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CAMPUS SOROCABA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA E MONITORAMENTO AMBIENTAL

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CONCENTRAÇÃO DE METAIS E METALOTIONEÍNAS EM TECIDOS DE GIRINOS DE RÃS-TOURO, *Aquarana catesbeiana* (Shaw, 1802), EXPOSTOS ÀS ÁGUAS DO RIO SOROCABA

Sorocaba
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RESUMO

O monitoramento ambiental por meio de bioindicadores é uma estratégia essencial para compreensão dos seus impactos nos corpos hídricos. Os girinos de rãs-touro foram utilizados para verificar o efeito da exposição por metais presentes nas águas de rios, verificando o potencial para o biomonitoramento de corpos hídricos. Foi realizada a coleta de água em 2 pontos do Rio Sorocaba, sendo uma região de coleta na cidade de Ibiúna, representado a nascente do rio (PI) e outra o Reservatório de Itupararanga (PIR), durante a época seca do ano. Os girinos de rã-touro foram expostos às águas por 96 horas e, ao fim do ensaio, os animais foram anestesiados e mortos para a coleta de brânquias, pele e músculo caudal, onde foram avaliados a concentração dos metais arsênio (As), bário (Ba), cádmio (Cd), chumbo (Pb), cobalto (Co), cobre (Cu), estrôncio (Sr), manganês (Mn), molibdênio (Mo), níquel (Ni) e zinco (Zn) e de metalotioneínas (MTs). Os metais Ba, Cu, Mn, Sr, Zn estavam em maior concentração nas amostras de brânquias e pele do grupo exposto às águas do Reservatório de Itupararanga em comparação com o controle. A concentração de metalotioneína, nas amostras de brânquias e músculo caudal, apresentou um aumento significativo nos girinos expostos às águas do Reservatório de Itupararanga, em relação ao controle e às amostras dos girinos expostos às águas coletadas em Ibiúna (PI). As brânquias apresentaram forte correlação entre MTs e os metais. Os resultados mostram diferentes efeitos nos tecidos e evidencia potencial do uso desse biomarcador e da brânquia, que constitui uma interface entre o ambiente aquático e o meio interno do animal, e se mostrou um órgão importante para o uso no biomonitoramento ambiental.

Palavras-chave: Bioindicadores; Poluição hídrica; Monitoramento Ambiental.

ABSTRACT

Environmental monitoring through bioindicators is an essential strategy for understanding the impacts that may occur on water bodies. Bullfrog tadpoles were used to verify the effect of exposure to metals present in river water, verifying the potential for biomonitoring of water bodies using this species. Water was collected at 2 points on the Sorocaba River, one in the city of Ibiúna, representing the source of the river (PI) and another in the Itupararanga Reservoir (PIR), during the dry season of the year. Rã-Touro tadpoles were exposed to the collected water for 96 hours and, at the end of the test, the animals were anesthetized and killed for the collection of samples of gills, skin and caudal muscle, where the concentration of metallothionein proteins (MTs) and the metals arsenic (As), barium (Ba), cadmium (Cd), lead (Pb), cobalt (Co), copper (Cu), strontium (Sr), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn). The concentration of metallothionein, in the samples of gills and caudal muscle, showed a significant increase in the tadpoles exposed to the waters of the Itupararanga Reservoir, in relation to the control and the samples of the tadpoles exposed to the waters collected in Ibiúna (PI). The metals Ba, Cu, Mn, Sr, Zn were in higher concentration in the gill and skin samples of the group exposed to the waters of the Itupararanga Reservoir compared to the control. Gills showed a strong correlation between MTs and metals. The results show different effects on the tissues and potential evidence of the use of this biomarker using the gills, which constitute an interface between the aquatic environment and the internal environment of the animal, which proved to be an important organ for use in environmental biomonitoring.

Keywords: Bioindicators; water pollution; Environmental monitoring.

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LISTA DE SIGLAS

As – Arsênio

Ba – Bário

Cd – Cádmio

Cd – Cádmio

Co – Cobalto

CONAMA – Conselho Nacional do Meio Ambiente

Cu – Cobre

Mn – Manganês

Mo – Molibdênio

MTs – Metalotioneínas

Ni – Níquel

Pb – Chumbo

PI -Ponto de coleta Ibiúna

PIR – Ponto Represa de Itupararanga

Zn – Zinco

SUMÁRIO

1 INTRODUÇÃO GERAL

Os corpos hídricos de água doce são essenciais para a manutenção da vida e para o funcionamento da sociedade. Os primeiros assentamentos humanos ocorreram ao redor desses corpos hídricos para que o uso da água tornasse possível a manutenção da vida e o estabelecimento de comunidades e, nos dias de hoje, o acesso à água de qualidade é essencial para que a população possa viver com dignidade e os processos produtivos que são inerentes às cidades possam ser mantidos (Frascareli *et al.*, 2016). Conforme as populações se estabelecem e as necessidades de uso da água e das bacias hidrográficas aumentam, maiores são os impactos ambientais que as populações humanas exercem sobre os rios e lagos (Cunha e Borges, 2015).

Como medida para controlar os impactos e monitorar os ambientes aquáticos o Governo Federal, por meio da lei 6938/81 que trata sobre a Política Nacional do Meio Ambiente, instituiu o Conselho Nacional do Meio Ambiente (CONAMA) como organismo responsável por deliberar as resoluções que guiam o uso, manejo, tratamento e outras disposições sobre os ambientes naturais. O monitoramento ambiental recebe os primeiros parâmetros para análise através da Resolução CONAMA nº 274, de 29 de novembro de 2000, que classifica e define padrões de qualidade para águas de contato primário, com níveis estabelecidos para a balneabilidade. Nesta resolução apenas alguns aspectos físico-químicos e bacteriológicos foram contemplados, porém, em 2005, na resolução CONAMA nº 357, que trata sobre a classificação de corpos hídricos e as condições para lançamento de efluentes, parâmetros mais complexos, como metais totais, metais dissolvidos e substâncias orgânicas receberam valores máximos permitidos para avaliação e controle. Neste momento é percebida a compreensão do CONAMA sobre a necessidade da utilização de organismos e/ou comunidades aquáticas para avaliação das águas por meio de testes ecotoxicológicos e não apenas de testes físico-químicos. As análises convencionais baseadas em características físicas e químicas não são suficientes para atender os múltiplos usos da água, pois são o retrato do ambiente sobre o momento em que foram coletadas, como uma fotografia, e a avaliação mais completa demandaria sequências grandes de coletas, análises e avaliações (Souza, 2015).

A avaliação de ambientes naturais por meio de parâmetros avaliáveis em seres vivos pode fornecer respostas sobre um histórico do ambiente estudado, já que os organismos, sendo expostos ao ambiente, são impactados por situações de descartes e distúrbios eventuais que acontecem naquele meio durante o tempo em que ali habitam, permitindo a avaliação de toxicidade aguda e crônica. Essa característica é importante e inovadora, já que a avaliação da presença, aumento ou diminuição de compostos do metabolismo responsáveis por responder à estressores ambientais nos seres vivos permite inferir o estado do ambiente estudado e como ele impacta os seres que ali habitam (Souza, 2015).

Ainda que o acompanhamento da situação ambiental seja realizado rotineiramente com o uso de técnicas clássicas de coleta de amostras de água e solo e quantificação de compostos presentes por instrumentos analíticos, o biomonitoramento, na esfera da avaliação ambiental de corpos hídricos, complementa a avaliação e é indicado mesmo em situações em que os lançamentos de efluentes e demais descartes sejam realizados em concentrações permitidas na legislação. Os compostos que não são excretados ou metabolizados, como certos pesticidas e metais, podem biomagnificar e passar para um nível superior na cadeia trófica (Miller, Hamann e Kroon, 2020). Esses compostos podem impactar a saúde animal devido os efeitos tóxicos e gerar perigo para a população humana se o ambiente for utilizado como local de pesca/captura para consumo ou recreação (Souza, 2015; Suriano *et al.*, 2011).

Os metais estão naturalmente presentes na natureza, porém ações como a lixiviação natural dos solos e ações antropogênicas podem aumentar a concentração destes a níveis superiores aos valores máximos permitidos nas resoluções (Lollo, 2016). Metais como arsênio (As), cádmio (Cd), cobre (Cu), cromo (Cr), estanho (Sn), chumbo (Pb), molibdênio (Mo), níquel (Ni) e outros são comumente encontrados em níveis de traço em águas superficiais e são conhecidos como metais potencialmente tóxicos (Kumar *et al.*, 2019). Anteriormente chamados de metais pesados, estes elementos são fonte de grande preocupação em toda a sociedade devido ao alto potencial de acumular no ambiente e biomagnificar na rede trófica, podendo chegar a níveis tóxicos e impactar a vida animal e humana no local (Jaishankar *et al.*, 2014).

As principais fontes de aumento de concentração e/ou contaminação ambiental por metais potencialmente tóxicos nos corpos hídricos são os processos naturais que envolvem a precipitação atmosférica, intemperismo geológico e a lixiviação (Lollo, 2016). Porém a maior preocupação consiste no campo dos impactos de origem antropogênica, onde descartes de resíduos e rejeitos de processos produtivos, processos agrícolas e dejetos urbanos sem tratamento adequado podem alterar rapidamente a estrutura de uma comunidade aquática, alterando as frequências populacionais e até inviabilizando a vida no local (Lollo, 2016). Mesmo em níveis considerados seguros, os metais no ambiente podem ocasionar alterações metabólicas em espécies com menores tolerâncias e alterarem padrões de distribuição e dinâmica de uma biota local (Kumar *et al.*, 2019; Zhou *et al.*, 2007; Silveira, 2004).

Segundo Zhou *et al.* (2007), o uso de bioindicadores está condicionado à possibilidade de analisar os efeitos e marcações que as substâncias químicas refletem nos tecidos dos organismos, podendo ser produtos de reação a partir da substância estrangeira que adentra o organismo, mudanças no próo bioindicador ou a presença da substância química no ser. Conforme descrito pelos autores, o bioindicador ideal para a poluição aquática por metais deve conter as seguintes características:

a. Pode acumular altos níveis de poluentes sem morrer.
b. Vive de forma séssil, de forma a representar a poluição de um local.
c. Possui abundância suficiente e ampla distribuição para que seja possível a realização de repetidas coletas e comparações.
d. Possui ciclo de vida longo o suficiente para que seja possível a comparação entre diferentes etapas da vida do ser vivo bioindicador.
e. Possui tecido ou célula alvo viável para estudos futuros variando a habitação do bioindicador em diferentes microcosmos, que são os sistemas de ecossistemas artificiais e simplificados usados para simular e prever o comportamento de ecossistemas naturais sob condições controladas.
f. Fácil coleta e criação em laboratório.
g. Se mantém vivo na água.
h. É possível observar efeito dose/resposta ao poluente (Zhou *et al.*, 2007).

Os bioindicadores para metais em água mais comumente utilizados são o plâncton, insetos, moluscos, peixes, plantas e outros (Gagnon e Rawson, 2017; Stibilij *et al.,* 2014) cada espécie/táxon de bioindicador possui características únicas, atendendo necessidades de análise específicas para diferentes situações. Sendo rigorosa a lista para eleição de bioindicador ideal, o atendimento a alguns dos requisitos já permite o uso do organismo como bioindicador de poluição aquática por metais (ZHOU *et al*, 2007).

Diversas são as técnicas disponíveis para a utilização de organismos como bioindicadores, sendo as mais comuns a verificação de alterações nas dinâmicas populacionais nos organismos expostos às águas poluídas, a determinação de concentração de metais nos tecidos dos organismos bioindicadores, análises de biomarcadores e observações histopatológicas (Freitas *et al.,* 2015; Stibilij *et al.*, 2014; Zhou *et al.*, 2007). Buscando encontrar organismos capazes de bioindicar poluição ambiental por metais, os anfíbios são candidatos por possuírem características específicas como a capacidade de respirar através da pele e a capacidade de poluentes penetrarem no corpo através do tecido epitelial, os tornando viáveis para utilização em diversas técnicas de pesquisa, como a observação morfológica, comportamental, metabólica e de análise de conteúdo total de metais nos tecidos (Zhou *et al.*, 2007).

Diversos estudos com girinos de anfíbios relataram alterações morfológicas (Veronez *et al.*, 2016), fisiológicas (Dal Médico *et al.*, 2014), bioquímicas (Utsunomiya *et al.*, 2022; Fernandes *et al.*, 2021) e comportamentais (Amaral *et al.*, 2017) após períodos de exposição à agentes químicos. Lin *et al.* (2015) ao exporem girinos de rã marrom de Zhenhai (*Rana zhenhaiensis*) à diferentes concentrações de Cu, Pb e Zn, observaram aumento das alterações bioquímicas em biomarcadores sanguíneos, como o aumento da frequência de núcleos de eritrócitos anormais em relação ao grupo controle à medida que as concentrações dos metais e o tempo de exposição aumentavam. Esses conhecimentos adquiridos sugerem potencial de utilização destes animais como bioindicadores de poluição por metais nos ambientes aquáticos (Marcantonio *et al*., 2022; Murthy *et al*, 2022; Lin *et al.*, 2015).

