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INSTITUTO DE PATOLOGIA TROPICAL E SAÚDE PÚBLICA (IPTSP)
PROGRAMA DE PÓS-GRADUAÇÃO BIOTECNOLOGIA E BIODIVERSIDADE

FERNANDA NEVES ESTRÊLA RESENDE

Múltiplos endpoints da toxicidade dos nanoplásticos de poliestireno e das nanopartículas de óxido de zinco em peixes e mamíferos

GOIÂNIA
2021

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FERNANDA NEVES ESTRÊLA RESENDE

Múltiplos endpoints da toxicidade dos nanoplásticos de poliestireno e das nanopartículas de óxido de zinco em peixes e mamíferos

Tese apresentada à Pós-Graduação em Biotecnologia e Biodiversidade, da Faculdade à Universidade Federal de Goiás (UFG), como requisito para obtenção do título de Doutor em

Área de concentração: Biotecnologia
Linha de Pesquisa: Bioeconomia e Conservação dos Recursos Naturais

Orientador: Professor Dr. Guilherme Malafaia Pinto
Coorientador: Professor Dr. Paulo Sérgio Pereira

GOIÂNIA
2021

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XI, 69 f.: il.

Orientador: Prof. Dr. Guilherme Malafaia Pinto; co-orientador Dr. Paulo Sérgio Pereira.
Tese (Doutorado) - Universidade Federal de Goiás, Instituto de Patologia Tropical e Saúde Pública (IPTSP), Programa de Pós graduação em Biotecnologia e Biodiversidade, Goiânia, 2021.
Bibliografia. Anexos.
Inclui lista de figuras, lista de tabelas.

1. Mistura de poluentes. 2. Nanotoxicidade. 3. Água doce. 4. Ambiente terrestre. 5. Vertebrados. I. Pinto, Guilherme Malafaia, orient. II. Título.

CDU 60

UNIVERSIDADE FEDERAL DE GOIÁS

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ATA DE DEFESA DE TESE

ATA DA REUNIÃO DA BANCA EXAMINADORA DA DEFESA DE TESE DE FERNANDA NEVES ESTRÊLA RESENDE - Aos vinte dias do mês de outubro do ano de 2021 (20/10/2021), às 13h00min, reuniram-se os componentes da Banca Examinadora: Profs. Drs. **Guilherme Malafaia Pinto** (IFGoiano) (orientador), **Fernando Postalli Rodrigues** (IFGoiano), **Letícia Paiva de Matos** (IFGoiano), **Ives Charlie da Silva** (USP) e **Vanessa Bezerra de Menezes Oliveira** (UFT) para, sob a presidência do primeiro, e em sessão pública por Webconferência, procederem à avaliação da defesa de tese intitulada: "**MÚLTIPLOS ENDPOINTS DA TOXICIDADE DOS NANOPLÁSTICOS DE POLIESTIRENO E DAS NANOPARTÍCULAS DE ÓXIDO DE ZINCO EM PEIXES E MAMÍFEROS**", em nível de **DOUTORADO**, área de concentração em **BIOTECNOLOGIA**, de autoria de **FERNANDA NEVES ESTRÊLA RESENDE**, discente do PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA E BIODIVERSIDADE, da Universidade Federal de Goiás. A sessão foi aberta pelo orientador da discente, Prof. Dr. GUILHERME MALAFAIA PINTO, que fez a apresentação formal dos membros da Banca e orientou a Candidata sobre como utilizar o tempo durante a apresentação de seu trabalho. A palavra a seguir, foi concedida à autora da tese que, em 30 minutos procedeu à apresentação de seu trabalho. Terminada a apresentação, cada membro da Banca arguiu a Candidata, tendo-se adotado o sistema de diálogo sequencial. Terminada a fase de arguição, procedeu-se à avaliação da defesa. Tendo-se em vista o que consta na Resolução nº. 1181/2013 do Conselho de Ensino, Pesquisa, Extensão e Cultura (CEPEC), que regulamenta o Programa de Pós-Graduação em Biotecnologia e Biodiversidade a Banca, em sessão secreta, expressou seu Julgamento, considerando a candidata **Aprovada** ou **Reprovada**:

Banca Examinadora	**Aprovada / Reprovada**
Prof. Dr. **Guilherme Malafaia Pinto**	**__Aprovada___**
Prof. Dr. **Fernando Postalli Rodrigues**	**___Aprovada___**
Profa. Dra. **Letícia Paiva de Matos**	**__Aprovada____**
Prof. Dr. **Ives Charlie da Silva**	**__Aprovada____**
Profa. Dra **Vanessa Bezerra de Menezes Oliveira**	**___Aprovada_**

Em face do resultado obtido, a Banca Examinadora considerou a candidata **_Habilitada____**, (**Habilitada ou não Habilitada**), cumprindo todos os requisitos para fins de obtenção do título de **DOUTORA EM BIOTECNOLOGIA E BIODIVERSIDADE**, na área de concentração em **BIOTECNOLOGIA**, pela Universidade Federal de Goiás. Cumpridas as formalidades de pauta, às 17_h 20_min, a presidência da mesa encerrou esta sessão de defesa de tese e para constar eu, HELOÍSA DE SOUSA VIEIRA, secretária do Programa de Pós-Graduação em Biotecnologia e Biodiversidade lavrei a presente Ata que depois de lida e aprovada, será assinada pelos membros da Banca Examinadora.

TÍTULO SUGERIDO PELA BANCA

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DEDICATÓRIA

"Aos que se tornaram familiares, aos que nasceram familiares e
aos que conheci antes de ontem;
Aos que me deixaram louca e aos que enlouqueci;
Aos que me criticaram em tudo e a um ou outro que aturou minha 'chatura';
Aos amigos que passaram e aos que estagnaram em mim;
Aos que me consideram muito e aos que com razão fizeram pouco;
Aos que conhecem o que penso e aos que só conhecem o que
faço;
Aos que passam o dia todo comigo e aos que estão o tempo todo em mim.
Esse trabalho é a soma de todos vocês.
E se não é melhor, é por falta de memória, mas não por falta de amigos."
(Richard Primack &Efraim Rodrigues)

Em memória á meu pai.

AGRADECIMENTOS

Primeiramente agradeço à Deus, pela vida, por todo seu amor e pelo seu cuidado em todas as etapas da minha vida.

À minha mãe, pelo apoio e incentivo aos estudos, por seu esforço sem medida e por me ensinar que tudo na vida é uma questão de vontade, trabalho árduo e determinação. Agradeço também aos meus irmãos, Janaina e Paulo, Fernando e Júnior (irmãos que a vida me deu) pela força, torcida, carinho, amor e palavras de incentivo. Àminha sobrinha Elena eu agradeço pelos momentos de alegria, amor e distração. Agradeço também à minha segunda família pela acolhida, confiança, ajuda e incentivo, em especial à minha sobrinha Yasmin e minha concunhada Juzimar, pelas palavras de incentivo e apoio, meu tio José Antônio, pela torcida e aos meus queridos sogros por tudo que fizeram por mim.

Ao meu amado esposo, Pedro Henrique, eu agradeço por tudo! Quantos foram os momentos de paciência, amor, dedicação, abraços acolhdores, palavras de motivação e de alicerce. Serei eternamente grata a você!

A meu orientador, Guilherme Malafaia, minha eterna GRATIDÃO! Você não imagina o quanto te admiro, por ser esse profissional e ser humano extraodinário. Obrigada por cada palavra, por todo ensinamento, pelas valiosas ajudas, incentivo, apoio e, sobretudo, por não me deixar desanimar e desistir nos momentos mais difíceis. Nunca esquecerei tudo que você fez por mim. Muito obrigada

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Ao programa de Pós-Graduação em Biotecnologia e Biodiversidade, por possibilitar um novo mundo de aprendizados! À banca pela disponibilidade e contribuições.

Ao Instituto Federal Goiano – Câmpus Urutaí, em especial ao Laboratório de Pesquisas Biológicas, assim como ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG) pela concessão de recursos que direta ou indiretamente foram utilizados na condução do meu estudo. Além disso, agradeço ao Laboratório Multiusuários de Microscopia de Alta Resolução (LabMic) da Universidade Federal de Goiás, pela caracterização das nanopartículas e nanoplásticos utilizados em meu trabalho.

Por fim, agradeço a todos que, mesmo em suas mais breves passagens ou curtas convivências me ofereceram sua contribuição, de qualquer natureza, para a realização deste trabalho.

Obrigada!

LISTA DE FIGURAS

Figure 3. (A) Total soluble carbohydrates, (B) total proteins and (C) triglycerides in the liver; and (D–E) total soluble carbohydrates and total proteins in the brain (respectively) of *Ctenopharyngodon idella* juveniles exposed to different treatments. Bars indicate mean + standard deviation. Parametric data were subjected to one-way ANOVA and the non-parametric ones were subjected to Kruskal-Wallis test, and to Tukey and Dunn's post-tests, both at 5% probability level. Statistical summary is shown at the top of each figure. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). Concentrations of both nanomaterials: 760 μg/L, each. 6

Figure 4. Total glutathione (A), DPPH radical scavenging activity (B) and superoxide dismutase enzyme (C) activity in the brain of s *Ctenopharyngodon idella* juveniles exposed to different treatments. Bars indicate mean + standard deviation. Parametric data were subjected to one-way ANOVA and the non-parametric ones were subjected to Kruskal-Wallis test, and to Tukey and Dunn's post-tests, both at 5% probability level. Statistical summary is shown at the top of each figure. Different lowercase letters indicate differences between experimental groups. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). AA: Ascorbic acid. Concentrations of both nanomaterials: 760 μg/L, each. 7

Figure 5. Thiobarbituric acid reactive substances (A), hydrogen peroxide (B) and nitrite levels (C) in the brain of *Ctenopharyngodon idella* juveniles exposed to different treatments. Bars indicate mean + standard deviation. Parametric data were subjected to one-way ANOVA and the non-parametric ones were subjected to Kruskal- Wallis test, and to Tukey and Dunn's post-tests, both at 5% probability level. Statistical summary is shown at the top of each figure. Different lowercase letters indicate differences between experimental groups. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). Concentrations of both nanomaterials: 760 µg/L, each.

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Figure 6. (A) Tail length (B) DNA percentage in the tail, (C) Olive tail moment nitrite and (D) photomicrographs representing *Ctenopharyngodon idella* juveniles in (D) the control and (E) pollutant-exposure groups subjected to comet assay. Bars indicate mean + standard deviation. Data were subjected to Kruskal-Wallis test, and to Tukey and Dunn's post-tests, at 5% probability level. Statistical summary is shown at the top of each figure. Different lowercase letters indicate differences between experimental groups. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). Concentrations of both nanomaterials: 760 µg/L, each.

9

Figure 7. Estimated Zn (A) and PS NPs concentrations in the brain of *Ctenopharyngodon idella* juveniles exposed to different treatments. Bars indicate mean + standard deviation. In "A", data were subjected to Kruskal-Wallis test, and to Tukey and Dunn's post-tests, at 5% probability level. In "B", Student's t-test was used to compare parametric data. Statistical summary is shown at the top of each figure. Different lowercase letters indicate differences between experimental groups. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). Concentrations of both nanomaterials: 760 µg/L, each. 10

Figuras do Capítulo IV – A paginação segue aquela presente no artigo publicado

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Figure 1. Schematic design of the "triple test" model adopted in the present study. (A-F) Animals' acclimation to the testing room (30 min), (B) open field test, (C-D) object recognition test and (E) forced swimming test. 4

Figure 2. Locomotor activity parameters: (A) distance covered, (B) locomotion speed and (C) obtained Basso Mouse Scale for Locomotion and (D) anxiety index recorded for male Swiss mice exposed, or not, to different nanomaterials. Bars represent data mean ± standard deviation (n ¼ 12 animals/group). Parametric data were subjected to one-way ANOVA and non-parametric data were analyzed through Kruskal-Wallis test, both at 5% probability level. Statistical summaries are shown at the top of the graphs. C: control group; ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: animals exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnONP þ PS NaP: mice simultaneously exposed to both nanomaterials (at equitable doses).

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Figure 3. (A-B) Object recognition index used to test validation based on Swiss mice in the control group; (C) recorded new-object recognition index, which was calculated based on models' exposure to TOS, at different treatments. Bars represent data mean þstandard deviation (n ¼ 12 animals/group). Data in "A and B" were analyzed through Kruskal-Wallis test (with Dunn's post-test), at 5% probability level. Statistical summaries are shown at the top of the graphs. Different lowercase letters represent significant differences between means recorded for the samples. C: control group; ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: animals exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnONP þ PS NaP: mice exposed to both nanomaterials (at equitable doses).

6

Figure 4. Serum (A) albumin, (B) total proteins, (C) globulins, (D) total cholesterol and (E) triglycerides levels recorded for Swiss mice exposed, or not, to ZnO NPs and PS N aPs. Bars represent data mean ± standard deviation (n ¼ 12 animals/group). Parametric data were subjected to one-way ANOVA (with Tukey's post-test) and non-parametric data were analyzed through Kruskal-Wallis test (with D'nn's post-test), both at 5% probability level. Statistical summaries are shown at the 7
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Figure 5. (A) Thiobarbituric acid reactive species (TBARS), (B) nitrite concentrations and (C) brain homogenate acetylcholinesterase activity in Swiss mice exposed, or not, to ZnO NPs and PS NaPs. Bars represent data mean þstandard deviation (n ¼ 12 animals/group). Parametric data were subjected to one-way ANOVA (with Tukey's post-test) at 5% probability level. Non-parametric data were analyzed through Kruskal-Wallis test (with Dunn's post-test), at 5% probability level. 8
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Figure 6. (A) Tail length (TL), (B) DNA percentage in the tail (% DNA) and (C) Olive tail moment (OTM) recorded at comet assay applied to circulating erythrocytes of Swiss mice exposed, or not, to ZnO NPs and PS NaPs. Bars represent data mean þstandard deviation (n = 12 animals/group). Data were analyzed through Kruskal-Wallis test (with Dunn's posttest) at 5% probability level. Statistical summaries are shown at the top of the graphs. Different lowercase letters represent significant differences between the means recorded for the samples. C: control group; ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: mice exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnONP + PS NaP: mice exposed to both nanomaterials (at equitable doses)

9

Figure 7. (A) PS NaPs and zinc (B) accumulation in the brains of Swiss mice exposed, or not, to ZnO NPs and PS NaPs. Bars represent data mean þstandard deviation (n = 12 animals/group). Data were compared through Student's t-test, at 5% probability level. Statistical summaries are shown at the top of the graphs. Different lowercase letters represent significant differences between the means recorded for the samples. C: control group; ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: mice exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnO NP + PS NaP: mice exposed to both nanomaterials (at equitable doses).

LISTA DE QUADROS E TABELAS

RESUMO

ESTRELA. F. N. Múltiplos endpoints da toxicidade combinada de nanoplásticos de poliestireno e nanopartículas de óxido de zinco em vertebrados aquáticos e terrestres. 2021. Tese (Doutorado em Biotecnologia e Biodiversidade) – Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, 2021.

A toxicidade das nanopartículas de óxido de zinco (ZnO NPs) e dos nanoplásticos de poliestireno (PS NaPs) tem sido demonstrada em diferentes modelos animais. No entanto, o conhecimento sobre o impacto desses poluentes é incipiente, ainda no tocante de uma combinação desses poluentes. Assim, objetivamos avaliar os efeitos desses nanomateriais em camundongos Swiss (*Mus musculus)* e carpa capim (*Ctenopharyngodon idella)* após exposição individual e em combinação binária. Foi investigado se a curta exposição (três dias) à dose ambientalmente relevante (760 µg/L) dos poluentes, seria capaz de causar efeito neurotóxico, bioquímico e genotóxico nos animais. Nossos dados revelam que, embora as exposições não tenham provocado alterações locomotoras e anxiogênica ou ansiolítica aos animais expostos aos poluentes, observamos algumas diferenças específicas nos testes comportamentais. Os peixes expostos aos PS NaPs apresentaram déficit na resposta ao teste do espelho tanto em separado, quanto em combinação com nanopartículas. Por outro lado, todos os tratamentos induziram igualmente a inatividade em relação a substâncias de alarme (artigo 1). Já nos camundongos pudemos observar prejuízo cognitivo no teste de reconhecimento de objetos (individualmente). No entanto, tais parâmetros não diferiram entre os animais do grupo controle e aqueles expostos à combinação binária dos poluentes. Por outro lado, as exposições (individuais e em combinação) induziram equitativamente danos no DNA eritrocitário. Houve aumento do estresse oxidativo, principalmente nos grupos expostos a PS NaPs (em combinação, ou não, com nanopartículas). Embora aumentados, os níveis de antioxidantes avaliados não parecem ser suficientes para inibir os efeitos da produção de radicais livres induzida pelo tratamento (artigo 2). Tomados em conjunto, nossos estudos confirmam o potencial toxicológico das NPs ZnO e NaPs PS nos modelos estudados e instigam novos estudos a investigarem mais profundamente os fatores que elucidam a ausência de efeitos aditivos ou sinérgicos advindos da exposição combinada aos poluentes.

Palavras-chave: Mistura de poluentes, Nanotoxicidade, Água doce, Ambiente terrestre, Vertebrados.

ABSTRACT

The toxicity of zinc oxide nanoparticles (ZnO NPs) and polystyrene nanoplastics (PS NaPs) has been demonstrated in different animal models. However, knowledge about the impact of these pollutants is incipient, even in terms of a combination of these pollutants. Thus, we aim to evaluate the effects of these nanomaterials in Swiss mice (*Mus musculus*) and grass carp (*Ctenopharyngodon idella*) after individual exposure and in binary combination. It was investigated whether the short exposure (three days) to the environmentally relevant dose (760 µg/L) of the pollutants would be able to cause neurotoxic, biochemical, and genotoxic effects in animals. Our data reveal that although the exposures did not cause locomotor and anxiogenic or anxiolytic changes to animals exposed to pollutants, we observed some specific differences in behavioral tests. Fish exposed to PS NaPs showed a deficit in the response to the mirror test, both separately and in combination with nanoparticles. On the other hand, all treatments also induced inactivity in relation to alarm substances (paper #1). In mice, we could observe cognitive impairment in the object recognition test (individually). However, these parameters did not differ between animals in the control group and those exposed to the binary combination of pollutants. On the other hand, exposures (individual and in combination) equitably induced damage to erythrocyte DNA. There was an increase in oxidative stress, especially in groups exposed to PS NaPs (in combination, or not, with nanoparticles). Although increased, the levels of antioxidants evaluated do not appear to be sufficient to inhibit the effects of treatment-induced free radical production (paper #2). Taken together, our studies confirm the toxicological potential of NPs ZnO and PS NaPs in the models studied and encourage new studies to further investigate the factors that elucidate the absence of additive or synergistic effects arising from combined exposure to pollutants.

Keywords: Mixture of pollutants, Nanotoxicity, Fresh water, Terrestrial environment, Vertebrates.

SUMÁRIO

INTRODUÇÃO GERAL

É notório que o crescimento exponencial da população tem aumentado a demanda por recursos naturais e tecnologias (Crist et al., 2017), expondo-nos a diversos novos agentes/poluentes físicos, químicos e biológicos, advindos especialmente do descarte incorreto dos recursos tecnológicos. Porém, o potencial toxicológico de muitos desses agentes sobre o sistema biológico e ambiental ainda não é bem discriminado e, por vezes, ainda desconhecido (Paschoalino et al., 2010; Bessa et al.,2020).

As tecnologias e a ciência vêm passando por uma revolução desde a descoberta que um material em escala nanométrica, apresenta propriedades e comportamentos diferentes daqueles obtidos em escala macroscópica (Durán et al., 2006; Ni et al., 2018), ocasionando, um aumento nas últimas décadas, do número de estudos abarcando o referido tema. O termo "nano" é utilizado para processos, materiais e produtos na escala que varia de 1 a 100 nm e, devido às suas inúmeras aplicações e benefícios, seu uso tem aumentado consideravelmente, bem como sua produção e incorporação à diferentes matérias primas (Hansen et al., 2016). As nanopartículas (NPs) podem ser polímeros orgânicos ou inorgânicos, como aquelas constituídas de óxidos metálicos e sais metálicos (Nel et al., 2006; Wallyn et al.,2019). As nanopartículas de oxido de zinco (NPs de ZnO), por exemplo, têm sido amplamente produzidas e aplicadas em diferentes setores, especialmente devido às suas propriedades ópticas, fotocatalíticas, semicondutoras, piezoelétricas e magnéticas (Xiong et al., 2017, Balram et al., 2020). Os nanoplásticos (NaPs), por sua vez, também são nanomateriais cuja utilização tem crescido atualmente, especialmente devido às suas aplicações em uma ampla variedade de produtos, além de serem versáteis, de baixo custo, simplicidade de fabricação e resistentes à água e a produtos químicos. Entre os principais tipos de plásticos utilizados nas indústrias, podemos citar o polietileno, polipropileno, poliuretano, cloreto de polivinila, poliamida e poliestireno (Spinacé & De-Paoli, 2005; Shetti et al.,2019).

Contudo, as mesmas características que conferem às NPs e aos NaPs alta aplicabilidade tem sido relacionadas à diferentes efeitos toxicológicos, quando presentes e dispersos no ambiente (Biswas & Sarkar, 2019). Além disso, devido ao pequeno tamanho, alta resistência e persistência no ambiente, esses nanomateriais podem adsorver outros poluentes, por meio de um processo conhecido como "efeito cavalo de tróia" (O'donovan et al., 2018; Rist et al., 2018). Assim, o presente estudo visou ampliar o conhecimento sobre os efeitos induzidos por esses materiais nos animais, enfocando na avaliação isolada ou combinada das NPs de ZnO e NaPs de poliestireno, utilizando roedores *Mus musculus* (camundongos Swiss) e peixes da espécie *Ctenopharyngodon idella* (carpa capim), como modelos experimentais representativos dos grupos dos vertebrados

terrestres e aquáticos, respectivamente. Acreditamos que este estudo possa ser útil não apenas para identificação do potencial (eco)toxicológico das NPs e NaPs, mas também como subsídio científico para a proposição de medidas de mitigação e remediação da poluição plástica em ambientes dulcícolas receptores de alta carga de poluentes.

1.1. Conhecendo os nanomateriais: conceitos, benefícios e preocupações ambientais

O crescimento emergente da nanotecnologia tem ganhado destaque no meio científico e industrial. Sua primeira aplicação ocorreu com Michael Faraday, em 1857, ao trabalhar com amostras de "ouro coloidal". O objetivo de suas investigações era observar a interação da luz com partículas de metal através da cor rubi, produzida em "solução" por finas partículas de ouro que são "muito diminutas em suas dimensões". Em 1959, Richard Feynman trouxe à tona diferentes questões ligadas à nanotecnologia, especialmente, ao questionar a hipótese de que não existe obstáculo teórico à construção de pequenos dispositivos compostos por elementos muito pequenos. Muitas de suas previsões são agora técnicas bem estabelecidas na área, embora ele não tenha cunhado a terminologia (Feynman, 1960, Feynman, 2003, Zaib & Iqbal, 2019). A termologia foi introduzida por Norio Taniguchi em 1974, um educador da Tokyo University of Science (Japão), o qual definiu a nanotecnologia como a área que "consiste principalmente no processamento de separação, consolidação, e deformação de materiais por um átomo ou uma molécula" (Taniguchi, 1974, Zaib & Iqbal, 2019).

Atualmente, o referido termo tem sido utilizado para caracterização do uso de qualquer tecnologia na escala nanométrica, desde a fabricação até suas aplicações nos mais diferentes sistemas (biológicos, físicos e químicos), em escalas que variam de moléculas ou átomos distintos às dimensões visíveis por microscopia (Kabir, et al., 2018). Os nanomateriais compreendem materiais que contêm pelo menos 50% das partículas (por número) na faixa de 1–100 nm (Figura 1). No entanto, essa definição pode apresentar imprecisões na quantificação dos componentes na extremidade inferior dessa faixa. Dessa forma, é recomendada a adoção de um limite baseado em peso para a distribuição de tamanho, em vez de uma abordagem baseada em números (Bleeker et al., 2013, Release et al., 2019, Chaudhary et al., 2020).

