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Amanda Pereira da Costa Araujo

AVALIAÇÃO DA TOXICIDADE DO MICROPLÁSTICO POLIETILENO EM CONJUNTO COM DISTINTOS POLUENTES EMERGENTES EM VERTEBRADOS AQUÁTICOS

GOIÂNIA
2024

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AVALIAÇÃO DA TOXICIDADE DO MICROPLÁSTICO POLIETILENO EM CONJUNTO COM DISTINTOS POLUENTES EMERGENTES EM VERTEBRADOS AQUÁTICOS

Tese apresentada ao Programa de Pós-Graduação em Ciências Ambientais, do Instituto de Estudos Socioambientais, da Universidade Federal de Goiás (UFG) como requisito parcial para obtenção do título de Doutora em Ciências Ambientais.

Área de concentração: Estrutura e Dinâmica Ambiental
Linha de pesquisa: Monitoramento e Análise de Recursos Naturais

Orientadora: Profa. Dra. Daniela de Melo e Silva
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UNIVERSIDADE FEDERAL DE GOIÁS

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ATA DE DEFESA DE TESE

Ata Nº **004/2024D** da sessão de Defesa de Tese de **Amanda Pereira da Costa Araujo** que confere o título de Doutora em **Ciências Ambientais**, na área de concentração em **Estrutura e Dinâmica Ambiental.**

Aos **nove dias do mês de fevereiro do ano de 2024**, a partir das **8h30min.**, na sala da plataforma **Google Meet**: **https://meet.google.com/cnp-cxoj-uyc**, cuja participação ocorreu por videoconferência, realizou-se a sessão pública de Defesa de Tese intitulada **"AVALIAÇÃO DA TOXICIDADE DO MICROPLÁSTICO POLIETILENO EM CONJUNTO COM DISTINTOS POLUENTES EMERGENTES EM VERTEBRADOS AQUÁTICOS"**. Os trabalhos foram instalados pela Orientadora, Professora Doutora **Daniela de Melo e Silva (ICB/UFG)**, com a participação dos demais membros da Banca Examinadora: Professor Doutor **Marcelino Benvindo de Souza (UEG/RENAC)**, membro titular externo; Professora Doutora **Maria Betania Melo de Oliveira (UFPE)**, membro titular externo; Professora Doutora **Karla Maria Silva de Faria (IESA/UFG)**, membro titular interno; Professor Doutor **Flávio Manoel Rodrigues da Silva Júnior (FURG)**, membro titular externo. Durante a arguição os membros da banca **sugeriram** alteração do título do trabalho. A Banca Examinadora reuniu-se em sessão secreta a fim de concluir o julgamento da Tese tendo sida a candidata **aprovada** pelos seus membros. Proclamados os resultados pela Professora Doutora **Daniela de Melo e Silva**, Presidente da Banca Examinadora, foram encerrados os trabalhos e, para constar, lavrou-se a presente ata que é assinada pelos Membros da Banca Examinadora, aos **nove dias do mês de fevereiro do ano de 2024.**

TÍTULO SUGERIDO PELA BANCA

"AVALIAÇÃO DA TOXICIDADE DO MICROPLÁSTICO POLIETILENO ASSOCIADO COM DISTINTOS POLUENTES EMERGENTES EM ANFÍBIOS ANUROS E PEIXES"

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ARAUJO, A. P. C. **AVALIAÇÃO DA TOXICIDADE DO MICROPLÁSTICO POLIETILENO EM CONJUNTO COM DISTINTOS POLUENTES EMERGENTES EM VERTEBRADOS AQUÁTICOS**. 2024. 176 f. Tese (Doutorado em Ciências Ambientais) - Universidade Federal de Goiás, Goiânia, 2024.[1]

A crescente presença de microplásticos (fragmentos < 5mm) tem gerado preocupações globais devido ao seu potencial impacto na saúde humana e no meio ambiente. Esses pequenos poluentes emergentes têm a capacidade de se associar a outros contaminantes, como metais e agrotóxicos, aumentando sua toxicidade e provocando efeitos mais graves. Uma análise do estado atual do conhecimento científico até o ano de 2020 revelou que a maioria dos estudos sobre a toxicidade dos microplásticos em anfíbios está concentrada no Brasil, Itália e China, com ênfase na ordem Anura. Dessa análise, surgiram recomendações importantes, como a necessidade de ampliar a diversidade de espécies estudadas, avaliar a sensibilidade das espécies considerando o tamanho micro e nano das partículas, investigar as contribuições dos microplásticos para a intensificação dos efeitos causados por estressores ambientais e explorar os efeitos das partículas de plástico em associação com outros poluentes, uma vez que no ambiente esses poluentes raramente estão isolados. Para abordar algumas dessas lacunas, esta tese avaliou os possíveis efeitos da exposição de girinos da espécie *Physalaemus cuvieri* e de adultos de *Danio rerio*, tanto em exposição isolada aos microplásticos quanto em associação com diferentes compostos químicos [i.e., farmacêuticos, resíduos agroindustriais, hidrocarbonetos, hormônios sintéticos, fertilizantes, agrotóxicos, dentre outros] em concentrações ambientalmente relevantes. Após a exposição dos espécimes, foram analisadas a captação e acumulação de microplásticos, além de diversos biomarcadores. Nos girinos de *P. cuvieri*, foram investigados índices morfológicos, biométricos e de desenvolvimento, parâmetros comportamentais, mutagenicidade, citotoxicidade, respostas antioxidantes e colinesterásicas. Nos adultos de *Danio rerio*, foram analisados biomarcadores de genotoxicidade, mutagenicidade e desequilíbrio redox. Os resultados indicaram mudanças notáveis nos padrões fisiológicos e bioquímicos das espécies avaliadas (desequilíbrio de atividades pro-antioxidantes – inferido pelos níveis de nitrito, superóxido dismutase e catalase, por exemplo), além de confirmar a translocação dessas partículas para o interior dos animais. Esse cenário destaca os potenciais efeitos adversos resultantes da presença de diversos poluentes nos ecossistemas de água doce.

Palavras-chave: Poluição plástica, ecotoxicologia, poluição aquática, biomarcadores, toxicidade de misturas.

[1]Orientadora: Profa. Dra. Daniela de Melo e Silva. ICB - LabMUT – UFG.

ARAUJO, A. P. C. **EVALUATION OF THE TOXICITY OF THE MICROPLASTIC POLYETHYLENE IN ASSOCIATION WITH DISTINCT EMERGING POLLUTANTS IN AQUATIC VERTEBRATES**. 2024. 176 f. Thesis. (PhD in Environmental Sciences) - Goias Federal University, Goiânia, 2024.[1]

The growing presence of microplastics (fragments <5mm) has generated global concerns due to their potential impact on human health and the environment. These small emerging pollutants can associate with other contaminants, such as metals and pesticides, increasing their toxicity and causing more serious effects. An analysis of the current state of scientific knowledge up to 2020 revealed that most studies on the toxicity of microplastics in amphibians are concentrated in Brazil, Italy, and China, with an emphasis on the order Anura. From this analysis, important recommendations emerged, such as the need to expand the diversity of species studied, evaluate the sensitivity of species considering the micro and nano size of particles, investigate the contributions of microplastics to the intensification of the effects caused by environmental stressors and explore the effects of plastic particles in association with other pollutants, since these pollutants are rarely isolated in the environment. To address some of these gaps, this thesis evaluated the possible effects of exposure of tadpoles of the species *Physalaemus cuvieri* and adults of *Danio rerio*, both in isolated exposure to microplastics and in association with different chemical compounds [i.e., pharmaceuticals, agro-industrial waste, hydrocarbons, synthetic hormones, fertilizers, pesticides, among others] in environmentally relevant concentrations. After exposing the specimens, the uptake and accumulation of microplastics, in addition to several biomarkers, were analyzed. In *P. cuvieri* tadpoles, morphological, biometric, and developmental indices, behavioral parameters, mutagenicity, cytotoxicity, antioxidant, and cholinesterase responses were investigated. In adults of *Danio rerio*, biomarkers of genotoxicity, mutagenicity and redox imbalance were analyzed. The results indicated notable changes in the physiological and biochemical patterns of the species evaluated [imbalance of pro-antioxidant activities – inferred by the levels of nitrite, superoxide dismutase and catalase, for example], in addition to confirming the translocation of these particles into the animals. This scenario highlights the potential adverse effects resulting from the presence of various pollutants in freshwater ecosystems.

Keywords: Plastic pollution, ecotoxicology, water pollution, biomarkers, toxicity of mixtures.

[1]Advisor: Profa. Dra. Daniela de Melo e Silva. ICB - LabMUT – UFG.

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ABREVIAÇÕES

AChE	Acetylcholinesterase
CAPES	Coordination For The Improvement Of Higher Education Personnel
DDE	1,1-Dichloro-2,2-Bis P-Chlorophenylethylene
DDT	Dichloro Diphenyl Trichloroethane
ENAs	Erythrocyte Nuclear Abnormalities
HO-1	Heme Oxygenase-1
NeE	Percentage Of Necrotic Erythrocytes
NO	Nitric Oxide
NSFC/China	Natural Science Foundation Of China National
PAH	Polycyclic Aromatic Hydrocarbons
PE	Polyethylene
PP	Polypropylene
PS NPs	Polystyrene Nanoparticles
ROS	Reactive Oxygen Species
SD	Standard Deviation
SISBIO	Brazilian Biodiversity Authorization And Information System
ST	Swimming Time
WWTP	Wastewater Treatment Plant
ACh	Acetylcholine
ANOVA	Analysis Of Variance
AOPs	Adverse Outcome Pathways
As	Arsenic
ASG	Amphibian Specialist Group
CAT	Catalase
CNPq	National Council For Scientific And Technological Development
CPE	Chlorinated Polyethylene
Cr	Chromium
DCF-DA	Dichlorofluorescein-Diacetate
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Eosinophil Peroxidase
EVA	Ethylene-Vinyl Acetate
FTIR	Fourier Transform Infrared Spectroscopy
GAA	Conservation Union Global Amphibian Assessment
GPX	Glutathione Peroxidase
H2O2	Hydrogen Peroxide
HMWPE	High
IBR	Integrated Biomarker Response
IP	Intestinal Position Score
KOH	Potassium Hydroxide Solution
LPO	Lipid Peroxidation
MIX	Mistura De Poluentes
MN	Micronucleus
Mn	Manganese
MPs	Microplastics
ND	Not Detected
NI	Not Informed

NO	Nitric Oxide
NO2-	Nitrite
NPs	Nanoplastics
PA	Polyacrylic
PAm	Polyamide Nylon
PAST	Palaeontology Statistic
PB	1,2-Polybutadiene
PBS	Phosphate-Buffered Saline
PCA	Component Analysis
PEI	Polyetherimide
PES	Polyester
PET	Polyethylene Terephthalate
PI	Polyisopren
PS	Polystyrene
PSA	Pressure-Sensitive Adhesives
PU	Polyurethane
PVA	Polyvinyl Alcohol
PVC	Polyvinyl Chloride
PVP	Polyvinylpyrrolidone
RY	Raylon
SBR	Styrene-Butadiene.
SOD	Superoxide Dismutase
SSC	Species Survival Commission
TBARS	Thiobarbituric Acid Reactive Substances
TR	Teeth Row Score
UHMWPE	Ultra-High Molecular Weight Polyethylene
ULMWPE	Ultra-Low Molecular Weight Polyethylene

1 REVISÃO DE LITERATURA

1. Questões ambientais atuais

A partir da publicação de Silent Spring em 1962, a ameaça da poluição ambiental aos ecossistemas e à biota (incluindo humanos) vem chamando a atenção de governos e cientistas do mundo todo (Arp et al., 2023). Nos últimos anos, particularmente, as preocupações relacionadas às questões ambientais veem aumentando na mesma proporção do aumento das atividades humanas – quase sempre vinculadas ao uso não sustentável dos recursos naturais – haja vista os riscos que a poluição do ar, água e solo representam para a saúde humana. Conforme discutido por Egbueri & Enyigwe (2020) e, mais recentemente, por Egbueri et al. (2023), tem sido notório que a industrialização, a urbanização, a densidade populacional, a utilização de recursos naturais e as atividades de mineração aumentaram exponencialmente nos últimos anos. Logo, isso implica diretamente em impactos negativos que afetam aspectos relacionados ao uso das águas, seja pelos humanos, seja por diferentes outros organismos. Estudos vêm confirmando que a disponibilidade de água potável de qualidade reduziu significativamente no mundo, tanto em padrões de qualidade como de quantidade (Javed et al. 2017; Boretti & Rosa, 2019; Rashid et al., 2019; Rao et al., 2021; Mukhopadhyay et al., 2022; Mishra, 2023).

Parte desse problema pode ser associado à diversidade e às crescentes concentrações de poluentes e contaminantes (de variadas fontes – Figura 1) nos mais variados ecossistemas aquáticos (Yu et al., 2024). Nos últimos anos, tem sido dada grande atenção aos contaminantes de preocupação emergente [do inglês, *contaminants of emerging concern* (CECs)] que, em termos gerais, se referem a substâncias não regulamentadas detectadas no ambiente que podem representar um risco para a saúde humana, a vida aquática ou o ambiente, e para as quais a compreensão científica dos riscos potenciais ainda está em evolução (Raman et al., 2023). Os CECs incluem diferentes tipos de produtos químicos e substâncias manufaturadas [e.g., produtos agrícolas, micro(nano)materiais, produtos farmacêuticos, produtos químicos industriais etc.], bem como substâncias que ocorrem naturalmente [e.g., toxinas de algas, microorganismos, hormônios] (Wu et al., 2023; Shi et al., 2023). Em contraste, os poluentes "legislados" (do inglês, *legacy pollutants*) são uma preocupação de saúde pública devido à sua persistência no ambiente, à sua toxicidade para os ecossistemas e biota e ao potencial para transporte de longas distâncias (Souza et al., 2022). Exemplos de poluentes "legislados" incluem agrotóxicos organoclorados, bifenilas policloradas e hidrocarbonetos policíclicos aromáticos (Cantoni et al., 2023).

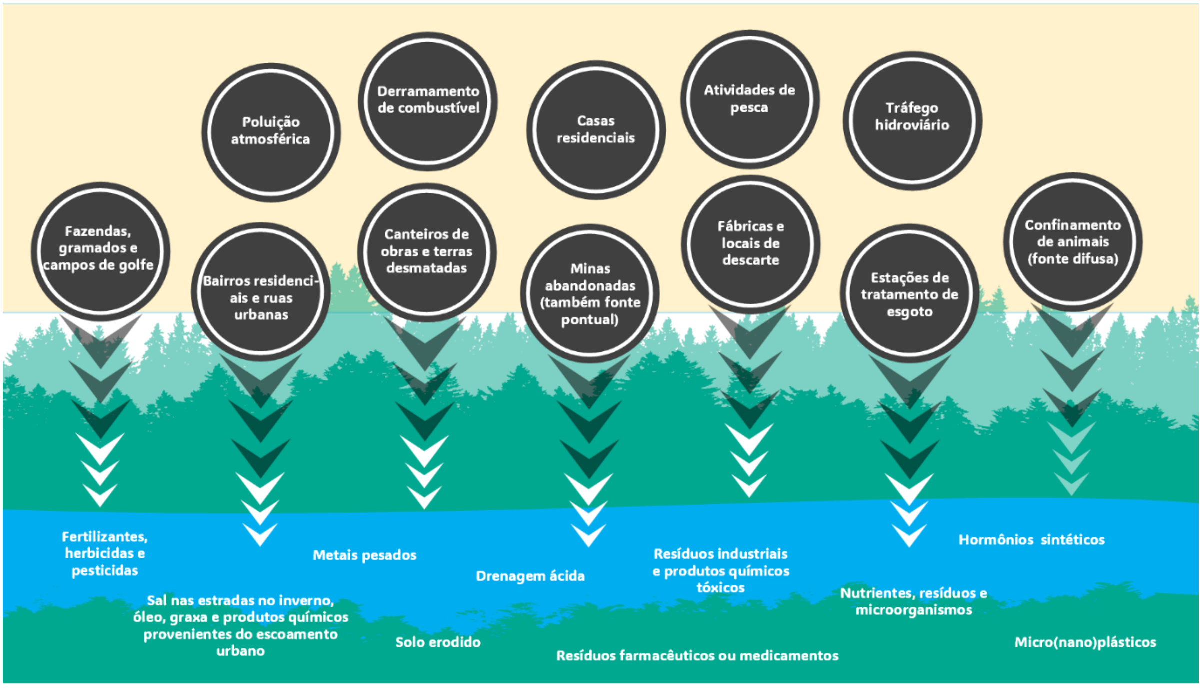

Figura 2. Representação esquemática das diferentes fontes de poluição/contaminação aquática.

Em face disso, tem sido notado um esforço acadêmico-científico para o desenvolvimento de estudos relacionados ao desenvolvimento e proposição de medidas, ações ou tecnologias que visam remediar ou controlar os atuais níveis de poluição. Em termos de remediação da poluição das águas fluviais, conforme destacado por Md-Anawar & Chowdhury (2020), diferentes métodos podem ser aplicados, os quais são categorizados principalmente em técnicas físicas, biológicas, químicas e ecológicas. Entretanto, em muitos casos, a adoção de um único método é insuficiente para a "purificação" de águas fluviais altamente contaminadas/poluídas. Logo, técnicas híbridas, que combinam dois ou mais métodos únicos, são mais amplamente recomendadas (Ugrina & Jurić, 2023).

Enquanto as medidas gerais de remediação física da poluição das águas fluviais incluem aeração artificial, interceptação da poluição, transferência e descarga de água e dragagem de rios (Gunjyal et al., 2023); na remediação biológica os poluentes são removidos ou imobilizados utilizando agentes biológicos (Kanaujiya et al., 2019), incluindo plantas (especialmente das famílias Brassicaceae, Fabaceae, Lamiaceae, Poaceae e Euphorbiaceae) (Boi et al., 2023) e microrganismos [principalmente bactérias (e.g., *Bacillus* e *Rhodobacter*) (Kumar et al., 2023) e fungos (e.g., *Aspergillus* e *Penicillium*)] (Chaurasia et al., 2023). Em contrapartida, o processo de remediação química é realizado pela adição de produtos químicos nas águas poluídas ou pelo uso de adsorventes para alterar o potencial redox, pH, bem como promover a adsorção e precipitação de substâncias suspensas e matéria orgânica na água (Kordbacheh & Heidari, 2023). As principais técnicas empregadas nesse tipo de remediação incluem remediação eletroquímica (Islam, 2023; Brosler et al., 2023), estabilização/solidificação (Tian et al., 2022; Xiong et al., 2022), barreira reativa permeável (Budania & Dangayach, 2023; Singh et al., 2023) e fotocatálise (Friedmann, 2023; Mutalib & Jaafar, 2023). Já a restauração dos ecossistemas fluviais e o tratamento das águas de rios podem ser realizados mediante várias técnicas, como a implementação de zonas ripárias, aeração, processos de sedimentação para a absorção de nitrogênio e fósforo e *wetlands* flutuantes. (Peilin., 2019; Shen et al., 2022).

Apesar dessa gama de possibilidades para o tratamento das águas e remediação de poluentes, a aplicação prática das técnicas atualmente disponíveis esbarra em dificuldades e limitações em termos de eficiência, viabilidade, flexibilidade e requisitos energéticos. Conforme discutido por Ugrina & Jurić (2023), em muitas situações, a baixa eficiência do processamento e os elevados custos operacionais acabam por demandar técnicas alternativas que atendam aos requisitos de eficiência e tempo de remediação. Além disso, a limitação metodológica/analítica atual para identificar e quantificar componentes de misturas complexas de substâncias/compostos químicos (i.e., não detectados por técnicas analíticas rotineiras e convencionais) (Gibson et al., 2019) também constitui um aspecto limitador que precisa ser ultrapassado. A avaliação da eficiência remediadora, seja de qual for o processo/técnica, demanda que sejamos capazes de identificar os poluentes, seja *in situ* ou em nível laboratorial, especialmente ao considerarmos que os sistemas aquáticos recebem insumos químicos de fontes diversas, conforme sumarizado na Figura 1.

Nesse sentido, vários estudos têm sido realizados a fim de contribuir para o desenvolvimento de técnicas de remediação híbrida que, concomitantemente, apresentem alta eficiência, rápida remediação e que utilizem procedimentos mais “amigos do ambiente” (do inglês, *environmentally friendly*) (Bener et al., 2020; Ali et al., 2021; Ahmed et al., 2021; Zhao et al., 2021; Rajpurohit et al., 2022; Rigoletto et al., 2022; Zubair et al., 2022; Zhou et al., 2023; Mukherjee et al., 2023). Entretanto, grande parte das investigações ainda tem sido focada em testes/ensaios conduzidos em laboratórios, o que limita nossa compreensão sobre a adequação, sustentabilidade e aplicabilidade destas técnicas para fins práticos (reais). Conforme discutido por Ugrina & Jurić (2023), a superação das limitações atuais poderá ser alcançada, principalmente, a partir da condução de novos estudos que testem a combinação simultânea ou consecutiva de diferentes técnicas de remediação, que confirmem a viabilidade de técnicas de remediação híbrida em amostras reais e em campo, que utilizem materiais “verdes” (sem afetar o ambiente) e que considerem os custos necessários para suas implementações.

2. Ecotoxicologia: benefícios e limitações

Enquanto a remediação da poluição dos ecossistemas fluviais ainda constitui uma questão desafiadora, a ecotoxicologia aquática emerge como uma ciência que tem provido importante suporte no enfrentamento dos problemas relacionados à contaminação/poluição dos ecossistemas fluviais (Gross, 2022; Connell, 2022; Romero-Blanco & Alonso, 2022; Rosner et al., 2023). Conforme destacado por Olker et al. (2022), as técnicas, instrumentos ou ferramentas de análise utilizados na área auxiliam na predição da toxicidade de uma ampla gama de substâncias ou compostos químicos presentes e dispersos nos mais variados ambientes aquáticos e, assim, sinalizam seus potenciais efeitos ecotoxicológicos, bem como seus possíveis mecanismos de toxicidade. Somado a isso, a ecotoxicologia tem sido considerada essencial na avaliação de impacto ambiental ou em qualquer avaliação ambiental pelas seguintes razões inter-relacionadas que, juntamente com as advertências e precauções fornecidas, contribuem direta ou indiretamente para a mitigação dos problemas ambientais atuais:

- ✓ os testes de toxicidade e de bioacumulação fornecem informações reativas e proativas (Chapman, 1993; Chapman, 1995);
- ✓ os testes ecotoxicológicos fornecem subsídios importantes para a regulamentação e a tomada de decisões baseadas em efeitos (Amiard-Triquet et al., 2015; De-Boeck et al., 2022; Neale et al., 2022). Conforme destacado por Magalhães & Ferrão-Filho (2008), os resultados das análises tradicionais utilizadas no monitoramento da qualidade das águas (e.g., físico-químicas, químicas e microbiológicas), por si só, não retratam o impacto causado pelos contaminantes/poluentes, uma vez que não demonstram os efeitos sobre o ecossistema;
- ✓ ao utilizarem táxons-chave, os ensaios ecotoxicológicos contribuem para a redução das incertezas inerentes às avaliações de risco (Breitholtz et al., 2006; Baun & Grieger, 2022);
- ✓ se forem utilizados parâmetros apropriados, os ensaios de ecotoxicidade ao nível de indivíduo podem fornecer informações sobre a estabilidade de suas populações e dos níveis mais elevados de organização biológica (Beyer et al., 2014);
- ✓ sendo realizados nas condições preditivas e de pior caso, os ensaios ecotoxicológicos podem prover informações para determinar claramente uma conclusão sem impacto significativo (Magalhães & Ferrão-Filho, 2008);
- ✓ testes adequadamente conduzidos e interpretados de forma holística proporcionam a priorização de ações e respostas, incluindo a melhoria dos efeitos, e uma base para o monitoramento (Rosner et al., 2023);

- ✓ e por fim, testes ecotoxicológicos podem ser utilizados tanto antes, quanto após um determinado evento de poluição ter ocorrido. Se realizado antes do evento, tais testes fornecem informações sobre os potenciais efeitos/riscos ecológicos relacionados à introdução de substâncias/compostos nos ambientes naturais. Já se realizados após a ocorrência de eventos poluidores, os testes ecotoxicológicos serão úteis para avaliação do efeito adverso decorrente da introdução dessas substâncias/compostos (Arvidsson et al., 2022).

Nesse sentido, uma vasta gama de estudos ecotoxicológicos tem sido conduzida e provido evidências que confirmam os impactos dos mais variados tipos de CECs e poluentes "legislados" sobre os organismos. Nos peixes de água doce, por exemplo, tais estudos já demonstraram o quanto os agrotóxicos (Kumari, 2020; Rohani, 2023), metais (Kal-Taee et al., 2020; Shahjahan et al., 2022), efluentes industriais (Li et al., 2023; Ishaq et al., 2023), esgotos domésticos (Bhanot & Hundal, 2019), fertilizantes químicos (Nadarajan & Sukumaran, 2021), resíduos farmacêuticos (Godoy et al., 2015; Yang et al., 2020; Porretti et al., 2022) e micro(nano)materiais (Zafar et al., 2023; Yahya et al., 2023) afetam a saúde e a vida desses animais. Similarmente, abordagens ecotoxicológicas também têm sido adotadas na avaliação dos poluentes/contaminantes sobre os anfíbios, as quais alertam para o iminente risco desses animais e de suas populações serem drasticamente afetados caso os níveis de poluição/contaminação não reduzam (Salla et al., 2023; Tsukada et al., 2023; Peluso et al., 2023; De-Almeida & Freitas, 2024).

Por outro lado, assim como em qualquer área, a ecotoxicologia aquática possui desafios a serem superados, especialmente se quisermos aprimorar nossa capacidade de predição do impacto causado pelos poluentes/contaminantes, além de melhor vincular os achados reportados em laboratório aos efeitos reais. O uso de testes de toxicidade aguda (de curto período de exposição – 0 a 96h), por exemplo, pouco contribui para avaliarmos de que maneira a mortalidade aumentará após as exposições, já que, em alguns casos, o efeito adverso pode aparecer apenas depois de um período de latência (Magalhães & Ferrão-Filho, 2008). Ensaios conduzidos com uma única espécie modelo não representa fielmente o que ocorre ou o que poderá ocorrer quando os agentes tóxicos interagirem com os organismos em um contexto de multiespécies (Straub et al., 2020). Nos ambientes naturais, as espécies compartilham habitats e recursos e, assim, as substâncias/compostos podem ser transferidas via cadeia alimentar, ser biomagnificadas e, consequentemente, resultar em níveis de exposição maiores do que os que causam mortalidade dos indivíduos a partir de uma dada concentração na água (Saidon et al., 2024). Além disso, a sensibilidade de um organismo a determinado poluente/contaminante é espécie-dependente (Posthuma et al., 2019; Spurgeon et al., 2020; Van-den-Berg et al., 2021), o que significa que um nível seguro para uma espécie pode não ser para indivíduos de outra espécie que integram a mesma comunidade biológica. Somado a isso, tem sido observado que em muitos estudos ecotoxicológicos o efeito dos poluentes/contaminantes tem sido avaliado apenas em um determinado estágio de desenvolvimento da espécie (Magalhães & Ferrão-Filho, 2008). Conforme discutido por Mohammed (2013), a sensibilidade ou tolerância das espécies aos estressores ambientais (incluindo poluentes/contaminantes) é dependente do estágio de desenvolvimento, sendo – geralmente – as fases embrionária, larval e juvenil de uma determinada espécie mais sensíveis do que a fase adulta.

Por outro lado, a abordagem ascendente adotada por muitos ecotoxicologistas — i.e., investigar os efeitos tóxicos no nível de suborganismo e/ou organismo para extrapolar para os níveis de populações e comunidades — precisa ser analisada com cautela (Segner, 2007). Isso se justifica principalmente em razão do efeito dos poluentes/contaminantes não se propagar linearmente. Contrariamente, deve-se ponderar que em cada nível da hierarquia biológica novas propriedades podem surgir, as quais não são previsíveis a partir das propriedades do nível inferior (Figura 2). Há 25 anos atrás, Segner & Braunbeck (1998) já instigavam reflexões sobre essa questão ao demonstrar que um efeito celular tóxico não conduz necessariamente a uma resposta tóxica do organismo, considerando a existência de mecanismos compensatórios a nível supracelular.

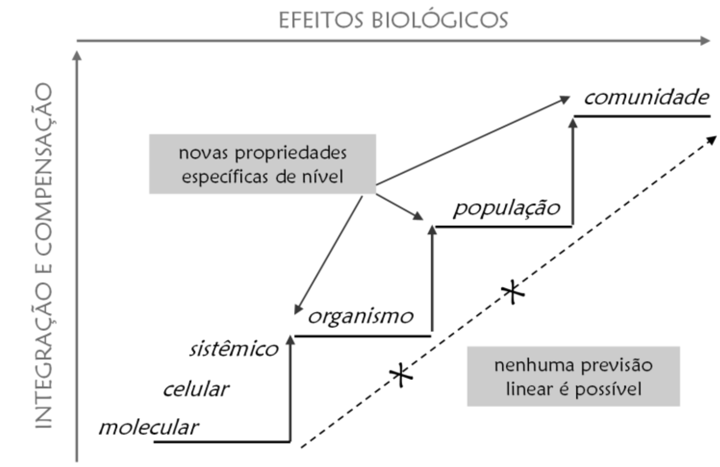

Figura 2. Representação de uma curva que mostra que a propagação dos efeitos tóxicos ao longo dos níveis da hierarquia biológica não segue uma forma linear e determinística. Adaptado de Segner (2007).

Portanto, emerge dessas limitações o consenso atual de que os ensaios ecotoxicológicos devem ser conduzidos a partir de abordagens mais realísticas, de modo que as avaliações realizadas em laboratórios permitam extrapolações mais seguras sobre “o que” e “como”, de fato, os poluentes/contaminantes afetam a biota. Muitos ecotoxicologistas ainda têm concentrado esforços na avaliação dos efeitos de poluentes ou contaminantes específicos (i.e., isolados) adotando concentrações ou doses muito superiores às encontradas no meio ambiente. Conforme discutido por Souza et al. (2018), a priorização de estudos que não representem situações reais ou que investiguem os efeitos de um único poluente/contaminante na biota, negligencia o fato de que uma grande diversidade de substâncias ou compostos (oriundos de fontes múltiplas – Figura 1) são encontradas no meio ambiente. Dessa forma, os organismos estão sujeitos à exposição a um verdadeiro "coquetel". Além disso, deve-se considerar que determinadas combinações de poluentes podem interagir e produzir efeitos tóxicos diferentes daqueles atribuídos a um tipo específico de substância ou composto.

2.1. Ecotoxicidade de misturas

Uma das formas de contribuir para que a ecotoxicologia aquática gere subsídios mais práticos e realísticos é investir em estudos que identifiquem e caracterizem os efeitos dos poluentes/contaminantes utilizando abordagens que superam os esforços centrados em produtos químicos individuais. Na revisão de Kabir et al. (2015), os autores reforçam essa discussão ao demonstrarem que a exposição dos organismos a um único produto químico (ainda que em baixas concentrações ou doses) reflete em efeitos diferentes quando combinados com a gama de substâncias ou compostos presentes e dispersos nos ambientes.

Nesse contexto, um campo de pesquisa em ascensão refere-se à toxicidade da combinação entre os microplásticos (MPs) e diversos outros CECs e/ou poluentes “legislados”. Definidos como partículas plásticas [< 5 mm – (Betts, 2008; Fendall & Sewell, 2009; Hidalgo-Ruz et al., 2012)], os MPs podem provir da fragmentação de objetos maiores descartados no ambiente ou podem adentrar os sistemas fluviais como partículas inicialmente sintetizadas já em pequenos diâmetros (Li et al., 2018; Wang et al., 2022; Wang et al., 2023), presentes, por exemplo, em pellets de resinas plásticas e em forma de *microbeads* utilizadas em produtos de higiene e cuidados pessoais (Fendal & Sewell, 2009).

Embora os MPs tenham sido identificados na década de 1970, o conhecimento sobre seus efeitos em organismos de água doce (especialmente vertebrados) ainda é limitado quando comparado ao conhecimento que temos sobre sua presença e efeitos no ambiente marinho (Li et al., 2023). Aliado a essa carência, outra questão desafiante refere-se à contribuição toxicológica desses poluentes na toxicidade de misturas de substâncias ou compostos químicos que podem estar presentes nos ambientes aquáticos. Estudos mais recentes têm demonstrado que os MPs podem associar a vários outros poluentes/contaminantes [vide revisões de Mei et al. (2020), Xiang et al. (2022), Yang et al. (2022) e Sun et al. (2023)], cujas consequências ecotoxicológicas são ainda desconhecidas para a grande maioria dos organismos aquáticos ou terrestres. Conforme discutido por Rist & Hartmann (2018), devido às suas dimensões e propriedades químicas, a associação dos MPs a outras substâncias químicas pode afetar a mobilidade e a disponibilidade dos xenobióticos no ambiente natural e, em um cenário pessimista, pode favorecer a entrada dos poluentes nos organismos, exacerbando seus efeitos prejudiciais nos organismos.

Na Tabela 1, podemos notar que a toxicidade da combinação dos MPs com outros poluentes, em peixes, tem sido avaliada – nos últimos três anos (2021 a 2023) – a partir, majoritariamente, de combinações binárias envolvendo compostos orgânicos [e.g., Zhang et al. (2021a), Zhang et al. (2021b), Xu et al. (2021), He et al. (2021), Wang et al. (2022a), Li et al. (2022a) e Sim et al. (2023)], medicamentos [e.g., Huang et al. (2021), Li et al. (2022a), Zhang et al. (2023a), Liao et al. (2023), Banaee et al. (2023a), Yu et al. (2023), Shi et al. (2023), Banaee et al. (2023b) e Zhao et al. (2023)], metais [e.g., Santos et al. (2021b), Zhang et al. (2022), Chen et al. (2022), Hoseini et al. (2022), Wang et al. (2021), Soliman et al. (2023), Wei et al. (2023), Yang et al. (2023), Hu et al. (2023)] e agrotóxicos [e.g., Hanachi et al. (2021), Karbalaei et al. (2021), Lajmanovich et al. (2022), Montero et al. (2022), Wang et al. (2023a), Zhag et al. (2023b)]. Obviamente, esses estudos têm contribuído enormemente para a ampliação do conhecimento sobre os riscos que os poluentes/contaminantes combinados com os MPs representam para a vida dos organismos. Em alguns desses estudos tem sido reportado um efeito sinérgico entre os MPs e os poluentes/contaminantes [e.g., Xu et al. (2021), Yu et al. (2023) e Banaee et al. (2023ab)], efeito aditivo [e.g., Liao et al. (2023) e Zhang et al. (2023a)], antagônico [e.g., Hu et al. (2023) e Yang et al. (2023)] e em alguns a toxicidade isolada dos componentes, quando misturados, não foi alterada [e.g., Santana et al. (2022)].

Tabela 1. Sumário de alguns estudos conduzidos entre 2021 e 2023 que deram enfoque na avaliação da toxicidade de combinações binárias entre os microplásticos e diversos outros poluentes/contaminantes.

