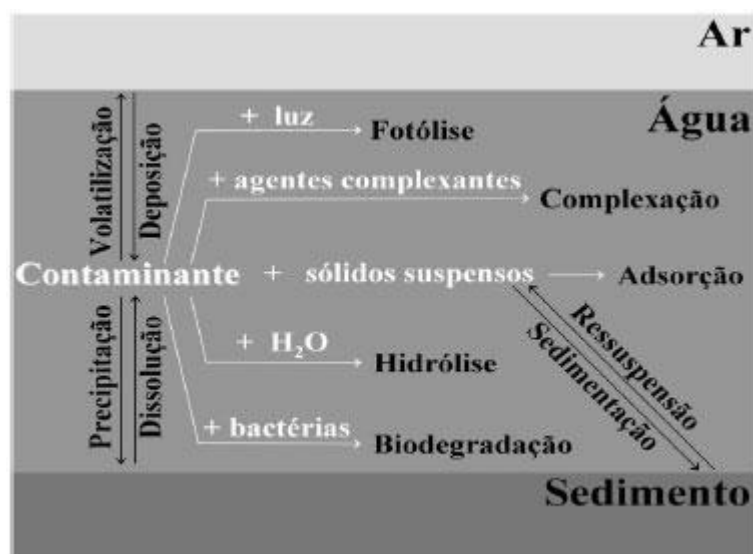


Figura 2: Processos de transporte e transformação de contaminantes que podem ocorrer no ambiente aquático. Fonte: Costa et al., (2008).

Em relação à toxicidade de misturas, como efluentes domésticos, compostos por uma variedade de substâncias, e mananciais contaminados por estes efluentes e outras diversas classes de contaminantes, as interações entre os químicos podem levar a efeitos

aditivos, sinérgicos, antagônicos ou potenciais. O efeito é aditivo quando o efeito da mistura de contaminantes é igual à soma de seus efeitos isoladamente. O efeito sinérgico ocorre quando o efeito de uma mistura é maior que a soma das toxicidades dos contaminantes isoladamente. Quando o efeito da mistura é menor que a soma das toxicidades dos contaminantes isoladamente há um efeito antagônico. Por fim, a potencialização é vista quando o contaminante só tem efeito ao se unir a outro (Rand et al., 2003).

Uma vez no ambiente aquático, os contaminantes podem afetar organismos de diferentes níveis tróficos e causar efeitos deletérios aos ecossistemas, como modificações nas características e na dinâmica das populações. Além disso, podem acarretar mudanças na estrutura e função das comunidades de modo a alterar a diversidade de espécies e levar a modificações na relação predador-presa (Connell e Miller., 1984). De fato, o declínio da biodiversidade de diferentes ecossistemas tem sido influenciado pela contaminação, sobretudo em ambientes dulcícolas (Young et al., 2016).

Ecotoxicologia e monitoramento de ambientes aquáticos

A ecotoxicologia surgiu na década de 60 e é a ciência que permite estudar e avaliar os efeitos danosos de substâncias químicas sobre os organismos (Rocha et al., 2010, Zagatto e Bertoletti, 2008). Os estudos em ecotoxicologia aquática tem muitas aplicações, sendo utilizados para se estabelecer padrões de qualidade da água, limites para lançamento de efluentes nos corpos hídricos, além de planos de monitoramento ambiental e manejo (Zagatto e Bertoletti, 2008).

Análises ecotoxicológicas podem ser realizadas em laboratório, com organismos vivos, para testar os efeitos dos contaminantes e, assim, gerar conhecimento sobre os riscos das substâncias químicas nos animais e nos ambientes aquáticos receptores de contaminantes. Estes ensaios também podem ajudar a entender alguns fatos relacionados às relações tróficas, à sensibilidade dos organismos e à tolerância a determinados contaminantes (Filser, 2007). Os ensaios de toxicidade podem ser agudos ou crônicos. Os ensaios agudos permitem que sejam observados efeitos rápidos e severos, como mortalidade e imobilidade dos organismos, em decorrência da exposição ao contaminante. Já os ensaios crônicos buscam avaliar efeitos em níveis subletais, como taxa de reprodução e crescimento dos organismos. Os ensaios agudos e crônicos possuem duração variável e estão associados ao tempo de vida / desenvolvimento dos organismos (Magalhães et al., 2008).

O monitoramento ambiental pode envolver análise *in situ*, bem como testes laboratoriais como os ensaios de toxicidade mencionados acima, utilizando amostras coletadas em corpos d'água. Visto que a biota aquática está sob constante exposição à uma variedade de substâncias tóxicas lançadas no ambiente, a avaliação dos recursos hídricos a partir de organismos bioindicadores é recomendada para um monitoramento ambiental eficaz e, de forma complementar à determinação das condições físico-químicas e de contaminação da água (Costa et al., 2008).

Enquanto a investigação química de contaminantes identifica e quantifica as concentrações de diferentes substâncias na amostra, as análises ecotoxicológicas avaliam os efeitos destas substâncias sobre os sistemas biológicos. Assim, as análises químicas, físico-químicas e toxicológicas se complementam em um monitoramento ambiental, trazendo um melhor diagnóstico da qualidade do ambiente em estudo (Zagatto e Bertoletti, 2008).

A resolução CONAMA 357/2005 que dispõe sobre a classificação dos corpos de água e diretrizes ambientais para o seu enquadramento, prevê limites máximos permitidos para parâmetros como cor, turbidez, níveis de sólidos totais, temperatura, pH, oxigênio dissolvido, fósforo, nitratos, cloretos, dentre outros. O monitoramento de concentrações de coliformes termotolerantes em corpos hídricos também está regulamentado pela CONAMA 357/2005 e sua relevância se dá por ser uma medida que representa bactérias presentes em fezes humanas e de animais homeotérmicos, podendo, dessa forma, indicar contaminação por efluentes domésticos.

Uma melhor compreensão dos efeitos de poluentes para os ecossistemas aquáticos se dá com o uso de uma variedade de espécies representantes da biota aquática (Figura 3). Isso se faz importante devido à diferença na sensibilidade desses organismos quando expostos aos contaminantes, o que permite estimar com mais segurança o impacto do contaminante no meio (Sponza, 2002; Pane et al., 2008). Para o monitoramento de um corpo d'água ou efluente é recomendado, no mínimo, a utilização de três espécies pertencentes a diferentes níveis tróficos (Goldstein, 1988, Costa et al., 2008). Nesse sentido, a Resolução CONAMA 430/2011 que dispõem sobre as condições e padrões de lançamento de efluentes, determina que os ensaios ecotoxicológicos realizados em efluentes utilizem organismos aquáticos de pelo menos dois níveis tróficos diferentes. Com relação a sensibilidade dos organismos, no estudo de Hernando et al. (2005) foram realizados testes de toxicidade com os organismos, bactéria *Vibrio fischeri*, microcrustáceo *Daphnia magna* e microalga *Pseudokirchneriella subcapitata* e

verificou-se que os organismos apresentavam respostas diferentes quando expostos ao mesmo efluente doméstico, visto que a bactéria *V. fischeri* não apresentou toxicidade, enquanto para os demais organismos o efluente apresentou-se tóxico. Por outro lado, no trabalho de Paredes et al. (2014), foi avaliada a toxicidade do filtro solar Benzofenona-3 para os organismos, microalga *Isochrysis galbana*, bivalve *Mytilus galloprovincialis*, crustáceo *Siriella armata* e ouriço-do-mar *Paracentrotus lividus* e observou-se que o filtro solar é mais tóxico para a microalga *I. galbana* do que para os demais organismos analisados.

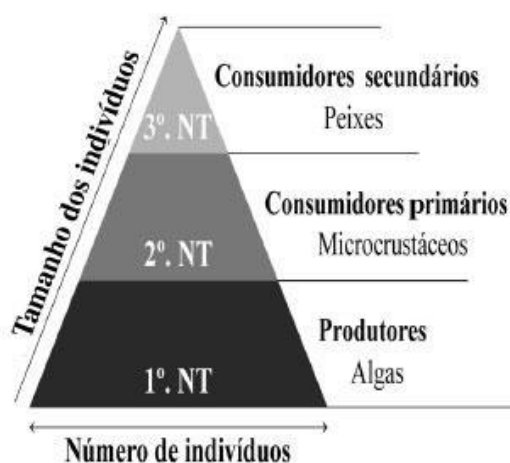


Figura 3: Exemplo de cadeia alimentar do ecossistema aquático constituída por organismos representantes de três níveis tróficos (NT). Fonte: Costa et al., (2008).

Diferentes organizações e agências de normatização nacionais e internacionais recomendam procedimentos e espécies diferentes para ensaios de toxicidade, dentre elas estão: *United States Environmental Protection Agency* (USEPA), *Association française de Normalisation* (AFNOR), *International Organization for Standardization* (ISO), *Organization for Economic Co-operation and Development* (OECD) e, no Brasil, Associação Brasileira de Normas Técnicas (ABNT). A utilização de testes padronizados é recomendada e vantajosa, pois facilita a comparação dos dados, permite a reprodutibilidade dos ensaios e apresenta resultados que podem servir de guia para agências regulamentadoras derivem critérios de qualidade de água. As normas também trazem outras informações sobre como cultivar os organismos que serão utilizados nos ensaios, como devem proceder os testes de toxicidade em laboratório e descrevem os testes com substância referência, que devem ser realizados regularmente com o intuito de avaliar a saúde dos organismos que serão utilizados nos ensaios de toxicidade. Além disso, visto que os ensaios de toxicidade podem não apresentar efeitos agudos ou

crônicos, o uso de biomarcadores é recomendado para auxiliar nos monitoramentos ambientais, analisando biomarcadores em organismos expostos as amostras dos locais de monitoramento ou diretamente na biota local (Van der Oost et al., 2003; Vieira et al., 2022).

Biomarcadores bioquímicos e comportamentais

Os biomarcadores são definidos como alterações comportamentais, bioquímicas, celulares, moleculares, mudanças fisiológicas nas células, fluidos corpóreos, tecidos ou órgãos de um organismo que são indicativos da exposição ou efeito de um contaminante e são antecipatórias do ponto de vista ecotoxicológico (Lam e Gray, 2003). De fato, uma amostra pode não apresentar toxicidade nos ensaios toxicológicos, porém essa exposição pode causar efeitos futuramente deletérios aos organismos, como alterações bioquímicas e comportamentais que comprometam a saúde e em última instância, sobrevivência do indivíduo (Scott e Sloman, 2004).

Os biomarcadores bioquímicos são capazes de relacionar o efeito do contaminante a nível subcelular. Dentre os biomarcadores bioquímicos mais utilizados para avaliação de contaminação por agrotóxicos está a inibição da atividade da Acetilcolinesterase (AChE), enzima responsável pela degradação do neurotransmissor acetilcolina (ACh) liberado nas fendas sinápticas, em colina e ácido acético, finalizando a transmissão dos impulsos nervosos (Figura 4) (Rang, 2001; Hill et al., 2012). Nestas sinapses a ACh atua transmitindo a mensagem de um neurônio a outro no sistema nervoso central e periférico ou para células musculares, sendo importante para a manutenção de inúmeras funções fisiológicas. A AChE quando bloqueada é incapaz de hidrolisar a ACh, assim este neurotransmissor tende a permanecer ativo por um período maior na fenda sináptica (ou junção neuromuscular) e, conseqüentemente, observa-se um colapso do sistema nervoso central, causando perda do controle muscular, convulsões e morte por parada cardiorrespiratória como um efeito mais agressivo (Guyton e Hall, 2006).

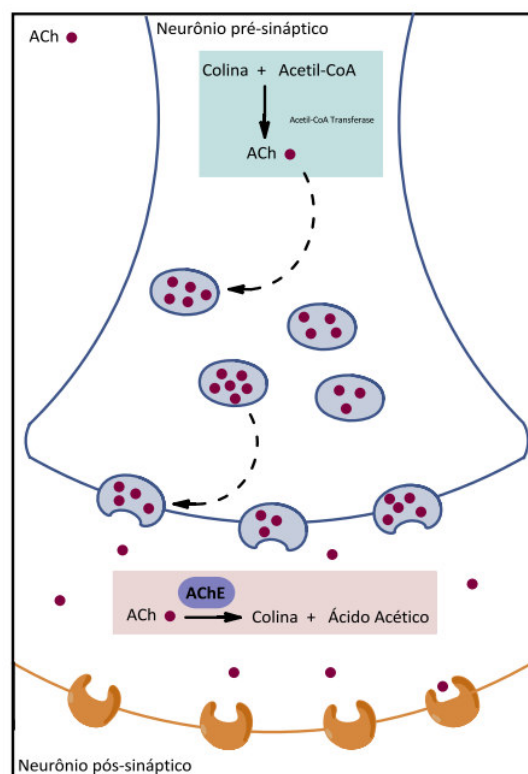


Figura 4: Síntese, liberação e degradação da acetilcolina. Fonte: Araújo et al., (2016).

A inibição da AChE é descrita como o principal mecanismo de toxicidade de pesticidas organofosforados e carbamatos, tanto para invertebrados quanto para vertebrados (Monserat et al., 2002). Porém, estudos também tem demonstrado que o glifosato causa a inibição desta enzima em peixes e microcrustáceos (Zongming et al., 2015; Lopes et al., 2022; Ames et al., 2023). Outras classes de contaminantes ambientais, como misturas complexas de poluentes, metais e detergentes também podem causar inibição da AChE (Diamantino et al., 2003, Frasco et al., 2005, Guilhermino et al., 1998, Payne et al., 1996), fazendo dela um bom biomarcador para avaliar a contaminação dos ambientes aquáticos em geral.

Proteínas associadas ao processo de desintoxicação também tem sido bastante usadas para o monitoramento de corpos hídricos. O processo de detoxificação ocorre em três fases, estritamente relacionadas (Figura 5). Na fase I, geralmente se observa a transformação do xenobiótico através das citocromo P450 (CYP), que são tipicamente envolvidas na fase I e conjugação do xenobiótico na fase II, a fim de torná-lo mais solúvel em água, além de diminuir sua toxicidade. Na fase III, estes metabólitos são transportados para o exterior da célula e então excretados (Huber et al., 2008).