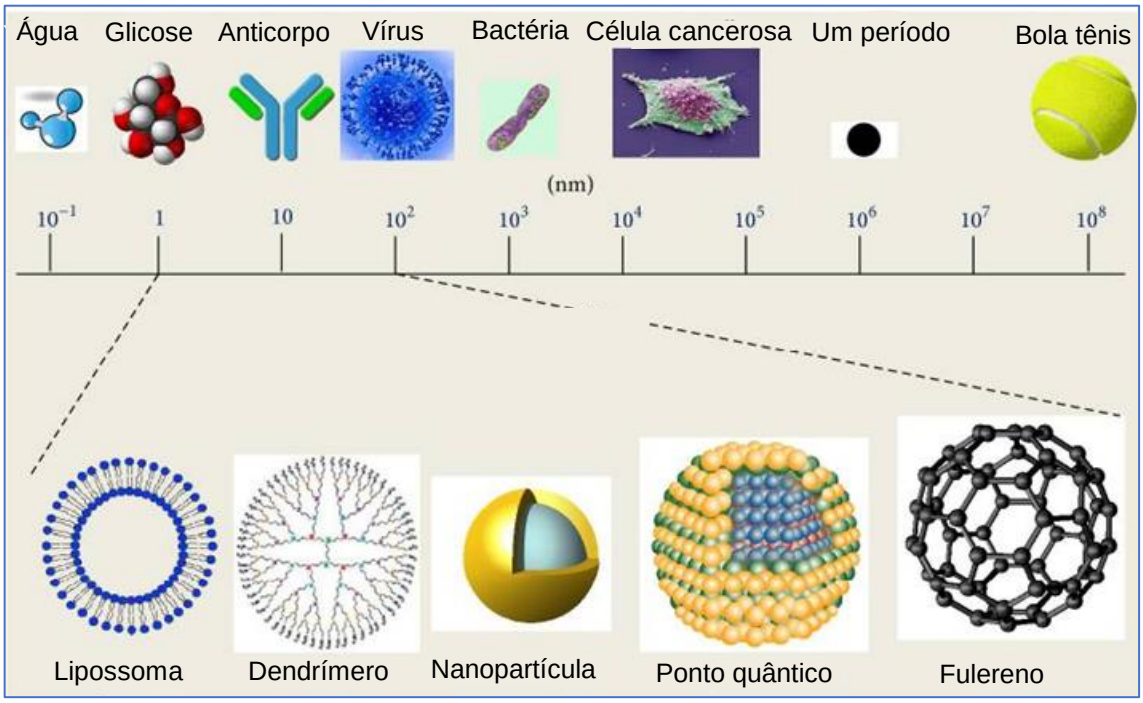

Figura 1. Representação esquemática de uma escala manométrica, demonstrando diferentes nanomateriais em comparação com outras moléculas, estruturas, células e objetos (de tamanhos superiores). Adaptada de Pradhan et al. (2015).

Quanto à origem dos nanomateriais (NMs), estes podem ser oriundos de diferentes fontes, tais como aquelas "naturais", em que as partículas são formadas por meio de processos (bio)geoquímicos ou mecânicos, sem conexão direta ou indireta com uma atividade humana ou processo antropogênico. Atividades vulcânicas, poeiras cósmicas, partículas eólicas derivadas de intemperismo, erosão eólica, precipitação química, biomineralização e combustão de biomassa são alguns exemplos de fontes naturais de NMs (Hochella et al.,2019). Por outro lado, eles também são produzidos por meio de processos industriais ou projetados intencionalmente, para atender demandas específicas dos seres humanos ou incidentais, produzidos como resultado de qualquer forma de influência humana, direta ou indireta (Bleeker et al., 2013, Hochella et al.,2019, Chaudhary et al., 2020, Saleh, 2020, Khan, 2020). Quanto ao formato e à estrutura dos NMs, estes possuem uma variabilidade de subdivisões incluindo as (i) unidimensionais [como nanotubos; (Yoo at al., 2018) e nanofios (Waqar et al. 2015)] (ii) bidimensionais, tais como as nanofolhas, produzidas a partir da quebra contínua de nanocamadas e, por fim, (iii) tridimensionais, cujas diferenças estão relacionadas a dimensionalidade [e.g.: grafenos (Vaseghi & Nematollahzadeh et al., 2020).

Quanto à sua composição, os NMs podem ser categorizados em orgânico-lipossomais, nanoemulsões, dendrímeros, micelas poliméricas, assim como em inorgânicos, incluindo as nanopartículas metálicas (e.g.: ouro, prata, cobre, níquel, zinco, etc.), nanopartículas de óxidos (óxido de cobre, óxido de zinco, óxido de magnésio, óxido de níquel, sílica, magnetite, etc.), nanopartículas

de carbono e grafeno (Khan, 2020), dentre outras. Além disso, os NMs podem apresentar composição híbrida (orgânico-inorgânico), tais como os nanocompósitos (Vaseghi & Nematollahzadeh et al., 2020), os quais são formados por uma matriz polimérica reforçada com uma carga nanométrica. Os principais nanocompósitos são utilizados em engenharias, produção de plásticos, equipamentos esportivos, borrachas, revestimentos, materiais eletrônicos e ópticos no setor petrolífero, gás e mineração, energias renováveis e setor automotivo (Moraes et al., 2014, Ates et al., 2020), etc.

Independentemente do formato e composição, os NMs apresentam características diferentes e únicas daquelas dispostas em tamanho macro. Sua área de superfície, por exemplo, é maior, permitindo assim a inclusão de grupos funcionais e arranjos específicos para promover propriedades físicas, químicas, ópticas, elétricas, magnéticas, de transporte, catalíticas entre outras de interesse humano. Tais propriedades possuem uma dependência com o tamanho das partículas, manifestando-se a partir de um determinado tamanho crítico com a relação de volume, o que permite a melhoria de algumas propriedades de interesse, tais como sensibilidade, especificidade, permeabilidade e reatividade (Khan et al., 2019, Rawtani et al., 2019).

Nesse sentido, devido à versatilidade e aplicabilidades gerais, é notório que a nanotecnologia constitui um dos ramos da ciência que mais tem se desenvolvido na última década (Kolahalam et al.,2019). Seu uso tem agregado valor aos mais diferentes setores da sociedade, apresentando como principal vantagem à possibilidade de projetar uma infinidade de propriedades desejáveis nos campos tecnológicos, industriais e biomédicos, bem como seu uso extensivo em vários produtos de consumo (Raha & Ahmaruzzaman, 2020). Já é possível identificar, por exemplo, a presença de NMs em mais de 4000 produtos, incluindo os cosméticos e produtos de higiene pessoal, alimentícios, bebidas, produtos agrícolas, vestuários, eletrônicos, automotivos, aplicados ao esporte, materiais de construção, bioenergia, purificação de água, além de diferentes produtos com aplicações medicinais (Wright, 2016; Chaurasia, 2017; Bazrafshan et al., 2017; Rai & Biswas, 2018; Khan et al., 2019; The Nanodatabase, 2020; Halder et al., 2020; Kidd et al., 2020; Attia & Elsheery, 2020; Srivastava et al., 2020; Barik, et al., 2021; Carvalho & Conte-Junior, 2021).

Contudo, o uso dos NMs deve ser pesado contra seus riscos ambientais potenciais, uma vez que o desenvolvimento de métodos para avaliar sua segurança (eco)toxicológica não acompanha sua rápida utilização/comercialização (Johnston et al., 2020; Neves et al., 2020). Embora seja claro que muitos NMs têm efeitos benéficos aos seres humanos, propriedades prejudiciais também são observadas em diferentes organismos (Figura 2), dada suas interações oportunizadas por meio da inalação, ingestão, absorção, contato dérmico, dentre outros (Oberdörster et al., 2005, Barik et al., 2021). As células animais, por exemplo, têm tamanho aproximado de 10.000 a 20.000 nm e, assim, os NMs podem adentrar células e organelas e interagir com o DNA e diferentes proteínas celulares (Anoop et al., 2020), o que pode explicar seus diferentes efeitos danosos. Ademais, conforme discutido

por Hochella et al. (2019), os NMs podem ocasionar diferentes impactos ambientais, seja em nível aquático, atmosférico e terrestre.

Entretanto, é importante salientar que ainda são escassas, pouco disponíveis e padronizadas as ferramentas analíticas capazes de quantificar os NMs nos ambientes naturais, estudar seus tamanhos e formas, bem como suas composições e reatividades com outros materiais/elementos/poluentes. Portanto, é de suma importância que a ciência avance nessa área, uma vez que o conhecimento sobre o comportamento distinto desses poluentes no ambiente contribuirá para a proposição de estratégias de remediação e mitigação de seus impactos ecossistêmicos (Hendren et al., 2015).

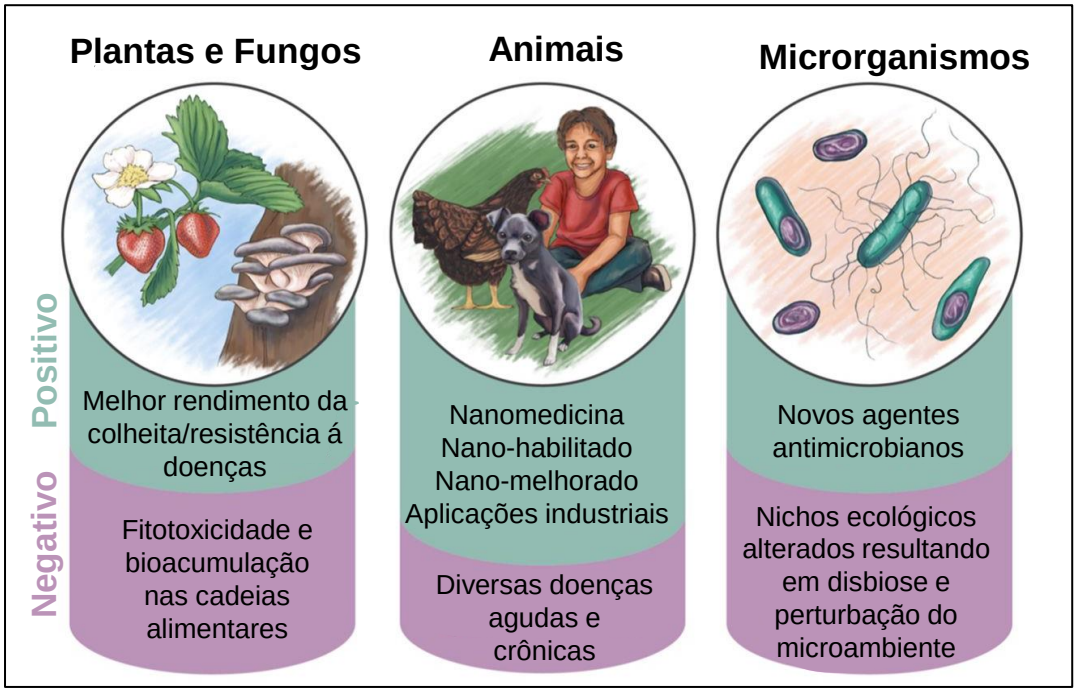

Figura 2. Distribuição dos nanomateriais antropogênicos e naturais por meio de múltiplas rotas demonstrando impactos positivos e/ou negativos. Adaptado de Hochella et al. (2019).

1.2. Nanoplásticos: um nanomaterial emergente e com alto potencial (eco)toxicológico

Poluentes emergentes é a terminologia empregada, comumente, para descrever poluentes químicos ou naturais, que estão presentes em vários ecossistemas, e cuja toxicidade e / ou persistência é capaz de alterar o metabolismo de um ser vivo. Podemos classifica-los em três categorias. A primeira consiste em compostos que são introduzidos recentemente no meio ambiente. A segunda categoria inclui compostos que foram introduzidos no meio ambiente ao longo de um período substancial de tempo e, no entanto, só foram detectados recentemente. A terceira categoria consiste em compostos que são conhecidos e medidos há algum tempo e são reconhecidos como potencialmente causadores de efeitos adversos nos ecossistemas e / ou humanos. Podemos citar como exemplos- a contaminação

por chumbo, dicloro-difenil-tricloroetano (DDT) e clorofluorocarbonetos, resíduos de origem farmacêuticas, produtos de cuidados pesoais, retardadores de chama, drogas icilítas, defensivos agrícolas, aditivos alimentares e NMs projetados (Peña-Guzmán et al.,2019;Sauvé & Desrosiers, 2014). Entre os NMs, salienta-se a poluição por nanoplásticos (NPs), a qual apresenta-se como um desafio e problemática, que precisam ser investigados, debatidos e enfrentados por diversos setores da nossa sociedade, como: governo, empresas, cientistas e cidadãos (Haward, 2018; Landon-Lane, 2018; Mitrano & Nowack 2021).

Plásticos têm sido empregados em uma ampla gama de necessidades humanas, além de diversos setores industriais (Cai et al., 2021). Entretanto, seu potencial e evidente prejuízo eco(toxicológico) emerge como uma das principais preocupações atuais, já que partículas plásticas maiores podem ser degradadas a partículas micro- e nanoplásticas (Kogel et al., 2020). As principais composições químicas dos plásticos já identificados nos ambientes incluem o polipropileno (PP), polietileno (PE), cloreto de polivinila (PVC), tereftalato de polietileno (PET) e poliestireno (PS) (Zhang & Xu, 2020). O PS é um homopolímero amorfo e transparente polimerizado. Através das caracteristicas do estireno (vinil benzeno), flexibilidade ou moldabilidade sob a ação do calor, são isolantes térmico e elétrico, resistentes ao impacto e possuem baixo custo, portanto, apresentam uma larga faixa de aplicações está entre os plásticos mais utilizados na fabricação de embalagens e utensílios descartáveis como potes para iogurtes, sorvetes, doces, frascos, bandejas de supermercados, geladeiras (parte interna da porta), pratos, tampas, copos descartáveis, e brinquedos. O PP utilizado em embalagens, cordas, fios e cabos, frascos, caixas de bebidas, fibras para tapetes, o PE são utilizados embalagens de detergente e óleos automotivos, sacolas de supermercados, tampas, bolsa para soro medicinal e sacos de lixo, o PVC embalagens para água mineral, óleos comestíveis, tubulações de água e esgoto, mangueiras, embalagens para remédios e o PET normalmente compõe frascos e garrafas para uso alimentício/hospitalar, cosméticos, bandejas para micro-ondas e fibras têxteis (Redondo-Hasselerharm et al., 2018;Yang et al.,2021).

Quanto à origem dos NPs, esta pode ser dividida em primária e secundária (Figura 3). Aqueles de origem primária são os adquiridos ou extraídos de produtos para os quais são produzidos e adicionados intencionalmente, como revestimentos, produtos biomédicos, cosméticos, administração de medicamentos, diagnósticos médicos, eletrônicos, magnéticos e optoeletrônicos (Koelmans et al., 2015 , Mitrano et al., 2019). Já aqueles de fonte secundária, derivam da quebra dos plásticos com diâmetro maior (i.e.: macroplásticos – ≥ 5 mm (Lechthaler et al.,2020), através da degradação natural (não intencional) - desgaste mecânico (Ekvall et al., 2019), o calor (Hernandez et al., 2019), a degradação UV (Lambert & Wagner, 2016) e fatores biológicos (Dawson et al., 2018; Gigault et al., 2021). Tais processos, torna-os mais heterogêneos, altamente polidispersos, com formatos diferentes e carregados negativamente (Gaylarde & da Fonseca, 2020; El-Hadri et al., 2020), o que pode aumentar

sua toxicidade.

Por outro lado, é importante destacar que esses nanopoluentes tem sua abundância relacionada à mair densidade antrópica. Com a utilização cada vez mais presente no dia a dia, aumenta-se também a quantidade de resíduos produzidos e descartados no ambiente. Os detritos de plástico, podem ser considerados como uma questão ambiental global e o gerenciamento/descarte inadequados resultam em níveis crescentes no meio ambiente (Yu, et al.,2019). Há registro da sua detecção em locais remotos longe das atividades humanas, como águas polares árticas e fundos marinhos profundos (Peng et al., 2020), lagos menos povoados (Free et al., 2014) e bacias de montanhas intocadas (Allen et al., 2019). Além disso, o transporte de longa distância e a transferência entre compartimentos ambientais, são processos cruciais em seus comportamentos ambientais, que respondem pela distribuição mundial de NPs (Zhang & Xu, 2020).

O plástico se acumula no sistema terrestre devido a fontes comuns, como sistemas de gerenciamento de resíduos defeituosos, espalhamento de lodo de esgoto, uso de cobertura morta de plástico e outros produtos plásticos na agricultura e partículas de desgaste de pneus (Carr et al., 2016 ; He et al. , 2018; Liu et al., 2019). Através das emissões de fontes terrestres, essas podem migrar primeiramente para aos ambientes de águas doces por meio do transporte fluvial (Rech et al., 2014). Os sistemas de água doce podem ser particularmente contaminados, já que muitos poluentes acabam nos rios, lagos e riachos, seja por meio da entrada direta de resíduos ou como escoamento da terra ou precipitação do ar. Entretanto, os estudos que examinaram o impacto da liberação desses NMs nesse sistema e como eles interagem com a biota são bem menos numerosos do que aqueles com foco no ambiente marinho (Birch et al.,2020; Redondo-Hasselerharm et al.,2020; Kukkola et al.,2021).

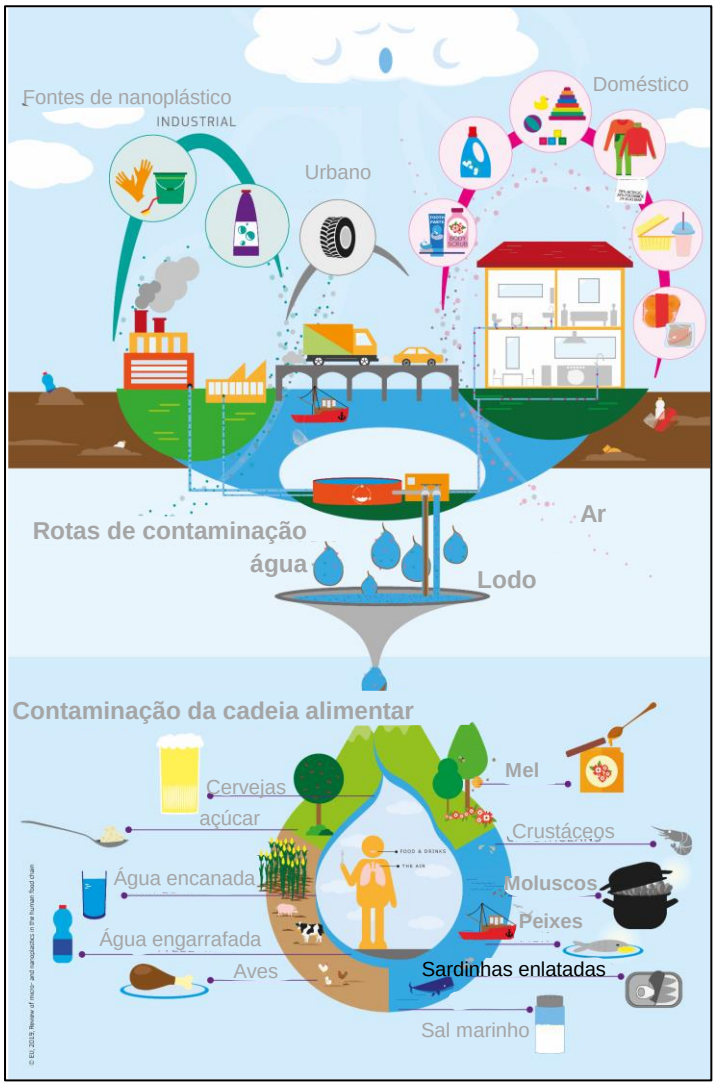

Figura 3. Fontes e nanoplástico no meio ambiente e rotas de contaminação. Adaptado de Toussaint et al. (2019).

Os NPs tem se mostrado como uma potencial ameaça mais grave para a biota do que os microplásticos (MPs), devido ao impacto de seu pequeno tamanho em seu destino ambiental, que permite outros cenários de exposição e a invocação de outros efeitos biológicos (Ekvall et al., 2019), adentrando mais facilmente aos diferentes compartimentos biológicos (Cai et al., 2021). A elucidação e compreensão dos potenciais efeitos adversos dos NPs sobre a biota, é um desafio emergente em diferentes níveis (Town & Van Leeuwen, 2020). No Quadro 1 são sumarizados alguns dos estudos recentes (2020-2021) com foco na ecotoxicidade dos nanoplástico de poliestireno (NPs de PS), envolvendo peixes e mamíferos, uma vez que há importantes lacunas na literatura sobre o grupo. Em suma, tais estudos mostram que os NPs se acumulam nos estágios iniciais do desenvolvimento, atingem diferentes órgãos ao longo do desenvolvimento e têm o potencial de causar distúrbios fisiológicos. Além disso, é possível observar que os NPs induzem efeitos deletérios em uma ampla gama de espécies e em vários níveis de organização biológica.

Quadro 1. Informações gerais sobre alguns dos estudos recentes (2020-2021), envolvendo a toxicidade dos nanoplásticos de poliestireno em peixes e mamíferos (*in vivo* ou *in vitro*).

Estudos envolvendo representantes do grupo dos peixes					
Referências	**Modelo experimental**	**Tamanho do nanoplásticos**	**Concentrações testadas**	**Tempo de exposição**	**Principais conclusões**
Elizalde-Velázquez et al. (2020)	*Pimephales promelas*	50 nm	Injecção 0,1 mL - 50 nm (5 µg/L)	2 dias	NPs PS induzem imunotoxicidade em peixe através de um percurso de exposição ambiental relevante, interferirem na síntese e função de neutrófilos, macrófagos em seus principais tecidos hematopoiéticos.
Sökmen et al. (2020)	*Danio rerio*	20 nm	injeção 1% de partículas sólidas, 0,027% (~ 270 ppm)	5 dias	Observou-se aumento da mortalidade e anormalidades nos animais, além do aumento na produção de espécies reativas de oxigênio e do processo de apoptose, especialmente no cérebro.
Brandts et al. (2020)	*Danio rerio*	65 nm	50 mg/L[1]	2 dias	NPs PS induzem resposta imune, porém, sobrevivência dos animais não é afetada em relação a larvas não expostas.
Pedersen et al. (2020)	*Danio rerio*	50 e 200 nm	10, 100, 1000 ou 10.000 partes por bilhão (ppb)	6 h a 5 dias pós fertilização	Observou-se acúmulo de NPs PS dose-dependente, coincidindo com uma resposta comportamental alterada. No entanto, os NPs não afetaram a sobrevivência, taxa de eclosão dos ovos ou induziram deformidades morfológicas evidentes. A análise transcriptômica sugere neurodegeneração e disfunção motora em concentrações altas e baixas.

Quadro 1. *Continuação.*

Estudos envolvendo representantes do grupo dos peixes					
Referências	Modelo experimental	Tamanho do nanoplásticos	Concentrações testadas	Tempo de exposição	Principais conclusões
Zhang et al. (2020)	*Danio rerio*	42 nm	1000, 3000, 5000, 0,5 e 5 ppm	4 dias pós-fertilização	Foi observado que exposição aquosa ocasionou um aumento no acúmulo nos olhos, evidenciando a inibição do crescimento, aumento da frequência de malformações morfológicas e hipoatividade. Ambas as rotas de exposição causaram mudanças na expressão de genes neurais e oculares, mas apenas a via aquosa causou uma regulação negativa de genes relacionados a antioxidantes. As concentrações testadas nesses experimentos são altas e representam o pior cenário possível.
Duan et al. (2020)	*Danio rerio*	100 nm	250 itens de μ-PS ou 2×10^4 itens de n-PS suspendido em 50 mL de solução de exposição	até 4 dias hpf.	Foi observado que a adsorção de n-PS e μ-PS na superfície externa do córion alterou as propriedades mecânicas dessa estrutura, podendo levar a um microambiente hipóxico nos embriões. A variação da frequência cardíaca, velocidade do fluxo sanguíneo e taxa de eclosão dos embriões indicam a toxicidade do desenvolvimento induzida pela exposição a n-PS e μ-PS em embriões. Além disso, os resultados da metabonômica mostram que ela está relacionada às alterações do sistema antioxidante do embrião.

Quadro 1. *Continuação.*

Estudos envolvendo representantes do grupo dos peixes					
Referências	Modelo experimental	Tamanho do nanoplásticos	Concentrações testadas	Tempo de exposição	Principais conclusões
Sarasamma et al. (2020)	*Danio rerio*	~ 70 nm	0,5 ppm, 1,5 ppm e 5 ppm	aguda (~ 7 dias) e crônica (~ 30 dias e ~ 7 semanas)	NPs PS se acumularam nas gônadas, intestino, fígado e cérebro, além de induzir distúrbios do metabolismo energético e no comportamento dos animais, especialmente relacionados à atividade de locomoção, agressividade, formação de cardumes e comportamento de esquiva de predadores.
Pang et al. (2021)	*Oreochromis mossambicus*	100 nm	20 mg/L	7 dias	NPs PS induziram alterações no metabolismo de glicolipídeos e aminoácidos. Além disso, os NPs causaram sinalização molecular perturbada, como sugerido pelos resultados transcriptômicos.
Guimarães et al. (2021)	*Ctenopharyngodon idella*	23,03 ± 0,266 nm e 80% na faixa de 20 a 26 nm	0,04 ng/L, 34 ng/L e 34 µg/L	20 dias	Observou-se déficit de resposta anti-predatória, alteração de índices viscerais somáticos e hepatossomáticos, além de estresse oxidativo e diminuição da resposta antioxidante.
Sendra et al. (2021)	*Danio rerio*	50 nm e 1 µm	10 mg/L^{-1}	1, 3, 4 e 5 dias após a fertilização	Distribuição celular diferiu dependendo do tamanho da partícula. Mecanismos endocíticos para a captação de NPs foram a internalização dependente de dinâmina para 50 nm e a fagocitose para as nanopartículas de 1 µm. Além disso, observou-se acúmulo principalmente no intestino, onde desencadeiam espécies reativas de oxigênio, diminuem a expressão do gene

					antioxidante catalase e induzem a migração de células do sistema imune.