Referências	**Composição química polimérica dos plásticos**	**Tamanho dos MPs (diâmetro)**	**Concentrações dos MPs**	**Poluentes ou contaminantes testados**	**Concentrações dos poluentes ou contaminantes**	**Período de exposição**	**Espécies modelos**	**Fase de vida**
Santos et al. (2021a)	Polímero de composição não reportada	1–5 µm	2 mg/L	Sulfato de cobre pentahidratado	60 µg/L e 125 µg/L	14 dias	*Danio rerio*	2 hpf a 14 fpf
Zhang et al. (2021a)	Polietileno	100-150 µm	10 mg/L e 40 mg/L	9-nitroantraceno	5 µg/L e 500 µg/L	4, 7 e 21 dias	*Danio rerio*	Adultos
Santos et al. (2021b)	Polímero de composição não reportada	1-5 µm	2 mg/L	Cobre	60 e 125 µg/L	14 dias	*Danio rerio*	Embriões
Huang et al. (2021)	Poliestireno	5 µm	10 µg/L	sulfametoxazol (SMX) e o β-bloqueador propranolol (PRP)	50 µg/L	14 dias	*Oreochromis niloticus*	11 meses de idade
Zhang et al. (2021b)	Poliestireno	1 µm	20 µg/L	Fosfato de trifenil	20 e 100 µg/L	7 dias	*Oryzias melastigma*	Larvas
Xu et al. (2021)	Poliestireno	150 µm	3 mg/L	Fenantreno	0,2 mg/L	12 e 24 dias	*Danio rerio*	Adultos
He et al. (2021)	Poliestireno	5,8 µm	2 mg/L	Fosfato de trifenil	80 µg/L	21 dias	*Danio rerio*	Adultos
Wang et al. (2022a)	Poliestireno	10 µm	2, 20 e 200 µg/L	Bifenilas policloradas	500 ng/L	21 dias	*Paralichthys olivaceus*	Juvenis

Tabela 1. *Continuação.*

Referências	Composição química polimérica dos plásticos	Tamanho dos MPs (diâmetro)	Concentrações dos MPs	Poluentes ou contaminantes testados	Concentrações dos poluentes ou contaminantes	Período de exposição	Espécies modelos	Fase de vida
Karbalaei et al. (2021)	Poliestireno	21,89 a 466,7 µm	30 ou 300 µg/L	Clorpirifós	2 ou 6 µg/L	96 h	*Oncorhynchus mykiss*	Juvenis
Hanachi et al. (2021)	Poliestireno	21,89 a 466,7 µm	30 ou 300 µg/L	Clorpirifós	2 ou 6 µg/L	96 h	*Oncorhynchus mykiss*	Juvenis
Wang et al. (2021)	Poliestireno	80 nm e 0,5 µm	200 µg/L	Cádmio	50 µg/L	24, 48 e 96 h	*Channa argus*	Juvenis
Wang et al. (2022b)	Poliestireno	2 µm	2, 20, 200 µg/L	17α-etinilestradiol	10 ng/L	28 dias	*Oryzias melastigma*	3 meses de idade
Santana et al. (2022)	Polietileno	64-125 µm	0,1, 1,0 e 10,0 mg/L	Hidróxidos duplos lamelares de Cu/Al	0.33, 1.0 e 3.33 mg/L	3 horas	*Solea senegalensis*	Larvas
Montero et al. (2022)	Polipropileno	0,7-1mm	Razão de MPs/zooplâncton de 0,1 em peso fresco	Diclorodifenildicloroetileno Clorpirifós Benzofenona-3	1000 ng/g 100 ng/g 300 ng/g	60 dias	*Dicentrarchus labrax*	Juvenis
Li et al. (2022a)	Poliestireno	9–10 µm 50 –70 µm	0,1, 1, 10, 50 e 100 mg/L	Difenoconazol	1,30–1,74 mg/L	4-8 dias	*Danio rerio*	Adultos
Zhang et al. (2022)	Poliestireno	0,1 µm/100 nm	1 mg/L	Cobre	0,5, 1, e 2 mg/L	14 dias	*Oreochromis niloticus*	Juvenis
Chen et al. (2022)	Poliestireno	5,0 µm	500 µg/L	Cádmio	5 µg/L	30 dias	*Danio rerio*	Embriões

Tabela 1. *Continuação.*

Referências	Composição química polimérica dos plásticos	Tamanho dos MPs (diâmetro)	Concentrações dos MPs	Poluentes ou contaminantes testados	Concentrações dos poluentes ou contaminantes	Período de exposição	Espécies modelos	Fase de vida
Li et al. (2022a)	Poliestireno	13 µm	2, 20 e 200 µg/L	Fenantreno	50 µg/L	60 dias	*Oryzias melastigma*	Fêmeas adultas e embriões
Hoseini et al. (2022)	Cloreto de polivinila	140 µm	0,5 mg/L	Cobre	0,25 mg/L	14 dias	*Cyprinus carpio*	Juvenis
Lajmanovich et al. (2022)	Polietileno	40-48 µm	60 mg/L	Glifosato e glufosinato de amônio	1,56, 3,12, 6,25, 12,5, 25, 50 e 100 mg/L	48 h	*Scinax squalirostris*	Girinos - 26 a 30G
Zhang et al. (2023a)	Poliestireno	2 µm	0,44 mg/L	Amitriptilina	2,5 µg/L	7 dias	*Danio rerio*	5 meses de idade
Sim et al. (2023)	Poliestireno	0,2, 1,0, e 10 µm	20 µg/mL	Benz[a]antraceno	1, 5, e 10 ppm	96 hpf	*Danio rerio*	Larvas
Liao et al. (2023)	Poliestireno	10 µm	10 µg/L	Tetraciclina	50 µg/L	96 hpf	*Oryzias melastigma*	Adultos
Wang et al. (2023a)	Polietileno	5 µm	1 mg/L	Acetocloro	0,10, 0,20, 0,40, 0,80, 1,60 mg/L e 0,05 mg/L	30 dias	*Danio rerio*	Adultos
Banaee et al. (2023a)	Polietileno	15–25 µm	1000 e 2000 mg/Kg	Enrofloxacina	1.35 e 2.7 ml/Kg	96 h e 21 dias	*Oncorhynchus mykiss*	-
Zhag et al. (2023b)	Poliestireno	200 nm	0.5 mg/L	Trifenilestanho	1 µg/L	21 dias	*Cyprinus carpio*	Juvenis
Yu et al. (2023)	Polietileno	50 – 300 nm e 10 – 200 µm	100 µg/L	Oxitetraciclina	100 µg/L	42 dias	*Danio rerio*	6 meses de idade

Tabela 1. *Continuação.*

Referências	Composição química polimérica dos plásticos	Tamanho dos MPs (diâmetro)	Concentrações dos MPs	Poluentes ou contaminantes testados	Concentrações dos poluentes ou contaminantes	Período de exposição	Espécies modelos	Fase de vida
Banaee et al. (2023b)	Polietileno	200–250 µm	100 µg/L, 200 µg/L	Nanopartículas de óxido de zinco	50 µg/L	14 dias	*Gambusia holbrooki*	-
Shi et al. (2023)	Poliestireno	2–4 µm	440 µg/L	Amitriptilina	2.5 µg/L	21 dias	*Danio rerio*	5 meses de idade
Soliman et al. (2023)	Polietileno	-	100 mg/L	Chumbo	1 mg/L	15 dias	*Clarias gariepinus*	-
Wei et al. (2023)	Poliestireno	100 ± 0.4 nm	1 mg/L	Cádmio	5 mg/L	96 h e 21 dias	*Carassius carassius*	Juvenis
Yang et al. (2023)	Polipropileno	5.12-398 µm	10 mg/L e 100 mg/L	Boro	30 mg/L e 70 mg/L	21 dias	*Oreochromis niloticus*	Juvenis
Wang et al. (2023b)	Poliestireno	80 nm e 8 µm	700 µg/L	Androstenediona	1 µg/L	48 h e 96 h	*Gambusia affinis*	Adultos
Hu et al. (2023)	MPs cloreto de polivinila	6.5 µm	0.2 mg/L e 2 mg/L	Chumbo	250 µg/L	21 dias	*Cyprinus carpio*	-
Zhao et al. (2023)	Poliestireno	10 µm	0.6–1.2 × $10^8/m^3$	Tetraciclina	50 µg/L	28 dias	*Oryzias melastigma*	Adultos

Aparte dos processos e interações que explicam os resultados reportados nas investigações supramencionadas, é inegável que esses estudos têm alterado o direcionamento da ecotoxicologia aquática, mostrando uma clara tendência para a adoção de abordagens mais realísticas e passíveis de maiores extrapolações para o contexto real. Entretanto, ainda são muito incipientes as pesquisas com foco na toxicidade de misturas mais complexas e diversificadas, a exemplo dos trabalhos de Freitas et al. (2022) e Freitas et al. (2023), nos quais a toxicidade de peptídeos do SARS-CoV-2 combinados com um mix de poluentes/contaminantes foi avaliada em larvas de *Cloeon dipterum* e adultos de *D. rerio*, respectivamente. Enquanto nas larvas nenhum efeito aditivo, sinérgico ou antagônico foi observado a partir da mistura dos componentes da mistura (Freitas et al., 2022), a exposição de *D. rerio* a fragmentos virais, associados ao mix de poluentes/contaminantes, induziu uma toxicidade mais proeminente quando comparada àquela proveniente da exposição aos componentes isolados (Freitas et al., 2023).

Deste modo, esse cenário reforça a iminente necessidade de avançarmos nas pesquisas voltadas à ecotoxicidade de misturas, especialmente em razão da poluição dos ecossistemas fluviais por metais (Muhammad & Usman, 2022; Liu et al., 2023), surfactantes (Al-Ani et al., 2020; Lobotková et al., 2023), compostos fenólicos (Ramos et al., 2021; Wang et al., 2023), petróleo (Edori e Edori, 2021; Rahimi-Moazampour et al., 2023), resíduos farmacêuticos (Quincey et al., 2022; Castaño-Ortiz et al., 2024), agrotóxicos (Kalantary et al., 2022; Sultan et al., 2023), produtos de cuidados pessoais (Liu et al., 2021), microplásticos (Talbot & Chang, 2022), estar sendo cada vez mais documentada.

Assim, questiona-se se as novas propriedades necessárias para a tecnologia e inovação, como a restrição espacial de propriedades eletrônicas (i.e., mudanças nas propriedades elétricas de materiais devido a seu tamanho) e a alta área superficial específica dos micromateriais podem causar novos e adicionais efeitos ambientais e biológicos. Ou esses micromateriais podem atuar como remediadores da poluição, diminuindo a mobilidade e disponibilidade dos variados xenobióticos nas águas e, por conseguinte, seus efeitos negativos sobre a biota? A compreensão dessas questões pode contribuir para o direcionamento de estratégias específicas de remediação e mitigação dos impactos causados por esses micromateriais.

Portanto, é importante destacar que investigar os efeitos isolados desses micropoluentes e, sobretudo em conjunto com aqueles que podem ser encontrados em águas superficiais, constitui uma questão atual, relevante e que nos permite aproximar os estudos laboratoriais a um contexto ambiental mais realista.

Além disso, a avaliação dos efeitos da exposição de organismos de água doce a uma variedade de compostos químicos, fornece informações que não apenas podem servir como base para orientar a atuação das agências reguladoras e aprimorar o monitoramento da qualidade da água, incluindo a elaboração de normas ambientais, definição de valores guia e padronizações, bem como estratégias de remediação e mitigação de impactos. Essas contribuições não se limitam apenas ao âmbito local ou nacional, mas também têm o potencial de contribuir para o cumprimento das metas estabelecidas na agenda global de desenvolvimento sustentável.

3 OBJETIVOS

3.1. Objetivo geral

Nesse sentido, o principal objetivo deste estudo foi investigar os impactos potenciais da exposição ao microplástico de polietileno, tanto isoladamente quanto em combinação com diversas substâncias e compostos químicos nocivos, sobre os vertebrados aquáticos.

3.2. Objetivos específicos

a) Realizar uma revisão cientométrica e sistemática da produção científica atual acerca dos efeitos das partículas plásticas na saúde dos anfíbios;

b) Avaliar se a exposição de *Danio rerio* (zebrafish) e girinos de *Physalaemus cuvieri* ao microplástico polietileno – isolados ou em associação a distintos poluentes emergentes – induz alterações nos biomarcadores avaliados, partindo da hipótese de que a co-exposição potencializaria os efeitos observados;

c) Investigar possíveis alterações mutagênicas em girinos de *P. cuvieri* após 30 dias de exposição, bem como analisar parâmetros morfológicos, comportamentais, respostas antioxidantes e atividade colinesterásica, os quais podem ser associados com eventual absorção e acúmulo de microplásticos nos animais;

d) Investigar possíveis efeitos adversos a partir de biomarcadores de genotoxicidade, mutagenicidade e de desequilíbrio redox em zebrafish adulto (*Danio rerio*), após 15 dias de exposição aos poluentes e/ou contaminantes;

e) E por fim, avaliar as contribuições toxicológicas específicas dos microplásticos quando são parte integrante de uma mistura de xenobióticos para organismos que compõem a biota aquática.

4 ESTRUTURA DA TESE

Nesse contexto, essa tese foi subdivida em três capítulos principais. No primeiro capítulo apresentamos e discutimos o estado do conhecimento científico (até o ano de 2020) envolvendo anfíbios e partículas plásticas. Esse levantamento deu origem ao artigo "Micro(nano)plastics as an emerging risk factor to the health of amphibian: A scientometric and systematic review", publicado no periódico *Chemosphere*, aceito em junho de 2021, disponível em: https://doi.org/10.1016/j.chemosphere.2021.131090.

A escassez de dados disponíveis sobre a toxicidade de partículas plásticas em anfíbios e ausência de estudos envolvendo a co-exposição de microplásticos com outros poluentes emergentes, resultaram nos dois capítulos seguintes.

O capítulo dois refere-se ao artigo "Toxicity assessment of polyethylene microplastics in combination with a mix of emerging pollutants on *Physalaemus cuvieri* tadpoles", publicado no periódico *Journal of Environmental Sciences*, aceito em maio de 2022, disponível em https://doi.org/10.1016/j.jes.2022.05.013. Esse artigo teve como objetivo avaliar a possível toxicidade dos microplásticos de polietileno (sozinhos ou em combinação com uma mistura de poluentes) sobre a saúde de girinos de *Physalaemus cuvieri*.

O terceiro capítulo corresponde ao artigo "Toxicity evaluation of the combination of emerging pollutants with polyethylene microplastics in zebrafish: Perspective study of genotoxicity, mutagenicity, and redox unbalance", publicado no periódico *Journal of Hazardous Materials*, aceito em março de 2022, disponível em: https://doi.org/10.1016/j.jhazmat.2022.128691. O artigo teve como objetivo testar a hipótese de que a associação de microplásticos de polietileno a uma mistura de poluentes emergentes induziria mais efeitos adversos em adultos de *Danio rerio* expostos por um período considerado subcrônico. Esse trabalho foi desenvolvido durante a disciplina de Oficinas em Análise Ambiental, sob supervisão do prof. Dr. Thiago Lopes Rocha, sendo pré-requisito para a aprovação na disciplina a submissão e/ou publicação do artigo em revista científica.

CAPÍTULO I

MICRO(NANO)PLASTICS AS AN EMERGING RISK FACTOR TO THE HEALTH OF AMPHIBIAN: A SCIENTOMETRIC AND SYSTEMATIC REVIEW[1]

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Micro(nano)plastics as an emerging risk factor to the health of amphibian: A scientometric and systematic review

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ABSTRACT

Although the toxicity of microplastics (MPs) and nanoplastics (NPs) is recognized at different trophic levels, our know-how about their effects on amphibians is limited. Thus, we present and discuss the current state on studies involving amphibians and plastic particles, based on a broad approach to studies published in the last 5 years. To search for the articles, the ISI Web of Science, ScienceDirect, and Scopus databases were consulted, using different descriptors related to the topic of study. After the systematic search, we identified 848 publications. Of these, 12 studies addressed the relationship "plastic particles and amphibians" (7 studies developed in the laboratory and 5 field studies). The scientometric analysis points to geographic concentration of studies in Brazil and China; low investment in research in the area, and limited participation of international authors in the studies carried out. In the systematic approach, we confirm the scarcity of available data on the toxicity of plastic particles in amphibians; we observed a concentration of studies in the Anura order, only one study explored the toxicological effects of NPs and polystyrene, and polyethylene are the most studied plastic types. Moreover, the laboratory tested concentrations are distant from those of the environmentally relevant; and little is known about the mechanisms of action of NPs/MPs involved in the identified (eco)toxicological effects. Thus, we strongly recommend more investments in this area, given the ubiquitous nature of NPs/MPs in aquatic environments and their consequences on the dynamics, reproduction, and survival of species in the natural environment.

Keywords: emerging pollutants, vertebrates, freshwater environment, tadpoles, frog, plastic pollution.

1. INTRODUCTION

Amphibians are one of the most endangered animal groups (Beebee and Griffiths, 2005; Green et al., 2020). The decline and disappearance of their populations have been part of scientific discussions for some time, especially since the late 1980s (Wake, 1991, 1998). However, more recently, concerns about their risk of mass extinction have increased considerably (Green et al., 2020; Bolochio et al., 2020). The latest report from the World Conservation Union Global Amphibian Assessment (GAA-2004) indicated that up to a third of the cataloged species have suffered severe declines or extinction (Stuart et al., 2004) and that neotropical species, in particular, that inhabit streams and lentic environments are even more threatened. Currently, researchers have been working on updating this report (2019–2020) and the prospects are even more discouraging (GAA, 2021). The challenge for the initiative led by the IUCN Species Survival Commission (SSC) Amphibian Specialist Group (ASG) is to update all 6260 species cataloged and evaluated in the GAA report, version 2004, and their updates for 2006 and 2008, along with more than 1700 species that were not evaluated in the first version of this report. Although habitat loss is considered a major cause of the amphibian decline crisis, more recent research has linked the effects of UV-B irradiation (Lundsgaard et al., 2020; Morison et al., 2020), the emergence of emerging diseases (Blaustein et al., 2018; Ruthsatz et al., 2020; Fisher and Garner, 2020; Brannelly et al., 2021), increased introduction of non-native species (Nunes et al., 2019), climate change (Bucciarelli et al., 2020), as well as the increase in water pollution (Wesner et al., 2020; Meindl et al., 2020; Lent et al., 2020) with the factors that drastically threaten amphibians.

These studies increase knowledge about the impact of anthropogenic activities on the decline of amphibians. However, studies focused on the effects of pollutants on the biology of these animals have been dedicated, in their majority, to the identification of the bioaccumulation and impacts caused by classic chemical compounds. These include pesticides and their degradation products, heavy metals, nitrogen-based fertilizer, among others [see review by Blaustein et al. (2003)]. Moreover, being fewer those that address the impacts of emerging pollutants, such as chemicals (drugs, cosmetics and personal care products), micro- and nanomaterials [see review by McConnell et al. (2010), Egea-Serrano et al. (2012) and Amaral et al. (2019)]. Conceptually, emerging pollutants include a vast list of chemicals (synthetic or natural) that are not part of the list of those included in environmental monitoring programs (national or international); but which have great potential to enter various environmental compartments and cause ecological and/or human health effects (Geissen et al., 2015; Calvo-Flores et al., 2018).

Among the emerging pollutants, microplastics (MPs) and nanoplastics (NPs) can be considered as major emerging pollutants worldwide (Kwach and Shikuku, 2020). Such materials are transported and dispersed in the most varied environmental compartments (Du et al., 2021; Li et al., 2021). The persistence, high stability, ubiquity and its comprehensive and negative effects on biota and the functioning of ecosystems, are the main reasons for the increase in current concerns about global plastic pollution. Conceptually, as highlighted by the Panel on Contaminants in the Food Chain (CONTAM) (EFSA, 2016), there is no internationally recognized definition of microplastics. Commonly, "microplastics" are defined by most authors as a heterogeneous mixture of differently shaped materials referred to as fragments, fibers, spheroids, granules, pellets, flakes or beads whose longest diameter is < 5 mm. This the upper limit is generally accepted because this size can include a range of small particles that can be readily ingested by organisms (Wagner and Lambert, 2018). On the other hand, nanoplastic particles are defined as pieces of plastic between 1 and 100 nm in diameter (Mendoza et al., 2018; Sorensen and Jovanović, 2021). In addition, MPs/NPs can be classified into primary and secondary plastic particles. While primary MPs/NPs are those that were originally manufactured to be that size, secondary MPs/NPs originate from fragmentation of larger items (e.g.: plastic debris) (Sorensen and Jovanović, 2021).

Recent reviews have gathered compelling information about how much these particles threaten the health of terrestrial (Boyle and ¨Ormeci, 2020; Wong et al., 2020; Wu et al., 2020; Dioses-Salinas et al., 2020; Qi et al., 2020; Zhou et al., 2020; Huang et al., 2020; Xu et al., 2020a, 2020b; Zhang et al., 2021), marine (Coyle et al., 2020; Sana et al., 2020; Pirsaheb et al., 2020; Xu et al., 2020c; Wang et al., 2021; Ng et al., 2021) and freshwater ecosystems (Boyle and ¨Ormeci, 2020; Li et al., 2020; Naqash et al., 2020; Peller et al., 2020; Yang et al., 2021; Miloloza et al., 2021; Darabi et al., 2021). In these studies, there is a consensus that, unlike microplastics, MPs and NPs can translocate through tissues of different organs and cause damage through physical, chemical, and biological changes, at different biological organization levels.

However, our knowledge of how MPs and NPs affect the health of freshwater organisms is much more limited than in marine organisms (both in the number of research conducted and in the number of species investigated) (Wagner et al., 2014; Eerkes-Medrano et al., 2015; Wagner and Lambert, 2018; Anbumani and Kakkar, 2018; Ma et al., 2019; Xu et al., 2020c). The most studied groups were microcrustaceans and fish (Rezania et al., 2018; Triebskorn et al., 2019; Collard et al., 2019; Sorrentino et al., 2020; Wang et al., 2020a, 2020b). In general, these studies have shown that exposure to MPs and NPs cause a wide range of toxic effects from producers to consumer trophic level, such as feeding disruption, reproductive efficiency, physical adsorption, energy metabolism disruption, changes in liver physiology, synergistic, and antagonistic activity of certain persistent organic pollutants [e.g.:, polycyclic aromatic hydrocarbons (PAH) (Chen et al., 2019), polychlorinated biphenyls (PCBs) (Yeo et al., 2020) and organochlorine pesticides [such as dichloro diphenyl trichloroethane (DDT), 1,1-dichloro-2,2-bis (p-chlorophenyl)ethylene) (DDE), chlordane, heptachlor, endosulfan, aldrin, dieldrin, and endrin, among others], indicating "Trojan Horse Effects" [see more details in Rodrigues et al. (2019) and Zhang and Xu (2020)].

Therefore, studies to identify, evaluate and characterize the ecotoxicological impacts of MPs and NPs on amphibians are scarce. The knowledge concerning the extent to which these pollutants are acting insidiously with other driving forces (such as deforestation, burning, UV radiation, emerging diseases, etc.) that have caused the global amphibian decline phenomenon, depends on studies that assess their impacts on the biology of these animals. The way of life of these animals is, in particular, an aspect that makes them highly vulnerable to plastic pollution. While eggs, larvae, and tadpoles remain in streams, ponds, and swamps for up to months; after their complete metamorphosis, juveniles are often dispersed from their home environments and start to occupy environments with characteristics very different from those where they developed and grew up (Chelgren et al., 2006; Petranka et al., 2007; Szekely et al., 2020). As adults, many species of anurans and urodele amphibians migrate seasonally between terrestrial (where they take refuge and/or forage) and aquatic (where they breed) habitats (Muths et al., 2018; Holmes et al., 2020; Zhu et al., 2020; Zheng et al., 2020). Therefore, as highlighted by Becker et al. (2007), the quality of the environment, both in and around breeding sites, is an important determinant of the persistence of amphibians in their natural habitats. However, this evidence has not been sufficient to leverage studies involving MPs/NPs and these animals, which categorizes them as a group severely underrepresented in ecotoxicological research (Green et al., 2020). Thus, the current study provides a comprehensive review on the impacts of plastic particles (both MPs and NPs) in amphibian health, from a scientometric and systematic approach. It provides an in-depth discussion on bioaccumulation and both short- and long-term effects of MPs/NPs, seeking to establish a basic understanding of the toxic effects of these pollutants on this animal group. Furthermore, the data about the experimental conditions, such as exposure time, concentrations, specific physiological responses, amphibians' species and MPs/ NPs properties are discussed, as well as significant research gaps and recommendations for future research are indicated, that remained poorly covered by the recently published critical reviews on similar topics.

Thus, the current study provides a comprehensive review on the impacts of plastic particles (both MPs and NPs) in amphibian health, from a scientometric and systematic approach. It provides an in-depth discussion on bioaccumulation and both short- and long-term effects of MPs/NPs, seeking to establish a basic understanding of the toxic effects of these pollutants on this animal group. Furthermore, the data about the experimental conditions, such as exposure time, concentrations, specific physiological responses, amphibians' species and MPs/ NPs properties are discussed, as well as significant research gaps and recommendations for future research are indicated, that remained poorly covered by the recently published critical reviews on similar topics.

2. METHODOLOGICAL APPROACH

2.1. General design

This review was conducted on the ISI Web of Science, ScienceDirect, and Scopus databases (Fig. 1). The keywords "microplastics", "nanoplastics" and "microbeads" have been combined with "amphibian", "tadpole", "frog", "toads", "salamanders" and "caecilians", in both singular and plural. Studies that did not fit the objectives of this review, as well review articles, duplicate publications were excluded from the research. After, titles and abstracts were selected by individual reading (or the full text in some cases), ensuring that the results were relevant to the subject of the study.

For the scientometric evaluation, different indicators were used [defined based on Vinkler (2010)], which included (i) number of publications and growth trend; (ii) contributing countries [geographical distribution (corresponding author's location)], and (iii) study funding agencies. The information regarding the indicators (i) authors; (ii) cited reference count; (iii) times cited, WoS core; (iv) times cited (all databases), (v) 180 days usage count; (vi) since 2013 usage count, and (vii) publisher were obtained from ISI Web of Science (on 31 Dec. 2020).

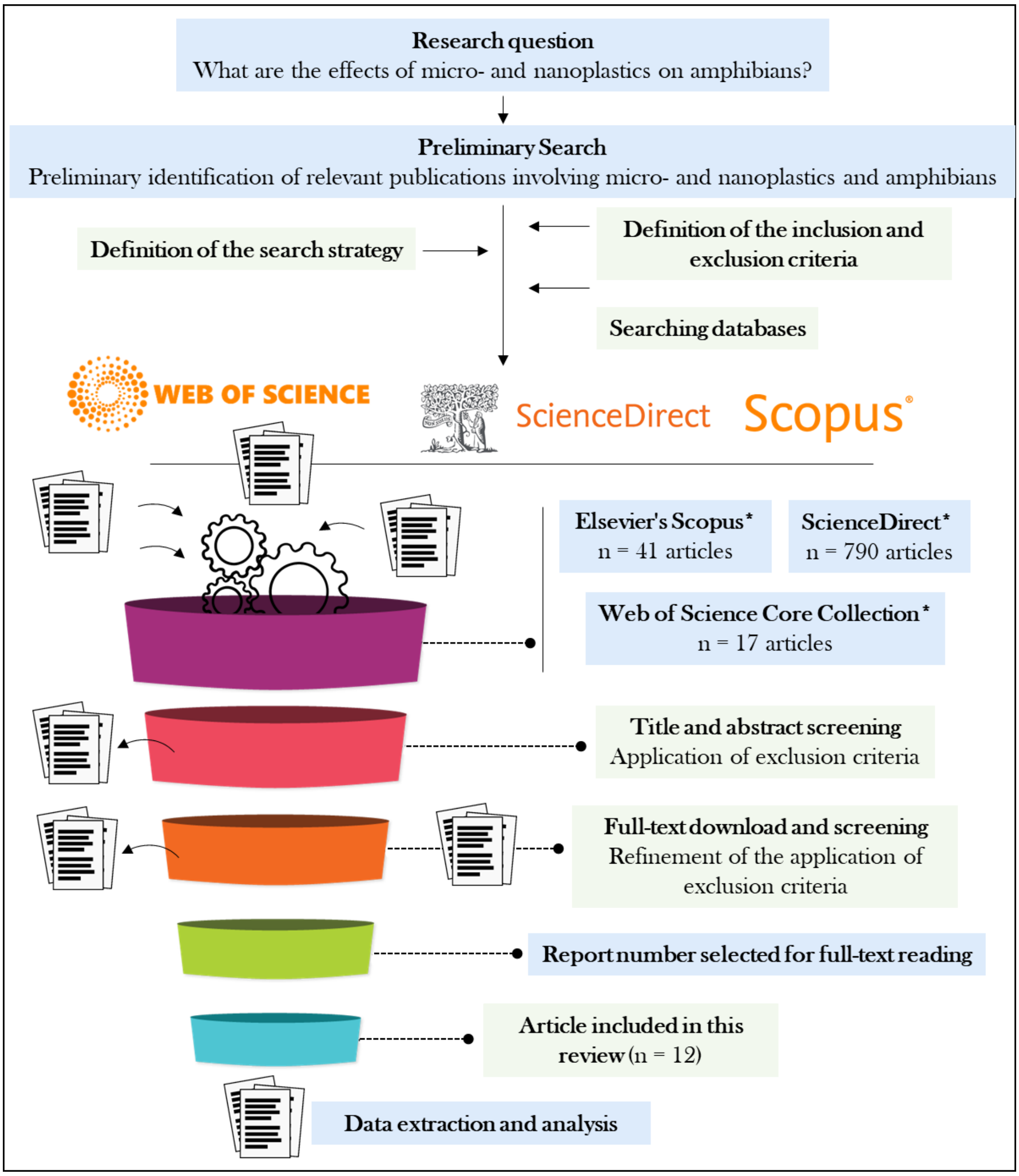

Fig. 1. The research methodology used to find and analyze studies concerning the ecotoxicity of micro(nano)plastics in amphibians.

Regarding the specific content of the articles, the summary information included plastic particle properties, amphibian species, and experimental conditions, methods used to assess possible biological damage and the main findings of each study. Particularly, concerning the pollutants, the following parameters were extracted: (i) particle type; (ii) particle origin (commercial or environmental); (iii) size and shape; (iv) tested concentrations; (v) method of quantifying and processing the samples and (vii) staining. The experimental design adopted by each study was evaluated based on information about (i) taxonomic classification of the individuals studied; (ii) developmental stage of the animals at the beginning of experiments or field evaluations [according to Gosner (1960)]; (iii) sample number and replicates; (iv) exposure conditions (such as time and route); (v) exposure system (static or semi-static) and (vi) and type of research (laboratory or field). Besides, we recorded the biomarkers used and the main conclusions of the studies. Information on the conservation status of the species was obtained by consulting the IUCN Red List of Threatened Species™ (Version 2021-1 - https://www.iucnredlist.org) and that concerning the anatomy, physiology, habitats, and geographic distribution of the species studied were obtained from specific publications available in the literature.

2.2. Data analysis

The numerical data were analyzed using descriptive statistics (including means, standard deviations, frequencies, and percentages) and data on number publications evaluated in this review, were subjected to linear regression analysis. GraphPad Prism Software Version 7.0 (San Diego, CA, USA) was used to perform the statistical analysis and create the graphs.

3. RESULTS

3.1. Growth trend analysis and article metrics

After the systematic search for articles in the different selected databases, we identified 41 publications in Scopus, 790 in ScienceDirect, and 17 in the Web of Science Core Collection (total of 848 publications). After following the steps presented in Figure 1, we identified 12 studies [from 2015 (year of first registration) to December 2020] that addressed the relationship "plastic particles and amphibians" (7 studies developed in the laboratory and 5 field studies), which corroborates the hypothesis that this area has been very little explored. Applying the criteria defined in our study, we observed that the first study concerning the ecotoxicological impact of plastic particles on amphibian was conducted by Tusselino et al. (2015), which were the only ones that used a NP in the experimental design. The authors studied the effects of exposing *Xenopus laevis* to 50-nm-uncoated polystyrene nanoparticles (PS NPs), via contact exposure or microinjections. At the time, it was reported that the embryos mortality rate is dose dependent and that the survived embryos showed high percentage of malformations (e.g.: display disorders in pigmentation distribution, malformations of the head, gut and tail, edema in the anterior ventral region, etc.). In addition, the authors reported changes in the expression of different mesoderm markers, such as *bra*, *myod1*, and of neural crest marker *sox9* genes.

However, the largest registration of studies occurred in 2020 (Fig. 2A), which is equivalent to almost 58.4% of the publications found, which may justify the low metric indexes of the evaluated publications, such as the number of times that the full texts of articles were accessed or saved in the last 180 days and the frequency with which the articles were cited in other publications of the Web of Science and other databases (Fig. 2B). Also, we observed that the journal diversity was small, with the Journal Hazardous Materials and Chemosphere being those where we identified the largest number of publications (50% of the articles evaluated) (Fig. 2C). The average of its impact factor, reported by the Journal Citation Reports (2019), was equal to 4.345 ± 1.245 (mean ± SEM) (from 2.132 to 9.038) and the most frequent publishers were Springer and Elsevier (Fig. 2C).

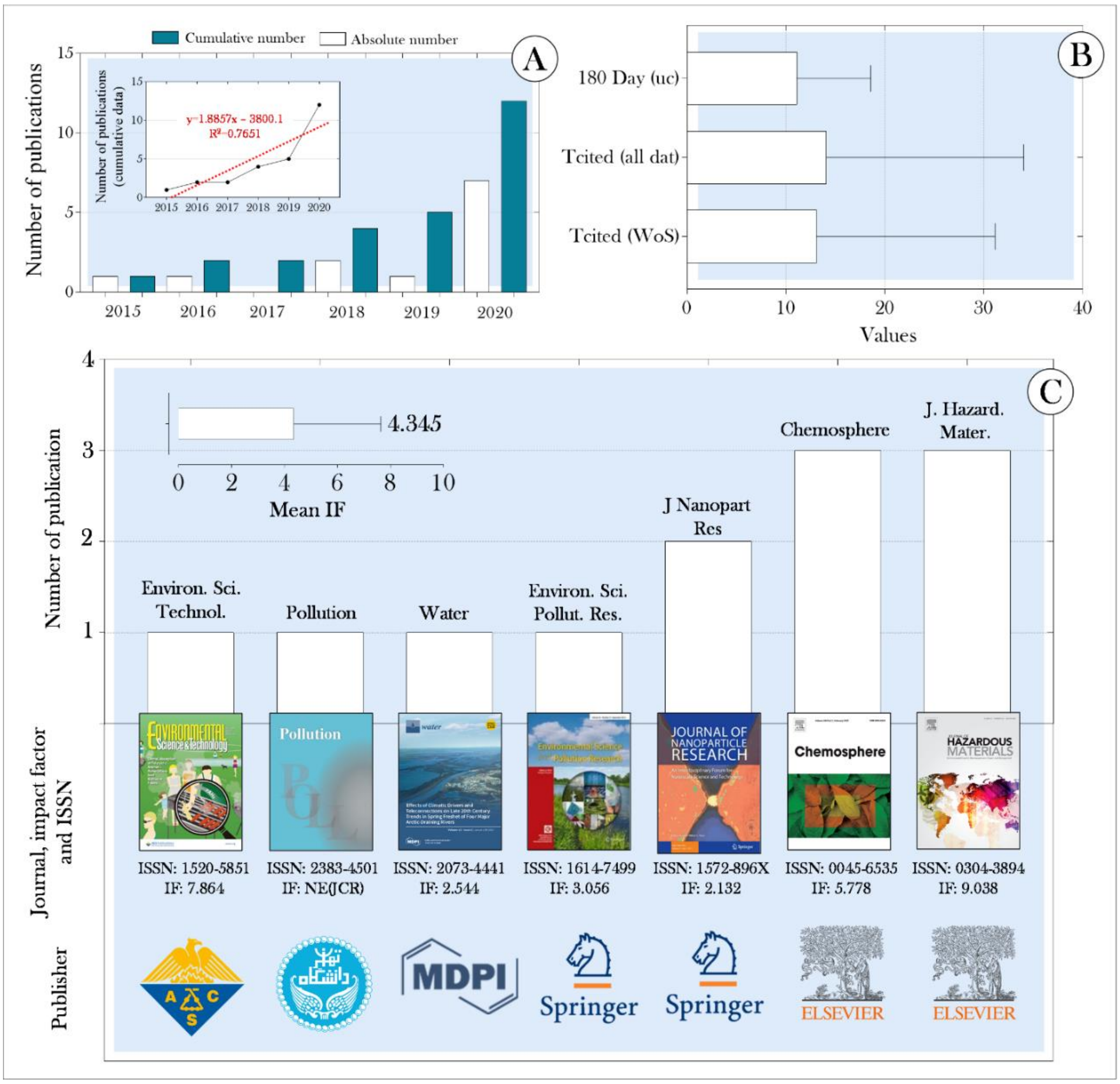

Fig. 2. (A) Number, (B) metrics, and (C) information about the publications evaluated in this review, including journal name and publishers. In "B" and "C" (in the upper left graph), the bars represent the mean + standard deviation. ACS: American Chemical Society; (دانشگاه تهران): Tehran University; MDPI: Multidisciplinary Digital Publishing Institute. IF: impact factor; ISSN: International Standard Serial Number. Environ. Sci. Technol: Environmental Science & Technology (https://pubs.acs.org/journal/esthag); Pollution (https://jpoll.ut.ac.ir/); Water (https://www.mdpi.com/journal/water); Environ. Sci. Pollut. Res.: Environmental Science and

Pollution Research (https://www.springer.com/journal/11356); J Nanopart. Res.: Journal of Nanoparticle Research (https://www.springer.com/journal/11051); Chemosphere (https://www.journals.elsevier.com/chemosphere), and J. Hazard. Mater.: Journal of Hazardous Materials (https://www.journals.elsevier.com/journal-of-hazardous-materials).

3.2. Geographic distribution, funding, and research centers

Our data also demonstrate that few countries (n = 7) have contributed so far to the publication of studies involving the theme of this review, with Brazil and Italy (25%), followed by China (16.6%) being those in which the largest number of research has been carried out (Fig. 3A). However, less than half of the studies received funding from any funding agency or institution, with the National Council for Scientific and Technological Development (CNPq/Brazil) and the Natural Science Foundation of China National (NSFC/China) (both in the public sector), those that invested the most in studies involving plastic particles and amphibians (Fig. 3B).

We identified that 52 authors (approximately 4 authors/article) contributed to the field of toxicity of plastic particles in amphibians, coming from 23 institutions, with Instituto Federal Goiano (Brazil) (n = 3; 25% of publications) and East China Normal University (China) (n = 2; 16.6% of publications) those linked to the largest number of publications (Fig. 4A). The University of L'Aquila (Italy) was the institution with the highest number of authors (n = 8; which is equivalent to 15.3% of the total) (Fig. 4B). In Fig. 4C–E we can see the top 10 authors who presented, at the time of the research, the largest number of publications and H-index (respectively) and the variations of these parameters, considering all authors, are shown in Fig. 4D–F (respectively) [H-index: 13.08 (mean) ± 1.54 (SEM) and 9.5 (median); number of publications: 48.92 (mean) ± 10.00 (SEM) and 22 (median)].

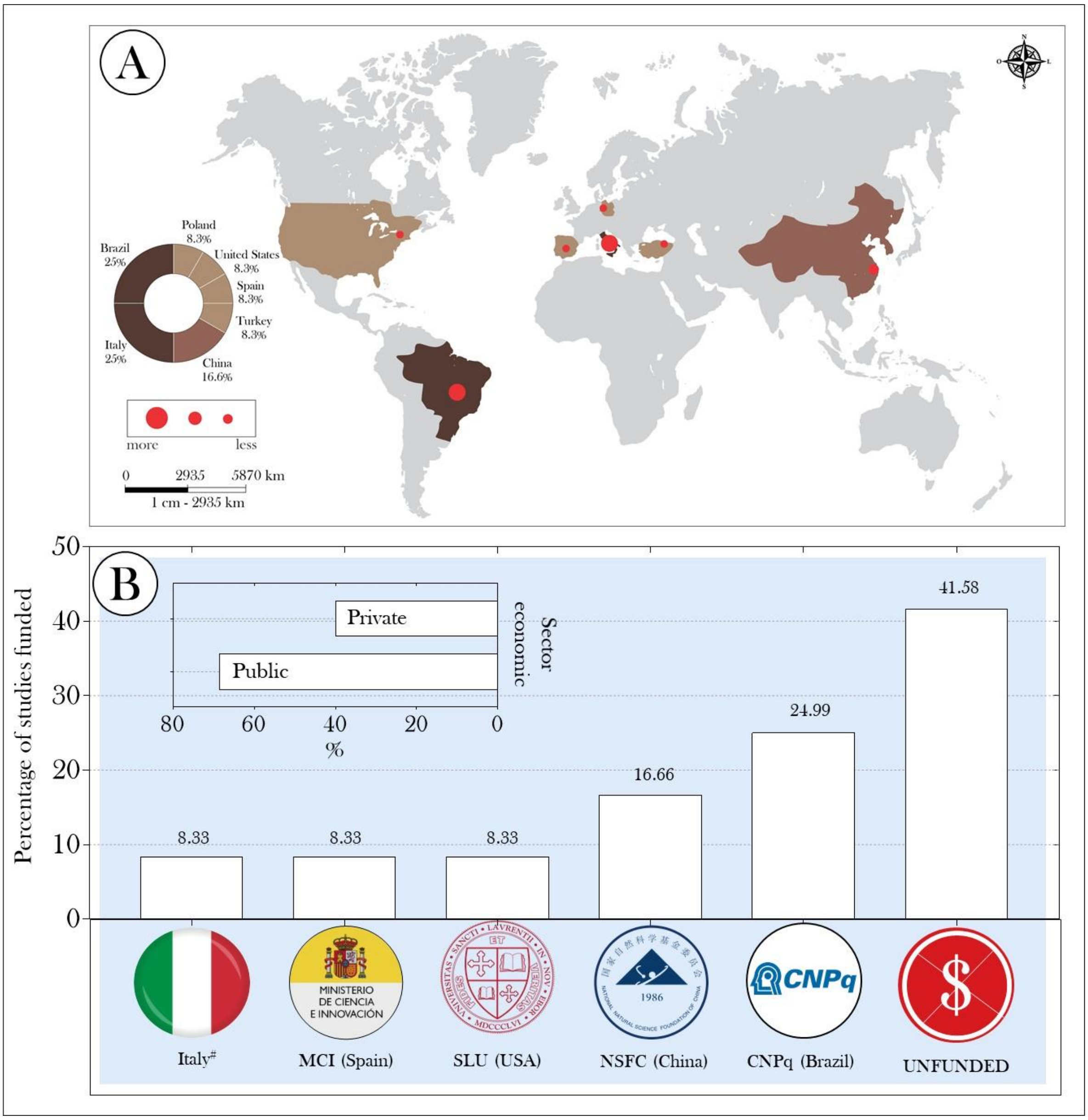

Fig. 3. (A) Worldwide distribution of articles concerning the ecotoxicity of micro(nano)plastics in amphibians and (B) percentage of studies funded. MSI: Ministry of Science and Innovation; SLU: St. Lawrence University; NSFC: National Natural Science Foundation of China; CNPq: National Council for Scientific and Technological Development; UNFUNDED: unfunded studies; #: This work was supported by the Grant FARO (Finanziamento per l'Avvio di pROgetti Speciali) and by the departmental research funding (Project: A10113.CRRDI; F.S.2.18.03). Numbers above the bars indicate the corresponding percentages.

