Universidade Federal de Uberlândia

Instituto de Biologia

Programa de Pós-Graduação em Ecologia,
Conservação e Biodiversidade

AVALIAÇÃO DO POTENCIAL ECOTOXICOLÓGICO DAS NANOFIBRAS DE CARBONO UTILIZANDO ORGANISMOS DO GRUPO DOS INVERTEBRADOS E VERTEBRADOS

Mateus Flores Montalvão

2023

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Avaliação do potencial ecotoxicológico das nanofibras de carbono utilizando organismos do grupo dos invertebrados e vertebrados

Tese apresentada à Universidade Federal de Uberlândia, como parte das exigências para obtenção do título de Doutor em Ecologia, Conservação e Biodiversidade".

Orientador

Prof. Dr. Guilherme Malafaia

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ÍNDICE

RESUMO GERAL

A presente tese foi dividida em capítulos, nos quais diferentes objetivos foram almejados. No primeiro capítulo, foi trazemos a tona os principais meios de utilização das nanofibras de caborno, bem como as principais formas de produção, trabalhos cientificos mais recentes. No segundo capítulo testamos a hipótese de que a exposição às nanofibras de carbono (NFCs), em concentração ambiental, por um curto período (48 h), causa distúrbios bioquímicos preditivos de estresse oxidativo, déficit antioxidante e neurotoxicidade em larvas de libélula (*Aphylla williamsoni*). As libélulas pertencem à ordem Odonata, que possui um dos maiores números de espécies aquáticas já catalogadas, apresentando importantes funções ecológicas, como o controle populacional de diversos outros organismos. Até onde sabemos, o referido capítulo constitui o primeiro estudo a relatar o impacto das NFCs em uma espécie pertencente a esta ordem taxonômica, provendo um *insight* sobre os riscos ecotoxicológicos que esses poluentes representam para a fauna de macroinvertebrados bentônicos que vivem em ecossistemas de água doce. No terceiro capítulo, objetivamos avaliar os possíveis efeitos da exposição prolongada (90 dias) de juvenis de zebrafish (*Danio rerio*) à diferentes concentrações de NFCs. O zebrafish é um dos peixes de água doce mais utilizados como modelo ecotoxicológico para perfis de toxicidade induzida por nanomateriais e possui importantes funções ecológicas em seus ambientes naturais, o que justifica sua escolha como sistema modelo neste trabalho. Partimos da hipótese de que mesmo em pequenas concentrações, as NFCs são ingeridas pelos animais, induzem alterações comportamentais, bem como na densidade de mecanorreceptores chamados neuromastos (órgãos da linha lateral), além de alterações bioquímicas cerebrais, anormalidades nucleares eritrocitárias, danos no DNA e comprometimento do crescimento /desenvolvimento animal. No quarto capítulo, avaliamos, pela primeira vez, se a exposição de minhocas *Lumbricus terrestris* naturalmente infectadas por *Monocystis* sp. às NFCs, em concentrações ambientalmente relevantes, potencializa a infecção parasitária e induz efeitos adversos na saúde dos animais. As minhocas apresentam diversos serviços ecossistêmicos através da pedogênese, desenvolvimento da estrutura do solo, regulação da água, ciclagem de nutrientes, produção primária, regulação do clima, remediação da poluição e serviços culturais. Logo, acreditamos que nosso estudo contribuirá para a compreensão dos riscos ecotoxicológicos das NFCs para os organismos edáficos. Por fim, o capítulo "Conclusões e considerações finais" apresenta uma visão geral da ecotoxicidade das NFCs reportadas nesta tese, bem como lança luzes sobre os campos de investigações nesta área que podem ser melhor explorados futuramente.

Palavras-chave: *Aphylla williamsoni*; *Danio rerio*; *Lumbricus terrestris*; ecotoxicologia; biomarcadores, nanomateriais.

GENERAL ABSTRACT

This thesis was divided into chapters, in which different objectives were pursued. In the first chapter, we look at the main ways in which carbon nanofibers are used, as well as the main forms of production and the most recent scientific work. In the second chapter, we tested the hypothesis that exposure to carbon nanofibers (CNFs) at environmental concentrations for a short period (48 h) causes biochemical disturbances predictive of oxidative stress, antioxidant deficit and neurotoxicity in dragonfly larvae (Aphylla williamsoni). Dragonflies belong to the order Odonata, which has one of the largest numbers of aquatic species ever cataloged, and has important ecological functions, such as population control of various other organisms. As far as we know, this chapter is the first study to report the impact of NFCs on a species belonging to this taxonomic order, providing an insight into the ecotoxicological risks that these pollutants pose to the fauna of benthic macroinvertebrates living in freshwater ecosystems. In the third chapter, we aimed to evaluate the possible effects of prolonged exposure (90 days) of juvenile zebrafish (Danio rerio) to different concentrations of NFCs. . The zebrafish is one of the freshwater fish most commonly used as an ecotoxicological model for nanomaterial-induced toxicity profiles and has important ecological functions in its natural environments, which justifies its choice as a model system in this work. We started from the hypothesis that even in small concentrations, when NFCs are ingested by animals, they induce behavioral changes, as well as changes in the density of mechanoreceptors called neuromasts (lateral line organs), in addition to brain biochemical changes, erythrocyte nuclear abnormalities, DNA damage and impaired animal growth/development. In the fourth chapter, we assess, for the first time, whether the exposure of Lumbricus terrestris earthworms naturally infected with Monocystis sp. to NFCs, at environmentally relevant concentrations, potentiates the parasitic infection and induces adverse effects on the animals' health. Earthworms provide various ecosystem services through pedogenesis, soil structure development, water regulation, nutrient cycling, primary production, climate regulation, pollution remediation and cultural services. We therefore believe that our study will contribute to understanding the ecotoxicological risks of NFCs for edaphic organisms. Finally, the chapter "Conclusions and final considerations" presents an overview of the ecotoxicity of the NFCs reported in this thesis, as well as shedding light on the fields of research in this area that can be better explored in the future.

Keywords: *Aphylla williamsoni*, *Danio rerio*, *Lumbricus terrestris*, ecotoxicology, biomarkers, nanomaterials.

CAPÍTULO 1 - REVISÃO DE LITERATURA

1. CARACTERÍSTICAS E APLICAÇÕES DAS NANOFIBRAS DE CARBONO

As nanofibras de carbono são um material extremamente interessante para aplicações em diversas áreas, como engenharia de materiais, biomedicina, energia, eletroquímica e eletrônica, devido às suas propriedades físicas e químicas únicas (Mohamed, 2019). A história do desenvolvimento das nanofibras de carbono (NFCs) remonta ao final do século XX, quando uma nova classe de materiais carbonáceos, conhecidos como nanotubos de carbono, foi descoberta por Sumio Iijima em 1991 (Iijima, 1991). Essa descoberta constituiu um marco importante no campo da nanotecnologia e abriu caminho para a pesquisa e o desenvolvimento de outros materiais baseados em carbono, incluindo as NFCs.

As características das NFCs e suas aplicações têm sido extensivamente estudadas desde então. Tais nanomateriais são compostas por fibras cilíndricas com diâmetro inferior a 100 nm e comprimento variável (Darne et al., 2010; Mohamed, 2019). Elas são produzidas a partir de diversas fontes de carbono, como fibras de carbono, grafite, fulerenos e nanotubos de carbono (Mohamed, 2019), além de apresentarem alta resistência mecânica, baixa densidade, alta condutividade térmica e elétrica, alta estabilidade química e uma grande área superficial, o que as tornam um material promissor para diversas aplicações (Chen et al., 2015; Mohamed, 2019).

As NFCs são empregadas em diferentes áreas. Na engenharia de materiais, as nanofibras são utilizadas para aumentar a resistência mecânica e a condutividade térmica e elétrica de materiais compósitos (Yu et al., 2014; Sagdic et al., 2022). Na biomedicina, elas são aplicadas em suporte para o crescimento de células e tecidos, sensores biológicos e liberação controlada de medicamentos (Wei et al., 2002; Owida et al., 2023; Liu et al., 2023). No campo energético, as NFCs são utilizadas em células solares, baterias e supercapacitores devido, especialmente, à sua alta condutividade elétrica (Guo et al., 2021). Na eletroquímica, as NFCs são empregadas como eletrodos em reatores eletroquímicos e em células de combustível (Wick et al., 2014) e, no setor eletrônico, elas têm sido utilizadas na fabricação de dispositivos diversos, como transistores e capacitores de alta capacidade (Pietroiusti et al., 2011).

2. SÍNTESE DAS NANOFIBRAS DE CARBONO

Atualmente, as NFCs podem ser sintetizadas principalmente por dois métodos: o crescimento catalítico por deposição de vapor químico térmico, e eletrofiação seguida de tratamento térmico.

2.1. Síntese de nanofibras de carbono por crescimento de deposição de vapor química térmica catalítica

Dois tipos de NFCs podem ser sintetizadas por deposição catalítica de vapor químico térmico, a saber: as NFCs empilhadas em copo e as NFCs plaquetárias. As NFCs empilhadas, também chamadas de NFCs cônicas, foram descritas pela primeira vez por Ge & Sattler (1994). As Figuras 1A-C apresentam esquemas da formação das NFCs empilhadas em forma de copo, e a Figura 1D apresenta a ilustração esquemática das estruturas plaquetárias das NFCs.

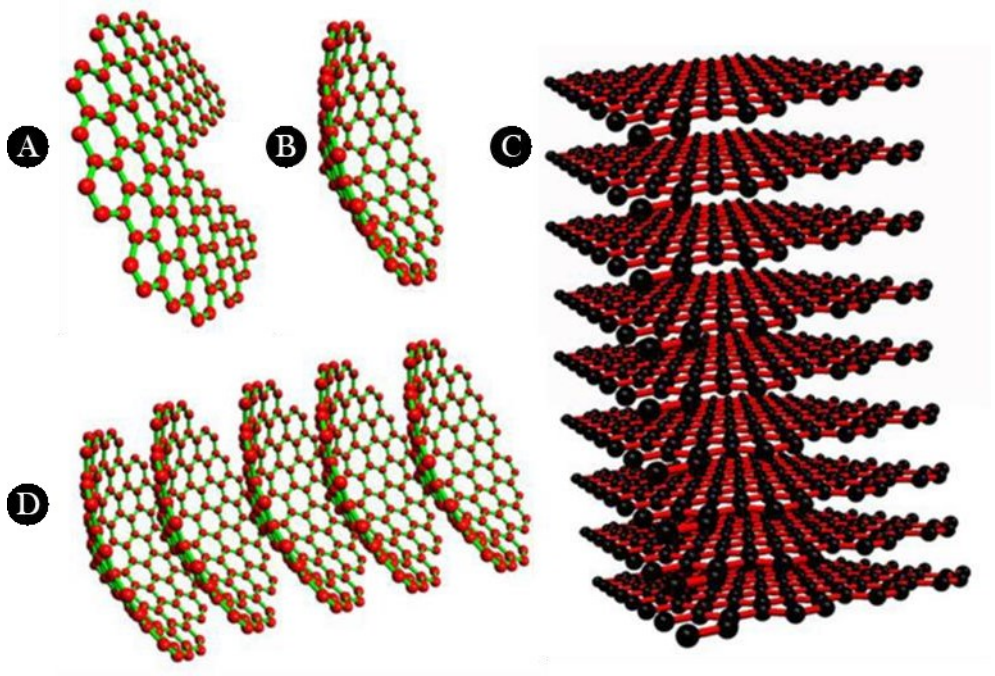

Figura 1. Demonstração esquemática da formação (A-C) da estrutura das nanofibras de carbono (NFCs) empilhadas em copos e em (D) estrutura plaquetária. Fonte: adaptado de Feng et al. (2014).

Para a preparação das NFCs pela abordagem de crescimento catalítico por deposição a vapor, vários tipos de metais ou ligas, que são capazes de dissolver carbono para formar carboneto de metal, têm sido utilizados como catalisadores, incluindo ferro, cobalto, níquel, cromo e vanádio. Adicionalmente, o molibdênio, metano, monóxido de carbono, gás de síntese (H_2/CO), etino ou eteno são utilizados para fornecer as fontes de carbono na faixa de temperatura de 700 a 1200 K (De Jong & Geus, 2000). Geralmente, as estruturas das NFCs são regidas pelas formas

das partículas metálicas nanométricas catalíticas. O mecanismo de crescimento foi comprovado como a deposição dos hidrocarbonetos dissolvidos na partícula de metal e precipitados na superfície do metal como carbono grafítico (Kim et al., 2013). As Figuras 2 e 3 demonstram a ilustração esquemática do mecanismo de crescimento típico das NFCs em forma de copo e de plaquetas (Terrones et al., 2001; Zheng et al., 2006). A Figura 4 mostra a imagem do microscópio eletrônico de transmissão de alta resolução (HRTEM) das NFCs em forma de copo e das NFCs em forma de plaquetas (Terrones et al., 2001; Zheng et al., 2006).

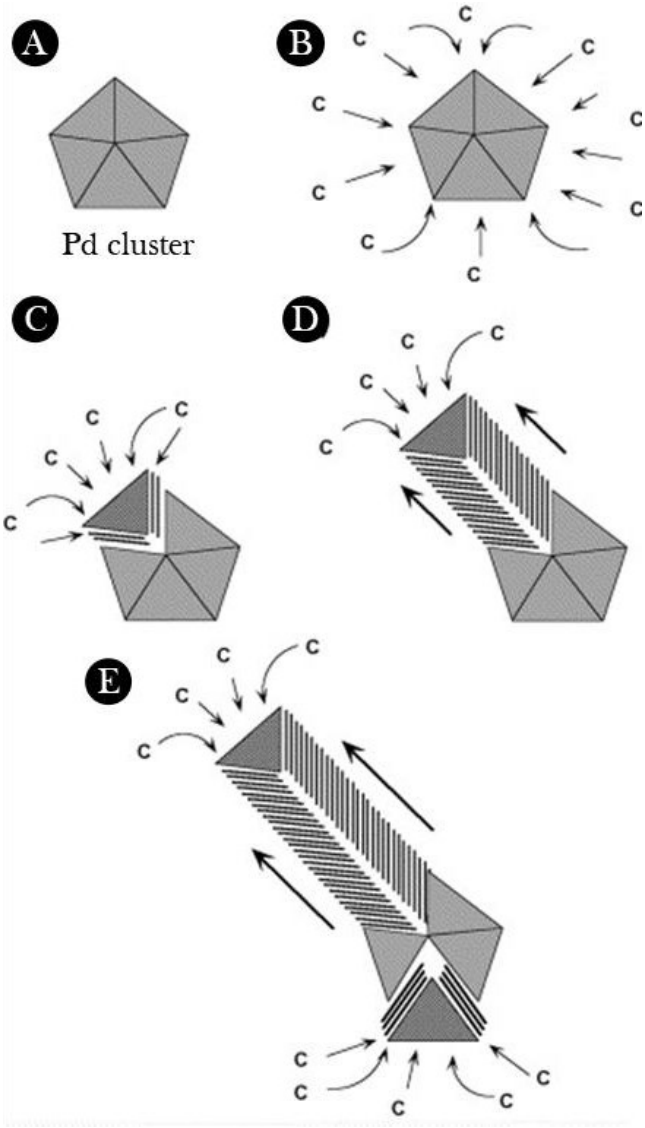

Figura 2. Possível mecanismo de crescimento para as nanofibras cônicas: (A) Clusters de Pd se formam e se agregam para criar partículas de geminação múltipla; (B) as espécies de carbono interagem com o aglomerado poligonal de Pd quente e se difundem ao longo da superfície exposta e entre os contornos de grão, fragmentando assim os cristais gêmeos em partículas de Pd semelhantes a tetraédricas e em forma de diamante; (C) o carbono se difunde, nas partículas cônicas de Pd expostas, e precipita na outra extremidade, formando cones de grafite; (D-E) o carbono continua a se difundir, criando cones 'frescos', que deslocam os cones formados anteriormente, liberando a tensão entre os cones e a partícula de Pd, criando assim as nafibras cônicas. Fonte: adaptado de Terrones et al. (2001).

Figura 3. Ilustração esquemática do mecanismo típico de crescimento por deposição de vapor químico das nanofibras de carbono (NFCs) plaquetárias. Fonte: Zheng et al. (2006).

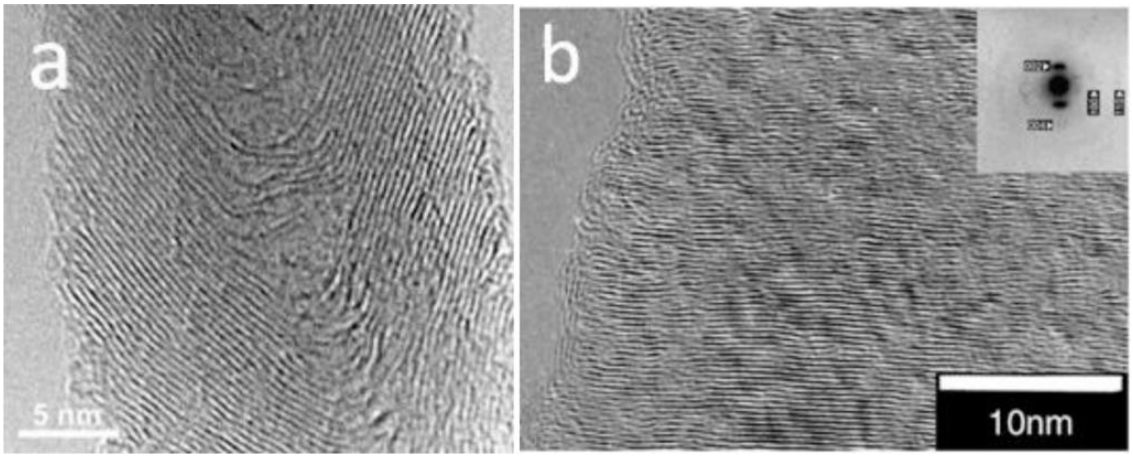

Figura 4. Imagens de microscopia eletrônica de transmissão de alta resolução de (A) nanofibras de carbono (NFCs) empilhada e (B) plaquetárias. Adaptado de Terrones et al. (2001) e Zheng et al. (2006).

2.2. Síntese de nanofibras de carbono por eletrofiação

Eletrofiação é outro método amplamente utilizado para a fabricação das NFCs, no qual as nanofibras poliméricas são preparadas como precursores das NFCs. As propriedades das NFCs finais são decididas pelos tipos de solução polimérica e pelos parâmetros de processamento. Poliacrilonitrila de alta resistência ou piche mesofásico são os polímeros mais utilizadps. Além disso, também foram utilizados poli(álcool vinílico), poliimidas (PIs), polibenzimidazol e poli(fluoreto de vinilideno) (resina fenólica e lignina (Inagaki et al., 2012). Uma vez que as nanofibras poliméricas tenham sido preparadas com sucesso, o tratamento térmico é aplicado para carbonizar as nanofibras, formando as NFCs. A morfologia, pureza, cristalinidade, diâmetros e porosidade são regidos pelos parâmetros do processo de

tratamento térmico, como pressão e temperatura. Na Figura 5 é apresenda uma demonstração esquemática do dispositivo de eletrofiação utilizado na fabricação das NFCs

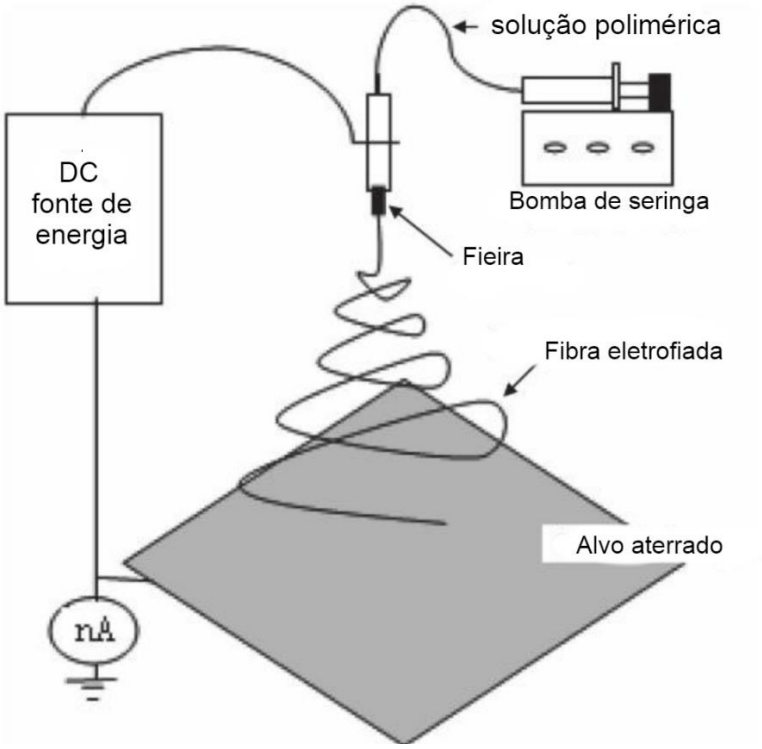

Figura 5. Demonstração esquemática da configuração de eletrofiação para fabricação de nanofibras de carbono (NFC). Adaptado de Inagaki et al. (2012).

Na maioria dos casos, as NFCs preparadas pelo método de eletrofiação são propensas a formar estruturas de teia ou manta. Esta estrutura é uma boa forma de ser usada como material de eletrodo para baterias ou supercapacitores.

3. Ecotoxicidade das nanofibras de carbono

A ampla utilização das NFCs está resultando no aumento da produção, com consequente liberação desses materiais no ambiente, podendo afetar os organismos e a homeostase ecossistêmica (Kisin et al., 2011). Entretanto, pouco sabemos sobre o potencial impacto das NFC na biota. Animais presentes em ambientes contaminados nos estágios iniciais de desenvolvimento podem, por exemplo, ter seu desenvolvimento afetado, resultando em taxas reduzidas de sobrevivência e reprodução (Pechenik, 2006). A quantidade de NFCs depositadas em sedimentos em ecossistemas aquáticos e terrestres pode apresentar ordem de grandeza superior à das águas circundantes, se levarmos em consideração a natureza química desses nanomateriais e a possibilidade de agregação de partículas em suspensão (Freixa et al., 2018; Mueller & Nowack, 2008). Assim, avaliar a possível toxicidade de desses nanomateriais em distintos grupos animais

é essencial para entendermos a magnitude de seu impacto nos ecossistemas. Conforme discutido por Ma et al. (2020), pequenas alterações na biota podem ser suficientes para causar distúrbios nos ecossistemas, os quais podem ir muito além dos impactos observados em nível de indivíduo (Ma et al., 2020).

Na literatura, alguns trabalhos já relataram a toxicidade dos nanomateriais baseados em carbono em sistemas biológicos (Smolka et al., 2019; Mehrabi et al., 2020; Salesa et al., 2020; Nekounam et al., 2020) e *in vitro* (Magrez et al., 2006; Mittal et al., 2017; Jensen et al., 2012; Kalman et al., 2019; Samadian et al., 2020). Estudos recentes têm demonstrado vários efeitos adversos desses nanomateriais nos organismos, incluindo impactos genotóxicos (Horibata et al., 2022; Ince- Yardimci et al., 2022; Di- Ianni et al., 2022), mutagênicos (Da-Costa- Siqueira et al., 2023), bioquímicos (Minchenko et al., 2022; Mortensen et al., 2022; Witkowska et al., 2022), histopatológicos (Duo et al., 2022; Bubols et al., 2023; Kim & Cho, 2023), comportamentais (Demir et al., 2022), dentre outros. Quanto às NFCs, em particular, alguns estudos apontam para baixa toxicidade ou inofensividade nos sistemas biológicos em que foram testados (por exemplo, Smolka et al., 2019; Mehrabi et al., 2019; Salesa et al., 2020; Nekounam et al., 2021; Gomes et al., 2021; Montalvão et al., 2023). Já outros, demonstram que a exposição dos organismos às NFCs induz alterações drásticas na sua saúde dos organismos. A Tabela 1 resume alguns dos trabalhos mais recentes envolvendo a interação e os efeitos das NFCs em diferentes grupos taxonômicos.

Tabela 1. Artigos que avaliaram os efeitos dos nanofibras de carbono em modelo animal *in vivo*.

Modelo experimental	Concentrações testadas	Tempo de exposição	Principais efeitos	Referências
Camundongos (*Mus musculus*)	120 µg/camundongo	1, 7 e 28 dias	Os autores evidências de que a área de superfície efetiva juntamente com a dose de massa, em vez da área de superfície específica ou número de partículas, estão significativamente correlacionadas com as respostas toxicológicas às nanopartículas fibrosas carbonáceas.	Murray et al. (2012)
Camundongos (*Mus musculus*)	40 e 120 µg/camundongo	5h/dia por 4 dias. Avaliação após 1 ano a partir da última aplicação.	Sem aumento de incidência de câncer no pulmão após 1 ano de aplicação. Os pesquisadores sugerem que a toxicidade do NFC, e definida, não apenas por sua composição química, mas também pela área de superfície e tipo de exposição.	Shvedova et al. (2014)
Mexilhão (*Mytilusedulis*).	0,01, 0,1 e 1 mg/L	24 horas	Não apresentou efeitos tóxicos significativos.	Barrick et al. (2019)
Microalgas marinhas (*Attheya ussuriensis*, *Chaetoceros muelleri*, *Heterosigma akashiwo*, e *Porphyridium purpureum*)	50, 100, 150, 200, 250 mg/L	3h e 24h; 6h e 24h; 96h e 7 dias	Alta sensibilidade apenas da microalga vermelha *Porphyridium purpureum* aos CNFs, sugerindo que esses nanomateriais podem se ligar a membranas de microrganismos por interação hidrofóbica e pontes de hidrogênio formadas entre superfícies de células e áreas defeituosas de CNFs.	Pikula et al. (2020)
Ave (*Gallus gallus domesticus*)	50 µg/g embrião		NFCs com sílica mesoporosa resulta em uma redução significativa na toxicidade de NFCs na embriogênese.	Abdo et al. (2021)
Invertebrados de água doce (*Diamesa* sp., *Drunella cryptomeria* e *Gammarus suifunensis*)	100 mg/L	7 dias	Os autores deste trabalho não identificaram efeito tóxico significativo.	Chaika et al. (2020)
Juvenis *Podocnemis expansa*	1 e 10 mg/L	7,5 meses	Foram identificadas alterações bioquímicas, mutagênicas, genotóxicas, citotóxicas e neurotóxicas.	Guimarães & Malafaia (2021)
Girinos *Physalaemus cuvieri*	1 e 10 mg/L	48 horas	Efeitos tóxicos.	Guimarães et al. (2021)
Tilápia (*Oreochromis*	500 µg/g	Fase I: 7 dias;	O presente estudo confirmou a hipótese inicial, uma vez	Gomes et al. (2021)

niloticus)	Fase II e III 48h.	que forneceu evidências de acúmulo de NFCs em diferentes níveis tróficos da cadeia experimental (*Eisenia fetida* → *Danio rerio* → *Oreochromis niloticus*) e observou efeito mutagênico e citotóxico em animais no último nível trófico (*O. niloticus*).

4. INTEGRANDO ECOLOGIA E TOXICOLOGIA: ECOTOXICOLOGIA

A ecologia de modo geral centra-se nas interações entre organismos, em sua distribuição e abundância, funcionamento das populações e comunidades biológicas e nos processos que afetam todos estes parâmetros (Andrewartha e Birch, 1954). Os ecologistas estudam as interações entre os organismos e o seu ambiente a todos os níveis, desde o organismo individual até ao ecossistema. Isto inclui os fatores que regem as distribuições geográficas das espécies e que influenciam a abundância e distinções das populações individuais. O principal objetivo das investigações ecológicas é "compreender e explicar os fenómenos naturais, assim como processos ecológicos e, por conseguinte, os padrões resultantes de distribuição, abundância, diversidade e interações das espécies" (Underwood et al., 2000).

O início da ecologia começou com observações simples (história natural e descrição de individuais), que foram depois complementadas por investigações planeadas e, mais tarde, por manipulações experimentais; que reflete no progresso da toxicologia (ou seja, de apenas exposições simples em laboratório para experiências complementares complexas in situ. Um dos principais objetivos da ecologia são os princípios gerais que estruturam as comunidades naturais (Menge, 2000).

Foram realizadas experiências clássicas para testar hipóteses relativas, por exemplo: à competição, à predação, à sucessão, à perturbação, à resiliência e à riqueza das espécies. Para além das experiências manipulativas, a gama de abordagens de estudo utilizadas pelos ecologistas inclui observações descritivas, experiências laboratoriais e modelos matemáticos (Chapman et al., 2002).

Mas, existe uma conexão entre a ecologia e a toxicologia aquática está relacionada com a utilização de experiências de "quase-campo" e de campo. Onde os pesquisadores tentam tentaram tornar as condições laboratoriais mais realistas. Por exemplo, os toxicologistas aquáticos utilizam microcosmos e mecocosmos (Solomon, 2002); os ecologistas também transferem animais para o laboratório com amostras de habitat natural (Della Santina e Naylor, 1994), levam o laboratório para o campo (Colombini et al., 1994) ou realizam experiências de transplante (Underwood, 2000). Os argumentos relativos às avaliações descendentes em oposição às avaliações ascendentes são tão frequentes na ecologia como na toxicologia aquática (Baird et al., 1996; Menge, 2000; Underwood, 2000).

Já outro ponto chave, embasa a toxicologia ambiental e ecotoxicologia. O domínio da toxicologia em geral engloba duas áreas (incluindo a toxicologia ambiental e a ecotoxicologia) a compreensão dos tipos de efeitos causados pelas substâncias químicas, os processos bioquímicos e fisiológicos responsáveis por esses efeitos, as sensibilidades relativas dos diferentes tipos de organismos às exposições químicas e as toxicidades relativas das diferentes substâncias e classes químicas. Embora as experiências laboratoriais controladas que utilizam espécies "indicadoras" únicas tenham servido bem no passado e continuem a constituir um pilar da toxicologia (por exemplo, a identificação de substâncias potencialmente prejudiciais), estudos mais complexos e uma melhor escolha das espécies de ensaio são complementos essenciais para os estudos atuais e abordagens futuras, se quisermos prever a toxicidade para os organismos selvagens em condições de exposição reais.

Por fim, existe uma dependência entre ambas, pois somente a ecologia não é capaz, por si só, de determinar o que está a ocorrer no ambiente relacionado com a contaminação. Em particular, a ecologia não pode, por si só, determinar: as relações entre os organismos e os contaminantes (ou outros fatores de stress); a forma como os contaminantes e outros fatores de stress alteram a estrutura da comunidade em termos de efeitos diretos (toxicidade) e indiretos (cadeia alimentar). Para avaliar e proteger adequadamente a qualidade ambiental, é extremamente importante determinar de que forma os fatores de tensão afetam os diferentes organismos e populações. Essa informação não provém apenas da toxicologia ambiental ou da ecologia, mas sim da sua combinação numa toxicologia ecológica (Chapman et al., 2002).

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CAPÍTULO 2 - CARBON NANOFIBERS ARE BIOACCUMULATED IN *Aphylla williamsoni* (ODONATA) LARVAE AND CAUSE REDOX IMBALANCE AND CHANGES OF ACETYLCHOLINESTERASE ACTIVITY[1]

ABSTRACT

Carbon-based materials have been considered very promising for the technological industry due to their unique physical and chemical properties, namely: ability to reduce production costs and to improve the efficiency of several products. However, there is little information on what is the level of exposure that leads to adverse effects and what kind of effects is expected in aquatic biota. Thus, the aim of the present study was to evaluate the toxicity of carbon nanofibers (CNFs) in dragonfly larvae (Aphylla williamsoni) based on predictive oxidative-stress biomarkers, antioxidant activity reduction and neurotoxicity. After ephemeral models' exposure to CNFs (48 h; at 500 μg/L), data have shown that these pollutants did not change larvae's nutritional status given the concentration of total soluble carbohydrates, total proteins and triglycerides in them. However, the levels of both nitric oxide and substances reactive to thiobarbituric acid (lipid peroxidation indicators) have increased and the antioxidant activity based on total thiol levels and on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (%) has reduced, and it suggests REDOX imbalance induction by CNFs. In addition, larvae exposed to these pollutants showed significant acetylcholinesterase activity reduction in comparison to the control group. Thus, the present study has brought further knowledge about how carbon-based materials can affect benthic macroinvertebrates and emphasized their ecotoxicological potential in freshwater environments.

Keywords: Water pollution, Nanopollutants, benthic macrofauna, carbon-based nanomaterials.

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1. INTRODUCTION

Nanoscience and nanotechnology brought along the sensitive changes in matter structure at nanometer level, which have triggered changes in the chemical, physical and mechanical properties of materials. The need of controlling such changes has led to the development of new materials that present properties never observed before. Carbon-based nanomaterials (CNs), which have wide potential to be applied in different sectors (Fu et al., 2019; Zheng et al., 2019; Liu et al., 2019; Yoosefian and Jahani, 2019; Zhuang et al., 2019; Bhandari, 2019; Zhu et al., 2019), stand out among nanotechnological products due to their excellent thermal and electrical properties, and high contact surface (Farré et al., 2011). However, one must take into account that their increased use almost inevitably leads to their discharge into the environment and, consequently, to (eco)toxicological risk (Cheng et al., 2007; Du et al., 2013; Myojo and Ono-Ogasawara, 2018). This has been demonstrated in different studies, involving different organisms (e.g.: microorganisms, animal models (including humans) and plants (Filho et al., 2014; Andrade et al., 2014; Maes et al., 2014; Ghosh et al., 2015; Girardi et al., 2017; Beard et al., 2018; Chen et al., 2018; Snyder-Talkington et al., 2019; Requardt et al., 2019; Zhao et al., 2019; Adeyemi et al., 2019; Lee et al., 2019; Knudsen et al., 2019; Farombi et al., 2020; Cheng et al., 2020; Pandey et al., 2020; Gomes et al., 2021)). Overall, these studies gather sufficient evidence to justify the need of paying closer attention to the potential toxicological risks posed by NCs.

However, studies referring to carbon nanotubes (CNTs) are much more abundant, when compared with those that focused on carbon nanofibers (CNFs) (Freixa et al., 2018; Gomes et al., 2021). According to Feng et al. (2014), the CNFs demand lower production cost than CNTs, although their flat edges (along their surface) make physical and chemical interactions with other substances in the environment easier. Therefore, assessing the possible impacts of CNFs on organisms is essential in order to better understand how these nanomaterials can affect natural ecosystems and biota.

Despite this, the study on the effects of CNs on aquatic organisms at early development stages of freshwater species remains a poorly assessed field (Freixa et al., 2018). Exposure to pollution at the early developmental stages can compromise their later life stages and result in reduced survival and reproduction rates (Pechenik, 2006). Knowledge about the toxicity of CNs (especially CNFs) in some groups of animals, such as freshwater benthic macroinvertebrates, still presents a gap. The amount of CNFs deposited in sediments in aquatic ecosystems can present order of magnitude higher than that of the surrounding waters, if one takes into consideration the chemical nature of these nanomaterials and the possibility of particle aggregation in suspension

(Freixa et al., 2018). This process can account for CNF bioavailability increase for organisms living in sediments and for the expansion of their toxicological potential. Previous studies have shown that the impact of pollutants on benthic invertebrates, both in marine and freshwater habitats, is of particular concern, since these organisms correspond to 90% of fish prey biomass (Schindler and Scheuerell, 2002; Weber and Traunspurger, 2015). Thus, evaluating the possible toxicity of nanomaterials in benthic macroinvertebrates is essential to understand the magnitude of their impact on matter's cycle, energy flow and on the primary production of freshwater ecosystems. Small changes in these organisms may be enough to cause ecosystem disturbances that go beyond impacts observed at individual level (Ma et al., 2020).