Quadro 1. *Continuação.*

Estudos envolvendo representantes do grupo dos mamíferos					
Referências	Modelo experimental	Tamanho do nanoplásticos	Concentrações testadas	Tempo de exposição	Principais conclusões
Fournier et al. (2020)	Fêmeas de ratos Sprague Dawley	O tamanho das nanopartículas foi medido com a óptica não invasiva de retroespalhamento (NIBS) usando um laser de 4 mW e 633 nm. Grânulos de poliestireno marcados com rodamina revelou um tamanho médio de aglomerado de partícula de 21,86 nm ± 0,026	Intratraqueal 300 µL ($2{,}64 \times 10^{14}$ partículas) de suspensão de nanopoliestireno	24 h	Foi relatado que os pesos fetal e placentário foram significativamente menores (7 e 8%, respectivamente) em comparação com os controles. Partículas de nanopoliestireno foram detectadas no pulmão materno, coração e baço.
Domenech et al. (2020)	In vitro: células intestinais Caco-2/HT29 diferenciadas e células Caco-2/HT29 + Raji-B	nPSNPs, PP-008-10) com um tamanho nominal variando de 0,05 a 0,1 µm usados em todos os experimentos. PSNPs amarelos fluorescentes (y-nPSNPs, FP-00552-2), com um tamanho nominal variando de 0,04 a 0,09 µm, experimentos que analisam a absorção celular ou a translocação de barreira.	Caco-2 / HT29 ou Caco-2 / HT29 + Raji-B foram expostas a 0, 1, 25, 50 e 100 µg/mL de nPSNPs (ou y-nPSNPs) por 24 h no 21º dia	As células foram co-cultivadas por 21 dias	Os resultados mostraram que os NPs podem entrar e cruzar a barreira epitelial do sistema digestivo. O controle positivo de cloreto de benzalcônio foi capaz de interromper as monocamadas Caco-2 / HT29 levando a um acúmulo significativo de LY para o lado basolateral, um efeito também observado nas inserções livres de células.

Quadro 1. *Continuação.*

Estudos envolvendo representantes do grupo dos mamíferos					
Referências	**Modelo experimental**	**Tamanho do nanoplásticos**	**Concentrações testadas**	**Tempo de exposição**	**Principais conclusões**
Tan et al. (2020)	Células RAW264.7 (macrófago de monócito leucêmico de camundongo)	300 nm	150 μg/mL	24 h	Observou-se efeito diferencial de NPs PS sobre a citotoxicidade de RAW264.7 na presença e ausência de meio sérico. Ao revestir a proteína BSA individual, foi demonstrado que, com a degradação dos revestimentos protéicos nos lisossomos, observou-se a recuperação da citotoxicidade dos NPs PS.
Jung et al. (2020)	Camundongos CD-1	100 nm	2,5% de sólidos [p/v], $4{,}55 \times 10^{13}$ partículas/mL. Os nanoplásticos de PS foram diluídos em ddH_2O autoclavado e os nanoplásticos de 0, 50, 100 ou 200 mg/L	48 h	Concentrações elevadas de NPs PS induzem toxicidade para células neuronais em cultura, evidenciada pelos biomarcadores de viabilidade celular, análise de imunofluorescência, imunoblot e PCR de transcrição reversa quantitativa.
Lim et al. (2021)	Ratos Sprague-Dawley	0,10 μm=100nm	Inalação: baixa $0{,}75 \times 10^{5}$ partículas/cm^3 ± 20%), grupo de concentração média ($1{,}50 \times 10^{5}$ partículas/cm^3 ± 20%) e grupo de alta concentração ($3{,}00 \times 10^{5}$ partículas/cm^3 ± 20%).	14 dias	Observou-se efeito mais pronunciado no nível molecular do que no orgânico, sugerindo que se a exposição for mantida, alterações no nível molecular podem levar a alterações subsequentes nos níveis mais elevados e, conseqüentemente, os riscos à saúde dos animais A expressão de proteínas inflamatórias (TGF-β e TNF-α) aumentou no tecido pulmonar de forma dependente da concentração de exposição.

Quadro 1. *Continuação.*

Estudos envolvendo representantes do grupo dos mamíferos					
Referências	Modelo experimental	Tamanho do nanoplásticos	Concentrações testadas	Tempo de exposição	Principais conclusões
Xu, et al. (2021)	Camundongos BALB/c	100 nm	100 µL de PS, PS-COOH e PS-NH_2 NPs (10 mg/mL)	28 dias	Observou-se acúmulo de NPs no fígado, baço, pulmões, rins, intestino delgado, intestino grosso, testículos e cérebro de camundongos após a exposição oral, levando a lesões no sistema hematológico e distúrbios no metabolismo lipídico. Além disso, os autores evidenciaram que era mais fácil para PS-NH_2 e PS-COOH entrarem nas células e desencadear toxicidade em comparação com NPs PS.
Ding et al. (2021)	Camundongos C57BL/6 SPF	60 nm	50 µg/mL	3 dias	Foi observado os seguintes resultados: NPs PS foram visíveis nos tecidos gástrico, intestinal e hepático de camundongos após 3 dias; aentrada dos poluentes nas células GES-1 foi tempo-dependente e NPs PS regulam a endocitose através da via de sinalização RhoA/F-actina e ao entrar nas células, os NPs inibiram a proliferação celular, promoveram a apoptose e induziram a autofagia.

Quadro 1. *Continuação.*

Estudos envolvendo representantes do grupo dos mamíferos					
Referências	Modelo experimental	Tamanho do nanoplásticos	Concentrações testadas	Tempo de exposição	Principais conclusões
Han & Ryu (2021)	Fibroblastos embrionários de camundongo ICR	100 nm	200 mg / L	Até 6 dias	Os resultados indicam que NPs vacumulados são removidos ou exportados para fora das células. Portanto, os nanoplásticos de PS no citoplasma afetam as funções celulares, mas são temporais e os fibroblastos embrionários de camundongo podem superar o estresse causado pela exposição aos poluentes.
Qiao et al. (2021)	Camundongos C57BL/6J	70 nm	2 mg/kg e 0,2 mg/kg	28 dias	Essa pesquisa destaca a importância da microbiota intestinal na toxicidade da exposição aos poluentes na função de barreira intestinal.
Kik et al. (2021)	Células mononucleares do sangue periférico humano	29, 44 e 72 nm	0,001,0,01,0,1,1,10 e 100 μg/mL	1 dia	Foi observado que a toxicidade e as propriedades oxidativas dos NPs PS examinados dependiam em grau significativo de seu diâmetro.

1.3. Nanoplástico e o impacto das interações com outros poluentes

Um aspecto importante que tem sido pouco abordado, refere-se ao potencial (eco)toxicológico dos NPs em associação com outros poluentes. Embora os estudos na forma isolada, tenham contribuído para a compreensão dos efeitos causados por nanopoluentes nos ecossistemas, é notável a carência de estudos dedicados à toxicidade comparativa e combinada de diferentes substâncias como partículas na faixa de nanoescala (Tong et al., 2015 , Minigalieva et al., 2015), incluindo aquelas orgânico-orgânicas, metal-metal e de metal-orgânico. Logo, essas interações precisam ser melhor investigadas, para uma melhor compreensão da poluição combinada, de plásticos com outras substâncias (LI et al., 2020; Bhagat & Shimada 2020), uma vez que nos ambientes naturais os NPs não se encontram isolados, mas sim combinados com outros poluentes aquáticos (Bellingeri et al., 2019).

Os efeitos combinados de plásticos com outras substâncias são amplamente dependentes dos tipos e características dos plásticos (como tamanho, área de superfície, estruturas, etc.), propriedades de produtos químicos adsorvidos e fatores ambientais. Além disso, uma vez ocorrida a formação dos NPs, ainda se discute se os riscos que elas representam serão aumentados ou não. Mudanças no perfil de acumulação durante uma exposição combinada foram associadas, por exemplo, a efeitos sinérgicos (Zhou et al., 2020; Qiao et al., 2019; Lee et al., 2019), antagônicos (Oliveira et al., 2018) ou aditivos (Ma et al., 2016a). De acordo com diferentes autores, as interações com co-contaminantes, podem causar alterações substanciais nas propriedades de superfície dos NPs interferindo, não apenas na sua toxicidade, mas também na formação de agregados, com implicações na biodisponibilidade desses poluentes (Barboza et al., 2018a; Huang et al., 2019; Zhou et al., 2020).

Estudos com misturas de poluentes evidenciaram a interação de NPs PS com diferentes poluentes. Bellingeri et al. (2019) observaram, por exemplo, alteração da carga superficial dos NPs PS-COOH e do seu diâmetro hidrodinâmico, reforçando a formação da eco-corona em algas *Raphidocelis subcapitata*. Já em cianobactérias, Zhang et al. (2018) avaliaram a toxicidade combinada de NPs e glifosato, tendo demonstrado efeito inibitório no seu crescimento. Já a exposição combinada de bifenilos policlorados (PCBs) e PNs PS em *Daphnia magna*, por outro lado, reduziu a toxicidade do PCB-18, diminuindo sua concentração biodisponível aos animais (Lin et al.,2019). NPs de poliestireno-benzopireno (PS@ Bap) alteraram significativamente o destino dos poluentes nas células epiteliais do pulmão (linhagem celular A549) (Ji et al.,2021). Também os efeitos dos nanoplásticos em *Mytilus galloprovincialis* após exposição individual e combinada com carbamazepina, foram evidenciados pelas mudanças na expressão de genes relacionados ao sistema imunológico. A exposição ao PS inibiu a atividade da colinesterase na hemolinfa e a integridade do DNA diminuiu após a exposição a PS, carbamazepina (Cbz) e PS + Cbz.PS (0,05 mg/L) em bivalves *Mytilus galloprovincialis* (Brandts et al., 2018). Já Trevissan et al. (2020) investigando o papel potencial do efeito do Cavalo de Tróia na toxicidade dos nanoplásticos, para o organismo modelo de vertebrados, peixe-zebra (*Danio rerio*)

evidenciaram que os nanoplásticos agiram como transportadores de poluentes ambientais principalmente no saco vitelino, além da toxicidade dos nanoplásticos para as mitocôndrias por meio da diminuição de sua eficiência em produzir energia, a presença de PAHs em sua superfície pode afetar potencialmente o sistema nervoso e a capacidade dos organismos de operar em cenários de alta demanda de energia. O quadro 2 apresenta o sumário dos estudos supramencionados, permitindo-nos observar aspectos específicos de seus delineamentos experimentais.

Quadro 2. Informações gerais sobre alguns dos estudos (2018-2021), envolvendo a toxicidade dos nanoplásticos de poliestireno em combinaçãos com outros poluentes e em diferentes modelos experimentais

Referências	Poluentes	Concentrações testadas/combinadas		Sistema modelo
		Poluente A	Poluente B (X	
Bellingeri et al. (2019)	NP PS carboxilado (PS-COOH NPs) + cobre (Cu)	NP PS (0,5, 1, 2,5, 5, 10, 50 mg PS/L)	Cobre (1, 5, 10, 15, 20, 25, 50, 100, 200 µg Cu/L)	*Raphidocelis subcapitata*
Zhang et al. (2018)	PS-NH_2 + glifosato	nPS-NH_2 1, 5, 9, 11 e 15 mg/L	Glifosato 5 mg/L	*Microcystis aeruginosa*
Lin et al. (2019)	NP PS+ bifenilas policloradas (PCBs)	0, 1, 5, 10, 20, 50 e 75 mg/L[1]	PCBs 0, 100, 200, 300, 350, 500, 600, 700, 900, 1500 µg/L	*Daphnia magna*
Ji et al. (2021)	NPs +NPs de poliestireno-benzopireno (PS @ Bap)	baixa dose (50 mg/kg), e alta dose (100 mg/kg)	Bap de baixa dose (1 mg/kg), alta dose (2 mg/kg), baixa dose de PS @ Bap (50 mg/kg de PS e 1 mg/kg de Bap), alta dose de PS @ Grupo de tratamento com Bap (100 mg/kg de PS e 2 mg/kg de Bap).	Camundongos BALB/c *Mus musculus*
Brandts et al. (2018)	NP PS+ carbamazepina	0,05 e 50 mg/L	Carbamazepina (6,3 µg/L)	*Mytilus galloprovincialis*
Trevissan et al. (2020)	NP PS+ hidrocarbonetos policíclicos aromáticos (PAHs)	1 ppm	PAHs (5.073 ng/mL)	*Danio rerio*

Nanopartículas são produzidas e usadas em grandes volumes e, portanto, a ocorrência de NPs descarregados em ambientes aquosos e terrestres é uma preocupação emergente. Evidenciamos a partir da revisão bibliográfica, que o impacto da coexistência entre nanoplásticos e nanopartículas e seus efeitos combinados em ambientes duclcícolas e solo são relativamente desconhecidos, de tal forma, fica evidente a necessidade dos avaliadores de nanoriscos de suporte científico para avaliar sistemas multi-nanoparticulados (Abdelazim et al.,2018; Ye et al.,2017).

As nanopartículas estão entre os nanomateriais com crescimento exponencial, principalmente no que refere-se as métalicas. Entre as NPs de óxido de metal, o óxido de zinco (ZnO) está entre os maiores volumes de produção, apresentam baixo custo de produção, capacidade de formar estruturas diversas e várias aplicações biológicas. São usados principalmente como aditivo de dispersão da luz UV em cosméticos, como filtros solares, cremes dentais e produtos de beleza, na fabricação de borracha, produção de células solares (como branqueador), eletrônica e têxtil, é um ingrediente essencial em quase todos os tipos de tintas antiincrustantes e propriedades antibacterianas (Bandeira et al.,2020; García -Gómez etal.,2019; Król et al.,2017). Justamente por sua ampla produção e grande utilização procpiciam a possibilidade de induzir perigos, e preocupações devido à sua disponibilidade mundial e acúmulo no meio ambiente, além do impacto no sistema biológico são mal compreendidos.

Nesse sentido, utilizamos roedores Mus musculus (camundongos Swiss) usados normalmente como organismos de teste, podem ser mantidas com eficiência em laboratório, e são considerados sensíveis a um amplo espectro de tóxicos Legradi et al.,2018). E peixes da espécie Ctenopharyngodon idella (carpa capim) nativo da Ásia Oriental e é amplamente distribuído do norte do Vietnã ao rio Amur, na fronteira entre a China e a Sibéria (Cudmore & Mandrak, 2004). A espécie foi selecionada como modelo experimental devido a sua boa adaptabilidade a ambientes de laboratório, resistência a doenças e variações climáticas, cosmopolita abragendo inclusise áreas com altos níveis de poluição por plásticos e nanoparticulas, bem como por ter sido previamente utilizada em diferentes estudos sobre toxicologia ambiental (Ding, 2017; Baldissera et al., 2020).

CAPÍTULO II - JUSTIFICATIVA E OBJETIVOS

2.1. Justificativa

A alta demanda por bens e produtos e a necessidade de suprimento alimentício da população humana, têm impulsionado setores industriais, farmacêuticos, petrolíferos, agropecuários e agrícolas de várias regiões do mundo. Se por um lado essas atividades favorecem o crescimento econômico de muitos países, de outro, têm sido associadas à degradação ambiental e à perda da biodiversidade sem precedentes (Nathaniel et al., 2021). Nesse cenário, é sabido que uma das formas de mitigar e remediar os impactos das atividades humanas sobre o ambiente, é conhecer os efeitos dos poluentes sobre a biota (Provencher et al., 2020). A identificação e a caracterização desses efeitos (especialmente precoces) constitui, portanto, uma das etapas iniciais que subsidiam a elaboração de propostas de mitigação, remediação e de monitoramento ambiental de diferentes biomas e fitofisionomias.

A quantidade de pesquisas em ecotoxicidade com foco nos efeitos tóxicos dos nanomateriais é crescente; contudo, várias lacunas ainda necessitam ser preenchidas (Ganguly & Pillai, 2018). A identificação individual dos poluentes no ambiente natural, tem sido cada vez mais difícil devido à limitação metodológica/analítica, bem como à enorme quantidade de substâncias (sintéticas) potencialmente tóxicas, cuja diversidade tem crescido exponencialmente nos últimos anos (Krewski et al.,2020; Dey et al., 2019; Halstead et al.,2018). Nesse sentido, a avaliação da toxicidade de misturas de poluentes em laboratório passa a ter importância, uma vez que seus resultados podem se aproximar de um cenário mais realista, em que contaminantes de fontes difusas não se encontram isolados, mas associados a outros tantos xenobióticos (Oriekhova & Stoll, 2018), podendo o impacto dessas misturas acarretar em resultados divergentes dos encontrados em contextos de exposição individual (Li et al., 2018).

Estudos prévios já demonstraram que os nanoplásticos podem associar a outros contaminantes (via processos de adsorção) (Li et al., 2018), com consequências desconhecidas na grande maioria dos vertebrados aquáticos ou terrestres. Conforme discutido por Rist & Hartmann (2018), devido às suas dimensões e propriedades químicas, a associação dos nanoplásticos a outras substâncias químicas pode afetar a mobilidade e a disponibilidades dos xenobióticos no ambiente natural. Em um cenário pessimista, essa associação pode favorecer a entrada dos poluentes nos organismos, exacerbando seus efeitos prejudiciais nos organismos. Portanto, é iminente a necessidade de avaliarmos as contribuições toxicológicas dos NPs associados a outros poluentes nos ecossistemas de água doce e terrestres, uma vez que a presença desses poluentes pode implicar em aumento do risco para a biota, através de interações que conduzem à reatividade extra, aumento de transporte e interação dos xenobióticos com distintos componentes subcelulares, o que pode levar a efeitos adversos mais graves nos organismos.

2.2. Objetivos

2.2.1. Objetivo geral

Com base no que foi exposto, nosso objetivo geral foi avaliar os efeitos da curta exposição de modelos experimentais representativos do grupo dos Vertebrados (aquáticos e terrestres) à nanomaterias isolados e em combinação com as NPs ZnO em concentrações preditivas ambientamente relevantes.

2.2.2. Objetivos específicos

Para alcançar o objetivo geral, almejamos os seguintes objetivos específicos:

a) Avaliar se a exposição de *Ctenopharyngodon idella* (carpa capim) e *Mus musculus* (camundongos Swiss) ao NPs PS – isolados ou em combinação com as NPs ZnO – ocasiona distúrbios comportamentais;
b) Investigar possíveis alterações bioquímicas em tecido encefálico que podem ser correlacionadas com os efeitos neurotóxicos dos tratamentos;
c) Avaliar os possíveis efeitos genotóxicos da exposição aos poluentes, utilizando o teste cometa para idenificação de possíveis danos da exposição DNA;
d) Análise da possível absorção dos poluentes e translocação para cérebro dos animais;
e) E por fim, avaliar as contribuições toxicológicas específicas dos NPs quando isolados e em uma mistura (com as NPs ZnO) para a biota de vertebrados terrestres e de ambientes dulcícolas.

CAPÍTULO III – EFEITOS DOS NANOPLÁSTICOS DE POLIESTIRENO SOBRE *Cteopharyngodon idella* (CARPA CAPIM) APÓS EXPOSIÇÃO INDIVIDUAL E COMBINADA COM NANOPARTÍCULAS DE ÓXIDO DE ZINCO

QUALIS CAPES

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Effects of polystyrene nanoplastics on *Ctenopharyngodon idella* (grass carp) after individual and combined exposure with zinc oxide nanoparticles

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ABSTRACT

The toxicity of polystyrene nanoparticles (PS NPs) and ZnO nanoparticles (ZnO NPs), in combination is poorly known. Thus, the aim of the current study was to evaluate the effects of PS NPs (760 μg/L) on *Ctenopharyngodon idella* exposed to it, both in separate and in combination with ZnO NPs (760 μg/L), based on behavioral, biochemical and genotoxic biomarkers. Current data have indicated that PS NPs, for a short exposure period (3 days), both in separate and in combination with nanoparticles, have affected animals' response to the mirror test. On the other hand, all treatments have equally induced *C. idella* inactivity towards alarm substances and DNA damage. There was increased oxidative stress, mainly in groups exposed to PS NPs (in combination, or not, with nanoparticles); although increased, the evaluated antioxidant levels did not appear to be enough to inhibit the effects of treatment-induced production of free radicals. Together, these results are likely co-responsible for the observed changes. The current study did not observe antagonistic, synergistic or additive effect on animals exposed to the combination between PS NPs and ZnO NPs; however, this outcome should not discourage the performance of similar studies focused on assessing the (eco)toxicity of pollutant mixtures comprising nanomaterials.

1. Introduction

Nowadays, there is consensus that nanomaterials are increasingly becoming part of individuals' daily lives, since they are heavily used in products such as sunscreens (titanium dioxide/zinc oxide nanoparticles), sporting goods (carbon nanotubes, graphene, among others), automotive composites (nanotubes, cellulose nanofibers, among others) and high-definition TVs (quantum dots) (Wright, 2016; Chaurasia, 2017). Such uses are certainly going to increase due to continuous industrial demand for nanomaterials to produce current- and next-generation batteries, biomedical imaging and flexible electronics (Rai and Biswas, 2018). Thus, it is clear that these materials already have, and will continue to have, decisive influence on individuals' lives, since they can improve the quality of life and life expectancy of different populations. However, it is clear that a scarce amount of the total amount of money invested in nanotechnology is used in studies focused on investigating the toxicity of these nanomaterials in different organisms (Paschoalino et al., 2010). The growing production and application of nanomaterials raise significant concern about their potential risk to the environment and to different organisms (Scheringer, 2008; Ray et al., 2009; Keller et al., 2017), mainly due to intrinsic nanoparticle features such as size, surface area and agglomeration/dispersion ability. These factors can favor nanoparticles' translocation into environmental/biological partitions, as well as harm individuals and damage the dynamics of their populations, in a cumulative way (Montano et al., 2014; Lead et al., 2018).

In addition, there is concern that these nanomaterials are not isolated in natural environments, since they can join different aquatic pollutants (from diffuse sources) and cause unprecedented environmental damage. Previous studies have already shown that rivers and streams receive a

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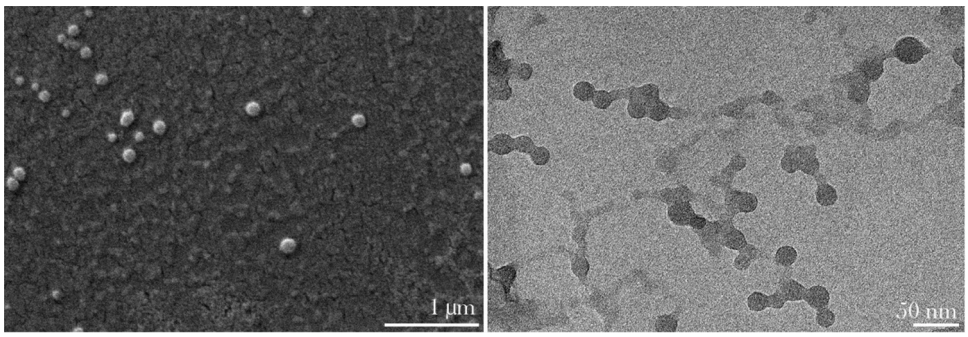

Fig. 1. Scanning transmission electron microscopy of PS NPs used in the exposure of *Ctenopharyngodon idella* (grass carp).

wide range of pollutants such as domestic and industrial effluents (da Silva Ferreira et al., 2020; Zhao et al., 2020), agricultural pesticides (via runoff) (from – Castro-Lima et al., 2020; Curchod et al., 2020), pharmaceutical waste (K'oreje et al., 2016; Mandaric et al., 2019; De-Oliveira et al., 2020), personal hygiene products (Liu and Wong, 2013), among others. In addition to these pollutants, there are those deriving from the degradation of larger amounts of improperly disposed waste, such as plastics. The presence of nanoplastics (NPs) in river ecosystems, for example, was identified in different studies that have warned about the toxicological potential of these nanomaterials (Mattsson et al., 2018; Anbumani and Kakkar, 2018; Sullivan et al., 2020). Therefore, freshwater is an important source of marine pollution caused by riverine transport (Rech et al., 2014).