A glutathione S-transferase (GST) é uma enzima de fase II que catalisa reações de conjugação entre os xenobióticos e o tripeptídeo glutathione (GSH), a fim de diminuir sua toxicidade e torná-los mais solúveis em água favorecendo sua degradação e excreção (Hayes e Pulford, 1995, Carletti et al. 2008). Desta forma, o aumento na atividade dessa enzima após a exposição a poluentes pode indicar um aumento no processo de desintoxicação dos organismos (Wang et al., 2009; Qi et al. 2013; Iummato et al., 2018; Iummato et al., 2019).

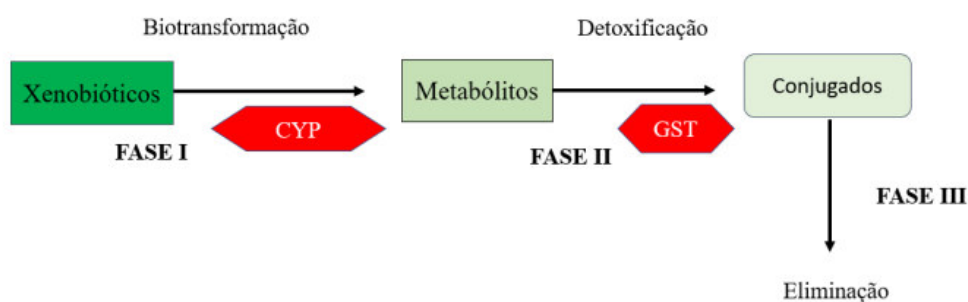


Figura 5: Esquema das fases de biotransformação enzimática de xenobióticos. Fonte: Castro, 2022.

Por outro lado, os biomarcadores comportamentais trazem informações em nível hierárquico maior, dependentes de alterações mais sistêmicas, cujas consequências estão relacionadas ao condicionamento individual do animal. Recentemente trabalhos tem explorado os efeitos dos contaminantes nos padrões comportamentais dos organismos, indicando a análise do comportamento como uma excelente ferramenta para a avaliação ecotoxicológica. O padrão de comportamento dos animais amplia sua capacidade de lidar com o ambiente e responder a diferentes condições e é controlado por vias genéticas, bioquímicas e fisiológicas (Lopes et al., 2022). Mudanças nas respostas comportamentais a um estímulo fisiológico e ambiental, devido a efeitos tóxicos de contaminantes presentes no ambiente aquático, podem tornar os organismos propensos à predação, interferir na busca de alimentos, afetar a reprodução, entre outros efeitos, comprometendo sua perpetuação e sobrevivência (Scott e Sloman, 2004; Lopes et al., 2022).

Neonatos de *Daphnia magna* e larvas de *Danio rerio* têm sido considerados como organismos-modelo na ecotoxicologia aquática a fim de avaliar os efeitos dos contaminantes nos ecossistemas aquáticos. Estes animais possuem algumas vantagens, tais como, genoma sequenciado, tamanho pequeno, manutenção fácil e barata,

desenvolvimento rápido e alta taxa de reprodução. Além disso, apresentam padrões comportamentais bem estabelecidos e são muito sensíveis a produtos químicos (Freiry et al., 2014). Sendo assim, estes organismos são muito utilizados em ensaios de toxicidade aguda e crônica, e vêm sendo utilizados em trabalhos envolvendo avaliação de biomarcadores bioquímicos e comportamentais (Hussain et al., 2020; Bellot et al., 2021; Lopes et al., 2022).

Abordagens integrativas em estudos de monitoramento ambiental

A utilização de análises integrativas multivariadas vem sendo empregadas em monitoramentos ambientais, para integrar fatores bióticos e abióticos, apresentando um panorama geral dos fenômenos ambientais envolvidos, os quais, na maioria das vezes, não são percebidos por ferramentas convencionais (Zhao et al., 2012). Os programas de monitoramento ambiental geralmente incluem, coletas frequentes nos mesmos pontos de amostragem e análise em laboratório de grande número de variáveis (diferentes ensaios de toxicidade e parâmetros físico-químicos, por exemplo), resultando em uma matriz de grandes dimensões e complexa interpretação (Simeonov et al., 2003).

Deste modo, tem sido muito utilizado abordagens multivariadas, provando ser ferramentas muito eficientes na sumarização de matrizes complexas, em diversos trabalhos de monitoramento ambiental, como Vieira et al. 2022 e Marques et al. 2022, que avaliaram os efeitos espaço-temporal dos metais oriundos do rompimento da barragem de Fundão em Mariana (MG) sobre biomarcadores em peixes dulcícolas e comunidades planctônicas de áreas marinhas, respectivamente. Além disso, Do Amaral et al. 2020 avaliou o efeito sazonal de parâmetros físico-químicos, pesticidas e metais-traço sobre biomarcadores bioquímicos em brânquias, cérebro, fígado e músculo de peixes coletados no Reservatório Passo Real, localizado no Rio Grande do Sul, que sofre influência agrícola e urbana, entre outros estudos de monitoramento (Stock et al., 2022; Maraschi et al., 2022; Martínez-Durazo et al., 2023).

Além disso, para os biomarcadores o uso do índice de Resposta Integrada de Biomarcadores (IBR), um método multivariado simples que permite integrar um conjunto de respostas de alerta precoce (Beliaeff e Burgeot, 2002). Além do valor único de todos os efeitos dos biomarcadores, o uso do gráfico de radar pode ser utilizado para auxiliar na interpretação do IBR. Por ser um diagrama que contempla dados multivariados na forma de um gráfico bidimensional, o gráfico radar permite a integração visual de um conjunto de respostas de três ou mais variáveis quantitativas em eixos que partem do

mesmo ponto, permitido observar quais biomarcadores contribuíram mais para o valor do índice, de forma independente (Beliaeff e Burgeot, 2002).

Local do estudo: Lagoa Mirim

A Bacia Hidrográfica da Lagoa Mirim (Figura 6) está localizada na fronteira entre o Uruguai e o estado do Rio Grande do Sul, no extremo sul do Brasil, entre as coordenadas geográficas de 31° 30' a 34° 35' de latitude Sul e 53° 31' a 55° 15' de longitude Oeste, possuindo uma área de superfície em torno de 62.250 km². Aproximadamente 47% da sua área encontra-se em território brasileiro e 53% em território uruguaio. É considerada a segunda maior lagoa do Brasil e está ligada à Lagoa dos Patos através do Canal São Gonçalo, formando o maior sistema lagunar da América do Sul. O Canal São Gonçalo em 1977 recebeu a construção de uma barragem para impedir o aporte das águas salinas vindas do estuário da Lagoa dos Patos para o Canal São Gonçalo (em aproximadamente 80%) e em toda área da Lagoa Mirim, pois a entrada de água salobra da Lagoa dos Patos acabava impedindo a utilização de suas águas para abastecimento público e irrigação de plantação de arroz, abundantes na região (Oliveira et al., 2015).

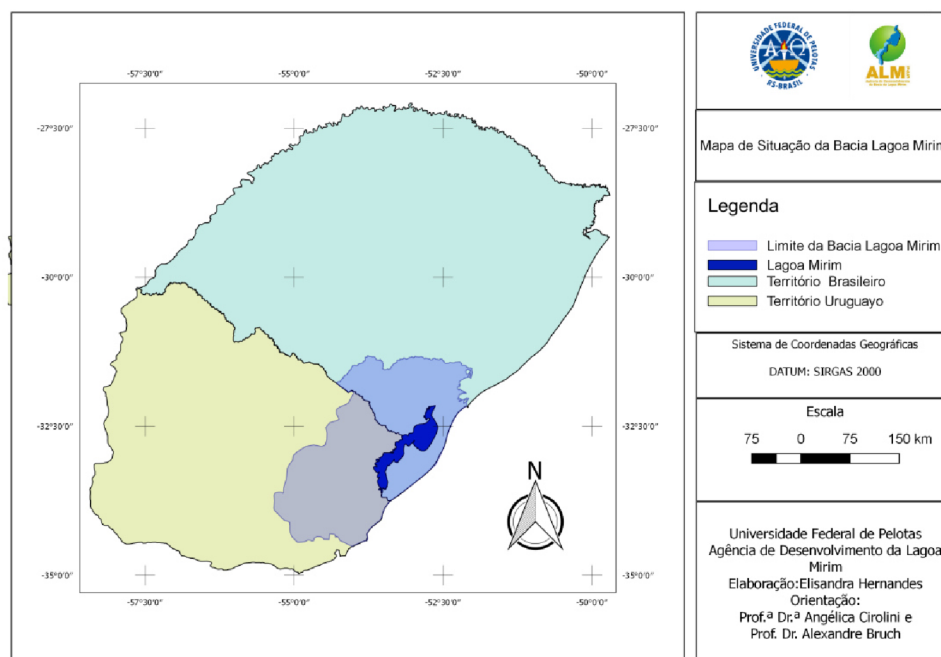


Figura 6: Mapa de localização da Bacia Hidrográfica da Lagoa Mirim. Fonte: Agência de Desenvolvimento da Lagoa Mirim (ALM), 2022.

Sob o ponto de vista econômico, a Lagoa Mirim é uma valiosa fonte de recurso pesqueiro, principalmente, para a população que vive de pesca artesanal. Além disso, é

manancial de captação de água para irrigação de plantações de arroz, atividade que impulsiona o desenvolvimento econômico da região e, também é manancial de captação de água para abastecimento público da cidade de Pelotas e Rio Grande através do Canal São Gonçalo (Steinke e Saito, 2008). Do ponto de vista ecológico, a Lagoa Mirim tem um importante papel na manutenção da umidade dos banhados do Taim, reconhecidos como Reserva da Biosfera pela UNESCO e uma das mais importantes áreas de conservação. Ademais, preserva banhados, lagoas, campos, dunas e matas e abriga uma grande diversidade de flora e fauna, servindo como ponto de alimentação e nidificação de aves migratórias (Santos et al., 2003; ALM, 2022). A Lagoa Mirim também possui grande importância para a reserva Humedales Del Este, a qual é composta por áreas úmidas localizadas no Uruguai, que fazem parte da Lista da Convenção Sobre Áreas Úmidas de Importância Internacional (Convenção Ramsar), formada por um ecossistema de, aproximadamente, 560 mil hectares que inclui cerca de 10 áreas protegidas para Reserva da Biosfera (ALM, 2022).

Entretanto, a bacia da Lagoa Mirim vem sofrendo degradação ao longo dos anos e a principal fonte de contaminação advém da utilização de agrotóxicos e fertilizantes (Grützmacher et al., 2008, Corrêa et al., 2022 Corrêa, 2023). A água da Lagoa Mirim é captada para irrigação das lavouras de arroz irrigado, sendo drenada para a lagoa novamente ao final do período de crescimento do arroz, mas agora com resíduos de agrotóxicos e fertilizantes aplicados nas lavouras durante a plantação (Torman, et al., 2017).

Estima-se que aproximadamente 2,7 milhões de toneladas de agrotóxicos sejam usadas por ano na agricultura em todo o mundo (FAO, 2022). Somente no Brasil em 2021, a comercialização de agrotóxicos foi cerca de 720 mil toneladas, das quais cerca de 76 mil toneladas foram comercializadas no Rio Grande do Sul (RS) (IBAMA, 2022). No RS, as culturas de arroz são consideradas como as principais responsáveis pelo lançamento de herbicidas nos corpos d'água, uma vez que o Estado concentra 70% da rizicultura irrigada do país (Conab, 2016). Os agrotóxicos mais utilizados no RS no ano de 2021 foram os herbicidas Glifosato, 2,4-D e Atrazina, os fungicidas Mancozebe e Clorotalonil e o inseticida Acefato (IBAMA, 2022), alguns atualmente banidos ou severamente restritos na União Européia (Braga et al., 2020), e que já foram detectados na Lagoa Mirim (Corrêa et al., 2022; Corrêa, 2023).

No Brasil, a resolução CONAMA 357/2005 prevê limites máximos permitidos para um número pequeno de agrotóxicos nos corpos d'água. Para as águas de classe 2

como a Lagoa Mirim, é permitido até 65 µg/L de Glifosato, 2 µg/L de Atrazina, 4 µg/L de 2,4-D, 0,1 µg/L de Malation, 0,04 µg/L de Paration e 10 µg/L de Metolacoloro.

Em 1994 foi criada a Agência de Desenvolvimento da Lagoa Mirim (ALM), vinculada a Universidade Federal de Pelotas (UFPEL), que atua para o desenvolvimento da bacia da Lagoa Mirim. Cabe à ALM a responsabilidade pela operação e manutenção da Barragem Eclusa do Canal São Gonçalo e de avaliar a qualidade da água da bacia, através de coletas mensais em vários pontos ao longo da lagoa, que vão desde Pelotas através do Canal São Gonçalo até Santa Vitória do Palmar, onde são analisados parâmetros físico-químicos e microbiológicos (ALM, 2022). Além do monitoramento pela ALM estudos independentes ou vinculados à ela foram desenvolvidos na bacia da Lagoa Mirim abordando aspectos hidrológicos (Oliveira et al., 2015), avaliação da qualidade da água com base em parâmetros físico-químicos gerais (Souza, 2015; Drose et al., 2020; Valentini et al., 2021), quantificação de metais com evidência de altos níveis de Chumbo (Valadão et al., 2016), Zinco e Cádmio em água superficial (Friedrich et al., 2006), quantificação de agrotóxicos, como os herbicidas Clomazona, Imazapyr e Quinclorac, inseticidas como o Carbofuran e Tiametoxam, além de fungicidas como Metalaxil e Epoxiconazole (Grützmacher et al., 2008; Corrêa et al., 2022). No entanto, a maioria dos trabalhos para esta região foca em análises químicas, ao passo que abordagens de caráter ecotoxicológico são escassas. Nesse contexto, o objetivo desse trabalho foi investigar a qualidade da bacia da Lagoa Mirim do ponto de vista ecotoxicológico, analisando amostras de água superficial coletadas em diferentes pontos da lagoa e afluente com o intuito de avaliar este importante sistema lagunar. Assim, as hipóteses deste trabalho foram: que amostras coletadas no período de março seriam mais tóxicas para os organismos testados, visto que neste período ocorre a drenagem da água utilizada na lavoura para a lagoa; e que na estação de inverno (junho) a toxicidade seria menor pois os contaminantes ainda não degradados e metabólitos estariam diluídos, já que essa estação é caracterizada por alta taxas pluviométricas na região da Lagoa Mirim e não ocorre aplicação de agrotóxicos.