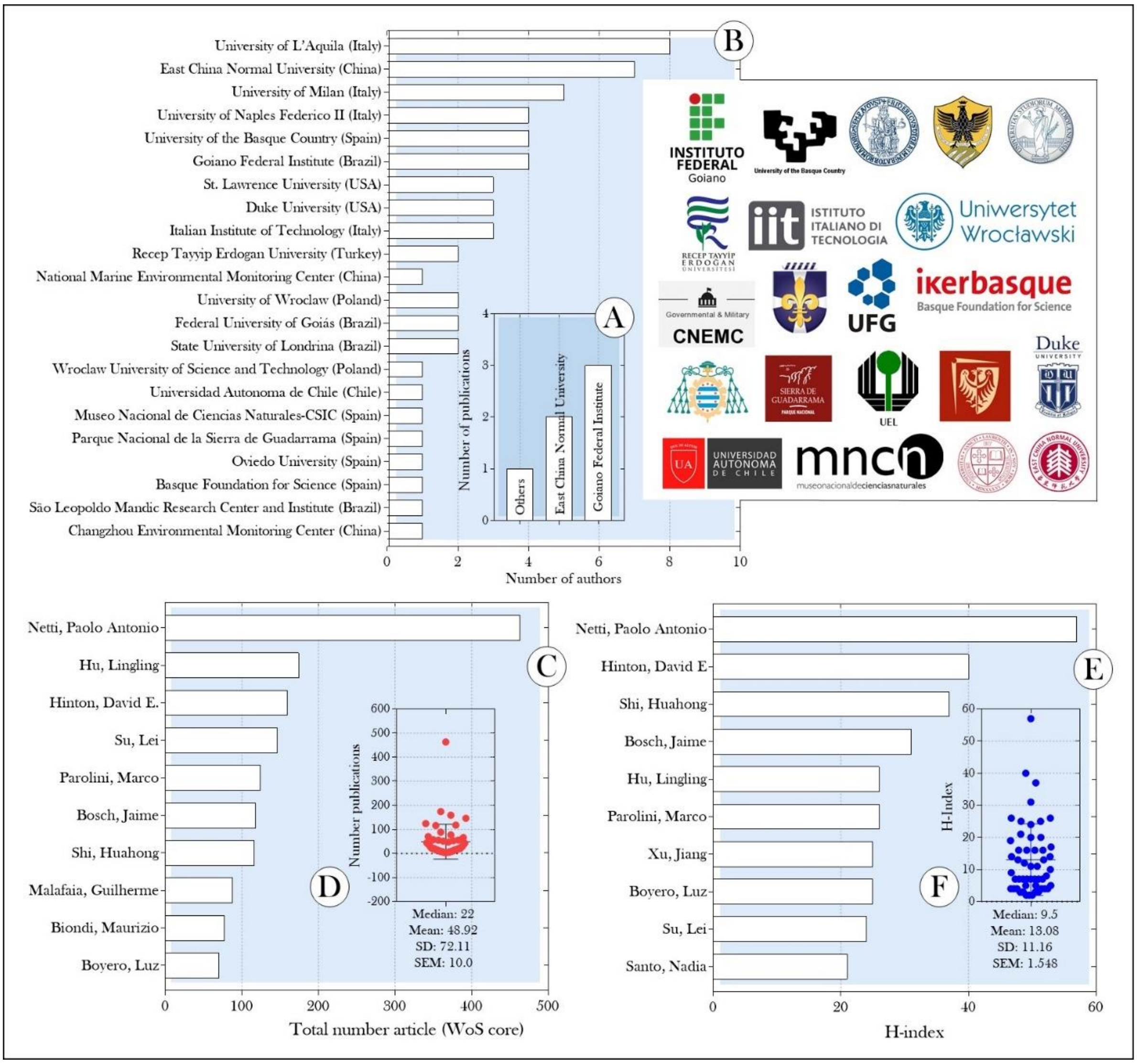

Fig. 4. (A) Institutions and their (B) logos to which the authors of articles concerning the ecotoxicity of micro (nano) plastics in amphibians are linked. (C–F) 10-top authors regarding the number of publications and H-index, respectively. The results presented in "A and B" refer to the institutional bond of all authors (n = 52) identified in the analyzed papers. Dr. Netti, Paolo Antonio.: University of Naples Federico II (Italy); Dr. Hu, Lingling: Sun Yat-sen University (China); Dr. Hinton, David E: Duke University (United States); Dr. Su, Lei.: East China Normal University (China); Dr. Parolini, Marco: Università degli Studi di Milano (Italy); Dr. Bosch, Jaime: University of the Basque Country (UPV/EHU) (Spain); Dr. Shi, Huahong: East China Normal University (China); Dr. Malafaia, Guilherme: Federal Institute Goiano (Brazil); Dr. Biondi, Maurizio: University of L'Aquila (Italy); and Dr. Boyero, Luz: University of the Basque Country (UPV/EHU) (Spain).

3.3. Amphibian species

The ecotoxicological impact of plastic particles was reported for 21 amphibian species (Fig. 5). In laboratory studies, MPs (n = 6 species) were more studied compared to NPs (n = 1 species). The species *Physalaemus cuvieri* (Anura, Leptodactylidae) and *Xenopus laevis* (Anura: Pipidae) being the most prevalent in the publications (25% and 16.6% of the articles, respectively). Although all species have had their conservation status classified as “least concern (LC)”, 33.3% currently have a population decline, according to the IUCN Red List of Threatened Species (*Alytes obstetricans*, *Hyla arborea*, *Lithobates pipiest*, *Pelobates fuscus*, *Pelophylax nigromaculatus*, *Rana macrocnemis* and *Triturus carnifex* – Fig. 5). A population growth trend has been observed only for *Pelophylax ridibundus* and *X. laevis* (i.e., 9.5% of species studied) (Table 1).

On the other hand, we observed that the studied species, taken together, have a wide geographical distribution, covering different tropical and subtropical continents (Fig. 6). However, the number of species studied, when compared to the number of species already cataloged [total 8273 amphibian species (Jan 21, 2021) https://amphibiaweb.org/amphibian/speciesnums.html], represents a tiny portion (approximately 0.241%). While no species of the order Gymnophiona were addressed in the studies evaluated, the order Anura had a higher prevalence (91.66% of studies), whose percentage concerning the total of species cataloged in this order does not exceed 0.3% (Fig. 7). As for the Urodela, only one species (*Triturus carnifex*) was identified in the studies evaluated, which corresponds to approximately 0.13% of the total species already cataloged in that order (Fig. 7).

Fig. 5. Representative images of amphibian species addressed in studies on the impact of micro(nano) plastics. All images have Creative Commons Licenses.

Table 1. General information about the amphibian species studied.

Species	Common name	Current population trend	IUCN status	References
Alytes obstetricans	Common Midwife toad	↓ Decreasing	LC	Bosch et al. (2009)
Anaxyrus americanus	American toad	— Stable	LC	AmphibiaWeb (2012)
Bufo bufo	Common toad	— Stable	LC	Agasyan et al. (2009)
Bufo gargarizans	Asiatic toad	— Stable	LC	AmphibiaWeb (2012a)
Fejervarya limnocharis	Asian grass frog	— Stable	LC	van Dijk et al. (2004)
Hyla arborea	European tree frog	↓ Decreasing	LC	Kaya et al. (2009)
Lithobates palustris	Pickerel frog	— Stable	LC	Fenolio et al. (2005)
Lithobates pipiens	Northern leopard frog	↓ Decreasing	LC	Griego (2015)
Lithobates septentrionalis	Mink frog	— Stable	LC	AmphibiaWeb (2001)
Microhyla ornata	Ant frog	— Stable	LC	Dutta et al. (2008)
Pelobates fuscus	Common spadefoot toa	↓ Decreasing	LC	Agasyan et al. (2009)
Pelophylax esculentus	Edible frog	NE	NE	NE
Pelophylax nigromaculatus	Black-spotted pond frog	↓ Decreasing	LC	Kuzmin et al. (2004)
Pelophylax ridibundus	Marsh frog	↑ Increasing	LC	Kuzmin et al. (2009)
Physalaemus cuvieri	Barker frog	— Stable	LC	Mijares et al. (2010)
Rana clamitans	Green frog	— Stable	LC	ASG (2015)
Rana macrocnemis	Bruna frog	↓ Decreasing	LC	Kuzmin et al. (2009)
Rana temporaria	European common brown frog	— Stable	LC	Kuzmin et al. (2009)
Triturus carnifex	Italian crested newt	↓ Decreasing	LC	Romano et al. (2009)
Xenopus laevis	African clawed frog	↑ Increasing	LC	AmphibiaWeb (2019)
Xenopus tropicalis	Tropical clawed frog	— Stable	LC	AmphibiaWeb (2010)

NE: Not evaluated. The species has not been evaluated by the IUCN criteria.

3.4. Ecotoxicological impact of micro(nano)plastics in amphibians

Initially, we registered the types of plastic particles that were part of the experimental design or that were identified in the evaluated studies, as can be seen in Fig. 8. In the laboratory studies, the particles studied were those of polyethylene (PE) and polystyrene (PS), the latter being the prevalent type (57.1%) (Fig. 8A). In the field studies, we observed a wide variety of plastics (19 types), with PE, nylon (polyamide), and polypropylene (PP) particles being more prevalent, which correspond to 16%, 12%, and 8% (respectively) of the total variety of MPs identified in tadpoles (Fig. 8B). Schessl et al. (2019) not identified plastic particles in the animals evaluated. Regarding the particle size, we observed that in studies conducted in the laboratory, the diameter varied from 50 nm to 35.46 µm and, in field studies, this variation was even greater (from 0.1 mm to 36.4 mm). As already pointed out in this article, only by the study Tussellino et al. (2015) addressed the toxicity of particles in nanometric size (i.e.: 50-nm-uncoated polystyrene nanoparticles (PS NPs), using the Frog Embryo Teratogenesis Assay-*Xenopus* test during the early stages of larval development of *Xenopus laevis*), which shows the lack of studies on the toxicity of NPs in amphibians (Table 2).

We also observed a wide variation in the particle concentrations used in the animals' exposures under laboratory conditions, as well as those identified in the tadpoles (by field studies), both numerically and with the units of measurement used. In studies conducted in the laboratory, concentrations were expressed in "mg/L" and "particles/mL", ranging from 0.125 to 60 mg/L (15.05 ± 7.86 mg/L; mean ± SEM) and from 0.1 to 10^5 particles/mL (1471 x 10^4 ± 1421 x 10^4 particles/mL; mean ± SEM), respectively (Figure 8C-D). In the field studies, the number of particles identified in amphibians ranged from 2.44 to 306.7 items/g (94.44 ± 43.37; mean ± SEM) (expressed in "items/g wet weight") and 0.2 to 18 items/individuals (3.13 ± 1.7 items/individual) (expressed in "items/individuals") (Figure 8E-F, respectively). As for the exposure period adopted in the studies conducted in the laboratory, the average was 65.68 ± 31.1 h (mean ± SEM), ranging from 1 h to 336 h (14 days). One of the studies (De Felice et al., 2018) did not specify the exposure time, emphasizing that the animals were exposed to the treatments of 36-37 stages until they reached 46 stages, according to the classification proposed by Nieuwkoop & Faber (1994).

In the studies conducted in the laboratory, we also evaluated the characteristics of the experimental design, having observed that the number of animals per group varied according to the objectives of the study. On average, 67.33 animals/group (including tadpoles or embryos) were used (Fig. 9A), with the studies by Araujo et al. (2020a, b) and Tussellino et al. (2015) those who used the smallest (20 tadpoles; *P. cuvieri*) and the largest (n = 144 embryos; *X. laevis*) number of individuals/groups, respectively. Only in the study by Boyero et al. (2020) the number of individuals used in each evaluated group was not identified. However, in more than half of the studies, the design did not have experimental replicates/group and, in those that adopted replicas, the average was 2.7 replicates/group (Fig. 9B). In the field studies, the mean of tadpoles analyzed was 135.3 ± 94.24 (mean ± SEM) (in three publications) and of adult individuals (in two publications) was 136 ± 105 (mean ± SEM). Regarding the developmental stage of the animals evaluated, none of the studies conducted in the laboratory used adult individuals and, in most of these publications, tadpoles were evaluated in stages 25–29, according Gosner (1960) and Faber and Nieuwkoop (2020) (specific classification for *X. laevis*) (Fig. 9C). On the other hand, field studies that evaluated tadpoles did not specify the stage of larval development. In this case, the authors standardized the samples using the length and biomass of the animals collected in different sample sites.

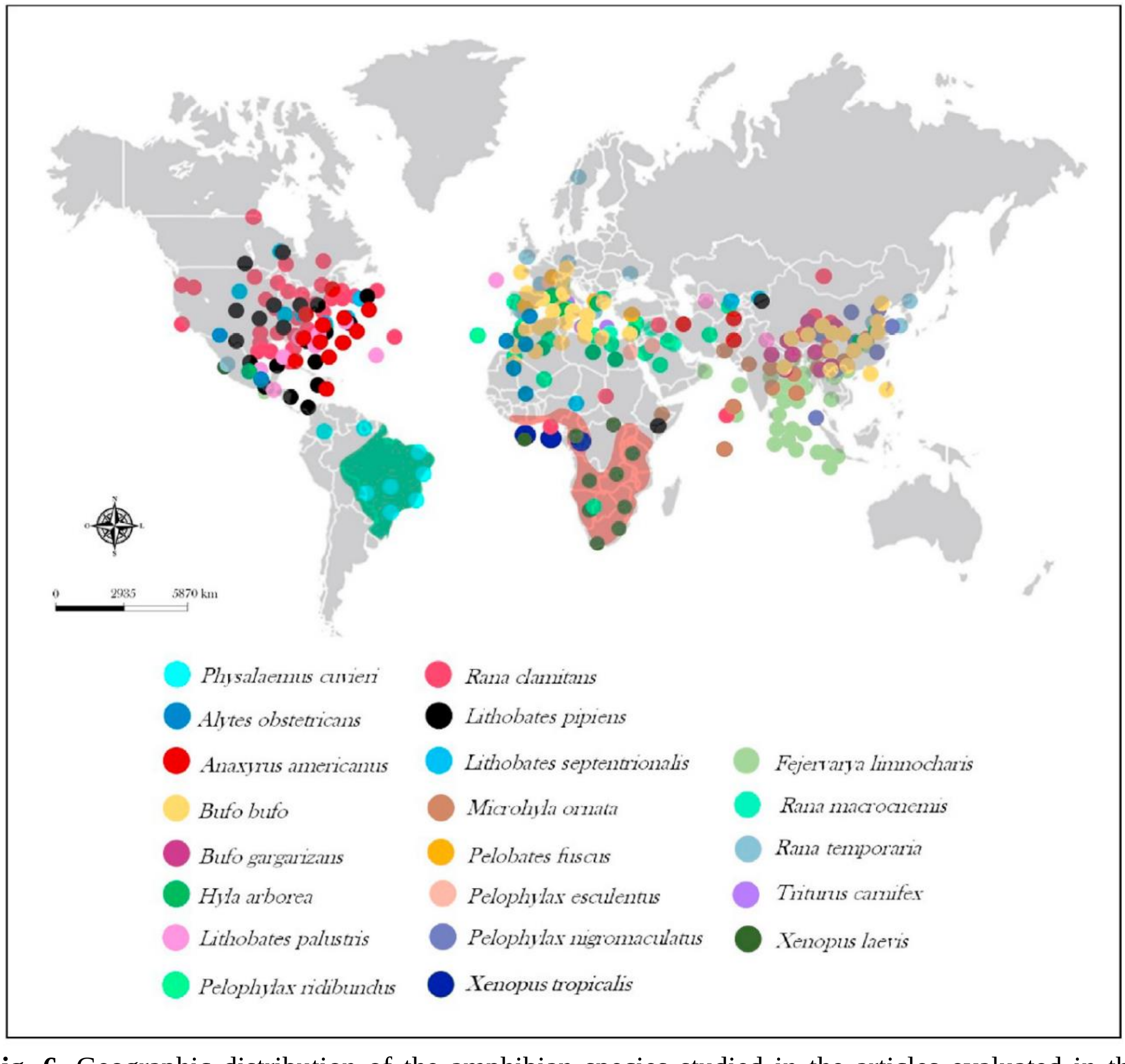

Fig. 6. Geographic distribution of the amphibian species studied in the articles evaluated in this review. The green and red areas indicate the distribution of the species *P. cuvieri* e *X. laevis*,

respectively. Source of information: https://amphibiaweb.org/. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

We also observed that the uptake and/or accumulation of plastic particles were addressed in all studies analyzed (in the field and laboratory), and in the research conducted in the laboratory, such parameters were associated with different toxicity biomarkers (Fig. 10A). In these studies, assessments of body morphology, behavioral effects and mortality rate in tadpoles exposed to microplastics were more frequent (i.e.: 57.14%, 28.57% and 28.57% of laboratory studies analyzed in our study) (Fig. 10A). Predictive biomarkers of teratogenicity, mutagenicity, cytotoxicity, as well as histopathological, genetic, and dietary changes were less addressed in the studies (14.28% of the studies/each). The study by Tussellino et al. (2015) - who evaluated the effects of PS NPs on *X. laevis* - was the one who explored the largest number of biomarkers/ parameters in the evaluation of the toxicity of the experimental model used (Fig. 10B). On the other hand, in the field studies, the evaluations were restricted to the uptake and/or accumulation of MPs in the collected amphibians (Fig. 10A), with this parameter not being associated with any toxicity biomarker. Table 3 presents details of the results obtained in the evaluation of the biomarkers evaluated and in Table S1, complementary information about the articles analyzed in our study are provided.

4. DISCUSSION

4.1. Historical review, growth trend and contributing countries analysis

Initially, from a scientometric analysis involving the toxicity of micro (nano)particles in amphibians, we register different indicators that confirm the lack of research in this area and, therefore, our limited understanding of how these pollutants can affect this animal group, as well as the still incipient impact of the publications analyzed. The low number of articles identified in the databases consulted (n = 12, until 31 Dec. 2020) complements the information made available in similar reviews, which already warned of the scarcity of studies involving the effects of plastic microparticles in some groups of vertebrates, including amphibians (De Sá et al., 2018; Granek et al., 2020). Therefore, we know little about the contributions of these pollutants to the population decline of these animals and how they can intensify the most varied stressful events that have disturbed the balance of their natural populations, contrasting them with the numerous studies that have been dedicated to identifying and understanding the impacts of MPs in other groups of freshwater animals, such as fish and some micro-and macroinvertebrates (Boyle & Örmeci, 2020; Li et al., 2020; Ma et al., 2020; Naqash et al., 2020; Peller et al., 2020; Yang et al., 2021; Miloloža et al., 2021; Darabi et al., 2021).

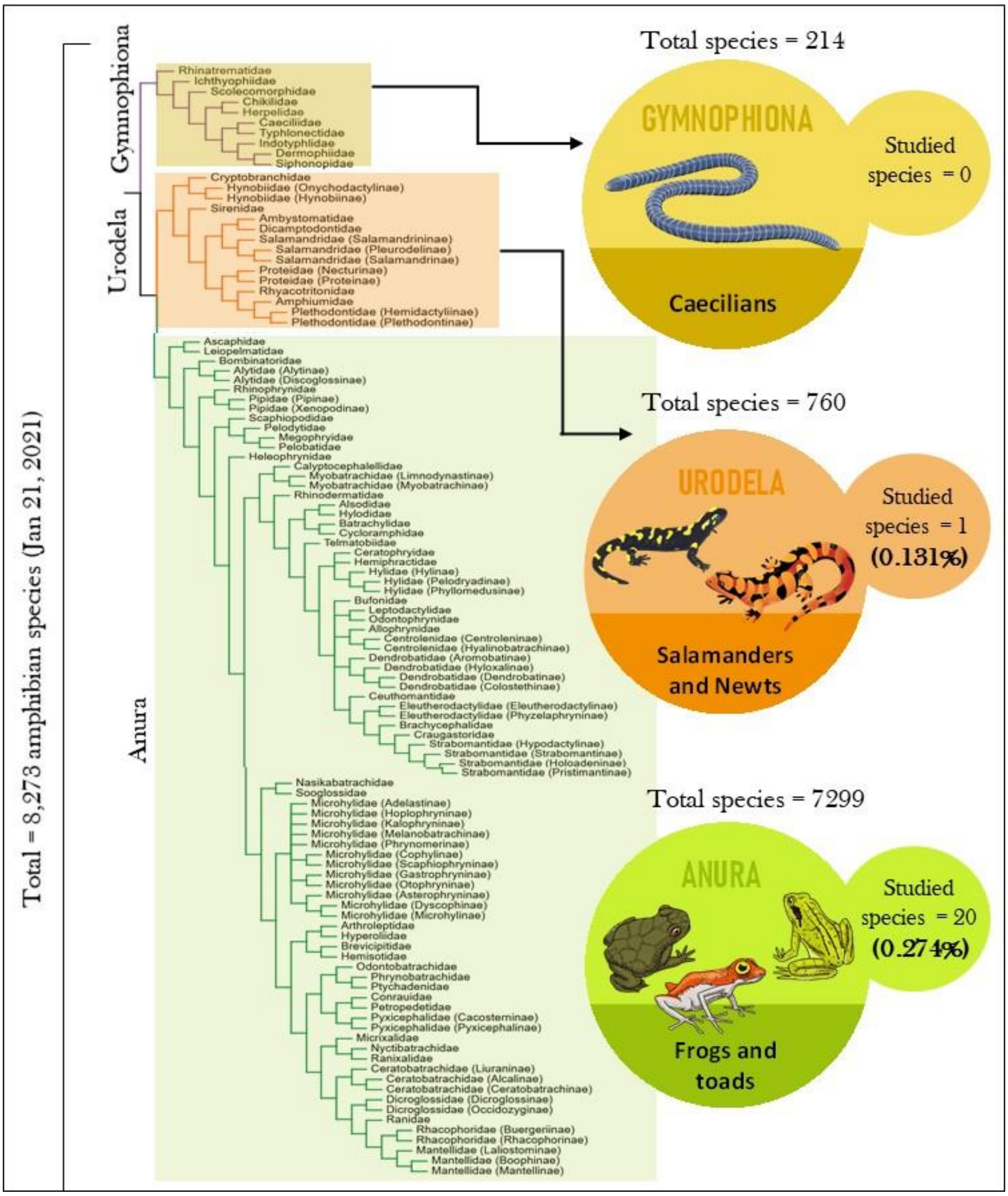

Fig. 7. Scheme representing the taxonomic diversity of the amphibian group and the percentages referring to the number of species studied in the articles concerning the ecotoxicity of micro(nano)plastics.

On the other hand, there is a growing trend in studies focusing on the effects of plastic particles on amphibians (Fig. 2A), which indicates that scientific efforts in this area may intensify in the years to come, especially due to the expansion the global discussions about these pollutants (Rochman and Hoellein, 2020; Rochman and Hoellein, 2020) and the fact that the studies, so far, have been published in renowned journals (Fig. 2C). The scientific influence and the prestige of the journals where the publications were published certainly contribute to the visibility of the studies, increasing the expectation that they will serve as motivation for the conduct of new investigations. The two journals that published the most studies relating MPs/NPs to amphibians (Journal of Hazardous Materials and Chemosphere) (Fig. 2C), for example, are among the 100 journals that received the most citations in the last three years (i.e.: > 23,675), by SC Imago Journal Rank (SJR, 2021). However, the modest H-index (median: 9.5) and the number of publications (median: 22) of the authors (Fig. 4C–F) indicates that few researchers with high international impact have participated in studies involving amphibians and plastic particles, demonstrating the need for new studies to expand their geographic areas and involve more researchers who can leverage publications in the area, in addition to contributing greatly to the conduct of research.

We also observed that the publications analyzed in this review came from studies conducted, mostly, in Brazil, China, and Italy (corresponding to 66.6% of the publications), which are among those whose accumulation of mismanaged plastic waste, together, exceeds 20 Mt/y (Lebreton and Andrady, 2019). Therefore, this may explain the greater interest (albeit incipient) of researchers from these countries for investigations that seek to contribute to the identification and understanding of the aspects that link plastic pollution to the decline of amphibians. On the other hand, although we have observed greater participation of countries considered to be economically developed (Italy, USA, Poland, and Spain) (UN, 2020) in conducting the studies, the number of publications linked to them does not exceed those from developing countries (i.e., Brazil, China, and Turkey). Furthermore, paradoxically, Brazil and China were the ones that most invested in the development of studies aimed at the identification of plastic particles in amphibians or on their impacts on these animals, which reinforces the need for greater support in this area by countries with high economic and social development, as well as greater efforts by researchers to establish partnerships with research groups in these countries. In addition, it is essential that future studies on the impact of plastic particles on amphibians are also conducted in countries (besides Brazil, China, and United States) where the amphibian diversity is high (with a considerable percentage of endemism), such as Colombia, Ecuador, Mexico, Madagascar, Australia, India, Sri Lanka, Indonesia, Papua New Guinea, Malaysia, and Philippines' [vide cartogram of the world's amphibian diversity in Koo et al. (2013)]. Within the time limit established in this study, no investigation (in the field or in the laboratory) has been identified in these countries.

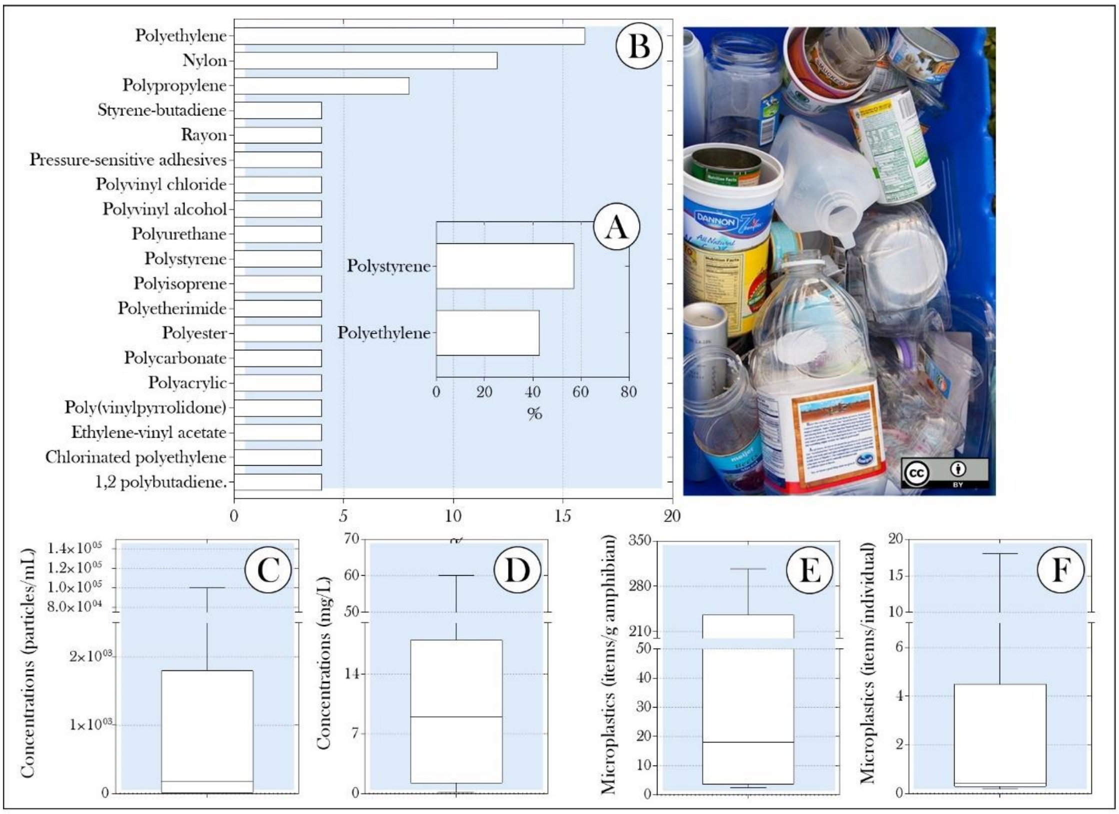

Fig. 8. (A) Types of micro(nano)plastics evaluated in the laboratory (B) and identified in the field and (C-F) boxplots of their concentrations tested in the laboratory (C-D) and observed in the field studies (E-F).

4.2. Amphibian species and plastic particle type

Regarding the content of the analyzed publications, we observed that the effects or the presence of plastic particles were investigated in just over 20 species of amphibians (Table 1), which represents a very small portion of the number of species already cataloged (0.241%) (Fig. 7). What strikes us is the fact that the studies conducted in the laboratory have used, mainly, two species of amphibians (*P. cuvieri* and *X. laevis*), and future studies must address other species of the anuran order, as well as the Urodela orders (explored in only one field study) and Gymnophiona (not covered in any study). Although *X. laevis* and *P. cuvieri* are considered a suitable model for amphibians (Qin and Xu, 2006; Horb et al., 2019), their natural occurrences are restricted to the African continent and South America, respectively (Fig. 6) (Tinsley et al., 1996; Poynton, 1999; Channing and Howell, 2006). Therefore, the use of other species is essential to understand how the levels of plastic pollution observed in different countries have influenced their natural populations of amphibians.

We note that studies of Hu et al. (2018), Schessl et al. (2019), and Kolenda et al. (2020), conducted in Shanghai/Zhejiang (China), New York (USA), and Wroclaw (Poland), respectively, covered a greater number of species, as all individuals found during field campaigns were captured for later analysis. However, it is worth noting that the lack of detailed information about the sampling methods used is a common characteristic among these studies, which makes it difficult to assess the sampling effort undertaken by the authors in the search and capture of the animals evaluated. Although this information is most commonly provided in biodiversity monitoring studies (or inventories), the ability to detect reptiles and amphibians is influenced by environmental and behavioral variables and detection probabilities (Hutchens and DePerno, 2009). According to Williams and Berkson (2004), environmental variables, such as temperature, humidity, wind, and seasonality can influence the activity and detectability of individuals. Likewise, sedentary. and fossorial behaviors and cryptographic features can limit the detectability of certain species (Flint and Harris, 2005). In tropical regions, in general, amphibians are more active during wet seasons (Brasileiro et al., 2005; López et al., 2011; Galoyan et al., 2017; Muller et al., 2018); however, the capture of individuals must also cover the cold and dry seasons, due to seasonal differences in the composition of the communities, with the possibility of meeting individuals, of different age groups or physiological states from the samples obtained in rainy seasons. Besides, the use of few sampling methodologies greatly limits the probability of species detection. Therefore, future field studies involving the detection of plastic particles in amphibians must take these variables into account, as the absence of a detailed sampling plan can generate underestimated results, which will limit our understanding of the extent of the impact of these pollutants on biodiversity of amphibian fauna.

Another interesting aspect refers to the variation in the composition of plastic particles evaluated. While in laboratory work, only polystyrene (PS) and polyethylene (PE) were used; in field studies, a wide variety of plastic particles was identified in animals (Fig. 8B), revealing the need for greater compatibility between the toxicological effects of plastic particles evaluated in the laboratory and those identified in natural environments, as previously recommended by De Sá et al. (2018). On the other hand, it is not surprising that the plastic debris most commonly identified in amphibians belongs to PE (Fig. 8B), since this material is used in a wide variety of products, including plastic bags; bottles for milk, water, shampoo, or motor oils; toys; food packaging like yogurt cans, margarine containers or cereal box liners; irrigation and drainage pipes; various medical and cosmetic products (Peacock, 2000; McLain, 2007). However, new investigations must consider the different types of PE (ultra-high, high, or ultra-low molecular weight PE (UHMWPE, HMWPE or ULMWPE, respectively) and the fact that these materials have a density lower than water (0.91 – 0.97 g/mL – Lambert et al., 2018). This characteristic may be preponderant for its toxicity, as it is uncommon for them to be found in deeper layers of aquatic ecosystems, where tadpoles normally rest and forage.

Table 2. Properties of micro(nano)plastics from the studies concerning the ecotoxicity of plastic particles in amphibians.

Composition	Size	Shape	Origin	References
Laboratory studies				
PS	50 nm	NI	Commercial	Tussellino et al. (2015)
	1 e 10 µm	Spherical	Commercial	Hu et al. (2016)
PS	3 µm	Granules	Commercial	De Felice et al. (2018)
PE	10 µm	Spherical	Commercial	Boyero et al. (2020)
	35.46 ± 18,17 µm		Commercial	Araujo et al. (2020)
	35.46 ± 18,17 µm		Commercial	Araujo et al. (2020b)
	35.46 ± 18.17 µm		Commercial	Araujo & Malafaia (2020)
Field studies				
Nylon (polyamide, PAm), PEI, PP, RY, PC, PE, PES, PS, and PVC.	> 5 µm	Fibers, fragments, and granules	Natural environment	Hu et al. (2018)
ND	ND	ND	ND	Schessl et al. (2019)
PE, PA, and PSA.	3.7 mm 3.1 mm 1.8 mm 1.2 mm 1.44 mm 0.1 mm	Fibers and chopped fragments	Natural environment	Iannella et al. (2020)
PAm, PU, PI and PB.	23.4 ± 2.1 mm 27.5 ± 2.7 mm 26.8 ± 1.4 mm 27.3 ± 1.8 mm 18.9 ± 2.5 mm 36.4 ± 9.9 mm 25.9 ± 2.2 mm 30.1 ± 3.85 mm	Fibers and fragments	Natural environment	Kolenda et al. (2020)
PE, PET, PA, PAm, CPE, PVP, EVA, PVA and SBR.	132 µm a 1190 µm	Fibers, fragments, and granules	Natural environment	Karaoglu & Gul (2020)

Legend: PS: polystyrene; PE: polyethylene; NI: not informed; PEI: polyetherimide; PP: polypropylene; RY: raylon; PES: polyester; PVC: polyvinyl chloride; ND: not detected; PA: polyacrylic; PAm: polyamide (nylon); PSA: pressure-sensitive adhesives; PU: polyurethane; PI: polyisopren; PB:1,2-polybutadiene; PET: polyethylene terephthalate; CPE: chlorinated polyethylene; PVP: poly(vinylpyrrolidone); EVA: ethylene-vinyl acetate; PVA: polyvinyl alcohol; SBR: styrene-butadiene.

As highlighted by Vaz-Silva et al. (2020), the tadpoles' own anatomy (including the presence of sinister spiracles and ventral oral discs) is adapted for the permanence of these animals in soft sediments at the bottom of watercourses. Thus, the probability of contact between tadpoles and plastic particles of lower density is limited and depends on amphibian species, developmental stages, and environmental conditions.

It is also necessary to expand the performance of laboratory studies involving MPs/NPs of nylon (polyamide) and PP, which were also identified in most of the studies conducted in the field (Fig. 8B). PP is also widely used (as packaging material; medical and electronic equipment; furniture production; textile and automotive industries (Bond et al., 2018), with its high resistance to water, inorganic chemicals, organic solvents, and lubricants (Hrnjak-Murgic, 2015); as well as its low biodegradability (Krueger et al., 2015) factors that are preponderant for its toxicity in aquatic organisms, which should be better explored in studies involving amphibians, while the presence of nylon (polyamide) microfibers in aquatic environments it is generally attributed to the release of fibers during the washing of clothes (Browne et al., 2011; Xu et al., 2020a) and to the fragmentation of materials used in the manufacture of carpets, cables, textiles intended for fishing (lines, nets, and ropes), among others (Andrady and Neal, 2009). The abundance of these materials in the amphibians collected in the studies we evaluated associated with their high proportions, biopersistence and small size, constitutes key attributes in the toxicity of these materials. Therefore, the absence of studies on the toxicity of MPs and NPs of PP and microfibers/ nylon granules in amphibians reinforces the need to expand investigations in this field. The association between the bioaccumulation of plastic particles in amphibians and their possible physiological consequences is crucial to identify and understand their impacts on these animals.

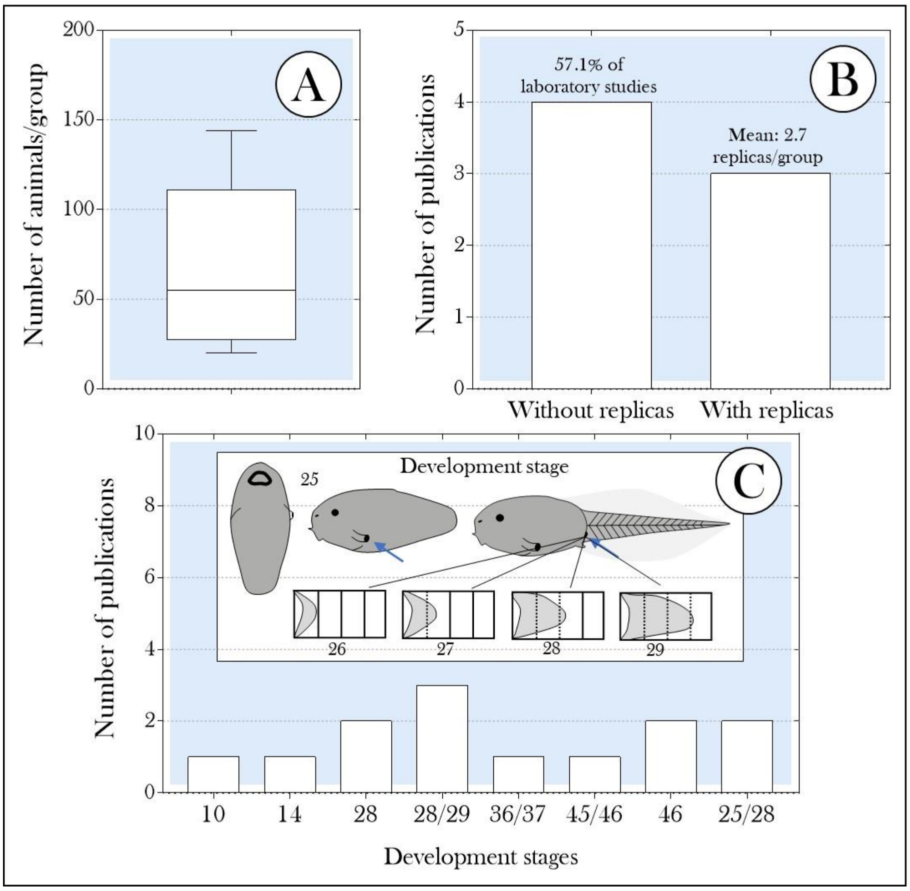

Fig. 9. (A) Number of animals per group, (B) number of publications (with and without replicates in their experimental designs), and (C) number of publications that used tadpoles in distinct stages. In "C", the sum of the number of publications exceeds the total number of articles reviewed in this study, as some studies have evaluated tadpoles from different stages of development. In addition, tadpoles were evaluated in stages 25–29, according Gosner (1960) and Faber and Nieuwkoop (2020).

4.3. Experimental design

Another aspect that caught our attention refers to the concentrations of plastic particles used in animal exposures in studies conducted in the laboratory, either by the wide variation observed (0.125–60 mg/L or 0.1 to 10^5 particles/mL) or by the units used (mg/L and particles/L). Although in some studies, the authors have argued the choice of tested concentrations based on their possible occurrences in the natural environment (e.g., Araujo et al., 2020a, 2020b), in general, the concentrations used are substantially above typical levels found in most ecosystems [see review by Li et al. (2018); Jiang et al. (2018); Triebskorn et al. (2019); Wang et al. (2021); Du et al. (2021)]. In the studies by Tussolino et al. (2015), Hu et al. (2016), De-Felice et al. (2018), and Boyero et al. (2020) no justification for the choice of concentrations was identified, which shows an important limitation of these studies. Thus, as discussed by Cunningham and Sigwart (2019), the concentrations tested in the laboratory must be justified and consistent with those identified in natural environments, since the use of high concentrations can overestimate the ecotoxicological effects, in addition to limiting the extrapolation of results for the natural environment. On the other hand, the lack of consistent units (Fig. 8C–F) impedes real comparisons among extraction studies, which provide the baseline to determine what qualifies as environmentally relevant concentrations of microplastic pollution (Burns and Boxall, 2018). Also, Cunningham & Sigwart (2019) emphasize that unified measure of particles/L of water does not guarantee comparability in the concentration or the properties of plastic pollution and, therefore, this issue needs attention in planning future studies involving not only amphibians but also other organisms.

