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Toxicity assessment of SARS-CoV-2-derived peptides in combination with a mix of pollutants on zebrafish adults: A perspective study of behavioral, biometric, mutagenic, and biochemical toxicity

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HIGHLIGHTS

• SARS-CoV-2 peptide fragments and a mix of pollutants are evaluated in D. rerio adults.
• NO and MDA production in the brain, gills, and muscle did not differ between groups
• SOD activity was equitably reduced in animals from “PSPD”, “Mix” and “Mix+PSPD” groups.
• Increase in catalase activity and a reduction in DPPH radical scavenging activity were observed in the brains of D. rerio.
• Exposure to viral fragments, associated with the mix of pollutants, induced more significant toxicity in zebrafish adults.

ABSTRACT

The dispersion of SARS-CoV-2 in aquatic environments via the discharge of domestic and hospital sewage has been confirmed in different locations. Thus, we aimed to evaluate the possible impacts of zebrafish (Danio rerio) exposure to SARS-CoV-2 peptide fragments (PSPD-2001, 2002, and 2003) alone and combined with a mix of emerging pollutants. Our data did not reveal the induction of behavioral, biometric, or mutagenic changes. But we noticed an organ-
1. Introduction

COVID-19 (Coronavirus Disease-2019), caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), has caused worldwide impacts unprecedented in recent human history. Such consequences encompass the world economy (Ozili and Aran, 2023), education (Mehta et al., 2022; Reimers, 2022; Panakaje et al., 2022), social aspects (Kiran, 2020), travel behavior, and community living (Park et al., 2022), petroleum consumption (Wang et al., 2022), sports governance and management (Byers et al., 2022), as well as the health of populations, including aspects of mental health (Sanjii et al., 2022; Banna et al., 2022), eating disorders (Linardon et al., 2022), and life expectancy (Aburto et al., 2022), among others. Regarding mortality, as of September 5, 2022, the WHO Coronavirus (COVID-19) Dashboard has 6,460,493 deaths (WHO, 2022).

However, recent studies have also pointed to multiple impacts (direct and indirect) of the COVID-19 pandemic on the environment, which have been associated with increased use of chemicals (particularly detergents, soap, and sanitizers (Chirani et al., 2021; Dhama et al., 2021), increase in solid waste generation (e.g., hospital, biomedical (Ye et al., 2022; Parida et al., 2022) and domestic (Jebaranjitham et al., 2022; Sharma et al., 2022), and climate change (Marazziti et al., 2021). In addition, the consumption of plastic-based products has increased considerably during the COVID-19 pandemic (Silva et al., 2021; Benson et al., 2021), especially those related to petrochemical-based synthetic fibers, which are typically used for single-use protective clothing (Uddin et al., 2022), as well as personal protective equipment kits, face masks, and gloves (Kumar et al., 2021).

Thus, it has been a consensus that the COVID-19 pandemic has been intensifying water pollution, already noted for some time, in terms of heavy metals (Muhammad and Usman, 2022), surfactants (Al-Ani et al., 2020), phenolic compounds (Ramos et al., 2021), petroleum (Edori and Edori, 2021), pharmaceutical residues (Quincey et al., 2022), pesticides (Kalantary et al., 2022), personal care products (Liu et al., 2021) and microplastics (Talbot and Chang, 2022), among others.

Associated with this, the identification of SARS-CoV-2 or its fragments in hospital and domestic sewage (Dharmadhikari et al., 2022; Pellegrinelli et al., 2022; Yang et al., 2022; Zhao et al., 2022) and in natural aquatic environments (e.g., rivers – De-Oliveira et al., 2021; Fengaro et al., 2021; Rocha et al., 2022; Fonseca et al., 2022) have raised concerns about possible secondary transmission of SARS-CoV-2 (Ahmed et al., 2022) and its impacts on non-target organisms (Charlie-Silva and Malafia, 2022). In this regard, studies conducted in the laboratory have already shown that exposure to peptide fragments of SARS-CoV-2 induces changes in the health of different animal species, such as Physarum salivari tadpoles (Charlie-Silva et al., 2021), Culex quinquefasciatus larvae (Mendonça-Gomes et al., 2021), Pocilga reticulata juveniles (Malafaia et al., 2021; Gonçalves et al., 2022), Danio rerio (Fernandes et al., 2022; Kraus et al., 2022), and mice (Luz et al., 2022). Although these studies represent preliminary and incipient findings on the potential effects of SARS-CoV-2, considering not only the great diversity of viral fragments dispersed in aquatic environments but also the effects of exposure to SARS-CoV-2 itself in situ have not yet been evaluated, they certainly “shed light” on the (eco)toxicological potential of the spread of the new coronavirus on non-target organisms.