OBJETIVOS

Objetivo Geral

Avaliar a qualidade da Lagoa Mirim e afluyente através de testes ecotoxicológicos com organismos bioindicadores de diferentes níveis tróficos e múltiplos biomarcadores, apresentados de forma simples e integrativas.

Objetivos específicos

- Determinar a toxicidade da água superficial da Lagoa Mirim e afluyente a partir de ensaios agudos e crônicos utilizando organismos de diferentes níveis tróficos em diferentes períodos amostrais;
- Avaliar os efeitos em biomarcadores bioquímicos e comportamentais em neonatos de *Daphnia magna* e larvas de *Danio rerio* expostos as amostras de água superficial da Lagoa Mirim e afluyente coletadas em diferentes períodos amostrais;
- Avaliar a interação dos parâmetros físico-químicos sobre os biomarcadores a partir de análises multivariadas;
- Aplicar uma ferramenta integrativa de avaliação da qualidade da água a partir do Índice de Resposta Integrada (IBR);
- Fornecer informações pioneiras sobre a qualidade da lagoa do ponto de vista ecotoxicológico e contribuir com o monitoramento realizado pela ALM;

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CAPÍTULO 1

The use of integrative tools and multiple models for aquatic environmental quality assessment: Mirim Lagoon, an example of study in Southern Brazil

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Abstract

This study performed toxicity assays with microalgae, microcrustaceans, and fish as well as evaluated biochemical and behavioral biomarkers in fish and microcrustaceans to assess the quality of surface water of Mirim Lagoon, one of the world's largest watershed located in Southern Brazil. Three distinct sampling periods were chosen (January, March, and June 2022) based on the rice plantation dynamics which is the main activity surrounding the lagoon. In January the plantation is irrigated; in March the water is drained to the Mirim Lagoon; and July is the off-season. Multivariate and integrative tools were used for a general overview of the effects caused to organisms spatially and temporally. Concerning toxicity tests, there was significant inhibition in microalgae growth when they were exposed to water collected in March, but no mortality was observed for the *Ceriodaphnia dubia*, *Daphnia magna* and *Danio rerio* at all. Regarding biomarkers, behavioral variables contributed more to the higher values of the Integrated Biomarker Response (IBR) index for both *D. magna* and *D. rerio*, in March. The Redundancy Analysis (RDA) indicated a correlation between the biomarkers for both organisms and abiotic parameters, mainly nutrients (total phosphorus and total nitrogen), fecal coliforms, total solids, and turbidity. Spatially, there was no difference during monitoring, but March was the period in which the most significant ecotoxicological effects were observed. Multivariate analysis and the IBR index proved to be useful tools for assisting in the monitoring of water bodies such as Mirim Lagoon.

Keywords: toxicity test; biomarker; aquatic monitoring; multivariate analysis; integrated biomarker response

Introduction

Population growth and anthropogenic activities have led to an increase in the diversity of commercially available products, and consequently their input into the environment (Freire et al., 2008). Contaminants can persist in the environment due to their resistance to degradation processes (long half-life) or even because a constant input (over the degradation rate). Given the complexity of the substances present in different ecosystems, they can interact with each other, altering their bioavailability. In addition, bioavailable compounds can be accumulated and cause toxicity to organisms, affecting the ecosystem as a whole (Connell and Miller, 1984; Holt, 2000).

The Mirim Lagoon watershed (Fig. 1) located in southeastern South America belongs to one of the largest lagoonal systems of the world. This lagoon is a valuable source of water both for irrigation of rice crops and public supply (Steinke and Saito, 2008). From an ecological point of view, the Mirim Lagoon plays an important role in maintaining the humidity of the Taim wetlands, recognized as a Biosphere Reserve by UNESCO, and serves as a feeding and nesting point for mammals, reptiles, and migratory birds (Santos et al., 2003; ALM, 2022). The intense agricultural activities surrounding the Mirim Lagoon, led to its contamination with pesticides that can reach the waters through leaching, surface runoff, precipitation, drifting from aerial sprays, and especially drainage from irrigated rice fields (Spadotto et al., 2010). It is estimated that approximately 2.5 million tons of pesticides are used in agriculture worldwide each year (FAO, 2022). In Brazil, around 720,000 tons of pesticides were commercialized in 2021, being 76,000 tons sold in the province of Rio Grande do Sul (RS) where the major rice plantations are and where Mirim Lagoon is located (IBAMA, 2022). The presence of pesticides in aquatic environments can be toxic to organisms from different trophic levels, including non-target ones (Miron et al., 2004; Thomas et al., 2020; Nogueira et al., 2022).

Monitoring activities are generally carried out to assess the environmental health / quality of the water bodies. Most of monitoring programs are based on physic-chemical analysis, however, it is essential to consider the ecotoxicity of the substances present in these waters. In this sense, the evaluation of water resources using bioindicator organisms is recommended by several regulatory agencies as a complementary tool for the effective environmental monitoring (Costa et al., 2008). The use of a variety of representative species of the aquatic biota and different trophic levels is recommended for the toxicological assays, allowing a more precise estimate of the impacts of the pollutants on

the ecosystem (Sponza, 2002; Pane et al., 2008). Several ecotoxicological assays are well-established and standardized by international organizations and standardization agencies, such as the United States Environmental Protection Agency (USEPA), Association française de Normalisation (AFNOR), International Organization for Standardization (ISO), Organization for Economic Co-operation and Development (OECD), and, in Brazil, the Associação Brasileira de Normas Técnicas (ABNT). They are useful tools for establishing limits of chemical substances in the aquatic environment, evaluating the effects of effluents on receiving waters, and assessing the quality of water sources (CETESB, 2008).

Besides the toxicity tests conducted with environmental samples (such as surfaces water, for example), the use of biomarkers has been also recommended for determining sublethal effects (Sureda et al., 2011; Marques et al., 2022; Maraschi et al., 2022; Vieira et al., 2022; Solomando et al., 2022; Barbarin et al., 2023). Biomarkers can be described as a response, as behavioral, biochemical, cellular, molecular, physiological changes in cells, body fluids, tissues or organs, of an organism that are indicative of exposure or the effect of a contaminant (Lam and Gray, 2003). They are able to give an early diagnosis of irreversible effects, that could, therefore, be prevented (Van der Oost et al., 2003). One of the most used biochemical biomarkers for pesticide contamination assessment is the inhibition of the Acetylcholinesterase (AChE) enzyme activity (Araújo et al., 2018; Bernal-Rey et al., 2020; Parrino et al., 2020; Pompermaier et al., 2022). This is one of the toxicity mechanisms for organophosphate and carbamate pesticides, both in invertebrates and vertebrates (Monserrat et al., 2002). On the other hand, enzymes involved in the detoxification process are also often used to assess the presence of contaminants in organisms, and this is the case of the enzyme glutathione S-transferase (GST) that conjugates xenobiotics to glutathione (GSH) for excretion, and also works as a scavenger of reactive oxygen species (ROS) generated by a wide range of cell stressors (Tsuchida and Yamada, 2014). Thus, an increase in the activity of GST may indicate the increase in the detoxification process of organisms, among others (Wang et al., 2009). Changes in behavioral patterns have been recently explored as biomarkers, and they seem to be quite responsive/sensitive. Behavior observations provide information about the consequences that contaminants can have on an individual animal's conditioning (Lopes et al., 2022).

The use of multivariate integrative analysis has been employed in environmental monitoring to integrate biotic and abiotic factors, providing an overview of the environmental phenomena involved. These phenomena are often not perceived by

conventional tools (Zhao et al., 2012). In this context, multivariate analyses are paramount for assessing complex datasets with several variables (Legendre and Legendre, 1998). Therefore, multivariate approaches have been extensively utilized and have proven to be highly effective tools in various environmental monitoring studies (Vieira et al., 2022; Marques et al., 2022; Maraschi et al., 2022; Martínez-Durazo et al., 2023).

Given the exposed above, this study analyzed the water quality of Mirim Lagoon through toxicity tests and biochemical and behavioral biomarkers, using for those standardized organisms from different trophic levels. Results were integrated with different periods of sampling, different sampling sites as well as water physical-chemical and pesticides analyzed in the same pond.

Materials and methods

Study area and water sampling sites

The Mirim Lagoon watershed (Fig. 1) is located on the border between Uruguay and the state of Rio Grande do Sul, in the southernmost region of Brazil, between the geographic coordinates of 31°30' to 34°35' East and 53°31' to 55°15' West, with a surface area of approximately 62.250 km². About 47% of its area is in Brazilian territory and 53% in Uruguay (Oliveira et al., 2015). Six sampling sites were selected for this study (see Fig. 1 and Table S1 at supplementary material), being all of them usually monitored by the Agência de Desenvolvimento da Lagoa Mirim (ALM), and chosen in a way that encompassed the lagoon as a whole and based on the pesticide concentrations quantified by Corrêa (2023). Three sites were located at the west side of the lagoon, in the municipalities of Rio Grande (P9) and Santa Vitória do Palmar (P11 and P12), and the other three sites were at the east side, in the municipalities of Jaguarão (P8) and Arroio Grande (P6 and P4). Except for the P4, which is a tributary of the lagoon, all other sites are located at the Mirim Lagoon.

The sampling was carried out in January, March, and June 2022. This sampling frequency was chosen based on the chronology of irrigated rice plantations that occurs around the entire lagoon, as previously reported (Corrêa, 2023). Usually, rice planting begins in September and between December and January, the plantations are irrigated by pumping water from Mirim Lagoon. Pesticides and fertilizers are applied in the crops according to the demand of the plantation, but they are commonly used. In March, the water is drained back into the lagoon before the grain harvest starts. June was selected

because there is no agricultural activity over this month or it is minimal, therefore, this would be considered a month of lower input of agricultural residuals. At each sampling, surface water was collected to polypropylene flasks, previously washed with nitric acid 1% and distilled water in abundance. Samples were immediately refrigerated (on ice) during transportation, and once in the laboratory, they were stored at -20 °C until the assays (ABNT NBR 15469, 2016).

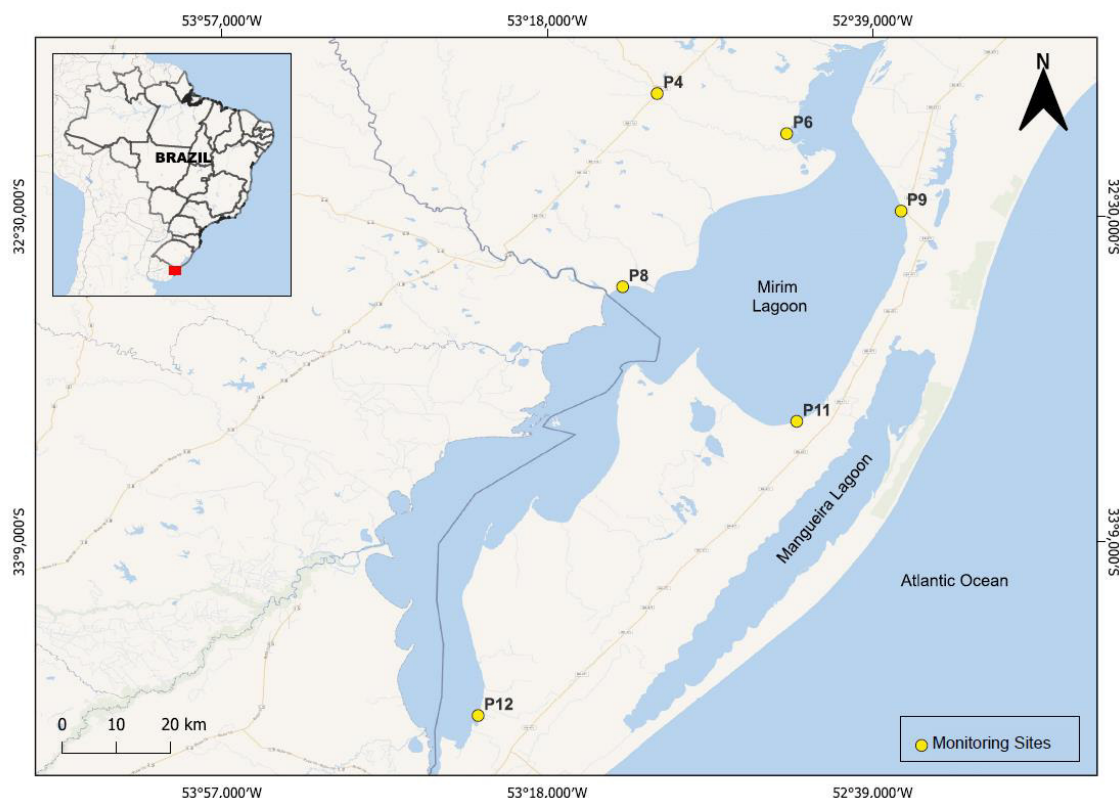


Fig. 1: Area of study with the 6 sampling stations (P4, P6, P8, P9, P11 and P12) along Mirim Lagoon and a tributary.

Water measurements

Water parameters analyzed in samples collected in this study were: turbidity, total phosphorus, total nitrogen, dissolved oxygen, chlorophyll α , biochemical oxygen demand, total solids, and thermotolerant coliforms. The pH, temperature, and conductivity values were measured at the time of collection using a K39-003PHC multiparameter meter (Kasvi), and other methods are described in Table S2 of the supplementary material. These measurements belong to ALM and can be accessed through the SIIRH (2021). These data were compared to the limits established by

Resolution n°. 357 of the Conselho Nacional do Meio Ambiente (CONAMA) for Class 2 waters (CONAMA, 2005), which is the one that regulates the study region.