Equally important is the use of replicates in experimental designs. Among the studies evaluated, more than half did not provide information on the number of replicates/treatments used in the experimental design (Fig. 9B), which characterizes them as typical cases of pseudoreceptors. This question is not recent, and several studies have already called attention to the importance of these aspects in the stages of experimental planning since pseudo-replication requires a statistical analysis that considers the dependence of the variables, which is not always observed in the studies (Ramírez et al., 2000). In the study by Araujo et al. (2020a, 2020b), for example, the authors distributed tadpoles of *P. cuvieri* into two groups (control and MPs), each being kept in an aquarium throughout the experimental period. In these cases, each animal constituted a replica of “tadpole”, but not replicas of the treatments (control and MPs groups). Therefore, the tadpoles that were exposed to pollutants in the same aquarium are not independent of each other and, therefore, are subject to interference (of a different nature), which can be a confusing factor that will skew the interpretation of the results. Any interference that has occurred in only one of the aquariums, may influence our perception of the animals’ response to pollutants.

The discussions on this subject are not exhaustive and other works provide further details on this (Clarke and Green, 1988; Queen et al., 2002) but, considering that studies involving MPs/NPs and amphibians are still incipient, it is important to raise discussions about the design to be adopted in future experiments. In some situations, it is possible, for example, that the use of pseudo-replicas is justified. This applies, especially, in studies involving the behavioral evaluation of animals, in which researchers must consider the effects of pseudo-replicates in the future analysis of results and those arising from the isolation of animals tested in replicates with reduced sample numbers. In Araujo and Malafaia (2020), for example, the effect of PE MPs on the defensive anti-predatory response of *P. cuvieri* tadpoles (n = 80 animals/group) was evaluated, based on the animals’ social aggregation behavior. In this case, considering that the social isolation of tadpoles during the exposure period could be an additional stress variable since the focus was precisely on the evaluation of gregarious behavior, the evaluation of the behavior of ten “schools” of eight animals/group, it seems plausible and mitigates the analytical limitations imposed by the pseudo-replication.

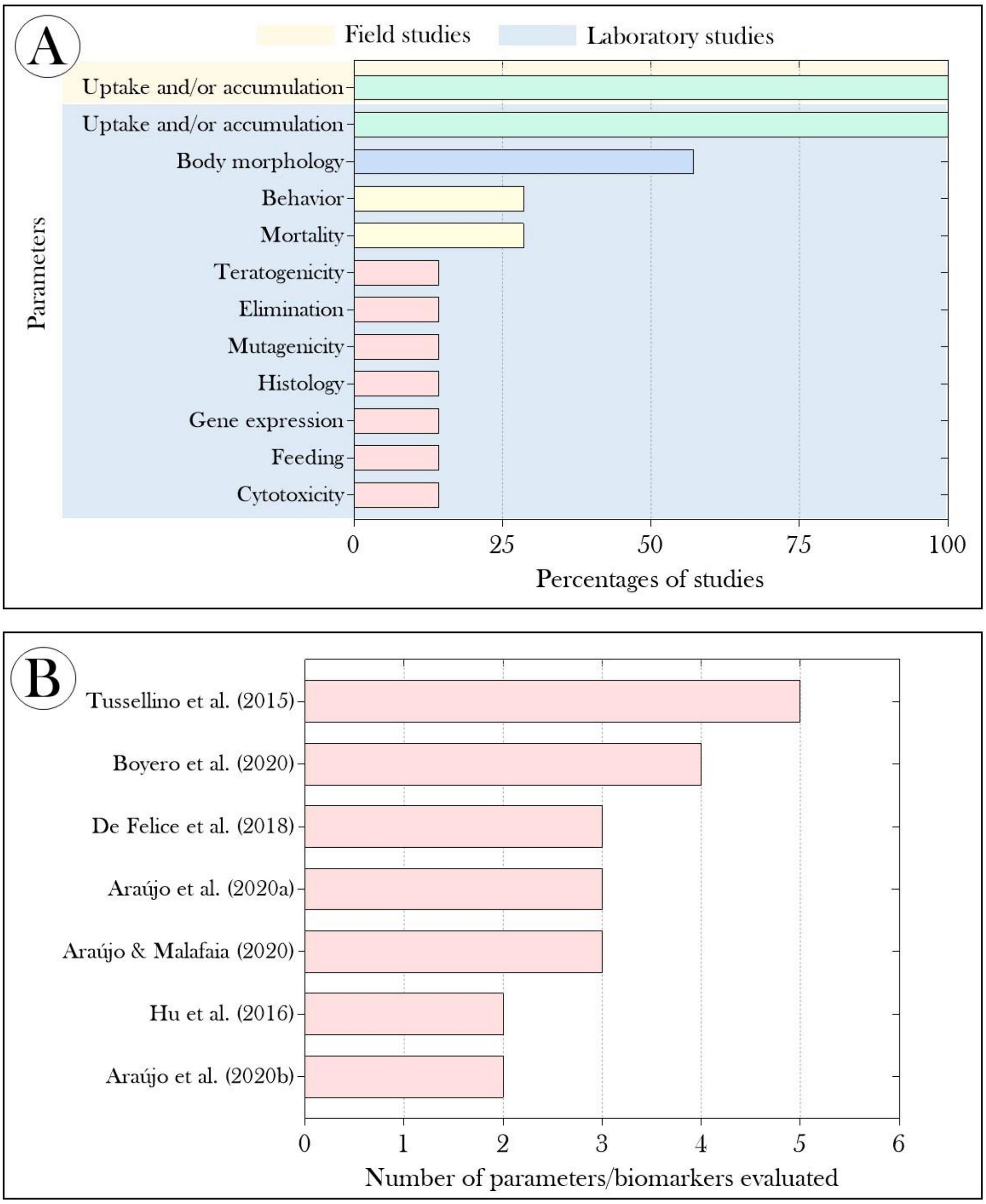

Fig. 10. (A) Types of parameters or biomarkers evaluated in the studies analyzed in this review [yellow background = field studies (n = 5 articles) and blue background: laboratory studies (n = 7 articles)] and (B) frequency of their approaches in laboratory studies (n = 7 articles). In “A”, the sum of the percentage of studies exceeds 100%, since some parameters or biomarkers of toxicity were evaluated in more than one article. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.4. Ecotoxicological effects of plastic particles on amphibian

We also observed that different effects of plastic particles were reported in studies conducted in the laboratory (Table 3), different from field studies, which were limited to identifying the presence of these materials in the animals studied. The uptake and/or bioaccumulation of plastic particles was the parameter evaluated in all studies, having been associated with different changes in the animals evaluated in the laboratory (Table 3). Although these biomarkers have also been used to assess the toxicity of MPs/NPs in other organisms, including especially fish and invertebrates (Azevedo-Santos et al., 2021), our data reveal that many investigative gaps can be better explored in the future. However, it is worth noting that, at the current stage, most studies have expanded efforts to identify and characterize the impact of a few types of MPs (PE and PS), and the mechanisms of action (responsible for the effects) were not the focus of these studies, which prevents the identification of a mechanistic tendency of the action of plastic particles on amphibians. The exception refers to the study by Tusselino et al. (2015), in which the embryotoxic and teratogenic effects observed in *X. laevis* tadpoles exposed to PS NPs (size: 50-nm; concentrations: 4.5, 9, or 18 mg/L) were associated with changes in the expression of *bra*, *myod1,* and *sox9* mRNAs.

On the other hand, we know nothing about the mechanisms that mediate mutagenic, cytotoxic, biometric, behavioral, and histopathological changes reported in *P. cuvieri* tadpoles exposed to PE MPs (diameter: 35.46 μm; concentration: 60 mg/L) for 7 days (Araujo et al., 2020ab; Araujo and Malafaia, 2020). The effects on feeding, growth, and body condition observed in *A. obstetricans* exposed to PS MPs (diameter: 10-μm polystyrene microspheres; concentration: 0 to 10^3 particles mL^{-1}) for 14 days (Boyero et al., 2020), nor the biological or environmental variables that influence the uptake, accumulation and elimination of PS MPs [(diameter: 1 and 10 μm; the tadpoles were exposed at three concentrations of each particle size: 10, 10^3 and 10^5 particles/mL (1 μm) and 0.1, 10 and 10^3 particles mL^{-1} (10 μm)] in *X. tropicalis* tadpoles exposed from 1 to 48 h (Hu et al., 2016). Besides, it is intriguing that the ingestion of polystyrene MPs (diameter: 3 μm; concentration: 0.125, 1.25, and 12.5 μg/mL) by *X. laevis* tadpoles did not cause changes in the development and swimming behavior of the animals, even though they were exposed to concentrations much higher than those found in natural environments (De Felice et al., 2018). Obviously, the fact that the authors have not investigated the mechanisms intrinsic to the effects does not disparage or disqualify the published studies, nor does it generate doubt about the validity of their results/conclusions. As discussed by Chapman (1995) and Lehtonen et al. (2017), the understanding of the magnitude of the impact of pollutants on the biota inevitably depends on the development of studies that identify and characterize (initially) the effects of pollutants on the biota, being - commonly - the mechanisms of action step to be performed at posterior. Our finding confirms the incipience of studies involving plastic particles and amphibians, as well as our limited understanding of how these pollutants affect the health of these animals and their impacts at the population and ecosystem level.

Table 3. Details of the main results obtained from the toxicity parameters or biomarkers evaluated in amphibians exposed to (nano) microplastics.

Parameters	Responses	Species	References
Laboratory studies			
Mortality	↑ Mortality rate	*Xenopus laevis*	Tussellino et al. (2015)[a]
Teratogenicity	↑ Percentage of malformations Pigmentation disorders		
Body morphology	↓ Body length ↓ Growth pace		
Uptake and/or accumulation	Presence of nanoparticles of the digestive gut cells		
Gene expression	Changes in gene expression of the mesoderm markers		
Uptake and/or accumulation	Presence of microspheres in the gills, digestive tract, and feces	*Xenopus tropicalis*	Hu et al. (2016)
Elimination	The presence of food ↓ absorption and ↑ elimination of microspheres		
Body morphology Behavior	No changes in development and swimming behavior were observed	*Xenopus laevis*	De Felice et al. (2018)
Uptake and/or accumulation	Presence of microplastics in the digestive tract		
Mortality	↑ Mortality rate in the highest concentration	*Alytes obstetricans*	Boyero et al. (2020)
Body morphology	↓ Body condition index ↓reduced growth		
Feeding	↓ Feed rate in the highest concentration		
Uptake and/or accumulation	Presence of microplastics in tadpoles and feces		
Mutagenicity	↑ frequency of erythrocyte nuclear abnormalities	*Physalaemus cuvieri*	Araujo et al. (2020)
Cytotoxicity	Change in erythrocyte shape and nucleus/cytoplasm ratio		
Body morphology	↑ Pigmentation rate ↓ Reduced ratio between total length and mouth-cloaca distance, caudal length, ocular area and mouth area		
Uptake and/or accumulation	Presence of microplastics in the intestine, gills, liver, and muscle tissues of the tail		
Histology	↑ histopathological changes (blood vessel dilation, infiltration, congestion, hydropic degeneration, hypertrophy, and hyperplasia)	*Physalaemus cuvieri*	Araujo et al. (2020b)
Uptake and/or accumulation	Presence of microplastic in liver tissue		
Behavior	↓ Locomotor ability		

Uptake and/or accumulation	↓ Social aggregation ↓ Anti-predator defense Presence of microplastic in tadpoles	*Physalaemus cuvieri*	Araujo & Malafaia (2020)
Field studies			
Uptake and/or accumulation	Presence of plastic particles in the tadpoles evaluated	*Microhyla ornata* *Rana limnochari* *Pelophylax nigromaculatus* *Bufo gargarizans*	Hu et al. (2018)
Uptake and/or accumulation	No microspheres were detected in any of the amphibian samples	*Rana catesbeiana* *Rana pipiens*	Schessl et al. (2019)
Uptake and/or accumulation	Presence of plastic particles in the stomach content of the species	*Triturus carnifex*	Iannella et al. (2020)
Uptake and/or accumulation	Presence of plastic particles in the tadpoles evaluated	*Bufo bufo* *Rana temporaria* *Pelophylax esculentus* *Pelobates fuscus* *Hyla arborea*	Kolenda et al. (2020)
Uptake and/or accumulation	Presence of plastic particles in the tadpoles evaluated	*Pelophylax ridibundus* *Rana macrocnemis*	Karaoglu & Gul (2020)

[a]Single study in which the effects of a nanoplastic were evaluated on an experimental model. See details in Table 2. ↓: decrease; ↑: increase.

5. CONCLUSIONS AND RECOMMENDATIONS

Based on what we have exposed, it is evident the need for studies such as those analyzed in this review to be continued, which may contribute to the understanding of how plastic particles affect the health of amphibians, how these pollutants intensify the effects of other stressors environmental factors, as well as for proposing mitigation measures or remediation of plastic pollution, aiming at the conservation and preservation of amphibian species. For this, we believe it is important that new investigations:

1) expand the diversity of species used in the studies (either in the laboratory or in the field), including representatives not only of the order Anura, but also Urodela and Gymnophiona.

2) assess the existence of variation in the sensitivity of species to MP and NP pollution, which can be done by focusing on native amphibians according to the environment.

3) assess how much the types of MPs/NPs properties (i.e., size, shapes, composition, among others) affect their toxicity to amphibians.

4) prioritize the use of concentrations of MPs/NPs, periods and routes of exposure that are ecologically relevant, which will bring the experimental designs closer to more realistic conditions.

5) expand the development of studies focusing on the effects of NPs, since MPs have been mostly addressed.

6) evaluate the effects of MPs/NPs over the entire aquatic life of amphibians and the extent to which exposures during the pre-metamorphosis phases can interfere in the adult life of these animals.

7) investigate the participation of amphibians in the transfer of MPs/NPs along the food chain (aquatic and terrestrial);

8) propose the investigation of the effects of plastic particles on amphibians, in association with other pollutants, since, in the environment, these pollutants are not found in isolation; but, associated with other contaminants/pollutants that may have their effects intensified or diminished.

9) invest in assessing the contributions of MPs/NPs to the intensification of the effects caused by environmental stressors that are known to have contributed to the population decline of amphibians (e.g., loss of habitats, emerging diseases, solar radiation, increased pollution by pesticides, among others), especially in species threatened with extinction.

10) pay attention to the influence of MPs/NPs on the quality of the exposure water, since small changes in physical-chemical parameters, such as pH, salinity, electrical conductivity, or the

increased availability of metal ions may be sufficient to induce changes in animals and, consequently, “mask” the effects of pollutants.

11) seek to associate the accumulation of MPs/NPs in amphibians captured in the field with possible physiological effects and in the dynamics of their natural populations, including impacts on social, reproductive, anti-predatory behaviors, among others.

12) address aspects of the toxicity of MPs/NPs, involving the tissue distribution, metabolism, and detoxification process of these pollutants in amphibians.

The inclusion of these and several other investigative aspects in the planning of novel studies requires that some challenges be overcome, be they of a financial/economic nature, or a technical-scientific nature. The use of diversified procedures for sampling, quantification, and analysis of MPs/NPs in biological samples, can compromise the association of the accumulation of these pollutants with the different effects observed, in addition to limiting the comparison between the results obtained. In this case, the establishment of national and international partnerships can be important not only for the alignment of the methods to be used, but also for the transfer of procedures that allow the more precise quantification of the MPs/NPs, either using fluorescent probes (linked to image processing analysis) or automated mapping technology to characterize polymers (for example, FTIR). Equally interesting, will be the investment in the creation of free access databases (e.g., repositories) and libraries of polymers, which will result in cost savings associated with the identification of polymer and again to allow the much-needed transparency for the meta-analysis.

Also, there is a great need to establish a consistent framework for assessing the ecological risk associated with micro(nano)plastic pollution from freshwater environments, where amphibian species mostly live. Frameworks such as those established for the determination of Adverse Outcome Pathways (AOPs) or AOP networks (Ankley et al., 2010; Knapen et al., 2018) have been in existence for up to a decade or more for different aquatic pollutants, including pesticides, industrial and pharmaceutical chemicals and, therefore, can also cover aspects related to MPs/NPs. In this case, as suggested by Granek et al. (2020), micro(nano)plastic researchers could use these existing constructs to inform experimental design for sampling efforts and exposure regimes, since information on endpoints relevant to fitness (e.g., growth, development, behavior, reproduction) are essential to predict potential effects at organism and population levels.

Finally, it is worth noting that the expansion of studies focused on the effects of MPs/NPs on amphibians and the overcoming of their inherent challenges, permeates the valorization of the group of amphibians in the scope of ecotoxicological assessments linked to plastic pollution, as well as community effort (including different segments of society) to curb the sharp population decline of these animals. In general, the studies evaluated in this review constitute only the "tip of an iceberg" that represents the effects of plastic pollution on the health of amphibians and, therefore, it is natural that the revised findings generate more new questions than the number of answers they provide. We hope that this review will help guide MPs/NPs research in the coming years, while we await future research directions catalyzed by the analyzed articles.

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7. ETHICAL CONSIDERATION AND DECLARATION OF COMPETING INTEREST

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, among others) have been completely observed by the authors. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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CAPÍTULO II

TOXICITY ASSESSMENT OF POLYETHYLENE MICROPLASTICS IN COMBINATION WITH A MIX OF EMERGING POLLUTANTS ON *Physalaemus cuvieri* TADPOLES[1]

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Toxicity assessment of polyethylene microplastics in combination with a mix of emerging pollutants on *Physalaemus cuvieri* tadpoles

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ABSTRACT

Studies in recent years have shown that aquatic pollution by microplastics (MPs) can be considered to pose additional stress to amphibian populations. However, our knowledge of how MPs affect amphibians is very rudimentary, and even more limited is our understanding of their effects in combination with other emerging pollutants. Thus, we aimed to evaluate the possible toxicity of polyethylene MPs (PE-MPs) (alone or in combination with a mix of pollutants) on the health of *Physalaemus cuvieri* tadpoles. After 30 days of exposure, multiple biomarkers were measured, including morphological, biometric, and developmental indices, behavioral parameters, mutagenicity, cytotoxicity, antioxidant, and cholinesterase responses, as well as the uptake and accumulation of PE-MPs in animals. Based on the results, there was no significant change in any of the parameters measured in tadpoles exposed to treatments, but induced stress was observed in tadpoles exposed to PE-MPs combined with the mixture of pollutants, reflecting significant changes in physiological and biochemical responses. Through principal component analysis (PCA) and integrated biomarker response (IBR) assessment, effects induced by pollutants in each test group were distinguished, confirming that the exposure of *P. cuvieri* tadpoles to the PE-MPs in combination with a mix of emerging pollutants induces an enhanced stress response, although the uptake and accumulation of PE-MPs in these animals was reduced. Thus, our study provides new insight into the danger to amphibians of MPs coexisting with other pollutants in aquatic environments.

Keywords: Amphibians, Environmental toxicology, Micropollutants, Emerging pollutants, Aquatic pollution, Biomarkers.

1. INTRODUCTION

It is known that although amphibians constitute the largest vertebrate biomass and contribute to the trophic dynamics of many ecological communities (Gao and Wang, 2022), declines in their populations and the richness of species have been reported since the 1980s (Halliday, 2021). The causes of this scenario are complex and comprehensive, with no consensus on the factors that have contributed more to the population decrease of these animals or their species-dependent effects (Green et al., 2020; Capdevila et al., 2022). However, recent research has listed significant habitat- loss impact (Moss et al., 2021; Rucker et al., 2021), an increase in UV-B irradiation (Lundsgaard et al., 2020, 2021), emerging diseases (Brannelly et al., 2021), non-native species introduction (Nunes et al., 2019), climate change (Bucciarelli et al., 2020; Sattar et al., 2021) and ecosystem pollution (Lent et al., 2021; Crnobrnja-Isailović et al., 2021) as the main factors that have affected amphibians. Despite this evidence, studies involving the possible (eco)toxicological effects of pollutant mixtures on amphibians are scarce in the literature, which makes it difficult to understand the real effect of multiple pollutants on these animals since they are not present alone in the aquatic environment (Souza et al., 2018). In amphibians and particularly in tadpoles, most studies have focused on evaluation of the toxicity of mixtures of a few pollutants (Amaral et al., 2019; Cory et al., 2019; Boccioni et al., 2021; Uçkun and Özmen, 2021; Usal et al., 2022) and some types of effluents (Marques et al., 2013; Montalvão et al., 2018; Amaral et al., 2018; Romero et al., 2019) without, however, considering the co-occurrence of a wide variety of emerging pollutants dispersed in freshwater ecosystems.

Another gap in our knowledge refers to the impact of microplastics (MPs) on amphibian health (Araujo et al., 2021), as well as their effects when combined with other pollutants (Araujo et al., 2022), such as heavy metals, pesticides, agricultural fertilizers, pharmaceutical residues, petroleum- derived pollutants, and surfactants, which have also been identified in different aquatic ecosystems (Karaouzas et al., 2021; Edori and Edori, 2021; Al-Ani et al., 2020; Campanale et al., 2021; Xiang etal., 2021; Tavengwa et al., 2022; Jayasiri et al., 2022). As discussed by Shruti et al. (2021), MP pollution has been considered a problem of global magnitude and increasing concern in recent decades (Shruti et al., 2021), with the origin of plastic microparticles being derived from two main sources: one is primary, from plastics developed to be smaller in size like nurdles or powders, and the other is secondary, resulting from the fragmentation of larger particles. While primary MPs are generated microscopically and are present in products for personal care like toothpaste, scrubbers, and other cosmetics, secondary MPs – which constitute most MPs formed in the aquatic environments– originate from the breaking up of larger plastics due to photodegradation and several other weathering processes (Kasmuri et al., 2022).

According to Liu et al. (2022), the input of MPs into the aquatic environment comes from a variety of sources including stormwater runoff, wastewater treatment plant effluent, and atmospheric deposition, and their distribution in rivers, in particular, is influenced by several factors. The proximity of the river to MP hotspots (e.g., wastewater treatment plants, urban stormwater discharge points, and runoff from open landfills), the flow rate due to dilution, seasonal variations, and climatic conditions are some of the factors that can influence the distribution, transport, and deposition of MP sin these environments (Koutnik et al., 2021). Regardless, when in contact with aquatic organisms, MPs can induce a wide range of toxic effects (see review by Zhang et al., 2022) and, therefore, interfere negatively with nutrient productivity and cycling, cause physiological stress in organisms, and threaten the ecosystem composition and stability (Ma et al., 2020).

If this were not enough, the co-existence of multiple pollutants with MPs in the aquatic system has recently been observed, and the interaction between plastic microparticles and the combined pollutants, as well as their toxicity, may further exacerbate the effects of MP pollution on the functioning of ecosystems (Tang, 2021; Zhang et al., 2022). Souza et al. (2018) discussed how different interactions between compounds/chemical elements can affect the mobility and availability of xenobiotics and, in a pessimistic scenario, favor the uptake of specific pollutants by organisms and exacerbate their toxicological effects. However, the impacts of amphibian exposure to MPs (alone or in combination with other pollutants) are still poorly understood (Araujo et al., 2021). So far, it is completely unknown how amphibians can be affected when exposed to a mix of pollutants (associated with MPs or not).

Thus, using a design that simulates aquatic contamination by a range of pollutants of diversified chemical nature, we aim to evaluate the possible effects of microplastics (MPs) and a mix of pollutants (alone and combined) on *Physalaemus cuvieri* tadpoles. For this, we used biomarkers of mutagenicity, oxidative stress, and cholinesterase effects. We hypothesized that MPs when interacting with other pollutants may induce more serious adverse effects on these animals. To the best of our knowledge, the present study is the first to evaluate the possible toxicity of MPs in association with several other emerging pollutants in amphibians, which complements previous reports on the toxicity of MPs in simple binary combinations with other pollutants in aquatic organisms (Ziajahromi et al., 2017; Barboza et al., 2018; Wakkaf et al., 2020; Li et al., 2020; Yang et al., 2020; Zhang et al., 2021; Thi et al., 2021).

2. MATERIAL AND METHODS

2.1. Microplastics

We utilized polyethylene MPs (PE-MPs), since currently, it is one of the most widely used polymers in various products (Horton et al., 2017), whose identification in different freshwater environments has been reported by several previous studies (Jones et al., 2020). Such MPs were obtained from Sigma Aldrich®, St. Louis, Missouri, United States (CAS number: 9002-88-4, purity 99%). As demonstrated in a previous study by our group (Araujo et al., 2020a), the PE-MPs have diameters of (35.46 ± 18.17) μm (mean ± SD) and heterogeneous shapes, with irregular surfaces, rough or smooth. The PE-MPs used in the exposures came from a stock solution prepared with ethanol (at 50%, *V/V*) (1×10^{12} particles/m^3). We emphasize that this dispersion and the exposure waters were free of any dispersant or preservative.

2.2. Mix of pollutants

To simulate aquatic contamination by different xenobiotics, we prepared a mix of 15 pollutants (in addition to those that makeup tannery effluent) in environmentally relevant concentrations (i.e., that were previously identified in surface waters). As can be seen in Appendix A **Table** S1, these components more realistically represent the diversity of pollutants that can enter freshwater ecosystems from diffuse or point sources, especially when including pesticides, agro-industrial effluent, pharmaceutics/hormones, agricultural fertilizers, surfactant, and constituent substances of petroleum. The physicochemical and inorganic characterization and the profile of organic compounds identified in this mix is described in Souza et al. (2018).

2.3. Animals and experimental design

In this study we used *P. cuvieri* tadpoles as a model system, hatched from eggs collected in a lentic environment surrounded by the native Cerrado–biome (Urutaí, GO, Brazil), under the approval of the Brazilian Biodiversity Authorization and Information System (SISBIO/MMA/ICMBio/Brazil) (License No. 73339–1). *P. cuvieri* is widely distributed in South America (Oliveira-Miranda et al., 2019) with populations that present stability and abundance in the areas of occurrence (McDonough, 2017). In addition, the species is considered a good experimental model for the evaluation of the MPs toxicity in amphibians (Araujo et al., 2021). After collection, the eggs were transported to the Biological Research Laboratory of the Federal Institute of Goiano - Campus Urutaí (GO, Brazil), and were kept under controlled conditions (12 hr:12 hr light-dark cycle, temperature 26 ± 1°C). Before and during the experiment, tadpoles were fed once a day (*ad libitum*) with commercial fish food (composition:

45% crude protein, 14% ether extract, 5% crude fiber, 14% mineral matter, and 87% dry matter). Upon reaching the 26G stage Gosner et al. (1960), 340 healthy individuals (i.e., no morphological deformity, apparent lesions, or locomotor alterations) were distributed into four experimental groups. While the group "PE-MPs" was composed of animals kept in water containing 2.7×10^8 PE-MPs particles/m^3, tadpoles exposed to the pollutant mix described above (Appendix A **Table** S1) (without the presence of MPs), composed the group "Mix". On the other hand, the group "PE-MPs + Mix" was composed of animals that were submitted to the combined exposure of PE-MPs with the mix of pollutants, at the same concentrations defined in the previous groups. In the "control" group, tadpoles were kept in dechlorinated water free of MPs or any component of the pollutant mix. We emphasize that the same amount of ethanol (up to 50%) used as a dilution vehicle for PE-MPs was added to the "control" and"Mix" groups, so the final dilution of ethanol in the exposure waters was 0.0125%. Each group was composed of five replicates (*n*=17 animals/replicate, totaling *n*=85 animals/group). Exposures were carried out in glass cylinders with a capacity of 2 L of water for 30 days in a semi-static system (i.e.,with water renewal every 72 hr). Such a period represents approximately 65% of the animal's total aquatic life (total of 45 days - Kwet and Di-Bernardo, 1999), which would be an ecologically relevant exposure period. Thus, the 30-day exposure time was classified as a chronic period.

2.3.1. Concentration of pollutants

In this study, the concentration~~s~~ of all pollutants used were defined based on previous studies that identified their presence in surface waters, bringing the study design close to realistic conditions. The studies on which the concentrations of the constituents of the mix are based are mentioned in Appendix A **Table** S1. The concentration of PE-MPs (2.7×10^8 particles/m^3) represents a pessimistic scenario of plastic pollution estimated by Koelmans et al. (2019), which from the compilation of different studies reported that concentrations of MPs can range from 1×10^{-2} to 10^8 particles/m^3 in individual freshwater samples (including rivers, lakes, bottled water, and wastewater treatment plant effluent/influent).

2.4. Biomarkers

2.4.1. Swimming activity

Initially, we evaluated the possible effect of pollutants on the swimming activity of tadpoles, assuming the influence of the pollutant mix and MPs (alone or in combination) on the mechanisms that regulate the neuro locomotion of animals. For this, 14 animals/group were submitted to the forced swimming test proposed by Amaral et al. (2018), with some modifications. This test was performed in a room containing acoustic isolation, temperature control (26°C), luminosity, and cameras coupled to external computers. Briefly, each tadpole was introduced into a glass beaker containing 500 mL of dechlorinated water (free of pollutants) and a magnetic bar (2.5 cm long; 2.5 mm thick), which was positioned on a magnetic stirrer. After 1 min of habituation, the magnetic stirrer was connected at 400r/min for 25 sec and swimming activity was recorded in the 15 sec following the shutdown of the equipment. The mobility time of each tadpole was recorded as the time the animal actively swims against the current created by the agitation of the water.

2.4.2. Biometrics and morphometry

To evaluate the possible effects of treatments on the development and growth of animals, different biometric biomarkers were evaluated. The stage of larval development of tadpoles was evaluated at the beginning (*n*=12 animals) and the end of the experiment (*n*=12 animals/group), based on the criteria described in Gosner (1960). At the end of the experiment (*n*=12 animals/group), we also evaluated body biomass, total body length, mouth-cloaca length, tail length, tail width (median and lower region), inter-nostril, interocular distance, nostril-eye, nostril-snout distance, head width before, and after eyes, as well as head and eye area. Except for body biomass, the other parameters were measured with the ImageJ software (https://imagej.nih.gov/ij/download.html) by processing photomicrographs of the animals obtained by stereoscopic microscopy. The results of these measuraments were expressed relative to the stages of larval development of the respective experimental groups. Some studies provided the background for the choice of morphological biomarkers (Costa and Nomura, 2016; Montalvão et al., 2018; Wang et al., 2019; Araujo et al., 2020).

We also evaluated possible alterations caused by the mix of pollutants and MPs (alone or in combination) on the dentition and pigmentation of the mandibular sheaths of tadpoles, according to Amaral et al. (2019) and Araujo et al. (2020). For this, 12 tadpoles/group were fixed in formaldehyde (at 4%) and evaluated in the ventral position under a stereoscopic microscope. Tadpoles with depigmented sheaths received a score of "0", animals with partially depigmented mandibular sheaths were assigned a score of "0.5"and those with dark mandibular sheaths (natural pattern of the species, according to Altig (1970)) received a score "1". The lower and upper mandibular sheaths were evaluated separately, and the results presented refer to the sum of the scores attributed to each of them. The tadpole dentition condition was evaluated from the five rows of anterior (A1 and A2) and posterior (P1 to P3) keratodonts typical of the species. The animals devoid of keratodonts received a score of "0", those with alterations received a score of "0.5" and the tadpoles that did not not present alterations in the rows of keratodonts were assigned the score "1". The results were expressed from the sum of the scores attributed to the rows of anterior and posterior regions. The intestinal condition was evaluated based on Araujo et al. (2020), from the position of the organ in the abdominal cavity (score "0": abnormal positioning – i.e.: shifted to the lateral of the abdomen; score "0.5": moderately displaced and score "1": no changes) and its characteristic winding (score "0": abnormal winding; score "0.5": moderately altered and score "1": no changes).

2.4.3. Mutagenicity

The micronucleus (MN) test and enumeration of other erythrocyte nuclear abnormalities (ENAs) were performed based on Araujo et al. (2020), aiming to evaluate possible mutagenic effects induced by the treatments. For this, 10 µL of blood (n=8 animals/group) were collected (via a cut in the tail) and used to perform a blood smear on a previously sanitized glass slide. After drying at room temperature, the slides were fixed in methanol (Dynamics®, São Paulo, Brazil, purity 98%, CAS number: 67-56-1) for 10 min and sequentially stained using a *Panótipo Rápido* kit (Laborclin®, Paraná, Brazil, code #620529), according to Estrela et al. (2021), for further analysis performed under an optical microscope at 100× magnification. A total of 1000 cells/slide was analyzed based on the criteria reported by Fenech (2007). In addition to evaluating the presence of MNs, other ENAs were also recorded, according to the nomenclatures adopted by Amaral et al. (2019).

2.4.4. Cytotoxicity

The possible induction of apoptotic and necrotic erythrocytic events by pollutants was evaluated via fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes, based on Ribble et al. (2005) and Guimarães et al. (2021b). For this, 1 µL of blood/animal was mixed with 200 µL phosphate-buffered saline (PBS) (pH 7.2; to 4°C) (Laborclin®, Pinheiros, Paraná, Brazil; ref. #590338) in a microtube (without anticoagulant). After that homogenization (in vortex agitator) was performed with 50 µL of acridine orange (AO) (Sigma Aldrich®, St. Louis, Missouri, United States;CAS number: 10127-02-3; Ref. #1003086365) and 50 µL of ethidium bromide (EB) (Dinâmica®, São Paulo, Brazil; CAS number: 1239-45-8; Ref. #3369-91) solutions (both at 1 µg/mL) followed by incubation at room temperature for 5 min. Subsequently, the cell suspension was analyzed by fluorescence light microscopy and the frequency of viable erythrocytes, apoptosis, and necrosis process was recorded (120 cells/animal; *n*=9 tadpoles/group; a total of 1080 cells/group). According to MacGahon et al. (1995), AO/EB staining was used to distinguish the viable cells from non-viable cells, based on erythrocyte membrane integrity. While living (viable) cells were green, apoptotic and necrotic cells were orange and red, respectively (Singla and Dhawan, 2013).

2.4.5. Biochemical assessments

Assuming the existence of a close relationship between the changes in the biomarkers described earlier and a possible unbalanced redox and anti- or cholinesterase-like effect induced by the mix of pollutants and MPs (alone or in combination), different biochemical parameters were evaluated. For this, tadpoles (*n*=10 animals/group) were macerated in 1 mL of PBS (pH 7.2; 4^{o}C) and centrifuged at 13,000 r/min for 5 min (at 4^{o}C), and the supernatant was used in post evaluations. We emphasize that similarly to Nascimento et al. (2021), entire bodies were used in the experiment due to the difficulty of isolating certain organs from small animals. In addition, the intestine of the animals was removes before being macerated to avoid possible bias in biochemical analysis caused by organic matter and/orby other particles consumed by tadpoles. The production of reactive oxygen species (ROS) and nitric oxide (NO) levels were defined as biomarkers of oxidative and nitrosative stress, respectively, similarly to other studies (He et al., 2021; Liu et al., 2021; Malafaia et al., 2021). The ROS generation was measured using dichlorofluorescein-diacetate (DCF-DA) (Sigma Aldrich®, St. Louis, Missouri, United States; CASnumber: 4091-99-0; Ref. #D6883) by a method modified from that of Zhao et al. (2013). For the NO dosage, we used the colorimetric reaction of Griess (Grisham et al., 1996), which consisted of the detection of nitrite ($NO2^-$), resulting from the oxidation of NO, similarly to Soneja et al. (2005) and Nascimento et al. (2021). Furthermore, we evaluated the activity of superoxide dismutase (SOD) according to Del-Maestro and McDonald (1987) and catalase (CAT) as proposed by Sinha et al. (1972), consider enzymes that make up the organisms' first line of antioxidant defense (Ighodaro andAkinloye, 2018). On the other hand, the levels of malondialdehyde (MDA) were useful for inference of the possible consequences of oxidative stress induced by pollutants, considering that the MDA is an indicator of lipid peroxidation (LPO) level produced by ROS (Grotto et al., 2009; Ayala et al., 2014).

On the other hand, the activity of acetylcholinesterase (AChE) – the action of which is crucialin the propagation of nerve impulses (Dunant, 2021) – was evaluated by assuming a possible effect of pollutants on the cholinesterase system of animals. For this, we adopted the procedures proposed by Ellman et al. (1961), with minor modifications described in

Nascimento et al. (2021). The results of the analysis of all biomarkers were expressed proportionally to the concentration of total proteins and evaluated according to the instructions of the commercial kit used (*Biotécnica*®, Varginha, Minas Gerais, Brazil; Ref. BT1000900).

2.5. Uptake and accumulation of polyethylene microplastics

To evaluate the possible uptake and accumulation of plastic particles in the animals of the groups "PE-MPs" and "PE-MPs + Mix", we followed the protocol proposed by Malafaia et al. (2021), with some modifications. Briefly, 8 animals/group were carefully washed in purified water (via reverse osmosis) and euthanized by cooling. Subsequently, the gastrointestinal system was removed and separated from the rest of the organs/tissues. Then, the samples were weighed (tadpoles without intestine: 0.030 g ± 0.004 and intestine: 0.010 ± 0.001 (mean ± SEM)), macerated in 500 µL of potassium hydroxide solution (KOH) (Vetec®, Rio de Janeiro, Brazil; CAS number: 1310-58-3; Ref.#105) (10%, *W/V*) and transferred to conical bottom tubes (previously sanitized and sterilized) whose volume was made up to 10 mL with the alkaline digestion solution. Then, the samples were incubated in a water bath at 60°C (for 17 hr) and, after that, aliquots of 200 µL of each sample (in duplicate) were introduced into an ELISA microplate (96 wells) and mixed with 50 µL of Nile red fluorescent dye solution (Sigma-Aldrich Company, Milwaukee, US, CAS number 7385-67-3 – at 100 µg/mL (in Acetone P.A.), based on Maes et al., 2017). After 5 min (at room temperature), the samples were read in an ELISA reader at 630 nm. The absorbance values of the samples were used to determine the concentration of MPs (in µg/mL), from the equation based on the standard curve previously made (equation: $y = 0.0055x - 0.1244$; A2: 0.9879). The uptake and accumulation of MPs were expressed in "(µg/mL)/g intestine" and "(µg/mL)/g body biomass (tadpole without intestine)", respectively. We emphasize that the uptake and accumulation of the components of the pollutant mix were not evaluated considering its high chemical complexity (see chemical characterization in Souza et al., 2018) and lack of knowledge on the components that exerted greater or lesser influence on the biomarkers evaluated.

2.6. Integrated Biomarker Response Index

To evidence the toxicity of the treatments, the results of all biomarkers evaluated were applied to the"Integrated Biomarker Response Index" (IBRv2), which is based on the principle of reference deviation between a disturbed and undisturbed state. For this, we adopted the procedures described in Beliaeff and Burgeot (2002), with some modifications proposed by Sanchez et al. (2013). In our study, the deviations between biomarkers measured in tadpoles exposed to MPs and the mix of pollutants (alone or in combination) were compared to those measured in tadpoles not exposed to pollutants ("control" group"). For each experimental group, the ratio between the mean value obtained for each biomarker evaluated and the respective reference control value was log-transformed (*Yi*). In the next step, a general mean (μ) and standard deviation (SD) were calculated, considering the *Yi* values of a given biomarker measured in each group. Subsequently, Yi values were standardized by Eq. (1) and the difference between *Zi* and *Z0* (control group) was used to define the biomarker deviation index (*A*) (i.e., A = *Zi* – *Z0*). To create a basal line centered on 0 and to represent biomarker variation according to this basal line, the mean of standardized biomarker response (Zi) and mean of reference biomarker data (Z0) are used to define a biomarker deviation index (A).