Thus, the concern that nanomaterials (including nanoparticles and nanoplastics) can significantly affect aquatic biota has encouraged the development of different studies, mainly in the last decade. The toxicity of metallic nanoparticles [e.g.: zinc oxide nanoparticles (ZnO NPs)] was already evidenced in different organisms; among the investigated effects, one finds behavioral changes in mammals (de Souza et al., 2018), mutagenic and cytotoxic effects on reptiles (Araújo et al., 2019) and birds (Vieira et al., 2019), impact on amphibians' growth and survival rates (Nations et al., 2011; Al Mahrouqi et al., 2018), as well as oxidative stress induction (Kaya et al., 2015), renal and liver dysfunction (Chupani et al., 2018), and histopathological and genotoxic damages (Shahzad et al., 2019) in fish. Other equally important studies have shown that the exposure of different organisms to nanoplastics, such as polystyrene (PS NP), triggers a wide variety of toxic damages such as food losses in fish (Jovanović, 2017), negative impacts on the reproductive performance of *Daphnia magna* (Besseling et al., 2014), *D. pulex* (Liu et al., 2019a) and *D. galeata* (Cui et al., 2017), energy metabolism disturbances in several fish species (Cedervall et al., 2012), immunological changes in *Pimephales promelas* (Greven et al., 2016), liver changes in Goldfish *Carassius auratus* larvae (Yang et al., 2020) and hormonal changes in *Danio rerio* larvae (Brun et al., 2019), among others.

However, it is necessary taking into consideration that most studies have investigated the impact of these nanomaterials based on laboratory tests, whose tested concentrations are often much higher than the ones potentially identified in natural environments; this difference may have resulted in overestimated toxicological risks. Another gap in the literature concerns the likely toxicological effects of the binary combination of these nanomaterials (e.g., NP PS and ZnO NP), since they can coexist in contaminated aquatic environments, mainly in urban and densely populated areas. Although some studies have evaluated the toxicity of some nanomaterials mixed with other pollutants [e.g. ZnO NP (Ye et al., 2017; Chen et al., 2020) and NP PS (Lee et al., 2019; Liu et al., 2019b)], the effects of pollutant combinations on the nanoscale remain poorly known. If one takes into consideration that different organisms are exposed to nanoplastics and to other environmental contaminants in their natural environment, it is essential better understanding the potential role played by these particles in the incorporation of other environmental contaminants and their effects (Brandts et al., 2018). In addition, one cannot deny the existence of a significantly disproportional number of studies about the toxicity of these nanomaterials in freshwater ecosystems in comparison to those aimed at investigating the marine environment (Koelmans et al., 2015; Mendoza et al., 2018; Peng et al., 2020). Therefore, it is necessary assessing the response of aquatic freshwater organisms exposed to nanomaterials, either in separate or in combination with each other.

Thus, the aim of the current study was to apply the current conceptual approaches adopted in combined toxicity studies to a site-specific mixture comprising ZnO NP and PS NP by using grass carp (*Ctenopharyngodon idella*) juveniles as representative models of the freshwater fish group. The importance of assessing the toxicity of these nanomaterials, either in separate or in combinations, is not only justified by their wide use to manufacture different products and, consequently, by their disposal in aquatic environments; but mainly because, assumingly, the coexistence of these materials in aquatic ecosystems can have different impacts than the ones observed in individual exposure contexts. Thus, the current study is based on the assumption that animals exposed to both nanomaterials, at environmentally relevant concentrations for a short period-of-time, present behavioral, biochemical and genotoxic changes due to synergistic or additive effect. Besides, it provides an insight into the toxicity of mixtures comprising nanomaterials from different polluting sources, as well as warns about the ecotoxicological risk posed by such mixtures.

2. Material and methods

2.1. Nanomaterials

2.1.1. Polystyrene nanoplastics

The current study used yellow-green fluorescent polystyrene plastics (PS NP) as representative of nanoplastics (λex = 470 nm and λem = 505 nm; (Sigma Aldrich, USA. Product n. L5155). Polystyrene plastics are among the most produced materials worldwide; it is used in products associated with food storage, wrapping, disposable cutlery and industrial packaging. Moreover, particles of this polymer are often found in aquatic environments due to its low reuse rate (Ericksen et al., 2014; Sá et al., 2018). Raman spectrometer (Horiba LabRam HR Evolution) equipped with a Single Mode Open Beam Laser Diode (Innovative Photonic Solutions) and operating at wavelength of 785 nm, coupled to a charge-coupled device (Horiba Synapse) was used to confirm the chemical composition of PS NPs (Fig. 1S). Images were captured under scanning electron microscopy and posteriorly analyzed in the ImageJ 1.52av software (https://imagej.nih.gov/ij/download.html) in order to measure the diameter of PS NPs (n = 350 particles) (23.03 ± 0.266 nm and 80 % in the range from 20 to 26 nm) (Fig. 1A). In addition, nanomaterials were observed in transmission electron microscope with "field emission" [i.e., field emission electron gun (FEG)] (Fig. 1B).

2.1.2. Zinc oxide nanoparticles

The ZnO NPs (purity > 99.99 % - Sigma Aldrich, Saint Louis, MO,

USA; CAS number 544906) used in the current study were nanocrystals without surface modifications (mean diameter 68.96 nm ± 2.519, mean ± SEM). The chemical characterization of these NPs (distribution of the individual diameter of each ZnO nanoparticle; X-ray diffraction patterns of the crystal quality of ZnO nanoparticles; and Fourier transform-infrared transmission spectra) was previously performed by our research group in de Souza et al. (2018).

2.2. Animals and experimental design

Initially, 128 *Ctenopharyngodon idella* juveniles (body biomass: 5.30 g ± 0.85 g; total length: 4.22 cm ± 0.68 cm; 1:1 ratio of male and female) were purchased in a commercial breeding site (Goiânia, GO); they were left to acclimate to the laboratory environment, for 15 days, under controlled light (12 h light/dark cycle) and temperature (25−26 °C) conditions. *C. idella* is a large herbivorous freshwater fish species belonging to family Cyprinidae; it is native to Eastern Asia and is widely distributed from Northern Vietnam to Amur River at the Siberian-Chinese border (Chilton and Muoneke, 1992). The species was herein selected as experimental model due to its good adaptability to laboratory environments, resistance to diseases and climatic variations, as well as because it had been previously used in different studies about environmental toxicology (Forouhar-Vajargah and Hedayati, 2017; Souza et al., 2019; Baldissera et al., 2020).

After the acclimation period was over, animals were distributed in four experimental groups (n = 32/each) (similar biomass and body size), namely: (i) control group, which comprised *C. idella* individuals kept in PS NPs and ZnO NPs-free exposure water; (ii) ZnO NP group, which comprised animals exposed to ZnO NPs at concentration of 760,000 ng/L; (iii) PS NPs group, whose animals were exposed to PS NPs at the same concentration used in the previous group; and (iv) ZnO NP + PS NP group, whose exposure water was added with equivalent concentrations of both nanomaterials (760 µg/L, each). The ZnO NPs concentration defined in the present study is compatible to one of the predictive concentrations suggested in the study by Boxal et al. (2007), via mathematical modeling, which was also tested by Vieira et al. (2019). This concentration was also defined for PS NPs, since it falls within the range of concentrations tested in different studies reviewed by Kögel et al. (2020) (0.8 µg/L to 2 g/L, or 1,000–1,800,000 p/L and 0.0003 % of plastics in water). Therefore, these concentrations are close to the ones eventually identified in surface water, mainly in points close to pollution sources, a fact that brings the current experimental design closer to realistic conditions.

Animal models were exposed to nanomaterials in static exposure system (i.e., without water renewal) for 72 h, which simulates ephemeral and uninterrupted exposure of animals to pollutants. Furthermore, this period is an acute exposure to pollutants, according to the Organization for Economic Co-operation and Development (OECD, 1992). For this, the animals were kept in aquariums filled with naturally de-chlorinated water (for at least 24 h) and continuously aerated with the aid of air compressors. Animals fed twice a day on commercial fish feed in the form of small pellets (guarantee levels: 45 % crude protein, 14 % ether extract, 5% crude fiber, 14 % mineral matter and 87 % dry matter) at the amount equivalent to 5% of their body biomass.

2.3. Toxicity biomarkers

2.3.1. Behavioral assessment

After 72 h of exposure, animals were subjected to several tests based on the assumption that the selected nanopollutants could affect their innate behavior under different stimuli. All tests were carried out in a specific room equipped with sound insulation, under controlled temperature, light and humidity conditions; the same climatic conditions adopted in the experimentation room were maintained when animals were exposed to pollutants. Tests aimed at assessing animals' behavioral performance in conflicting situations created by the open field, novel tank diving, mirror and alarm substance-response tests. The tests were performed in the order they are detailed below (from least to most stressful).

2.3.1.1. Open field test. Locomotor activity and anxiety-like behavior of *C. idella* (n = 16/group) individuals were assessed through open field test (a conflict-based novelty test). Although this test is an experimental paradigm often applied to rodents, it has been widely introduced in protocols focused on measuring behavioral aspects and locomotor skills in fish (Maximino et al., 2010a). The open field was represented by a circular arena with opaque walls (Ø = 15 cm; water depth = 3 cm) and followed pre-established protocol (Stewart et al., 2010), with minor modifications. The test started with the individual introduction of animals in the center of the arena; they were video recorded for 3 min to enable measuring parameters such as covered distance, swimming speed and percentage of time animals spent in the central quadrants of the apparatus. According to Prut and Belzung (2003), this proportion features the anxiety index, since high locomotion rates in peripheral quadrants can be interpreted as increased thigmotaxis behavior (time spent at the edge of the apparatus), which is largely associated with anxiety-like behavior in fish (Champagne et al., 2010; Maximino et al., 2010b; Schnörr et al., 2012; Baiamonte et al., 2016).

2.3.1.2. Novel tank diving test. The novel tank diving test (n = 16/group) was also used to assess the anxiety-like behavior of *C. idella* individuals exposed, or not, to nanomaterials. This test investigates the natural trend of fish to initially dive to the bottom of a novel experimental tank and to gradually increase vertical activity over time (Levin et al., 2007; Blaser and Rosemberg, 2012). Previous studies have compared the initial preference for the bottom of the novel tank to thigmotaxis behavior in rodents; the degree of 'bottom dwelling' has been interpreted as anxiety index (Stewart et al., 2012; Blaser and Rosemberg, 2012; DePasquale and Leri, 2018). Fish subjected to this test were individually transferred to test aquarium filled with 4.8 L of dechlorinated water (dimensions: 19 cm long x 14.5 cm wide x 20 cm high) where they were video recorded for 5 min, similarly to Chagas et al. (2019). The aquarium was virtually divided into two equal horizontal segments and, subsequently, the following parameters were evaluated to enable analyzing animals' anxiogenic-like behavior: latency (s) to rise to the top (upper half), permanence at the top (s) and immobility time. Such a behavior was only observed in the absence of animal movement, except for the eyes and branchial arches.

2.3.1.3. Mirror test. After the novel tank diving test was over, animals remained in the tank for 15 min in order to acclimate and, subsequently, they were subjected to the mirror test, based on the methodology adopted by Chagas et al. (2019). The mirror test conducted in the current study consisted in replacing the wallpaper on one side of the aquarium by a mirror positioned at the right angle; it reflected the image of the animals while they were swimming back and forth. Animals were monitored for 5 min [similarly to the study by Moretz et al. (2007)] and the following parameters were recorded: number of aggressive tail strikes against the mirror, fish-mirror interaction time (minimum distance of 1 cm) and fish immobility time (according to previously described criteria).

2.3.1.4. Alarm substance-response test. Animals' response to alarm substances (conspecific alarm pheromone) was evaluated based on procedures described by Sandahl et al. (2007) and Sovová et al. (2014). The tissue extract used as alarm substance was initially prepared. In order to do so, the solution comprising alarm substances from three non-family-related conspecific individuals (body size and biomass similar to those of the test animals), which were not included in the experimental design of the present study, was prepared. Euthanized animals had their viscera and head removed, whereas the remaining

carcasses were crushed in blender filled with 300 mL of purified water via reverse osmosis (1 individual/100 mL), for 30 s. Next, the resulting solution was filtered on filter paper (thickness: 20 μm; weight: 80 g/m^2; pores: 25–40 μm) and later used as solution for alarm substances.

The test consisted in individually acclimating animals in circular arena with opaque walls (Ø = 15 cm; water depth = 3 cm) for 5 min and, then, adding 1 mL of purified water in the central region of it; animals were video recorded for 3 min. Next, 1 mL of the solution added with alarm substances (previously prepared) was added to the apparatus and animals were evaluated for additional 3 min. Fish immobility time(s) was recorded and anxiety index was calculated based on the video recordings, as described in item "2.3.1.1".

2.3.2. Single-cell gel electrophoresis assay (comet assay)

The comet assay followed the alkaline method based on procedures described by Singh et al. (1988) and Mitkovska & Chassovnikarova (2020), with some modifications. The collected peripheral blood samples (10 μL) were diluted in fetal bovine serum at ratio (v/v) 1:19. Slides were prepared (two per sample) with 15 μL of cell suspension diluted in 120 μL of low melting point agarose (0.5 %) at 37oC and, subsequently, submersed in lysis solution (Triton X-100, DMSO and stock lysis solution) for 24 h at 6 °C - it was protected from light. Next, slides were electrophoresed at 300 mA and 25 V (0.90 V/cm) for 25 min under no light (Poletta et al., 2008). Positive controls were included in each of the carried out electrophoreses to show the electrophoresis conditions. The result of each electrophoresis was only taken into account when positive controls recorded positive results. After electrophoresis, slides were placed on staining tray, covered with neutralization buffer (0.4 M, Tris-HCl, pH 7.5) and kept in the dark for 5 min.

Slides were stained with ethidium bromide (10 μg/mL) and covered with coverslip for the analysis. In total, 50 nucleoids were analyzed per slide, thus totaling 100 nucleoids per sample (n = fish/group). Analysis was performed using the CaspLab software program to assess DNA damage, according to Gyori et al., 2014, Surniyantoro et al. (2020). The following parameters were used to assess DNA damage caused by pesticides: (i) tail length (TL), (ii) DNA percentage in the tail (% DNA) and (iii) Olive tail moment (OTM), as described by Collins (2004) and adopted in previous studies (Ramos et al., 2016; Gonçalves et al., 2017; Fernandes et al., 2018).

2.3.3. Biochemical assessment

2.3.3.1. Sample preparation. Animals were euthanized (by submersion in ice) to enable collecting liver and brain samples (10 mg), which were used to investigate the association between likely genotoxic/behavioral changes and biochemical changes. Next, samples were macerated in microtubes filled with 1 mL of phosphate buffered saline (PBS), in automated maceration system; subsequently, they were centrifuged at 13,000 rpm and 4 °C, for 5 min. The supernatant was transferred to other microtubes, which were used to evaluate the biochemical doses described below (they were kept on ice until experiment conduction).

2.3.3.2. Nitric oxide dosage. Griess colorimetric reaction was used to measure nitric oxide concentrations; this assay consisted in detecting nitrite resulting from NO oxidation, based on Ajjuri and O'Donnell (2013). Nitrite levels were measured based on the Griess method (Grisham et al., 1998), with some adaptations. According to this technique, 30 μL of each sample (in triplicate) was pipetted (in triplicate) into ELISA microplate wells filled with 150 μL of Griess reagent [2.3 % sulfanilamide - N- (1-naphthyl) was subsequently added to 0.12 % ethylenediamine and orthophosphoric acid (0.5 mol/L) in purified water via reverse osmosis]. Next, the Griess sample/reagent mixture was incubated at room temperature for 5 min and read in microplate reader (Heales, model MB-580) at 492 nm. Sodium nitrite solutions based on stock solution were prepared in triplicate at the concentrations of 50, 100, 200, 300, 400 and 500 μmol/L in order to find the standard curve. Linear regression analysis (y = 0.0014x + 0.0275; r^2 = 0.99) was used to calculate nitrite concentrations in the samples (μmol/L).

2.3.3.3. TBARS (thiobarbituric acid reactive species). TBARS (thiobarbituric acid reactive species) is widely adopted as lipid redox state measurement method (Draper and Hadley, 1990). TBARS test was performed in the current study based on Pothiwong et al. (2007) and Carvalho et al. (2019), with some modifications. First, 100 μL of tissue supernatants were mixed with 50 μL of trichloroacetic acid (TCA) (28 % w/v in 0.25 N HCl), 50 μL of thiobarbituric acid (TBA, 1% in 0.25 N acetic acid) and 25 μL of butylhydroxytoluene (BHT, 5 mM). Next, samples were incubated in water bath at 95 °C, for 15 min; subsequently, they were centrifuged at 13,000 rpm, at room temperature, for 10 min. Supernatants deriving from the samples were transferred to ELISA microplate (in triplicate) and read at 492 nm. Results were expressed in "nmol/L".

2.3.3.4. Hydrogen peroxide. Hydrogen peroxide measurement was based on the colorimetric method used to determine the millimolar quantities of hydrogen peroxide [similarly to Graf and Penniston (1980)], on iodide oxidation in the presence of ammonium molybdate, as well as on the photometry of the resulting blue starch -iodine complex, by using the commercial kit (Kit Elabscience, Cat.: E-BC-K 102-S. Lot: 731RIHZ1ES. Exp: 2020-06-16). Results were expressed in "mmol/L"

2.3.3.5. Total glutathione content (GSH + GSSG). The total glutathione content (reduced (GSH) + oxidized (GSSG)) was determined based on the method described by Griffith (1980), with modifications; similarly, to those adopted by Carvalho et al. (2018). Dosing was performed in ELISA microplate, whose wells were initially added with 5 μL of samples and, then, with 75 μL of working mixture (95 mM phosphate buffer, 0.95 mM EDTA, 48 μM NADPH, 0.031 mg/mL DTNB, 0.115 units/mL glutathione reductase and 0.24 % sulfosalicylic acid). Next, samples were incubated at room temperature for 5 min. Subsequently, 25 μL of NADPH (0.16 mg/mL) was added to the wells; absorbance was measured at 405 nm right after the reaction started. Five readings were performed at 1-min intervals between readings.

In addition, absorbance of serial dilutions of a standard reduced glutathione solution (0.25, 0.5, 1, 2 and 4 nmol/10 μL) was determined to find the calibration curve. Next, a graph was plotted based on the points in the standard curve, which corresponded to absorbance delta. The line equation (y = 0.0423x = 0.0037; R^2 = 0.9741) was determined after linear regression analysis, and it was used to determine total glutathione concentration (in nmols) in 5 μL of sample. This value was converted into "nmols/mL".

2.3.3.6. Diphenyl -1-picrylhydrazyl (DPPH) radicals' scavenging activity. DPPH stable radical assay was performed in compliance with Brand-Williams et al. (1995). The reaction mixture comprising 200 μL of DPPH · solution (prepared in 95 % ethanol) (100 mM), 50 μL of each test sample (supernatant) or 5 μL of Trolox (1000 μM) or 50 μL of vitamin C (80 μM) standard solution (prepared in 95 % ethanol) were incubated at room temperature for 30 min; absorbance was measured at 492 nm. Ethanolic DPPH solution (100 mM) was used as control and DPPH radical scavenging activity rate was calculated based on the following equation (Zarban et al., 2009; Chaves et al., 2019): DPPH radical scavenging activity (%) = [(control absorbance - sample absorbance)/ control absorbance] x 100.

2.3.3.7. Total superoxide dismutase (SOD) activity. Total superoxide dismutase (SOD) activity was measured in the supernatants, based on the method described by Dieterich et al. (2000), with some modifications. Measurements were based on SOD's ability to scavenge

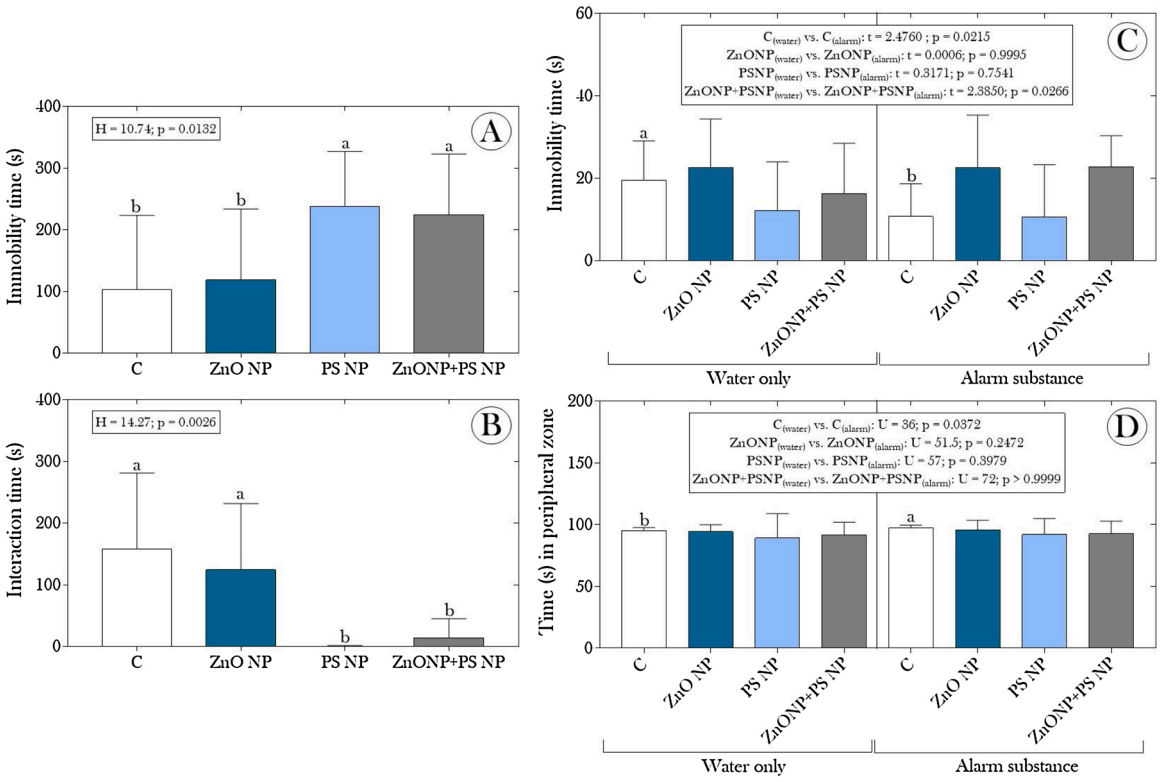

Fig. 2. A) Immobility time and (B) interaction of *Ctenopharyngodon idella* juveniles exposed to different treatments with their images in the mirror test. (C) Immobility time and (D) permanence in the peripheral zone of the apparatus where the alarm substance-response test was performed. Bars represent the mean + standard deviation. Data in "C" and "D" were analyzed through paired test because fish subjected to the "water" and "alarm" sessions were not independent. Student's *t*-test was used to compare parametric data and Mann-Whitney *U* test was used to compare non-parametric data (both at 5% probability level). Statistical summary is shown at the top of the graphs. Different lowercase letters indicate differences between experimental groups. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). Concentrations of both nanomaterials: 760 μg/L, each.

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. Results were expressed in U/mg of protein [determined based on the method by Lowry et al. (1951)].

2.3.3.8. Acetylcholinesterase (AChE) activity. Acetylcholinesterase (AChE) enzyme activity was also determined in microplate, based on Ellman's spectrophotometric method (Ellman et al., 1961), by using iodized acetylcholine substrate and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). Fifty (50) μL of each sample were added (in sequence) to each microplate well, as well as 100 μL of iodinated acetylcholine substrate solution (750 μg/mL) and 100 μL of DTNB solution (130 μg/mL). Next, absorbance was measured 30 s after reagents' homogenization and 3 min after the first reading in microplate reader, at 405 nm(Heales, model MB-580). Results were expressed in "μmol/min/mL".

2.3.3.9. Nutritional status assessment. Animals' nutritional status was assessed based on the assumption that the exposure to nanomaterials could affect their energy metabolism. Total proteins and carbohydrates, as well as triglycerides and total cholesterol levels were evaluated in liver and brain samples. Total tissue proteins were calculated based on the method by Lowry et al. (1951). The standard curve was built based on standard solution of total proteins (Quimiprot kit, Ebram Prods. Laboratoriais Ltda®, São Paulo, Brazil), whereas the equation of the *y* line ($y = 2.0057x + 0.0955$; $R^2 = 0.9834$) was used to determine the total protein concentration in supernatant fraction of tissue homogenate [(mg/mL)/g tissue]. The Folch method [using chloroform: methanol (2: 1 v/v)] (Folch et al., 1957) was used to calculate total lipids; this method has been widely used to extract lipids from animal, plant and algae tissues (Sheng et al., 2011). Total carbohydrate concentration was evaluated based on the methodology suggested by Dubois et al. (1956); anhydrous glucose solutions (Equiplex Indústria Farmacêutica Ltda, Aparecida de Goiânia, Brazil) were used to determine the standard curve and to find the straight equation, as seen in studies by Nielsen (2010) and Jain et al. (2017).