However, only one study (so far) has aimed to assess whether the coexistence of multiple pollutants with SARS-CoV-2 in the aquatic system constitutes an additional concern for aquatic species. On that occasion, Freitas et al. (2022) demonstrated that the combined exposure of mayfly larvae (Chenio diptera) to SARS-CoV-2-derived peptides (PSPD-2001, PSPD-2002, and PSPD-2003) with multiple emerging pollutants at ambient concentrations induces changes in the health of these animals. After six days of exposure, higher mortality of larvae exposed to SARS-CoV-2-derived peptides (alone or in combination with the pollutant mix) and a lower body condition index than those unexposed larvae were reported. Furthermore, in the “PSPD” and “Mix + PSPD” groups, reduced antioxidant activity, nitrosative stress, and anticholinesterase effects were reported. However, there was no evidence of synergistic or additive action between the viral peptides and the pollutants that composed the mix. Therefore, our knowledge of the global impacts of the COVID-19 pandemic, associated with current water pollution levels, under an ecological/environmental optimum is still minimal, which justifies further studies.

Therefore, in this study, we used Danio rerio adults (zebrafish) as a model system to assess the possible effects of combined exposure of SARS-CoV-2-derived peptides with multiple pollutants commonly identified in aquatic environments. D. rerio is a freshwater tropical teleost fish belonging to the Cyprinidae family, originally from South Asia (Grunwald and Eisen, 2002; Spence et al., 2006; Engeszer et al., 2007); having been used worldwide as an animal model in different ecotoxicological studies (Verma et al., 2021; Ribeiro et al., 2022; Da-Silva-Brito et al., 2022). Using biometric, antioxidant, nitrosative, cholinesterase, and behavioral biomarkers, we tested the hypothesis that co-exposure to viral peptides and the pollutant mix induces more intense impacts on animals than those exposed to peptides and the pollutant mix alone. Conducting studies like ours is essential to understanding the magnitude of the effects of the COVID-19 pandemic on wild animals. It supports the planning and proposition of actions to reduce its impact on non-target organisms. Although the COVID-19 pandemic has been controlled in recent months (due to advances in peptide discovery (MubarakAli et al., 2021), vaccine strategies (Jafari et al., 2022; Kumar et al., 2022), therapeutics (Yin et al., 2022; Menéndez, 2022), and clinical outcomes (Al-Musa et al., 2022)), the persistence of SARS-CoV-2 in aquatic environments even after the end of the pandemic (Yang et al., 2022) may pose a risk to wild biota, which may be even greater if associated with the different pollutants present in aquatic ecosystems.

2. Material and methods

2.1. SARS-CoV-2-derived peptides

In this study, we used the peptides of the Spike protein of SARS-CoV-2 synthesized, purified, and characterized (called PSPD-2001, PSPD-2002, and PSPD-2003) in a previous study of our research group (Charlie-Silva et al., 2021) (Fig. 1). Such peptides were synthesized via the solid phase peptide synthesis method following the Fmoc strategy, according to Behrendt et al. (2002). The resin used for synthesis were Fmoc-Cys (Trt)-Wang, Fmoc-Thr (Tbu)-Wang, and Fmoc-Asn (Trt)-Wang for peptides Arg-Val-Tyr-Ser-Ser-Wing-Asn-Asn-Cys-COOH (PSPD-2001); Gin-Cys-Val-Asn-Leu-Thr-Arg-Thr-COOH (PSPD-2002) and Asn-Asn-Ala-Thr-Asn-COOH (PSPD-2003). The purification of peptides was performed via high-performance liquid chromatography [based on Klaffen et al., 2019]. Only compounds with purity equal to or >95 % were used in the present study, as determined by the
National Health Surveillance Agency (ANVISA/Brazil) and Food and Drug Administration (FDA/USA).

In our study, the concentration of SARS-CoV-2-derived peptides used in animal exposure (see details in section "2.3") aimed to simulate the presence of viral particles at a predicted environmental concentration (222.6 ng/L), considering the complete absence, to date, of studies aimed at identifying and quantifying viral protein fragments in freshwater ecosystems. In addition, the tested concentration corresponds to approximately 15% of the highest urinary level of SARS-CoV-2 nucleocapsid protein (SARS-CoV-2-N) in patients with confirmed SARS-CoV-2 infection admitted to the Emergency and Intensive Care Medicine at the University Medical Center Göttingen (Germany) (Tampe et al., 2021). Therefore, this percentage constitutes a plausible dilution of SARS-CoV-2 in areas near hospital sewage disposal sites (untreated) in a small watercourse.