We aim at testing the hypothesis that environmental concentration of CNFs in aquatic environments for a short period-of-time (48 h) can cause biochemical disorders predictive of oxidative stress, deficit of antioxidant defenses and neurotoxicity in dragonfly larvae (Aphylla williamsoni), as well as bioaccumulate in them. Small biochemical changes can damage the development of this animal model, its fitness and populations (Walker, 1992). Dragonflies belong to order Odonata, which holds one of the largest numbers of ever-cataloged aquatic species (Dijkstra et al., 2014; Juen et al., 2014), presenting important ecological functions, such as population control of several other organisms (Barzoki et al., 2020; Cudera et al., 2020). To the best of our knowledge, the present study is the first to report the impact of CNFs on a species belonging to this taxonomic order, it is an insight on the ecotoxicological risks these pollutants pose to the fauna of benthic macroinvertebrates living in freshwater ecosystems.

2. MATERIALS AND METHODS

2.1. Carbon nanofibers and their featuring

Pyrolytically stripped CNFs (i.e., polyaromatic hydrocarbons removed from fiber surface) were provided by Sigma-Aldrich - San Luis, Missouri, USA. Their detailed chemical features were reported by Gomes et al. (2021). These pollutants are a mixture of different size and shape CNFs [from 60 to 100 nm (mean: 86.85 ± 1.80 nm)], including the ones with open tips (Fig. 1A) and clearly curved (Fig. 1B). In addition, according to the manufacturer and as seen in the photoelectric micrographs taken during the transmission electron microscopy analysis, the assessed CNFs have different metallic particles (Ca, Si, S, Na, Mg and Fe), which are used as catalysts (Fig. 1C).

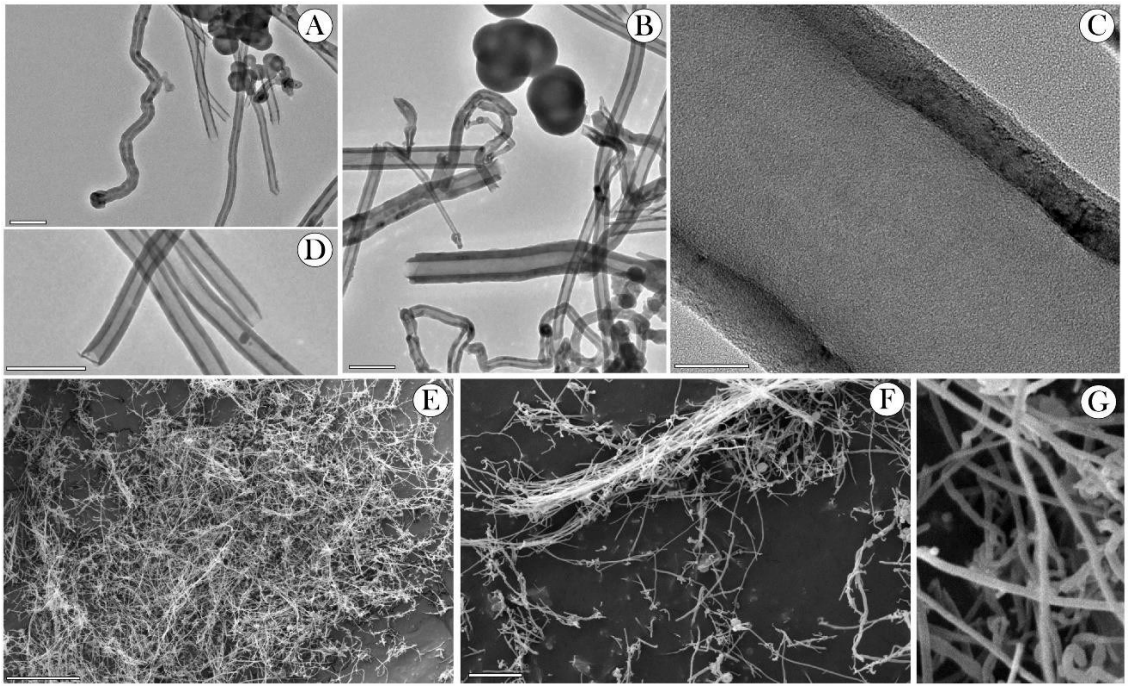

Fig 1 (A–D) Transmission electron microscopy images and (E–G) scanning electron microscopy images of a CNF film, at different magnifications. Scale bars in "A, B and D": 250 nm, in "C": 25 nm; "E": 20 μm; "F": 5 μm and "G": 1 μm. White arrows indicate the presence of metallic particles inside the CNFs or surrounding their surfaces.

2.2. Animal models and experimental design

Aphylla williamsoni larvae (biomass: 0.1025 g ± 0.0089; length: 2.55 cm ± 0.63) were collected in a stream in a permanent preservation area of Federal Goiano Institute (IF Goiano) - Campus Urutaí (GO, Brazil) (Fig. S1 – "Supplementary material"). A. williamsoni, often known as "two-striped forceptail", is a clubtail species belonging to family Gomphidae (Class: Insecta, Order: Odonata) (Gloyd, 1936) and its use in this study is due to its important ecological functions, as described in several previous publications (Mikolajewski and Johansson, 2004; Córdoba-Aguilar, 2008; Bybee et al., 2016; Eagles-Smith et al., 2020). In addition, this species has been considered an interesting animal model in recent (eco)toxicological studies (Chagas et al., 2020; Guimarães et al., 2021). The nymphs were collected, as recommended by Samanmali et al. (2018). The collected nymphs were recorded and transferred to sampling jars filled with water from the same waterbody. The sampling jars were carefully taken to the laboratory and kept in room at 27 °C (±1 °C) under 14/10 h light/dark photoperiod, similarly to Guimarães et al. (2021). The individuals were allowed to acclimate for 10 h in climatic chamber before the experiment, based on Guimarães et al. (2021). This acclimation time regards rough natural conditions. Then, larvae were distributed into two experimental groups (n = 24 replicates, each): (i) control - whose exposure water was CNF-free and (ii) CNF - formed by animals kept under

the same conditions as the control group, but in water added with CNFs, at predictive concentration of 500 μg/L. Such a concentration was defined based on aquatic concentrations of CNTs, given the absence of studies focusing on the determination of environmental concentrations of CNFs. In this case, we based on previous studies that evaluated the toxicity of CNTs in different experimental models, using concentrations ranging from 0.1 to 100 mg/L (Mouchet et al., 2007, 2009, 2010, 2011; Bourdiol et al., 2013; Saria et al., 2014; Verneuil et al., 2015; Zhao et al., 2020; Tavabe et al., 2020). In addition, we consider the monitoring data for MWCNTs in aquatic environments, obtained by Nezhadheydari et al. (2019) and used in the experimental design proposed by Tavabe et al. (2020). According to the authors, the concentrations of these materials vary enormously (ng/L to mg/L), with a concentration of up to 20 mg/L having been identified. Therefore, the concentrations tested in our study are considered environmentally relevant, which approximates our experimental design to a realistic condition of pollution by CNFs.

Animals were exposed to the pollutants for 48 h, in a static system (i.e., without water renewal), in order to simulate ephemeral exposure to them. Each larva (i.e., each replica) was isolated in a beaker filled with 50 mL of de-chlorinated tap water. Individual exposure aimed at avoiding cannibalistic behavior, which is common to the species (Van-Buskirk, 1989). Larvae were not fed during the 48-h exposure time in order to avoid energetic carry-over effects caused by changes in food intake from exposure period to post-exposure period, based on Tollett et al. (2009) and Jinguji et al. (2018). Larvae were weighed and separated in previously-cleaned microtubes at the end of the experiment for further storage in ultra-freezer (−80 °C) until biochemical analysis and pollutant quantification time - 24 h and 48 h after the end of the experiment, respectively.

2.3 Toxicity biomarkers

Predictive biomarkers of nutritional deficit, oxidative stress, interferences in animals' antioxidant systems, and neurotoxicity (via AChE activity) were listed to assess the toxicity of CNFs. Samples were prepared based on Meyer et al. (1986), with modifications. Briefly, each larva was macerated in 1 mL of phosphate-buffered saline (PBS, pH 7.2) (using semi-automatic cell disruptor), centrifuged at 13,000 rpm for 5 min at 4 °C; supernatants were separated in aliquots that were used for biochemical evaluation. All tests were performed in ELISA microplate (96 wells), as detailed below.

2.3.1 Nutritional status assessment

The nutritional status of the animals was assessed in terms of the major macronutrient profiles, which directly correlate to the dietary requirements of larvae. Therefore, similarly to the study by Reeves et al. (2018), we evaluated the levels of total proteins [via Lowry method (Lowry et al., 1951)], triglycerides [via colorimetric method by using glycerol-3-phosphate oxidase (GPO) (Kalia and Pundir, 2004)], and total soluble carbohydrates [via methodology proposed by Dubois et al., 1956] in the supernatant samples obtained previously. According to Guimarães et al. (2021), changes in these parameters may be indicative of metabolic changes essential for the functioning of physiological systems.

2.3.2 Oxidative stress-related parameters

Due to its short lifetime (few milliseconds) and low sub-nanomolar concentration, direct reliable measurements in vivo of NO present great technical difficulties. Thus NO availability is usually estimated based on the amount of its oxidation products, such as nitrite (Piknova et al., 2016). In present study, we used the Griess colorimetric method to quantify the production of nitrite in the samples [see details in Grisham et al. (1998) and Ajjuri and O'Donnell, 2013]. The thiobarbituric acid reactive species (TBARS) test was adopted to measure the lipid redox state, which was performed as detailed in previous studies (Draper and Hadley, 1990; Pothiwong et al., 2007; Carvalho et al., 2019).

2.3.3 Predictive parameters of antioxidant activity and neurotoxicity changes

Total superoxide dismutase (SOD) activity was assessed based on the method proposed by Dieterich et al. (2000) and detailed in Guimarães et al. (2021). The scavenging activity of diphenyl-1- picrylhydrazyl (DPPH) radicals followed the method by BrandWilliams et al. (1995). Trolox (6-Hydroxy-2,5,7,8-tetramethylchro mane-2-carboxylic acid 97, to 1000 μM) was used as positive control, according to Estrela et al. (2021). The enzymatic activity of acetylcholinesterase (AChE) was defined as the predictive neurotoxicity parameter based on the spectrophotometric method by Ellman et al. (1961) [see detailed procedures in Estrela et al., 2021]. Total thiols (nonenzymatic antioxidant) were determined based on procedures described by Santos et al. (2015).

2.4 CNF quantification in Aphylla williamsoni larvae

Considering that the specific quantification of CNs in environmental and biological samples is a great challenge, with no accessible standard methods for quantifying these nanomaterials (Wang et al., 2013; Chang et al., 2014; Bourdiol et al., 2015; Petersen et al., 2016), the accumulation of CNFs was estimated by determining the concentrations of total organic

carbon (TOC), based on Schwab et al. (2011). A detailed description of the procedures adopted can be obtained in Gomes et al. (2021). Furthermore, the bioaccumulation factor (BAF) was computed to investigate likely translocation of CNFs in the exposure water (expressed in kg/L), according to Guimarães et al. (2021).

2.5 Statistical analysis

All collected data were analyzed for normality and homoscedasticity of variances through Shapiro-Wilk and Bartlett tests, respectively. Pairwise comparisons were performed through Student's t-test (when parametric) or Mann-Whitney U test (when data were nonparametric), at 5% probability level. Data about the scavenging activity of DPPH radicals were subjected to one-way ANOVA, with Tukey's post-test, at 5% probability level. Correlation analyses were performed through Pearson (parametric data) or Spearman (non-parametric data) tests, at 5% significance level. All analyses and graphic plotting were performed in GraphPad Prism software (version 7.0).

3. RESULTS

Nutritional status assessment showed that CNFs did not change total soluble carbohydrate (Fig. 2A), total protein (Fig. 2B) and triglycerides (Fig. 2C) concentrations in the larvae, and this finding suggested that these pollutants did not affect their energy metabolism. On the other hand, nitric oxide and thiobarbituric acid reactive species concentrations evidenced that A. williamsoni larvae exposed to the assessed pollutants showed increased oxidative stress reactions (Fig. 3A–B, respectively). These animals also presented increased activity of enzyme superoxide dismutase (Fig. 4A), as well as reduced total thiol levels (Fig. 4B) and scavenging activity of DPPH radicals (%) (Fig. 4C), which indicates likely REDOX imbalance caused by exposure to CNFs.

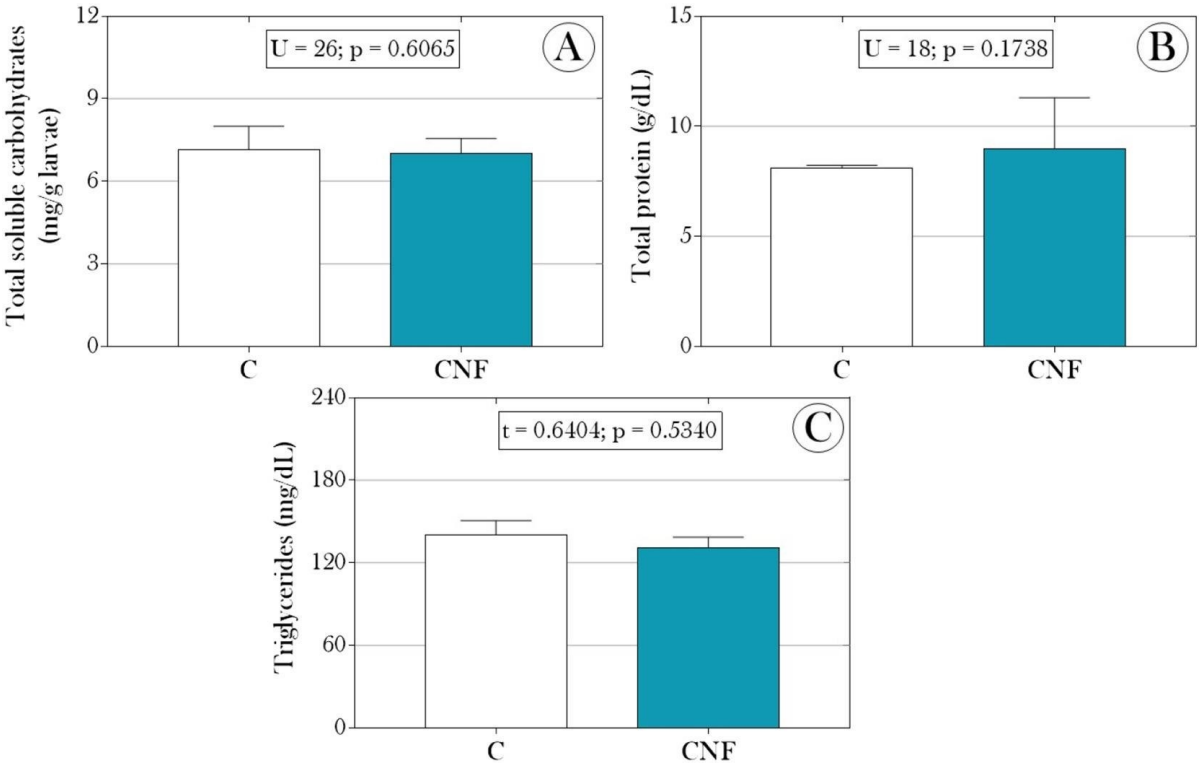

Fig 2. Concentrations of (A) total soluble carbohydrates, (B) total proteins and (C) triglycerides in *A. williamsoni* larvae exposed, or not, to CNFs. Bars indicate mean + standard deviation. Statistical summaries are shown at the top of the graphs. C: control group; CNF: group of *A. williamsoni* larvae exposed to CNFs at concentration of 500 µg/L. *n* = 24 animals/group.

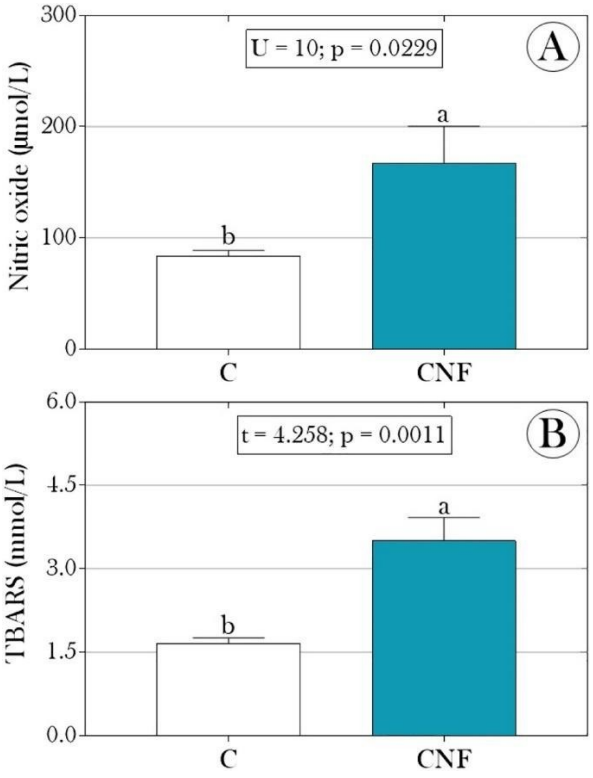

Fig 3. (A) Concentrations of nitric oxide and (B) thiobarbituric acid reactive species (TBARS) in *A. williamsoni* larvae exposed, or not, to CNFs. Bars indicate mean + standard deviation. Statistical summaries are shown at the top of the graphs. Different letters indicate significant difference between experimental groups. C: control group; CNF: group of *A. williamsoni* larvae exposed to CNFs at concentration of 500 µg/L. n = 24 animals/group.

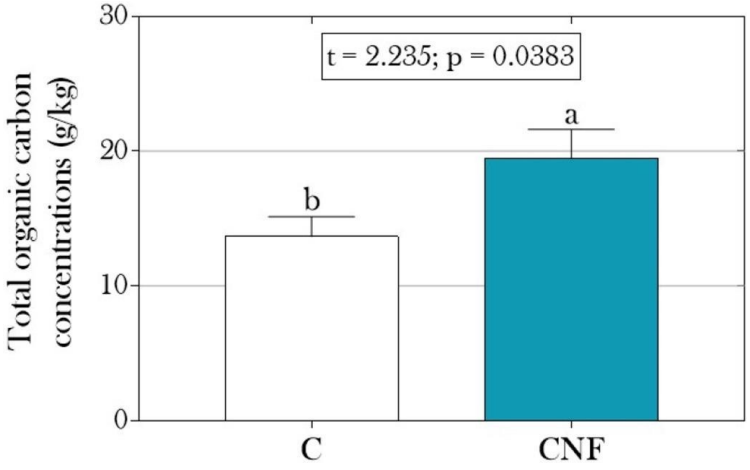

Fig 4. Concentrations of total organic carbon in *A. williamsoni* larvae exposed, or not, to CNFs. Bars indicate mean + standard deviation. The statistical summary is shown at the top of the graph. C: control group; CNF: group of *A. williamsoni* larvae exposed to CNFs at concentration of 500 µg/L. n = 24 animals/group.

Larvae exposed to CNFs showed significant reduction in the activity of enzyme AChE in comparison to the control group, and it suggests neurotoxic effect. The correlation analyses

showed significant correlation between the reported biochemical changes (Table 1) and total organic carbon concentration in the assessed larvae. This finding suggests the accumulation effect of nanomaterials on evaluated biomarkers of animals in the CNFs group (Fig. 5). BAF values in dragonfly larvae ranged from 22,819 to 66,052, per individual (mean BAF value: 38,951 ± 5418), and these numbers indicate high pollutant bioaccumulation in animals.

Table 1. Summary of the correlation analysis between total organic carbon concentrations and predictive REDOX imbalance biomarkers evaluated in *A. williamsoni* larvae exposed to CNFs.

Toxicity biomarkers	Statistical summary[a]			
	Correlation coefficient (r)	95% confidence interval	*P* value (two-tailed)	Significant? (alpha = 0.05)
TOC vs. NO	0.6606 (Spearman)	0.3394 to 0.8438	0.0004	Yes
TOC vs. TBARS	0.5299 (Spearman)	0.1486 to 0.7741	0.0077	Yes
TOC vs. SOD	0.4992 (Spearman)	0	0.0130	Yes
TOC vs. DPPH	−0.4523 (Spearman)	−0.7296 to −0.0471	0.0265	Yes
TOC vs. Thiol	−0.5779 (Spearman)	−0.8004 to −0.2155	0.0031	Yes
TOC vs. AChE	0.4640 (Pearson)	0.0745 to 0.7306	0.0224	Yes

Legend: TOC: total organic carbon; NO: nitric oxide; TBARS: thiobarbituric acid reactive species; DPPH: scavenging activity of DPPH radicals (%); Thiol: total thiol; AChE: activity of enzyme acetylcholinesterase.

[a] The statistical summaries presented refer to significant correlations.

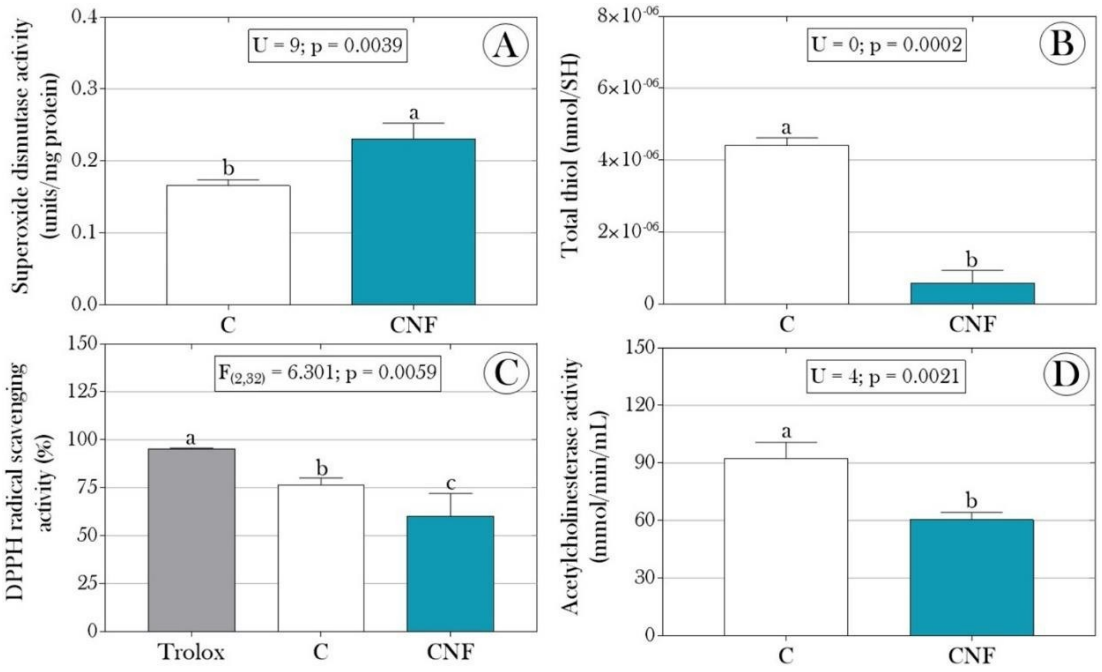

Fig. 5. (A) Superoxide dismutase activity, (B) total thiol levels, (C) scavenging activity of DPPH radicals (%) and (D) acetylcholinesterase activity of *A. williamsoni* larvae exposed, or not, to CNFs. Bars indicate mean + standard deviation. Statistical summaries are shown at the top of the graphs. Different letters indicate significant difference between experimental groups. C: control group; CNF: group of *A. williamsoni* larvae exposed to CNFs at concentration of 500 µg/L. *n* = 24 animals/group.

4. DISCUSION

Identifying the impacts of pollutants on the biota is an important step towards evaluating their (eco)toxicological risks (Landis et al., 2003). Evidences in the present study suggest strong association between CNF accumulation and biochemical changes in A. williamsoni larvae. Such evidences broaden the knowledge about how these nanomaterials affect the fauna of freshwater benthic macroinvertebrates. Moreover, they help inferring the impact of these pollutants on the health of the assessed larvae. Assumingly, their effects are not limited to the larval stage of this species.

The high CNF accumulated in the larvae is a particularly interesting fact, since it indicates that these nanomaterials can be absorbed by A. williamsoni when they are dispersed in water. According to Barzoki et al. (2020) and Cudera et al. (2020), dragonflies are essentially carnivores and lack of food during the experimental period strongly suggested that these pollutants have entered the organisms through water intake or rectal tracheal ramifications, during water entry and exit through the anus, mainly during events causing the fast propulsion of these animals' bodies. Therefore, these entry routes correspond to passive exposure to CNFs, and it had never been reported in the literature, before. Although there are no reports similar to the present one in the literature, the passive absorption of other pollutants by dragonfly larvae is common

pathways for the absorption of different pollutants. Consequently, this process can change the physiological functions of these animals, as observed in studies involving Tramea cophysa larvae exposed to different metals (Dos-Santos-Lima et al., 2019), Somatochlora cingulata exposed to aluminum (Correa et al., 1985), Neurocordelia virginiensis exposed to insect larvicide abate® (temephos) (Anadu et al., 1996) and Bradinopyga geminata exposed to different textile dyeing effluents (Thangaraj et al., 2017). Unlike the present study, and the studies mentioned above, dragonflies can be exposed to chemicals through water, air, soil and diet within natural environments. Therefore, future investigations involving their exposure to CNFs through different exposure pathways are necessary in order to find out how these pathways favor the accumulation of these nanomaterials.

Based on the current data, CNFs absorbed by A. williamsoni larvae have entered different cells and led to the herein observed biochemical responses. According to Møller et al. (2014), there is substantial evidence that CNs can enter the cells either through active processes (e.g., phagocytosis) or passively, by piercing. Therefore, the fat-soluble nature of CNFs favors their penetration through cell membrane and generates intracellular oxidative stress (Mohanta et al., 2019). Assumingly, CNFs in the cytoplasm (free material or in membrane envelopes - vacuoles) induced oxidative stress increase, which was herein inferred based on the high nitric oxide (Fig. 3A) and TBARS (Fig. 3B) levels recorded for larvae in the CNF group. These data corroborate those in other studies (in vitro and in vivo) that have observed oxidative stress induction as one of the possible causes of CN toxicity (Migliore et al., 2010; Shvedova et al., 2012; Long et al., 2012; Hsieh and Jafvert, 2015; Alarif and Ali, 2015; Mohanta et al., 2019). The oxidation of nucleic acids (DNA and RNA), proteins and cellular lipids can have different harmful effects on animals. Reduced total thiol levels (non-enzymatic antioxidant) (Fig. 4B) and DPPH radical scavenging activity (%) (Fig. 4C) are factors reflecting REDOX imbalance caused by the assessed pollutants. The increased SOD activity (Fig. 4A) was not enough to mitigate oxidative stress induced by CNFs. Cells employ primary strategies, such as antioxidant system induction (Monserrat et al., 2007) - which does not seem to have occurred in larvae exposed to CNFs - to preserve overall homeostatic redox balance in the presence of some xenobiotic substances. According to Palma et al. (2020), SOD enzymes catalyze the dismutation of superoxide radical into hydrogen peroxide and molecular oxygen, and consequently, present an important defense mechanism against superoxide radical toxicity. Few studies have evaluated oxidative stress induction in vivo after exposure to CNs, which makes it difficult comparing data in the present study to those in previous reports. According to Møller et al. (2014), the exposure to CNs has been associated with antioxidant depletion, increased intracellular production of reactive oxygen

species and proinflammatory signaling. The mostly used model systems in experimental designs performed in vitro were immune, epithelial, endothelial and stromal cells (Shvedova et al., 2003; Manna et al., 2005; Shvedova et al., 2005; Sarkar et al., 2007; Pacurari et al., 2008; H. Yang et al. 2008; Jacobsen et al., 2008; Thurnherr et al., 2011; Tsukahara et al., 2013; Vesterdal et al., 2014). Oxidative stress investigations with animals are fewer in number and often use CN exposure routes different from that in natural environments. For example, it is unlikely to be exposed to CNFs through intra-nasal instillation (Crouzier et al., 2010) or through intravenously (Ji et al., 2009; S.T. Yang et al. 2008) and intraperitoneal routes (Patlolla et al., 2010; Nagai et al., 2013), as previously evaluated. Such studies are essential to broaden the knowledge about the action mechanisms of CNs and about how these pollutants enter the cells. However, their applications to real pollution contexts are very limited. This statement allows including the present study in the group of few studies about oxidative stress induction in vivo after models' exposure to CNs at ecologically relevant conditions. Other interesting data in the present study refer to AChE reduction in larvae exposed to CNFs, which suggests the anticholinesterase action of these pollutants - this finding corroborates reports in studies with other CN types and model systems, either in vitro (J. Wang et al., 2009; Z. Wang et al., 2009) or in vivo (Da-Rocha et al., 2019). According to Colovic et al. (2013), this enzyme hydrolyzes the neurotransmitter acetylcholine (ACh), which is one of the most crucial enzymes for nerve response and function. Therefore, acetylcholine (ACh) inhibition causes Ach accumulation, which influences the control of many physiological and behavioral responses in insects and, eventually, can lead to respiratory failure and death (Breer and Sattelle, 1987; Breer et al., 1989; Gauthier, 2010; Grünewald and Siefert, 2019). The literature is rich in studies that have identified AChE reduction induced by different xenobiotics in aquatic organisms exposed to them (Rickwood and Galloway, 2004; Brahma and Gupta, 2020; Barreto et al., 2020; Umar and Aisami, 2020; Dahms-Verster et al., 2020), including dragonfly

larvae (Guimarães et al., 2021). The action mechanisms linked to the herein observed inhibition is preliminary, therefore, future investigations are important to broaden knowledge on the topic. However, previous reports have shown CNs' compatibility to AChE (Liu and Lin, 2006, Yan et al., 2013, Chen et al., 2017) and the ability of AChE to attach to the surface of these nanomaterials. Studies conducted in vitro by J. Wang et al. (2009) and Z. Wang et al. (2009) have confirmed the present findings and showed that CNs-AChE adsorption reduced by 76%– 88% the activity of this enzyme, and this outcome suggests that a similar process may explain the AChE reduction in the larvae in the CNF group. On the other hand, it is tempting speculating that AChE reduction is related to endocrinological changes [see details in Rattner and Fairbrother (1989)]

indirectly caused by CNFs. Moreover, one cannot neglect the hypothesis of CNF's influence on gene expressions linked to AChE synthesis and release, such as ace genes (Ye et al., 2017). According to Kim and Lee (2013), the biological functions of the two ace genes have been assessed in insects. Overall, AChE1 is the major enzyme in insects, it is even more abundant than AChE2. The expression of ace1 is higher than that of ace2 in some insect species (Kim et al., 2006; Jiang et al., 2009; B.M. Kim et al., 2015; J.S. Kim et al., 2015), whereas the opposite is also true, in other species. It is necessary taking into consideration that, regardless of CNF's action mechanisms, any anticholinesterase effect on insects is alarming, given the central role of AChE in controlling different physiological functions. AChE expression silencing in different experimental insect models (Kumar et al., 2009; Revuelta et al., 2009; Hui et al., 2011; He et al., 2012) caused, among other factors, high mortality, growth inhibition, malformations, drastically reduced fertility and behavioral disorders. Such effects, in addition to affect the larval development in these animals, can drastically affect individuals' fitness and the dynamics of populations living in polluted areas.

5. CONCLUSION

The initial hypothesis that ephemeral exposure to CNFs leads to their accumulation in A. williamsoni larvae, as well as to biochemical changes predictive of REDOX imbalance and neurotoxicity (inferred by reduced AChE activity), even at low concentration (500 μg/L), was confirmed. Although it is just a preliminary study, its data are valuable in order to better understand the magnitude of (eco)toxicological impacts of CNFs on benthic macroinvertebrates, mainly due to their potential consequences at biochemical/physiological and behavioral level. Future studies with new investigative approaches (e.g.: mutagens, genotoxic, behavioral and molecular), both in larval stage and in adults, will help broadening the knowledge on how the macrofauna of benthic invertebrates is affected by NCs, which would provide subsidies for the proposition and/or adoption of pollution mitigation and/or remediation strategies. Supplementary data to this article can be found online at https://doi. org/10.1016/j.scitotenv.2020.143991.

6. CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

The authors indicated in parentheses made substantial contribution to the following tasks of research: initial conception (Malafaia G.); design (Malafaia G; Montalvão MF); provision of

resources (Malafaia G; Rodrigues ASL), collection of data (Montalvão MF, Guimarães ATB), analysis and interpretation of data (Malafaia G), writing and revision of paper (Montalvão MF, Guimarães ATB, Rodrigues ASL, Malafaia G).

7. 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.

8. ACKNOWLEDGMENT

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9. COMPLIANCE WITH ETHICAL STANDARDS

Ethical approval: All experimental procedures were carried out in compliance with ethical guidelines related to animal experimentation. 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.

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CAPÍTULO 3 - LONG-TERM EXPOSURE OF ZEBRAFISH JUVENILES TO CARBON NANOFIBERS AT PREDICTED ENVIRONMENTALLY RELEVANT CONCENTRATIONS: OUTSPREADING WARNS ABOUT ECOTOXICOLOGICAL RISKS TO FRESHWATER FISH[2]

ABSTRACT

Although carbon-based nanomaterials (CNMs) toxicity has already been demonstrated in some animal models, little is known about the impact of carbon nanofibers (CNFs) on aquatic vertebrates. Thus, we aimed to evaluate the possible effects of long-term exposure of zebrafish (*Danio rerio*) juveniles (90 days) to CNFs in predicted environmentally relevant concentrations (10 ng/L and 10 µg/L). Our data revealed that exposure to CNFs did not affect the growth and development of the animals, in addition to not having induced locomotor alterations or anxiety-like behavior. On the other hand, we observed that zebrafish exposed to CNFs showed a response deficit to the vibratory stimulus test, alteration in the density of neuromasts recorded in the final ventral region, as well as an increase in thiobarbituric acid reactive substances levels and a reduction in total antioxidant activity, nitric oxide, and acetylcholinesterase activity in the brain. Such data were directly associated with a higher concentration of total organic carbon in the brain, which suggests the bioaccumulation of CNFs. Furthermore, exposure to CNFs induced a picture suggestive of genomic instability, inferred by the increased frequency of nuclear abnormalities and DNA damage in circulating erythrocytes. Although the individual analyses of the biomarkers did not point to a concentration-dependent effect, the principal component analysis (PCA) and the Integrated Biomarker Response Index (IBRv2) indicate a more prominent effect induced by the higher CNFs concentration (10 µg/L). Therefore, our study confirms the impact of CNFs in the studied model (*D. rerio*) and sheds light on the ecotoxicological risks of these nanomaterials to freshwater fish. Based on the ecotoxicological screening provided by our study, new horizons are opened for investigations into the mechanisms of action of CNFs, which will help understand the magnitude of the impact of these materials on aquatic biota.