Toxicity tests

The toxicity of the water samples, following standardized protocols, were performed with the microalgae *Pseudopedriastrum boryanum*, the cladocerans *Ceriodaphnia dubia* and *Daphnia magna*, and the fish *Danio rerio* at the embryo-larval stage. All assays included controls. Reference substance was tested as well to ensure adequacy (health) of the organism used for the tests. Temperature, pH, conductivity, and dissolved oxygen were measured at the beginning and end of each test. The measurements were in accordance with the standards required for the species used in this study and the organisms were obtained from established cultures at the Biological Sciences Institute of the Federal University of Rio Grande (FURG).

Microalgae assay

Microalgae used for toxicity test were obtained from the Collection of Freshwater Microalgae Cultures of the Biological Sciences Institute, Federal University of Rio Grande (algae registration code: CH007). They were exposed water samples and a control group for 72 h and the algal growth rate was estimated (ABNT NBR 12684, 2011). The WC culture medium (Guillard and Lorenzen, 1972) was used for cultivation and for the control group. The tests were carried out in 100 mL erlenmeyer flasks filled with 50 mL of the test solution. Cell densities of the pre-cultures and over the test were determined by counting the cells using a Neubauer chamber and an optical microscope. The density at the begin of the test was between 10^4 and 10^5 cells/mL. Experimental conditions were: temperature at $23 \pm 2^\circ\text{C}$ and constant light (>4500 lux). The tests were performed in quadruplicate, under constant agitation (100 to 175 rpm) to keep the cells in suspension. At the end of the tests, the microalgae were aliquoted into Eppendorf tubes and preserved with a 4% formaldehyde solution for later evaluation of the growth rate through cell counting, according to the following equation:

$$\text{Growth rate: } (cf - ci) / (tf - ti)$$

Where,

cf/ci: final and initial cell density

tf/ti: final and initial time, respectively

Results are expressed as percentage of growth over 72 h of experiment, considering the control growth as 100%.

Microcrustaceans assays

Toxicity of water samples was also evaluated by acute exposure of *D. magna* and chronic exposure of *C. dubia* following standardized methods (ABNT NBR 12713, 2022 and ABNT NBR 13373, 2017, respectively). However, in these tests, the animals were exposed only to the water collected in the sampling sites and to the control media, which were same used for the cultivation of animals.

D. magna were maintained in 2 L beakers filled with 1.5 L of mineral water at pH 7.6 to 8.0 (Água da Pedra®) with 50 organisms, and they were fed daily with *Raphidocelis subcapitata* microalgae at a density of 1×10^6 cells per organism. The cultures and the assays were kept inside a B.O.D incubator with controlled at $20 \pm 2^\circ\text{C}$ and photoperiod of 16 h light/08 h dark. For the tests, neonates aged 2 to 26 h were distributed into 4 replicates per treatment (water from the lagoon and mineral water) containing 5 organisms per replicate and 20 mL of the test solution (control [mineral water] and non-diluted water samples). The tests lasted 48 h under static conditions without feeding. At the end of exposure time, immobilized organisms were quantified as a measure of toxicity and expressed as percentage.

The *C. dubia* were cultivated in 600 mL beakers containing 500 mL of reconstituted water, composed of salts (sodium bicarbonate, calcium sulfate dihydrate, potassium chloride, and magnesium sulfate heptahydrate) at pH 7.0 to 7.6 (ABNT, 2017). Fifty organisms were kept per beaker and fed daily with *R. subcapitata* microalgae at a density of 1×10^5 cells per organism. In both the cultures and the tests, the organisms were kept inside a B.O.D incubator with temperature adjusted at $25 \pm 2^\circ\text{C}$ and photoperiod of 16 h light/8 h dark. For the assays, neonates aged 6 to 24 h were distributed into 12 replicates per treatment containing one organism per replicate. As mentioned above, the animals were exposed to water samples and a control group (reconstituted water). The assays lasted for 7 days under semi-static conditions, and the organisms were fed with *R. subcapitata* (1×10^5 cells per animal) at the beginning of the test and at each water renewal (days 3 and 5). At the end of the test neonates were removed and counted. Also, immobility was observed. Results were expressed as percentage of immobile animals or neonates born during the experiment.

***Danio rerio* embryo-larval assay**

Chronic short-term embryonic-larval toxicity tests with zebrafish (OECD 236, 2013). Embryos were obtained through the breeding of adult animals (1 male: 2 females). Eggs were selected under a stereomicroscope and those with fertilization rates higher than 90% were used in the assays. In the assays embryos 3 hpf (hours post fertilization) were exposed to water samples or control condition (E3 medium) over 7 days. Media were completely renewed within 72 h of exposure (semi-static condition). The exposures were performed in 24-well plates, and for each sample, the control group had 24 replicates with 1 embryo in each well, filled with 2 mL of the test solution. Mortality was evaluated by immobility of the larvae, and surviving organisms at the end of the test were euthanized by freezing at -80°C.

The Ethics Committee for Animal Use at the Federal University of Rio Grande (CEUA - FURG; reference PQ014/2022) approved the protocols performed in this study with fish.

Biomarkers

Because no mortality / immobility was observed in the toxicity tests (see Results section), we analyzed biomarkers as more sensitive tools to determine effects of water samples from Mirim Lagoon on the *D. rerio* embryo-larvae (3hpf) and *D. magna* neonates (2 to 24 h).

Behavioral biomarkers

For behavioral analysis, *D. rerio* embryo-larvae (3hpf) (n = 24) and *D. magna* neonates (2 to 24 h) (n = 20) were exposed to water samples or control solution over same time and conditions used in the toxicity tests, being 7 d for the fish larvae and 48 h for the *D. magna* neonates. At the end of the exposure period, animals were transferred to a 96-well plate containing 200 µL of the exposure solution (1 organism per well) and, after 10 minutes of habituation, behavior was recorded over 30 min in the dark, using the Daniovision system (Noldus, Netherlands). The Ethovision XT 11.5 software was used to analyze the following parameters: total distance travelled and mean swimming velocity.

Biochemical biomarkers

The same exposure protocols used to analyze the behavioral biomarkers and toxicity tests were used in the tests to analyze the biochemical biomarkers, except for the total number of animals, which in the case of *D. magna* were 160, divided in 16 replicates,

and *D. rerio* larvae were 80, divided in 8 replicates per treatment (water samples and controls). At the end of the exposure period (48 h of *D. magna* and 7 d for *D. rerio*), organisms were euthanized by rapid freezing in liquid nitrogen and stored at -80°C. For the analyses, animals were thawed on ice and then homogenized (*D. magna* pool of 40 organisms and *D. rerio* pool of 20 organisms, n=4 for both organisms), for about 40 s, at the lowest amplitude, using the Ika T10 basic Ultra Turrax homogenizer in 0.1 M phosphate buffer at pH 7. The homogenates were centrifuged at 10.000g for 20 minutes, and the supernatant was separated for the Acetylcholinesterase (AChE) and Glutathione S-transferase (GST) enzymes assays.

The AChE activity was determined according to the method of Ellman et al., (1961). Enzymatic activity was measured using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and acetylthiocholine iodide as substrates. The yellow color produced by thiocholine after its reaction with DTNB was quantified by spectrophotometer at 412 nm (2 readings, with a 30 minutes interval for *D. magna* and a 5 minutes interval for *D. rerio*). The results were expressed in μmol of acetylcholine iodide hydrolyzed/minute/mg of protein.

The GST activity was determined according to Keen et al., (1976) by complexing reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB), quantified by spectrophotometry at 340 nm (2 readings, with a 5 minutes interval for both organisms). The results were expressed as μmol of GSH/minute/mg of protein.

Total protein content was determined following the Bradford (1976) method. The assay is based on the binding of the Bradford reagent (dye) to the total protein, which forms a stable protein-dye complex causing a visible color change. The quantification of this complex was measured by spectrophotometry at a wavelength of 595 nm.

Statistical analyses

For differences in microalgae growth rate, a student t-test was performed to evaluate statistical differences between the control group and the samples. For the reproduction assays with *C. dubia* and the biochemical and behavioral biomarkers, comparisons between the mean values were made using one-way analysis of variance (ANOVA - one way) followed by Dunnett's post-hoc test to determine differences between control and water samples. Data that did not fit ANOVA assumptions were analyzed using the Kruskal-Wallis's test followed by Dunn's post-hoc test. Such analysis

was carried out since the normality and homoscedasticity of data was proven and Significance level adopted was 95% ($p < 0.05$) (Zar, 2010).

To integrate the multiple biomarkers into an overall index of physiological stress, we used the Integrated Biomarker Response (IBR). It was calculated for the biochemical and behavioral biomarkers analyzed for *D. magna* and *D. rerio*, separately by organism. For a better interpretation of the data, the index was calculated for each monitoring period (January, March, and June). The calculations followed the method of Beliaeff and Burgeot (2002) with modifications suggested by Devin et al., (2014) and were performed using the interface of the 'CALculate IBR Interface' website (Calibri, <https://liec-univ-lorraine.shinyapps.io/calibri/>) created by the Laboratory of Continental Environments (LIEC) at the University of Lorraine (France). More details about the calculations can be found in Devin et al. (2014) and Silva et al. (2018).

Redundancy Analyses (RDA) were applied to analyze the relationship between biomarker response and physical-chemical and thermotolerant coliforms parameters in *D. magna* and *D. rerio*, separately. The significance of the models, and the correlation between the abiotic data matrix and the biomarker matrix, were tested through permutation analyses (PERMANOVA, number of permutations = 1000), and the total variance explained by the ordination was represented as adjusted R^2 . Subsequently, the influence of spatial variables (sampling sites) and temporal variables (sampling period), and the interaction between them, was tested through PERMANOVA (number of permutations = 1000). A Principal Component Analysis (PCA) was performed to evaluate the integration between sampling sites and periods with pesticide analysis carried out by Corrêa (2023) with water samples collected at the sites and periods, used for this study, but in 2019; and a PERMANOVA was carried out to verify if the quantified pesticides had an effect on the biomarkers measured for *D. magna* and *D. rerio* (Legendre & Ledengre, 1998). Further details about the quantified pesticides can be found in Corrêa (2023). The analyses were performed using Sigma-Plot 14.5 and R Studio (RStudio Team, 2021), with the “vegan” package.

Results

Physic-chemical and thermotolerant coliforms parameters

Physic-chemical and thermotolerant coliforms analyses carried out on surface water samples collected in January, March, and June 2022 at different sampling site of the Mirim Lagoon watershed are presented in Table 1.

The total phosphorus equal to or higher than 0.12 mg/L at all sampling sites at some point during monitoring, disagreeing with the legislation. Furthermore, for P4, the total nitrogen was higher compared to the other sampling sites with an average of 1.55 mg/L, while for the other the average was 0.22 mg/L. Dissolved oxygen was also below the limit of ≥ 5 mg/L indicated by the legislation, in the months of January and March (4.81 and 4.38 mg/L, respectively) at P4. The mean ratio N:P (total nitrogen:total phosphorus) for each monitoring period was higher in January (5.18). For March and June, the mean ratio remained similar between months (0.85 and 0.99, respectively). Again, the site P4 had the highest ratio value (27.33) in January, agreeing with the high amount of total nitrogen. Thermotolerant coliforms also presented higher values for sampling site P4 in all monitoring period (7,900, 13,000 and 4,900 NMP/100mL, respectively) and for the P9 only in January (4,900 MPN/100mL).

Table 1: Physical-chemical parameters determined in surface water samples at different sites in the Mirim Lagoon watershed, in January, March, and June 2022, and reference values for CONAMA 357/2005. Highlighted values are above CONAMA 357/2005.

Period	Sites	Temperature (°C)	Dissolved oxygen (mg/L O ₂)	Conductivity (µS/cm)	pH	Turbidity (UNT)	Total solids (mg/L)	Total phosphorus (mg/L P)	Total Nitrogen (mg/L N)	Chlorophyll α (µg/L)	Biochemical oxygen demand (mg/L O ₂)	Thermotolerant coliforms (MPN/100mL)	N:P ratio
CONAMA limits - Class 2		≤ 40,0	≥5,0	-	6,0 -9,0	≤ 100	≤ 500	≤ 0,03	-	≤ 30	≤5	≤1000	-
January	P4	29.2	4.81	65	8.2	29.8	109.2	0.12	3.29	1.34	2.04	7.900	27.33
	P6	29.8	6.01	104	8.8	58.3	181.0	0.12	0.30	25.39	0.75	33	2.50
	P8	31.5	8.22	66	9.1	27.3	121.5	0.32	0.19	NR	3.20	27	0.50
	P9	27.2	7.72	84	8.5	36.6	123.5	1.57	0.08	23.30	2,35	1.600	0.05
	P11	29.7	7.26	97	9.0	41.6	171.5	0.64	0.19	16.40	1.26	170	0.29
	P12	27.6	5.67	87	8.2	40.8	161.2	0.57	0.25	21.16	1.37	49	0.43
March	P4	22.1	4.38	114	7.5	4.21	128.5	0.41	1.06	1.07	1.44	13.000	2.59
	P6	21.3	6.73	241	8.1	15.0	116.5	ND	0.19	12.62	0.10	130	0.90
	P8	26.0	8.56	92	8.4	18.4	142.2	ND	0.18	2.17	0.31	6.1	0.90
	P9	15.0	8.41	138	8.4	8.4	244.5	1.49	0.34	22.98	2.41	590	0.22
	P11	17.0	6.77	382	7.9	7.9	344.0	1.28	0.29	4.90	1.18	540	0.22
	P12	17.3	8.18	173	8.6	8.6	166.0	0.97	0.30	31.65	3.00	ND	0.30
June	P4	8.0	7.55	61	8.1	35.6	147.7	0.43	0.30	1.19	1.96	4.900	0.70
	P6	9.4	7.45	224	7.8	19.1	223.0	0.35	0.16	5.35	2.27	11	0.45
	P8	10.2	9.27	102	7.8	31.6	273.7	0.51	0.23	3.91	1.34	33	0.45
	P9	10.0	6.68	250	7.6	24.6	182.0	0.12	0.23	10.06	0.93	130	1.91
	P11	9.7	8.27	330	7.7	14.8	293.5	ND	0.19	4.28	0.91	450	0.95
	P12	9.0	8.18	94	7.9	21.3	137.7	0.12	0.18	18.37	0.91	10	1.50

ND= Not detected and NR= Not realized

Toxicity tests

According to the results presents on Fig. 2, water collected at P11 caused a significant microalgae growth inhibition along to the 3 months monitored, but water collected in March at the P11 and in June at the P6 and P9 were also cause significant growth inhibition. Moreover, it can be observed that in March, there were samples that cause a significant increase in the microalgae growth rate, as the one collected at P6, P8, and P9.