2.4. ZnO NPs quantification

Zinc (Zn) was quantified through direct colorimetric assay by using nitro-PAPS and microwell plates, based on Makino (1991), with some modifications. Brain fragments (30 mg) were initially collected and weighed; they were macerated and digested in screw-cap sample beaker filled with 2 mL of wet-digest solution (75 % nitric acid (HNO_3): 70 % perchloric acid ($HClO_4$) = 1: 1 v/v). Samples were placed on hot plate at 100 °C for 2 h. After sample digest was clear, the cap of the beaker was removed and the heating process continued at 80 °C until the sample was totally dry. Five percent (5%) of HNO_3 was added to dissolve the sample digest residue; thus, the final volume of it dropped to 5 mL.

Next, 5 μL of each digested and standard sample (zinc nitrate solution, 200 μg/dL) was mixed to 80 μL of buffer solution (borate buffer pH 8.2; salicylaldoxime and dimethylglyoxime) in ELISA microplate and incubated at 37 °C for 5 min. Subsequently, 40 μL of nitro-PAPS solution (2- (5-Nitro-2-pyridylazo) -5-(N-propyl-*N*-sulfopropylamino) phenol) were added to each microplate well and incubated again at 37 °C for 5 min; this procedure was followed by reading in ELISA reader, at 492 nm. Nitro-PAPS solution (0.6 mmol/L) was prepared by dissolving 30.2 mg of nitro-PAPS (mol wt: 503.44, from Wako) in 90 mL of water. Solution

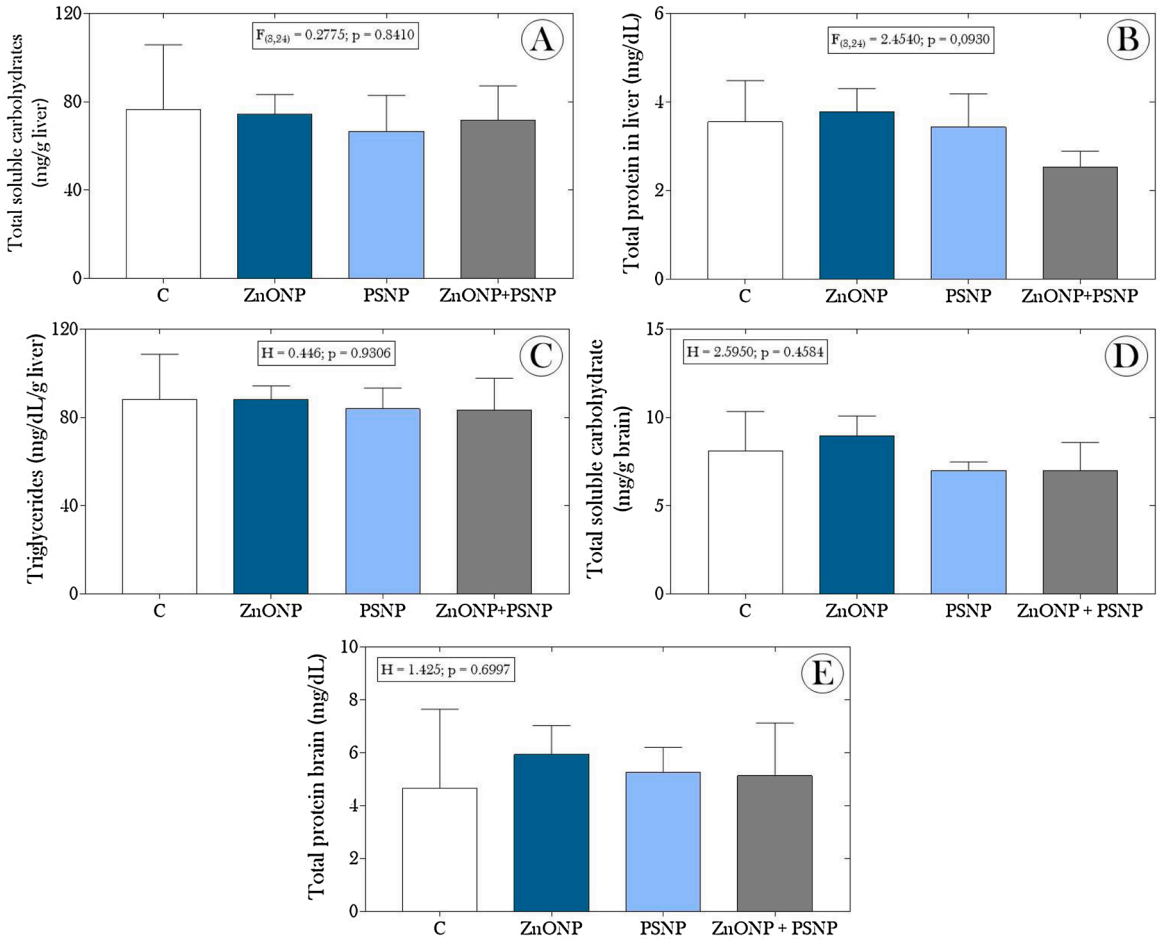

Fig. 3. (A) Total soluble carbohydrates, (B) total proteins and (C) triglycerides in the liver; and (D—E) total soluble carbohydrates and total proteins in the brain (respectively) of *Ctenopharyngodon idella* juveniles exposed to different treatments. Bars indicate mean + standard deviation. Parametric data were subjected to one-way ANOVA and the non-parametric ones were subjected to Kruskal-Wallis test, and to Tukey and Dunn's post-tests, both at 5% probability level. Statistical summary is shown at the top of each figure. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). Concentrations of both nanomaterials: 760 μg/L, each.

pH was adjusted to 8.1 by adding 1 mol/L of NaOH or 1 mol/L of HCl to it and by diluting it with 100 mL of water. Zinc and nitro-PAPS formed a colored complex, which was proportional to the amount of zinc in the sample - it was determined based on the following equation:

$$\text{Zinc } (\mu g \Big/ dL) = \frac{\text{Sample Absorbance}}{\text{Standard absorbance}} \; x\; 200 \qquad (1)$$

wherein "200″ refers to the final concentration of the standard (in μg/dL). Results were converted into "μg/g of tissue", by taking into consideration the biomass of the analyzed organs.

2.5. PS NPs quantification

PS NPs accumulation was estimated based on a methodology similar to the one adopted by Katzenberger and Thorpe (2015), Boyero et al. (2020) and Sarasamma et al. (2020), with some modifications. The adopted methodology consisted in collecting animals' brains, weighing them on analytical scale and macerating them in PBS by using conical bottom microtubes. Next, 100 μL of homogenized sample were added to ELISA microplate wells (in triplicate), mixed to 100 μL of purified water and read in microplate reader (Heales, model MB-580) at 450 nm. Standard sample absorbance was generated based on fluorescence PS NPs suspension (0.13 μg/μL). The background luminescence of tissues collected from fish who were not exposed to PS NPs was detected and subtracted from that of PS-NPs-exposed samples. The estimated PS NPs concentrations were expressed in μg/mg tissue.

2.6. Data analysis

Initially the normalcy of all data obtained was tested using the Shapiro-Wilk, Anderson-Darling and Kolmogorov-Smirnov tests. The data were compared through one-way ANOVA (parametric data) or through the Kruskal-Wallis test (non-parametric data), also at 5% probability. On the other hand, data of the alarm substance-response test were analysed as paired test, because the fishes in the first and second sessions were the same. In these cases, the Student's t-test was used to compare the parametric data and the Mann-Whitney test was applied to the non-parametric data. The videos were analysed using PlusMZ and all analyses and graphic elaborations were performed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA).

Fig. 2.

3. Results

Treatments neither influenced the locomotion of test animals in the open field test, nor induced anxiety-like behavior in them. Swimming speed, covered distance and anxiety index did not differ between treatments (Fig. 2S-A-C, see "Supplementary material"). In addition, there were no differences in latency to go to the top, immobility time and time spent at the top of the novel tank between groups (Fig. 2S-D-F, see

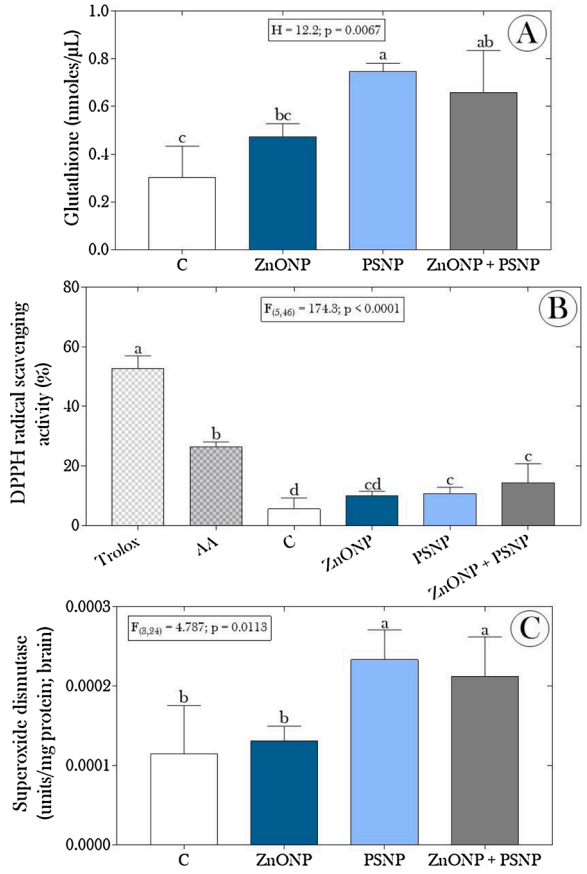

Fig. 4. Total glutathione (A), DPPH radical scavenging activity (B) and superoxide dismutase enzyme (C) activity in the brain of *s Ctenopharyngodon idella* juveniles exposed to different treatments. Bars indicate mean + standard deviation. Parametric data were subjected to one-way ANOVA and the non-parametric ones were subjected to Kruskal-Wallis test, and to Tukey and Dunn's post-tests, both at 5% probability level. Statistical summary is shown at the top of each figure. Different lowercase letters indicate differences between experimental groups. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). AA: Ascorbic acid. Concentrations of both nanomaterials: 760 µg/L, each.

"Supplementary material"). This outcome reinforced the perception that treatments did not cause anxiety- or anxiogenic-like behavior in the assessed animals.

On the other hand, although any animal in the experimental groups presented aggressive behavior against the mirror (mirror test), fish in the "PS NP" and "ZnO NP + PS NP" groups recorded longer immobility time and shorter interaction with their images in the mirror (Fig. 2A-B, respectively). In addition, treatments (in separate or in combination) affected animals' response to alarm substances in the tissue extract. Based on Fig. 2C, it is possible seeing that only animals in the control group changed their locomotor behavior in the second session of the alarm solution-response test, which indicated that their reaction may have been motivated by the perception of substances added to the test apparatus. Moreover, only non-exposed animals (unlike the other groups) presented significant thigmotaxis behavior in the presence of alarm solution, as inferred based on the longer time they spent in the peripheral zone of the apparatus (Fig. 2D).

Biochemical data have indicated that treatments did not change nutritional parameters evaluated in the liver (total carbohydrates and proteins, and triglycerides, Fig. 3A–C) and brain (total carbohydrates and proteins, Fig. 3D-E) of the investigated animals. Therefore, this outcome suggests that the previously reported behavioral changes are not associated with likely impacts of treatments on animals' energy metabolism.

On the other hand, PS NPs, either used in separate or in association with ZnO NPs, have stimulated antioxidant activity in the brain of *C. idella* individuals, as inferred through the significant increase in total glutathione concentrations, SOD activity and DPPH radical scavenging activity (Fig. 4A –C, respectively). In addition, these nanoparticles led to increased thiobarbituric acid reactive substances and hydrogen peroxide production in the brains of these very same animals, although they did not have effect on nitric oxide production (Fig. 5A–C, respectively). Furthermore, PS NPs, either used in separate or in combination with ZnO NPs, have induced significant increase in AChE activity (Fig. 5D).

The single-cell gel electrophoresis assay (comet assay) has shown equitable effects between treatments. The exposure of animals to ZnO NPs and PS NPs, be them in separate or in binary combination of both of them, has induced significant changes in the DNA of the evaluated erythrocytes, as inferred through the increased TL, %DNA and OTM (Fig. 6). Current data have shown increased Zn concentration in the brain of animals exposed to ZnO NPs (in separate) and to the ZnO NPs/PS NPs combination (ZnONP + PSNP group), which suggests the accumulation of this nanomaterial in the evaluated organs (Fig. 7A). PS NPs recorded equivalent concentrations in the brain of animals exposed to the pollutant (in separate) and to the PS NPs/ZnO NPs combination (Fig. 7B).

4. Discussion

It is essential understanding how pollutants affect aquatic biota before making decisions about it, as well as proposing and implementing, mitigation measures (Wathern, 2013; Mareddy et al., 2017). Thus, data presented in the current study have confirmed the behavioral, biochemical and genotoxic toxicity of the investigated pollutants, as well as that the combined exposure to ZnO NPs and PS NPs induced different effects from those observed in animals exposed to these pollutants, in separate. There was clear association between behavioral changes in animals exposed to treatments and likely imbalance in, or inefficiency of, redox reactions; neurotoxic effect was inferred through increased AChE activity.

Based on the mirror test, the greater immobility and reduced interaction with the mirror recorded for *C. idella* individuals exposed to PS NPs (in separate), and to their combination with ZnO NPs, may be associated with impairments in neuronal mechanisms responsible for regulating animals' recognition/interaction processes, likely due to changes linked to oxidative stress. According to Gerlai et al. (2000), this interaction plays an important role in establishing dominance hierarchies between individuals, as well as in the formation of shoals to minimize the risk of predation (Kalueff et al., 2016). The herein evaluated increased antioxidant levels support the hypothesis that the investigated pollutants induced oxidative stress, which may have affected the display of this important behavioral category. Glutathione is the most abundant thiol compound in cells of all organs and it plays an important protective role against oxidative stress in the brain (Cheng et al., 2017; Adeoye et al., 2018). Overall, H_2O_2 is inactivated by catalase or by glutathione peroxidase in a reaction where GSH is used as co-substrate (Schulz et al., 2000); this process explains the increased GSH levels and decreased hydrogen peroxide production in the brains of animals belonging to groups "PS NP" and "ZnO NP + PS NP" (Fig. 5B). The SOD activity observed in animals exposed to PS NPs (in separate), and to their combination with ZnO NPs, suggests increased superoxide radical ($O2^{\bullet-}$) scavenging activity and its subsequent conversion into hydrogen peroxide, which appears to have been catalyzed by GSH.

On the other hand, increased GSH levels did not seem to have been enough to interrupt the chain breaking associated with lipid peroxidation processes observed in animals' brains (Fig. 5A). Thus, it is tempting speculating that it may have happened due to excessive increase in lipid peroxidation processes, or to direct or indirect interference of pollutants

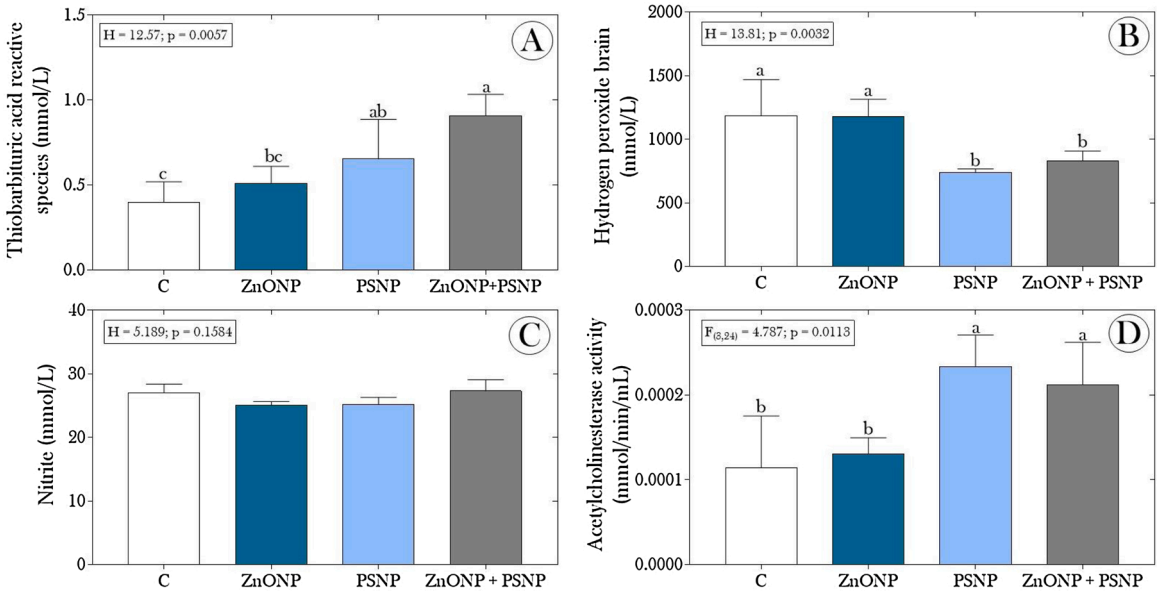

Fig. 5. Thiobarbituric acid reactive substances (A), hydrogen peroxide (B) and nitrite levels (C) in the brain of *Ctenopharyngodon idella* juveniles exposed to different treatments. Bars indicate mean + standard deviation. Parametric data were subjected to one-way ANOVA and the non-parametric ones were subjected to Kruskal-Wallis test, and to Tukey and Dunn's post-tests, both at 5% probability level. Statistical summary is shown at the top of each figure. Different lowercase letters indicate differences between experimental groups. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). Concentrations of both nanomaterials: 760 μg/L, each.

in biochemical reactions in GSH antioxidant action, which comprise peroxide elimination, vitamin E maintenance - in its reduced and functional form - and, finally, detoxification of reactive aldehydes generated during lipid peroxidation (Adeoye et al., 2018). Nevertheless, results recorded for DPPH radical scavenging activity have shown increased number of antioxidant compounds in samples collected from animals in the "PS NP" and "ZnO NP + PS NP" groups to capture DPPH radicals, differently from what was observed in the other experimental groups ("Control" and "ZnO NP") (Fig. 4B).

In physiological terms, the hypoactivity of animals exposed to PS NPs, both in separate and in combination with ZnO NPs - which was associated with lesser animal interaction with the mirror - may be suggestive of flaw in the interpretation of specific signals that mediate the recognition of the reflected image as co-specific individuals. Unlike the expectations, the image reflected in the mirror appears to have induced decreased locomotor activity, which has been used as anxiety behavior marker in fish (Aponte and Petrunich-Rutherford, 2019). One explanation for this behavioral response may be associated with likely direct or indirect effects of oxidative stress on animals' vision, which is a dominant sensory system used for social recognition purposes. Fernald and Hirata (1979) have already shown that brightly colored spatial and chromatic patterns used by *Haplochromis burtoni* for social signaling - in their natural habitat - are compatible to chromatic transmission properties of the water they live in, a fact that reinforces the role played by the visual system in animals' social recognition. In fact, previous studies have already shown the impact of exposure to MPs, PS NPs and nanoparticles on the optical physiology of different fish species, such as *D. rerio* exposed to AuNPs with N, N, N-trimethylammonium ethanethiol ligand (TMAT-AuNPs) (Kim et al., 2013), *Oryzias latipes* exposed to Ag NPs (Wu et al., 2010) and *D. rerio* larvae exposed to nano- and microplastics (Chen et al., 2017; Zhang et al., 2020). However, oxidative stress observed in fish exposed to PS NPs, either in separate or in combination with ZnO NPs, as well as the increased AChE activity in animals' brains (Fig. 5D) may explain their visual impairment, as recently highlighted by Barboza et al. (2020).

Although this increase, in particular, differs from those in previous studies, which reported inhibition of AChE activity induced by MPs and NPs (Oliveira et al., 2013; de Sá et al., 2015; Ferreira et al., 2016), it is likely that lipid oxidative stress (inferred in the present study through increased TBARS level - Fig. 5A) has caused the rupture of vesicle membranes containing acetylcholine in the presynaptic neurons of *C. idella* individuals exposed to PS NPs, either in separate or in combination with ZnO NPs. This process resulted in increased neurotransmitter release in cholinergic synaptic clefts and in over-stimulation of postsynaptic receptors [see details about the molecular and cellular biology of cholinesterases in Massoulié et al. (1993)]. In addition, it is likely that AChE production was induced to deal with oxidative stress and with the damage caused by these pollutants, since damaged cells and tissues are associated with greater amount of acetylcholine than healthy ones (Gambardella et al., 2017). Barboza et al. (2020) reported increased AChE activity and lipid peroxidation (measured through TBARS) in the brain of *Dicentrarchus labrax*, *Trachurus trachurus* and *Scomber colias* individuals presenting MPs in the intestine, a fact that reinforces the current hypothesis. In addition, it is not possible ruling out the hypothesis that the interaction between pollutants and acetylcholine receptors leads to increased AChE synthesis in order to decompose the highest neurotransmitter levels, as acute response. The hypothesis that increased AChE activity in these animals was associated with positive regulation of the AChE gene, due to the inhibitory effect of pollutants; it should be tested in future studies, although the current data have evidenced DNA damage caused by pollutants (both in separate and in combination with each other - Fig. 6).

Based on the alarm substance-response test, it is likely that the evaluated pollutants (ZnO NPs and PS NPs, either in separate or in combination each other) have caused effects that go beyond the social recognition failures suggested in the mirror test. As shown in Fig. 3C, animals in the control group were the only ones reacting to the presence of alarm substances, since they remained longer in the peripheral zone of the apparatus (Fig. 3D). Reduced immobility (i.e.: increased locomotor activity) of animals in the control group can be interpreted as defensive response (Mirza and Chivers, 2003; McIntyre et al., 2012), whereas the longer time they stayed in the periphery of the apparatus indicated typical thigmotaxis behavior, which has been associated by several studies with anxiety-like behavior (Richendrfer et al., 2012). In this case, it is plausible assuming that the investigated pollutants have affected the mechanisms regulating the detection and/or processing of

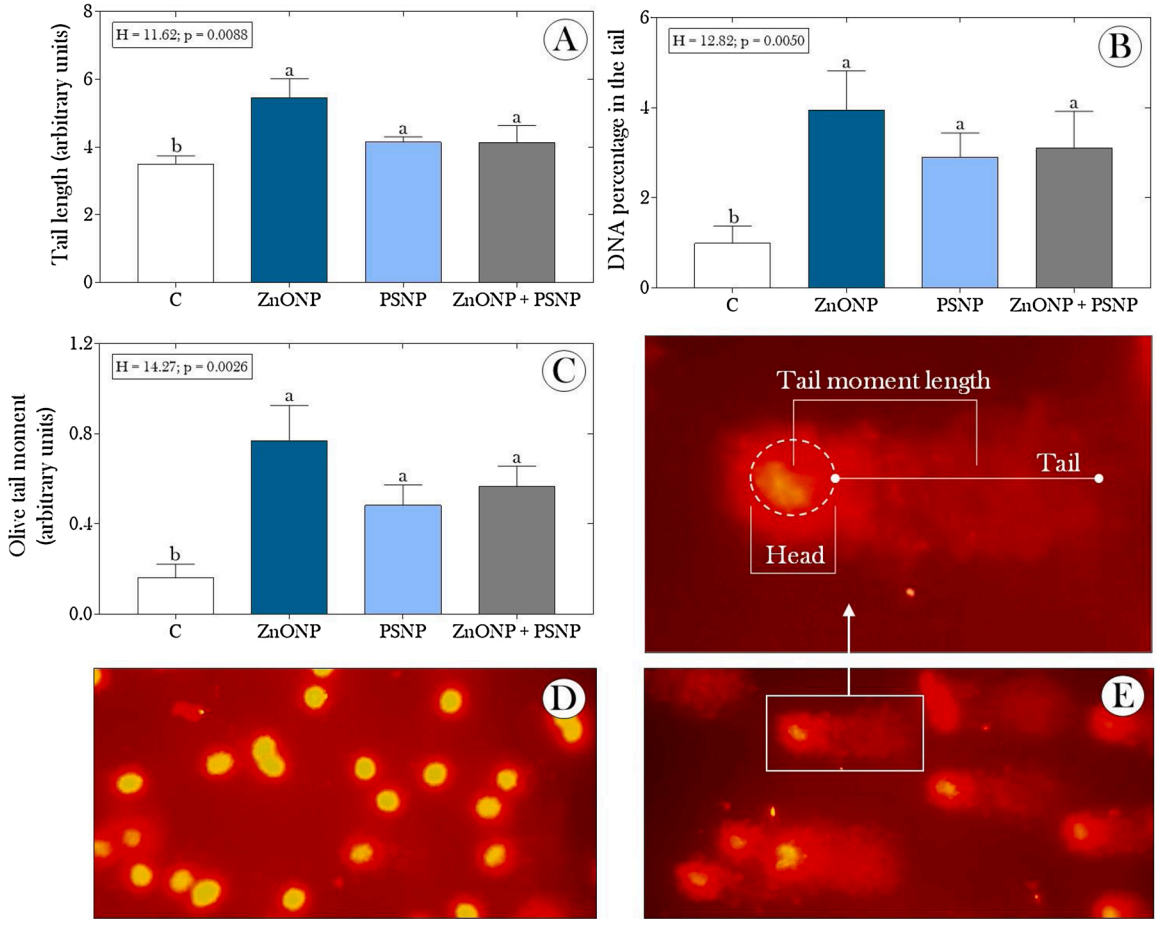

Fig. 6. (A) Tail length (B) DNA percentage in the tail, (C) Olive tail moment nitrite and (D) photomicrographs representing *Ctenopharyngodon idella* juveniles in (D) the control and (E) pollutant-exposure groups subjected to comet assay. Bars indicate mean + standard deviation. Data were subjected to Kruskal-Wallis test, and to Tukey and Dunn's post-tests, at 5% probability level. Statistical summary is shown at the top of each figure. Different lowercase letters indicate differences between experimental groups. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). Concentrations of both nanomaterials: 760 μg/L, each.

chemical signals stimulated by the alarm substances introduced in the apparatus in different ways. For example, pollutants may have precipitated or permanently complexed in the mucous environment of animals' nasal cavities and affected water flow to olfactory sensory cells, thus impairing the capture of stimuli triggered by alarm substances. The authors of a study about the effects of Cu NPs on *Oncorhynchus mykiss* juveniles' response to alarm substances have shown that animals' responsive deficit remained even after they were transferred to pollutant-free water. This deficit was associated with damage in animals' olfactory epithelium (Sovavá et al., 2014), a fact that corroborates the current hypothesis. However, this interference (whether it is permanent, or not) appears to depend on pollutant type, as suggested by Bilberg et al. (2011), who showed that olfactory rosette irrigation promoted by contaminant-free water has immediately restored the electro-olfactory response of *Carassius carassius* individuals previously exposed to Ag NPs. Therefore, future investigations may help better understanding the mechanisms involved in these effects.