Keywords: carbon-based nanomaterials, *Danio rerio*, ecotoxicology, biomarkers, aquatic pollution.

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1. INTRODUCTION

Currently, carbon nanomaterials (CNMs) – defined as materials with sizes ranging from 1 to 100 nm – have received significant interest in nanotechnology, considering their unique properties and flexible dimensional structure (Gaur et al., 2021; Sridharan et al., 2022; Boruah & Chowdhury, 2023). As highlighted by Musa et al. (2023), the improvement of the characteristics of CNMs (e.g., optical activity, multifunctional surface morphology, surface area, drug loading efficiency, biocompatibility, and immunogenicity) allows the use of these materials in several sectors, including biomedicine (Jiang et al., 2023; Owida et al., 2023), gas sensors (Hejazi et al., 2023), water purification (Fan et al., 2023), imaging (Mohammadi et al., 2022), and bioelectrochemical applications (Holzinger et al., 2023), etc.

Among the CNMs, Gaur et al. (2021) point out that carbon nanofibers (CNFs) – diameter range, 3–100 nm; length range, 0.1–1000 μm (De-Jong & Geus, 2000) – are ideal candidates for next-generation on-chip connection materials, as well as potential immobilization substrates, due to their unique chemical and physical characteristics. In addition, when compared to carbon nanotubes (CNTs), CNFs are less expensive, preferred for electrical and thermal conductivity (since they have a higher degree of crystallographic alignment) in addition to having a low defect rate, large proportion, and a vast surface area (Ozkan et al., 2010). Its use in biosensors, tissue engineering, wound dressings, energy conservation, nano-monitoring devices, the textile industry, gas and pressure sensors, batteries, and supercapacitors has been reported in different studies [see review by Yadav et al. (2020)].

However, the increasing use of CNFs in manufacturing different materials implies an increase in their disposal and potential release in natural ecosystems. Although some studies point to the low-toxicity or non-toxicity of CNFs in the biological systems in which they were tested (e.g., Smolka et al., 2019; Mehrabi et al., 2019; Salesa et al., 2020; Nekounam et al., 2020); the toxicity of these nanomaterials has been reported *in vitro* studies involving different cell types [e.g., lung tumor cells (Magrez et al., 2006; Mittal et al., 2017), RBE4 (rat brain endothelial cells) (Jensen et al., 2012); topminnow fish hepatoma cell line (PLHC-1) and on carp leukocyte cell line (CLC) (Kalman et al., 2019); human THP 1 cell line (Brown et al., 2007); and MG-63 cells (Samadian et al., 2020), among others]. However, the potential ecotoxicological effects of CNFs have only been reported more recently, with little ecological representation of the organisms tested so far. The vast majority of ecotoxicological studies involving CNMs were developed with CNTs, which differ from CNFs in several characteristics and, therefore, can also induce

differentiated ecotoxicological effects (Mouchet et al., 2007; Pérez et al., 2009; Petersen et al., 2012; Eckelman et al., 2012; Jackson et al., 2013; Campos-Garcia et al., 2015; Cerrillo et al., 2015; Calisi et al., 2016; Cerrillo et al., 2016; Zhang et al., 2021; Zhao et al., 2021; Gamoń et al., 2022; Das et al., 2023).

Pikula et al. (2020), when evaluating the potential biochemical effects induced by CNFs in four marine microalgae species, reported high sensitivity only of the red microalgae *Porphyridium purpureum* to CNFs, suggesting that these nanomaterials can bind to membranes of microorganisms by hydrophobic interaction and hydrogen bonding formed between surfaces of cells and defect areas of CNFs. In Guimarães & Malafaia (2021), the authors demonstrate the chronic effects (7.5 months) of 1 and 10 mg/L of CNF on *Podocnemis expansa* (Amazon turtle) juveniles based on biochemical, mutagenic, genotoxic, cytotoxic, and neurotoxic biomarkers. Furthermore, negative impacts of CNFs on the health of *Physalaemus cuvieri* tadpoles [after 48-h exposure, at 1 and 10 mg CNFs/L – Guimarães et al. (2021b)] and in *Aphylla williamsoni* larvae [after ephemeral models' exposure to CNFs (48 h; at 500 µg/L) (Montalvão et al., 2021)]. Furthermore, Gomes et al. (2021) observed that CNFs are transferred along the food chain, causing mutagenic and cytotoxic damage at the upper trophic level (*Oreochromis niloticus*). On the other hand, histological examinations performed on three freshwater invertebrate species (*Diamesa* sp., *Drunella cryptomeria*, and *Gammarus suifunensis*) exposed to CNFs (at 100 mg/L for seven days) did not reveal any toxic effect of nanofibers (Chaika et al., 2020). In mussels (*Mytilus edulis*) exposed to the CNFs (0.01, 0.1, and 1 mg/L, for 24 h), limited statistical significance was observed between the control, suggesting little effects of the tested CNFs (GANF, GATam, and GANFg) (Barrick et al., 2019). Therefore, this inconclusive scenario denotes the need to expand studies on the ecotoxicity of CNFs to assess the risks associated with the interaction of these nanomaterials with biota and, therefore, to determine their eco-safety.

Thus, aiming to contribute to increasing knowledge about the impacts of CNFs on freshwater fish, we aimed to evaluate the possible effects of prolonged exposure (90 days) of juvenile zebrafish (*Danio rerio*) to different concentrations of CNFs. As highlighted by Verma et al. (2021), zebrafish is one of the most used freshwater fish as an ecotoxicological model for nanomaterial-induced toxicity profiling and having important ecological functions in their natural environments (Spence et al., 2008), which justifies their choice as a system model in this study. We start from the hypothesis that even in small concentrations, CNFs are captured by zebrafish, induce behavioral changes, as well as in the density of mechanoreceptors called neuromasts (lateral line organs), brain biochemical changes, erythrocyte nuclear abnormalities, DNA damage

and growth impairment/animal development.

2. MATERIALS AND METHODS

2.1 Carbon nanofibers

In the present study, pyrolytically stripped CNFs, purchased at Sigma-Aldrich (San Luis, Missouri, USA, PR-25-XT-OS, MDL number: MFCD00133992) were used. The characterization of the CNFs [presented in a previous study by our group (Gomes et al., 2021)] revealed that the CNFs presented distinct formats (including the ones presenting open ends and with pronounced curvatures) and a mean diameter of 86.85 ± 1.80 nm [mean ± standard error of the mean (SEM)]. Raman spectroscopy analysis revealed the presence of the D band, which refers to defective graphite structures (1336 cm^{-1}), and the G band (1750 cm^{-1}), which refers to graphite crystalline structures, in addition to the G′ band (2654 cm^{-1}). In addition, the scanning electron microscopy technique showed the presence of metallic particles used as catalysts [Ca (140 ppm), Si (30 ppm), S (10,200 ppm), Na (40 ppm), Mg (40 ppm), and Fe (11,372 ppm) [see characterization details in Gomes et al. (2021)].

2.2 Animals and experimental design

This study was conducted at the Laboratory of Toxicology Applied to the Environment of the Instituto Federal Goiano - Campus Urutaí (GO, Brazil), using mixed-sex juvenile zebrafish (D. rerio – wild lineage), aged approximately 3-4 months and average body weight of 0.27 ± 0.07 g. A commercial farm provided fish specimens (Goiânia, GO, Brazil) and, upon arrival at the laboratory, were acclimatized for 15 days. During the acclimatization period, the animals were kept collectively in aquariums (dimensions: 85 cm long x 40 cm wide x 40 cm high) filled with 19 L of naturally dechlorinated water, with constant aeration, light/dark photoperiod of 12 hours:12h and temperature of 26±1°C. The animals were fed twice a day, with commercial fish feed, corresponding to 2.5% of their live body biomass, according to Lawrence et al. (2012). Aquarium waters were completely replaced every three days.

After the acclimatization period, 90 healthy zebrafish (male: female ratio 1:1) (i.e., presenting normal swimming behavior and no morphological deformities or apparent lesions) of similar size and body weight were distributed into three experimental groups [each group was composed of four replicates (n=10 animals/replica)]. The “control” group consisted of zebrafish kept in dechlorinated water free of CNFs. In the “CNF-I” and “CNF-II” groups, CNFs were

added to the exposure water at 10 ng/L and 10 µg/L, respectively. Such concentrations were based on studies on multi-walled carbon nanotubes (MWCNTs) monitoring in aquatic environments. To different wastewater types, the concentrations of these nanomaterials can have high variations – up to 20 mg/L (Nezhadheydari et al., 2019; Tavabe et al., 2020). We also emphasize that the decision to base the tested concentrations on CNT concentrations is justified because there is no study, so far, that has identified environmental concentrations of CNFs in aquatic environments. Therefore, the concentrations tested here are "predictive environmentally relevant concentrations", making our experimental design closer to realistic pollution conditions by CNFs. The experimental groups were exposed to the previously described conditions in glass aquariums (dimensions: 46 cm long x 31 cm wide x 16 cm high) containing 19 L of dechlorinated water with ("CNF-I" and "CNF-II" groups) or without the CNFs ("control" group). The animals remained exposed to the nanomaterials for 90 days (long-term exposure) in a semi-static condition, i.e., with the complete renewal of the exposure water every three days.

2.3 Toxicity biomarkers

2.3.1 CNFs-induced neurotoxicity

2.3.1.1 Locomotion and anxiety-like behavior

To evaluate the possible effect of exposure to CNFs on the behavior of the animals, at the end of the 90 days of exposure, 18 animals from each group were submitted to the open field test (OFT), according to Chagas et al. (2021), with some modifications. Briefly, the test consisted of introducing each animal individually into a circular arena with opaque walls (Ø = 15 cm) containing dechlorinated water without any pollutant (at 26±1°C) (water depth = 3 cm) and following pre-established protocol (Estrela et al., 2021b). The behavior of each animal was filmed for 3 min from a camera (coupled to a computer external to the test room) installed 1.5 m above the arena. The test room had acoustic insulation, luminosity, and temperature (26 ± 1°C) controlled. From the footage, the time the animal spent in proximity to the wall of the experimental arena was quantified and used to calculate the anxiety index (Equation 1), aiming to evaluate the possible induction of anxiogenic or anxiolytic effects in the animals by the CNFs. The total number of quadrant crossings virtually traced on the computer screen was recorded as locomotor/exploratory activity of the animals. PlusMZ software was used for behavior recording, like Freitas et al. (2023).

$$\text{Anxiety index} = \left[\frac{\left(\frac{\text{Time spent peripheral zone (s)}}{\text{Total test time (300s)}}\right) \text{x } 100}{\text{Total crossing}}\right] \text{x } 100$$

2.3.1.2 Response to vibratory stimulus

Five minutes after the OFT, we evaluated whether exposure to CNFs was able to induce changes in the animals' mechanisms for detecting vibration stimuli mediated by the lateral line system, i.e., an additional sensory organ for vibration detection (Lush & Piotrowski, 2014; Wang et al., 2021). For this, we adopted the procedures detailed in Guimarães et al. (2021d) (with minor modifications), in which an electromagnetic compressor (model ACQ-003, pressure: 0.028 Mpa; airflow: 50 L/min; dimensions: 12 cm length x 10 cm width and 12 cm height) was used as a provider of the vibratory stimulus. This compressor was installed 10 cm from the apparatus used in the OFT. The test consisted of turning on the air compressor thrice, for 10 s, at intervals of 1 min. Subsequently, the locomotor activity of the animals was recorded before (i.e., during the 60 s preceding the first vibratory stimulus) and after each subsequent vibratory stimulation (1st min, 2nd min, and 3rd min).

2.3.1.3 Response to vibratory stimulus

The possible impact of exposure to CNFs on neurosensory functions was assessed by quantifying neuromasts in different regions of the body of zebrafish (n=10 animals/group), which are considered an excellent system for studying ototoxicity (damage of the ear) since their peripheral sensory neurons are in direct contact with the surrounding water containing pollutants (Froehlicher et al., 2009). For this, we adopted the procedures described in detail in the study by Guimarães et al. (2021a), using 4-(4-diethylaminostyryl)-1-methylpyridinium iodide (4-Di-2-ASP) for specific labeling of neuromasts. Neuroblasts positive for 4-Di-2-ASP were counted in the region corresponding to the terminal neuromasts (T1, T2, and T3) of each animal's lateral caudal line system using an epifluorescence microscope. In addition, the neuromasts of the ocular region and the final ventral region of the animals were counted. After that, the indices listed in Table 1 were calculated.

Table 1. Neuromasts index calculated in zebrafish exposed or unexposed to carbon nanofibers.

Index	Formula
Penduncular neuromasts index	$\frac{\text{Number of neuromasts penduncular region (T1 + T2 + T3)}}{\text{Total length (cm)}}$
Ocular neuromasts index	$\frac{\text{Number of neuromasts ocular region}}{\text{Head length}}$
Final ventral region neuromasts index	$\frac{\text{Number of neuromasts (final ventral region)}}{\left[\frac{\text{Body depth + penduncle depth}}{2}\right]}$

2.3.1.4 Brain biochemical assessments

2.3.1.4.1. Sample processing

Different biochemical biomarkers in the brain of animals were evaluated, aiming to associate the possible biochemical changes induced by exposure to CNFs in the behavioral response of animals. Thus, the previous sample preparation consisted of macerating 0.0040 g of the brain of the animals (n=16 fish/group) in phosphate-buffered saline (PBS), like Guimarães et al. (2021a). Afterward, the samples were centrifuged at 13000 rpm for 10 min at 4oC, and the supernatants were collected and stored at -80oC until use. Sample processing and biochemical analyzes were performed in the dark.

2.3.1.4.2. Biochemical biomarkers

Predicting some association between exposure to CNFs and the induction of oxidative stress in the brain of animals, the thiobarbituric acid reactive substances (TBARS) levels and the total antioxidant capacity were evaluated. Oxidative lipid stress (inferred through increased TBARS levels) was assessed based on the method proposed by Pothiwong et al. (2007), modified by Guimarães et al. (2021d). The total antioxidant activity in the brain was estimated by the DPPH (2,2-diphenyl-1-picryl-hydrazine-hydrate) free radical method, as described in Guimarães et al. (2023). Furthermore, nitric oxide production was evaluated (aiming to infer possible CNF-induced nitrosative stress) based on the method by Grisham et al. (1998), adapted by Araújo et al. (2022). On the other hand, the potential anticholinesterase effect associated with exposure to CNFs was assessed using acetylcholinesterase (AChE) activity, according to Ellman's spectrophotometric method (Ellman et al., 1961).

2.3.2 CNFs-induced mutagenicity

The possible CNFs-induced mutagenic effect was evaluated by the micronucleus and other nuclear abnormalities of erythrocytes test, according to procedures described in Guimarães et al. (2021c). The test consisted of collecting 3 µL of blood (cutting the caudal peduncle after ice anesthesia) and making a blood smear on a previously sanitized glass slide. Subsequently, the slides were dried at room temperature and fixed with methanol P.A. Then, the slides were stained with the Panotic Rapid® kit (Laborclin®, Paraná, Brazil, code #620529 – reagent #1: triarylmethane, at 1 g/L in methanol; reagent #2: xanthenes, at 1 g/L in deionized water, and reagent #3: thiazines, at 1 g/L in deionized water), like Araújo et al. (2023). In an optical microscope at 100 × magnification, 1000 cells/slide (totaling 13000 erythrocytes/group analyzed; n=13 animals/group) were based on the criteria reported by Fenech (2007). In addition, the presence of micronuclei and other erythrocyte nuclear abnormalities were recorded, according to the nomenclatures adopted by Braham et al. (2017) and Araújo et al. (2022).

2.3.3 CNFs-induced genotoxicity

Assuming that CNFs also induce DNA damage, blood samples from nine animals/group were subjected to alkaline comet assay (single-cell gel electrophoresis), which is considered a sensitive method for assessing DNA damage at the single-cell level (Pu et al., 2015) and used to assess the genotoxicity of different pollutants in zebrafish [see review by Canedo & Rocha (2021)]. We adopted the procedures detailed in Araújo et al. (2022) for this. Fifty randomly selected nucleoids per animal (totaling 450 nucleoids/group) were evaluated from photomicrographs processed in the Casp Lab software (version 1.2.3) [like Souza et al. (2017)]. The parameters "tail DNA %" (tail DNA content as a percentage of comet DNA content), "tail length" (length of the tail in pixels), and "Olive tail moment", calculated as OTM = (Tail. Mean – Head .mean) × (Tail %DNA)/100 (Wang et al., 2013).

2.3.4 CNF-induced growth and developmental toxicity

To assess whether exposure to CNFs altered the growth/development of the animals, at the end of the experiment, 12 animals from each group were euthanized (on ice), fixed in formaldehyde (at 10%) for 24 h, and then photographed using a microscope stereoscope. After that, the following were measured: total, fork, and standard length, predorsal length, head length, body depth, peduncle depth, preorbital length, and eye diameter, using ImageJ software, like

Chagas et al. (2021). Such measurements were used to calculate different body condition indices (Table 2). arm in the Goiás state, Brazil, during December 2017 – January 2021. The region has a seasonal climate (AW in the Köppen-Geiger classification), with a rainy summer and a dry winter (Alvares et al. 2013).

Table 2. Biometric indices calculated in zebrafish exposed or unexposed to carbon nanofibers.

Index	**Formula**
Total length index	$\frac{\text{Total length (cm)}}{\text{Body biomass (g)}}$
Fork length index	$\frac{\text{Fork length (cm)}}{\text{Body biomass (g)}}$
Standard length index	$\frac{\text{Standard length (cm)}}{\text{Body biomass (g)}}$
Predorsal length index	$\frac{\left[\frac{\text{Predorsal length (cm)}}{\text{Average total, fork, and standard length (cm)}}\right]}{\text{body biomass}}$
Head length index	$\frac{\left[\frac{\text{Head length (cm)}}{\text{Average total, fork, and standard length (cm)}}\right]}{\text{body biomass}}$
Body depth index	$\frac{\left[\frac{\text{Body depth (cm)}}{\text{Average total, fork, and standard length (cm)}}\right]}{\text{body biomass}}$
Peduncle depth index	$\frac{\left[\frac{\text{Penduncle length (cm)}}{\text{Average total, fork, and standard length (cm)}}\right]}{\text{body biomass}}$
Preorbital length index	$\frac{\left[\frac{\text{Preorbital length (cm)}}{\text{Head length (cm)}}\right]}{\left[\frac{\text{Average total, fork, and standard length (cm)}}{\text{Body biomass (g)}}\right]}$
Eye diameter index	$\frac{\left[\frac{\text{Eye diameter (cm)}}{\text{Head length (cm)}}\right]}{\left[\frac{\text{Average total, fork, and standard length (cm)}}{\text{Body biomass (g)}}\right]}$
Body condition score	(Eq 1 + Eq 2 + Eq 3 + Eq 4 + Eq 5 + Eq 6 + Eq 7 + Eq 8 + Eq 9)

2.4 Uptake of carbon nanofibers

Aiming to associate the response of the animals to the biomarkers previously described to the uptake of CNFs, the total organic carbon (TOC) concentrations in zebrafish (n=12 animals/group) were considered an indirect measure of the uptake of CNFs. For this, the Walkley-Black method was used, based on using dichromate (Cr_2O7^{2-}) (Cr VI) as an oxidizer in an acid medium (Walkley & Black, 1934), according to procedures described in detail in Gomes et al. (2021) and Guimarães & Malafaia (2021).

2.5 Data analysis

The comparison of the means of the results obtained between the experimental groups was performed using the one-way ANOVA test (with Tukey post-test) or Kruskal-Wallis test (with Dunn's post-test), defined based on the distribution of residual data (assessed using the Shapiro-Wilk test) and homogeneity of variances (assessed using the Bartlett test). Furthermore, correlation analyses (via Spearman or Pearson coefficients) and linear regression were performed. Significance levels were set at Type I error (p) values lower than 0.05, and GraphPad Prism software Version 9.0 was used to perform the statistical analyzes. Biomarker data were also explored based on principal component analysis (PCA) using GraphPad Prism software Version 9.0, like the procedures described in Freitas et al. (2023). Such an analysis was essential to assess whether certain combinations of key variables account for differences between, in our case, experimental groups. In all PCA analyses in this work, the outliers' values (identified via the Grubbs test) were excluded from the original data and sequentially logarithmized before PCA analysis. Ward's hierarchical clustering method was also used to identify the group distributions according to the variables on the PCA results. Furthermore, the results of all biomarkers were applied to the second-generation "Integrated Biomarker Response" index (IBRv2) to transform the different responses of animals to treatments from the different biomarkers evaluated into a single value, allowing them to analyze their sensitivities to each treatment comparatively. For this, we used the method adopted by Malafaia et al. (2022), based on Sanchez et al. (2013).

3. RESULTS

Initially, no animal deaths were observed throughout the experimental period in the different experimental groups, which confirms that exposure to CFNs did not induce a lethal effect on zebrafish. Furthermore, the locomotor activity of animals exposed to CNFs did not differ from that recorded in unexposed zebrafish ("control" group) (Figure 1A). Furthermore, using OFT, we did not observe results suggestive of the induction of anxiety- or anxiolytic-like behavior in animals from the "CNF-I" and "CNF-II" groups (Figure 1B). On the other hand, we noticed that exposure to CNFs induced a different behavior in the vibratory response test. As shown in Figure 1C, only the animals in the "control" group decreased locomotion activity throughout the test, following a simple linear regression model. In these animals, the locomotor activity evaluated before and after the vibratory stimulus differed significantly, which was not observed in the other experimental groups (Figure 1D). We also observed that, although the

number of neuromasts in the ocular (Figure 2A-B) and peduncular (Figure 2C-D) regions did not differ between the experimental groups, a greater number of neuromasts in the final ventral region was observed in animals exposed to CNFs (Figure 2E-F).

Regarding the biochemical evaluations carried out in the animals' brains, we observed a significant increase in MDA levels induced by exposure to CNFs (Figure 3A), a suppression of total antioxidant activity [inferred by the lower DPPH radical scavenging activity (Figure 3B)], as well as of NO production (Figure 3C). Furthermore, a CNFs-induced anticholinesterasic effect was observed in animals from the "CNF-I" and "CNF-II" groups, which was marked by a significant reduction in AChE activity (Figure 3D). Such results were significantly correlated with the TOC concentrations observed in the animals, which were higher in animals exposed to CNFs, which reinforces the association between the uptake of CNFs and the reported biochemical changes (Figure 4).

On the other hand, we observed a mutagenic effect (concentration-dependent) induced by exposure to CNFs, inferred by the linear increase in the frequency of erythrocyte nuclear abnormalities in zebrafish (Figure 5A-B). Although micronucleus formation was not identified, different types of other nuclear abnormalities were identified in animals exposed to nanomaterials, including constricted nuclei (Figures 5D and 5G), blebbed nuclei (Figure 5E), kidney-shaped nuclei (Figure 5F), notched nuclei (Figure 5H), moved nuclei (Figure 5I), and nuclear vacuoles (Figure 5J) (Table 3). Furthermore, we observed in the "CNF-I" and "CNF-II" groups an increase in all the parameters evaluated in the comet assay without, however, having observed a concentration-dependent effect (Figure 6).

As for the biometric biomarkers, we noticed that the animals in all experimental groups showed an increase in body biomass at the end of the exposure period compared to the initial biomass (Figure 7A). However, we did not observe differences between the experimental groups regarding biomass (Figure 7A) and body condition score (Figure 7B) evaluated at the end of the experiment. In addition, the body condition indices evaluated separately did not differ between the groups exposed or unexposed to CNFs (Table 4), suggesting that exposure to nanofibers did not affect the growth/development of the animals.

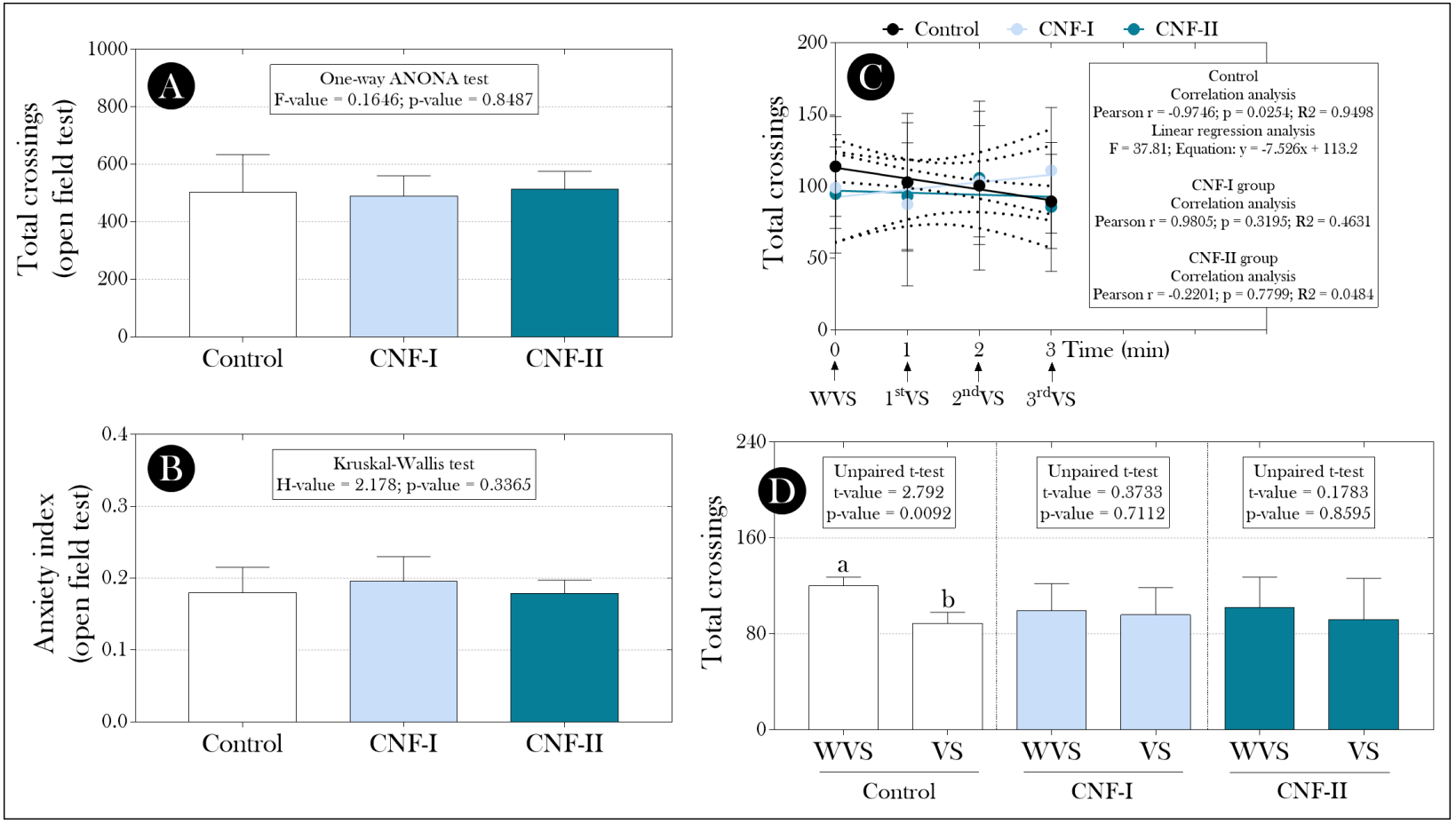


Figure 1. Concentrations of total organic carbon in *A. williamsoni* larvae exposed, or not, to CNFs. Bars indicate mean + standard deviation. The statistical summary is shown at the top of the graph. C: control group; CNF: group of *A. williamsoni* larvae exposed to CNFs at concentration of 500 µg/L. n = 24 animals/group.

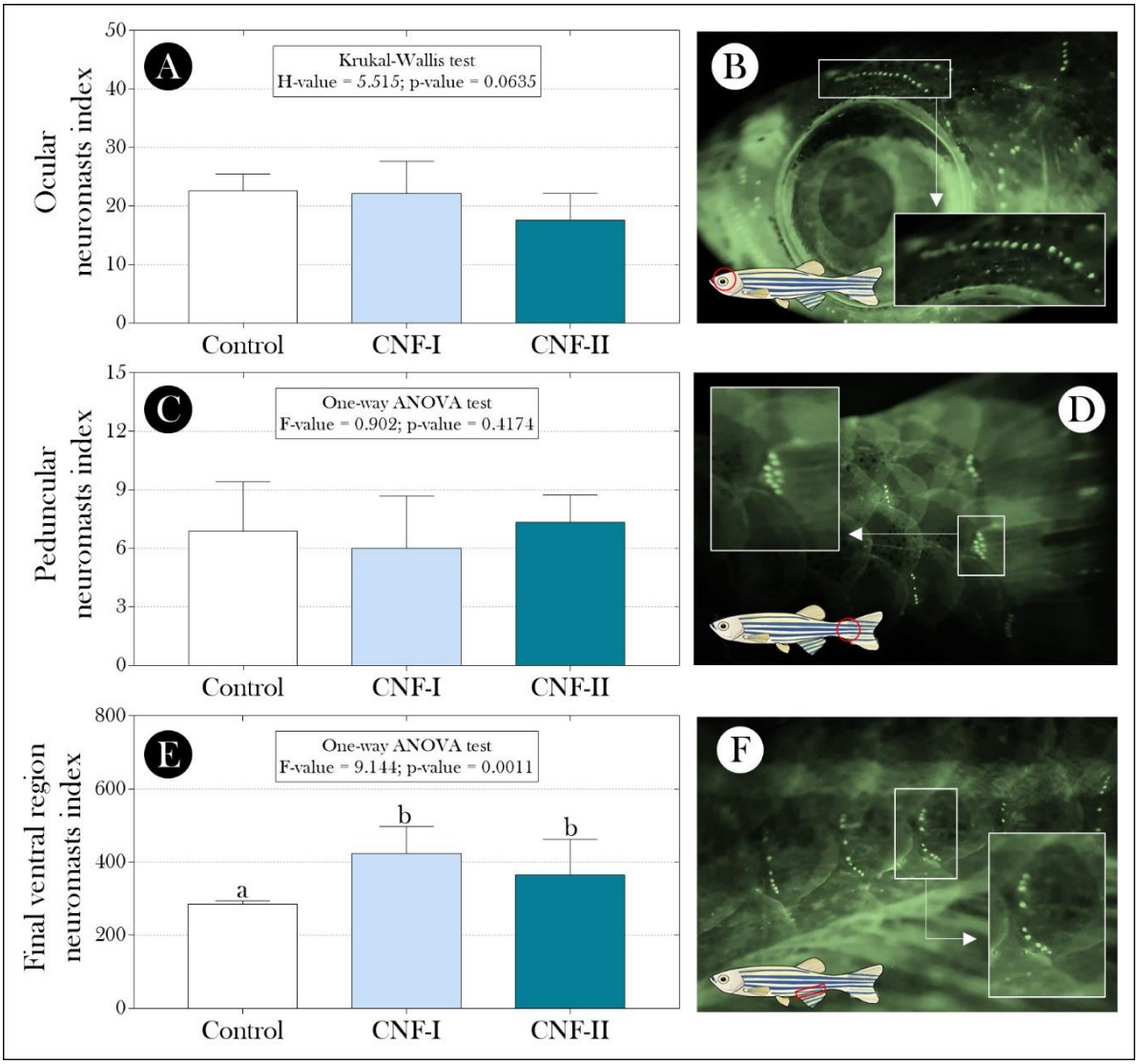


Figure 2. Neuromasts index in different regions of *Danio rerio* (zebrafish) unexposed or exposed to carbon nanofibers (CNF) at different concentrations. (A-B) Ocular, (C-D) peduncular, and (E-F) final ventral region. Parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Statistical summaries are displayed at the top of the graphs. Distinct lowercase letters indicate significant differences. CNF-I and CNF-II: composite groups of zebrafish exposed to CNFs at 10 ng/L and 10 µ/L, respectively.