1.1 HIPÓTESE

O Rio Sorocaba está localizado em uma região densamente povoada e recebe descartes de efluentes em várias partes do seu percurso. Desta forma, as hipóteses que este estudo busca avaliar é que girinos de rãs-touro expostos às águas dos percursos finais do rio, do Reservatório de Itupararanga, apresentarão maiores concentrações de MTs e de metais em seu tecido, evidenciando a piora na qualidade de água do Rio Sorocaba no Reservatório de Itupararanga em relação à região de nascentes em Ibiúna, assim como avaliar a diferença na concentração de MTs e de metais nos diferentes órgãos, testando a hipótese de que os órgãos que desempenham funções relacionadas à interface meio externo e interno animal apresentarão maiores quantidades de MTs e metais em relação aos órgãos mais internos do organismo.

1.2 OBJETIVOS

Dessa forma, este estudo tem o objetivo de verificar se a exposição de girinos de rã-touro (*Aquarana catesbeiana* syn. *Lithobates catesbeianus,* Shaw 1802) por 96 horas às águas de dois pontos de coleta do Rio Sorocaba no período de estiagem (seco) é capaz de causar alterações nas concentrações de metais nas brânquias, músculos da cauda e na pele destes animais; verificar se existe alteração na concentração das metalotioneínas (MTs) nos músculos e brânquias; e verificar se existe correlação entre as metalotioneínas e a concentração de metais nos músculos e brânquias dos girinos, utilizando métodos analíticos espectrofotométricos e espectrometria de absorção atômica.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 POLUIÇÃO

A poluição pode ser definida como alteração prejudicial na dinâmica de ambientes, na dinâmica de seres-vivos e ambientes ou impactos a recursos naturais como os solos e a água. em que qualquer dos envolvidos sofram prejuízo ou danos (Owa, 2013). A alteração de características físico-químicas e a inviabilização do uso do solo ou água para o florestamento, agricultura, pesca e/ou usos da água também podem caracterizar poluição ambiental quando prejudica a vida e a comunidade (Owa, 2013; Nass, 2002). A poluição dos corpos hídricos está intimamente ligada ao desenvolvimento das comunidades humanas e ao rápido desenvolvimento industrial, onde a principal causa de alterações ecológicas nos lagos e rios é o descarte indevido de poluentes, lançando substâncias tóxicas, metálicas e/ou orgânicas no ambiente, que podem ocasionar a eutrofização e/ou elevar o nível de toxicidade da água, prejudicando toda comunidade local (Wang e Yang, 2016). No levantamento realizado por Wang e Yang (2016), avaliando o uso da água em diversas comunidades humanas na China os autores demonstraram conexões entre o uso de águas poluídas e o aumento de incidência de doenças veiculadas através da água, juntamente com maiores taxas de ocorrências de doenças carcinogênicas na população humana.

2.1.1 Poluição Por Metais Na Água

A poluição por metais na água pode ter origem em diversos processos produtivos humanos, principalmente em processos de mineração e industriais. Esses processos produzem rejeitos que, quando não tratados corretamente, podem causar grandes impactos no meio ambiente (Sakakibara *et al*, 2011). Alguns metais potencialmente tóxicos, como o Cu, Mn e cromo (Cr), quando em baixas concentrações, são elementos essenciais para os organismos. Outros metais e metalóides como o arsênio (As), mercúrio (Hg), cádmio (Cd) e chumbo (Pb) são altamente tóxicos mesmo em baixas concentrações (Saha, 2016).

A resolução do Conselho Nacional do Meio Ambiente (CONAMA) nº 350, de 17 de março de 2005, fornece a classificação dos corpos de água e as diretrizes para o enquadramento, dividindo as águas doces em 4 classes, com base nas suas necessidades de uso. As águas classificadas como classe 1 ou especial, que possuem uso mais fino como o abastecimento humano após tratamento primário, recreação de contato primário e irrigação de hortaliças que são ingeridas cruas, possuem parâmetros mais exigentes de controle de qualidade, com valores máximos permitidos para metais em concentrações de níveis traço. Rios e lagos que são utilizados para o abastecimento humano após tratamento convencional (floculação, decantação, filtração, desinfecção e fluoretação) em sua maioria são enquadrados nas classes 2 ou 3, onde a classe 3, menos exigente, é a classe de águas destinadas para a irrigação, pesca amadora, recreação de contato secundário e dessedentação de animais, além do abastecimento humano após tratamento convencional ou avançado.

Os valores máximos permitidos para parte dos metais na Resolução CONAMA 357/2005, para águas de classe 2 envolvidos nesta pesquisa são apresentados na tabela 1. Os valores máximos permitidos de concentração para metais e metaloides totais são a soma da carga de metais solubilizada e insolubilizada presente no ambiente (Ribeiro, 2012).

Tabela 1 – Valores máximos permitidos para parte dos metais na Resolução do Conselho Nacional do Meio Ambiente (CONAMA) 357/2005, para águas de classe 2

METAIS	VALOR MÁXIMO PERMITIDO (VMP)
Arsênio Total	0,01 mg/L
Bário Total	0,7 mg/L

Cádmio Total	0,001 mg/L
Chumbo Total	0,01 mg/L
Cobalto Total	0,05 mg/L
Cobre Dissolvido	0,009 mg/L
Cromo Total	0,05 mg/L
Manganês Total	0,1 mg/L
Níquel Total	0,025 mg/L
Zinco Total	0,18 mg/L

Fonte: Adaptado de CONAMA nº 357, de 17 de março de 2005.

Com o curso de água se iniciando na cidade de Ibiúna, o Rio Sorocaba é o principal curso d’água da Bacia do Alto Sorocaba, e é formado pela união dos rios Una, Sorocabuçu e Sorocamirim (Fernandes *et al.*, 2021). Passando por 9 cidades da região sudeste do Estado de São Paulo e terminando o curso represado no reservatório de Itupararanga, as águas da Bacia do Alto Sorocaba enfrentam diversos lançamentos de descartes domésticos e industriais, principalmente de industrias produtoras de cimento, agropecuárias e outras, até a chegada ao reservatório final. Diversas pesquisas investigaram a presença de metais no Rio Sorocaba (Fernandes *et al.,* 2021; Conceição *et al*., 2016; Pedrazzi *et al.,* 2014; Fernandes, 2012; Conceição *et al*., 2011), mas ainda faltam estudos sobre a análise de metais nestas águas por meio de bioindicadores. Por exemplo, no trabalho de Castro (2002), com as espécies de peixes cascudo (*Hypostomus margaritifir*), bagre (*Rhamdia sp.*), mandi (*Iheringichtys labrosus*) e outras espécies coletadas do Rio Sorocaba, foram registradas a presença de cromo, níquel e mercúrio em amostras de músculo da região lombar destes animais.

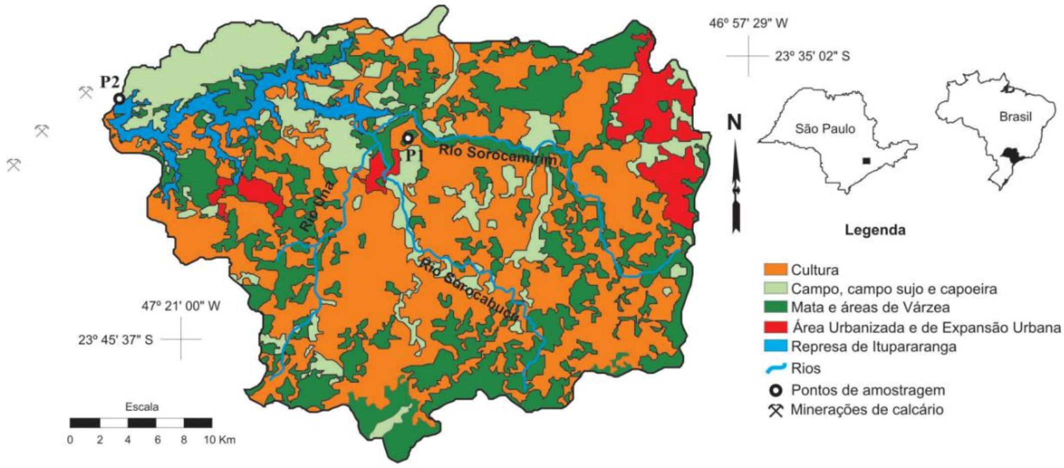
2.2 CARACTERIZAÇÃO DO RIO SOROCABA

Formado pela união das cabeceiras dos rios Sorocamirim, Sorocabuçu e Una nos munícipios Ibiúna, Cotia, Vargem Grande Paulista e São Roque, o Rio Sorocaba é um importante manancial que abastece cerca de um milhão de pessoas nos municípios de Ibiúna, Sorocaba, Mairinque e Votorantim e está a montante do Reservatório de Itupararanga (Conceição *et al.*, 2015).

Os rios Sorocamirim, Sorocabuçu e Una formam a bacia do Alto Sorocaba, que é uma das seis sub-bacias que formam a bacia do Sorocaba médio Tietê. Este rio percorre cerca de 71km^2 de área urbanizada, 35 km de área utilizada por chácaras e 393 km^2 de área utilizada para agricultura, com intensa atividade agrícola. Está situada na região sudeste do Estado de São Paulo (Brasil) (23°45’37’’ e 23°35’02’’de latitude S e 47°21’00’’ e 46°57’29’’ de longitude W) (Conceição *et al.*, 2015; Conceição *et al.*, 2011).

Um dos pontos iniciais de formação do Rio Sorocaba está localizado na região da cidade de Ibiúna, e um ponto de grande interesse é o Reservatório de Itupararanga, onde o rio fica represado com fins de abastecer a população da região de Sorocaba (Sorocaba, Ibiúna, Votorantim, São Roque e outras cidades vizinhas), estando no caminho entre estes dois pontos indústrias, fábricas de mineração e cimento, áreas de atividades agrícolas e regiões urbanizadas (Conceição *et al.*, 2011).

Figura 1 – Mapa de localização dos pontos de coleta Ibiúna (PI) e Reservatório de Itupararanga (PIR) do Rio Sorocaba e identificação de uso e ocupação de solo da bacia do Alto Sorocaba. Itupararanga.



Fonte: Adaptado de Conceição *et al.*, 2011.

Em relação a este rio, destaca-se o trabalho realizado por Fernandes (2012) em 20 amostras de água coletadas do Rio Sorocaba, no período de março de 2009 a novembro de 2010. Em uma das nascentes localizada no planalto de Ibiúna (23°45’37’’ e 23°35’02’’ S e 47°21’00’’ e 46°57’29’’ W), dentre os 4 metais dissolvidos avaliados (Fe, Al, Mn e Sr) o Fe se apresentou em maior concentração (1,70 mg L^{-1}), seguido pelo Al (0,17 mg L^{-1}), Sr (0,05 mg L^{-1}) e Mn (0,04 mg L^{-1}). Enquanto na cidade de Votorantim (23º32’41” S, 47º26’47” O), localizada a jusante do Reservatório de Itupararanga, o rio apresentou maior concentração de Fe (0,63 mg

L^{-1}), seguido do Mn (0,12 mg L^{-1}), Al (0,097 mg L^{-1}) e Sr (0,05 mg L^{-1}). Neste ponto o autor descartou a influência urbana, pois o local de coleta em Votorantim estava a menos de 500 metros de onde o rio adentra a cidade. Avaliando o relatório CETESB de águas interiores de 2019, ano em que as amostras desta pesquisa foram coletadas, foi verificado que o índice de qualidade de água do Reservatório de Itupararanga foi classificado como regular, devido ao aumento nas concentrações de fósforo e clorofila *a* na água. Esse índice demonstra uma piora na qualidade de água em relação ao ano anterior, 2018, classificado como qualidade boa.

Outro estudo realizado por Fernandes *et al.* (2021) evidenciou características mais atuais dos dois pontos de coleta no período de estiagem (na região próxima à nascente do Rio Sorocaba em Ibiúna e na Represa de Itupararanga). Os autores registraram a presença de Al, Cu, Mn e Zn nos dois pontos do rio e no sedimento e, mesmo em níveis de concentração considerados seguros pela resolução CONAMA 454/12, foi observado alteração em enzimas relacionadas ao estresse oxidativo em girinos de rãs-touro, indicando que estes efeitos foram provocados pela exposição ao rio Sorocaba. Segundo Moraes (2016) os metais nos sedimentos em suspensão podem indicar a erosão mecânica nas bacias ou evidenciar o descarte de efluentes no percurso dos rios.

Assim, a presença dos metais pode trazer risco à comunidade local, já que podem acumular em diversos órgãos dos animais e biomagnificar para níveis superiores das cadeias tróficas (Miller, Hamann e Kroon, 2020).

Nessa perspectiva, essa dissertação vai apresentar a análise de metais nos órgãos de girinos de rãs-touro e a concentração da metalotioneína após exposição as águas do Rio Sorocaba. Para este trabalho, os pontos de coleta de água são os mesmos dos utilizados por Fernandes *et al.* (2020) em sua pesquisa e representa uma continuidade deste estudo e de Fernandes *et al.* (2021) e Utsunomiya *et al*., (2022).

2.3 BIOMARCADORES

Os biomarcadores podem ser definidos como sendo os compostos bioquímicos ou as alterações nos tecidos que indicam alteração na fisiologia do animal e podem indicar a presença de um agente externo causador das alterações (Hook *et al*, 2014). Os biomarcadores são divididos em 3 classes: biomarcadores de exposição, efeito ou susceptibilidade (Hughes, 2006).