However, one cannot neglect the hypothesis that ZnO NPs and PS NPs have affected other brain centers, although we have not assessed the impact on animals' olfactory epithelium. This outcome is corroborated by Mirza et al. (2009), who investigated *Perca flavescens* individuals living in native ponds contaminated with metals in Canada. The aforementioned authors reported intact and electrophysiological olfactory epithelium, without significant loss of olfactory sensory neurons, which was not enough for animals to show responsive behavior to the tested alarm substances. Therefore, the oxidative stress induced by the treatments may have led to changes in neural circuits responsible for regulating olfactory response, since the antioxidant mechanisms necessary to maintain animals' olfactory function were not enough to compensate the damage caused by ZnO NPs and PS NPs (either in separate or in combination with each other). This hypothesis is also corroborated by Razmara et al. (2019), who suggested that the olfactory antioxidant activity of *Oncorhynchus mykiss* individuals exposed to Cu NPs was not enough to prevent damages caused by nanoparticles in animals' olfactory response.

With respect to likely genotoxic effects, pollutants had equitoxic effects on the DNA of the erythrocytes of the evaluated animals. Increased TL, %DNA and OTM levels in animals exposed to ZnO NPs and PS NPs (either in separate or in combination with each other) is consistent with levels reported by other studies that have associated the increase in these parameters with damage to DNA integrity (Gopinath et al., 2019; Hong et al., 2020; Shah et al., 2020). These results may be related to the oxidative product accumulation caused by the treatments, which have the potential to attack DNA and lead to clastogenic and molecular damages. Although similar observations have already been reported in the literature, both in fish exposed to ZnO NPs (Boran and Ulutas, 2016; Shahzad et al., 2019) and to PS NPs (Sökmen et al., 2020), the present study did not evidence synergistic, antagonistic or additive action of pollutant combination. Therefore, the likely DNA damage observed in the current study derived from single DNA strand breaks, double DNA strand breaks, DNA adduct formation and from DNA-DNA and DNA-protein

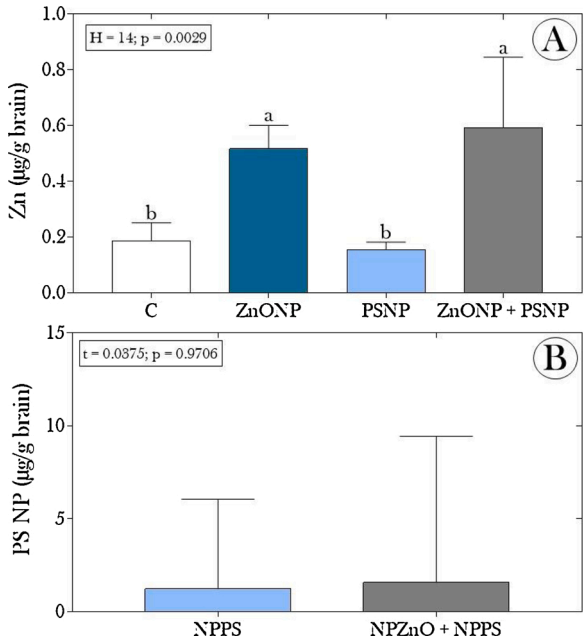

Fig. 7. Estimated Zn (A) and PS NPs concentrations in the brain of *Ctenopharyngodon idella* juveniles exposed to different treatments. Bars indicate mean + standard deviation. In "A", data were subjected to Kruskal-Wallis test, and to Tukey and Dunn's post-tests, at 5% probability level. In "B", Student's *t*-test was used to compare parametric data. Statistical summary is shown at the top of each figure. Different lowercase letters indicate differences between experimental groups. C: control group; ZnO NP: group exposed to zinc oxide nanoparticles; PS NP: group exposed to polystyrene nanoplastics; and ZnO NP + PS NP: group exposed to both nanomaterials. (n = 16 animals/group). Concentrations of both nanomaterials: 760 µg/L, each.

cross-linking that has resulted from the pollutants (or their metabolites)/DNA interaction. All these mechanisms are consistent with the damage often reported in the comet assays applied to different model systems (Hackenberg et al., 2011; Khan et al., 2015; Brandts et al., 2018; Poma et al., 2019; Yong et al., 2020; Cortés et al., 2020). An alternative lies on assuming that the investigated particles have interfered in several DNA damage repair mechanisms. Accordingly, proteolytic enzymes, such as Proteasome and mitochondrial Lon protease, can recognize and degrade oxidatively-damaged polypeptides and remove them from cells before they aggregate and cross-link (Bota and Davies, 2002; Cadet and Davies, 2017), whereas lipolytic enzymes, such as phospholipase A2, can selectively recognize themselves and remove oxidized phospholipids from biological membranes (van Kuijk et al., 1987). In addition, heavily oxidized proteins and lipids (often in cross-linked mixed-form aggregates) that have escaped the proteolytic and lipolytic enzymes can be engulfed by phagocytic cells and degraded through the process called autophagy.

Finally, it is necessary acknowledging that the current study did not exhaust the topic; therefore, it presents some limitations that can be used as starting point in future investigations. For example, it is worth investigating whether the effects observed in the current study are gender-specific or species-dependent, whether they change in older animals or whether the behavioral responses of adult animals are similar to those observed in the herein performed tests. In addition, can longer exposure times (via direct exposure or via the food chain) lead to more severe effects? Obviously, these are broad questions yet to be answered. Therefore, further research should be carried out in order to feature and evaluate the impact of the combination of different nanomaterials on fish health, which is certainly important to help better understanding the real magnitude of their impact on aquatic ecosystems.

5. Conclusion

The current study has shown that the toxicity of ZnO NPs and PS NPs in *C. idella* juveniles was biomarker-dependent. On the one hand, ZnO NPs were harmless to the behavioral response of fish in the mirror test, but all pollutants have equally affected animals' response to alarm substances and had genotoxic effect on the assessed erythrocytes. This outcome was associated with the neurotoxicity of PS NPs, oxidative stress induction, as well as with the likely interferences in DNA integrity. Therefore, the animal model subjected to the herein adopted experimental conditions did not show synergistic, antagonistic or additive effects deriving from their exposure to the ZnO NPs/PS NPs combination.

Ethical approval

All procedures experimentais foram realizados em conformidade com as orientações éticas relacionadas à experimentação animal. Meticulous efforts were made to assure that animals suffered the least possible and to reduce external sources of stress, pain and discomfort. The current study did not exceed the number of animals necessary to produce trustworthy scientific data. This article does not contain any studies with human participants performed by any of the authors.

Author's contribution

Fernanda Neves Estrela: Research Conception/Design, Data Acquisition, Data Analysis/Interpretation, Manuscript Preparation, Final Approval.

Abraão Tiago Batista Guimarães: Research Conception/Design, Data Acquisition, Final Approval.

Fabiano Guimarães Silva: Research Conception/Design, Manuscript Preparation, Final Approval.

Thiarlen Marinho da Luz: Data Acquisition, Final Approval.

Abner Marcelino Silva: Data Acquisition, Final Approval.

Paulo Sergio Pereira: Manuscript Preparation, Final Approval.

Guilherme Malafaia: Research Conception/Design, Data Acquisition, Data Analysis/Interpretation, Manuscript Preparation, Final Approval.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.123879.

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Toxicity of polystyrene nanoplastics and zinc oxide to mice

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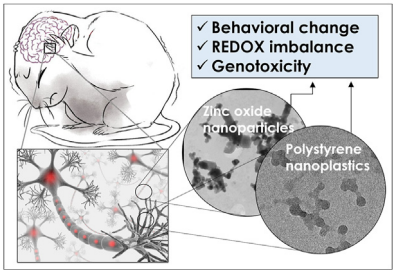
HIGHLIGHTS

- ZnO NPs and PS NaPs do not induce locomotor changes or anxiety-like behavior in mice.
- Exposure to ZnO NPs and PS NaPs, in separate, induces cognitive impairment during the object recognition test.
- Mice exposed to nanomaterials, in separate, show REDOX imbalance.
- ZnO NPs and PS NaPs, in separate, suppresse the AChE activity.
- DNA damage has been observed in mice exposed to nanomaterials (in separate, or in combination).

GRAPHICAL ABSTRACT



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ABSTRACT

The toxicity of zinc oxide (ZnO NPs) and polystyrene nanoplastics (PS NaPs) has been tested in different animal models; however, knowledge about their impact on mice remains incipient. The aim of the current study is to evaluate the effects of these nanomaterials on Swiss mice after their individual exposure to a binary combination of them. The goal was to investigate whether short exposure (three days) to an environmentally relevant dose (14.6 ng/kg, i.p.) of these pollutants would have neurotoxic, biochemical and genotoxic effects on the modelss. Data in the current study have shown that the individual exposure of these animals has led to cognitive impairment based on the object recognition test, although the exposure experiment did not cause locomotor and anxiogenic or anxiolitic-like behavioral changes in them. This outcome was associated with increased nitric oxide levels, thiobarbituric acid reactive species, reduction in acetylcholinesterase activity and with the accumulation of nanomaterials in their brains. Results recorded for the assessed parameters did not differ between the control group and the groups exposed to the binary combination of pollutants. However, both the individual and the combined exposures caused erythrocyte DNA damages associated with hypercholesterolemic and hypertriglyceridemic conditions due to the presence of nanomaterials. Based on the results, the toxicological potential of ZnO NPs and PS NaPs in the models was confirmed and it encouraged further in-

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depth investigations about factors explaining the lack of additive or synergistic effect caused by the combined exposure to the assessed pollutants.

1. Introduction

Nowadays, technological advances account for the development of materials adopted by different sectors and nanoparticles (NPs) stand out among them. These NPs bring several benefits, including their addition to the formulation of new medicines, allow higher-quality medical services and are used in encapsulating pesticides for agricultural enhancement, as well as reduce the toxicological effects of different chemicals (Khan et al., 2019). They work as compounds in several products, with emphasis on sunscreens (titanium dioxide/zinc oxide nanoparticles), sports goods (carbon nanotubes, graphene, among others), car parts (nanotubes, cellulose nanofibers, among others) and high-definition TV sets (quantum dots) (Chaurasia, 2017); therefore, their features can be adjusted to different market demands (Wright, 2016; Chaurasia, 2017).

However, their growing production and application raise concerns about their (eco)toxicological risks (Scheringer, 2008; Ray et al., 2009; Keller et al., 2017). NPs' small size, wider surface area and agglomeration/dispersion ability contribute to their translocation into environmental/biological compartments. Such a process can damage individuals and affect the dynamics of their populations due to nanoparticle accumulation (Montano et al., 2014; Lead et al., 2018; Khan et al., 2019). Studies with zinc oxide nanoparticles (ZnO NPs) provide strong evidence of their toxicological potential in different terrestrial Falfushynska et al. (2017) (*Pelophylax ridibundus*); Mesak et al. (2018) (*Gallus gallus domesticus*); Khoobbakht et al. (2018) (*Coturnix Coturnix japonica*); Vieira et al. (2019) (*G. gallus domesticus*); Goma et al., 2021 (*Mus musculus*); Luz et al. (2020) (*M. musculus*) and aquatic organisms (Bhuvaneshwari et al., 2018; De-Campos et al., 2019; Munir et al., 2020); therefore, given their high toxicity, their use as raw material in different manufactures must be based on their ecological risks to natural environments (Keerthana & Kumar, 2020).

Nanomaterials derive from the degradation of plastics discharged in the environment. Nanoplastics (NaPs) have been the topic of several studies focused on their risk as signficant pollution *input* to aquatic ecosystems (Mattsson et al., 2018; Anbumani and Kakkar, 2018; Sullivan et al., 2020), mainly to the marine environment (Rech et al., 2014). According to previous studies, NaPs affect organisms, mainly at cell level, as well as impaire species' population dynamics (Kögel et al., 2020; Yong et al., 2020; Jiang et al., 2020; Barría et al., 2020).

Studies with NPs and NaPs broaden the knowledge about nanomaterials' impacts on the biota, although their tested pollutant concentrations are often higher than the ones observed in natural environments. The effects of pollutants used in the aforementioned studies were overstated, a fact that makes it difficult conducting more accurate analysis about their real impact on ecosystems. The likely toxic effects of exposing these animals to the herein assessed nanomaterials is another concerning gap in the literature (Estrela et al., 2021), since these elements can coexist in different environments. Based on De-Souza et al. (2018a,b), the combination of several xenobiotics in a given environment can reinforce their biological effects. Although previous studies have assessed the toxicity of nanomaterial/pollutant combination [e.g.: ZnO NP (Ye et al., 2017; Chen et al., 2020) and PS NaP (Lee et al., 2019; Liu et al., 2019)], still, the literature does not address its impact at nanoscale.

Grass carp (*Ctenopharyngodon idella)* juveniles exposed to ZnO NPs and PS NaPs have recently shown to be biomarker-dependent. ZnO NPs were harmless to fish behavioral response in the mirror test; however, the binary combination of pollutants has affected their anti-predatory defensive response, since the models presented erythrocyte genotoxic effect caused by them. These changes were associated with the neurotoxicity of these nanomaterials, with increased oxidative stress reactions and with pollutant accumulation in animals' brains. Except for the aforementioned study, no other study has assessed the effects of metallic NP and NaP co-exposure. A previous study conducted by our research team rose questions about the effects of exposing a terrestrial organism to a combination of pollutants; assumingly, the tested terrestrial organism contact with the pollutants was much lower than that observed in exclusively aquatic organisms.

Accordingly, the aim of the current study was to identify and understand whether the coexistence of these nanomaterials in freshwater ecosystems can affect the health of terrestrial organisms. Male Swiss mice were used as experimental model, since they have been used in several environmental toxicology studies (Guimarães et al., 2019; Su et al., 2019; Luz et al., 2020; Bano and Mohanty, 2020). The exposure (even in the short-term) of mice to isolated or combined nanomaterials was based on the assumption that such pollutants would accumulate in them and induce behavioral, biochemical and genotoxic changes due to synergistic or additive effects. To the best of our knowledge, the present study is the first to report the effects of exposing mice to a ZnO NPs/PS NaPs combination.

2. Material and methods

2.1. Nanomaterials

Yellow-green fluorescente polystyrene nanoplastic (PS NaP) was used in the study ($\lambda_{ex} = 470$ nm and $\lambda_{em} = 505$ nm (Sigma Aldrich, USA. Produto n. L5155); to represent the most produced nanoplastic types worldwide. This nanoplastic is used to produce food storage, wrapping and disposable cutlery goods, and to industrial packaging; therefore, it has been commonly found in aquatic environments due to the inappropriate discharge of such goods (Eriksen et al., 2014a,b). These NaPs are round-shaped and measure 23.03 ± 0.266 nm in diameter [mean ± standard error of the mean (SEM)]. Their full chemical featuring was shown in previous study conducted by our research group (Guimarães et al., 2020). However, the possible effect of fluorescent dye on the toxicity of PS NaP was not evaluated in the current study.

ZnO NPs (purity > 99.99%) used in this study were purchased at Sigma Aldrich, Saint Louis, MO, USA; CAS number 544906. They were nanocrystals without any surface modification (mean diameter of 68.96 nm ± 2.519, mean ± SEM). Their chemical featuring, the individual diameter distribution of each ZnO nanoparticle, X-ray diffraction patterns of the crystal quality of ZnO nanoparticles and the Fourier-transform infrared transmission spectra can be observed in previous studies carried out by our research team (De-Souza et al., 2018a,b).

2.2. Animals and experimental design

Initially, 48 male Swiss mice (32.1 ± 0.8 g; 4–6 weeks of life) were obtained from matrices at the bioterium of Laboratory of Biological Research (Goiano Federal Institute - Urutaí Campus). Male mice were chosen because female mice present hormonal variations during the estrous cycle, and it could influence their responses to behavioral tests. The models were kept in ventilated shelves under controlled temperature (22–24 °C) and lighting (12 h light/dark cycle), at 58% ± 3% humidity. They had access to water and standard feed for rodents *ad libitum,* throughout the experiment, and were divided into four experimental groups after 30-day acclimation. Group division was based on their biomass, age and counterbalanced size (m = 12/each): (i) control group - mice who were not exposed to pollutants; (ii) ZnO NP group - mice who were exposed to ZnO NPs at predictive environmentally relevant dose (14.6 ng/kg); (iii) PS NaP group – mice who were exposed to PS NaPs at dose equivalent to that of the previous group and (iv) ZnO NP + PS NaPs group - animals who were exposed to the binary combination of nanomaterials. Models were exposed to the treatmemts for three days, with daily intraperitoneal administration of ZnO NPs and/or PS NaPs at dose equivalent to 14.6 ng/kg of body biomass diluted in purified water (via reverse osmosis). Each model was weighed on a daily basis, so the pollutant dose could be accurately adjusted to it. Animals in the control group were treated with pollutant-free purified water. Pollutants were administered via intraperitoneal route to ensure that equal doses would be delivered, similar to the method by De-Souza et al. (2018a,b), Vieira et al. (2019). Although the oral route would be closer to a realistic exposure condition, the provision of water added with ZnO NP and/or PS NaPs in drinking fountains would not necessarily result in equitable dosing, since water intake among individuals can vary and, consequently, each individual could intake different amounts of pollutant.

The study by Boxall et al. (2007) was the reference for the adopted doses. They used logarithms to estimate potential NP concentrations in surface waters, based on their use in cosmetics, hygiene products and paint formulations. Boxall et al. (2007) estimated ZnO NPs concentation of 760 μg/L - which is assumingly found in fresh surface water – based on a conservation scenario without NPs processing, deposition or removal from rivers. The same concentration range tested in different publications reviewed by Kögel et al. (2020) (0.8 μg/L to 2 g/L, or 1000–1,800,000 p/L and 0.0003% plastic in water) was adopted for the PS NaPs. These concentrations were close to the ones found in fresh water, mainly in points close to pollution sources. This profile took the current experimental design closer to real environmental conditions.

Animals were exposed to ZnO NPs or PS NaPs amounts similar to the ones they would intake on a daily basis. Water was exclusively captured from a source contaminated with these nanomaterials, at amounts based on estimates by De-Souza et al. (2018a,b), Mesak et al. (2018) and Vieira et al. (2019). Exposure dose calculation took into account models' biomass (32.1 ± 0.5 g) and mean daily water intake volume (±6 mL/day - measured days before the beginning of the experiment). Daily dose reached 14.6 ng/kg and it was applied at volume of 2 mL of purified water/30 g of body biomass.

2.3. Toxicity biomarkers

2.3.1. Behavioral assessment

Assumingly, short-term exposure to ZnO NPs and PS NaPs, either in separate or in combination, would account for neurobehavioral effects. Therefore, the models were subjected to "subsequent triple tests" (Fig. 1) 24 h after the last administration of pollutant dose. It was done to assess their locomotor activity, anxiety-like behavior, memory impairment and depressive-like behavior, based on Ramos (2008) and De-Souza et al. (2018a,b). All tests were performed in soundproof room under controlled temperature (22–24 °C), humidity (58 ± 3%) and lighting (100 lux) conditions. The behavior of the models was analyzed in Plus MZ v1 software. All models were allowed to acclimate to the test room for 30 min, prior to the tests (Fig. 1A).

2.3.1.1. Open field test. The open field test was performed based on procedures adopted by De-Souza et al. (2017) to assess the likely effect of the treatments on models' locomotor skills and anxiety-like behavior. The test consisted in placing the model in the center of a polyethylene box (size: 30 cm, in length x 20 cm, in width x 13 cm, in height) with opaque white walls. They were allowed to freely explore the environment for 5 min (Fig. 1B). The total distance traveled (cm) by the models at walking speed (cm/s) was recorded and the anxiety index was calculated based on the following equation, according to the study by Estrela et al. (2015).

$$\text{Anxiety Index: } \frac{Time\ (s) of\ animal's\ permanence\ in\ the\ central\ zone\ of\ the\ apparatus}{Total\ test\ time\ (360\ s)} \quad (1)$$

The assessmen of biomechanical attributes through the open field test was related to 'models' locomotion, since it could have been changed by the treatments. Their locomotor-function features were analyzed based on the scores defined by the Basso Mouse Scale for Locomotion (BMS) (Basso et al., 2006), similar to the evaluation carried out by Oliveira et al. (2017). Locomotion events included assessing forelimb and hindlimb coordination during sustained locomotion, trunk instability, paw orientation and tail position, among others. Locomotion rates recorded for the treatment group were simultaneously scored by two blind-observers.

2.3.1.2. Object recognition test. The object recognition test followed procedures adopted by Rabelo et al. (2016) and Guimarães et al. (2016); it was used to assess possible object recognition memory deficit induced by the treatments. The training session started right after the end of the open field test. It consisted in momentarily removing the model from the apparatus, introducing two identical objects (LEGO, plastic building-block toys - at the same size, shape and color of F1 and F2) in opposite positions and, taking the model back to the apparatus (Fig. 1C) - the model was allowed to operate in the environment for 5 min. Next, the model was once again removed from the apparatus and one of the objects was replaced by a new object (N) (cylindrical object presenting size, shape and color different from that of the previously used objects) (Fig. 1D). The model was taken back to the box and its exploratory behavior was video recorded for 3 min.

The model was placed in the center of the apparatus at the beginning of each session, its snouts had to face the wall opposite to the objects. Objects were sanitized with alcohol 70% (w/v) and dried in a fume cupboard between sessions. The time model spent

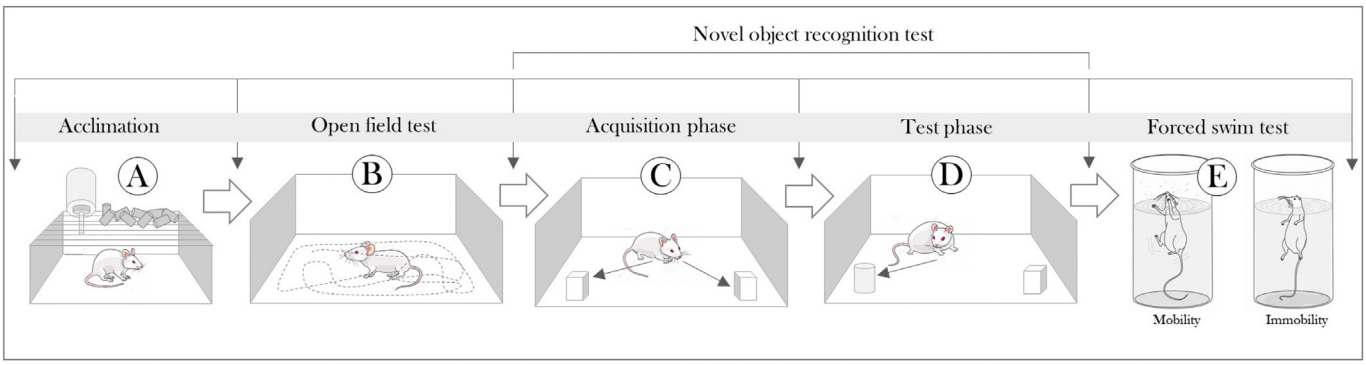

Fig. 1. Schematic design of the "triple test" model adopted in the present study. (A–F) Animals' acclimation to the testing room (30 min), (B) open field test, (C–D) object recognition test and (E) forced swimming test.

exploring each object was video recorded. Crossover design was used in all sessions, and new and familiar objects were placed in alternating positions to rule out the possibility of having interference from model's preference for a particular spatial location for the objects in the apparatus. Object exploration was only taken into consideration when the animal smelled and/or touched (intentionally) the objects and stood still up to 2 cm from the objects. Recognition rates recorded for both training sessions were determined after the video recording was over. Calculations were based on the equations by Rabelo et al. (2016) and Guimarães et al. (2016) (Eqs. (2) and (3)).

$$\text{Recognition index}(\%)\ (\text{training}): \quad \frac{\textit{Object exploration time}\ (n)}{\textit{Total exploration time of objects F1 and F2}} * 100 \tag{2}$$

$$\text{Recognition index}(\%)\ (\text{test}): \quad \frac{\textit{Time exploring the new object}}{\textit{Total time exploring objects F and N}} * 100 \tag{3}$$

wherein, "F (n)" is the time operating objects F1 or F2 (in session training) and "F" and "N" indicate the time operating familiar and new objects during "the test session", respectively.