To obtain integrated multiple biomarker responses, similarly to García-Medina et al. (2022), the value of *A* of each biomarker was calculated for every exposed group and IBRv2 was calculated for each group by the sum of the absolute values of *A*. The biomarker response scores were plotted as radar graphs. The area above 0 reflects biomarker induction.

$$Zi = (Yi - \mu)/\mathrm{SD} \qquad (1)$$

2.7. Statistical analyses

2.7.1. Comparison means

To assess the relative size of variance among group means (between-group variance) compared to the average variance within groups (within-group variance), initially, all data obtained were evaluated regarding the assumptions for the use of parametric models. For this, we used the Shapiro-Wilk test to assess the distribution of residual data, and the Bartlett test was used to assess the homogeneity of variances. The data that met the assumptions for the use of parametric models were analyzed via a one-way ANOVA test (with Tukey post-test) and the non-parametric data were compared via the Kruskal-Wallis test (with Dunn's post-test). The average concentrations of MPs in the groups "MP" and "MP+Mix" were compared by Student's *t*-test, at 5% probability. GraphPad Prism Software Version 9.0 was used to perform these statistical analyses. Significance levels were set at Type I error (*p*) values lower than 0.05.

2.7.2. Principal component analysis (PCA)

PCA was performed to explore correlations between treatments, based on the average value of each biomarker evaluated. Before performing PCA, the variables were log-transformed to adjust the distribution to normality. Then, the values were centered at zero for PCA and an ellipse was drawn to indicate the 95% confidence interval assuming a Student distribution of the main components (PCs). After PCA, we used the proportions of variance plot, scree plot, loading plot, PC1 score plot as well as loading values and correlation (or covariance) matrix between variables generated in GraphPad Prism Software Version 9.0. Ward's hierarchical clustering method was also used to identify groups distribution according to the variables on the PCA results (Eszergár-Kiss and Caesar, 2017).

3. RESULTS

During the experimental period, we did not record tadpole deaths in any experimental group. However, the behavioral analysis revealed that the treatments influenced the swimming activity of the animals. We observed that the *P. cuvieri* tadpoles exposed to "MPs" and "pollutant mix" (separately) showed lower locomotor activity than non-exposed animals (reduction of 69.0% and 67.7%, respectively). On the other hand, when exposed to the combination of pollutants (MP + Mix group), the swimming activity of the animals was, surprisingly, 48.8% higher than that observed in the animals of the control group (**Fig.** 1a). We also observed advances in the larval development of tadpoles of all experimental groups, when comparing the initial larvae stage (stage 26.25 ± 0.13G, mean ± SEM) and at the end of the experiment (**Fig.** 1b). After 30 days of exposure, the tadpoles of the experimental groups showed, on average, an increase of 24% relative to the larval stage initially registered (**Fig.** 1b and Appendix A **Fig.** S1). However, we observed that tadpoles exposed to pollutants (alone or in combination) showed a more accelerated tendency to develop larval (control: 30.58 ± 0.54G *vs.* the average of the other groups: 33.22 ± 0.51G) and increased body biomass/development stages index (control: 0.0020 ± 0.0001 g/G *vs.* the average of the other groups:0.0031 ± 0.0002 g/G). In the tadpoles of the "Mix" group, in particular, the stage of development was higher than that observed in the "control" group, representing an increase of 12% (**Fig.** 1b). The body biomass/development stage index of animals exposed to PE-MPs (alone) was 57.94% higher than that observed in non-exposed tadpoles (**Fig.** 1c).

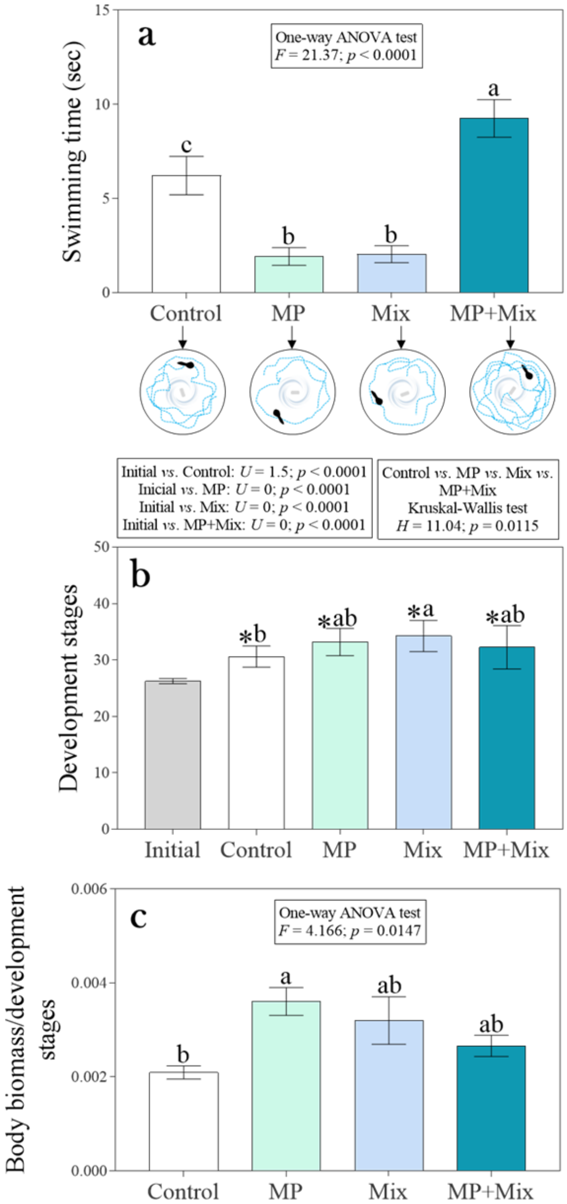

Fig. 1 (a) Swimming time (*n*=14 tadpoles/group), (b) development stages according to Gosner (1960) (*n*=12 tadpoles/group) and (c) "body biomass/development stages" index (*n*=12 tadpoles/group) of *P. cuvieri* tadpoles exposed or unexposed to the polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants) after a 30-day exposure. Bars represent mean ± SD. Statistical summaries are presented at the top of the charts. Distinct lowercase letters indicate significant differences between "Control" *vs.* "MP" *vs.* Mix *vs.* "MP+Mix" groups. In "b" the asterisks indicate significant differences between the mean of the development stages observed at the beginning of the experiment (initial group) and each experimental group at the end of the study. "MP" refers to the group composed of animals exposed only to PE-MPs (at 2.7×10^8 PE-MPs particles/m^3); "Mix", those exposed to the mix of pollutants (see concentrations in Appendix Table S1), and "MP+Mix" include animals exposed to PE-MPs in combination with the mix of pollutants (*n*=12 tadpoles/group).

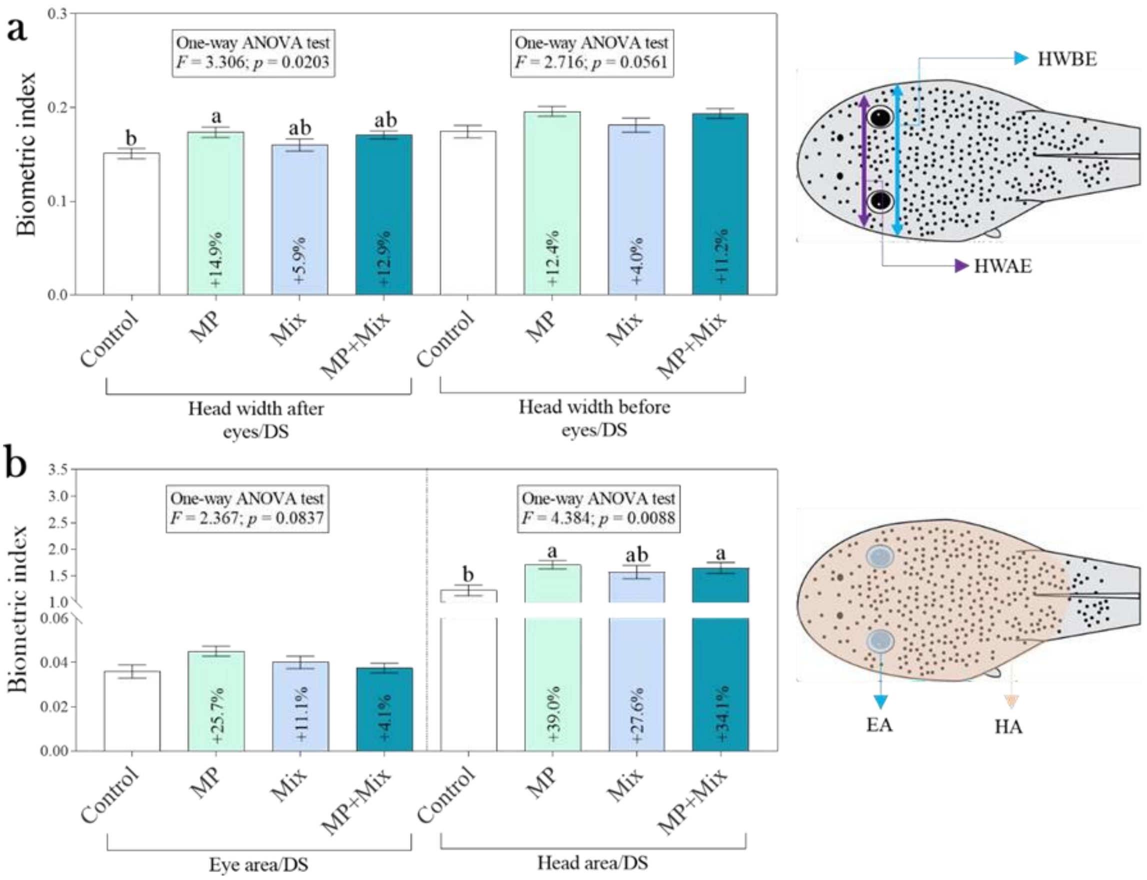

Fig. 2 Biometric index (head width (a) after and (b) before eyes, (c) eye, and (d) head area evaluated for *Physalaemus cuvieri* tadpoles exposed or unexposed to the polyethylene microplastics (PE-MPs) (alone or alone or in combination with the mix of pollutants) after a30-day exposure. Bars represent mean ± standard deviation. Statistical summaries are presented at the top of the charts. Distinct lowercase letters indicate significant differences between "Control" *vs*. "MP" *vs*. "Mix" *vs*. MP+Mix" groups. The percentages presented at the base of the bars refer to the increase in biomarkers observed in animals, compared to the "control" group. "MP+Mix" includes animals exposed to PE-MPs in combination with the mix of pollutants (n=12 tadpoles/group). "MP" and "Mix" are the same as Fig. 1.

Regarding the biometric evaluation, we also observed a tendency of biomarkers in tadpoles to increase when exposed to pollutants (alone or in combination), when compared to the control group.Although for some measures this trend has not been statistically confirmed, increases greater than 10%-12% were observed in tadpoles exposed to pollutants (**Fig.** Appendix A S2a-c and **Fig.** 2). However, we noticed even greater increases in tadpoles exposed to pollutants in the combination ("MP+Mix" group) (e.g., total body and tail length: >23%; mouth-cloaca length: >30%; tail width(median region): 40%; inter-nostril distance: >21%, nostril-eye distance: >12% (Appendix A **Fig.** S2), as well as significant reduction of scores related to dentition condition (19.4%), pigmentation of the mandibular sheath (17.4%, bowel tube winding (14.6%) (**Fig.** 3a-c, respectively). On the other hand, the tadpoles of the "MP" and "Mix" groups presented a reduction in the intestinal position score greater than 50%, relative to the "control" group (**Fig.** 3d).

In terms of mutagenic evaluation, we observed that most of the tadpoles' erythrocytes were typically elliptical (while only a few were found to be circular) with centrally placed nuclei (**Fig.** 4a). However, in animals exposed to pollutants (alone or in combination) a variety of ENAs was observed, including binucleated erythrocytes (**Fig.** 4b), multilobulate nucleus (**Fig.** 4c-d), notched nucleus (**Fig.**4e-g), kidney-shaped nucleus (**Fig.** 4h-i and Q), nuclear bud (**Fig.** 4j), blebbed nucleus (**Fig.** 4k), micronuclei (**Fig.** 4l-n), symmetric (**Fig.** 4o) and asymmetric constriction (**Fig.** 4p), in addition to other NATs with unconventional nomenclature (**Fig.** 4r-y). The total sum of ENAs in tadpoles exposed to pollutants was, on average, 106.2% higher than that found in control group animals (**Fig.**4z). On the other hand, the cytotoxicity assay revealed a significant reduction in the frequency of viable erythrocytes in the "MP+Mix" group (Appendix A **Fig.** S3) and an increase greater than 800% of cells in a process suggestive of necrosis, compared to unexposed tadpoles. In addition, we noticed that the animals of the groups "Mix" and "MP+Mix" presented a higher frequency of apoptotic erythrocytes and a greater sum of cells in apoptosis and necrosis (Appendix A **Fig.** S3), and such increases were even higher in tadpoles exposed to the combination of pollutants (PE-MPs and mixed components).

Our data also revealed that pollutants (alone or in combination) induced a significant increase in nitrite production (on average 23.2% - Appendix A **Fig.** S4a), ROS (15% - Appendix A **Fig.** S4b),

as well as SOD activity (19.1%), and CAT (15.3%) (Appendix A **Fig.** S4c-d, respectively), compared to the control group. On the other hand, TBARS levels higher than 19% were observed in animals of the groups "Mix" and "MP + Mix" (Appendix A **Fig.** S4e). Similarly, the activity of AChE was increased on average 21.6% in the animals of the groups "MP" and "Mix" and approximately 70% in tadpoles exposed to the combination of pollutants (Appendix A **Fig.** S4f), which suggests a cholinesterase effect. The quantification of MPs in tadpoles and intestines confirmed that the tadpoles of the "MP" and "MP + Mix" groups were able to take up and accumulate the PE-MPs dispersed in the exposure waters (**Fig.** 5). However, we noticed that the concentration of PE-MPs in animals exposed to the combination of PE-MPs with the mix of pollutants was significantly lower than that observed in tadpoles of the "MP" group. In the "MP + Mix" group, the accumulation of PE-MPs was 17% lower than that observed in the "MP" group, and, in their intestines, the difference was even greater (25.5%). In the "control" and "Mix" groups, we did not record the presence of PE-MPs, as expected.

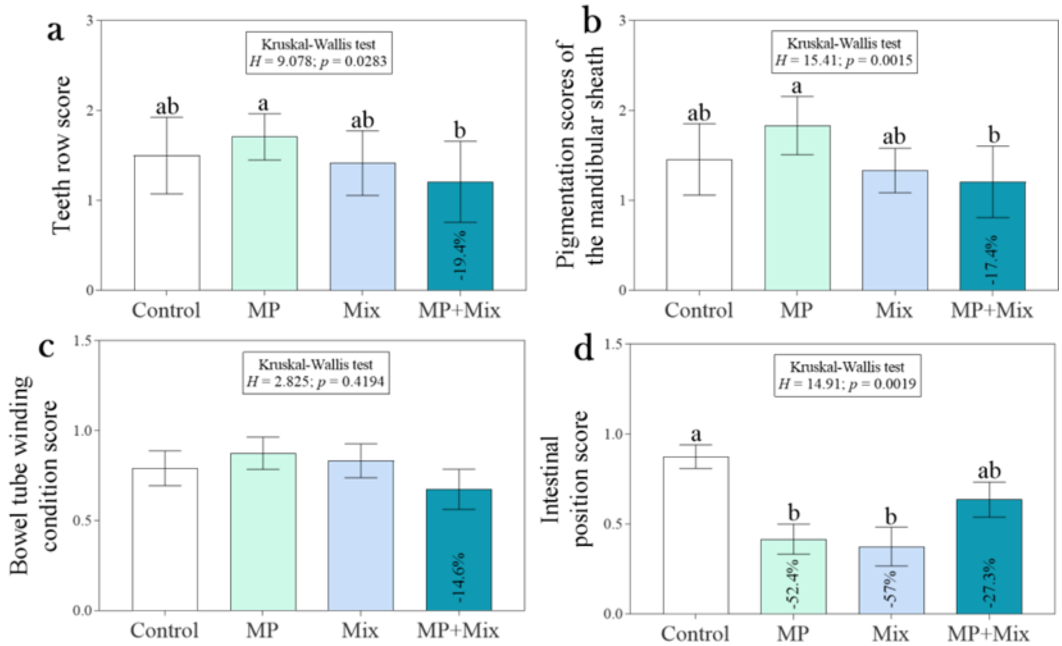

Fig. 3 (a) Teeth row score, (b) pigmentation scores of the mandibular sheath, (c) bowel tube winding condition, and (d) intestinal position score of *Physalaemus cuvieri* tadpole exposed or unexposed to the polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants) after a 30-days exposure. Bars represent mean ± SD. Statistical summaries are presented at the top of the charts. Distinct lowercase letters indicate significant differences between groups. The percentages presented at the base of the bars refer to the decrease in biomarkers observed in the animals of the "Mix" group, compared to the "control" group. "MP+Mix" includes animals exposed to PE-MPs in combination with the mix of pollutants (n=12 tadpoles/group). "MP" and "Mix" are the same as Fig. 1.

To show an overview of the results and correlations between the groups and the variables evaluated in this study, the data obtained were subjected to PCA. According to this analysis, the first two main components (PC1 and PC2) cumulatively explained more than 86% of the total variation (Appendix A **Fig.** S5a), with the eigenvalues of PC1 and PC2 being higher than 9.0 (Appendix A **Fig.**S5b). The loadings plot (which shows the relationship between the PCs and the original variable – Appendix A **Fig.** S5c) and **Table** 1 show that most of the biomarkers evaluated were positively associated with PC1 and that PC2 was negatively determined by most of the variables investigated. As expected, biomarkers related to animal size (total body, mouth-cloaca, and tail length), cytotoxicity assay (frequency of cells in the apoptotic and necrotic process), and antioxidant activity (SOD and CAT) were strongly correlated (Appendix A **Table** S1).

We also noticed that the groups exposed to pollutants (alone or in combination) were clearly separated from the "control" group by PC1, the latter having presented a negative score

(-6.43) (Appendix A **Fig.** S5d and S6a), and the other positive score groups, which confirms the trends observed in the tests previously presented (one-way ANOVA or Kruskal-Wallis tests), through which we noticed that tadpoles exposed to pollutants presented significant differences from non-exposed tadpoles. In addition, CP1 allowed the separation of experimental groups into three subgroups. The animals of the "control" and "MP+Mix" groups were separated into two different subgroups and those exposed to PE-MPs and the pollutant mix (separately) were grouped into a single subgroup (AppendixA **Fig.** S5a-b). The greater distance between the PC1 scores of the "control" and "MP+Mix" groups reinforces the conclusion that the responses of tadpoles in the latter group were differentiated, suggesting that the association between pollutants intensified its effects. The proximity, for example,of the PC score of the "MP+Mix" group with the vector loads of the variables evaluated in the cytotoxicity assay (percentage of necrotic and apoptotic erythrocytes) and the AChE corroborate the higher intensity of the effects of the combination of pollutants on animals, which was also evidenced in **Fig.** 5Sc-d and 6a, respectively. On the other hand, the greater distance between the PC1 scores of the "MP+Mix" group and the vector burdens of the intestinal condition (IP) and the percentage of viable cells (VE) (Appendix A **Fig.** S5b) confirm that the pollutants (in combination) more intensely affected the positioning of the gastrointestinal system in the abdominal cavity of tadpoles (**Fig.** 3c), as well as erythrocytic viability (**Fig.** 5Sb).

To complement the information provided by the statistical analyses previously performed, we used data from all biomarkers evaluated to determine the IBRv2 value. The

results of the IBRv2 values calculated for each treatment group are shown in Fig. 6a-c and confirmed as differences among the groups from the integrated visualization of the animal response set. We noticed that the group "MP+Mix" presented the highest IBRv2 value (4.909) (Fig. 6). The groups "MP" and "Mix" presented similar IBRv2 values (3.480 and 3.977, respectively – Fig. 6a) and the IBRv2 of the group "MP + Mix" was 4.909. The star plots indicate that the induction of cytotoxicity biomarkers (percentage of necrotic erythrocytes (NeE) and percentage of apoptotic + necrotic erythrocytes (AENE)) and da AChE activity, together with the reduction in intestinal position score (IP) and time (ST), were the most representative biomarkers to differentiate the "MP+Mix" group from the "MP" and "Mix" groups.

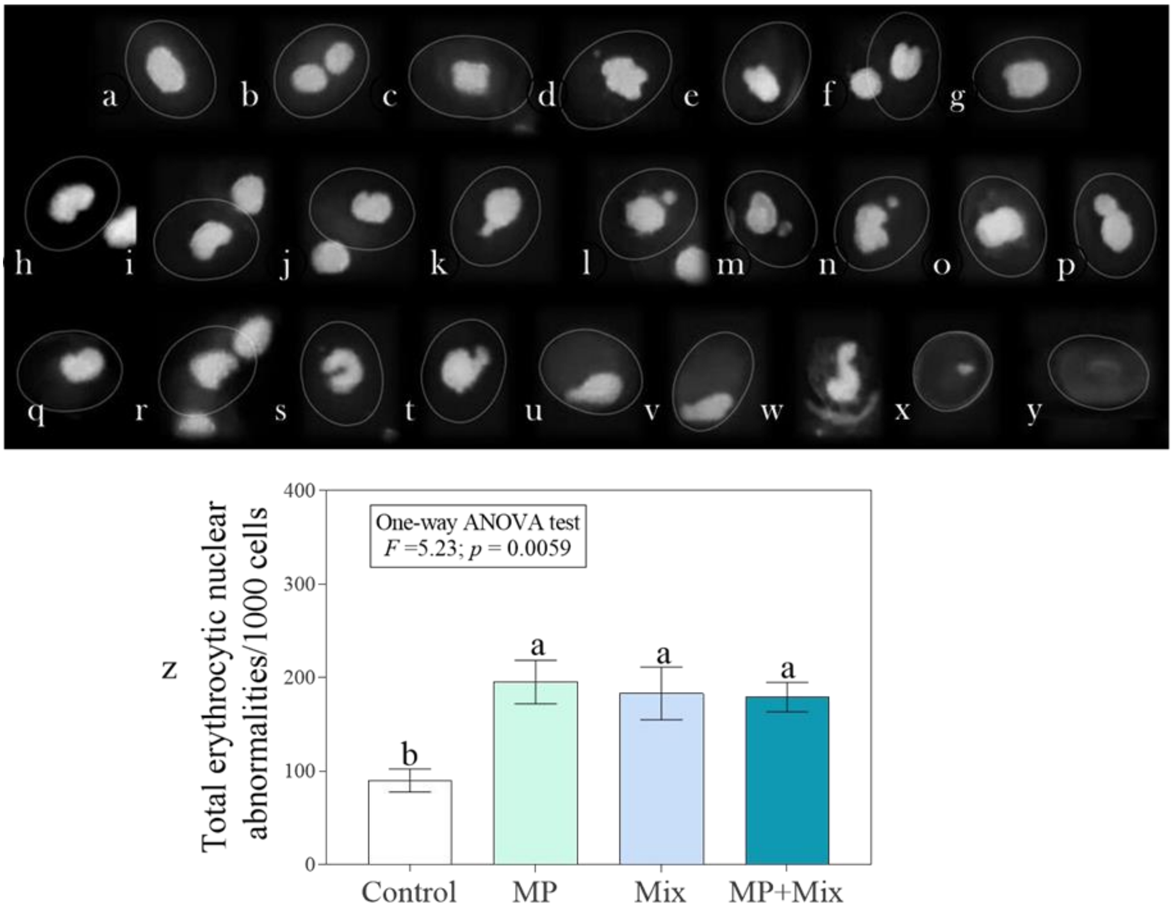

Fig. 4 Photomicrographs representative of different types of erythrocyte nuclear abnormalities (ENAs) observed in *Physalaemus cuvieri* tadpole exposed or unexposed to the polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants) after a 30-day exposure. (a) erythrocyte with a normal nucleus; (b) binucleated; (c-d) multilobulate; (e-g) notched; (h-i and q) kidney-shaped; (j) nuclear bud, (k) blebbed; (l-n) micronucleated; symmetric and (p) symmetric and (r-y) other ENAs with non-conventional nomenclature and (r-y) constriction. (z) Total erythrocytic nuclear abnormalities were observed in *P. cuvieri* exposed to different treatments. Bars represent mean ± SD. The statistical summary is presented at the top of the chart. Distinct lowercase letters indicate significant differences (p<0.05) between groups (n=8 animals/group). MP", "Mix" and "MP+Mix" are the same as Fig. 1.

4. DISCUSSION

It is generally agreed that one of the major challenges of (eco)toxicologic evaluation studies is to understand how pollutants/contaminants are distributed and dispersed in natural environments, their potential interactions, and their mechanisms of action in organisms. This becomes even more challenging considering that chemical contamination results from multiple sources and contaminated sites have many classes of chemicals in their compartments (from a few tens to thousands of compounds, constituting a true toxic "cocktail"). However, toxicological assessment of the effects of pollutants on organisms has not been fully addressed in many investigations. Previous studies have highlighted the type and adsorption of chemical compounds on the surfaces of MPs, and the factors that can increase the bioavailability of pollutants, therefore, potentiate their harmful effects in various environments (Verla et al., 2019; Bhagat et al., 2021; Marchant et al., 2022). On the other hand, it has been demonstrated that MPs can also mitigate the toxicity of various chemical compounds, especially through strong and irreversible bonds that make them less available (Rehse et al., 2018; Kleinteich et al., 2018, Yang et al., 2020). In addition, some reports suggest an apparent absence of combined effects of MPs with other pollutants, such as persistent organic pollutants (Gerdes et al., 2019), fluoranthene (Magara et al., 2019), and the pesticide methiocarb (Schimieg et al., 2020).

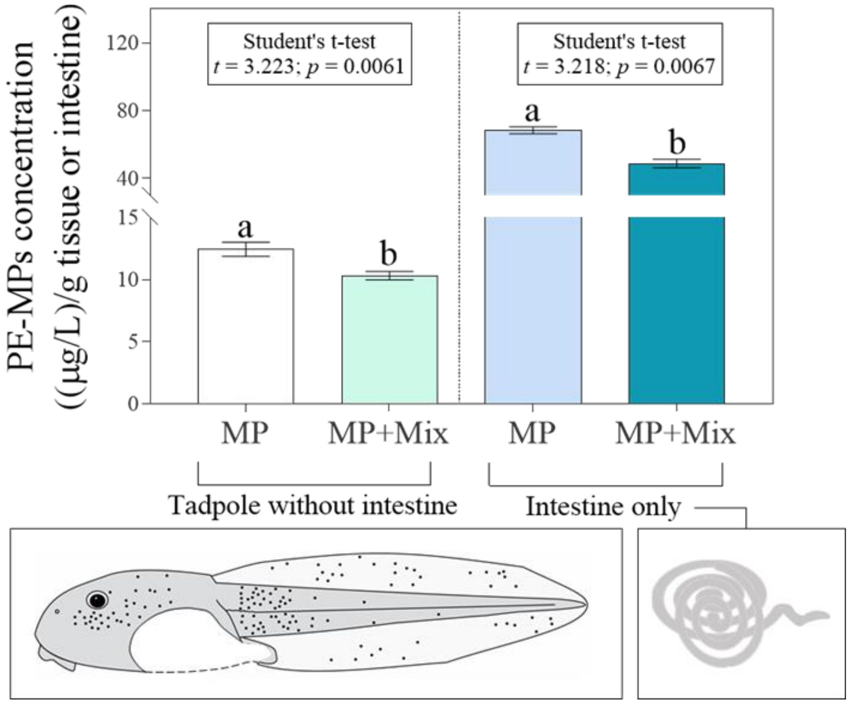

Fig. 5 Concentration of polyethylene microplastics (PE-MPs) in tissues and intestine of *Physalaemus cuvieri* tadpole exposed to polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants) after a 30-day exposure. Bars represent mean ± SD. Statistical summaries are presented at the top of the chart. Distinct lowercase letters indicate significant differences between groups. "MP+Mix" includes animals exposed to PE-MPs in combination with the mix of pollutants (*n*=8 tadpoles/group). "MP" is the same as Fig. 1.

In a single study, we showed that the combination of pollutants with MPs induced a differentiated response in the animals, which can be considered as one of the biomarkers, and this intensifies the current discussions on the impact of MPs as mediators in aquatic organisms. On the other hand, our data also reinforce the discussion promoted by Bartonitz et al. (2020) and Marchant et al. (2022), who point out that the influence of MPs on the toxicity of other pollutants may also differ from other (eco)toxicological endpoints evaluated. In **Fig.** 1a, it is clearly shown that the reduction of natatory activity of tadpoles exposed to MPs and Mix was not observed in animals exposed to the combination of pollutants alone. Contrary to what we expected, the swimming activity of tadpoles in the "MP+Mix" group was significantly higher than in the other experimental groups. Moreover, at the end of the experiment, only the animals of the "MP+Mix" group presented higher total body length and tail width (median region) compared to unexposed tadpoles (Appendix A **Fig.** S2a-b, respectively). In the cytotoxicity assay, the combination of MPs and the pollutant mix increased the frequency of unviable erythrocytes (necrosis+apoptosis) recorded in the animals of the MP group by more than 90% (**Fig. S**3e). On the other hand, the ROS levels detected in the "MP+Mix"group were statistically equivalent to those observed in the control group, which were different from the oxidative stress induced by the pollutant mix alone (Appendix A **Fig.** S4b).

The proposition of any hypothesis for the induction of these differentiated effects will require the development of future investigations, especially regarding the mechanisms involving the interactions between the PE-MPs and the other pollutants of the mix, as well as their potential (eco)toxicological effects. However, speculation in this field is welcome and may direct further studies. The increase in the swimming activity of tadpoles in the "MP+Mix" group may be related, for example, to possible changes in AChE activity induced by pollutants (Appendix A **Fig.** S4F). It is known that AChE is mainly found in neuromuscular junctions and in chemical synapses of the cholinergic type, where its activity serves to terminate synaptic transmission of the cholinergic system (Massoulié et al., 1993; Zimmerman and Soreq, 2006), which play key roles in many functions in the central nervous system, including the control of locomotor functions (Anglister et al., 2008, Silman and Sussman, 2008). In the "MP + Mix" group, the increase in the natatory activity of tadpoles was coincident with the cholinesterase effect inferred by the significant increase in AChE activity (Appendix A **Fig.** S4f) observed in these animals, which has also been reported in previous studies involving the exposure of animals to different chemical compounds (Bonansea et al., 2016; Damazio et al., 2017; Kowalczyk et al., 2020). This suggests that the pollutants may have activated cholinergic receptors by the accumulation of acetylcholine (ACh), demanding a subsequent increase in AChE activity, or caused via yet unknown mechanisms and the disruption of vesicles containing ACh in pre-synoptical neurons, which would have resulted from increased release of neurotransmitters in cholinergic synaptic clefts and overstimulation of post-synaptic receptors.

Table 1 Rotated loading (coefficient) matrix provided by the multivariate analysis to define factors or main components PC1 and PC2.

Biomarkers	**Abbreviation**	**Principal components**[a]	
		PC1	**PC2**
Development stages	DS	0.700	-0.447
Teeth row score	TR	-0.397	**-0.910**[b]
Pigmentation scores of the mandibular sheath	PMS	-0.210	**-0.920**[b]
Bowel tube winding condition score	BTB	-0.286	**-0.905**[b]
Intestinal position score	IP	-0.695	0.600
Body biomass/development stages	BB	0.614	-0.775
Swimming time (sec)	ST	0.029	**0.874**[b]
PE-MPs concentration ((mg/L)/g tissue) - tadpole	MPCT	0.601	-0.292
PE-MPs concentration ((mg/L)/g tissue) - intestine	MPCI	0.564	-0.384
Total body length/DS	TBL	**0.964**[b]	0.266
Mouth-cloaca length/DS	MCL	**0.971**[b]	0.236
Tail length/DS	TL	**0.977**[b]	0.186
Tail width/DS	TW	**0.893**[b]	-0.250
Tail width (median region)/DS	TEM	**0.991**[b]	-0.124
Tail width (lower region)/DS	TWL	**0.837**[b]	-0.489
Internostril distance/DS	IN	0.713	0.700
Interocular distance/DS	IO	0.955	-0.261
Nostril-eye distance/DS	NE	**0.829**[b]	0.011
Nostril-snout distance/DS	NS	0.371	-0.693
Head width after eyes/DS	HWA	**0.823**[b]	-0.348
Head width before eyes/DS	HWB	0.780	-0.331
Eye area	EA	0.352	**-0.934**[b]
Head area	HA	**0.919**[b]	-0.371
Erythrocyte nuclear abnormalities	ENA	**0.917**[b]	-0.394
Percentage of viable erythrocytes	VE	-0.736	-0.675
Percentage of apoptotic erythrocytes	ApE	0.761	0.618
Percentage of necrotic erythrocytes	NeE	0.643	0.635
Percentage of apoptotic + necrotic erythrocytes	AENE	0.752	0.658

Nitrite	Ni	**0.975**[b]	-0.061
Reactive oxygen species	ROS	0.758	-0.013
Superoxide dismutase	SOD	**0.958**[b]	0.050
Catalase	CAT	**0.962**[b]	0.032
Thiobarbituric acid reactive species	TBARS	**0.972**[b]	0.141
Acetylcholinesterase	AChE	**0.832**[b]	0.484

[a]Only components with eigenvalues >1 and that explain > 10% of the total variance were considered.
[b]Biomarkers with significant projections on the principal components.

On the other hand, the decrease in swimming activity observed in the tadpoles of the "MP" and "Mix" groups (**Fig.** 1a) may be associated with other mechanisms regulating the motor activity of animals that do not necessarily involve the participation of the cholinergic system, since the AChE activity in these animals did not differ from that of the control group (Appendix A **Fig.** S4f). It has been demonstrated that, for example, such plastic particles (Li et al., 2021; Wu et al., 2021) and various chemical compounds present in the "mix of pollutants" (e.g., heavy metals (Viarengo and Nicotera, 1991; Viarengo, 1994)) can interfere with the release of Ca^{2+} from the endoplasmic reticulum and thus perturb Ca^{2+} homeostasis, which mediates a wide variety of intra and extracellular processes, such as motor function (Berridge et al., 2003). According to Chin and Allen (1996) and Baylor and Hollingworth (2012), in skeletal muscle fibers the status of the excitation-contraction–relaxation cycle is determined by the cytosolic Ca^{2+} levels. While high Ca^{2+} levels are crucial for triggering muscle contraction, low levels are critical for initiating muscle relaxation (Berridge et al., 2003). Alternatively, we cannot rule out the hypothesis of the reduction in swimming activity of tadpoles in the "MPs" and "Mix" groups related to possible sensorimotor alterations, which may have altered the reception and neuronal processing information of the current flow (water). Previous reports show that different pollutants can affect the mechanosensory lateral line system of tadpoles (Schmidt et al., 2011; Guimarães et al., 2021a; Nascimento et al., 2021), as well as fish (Faucher et al., 2006; Sonnack et al., 2015; Young et al., 2018; Guimarães et al., 2021b), consequently inducing changes in their behavioral responses, which reinforce our hypothesis.

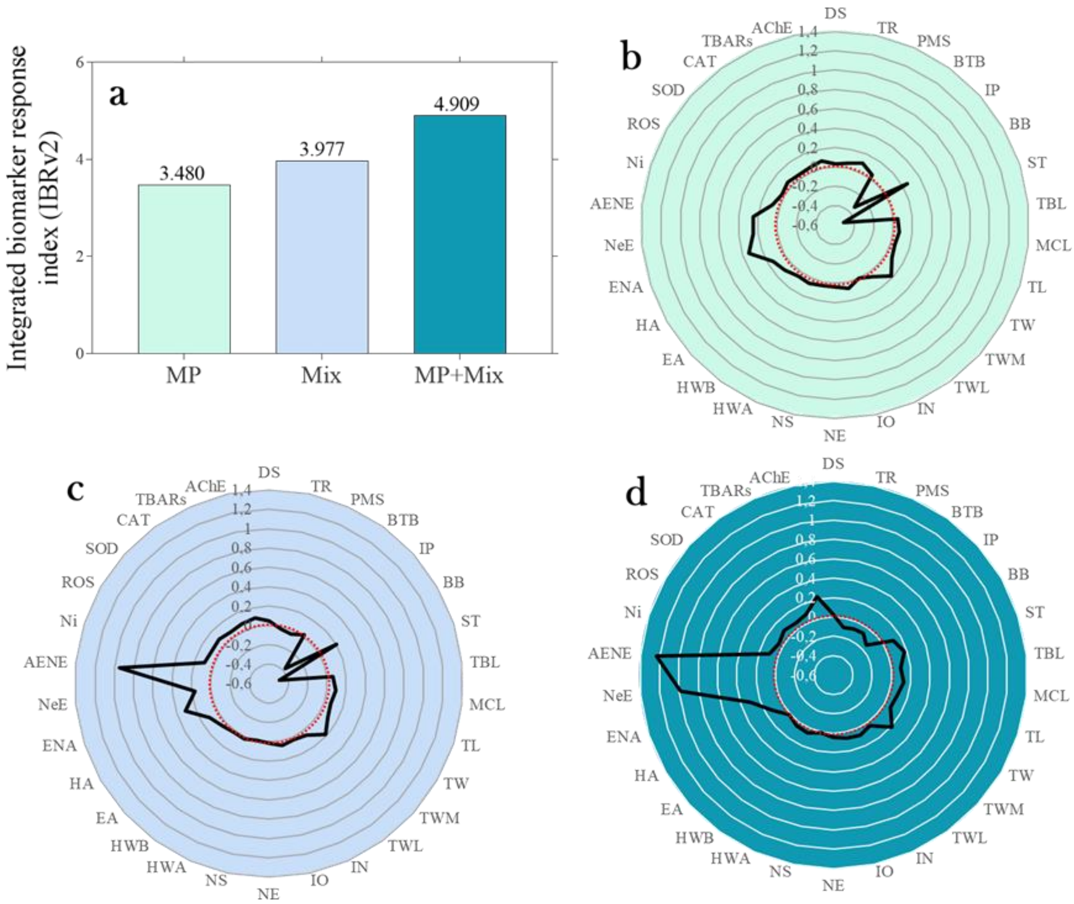

Fig. 6. (a) Results of integrated biomarker response index (IBRv2) calculations and star plots for groups (b) "MP", (c) "Mix", and (d) "MP+Mix". A value calculated for each biomarker was reported in the star plot in a reference deviation of each investigated biomarker. Biomarker results represented the baseline group. The area above 0 reflects induction of the biomarker and below 0 indicates a reduction of the biomarker. DS: development stages; TR: teeth row score; PMS: pigmentation scores of the mandibular sheath; BTB: bowel tube winding condition score; IP: intestinal position score; BB: body biomass/development stages; ST: swimming time; TBL: total body length/DS; MCL: mouth-cloaca length/DS; TL: tail length/DS; TW: tail width/DS; TWM: tail width (median region)/DS; TWL: tail width lower region)/DS; IN: internostril distance/DS; IO: interocular distance/DS; NE: nostril-eye distance/DS; NS: nostril-snout distance/DS; HWA: head width after eyes/DS; HWB: head width before eyes/DS; EA: eye area; HA: head area; ENA: nuclear erythrocyte abnormalities; NeE: percentage of necrotic erythrocytes; AENE: percentage of apoptotic + necrotic erythrocytes; Ni: nitrite; ROS: reactive oxygen species; SOD: superoxide dismutase; CAT: catalase; TBARS: thiobarbituric acid reactive substances; AChE: acetylcholinesterase. MP", "Mix" and "MP+Mix" are the same as Fig. 1.