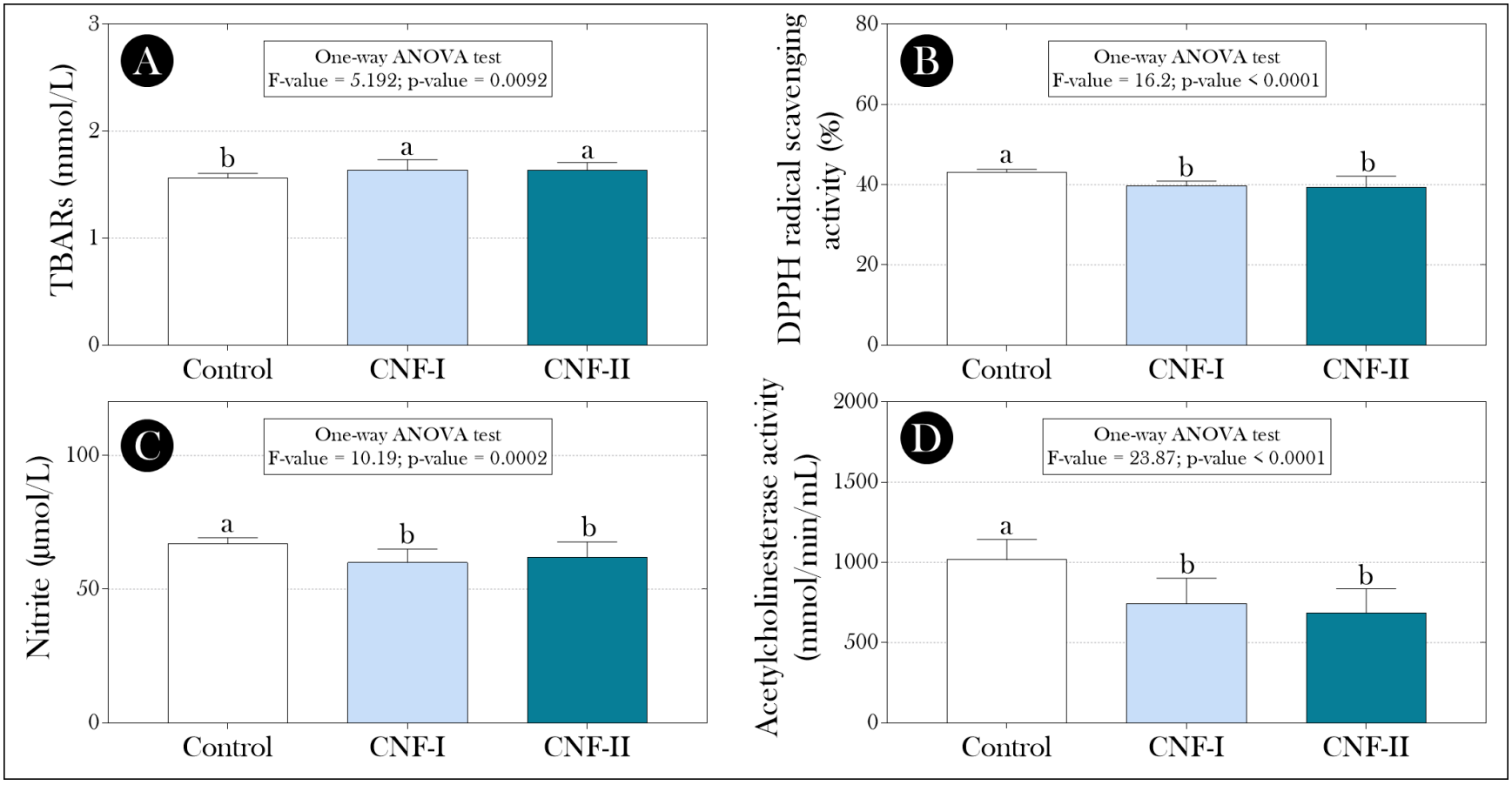
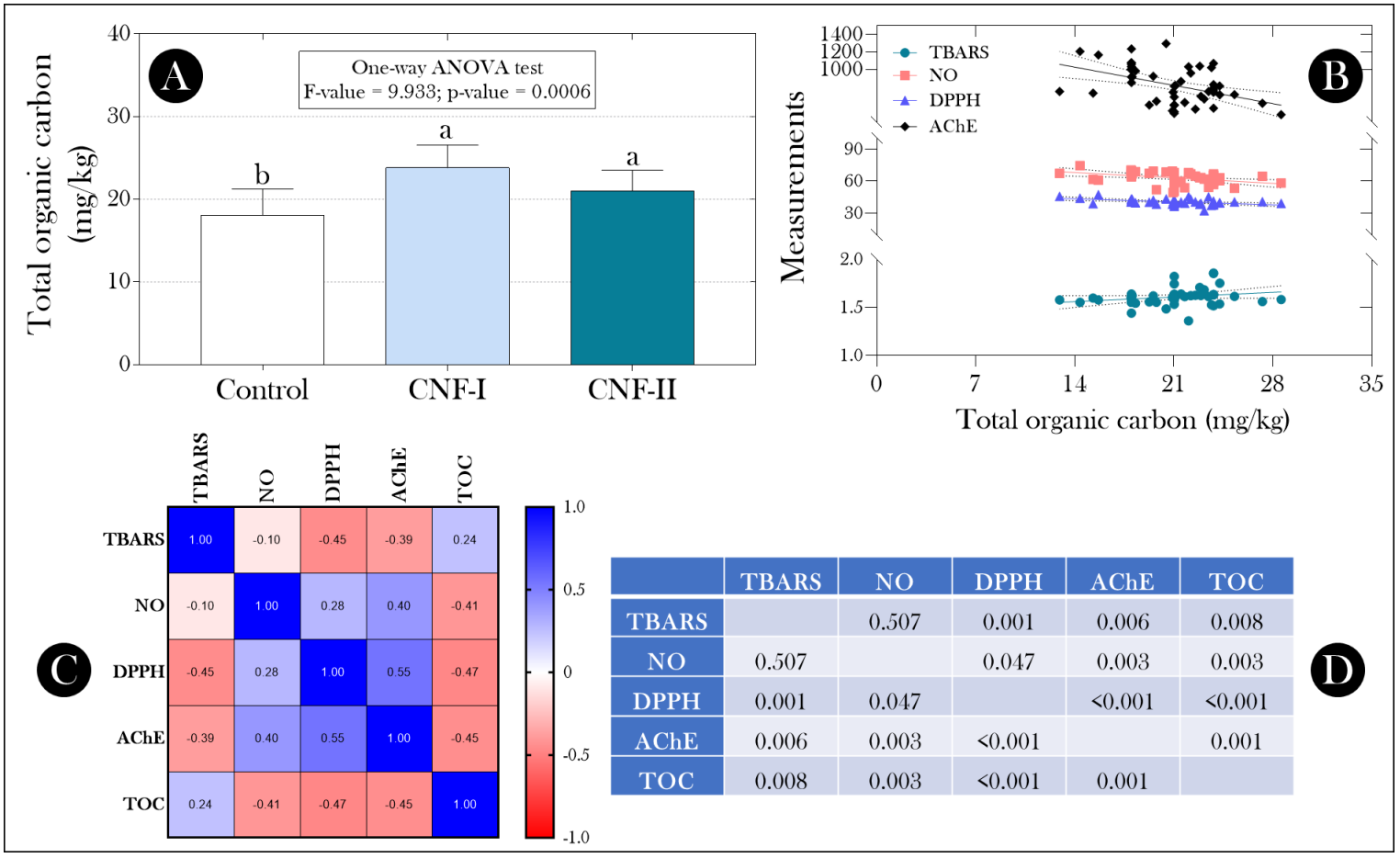


Figure 3. (A) Thiobarbituric acid reactive substances (TBARS) levels, (B) DPPH radical scavenging activity, (C) nitrite levels, and (D) acetylcholinesterase activity in the brain of *Danio rerio* (zebrafish) unexposed or exposed to carbon nanofibers (CNF) at different concentrations. Parametric data are presented by the mean + standard deviation. Statistical summaries are displayed at the top of the charts. Distinct lowercase letters indicate significant differences. CNF-I and CNF-II: composite groups of zebrafish exposed to CNFs at 10 ng/L and 10 µ/L, respectively.



	TBARS	NO	DPPH	AChE	TOC
TBARS		0.507	0.001	0.006	0.008
NO	0.507		0.047	0.003	0.003
DPPH	0.001	0.047		<0.001	<0.001
AChE	0.006	0.003	<0.001		0.001
TOC	0.008	0.003	<0.001	0.001	

Figure 4. (A) Total organic carbon (TOC) in the brain of *Danio rerio* (zebrafish) unexposed or exposed to carbon nanofibers (CNF) at different concentrations. (B) Correlation analysis and linear regression between "biochemical biomarkers vs. TOC" and (B-C) correlation matrix, indicating (C) Pearson correlation coefficient (r-values) and (D) p-values. In "A", parametric data are displayed by the mean + standard deviation, and the statistical summary is displayed at the top of the graph. Distinct lowercase letters indicate significant differences. CNF-I and CNF-II: composite groups of zebrafish exposed to CNFs at 10 ng/L and 10 µ/L, respectively. AChE: acetylcholinesterase activity (mmol/min/mL); TBARs: thiobarbituric acid reactive substances (mmol/L); NO: nitric oxide (µmol/L); and DPPH: DPPH (2,2-diphenyl-1-picryl-hydrazine-hydrate) radical scavenging activity (%).

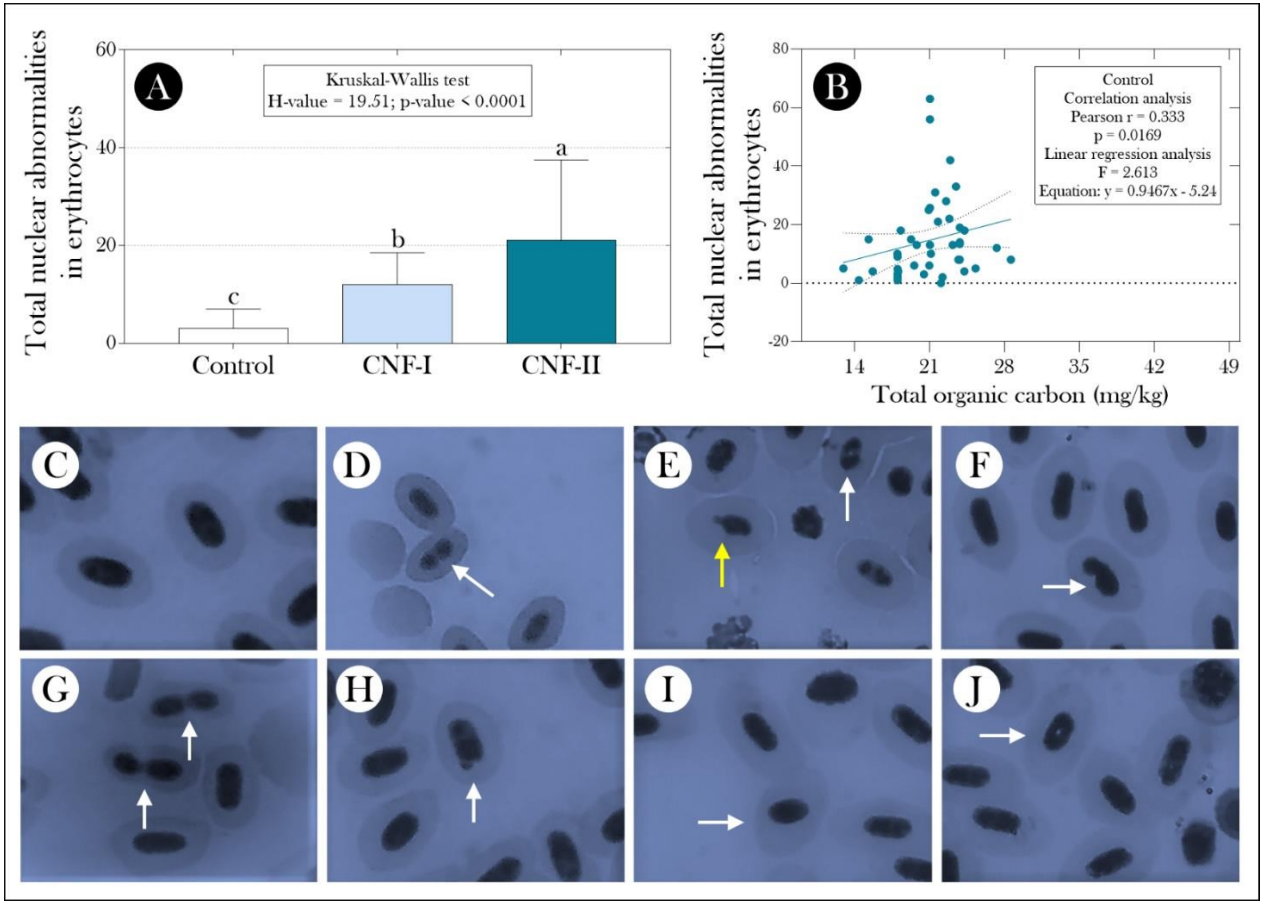


Figure 5. (A) Total nuclear abnormalities in erythrocytes of *Danio rerio* (zebrafish) unexposed or exposed to carbon nanofibers (CNF) at different concentrations. (B) Correlation and linear regression analysis between "total organic carbon *vs*. total nuclear abnormalities". (C-J) Representative images of erythrocytes identified in animals [(C) erythrocytes with normal nuclei, (D and G) constricted nuclei (white arrows), (E) blebbed nucleus (yellow arrow), (F) kidney-shaped nucleus (white arrow), (H) notched nuclei (white arrow), (I) moved nuclei (white arrow), and (E and J) nuclear vacuoles (white arrows)]. In "A", non-parametric data are presented by the median and interquartile range. Statistical summaries are displayed at the top of the graphs. Distinct lowercase letters indicate significant differences. CNF-I and CNF-II: composite groups of zebrafish exposed to CNFs at 10 ng/L and 10 µ/L, respectively.

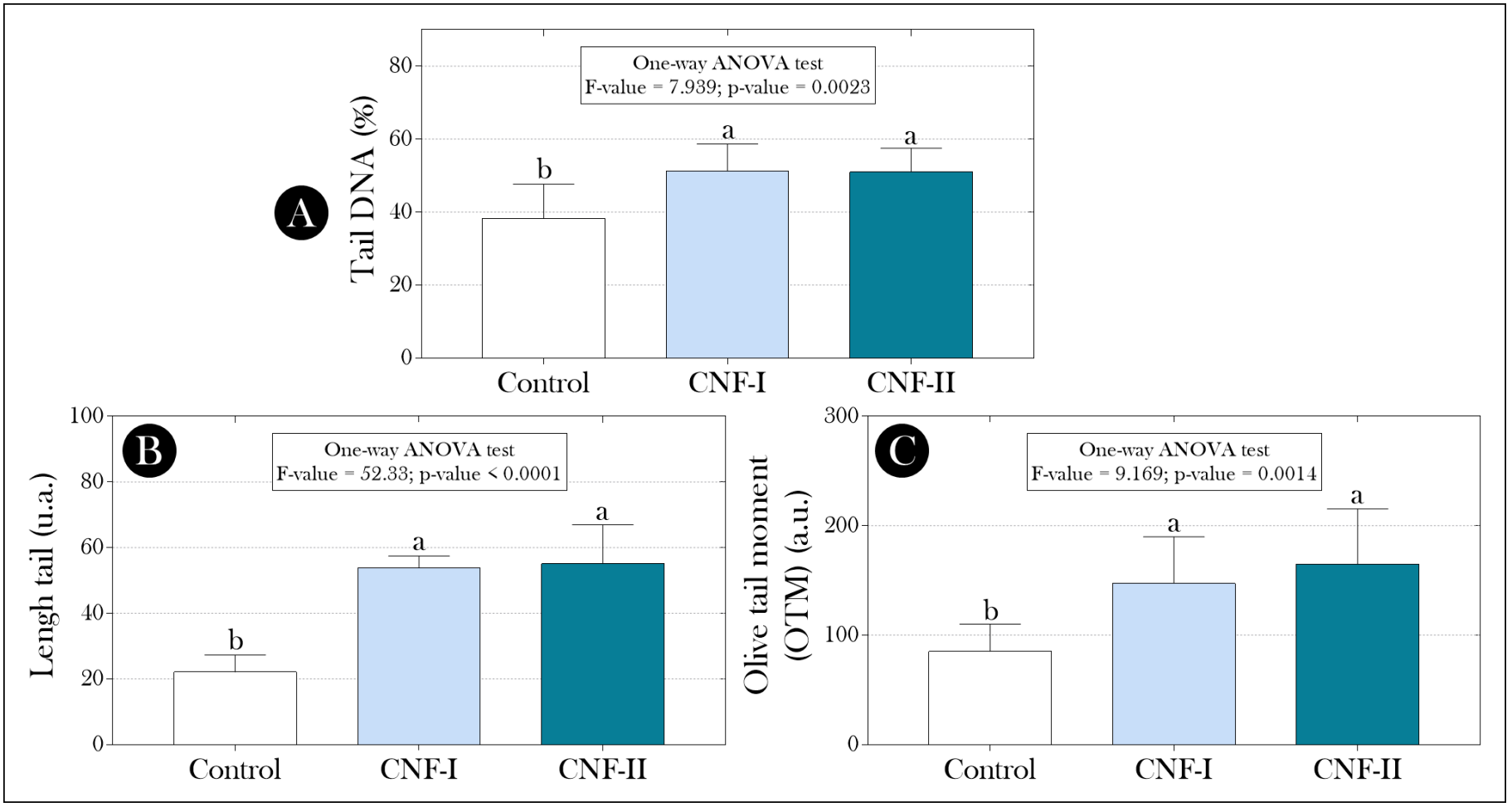


Figure 6. Parameters evaluated in the comet assay of *Danio rerio* (zebrafish) unexposed or exposed to carbon nanofibers (CNF) at different concentrations. (A) Tail DNA (%), (B) length tail, and (C) Olive tail moment (OTM). Parametric data are presented by the mean + standard deviation. Statistical summaries are displayed at the top of the graphs. Distinct lowercase letters indicate significant differences. CNF-I and CNF-II: composite groups of zebrafish exposed to CNFs at 10 ng/L and 10 µ/L, respectively. a.u.: arbitrary unit.

Table 1. Summary of the correlation analysis between total organic carbon concentrations and predictive REDOX imbalance biomarkers evaluated in *A. williamsoni* larvae exposed to CNFs.

Toxicity biomarkers	**Control**	**CNF-I**	**CNF-II**	**Sumário estatístico**
Blebbed *nucleus*	0.0 (0±0)[b]	0.0 (1.18±2.58)[ab]	2.0 (1.68±1.58)[a]	H-value=15.61; p-value=0.0004
Kidney-shaped nucleus	0.0 (0.50±1.50)[b]	0.0 (1.12±1.58)[ab]	2.0 (2.62±2.68)[a]	H-value=12.26; p-value=0.0022
Notched nucleus	0 (0.35±0.88)[b]	1.5 (2.00±2.68)[ab]	3.5 (4.06±3.37)[a]	H-value=16.08; p-value=0.0003
Constriction nuclear	1.0 (1.37±1.74)[b]	1.5 (2.12±2.30)[b]	5.0 (9.00±10.58)[a]	H-value=11.89; p-value=0.0026
Moved nucleus	2.0 (1.85±1.48)[b]	4.5 (5.68±5.17)[a]	4.0 (4.62±4.01)[ab]	H-value=6.306; p-value=0.0427
Nuclear vacuole	0.0 (0.12±0.50)[a]	0.0(0.37±0.80)[ab]	1.0 (1.00±1.18)[a]	H-value=11.41; p-value=0.0033
Eritrócito binucleado	0.0 (0±0)[b]	0.0 (0.06±0.25)[ab]	0.0 (1.13±2.57)[a]	H-value=8.076; p-value=0.0176

Note – CNF-I and CNF-II: groups composed of zebrafish exposed to carbon nanofibers at 10 ng/L and 10 μg/L, respectively. Values are presented as "median (mean ± standard deviation)". Distinct lowercase letters on the lines indicate significant differences between experimental groups.

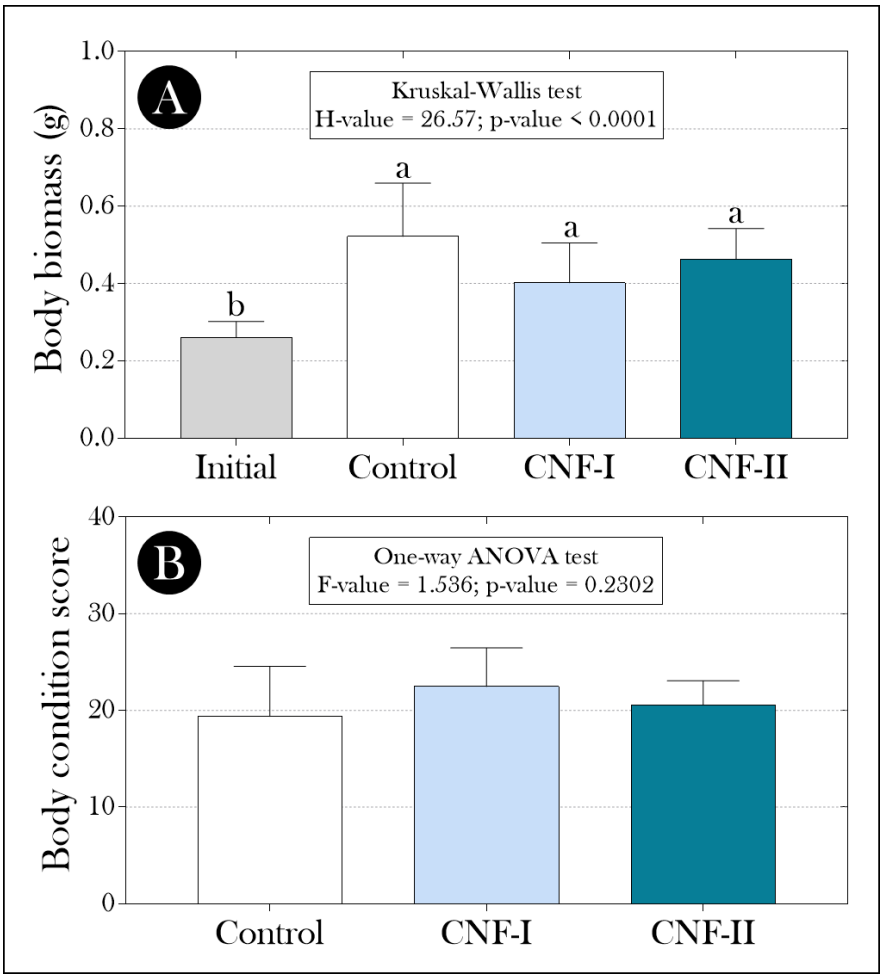


Figure 7. (A) Body biomass and (B) body condition score of *Danio rerio* (zebrafish) unexposed or exposed to carbon nanofibers (CNF) at different concentrations. Parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Statistical summaries are displayed at the top of the charts. Distinct lowercase letters indicate significant differences. CNF-I and CNF-II: composite groups of zebrafish exposed to CNFs at 10 ng/L and 10 µ/L, respectively.

Table 4. Biometric indices evaluated in adults Danio rerio (zebrafish) unexposed or exposed to carbon nanofibers (CNF) at different concentrations.

Abnormality	Control	CNF-I	CNF-II	Statistical summary
Total length index	6.80 ± 1.1	7.65 ± 1.30	7.00 ± 0.88	F-value = 1.3820; p-value = 0.2653
Fork length index	6.44 ± 1.56	7.28 ± 1.33	6.71 ± 0.88	F-value = 1.3380; p-value = 0.2763
Standard length index	5.45 ± 1.31	6.40 ± 1.53	5.69 ± 0.77	F-value = 1.8960; p-value = 0.1661
Predorsal length index	0.54 ± 0.02	0.52 ± 0.03	0.54 ± 0.09	H-value = 1.4640; p-value = 0.4810
Head length index	0.21 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	F-value = 2.1250; p-value = 0.0980
Body depth index	0.23 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	F-value = 0.9613; p-value = 0.3928
Penducle depth index	0.11 ± 0.01	0.10 ± 0.009	0.11 ± 0.001	F-value = 0.2903; p-value = 0.7499
Preorbital length index	0.02 ± 0.01	0.01 ± 0.009	0.02 ± 0.008	H-value = 1.6280; p-value = 0.4431
Eye diameter index	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	H-value = 1.5720; p-value = 0.4556

Note – CNF-I and CNF-II: groups composed of zebrafish adults exposed to carbon nanofibers at 10 ng/L and 10 µg/L, respectively. Values are presented as mean ± standard deviation.

To evaluate the general response of the animals, considering all the biomarkers being assessed, the data obtained in the "control", "CNF-I", and "CNF-II" groups were submitted to the PCA. We noticed that the first two principal components (PC1 and PC2) cumulatively explained 100% of the total variation (PC1: 71.18% and PC2: 28.82%), the PCs-eigenvalues were greater > 8.5 (PC1: 22.06 and PC2: 8.93), and most of the analyzed variables were positively associated with PC1 and PC2 (Figure 8A and Table 5). Furthermore, we observed that the experimental groups were clearly separated into two subgroups. While the "control" group was positioned in the negative quadrant of PC1 (PC1 score: -5.349), the "CNF-I" and "CNF-II" groups were positioned in the positive quadrants (i.e., opposed) of this same PC [PC1 score (CNF-I) = 1,898 and PC1 score (CNF-II) = 3,451] (Figure 8B), which indicates a certain similarity in the response of animals to exposure to CNFs, which was also noted in the hierarchical clustering analysis (Figure 9). However, the positioning of the "CNF-I" and "CNF-II" groups in opposite quadrants in PC2 (Figures 8B-C), as well as the different values reported by IBRv2

(difference of 21.9% between the IBRv2-values - Figure 10), demonstrate that exposure to CNFs at 10 µg/L, taking the set of animal responses, induced greater toxicity. Through Figure 10B, it is possible to notice that this greater toxicity was determined especially by the mutagenic, genotoxic, and AChE activity biomarkers, which showed greater deviations from the "control" group.

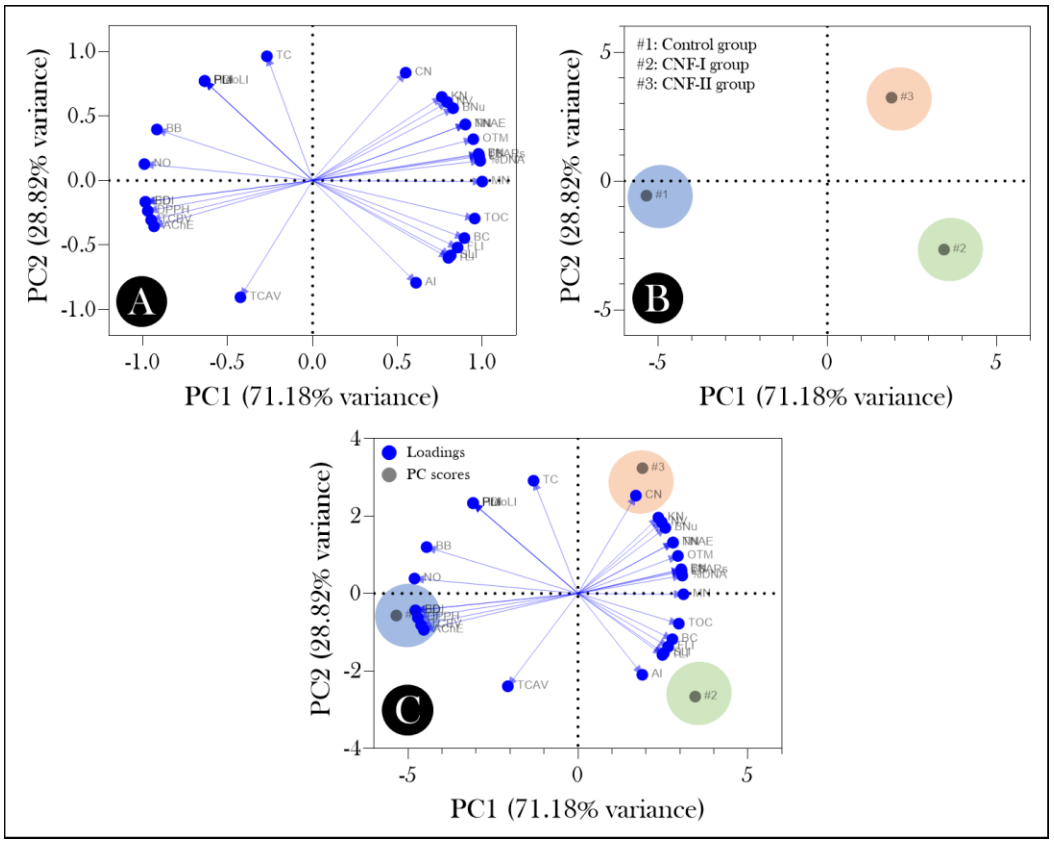


Figure 8. (A) Loadings plot of the investigated variables, (B) PC score plot, and (C) PCA biplot of the first two principal components (PCs) that simultaneously shows scores of experimental groups (gray points) and loadings of explanatory variables (vectors – arrows). See the meanings of the acronyms in Table 5. CNF-I and CNF-II: groups composed of zebrafish exposed to carbon nanofibers at 10 ng/L and 10 µg/L, respectively.

Table 5. Loading (coefficient) matrix provided by the multivariate analysis to define factors or principal components (PC1 and PC2).

Biomarkers	Abbreviations	Principal components	
		PC1	PC2
Anxiety index	AI	0,610	-0,793
Total crossings	TC	-0,269	0,963
Total crossings (before the vibration)	TCBV	-0,952	-0,306
Total crossings (after the vibration)	TCAV	-0,426	-0,905
Total nuclear abnormalities in erythrocytes	TNAE	0,901	0,434
Blebbed nucleus	BN	0,978	0,207
Kidney-shaped nucleus	KN	0,762	0,648
Notched nucleus	NN	0,900	0,435
Constriction nucleus	CN	0,549	0,836
Moved nucleus	MN	1,000	-0,008
Nuclear vacuole	NV	0,794	0,608
Binucleated erythrocyte	BNu	0,828	0,560
Tail DNA content as a percentage of comet DNA content	%DNA	0,988	0,153
Length of the tail in pixels	LT	0,982	0,187
Olive tail moment	OTM	0,947	0,320
Acetylcholinesterase activity	AChE	-0,935	-0,355
Thiobarbituric acid reactive substances	TBARs	0,980	0,199
Nitric oxide	NO	-0,992	0,127
DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical scavenging activity	DPPH	-0,972	-0,235
Body biomass	BB	-0,918	0,396
Body condition	BC	0,895	-0,446
Total length index	TLI	0,800	-0,599
Fork length index	FLI	0,855	-0,519
Standard length index	SLI	0,814	-0,580
Predorsal length index	PLI	-0,636	0,771
Head length index	HLI	-0,636	0,771
Body depth index	BDI	-0,986	-0,165

Penducle depth index	PDI	-0,636	0,771
Preorbital lenght index	PreoLI	-0,636	0,771
Eye diameter index	ED	-0,986	-0,165
Total organic carbon	TOC	0,956	-0,295

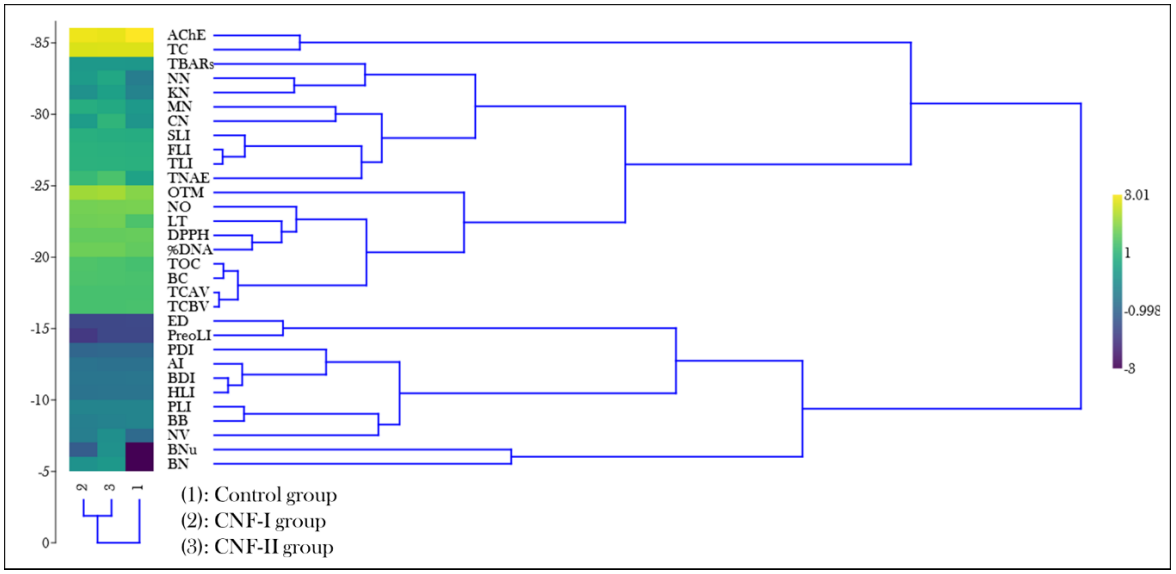


Figure 9. Cluster analysis dendrogram of different biomarkers evaluated in Danio rerio (zebrafish) unexposed or exposed to carbon nanofibers (CNF) at different concentrations. CNF-I and CNF-II: groups composed of zebrafish exposed to carbon nanofibers at 10 ng/L and 10 µg/L, respectively. See the meanings of acronyms in Table 5.

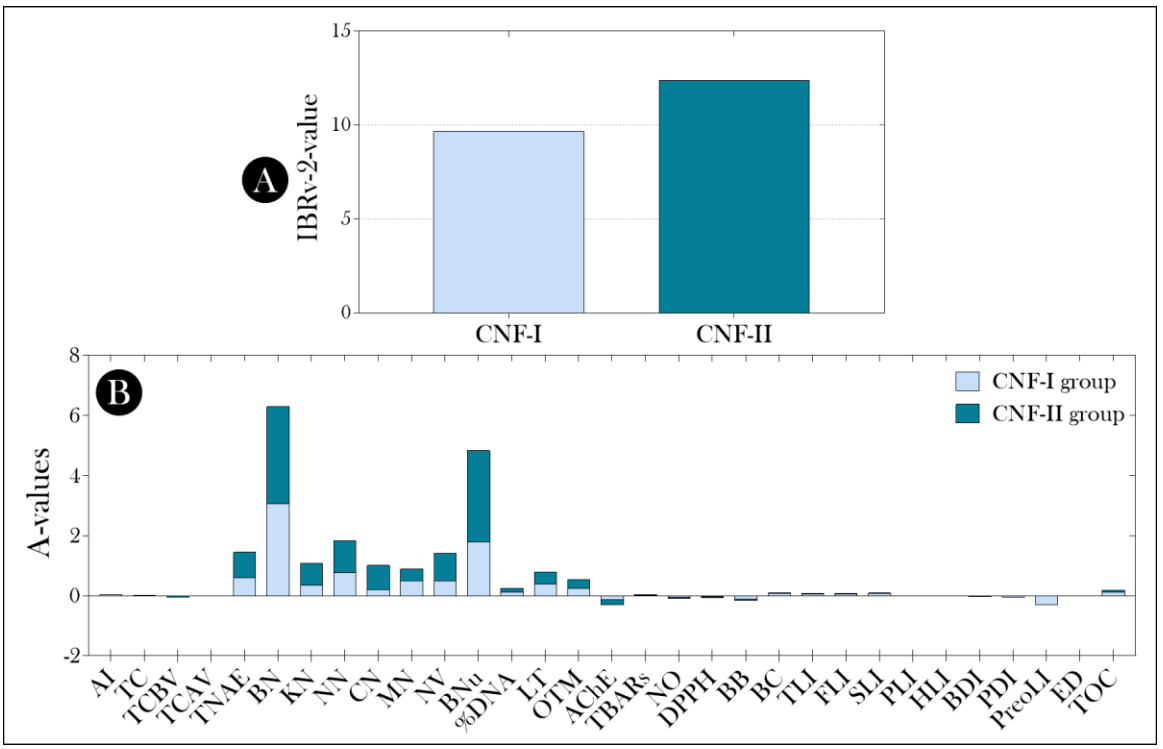


Figure 10. (A) "Integrated Biomarker Response" index (IBRv2) calculated for groups composed of *Danio rerio* (zebrafish) unexposed or exposed to carbon nanofibers (CNF) at different concentrations. (B): A-values derived from the IBR obtained for the respective groups exposed to the treatments. CNF-I and CNF-II: groups composed of zebrafish exposed to CNFs at 10 ng/L and 10 µg/L, respectively.

4. DISCUSSION

The increased demand and consumption of carbon-based products raise concerns about their disposal and the impacts their constituents cause on the environment and wild biota. In our study, we demonstrated for the first time that exposure of zebrafish to CNFs may constitute a risk to the health of these animals, which sheds light on the ecotoxicity of these nanomaterials. Although we did not notice the effect of CNFs on the growth/development of the animals (Figure 7 and Table 4), as well as changes in locomotor behavior (Figure 1A) or induction of anxiety-like behavior (Figure 1B), our data revealed the impact of treatments in response to the vibratory response test.

As shown in Figures 1C and 1D, the vibratory stimulus provided in the exposure water did not elicit a similar response between the different experimental groups. While in the unexposed animals, the vibratory stimulus induced a decrease in locomotor activity throughout the test, in the "CNF-I" and "CNF-II" groups, this was not observed (Figure 1C). One of the plausible explanations for this finding may be related to the biochemical changes reported in the

brain of animals exposed to nanomaterials [inferred especially by the increase in lipid peroxidation processes (Figure 3A) and reduction in total antioxidant activity (Figure 3B)], which it was also observed in tadpoles exposed to CNFs (Guimarães et al., 2021b). As discussed by Kopp et al. (2018), zebrafish are highly sensitive to vibrations in the water, whose regulation occurs through adaptive responses to stress by the hypothalamus-pituitary-interrenal axis, similar to what occurs in humans (Cachat et al., 2010). Therefore, it is possible that neural circuits have suffered negative impacts due to the reported biochemical changes, which would have, consequently, affected the regulation of the response to the vibratory stimulus mediated by the hypothalamus-pituitary-interrenal axis, whose intrinsic mechanisms of these changes need to be better investigated.