2.3.2. Biochemical assessments

Models were euthanized (by decapitation) for blood collection and brain extraction after the tests. It was done to associate possible behavioral changes induced by treatments with biochemical changes. Serum was used to evaluate total protein, albumin, globulin, total cholesterol and triglycerides levels. However, brain samples were used to quantify the pollutants and to evaluate likely oxidative stress induction by treatments, as well as the interference of acetylcholinesterase (AChE) activity levels.

2.3.2.1. Serological assessments. The aliquot of 600 μL of blood was collected and immediately transferred to previously sterilized microtubes for blood sample preparation. Samples were centrifuged at 3000 rpm, for 10 min, at 4° C, and serum (supernatant) was transferred to other microtubes for further analysis. Total cholesterol and triglycerides levels were determined by using commercial kits (Biotécnica®, Varginha, MG, Brazil - Ref. 10.004.00 and 10.010.00, respectively). Although these parameters were considered sensitive nanomaterial-contamination biomarkers (Sulaiman et al., 2015; Alya et al., 2015; Lotfi et al., 2017), they were also useful to assess the possible effect of treatments on models' nutritional status.

2.3.2.2. Brain biochemical assessments. The collected brains were previously macerated in conical bottom microtubes filled with 1 mL of PBS for brain biochemical assessment. Samples were centrifuged at 13,000 rpm, for 5 min, at 4° C. Supernatants were transferred to other microtubes, for further analysis. They were kept on ice until their use.

2.3.2.2.1. Oxidative stress. The concentrations of nitric oxide and thiobarbituric acid reactive species (TBARS) were determined to assess possible oxidative stress induction by treatments. Griess colorimetric reaction was used to measure nitric oxide (Grisham et al., 1996). This reaction consisted in detecting nitrite (NO^{2-}) resulting from NO oxidation, based on Ajjuri and O'Donnell (2013) and Guimarães et al. (2020). Under normal conditions, nitric oxide (NO) is an important physiological signaling molecule in the central nervous system (Schmidt and Walter, 1994). However, NO also displays neurotoxicity when it is excessively produced (Wei et al., 2000).

TBARS concentrations were adopted as lipoperoxidation indicators (De-Azevedo-Gomes et al., 2003; Borges, 2020). They were determined based on Carvalho et al. (2018), with some modifications. Initially, 100 μL of supernatant (see item "2.3.2.2") was mixed to 50 μL of tricloacetic acid (TCA) (28% w/v in 0.25 N HCl), 50 μL of thiobarbituric acid (TBA, 1% in 0.25 N acetic acid) and 25 μL of butylhydroxytoluene (BHT, 5 mM). Then, samples were incubated in water bath at 95 °C, for 15 min and, later, centrifuged for 10 min, at 13,000 rpm, at room temperature. Supernatant was transferred to ELISA microplate (in triplicate) and read at 492 nm.

2.3.2.2.2. Neurotoxicity. The enzymatic activity of AChE was used as specific biomarker for the possible neurotoxic action of nanomaterials, as previously described in studies with ZnO NPs such as PS NaPs (Chen et al., 2017a; Lei et al., 2018; Sawicki et al., 2019; Guimarães et al., 2020). The Ellman's spectrophotometric method (Ellman et al., 1961) adapted for ELISA microplate analysis was used to determine AChE activity. Iodinated acetylcholine substrate and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were used for this purpose. In total, 50 μL of each sample, 100 μL of iodinated acetylcholine substrate solution (750 μg/mL) and 100 μL of DTNB solution (130 μg/mL) were sequentially added to each microplate well. Absorbance was recorded 30 s after reagent homogenization and 3 min after the first reading, at 405 nm, in microplate reader.

2.3.3. Genotoxic evaluation

If one takes into consideration that the same action mechanisms of nanomaterials induce oxidative stress and have neurotoxic effect on mice, it is possible assuming that they can account for DNA damages. Therefore, the likely genotoxicity of nanopoluents in mice

was assessed. A single-cell gel electrophoresis assay (comet assay) was perfomed based on procedures described by Singh et al. (1988) and De-Oliveira et al. (2020), with some modifications. Peripheral blood samples (10 μL) were diluted in PBS at ratio (v/v) of 1:19. Slides were prepared (two per sample) with 15 μL of cell suspension diluted in 120 μL of low melting point agarose (0.5%) at 37° C and, subsequently, submersed in lysis solution (Triton X-100, DMSO and stock lysis solution) for 24 h, at 6 °C, in the dark. Next, slides were electrophoresed at 300 mA and 25 V (0.90 V/cm), for 25 min, in the dark (Poletta et al., 2009). After electrophoresis, slides were placed on staining tray, covered with neutralization buffer (0.4 M, Tris-HCl, pH 7.5) and kept in the dark for 5 min.

Slides were stained with ethidium bromide (10 μg/mL) and covered with coverslip for the analysis. In total, 50 nucleoids were analyzed per slide, thus totaling 100 nucleoids per sample (n = mice/group). The analysis was performed in CaspLab software to assess DNA damage, based on Gyori et al. (2014) and Yusuf et al. (2018). The following parameters were used to assess DNA damage caused by pesticides: (i) tail length (TL), (ii) DNA rate in the tail (% DNA) and (iii) Olive tail moment (OTM) - as described by Collins (2004) and adopted in previous studies (Ramos et al., 2016; Gonçalves et al., 2017; Fernandes et al., 2018).

2.3.4. Accumulation of nanomaterials

2.3.4.1. Zinc oxide nanoparticles. Zinc (Zn) quantification was performed with the aid of fluorescent zinc probes by using fluorescein dye, based on Brannon and Magde (1978), Huang and Lippard (2012) and Saha et al. (2017), with some modifications. Initially, 100 mg of brain tissue was subjected to chemical digestion, as described in De-Souza et al. (2018a). Then, 150 μL of final digestion solution was pipetted into ELISA microplate. Next, 50 μL of fluorescein stock solution (1 mg/mL in purified water) was added to the samples and homogenized. Standard solution (750 μg/mL) readings were performed. Samples were read at 405 nm in microplate reader after incubation, at room temperature, for 5 min. Readings were taken in triplicate and Zn concentrations in the samples were determined based on the equation below. Background luminescence applied to the tissue of mice who were not exposed to ZnO NPs was detected and subtracted from that of ZnO NPs-exposed samples.

$$\text{Zinc } (\mu g/mL)\ \frac{\text{Sample Absorbance}}{\text{Absorbance of the standard}}\ x\ 750 \qquad (4)$$

wherein, " 750 " is the final concentration of the standard (in μg/mL).

2.3.4.2. Polystyrene nanoplastic. PS NaPs accumulation was estimated based on the methodology adopted by Katzenberger and Thorpe (2015), Boyero et al. (2020) and Sarasamma et al. (2020), with some modifications. This method consists in collecting fragments of brain, weighing them on analytical scale and steeping them in PBS in conical bottom microtubes. Then, 100 μL of homogenized sample was added to the wells in the ELISA microplate (in triplicate) and mixed to 100 μL of purified water - it was read at 450 nm, in microplate reader. Standard sample absorbance was generated by using PS NaPs suspensions (0.13 μg/μL). Readings were performed in triplicate and the concentrations of PS NaPs in the samples were determined based on Eq. (5). Background luminescence of tissues from mice who were not exposed to PS NaPs was detected and subtracted from that of PS NaPs-exposed samples.

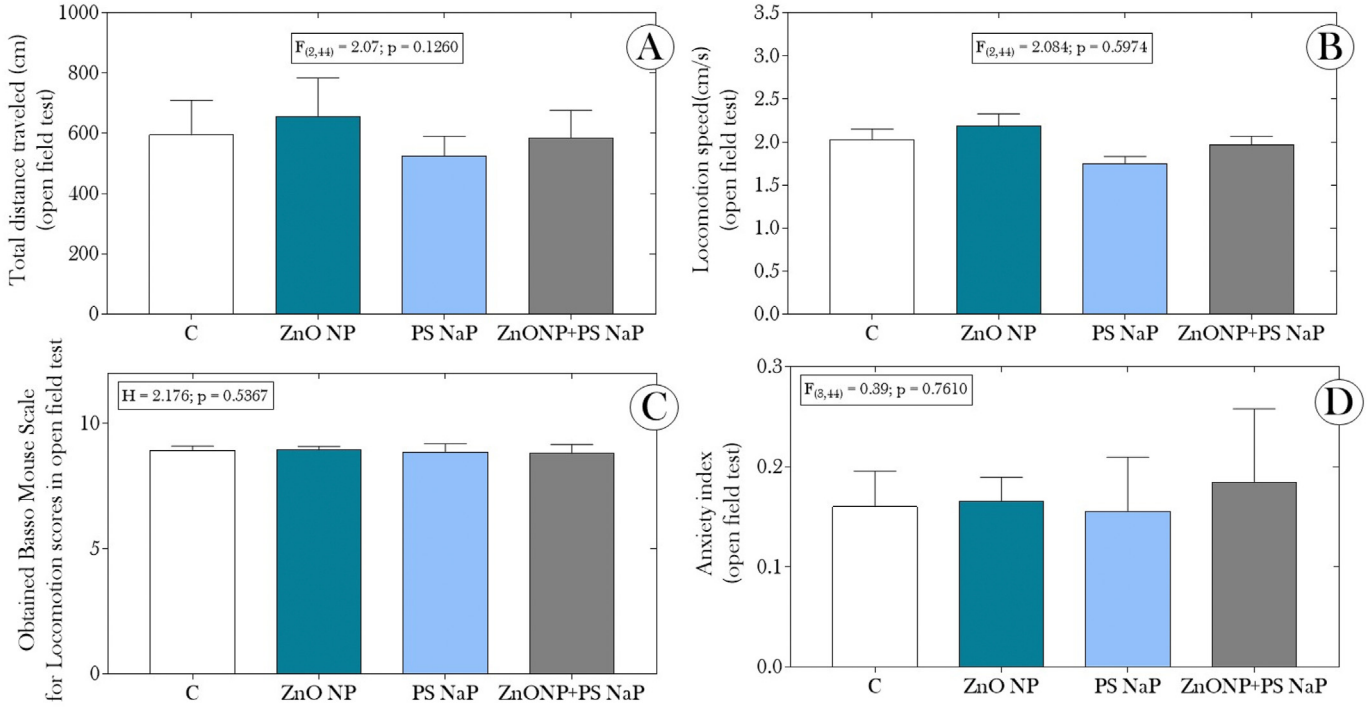

Fig. 2. Locomotor activity parameters: (A) distance covered, (B) locomotion speed and (C) obtained Basso Mouse Scale for Locomotion and (D) anxiety index recorded for male Swiss mice exposed, or not, to different nanomaterials. Bars represent data mean +standard deviation (n = 12 animals/group). Parametric data were subjected to one-way ANOVA and non-parametric data were analyzed through Kruskal-Wallis test, both at 5% probability level. Statistical summaries are shown at the top of the graphs. C: control group; ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: animals exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnONP + PS NaP: mice simultaneously exposed to both nanomaterials (at equitable doses).

$$\text{PS NaPs } (\mu g/mL) = \frac{\text{Sample Absorbance}}{\text{Absorbance of the standard}} x\ 0.13 \qquad (5)$$

wherein, "0.13" is the final concentration of the standard (in μg/mL).

2.4. Data analysis

Data residual normality was assessed through Shapiro-Wilk test. Bartlet test was applied to assess variance homocesticity. Parametric data were subjected to one-way ANOVA (with Tukey's test). Kruskal-Wallis test was used to compare means of non-parametric data through post-test and Dunn's test, both at 5% probability level. Data regarding object recognition test validation were subjected to Mann-Whitney *U* test, at 5% probability level. In addition, the correlation analysis was applied to the different biomarkers by taking into account Spearman (non-parametric data) and Pearson's (parametric data) correlation coefficients at 5% probability level. All statistical analyses and graph plotting were performed in GraphPad Prism software (version 7.0).

3. Results

Initially, the treatments did not have negative influence on models' locomotion in the open field test. The distance covered by them (Fig. 2A), locomotion speed (Fig. 2B) and the obtained Basso Mouse Scale for Locomotion scores did not differ between experimental groups (Fig. 2C). This outcome suggested that treatments did not change the biomechanical attributes necessary for models' normal locomotion. In addition, the exposure to ZnO NPs and PS NaPs (individual or in combination) did not induce anxiety- or anxiogenic-like behavior in the models - anxiety levels were similar between the different groups (Fig. 2D).

The object recognition test showed that recognition indices recorded for the familiar objects (F1 and F2) in the training session with the control group differed from zero and did not present significant differences (Fig. 3A). Similar to results in the research by Rabelo et al. (2016) and Silva et al. (2016), this outcome is one of the test validation indicators, as it evidences that the random exploration of objects in the training session results in equal exploration of both objects. It also excludes the potential preference for a particular spatial location of the objects in the test box. Furthermore, the models in the control group recorded higher recognition indices for the new object in the test session, which was performed right after the training session (phase that acquired memory). This finding indicates the success in keeping the memory about the familiar object. These data also represent another validation indicator of the performed test (Fig. 3B). However, models exposed to the pollutants (in separate) recorded lower new-object recognition rates than the ones of the other experimental groups. This outcome suggests the possible interference of ZnO NPs and PS NaPs (individually) in models' cognitive abilities. However, when models were exposed to the combination of both nanomaterials, new-object recognition rates increased, and it indicated that the effect observed in exposed individuals was inhibited (Fig. 3C).

Although there were no changes in albumin levels (Fig. 4A), serum levels recorded for total proteins and globulins (Fig. 4B and C, respectively) have significantly increased in models exposed to nanopoluents, either in separate or in combination. Similar results were observed in total cholesterol and triglycerides levels (Fig. 4D and E, respectively), which were higher in models exposed to different treatments than in the control group.

TBARS concentrations (Fig. 5A) and nitrite levels (Fig. 5B) suggestedincrease in oxidative stress reactions in models exposed to

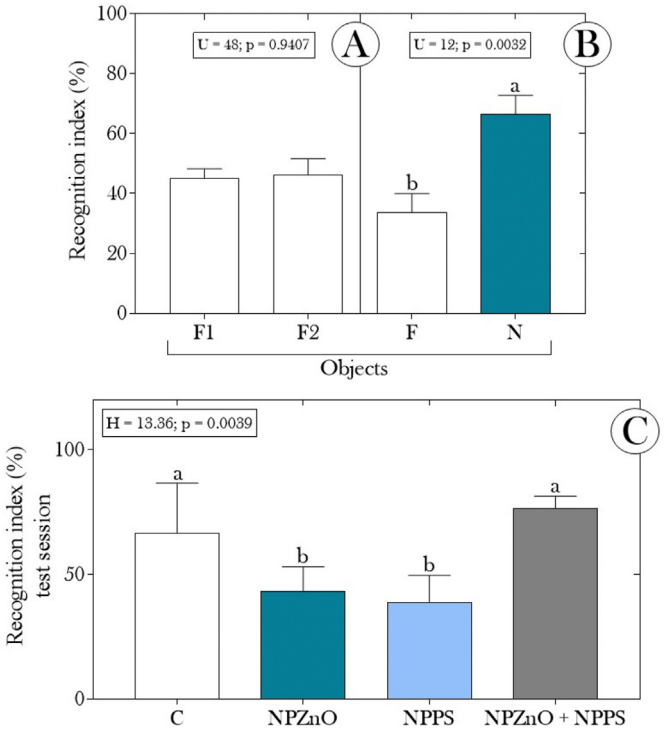

Fig. 3. (A–B) Object recognition index used to test validation based on Swiss mice in the control group; (C) recorded new-object recognition index, which was calculated based on models' exposure to TOS, at different treatments. Bars represent data mean +standard deviation (n = 12 animals/group). Data in "A and B" were analyzed through Kruskal-Wallis test (with Dunn's post-test), at 5% probability level. Statistical summaries are shown at the top of the graphs. Different lowercase letters represent significant differences between means recorded for the samples. C: control group; ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: animals exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnONP + PS NaP: mice exposed to both nanomaterials (at equitable doses).

ZnO NPs and PS NaPs, in separate, but it was not observed at combined exposure. Similar results were observed for AChE acctivity. However, individual exposures to pollutants reduced the activity of this enzyme, but modelss exposed to the binary combination of nanomaterials showed equitable activity in comparison to the control group (Fig. 5C).

Based on the current data, pollutants act in the erythrocyte DNA of models exposed to ZnO NPs and PS NaP, in separate, and of the ones exposed to the binary combination of pollutants in the single-cell gel electrophoresis assay (comet assay). Tail length (TL), DNA percentage in the tail (% DNA) and Olive tail moment (OTM) values were higher in animals exposed to pollutants than in the ones who were not exposed to them (control group) - with emphasis on animals exposed to PS NaPs, who recorded milder genotoxic effect (Fig. 6).

Pollutant accumulation in models' brains was influenced by the binary combination of nanomaterials. Both PS NaPs and Zn concentrations were lower in the brains of models in the "ZnO NP + PS NaP" group (Fig. 7). Table 1 summarizes the performed correlation analyses, which suggested connection between oxidative stress induction and pollutant accumulation due to differences in the herein evaluated parameters.

4. Discussion

The literature has provided information about pollutants dispersed in the environment (Amin et al., 2014; LeFevre et al., 2015; Xu et al., 2020); however, awareness on how much these

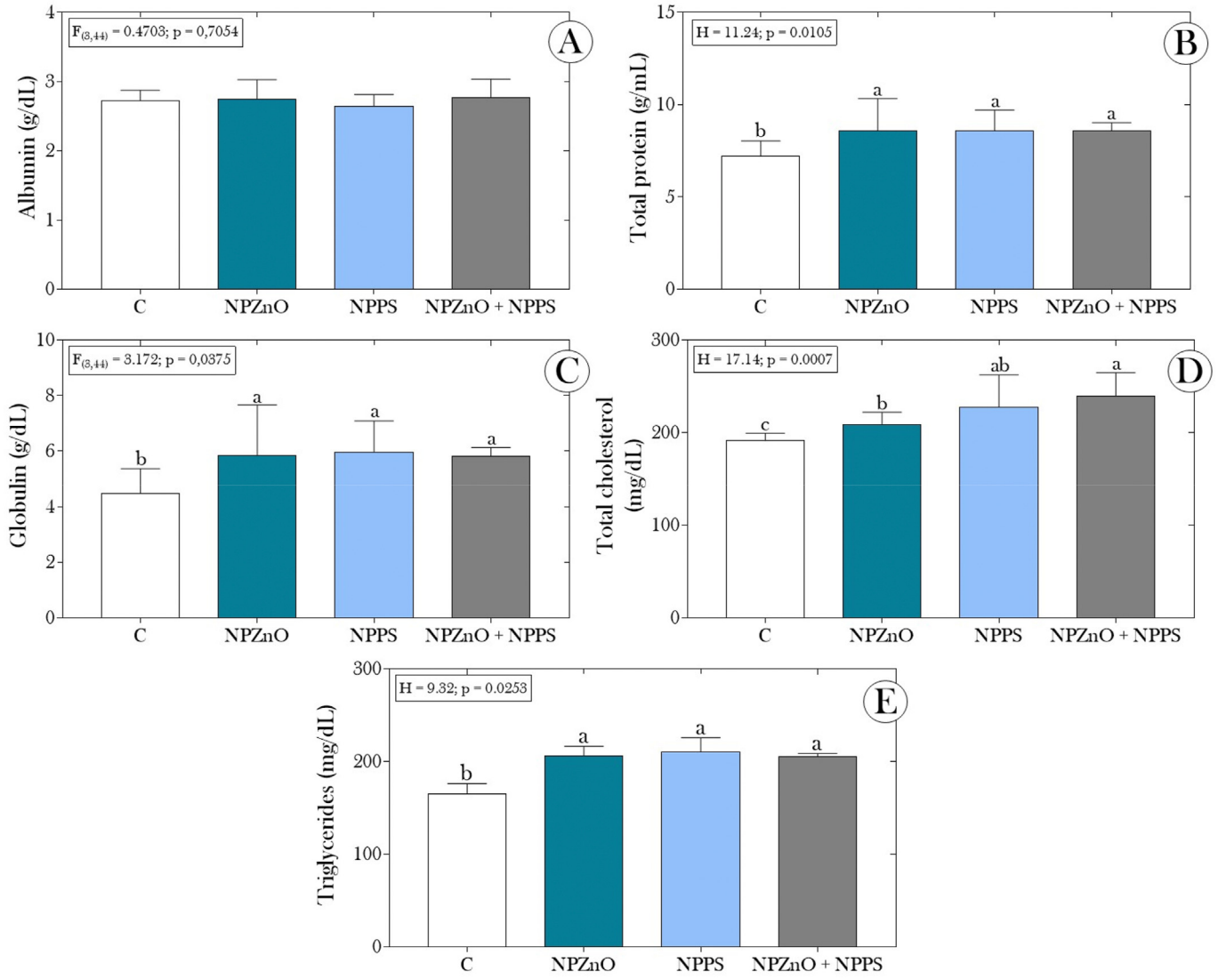

Fig. 4. Serum (A) albumin, (B) total proteins, (C) globulins, (D) total cholesterol and (E) triglycerides levels recorded for Swiss mice exposed, or not, to ZnO NPs and PS N aPs. Bars represent data mean +standard deviation (n = 12 animals/group). Parametric data were subjected to one-way ANOVA (with Tukey's post-test) and non-parametric data were analyzed through Kruskal-Wallis test (with D'nn's post-test), both at 5% probability level. Statistical summaries are shown at the top of the graphs. Different lowercase letters represent significant differences between the means recorded for the samples. C: control group; ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: animals exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnONP + PS NaP: mice exposed to both nanomaterials (at equitable doses).

pollutants affect the aquatic and terrestrial biota remains limited. ZnO NPs and PS NaPs have been used as promising materials in several sectors to improve the quality and features of countless products. However, using these materials demands previous (eco) toxicological assessments that must be simple and involve different agents, because they can enter different environmental compartments. Accordingly, the contribution of the current study lies on broadening the knowledge about the (eco) toxicological potential of ZnO NPs and PS NaPs, mainly when it shows that Swiss mice's exposure to them (even short-term exposure) is enough to induce different physiological changes in these models.