Interestingly, we also observed a higher total body, tail length and tail width (median region) in tadpoles exposed to the combination of pollutants (MP+Mix) (Appendix A **Fig.** S2a-b) when compared to the control group, which was not expected in our study since growth/development delays have already been reported in tadpoles exposed to several other pollutants (Relyea, 2004; Lavorato et al., 2013; Bach et al., 2016; Svartz et al., 2016; Montalvão et al., 2018; Babalola and van-Wyk, 2021; Boccioni et al., 2021; Cary and Karasov, 2022). In this regard, it is plausible to assume that the abnormal growth of the body and tail of the animals, as well as the median caudal width, is associated with compensatory mechanisms to maintain the regular locomotor capacity of the animals. The interaction of MPs with different pollutants in the mix may have increased aggregation between dispersed particles in water, with a consequent increase in water resistance, which would require the animals to have a stronger propulsion force to overcome the trawling pressure. In this case, the development of the caudal muscles of tadpoles exposed to a combination of pollutants (inferred by the increase in tail width (median region)), along with increased tail body and tail length, corroborate this hypothesis together with the studies by Baboza et al. (2018) and Araujo et al. (2020), involving *Dicentrarchus labrax* and *P. cuvieri* tadpoles, respectively.

Regarding the cytotoxicity assay, we showed that both exposures to “Mix” and “MP+Mix” induced a significant increase in the frequency of erythrocytes during the process of apoptosis and necrosis (**Fig.** S3e). On average, the total sum of these cell types observed in the "Mix" and "MP-Mix" groups was 1627% higher than in the unexposed tadpoles, which may be related to the increase in TBARS levels (LPO indicators) in these same animals (Appendix A **Fig.** S4e), whose relationship with apoptosis and necrosis processes is well described in the literature (Chukhlovin, 1996; Das, 1999; Kannan and Jain, 2000; Franco et al., 2009). However, it is interesting to note that the combination of PE-MPs with the mix of pollutants appeared to intensify the cytotoxic effect of the chemical constituents of the mix. The analysis presented in **Fig.** S3e allows us to observe that in the "MP+Mix" group, the percentage of apoptotic + necrotic erythrocytes was 92.9% higher than the frequency of these cells in tadpoles exposed to the pollutant mix alone. Although we did not observe statistical differences between these groups, these results cannot be neglected from a biological point of view. On the other hand, it is unlikely that the slight increase (i.e., 2.6%) in the TBARS levels in the "MP+Mix" group compared to the "Mix" group (Appendix A **Fig.** S4e) was responsible for this significant difference between the groups. In this case, the induction of erythrocytic alterations may have been modulated by other mechanisms such as induction of apoptosis and necrosis caused by the different pollutants to which the tadpoles of the "MP+Mix" group were exposed. As highlighted by Zhu et al. (2000) and Föller et al. (2008), alterations in Ca^{2+} homeostasis, which may be related to the locomotor behavior observed in the animals of this group (**Fig.** 1a), are also commonly observed during apoptosis or necrosis. However, future studies should be conducted to expand our understanding of how PE-MPs (alone or in combination with the mix of pollutants) act to induce these processes in erythrocyte cells and whether their action mechanisms are similar to those of other pollutants responsible for increasing the density of non-viable cells in the tadpole.

By contrast, the ROS production in animals exposed to "MP+Mix" was equivalent to that observed in the animals of the control group (Appendix A **Fig.** S4b), which suggests a mitigating effect of MPs on the oxidative stress induced by the mix of pollutants alone. This result is intriguing since the same response pattern was not observed for TBARS levels (Appendix A **Fig.** S4e) or in the activity of antioxidant enzymes evaluated in our study (SOD and CAT) (Appendix A **Fig.** S4c-d). A possible explanation for this may be related to the fact that this evaluation constitutes a portrait of the biochemical response in animals evaluated after 30 days of exposure, whose ROS production may have been increased in the MP+Mix group in the period before the evaluation. Therefore, the equivalence of ROS levels in this exposed and the control group may represent a reaction of the antioxidant system in the animals exposed to the combination of pollutants. On the other hand, it is also possible that this attenuation is a consequence of other components (enzymatic and non- enzymatic) in the antioxidant system of animals. As highlighted by Mates (2000) and He et al. (2017), several biologically important compounds have been reported to have antioxidant functions, including vitamins (e.g. vitamin C, E, and A, β-carotene, metallothionein, polyamines, melatonin, NADPH, adenosine, coenzyme Q-10, urate, ubiquinol, polyphenols, flavonoids, phytoestrogens, cysteine, homocysteine, taurine, methionine, s-adenosyl-l-methionine, resveratrol, nitroxides, GSH, glutathione peroxidase (GPX), thioredoxin reductase, nitric oxide synthase (NOS), heme oxygenase-1 (HO-1) and eosinophil peroxidase (EPO). In this sense, future studies are needed to evaluate the impact of these components on the antioxidant defense system of tadpoles exposed to the combinationof pollutants (MP+Mix).

Another intriguing issue observed in our study refers to the lower uptake and accumulation of PE-MPs in tadpoles exposed to the combination of pollutants (**Fig.** 5), which suggests that the interaction between plastic particles and other distinct compounds/chemical elements may have decreased the bioavailability of the MPs in the exposure waters. In this case, the complexity of the chemical processes can explain these results and, therefore, new studies are needed. However, one possibility would be related to an increase in the density of PE-MPs (with a consequent decrease in their dispersion in the water column) when interacting (via sorption/desorption) with other pollutants, as suggested by Wang et al. (2017). In addition, the complexation of MPs with other pollutants, mainly through hydrophobic and electrostatic interactions, pore-filling, and π-π interactions, which are largely governed by the nature of the microplastic polymer, chemical properties of the compounds, and environmental conditions (Wang et al., 2020; Torres et al., 2021; Menéndez-Pedrizaand Jaumot, 2021), could have caused greater aggregation between MPs and other compounds, consequently hindering their intestinal absorption, culminating in the lower translocation of MPs to the hepatic-portal circulation. In this case, even if ingested by the animals, the unabsorbed PE-MPs would have been excreted in the feces.

Finally, it is important to consider the natural difficulty in understanding the possible effects observed in animals exposed to the mix of pollutants (alone or in combination with PE-MPs), considering the complexity of the chemical composition of these pollutants as well as the diversity of interactions that may occur between them. This also explains the lack of studies involving the (eco)toxicity of complex mixtures of pollutants and the absence of definitive conclusions on the additive, synergistic or antagonistic effects of MPs. As recently discussed by Bhagat et al. (2021), several factors can affect the interactions between pollutants and MPs (e.g., polymer types, physical or chemical properties of MPs and the contaminants, particle size, color, surface roughness, and functional groups) and therefore can play decisive roles in all the toxicological effects observed. In this sense, future investigations should focus on finding out how much the chemical composition of the mixture and the individual mechanisms of action of its pollutants support the toxicological results. In addition, the diversity of experimental models to be tested should be increased, not only to increase the environmental representativeness of the studied taxonomic groups but also because the choice of the test organism also plays an important role in the (eco)toxicity of the MPs (alone or in combination with other pollutants). Equally important is to consider the stability of MPs when mixed with different pollutants, as well as to investigate whether pollutants adsorbed in MPs are desorbed within the body, and lead to greater exposure. If, on the one hand, these questions expose some limitations of our study, on the other hand, we need to consider the pioneering aspects of our research and that the effects induced by pollutants (MP+Mix) can be as complex as their chemical constitution.

4. CONCLUSIONS

In conclusion, the data obtained in our study, taken together, confirm the hypothesis that the exposure of *P. cuvieri* tadpoles to PE-MPs in combination with a mix of emerging pollutants induces an enhanced stress response considering the multiple biomarkers evaluated through IBRv2 and PCA analysis. On the other hand, our study also shows that the effects of pollutants (PE-MPs and mixture of pollutants, alone or in combination) in animals can be considered biomarker-dependent, with some being more sensitive for the observation of synergistic effects, others showing antagonistic effects, and others showing no synergistic, antagonistic, or additive interactions. In any case, it is crucial to consider that our study is not exhaustive and that the continuity of investigations in this area is fundamental for understanding the real impact of the different pollutants dispersed in freshwater ecosystems, as well as the contribution of MPs to their harmful effects on aquatic biota.

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Dados suplementares

Capitulo II) Toxicity assessment of polyethylene microplastics in combination with a mix of emerging pollutants on the *Physalaemus cuvieri* tadpole

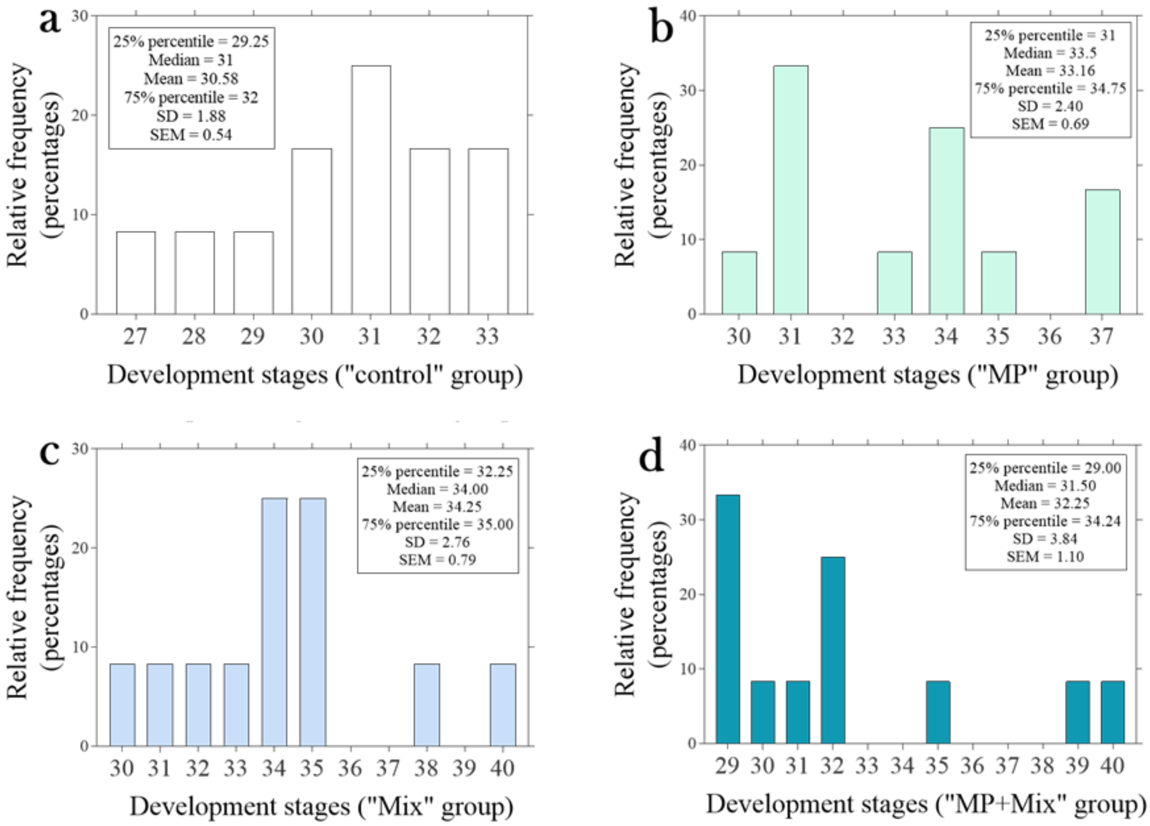

Fig. S1 Histogram distribution of the larval development stages of *Physalaemus cuvieri* tadpole exposed or not to the polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants) after a 30-day exposure. "MP" refers to the group composed of animals exposed only to PE-MPs (at 2.7×10^8 PE-MPs particles/m^3); "Mix", those exposed to the mix of pollutants (see concentrations in Table S1) and "MP+Mix" include animals exposed to PE-MPs in combination with the mix of pollutants.

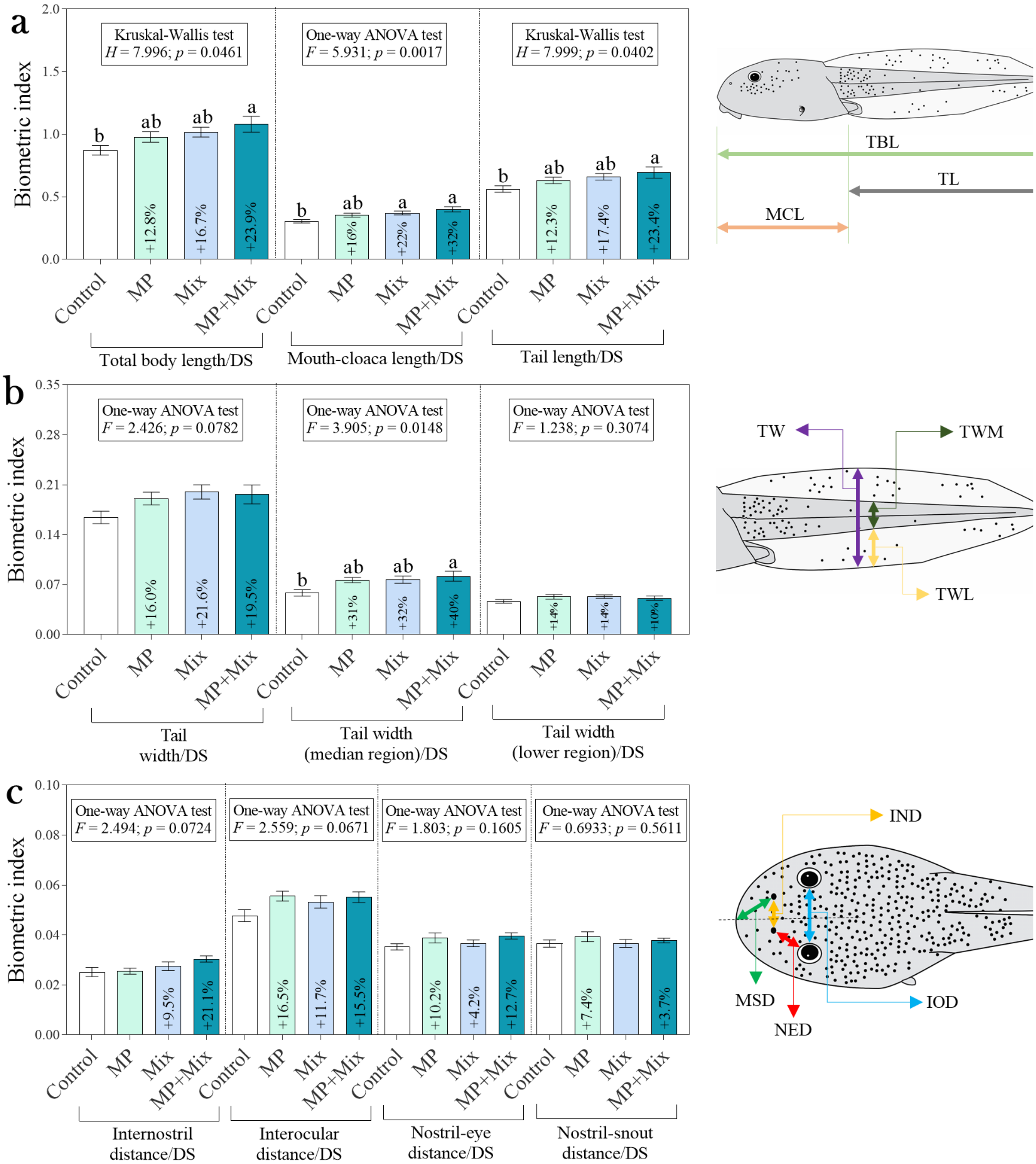

Fig. S2 Biometric index evaluated *in Physalaemus cuvieri* tadpole exposed or unexposed to the polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants) after a 30-days exposure. Bars represent mean ± SD. Statistical summaries are apresenados at the top of the charts. Distinct lowercase letters indicate significant differences between "Control *vs*. MP *vs*. Mix *vs*. MP+Mix" groups. The percentages presented in the base of the bars refer to the increase in biomarkers, compared to the "control" group. "MP" refers to the group composed of animals exposed only to PE-MPs (at 2.7 × 10^8 PE-MPs particles/m^3); "Mix", those exposed to the mix of pollutants (see concentrations in **Table** S1) and "MP+Mix" include animals exposed to PE-MPs in combination with the mix of pollutants (*n*=12 tadpoles/group).

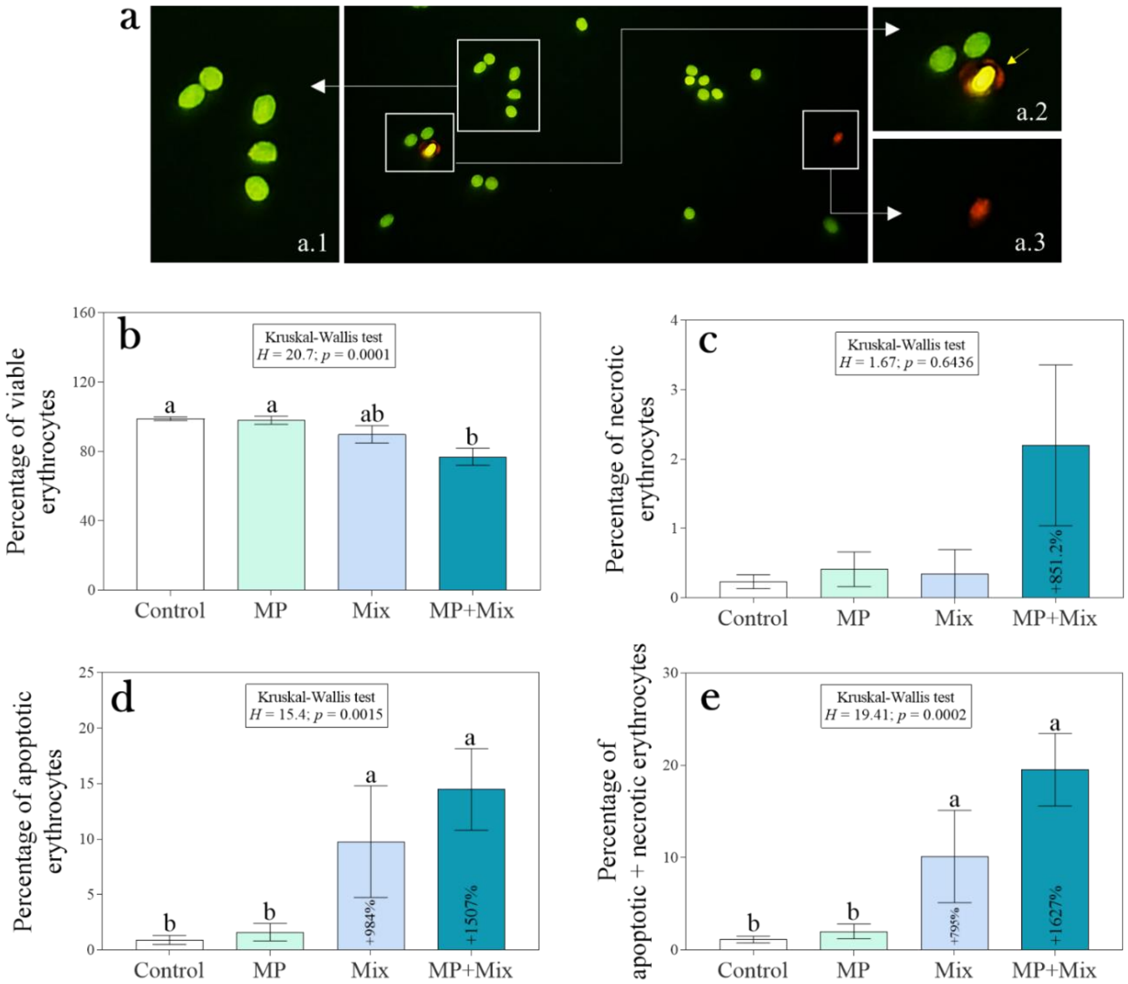

Fig. S3 (a) Photomicrographs representative of erythrocytes evaluated via fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes (a.1: viable erythrocytes; a.2: necrotic erythrocyte (yellow arrow) and a.3: apoptotic erythrocyte). Percentage of (b) viable, (c) necrotic, (d) apoptotic, and (e) necrotic+apoptotic erythrocytes of *Physalaemus cuvieri* tadpole exposed or not to the polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants) after a 30-day exposure. Bars represent mean ± SD. From "b" to "e", statistical summaries are presented at the top of the graphics and the distinct lowercase letters indicate significant differences between the groups. The percentages presented in the base of the bars refer to the increase of biomarkers in the respective groups, compared to the control group. "MP" refers to the group composed of animals exposed only to PE-MPs (at 2.7 × 10^8 PE-MPs particles/m^3); "Mix", those exposed to the mix of pollutants (see concentrations in **Table** S1), and "MP+Mix" include animals exposed to PE-MPs in combination with the mix of pollutants (n=9 tadpoles/group).

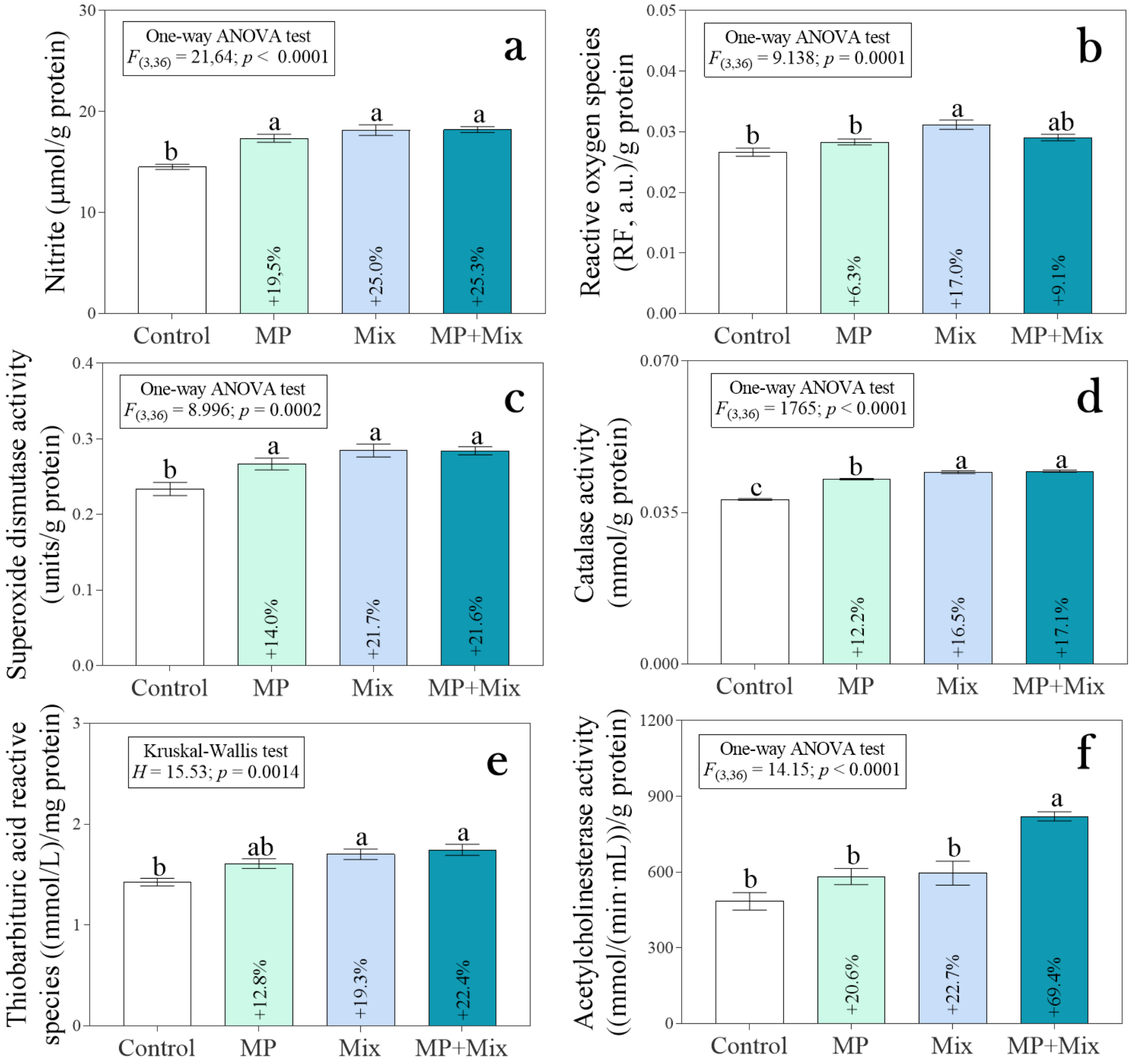

Fig. S4 Levels of (a) nitrite, (b) reactive oxygen species (ROS), (c) superoxide dismutase (SOD), (d) catalase (CAT), (e) thiobarbituric acid reactive species (TBARS), and (f) acetylcholinesterase (AChE) activity in *Physalaemus cuvieri* tadpoles exposed or not to the polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants) after a 30-day exposure. Bars represent mean ± SD. Statistical summaries are presented at the top of the charts. Distinct lowercase letters indicate significant differences between groups. The percentages presented at the base of the bars refer to the increase of biomarkers in the respective group, compared to the control group. "MP" refers to the group composed of animals exposed only to PE-MPs at 2.7×10^8 PE-MPs particles/m^3); "Mix", those exposed to the mix of pollutants (see concentrations in **Table** S1), and "MP+Mix" include animals exposed to PE-MPs in combination with the mix of pollutants (*n*=10 tadpoles/group).

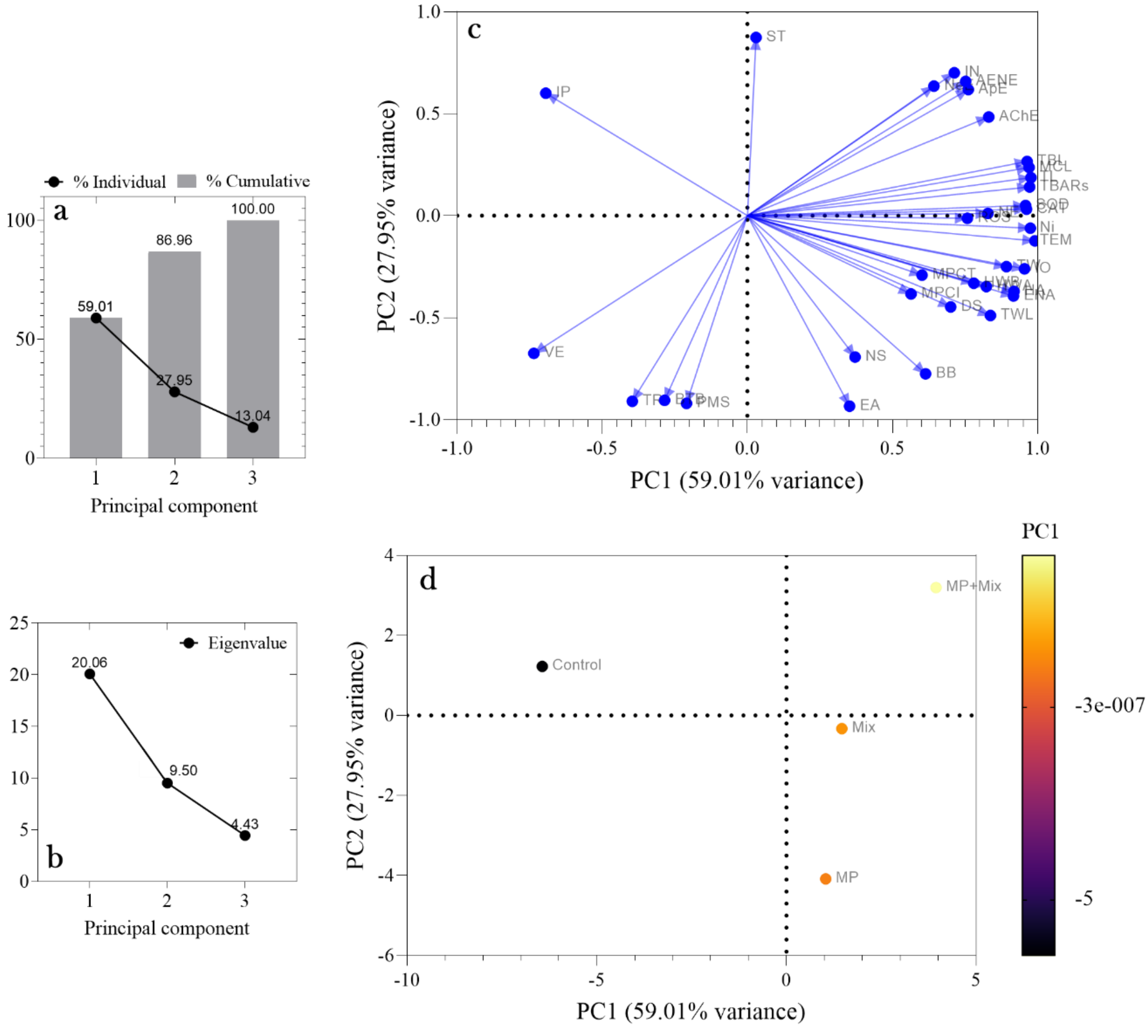

Fig. S5 (a) Proportion of variance and (b) eigenvalues of the main components, (c) loading plot of the variables investigated, and (d) PC1 scores of experimental groups. "MP" refers to the group composed of animals exposed only to PE-MPs (at 2.7× 10^8 PE-MPs particles/m^3); "Mix", those exposed to the mix of pollutants (see concentrations in **Table** S1) and "MP+Mix" include animals exposed to PE-MPs in combination with the mix of pollutants.

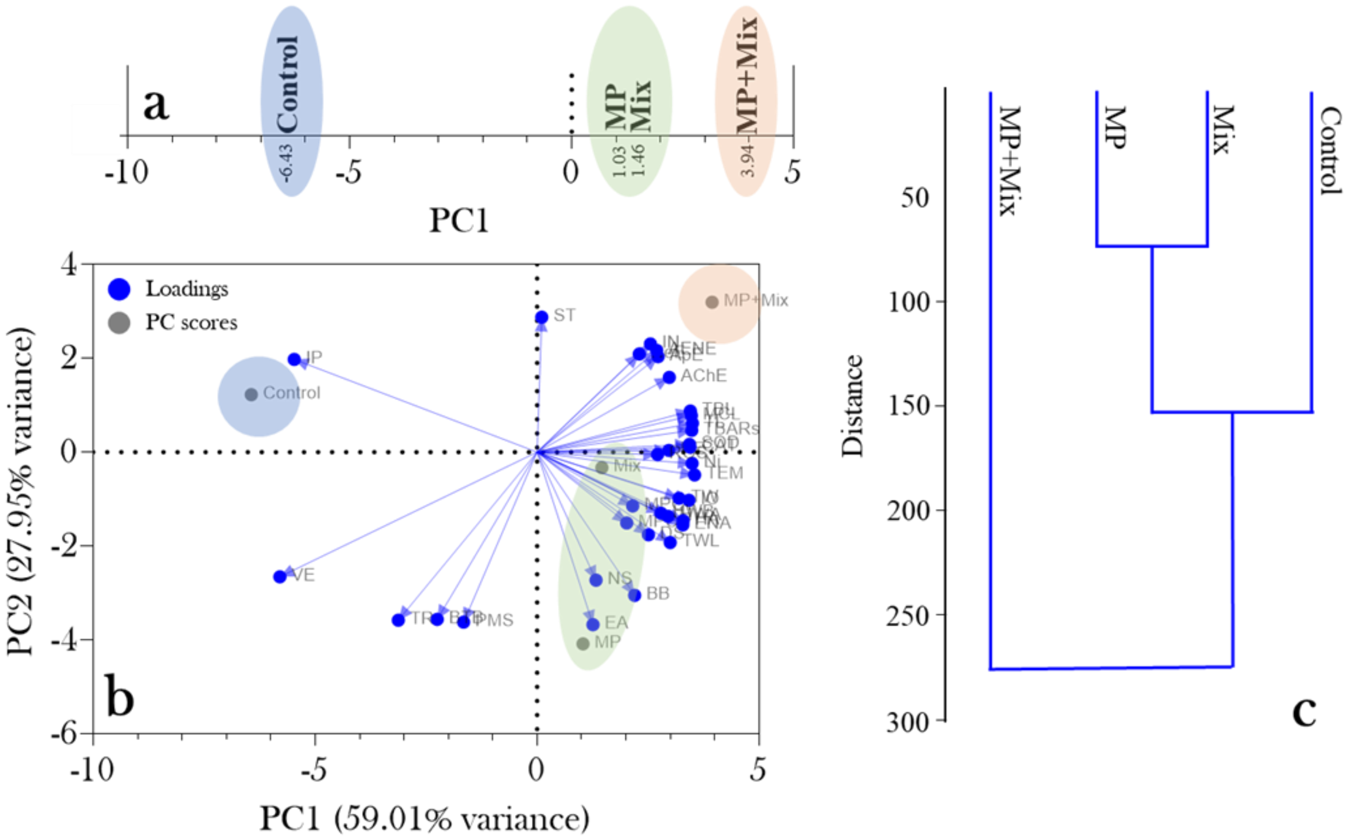

Fig. S6. (a) PC1 scores of experimental groups, (b) PCA biplot of the first two main components simultaneously showing the PC scores of experimental groups (points in gray) and loadings of explanatory variables (vectors – blue arrows) and (c) cluster analysis dendrogram. The meanings of the abbreviations presented in "B" are presented in **Table** 2. "MP" refers to the group composed of animals exposed only to PE-MPs (at 2.7 × 10^8 PE-MPs particles/m^3); "Mix", those exposed to the mix of pollutants (see concentrations in **Table** S1) and "MP+Mix" include animals exposed to PE-MPs in combination with the mix of pollutants.

Table S1 General information about the mixed emerging pollutants used in our study.

Components	IUPAC names	Molecular formula	Concentrations	References
Amoxicilin[1]	(2S,5R,6R)-6-[[(2R)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid	$C_{16}H_{19}N_3O_5S$	0.0045 µg/L	Sodré et al. (2010)
Acetylsalicylic acid[2]	2-acetyloxybenzoic acid	$C_9H_8O_4$	0.34 µg/L	Ternes (1998)
Sodium diclofenac[3]	sodium; 2-[2-(2,6-dichloroanilino)phenyl]acetate	$C_{14}H_{10}Cl_2NNaO_2$	1.8 µg/L	Hoeger et al. (2005)
Ibuprofen[4]	2-[4-(2-methyl propyl)phenyl]propanoic acid	$C_{13}H_{18}O_2$	2.7 µg/L	Flippin et al. (2007)
Fluoxetine[5]	N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine	$C_{17}H_{18}F_3NO$	0.030 µg/L	Perreault et al. (2003)
Clonazepam[6]	5-(2-chlorophenyl)-7-nitro-1,3-dihydro-1,4-benzodiazepin-2-one	$C_{15}H_{10}ClN_3O_3$	0.053 µg/L	Ternes et al. (2001)
Dipyrone monohydrate[7]	sodium;[(1,5-dimethyl-3-oxo-2-phenyl pyrazole-4-yl)-methylamino]methanesulfonate;hydrate	$C_{13}H_{18}N_3NaO_5S$	5 µg/L	Pamplona et al. (2011)
Ranitidine[8]	(E)-1-N'-[2-[[5-[(dimethylamino)methyl]furan-2-yl]methylsulfanyl]ethyl]-1-N-methyl-2-nitroethene-1,1-diamine	$C_{13}H_{22}N_4O_3S$	10 ng/L	Boxall (2004)
Benzene[9]	Benzene	C_6H_6	0.005 mg/L	Souza et al. (2018)
Tannery effluent[10]	-	-	1%	Rabelo et al. (2016)
Estradiol cypionate[11]	[(8R,9S,13S,14S,17S)-3-hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthren-17-yl] 3-cyclopentylpropanoate	$C_{26}H_{36}O_3$	2.6 µg/L	Jardim et al. (2012)
Nitrogen[12]	Molecular nitrogen	N_2	2.4 mg/L	Xu et al. (2014)
Glyphosate[13]	2-(phosphonomethylamino)acetic acid	$C_3H_8NO_5P$	0.70 mg/L	Peruzzo et al. (2008)
Abamectin[14]	(1'R,2R,3S,4'S,6S,8'R,10'E,12'S,13'S,14'E,16'E,20'R,21'R,24'S)-2-butan-2-yl-21',24'-dihydroxy-12'-[(2R,4S,5S,6S)-5-[(2S,4S,5S,6S)-	$C_{95}H_{142}O_{28}$	0.004 mg/L	Vasconcelos et al. (2016)

	5-hydroxy-4-methoxy-6-methyloxan-2-yl]oxy-4-methoxy-6-methyloxan-2-yl]oxy-3,11',13',22'-tetramethylspiro[2,3-dihydropyran-6,6'-3,7,19-trioxatetracyclo[15.6.1.14,8.020,24]pentacosa-10,14,16,22-tetraene]-2'-one;(1'R,2R,3S,4'S,6S,8'R,10'E,12'S,13'S,14'E,16'E,20'R,21'R,24'S)-21',24'-dihydroxy-12'-[(2R,4S,5S,6S)-5-[(2S,4S,5S,6S)-5-hydroxy-4-methoxy-6-methyloxan-2-yl]oxy-4-methoxy-6-methyloxan-2-yl]oxy-3,11',13',22'-tetramethyl-2-propan-2-ylspiro[2,3-dihydropyran-6,6'-3,7,19-trioxatetracyclo[15.6.1.14,8.020,24]pentacosa-10,14,16,22-tetraene]-2'-one			
Detergent[15]	-	-	740 μg/L	Mortatti et al. (2012)

[1]TEUTO, *Laboratório Teuto Brasileiro* S/A, Anápolis, GO, Brazil; [2]*Brasterápica Farmacêutica*, Atibaia, SP, Brazil; [3]*BrainFarma Indústria Química & Farmacêutica* Ltda, Anápolis, GO, Brazil; [4]*GeoLab Indústria Farmacêutica* S/A, Anápolis, GO, Brazil; [5]*Hipolabor Farmacêutica ltda*, Belo Horizonte, MG, Brazil; [6]*GERMED Farmacêutica*, Hortolândia, SP, Brazil; [7]*Farmace ind. Químico-Farmacêutica Vearense*, Barbalha, CE, Brazil; [8]*Medquímica Indústria Farmacêutica*, Juiz de Fora, MG, Brasil; [9]*Proquímicos*, Rio de Janeiro, RJ, Brazil;; [11]ZOETIS PFIZER, Itapevi, SP, Brazil; [12]USI FERTIL, Maruim, SE, Brazil; [13]UPL, Ituvebara, SP, Brasil; [14]Bayer CropScience Ltda, Belford Roxo, RJ, Brazil; [15]*Química Amparo* (Ypê), Figueira, SP, Brazil. (-): Complex chemical composition because the pollutant is formed by multiple xenobiotics.