2.3.1 Biomarcadores de Exposição

Os biomarcadores de exposição (ou dose interna) representam respostas rápidas à exposição do animal ao composto tóxico por surgimento de respostas bioquímicas ou alterações na fisiologia do animal (Van Der Oost *et al.*, 2003). Como exemplos podemos citar a citocromo P450 1A que foi induzida na espécie de coral *Porites lobata* na presença de óleo derramado por navios (Hook, 2014), após o desastre ambiental ocorrido na Micronésia, conjunto de ilhas na Oceania, em 2002 (Downs *et al.*, 2006). Esta enzima está envolvida em processos de biotransformação de xenobióticos, tornando-os mais solúveis e fáceis de serem excretados pelo organismo. Outro exemplo é a metalotioneína (MT), proteína que se liga aos metais para manutenção da homeostase dos metais essenciais e desta forma também atua como um sistema protetor do organismo contra o aumento de metais e estresse oxidativo. As MTs são classificadas como biomarcadores de exposição para metais, já que são expressas em maior quantidade quando os animais são expostos a quantidades maiores de metais, agindo de forma a desintoxicar o organismo (Fernandes *et al*., 2021; Movahedinia *et al*, 2017; Hook *et al*, 2014).

2.3.2 Biomarcadores de Resposta ou Efeito

Os biomarcadores de resposta ou efeito refletem a interação dos compostos químicos com receptores celulares, resultando em alteração bioquímica ou morfológica (Van Der Oost *et al.*, 2003), sendo uma resposta direta ao efeito do contaminante, como dano genético ou inibição da acetilcolinesterase (Hook *et al.*, 2014). Por fundamento, a presença de um biomarcador de resposta mostra prejuízo à saúde ou bem estar animal devido a presença do contaminante, porém esta associação pode ser difícil de demonstrar quantitativamente (Hook *et al.*, 2014).

2.3.3 Biomarcadores de susceptibilidade

Biomarcadores de susceptibilidade são indicadores de sensibilidade aos efeitos de um agente químico ou tóxico específico, que podem indicar indivíduos ou populações que estão suscetíveis a desenvolver certas condições (Califf , 2018; Van Der Oost *et al.*, 2003) e envolvem fatores genéticos e alterações em receptores celulares (Van Der Oost *et al.*, 2003). Estes biomarcadores podem indicar as variações na susceptibilidade de um indivíduo ou população em desenvolver patologias, como no caso de altos níveis do colesterol LDL (*low density* *lipoprotein* – lipoproteína de baixa densidade em tradução livre) e que está ligada a altas probabilidades de doenças cardíacas (Califf, 2018; Hook *et al.*, 2018).

2.3.4 Biomarcadores para Metais

A poluição nos corpos hídricos está frequentemente ligada aos descartes urbanos e de industrias de mineração ao longo do percurso dos rios. As avaliações de qualidade de água utilizando parâmetros biológicos frequentemente utilizam peixes, como a carpa (*Cyprinus carpio*) (Zhou, 2007) e o peixe-zebra (*Danio rerio*) (Udiba, 2014). Crustáceos e macroinvertebrados também são utilizados em avaliações de biomonitoramento ambiental, possuindo vantagens em relação ao tempo menor de vida e alta resposta a alterações ambientais (Zhou, 2007). Os macroinvertebrados bentônicos apresentam uma vantagem de permanecerem fixos no ambiente e representar com maior precisão a situação da região de coleta (Souza, 2015). No que se refere aos anfíbios, a utilização de girinos de anuros para biomonitoramento ambiental está em expansão, já que possuem características únicas de pele semipermeável a diversos compostos, parte da vida em ambiente aquático e possibilidade de criação e manutenção em ambiente controlado de laboratório. A utilização destes animais no biomonitoramento ambiental é realizada por meio da avaliação de biomarcadores (Hook *et al.*, 2014), como a avaliação das enzimas colinesterase (ChE) (Fernandes *et al*., 2021), enzimas antioxidantes (Veronez *et al.*, 2016, Boiarski *et al.*, 2020), proteases e lactato desidrogenase (Pinto-Vidal *et al.*, 2022).

A quantificação molecular dos metais presentes nas proteínas dos animais utilizando instrumentos como espectrômetros de absorção atômica, emissão ótica e de massas também é frequentemente utilizada (Lin *et al.*, 2015). Este método de avaliação está bem estabelecido (Aurelio e Ramiro, 2020; Jofré, Antón e Vidal, 2012) e diferentes órgãos com diferentes funções podem ser analisados a fim de serem obtidas informações relevantes para pesquisa, como a análise das brânquias e pele de girinos de anfíbios, que formam os locais onde acontece a maior parte das trocas gasosas (Brunelli, Perrotta, Tripepi, 2004). A avaliação do comportamento e do desenvolvimento corporal também podem serem utilizadas como biomarcadores (Kadim e Risjani, 2022; Udiba *et al*., 2014).

A avaliação da concentração de proteínas do tipo das metalotioneínas, que são as proteínas que se ligam aos metais nos casos de exposição à ambientes com concentrações elevadas destes elementos, já é bem estabelecida em peixes (Sakuragui *et al*., 2013; Olsvik *et* *al.*, 2000; Schlenk *et al.*, 1995; Hylland *et al.*, 1992; Olsson *et al.*, 1986), bivalves e poliquetas (Hook *et al.*, 2014). As MTs possuem a característica de se ligar à moléculas de metais por meio do grupo tiol (-SH, sulfidrila) devido a grande quantidade de aminoácidos Cys na sua estrutura (Kadim e Risjani, 2022; Ahmad *et al*., 2014; Viarengo *et al*., 1997). A indução dessas proteínas depende da quantidade de metais potencialmente tóxicos e da concentração de glicocorticoides no corpo do animal (Viarengo *et al*., 1997). As maiores concentrações destas proteínas são encontradas no fígado e nos rins dos animais (Movahedinia *et al.*, 2017) e como já descrito anteriormente a ligação das MTs aos metais impede a ligação destes com outras proteínas e estruturas, prevenindo efeitos tóxicos e adversos, dessa forma esta proteína atua no controle de concentrações de Zn e Cu, mantendo a homeostase dos metais e em processos de desintoxicação de metais como Cd e Hg (Kadim e Risjani, 2022; Fernandes *et al*., 2021; Hayati, 2017; Ahmad *et al*., 2014).

3 ARTIGO 1

Metals in skin of bullfrog tadpoles, *Aquarana catesbeiana* (Shaw, 1802), exposed to the waters of the Sorocaba River

Metais na pele de girinos de rãs-touro, *Aquarana catesbeiana* (Shaw, 1802), expostos às águas do Rio Sorocaba

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Summary: Aim: In this study, the recent bibliography on the use of the bullfrog species (*Aquarana catesbeiana*, Shaw, 1802) as an environmental bioindicator of pollution was verified, and the total concentration of metals in the skin of bullfrog tadpoles exposed to the waters of the Sorocaba River was evaluated in order to analyze the potential use of the tissue as a tool for environmental biomonitoring and to verify the disponibility of metals in the Sorocaba River to animals. **Methods:** The recent bibliography was evaluated through the Scopus and Pubmed platforms using the terms ¨*Lithobates catesbeianus* biomarkers¨ and ¨*Rana catesbeiana* biomarkers¨. Tadpoles were exposed for 96 hours to the waters of 2 points of the

Sorocaba River, with the point Ibiúna (PI) representing the source of the river Sorocaba and the point Ituparanga Reservoir (PIR) representing an important point of water supply to region of the city of Sorocaba-SP and using contaminant-free deionized water as a control. The evaluation of the concentration of metals Ba, Cu, Mn, Sr and Zn in skin samples (n=30) was carried out after digestion of the samples in a closed system using HNO_3 and HCl and determination in Microwave Plasma Atomic Emission Spectrometer (MP-AES). **Results:** The review of bibliographical production over the last 10 years resulted in 35 articles on the topic, where metals were the second most studied contaminant using this species (around 34% of articles), behind only agricultural pesticides (around 43% of articles). The concentration of Ba and Zn metals in the group PI varied in relation to the control group, and the concentration of Mn in the PIR group varied in relation to the control group and in relation to the group exposed to the waters from group PI. For the metals Cu and Sr there was no significant variation between the groups. **Conclusions:** The existing literature demonstrates the ability to use various tissues from bullfrog tadpoles as environmental bioindicators. An increase in Ba (12%) and Mn (54%) was identified in the PIR group in relation to the PI group in the tadpoles' skin. For the metals Cu, Sr and Zn there was no difference between the groups. As, Cd, Co, Mo, Ni and Pb had results below the limit of quantification in the groups studied. The increased concentration of Mn in the skin of bullfrog tadpoles exposed to the waters of the PIR in relation to the group PI may indicate the accumulation of this metal, pointing to a worsening of water quality in relation to the initial point of the river's course.

Keywords: Environmental monitoring; metallic pollution; amphibians.

Resumo: Objetivo: Neste estudo foi avaliada a bibliografia recente sobre o uso da espécie de rã-touro *Aquarana catesbeiana* (Shaw, 1802) como bioindicador ambiental e a concentração total de metais na pele de girinos de rã-touro expostos às águas do rio Sorocaba, a fim de avaliar o potencial uso do tecido como ferramenta de biomonitoramento ambiental e a disponibilidade de metais no Rio Sorocaba para os animais. **Métodos:** A bibliografia recente foi avaliada através das plataformas *Scopus* e *Pubmed* utilizando os termos ¨*Lithobates catesbeianus biomarkers*¨ e ¨*Rana catesbeiana biomarkers*¨. Os girinos foram expostos por 96 horas às águas de 2 pontos do Rio Sorocaba, sendo o ponto Ibiúna (PI) representando a nascente do Rio Sorocaba e o ponto Represa de Itupararanga (PIR) representando um importante ponto de abastecimento de água para região da cidade de Sorocaba-SP. Foi utilizada água deionizada livre de contaminantes como controle. A avaliação da concentração dos metais Ba, Cu, Mn, Sr e Zn nas amostras de pele (n=30) foi realizada após digestão das amostras em sistema fechado utilizando HN_3 e HCl e determinação em Espectrômetro de Emissão Atômica por Plasma de Microondas (MP-AES). **Resultados:** A revisão da produção bibliográfica dos últimos 10 anos resultou em 35 artigos sobre o tema, onde os metais foram o segundo contaminante mais estudado utilizando esta espécie (cerca de 34% dos artigos), atrás apenas dos defensivos agrícolas (cerca de 43% dos artigos). A concentração dos metais Ba e Zn no grupo PI variou em relação ao grupo controle, e a concentração de Mn no grupo PIR variou em relação ao grupo controle e em relação ao grupo exposto às águas do grupo PI. Para os metais Cu e Sr não houve variação significativa entre os grupos. **Conclusões:** A bibliografia existente demonstra a capacidade de utilização de diversos tecidos de girinos de rã-touro como bioindicadores ambientais. Foi identificado o aumento de Ba (12%) e Mn (54%) no grupo PIR em relação ao grupo PI na pele dos girinos. Para os metais Cu, Sr e Zn não houve diferença entre os grupos. As, Cd, Co, Mo, Ni e Pb tiveram resultados abaixo do limite de quantificação nos grupos estudados. O aumento da concentração de Mn na pele dos girinos de rã-touro expostos às águas do reservatório de Itupararanga em relação ao grupo exposto às águas de Ibiúna pode indicar o acúmulo deste, apontando para piora na qualidade da água em relação ao ponto inicial do curso do rio.

Palavras-chave: Monitoramento ambiental; poluição metálica; anfíbios.

1 Introduction

Fresh water bodies are essential for the functioning of human and animal communities, as well as for supplying the productive processes that enable the operations of cities (Frascarelli *et al*, 2016). Access to quality water is essential for a dignified life and is a toll for disease prevention, as the disposal of emissions into water without proper care and treatment can lead to increased water toxicity due to the accumulation of metals and other pollutants, causing harm to both the local fauna and flora, and the population that uses the water (Wang; Yang, 2016). Biological processes, species or communities can be used to evaluate the quality of the environment and the changes that occur in the location over time, through the evaluation of physical or chemical changes in parameters found in animals exposed to the environment and even monitoring the changes in the diversity of local fauna and flora (Holt; Miller, 2011).

As described by Zhou *et al*. (2007), the use of bioindicators is conditioned by the possibility of analyzing the effects and markings that chemical substances reflect on the tissues of organisms, which may be reaction products from the foreign substance that enters the organism, changes in the bioindicator itself or the presence of the chemical substance itself in being. The most commonly used bioindicators for metals in water are plankton, insects, molluscs, fish, plants and others (Gagnow and Rawson, 2017; Stibilij *et al*., 2014). Each bioindicator species/taxon has unique characteristics, meeting analysis needs specific to different situations. There are several techniques available for using organisms as bioindicators, the most common being the verification of changes in population dynamics in organisms exposed to polluted waters, the determination of metal concentrations in the tissues of bioindicator organisms, biomarker analyzes and histopathological observations (Freitas *et al*., 2015; Stibilij *et al*., 2014; Zhou *et al*., 2007). Seeking to find organisms capable of bioindicating environmental pollution by metals, amphibians are candidates because they have specific characteristics such as the ability to breathe through their skin and the ability for pollutants to penetrate the body through epithelial tissue, making them viable for use in various research techniques, such as morphological, behavioral, metabolic observation and analysis of total metal or pollutant content in tissues (Fernandes *et al*, 2021; Prokic *et al*., 2016; Zhou *et al*., 2007).