On the other hand, it is plausible to assume that CNFs may have interfered with the lateral line system of animals, which acts as an additional sensory organ for detecting vibrations in the aquatic environment (Mogdans, 2019). Although the number of neuromasts observed in the final ventral region of zebrafish from the “CNF-I” and “CNF-II” groups was greater than those reported in unexposed animals, this increase may be indicative of a compensatory response to try to maintain functions of potentially damaged mechanoreceptors, which are composed of the nerve mound (located on the surface) and the duct nerve mound (located in the lower lateral duct of the skin) (Metcalfe et al., 1985). In this case, it is possible that the CNFs dispersed in the water, when interacting with the neuromasts, affected the structures that compose them (e.g., sensory hair cells and surrounding support cells), similar to what was proposed in the study by Guimarães et al. (2021a), which correlated locomotor changes in zebrafish exposed to microplastics to possible interferences in these systems. Furthermore, it is tempting to speculate that the CNFs have accessed the fluid within the neuromast channels, culminating in changes in their functionality, whose increased density of neuromasts in the final ventral region would not have been sufficient for a response to the vibratory stimulus provided.

Alternatively, we cannot neglect the hypothesis that the biochemical alterations reported in the animals' brains have culminated in modifications in the transmission of information between afferent neurons from the lateral line ganglia to the brain and efferent neurons that carry information from the brain to the neuromasts. The anticholinesterase effect observed in animals exposed to CNFs (Figure 3D) indicated how this information transmission network might have been altered. While the reduction in cerebral AChE activity may have been insufficient for a negative effect on the animals' locomotion, the average decrease of 28.8% in the activity of this enzyme in the “CNF-I” and “CNF-II” groups may have been able to induce alterations in the mechanisms of transmission of neuronal information necessary for the response of animals to

vibrations in the water. Anyway, even though the hypotheses raised here need to be confirmed, the absence of response to the vibratory stimulus suggests a harmful effect on the animals, especially when we consider that the perception of vibrations in the water is essential for determining the direction of the water flow and the positioning of objects (Engelmann et al., 2000), as well as other fish in schools and potential predators (Thomas et al., 2015; Haehnel-Taguchi et al., 2018; Lunsford et al., 2019).

Interestingly, the anticholinesterase effect reported in our study does not coincide with the reports by Guimarães et al. (2021b) and Guimarães & Malafaia (2021) when exposing *P. cuvieri* tadpole and *P. expansa* juveniles to CNFs, respectively. At the time, both studies reported a significant increase in AChE activity, which was suggested to be a consequence of increased oxidative stress induced by CNFs. In our study, it is possible that the reduction in AChE activity was due to its interaction with CNFs, possibly due to the competition between the nanomaterials and the substrate (acetylthiocholine) for the active sites of AChE. This hypothesis is especially reinforced by the study by Cabral et al. (2013), in which the possible effect of covalent immobilization of AChE on CNTs using kinetic parameters from the Michaelis–Menten equation was investigated. At the time, the authors observed that the Vmax value of AChE decreased about 45-fold due to the substrate's inability to reach the active enzyme site by immobilizing the CNTs.

Our results also demonstrated that exposure to CNFs induced a prominent mutagenic and genotoxic effect in the animals, marked by an increase in the number of erythrocyte nuclear abnormalities (Figure 5) and DNA damage (Figure 6), similar to that reported by Guimarães & Malafaia (2021) in *P. expansa* juveniles long-term exposed to CNFs (at 1 and 10 mg/L) and by Gomes et al. (2021), in *O. niloticus* fed with *D. rerio* that ingested *Eisenia fetida* exposed to CNFs. Such results may be associated with the consequences of the redox imbalance suggested by the biochemical analyzes carried out in the animals' brains, which may have been more systemic and, therefore, not restricted to brain regions. The increase in TBARS levels (Figure 3A), e.g., strongly suggests oxidative stress with a consequent increase in lipid peroxidation processes induced by CNFs, which can further intensify ROS production and lead to cell and DNA damage. Our data suggest that the antioxidant activity of zebrafish was insufficient to counteract this oxidative stress. At the same time, the reduction in NO production (Figure 3C) may have contributed to increased lipoperoxidation processes. As shown by different authors, paradoxically, NO can act as a potent inhibitor of lipid peroxidation chain reaction (Yates et al., 1992; Hogg and Kalyanaraman, 1999; Lee et al., 2000; Girotti & Korytowski, 2020).

Although mutagenic or genotoxic changes induced by direct exposure to CNFs have not

been evidenced in previous studies, similar mutagenicity/genotoxicity have been reported *in vitro* studies with other carbon-based nanomaterial types, including, e.g., V79 cells and single-walled carbon nanotubes (SWCNTs) (Kisin et al., 2007), epithelial cells and MWCNTs (Muller et al., 2008), human cells and MWCNTs (Cveticanin et al., 2010), RAW 264.7 cells and CNTs (Migliore et al., 2010), human alveolar epithelial cells (A549) and MWCNTs (Kato et al., 2013; Di Ianni et al., 2021), BEAS 2B cells and CNTs (Lindberg et al., 2013), among others (Møller et al., 2021). On the other hand, the absence of mutagenic or genotoxic effects reported by *in vivo* studies involving other CNMs and amphibians (Mouchet et al., 2010) and rodents (Naya et al., 2011; Ema et al., 2013; Kim et al., 2015; Horibata et al., 2017) encourages the development of future research about this topic.

In any case, the consequences of the mutagenic and genotoxic effects reported in zebrafish exposed to CNFs in our study could be broad and culminate in systemic negative impacts. The presence of erythrocytes with nuclear abnormalities (e.g., blebbed, kidney-shaped and notched nuclei) has been associated with alterations in the cell cycle and events that precede the formation of micronuclei (Shimizu et al., 2000; Lindberg et al., 2007; Kalsbeek & Golsteyn, 2017; Hintzsche et al., 2017), which can lead to cell death, cause genomic instability and/or induce cancer development (Alimba & Bakare, 2016; Souza et al., 2017). Furthermore, DNA damage can initiate a cascade of harmful biological consequences that, in association with other physiological changes, may affect animal survival, reproduction, or genetic heritage.

Finally, it is essential to point out that our study does not exhaust the numerous possibilities for investigations to be carried out to advance knowledge about the potential ecotoxicological effects of CNFs on zebrafish and aquatic biota in general. Addressing more specific aspects of the mechanisms of action of CNFs will be helpful for us to understand the causes of the changes reported in the evaluated zebrafish, as well as the absence of treatment effects on some of the biomarkers being assessed (e.g., biometric parameters, locomotor activity and behavior of animals in the OFT). Regarding this aspect, we believe that exposures in different periods (> or < 90 days) can also induce different effects and that the use of biomarkers other than those used in our study (e.g., molecular, histological, mutagenic, neurological, hematological, etc.) may reveal other endpoints of the ecotoxicity of CNFs. The absence of a concentration-response effect in many of the biomarkers evaluated in our study may be associated with a greater agglomeration of CNFs dispersed in water (when in higher concentration) and, consequently, with a decrease in their bioavailability or greater difficulty in absorption by the animals. Although the general response of the animals to the treatments was considered concentration-dependent by IBRv2 (Figure 10), this was especially influenced by mutagenic and genotoxic biomarkers, which

was not observed in the other evaluated animals. Therefore, we strongly suggest developing studies on the factors that modulate the behavior of CNFs in aquatic environments and their bioavailability to the biota.

Equally important will be the development of studies involving other aquatic species (vertebrates and invertebrates) to expand the ecological representativeness of the tested models, allowing us to know the magnitude of the impacts caused by the CNFs. As discussed by Moermond et al. (2016), pollutants' ecotoxicity depends on several factors, including the tested organism. It is necessary to diversify the exposure conditions of zebrafish and other animal models, which can elicit dependent responses, e.g., on the size, shape, and concentrations of CNFs, as well as time exposure of animals to treatments. In addition, investigations related to the mechanisms of absorption of CNFs by organisms (via gills, skin, or gastrointestinal tract), as well as those associated with their bioaccumulation in vital organs, such as the one reported in the brain of zebrafish (Figure 5A) are essential for us to understand the toxicokinetic and toxicodynamic of these nanomaterials.

5. CONCLUSION

Based on what has been exposed, our study confirms the ecotoxicological potential of CNFs in the studied model (zebrafish) marked, especially by the inability of the animals to respond to the vibratory stimulus test, by the alteration in the density of neuromasts recorded in the final ventral region of the animals. , by the biochemical alterations reported in the brain and the mutagenic and genotoxic effects evidenced in circulating erythrocytes. On the other hand, contrary to what we expected, exposure to CNFs, at the concentrations tested and during the period of exposure evaluated did not affect the growth and development of the animals, in addition to not having induced locomotor alterations or anxiety-like behavior. We believe that studies like ours, i.e., focused on identifying and characterizing impacts caused by CNMs, provide applicable scientific subsidies for conducting investigations on their mechanisms of action, in addition to reinforcing the need to assess the bio- and eco-safety of these nanomaterials and plan actions that can mitigate their impacts.

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7. DECLARATION OF COMPETING INTEREST

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

8. ETHICAL ASPECTS

All experimental procedures were performed in accordance with the ethical standards for animal experimentation, and meticulous efforts were made to ensure that the animals suffered as little as possible and to reduce external sources of stress, pain, and discomfort. The current study has not exceeded the number of animals needed to produce reliable scientific data. This article does not refer to any study with human participants performed by any authors.

9. AUTHOR CONTRIBUTION STATEMENTS

Mateus Flores Montalvão: designed and performed experiments, analyzed data, and co-wrote the paper. **Thales Quintão Chagas**: performed experiments. **Aline Sueli de Lima Rodrigues**: revised the article critically for important intellectual content. **Abraão Tiago Batista Guimarães**: performed experiments. **Guilherme Malafaia**: designed and performed experiments, analyzed data, co-wrote the paper, supervised the research, provided funding acquisition, project administration, and resources.

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CAPÍTULO 4 - TOXICITY OF CARBON NANOFIBERS IN EARTHWORMS (*Lumbricus terrestris*) NATURALLY INFECTED WITH *Monocystis sp.*[3]

ABSTRACT

Although the ecotoxicity of carbon-based nanomaterials (CBNs) is known, the potential effect of carbon nanofibers (CNFs) on edaphic organisms has been insufficiently explored. Thus, we aimed at the ecotoxicity of CNFs (at 10 and 100 mg/kg) in *Lumbricus terrestris* earthworms naturally infected with *Monocystis* sp. After 28 days of exposure, treatments did not affect the survival rate. However, we observed a significant loss of body biomass, and *Monocystis* sp. infection in seminal vesicles was potentiated by exposure to CNFs. Earthworms exposed to CNFs showed a redox imbalance in the seminal vesicle, muscle, and intestine and an alteration in nitric oxide production in these organs. In muscles, we also noticed a significant reduction in AChE activity in earthworms exposed to CNFs. The histopathological analyses revealed the treatments' significant effect on the structures of the different evaluated tissues. Although we did not notice a concentration-response for several of the biomarkers, when taken together and after the application of Integrated Biomarker Response (IBR) and principal component analysis (PCA), we noticed that the response of earthworms to CNFs at 100 mg/kg showed a more significant deviation from the unexposed group. This was mainly determined by inhibiting antioxidant activity in the seminal vesicle, biochemical biomarkers assessed in muscle and intestine, and histomorphometric muscle biomarkers from earthworms exposed to CNFs at 100 mg/kg. Thus, we demonstrate that CNFs increase the parasite load of *Monocystis* sp. of adult *L. terrestris* earthworms and induce biochemical and histopathological changes, especially at 100 mg/kg. Our results point to the additional impact these nanomaterials can have on the health of earthworms, signaling the need for greater attention to their disposal and ecotoxicological effects on soil organisms.

Keywords: Carbon-based nanomaterials, edaphic organisms, IBR, soil pollution, ecotoxicity.

[3] O presente capítulo está em processo de revisão na ***Science of The Total Environment***, Qualis/CAPES A1 e fator de impacto de 9,8.

1. INTRODUCTION

It has been agreed that, in recent years, the development of nanoscience and nanotechnology has promoted a new frontier in the study of matter, allowing materials already known to have their properties rediscovered and investigated at the molecular level (Ariga, 2023; Singh & Kaur, 2023). Thus, nanomaterials, whose dimensions are of the nanometric order, have shown great relevance due to their various applications in important sectors of modern society (Singh et al., 2023; Malik et al., 2023). Carbon-based nanomaterials (CBNs), including carbon nanotubes and nanofibers (CNTs and CNFs, respectively), are endowed with several properties that allow them to be used in the development and application in products, processes, and devices that benefit several areas, such as food (Amenta et al., 2020), medical (Owida et al., 2023; Liu et al., 2023), agribusiness (Chen et al., 2022; Zhu et al., 2022), cosmetics (Singh, 2021; Dalla-Colletta et al., 2022), among others. Furthermore, the potential of using these CBNs in manufacturing smart materials for biotechnological applications has been recently investigated (Sagdic et al., 2022). However, the increasing use of these CBNs also implies an increase in their disposal and release in environments.

Although studies involving the impacts of CBNs on soils are limited, it has been demonstrated that the addition of CNTs and CNFs, even in small concentrations, can alter soil properties. Taha & Alsharef (2018), for example, when evaluating the effects of adding CBNs (at a concentration lower than 0.2%) in clayey soil, observed that the specific gravity and pH of the mixtures increased with the addition of nanomaterials. Although changes in the soil plasticity index were more evident in the mixture containing CNTs, analyses of hydraulic conductivity showed that soil samples containing CNFs had a greater reduction in hydraulic conductivity. Furthermore, Alsharef et al. (2017) reported significant increases in peak and residual shear strength in soil samples containing CNFs. However, assessing the impacts of CBNs on soils is still a challenge, mainly due to the lack of precise techniques and methods for identifying and quantifying these nanomaterials in natural soils. Environmental concentrations of CBNs in soils

On the other hand, studies conducted in laboratories using predictive concentrations of CBNs (in water and/or soil) have raised concerns about the risks of these materials affecting biodiversity (Gamoń et al., 2022). Recent studies involving ecotoxicity have demonstrated several adverse effects of CNTs on organisms, such as cyto-genotoxic impact in plants (*Allium cepa*) [e.g., Ince-Yardimci et al. (2022) and animal cells (Di-Ianni et al., 2022), chromosomal aberrations and changes in the methylation patterns of *Lactuca sativa* L. (Da-Costa-Siqueira et al., 2023), alterations in the expression of microRNA in zebrafish (*Danio rerio*) (Minchenko et al., 2022),

as well as in the urinary biochemical profile of female rates (Mortensen et al., 2022). Furthermore, histological damage in the lungs, liver and kidneys of Sprague Dawley female rats was observed after exposure to multi-walled carbon nanotubes (MWCNT) (Bubols et al., 2023), as well as behavioral changes in organisms exposed to these materials [e.g., *Drosophila melanogaster* (Demir et al., 2022) and *Caenorhabditis elegans* (Zhao et al., 2022)].

Regarding the CNFs, some investigations point to low-toxicity or non-toxicity in the biological systems in which they were tested, like the studies conducted by Mehrabi et al. (2019) (using human umbilical vein endothelial cells), Salesa et al. (2020) (human keratinocyte HaCaT cells), and Nekounam et al. al. (2020) (MG-63 cell line human). On the other hand, several other evidence pointed to the risk that the dissemination of these nanomaterials may represent for the health of organisms. For example, Pikula et al. (2020) showed that exposure to CNFs – for seven days – significantly inhibited the growth rate of seaweed *(Attheya ussuriensis* and *Heterosigma akashiwo*). In Montalvão et al. (2021), it was shown that CNFs caused redox imbalance and changes in acetylcholinesterase activity in *Aphylla williamsoni* (Odonata) larvae after only 48 h of exposure. In zebrafish (*D. rerio*) juveniles, the accumulation of CNFs in animals was directly associated with an increase in the frequency of nuclear abnormalities and DNA damage in circulating erythrocytes (Montalvão et al., 2023). Guimarães et al. (2021) and Gomes et al. (2021) evidenced the toxicity of CBNs in Amazon turtle juveniles (*Podocnemis expansa*) and Nile tilapia (*Oreochromis niloticus*), respectively, through multiple biomarkers, including those predictive of changes in redox homeostasis and mutagenic effect.

Thus, it can be noted that most studies involving CNFs have focused on investigating their ecotoxicity when dispersed in the aquatic environment. However, Mueller & Nowack (2008) estimate that the concentrations of CBNs may be higher in soil than in water, which may be related to the increasing use in agricultural practices. The authors estimated the predicted environmental concentrations (PEC) based on a substance flow analysis from products to air, soil, and water in Switzerland, using several parameters as model inputs (e.g., estimated worldwide production volume, allocation of the production volume to product categories, particle release from products, and flow coefficients within the environmental compartments). At the time, two scenarios were modeled [realistic scenario (RS) and high emission scenario (HS)] and, in both, the PEC-values in the soil (RS: 0.01 and HS: 0.02) were higher than the predicted ones. for the aquatic environment (RS: 0.0005 and HS: 0.0008). In fact, different studies have shown, for example, that CNFs embedded in fertilizers can suppress plant disease and enhance plant growth via the promotion of water and nutrition uptake (Ashfaq et al., 2017; Kumar et al., 2018; Gupta et al., 2019; Alquraan et al., 2021; Gahoi et al., 2021; Pandey et al., 2022). Therefore, intentionally

introducing CNFs into agricultural ecosystems may threaten terrestrial habitats and their biodiversity. However, the effects of soil contamination with CNFs have not yet been the subject of previous investigations. Our knowledge of the ecotoxicity of CBNs in soil fauna is restricted to findings from studies involving exposure of earthworms to CNTs [e.g., Petersen et al. (2009), Petersen et al. (2011), Hu et al. (2013), Li et al. (2013), Zhang et al. (2014), Calisi et al. (2016), Yang et al. (2021), Xu et al. (2021), Yang et al. (2022)]. Only Gomes et al. (2021) exposed *Eisenia fetida* to CNFs to serve as food at a higher trophic level without evaluating their responses to nanomaterials.

Another important shortcoming refers to the fact that previous studies concentrated their assessments on theoretically healthy organisms without considering or mentioning any aspect of their disease status that, according to Hoffman & Henniget al. (2017) and Fuller et al. (2022), can influence the toxic effects of pollutants. In the earthworm community, in particular, there is a large natural incidence of gregarine infection in seminal vesicles, being the infection of *Lumbricus terrestris* earthworms by *Monocystis* sp. (Apicomplexa: Gregarinidae), well-known host–parasite system (Field & Michiels, 2005; Stroud, 2022). As highlighted by Field & Michiels (2006), *Monocystis* sp. is perhaps the most common and best-known parasite of *L. terrestris*, being so ubiquitous that samples containing 100% infected individuals are not uncommon. Although parasitic transmission between earthworms can occur in other ways, ingesting soil contaminated with sporocysts is considered the main way the parasite's life cycle begins (Field & Michiels, 2005). After ingestion, the sporozoites enter the circulatory system and invade the sperm vesicle lumen, where they mature as trophozoites and, consequently, affect the development of spermatocytes. In the sexual phase, gamonts undergo syzygy and form a gametocyst and, after several nuclear divisions, result in a zygote that secretes an oocyst membrane. Then, the oocyst membrane hardens further, resulting in sporocysts that, after 2-3 cell divisions, form 8 sporozoites inside one spore. At this point, the gametocyst ruptures, releasing the many sporocysts into the seminal fluid and into the environment to repeat the life cycle (Velavan et al., 2009). According to Stroud (2022), infection by Monocystis sp. may result in reproductive inefficiency due to hypertrophy of male funnel cells and parasitism of spearm moralae, while high parasitic loads lead to parasitic castration by destroying the testes and by male ducts to atrophy.

Thus, we aimed, for the first time, to assess whether the exposure of *L. terrestris* earthworms naturally infected with *Monocystis* sp. to soil contaminated with CNFs at environmentally relevant concentrations potentiates parasitic infection and induces adverse effects of these nanomaterials on animal health. For this, biometric, reproductive, biochemical, and histological biomarkers were evaluated in *L. terrestris* earthworms after 28 days of exposure

to CNFs in the soil. We start from the hypothesis that CNFs, by inducing adverse effects on earthworms, increase the parasite load of *Monocystis* sp. in seminal vesicles, which would represent an additional indirect impact of these pollutants on naturally infected earthworm populations. By adopting a more realistic experimental design, we believe our study will contribute to understanding the ecotoxicological risks of CNFs to edaphic organisms.

2. MATERIAL AND METHODS

2.1. Carbon nanofibers

Commercially obtained pyrolytically stripped CNFs (Sigma-Aldrich, San Luis, Missouri, USA - MDL number: MFCD00133992) were used in this study, whose chemical and morphometric characterization was previously presented (Gomes et al., 2021). Briefly, the characterization showed that the CNFs used have a heterogeneous shape (including the ones presenting open ends and pronounced curvatures) and a diameter of 86.85 ± 1.80 nm (media ± SEM). The Raman spectroscopy analysis revealed the presence of the D band, which refers to defective graphite structures (1336 cm^{-1}), and the G band (1750 cm^{-1}), which refers to graphite crystalline structures, in addition to the G′ band (2654 cm^{-1}). In addition, the Scanning Electron Microscopy/Energy Dispersive Spectroscopy (SEM-EDS) analysis pointed to the presence of metallic particles used as catalysts [iron (11,372 ppm), silicon (30 ppm), magnesium (40 ppm), sulfur (10,200 ppm), calcium (140 ppm), and sodium (40 ppm)] [see more details in Gomes et al. (2021)].

2.2. Model system and experimental design

The present study was carried out at the Laboratory of Toxicology Applied to the Environment of the Instituto Federal Goiano - Campus Urutaí (GO, Brazil), using adult *Lumbricus terrestris* earthworms (Oligochaeta, Annelida) as a model system. This species was chosen based on its ecological/environmental importance, acting as an essential soil biota that can improve soil structure and hydraulic properties by burrowing (Edwards et al., 1990; Lee & Foster, 1991). Furthermore, the species has been considered a good experimental model in previous ecotoxicological studies [e.g., Puddephatt et al. (2022), Fawzy-Salman et al. (2022), Ju et al. (2023), and Peña et al. (2023)].

All earthworms used in the experiment were obtained from a single breeding site, with a previous evaluation of 10 random individuals confirming infection by *Monocystis* sp.

(Apicomplexa: Gregarinidae). The seminal vesicles of these individuals were dissected, macerated under glass slides, and analyzed under an optical microscope. *Monocystis* sp. sporocysts of varying sizes were identified on all slides, depending on their life cycle stages. Overall, the sporocysts had a characteristic biconic shape with a mucoid plug at each end, in line with the descriptions by Mackinnon & Hawes (1961) and Field et al. (2003).

In the laboratory, the earthworms were acclimatized (in the dark) for 15 days before the start of the experiment under conditions similar to those described in Fawzy-Salman et al. (2022), Gospodarek et al. (2022), and Ju et al. (2023) [temperature: 22°C (i.e., the same as the animals were being kept at the breeding site), soil mixture with cattle manure (up to 25%), and humidity: 60-70%]. The acclimatization period was important to monitor the locomotor activity of the animals, as well as to isolate dead or inactive earthworms. In addition, the animals were kept in the dark since earthworms have been shown to display relatively well-defined activity rhythms and light-withdrawal reflexes, and, therefore, we avoided the effect of artificial lighting on the animals' physiology and behavior.

After acclimatization, 150 adult earthworms with well-developed clitella without evident alterations (in terms of locomotion or visible lesions on the skin) were rinsed with purified water, wiped dry using absorbent paper, weighed (1.30 g ± 0.15 g – mean ± SD), and distributed into three experimental groups (5 replicates with ten individuals/each, totaling 50 earthworms/group). Each replica of the "control" group was composed of individuals kept in a polypropylene container containing 500 g of CNF-free soil. Such soil was collected (depth: 0–20 cm) in a permanent preservation area on the Goiano Federal Institute – Campus Urutaí (Urutaí, GO, Brazil). The "CFN-10" and "CFN-100" groups were composed of individuals maintained under the same conditions described in the "control" group; however, CNFs were added to soils at concentrations of 10 and 100 mg/kg, respectively. Such concentrations are considered predictive since there are still no efficient and accessible techniques or methods for detecting CBNs (CNTs or CNFs) in environmental matrices, including soils. However, environmental concentrations of CBNs have been estimated to range from µg/kg to mg/kg by mathematical model [e.g., Gottschalk et al. (2009) and Sun et al. (2015)]. These studies extended the probabilistic material flow modeling approach to include temporal modeling of engineered nanomaterials production and biosolids handling and transfer onto soils, including CBNs. Thus, the concentrations tested in our study simulate soil contamination in different orders of magnitude, with the 100 mg/kg concentration representing a pessimistic pollution scenario. The soil used in all groups was of the Typic Dystrophic Red Latosol type [according to EMBRAPA (1999)], having been previously dried and sieved through a 2 mm mesh and sterilized by autoclaving (temperature: 121°C,

pressure: 110 kPa, and time: 50 min). The basic physical, physical-chemical, and chemical properties of the soil used are presented in the "results" section. Furthermore, it is important to highlight that the soil was previously autoclaved [like Zalleer et al. (2013)] to exclude the influence of their biological composition (microorganisms, plants, parasites, etc.) on earthworms.

2.2.1. Maintenance conditions

All experimental replicates were kept in a climate chamber under a controlled temperature (22° C) without light, like Yang et al. (2022), for 28 consecutive days. Each container was covered with perforated lids to limit water loss and serve as a barrier against possible escape by the animals. To keep the humidity of the experimental replicate constant (i.e., 25%), 5 mL of purified water was added to each container every three days, as Yang et al. (2021). Additionally, 5 g of dried cow dung powder (previously autoclaved - temperature: 121°C, pressure: 110 kPa, and time: 50 min) was added to containers as food for earthworms weekly, based on Yang et al. (2022).

2.3. Parasitic load in the seminal vesicles of earthworms

To evaluate the possible effect of CNFs on *Monocystis* sp. infection, after 28 days of exposure, the seminal vesicles of ten *L. terrestris* earthworms (n=2/replica) were extracted and processed, according to Schall (2021), with some modifications. Briefly, after extraction, the seminal vesicles were weighed and homogenized in phosphate-buffered saline [(PBS, pH 7.4), containing NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (8 mM), and KH_2PO_4 (2 mM)], using an automatic homogenizer (3420 rpm, 60 s, with 2 cycles of 20 s interval; five spheres – diameter: 2 mm) (Loccus, L-Beader-6). Then, a 10 µL aliquot of the homogenate was introduced into a Neubauer chamber to count the number of sporozoites in four quadrants of the chamber [areas in red in Figure 1S (see "Supplementary Material")]. The analysis was performed in duplicate, using an optical microscope with a 400x magnification objective, following the direction shown in Figure 1S (see "Supplementary Material"). The number of sporozoites/mL was recorded, considering that the total volume of these quadrants is 0.4 mm^3 (i.e., 0.4 µL). After that, the number of sporozoites was relativized with the biomass of the seminal vesicles, and the results were expressed in "sporocysts number/mg seminal vesicle." Furthermore, the parameters "gametocysts number with sporocysts/mm^2", "*Monocystis* sp. encapsulated gametocysts area (μm^2)", and "occupation area (%) of gametocysts in the seminal vesicle" were evaluated as

provided in section 2.4.2.

2.4. Toxicity biomarkers

2.4.1. Biomass and reproductive performance

At the end of the exposure period, the number of cocoons produced, the number of live and dead adult earthworms, and their weights were recorded, as Cañas et al. (2011). Particularly, cocoons were recovered from the soil using two 500 mm sieves (diameter ¼ 30 cm). A stream of tap water was washed through these sieves, allowing the soil to be separated from the cocoons. Soil was washed through the top sieve, leaving behind the cocoons. Cocoons were then counted.

2.4.2. Biochemical evaluations

Different biochemical biomarkers were evaluated to evaluate treatments' possible health effects on earthworms. For this, at the end of the experiment, ten individuals/group (n=2/replicate) were euthanized to extract seminal vesicles and fragments of the intestine and muscles. Intestinal and muscle fragments were collected between body segments n. 41 and 60. After extraction, all fragments were immediately washed in PBS (at 4°C, pH 7.4) before processing. Three sequential washes were performed on each fragment, thus reducing interference from intestinal contents in later biochemical tests. Right away, the tissues were mixed in 1 mL of PBS (pH 7.4) and submitted to maceration in a cell and tissue disruptor (4000 rpm, 60 s, with 2 cycles of 20 s interval; diameter spheres: 2 mm) (Loccus, L-Beader-6). Afterward, the samples were centrifuged (13,000 rpm, 10 min at 4°C), and the supernatants were filtered through a cellulose nitrate filter membrane (pore: 0.45 μm) and subsequently stored at -80°C until use. In Table 1S (see "see "Supplementary Material"), the justifications for the choice of each evaluated biomarker are presented [reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), nitric oxide (NO), acetylcholinesterase (AChE), and total protein], as well as the references used as a basis for carrying out biochemical assays.

2.4.3. Histopathological analyzes

After exposure of the earthworms, ten individuals/group (n=2/replica) were rinsed with purified water, euthanized, and had their seminal vesicles and body part (fragment between body segments n. 36 to 40) collected. Sequentially, tissues were fixed with 10% formaldehyde, and after

24 h, formaldehyde was replaced with 70% ethanol. Then, the tissues were embedded into the paraffin and sliced to 5 μm thick with a semiautomatic microtome. At least five sections of each tissue were mounted on glass microscope slides using standard histopathological techniques and stained with hematoxylin-eosin (H&E), like Xu et al. (2023) and Zhao et al. (2023). In addition, sections of the animals' bodies were stained with Masson's Trichrome, like Amaroli et al. (2018), aiming to characterize and discriminate different connective tissues and soft tissue components. In this type of staining, smooth muscle is stained pink to red, collagen fibers in blue or green, and elastic fibers in black. It should be noted that all tissues were prepared simultaneously in batches containing tissues from control animals and the treatments, like Lahive et al. (2014). This procedure aimed to eliminate differences in fixation or staining artifacts between treatments.

The possible impact of treatments on tissues was also evaluated through histomorphometric analyses, whose measured parameters are summarized in Table 2S (see "Supplementary Material") and exemplified in Figure 1S (see "Supplementary Material)." The slides were photographed at different magnifications and analyzed using ImageJ software. The histomorphometric parameters analyzed in the seminal vesicles were also used to infer about *Monocystis* sp. load on these organs. In addition, a qualitative assessment was performed for possible identification of tissue changes based on different previous studies (Reddy & Rao, 2008; Gobi & Gunasekaran, 2010; Oluah et al., 2010; Kılıç, 2011; Lourença et al., 2011; Van-Der-Ploeg et al., 2013; Lahive et al., 2014; Babić et al., 2016; Samal et al., 2017; Nayak et al., 2018; Li et al., 2020; Jiang et al. al., 2020; Wang et al., 2020; Duo et al., 2022; Xu et al., 2023; Boughattas et al., 2023; Arabi & Mahmoodian, 2023).

2.5. Uptake of carbon nanofibers

To evaluate the possible ingestion/uptake of CNFs, the total organic carbon (TOC) concentrations of the intestinal contents of ten individuals from each group (n=2/replica) were analyzed according to the Walkley-Black method described in Gomes et al. (2021) and Guimarães & Malafaia (2021). This method is based on using dichromate ($Cr_2O_7^{2-}$) (CrVI) as an oxidizer in an acid medium (Walkley & Black, 1934) and has been used to infer the uptake or bioaccumulation of CNFs in other experimental models (fish: Gomes et al. (2021) and Montalvão et al. (2023); dragonfly larvae: Montalvão et al. (2021), turtle: Guimarães & Malafaia (2021), and tadpoles (Guimarães et al., 2021c). After euthanasia, the final body portion of the animals was dissected, and the intestinal contents were extracted using surgical instruments and a stereoscopic microscope.

Then, aliquots were processed and analyzed, according to Gomes et al. (2021). Briefly, intestinal contents were weighed and transferred to glass Erlenmeyer flasks, and 4 mL of potassium dichromate solution was added. Subsequently, 8 mL of sulfuric acid was added to the solution. After 30 min, 2 mL of orthophosphoric acid, 80 mL of purified water, and three drops of diphenylamine indicator were added to the system. Subsequently, the system was titrated using ferrous ammonium sulfate solution until its color changed from blue to green. Furthermore, a blank sample (solution without the sample) was also titrated. Finally, the formulas presented by Gomes et al. (2021) were applied to quantify TOC.

2.6. Soil characterization

To evaluate the influence of treatments on soil quality, physical, physical-chemical, and chemical analyses were carried out according to the analysis methods manual of the Brazilian Agricultural Research Corporation (Embrapa, 1997). Analyzes were performed in triplicate, using samples collected at the end of the experiment. The reading of chemical elements was determined by atomic absorption spectrophotometry, like Kouadio et al. (2023).

2.7. Data analysis

2.7.1. "Integrated Biomarker Response" index (IBRv2)

To transform the different responses of *L. terrestris* earthworms exposed to CNFs into a single value, the results of all biomarkers were applied to the second-generation "Integrated Biomarker Response" index (IBRv2). For this, the method proposed by Sanchez et al. (2013) is described in detail in Malafaia et al. (2022). According to these authors, the IBRv2 index considers the concept of reference deviation based on the deviation between the response of animals exposed to treatments and an undisturbed state (control group).

2.7.2. Statistical analyzes

The one-way ANOVA test (with Tukey post-test) or Kruskal-Wallis test (with Dunn's post-test) was applied to compare the means of the biomarkers evaluated in the different experimental groups. In this case, the Shapiro-Wilk test was used to evaluate the distribution of residual data, and the Bartlett test was applied to evaluate the homogeneity of variances. Significance levels were

set at Type I error (p) values lower than 0.05, and GraphPad Prism software Version 9.0 was used to perform the statistical analyses. Biomarker data were also explored based on principal component analysis (PCA) using GraphPad Prism software Version 9.0, like the procedures described in Montalvão et al. (2023).

3. RESULTS

Initially, our data revealed that the survival rate of the animals at the end of the experiment did not differ between the experimental groups, whose average observed in the different groups was greater than 90% (Figure 2SA - see "Supplementary Material"). Furthermore, we did not observe differences in final biomass between earthworms from the "control," "CNF-10", and "CNF-100" groups (Figure 2SB - see "Supplementary Material"). However, a significant reduction in the final weight of the animals was observed in all groups, compared to the beginning of the experiment, with such reduction being more prominent in the animals exposed to CNFs (Figures 2SB-C - see "Supplementary Material"). Furthermore, as expected, the reproductive performance of the animals was considerably low, with no differences being observed between the experimental groups (control: 0.6 ± 0.4 cocoon/individual/replica; CNF-10: 0.4 ± 0.4 cocoon/individual/replica, and CNF-100: 0.6 ± 0.2 cocoon/individual/replica – mean ± SEM – H-value: 0.6723; p-value: 0.8002).

Regarding *Monocystis* sp. infection in the seminal vesicles of the earthworms evaluated at the end of the experiment, we noticed a higher parasite load in the animals of the "CNF-10" and "CNF-100" groups, measured by the sporocysts number (Figures 1A-B) and gametocysts number with sporocysts (Figure 1C). The area of *Monocystis* sp. encapsulated gametocysts observed in the vesicles of animals exposed to CNFs was, on average, 26.3% larger than that recorded in the "control" group (Figure 1D), culminating in a larger area of occupation of the organ (Figure 1E). Figures 1F-H show representative photomicrographs of the seminal vesicle of earthworms from different experimental groups, and Figures 1I-L show details of *Monocystis* sp. encapsulated gametocysts and sporocysts. In addition, we observed that exposure to CNFs induced a redox imbalance in the seminal vesicle of the animals [marked by reduced SOD and CAT activity (Figures 2A-B, respectively), increased production of ROS and MDA (Figures 2C-D, respectively)], as well as nitrosative stress [inferred by increased nitrite levels in this organ (Figure 2E)].