CAPÍTULO III

TOXICITY EVALUATION OF THE COMBINATION OF EMERGING POLLUTANTS WITH POLYETHYLENE MICROPLASTICS IN ZEBRAFISH: PERSPECTIVE STUDY OF GENOTOXICITY, MUTAGENICITY, AND REDOX UNBALANCE[1]

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Toxicity evaluation of the combination of emerging pollutants with polyethylene microplastics in zebrafish: perspective study of genotoxicity, mutagenicity, and redox unbalance

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ABSTRACT

Despite the toxicity of microplastics (MPs) in freshwater fish has been demonstrated in previous studies, their effects when mixed with other pollutants (organic and inorganic) are poorly understood. Thus, we aimed to test the hypothesis that the association of polyethylene MPs (PE-MPs) to a mix of emerging pollutants induces more adverse genotoxic, mutagenic, and redox unbalance effects in adult zebrafish (*Danio rerio*), after 15 days of exposure. Although the accumulation of MPs in animals was greater in animals exposed to PE-MPs alone, erythrocyte DNA damage (comet assay) and the frequency of erythrocytic nuclear abnormalities (ENAs) evidenced in zebrafish exposed to PE-MPs alone were as pronounced as those observed in animals exposed to the mix of pollutant (alone or in combination with MPs), which constitutes the big picture of the current study. Moreover, we noticed that such effects were associated with an imbalance between pro-and antioxidant metabolism in animals, whose activity of superoxide dismutase (SOD) and catalase (CAT) was assessed in different organs which were not sufficient to counterbalance the production of reactive oxygen species [hydrogen peroxide (H_2O_2)] and nitrogen [nitric oxide (NO)] evaluated. The principal component analysis (PCA) also revealed that while the antioxidant activity was more pronounced in the brain and liver of animals, the highest production of H_2O_2 was perceived in the gills and muscles, suggesting that the biochemical response of the animals was organ dependent. Thus, the present study did not demonstrate antagonistic, synergistic, or additive effects on animals exposed to the combination between PE-MPs and a mix of pollutants in the zebrafish, which reinforces the theory that interactions between pollutants in aquatic ecosystems may be as complex as their effects on freshwater ichthyofauna.

Keywords: Water pollution, *Danio rerio*, plastic particles, mixture toxicity, ecotoxicology, biomarkers

1. INTRODUCTION

Water pollution has extended outstanding levels in history, which has been associated, particularly, with population growth and the increase in production, consumption, and disposal of different products in natural environments (Inyinbor Adejumoke et al., 2018). Previous studies have confirmed the presence of different pollutants/contaminants in freshwater ecosystems, which confirms that anthropogenic activities greatly alter the natural composition of these environments. Recent reports of the presence of heavy metals (Ohiagu et al., 2021; Karaouzas et al., 2021), phenolic compounds (Ramos et al., 2021a,b), petroleum (Daniel & Nna, 2016; Edori & Edori, 2021), surfactants (Becker et al., 2008; Al-Ani et al., 2020), pesticides (De-Souza et al., 2020; Shah & Parveen, 2021; Campanale et al., 2021), pharmaceutical residues (including drugs for human health and disinfectants) (Rico et al., 2021; Xiang et al., 2021), organophosphate flame retardants (Han et al., 2021; Li et al., 2021), personal care products (Singh & Suthar, 2021; Liu et al., 2021), in addition to microparticles (MPs) arising from the degradation of larger plastic products (Huang et al., 2021; Napper et al., 2021), confirm the anthropogenic effects on freshwater quality.

Regarding MPs, a vast collection of studies has already shown how these materials can affect the health of organisms (Anbumani & Kakkar, 2018; Huang et al., 2021; Vo & Pham, 2021), including bacteria (Sun et al., 2018), fungi (Yang et al., 2021; Li et al., 2022), protists (Li et al., 2021; Sun et al., 2021), plants (Rillig et al., 2019; Jiang et al., 2019) and animals (Meaza et al., 2021; Yong et al., 2020; Wang et al., 2020; Wang et al., 2021; Araujo et al., 2021). Among the alterations induced in fish by MPs, particularly, those related to teratogenic effects (Malafaia et al., 2020; Xia et al., 2022), histopathological (Abarghouei et al., 2021; Varó et al., 2021), biochemical/immunological (Hamed et al., 2020; Wang et al., et al., 2021), mutagenic and genotoxic (Araujo et al., 2020; Hamed et al., 2020; Pannetier et al., 2020), hematological (Hamed et al., 2019; Kim et al., 2021), cytotoxic (Guimarães et al., 2021), endocrine (Wang et al., 2022), neurological/behavioral (Kim et al., 2021), as well as reproductive damage (Hou et al., 2021), among others.

Studies with isolated MPs are opportune and important to identify and characterize the impact of these particles on the ichthyofauna. However, the possible extrapolation of their results to the real environmental context is limited, especially considering the use of concentrations much higher than those identified in nature and the conduction of research that focuses only on the isolated effects of MPs. As discussed by Souza et al. (2018), when prioritizing designs that are distant from the real pollution scenario and/or that investigate the toxicological effects of only a given pollutant, the enormous variety of xenobiotics that co-occur in aquatic ecosystems is neglected. Furthermore, our knowledge about the effects of MPs when associated with different aquatic pollutants is still very limited. As discussed by Rist & Hartmann (2018), due to their dimensions and chemical properties, the association of MPs with other emerging pollutants can affect the mobility, availability, and fate of xenobiotics and, in a pessimistic scenario, favor the entry of these pollutants into organisms and exacerbate its harmful effects.

In this regard, studies involving mixes of pollutants have been, for the most part, restricted to evaluating the toxicity of the mix of a few pollutants (e.g.: Ambreen & Javed, 2015; Do-Amaral et al., 2018; Gopinathan & Binukumari, 2021) and effluents without, however, considering the co-occurrence of MPs [e.g.: tannery effluents (Souza et al., 2017; Montalvão et al., 2018; Quintão et al., 2018; Amaral et al., 2018; Chagas et al., 2019; Dos-Reis-Sampaio et al., 2019; Tamilmathi et al., 2021), effluent distillery (Tripathi et al., 2021), effluent pharmaceutical industry wastewater treatment plant (WWTP) (Tardy et al. al., 2021), textile effluent (Saxena et al., 2021; Methneni et al., 2021), refinery effluent (Onwumere et al., 1990; Krishnakumar et al., 2007), as well as those involving water-soluble fraction from crude oil (Meador & Nahrgang, 2019)]. On the other hand, investigations into the toxicity of MPs in association with other pollutants have been limited to the study of the effects of simple binary combinations on organisms (Ziajahromi et al., 2017; Barboza et al., 2018; Wakkaf et al., 2020; Li et al., 2020; Yang et al., 2020; Zhang et al., 2021; Thi et al., 2021).

Thus, from a design that simulates the contamination of water by a range of emerging pollutants of a diversified chemical nature, we aimed to evaluate the possible alone and combined effects of MPs and a mix of pollutants [based on Souza et al. (2018)] on zebrafish (*Danio rerio*) adults, using biomarkers of mutagenicity, genotoxicity, and oxidative stress. Zebrafish have been indicated as an excellent animal model for the toxicity assessment of plastic particles (see review by Bhagat et al., 2020). In this study, we started from the hypothesis that MPs, when interacting with other pollutants, can induce more serious adverse effects in animals. To the best of our knowledge, this study is the first to address the toxicity of MPs in association with several other pollutants in a vertebrate model and, therefore, expands our knowledge of the real magnitude of the impacts of these particles on the freshwater ichthyofauna.

2. MATERIAL AND METHODS

2.1. Microplastics

We utilized polyethylene (PE) microparticles (PE-MPs) as representative of MPs, as they are one of the most used polymers currently in the production of different products (Horton et al., 2017). Such MPs were obtained from Sigma-Aldrich (CAS number: 9002-88-4) and, as demonstrated in a previous study by our group (Araujo et al., 2020a), have 35.46 μm ± 18.17 μm of diameter (mean ± SD) and heterogeneous shape, with irregular surfaces, rough or smooth. The PE-MPs used in the exposures came from a stock solution prepared with ethanol (at 50% v/v) (24 g/L), which is equivalent, according to the conversion proposed by Leusch & Ziajahromi (2021), to 1 x 10^{12} particles/m^3. We emphasize that this dispersion and the exposure waters were free of any dispersant or preservative.

2.2. Mix of pollutants

To simulate the aquatic contamination by different xenobiotics, we prepared a mix composed by the addition of 15 pollutants in environmentally relevant concentrations (i.e.: that were previously identified in surface waters). As can be seen in Table 1, these components more realistically represent the diversity of pollutants that can enter freshwater ecosystems from diffuse or point sources, especially when including pesticides, agro-industrial effluent, pharmaceutics/hormone, agricultural fertilizers, surfactant, and constituent substance of petroleum. The physicochemical and inorganic characterization and the profile of organic compounds identified in this mix can be observed in Souza et al. (2018).

Table 1. General information about the mixed emerging pollutants used in our study.

Group	Class of constituents	Active ingredient	CAS number(*)	Brands	Concentrations	References
Pharmaceutical	Antibiotic	Amoxicilin	26787-78-0	TEUTO, Laboratório Teuto Brasileiro S/A, Anápolis, GO, Brazil	0.0045 μg/L	Sodré et al. (2010)
	Anti-inflammatory	Acetylsalicylic acid	50-78-2	Brasterápica Farmacêutica, Atibaia, SP, Brazil	0.34 μg/L	Ternes (1998)
		Sodium diclofenac	15307-79-6	BrainFarma Indústria Química e Farmacêutica Ltda, Anápolis, GO, Brazil	1.8 μg/L	Hoeger et al. (2005)
		Ibuprofen	15687-27-1	GeoLab Indústria Farmacêutica S/A, Anápolis, GO, Brazil	2.7 μg/L	Flippin et al. (2007)
	Antidepressant	Fluoxetine	54910-89-3	Hipolabor Farmacêutica ltda, Belo Horizonte, MG, Brazil	0.030 μg/L	Perreault et al. (2003)
	Anxiolytic	Clonazepam	1622-61-3	GERMED Farmacêutica, Hortolândia, SP, Brazil	0.053 μg/L	Ternes et al. (2001)
	Analgesic	Dipyrone monohydrate	5907-38-0	Farmace ind. Químico-Farmacêutica Vearense, Barbalha, CE, Brazil	5 μg/L	Pamplona et al. (2011)
	Antiacid	Ranitidine	66357-35-5	Medquímica Indústria Farmacêutica, Juiz de Fora, MG, Brasil	10 ng/L	Boxall (2004)
Hydrocarbon	Benzene	Benzene	50-00-0	Proquímicos, Rio de Janeiro, RJ, Brazil	0.005 mg/L	Brasil (2004)

Agroindustrial waste	Tannery effluent	-	-	-	1%	Rabelo et al. (2016)
Synthetic hormone	Estradiol	Estradiol cypionate	313-06-4	ZOETIS PFIZER, Itapevi, SP, Brazil	2.6 µg/L	Jardim et al. (2012)
Fertilizer	Nitrogen	Nitrogen	7727-37-9	USI FERTIL, Maruim, SE, Brazil	2.4 mg/L	Xu et al. (2014)
Pesticide	Glyphosate	Glyphosate	1071-83-6	UPL, Ituvebara, SP, Brasil	0.70 mg/L	Peruzzo et al. (2008)
	Kraft 36EC	Abamectin	71751-41-2	Bayer CropScience Ltda, Belford Roxo, RJ, Brazil	0.004 mg/L	Vasconcelos et al. (2016)
Surfactant	Detergent	-	-	Química Amparo (Ypê), Figueira, SP, Brazil	740 µg/L	Mortatti et al. (2012)

(*): Source: ChemIDplus (https://chem.nlm.nih.gov/chemidplus/chemidlite.jsp)

2.3. Model system and experimental design

Zebrafish (*D. rerio*) adults (wild-type strain; biomass: 0.58 g ± 0.12 g, body length = 3.06 cm ± 0.17 cm; mean ± SD), of both sexes (ratio 1:1), were obtained from commercial breeding (Goiânia, GO, Brazil), which were acclimated to laboratory conditions for 21 days. In the laboratory, the animals were kept in aquatics containing dechlorinated water (naturally), with constant aeration, the temperature of 26°C, light/dark cycle of 12-h/12-h and with food provided twice a day, with commercial feed for fish (Mix Fish®, Agromix, Jaboticabal, São Paulo, Brazil).

After the acclimatization period, 84 healthy individuals (i.e.: without morphological deformity, apparent lesions, and locomotor alterations) were distributed into four experimental groups. While the "PE-MPs" group was composed of animals kept in water containing 2.7 x 10^8 PE-MPs particles/m^3; zebrafish exposed to the mix of pollutants described above (see Table 1) (without the presence of MPs), composed the "Mix" group. On the other hand, the "PE-MPs + Mix" group comprised the animals that were subjected to combined exposure of MPs with the mix of pollutants, at the same concentrations defined in the previous groups. In the negative "control" group, zebrafish were kept in dechlorinated water free from any of the pollutants previously mentioned. We emphasize that to the "Control" and "Mix" groups we added the same amount of ethanol (up to 50%) used as a diluent for PE-MPs, so the final dilution of ethanol in the exposure waters was 0.0125%. Each group was composed of three replicates (n=7 animals/replica, totaling n=21 animals/group). The exposures took place for 15 days in a semi-static system (i.e., with water renewal every 72 h), in glass cylinders with a capacity of 2 L of water. Such exposure period represents 1.1% to 0.8% of zebrafish average life expectancy [3.5-5 years; Gerhard et al. (2002)] and, therefore, constitutes an ephemeral exposure to pollutants. During the exposures, the animals were fed twice a day, with commercial fish food, and were kept under the same conditions to which they were acclimated.

2.3.1. Pollutant concentrations

The concentrations of all pollutants that were used were defined based on previous studies that identified their presence in surface waters, which brings our design closer to a more realistic condition. The studies that supported the definition of the concentrations of the mix constituents are mentioned in Table 1. Furthermore, the concentration of PE-MPs (2.7 x 10^8 particles/m^3), on the other hand, represents a pessimistic plastic pollution scenario estimated by Koelmans et al. (2019), who from the compilation of different studies reported that concentrations of MPs can range from 1 x 10^{-3} to 10^8 particles/m³ in individual freshwater samples (including rivers, lakes, bottled water, and wastewater treatment plants effluent/influent).

2.4. Accumulation of polyethylene microplastics

To evaluate the possible accumulation of plastic particles in animals in the "PE-MPs" and "PE-MPs + Mix" groups, we followed the protocol proposed by Malafaia et al. (2021). Briefly, six random animals from the respective groups were carefully washed in purified water (via reverse osmosis) and euthanized by cooling. Subsequently, the gastrointestinal system was removed (avoiding overestimating the accumulation of MPs in other tissues due to the presence of particles in the stomach and intestinal lumen) and the animals were weighed (0.145 g ± 0.001 g – mean ± SD) and macerated in 1 mL of KOH solution (to 10% wt/v) and transferred to conical bottom tubes (previously sanitized and sterilized) whose volume was made up to 50 mL of alkaline digestion solution. Then, the samples were incubated in a water bath at 60°C (for 18 h) and 80°C (for 2 h) for further filtration in a vacuum pump, using nitrocellulose membranes (pore: 0.45 µm). After that, the membranes were immersed in 3 mL of acetonitrile P.A. and homogenized until all material was completely dissolved. Afterward, the aliquots of each sample were introduced into a Neubauer chamber to count the PE-MPs. For each animal, six chambers (i.e.: 54 quadrants/animal) were analyzed, totaling 324 quadrants/group. The quantification of PE-MPs in the samples was expressed as the number of PE-MPs particles/mg fish.

2.5. Biomarkers

2.5.1. Genotoxicity

Considering that the exposure of animals to PE-MPs (alone or in combination with a mix of pollutants) could induce changes predictive of DNA damage, we carried out the comet assay according to procedures described in Singh et al. (1988) and Estrela et al. (2021), with minor modifications. Briefly, after cooling anesthesia, a cut in the caudal peduncle was performed and the animals have immediately immersed in 200 µL of phosphate-buffered saline (PBS; 4°C, pH 7.2) for 5 min (sufficient time for blood to flow). Then, 15 µL of the blood obtained in the dilution was mixed with 120 µL of low melting point agarose (at 0.5%, wt/v) pre-warmed at 37°C in a water bath. The homogenate was then transferred to slides pre-coated with standard agarose (at 1.5%, wt/v), which were then covered with a coverslip until the material solidified when they were then submerged in lysis solution for 2 h (at 4°C, in a dark environment). Then, the slides were subjected to electrophoresis [at 300 mA and 25 V (0.90

V/cm)], for 30 min, and at the end, the slides were incubated in a neutralizing buffer solution (Tris-HCl, pH 7.5; for 5 min), washed with purified water and then dried at room temperature. Posteriorly, the slides were fixed in ethanol P.A. (for 10 min), stained with ethidium bromide (at 10 µg/mL), and covered with a coverslip for analysis under fluorescence microscopy (BEL Engineering®, model FLUO3, São Paulo, Brazil). One hundred randomly selected nucleoids per animal (totaling 800 nucleoids/group) were evaluated [similarly to Castro et al. (2021)] from photomicrographs processed in the ImageJ software (OpenComet), which recorded the tail intensity (u.a.) of comets, considered a valid metric for assessing the amount of DNA in the tail region (Martins & Costa, 2020; Eid et al., 2021). On the other hand, the calculation of the erythrocyte DNA damage index (performed from the qualitative assessment of the nucleoids) was performed based on the attribution of one of the damage scores (0 to 4), according to the protocol established by Collins et al. (1995). Previous studies have shown that both visual (qualitative) and automated analyzes are important to provide the degree of DNA damage induced by pollutants (Collins, 2004; García et al. 2004; Villela et al. 2006).

2.5.2. Mutagenicity

The micronucleus (MN) test and other erythrocyte nuclear abnormalities (ENAs) were performed [based on Guimarães et al. (2021)], aiming to evaluate a possible mutagenic effect induced by the treatments. For this, 10 µL of blood (n=8 animals/group) were collected (via a cut in the caudal peduncle) and used to perform the blood smear on a previously sanitized glass slide. After drying at room temperature, the slides were fixed in methanol P.A. for 10 min and sequentially stained using *Panótipo Rápido* kit (Laborclin®, Paraná, Brazil, code #620529), according to Pavan et al. (2021) and Estrela et al. (2021), for further analysis under an optical microscope, under 100x magnification. A total of 1,000 cells/slide was analyzed based on the criteria reported by Fenech (2000). In addition to evaluating the presence of MNs, other ENAs were also recorded, according to the nomenclatures adopted by Amaral et al. (2019) and Alvim et al. (2019).

2.5.3. Oxidative stress analysis

Aiming to associate the possible mutagenic/genotoxic effects with the induction of a redox imbalance, different biochemical biomarkers were evaluated. For this, muscle fragments (peduncular), brain, liver, and gills were extracted, macerated in PBS (at 4ºC, pH 7.2), and later centrifuged at 13,000 rpm, at 4°C, for 10 min. Then, the supernatants were collected and stored in an ultra-freezer (-80°C) until the moment of biochemical analysis. Assuming that the treatments could induce an increase in reactive oxygen and nitrogen species, we evaluated the levels of hydrogen peroxide (H_2O_2) and nitric oxide (NO) in the different collected organs.

2.5.3.1. Hydrogen peroxide production

For the measurement of H_2O_2, we adopted the procedures described in Elnemma et al. (2004), with some modifications. Briefly, 100 µL of PBS was added to 96-well microplates and preheated at 37ºC. Subsequently, 10 µL of supernatant and 100 µL of 0.5% ammonium molybdate solution (w/v in purified water) were added, mixed, and after 5 min ELISA reader reading at 405 nm. H_2O_2 concentrations in "mmol/L" were obtained from the standard curve previously obtained ($y = 0.0047x + 0.0126$, $R^2 = 0.9984$).

2.5.3.2. Nitrite production

For the determination of NO production (via nitrite production), we used the method proposed by Grisham et al. (1998), with some modifications. Initially, 30 µL of the overboard was mixed with 150 µL of Griess reagent in 96-well microplates. Griess reagent was prepared by mixing equal volumes of a solution of N-(1-naphthyl) ethylenediamine dihydro (1 mg/mL) and a sulfanilic acid (10 mg/mL) solution in 5% phosphoric acid. Subsequently, the sample was incubated for 5 min, at room temperature, and read at 540 nm. Nitrite concentrations were obtained via a standard curve previously made ($y = 0.0019x - 0.003$; $R^2 = 0.9996$).

2.5.4. Antioxidant response

The possible stimulatory or suppressive action of pollutants on the antioxidant activity of individuals was evaluated through the activity of superoxide dismutase (SOD) and catalase (CAT), taken as enzymes that make up the organisms' first line of antioxidant defense, acting mainly in suppressing or preventing the formation of free radicals or reactive species in cells (Ighodaro and Akinloye, 2018).

2.5.4.1. Superoxide dismutase activity

SOD activity in each analyzed organ was determined from the protocols established by Dieterich et al. (2000), with some modifications. Measurements were based on SOD's ability to scavenge superoxide radical anion, which decreases the overall pyrogallol autoxidation rate. One SOD activity unit was defined as the amount of enzymes inhibiting the pyrogallol autoxidation rate by 50%, which was determined at 630 nm.

2.5.4.2. Catalase activity

For the determination of catalase activity, it was determined from the protocols established by Sinha et al. (1972), with some modifications 16 µL of the supernatant were mixed (in 96-well microplates) with 240 µL of the reaction solution (PBS + glacial acetic acid P.A. + 5% potassium dichromate) and incubated for 20 min at 37ºC. Subsequently, the samples were read in ELISA reading at 630 nm. Catalase activity was obtained via a standard curve previously made ($y = 14.204x – 2.4088$; $R^2 = 0.9902$).

2.5.5. Determination of the protein level

All results of biochemical analyzes were expressed in unit/mg of protein in the samples. Thus, we used a commercial kit (Biotécnica Ind. Com. LTD, Varginha, MG, Brazil, code #10.009.00), whose total protein levels were determined based on the colorimetric reaction resulting from the reaction of Cu^{2+} ions and peptide bonds of proteins, giving rise to a blue color detected in an ELISA reader at 492 nm.

2.6. Visual screen on the aggregation of microplastics in the exposure waters

To evaluate whether the interaction between PE-MPs and the different components of the pollutant mix could induce greater aggregation between plastic particles, samples of the exposure waters of the “PE-MPs” and “PE-MPs + Mix” groups were filtered in a nitrocellulose membrane (pore: 0.45 µm), using a vacuum pump. Subsequently, the membranes were photographed under fluorescence microscopy to evaluate the formation of aggregates of MPs.

2.7. Statistical analysis

Initially, all data obtained in our study were evaluated for possible deviations from the normality of variance and residual homogeneity. Data normality was assessed using the Shapiro-Wilk test and homoscedasticity using the Bartlett test. Means were compared using the one-way ANOVA test, with Tukey's post-test (if parametric) or Kruskal-Wallis test, with Dunn’s post-test (if non-parametric). The averages referring to the number of plastic particles identified in the “PE-MPs” and “PE-MPs + Mix” groups were compared by the Mann-Whitney U test. The principal component analysis (PCA) was performed to explore correlations between treatments, based on the average value of each biochemical biomarker evaluated in different organs/tissues. In this regard, the number of principal components was selected based on scree plots (Jackson, 2004). Outliers’ values (identified by the Grubbs test) were excluded from all analyzes and, before the multivariate analysis, the data were logarithmized. Additionally, correlations were performed using Pearson's (for parametric data) or Spearman's (for non-parametric data) correlation coefficients, as well as hierarchical clustering analysis, based on Ward's method. Significance levels were set at Type I error (p) values lower than 0.05. GraphPad Prism Software Version 9.0 and PAST (PAlaeontology STatistic) software were used to perform the statistical analyses.

3. RESULTS

Initially, our data revealed that the animals were capable of uptake and accumulating the PE-MPs dispersed in the exposure waters, both when exposed to plastic particles alone and when exposed to the combination of MPs with the mix of pollutants (Figure 1). Although the number of particles identified between the animals in the "PE-MPs" and "PE-MPs + Mix" groups did not differ statistically (U-value = 8; p-value = 0.1320), the concentration of MPs in this last group was 53.7% lower than that observed in fish exposed only to microparticles (Figure 1A). This result was coincident with the formation of larger clusters of MPs (i.e.: > aggregation) when dispersed in the waters containing the pollutant mix (Figure 1B-C). In the "control" and "Mix" groups, we did not record the presence of PE-MPs, as expected.

On the other side, our data suggest that both PE-MPs and the mix of pollutants (alone or in association) were able to induce damage to the erythrocyte DNA of fish. According to Figure 2, the DNA damage index in these animals was on average 64% higher than that observed in the “control” group (Figure 2A) and the increase in tail intensity exceeded 60% compared to unexposed animals (Figure 2B). In terms of mutagenic evaluation, we did not record the occurrence of MNs in animals exposed to PE-MPs or a mix of pollutants (alone or in combination). However, several other ENAs were identified in the blood smears of animals exposed to the pollutants [including erythrocytes with notched nucleus (Figure 3A-B), kidney-shaped nucleus (Figure 3C), blebbed nucleus (Figure 3D), nuclear bud (Figure 3E), multilobulated nucleus (Figure 3F), moved nucleus (Figure 3G-H), binucleated erythrocytes (Figure 3I) and with nuclear constriction (Figure 3J)], whose sum did not differentiate between the "PE-MPs", "Mix" and “PE-MPs + Mix” groups (Figure 3K). Furthermore, Figure 3L demonstrates that the parameters evaluated in the comet assay were positively correlated with the total ENAs.

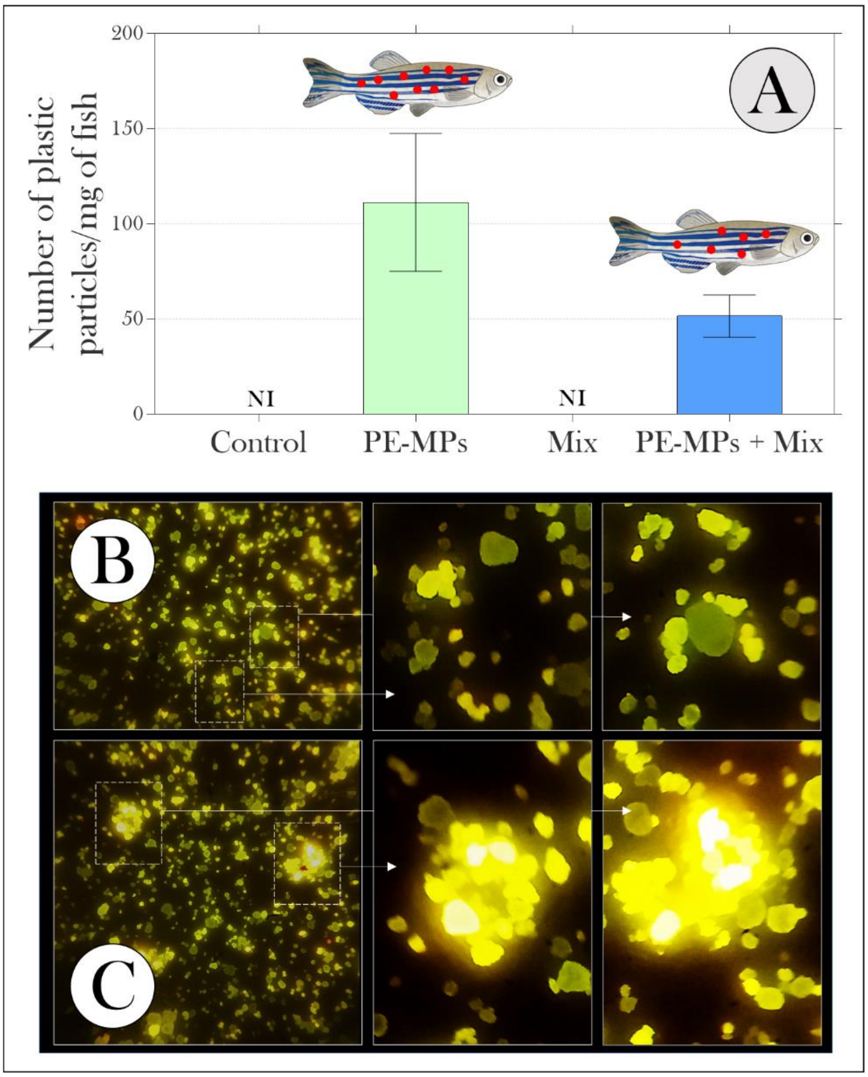

Figure 1. (A) Total microplastics recorded in *Danio rerio* adults exposed to the polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants) after a 15-day exposure. Bars represent mean ± SD. (B-C) A visual screen of PE-MPs dispersed in exposure waters without (B) and with (C) the mix of pollutants. Yellow staining refers to PE-MPs observed under fluorescence microscopy. “PE-MPs” refers to the group composed of animals exposed only to PE-MPs (at 2.7 x 10^8 PE-MPs particles/m^3); “Mix”, those exposed to the mix of pollutants (see concentrations in Table 1) and “PE-MPs + Mix” include animals exposed to PE-MPs in association with the mix of pollutants. NI: Unidentified PE-MPs. Each group was composed of three replicate tanks, containing seven animals/each (totaling n=21 animals/group). For evaluation of accumulation of MPs, 2 animals/replicates were randomly selected and evaluated, totaling 6 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: n=3 replicate tanks).

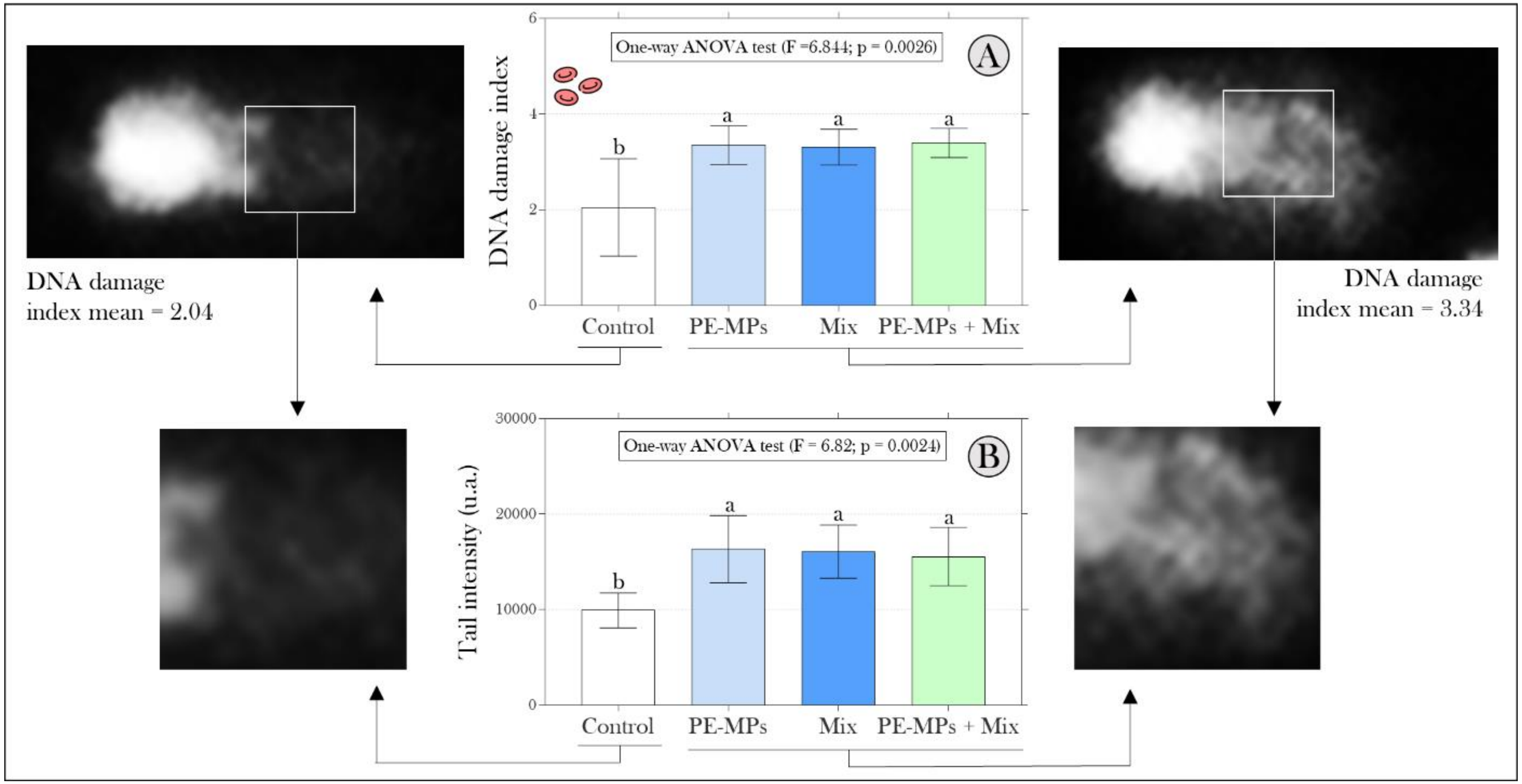

Figure 2. (A) DNA damage index and (B) tail intensity in erythrocytes of *Danio rerio* adults exposed or not to polyethylene microplastics (PE-MPs) (alone or in combination with a mix of pollutants) evaluated in the single-cell gel electrophoresis (comet assay). Bars represent mean ± SD and distinct lowercase letters indicate significant differences. The summaries of the statistical analyzes (one-way ANOVA test) are presented at the top of the graphs. “PE-MPs” refers to the group composed of animals exposed only to PE-MPs (at 2.7 x 10^8 PE-MPs particles/m^3); “Mix” to those exposed to the mix of pollutants (see concentrations in Table 1) and “PE-MPs + Mix” include animals co-exposed to PE-MPs in association with the mix of pollutants. Each group was composed of three replicate tanks, containing seven animals/each (totaling n=21 animals/group). In the comet assay, 2-3 animals/replicates were randomly selected and evaluated, totaling 8 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: n=3 replicate tanks).

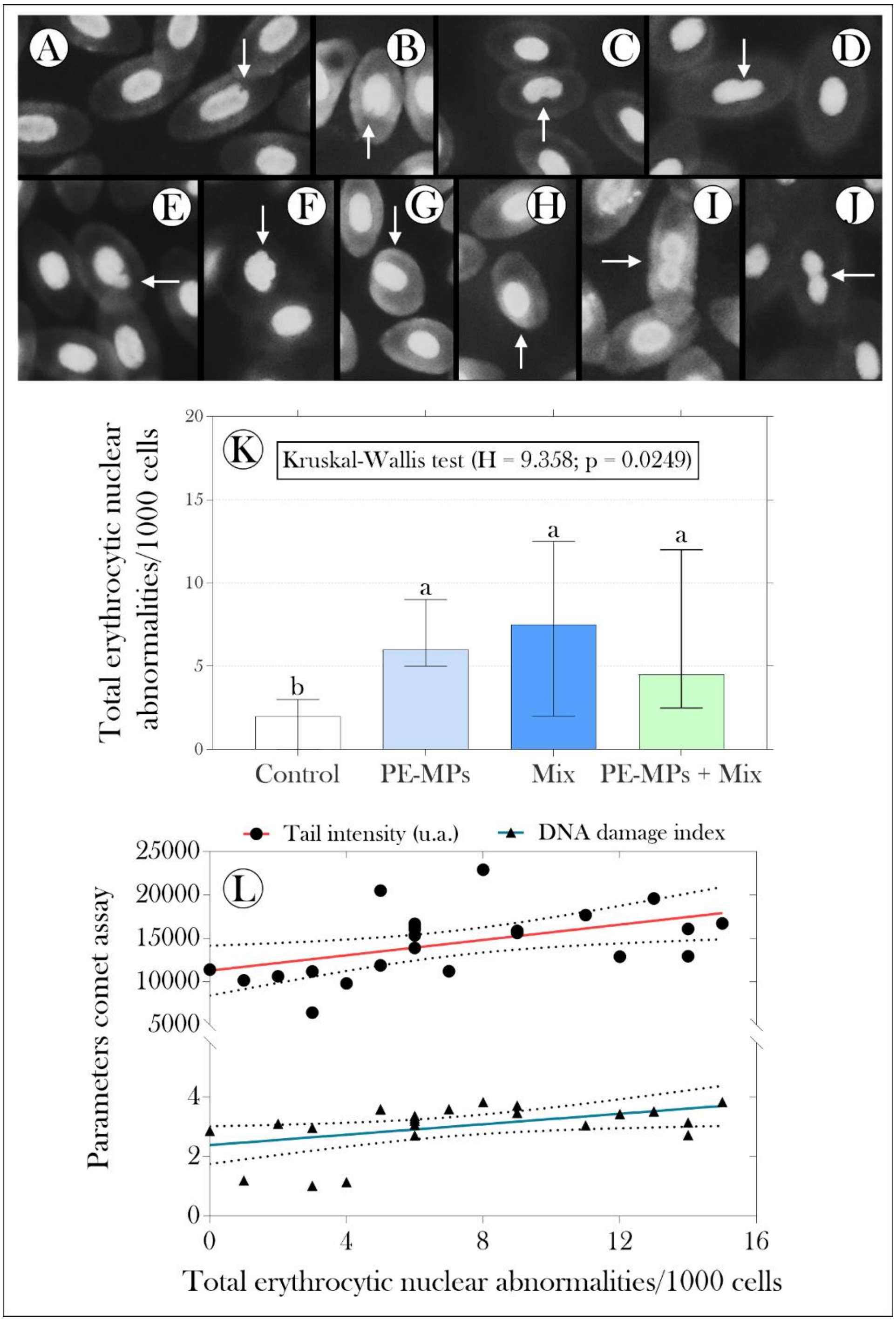

Figure 3. Representative photomicrographs of erythrocytes of *Danio rerio* adults exposed or not to polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants), with emphasis on cells with (A-B) notched nucleus, (C) kidney-shaped nucleus, (D) blebbed nucleus, (E) nuclear bud, (F) multilobulated nucleus, (GH) moved nucleus, (I) erythrocytes binucleate and with (J) nuclear constriction. (K) Total erythrocytic nuclear abnormalities (ENAs) were recorded in erythrocytes of *Danio rerio* adults exposed or not to polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants). Total ENAs considered the sum of the individual frequency of the identified nuclear abnormalities. In "K", data are presented by the median and interquartile range. Different lowercase letters indicate significant differences. The summary of the

statistical analysis (Kruskal-Wallis test) is presented at the top of the graph. (L) Analysis of correlation and linear regression between the parameters recorded in the comet assay and the total ENAs. DNA damage index *vs*. total ENAs (correlation analysis: $r = 0.4946$; $p = 0.0140$; linear regression: $F = 5.786$; equation: $y = 0.08748x + 2.384$); tail intensity *vs*. total ENAs (correlation analysis: $r = 0.4648$; $p = 0.0255$; linear regression: $F = 7.126$; equation: $y = 442.6x + 11261$). "PE-MPs" refers to the group composed of animals exposed only to PE-MPs (at 2.7×10^8 PE-MPs particles/m^3); "Mix", those exposed to the mix of pollutants (see concentrations in Table 1) and "PE-MPs + Mix" include animals exposed to PE-MPs in association with the mix of pollutants. n=8 animals/group. Each group was composed of three replicate tanks, containing seven animals/each (totaling n=21 animals/group). For MN and ENAs tests, 2-3 animals/replicates were randomly selected and evaluated, totaling 8 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: n=3 replicate tanks).