Due to their low mobility characteristics and part of the amphibian larval cycle taking place in an aquatic environment, these animals are vulnerable to discharges and pollutants that can occur in rivers and lakes, and knowledge about amphibian species from tropical and subtropical regions is still limited, which may affect the protection of species at greater risk of extinction. As stated by Fernández and Guillén (2021), bibliographic review articles are tools for evaluating recent existing knowledge on a given subject, which seek to identify problems, gaps or find answers about characteristics of the scientific knowledge already produced, being a fundamental tool for advancement in various fields of knowledge. Therefore, the search for work produced in the last 10 years on the use of bullfrogs as environmental bioindicators is essential to evaluate the prediction of biomonitoring work using these animals (Fernandes *et al*., 2021; Vidal *et al*., 2021; Ossana *et al*., 2017).

This work aims to evaluate the recent bibliography on the use of *Lithobates catesbeianus* syn. *Rana catesbeiana* species as bioindicators of exposure to contaminants and the concentration of metals present in the skin of bullfrog tadpoles exposed to the waters of two points of the Sorocaba River for 96 hours, with the aim of evaluating the feasibility of using this tissue as a tool for metal biomonitoring in water. The research seeks to analyze the hypothesis of tadpoles that were exposed to the waters of the Itupararanga Reservoir, representing an important water accumulation point for supplying the population of the region of the city of Sorocaba, would present higher concentrations of metals in their skin compared to tadpoles exposed to water collected in the city of Ibiúna, representing the source of the river and compared to the control group, which would demonstrate the worsening of the water quality of the Sorocaba River along its route.

2 Material and Methods

2.1 Literature review

Through the key text search systems of the main international scientific article platforms, the available scientific literature published in the last 10 years was evaluated by searching for the text ¨*Lithobates catesbeianus* biomarkers¨ and ¨*Rana catesbeiana* biomarkers¨ in PubMed and Scopus platforms, and scientific papers were categorized by contaminant, publication year and country of origin. Scientific articles outside the topic of exposure to contaminants for environmental monitoring studies or ecotoxicology were excluded.

2.2 Study area

The Sorocaba River is a river of great importance for the cities of Votorantim and the entire Metropolitan Region of the city of Sorocaba, in the State of São Paulo, as it feeds the Itupararanga Reservoir, which supplies water to the population of this region, comprised of around 1 million people and marks an environmental protection area (Oliveira and Amaral, 2022). Over 929 km^2 in length, the Sorocaba River crosses several areas where it suffers great anthropogenic influence on its banks, such as large planting areas and factories in the cement and mining areas, which can result in the transport of various agricultural defensives compounds and mining waste from the land to the river, whether through leaching or waste disposal (Oliveira and Amaral, 2022; Fernandes *et al*., 2021; Conceição *et al*., 2011). The Sorocaba River is formed by the union of the headwaters of the Una, Sorocabuçu and Sorocamirim Rivers in the cities of Cotia, Vargem Grande Paulista and São Roque and accumulates in the Itupararanga Reservoir, which is managed by Companhia Brasileira de Alumínio (CBA) (Oliveira and Amaral, 2022; Fernandes et al., 2021).

The water sample collection procedure was carried out using 50 liters capacity galons, 120 liters of water were collected from the Sorocaba River in the dry season of the year (August, 2019), near to the city of Ibiúna (PI sample point) (23º27'27" South latitude – 47º24'10.1" West longitude), representing the source of the Sorocaba River. The Itupararanga Reservoir (PIR sample point) (23º38'11.3" South latitude - 47º13'22.6" West longitude) was chosen as the collection point due to the distance between the two points and the importance in water supply for the regional population.

2.3 *Animal collection*

As described by Fernandes *et al.,* (2021), Bullfrog tadpoles *Rana catesbeiana* (syn. *Lithobates catesbeianus*) were obtained from Santa Rosa Frog Farm (Santa Barbara d'Oeste, SP, Brazil) (22º46'53.0"S/47º24'17.7"W). The animals (n=30) in stage 25 of Gosner (1980) were maintained in 80L tanks of dechlorinated water for 10 days, using a continuous flow of water (1.2 $L.h^{-1}$), continuous aeration (6 mg $O_2.L^{-1}$) and photoperiod 12 hours light, 12 hours dark, for acclimatization purposes before being exposed to the waters of the Sorocaba River. The animals were fed with commercial food (40% protein) and feeding was stopped 24 hours before exposure. The animals were divided into a control group (C), a group exposed to the waters of the city of Ibiúna (PI) and a group exposed to the waters of the Itupararanga Reservoir (PIR) through the random transfer of 12 animals to each group in 16-liter aquariums. The animals were exposed to water for 96 hours in static conditions, and the characteristics of the water were monitored: pH (7.2 to 7.6), dissolved oxygen (7.0 to 7.5 $mg.L^{-1}$), hardness (50 to 58 mg $CaCO_3$ $mg.L^{-1}$), conductivity 56-97 ± 0.02 µS/cm and ammonia concentration < 1 $mg.L^{-1}$. The experiment was conducted in triplicate.

After the end of the exposure period, the animals were anesthetized with 0.1% benzocaine and euthanized by cranial concussion, as recommended by the American Veterinary Medical Association (AVMA, 2001). The skins were separated and frozen in a biofreezer at -80ºC until analysis were carried out. The procedures were realized in accordance with the standards established by the American Society for Testing and Materials (ASTM, 2000) and were approved by the ethics committee of the Federal University of São Carlos (CEUA-UFSCar) through process #2578040219/2019.

2.4 Sample preparation

Arsenic (As), barium (Ba), cadmium (Cd), lead (Pb), cobalt (Co), copper (Cu), nickel (Ni), manganese (Mn), molybdenum (Mo), strontium (Sr) and zinc (Zn) were verified in samples of skin from bullfrog tadpoles. The samples were dried in a laboratory oven at 60ºC for 7 days, in order to eliminate moisture and determine total dry mass. This procedure results in a significant reduction in tissue mass. Therefore, skin samples from each group (control, PIR and PI) were combined to obtain a representative mass (Correa et al., 2016; Laurin et al., 2019). For this purpose, the samples were weighed at 0.15 g (dry mass).

Sample preparation was carried out using the procedure for digestion of fish tissues recommended by the manufacturer of the Anton Paar® Multi Wave Pro microwave digestion oven. The preparation consists of acid digestion of samples in a closed system through programming: Temp/Power: 650/1400, Ramp: 5/10 Hold: 10/10/15 Fan: 1/1/3, using 6 ml of 65% nitric acid (HNO3) and 1 ml of 32% hydrochloric acid (HCl) analytical grade per sample. After 1 hour of digestion in a microwave oven, the samples were digested and increased to 25 ml in a glass volumetric flask with a 5% HNO_3 solution, which consists of 50 ml of HNO_3 in 950 ml of ultrapure water. For the blank, 6 ml of HNO_3 was used with 1 ml of HCl, subjected to the digestion process described and subsequently increased to 25 ml with 5% HNO_3 solution in a volumetric flask.

2.5 Total metal concentration analysis

The microwave-induced plasma atomic emission spectrometry technique was used, where an Agilent MP-AES 4200 instrument with cyclonic nebulizer was operated to evaluate the concentration of As, Ba, Cd, Co, Cu, Pb, Mn, Mo , Ni, Sr and Zn. The results obtained in $\mu g.ml^{-1}$ calculated automatically by *Agilent MP Expert Software* were converted to $\mu g.g^{-1}$ with the aim of relating the sample mass to the concentration obtained through the following equation.

$$\frac{[Sample\ conc.-blank\ average\ conc.(\mu g.ml^{-1})]*\ vollumetric\ flask\ volumn\ (ml)}{Sample\ mass\ (g)} \qquad \text{Eq. 1}$$

The values obtained were expressed as mean ± standard deviation, and the results were compared between groups using the Student's T test, with the significance level of $p<0.05$ using *SigmaStat for Windows version 3.5, Copyright© 2006 Systat Software, Inc*.

3 Results and Discussion

3.1 Literature review

National and international scientific literature produced in the last 10 years reveals that there is interest in using the species *Lithobates catesbeianus* as a tool for environmental biomonitoring, as searches for the term ¨*Lithobates catesbeianus* biomarkers¨ and ¨*Rana catesbeiana* biomarkers" on the *PubMed* and *Scopus* platforms reveals bibliographies published every year since 2017. 36 works published in the last decade on the topic of biomarkers for environmental monitoring purposes using this species were verified. 15 works were excluded from the survey after critical analysis, due to the use of this species for other purposes that are beyond the scope of this research. It was verified that the existing bibliography on the topic is limited and can demonstrate that there may be a shortage of knowledge and also opportunities to be discovered.

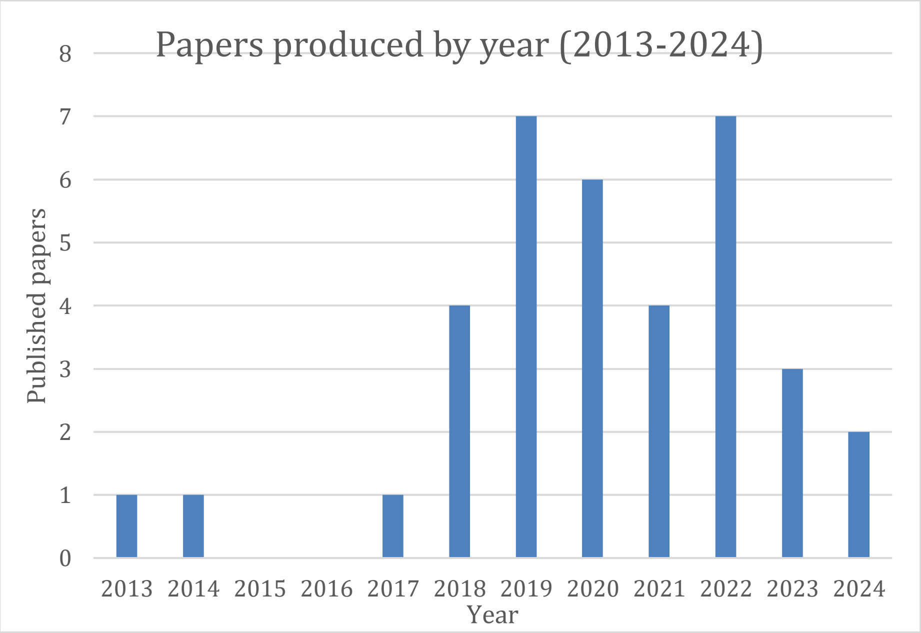

Figure 1. Number of papers published in the last decade with the term ¨*Lithobates catesbeianus* biomarkers¨ and ¨*Rana catesbeiana* biomarkers¨ in PubMed and Scopus platforms. 36 papers were found. 15 papers were excluded from the survey by topic avoidance. Source: From authors.

Of the 36 works evaluated on the topic, 2 articles originate in Argentina (Ossana *et al*., 2017; 2013), 1 paper originated from Canada (Jackman *et al.*, 2018), 1 paper is originated from Mexico (Péres-Alvarez *et al*., 2018), and 32 scientific articles from Brazil. These data reveal that the largest scientific production in the world on the topic of using *Lithobates catesbeianus* tadpoles as tools for aquatic biomonitoring is found in Latin America and demonstrates a research behavior that diverges from most other topics sought, which generally have greater production in countries such as China, the United States of America and India, countries that generally use species such as *Rana zenhaiensis* (Lin *et al*., 2015), *Bufo gargarizans* (Yao *et al*., 2019; Zhao et al., 2020), *Xenopus laevis* (Yologlu and Ozmen., 2015) and others for amphibian biomonitoring studies.

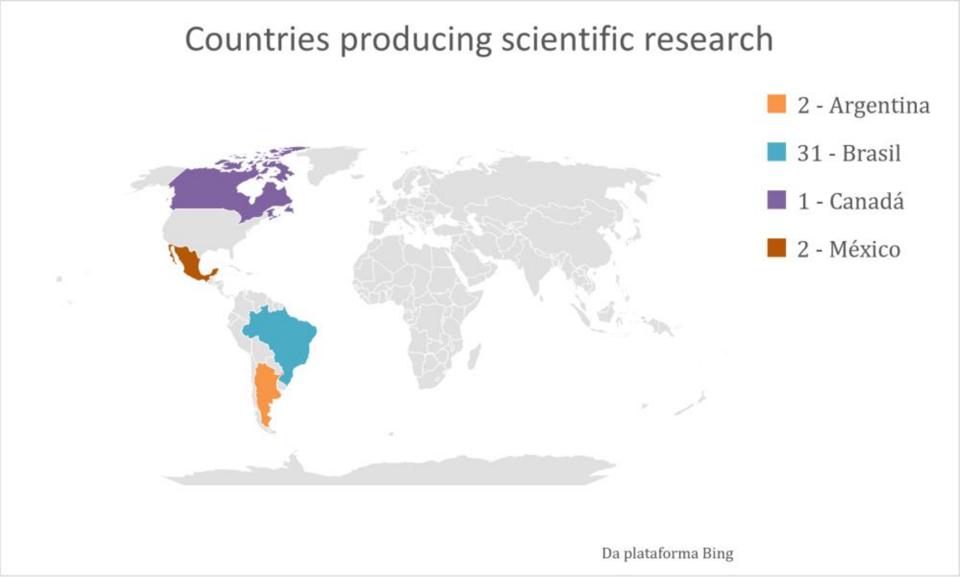

Figure 2. Scheme illustrating countries that had produced scientific papers about the theme ¨*Aquarana catesbeiana* biomarkers¨. Source: From authors. The ilustration was created in bing.com.