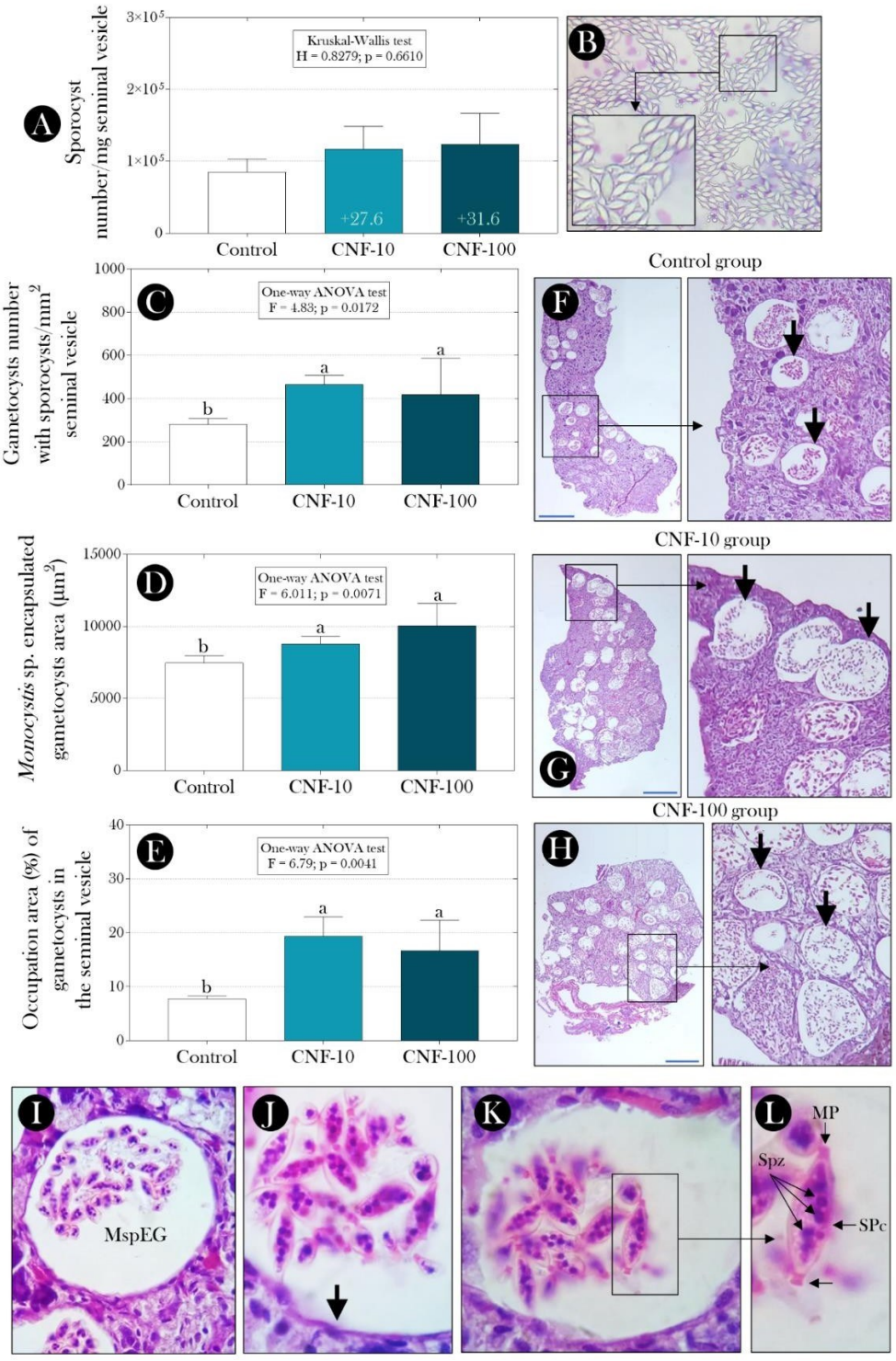

Figure 1. (A) Sporocysts number, (B) photomicrography representative of sporocysts, (C) gametocysts number with sporocyst, (D) *Monocystis* sp. encapsulated gametocysts area, (E) occupation area of gametocysts area in the seminal vesicle of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (F-H) Photomicrography representatives of the seminal vesicle of earthworms from the "control," "CNF-10", and "CNF-100" groups, respectively; and (I-K) details of *Monocystis* sp. encapsulated gametocysts and (L) sporocysts. Spz: developing sporozoites, MP: mucoid plug, Spc: sporocyst. In "A," "C," "D," and "E," parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. Statistical summaries are shown close to the graphs. C: control; CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. The sections of each tissue were stained with hematoxylin-eosin (H&E) using standard histopathological techniques.

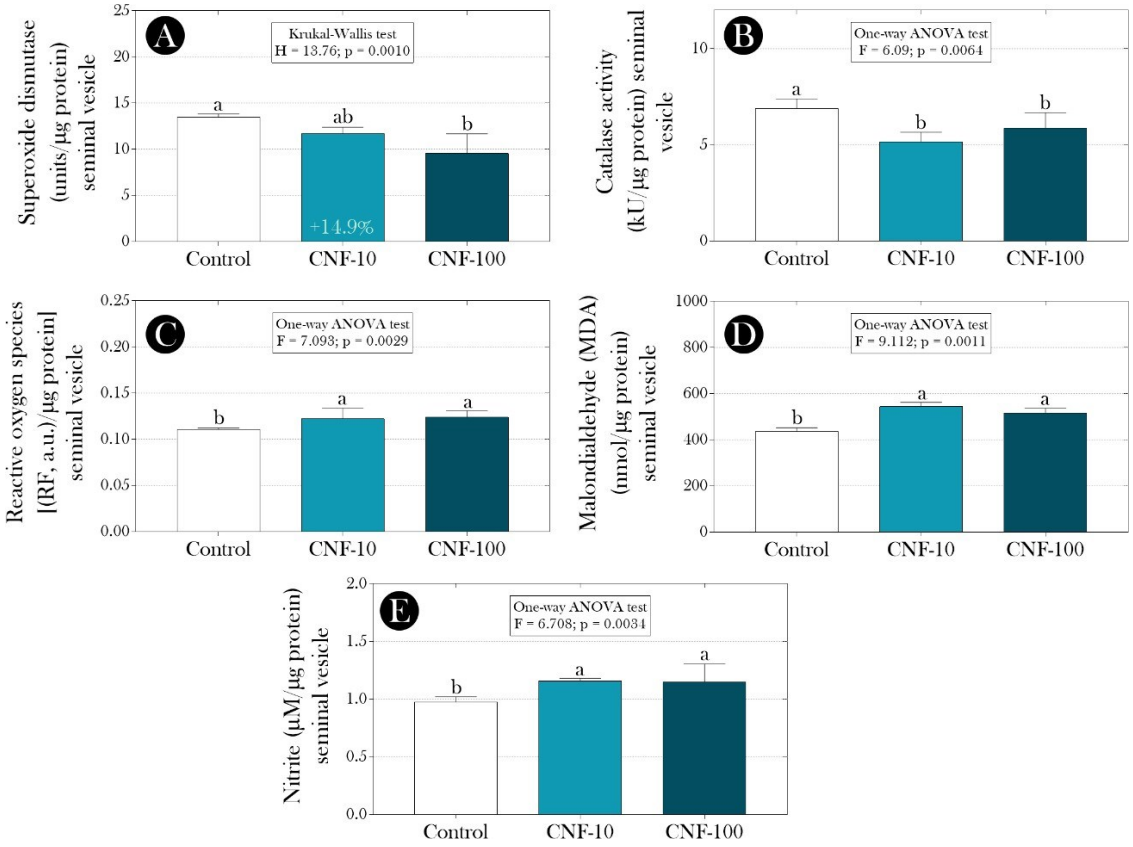

Figure 2. Biochemical biomarkers evaluated in the seminal vesicle of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (A) Superoxide dismutase (SOD) activity, (B) catalase (CAT) activity, (C) reactive oxygen species (ROS), (D) malondialdehyde (MDA), and (E) nitrite levels. Parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. Statistical summaries are shown close to the graphs. C: control; CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively.

In the muscles, we noticed that the increased SOD and CAT activity observed in the animals exposed to CNFs (Figures 3A-B, respectively) was not sufficient to prevent the increase in ROS and MDA production in these animals compared to the unexposed group (Figures 3C-D, respectively). In addition, we showed a significant reduction in nitrite production and AChE activity in the muscles of animals in the "CNF-10" and "CNF-100" groups (Figures 3E-F, respectively). On the other hand, exposure to both concentrations of CBNs induced an increase in the layer of circular muscle (Figure 3SA – see "Supplementary Materials") and a reduction in the layer of the longitudinal muscle of the earthworms of the "CNF-10" and "CNF-100" groups (Figure 3SB – see "Supplementary Materials"). Although a smaller thickness of the circular muscle fibers was observed only in earthworms exposed to the highest concentration of CNFs (Figures 4A-D), areas of deformation of the circular and longitudinal muscles were observed in the "CNF-10" and "CNF-100" groups. In the animals of these groups, we noticed the presence of

expansion of spaces between the muscular layers, circular/longitudinal muscle fibers, and irregular fibers (Figures 5E-H).

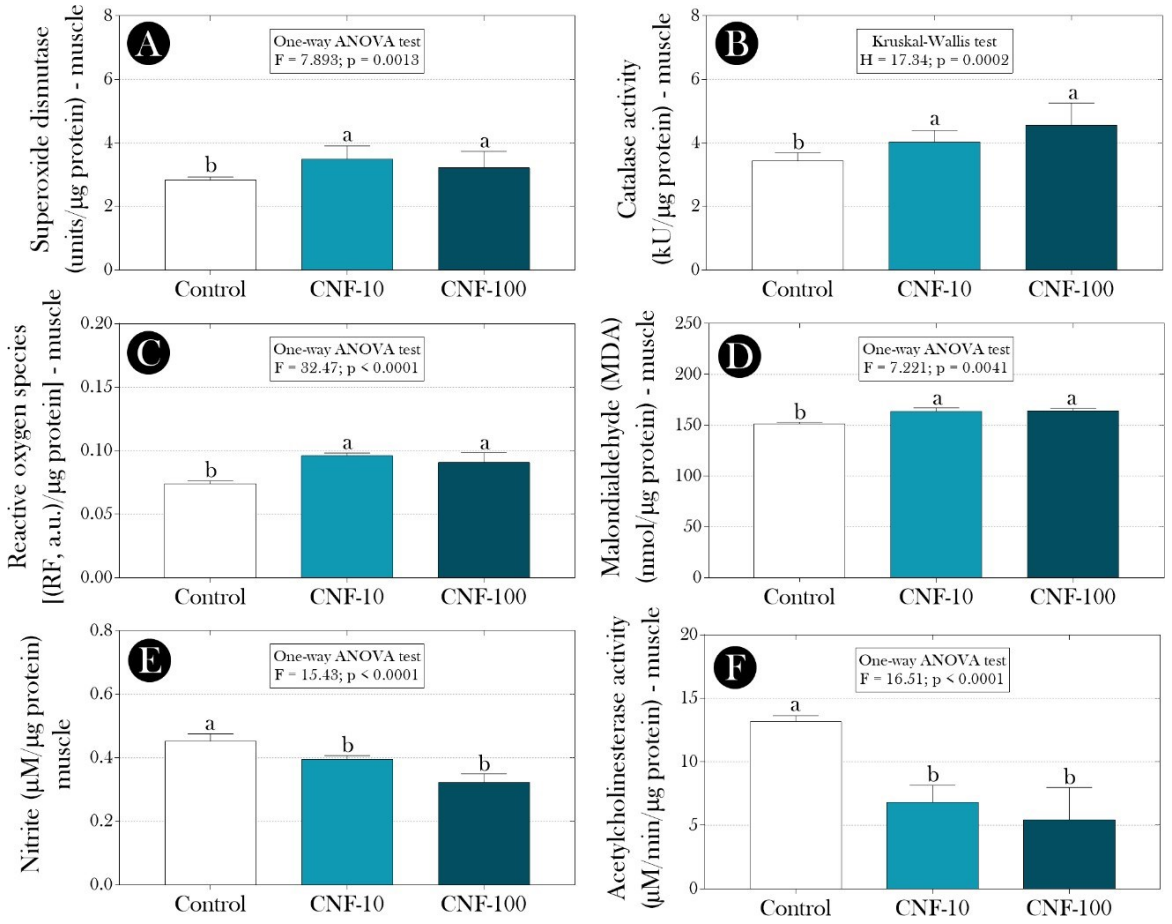

Figure 3. Biochemical biomarkers evaluated in muscles of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (A) superoxide dismutase (SOD) activity, (B) catalase activity (CAT), (C) reactive oxygen species (ROS), (D) malondialdehyde (MDA) and (E) nitrite levels, and (F) acetylcholinesterase (AChE) activity. Parametric data are presented by mean + standard deviation, while nonparametric data are presented by median and interquartile range. Distinct lowercase letters indicate significant differences. Statistical summaries are shown next to the graphs. C: control; CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively.

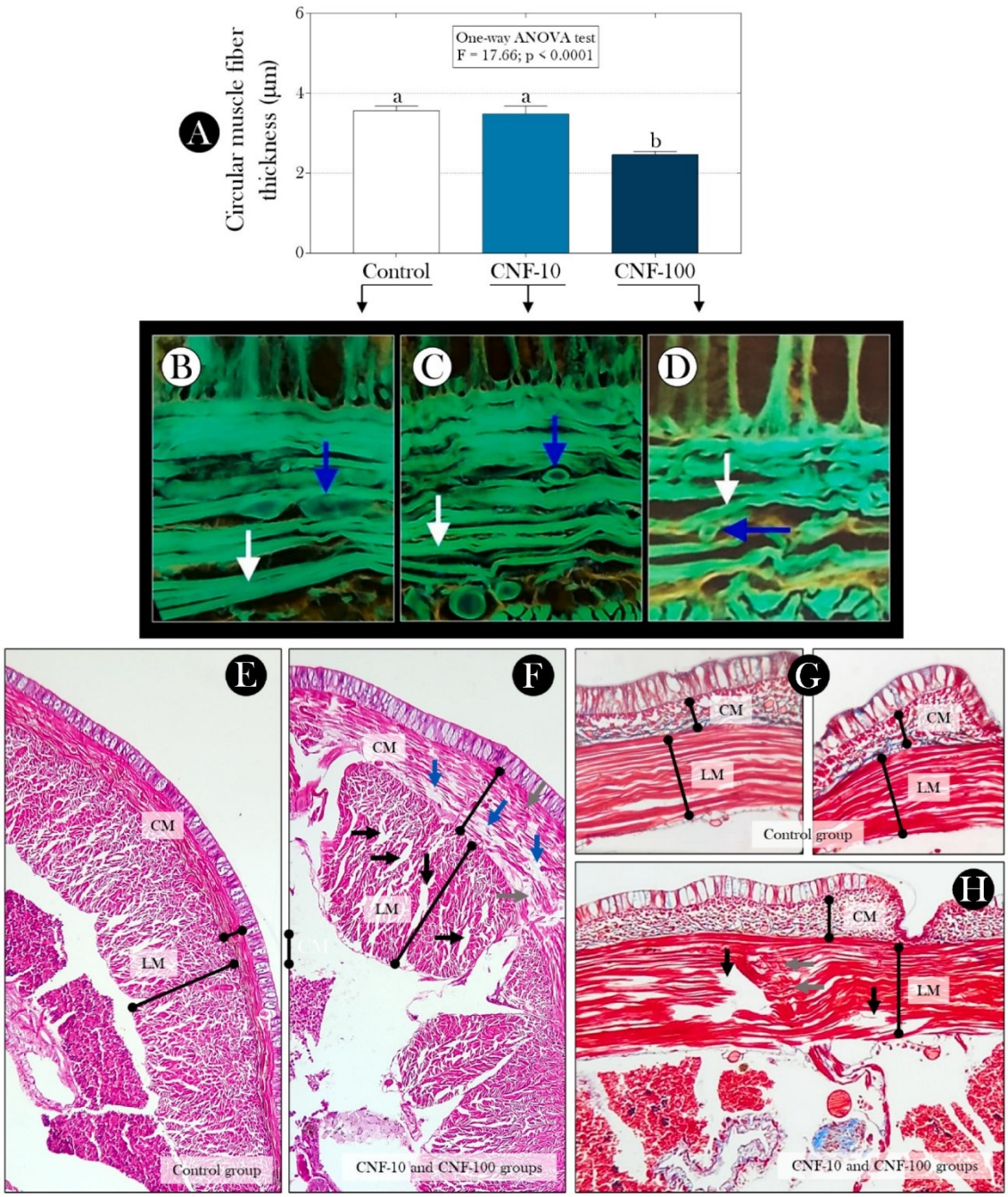

Figure 4. (A) Circular muscle fiber thickness of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations (B to D). Representative photomicrographs of muscular layers: (E-F) transversal and (G-H) longitudinal sections of the animals' bodies. In "A," parametric data are presented by the mean + standard deviation. Distinct lowercase letters indicate significant differences. Statistical summary is shown close to the graph. In "B to D," blue arrows indicate blood vessels and white arrows point to muscle fibers. The analyzed sections were stained with Masson's Trichrome using standard histopathological techniques. Photomicrographs were edited with a negative filter to allow better visualization of tissue structures. In "E" and "F" were stained with hematoxylin and eosin (H&E), and "G" and "H" with Masson's Trichrome, using standard histopathological techniques. Black and blue arrows indicate areas of expansion of spaces between the longitudinal and circular muscular layers, respectively. Gray arrows indicate the deformation/disorganization of muscle fibers. CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. CM: circular muscles and LM: longitudinal muscles.

In the intestine, we also noticed an increase in SOD activity in *L. terrestris* from the "CNF-100" group (Figure 5A), and, in relation to CAT, a concentration-dependent increase was evidenced (Figures 5B-C). Furthermore, a significant increase in oxidative stress biomarkers evaluated [i.e., ROS and MDA levels (Figures 5D-E)] was reported in earthworms exposed to CNFs. On the other hand, the reduction in nitrite production observed in animals from the "CNF-100" group suggests that pollutants (at 100 mg/kg) affected NO production (Figure 5F). In addition, histological analyses revealed the effect of exposure to CNFs on the tissue structure of the animals' intestines. As shown in Figures 4SA-D (see "Supplementary Materials") and 6A-D, the intestinal epithelium and muscle layer height in animals from the "CNF-10" and "CNF-100" groups were, on average, 25.8% and 15.8%, respectively, lower than that reported in unexposed animals. We noticed a typical enlargement of the intestinal lumen of earthworms exposed to CNFs, with the luminal area in these animals being, on average, 75% greater than that recorded in the "control" group (Figures 6E-H). In addition, lesions/ruptures of the intestinal epithelium and fibrous areas were observed in animals exposed to CNFs, with such alterations being more prominent in the "CNF-100" group (Figure 5S – see "Supplementary Materials"). The chloragogen cell layer height did not differ between the evaluated groups (Figure 6I).

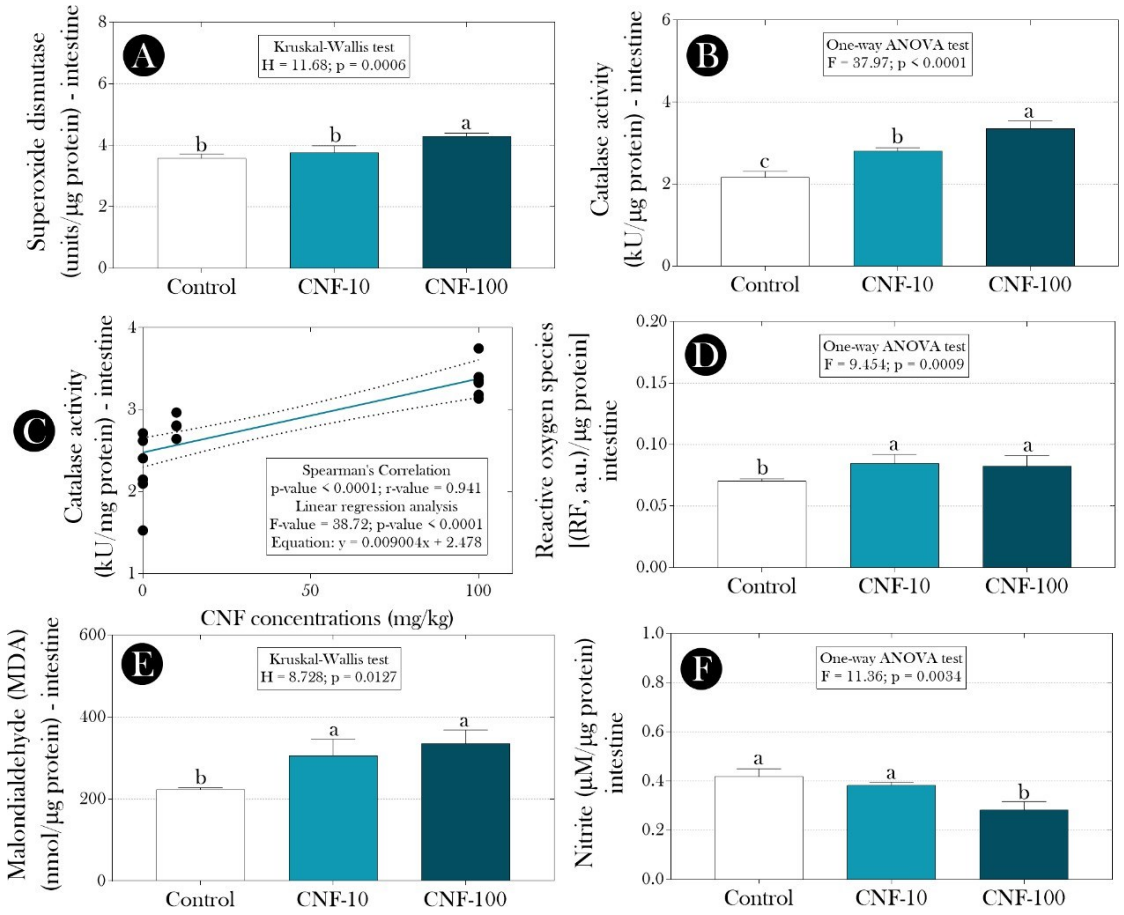

Figure 5. Biochemical biomarkers evaluated in the intestine of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (A) Superoxide dismutase (SOD) activity,

(B-C) catalase (CAT) activity, (D) reactive oxygen species (ROS), (E) malondialdehyde (MDA), and (F) nitrite levels. Parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. Statistical summaries are shown close to the graphs. C: control; CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively.

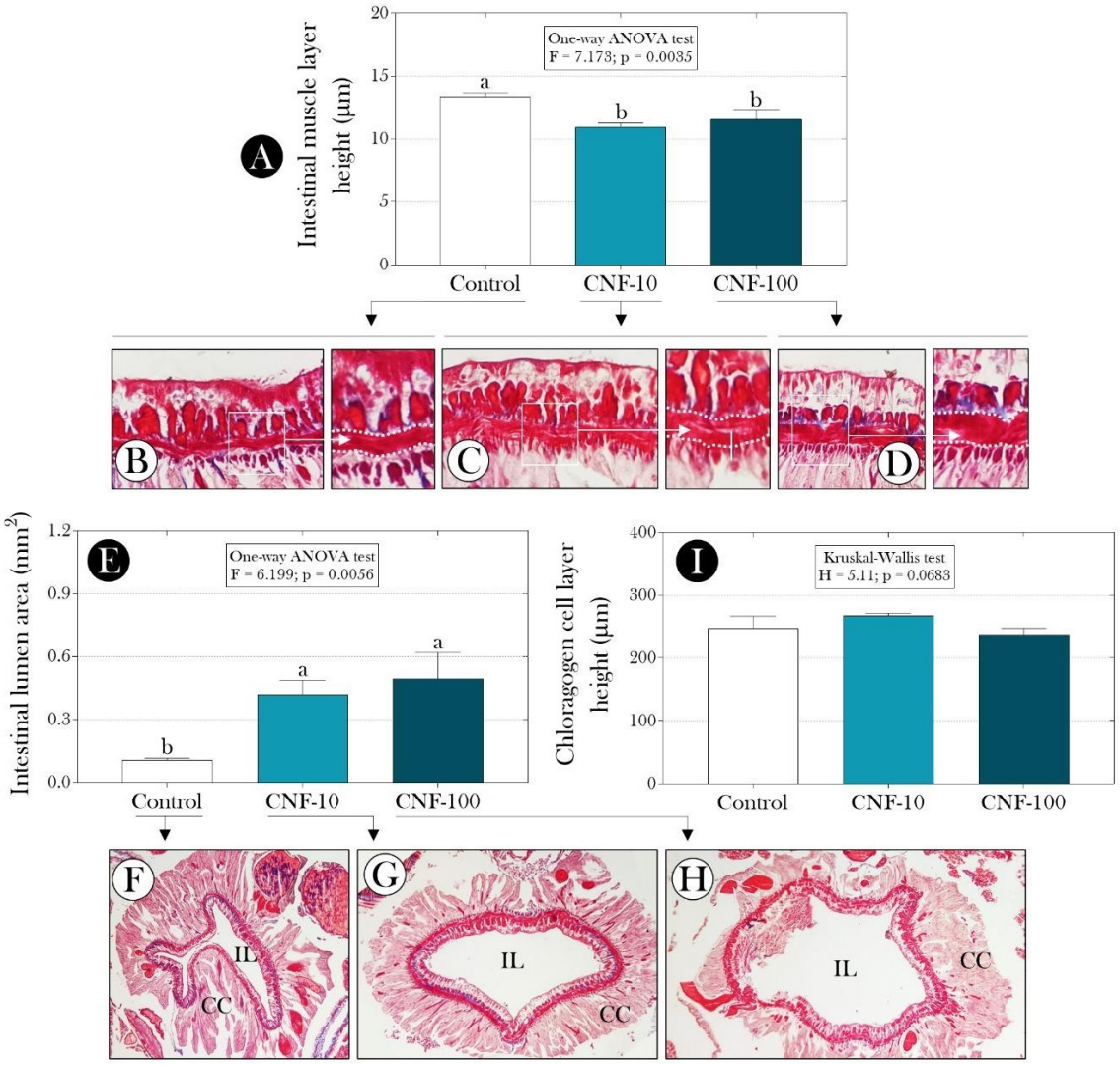

Figure 6. (A-D) Intestinal muscle layer height, (E) intestinal lumen area, and (I) chloragogen cell layer height of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (F to H) Representative photomicrographs of the intestinal tissues of earthworms from the "control," "CNF-10," and "CNF-100" groups, respectively. CC: chloragogen cell layer and IL: intestinal lumen. In "A," "E," and "I," parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. Statistical summaries are shown close to the graphs. C: control; CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. The analyzed sections were stained with Masson's Trichrome using standard histopathological techniques.

Our data also showed lower epidermal height in *L. terrestris* exposed to CNFs (with a concentration-dependent effect) (Figures 6SA-B, respectively – see "Supplementary Materials"), erosion areas in the epithelium (Figures 7A-B), reduced cuticular thickness (Figure 7S – see

"Supplementary Materials") and detachment of the cuticle in various regions of the body (Figures 7C-D). On the other hand, we found no effect of exposure to CNFs on large granule cells and reticular gland cell number (Figures 8SA-F - see "Supplementary Material").

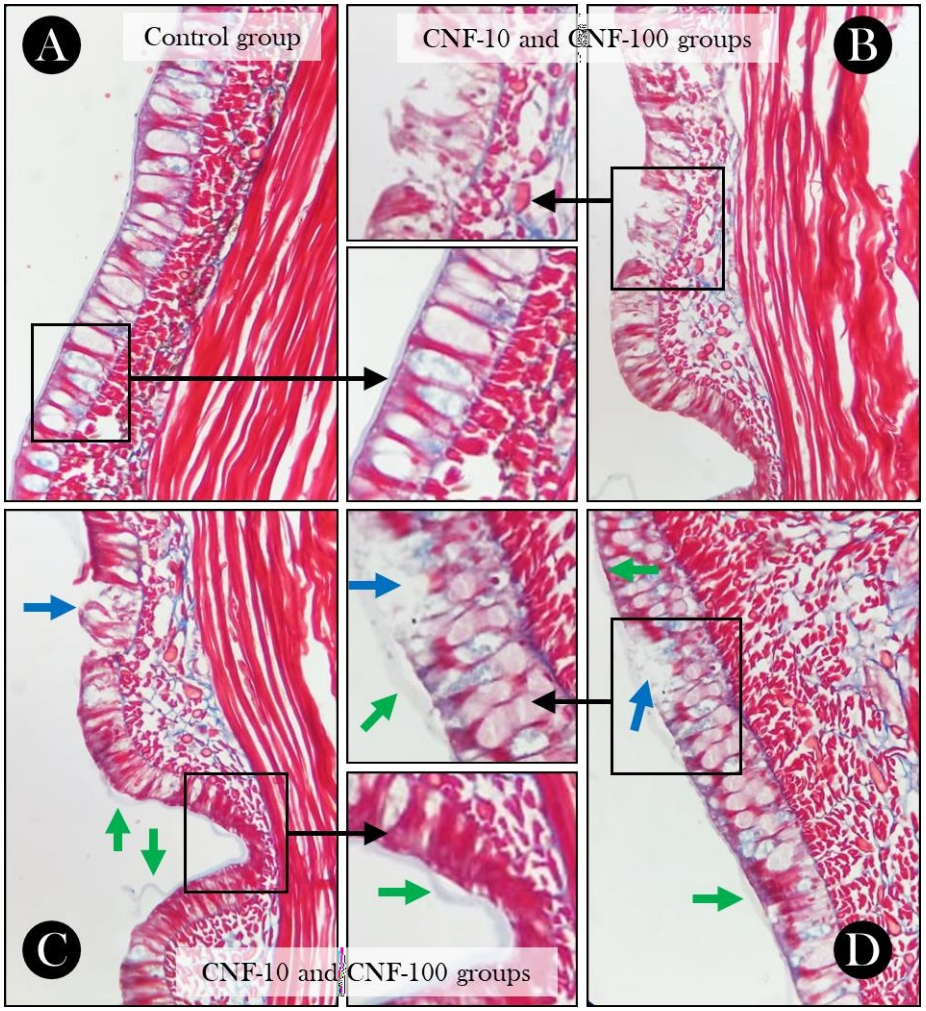

Figure 7. Representative photomicrographs of the epidermis (longitudinal section) of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. The sections were stained with Masson's Trichrome using standard histopathological techniques. Blue and green arrows indicate erosion areas in the epithelium and detachment of the cuticle, respectively.

Regarding the analysis of the exposure soils, Table 3S (see "Supplementary Material") confirms that the percentage of TOC concentration both before and after the experimental period was proportional to the amount of CNFs added in the experimental units. Furthermore, at the end of the experiment, we noticed an increase in the concentrations of Ca, Mg, CTC, P, K, Mn, and base saturation. On the other hand, Fe and Zn concentrations were reduced, and for the other attributes, no significant differences were evidenced (Table 3S). Furthermore, we noted a concentration-dependent increase in TOC concentration in the intestinal contents of *L.*

terrestris, which suggests that CNFs were uptaken by the animals (Figure 8).

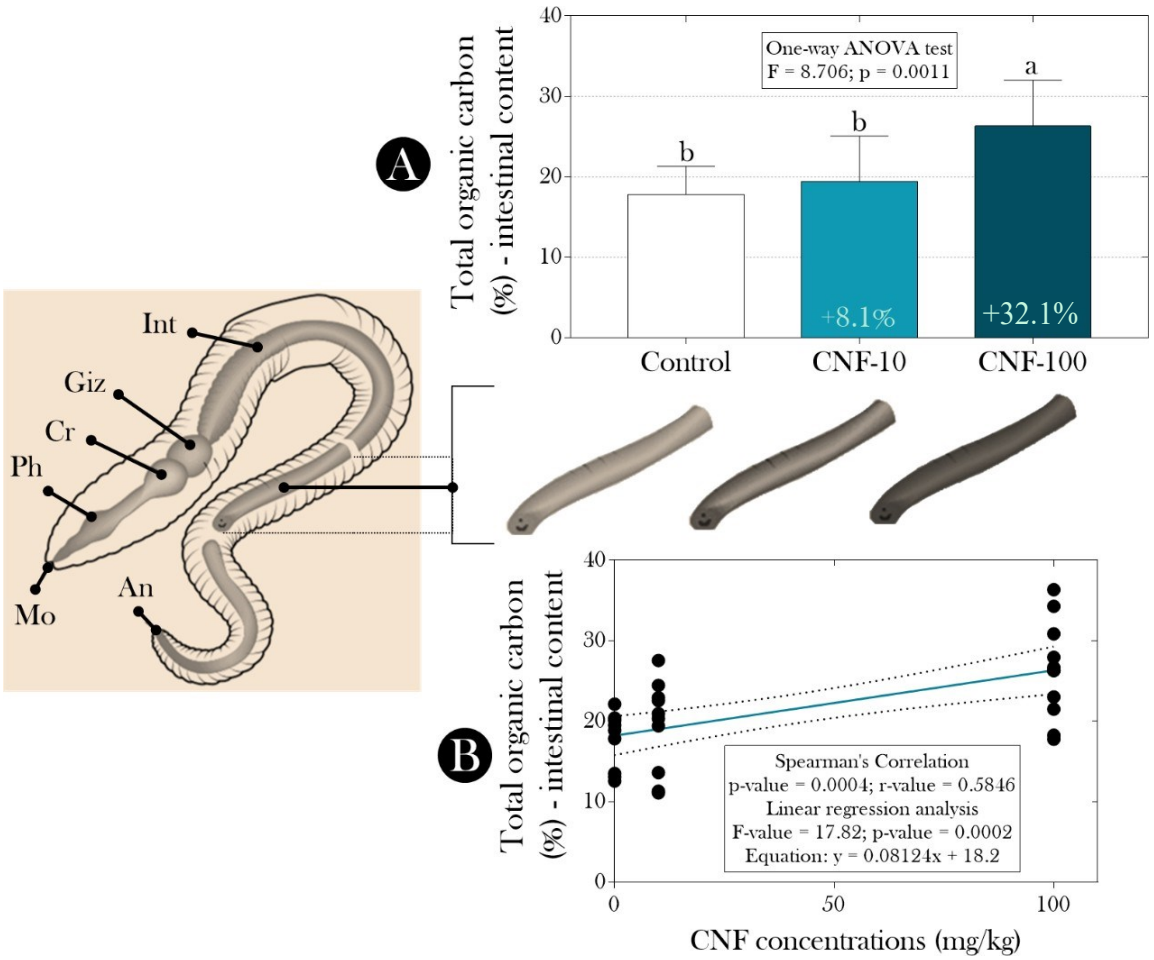

Figure 8. (A) Total organic carbon in intestinal content of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (B) correlation analysis and linear regression between the variables "OCD of intestinal content vs. concentrations of CNFs in soils." In "A," parametric data are presented by the mean + standard deviation. Distinct lowercase letters indicate significant differences. Statistical summaries are shown close to the graphs. CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. Int: intestine, Giz: gizzard, Cr: crop, Ph: pharynx, Mo: mouth, and An: anus.