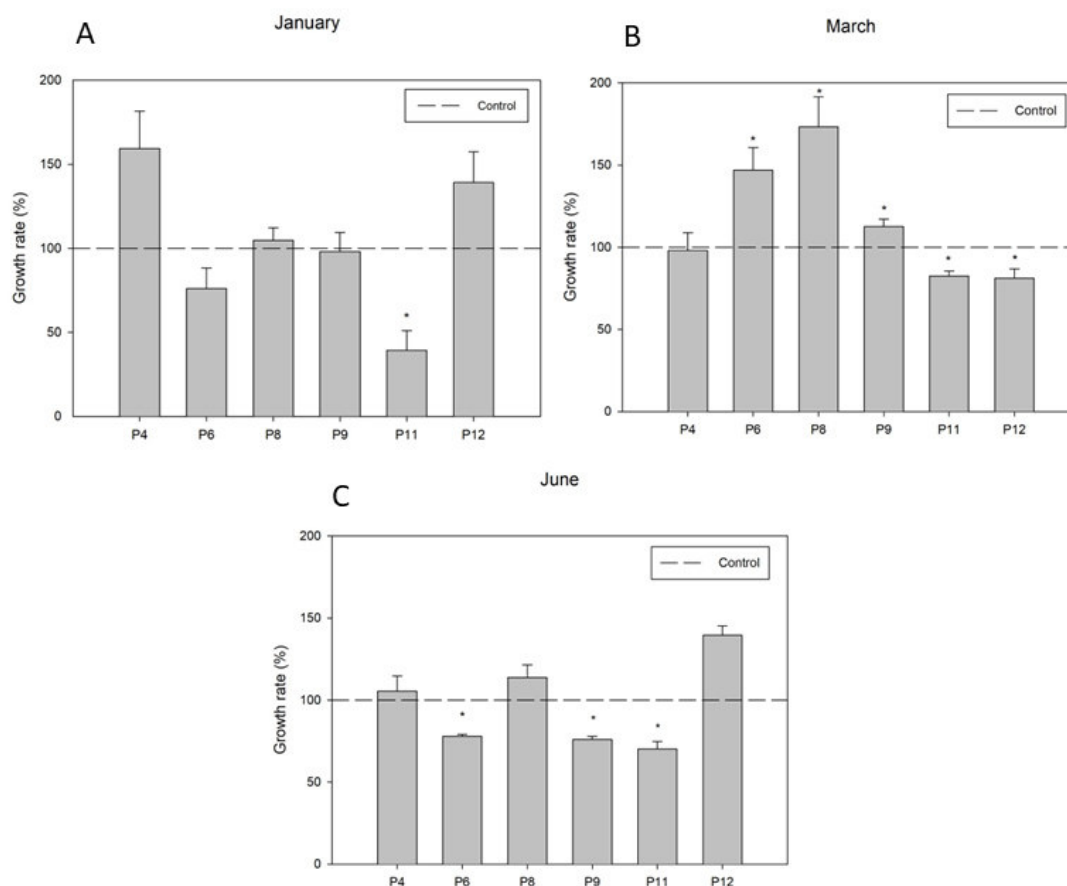


Fig. 2. Growth rate of the microalgae *P. boryanum* exposed to surface water samples collected in different sampling sites at Mirim Lagoon (see Fig. 1) for 72 h. Data were expressed as mean $\pm$ standard error. Statistical difference ($p < 0.05$) between control and treatments (sites) was represented by an asterisk.

The water samples from Mirim Lagoon did not cause acute toxicity for *D. magna* or chronic toxicity for *C. dubia*, since no mortality (immobility) or decrease in the number of newborns were observed, respectively. Instead, there was even an increase in the number of neonates from adult *C. dubia* exposed to P6 and P12 samples in March and P4, P9, and P11 samples in June. There was also no significant mortality of *D. rerio*

larvae. It is worth noting that guidelines used for the tests with *D. rerio* accept a mortality rate up to 20% in the control group (see Fig. S1 and Table S3 in supplementary material).

Biochemical and behavioral biomarkers

Fig. 3 shows that, overall, most significant behavioral effects for the neonates of *D. magna* were observed for samples collected in March, with the P4 samples showing significant hypoactivity in both parameters total distance travelled and mean swimming velocity. On the other hand, a significant increase in these parameters (hyperactivity) was observed when the neonates were acutely exposed to the P6, P8, and P12 samples. In June, hyperactivity was observed in neonates exposed to the P9 water samples. In January there was not a clear influence of the sampling site. Behavioral biomarkers measured in *D. rerio* showed hyperactivity of the organisms with a significant increase in total distance traveled and mean swimming velocity after the larvae were exposed to water from sites P4, P8, P11, and P12, only in June (Fig. 4).

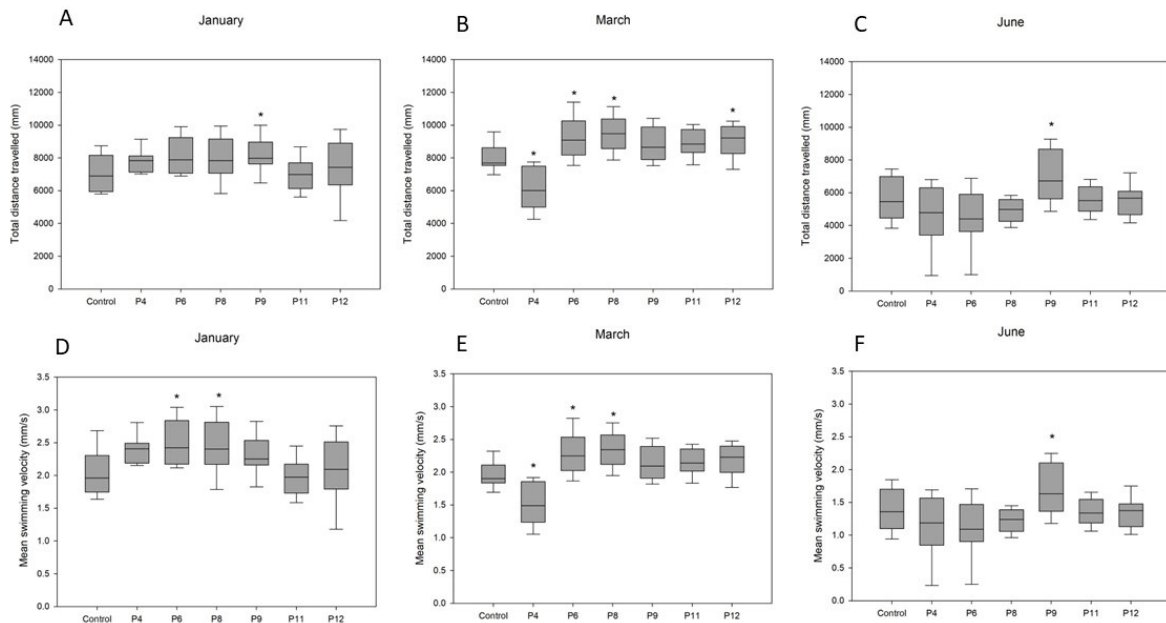


Fig. 3. Behavioral biomarkers: Total distance travelled (A, B, and C) and mean swimming velocity (D, E, and F), measured in *D. magna* exposed to surface water samples after 48 h of exposure. The boxplot shows the median, data distribution, and $\pm$ standard deviation (n=20). Statistically significant differences between control and treatments are represented with an asterisk ($p < 0.05$).

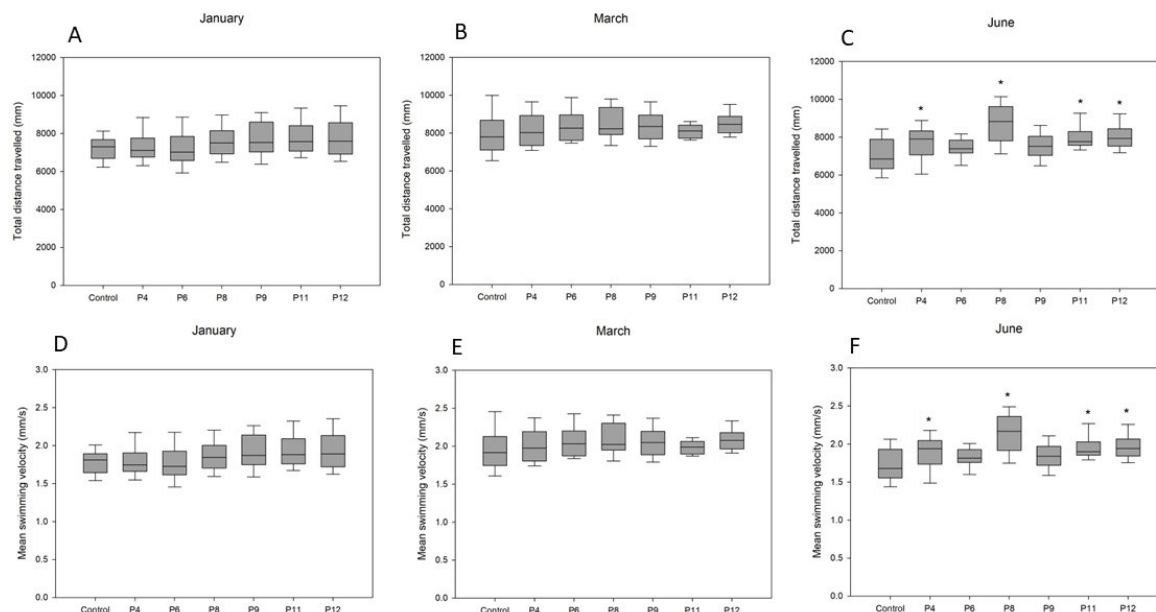


Fig. 4. Behavioral biomarkers: Total distance travelled (A, B, and C) and mean swimming velocity (D, E, and F), measured in *D. rerio* exposed to surface water samples after 168 h of exposure. The boxplot shows the median, data distribution, and $\pm$ standard deviation (n=24). Statistically significant differences between control and treatments are represented with an asterisk ($p < 0.05$).

Regarding the biochemical biomarkers (Fig. 5 and 6), the GST did not show any alteration neither in *D. magna* nor in *D. rerio*. The AChE activity was inhibited only in *D. magna* when exposed for 48 h to water collected at P6 in January and P4, P9, and P12 in March (Fig 5).

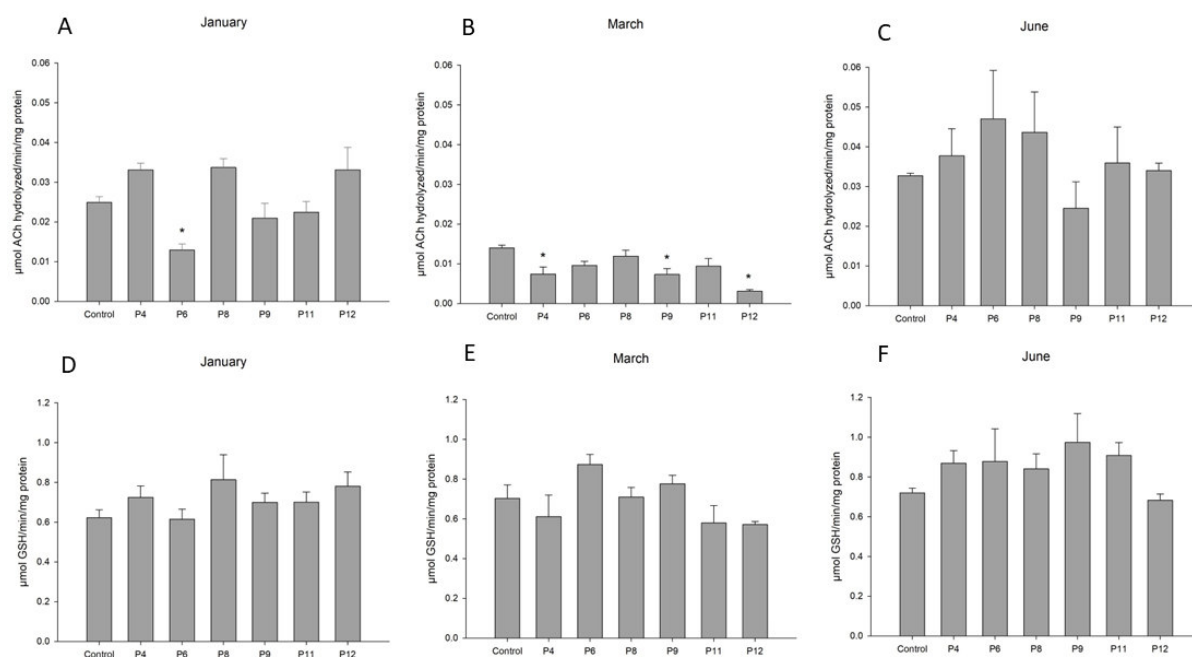


Fig. 5. Activity of Acetylcholinesterase (A, B, and C) and Glutathione S-transferase (D, E, and F) enzymes measures in *D. magna* exposed to surface water samples for 48 h. Data are expressed as mean $\pm$ standard error. Statistically significant differences between control and treatments were represented by an asterisk ($p < 0.05$).