As can be seen in figure 3, the majority of scientific articles that evaluated biomarkers in bullfrog tadpoles addressed the effects of exposure to agricultural pesticides in general (41.7%), such as herbicides (Dornelles and Oliveira, 2014; Grott *et al*., 2021; Wilkens *et al*., 2019), fungicides (Marcantonio *et al.*, 2022) and insecticides (Amaral *et al*., 2018; Nimet *et al*., 2021; Santos *et al*., 2021). Another relevant portion of work with bullfrog tadpoles was exposure to metals, where 36.1% of the articles address this topic (Fernandes *et al*., 2021; Motta *et al*., 2023; Vital *et al*., 2021). The exposition of animals to pharmacy drugs were carried out with the aim of identifying the effects that medicines can cause in the environment, coming through treated and untreated sewage networks, and consists of 16.7% of the studies found (Amaral *et al*., 2019;

Gregorio *et al*., 2019; Jackman *et al*., 2018). The smallest portion (5.6%) of studies found deals with the effects of surfactants on aquatic organisms through the exposure of bullfrog tadpoles to these components (Costa *et al*., 2018; Scaia *et al*., 2019).

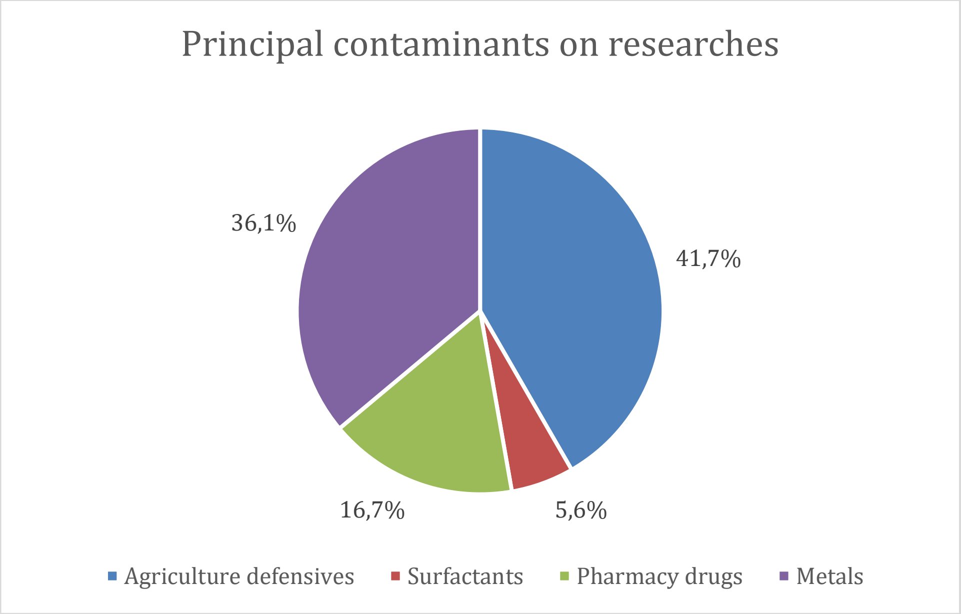

Figure 3. Scheme illustrating the proportions of principal contaminants on researches with *Aquarana catesbeiana* syn. *Lithobates catesbeianus* species. Source: From authors.

In all cases, the exposure of tadpoles to contaminants altered the physiological levels of the biomarkers analyzed in the exposed group in relation to the control group to some degree, suggesting that the species can be used as an environmental bioindicator. No articles that evaluated the skin of this species as a matrix for biomonitoring studies were found, but reports of the successful use of edible-frog skin (*Pelophylax esculentus*) (Prokic *et al*., 2016) as a tool for biomonitoring pollution of metals in water were examined.

Evaluating the articles that worked with the exposure of bullfrog tadpoles to metallic contaminants, it was found that the most used tissue to evaluate biomarkers was the liver, being used in 44.4% of researches. Next, the most used tissues in research were the kidneys (22.2%) and caudal muscle (22.2%), brain (5.55%) and assessments of the animal's entire body (5.55%). The majority of the work was carried out by exposing animals to metallic solutions of know concentration (66.7% of the work). Other studies used the method of collecting contaminated water from rivers and lakes, exposing the animals and, after a determined period of time, evaluating the effects on biomarkers. In 100% of the works evaluated, exposure to metals caused changes of some level in biomarkers evaluated by each author.

3.2 Metal concentration in skin

Calibration curves with good linearity were obtained ($r^2 > 0.99$) for all analytes. The detection and quantification limits can be seen in table 1.

Elements	Limit of Detection (LOD)	Limit of Quantification (LOQ)
As	64.35 mg.L^{-1}	214.48 mg.L^{-1}
Ba	0.33 mg.L^{-1}	1.16 mg.L^{-1}
Cd	0.58 mg.L^{-1}	1.93 mg.L^{-1}
Co	0.47 mg.L^{-1}	1.55 mg.L^{-1}
Cu	0.17 mg.L^{-1}	0.53 mg.L^{-1}
Pb	5.67 mg.L^{-1}	185.58 mg.L^{-1}
Mn	0.033 mg.L^{-1}	0.10 mg.L^{-1}
Mo	0.033 mg.L^{-1}	0.13 mg.L^{-1}
Ni	0.57 mg.L^{-1}	1.90 mg.L^{-1}
Sr	0.033 mg.L^{-1}	0.10 mg.L^{-1}
Zn	0.23 mg.L^{-1}	0.78 mg.L^{-1}

Table 1. Shows the limit of detection and limit of quantification defined for each element in this study. Source: From authors.

The metal concentration for skin samples from bullfrog tadpoles (*Aquarana catesbeiana syn. Lithobates catesbeianus*) can be seen in table 2.

(µg.g^{-1})	**SKIN**		
	Control	**PI**	**PIR**
Ba	122.27 ± 0.79	133.21 ± 1.13	↑**137.02 ± 0.78** (12.1%)
Cu	2.18 ± 0.08	2.22 ± 0.02	2.25 ± 0.08
Mn	8.73 ± 0.10	8.88 ± 0.08	↑**13.48 ± 0.01*** (54%)
Sr	5.67 ± 0.10	50.33 ± 0.18	53.16 ± 0.34
Zn	574.96 ± 6.21	↓**520.28 ± 1.82** (10.5%)	↓**515.12 ± 4.89** (11.7%)

Table 2 - Metal concentration (μg.g^{-1}) in the skin of bullfrog tadpoles. The results are expressed in the format of mean ± standard error. Group control; PI: Ibiúna sample point. PIR: Itupararanga Reservoir sample point. In bold means difference in relation to the control and * means significant difference in relation to the points. N=10 per group. As, Cd, Co, Mo, Ni e Pb were below the limit of quantification. Source: from the authors.

As can be seen in table 1, skin samples from the group PIR showed a concentration of Mn 54% higher than the group PI. The PIR group had a 12.1% higher concentration of Ba than the control group, but there was no significant difference between the two sample points (PI x PIR). The Zn concentration in both PI and PIR groups was significantly lower than the control group by at least 10%. The metals Cu and Sr had no statistical variation between the groups. The metals As, Cd, Co, Mo, Ni and Pb had results below the quantification limit of the method, defined at 0,1 mg.L^{-1}.

Fernandes *et al.*, (2021) verified the presence of metals at the beginning and at the end of the exposure of bullfrog tadpoles to water from the same points of the Sorocaba River for 96 hours. The results showed a reduction of 67.19% (PI) and 80.49% (PIR) in the concentration of Mn in the water at the end of the exposure. This phenomenon may indicate a process of metal absorption and accumulation in the exposed animals (Simoncelli et al., 2015; Girotto et al., 2020). Although the PI group did not differ from the control for this analyte in the skin samples, it is possible to state that there was an accumulation in the animal due to the reduction of the metal available in the water after the exposure period and, as the skin of amphibians has permeable characteristics, the metal can be absorbed and transported to other tissues. Furthermore, Veronez *et al.*, (2016) exposed *A. catesbeiana* to iron ore, Fe and Mn during metamorphosis and observed an increase in iron in the group exposed to iron ore and Fe and an increase in whole body Mn concentration in tadpoles exposed to iron ore and Mn.

The increased concentration of Ba and Mn in the skin of animals exposed to the waters of the Itupararanga reservoir demonstrates a worsening in water quality in relation to the starting point of one of the sources of the Sorocaba River, and this characteristic may be inducing oxidative stress in these animals. Péres-Alvarez *et al*. (2018) and Fernandes *et al*., (2021) discovered in their experiments that oxidative stress can occur even in conditions of metal concentration within the limits established by legislation. Oxidative stress in organisms can present changes in the expression and concentration of proteins, such as metallothionein (Carvalho *et al*., 2016), changes in enzymes such as superoxide dismutase (SOD) (Fernandes et al., 2021), glutathione S-transferase (GST) and catalase (CAT) (Veronez et al., 2016) as well as DNA damage and increased frequency of micronuclei (Veronez et al., 2016) and changes in animal behavior and growth (Péres-Alvarez *et al*., 2018). Other authors pointed to the need for attention to the water quality of this reservoir, such as Martins *et al*. (2021), who evaluated and concluded that the presence of metals such as Mn and Cu in this reservoir may be linked to agricultural and mining activities. Animals that inhabit the region may be more affected if they are more sensitive to these xenobiotics.

In the evaluation of the concentration of metals in various tissues of *Pelophylax kl. esculentus*, a species of frog widely used in food in Europe, the authors Prokic *et al.,* (2016) discovered that a high accumulation in the organism can occur even in environments where the concentration of metals in the water is low, even at concentrations below the limits of quantification, with the liver and skin being the tissue that accumulate the majority of metals, and collaborating with our results.

The differences between the accumulation of metals in tissues are due to the method of exposure to the metal, which may be through dermal absorption or through oral administration through food (Girotto *et al.*, 2020) The authors highlighted that the high level of metals in the liver and skin of these animals is linked to higher levels of activity in the antioxidant defense system, which are molecules that act collectively to protect against the damage that can be caused by free radicals to organelles, cells and tissues (Ighodaro *and* Akinloye, 2018) and indicate the use of these tissues as bioindicators of exposure to metals. With this information, we can state that, even with the physical-chemical analyzes of water samples from the Itupararanga Reservoir remaining within the levels permitted by Brazilian legislation, metal bioaccumulation processes in aquatic organisms may be occurring.

Monitoring metals in water is important due to the impacts that these substances can cause on aquatic animals and those who use the water. By exposing Zenhai brown frog tadpoles (*Rana zenhaiensis*) to 1/10 of the concentration calculated for acute toxicity (LC50) of Cu^{2+}, Pb^{2+} and Cd^{2+} for 18 days, the authors Wei *et al*., (2015) observed that chronic toxicity caused several differences between the control group and the exposed group, including size, mass and also affected the survival rate of the animals, which can seriously affect populations of animals with greater sensitivity.

Exposure to metals such as Cd can induce an increase in reactive oxygen species (ROS), which can result in oxidative stress, damaging the functioning of cells and structures (Simoncelli *et al*., 2015).The increased concentration of metals in the skin of animals exposed to the waters of the Itupararanga Reservoir may demonstrate that this region is more affected to anthropological pressures (Prokic *et al*., 2016), whether caused by industrial, agricultural or community waste, in comparation to Ibiúna source region. This demonstrates that the Sorocaba

River suffers a degradation in its quality along its route, making constant monitoring necessary and requiring greater care through public preservation policies.

3.3 Bioconcentration Factor (BCF)

The potential for bioaccumulation of contaminants is a factor that must be taken into account in studies on the implementation of environmental control policies, as substances with a high bioaccumulation factor can result in high internal concentrations in the biota and cause toxicity effects (Wassenaar *et al*., 2020). Substances that are very persistent in the environment and have the potential for bioaccumulation should also be monitored, as they are difficult to manipulate and can lead to serious toxicity effects, as they are not easily metabolized into products that are less toxic for organisms (Wassenaar *et al*., 2020).

There are several calculation methods for the bioconcentration factor in aquatic beings, having greater applicability in resolutions that deal with the acceptable limits of contaminants in fish used for human consumption (Wassenar *et al*., 2020; Gobas and Lee, 2019). This work uses the following equation (Eq. 2) to calculate the bioconcentration factor of the metals Cu, Mn and Zn in the skin of bullfrog tadpoles, using metal concentration data in water samples collected in the winter period obtained in the work of Fernandes *et al*., (2021).

$$BCF = \frac{average\ of\ metals\ in\ tissue\ (\mu g\,.\,g^{-1})}{average\ of\ metals\ in\ water\ (\mu g\,.\,l^{-1})}$$

Eq. 2

The results of the bioconcentration factor are presented in table 3. See metal concentration in water and sediment in supplementary material section.