Purposing to associate the effects previously reported to a possible redox unbalance induced by pollutants, as well as to evaluate the increase of oxidative processes in the sampled organs/tissues (which can lead to systemic physiological damage), different biochemical biomarkers were evaluated. As expected, the animals' biochemical response to the treatments was organ dependent. While in the muscle of animals exposed to PE-MPs in combination with the mix of pollutants ("PE-MPs + Mix" group) the production of H_2O_2 was lower than the other groups; in the brain, animals exposed to PE-MPs and the mix of pollutants (alone or in combination) showed higher production of this metabolite, when compared to the "control" group (Figure 4A). On the other hand, we observed greater production of H_2O_2 in the liver of animals exposed to PE-MPs (alone) and PE-MPs in combination with the mix of pollutants. In the gills, the reduction of H_2O_2 observed in animals exposed to PE-MPs and the mix of pollutants (both alone) was not observed when there was exposure to the combination of pollutants (Figure 4A).

Regarding NO production (inferred by nitrite levels), we observed a suggestive picture of brain nitrosative stress induced by pollutants (alone or in combination) and by a significant reduction in nitrite production in the gills of these same animals (Figure 4B). In muscle, the exposure of animals to the mix of pollutants (alone) induced an increase in nitrite production; but when exposed to PE-MPs and the mix of pollutants (in combination) the production of this metabolite was significantly reduced. In the liver, our data suggest nitrosative stress-induced in animals exposed to PE-MPs (alone) and the combination of pollutants ("PE-MPs + Mix" group), when compared to non-exposed animals (Figure 4B).

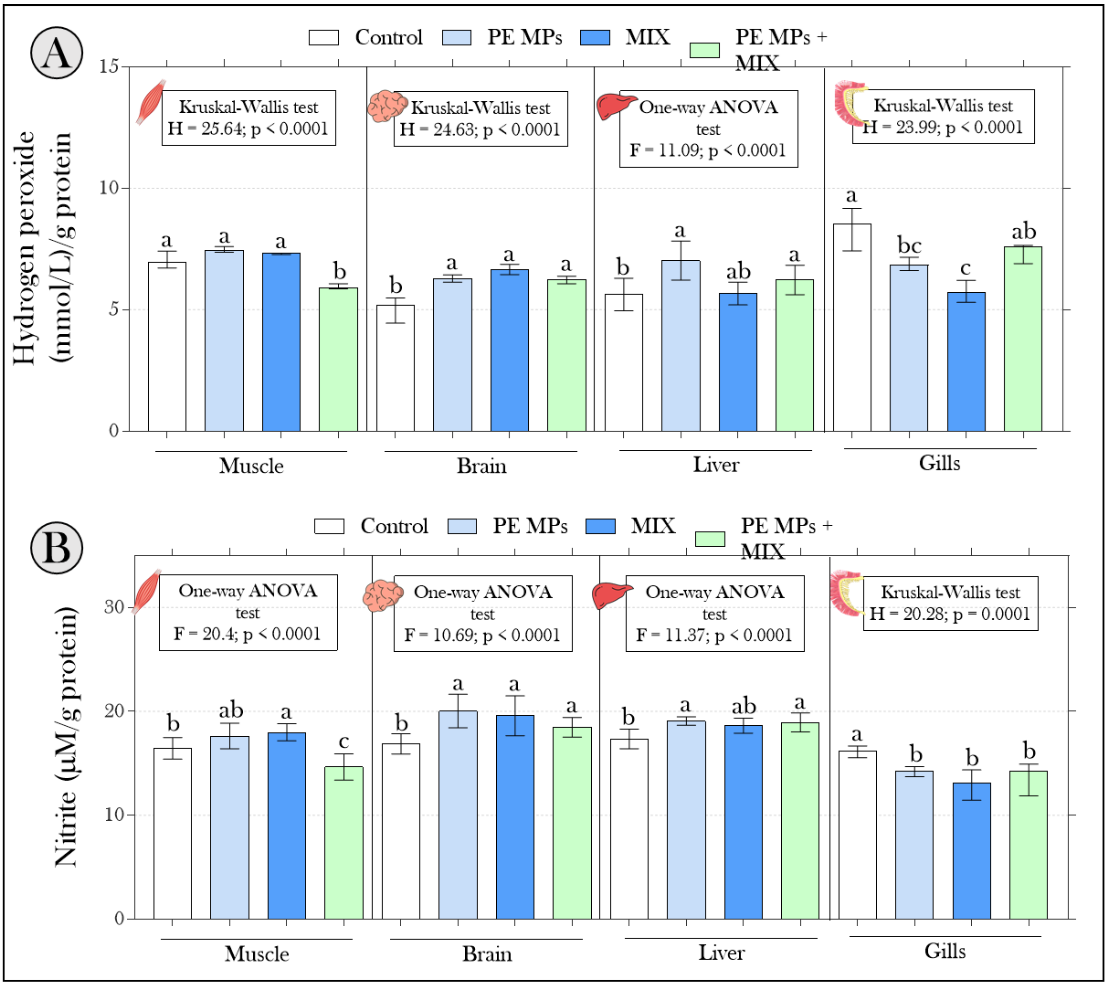

Figure 4. (A) Hydrogen peroxide and (B) nitrite levels in different organs/tissues of *Danio rerio* adults exposed or not to polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants). Parametric data are presented by the mean ± SD whereas non-parametric are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. Summaries of statistical analyzes (one-way ANOVA or Kruskal-Wallis test) are shown at the top of the graphs. "PE-MPs" refers to the group composed of animals exposed only to PE-MPs (at 2.7 x 10^8 PE-MPs particles/m^3); "Mix" to those exposed to the mix of pollutants (see concentrations in Table 1) and "PE-MPs + Mix" include animals co-exposed to PE-MPs in association with the mix of pollutants. Each group was composed of three replicate tanks, containing seven animals/each (totaling n=21 animals/group). For biochemical evaluations, 4-3 animals/replicates were randomly selected and evaluated, totaling 11 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: n=3 replicate tanks).

On the other side, the SOD activity in muscle, brain, and liver of experimental animals showed a response pattern like the production of H_2O_2. While a decrease in SOD was observed in the muscle only of animals exposed to the combination of pollutants; in the brain, exposure to pollutants (alone or in combination) equitably induced the SOD activity (Figure 5A). In the liver and brain, the increase in SOD activity in animals exposed to pollutants (alone or in combination) was on average 11.5% and 26.9%, respectively, whose results were significantly correlated with the production of H_2O_2 (Figure 5C -D). In the gills, we observed the suppression of SOD activity induced by exposure to PE-MPs (alone and in combination with the mix of pollutants) ("PE-MPs" and "PE-MPs + Mix" groups) when compared with the levels of this enzyme in animals in the “control” group (Figure 5A). Regarding CAT, we observed that in muscle there was a stimulatory effect on the activity of this enzyme in animals in the “PE-MPs” and “Mix” groups, as well as in the liver and brain of animals exposed to different treatments (Figure 5B). Additionally, we evidenced a positive and significant correlation between CAT activity and H_2O_2 levels evaluated in these organs (Figure 5C-D). In the gills, our data demonstrate that the pollutants did not induce a stimulatory or suppressive effect on the activity of this enzyme (Figure 5B).

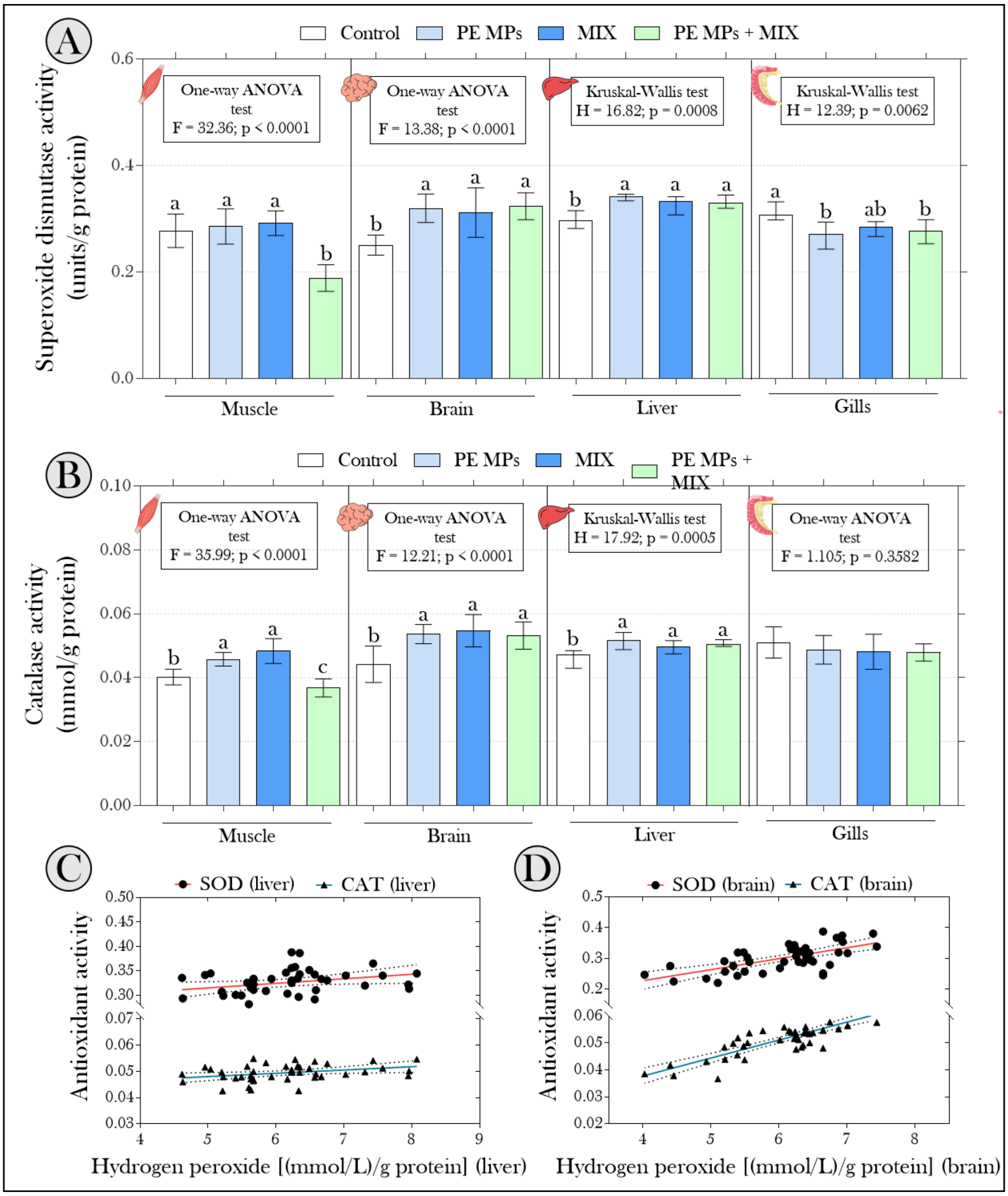

Figure. 5. (A) Superoxide dismutase (SOD) and (B) catalase (CAT) activity in different organs (muscle, brain, liver, and gills) of *Danio rerio* adults exposed or not to polyethylene microplastics (PE-MPs) (alone or in combination with the mix of pollutants). (C-D) Correlation analysis was performed between the production of hydrogen peroxide (H_2O_2) and the activity of SOD and CAT enzymes in the liver and brain, respectively. In "A" and "B", parametric data are presented by the mean ± SD whereas non-parametric are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. "PE-MPs" refers to the group composed of animals exposed only to PE-MPs (at 2.7 x 10^8 PE-MPs particles/m^3); "Mix", those exposed to the mix of pollutants (see concentrations in Table 1) and "PE-MPs + Mix" include animals exposed to PE-MPs in association with the mix of pollutants. Each group was composed of three replicate tanks, containing seven animals/each (totaling n=21 animals/group). For biochemical evaluations, 4-3 animals/replicates were randomly

selected and evaluated, totaling 11 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: n=3 replicate tanks).

To spectacle, an overview of the results and correlations between the treatments and the biochemical variables evaluated, the data obtained were submitted to a PCA. Based on this analysis, the first two principal components (PC1 and PC2) cumulatively explained more than 80% of the total variation (Figure 6A). The loadings plot (which shows the relationship between the PCs and the original variable – Figure 6B) demonstrated that all biomarkers were negatively associated with PC1 and that PC2 was positively determined by the variable “H_2O_2” and negatively by “nitrite” (Figure 6B), whose vectors are clearly opposite. Furthermore, we noticed that the scores arranged in the scatter plot were separated into five distinct subgroups (Figure 7A), which was also confirmed by cluster analysis (Figure 6C).

Subgroup 1, comprising the results obtained in the brain and liver of animals exposed to pollutants (alone or in combination), presents negative scores in PC1 (Figure 7B) and intermediate scores in PC2 (Figure 7C). Because the scores are closer to each other and the vector loads represented by the variables "SOD" and "CAT", this subgroup shows that the brain and liver of animals exposed to pollutants were the organs that presented a more pronounced antioxidant activity, whose differences with the “control” group, pointed out using tests (Figure 5A-B), are clearly evidenced in the PCA. Furthermore, the proximity between the vector loads of these two variables points to a strong positive correlation between the action of SOD and CAT in the animals' antioxidant systems.

In subgroup 2, the biochemical responses of the gills (from all experimental groups), the muscle of the animals in the “control” group, and those exposed to PE-MPs and the pollutant mix, in isolated form, were grouped (Figure 7A). This subgroup was the one with the highest positive scores on PC2 (Figure 7C) and the greatest proximity to the vector load of the “H_2O_2” variable, which indicates that the production of this metabolite was more pronounced in the gills and muscle of animals in these groups. However, about the gills, we evidenced greater proximity between the PC2 scores of the animals in the "control" group and those exposed to the combination of pollutants ("PE-MPs + Mix" group), differently from what was observed in zebrafish exposed to pollutants alone (“PE-MPs” and “Mix” groups). As a sequence, this demonstrates that the response of the gills was more sensitive when the animals were exposed to pollutants alone, meeting the results shown in Figure 4A. On the other hand, the greater distance between the PC1 score of the muscle of animals exposed to the combination of pollutants and the scores that make up subgroup 2 and the vector load of the variable "H_2O_2", clearly represents the suppressive effect of the production of this metabolite induced by the combination pollutants, thus characterizing it as an additional subgroup (i.e., subgroup 3) (Figure 7A).

Importantly, the greater distance between the PC2 scores of the variable "H_2O_2" and the liver of animals in the "control" and "Mix" groups justifies the classification of these scores in a separate subgroup from the others (subgroup 4). This subgroup had negative scores in both PC1 and PC2 (Figure 7B-C). As shown in Figure 4A, the production of H_2O_2 in the liver of these animals did not differ statistically. On the other hand, subgroup 5 (positive in PC1 and negative in PC2 - Figures 7B-C, respectively), formed by the brain score of the animals in the "control" group, well represents the results presented in Figure 4B, which show that the pollutants (PE-MPs and mix of pollutant, alone or in combination) upregulated nitrite production.

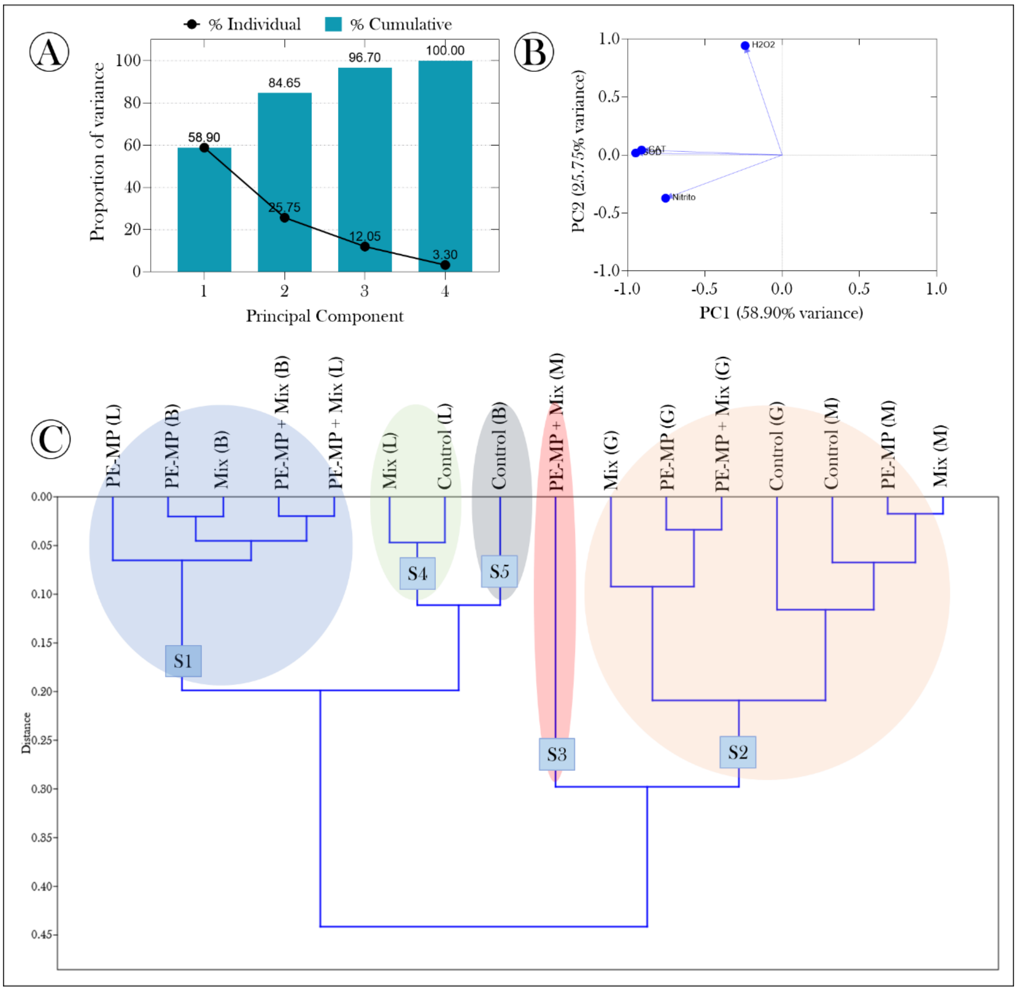

Figure 6. (A) Proportion of variance of the principal components 1 and 2 (PC1 and PC2), (B) loadings plot of the variables "hydrogen peroxide (H_2O_2)", "nitrite", "catalase (CAT)" and "superoxide dismutase (SOD)" and (C) cluster analysis dendrogram with emphasis on the formation of subgroups 1 to 5.

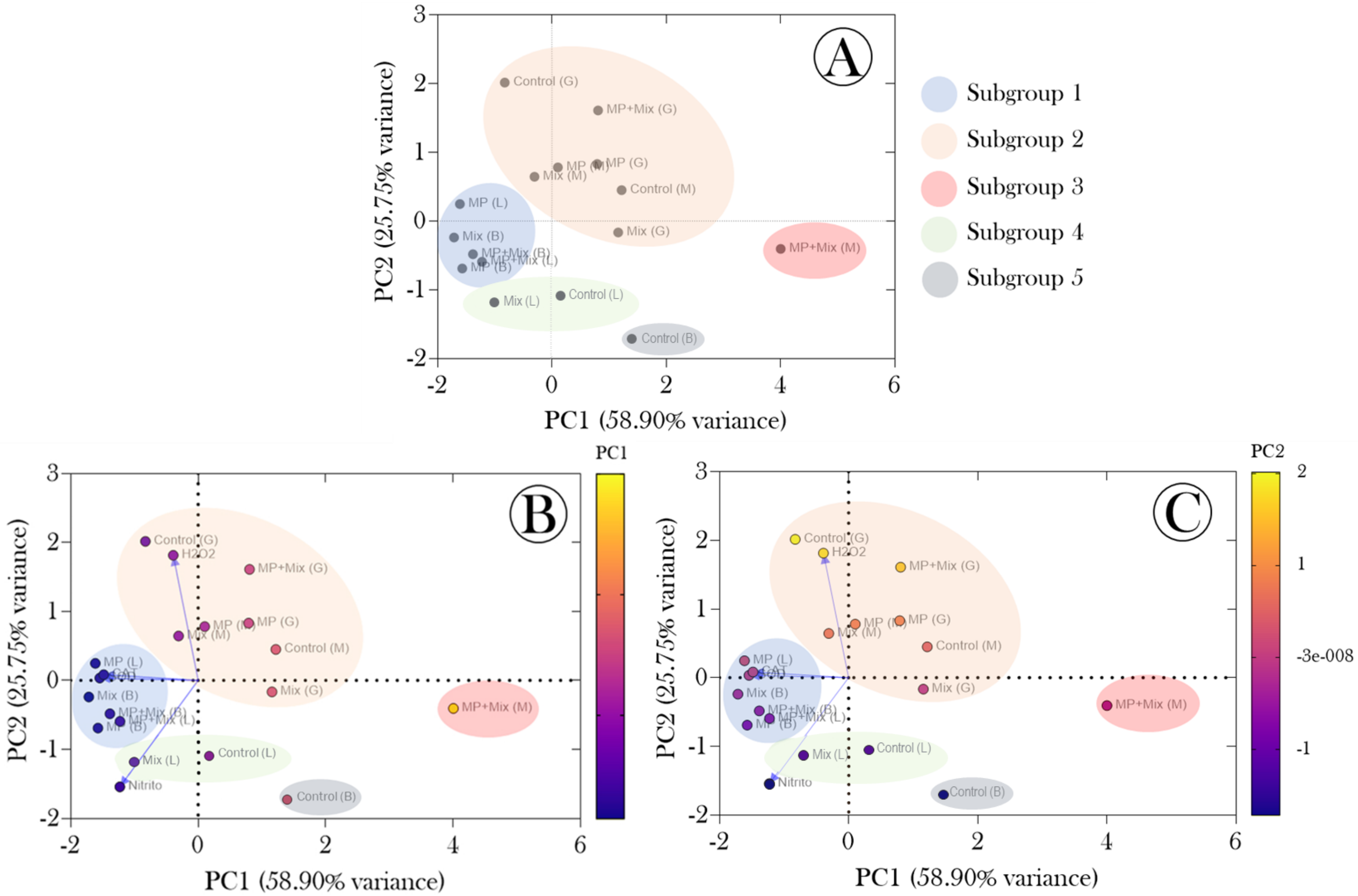

Figure 7. (A) Individual PC scores and (B-C) PCA biplot of the first two principal components that simultaneously show PC scores of the biochemical results of the different organs/tissues of the experimental groups (points) and loadings of explanatory variables (vectors – blue arrows). In "B" and "C", the built-in color maps represent the variation of the PC1 and PC2 scores (points), respectively.

4. DISCUSSION

In general, it is perceived that understanding the magnitude of the impacts of human actions on biota and natural ecosystems is directly dependent on conducting studies like ours, through which we identify how pollutants/contaminants affect aquatic organisms. Initially, the accumulation of plastic particles observed in the animals of the “PE-MPs” and “PE-MPs + Mix” groups (Figure 1) confirm previous studies, in which the uptake and accumulation of MPs by freshwater fish is a real possibility (Ding et al., 2018; Zhang et al., 2019; Zitouni et al., 2021; Guimarães et al., 2021; Abarghouei et al., 2021; McIlwraith et al., 2021). Although the mechanisms that mediate the accumulation of MPs by fish are not fully known (Roch et al., 2020) and even controversial (De-Sales-Ribeiro et al., 2020), the ingestion of PE-MPs by zebrafish has likely been the major uptake route. This hypothesis is especially supported by studies that point to the possibility of small plastic fragments (and not just free amino acids) being translocated through the gastrointestinal epithelium to the bloodstream, both in fish (Zitouni et al., 2020; Zitouni et al., 2021), and in other animal models (Von-Moos et al., 2012; Hu et al., 2016; Deng et al., 2017).

Interestingly, the PE-MPs accumulation was lower when the animals were exposed to the combination of pollutants (Figure 1), which reinforces the hypothesis that the interaction between plastic particles and other distinct chemical compounds/elements may have decreased the bioavailability of MPs in exposure waters. In this case, the complexity of chemical processes can explain the reduced accumulation of PE-MPs in the animals of the “PE-MPs + Mix” group. In our study, in particular, the proposition of any hypothesis on this topic is limited, as the mix we use is highly complex. In a previous study, we demonstrated that its chemical composition includes more than 350 organic compounds, in addition to high concentrations of heavy metals, including Pb, Ni, Zn, Cr, and Co (Souza et al., 2018). Therefore, several factors may have contributed to the reduction in the bioavailability of PE-MPs. One possibility would be related to the increase in their density (with a consequent decrease in their dispersion in the water column) when they interact (via sorption/desorption) with other pollutants, as suggested by Wang et al. (2017). Furthermore, the complexation of MPs with other pollutants [mainly hydrophobic and electrostatic interactions, pore-filling mechanism, and π-π interactions, which are larger by the nature of polymer of the microplastic, chemical properties of the compounds, and environmental condition (Wang et al., 2020; Torres et al., 2021; Menéndez-Pedriza & Jaumot, 2021)], could have provided greater aggregation between MPs and other compounds (as suggested in Figure 1C) and, consequently, hampered their intestinal absorption, culminating in lesser translocation of MPs for hepatic portal circulation. In this specific case, even if ingested by the animals, the unabsorbed PE-MPs would have been excreted in feces.

Despite the animals exposed to PE-MPs and the mix of pollutants (in combination) absorbing fewer MPs, the genotoxic (Figure 2) and mutagenic (Figure 3K) effects observed did not differ between the treated groups. In this case, the erythrocyte DNA damage (inferred by the comet assay) evidenced in zebrafish exposed to PE-MPs alone was as pronounced as those observed in animals exposed to the mix of pollutants (alone or in combination with MPs), which constitutes the big picture of the present study. Although previous studies have not focused on the potential genotoxicity and mutagenicity of mixtures of pollutants (including MPs) in freshwater fish, our results are like some studies that exposed different animal models to plastic particles. In Avio et al. (2015), for example, exposure of marine mussel *Mytilus galloprovincialis* to PE-MPs (alone or in combination with pyrene) induced genotoxic effects in hemocytes in terms of DNA strand breaks, MN frequency, and other nuclear alterations. Similarly, PE-MPs induced the formation of MN and other ENAs of zebrafish after feeding on fry of *Poecilia reticulata* (Araujo et al., 2020b), as well as in erythrocytes of *Physalaemus cuvieri* tadpoles (Araujo et al., 2020a) and early juvenile male tilapia (*Oreochromis niloticus*) (Hamed et al., 2021). Predominantly, such studies argue that these changes are indirectly induced by the formation of free radicals that interfere with DNA integrity, repair mechanisms [which would explain the DNA damage reported in these studies and our work (Figure 2)] and in the formation of the product of aneuploidy and/or disruption of cytokinesis [which would explain the increase in ENAs formation (Figure 3)].

While the increasing level in oxidative stress by PE-MPs may have been caused by the direct interaction between MPs and erythrocyte plasma membranes [as suggested by Araujo et al. (2020b) and Hamed et al. (2021)]; the action of xenobiotics present in the mix can be multiple. Previous studies reporting the harmful effects of various components of this mix (assessed alone) – especially in fish – provide an idea of their roles as inducers of mutagenic and/or genotoxic alterations [e.g.: As, Cr and Mn (Ramírez et al., 2005, Shaw et al., 2020; Rodrigues et al., 2020 - respectively) (present in the tannery effluent used in the mix of pollutant), steroids and hormones (Micael et al., 2007), abamectin (Hong et al., 2020); glyphosate (Ghaffar et al., 2021); benzene (Gomes, 2012); diclofenac (Ribas et al., 2014); dipyrone (Pamplona et al., 2011); ibuprofen (Mathias et al., 2018); and amoxicillin (Chowdhury et al., 2020)]. However, the individual contributions of each component of the mix in inducing these effects need to be further investigated in the future, since we cannot rule out the hypothesis that the effects also came from synergistic, additive, or potentiation interactions between pollutants.

In any case, it is important to highlight that although we have not identified MN in animals from any experimental group, several nuclear abnormalities reported in our study (Figure 3A-J) have already been associated with changes that are precursors to MN formation (Crott & Fenech, 2001). As discussed by Çavaş et al. (2005), the formation of ENAs may result from problems segregating tangled and attached chromosomes. In particular, the formation of cells with a blebbed nucleus (identified as a minor evagination of amplified DNA or affected chromatin material through the process of exocytosis – Figure 3D) has been associated with the amplification gene via the breakage–fusion–bridge cycle during the elimination of amplified DNA from the nucleus (Shimizu et al., 1998). On the other hand, notched nuclei (Figure 3A-B) are usually formed due to incomplete invagination or noticeable deepness inside the nucleus (Trivedi et al., 2021). According to Chondrou et al. (2018), the aneugenic activity induced by disturbances in tubulin polymerization constitutes one of the main reasons for the formation of this type of ENA. On the other hand, erythrocytes with a nuclear bud (Figure 3E) represent incomplete evagination of certain parts of genomic DNA and misrepair or telomere end-fusions, which suggests the induction (direct or indirect) by pollutants of failures in DNA repair and/or inactivation of essential mechanisms required for removal of misrepair complexes. In addition, the formation of binucleated erythrocytes (Figure 3I) has been associated with the difficulty of formation of mitotic fuse caused by aneugenic action of chemicals (as suggested by De-Campos-Ventura et al. (2018)] and the displacement of the nucleus (forming different rotation/displacement angles inside the cells - Figure 3G-H) to alterations in cellular structures, such as cytoskeletal or similar elements, which maintain the nucleus in a central position in the erythrocytes (Souza et al., 2017). Therefore, these ENAs provide a holistic view of how the PE-MPs and the mix of pollutants (single or in combination) may have induced (directly or indirectly) the genotoxic and mutagenic effects observed in our study. Moreover, both the genomic instability (inferred by the comet assay) and the mutagenic effects reported in the present study suggest that although the pollutants evaluated may have acted differently, their effects culminated in similar changes.

On the other side, the increased production of H_2O_2 and nitrite in the brain and liver of animals exposed to pollutants (Figure 4A), concomitant with the stimulation of SOD and CAT activity in these organs (Figure 5A-B, respectively) suggest the occurrence of a redox unbalance induced by exposure to PE-MPs and mix of pollutant (alone or in combination). In our case, particularly, the increase in the activity of the evaluated enzymes seems not to have been enough to counterbalance the production of the reactive oxygen (H_2O_2) and nitrogen (nitrite) species evaluated. These data support the hypothesis that the genotoxic and mutagenic effects observed in our study are associated with an imbalance between pro-and antioxidant metabolism in animals, followed by oxidative stress, which is in line with previous reports involving the exposure of animal models to both PE -MPs (e.g.: Alomar et al., 2017; Hamed et al., 2020; Zheng et al., 2021) and xenobiotics of complex chemical composition (Costa-Silva et al., 2015; Lakra et al., 2021; Chen et al., 2021). In contrast, we cannot disregard the hypothesis that nitrosative stress observed in the liver and brain of animals exposed to pollutants (alone or in combination) constitutes an adaptive response related to the antioxidant properties of NO. Contrary to the deleterious effects of the reactive nitrogen oxide species formed from either NO/O_2 and NO/O_2^-, it has been pointed out that NO shows antioxidant properties (Rubbo et al., 1994; Rubbo & Radi, 2001; Wink et al.., 2001). Since the biological chemistry of these molecules is dominated by free-radical reactions, the interaction of NO with other free-radical species could lead to either inhibition or potentiation of oxidative damage effect (Beckman et al., 1990).

However, to a better understanding of the mechanisms intrinsic to the effects observed in animals exposed to pollutants, new studies should be developed, since the biochemical response (predictive of redox unbalance) was organ/tissue-dependent, as revealed by the statistical analyzes performed (Figures 4, 5 and 7). While the increase in H_2O_2 production was more pronounced in the muscle of animals exposed to pollutants alone (PE-MPs and mix of pollutant) (possibly related to mitochondrial dysfunction induced by the pollutants); in the gills, the suppression of nitrite production was more evident (Figure 7B-C), presumably due to the continuous contact of this organ with the pollutants dispersed in the exposure waters. Such suppression, in addition to having potential implications for the redox homeostasis of fish (considering the important role of NO in the antioxidant system), can also signal systemic impacts on the immune response of animals caused by the pollutants, considering the multiple actions of NO in the regulation of immune and inflammatory cells (Coleman, 2001; Wink et al., 2011).

The brain and liver antioxidant response were more pronounced in animals exposed to pollutants, both alone and in combination (Figure 7B-C), which may be associated with factors related to the functionality of these organs. Previous studies have shown, for example, that the liver (or hepatopancreas in some fish species) is the central metabolic organ, being the main detoxification organ in vertebrates (Grant, 1991; Chiang, 2014), in addition to acting in the degradation of products potentially toxic metabolic agents (Lushchak et al., 2005). Thus, this organ is constantly challenged by many endogenous and exogenous free radicals, which require a more developed antioxidant system than in other organs. On the other side, the pronounced activity of SOD and CAT in the brain of animals exposed to pollutants is plausibly explained by the stimulation of the neuronal antioxidant system to avoid excessive oxidative insults. Although the brain generally presents a modest antioxidant defense mechanism, compared to the liver (Lee et al., 2020), depending on toxicological stimuli, it is possible to establish an adaptive response to avoid the increased generation of ROS, blocking and capturing the radicals generated by the action of pollutants.

Finally, it is important to emphasize that the pioneering nature of our study, combined with the complexity of the mix of pollutants used, reinforces the need to conduct new studies to better understand the impacts of exposure to PE-MPs in combination with various other emerging pollutants on freshwater ichthyofauna. The investigative perspectives suggested in the present study include, for example, assessments focusing on the individual contributions of the different constituents of the pollutant mix on the observed effects and on the investigation of possible differentiated animal responses to the combined exposure of the pollutant mix to other types of MPs (varying chemical nature, size, and shape), concentrations and exposure times. In addition, assessments considering other toxicity biomarkers (e.g.: behavioral, histopathological, endocrine, immunological, cytological, neurological, etc.) will be useful to better characterize the effects of the pollutants tested on animals.

5. CONCLUSION

To be concluded, our study confirms the toxicological potential associated with exposure of zebrafish adults to PE-MPs and a mix of pollutants containing distinct xenobiotics. However, the hypothesis that the association of MPs with the pollutant mix could induce more serious adverse effects in animals was not confirmed based on the evaluated toxicity biomarkers. The extent of erythrocyte DNA damage (assessed by the comet assay) and the induction of mutagenic changes (inferred by ENAs quantification) did not differ between animals exposed to pollutants (alone or in combination), indicating the genotoxic and mutagenic effects of MPs (alone or in the mixture) in freshwater fish. On the other hand, we showed a close relationship between the establishment of a redox imbalance and the observed genotoxicity and mutagenicity, although the pro-and antioxidant response was organ/tissue-dependent, with greater cerebral and hepatic antioxidant activity and higher production of H_2O_2 in muscle and gills. However, it must be borne in mind that our findings are just the “tip of an iceberg” represented by the potential negative effects caused by the exposure of freshwater fish to the diversity of pollutants/contaminants commonly dispersed in freshwater ecosystems. Therefore, it is highly recommended that further studies involving this issue be continued, especially considering that the impacts of PE-MPs in association with other xenobiotics can be as complex as the interactions between them.

6. ACKNOWLEDGMENTS

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7. DECLARATION OF COMPETING INTEREST

We confirm that there are no known conflicts of interest associated with this work and there has been no significant financial support for this work that could have influenced its outcome. We confirmed that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. Due care has been taken to ensure the integrity of the work.

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5 CONSIDERAÇÕES FINAIS

Nesta pesquisa, exploramos os efeitos da exposição a microplásticos em ambientes aquáticos, examinando sua influência, em conjunto ou não com outros poluentes, sobre a saúde de duas espécies de água doce. Em linhas gerais, os resultados obtidos destacam não apenas os efeitos diretos dos microplásticos na saúde das espécies estudadas, mas também a interação desses micropoluentes com outros xenobióticos.

Uma das descobertas mais notáveis foi a constatação de que a exposição de girinos de *P. cuvieri* aos PE-MPs, quando combinada com uma mistura de poluentes emergentes, desencadeia uma resposta de estresse intensificada. Ademais, observamos que algumas das alterações identificadas em animais expostos aos microplásticos se assemelham as manifestadas por aqueles submetidos à exposição à mistura de poluentes. Adicionalmente, percebemos que a expressão dos efeitos dos poluentes nos organismos aquáticos é altamente influenciada pelos biomarcadores utilizados e pelo órgão ou tecido específico analisado, destacando assim a complexidade das interações entre os poluentes e os organismos aquáticos.

Apesar de destacarmos como prioridade de pesquisa no capitulo I a necessidade de expandir a diversidade de espécies usadas nos estudos e incluir espécies representativas de outras ordens de anfíbios, nos preocupamos em aprofundar (no capítulo II) as pesquisas na ordem Anura e investigar os efeitos na espécie *Physalaemus cuvieri* pelos seguintes motivos elencados: poucos estudos que abordam os impactos de poluentes emergentes em comparação com compostos químicos clássicos; necessidade de avaliar e caracterizar os impactos toxicológicos (para além da identificação de bioacumulação), possibilidade de contato com micro(nano)plásticos em todo ciclo de vida.

Este estudo foi realizado no âmbito do Programa de Pós-Graduação em Ciências Ambientais da Universidade Federal de Goiás e está inserido na linha de pesquisa de “monitoramento e análise de recursos naturais”, abordando uma problemática que vinha sendo pouco discutida, mas que tem ganhado destaque a nível global: a questão da poluição plástica e a coexistência com outros compostos químicos nos ambientes naturais.

É preocupante que, embora a questão da poluição plástica e sua coexistência com outros compostos tenha atraído a atenção global, as normas legais brasileiras ainda não abordam adequadamente esta realidade. A falta de critérios de qualidade da água e de padrões que considerem a presença e o impacto dos micros(nano)plásticos, bem como de outros contaminantes, revela uma lacuna regulatória que precisa ser urgentemente preenchida.

Os micromateriais (a exemplo dos micros(nano)plásticos) são comprovadamente tóxicos para uma gama de seres vivos, persistentes no ambiente, facilmente dispersos, capazes de carrear outros compostos químicos, ocasionar bioacumulação e já foram encontradas, inclusive, em algumas amostras de água engarrafada e em diferentes compartimentos ambientais. Tudo isso indicaria, ao menos a princípio, a pertinência em considerá-las em programas de monitoramento ambiental e, portanto, no estabelecimento de valores guias e máximos permitidos.

A dinamicidade dos critérios de qualidade de água, influenciada por diversos fatores (pandemias (como foi o caso da Covid-19); o aumento de materiais plásticos; produção e comercialização de novos insumos e tecnologias) destaca a necessidade de uma legislação ambiental mais adaptável e que seja pautada na evolução do conhecimento científico, a fim de regulamentar novos produtos antes mesmo de liberá-los comercialmente.

Por fim, é importante ressaltar que a nossa pesquisa não é exaustiva e a continuação das investigações nesta área é essencial para uma compreensão do real impacto dos diversos poluentes que afetam os ecossistemas de água doce, bem como o papel dos microplásticos em amplificar esses efeitos adversos na vida aquática. A conservação e preservação da biodiversidade de ambientes de água doce requer um entendimento aprofundado e, portanto, destacamos a necessidade contínua de pesquisas interdisciplinares e ações de conservação para mitigar os impactos negativos da poluição por plásticos nos diferentes ecossistemas. Esperamos que as descobertas deste estudo sirvam como um recurso valioso em prol preservação dos recursos hídricos, da biodiversidade e saúde humana.

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