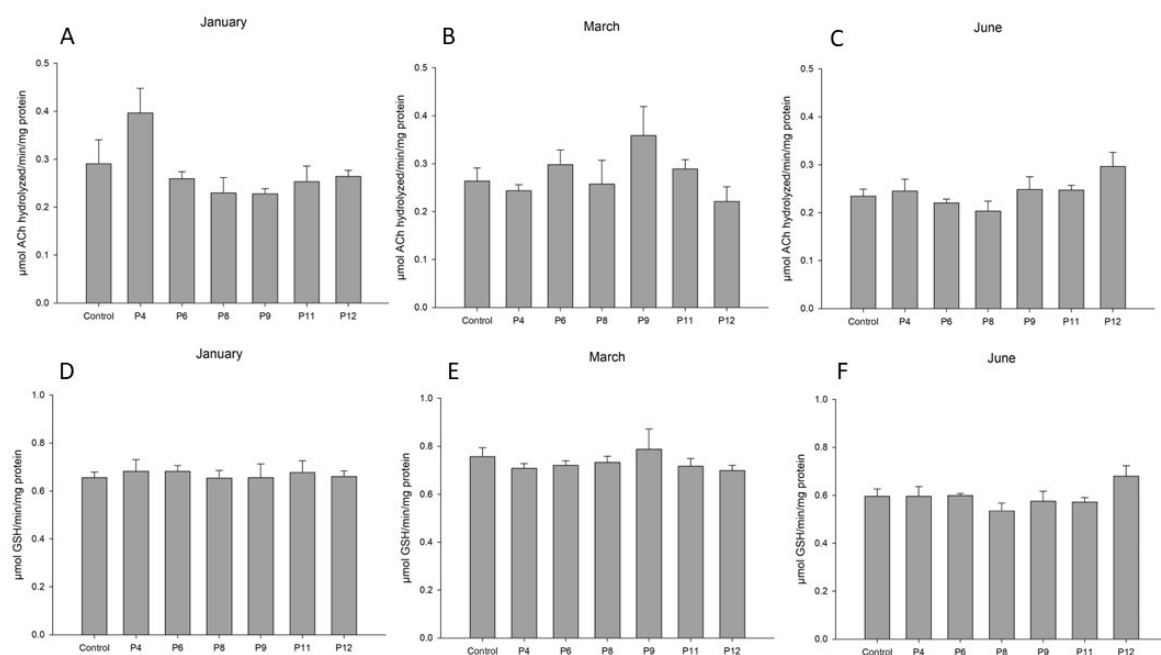


Fig. 6: Activity of the enzymes Acetylcholinesterase (A, B, and C) and Glutathione S-transferase (D, E, and F) enzymes measures in *D. rerio* exposed to surface water samples for 168 h. Data are expressed as mean $\pm$ standard error. Statistically significant differences between control and treatments were represented by an asterisk ($p < 0.05$).

Integrated Biomarker Response Index (IBR)

IBR integrated the behavior and biochemical biomarkers for the 3 sampling times, separately. For *D. magna*, the values were: IBR estimated for *D. magna* the values were 2.23, 3.3 and 0.0 and for *D. rerio* larvae they were 0.13, 3.99, 1.44 for January, March, and June, respectively. It is worth noting that highest IBR scores were attributes do March (3.3 for *D. magna* and 3.9 for *D. rerio*, respectively). According to the radar charts (Fig. 7), behavioral biomarkers contributed most to the IBR scores. In addition, for *D. magna*, AChE activity counted for the IBR score in March, and the GST activity had a high contribution to the *D. rerio* IBR score, in the same month.

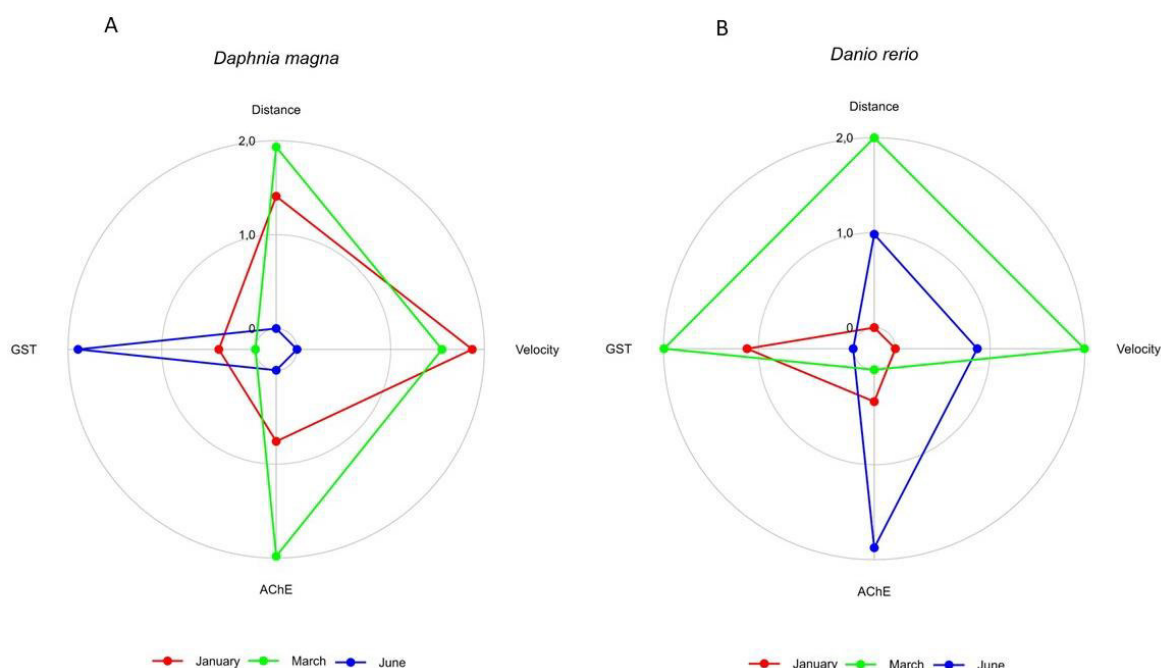


Fig. 7. Radar charts of the Integrated Biomarker Response (IBR) Index for *D. magna* (A) and *D. rerio* (B) exposed to water samples collected in the different sites of Mirim Lagoon in January, March, and June 2022.

Multivariate approach

Seasonal and spatial effects on water quality

The PERMANOVA showed that both variables (sampling period and sites), including their interaction, had a significant effect on the measured biomarkers for both *D. magna* and *D. rerio* (Table 2). However, most of the variability in the biomarkers was explained by the sampling period for both organisms *D. magna* and *D. rerio* ($R^2=0.642$ and $R^2= 0.366$, respectively). Although sampling sites had influenced the variability of the biomarkers, their importance was lower when compared to the other variables.

Table 2: Permutation analysis to assess whether the variables, sampling location, sampling period, and the interaction between location and period influence the behavioral and biochemical biomarkers for *D. magna* and *D. rerio*. Number of permutations = 1000.

Organism	Variable	R ²	F	P
<i>Daphnia magna</i>	Period	0.642	162.559	<0.001
	Sites	0.100	10.112	<0.001
	Period*Sites	0.152	7.702	<0.001
<i>Danio rerio</i>	Period	0.366	28.220	<0.001
	Site	0.136	4.205	0.004
	Period*Sites	0.147	2.263	0.002

Influence of physicochemical parameters on biomarkers

The redundancy analysis (RDA) applied to biomarker responses in *D. magna* (Fig. 8A) and abiotic and thermotolerant coliforms factors, as dependent and explanatory matrixes, was significant ($p=0.001$), indicating that the abiotic and thermotolerant coliforms factors influence the biomarkers matrix, and 26% of the constrained variability was explained by this model (adjusted $R^2=0.262$). The nutrients (N and P) and total solids were the factors influencing the first axis and turbidity and conductivity contributed to the second axis. The RDA was efficient in observing temporal trends, with the sampling periods were clustered. January and March were grouped in the left quadrant of the biplot, influenced by the nutrients N and P, and the behavioral biomarkers. January was particularly influenced by high nitrogen and low total solid levels. Also, mean swimming velocity was the main biomarker responsible for the clustering of the observations from this period. Similarly, total distance travelled was mostly associated with samples of March, which were positively influenced by phosphorus. On the other hand, June, which was ordered in the right quadrant and mostly influenced by low levels of nutrients but high levels of turbidity, coliforms, and total solids. IN addition, the biochemical biomarkers AChE and GST were considerably grouped and related to this sampling period.

The second RDA, applied to data recorded from *D. rerio* (Fig. 8B) showed a high overlap was of all periods, suggesting a more subtile differentiation between January, March and June biomarkers as a function of abiotic and microbiological factors. This was corroborated by the proximity of sampling observation to the center of the biplot. Still,

this model was statistically significant ($p=0.001$) and represented 23% of the data variability (adjusted $R^2=0.227$). The parameters that mostly influenced this ordination were turbidity, coliforms, total solids, and nutrients, similarly to those influencing the *D. magna* biomarkers. However, turbidity, phosphorous were the main factors contributing to the first axis, whereas nitrogen and coliforms contributed to the second axis. Again, phosphorous, and thermotolerant coliforms were positively associated to march, whereas turbidity was negatively related to this month. The behavioral biomarkers were also associated to this period and closely correlated. Regarding January and June, these periods were more aggregated, still, high levels of turbidity combined with low levels of phosphorous were the main factors influencing this month. Nitrogen, thermotolerant coliforms, and turbidity were also important factors influencing positively January samples.

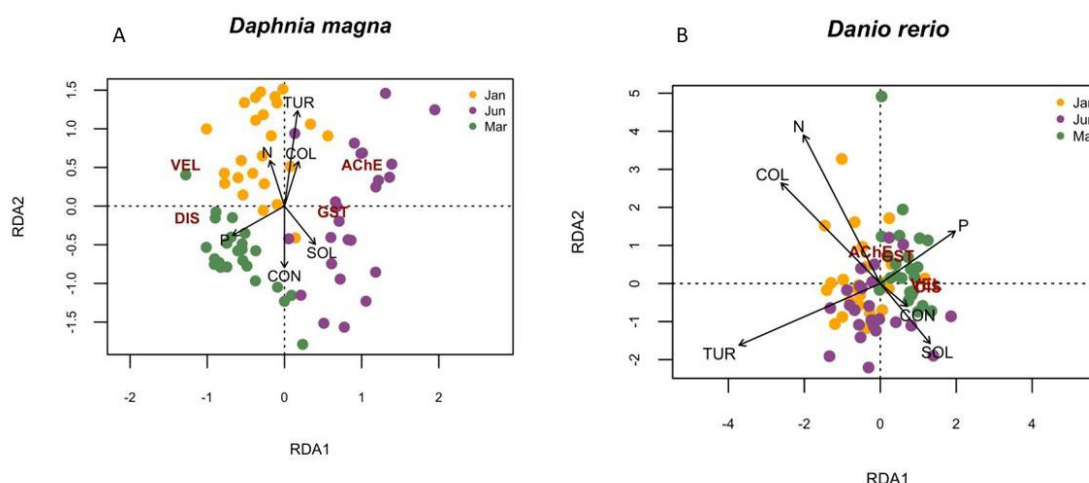


Fig. 8: Redundancy Analysis (RDA) using abiotic water parameters (P=total phosphorus, N=total nitrogen, TUR=turbidity, SOL=total solids, and CON=conductivity) and the behavioral and biochemical biomarkers (DIS=total distance travelled, VEL=mean swimming velocity, AChE=Acetylcholinesterase activity, and GST=Glutathione S-transferase activity) measured in *D. magna* (A) and *D. rerio* larvae (B). The sampling periods (January, March, and June) are represented by the orange, green, and purple, respectively.

Spatial and seasonal analysis of pesticide concentration

The Principal Component Analysis (PCA) integrating the sampling sites, periods of water sampling and pesticide concentrations determined by Corrêa (2023), revealed that axes 1 (PC1) and 2 (PC2) together summarized 54.76% of the total variation in the

results, where PC1 was responsible for 38.61% of the total variation in the analysis while PC2 accounted for 16.15% (Fig. 9). It is possible to observe the overlap of the sampling sites, corroborating with the results previously observed in PERMANOVA, where the sampling period is the predominant factor influencing the biomarker results (Table 2). Additionally, January was the month associated with most of the analyzed pesticides (Atrazine, Azoxystrobin, Clomazone, Chlorantraniliprole, Epoxiconazole, Flutriafol, Imazapyr, Imazethapyr, Metoxifenozone, Quinclorac, and Tricyclazole), while March and June were only positively associated with the herbicide Atrazine and the fungicide Epoxiconazole. It is important to say that we used only the pesticides that were detected in the samples, which were collected in 2019.

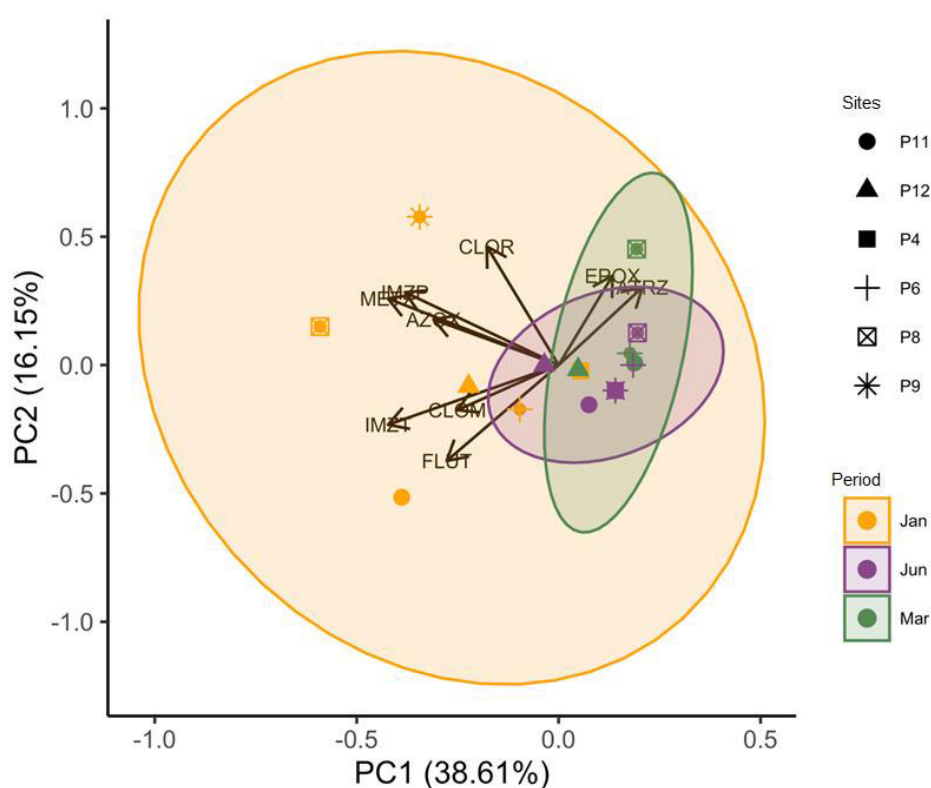


Fig. 9. Principal Component Analysis (PCA) considering the sampling sites and periods as well as pesticides determined in the same sampling sites by Corrêa (2023). Abbreviations: ATRZ=Atrazine, AZOX=Azoxystrobin, CLOM=Clomazone, CLOR=Chlorantraniliprole, EPOX=Epoxiconazole, FLUT= Flutriazole, IMZP=Imazapyr, IMZT=Imazethapyr, METX=Metoxifenozone, QUIN=Quinclorac, and TRIC=Tricyclazole.