	Control	**PI**	**PIR**
Cu	0,43	-	-
Mn	-	0,09	0,15
Zn	36,00	-	-

Table 3 – Results for the bioconcentration factor obtained. The BCF results with trace are due to the calculation not being possible, as the concentration of metals in the water was reported as ¨below the LQ¨ for the points mentioned. Source: from the authors.

The higher BCF bioconcentration factor for Zn in the control group is due to the fact that large concentrations of this metal were found in the skin samples of the group exposed to the control waters. The increase in BCF in the PIR group for Mn is related to the significant increase of this metal in samples from this group, indicating an increase in bioaccumulation rates in the waters of the PIR group compared to the PI group. Because most of the results for metals in water were reported as ¨less than the limit of quantification¨ in the work of Fernandes *et al*., (2021), further analysis on this topic was not possible.

4 Conclusion

Even with a low academic production in the last 10 years, the various studies analyzed point to the success in the use of bioindicators for environmental monitoring, especially for the use of African bullfrog tadpoles (*Aquarana catesbeiana*), in monitoring of metals and other xenobiotics in the waters of rivers and lakes, as they are shown to be sensitive organisms and it is possible to correlate the exposed dose with the response found in the organism in different methodologies. The use of the tadpole skin of this organism proved to be a tool to characterize the environment and demonstrated a worsening in the quality of the waters of the Sorocaba River along its route, as the organisms exposed to the waters of the PIR showed significantly higher concentrations of Ba and Mn than PI source region. The presence of these metals in the water of this reservoir and our results highlight the need for greater attention to the surroundings of the Sorocaba River, where farms and factories may be damaging the environmental quality of this region through the release of effluents and agricultural pesticides into the river.

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4 ARTIGO 2

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Metals and metallothionein in gills and caudal muscle of bullfrog tadpoles, *Aquarana catesbeiana* (Shaw, 1802), exposed to the waters of the Sorocaba River

Metais e metalotioneína em brânquias e músculo de girinos de rãs-touro, *Aquarana catesbeiana* (Shaw, 1802), expostos às águas do Rio Sorocaba

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Abstract: Objective: In this study, the concentration of metals and metallothioneins (MTs) was quantified in the gills and caudal muscle of tadpoles of the bullfrog *Aquarana catesbeiana* syn. *Lithobates catesbeianus* (Shaw, 1802), in order to evaluate the potential use of these tissues as tools for the biomonitoring of metals in water, correlating the data obtained, as well as evaluating the water quality at two points on the Sorocaba River. **Methods:** The tadpoles were exposed for 96 hours to the waters of 2 points on the Sorocaba River, with the Ibiúna point representing the source of the Sorocaba River and the Itupararanga Reservoir point representing an important water supply point for the region from the city of Sorocaba-SP. Contaminant-free deionized water was used as a control. The evaluation of the concentration of metals Ba, Cu, Mn, Sr and Zn in samples of gills (n=27) and caudal muscle (n=18) was determined using a Microwave Plasma Atomic Emission Spectrometer (MP-AES). The evaluation of MT was carried out using the method proposed by Viarengo *et al*., (1997) in samples from gills (n=18) and caudal muscle (n=18). Correlation of results was performed using Pearson correlation in *Sigma Stat v 3.5 software*, with a significance level of $p<0.05$. **Results:** Metals in the gills of animals exposed to the waters of the Itupararanga Reservoir increased in relation to the control and in relation to the animals exposed to the waters of the Ibiúna point, as well as MTs levels in this group were significantly higher in relation to the other groups, where it was possible to form strong correlations between the level of MTs and metals in the gills. The caudal muscle tissue showed lower concentrations of the metals evaluated in relation to the gills in the three groups, as well as lower concentrations of MT with no difference between the groups. **Conclusions:** The strong correlations obtained between the metals evaluated and MT in the gills of bullfrog tadpoles indicate that this tissue can be used as a tool for environmental biomonitoring. Caudal muscle of bullfrog tadpoles showed the lowest concentration of metals and MTs, as well as a low rate of strong correlations. The increase in the concentration of metals and MTs in tadpoles exposed to the waters of the Itupararanga Reservoir reveals the need to protect the waters of the Itupararanga Reservoir and the Sorocaba River, through quality monitoring, reduction of anthropogenic pressures and public policies that protect water quality in this location.

Keywords: biomonitoring; metal pollution; bioindicators; limnology.

Resumo: Objetivo: Neste estudo foi realizado a quantificação da concentração de metais e de metalotioneínas (MTs) nas brânquias e músculo caudal de girinos de rã-touro *Aquarana catesbeiana* syn. *Lithobates catesbeianus* (Shaw, 1802), a fim de avaliar o potencial de uso desses tecidos como ferramentas para o biomonitoramento de metais na água, correlacionando os dados obtidos, assim como avaliar a qualidade da água de dois pontos do Rio Sorocaba. **Métodos:** Os girinos foram expostos por 96 horas às águas de 2 pontos do Rio Sorocaba, sendo o ponto Ibiúna representando a nascente do Rio Sorocaba e o ponto Represa de Itupararanga representando um importante ponto de abastecimento de água para região da cidade de Sorocaba-SP. Foi utilizada água deionizada livre de contaminantes como controle. A avaliação da concentração dos metais Ba, Cu, Mn, Sr e Zn nas amostras de brânquias (n=27) e músculo caudal (n=18) foi determinada em Espectrômetro de Emissão Atômica por Plasma de Microondas (MP-AES). A avaliação das MTs foi realizada através do método proposto por Viarengo *et al*., (1997) em amostras de brânquias (n=18) e músculo caudal (n=18). A correlação de resultados foi realizada através de correlações de Pearson utilizando o *software Sigma Stat v. 3.5,* onde o nível de significância adotado foi de p<0,05. **Resultados:** Os metais nas brânquias dos animais expostos às águas da Represa de Itupararanga aumentaram em relação ao controle e em relação aos animais expostos às águas do ponto Ibiúna, assim como os níveis de MTs neste grupo foram significativamente maiores em relação aos outros grupos, onde foi possível formar fortes correlações entre o nível de MTs e metais nas brânquias. O tecido músculo caudal mostrou menores concentrações para os metais avaliados em relação às brânquias nos três grupos, assim como menores concentrações de MTs sem diferença entre os grupos. **Conclusões:** As fortes correlações obtidas entre os metais avaliados e MTs nas brânquias de girinos de rã-touro indicam que este tecido pode ser utilizado como uma ferramenta para o biomonitoramento ambiental. O músculo caudal de girinos de rã touro apresentou as menores concentrações obtidas para metais e MTs, assim como uma baixa taxa de fortes correlações. O aumento da concentração de metais e de MTs nos girinos expostos às águas da Represa de Itupararanga revela a necessidade de proteção das águas desta represa e do percurso do Rio Sorocaba, por meio do monitoramento da qualidade, diminuição das pressões antropogênicas e políticas públicas que protejam a qualidade de água neste local.

Palavras-chave: biomonitoramento; poluição por metais; bioindicadores; limnologia.

1 Introduction

Metal pollution in water can originate from various human production processes, mainly mining and industrial processes. These processes produce waste that, when not treated correctly, can cause major impacts on the environment (Sakakibara *et al.*, 2011). Some potentially toxic metals, such as copper (Cu), manganese (Mn) and chromium (Cr), when in low concentrations, are essential elements for organisms. Other metals and metalloids such as arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb) are highly toxic even at low concentrations (Saha, 2016).

The main sources of increased concentration and/or environmental contamination by potentially toxic metals in water bodies are the natural processes that involve the atmospheric precipitation, geological weathering and leaching (Lollo, 2016). However, the greatest concern is anthropogenic origin impact, where discards waste and rejects from production processes, agricultural processes and urban waste without proper treatment can quickly alter the structure of an aquatic community, changing population frequencies and even making life in the place unfeasible (Lollo, 2016; Bonecker *et al.*, 2022). Thus, the presence of metals can pose a risk to the local community, as they can accumulate in various animal organs and biomagnify to higher levels of the trophic chain (Miller, Hamann and Kroon, 2020).

Bioindicators are organisms that can represent, through physical, biochemical, metabolic or molecular characteristics, differences between environmental states (Van der Oost *et al*., 2003). There are several animals with potential for use as bioindicators, but important characteristics such as the ability to withstand high levels of pollutants without dying and the possibility of identifying a dose-response mechanism are necessary for the creation of bioindicators for environmental monitoring (Van der Oost *et al*., 2003, Zhou *et al*., 2007).

Amphibians are candidates because they have specific characteristics such as the ability to breathe through their skin and gills and the ability for pollutants to penetrate the body through epithelial tissue, making them viable for use in various research techniques, such as morphological, behavioral, metabolic observation and analysis of total metal or pollutant content in tissues (Zhou *et al*., 2007; Prokic *et al*., 2016; Prokic *et al.*, 2016b). Due to their low mobility characteristics and part of the amphibian larval cycle taking place in an aquatic environment, these animals are vulnerable to discharges and pollutants that can occur in rivers and lakes, and knowledge about amphibian species from tropical and subtropical regions is still limited, which may affect the protection of species at greater risk of extinction.

The evaluation of the concentration of proteins such as metallothioneins (MTs), which are the proteins that bind to metals in cases of exposure to environments with concentrations high levels of these elements, are already well established in fish (Olsson *et al*., 1986; Hylland *et al*., 1992; Schlenk *et al*., 1995; Olsvik *et al*., 2000, Sakuragui *et al*., 2013), bivalves and polychaetes (Hook, 2014). MTs have the characteristic of binding to molecules of metals through the thiol group (-SH, sulfhydryl) due to a large number of amino acids *Cys* in its structure (Viarengo *et al*., 1997).

The objective of this work is to evaluate the concentration of metals and metallothioneins in the gills and caudal muscle of bullfrog tadpoles exposed to the waters of two points of the Sorocaba River for 96 hours, with the aim of evaluating the feasibility of using these tissues as tools for metal biomonitoring in water. The research seeks to analyze the hypothesis of tadpoles that were exposed to the waters of the Itupararanga Reservoir, representing an important water accumulation point for supplying the population of the region of the city of Sorocaba, would present higher concentrations of metals in their gills and muscle compared to tadpoles exposed to water collected in the city of Ibiúna, representing the source of the river and compared to the control group, which would demonstrate the worsening of the water quality of the Sorocaba River along its route.

2 Material and Methods

2.1 Study area

The Sorocaba River is located in the State of São Paulo and is formed by the union of the headwaters of the Una, Sorocabuçu and Sorocamirim Rivers, which meet in the cities of Ibiúna, Cotia, Vargem Grande Paulista and São Roque. The Sorocaba River is approximately 929 km² long and runs through areas where it is subject to various anthropogenic influences, such as cement factories, mining and agricultural areas. Downstream, the Sorocaba River accumulates in the Itupararanga Reservoir, an important reservoir in an environmental protection area that supplies the population of the Sorocaba region (Conceição *et al*., 2011; Fernandes *et al*., 2021). Using 50 liters capacity galons, 120 liters of water were collected from the Sorocaba River in the dry season of the year (August, 2019), near to the city of Ibiúna (PI sample point) (23º27'27" South latitude – 47º24'10.1" West longitude), representing the source of the Sorocaba River. The Itupararanga Reservoir (PIR sample point) (23º38’11.3” South latitude - 47º13’22.6” West longitude) was chosen as the collection point due to the distance between the two points and the importance in water supply for the regional population.

2.2 Animal collection

As described by Fernandes *et al.*, (2021), bullfrog tadpoles *Aquarana catesbeiana* (syn. *Lithobates catesbeianus*) were obtained from Santa Rosa Frog Farm (Santa Barbara d’Oeste, SP, Brazil) (22º46’53.0”S /47º24’17.7”W). The animals (n=108) in stage 25 of Gosner (1980) were maintained in 80L tanks of dechlorinated water for 10 days, using a continuous flow of water (1.2 $L.h^{-1}$), continuous aeration (6 mg $O_2.L^{-1}$) and photoperiod 12 hours light, 12 hours dark, for acclimatization purposes before being exposed to the waters of the Sorocaba River. The animals were fed with commercial food (40% protein) and feeding was stopped 24 hours before exposure. The animals were divided into a control group (C), a group exposed to the waters of the city of Ibiúna (PI) and a group exposed to the waters of the Itupararanga Reservoir

(PIR) through the random transfer of 12 animals to each group in 16-liter aquariums. The animals were exposed to water for 96 hours in static conditions, and the characteristics of the water were monitored: pH (7.2 to 7.6), dissolved oxygen (7.0 to 7.5 $mg.L^{-1}$), hardness (50 to 58 mg $CaCO_3$ $mg.L^{-1}$), conductivity 56-97 ± 0.02 µS/cm and ammonia concentration < 1 $mg.L^{-1}$. The experiment was conducted in triplicate.