To assess the overall response of animals to treatments, we submitted all data to the PCA and applied IBRv2. Regarding PCA, we show that the first two principal components (PC1 and PC2) cumulatively explained 100% of the total variation (PC1: 77% and PC2: 23%), with PCs-eigenvalue > 8.0 for both PCs (PC1: 26.94 and PC2: 8.05). Furthermore, Figure 9A and Table 4S (see "Supplementary Material") show that most of the analyzed variables were positively associated with PC1 and PC2. According to IBRv2, exposure to CNFs at the highest concentration (100 mg/kg) induced a prominently more toxic response to earthworms (Figure 10A), which was determined especially by SOD activity in the seminal vesicle, CAT, and AChE muscular activities, nitrite levels in muscle, a layer of circular muscle (Figure 10B), circular muscle fiber thickness, intestinal CAT activity, intestinal nitrite levels (Figure 10C), as well as epidermal cuticle (Figure 10D), which showed more significant deviations from the "control" group.

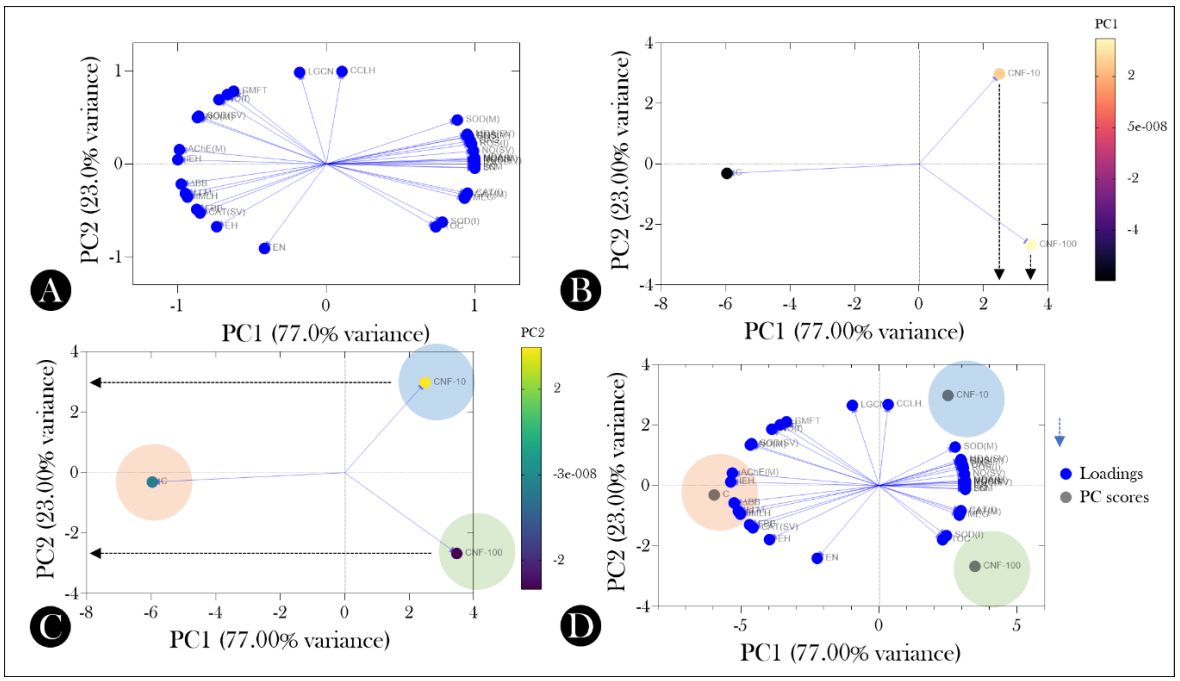

Figure 9. (A) Loadings plot of the investigated variables, (B-C) PC score plots (PC1 and PC2, respectively, and (D) PCA biplot of the first two principal components (PCs) that simultaneously shows scores of experimental groups [adults *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations]. See the meanings of the acronyms in Table 4S (see "Supplementary Materials"). In "B" and "C," the black and blue arrows point to the PC1 and PC2 scores of the experimental groups, respectively. C: control group, CNF-10, and CNF-100: adults *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively.

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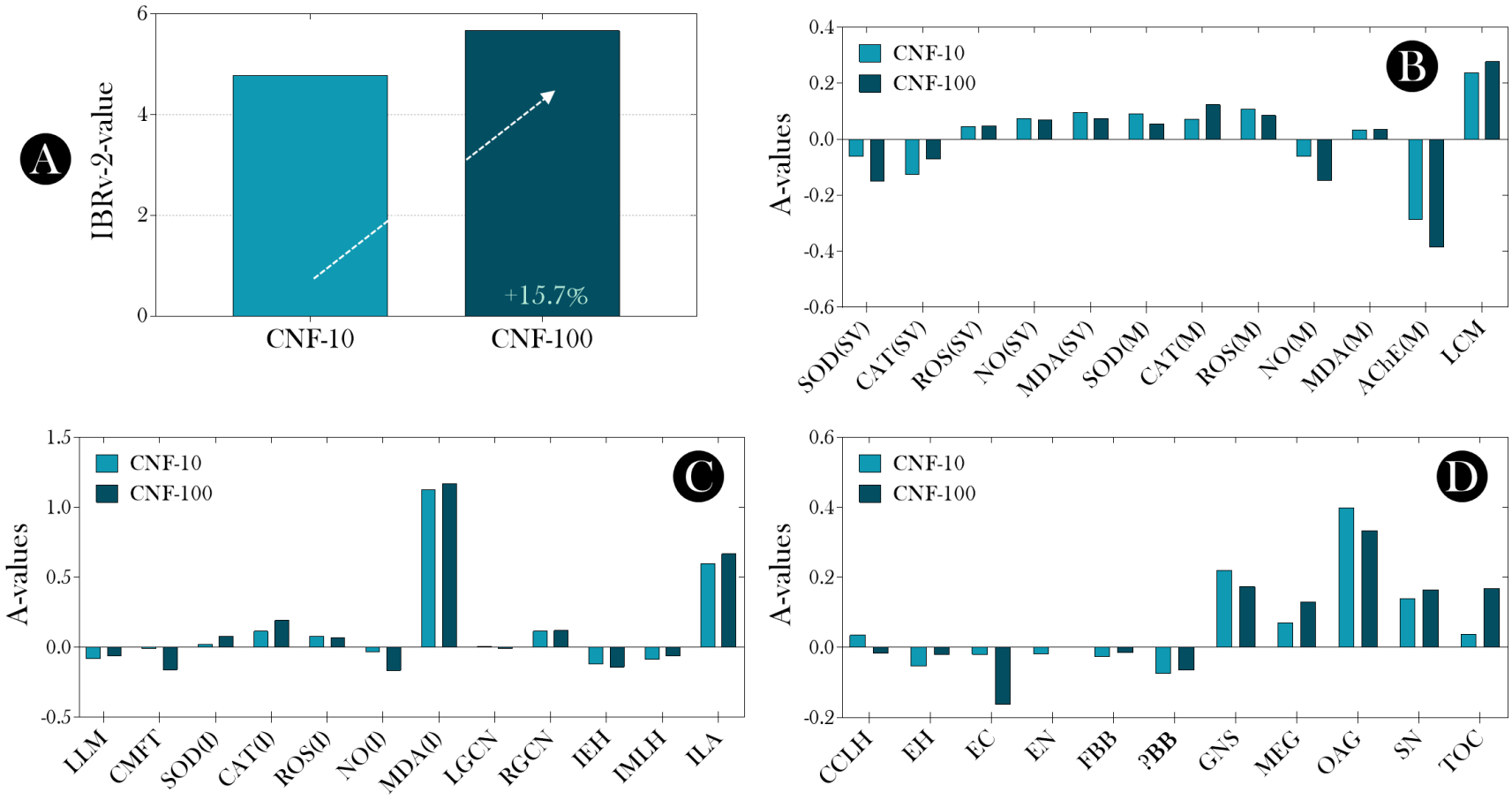

Figure 10. (A) The "Integrated Biomarker Response" index (IBRv2) was calculated for groups composed of adult *Lumbricus terrestris* earthworms unexposed to carbon nanofibers (CNFs) at different concentrations. (B to D): A-values derived from the IBRv2 obtained for the respective groups exposed to the CNFs. CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. See the meanings of the acronyms in Table 4S (see "Supplementary Material").

4. DISCUSSION

Assessing the possible impacts of pollutants on the biota is a crucial part of preventive risk assessment (identifying hazards early). It is considered one of the steps that precede the planning and proposition of mitigation or remediation strategies for the impacts caused by pollution. In this sense, our study provides pioneering evidence on how CNFs can impact earthworm health [one of the major macrofaunae among soil biota to maintain dynamic equilibrium and regulate soil fertility (Kale & Karmegam, 2010; Zuo et al., 2023)] and potentiate the effect of *Monocystis* sp. infection. Initially, we noticed that although the population density was not affected by the treatments (Figure 1SA - see "Supplementary Material"), earthworms from all groups showed a significant reduction in body biomass (Figure 1SB - see "Supplementary Material") at the end of the experiment, which may be a direct consequence of *Monocystis* sp. infection [as also evidenced in the study by Field & Michiels (2005)] potentiated by exposure to CNFs. Thus, it is possible that the weight loss (Figure 1SC - see "Supplementary Material") of earthworms from the "CNF-10" and "CNF-100" groups may be putatively explained by the high energetic costs of immune reaction to parasites in earthworms and/or an effect of exposure to CNFs. The reduction in energetic molecules (e.g., total soluble carbohydrate, total protein, and triglyceride levels in different tissues of the exposed animals) induced by exposure to nanomaterials may explain the reduction in the animals' body biomass, as reported in the study by Guimarães et al. (2021), involving *Physalaemus cuvieri* tadpoles exposed to the same CNFs used in the present study. Furthermore, as expected, we noticed a strong impact of *Monocystis* sp. infection on the reproductive performance of earthworms, like previous reports by Goldova & Breza (1999) and Field et al. (2004). As highlighted by Field & Michiels (2006), *Monocystis* sp. is an internal parasite in the reproductive system of earthworms, and when the sporozoites enter the circulatory system and invade the sperm vesicle lumen, destruction of spermatocytes occurs. Furthermore, *Monocystis* sp. infection may result in reproductive inefficiency due to hypertrophy of male funnel cells and parasitism of sperm morale (Stephenson, 1930; Stroud, 2022). Therefore, this constitutes a possible reason for the low cocoon production observed in all experimental groups. However, it is necessary to consider that this hypothesis must be confirmed in previous studies, mainly because our design did not predict control or at least controlled parasitic loading. Furthermore, we cannot neglect the hypothesis that the low reproductive performance in earthworms is related to the population density in the replicas, which may have been high for the experimental context of our study. Therefore, this constitutes another plausible investigative perspective to be evaluated in the future.

Our data also demonstrate that animals exposed to CNFs had the highest parasite load (Figure 1), suggesting that soil contamination by these nanomaterials is an environmental factor in earthworm parasitology. Although no studies are like ours, previous investigations involving other pollutants point to an analogous scenario. Purrini (1983), e.g., recorded a mean incidence of infection of 25% in earthworms from two polluted localities in Germany, and Purrini (1987), when comparing the parasitological results of earthworms captured in several localities, concluded that SO_2-deposits, industrial pollution, and pesticides are probably involved in the susceptibility of soil animals to disease and parasites. It was also supported by Pižl (1989) and Pižl (1991), who found strong correlations between earthworm infection by monocystid gregarines and heavy metals and herbicide treatment, assuming that the adverse effects of these other pollutants result in a decrease in the pathogen resistance of the animals. Furthermore, Pižl (1985) incubated uninfected earthworms with (and without) gregarine sporocysts in control and pesticide-treated soils for 26 weeks and observed that the earthworms in untreated control soils displayed *Monocystis* sp. infection rates of 64%, 36%, and 56% for epigeic, endogeic, and anecic earthworms, respectively. However, in pesticide-treated soils, the *Monocystis* sp. infection rates were considerably elevated to 100% for epigeic, 75% for endogeic, and 96% for anecic earthworms.

These findings, as in our study, can be attributed to the reduced immune response to *Monocystis* sp. infection induced by exposure of earthworms to pollutants. Previous *in vitro* and *in vivo* studies involving several nanomaterials (Bodó et al., 2020; Tang et al., 2021; Boraschi et al., 2023), including CBNs [see review by Yuan et al. (2019)], have already demonstrated that these pollutants can immunosuppress the immune response and, consequently, increase the susceptibility of earthworms to the parasitic infection, corroborating our hypothesis. However, based on our data, it can be assumed that the negative effect of CNFs on the mechanisms that regulate redox/nitrosative homeostasis and the observed histopathological changes can also explain the greater susceptibility of animals to *Monocystis* sp. infection. The increase in ROS and MDA production in the seminal vesicle (Figures 2C-D), muscles (Figures 3C-D), and intestine (Figures 5D-E) of earthworms exposed to CNFs strongly suggests the induction of oxidative stress by exposure to CNFs, whose increase in SOD and CAT activity – reported in muscle and intestine (Figures 3A-B and 5A-B, respectively) – was not sufficient to maintain redox homeostasis. In the seminal vesicle, in particular, we observed a suppression of antioxidant activity, especially in animals exposed to the highest concentration of CFNs (“CNF-100” group) (Figures 2A-B).

These results agree with previous reports that point to the induction of oxidative stress as

one of the most common mechanisms of CBN toxicity at the cellular level. In earthworms, mainly, some studies have observed increased ROS production even though the activity of SOD and CAT – essential antioxidant enzymes that play a crucial role in maintaining redox homeostasis (Kurutas, 2015) – has also increased. Xu et al. (2021), e.g., demonstrated that the SOD and CAT enzymes of *E. fetida* earthworms had their activities increased in response to exposure to single-wall carbon nanotube (SWCNT), which represents a cascade of events that reflected the effort and consequence made by the earthworms to deal with ROS. While the increase in SOD can be explained by the increased production of superoxide radical anion (later converted to H_2O_2), the increased CAT activity may have occurred due to the greater demand for H_2O_2 decomposition (i.e., a product of SOD action) and prevented further oxidative damage to proteins and lipids. However, other investigations demonstrate that the effect of CBNs on the antioxidant response of earthworms is dependent on the exposure concentration, as Yang et al. (2022) reported. At the time, the authors noted a significant increase in SOD, CAT, and glutathione-s-transferase (GST) in *E. fetida* earthworms exposed to multi-walled carbon nanotubes (MWCNTs) at 50 mg/kg. In comparison, suppression of these enzymes was noted up to 100 mg/kg.

On the other hand, the suppression of antioxidant activity observed in the seminal vesicle of earthworms exposed to CNFs (Figures 2A-B) may also be associated with *Monocystis* sp. infection. According to Sims & Gerard (1985) and Breidenback (2002), strong infections are known to destroy the seminal vesicle, culminating in the consequent reduction in the antioxidant response due to cell death. The increase in NO production (inferred by nitrite levels - Figure 2E) only in the seminal vesicle of earthworms exposed to CNFs may be linked to the immune response triggered in this organ against the parasites since it is the main target organ of *Monocystis* sp. infection. In this case, it is possible that the greater antigenic stimulation caused by the high parasite load in the seminal vesicle activated the expression of NO production signaling molecules, including pattern recognition receptors (PRRs) and pro-inflammatory cytokines. Prochazkova et al. (2019), for example, observed (via screening of the tissue expression profile) that Toll-like receptors (TLRs) - one of these PRRs expressed by various immune cells – was expressed primarily in *E. andrei* earthworm seminal vesicles and receptacles, infected by *Monocystis* sp. The recognition of pathogen-associated molecular patterns (PAMPs) by TLRs expressed in immune cells of the seminal vesicle of earthworms and, consequently, induction of inflammatory immune response (Vijay, 2018) constitutes, therefore, a reasonable explanation for the increase in NO production in “CNF-10” and “CNF-100” groups (Figure 2E). As discussed by Abdul-Cader et al. (2016), the TLR-PAMP interaction elicits an intracellular signaling cascade,

culminating in NO-mediated antigenic responses via, for example, inducible nitric oxide synthase (iNOS). This enzyme facilitates NO production at high levels, in which NO plays a role in host defense and immunological reactions (Colasanti & Venturini, 1998; Cook et al., 2015). On the other hand, the reduction of NO levels in muscle (Figure 3E) and intestine (Figure 5F) – especially in earthworms exposed to the highest concentration of CNFs (i.e., "CNF-100" group) may be associated with the impact of nanomaterials on the activity of iNOS, as suggested by Mohanta et al. (2019). However, new studies are required to understand better the factors associated with earthworms' biochemical response and immune response to *Monocystis* sp. infection and exposure to CNFs.

Interestingly, we also noticed that exposure to CNFs induced histopathological changes in different evaluated tissues. In the muscles, we observed a pathology characterized by alterations in the height of the muscular layers (circular and longitudinal) (Figures 3S and 3), the presence of expansion of spaces between the muscular layers (Figures 5E-H), and the irregular appearance of the circular and longitudinal muscles (Figures 5E-H) of earthworms exposed to nanomaterials. Therefore, this denotes these tissues' structural integrity loss, possibly induced by oxidative stress induced by CNFs (Figures 3C-D). The increased thickness of the circular musculature (Figure 3SA – see "Supplementary Materials"), in particular, could potentially also be a reflection of a general defense mechanism against toxic agents, as discussed by Bayner & Hodgson (2004) and Poleksić et al. (2010). This condition would increase the distance between the external environment (contaminated with CNFs) and the internal organs, acting as an additional barrier to the entry of nanomaterials. This hypothesis is especially reinforced when we note that exposure to CNFs induced important changes in the epidermis and cuticle of earthworms, as discussed later. Although more studies are required for a broader understanding of the mechanisms that led to these changes, it is known that damage to the circular and longitudinal muscle cells leads to disruptions in the peritoneum layer of an earthworm (Priyanka et al., 2018). Therefore, such a condition may lead to problems in functioning blood vessels, lymph vessels, and nerves, resulting in various degrees of histopathological alterations in the internal cells.

In the intestine, we noticed an enlargement of the lumen (Figure 6E) in both groups exposed to CNFs, as well as a reduction in the height of the intestinal epithelium (Figure 4SA – see "Supplementary Materials") and the underlying muscular lamina (Figure 6A). In addition, we observed alterations suggestive of lesions/ruptures of the intestinal epithelium, especially in animals from the "CNF-100" group (Figure 5S – see "Supplementary Materials"), which may be associated with the reaction of intestinal cells to direct contact with CNFs or their internalization, with oxidative stress and increased lipid peroxidation processes (Figures 5D-F) being plausible

explanations for the presence of severe tissue degradation. Furthermore, fibrotic areas in the intestinal region (Figure 5SE – see "Supplementary Materials") reinforce the relationship between these changes and the increase in inflammatory processes. Furthermore, the constant transit of CNFs in the intestinal lumen, their possible adhesion to the epithelial mucosa, and cellular internalization associated with the presence of *Monocystis* sp. parasites may have contributed to the induction of intestinal damage reported in our study. On the other hand, the accumulation of CNFs in the intestinal lumen (inferred by the highest TOC concentrations in the intestinal contents of earthworms exposed to nanomaterials – Figure 1S) may have affected the functioning of the underlying musculature (with reduced thickness). This may have occurred through the interference of CNFs in AChE activity on acetylcholine (ACh) (a neurotransmitter at the neuromuscular junction between the motor nerve and skeletal muscle) (Figure 3F), reducing the peristaltic movements of the intestinal muscles and leading to an accumulation of contents in the intestine with a consequent luminal enlargement (Figure 6E).

We also noted external physical effects associated with exposure to CNFs at the highest concentration (e.g., 100 mg/kg), such as epidermal eruptions (Figure 7) and cuticular damage, characterized by reduced cuticle thickness and slightly detached epithelium - Figure 7). In this case, the irregular and tapering shape of the CNFs could potentially damage – mechanically – the epidemic tissues and cause an increase in inflammatory processes that further aggravate the microlesions. Therefore, these damages also play a role in nanofibers' toxic effects on the studied earthworms, which can also trigger systemic effects. As discussed by Clauss (2001), the epidermis and cuticle represent a primary barrier that protects the body, in addition to participating in the transport of ions and allowing/blocking the entry of xenobiotics into the body of earthworms.

The soil analysis carried out at the end of the experiment showed an increase in the concentrations of some chemical elements, such as Ca, Mg, P, K, and Mn (Table 3S - see "Supplementary Material"), as well as in CEC and base saturation, which can be attributed to the weekly addition of dried cow dung powder throughout the experiment, as described in section 2.2.1. Furthermore, the reduction in Fe and Zn concentrations may be directly related to earthworm activity in the waste (cow dung powder) decomposition system and potential tissue bioaccumulation, as also suggested by Gupta et al. (2005) and Suthar & Singh (2009). However, such changes in the soil seem not to have influenced the response of the animals to the treatments since no significant differences were observed in the concentrations of chemical elements or other soil attributes between the different experimental groups (Table 3S - see "Supplementary Material").

On the other hand, it is important to consider that although our findings do not point to

a concentration-response for several of the evaluated biomarkers when taken together and after the application of IBRv2, we noticed that the response of earthworms exposed to soil contaminated with CNFs at 100 mg/kg showed greater deviation than the unexposed group (Figure 10A). As discussed by Lupi et al. (2020) and Hema et al. (2023), as a general stress, the IBRv2 index provides a simple interpretation of the level of response in a particular group, having recently been used to quantify the combined biological effects measured by a battery of biomarker induced by different pollutants [e.g., Ramesh et al. (2023), Shi et al. (2023), Freitas et al. (2023) and Oliveira et al. (2023)]. In our study, we used the IBRv2 index to integrate the responses of the 35 selected biomarkers (Table 4S – see "Supplementary material") to evaluate the effects of CNFs in L. terrestris. The investigated biomarkers exhibited a response that was induced or inhibited according to the different groups, as shown in Figure 10. However, the greater deviation of the response of the earthworms from the "CNF-100" group in relation to the unexposed group was mainly determined by the significant suppression of SOD activity assessed in the seminal vesicle, by biochemical biomarkers assessed in muscle (i.e., CAT and AChE activity and nitrite levels) and intestine (i.e., CAT activity and nitrite levels), as well as by muscle histopathological biomarkers (layer of circular muscle and circular muscle fiber thickness and epidermal cuticle of the animals in the "CNF-100" group (Figures 10B-D). We also observed that the experimental groups were clearly separated into distinct subgroups in the PCA (Figure 9), which was performed to explore correlations among experimental groups based on the average value of each biomarker evaluation, like Malafaia et al. (2022), Araújo et al. (2023) and Gomes et al. (2023). Although the "CNF-10" and "CNF-100" groups were positioned in positive quadrants of PC1, presenting similar PC scores (Figure 9B), such groups were positioned in opposite quadrants in PC2 and distinct from the "control" group (Figure 9C-D). Therefore, this indicates that the experimental groups presented a different response to the treatments.

However, future investigations may clarify whether similar results are also observed at different exposure times (both shorter, the longer they last), if the factor age/developmental stage of the animals and species are determinant in the toxicity of CNFs, and if the evaluations carried out only at the end of the 28 days of exposure would have "masked" the earlier effects. On the other hand, the inclusion of other biomarkers of toxicity to the experimental design (perhaps more sensitive, e.g., molecular, genetic, and immunological) may help evaluate the response of animals to CNFs. Another interesting approach refers to evaluating the depuration capacity of animals in situations of depollution of their environment, i.e., after cessation of exposure to CNFs.

5. CONCLUSIONS

In conclusion, our study confirms the hypothesis that CNFs increase the parasite load of *Monocystis* sp. in the seminal vesicles of *L. terrestris* earthworms, as well as induce biochemical and histopathological changes in animals after 28 days of exposure to pollutants, especially at a concentration of 100 mg/kg. Therefore, our findings point to the additional impact these nanomaterials can have on the health of earthworms and point to the need for greater attention to be given to their disposal and ecotoxicological effects on soil organisms. Although we have focused on the response at the individual level, our results shed light on the potential impact of CNFs on natural populations of *L. terrestris* earthworms and, therefore, advance knowledge on predicting the toxicity of CBNs in soils.

6. ACKNOWLEDGMENTS

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7. DECLARATION OF COMPETING INTEREST

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

8. ETHICAL ASPECTS

All experimental procedures were performed in accordance with the ethical standards for animal experimentation, and meticulous efforts were made to ensure that the animals suffered as

little as possible and to reduce external sources of stress, pain, and discomfort. The current study has not exceeded the number of animals needed to produce reliable scientific data. This article does not refer to any study with vertebrate animals (including human participants) performed by any authors.

9. AUTHOR CONTRIBUTION STATEMENTS

Mateus Flores Montalvão, Alex Rodrigues Gomes, Abraão Tiago Batista Guimarães, Letícia Paiva de Matos, Juliana dos Santos Mendonça, Thiarlen Marinho da Luz, and Stênio Gonçalves da Silva Matos: performed experiments and co-wrote the paper. **Aline Sueli de Lima Rodrigues, M. Safiur Rahman, Chinnasamy Ragavendran, Sengottayan Senthil-Nathan, Ajay Guru, Md. Refat Jahan Rakib, Nabisab Mujawar Mubarak, Md. Mostafizur Rahman, Thiago Lopes Rocha, and Abu Reza Md. Towfiqul Islam**: revised the article critically for important intellectual content. **Guilherme Malafaia**: designed and performed experiments, analyzed data, co-wrote the paper, supervised the research, provided funding acquisition, project administration, and resources.

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SUPPLEMENTARY MATERIAL

Table 1S. Biochemical biomarkers evaluated in different tissues of adult *Lumbricus terrestris* earthworms infected with *Monocystis* sp., unexposed or exposed to soil containing carbon nanofibers at different concentrations.

Biomarcadores	Brief description	References used as a basis for carrying out biochemical assays
Reactive oxygen species (ROS)	ROS are intimately involved in redox signaling but, in some situations, can also lead to *oxidative damage (Murphy et al., 2022). Therefore, ROS production was evaluated as a biomarker of oxidative stress.*	Zhao et al. (2013)
Malondialdehyde (MDA)	ROS causes tissue damage by various mechanisms, including lipid peroxidation (activating cyclooxygenases and lipooxygenases). MDA is one of the cells' final products of polyunsaturated fatty acids peroxidation (Gaweł et al., 2004).	Esterbauer & Cheeseman (1990) and Lushchak et al. (2005)
Superoxide dismutase (SOD)	SOD is one of the most effective intracellular antioxidant enzymes, acting on the conversion of superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Sheng et al., 2014; Carvalho-e-Martins et al., 2022). According to Perry et al. (2010). SOD is present in practically every cell in the body.	Marsh et al. (2006) and Deawati et al. (2017)
Catalase (CAT)	CAT constitutes an integral antioxidant defense system enzyme responsible for dismutating hydrogen peroxide (H_2O_2), converted to water and molecular oxygen (Carvalho-e-Martins et al., 2022). According to Gebicka & Krych-Madej (2019), the CAT is widely found in almost all eukaryotic cells, providing cell protection against the toxic effects of H_2O_2.	Hadwan & Abed (2016)
Nitric oxide (NO)	NO production (via sodium nitrite levels) may reflect nitrosative stress from the over-production of nitrogen-based free radicals (Stykel & Ryan, 2022). As highlighted by Wang et al. (2021), nitrosative stress can promote protein tyrosine nitration, resulting in the inactivation of functional enzymes, lipid peroxidation, cell membrane damage, DNA strand breaks, and activation of cascade signal responses of cell death.	Grisham et al. (1996), with modifications described in Guimarães et al. (2021a)
Acetylcholinesterase (AChE)	As highlighted by Dvir et al. (2010), the main biological role of acetylcholinesterase (AChE) is the termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (ACh).	Ellman et al. (1961), with modifications described in Guimarães et al. (2021b)
Total protein	The results of all biochemical biomarkers were relativized with the level of total proteins of the analyzed samples.	Bradford (1976)

Table 2S. Summary of histopathological biomarkers evaluated in different tissues of adult *Lumbricus terrestris* earthworms infected with *Monocystis* sp., unexposed or exposed to soil containing carbon nanofibers at different concentrations.

Tissues	Parameters evaluated	Description	Cutting direction
Epidermis	Epithelial layer height (µm) (Figure 1SA)	For each histological section, eight regions of the ventral (four regions) and dorsal (four regions) portions of the animals were evaluated (Figure 1SA)	Vertical
	Epidermal cuticle (thickness, µm) (Figure 1SA)		
	Large granule cell (or orthochromatic cell) number/500 µm (Figure 1SA) Reticular gland cell (or metachromatic cell) number/500 µm (Figure 1SA)	The cell number was evaluated in four regions of the epidermis of the ventral (two regions) and dorsal (two regions) portions of the animals (Figure 1SA)	
Muscle layer of the body wall	Longitudinal muscle layer height of body wall (µm) (Figure 1SC) Circular muscle layer height of body wall (µm) (Figure 1SC)	For each histological section, eight regions of the ventral (four regions) and dorsal (four regions) portions of the animals were evaluated (Figure 1SC)	
	Circular muscle fiber thickness (µm) (Figure 1SB)	For each histological section, eight regions of the ventral (four regions) and dorsal (four regions) portions of the animals were evaluated (Figure 1SB). In each region, all muscle fibers identified vertically were measured.	
Intestine	Intestinal epithelium height (µm) (Figure 1SD)	For each histological section, ten regions of the intestine (five in the ventral portion and five in the ventral portion) of the animals were evaluated (Figure 1SB)	
	Intestinal muscle layer height (µm) (Figure 1SE)		
	Intestinal lumen area (mm^2) (Figure 1SG)	The intestinal lumen of all histological sections was analyzed.	
Chloragogen cell layer	Chloragogen cell layer height (µm) (Figure 1SF)	For each histological section, ten regions of the chloragogen cell layer (five in the ventral portion and five in the ventral portion) of the animals were evaluated (Figure 1SF)	
Seminal vesicle	Gametocysts number with sporocysts/mm^2 *Monocystis* sp. encapsulated gametocysts area (μm^2) Occupation area (%) of gametocysts in the seminal vesicle	All regions of the histological section of the seminal vesicles were analyzed.	Horizontal

Table 3S. Physical, physical-chemical, and chemical attributes of the soils used in the present study before and after the experimental period.

Attributes	**Units**	**Control (initial)**	**CNF-10 (initial)**	**CNF-100 (initial)**	**Control (final)**	**CNF-10 (final)**	**CNF-100 (final)**
TOC	%	0.93 ± 0.01^{A}	1.33 ± 0.02^{A}	1.8 ± 0.03^{A}	0.7 ± 0.02^{B}	1.16 ± 0.01^{B}	1.33 ± 0.03^{B}
pH	Un.	5.5 ± 0.01^{A}	5.5 ± 0.02^{A}	5.6 ± 0.01^{A}	5.5 ± 0.01^{A}	5.6 ± 0.015^{A}	5.5 ± 0.01^{A}
Ca	cmolc/dm³	2.5 ± 0.3^{B}	2.3 ± 0.2^{B}	2.6 ± 0.1^{B}	4.7 ± 0.2^{A}	4.4 ± 0.1^{A}	4.4 ± 0.2^{A}
Mg	cmolc/dm³	0.7 ± 0.05^{B}	0.6 ± 0.04^{B}	0.5 ± 0.06^{B}	1.5 ± 0.04^{A}	1.2 ± 0.06^{A}	1.6 ± 0.02^{A}
Ca+Mg	cmolc/dm³	3.2 ± 0.1^{B}	2.9 ± 0.3^{B}	3.1 ± 0.1^{B}	6.2 ± 0.1^{A}	5.6 ± 0.4^{A}	6.0 ± 0.2^{A}
Al	cmolc/dm³	0 ± 0^{A}	0 ± 0^{A}	0 ± 0^{A}	0 ± 0^{A}	0 ± 0^{A}	0 ± 0^{A}
H+Al	cmolc/dm³	2.1 ± 0.05^{A}	1.8 ± 0.06^{A}	1.8 ± 0.03^{A}	2.2 ± 0.01^{A}	2 ± 0.02^{A}	1.8 ± 0.04^{A}
CTC	cmolc/dm³	5.73 ± 0.45^{B}	5.12 ± 0.61^{B}	5.36 ± 0.48^{B}	8.96 ± 0.64^{A}	8.14 ± 0.32^{A}	8.36 ± 0.47^{A}
P (Mehlich I)	mg/dm³	11.0 ± 2.2^{B}	14.0 ± 0.54^{B}	14.0 ± 0.12^{B}	39.0 ± 4.1^{A}	36 ± 3.2^{A}	43 ± 0.5^{A}
K	cmolc/dm³	0.435 ± 0.05^{B}	0.425 ± 0.02^{B}	0.46 ± 0.01^{B}	0.558 ± 0.01^{A}	0.537 ± 0.04^{A}	0.563 ± 0.02^{A}
Na	mg/dm³	2.0 ± 0.01^{A}	2.0 ± 0.02^{A}	1.8 ± 0.03^{A}	1.7 ± 0.02^{A}	1.8 ± 0.02^{A}	2.0 ± 0.01^{A}
S	mg/dm³	6.0 ± 0.02^{A}	6.0 ± 0.01^{A}	5.9 ± 0.03^{A}	6.0 ± 0.03^{A}	6.1 ± 0.04^{A}	5.8 ± 0.06^{A}
B	mg/dm³	0.26 ± 0.01^{A}	0.28 ± 0.04^{A}	0.26 ± 0.05^{A}	0.27 ± 0.01^{A}	0.25 ± 0.09^{A}	0.25 ± 0.12^{A}
Cu	mg/dm³	3.3 ± 0.014^{A}	2.5 ± 0.090^{A}	2.3 ± 0.110^{A}	1.9 ± 0.021^{B}	1.5 ± 0.075^{B}	1.5 ± 0.091^{B}
Fe	mg/dm³	78.0 ± 0.12^{A}	73.0 ± 0.23^{A}	75.0 ± 0.12^{A}	54.0 ± 0.24^{B}	56.0 ± 0.35^{B}	54.0 ± 0.45^{B}
Mn	mg/dm³	61.0 ± 0.70^{B}	64.0 ± 0.22^{B}	63.0 ± 0.09^{B}	71.0 ± 0.60^{A}	68.0 ± 0.07^{A}	69.0 ± 1.2^{A}
Zn	mg/dm³	2.5 ± 0.07^{A}	2.3 ± 0.04^{A}	2.3 ± 0.01^{A}	1.4 ± 0.05^{B}	1.5 ± 0.04^{B}	1.4 ± 0.02^{B}
Sat. Al	M%	0 ± 0^{A}	0 ± 0^{A}	0 ± 0^{A}	0 ± 0^{A}	0 ± 0^{A}	0 ± 0^{A}
Sat. base	V%	64.0 ± 1.2^{B}	65.1 ± 2.2^{B}	66.0 ± 1.0^{B}	76.9 ± 1.4^{A}	76.1 ± 2.1^{A}	78.0 ± 1.9^{A}

Note: TOC: total organic carbon, Ca: calcium, Mg: magnesium, Al: aluminum, H: hydrogen, CTC: cation exchange capacity, P: phosphorus, K: potassium, Na: sodium, S: sulfur, B: boron, Cu: copper, Fe: iron, Mn: manganese, Zn: zinc, Sat. Al: aluminum saturation, Sat. base: saturation by the base. Different capital letters on the same line indicate significant differences. Parametric data are presented by the mean ± standard deviation. CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively

Table 4S. Loading (coefficient) matrix provided by the multivariate analysis to define factors or principal components (PC1 and PC2).