The Permutation Analysis (Table S5 - supplementary material) was performed to verify whether the pesticides measured by Corrêa (2023), in January, March, and June of

2019, would influence the biomarkers measured for *D. magna* and *D. rerio* that was presented in this study. The biomarkers of *D. magna* were more prejudiced by pesticide concentrations than *D. rerio*. The biomarkers of *D. magna* responded to seven different pesticides, being the herbicides Atrazine, Imazethapyr, and Quinclorac, the fungicides Azoxystrobin, Epoxiconazole, and Tricyclazole, and the insecticide Metoxifenozone. On the other hand, in *D. rerio* the set of biomarkers responded only to Atrazine.

Discussion

Physic-chemical analysis and contamination

We will start this section by discussing the results of contamination of the Mirim Lagoon's waters published by Corrêa (2023) and the dynamics of pesticide application; then, we will link with the ecotoxicological effects measured by the present work. In Corrêa's study (2023), average pesticide concentrations detected in January were around 6 µg/L, with Quinclorac, Flutriazole, Imazapyr, Imazethapyr, Clomazone and Methoxyphenozone being the most detected compounds. In March, the average of pesticides was 3.5 µg/L, with Tricyclazole, Azoxystrobin, Epoxiconazole and Atrazine being the most representative. Meanwhile, the month of June showed an average below 1 µg/L corresponding basically to Tricyclazole, Imazethapyr, Azoxystrobin, and Atrazine. The pesticides mentioned above for the 3 months of monitoring are in a crescent order related to their concentration in the waters. It is worth saying that Corrêa (2023) considered 12 sampling sites in her study. As shown in the PCA (Fig. 9), there was a predominance of pesticides in the water collected January, while the months of March and June were represented mainly by the Epoxiconazole and Atrazine. This contamination profile corroborates with the chronology of pesticide application in the rice crops plantations and residual drainage to the Mirim Lagoon watershed.

Most of pesticides are used between December and January due to the need to protect the rice crops that are growing from pests and weeds until the grain harvest. In March, the water used for rice irrigation is drained back into the lagoon for harvesting, transporting residues and metabolites of pesticides and fertilizers (Grützmacher et al., 2008; SOSBAI, 2018; Corrêa et al., 2022). The late occurrence of pesticides in March and especially June is possibly associated with the pesticide's persistence or the presence to toxic metabolites, that can cause effects on organisms and are not usually measured (García-de la Parra et al., 2006; Gomes et al., 2022; Ivantsova et al., 2022; Bhatti et al., 2022). Corrêa (2023) showed a seasonal variation in contaminant concentrations for the

same sampling sites of this study, over 2019. In fact, these concentrations served as a basis for choosing the locations and months of sampling in the present study. We also considered that the dynamics of the irrigated rice crop surrounding Mirim Lagoon, which repeats every year. The same happens with the pesticides used in the plantations. There is usually a brand change, but the active principles of the formulas used remain the same. Additionally, the permutation analysis showed that the sampling period is a preponderant factor affecting biomarkers evaluated in *D. magna* and *D. rerio*. Thus, the discussion of this work turns around sample periods.

As mentioned before, physical-chemical and thermotolerant coliforms were compared with the local legislation (CONAMA 357, 2005) and some of them, mainly at P4 disagreed with the regulation. Some reference values of CONAMA 357/2005 are similar to those of international bodies, such as Environment Canada, for instance the nutrient and pH values are similar to those of CONAMA for the province of New Brunswick. Regarding nutrients, nitrogen and mainly phosphorous were found at elevated concentrations over the last 6 years of monitoring (2017 to 2022) (Table S4 - supplementary material). It may come from the application of fertilizers in the agricultural area surrounding the Mirim Lagoon and indicates that crop drainage water is possibly carrying fertilizers and contaminating the watershed (Grützmacher et al., 2008; Fia et al., 2009; Coradi et al., 2009; Droese et al., 2020; Valentini et al., 2021). When phosphorus and nitrogen are discharged in high concentrations into surface waters under good conditions of light, they can cause an increase in primary productivity and lead water bodies to eutrophication (Esteves, 2004; Aguiar et al., 2011; Soro et al., 2023). However, this was not observed in this study, as the O₂ concentration dissolved in water were mostly according to the regulation, and the high levels of phosphorus did not correspond to an increase in chlorophyll α , indicating low primary productivity in the lagoon. Cunha et al. (2013) evaluated the quality of Caiubá Lagoon, another watershed located in the southernmost part of Brazil and also used for irrigation of rice plantations, and they also found higher levels of phosphorus during monitoring and a low N:P ratio, like this study. This low ratio (N:P) could limit primary productivity due to nitrogen concentrations, available below what is needed by primary producers (Howarth et al., 2006; Howarth et al., 2021; Soro et al., 2023). Kratzer and Brezonik (1981) studying shallow tropical lakes in Florida, considered that N:P ratios less than 10 limits primary productivity by nitrogen. In this study the highest concentration we measured was 5.18 in January.

The amount of thermotolerant coliforms is an indicative of domestic sewage discharge and is one of the main parameters entering in the water quality (Sauvé et al., 2012; Ghildyal et al., 2018; Droese et al., 2020). The thermotolerant coliform at P4 which is out of Water Quality Criteria (WQC) and higher in comparison to the other sites, may be because it passes through the city of Arroio Grande, which does not have a domestic sewage treatment plant, before flowing into Mirim Lagoon (Godecke and Decker, 2014). In addition, the high amount of thermotolerant coliforms observed at P9 only in January could be due to the use of this location for recreational between December and February, over the summer. The decrease in thermotolerant coliform concentrations in all sites over the monitoring months may be associated with the high rainfall in austral winter period (June).

Ecotoxicological effects

Since the aquatic biota is constantly exposed to a variety of toxic substances released into the environment, the assessment of water resources through bioindicator organisms is recommended for environmental monitoring (Costa et al., 2008). Concerning toxicity assays, we can say that water collected at the different sites of Mirim Lagoon did not show acute or chronic toxicity when the endpoint was mortality as showed by toxicity tests with *D. magna*, *C. dubia* and *D. rerio* embryo-larvae. However, we observed effects on microalgae, mainly in March represented as growth or growth inhibition, and increased number of neonates of *C. dubia*. These effects might be associated with nutrients present in the water, and in the case of *C. dubia* with the primary biomass and water pH, which may contribute to the growth and reproduction of cladocerans (Santos et al., 2006; Gutseit et al., 2007). Among the organisms tested, microalgae proved to be the most sensitive organisms. Thus, the importance of using a variety of species representing the aquatic biota in ecotoxicological assays to estimate the impact of contaminants on the aquatic environment is supported (Sponza, 2002; Pane et al., 2008; Prato et al., 2011; Sulej-Suchomska et al., 2021). Microalgae are important for the balance of aquatic ecosystems, as they are primary producers found at the base of aquatic food chains. In this sense, effects caused in organisms at this level can compromise the perpetuation and survival not only of microalgae, but also of primary and secondary consumers, therefore triggering disturbances at the community level (Van der Oost et al., 2003; Cedervall et al., 2012). Moreover, each species has a specific role in the

ecosystem, keeping it in balance, whether as a predator, prey, decomposer, or producer. However, the excess or lack of a key species can trigger an imbalance in the ecosystem.

Despite the lack of toxicity to the *D. magna* and *D. rerio*, we observed effects of water samples, mainly collected at site P4, on behavioral and AChE activity of these animals. Behavioral responses were more sensitive than the biochemical ones, and it was mostly expressed as hyperactivity for both species, with the exception of the water collected at P4 which inhibited the total distance travelled and mean swimming velocity of *D. magna*. Furthermore, for *D. magna* the greater significant effects were observed in the behavioral biomarkers in March, while for *D. rerio* we observed significant effects only in July. It is important to note that although we saw more significant hyperactive effects in the organisms, the hypoactivity observed for *D. magna* should be concerning because this is deleterious to the organisms, making them prone to predation, interfering with food search, and affecting reproduction, compromising their perpetuation and survival in polluted aquatic environments (Weis et al., 2001; Giaquinto et al., 2017; Pompermaier et al., 2020; Sánchez et al., 2021; Lopes et al., 2022).

Most of literature analyses behavior as a biomarker for isolated contaminants or mixtures made in laboratory. For example, the study of Bridi et al. (2017) showed that 72 hpf *D. rerio* larvae exposed to Glyphosate, the most widely used herbicide in the world, including in the Brazilian rice crops plantations (IBAMA, 2022), and its formulation Roundup® reduced the distance travelled by the larvae when exposed to both in environmentally relevant concentration (0.01 mg/L). For *D. magna*, pesticides such as Imidacloprid, Fipronil, Carbaryl and Fenpropathrin reduced the locomotor activity of the microcrustacean, in sublethal concentrations of insecticides (Hussain et al., 2020).

As proposed by Lopes et al. (2022), there are some biochemical mechanisms that seems to be behind behavioral alterations caused by the presence of herbicides. Some of them, as organophosphorus, carbamates and glyphosate can inhibit the AChE (Gruber and Munn, 1998; Sturm et al., 1999; Monserrat et al., 2002). However, several authors have observed that the decrease in AChE activity is not only due these pesticides, but it is also inhibited by other classes of environmental contaminants, such as complex mixtures of pollutants, metals, and detergents (Payne et al., 1996; Guilhermino et al., 1998; Frasco et al., 2005; Diamantino et al., 2003; Simonato et al., 2016). This enzyme participates in neuronal communication, through the hydrolysis of the neurotransmitter acetylcholine in the synaptic cleft. The inhibitory action results in the accumulation of acetylcholine in the synapses of the central nervous system, neuromuscular junctions,

sympathetic and parasympathetic nerve endings (Guyton and Hall, 2006). For *D. magna*, the AChE inhibition and hypoactivity observed at point P4 in March stand out. The imbalance in cholinergic neurotransmission caused by the inhibition of the AChE enzyme can result in neuromuscular effects, such as the hypoactivity observed in this study (Khalil et al. 2017; Ogungbemi et al., 2019). Concerning the GST, the mixture of compounds present in the surface water of the Mirim Lagoon were not able to activate the detoxification process via GST. This non-activation may be somehow associated with the other effects observed in this study, such as AChE inhibition and behavioral changes.

The IBR index also indicated March as the month where most of the negative responses appear, for organisms. Indeed, the PERMANOVAs showed that the sampling period had a greater influence on biomarkers than sampling sites for both species. March, although not the month with the highest observed concentrations by Corrêa (2023), is characterized by water draining back to the lagoon, possibly carrying residues of pesticides, unmeasured metabolites, and fertilizers. Furthermore, in March and June 2019, Atrazine was detected in Mirim Lagoon, showing some resistance to degradation of the herbicide in the environment, which may be toxic to aquatic biota (Brodeur et al., 2013; Bautista et al., 2018; Cleary et al., 2019; Blahovaa et al., 2020; De Souza et al., 2023). Atrazine has been banned in the European Union since 2004, but European countries import commodities from Brazil, where atrazine is the fifth most used pesticide (Braga et al., 2020; IBAMA, 2022, MAP, 2022).

The hypothetical permutation analysis, using pesticide concentration data determined by Corrêa (2023), showed that the biomarkers measured in *D. magna* are more influenced by pesticide since it is influenced by seven pesticides, agreeing with the biomarker results that indicate *D. magna* as more sensitive than *D. rerio* larvae, already observed in other studies and emphasized by the shorter exposure time in this study (48 h for *D. magna* and 168 h for *D. rerio*) (Pavlaki et al., 2016; Reque et al., 2021; Yang et al., 2022).

Relate the physical-chemical parameters with biomarkers is important, since the fluctuation of these abiotic conditions directly alter physiological performance of the organisms (Do Amaral et al., 2020; Strode et al., 2023). Indeed, the RDA indicated that abiotic parameters have an influence on both biochemical and behavioral biomarkers. It was mainly observed an influence caused by nutrients (total phosphorus and total nitrogen), which are related to the entry of fertilizers in the water and presence of thermotolerant coliforms. The total solids values determined in Mirim Lagoon can affect

the water turbidity, a factor that significantly contributed to the RDAs, due to inorganic material leaching from the surrounding area and the action of wind resuspending sediment, especially in shallow lagoons such as the study area. Although we observed correlations between biomarkers and abiotic factors, we cannot rule out the effects of pesticides and domestic sewage because these compounds mix in the aquatic environment and can be potentiated by abiotic fluctuations, especially in subtropical regions with well-defined four seasons (Alvares et al., 2013; Do Amaral et al., 2020).

In order to assess the risk to aquatic ecosystems, the use of indexes such as IBR to integrate multiple biomarkers into an overall index of physiological stress (Beliaeff & Burgeot, 2002), together with multivariate approaches to integrate environmental contaminants and abiotic parameters, allows for a general overview of the effects caused to bioindicators after exposure to contaminated aquatic environments. Generally, monitoring of water bodies is carried out at various sites throughout the study area and at different times, and these tools allow for exploration of the spatiotemporal variation of measured biomarkers and the interaction between the observed effects with the sampling sites, periods, and physical-chemical parameters of the environment (Do Amaral et al., 2020; Maraschi et al., 2022; Vieira et al., 2022). Thus, biomonitoring programs using ecotoxicological assays and biomarker evaluation in these environments are important for detecting the effects on organisms, with this information being essential for future water resource management.