After the end of the exposure period, the animals were anesthetized with 0.1% benzocaine and euthanized by cranial concussion, as recommended by the American Veterinary Medical Association (AVMA, 2001). The organs were separated and frozen in a biofreezer at -80 ºC until analysis was carried out. The procedures were realized in accordance with the standards established by the American Society for Testing and Materials (ASTM, 2000) and were approved by the ethics committee of the Federal University of São Carlos (CEUA-UFSCar) through process #2578040219/2019.

2.3 Sample preparation

2.3.1 Metals

For determining the total concentration of metals and semimetals arsenic (As), barium (Ba), cadmium (Cd), lead (Pb), cobalt (Co), copper (Cu), nickel (Ni), manganese (Mn), molybdenum (Mo), strontium (Sr) and zinc (Zn) samples of gills (n=27) and caudal muscle (n=18) from bullfrog tadpoles were dried in a laboratory oven at 60 ºC for 7 days, in order to eliminate moisture and determine total dry mass. Due to the large reduction in tissue mass after they were dried in an oven, the sample pool strategy was used, which consists of combining samples from the same group for joint analysis, in order to obtain a sample with a representative mass (Correa *et al*., 2016; Laurin *et al*., 2019). Therefore, the samples were weighed in 0.15g (dry mass).

The samples were subjected to digestion in a Multi Wave Pro Anton Paar® microwave oven using the acid digestion method established by the manufacturer for fish samples. The method consists of programming Temp/Power: 650/1400, Ramp: 5/10 Hold: 10/10/15 Fan: 1/1/3, using 6 ml of 65% nitric acid (HNO_3) and 1 ml of 32% hydrochloric acid (HCl) analytical grade per sample. After 1 hour of digestion in a microwave oven, the samples were digested and increased to 25 ml in a glass volumetric flask with a 5% HNO_3 solution, which consists of 50 ml of HNO_3 in 950 ml of ultrapure water. For the blank, 6 ml of HNO_3 was used with 1 ml of HCl, subjected to the digestion process described and subsequently increased to 25 ml with 5% HNO_3 solution in a volumetric flask.

Metal concentration analyzes were carried out using the atomic emission spectrometer atomic by microwave plasma Agilent MP-AES 4200 equipped with cyclonic nebulization.

Metals and semimetals As, Ba, Cd, Co, Cu, Pb, Mn, Mo, Ni, Sr and Zn were evaluated. The limits of detectation and quantification can be seen in table 1.

Elements	Limit of Detection (LOD)	Limit of Quantification (LOQ)
As	64.35 mg.L^{-1}	214.48 mg.L^{-1}
Ba	0.33 mg.L^{-1}	1.16 mg.L^{-1}
Cd	0.58 mg.L^{-1}	1.93 mg.L^{-1}
Co	0.47 mg.L^{-1}	1.55 mg.L^{-1}
Cu	0.17 mg.L^{-1}	0.53 mg.L^{-1}
Pb	5.67 mg.L^{-1}	185.58 mg.L^{-1}
Mn	0.033 mg.L^{-1}	0.10 mg.L^{-1}
Mo	0.033 mg.L^{-1}	0.13 mg.L^{-1}
Ni	0.57 mg.L^{-1}	1.90 mg.L^{-1}
Sr	0.033 mg.L^{-1}	0.10 mg.L^{-1}
Zn	0.23 mg.L^{-1}	0.78 mg.L^{-1}

Table 1. Limit of detection and limit of quantification defined for each element in this study. Source: From authors.

The metal concentration results in mg.L^{-1} for the samples were calculated automatically by *Agilent MP Expert Software*. In order to relate the mass of the samples used with metal concentrations obtained in mg.L^{-1}, the results were converted to mg.Kg^{-1} from the following equation:

$$\frac{[Sample\ conc. - blank\ average\ conc.\ (mg.L^{-1})] * \ vollumetric\ flask\ volumn\ (L)}{Sample\ mass\ (kg)}$$

Eq. 1

The results were expressed as mean ± standard deviation obtained. Mean values for each parameter were compared with each other using the Student's T test. The significance level utilized was $p<0.05$. Statistical analyzes were performed using the software *SigmaStat for Windows version 3.5, Copyright© 2006 Systat Software, Inc.*

2.3.2 Metallothionein analysis

Analysis of metallothionein-type proteins was carried out based on the method proposed by Viarengo *et al* (1997) of sulfhydryl residues (SH) at from analytical curve equation obtained by diluting reduced glutathione (GSH) standard and determination in one spectrophotometer at absorbance of 412 nm. The extraction of metallothioneins (MTs) from bullfrog tadpole tissue samples was carried out by transfer of 300 µL of the supernatant of samples homogenized in phosphate buffered saline PBS (pH 7.2) into tubes of the type eppendorf with a capacity of 1 mL, where 342 µl of a solution of the mixture formed by 93% absolute ethanol with 7% chloroform refrigerated at -20 ºC were added. The samples were centrifuged at 12,000 g for 10 minutes at a temperature between 0 and 4 ºC and 490 µl of supernatant obtained was transferred to a new Eppendorf-type tube with a capacity of 2 ml, where 1502 µl of a solution of the mixture refrigerated at -20 ºC of 97.83% absolute ethanol with 2.17% concentrated hydrochloric acid (HCl) was added. The samples were shaken and kept at -20 ºC for 1 hour, where centrifugation of the samples at 6,000 g for 10 minutes at 0 to 4 ºC was subsequently performed and the supernatant obtained was discarded. The samples were resuspended by adding 1000 µl of a solution formed by 87% absolute ethanol, 1% chloroform and 12% TRIS-HCl buffer a 20 mM, which in turn consists of dissolving 1.576 g of TRIS-HCl in 500 ml of deionized water (pH 8.6). The samples were shaken, centrifuged at 6,000 G for 10 minutes in temperature from 0 to 4 ºC and the supernatant was discarded again. The samples were resuspended with the addition of 50 µl of sodium chloride (NaCl) at 250 mM and 50 µl of ethylenediaminetetraacetic acid (EDTA), formed from the dissolution of 0.0745g of EDTA in 45.06 ml of water deionized with 4.94 ml of HCl and then shaken. In each sample tube 1000 µl of Ellman's solution was added, which is prepared at the time by mixing 0.43 mM DNTB [5,5' dithio-bis (2-nitrobenzoic acid)] in 500 µl and then added 69.5 mL of sodium phosphate buffer (0.2M) saturated with sodium chloride at 2M (pH 8.0). The samples were shaken, centrifuged at 3,000 g for 5 minutes and 200 µl of supernatant was transferred to the spectrophotometer reading plate. The blank was 50 µl of NaCl solution (250 mM) that was used with 50 µl of EDTA solution (EDTA at 4mM with 1M HCl). The absorbance reading was performed at a wavelength of 412 nanometers in spectrophotometer.

2.4 Statistical analysis

The results were expressed as mean ± standard deviation obtained. The mean values for each parameter were compared with each other using the Student's T test or the Mann-Whitney U test, depending on data distribution. The level of significance established was $p<0.05$. Statistical analyzes were performed using the software *SigmaStat for Windows Version 3.5, 2006 Systat Software, Inc*. Finally, the metal results were correlated with MT results separately for the gill and muscle using Pearson correlation.

3 Results and Discussion

3.1 *Metal concentration in gills and caudal muscle*

The metal concentration for gills and caudal muscle samples from bullfrog tadpoles (*Aquarana catesbeiana*) can be seen in table 2.

		Gills	
mg.kg^{-1}	**Control**	**PI**	**PIR**
Ba	135.23 ± 1.53	↑ **175.59 ± 0.61** (29.8%)	↑ **194.46 ± 0.47*** (59.3%)
Cu	8.90 ± 0.42	8.69 ± 0.38	↑ **9.89 ± 0.19** (11 %)
Mn	3.56 ± 0.04	↑ **6.95 ± 0.04** (95%)	↑ **9.89 ± 0.01*** (178%)
Sr	4.74 ± 0.03	↑ **6.37 ± 0.02** (34%)	↑ **7.69 ± 0.06*** (62%)
Zn	281.73 ± 4,22	↑ **343.07 ± 5,18** (22%)	↑ **369.7 ± 2.37*** (31%)
		Caudal Muscle	
(mg.kg^{-1})	**Control**	**PI**	**PIR**
Ba	28.71 ± 0.27	↑ 31.50 ± 0.14 (9.7%)	↑ **92.99 ± 0.62*** (223.9%)
Cu	1.06 ± 0.01	↑ **1.66 ± 0.04** (56.6%)	↑ **1.54 ± 0.05** (45.2%)
Mn	3.19 ± 0.04	3.32 ± 0.04	↓ **1.54 ± 0.01*** (52%)
Sr	4.96 ± 0.05	↑ 6.08 ± 0.05 (22.6%)	↑ **8.73 ± 0.06*** (76%)
Zn	108.82 ± 1.53	↑ **156.39 ± 1.25*** (43.7%)	↓ **92.99 ± 0.64** (14.6%)

Table 2. Metal concentration (mg.kg^{-1}) in gills (N=27) and caudal muscle (N=18) samples. The results are expressed in the format of mean ± standard error. In bold means difference in relation to the control and * means significant difference in relation to the points. PI: Ibiúna sample point. PIR: Itupararanga Reservoir sample point. LOQ = limit of quantification. As, Cd, Co, Mo and Ni were below the limit of quantification. Source: from the authors.

The metals with the highest concentrations in the gills were Zn and Ba, followed by Cu, Mn and Sr. It is seen that, for all quantifiable analytes, the metals in the tadpole group PIR had the highest concentration among the 3 evaluated groups, which suggests deterioration in water quality along the river's route and a greater load of metals present in the waters of the Itupararanga Reservoir. Pedrazzi *et al*., (2014) in water samples from the Itupararanga Reservoir verified the presence of Ca, Mg, Na, K, Al, Fe, Mn, Ni, Pb and Zn with concentrations within the maximum permitted values for class 2 rivers (CONAMA Resolution, nº 357/05).

Caudal muscle samples do not show a pattern in results, with metals such as Ba and Sr present in greater concentration in PIR group, but presenting concentrations of Cu, Mn and Zn lower than the group exposed to water collected in Ibiúna (Table 2). Using the caudal muscle samples for analysis, the Mn concentration in the PIR group was 52% lower compared to the control group. The same condition was found for Zn, where the PIR group had results of Zn concentration 14.6% lower than the results obtained in the control group.

In this work it is seen that the external structures of the body as gills, present a higher concentration of metals compared to the concentrations found in bullfrog caudal muscle, suggesting the importance of this organ as a tool to biomonitoring. The different absorption rates of metals present in the environment may be related to functions performed by these organs (Simoncelli *et al*., 2015). Gills represents one of external structural barrier that interacts with the environment and its potential contaminants and the results revealed that amphibian gills react to stress induced by the presence of metals in the Sorocaba River.

The work of Severtsova *et al*. (2012), evaluated the accumulation of Fe and Pb in the gills, intestine, kidneys and tail of tadpoles of grass frog (*Rana temporaria* L.) and gray toad (*Bufo bufo L.*). It was found that the highest concentrations of metals were found in the intestine and liver of tadpoles of both species, and the accumulation increased according to the tadpole development. Furthermore, the authors also assessed that the main source of acquisition of metals by animals occurred orally through food. Studying the mechanisms of metal accumulation in various tissues of the European Edible Frog (*Pelophylax kl. esculentus*), the authors in Prokic *et al*., (2016) reported that the main means of metal absorption in amphibians is through dermal absorption and orally, through the feeding of these animals. It has been observed that, even at low concentrations in water, metals can bioaccumulate and show up in high intensity in aquatic organisms (Prokic *et al*., 2016).

The gills have structures that enable functions not only respiratory, but they are also sites for the exchange of ions such as Na$^+$ and Cl$^-$, however, in anuran tadpoles after metamorphosis the skin becomes the main site of transepithelial ionic transport (Thabah *et al*., 2018 apud Dietz & Alvarado, 1974; Diets & Alvarado, 1974). Mclndoe and Smith (1984) described the morphology and vascular anatomy of the internal gills of *Litoria ewingii* tadpoles by scanning electron microscopy. These are formed by four pairs of gills located in two gill baskets on each side of the heart being consisting of a gill arch with variable gill tufts that project ventrally and gill filters running dorsally. The gill tufts, formed by a complex three-dimensional network of capillary rings varying in length and diameter, suggest greater potential as gas exchangers than gill filters or skin. What also explains the higher concentration of metals found in this organ.

The accumulation of metal in tadpoles can vary in the organs according to the function they perform. These play and depend on the stage of development. Amphibian tadpoles pass through an intense process of metamorphosis, a particularly sensitive stage of life 42 (Krohn *et al*., 2020) where, among other modifications, the tail retracts with degradation of muscle tissue and may present adverse effects resulting from the presence of metals in water (Wang *et al*., 2023), such as delayed development, decreased size and length (Veronez *et al*., 2016). For example, *Bufo gargazians* tadpoles exposed at 416 mg.L^{-1} of Cr^{6+} until reaching Gosner stage 38 showed changes in the structure of intestinal tissue, decrease in total body length, wet body weight, intestinal length and wet weight (Yao et al., 2019). Furthermore, the same study revealed, by sequencing the 16S rRNA gene, a significant change in diversity and composition of the intestinal microbiota indicating that the metal affected metabolism with greater susceptibility to diseases.