The exposure to ZnO NPs and PS NaPs did not change models' locomotor responses, and this finding is indicative of their inability to have sedative or hyperactivity effect on them (Fig. 2A–C). Such data go against those in some studies carried out to assess the neurotoxicity of ZnO NPs and PS NaPs. According to Xie et al. (201 2), ZnO NPs causes significant motor impairments in groups treated with these elements at dose of 5.6 mg/kg. Male Swiss mice showed longer total time and turn time in the first week of treatment administration. This outcome was quite different in the second and third weeks of treatment in the group subjected to the highest pollutant dose in comparison to the control. Therefore, the time exposed to ZnO NPs and high doses of them may explain the differences between results in Xie et al. (2012) and the present findings. Similar locomotor impacts were reported for different animal models exposed to NaPs. Chen et al. (2017b) and Mak et al. (2019) reported damages to the locomotor ability of *D. rerio* representatives subjected to acute exposure to NaPs, as well as to *Achatina fulica* fed on *Vigna radiata* grown in soil added with carboxylate-modified polystyrene microspheres (Chae and An, 2020). The exposure of the nematode *Caenorhabditis elegans* to five different sizes of spherical polystyrene particles (0.1–5 μm) via culture medium (1 mg/L) resulted in excitatory toxicity in the locomotor behavior (Lei et al., 2018). This finding was indicative that the effect of NaPs depends on factors such as species, concentration, route and exposure time. Therefore, this statement may explain the

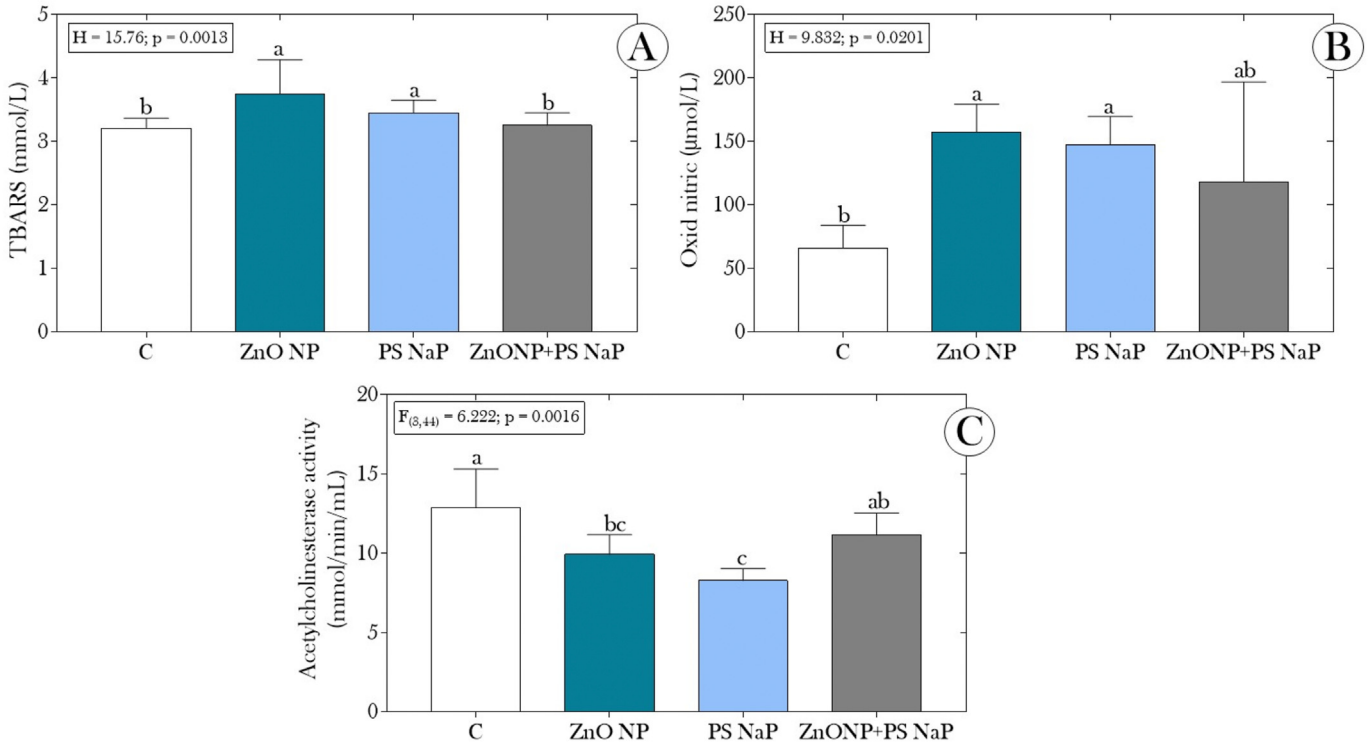

Fig. 5. (A) Thiobarbituric acid reactive species (TBARS), (B) nitrite concentrations and (C) brain homogenate acetylcholinesterase activity in Swiss mice exposed, or not, to ZnO NPs and PS NaPs. Bars represent data mean +standard deviation (n = 12 animals/group). Parametric data were subjected to one-way ANOVA (with Tukey's post-test) at 5% probability level. Non-parametric data were analyzed through Kruskal-Wallis test (with Dunn's post-test), at 5% probability level. Statistical summaries are shown at the top of the graphs. Different lowercase letters represent significant differences between the means recorded for the samples. C: control group: ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: animals exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnONP + PS NaP: mice exposed to both nanomaterials (at equitable doses).

differences between the current study and other publications in the literature. There was no anxiolytic or anxiogenic effect on models (Fig. 2D), and it suggests that these pollutants (in separate, or in combination) do not affect neural mechanisms accountable for regulating anxiety in mice. However, based on the literature, it is possible observing contradictory reports about anxiety-like behavior induction by ZnO NPs and PS NaPs. De-Souza et al. (2018a,b) have demonstrated the anxiogenic behavior of male Swiss mice exposed to the two doses of ZnO NPs (5625×10^{-5} mg/kg and 300 mg/kg, i.p.) in the open field test. However, anxiolytic effect was observed in the study by Torabi et al. (2013), who evidenced that the exposure of male albino Wistar rats to ZnO NPs (5, 10 and 20 mg/kg, i.p.) has induced anxiolytic-like behavior in the elevated plus maze test (EPM). Similar results were observed by Rafiee et al. (2018), who exposed male Wistar rats to different doses of PS NaPs. Overall, models treated with NaPs showed to have anxiolytic effect, which was measured based on the number (rate) of open-arms entries recorded during the EPM test. Anxiety-like behavior was observed in Swiss mice at the trophic transfer of plastic particles in experimental food chain (tadpole > fish > rodent) (Araújo and Malafaia, 2021). These findings confirmed that different factors may induce changes in the cholinergic, catecholaminergic and serotoninergic transmission systems, which are known to modulate the anxiety behavior (Sauvage and Steckler, 2001; Battaglia, 2002; Amilhon et al., 2010; Kaur and Singh, 2017).

There was memory deficit in models subjected to the exposure to nanomaterials, in separate (Fig. 3C), during the object recognition test. The same behavior was not noticed in models exposed to the binary combination of ZnO NPs and OS NaPs. These interesting data highlight lack of additive or synergistic effects induced in models exposed to nanomaterials, in separate. However, further studies in this field must be carried out, because the literature presents dubious results about the effects of these pollutants. Xie et al. (2012) and Tian et al. (2015) observed close correlation between increased cerebral oxidative stress and cognitive impairments in mice exposed to ZnO NPs; however, Amara et al. (2014) did not observe any change in the cognitive ability and norepinephrine, epinephrine, dopamine and serotonin levels in rats exposed to these pollutants.

There is tremendous scarcity of studies assessing the likely negative effects of PS NaPs on models' learning and memory skills. The study by Rafiee et al. (2018) is the only research (so far) focused on investigating the effects of exposure to PS NaPs on rodents. These authors analyzed the potential neurobehavioral effects of long-term exposure to PS NaPs on male Wistar rats (1, 3, 6, and 10 mg PS NaPs/kg, i.p.), but they did not find any significant change in rats' cognitive functions after they were subjected to the Y maze and passive avoidance tests. Assumingly, differences between the assessed PS NaPs sizes (20–26 nm *vs.* 25–50 nm), exposure routes (intraperitoneal vs. oral) and even differences between the assessed models (mice vs. rats) explain the divergence between data in the present research and those reported by Rafiee et al. (2018). Metabolic differences between rats and mice, and the possible formation of more toxic metabolites in mice (Mitchell et al., 1976; Nogueira et al., 2003; Meotti et al., 2003), are important hypotheses to explain the different effects observed in the tested species.

Significant correlation between models' behavioral performance in the object recognition test and increased NO and TBARS levels, and the decreased AChE activity (Table 1) observed in the current study has suggested that oxidative stress induction and AChE inhibition by pollutants can explain the memory deficit

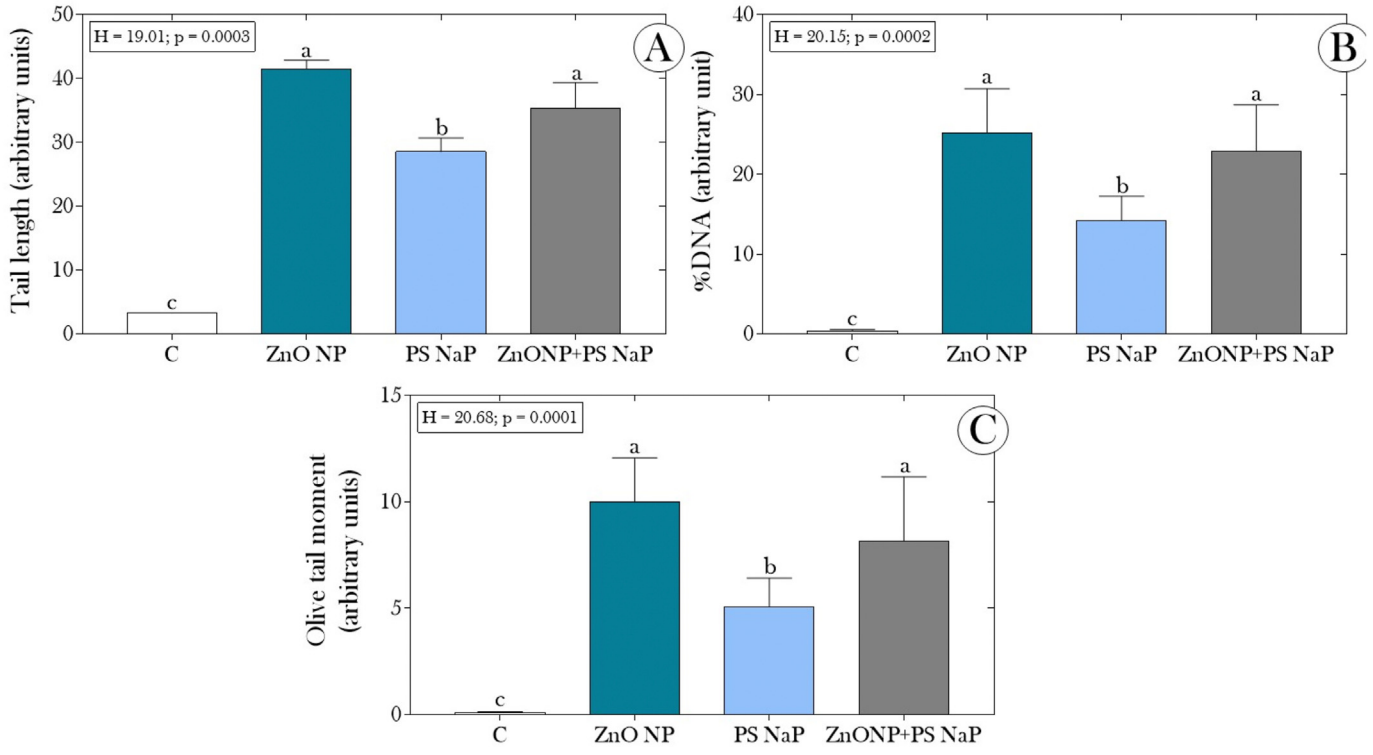

Fig. 6. (A) Tail length (TL), (B) DNA percentage in the tail (% DNA) and (C) Olive tail moment (OTM) recorded at comet assay applied to circulating erythrocytes of Swiss mice exposed, or not, to ZnO NPs and PS NaPs. Bars represent data mean +standard deviation (n = 12 animals/group). Data were analyzed through Kruskal-Wallis test (with Dunn's post-test) at 5% probability level. Statistical summaries are shown at the top of the graphs. Different lowercase letters represent significant differences between the means recorded for the samples. C: control group; ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: mice exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnONP + PS NaP: mice exposed to both nanomaterials (at equitable doses).

observed in models in the "ZnO NP" and "PS NaP" groups. Assumingly, the increased oxidative stress observed in brain tissue has reached specific brain regions accountable for modulating models' memory or for interconnecting neural circuits. Hippocampal regions, for example, are important for both the acquisition and recall of object recognition memory in murine models (Broadbent et al., 2010). Failures in Sprague-Dawley rats' behavioral performance during the object recognition test have been associated with several pathological changes in the hippocampus, including chronic inflammation typified by the presence of ED-1 + activated microglial cells and hypertrophy of astrocytes, persistently decreased neurogenesis (which is an important substrate for hippocampal-dependent cognitive and mood function) and by some neuron losses in the main hippocampal cell layers (Parihar et al., 2013).

Other studies have associated rodent's memory losses with reduced AChE activity in the brain (Liu et al., 2000; Gonçalves et al., 2010), and this finding reinforces the hypothesis that pollutants' neurotoxic action in enzyme activity contributes to memory deficit. There was signficant correlation between NO and TBARS levels, and AChE activity reduction (Table 1), and it suggests nanopolluents' interference in enzyme activity, which is likely caused by increased oxidative stress in the brain. The action mechanisms by ZnO NPs and PS NaPs in the cholinergic neurosystem need to be further investigated, since any anticholinesterase effect on mice would be alarming, if one takes into account the key role played by AChE in neurological functions such as controlling several physiological and behavioral aspects in these animals.

Both the exposure to binary combinations of pollutants, and the exposure to them in separate, have induced significant DNA damage in the evaluated erythrocytes during the comet assay (Fig. 6). This outcome suggests simple DNA strand breaks, DNA double strand breaks and DNA adduct formation and/or DNA-DNA and DNA-protein cross-links resulting from interactions between pollutants, or their metabolites, and DNA. All these mechanisms are consistent with reports on exposure procedures similar to those carried out in the present study (Estrela et al., 2021) and in different studies based on comet assay used as genotoxicity biomarker for different model systems (Sargsyan et al., 2019; Amara et al., 2020; Demir and Yavuz, 2020; Mansouri et al., 2020). However, pollutant combinations did not overcome the effect of exposure to pollutants, in separate, as the behavioral and biochemical biomarkers.

The high adsorption capacity recorded for both pollutants may explain the behavioral and biochemical data. Aggregation between different functional groups of particles can be mitigated by pollutant translocation to models' central nervous system, because the diameter of the aggregates exceeded that of individual particles. This hypothesis is mainly reinforced by the lower pollutant accumulation in the brains of models exposed to the binary combination ZnO NPs and PS NaPs in comparison to that observed in mice exposed to the nanomaterials, in separate (Fig. 7). Different studies related ZnO NPs (Sheela et al., 2012; Zhang et al., 2016; Yuvaraja et al., 2018; Gu et al., 2020; Sharifan et al., 2020) and PS NaPs ability (Zhang et al., 2018; Yu et al., 2019; Bellingeri et al., 2019) to adsorb the surface of different pollutants, and it reinforces the hypothesis of the current study. Assumingly, this aggregation did not prevent the activity of these pollutants (direct or indirect) in erythrocytes' DNA, given their continuous contact with circulating erythrocytes, which may have interfered in erythrocyte membrane functioning (Karlsson et al., 2013; Lesniak et al., 2013;

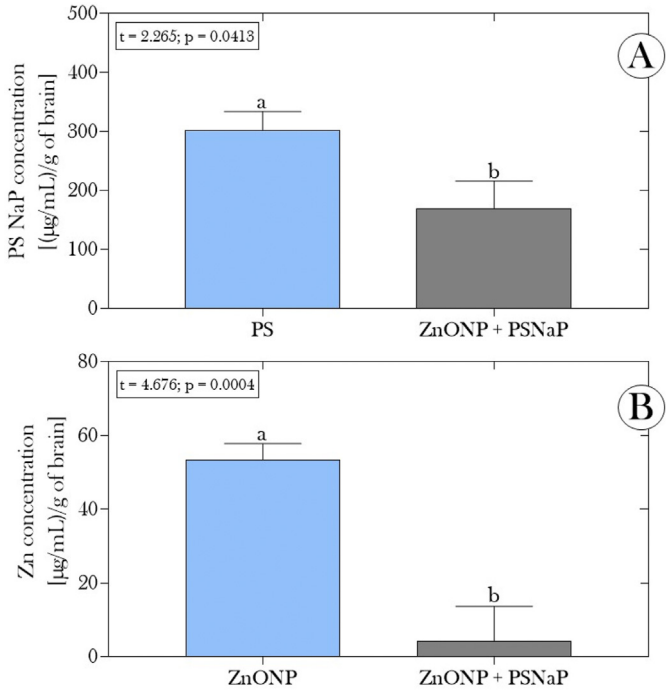

Fig. 7. (A) PS NaPs and zinc (B) accumulation in the brains of Swiss mice exposed, or not, to ZnO NPs and PS NaPs. Bars represent data mean +standard deviation (n = 12 animals/group). Data were compared through Student's *t*-test, at 5% probability level. Statistical summaries are shown at the top of the graphs. Different lowercase letters represent significant differences between the means recorded for the samples. C: control group; ZnO NP: mice exposed to zinc oxide nanoparticles (at dose of 14.6 ng/kg); PS NaP: mice exposed to polystyrene nanoplastics (at dose of 14.6 ng/kg) and ZnO NP + PS NaP: mice exposed to both nanomaterials (at equitable doses).

Contini et al., 2018; Hollóczki and Gehrke, 2019). This finding may explain the herein observed genotoxic effect.

There was significant correlation between genotoxic erythrocyte effects and high total cholesterol and triglycerides (Table 1) rates in models exposed to ZnO NPs and PS NaPs (in separate, or in binary combination). Thus, the hypothesis that intense DNA breaks are related to dysfunctions such as hipercolesterolemia and hipertrigliceridemia will not be neglected. Tonini et al. (2013) and Dalboni et al. (2012) have shown that increased cholesterol levels in C57Bl/6J (Apolipoprotein E-deficient) mice induced DNA damage in bone marrow cells and peripheral whole blood cells, respectively. This finding also reinforced the hypothesis in the curren research.

Table 1
Summary of the correlation analyses performed in the present study.

Biomarkers	Correlation coefficient (r)	P value	Significance (alpha = 0.05)
RI (%) *vs.* NO	Spearman r = −0.3512	0.0264	Yes
RI (%) *vs.* TBARS	Spearman r = −0.4722	0.0018	Yes
RI (%) *vs.* AChE	Pearson r = 0.5001	0.0010	Yes
AChE *vs.* NO	Spearman r = −0.5476	0.0003	Yes
AChE *vs.* TBARS	Spearman r = −0.4934	0.0010	Yes
LT *vs.* CHO	Spearman r = 0.4502	0.0086	Yes
%DNA *vs.* CHO	Spearman r = 0.4799	0.0047	Yes
OTM *vs.* CHO	Spearman r = 0.4526	0.0082	Yes
TL *vs.* TRI	Spearman r = 0.4297	0.0112	Yes
%DNA *vs.* TRI	Spearman r = 0.5334	0.0012	Yes
OTM *vs.* TRI	Spearman r = 0.5116	0.0020	Yes

IR (%): Recognition index (%) - test session; NO: nitric oxide; TBARS: thiobarbituric acid reactive species; AChE: acetylcholinesterase enzyme activity; TL: tail length; % DNA: DNA rate in the tail; OTM: Olive tail moment (OTM); CHO: cholesterol; TRI: triglyceride.

Based on the present results, excessive production of free radicals was induced by hypercholesterolemia (Dalboni et al., 2012) and hypertriglyceridemia (Bae et al., 2001); consequently, it promoted oxidative stress, a fact that can explain the genotoxic damages observed in the models. Although the action mechanisms by ZnO NPs and PS NaPs in mice's lipid metabolism are unknown, assumingly, oxidative stress induction by pollutants leads to cell cholesterol accumulation, increased cholesterol biosynthesis and esterification, as well as to decreased cholesterol ester hydrolysis and reduced cholesterol efflux, which can eventually potentiate the production of free radicals.

Finally, it is important noticing that our study is not exhaustive and that new investigative aspects must be explored in future studies. The influence of exposure to binary combinations of ZnO NPs and PS NaPs can be further assessed in studies focusing on evaluating other toxicity biomarkers, such as the histopathological, genetic and molecular ones, as well as whether similar results can be observed in other experimental models. Investigation about the persistence or recovery of harmful effects on exposed models would subsidize measures to be taken to mitigate plastic and metallic nanoparticles pollution in freshwater ecosystems. The possibility of broadening investigations in this field to extrapolate the present results is an essential factor to help deepening the understanding about the magnitude of the (eco) toxicological impact of these nanomaterials.

5. Conclusion

The hypothesis that both ZnO NPs and PS NaPs are absorbed by, and translocated to, models' brains, even under short-term exposure time (three days) and at small doses (14.6 ng/kg), was confirmed. Their accumulation caused behavioral, biochemical and genotoxic changes in the models. However, there was no additive or synergistic effect of binary combination of pollutants on the assessed toxicity biomarkers, as expected to happen. If, on the one hand, the combined exposure to nanomaterials proved to be harmless to models' cognitive abilities, to increase brain oxidative stress and to inhibit or increase AChE activity; on the other hand, erythrocyte DNA damage was similar among animals exposed to pollutants (both in separate, and in combination). To the best of our knowledge, the observed impacts of these nanopollutants have not been investigated in mice, before. Therefore, further investigations are essential to assess the ecological and health risks related to the exposure of terrestrial biota to the combination of nanomaterials dispersed in freshwater aquatic environments.

Author contributions

Fernanda Neves Estrela: Conceived and designed the analysis, Collected the data, Contributed data or analysis tools, Performed the analysis. Abraão Tiago Batista Guimarães: Collected the data, Contributed data or analysis tools, Performed the analysis. Amanda Pereira da Costa Araújo: Conceived and designed the analysis, Collected the data, Contributed data or analysis tools. Fabiano Guimarães Silva: Contributed data or analysis tools, Performed the analysis, Wrote the paper. Thiarlen Marinho da Luz: Collected the data, Contributed data or analysis tools, Performed the analysis. Paulo Sérgio Pereira: Contributed data or analysis tools, Performed the analysis, Wrote the paper. Guilherme Malafaia: Conceived and designed the analysis, Collected the data, Contributed data or analysis tools, Performed the analysis, Wrote the paper

Compliance with ethical standards

Ethical approval

All experimental procedures were carried out in compliance with ethical guidelines related to animal experimentation; project originating the present study was approved by the Ethics Committee on the Use of Animals (CEUA) of Goiano Federal Institute (Brazil) (protocol N. 1465260719). It should be noticed that meticulous efforts were made to ensure that models would suffer the least possible and to reduce external sources of stress, pain and discomfort. The current study did not exceed the number of animals necessary to produce trustworthy scientific data. This article does not contain any studies with human participants performed by any of the authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Em conclusão, nosso estudo confirma que as características típicas da poluição do plástico no meio ambiente podem ser resumidas como persistência, problemas globais, poluição combinada, ameaças potenciais aos organismos e o meio ambiente. Afirmamos nossa a hipótese de que tanto as NPs ZnO, quanto os NP Ps podem entrar direta ou indiretamente nos sistemas aquáticos e terrestres, migrando livremente entre eles de forma a serem absorvidos e translocados até o cérebro dos animais, ainda que a exposição tenha ocorrido por um curto período (três dias) e em pequena dose. Além disso, observamos que esse acúmulo ocasionou alterações comportamentais, bioquímicas e genotóxicas em ambos modelos utilizados. No entanto, não foi observado efeito aditivo ou sinérgico da combinação binária dos poluentes sobre os biomarcadores de toxicidade avaliados. O tema ainda apresenta lacunas importantes que precisam ser elucidadas através do prosseguimento de investigações nessa área para avaliarmos os riscos ecológicos relacionados à exposição da biota terrestre e aquática à uma combinação de nanomateriais dispersos no ambiente.

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CERTIFICADO

Certificamos que a proposta intitulada "BIOMARCADORES COMPORTAMENTAIS DE MODELOS EXPERIMENTAIS EXPOSTOS A NANOPARTÍCULAS.", protocolada sob o CEUA nº 1465260719 (ID 000825), sob a responsabilidade de **Fernanda Neves Estrêla Resende** *e equipe; Guilherme Malafaia Pinto; Alex Rodrigues Gomes ; Amanda Pereira da Costa Araújo ; Thales Quintão Chagas ; Letícia da Silva Cardoso ; Thiarlen Marinho da Luz ; Abraão Tiago Batista Guimarães* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Instituto Federal Goiano (CEUA/IF Goiano) na reunião de 13/02/2020.

We certify that the proposal "BEHAVIORAL BIOMARKERS OF EXPERIMENTAL MODELS EXPOSED TO NANOPARTICLES.", utilizing 15 Heterogenics mice (15 males), protocol number CEUA 1465260719 (ID 000825), under the responsibility of **Fernanda Neves Estrêla Resende** *and team; Guilherme Malafaia Pinto; Alex Rodrigues Gomes ; Amanda Pereira da Costa Araújo ; Thales Quintão Chagas ; Letícia da Silva Cardoso ; Thiarlen Marinho da Luz ; Abraão Tiago Batista Guimarães* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Goiano Federal Institute (CEUA/IF Goiano) in the meeting of 02/13/2020.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 05/2020 a 03/2021 Área: Ciências Biológicas

Origem:	Instituto Federal Goiano - Campus Urutaí						
Espécie:	Camundongos heterogênicos	sexo:	Machos	idade:	2 a 5 meses	N:	15
Linhagem:	Swiss			Peso:	44 a 50 g		

Local do experimento: Laboratório de Pesquisas Biológicas- Instituto Federal Goiano- Campus Urutai

Goiânia, 13 de fevereiro de 2020

[redacted]
Prof. Dr. Wallacy Barbacena Rosa dos Santos
Coordenador da Comissão de Ética no Uso de Animais
Instituto Federal Goiano

[redacted]
Profa. Dra. Adriana da Silva Santos
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Instituto Federal Goiano