Conclusion

Data presented in this study showed the greater sensitivity of microalgae concerning acute and short-term chronic effects, while for biomarker assessment, *D. magna* proved to be more sensitive than *D. rerio* to effects caused by water condition, highlighting the importance of evaluating organisms from different trophic levels in monitoring programs. In this context, behavioral biomarkers proved to be good indicators of deleterious effects on tested organisms. Regarding space-time effects, we observed that the collection period influenced more the analyzed biomarkers than the monitoring sites, in addition, in March more ecotoxicological effects were observed, mainly for microalgae and microcrustaceans. This study also demonstrated the applicability of evaluating toxicity and biochemical and behavioral parameters as complementary tools to the physical-chemical analysis for water quality assessments. The multivariate analyses and IBR index proved to be good approaches to assist in the monitoring of water bodies,

allowing us to observe a general spatial and temporal overview of the effects caused to organisms by contaminated environmental samples.

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Supplementary material

Table S1: Sampling stations defined for collection of surface water samples at different sites in the Mirim Lagoon watershed.

Identification	Sites	South latitude	West longitude
P4	Rio Arroio Grande	32°15'20.66"	53° 04'53.19"
P6	Praia do Pontal	32°20'04.02"	52°49'18.39"
P8	Fazenda São Francisco	32°38'24.95"	53° 08'57.77"
P9	Capilha	32°29'20.70"	52°35'35.36"
P11	Vila Anselmi	32°54'32.80"	52°48'05.90"
P12	Porto Santa Vitória	33°29'48.05"	33°29'48.05"

Table S2: Methods used to determine physical-chemical parameters in surface water samples at different sites in the Mirim Lagoon watershed.

Parameter	Analysis method	Reference	LOD
Chlorophyll α	Colorimetric - UV-Vis Spectroscopy	Cesteb, 2014	
Thermotolerant coliforms	Multiple Tubes	APHA,2012	1.8 (MPN/100mL)
Conductivity	Conductivimetric	APHA,2012	-
Biochemical oxygen demand	Incubation for 5 days	APHA,2012	0.05 mg L ⁻¹
Total Phosphorus	Colorimetric - UV-Vis Spectroscopy	APHA,2012	0.20 mg L ⁻¹
Total Nitrogen	Total Nitrogen Kjeldahl (NTK)	APHA,2012	5.0 mg L ⁻¹
Dissolved oxygen	Titrimetric	APHA,2012	-
pH	Potentiometric	APHA,2012	-
Total solids	Gravimetric	APHA,2012	1.0 mg L ⁻¹
Turbidity	Nephelometric	APHA,2012	0.1 UNT

UNT = Nephelometric turbidity unit

MPN = Most probable number

Table S3: Mortality percentage for *Ceriodaphnia dubia*, *Daphnia magna*, and *Danio rerio* exposed to surface water samples from Mirim Lagoon watershed.

Period	Point	Mortality percentage		
		<i>Ceriodaphnia dubia</i>	<i>Daphnia magna</i>	<i>Danio rerio</i>
January	Control	0	0	4.1
	P4	0	0	4.1
	P6	0	0	12.5
	P8	16.6	0	8.3
	P9	0	0	8.3
	P11	0	0	8.3
	P12	0	0	4.1
March	Control	0	0	4.1
	P4	9.0	0	12.5
	P6	0	0	8.3
	P8	0	0	4.1
	P9	0	0	16.6
	P11	8.3	0	16.6
	P12	0	0	4.1
June	Control	0	0	8.3
	P4	16.6	0	8.3
	P6	8.3	0	8.3
	P8	0	0	0
	P9	0	0	8.3
	P11	0	0	4.1
	P12	8.3	0	8.3

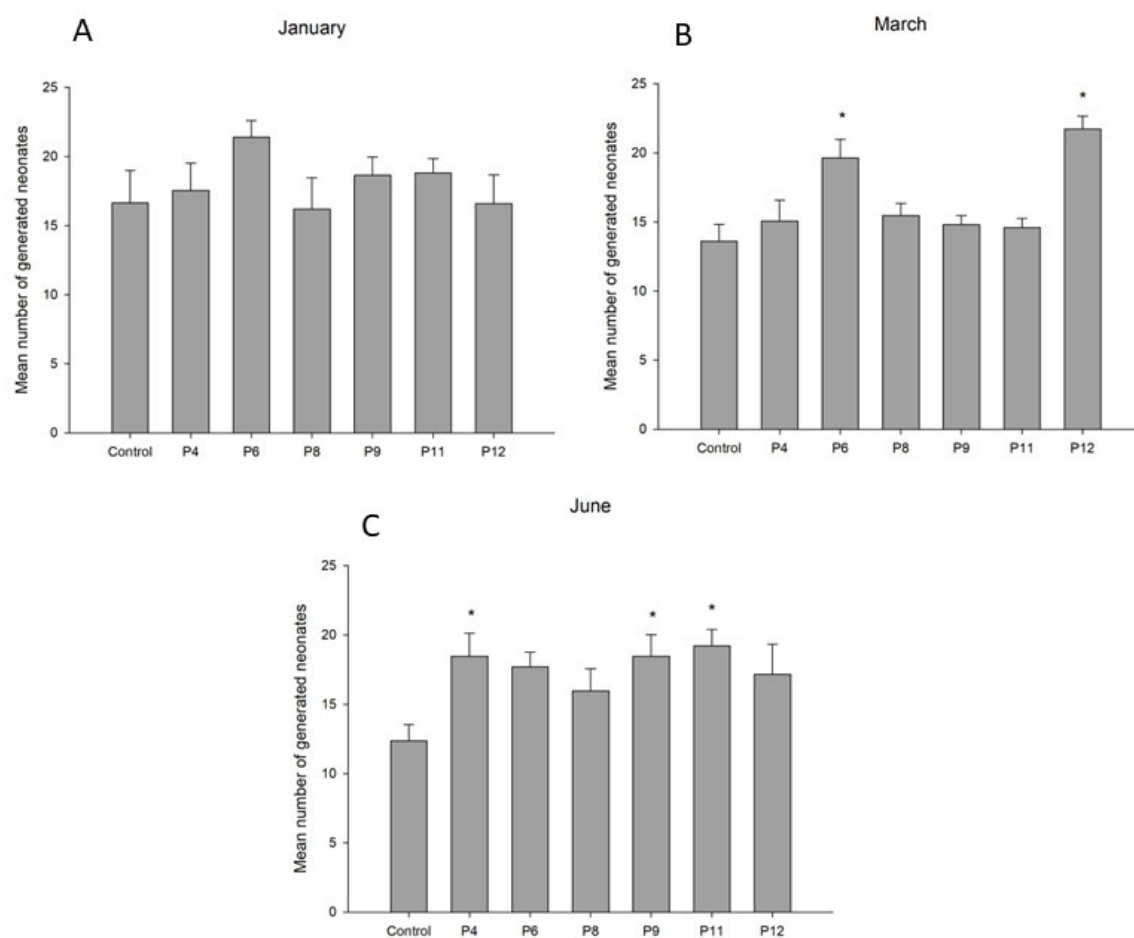


Fig. S1: Mean number of *C. dubia* neonates exposed to surface water samples at sites in Mirim Lagoon after 7 days of exposure. Mean $\pm$ standard deviation (n=12). Significant difference between control and treatments ($p < 0.05$) was represented with an asterisk.

Table S4: Mean values of total phosphorus and total nitrogen determined in surface water samples at different sites in the Mirim Lagoon watershed by the Agência de Desenvolvimento da Lagoa Mirim over the years.

	Year	P4	P6	P8	P9	P11	P12
Total phosphorus	2017	3.19	8.99	8.82	13.10	12.88	6.11
	2018	0.26	0.35	0.38	0.41	0.44	0.40
	2019	0.28	1.13	1.30	2.10	1.60	1.99
	2020	2.27	3.56	6.11	6.03	4.17	3.06
	2021	4.05	6.85	8.51	6.56	5.47	5.55
	2022	4.06	3.83	2.34	5.75	4.68	4.29
Total nitrogen	2017	7.63	5.94	4.88	5.59	4.48	6.80
	2018	3.44	2.56	3.70	4.47	7.68	4.86
	2019	2.03	4.34	6.09	5.42	4.21	5.56
	2020	1.07	4.67	2.16	1.72	0.41	1.87
	2021	1.07	2.39	3.05	0.65	0.81	0.84
	2022	6.57	1.40	1.05	1.01	1,65	1.21

Table S5: Permutation Analysis (PERMANOVA) between the pesticides detected by Corrêa (2023) and the biomarkers measured for *Daphnia magna* and *Danio rerio*. Number of permutations = 1000. * indicates statistical significance (p<0.05).

Pesticide	<i>Daphnia magna</i>			<i>Danio rerio</i>		
	R ²	F	P	R ²	F	P
Atrazine	0.022	4.993	0.035*	0.120	9.562	0.007*
Azoxystrobin	0.155	34.877	<0.001*	0.022	1.753	0.198
Clomazone	0.004	0.917	0.344	0.031	2.456	0.122
Chlorantraniliprole	0.001	0.076	0.841	0.007	0.547	0.464
Epoxiconazole	0.172	38.553	<0.001*	0.034	2.663	0.119
Flutriazole	0.006	1.354	0.26	0.001	0.081	0.782
Imazapyr	0.002	0.479	0.533	0.001	0.056	0.817
Imazethapyr	0.048	10.870	0.002*	0.001	0.067	0.794
Methoxyphenozide	0.039	8.796	0.002*	0.009	0.724	0.431
Quinchlorac	0.183	41.045	<0.001*	0.006	0.475	0.513
Tricyclazole	0.100	22.351	<0.001*	0.011	0.878	0.253

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Os dados apresentados na presente dissertação indicaram que as microalgas se mostraram mais sensíveis em comparação aos demais organismos testados (microcrustáceos e peixe). Em relação aos biomarcadores, os comportamentais se mostraram a mais sensíveis em comparação aos biomarcadores bioquímicos, que mostrou efeito significativo para a AChE apenas em *D. magna*. Além disso, observamos correlação entre os agrotóxicos quantificados nos mesmos pontos de coleta e meses, no ano de 2019 e os efeitos nos biomarcadores, principalmente de *D. magna*, se mostrando novamente mais sensível em relação ao *D. rerio*. Cabe ressaltar aqui que, apesar das análises dos agrotóxicos não terem sido feitas concomitantes com este estudo, entendemos que a comparação entre a presença e concentração destes agrotóxicos e os dados deste estudo é válida, pois a dinâmica de plantio de arroz, principal atividade do entorno da Lagoa Mirim, permanece a mesma bem como os produtos usados. Através de abordagens multivariadas foi percebido que o período de coleta influenciou mais sobre os biomarcadores analisados em *D. magna* e *D. rerio* do que os pontos monitorados. Além disso, no mês de março foi observado mais efeitos ecotoxicológicos, principalmente para as microalgas e microcrustáceos.

Evidenciamos a importância de avaliarmos organismos de diferentes níveis tróficos em monitoramentos, tanto para testes de toxicidade quanto para análise de biomarcadores, visto que estes organismos possuem sensibilidade diferentes quantos expostos a amostras ambientais contaminadas e assim conseguimos estimar com maior segurança o impacto dos contaminantes ao ambiente. Neste sentido, ressaltamos a importância do uso em conjunto de testes de toxicidade e diferentes biomarcadores para fornecer respostas mais completas acerca da saúde dos organismos. Além disso, salientamos a necessidade de avaliar diversos biomarcadores bioquímicos, visto que os contaminantes podem afetar diversas vias bioquímicas dos organismos e o investimento em biomarcadores comportamentais nos monitoramentos ambientais, visto que se mostraram bons indicadores de efeitos aos organismos testados, além de sempre fáceis e práticos de serem testados.

Atualmente, a Agência de Desenvolvimento da Lagoa Mirim (ALM) realiza o monitoramento mensal em diversos pontos ao longo da Lagoa Mirim e afluentes, mas somente com base de parâmetros físico-químicos e coliformes termotolerantes. Estudos assim, avaliando do ponto de vista ecotoxicológico são importantes, pois esses dados podem ser usados em propostas de avaliação de risco e regulamentação como maneiras de proteger a biota e os ambientes aquáticos. Além disso, as ferramentas multivariadas e integrativa se

mostraram bons instrumentos para auxiliar no monitoramento dos corpos hídricos como a Lagoa Mirim, permitindo observar de forma espacial e temporal um panorama geral dos efeitos causados aos organismos pelas amostras ambientais.

ANEXOS

COMISSÃO DE ÉTICA EM USO ANIMAL

Universidade Federal do Rio Grande
Pró-Reitoria de Pesquisa e Pós-Graduação - PROPESP
ceua@furg.br <http://www.propesp.furg.br>



CERTIFICADO Nº P014/2022

Certificamos que o projeto intitulado "Monitoramento ecotoxicológico da Lagoa Mirim - RS e relação com a contaminação por agrotóxicos", protocolo nº 23116.002705/2021-01, sob a responsabilidade de Camila de Martinez Gaspar Martins - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO RIO GRANDE (CEUA-FURG), em reunião de 30 de março de 2022 (Ata 003/2022).

A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

CEUA Nº	Pq017/2021
COLABORADORES AUTORIZADOS A MANIPULAR OS ANIMAIS	Josiane Araújo da Silva; Andressa Rubim Lopes; Jenifer Silveira Moraes; Márcio de Azevedo Figueiredo
VIGÊNCIA DO PROJETO	31/12/2023
ESPÉCIE/ LINHAGEM / RAÇA	<i>Danio rerio</i> (zebrafish)
NÚMERO DE ANIMAIS	120 Adultos (60 fêmeas e 60 machos); 5.760 Larvas
PESO/ IDADE	Adultos e Larvas
SEXO	60 Fêmeas e 60 Machos. Demais sexo indeterminado
ORIGEM	Toca dos Bichos, Rua General Neto, 367, Centro, Rio Grande, RS
ENVIO RELATÓRIO PARCIAL	Janeiro/2023
ENVIO DO RELATÓRIO FINAL	Janerio/2024

Rio Grande, 31 de março de 2022.

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