3.2 Metallothionein (MT)

The results for the concentration of metallothionein proteins can be seen in table 3. The analysis of MTs from the tadpoles' gills showed a significant variation (p = 0.017) with an increase of 98.7% in the PIR group compared to the control group and 89.5% higher (p =0.036) in the PI group. There was no significant difference (p = 0.742) between tadpoles in PI group and the control group (Table 3).

Organ	**Control**	**PI**	**PIR**
Gills	0.162 ± 0.014	0.169 ± 0.016	↑ **0.322 ± 0.059*** (98.7%)
Caudal Muscle	0.207 ± 0.041	0.249 ± 0.065	0.214 ± 0.046

Table 3. Concentration of metallothionein in samples (means ± Standard Error) of gill and muscle of bullfrog tadpoles (nmol SH . mg^{-1} protein). Data are presented as mean ± standard error. PI: Ibiúna sample point. PIR: Itupararanga Reservoir sample point. In bold means difference in relation to the control and * means significant difference in relation to the points. N=6 per group. Source: From authors.

The increase in MT concentrations in the gills is corroborated by the increase in the concentration of metals in this organ and suggests the presence of metals in the Sorocaba River, which can be absorbed by the local biota. The presence of potentially toxic metals in a reservoir that supplies the region and the potential for biomagnification in animals present at the site can also cause harm to the population.

In the caudal muscle, the concentration of MTs showed no significant difference ($P = 0.584$) in the PI group and the PIR group ($P = 0.657$) in relation to the control (Table 3).

MTs have an essential role in protecting against levels of metals capable of causing oxidative stress in cells and are strongly associated with the regulation of Zn levels in the body, where increased levels of free Zn induce the expression of MT through the factor transcriptional metal regulator 1 (MTF-1), an amino acid that responds directly to increased levels of free Zn (Kizek *et al*., 2013). Combinations of metals can increase the lethal potential of substances and increase the expression of MTs (Yologlu and Ozmen, 2015). *Xenopus laevis* exposed to Cd, Pb and Cu (96 h) showed increased levels of MTs with increased metal concentrations, demonstrating a dose and effect relationship (Yologlu and Ozmen, 2015). Furthermore, the authors determined the LC50-96h for these animals to be 5.81; 123.05 and 0.85 mg L-1 for Cd, Pb and Cu respectively.

The ion exchange function and direct contact with water present in the environment may explain the greater amount of metals and MTs in the gills compared to the muscle. Furthermore, the different distribution profiles of this protein may determine greater or lesser tolerance to metal toxicity by these animals. Thus, the exposure time, concentration and type of metal analyzed are important factors in inducing MTs between different organs. In tadpoles exposed to the waters of the Sorocaba River, metals were absorbed and distributed, and some were probably also excreted (Dobrovoljc *et al.*, 2012) and may be related to the detoxification process. That way, the high levels of Zn found in samples of gills and caudal muscle of *Aquarana catesbeiana* tadpoles, together with the identification of the metals Ba, Cu, Mn and Sr in the samples, may be the cause of high MT concentration in samples.

3.3 Metals and metallothionein correlation in samples

In Pearson correlation (Pearson's r), the linearity metric between variables is presented in numbers from -1 to +1. The closer to the extremes (-1 or +1), the greater the strength of the correlation. Values that are close to zero demonstrate a weak correlation. In this section the results of quantified metals are correlated with the concentrations of MTs obtained in the gills and muscle. The p-value obtained for the correlations between the results of mean concentration of Ba, Cu, Mn, Sr, Zn, and MT for gills and muscle was outside the significance range, with p-values greater than 0.05 being obtained (see table 4).

Analytes	**Pearson correlation coefficient**	***p* Value**	**Interpretation**
MT x Ba Gills	**0.770**	0.440	Strong correlation
MT x Cu Gills	**0.979**	0.129	Very strong correlation
MT x Mn Gills	**0.865**	0.335	Strong correlation
MT x Sr Gills	**0.855**	0.347	Strong correlation
MT x Zn Gills	**0.759**	0.451	Strong correlation
MT x Ba Muscle	-0.323	0.790	Weak correlation
MT x Cu Muscle	**0.764**	0.446	Strong correlation
MT x Mn Muscle	0.420	0.724	Weak correlation
MT x Sr Muscle	-0.074	0.953	No correlation
MT x Zn Muscle	**0.922**	0.254	Very strong correlation

Table 4. Correlations between metallothionein (MT) and metals in gills and caudal muscle of bullfrog tadpoles. Bold markings mean strong or higher correlation. Source: From authors.

The study by Carvalho *et al.*, (2017) demonstrated Pearson's correlation in bullfrog tadpole organs with MT and Zn in the kidneys (r=0.964), moderate correlation between Zn and MT in the muscle (r=0.530), however, no correlation of MT and Zn was observed in the liver. For Cu metal, strong correlations were found in liver, muscle and kidneys; for Cd there was a strong correlation in muscles while a strong negative correlation was demonstrated in the kidneys (r = -0.896) and liver (r = -0.988) indicating, in this case, that the increase in a variable (MT or metal) corresponds to a decrease of the other variable. The strong correlations found in the present study between Zn and MTs in gills (r=0.759) and muscle (0.922) in the control groups, the PI group and the PIR group, Cu and MTs in gills (r=0.979) and muscle (r = 0.764) demonstrate the function of a metal exposure bioindicator in this species of anurans tadpoles and corroborate the results of correlations obtained in the liver, kidney and muscle by Carvalho *et al.*, (2017). The Cd results could not be compared as they were obtained at concentrations below the detection limit in all organs analyzed in this research.

Caudal muscle samples did not show the same efficiency as an analysis matrix as a source of bioindicators, as they had the lowest concentrations of MTs and low rates of strong correlations between the analyzed metals and MTs proteins comparing to gills samples. The effect may be related to the fact that amphibians absorb metals mainly through their skin, in the process of acquiring oxygen in the water through the gills and orally, favoring the contact of metals PIRmarily in the outermost structures of the body (Prokic *et al*., 2016^{b}.

The exposure of tadpoles to different metals, under the experimental conditions of this study, produced a change in the intracellular distribution of metals and induced MT in the gills. Increased MT concentration (PIR), higher metal concentrations (PI and PIR), combined with the strong correlation between MT and Ba, Cu, Mn, Sr and Zn, indicates that this organ is viable tool for environmental biomonitoring of the Sorocaba River. More studies are needed to adapt the use of caudal muscle tissue to identify problems with water quality in the reservoir of the Itupararanga Reservoir, in order to prevent and treat contamination problems and bioaccumulation by metals to the local community, the population that uses this water, the fauna and the flora.

4 Conclusion

The use of bioindicators for environmental monitoring of metals brings several advantages to environmental monitoring. This could be observed in the use of bullfrog tadpoles (*Aquarana catesbeiana*) as bioindicators of metals in the waters of the Sorocaba River, where exposure for 96 hours was capable of causing changes in the concentrations of metals and MTs between animal exposure groups, especially when gills of bullfrog tadpoles were used as a matrix for evaluation, due to the significant change found in the concentration of MT in the gills of the PIR group in relation to the group exposed to the initial routes of the Sorocaba River (PI) and the control group, combined with higher rates for all the metals found, it was possible to demonstrate a worsening in the quality of the waters of the Sorocaba River along its route.

These animals proved to be highly viable for environmental biomonitoring studies of metals in water, as they accumulated large amounts of metals without the animal dying and it was possible to establish a dose-response relationship between metals in the environment and the concentration of MTs in the tissue, by finding of strong Pearson correlations.

These data reveal the need for greater attention to the surroundings of the Sorocaba River, where plantation farms, factories and domestic sewage may be damaging the environmental quality of this region through the release of effluents and the flow of agricultural pesticides for the river.

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5 CONSIDERAÇÕES FINAIS

A exposição dos girinos a diferentes metais, nas condições experimentais deste estudo, produziu uma alteração na distribuição intracelular dos metais e induziu a MT nas brânquias. O aumento da concentração de MT (PIR), as concentrações mais altas de metais (PI e PIR), aliado a correlação forte entre MT e Ba, Cu, Mn, Sr e Zn, indica que este órgão é um meio viável para o biomonitoramento ambiental do Rio Sorocaba, e os girinos satisfazem grande parte dos requisitos para um bioindicador proposto por Zhou *et al*., (2007), sendo eles:

> Pode acumular altos níveis de poluentes sem morrer;
>
> Possui abundância e ampla distribuição, permitindo comparação e repetição;
>
> Possui ciclo de vida longo;
>
> Fácil coleta e criação em laboratório;
>
> Se mantém vivo na água;
>
> É possível observar bom efeito dose/resposta ao poluente.
>
> (Zhou *et al.*, 2007)

A partir dos resultados obtidos foi possível identificar fortes correlações entre os resultados de concentração de metalotioneínas e os metais Ba, Cu, Mn, Sr e Zn obtidos nas brânquias que ativou respostas biológicas com indução da MT, sugerindo seu potencial papel como biomarcador de exposição aos metais. Esses dados podem auxiliar a validar o uso dos girinos de rã-touro como bioindicadores ambientais a exposição à metais. Por serem animais que passam por severas metamorfoses e alteram sua forma corporal, o músculo da cauda não apresentou alteração significativa para correlacionar metal e a indução da MT, entretanto, não podemos desconsiderar que o teste foi agudo (96h), talvez uma exposição a longo prazo possa apresentar efeitos observáveis neste tecido. A pele destes animais apresentou acúmulo de Zn, Ba, Sr em todos os grupos estudados (controle, PI e PIR), com destaque para o Sr que foi observado em concentração cerca de 10 vezes maior do obtido nas brânquias e no músculo do animal. Embora não tenha sido feita a análise da MT neste tecido, é importante considerar que os metais foram absorvidos e serão distribuídos no corpo do animal, podendo levar a prejuízos no desenvolvimento e sobrevivência dos girinos.

No que se refere ao músculo caudal de girinos de rã-touro, são necessários mais estudos e o uso de outros biomarcadores com fins de identificar os problemas com a qualidade das águas no reservatório da Represa de Itupararanga, de forma a prevenir e tratar problemas de contaminação e bioacumulação por metais à comunidade local, à população que faz uso desta água, a fauna e a flora.

A avaliação das águas do Rio Sorocaba por meio deste bioindicador mostrou resultados que apontam para uma piora na qualidade da água a partir do ponto coletado em Ibiúna, representando a nascente, e o ponto Represa de Itupararanga, representando a foz. Ademais, o estudo corrobora a hipótese de que os animais expostos aos percursos finais do Rio Sorocaba estão expostos à maiores concentrações de xenobióticos e demonstra que a carga de metais presentes nesse ambiente possui o potencial de bioacumular nos organismos, podendo prejudicar a vida local. Este estudo chama a atenção para o uso destas águas para o abastecimento da população e fornece uma base para uma melhor compreensão das respostas tecido-específicas de anfíbios como órgão-alvo à exposição aos metais e seu uso para testes de substâncias potencialmente nocivas presentes no ambiente.

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Supplementary Material

Água				
	Conama 357 Classe 2	*Controle*	*PI*	*PIR*
Temperatura (ºC)	-	24.0 ± 1.0	**13.7 ± 1.0**	**16.5 ± 1.5**
pH	6.0 – 9.0	6.7	7.3	**8.0**
Dureza (mg/l)	-	45	**28**	41#
Amônia (mg/l)	0,02	0.29 ± 0.04	**0.04 ± 0.00**	**0.02 ± 0.00**
Nitrato (mg/l)	10	0.20 ± 0.05	0.10 ± 0.01	**1.01 ± 0.23**
Nitrito (mg/l)	1	9.86 ± 1.30	7.79 ± 2.05	**2.08 ± 0.36#**
Fosfato (mg/l)	0,15	0.06 ± 0.02	**0.20 ± 0.00**	0.07 ± 0.00
Alumínio (µg/l)	100	<LQ	**111.79 ± 0.59**	**54.32 ± 0.59#↓**
Cádmio (µg/l)	1	<LQ	<LQ	<LQ
Cobre (µg/l)	9	4.97 ± 0.90	<LQ	<LQ
Manganês (µg/l)	100	<LQ	**93.64 ± 9.75**	**86.78 ± 6.65**
Zinco (µg/l)	180	15.97 ± 1.29	<LQ	<LQ
Sedimento				
Alumínio (mg/kg)	-	-	78.05 ± 4.6#	**66.41 ± 3.52#**
Cádmio (mg/kg)	0,6	-	<LQ	<LQ
Cobre (mg/kg)	35,7	-	0.49 ± 0.03#	**1.70 ± 0.23**
Manganês (mg/kg)	-	-	0.55 ± 0.03#	**0.95 ± 0.10#**
Zinco (mg/kg)	123	-	0.71 ± 0.04#	**0.57 ± 0.05#**

Quadro 1. Parâmetros físico-químicos e concentrações de metais (mg/L) em amostras de água coletadas no período de inverno em Ibiúna (PI) e nos reservatórios de Itupararanga (PIR) do Rio Sorocaba e os valores estabelecidos pelo Conselho Nacional do Meio Ambiente (CONAMA), resolução 357/2005. A diferença significativa é mostrada em negrito em comparação com o controle no mesmo período; # indica uma diferença significativa em comparação com PI e PIR. LQ = limite de quantificação. Fonte: Fernandes *et al.*, 2021.