Biomarkers	Abbreviations	Principal components	
		PC1	PC2
Superoxide dismutase activity in the seminal vesicle	SOD(SV)	-0,858	0,513
Catalase activity in the seminal vesicle	CAT(SV)	-0,850	-0,527
Levels of reactive oxygen species in the seminal vesicle	ROS(SV)	1,000	0,029
Nitrite levels in the seminal vesicle	NO(SV)	0,990	0,140
Malondialdehyde levels in the seminal vesicle	MDA(SV)	0,948	0,319
Superoxide dismutase activity in muscle	SOD(M)	0,881	0,473
Catalase activity in muscle	CAT(M)	0,942	-0,336
Levels of reactive oxygen species in muscle	ROS(M)	0,955	0,297
Nitrite levels in muscle	NO(M)	-0,867	0,498
Malondialdehyde levels in muscle	MDA(M)	0,999	0,051
Acetylcholinesterase activity in muscle	AChE(M)	-0,988	0,154
Layer of circular muscle	LCM	0,999	-0,040
Layer of longitudinal muscle	LLM	-0,948	-0,317
Circular muscle fiber thickness	CMFT	-0,623	0,782
Superoxide dismutase activity in the intestine	SOD(I)	0,780	-0,625
Catalase activity in the intestine	CAT(I)	0,949	-0,314
Levels of reactive oxygen species in the intestine	ROS(I)	0,977	0,215
Nitrite levels in the intestine	NO(I)	-0,722	0,691
Malondialdehyde levels in the intestine	MDA(I)	0,998	0,064
Large granule cell number	LGCN	-0,180	0,984
Reticular gland cell number	RGCN	0,999	0,044
Intestinal epithelium height	IEH	-0,999	0,045
Intestinal muscle layer height	IMLH	-0,934	-0,357
Intestinal lumen area	ILA	1,000	-0,002
Chloragogen cell layer height	CCLH	0,105	0,994
Epidermis height	EH	-0,738	-0,675
Epidermal cuticle	EC	-0,666	0,746
Earthworms number	EN	-0,416	-0,909
Final body biomass	FBB	-0,872	-0,489
Δ body biomass	ΔBB	-0,976	-0,217
Gametocysts number with sporocysts	GNS	0,956	0,294
Monocystis sp. encapsulated gametocysts area	MEG	0,930	-0,368
Occupation area (%) of gametocysts area in the seminal vesicle	OAG	0,969	0,247
Sporocysts number	SN	0,999	-0,046
Total organic carbon	TOC	0,736	-0,677

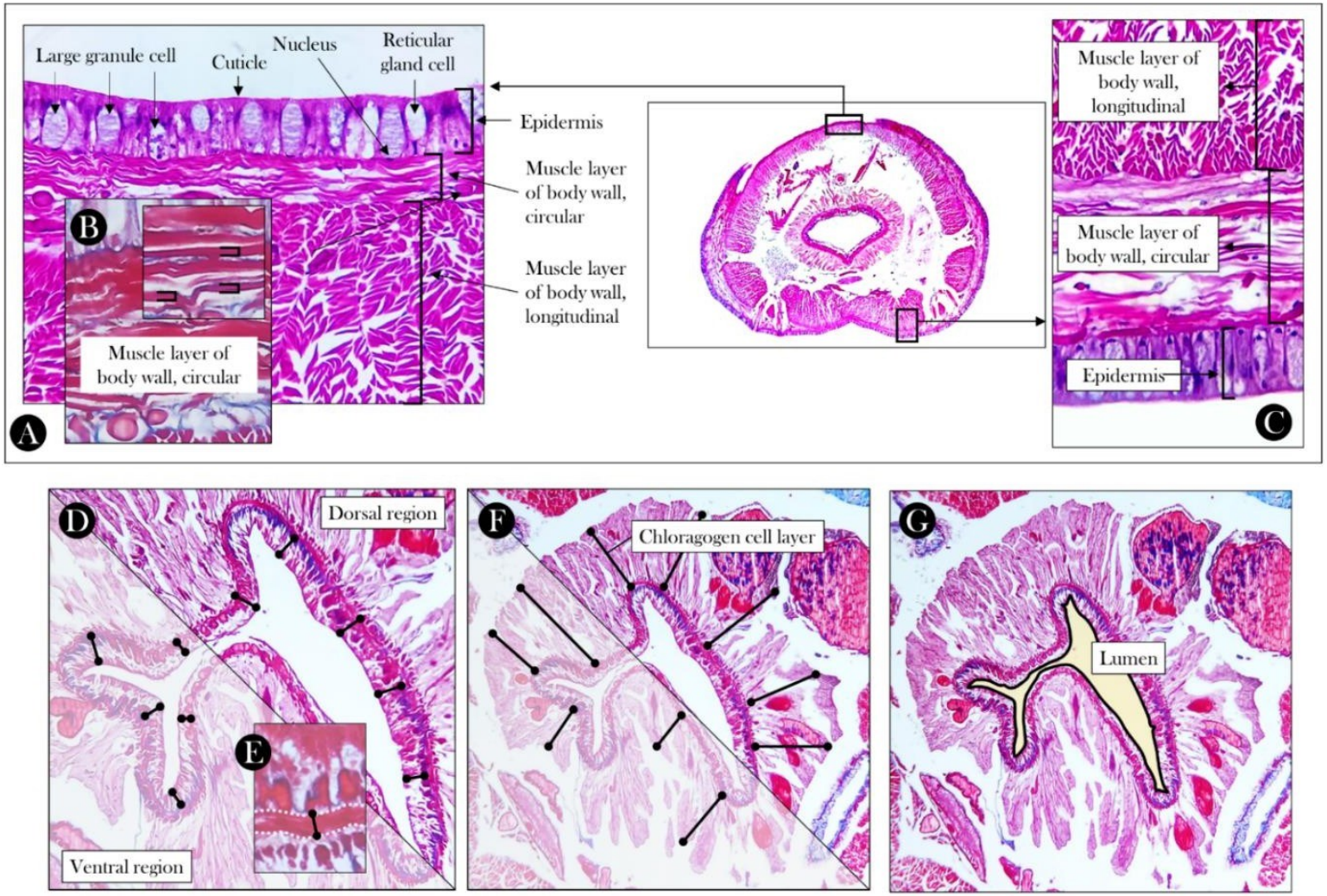

Figure 1S. Photomicrographs representative of the histopathological biomarkers evaluated in adult Lumbricus terrestris earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations, showing details of (A) epidermis, (B-C) muscle layers of body wall, (D) height of intestinal epithelium, (E) intestinal muscle layer height, (F) chloragogen cell layer height, and (G) intestinal lumen. The sections of each tissue were stained with hematoxylin-eosin (H&E) or Masson's Trichrome using standard histopathological techniques.

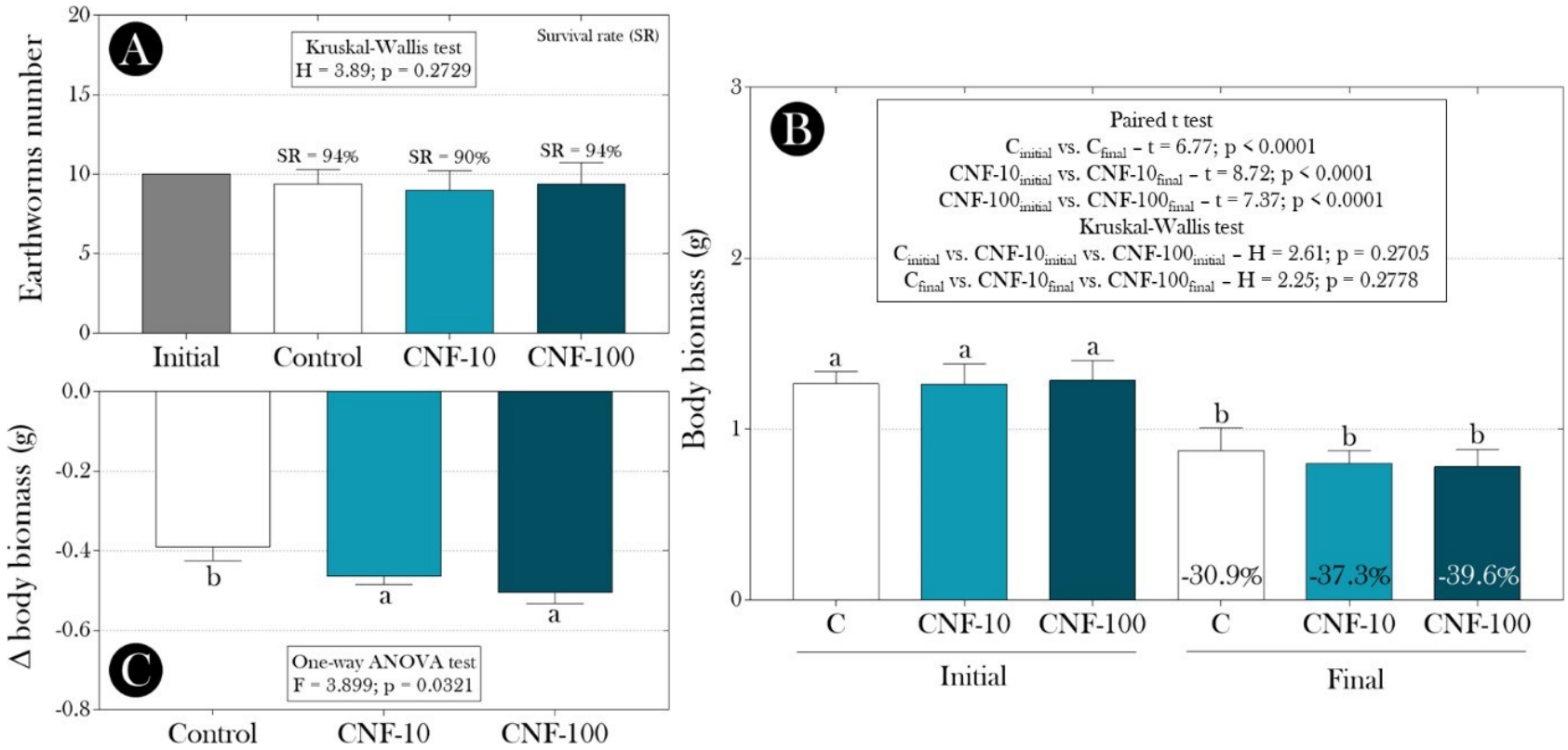

Figure 2S. (A) Population density (earthworms' number), (B) body biomass, and (C) delta (Δ) body biomass of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. Parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. Statistical summaries are shown close to the graphs. In "B," the first three columns refer to initial measurements, and the second three refer to the final. C: control; CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. SR: Survival rate.

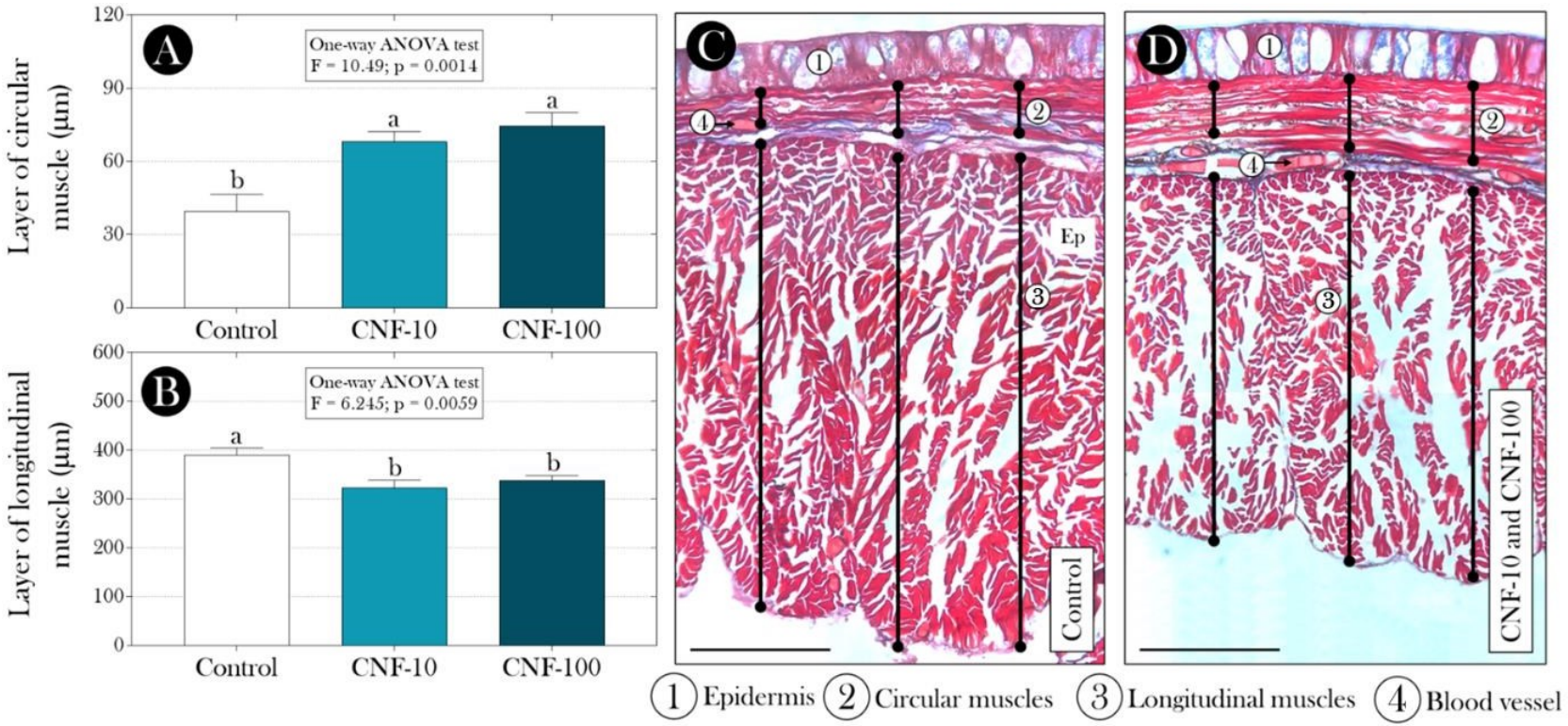

Figure 3S. Height of the (A) circular and (B) longitudinal muscle layers of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (C-D) Photomicrographs representative of the epidermis and muscle layers of the different experimental groups. Parametric data are presented as mean + standard deviation. Statistical summaries are shown next to the graphs. C: control; CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. The analyzed sections were stained with Masson's Trichrome using standard histopathological techniques.

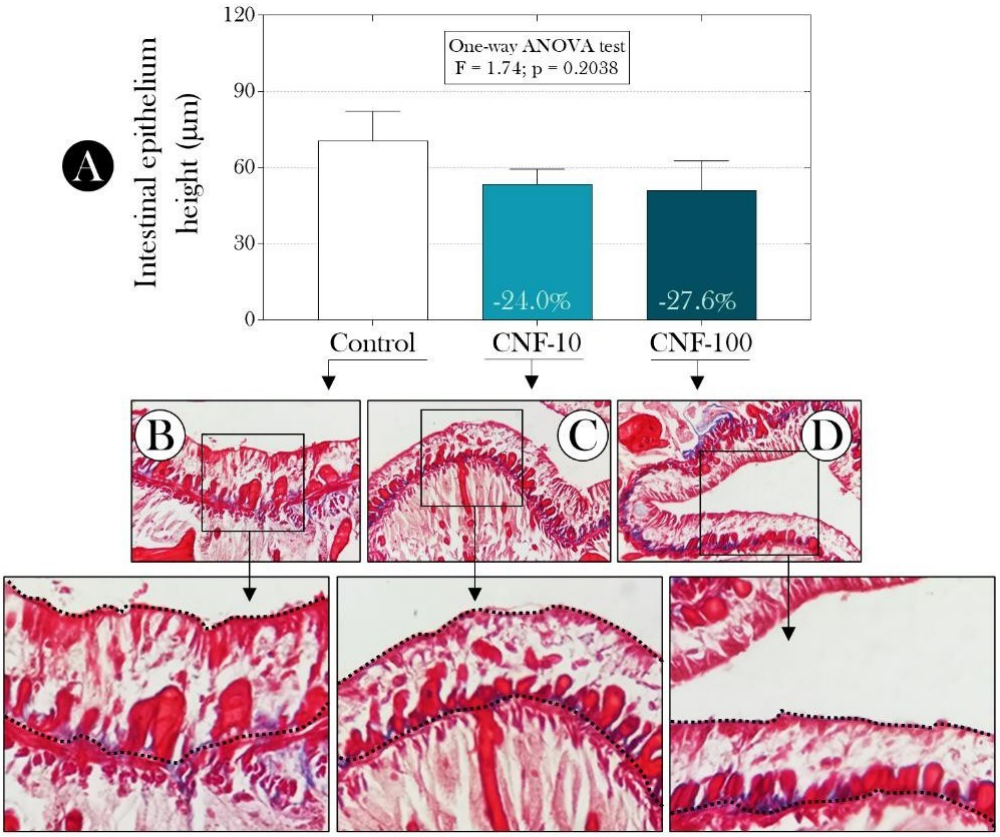

Figure 4S. (A) Intestinal epithelium height of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (B to D) Representative photomicrographs of the intestinal epithelium of earthworms from the "control," "CNF-10," and "CNF-100" groups, respectively. In "A," parametric data are presented by the mean + standard deviation. Statistical summary is shown close to the graph. C: control; CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. The analyzed sections were stained with Masson's Trichrome using standard histopathological techniques.

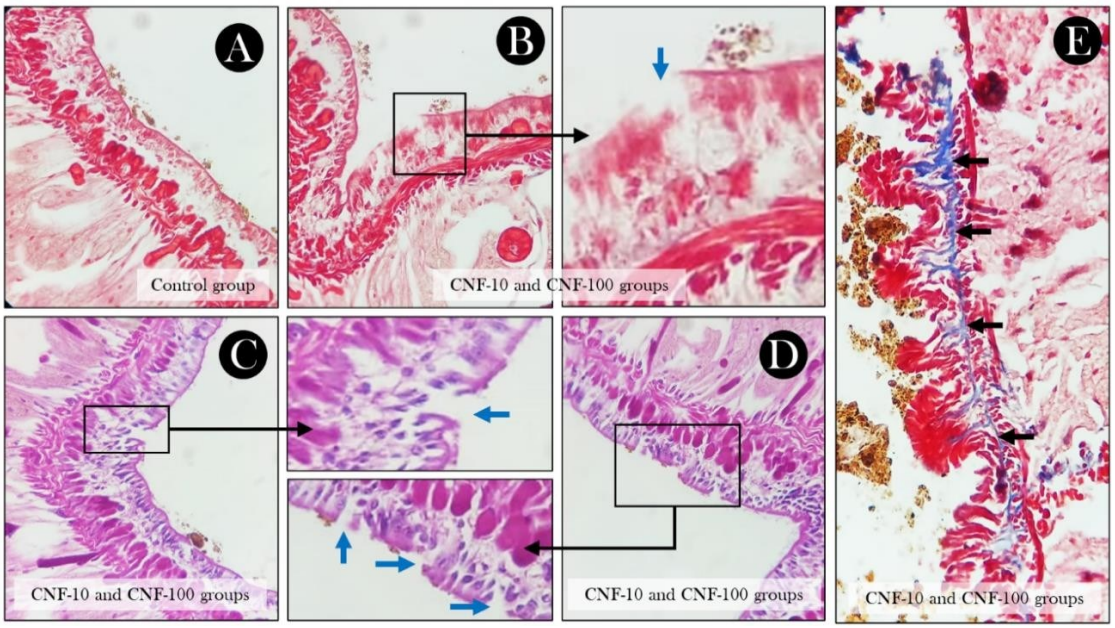

Figure 5S. Representative photomicrographs of the intestine of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (A to D) Transverse and (E) longitudinal section. CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. Sections "A," "B," and "E" were stained with Masson's Trichrome, and "C" and "D" with hematoxylin and eosin (H&E), using standard histopathological techniques. Blue and black arrows indicate lesions/ruptures of the intestinal epithelium and areas with fibrosis, respectively.

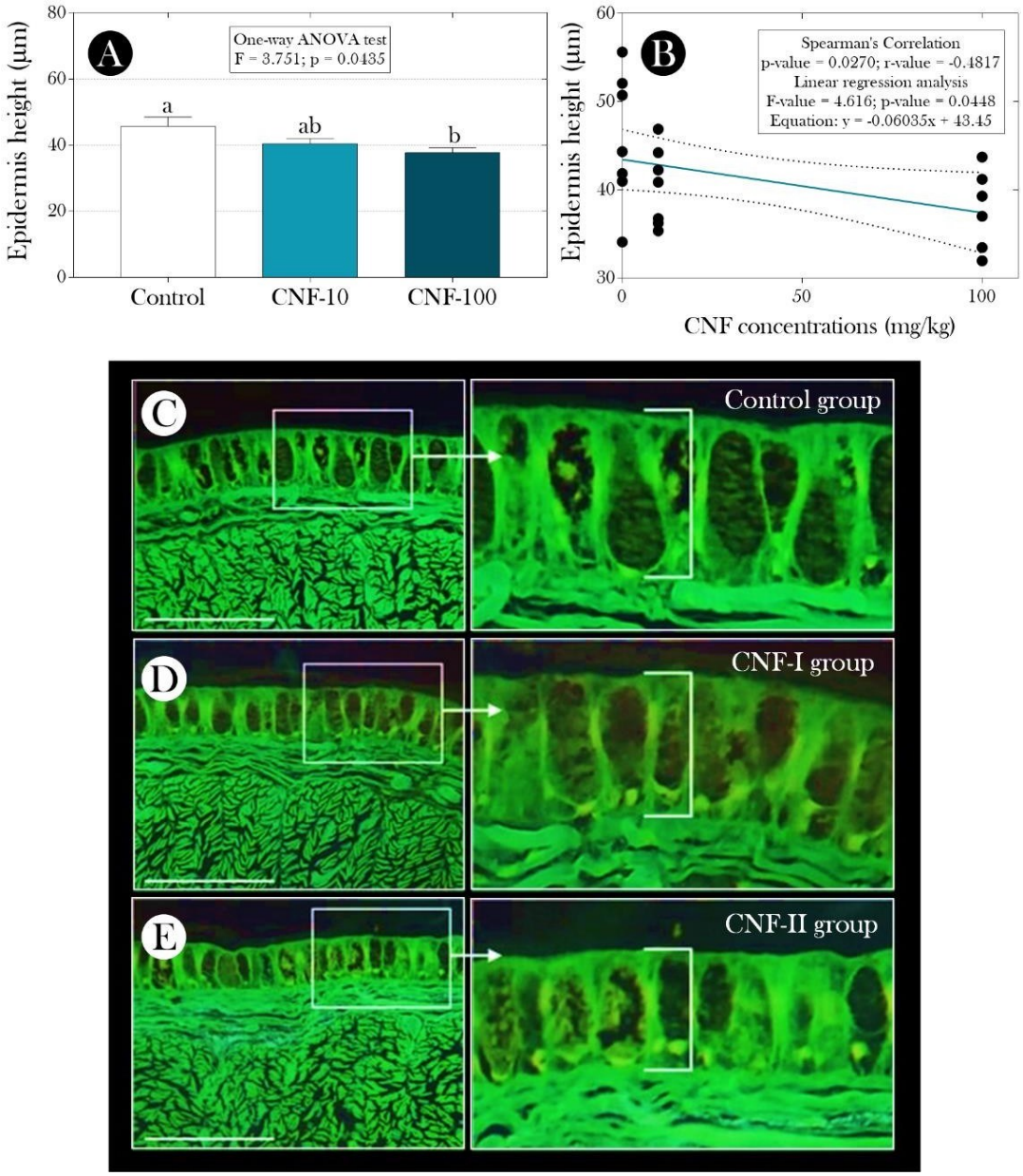

Figure 6S. (A) Epidermis height and (B) correlation analysis and linear regression between the variables "epidermis height vs. carbon nanofibers (CNFs)" of adult *Lumbricus terrestris* earthworms unexposed or exposed to CNFs at different concentrations. (C to E) Representative photomicrographs of the epidermis of earthworms from the "control," "CNF-10" (CNF-I group), and "CNF-100" (CNF-I group) groups, respectively. In "A," parametric data are presented by the mean + standard deviation. Distinct lowercase letters indicate significant differences. Statistical summaries are shown close to the graphs. CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. The analyzed sections were stained with hematoxylin and eosin (H&E) using standard histopathological techniques. However, the photomicrographs were edited with a negative filter to allow better visualization of tissue structures.

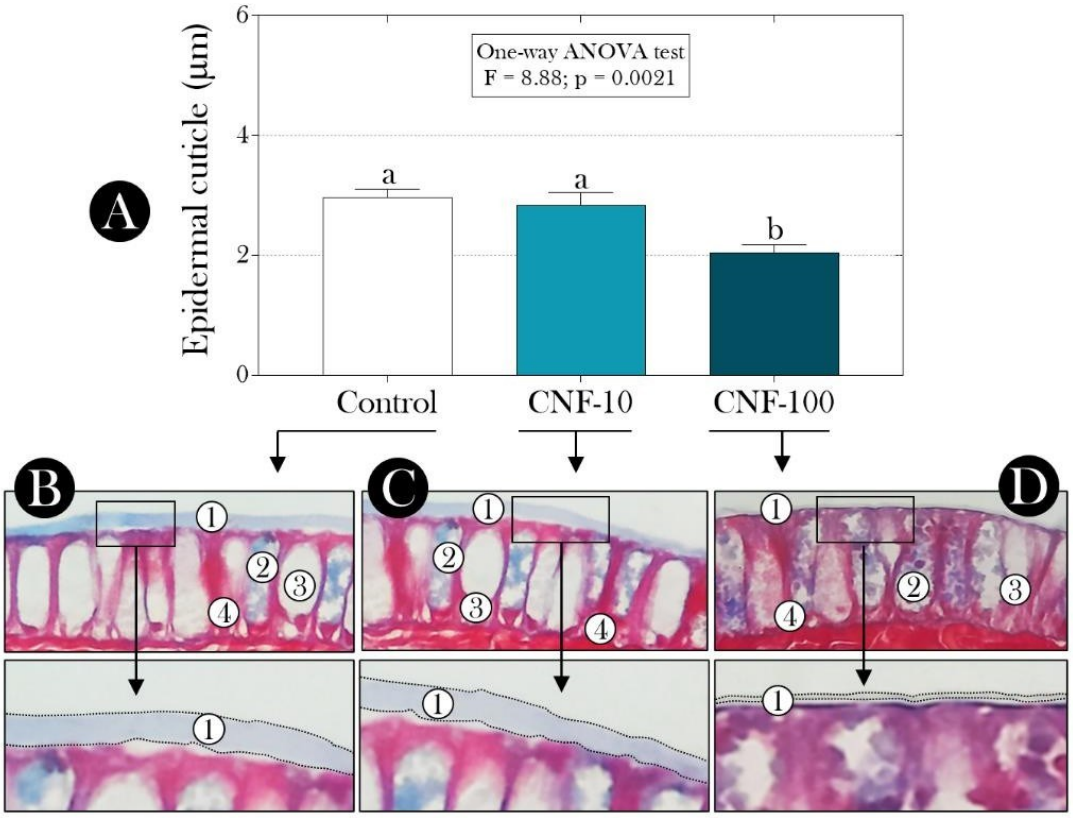

Figure 7S. (A) The epidermal cuticle of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (B-D) Representative photomicrographs of the epidermal cuticle of earthworms from the "control," "CNF-10," and "CNF-100" groups, respectively. In "A," parametric data are presented by the mean + standard deviation. Distinct lowercase letters indicate significant differences. Statistical summary is shown close to the graph. CNF-10 and CNF-100: adult *L. terrestris* earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. The analyzed sections were stained with hematoxylin and eosin (H&E) using standard histopathological techniques. In "B to D," (1): cuticle, (2): large granule cell, (3): reticular gland cell, and (4): supporting cell.

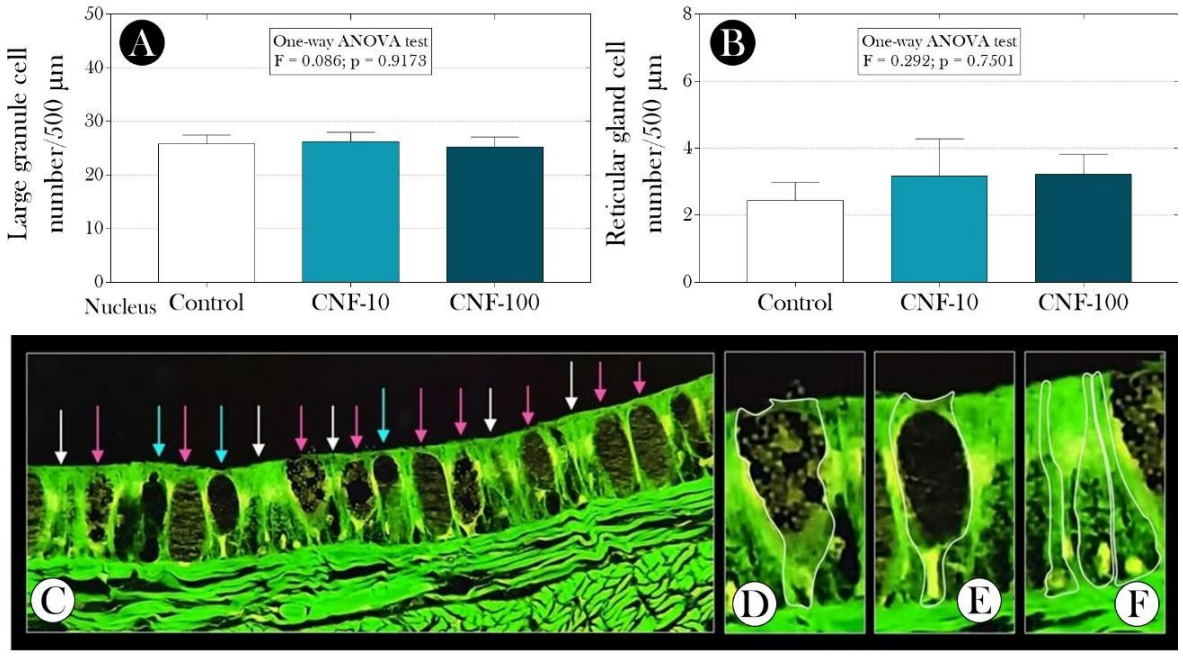

Figure 8S. (A) Large granule cell number and (B) reticular gland cell number of adult *Lumbricus terrestris* earthworms unexposed or exposed to carbon nanofibers (CNFs) at different concentrations. (C to F): Representative photomicrographs of the cell types that make up the epidermis of earthworms, with emphasis on (D) large granule cells, (E) reticular gland cells, and (F) supporting cells. In "A" and "B," parametric data are presented by the mean + standard deviation. Distinct lowercase letters indicate significant differences. Statistical summaries are shown close to the graphs. CNF-10 and CNF-100: adult L. terrestris earthworms exposed to soil contaminated with CNFs at 10 and 100 mg/kg, respectively. The analyzed sections were stained with hematoxylin and eosin (H&E) using standard histopathological techniques. However, the photomicrographs were edited with a negative filter to allow better visualization of tissue structures.

CAPÍTULO 5 - CONCLUSÕES GERAIS E CONSIDERAÇÕES FINAIS

Com base no nos resultados apresentados, nossa tese confirma o potencial ecotoxicológico dos NFCs em três diferentes modelos experimentais *A. williamsoni, D. rerio, L. terrestris* expostas. Os três modelos citdos acima foram expostos a diferentes concentrações de NFCs em diferentes meios, sendo na água (*A. williamsoni, D. rerio*) e solo (*L. terrestris*) em diferentes períodos de exposição. Podemos observar que os resultados obtidos atrávez da exposição, seja ela curta (*A. Williamsoni*) ou prolongada (*D. reriop, L. terrestris*) apresentaram certar semelhanças nos resultados apresentados, que cuminaram no desquilibrio redox desequilíbrio redox. Além disso, podemos notar que em todos os modelos testados tivemos maior concentração de carbono organico total, que podem ter sido responsáveis pela dinuição na atividade da AchE.

Portanto, no segundo capítulo, chegamos as seguintes conclusões, em que a hipótese inicial de que a exposição efêmera aos NFCs leva ao seu acúmulo em *A. williamsoni*, bem como alterações bioquímicas preditivas de desequilíbrio REDOX e neurotoxicidade (inferida pela redução da atividade da AChE), mesmo em baixa concentração (500 µg/L). Embora seja apenas um estudo preliminar, os seus dados são valiosos para melhor compreender a magnitude dos impactos (eco)toxicológicos dos CNFs nos macroinvertebrados bentónicos, principalmente devido às suas potenciais consequências a nível bioquímico/fisiológico e comportamental.

Já no terceiro capítulo, nosso estudo confirma o potencial ecotoxicológico dos NFCs no modelo estudado (peixe-zebra) marcado, principalmente, pela incapacidade dos animais em responder ao teste de estímulo vibratório, pela alteração na densidade dos neuromastos registrada no região ventral final dos animais, pelas alterações bioquímicas relatadas no cérebro e pelos efeitos mutagênicos e genotóxicos evidenciados nos eritrócitos circulantes. Por outro lado, ao contrário do que esperávamos, a exposição aos NFCs, nas concentrações testadas e durante o período de exposição avaliado não afetou o crescimento e desenvolvimento dos animais, além de não ter induzido alterações locomotoras ou comportamento semelhante à ansiedade.

E por fim, no último capítulo, nosso estudo confirma a hipótese de que os NFCs aumentam a carga parasitária de *Monocystis* sp. nas vesículas seminais de *L. terrestris* minhocas, além de induzir alterações bioquímicas e histopatológicas em animais após 28 dias de exposição a poluentes, principalmente na concentração de 100 mg/kg. Portanto, nossas descobertas apontam para o impacto adicional que esses nanomateriais podem ter na saúde das minhocas e apontam para a necessidade de

maior atenção ao seu descarte e aos efeitos ecotoxicológicos nos organismos do solo.

Acreditamos que estudos como o nosso, ou seja, focados na identificação e caracterização dos impactos causados pelos NFCs, fornecem subsídios científicos aplicáveis para a condução de investigações sobre seus mecanismos de ação, além de reforçar a necessidade de avaliação da bio e ecossegurança desses nanomateriais e planejar ações que possam mitigar seus impactos.Embora tenhamos nos concentrado na resposta em nível individual, nossos resultados esclarecem o impacto potencial dos NFCs nas populações naturais dos organismos testados, portanto, avançar no conhecimento sobre a previsão da toxicidade dos NFCsna água e solo são fundamentais para compreender a forma como esses nanomatérias agem em difentes organismos, além do seu tempo de exposição.