2.2. Mix of emerging pollutants

Similar to the diversity of emerging pollutants already identified in freshwater ecosystems, which can coexist with SARS-CoV-2 or its fragments, we add in the exposure waters distinct compounds/substances, including pesticides, agro-industrial effluent, pharmaceutics/hormone, agricultural fertilizers, surfactant, and constituent substances of petroleum. A total of 14 pollutants (in addition to those that make up the tannery effluent) were chosen based on the studies by Souza et al. (2018), Araújo et al. (2022), and Araújo et al. (2023), as well as their previous identifications in freshwater ecosystems (see references cited in Table 1).

2.3. Model systems and experimental design

In our study were used 48 adults of Danio rerio (zebrafish) (wild-type strain) of both sexes (ratio 1:1), aged between 5 and 6 months [body biomass of 0.238 g ± 0.042 g (mean ± standard deviation)]. These animals were obtained and maintained in the animal facility for aquatic organisms of the Laboratory of Toxicology Applied to the Environment of the Goiano Federal Institute – Campus Urutai (GO, Brazil). Seven days before the experiment, the animals were acclimatized in aquariums containing dechlorinated water at temperature (28.1 °C ± 0.24 °C, mean ± standard deviation) and controlled luminosity (12:12-h light:dark photoperiod). Before and during the experiment, the animals were fed once a day with commercial fish food. After the acclimatization period, the animals were distributed into four experimental groups (containing three replicates of 4 animals/group, for 12 animals/group). While the animals of the “control” group were not exposed to any component of the mix of pollutants or peptides of SARS-CoV-2; those of the “PSPD” group were exposed to an equimolar mixture of peptides PSPD-2001, PSPD-2002, and PSPD-2003, whose total concentration was 266.2 ng (PSPD/L). The “Mix” group was composed of animals exposed to the mix of emerging pollutants (at concentrations presented in Table 1) without the presence of SARS-CoV-2-derived peptides. On the other hand, in the group “Mix + PSPD” zebrafish were exposed to SARS-CoV-2-derived peptides with a mix of pollutants at the same concentrations defined in the previous groups. During the experimental period (30 days), the groups were kept in cylindrical glass aquariums containing 1.3 L of dechlorinated water added with the respective treatments (PSPD, Mix, or Mix + PSPD).

2.4. Toxicity biomarkers

2.4.1. Behavioral assessment

2.4.1.1. Locomotor activity and possible anxiety-like behavior. To evaluate the possible induction of locomotor alterations and anxiety-like behavior by treatments, to the 29th experimental day, the animals were submitted to the open field test, which has been widely used in behavioral tests involving zebrafish (Stewart et al., 2012; Godwin et al., 2012; Hamilton et al., 2021; Borba et al., 2022). Briefly, the test consisted of introducing the animals (individually) into a rectangular polycarbonate box (40 cm length x 30 cm width x 16.5 cm height; opaque white walls) containing 5 L of dechlorinated water (free of treatments, temperature: 28 °C) and evaluating their overall activity for 5 min, like Thompson et al. (2022). The test was performed in a room containing acoustic isolation, light, and temperature.

![Fig. 1. Structural models of peptides (PSPD-2001, PSPD-2002, and PSPD-2003) synthesized and used in the present study.](image)

Table 1

| Components                | Molecular formula | Concentrations References |
|---------------------------|-------------------|---------------------------|
| Amoxicilina               | C₁₇H₁₆N₄O₆S        | 0.0045 g/L Sodré et al. (2010) |
| Acetylsalicylic acid      | C₁₇H₁₇O₄        | 0.34 mg/L Ternes (1998) |
| Diclofenac Sodium         | C₁₆H₁₉N₂O₅        | 1.8 mg/L Hoeger et al. (2005) |
| Ibuprofen                 | C₁₇H₂₂N₂O₂        | 2.7 mg/L Flippin et al. (2007) |
| Fluoxetine                | C₁₇H₂₀F₂O₂        | 0.030 mg/L Perreault et al. (2003) |
| Clozaneapid               | C₁₇H₁ₙN₄O₅        | 0.053 mg/L Ternes et al. (2001) |
| Dicyclicenamide           | C₁₇H₁₅N₂O₅        | 5 mg/L Pamplona et al. (2011) |
| Ranitidine                | C₁₅H₂₁N₅O₄        | 0.01 mg/L Boixoil (2004) |
| Benzene                   | C₆H₆         | 5000 mg/L Brazil (2004) |
| Tannery effluent          | –                  | 1 % Rabelo et al. (2016) |
| Estradiol cipionate       | C₂₀H₂₂O₂        | 2.6 mg/L Jardim et al. (2012) |
| Nitrogen                  | N₂           | 2400 mg/L Xu et al. (2014) |
| Glycoluric acid           | C₇H₁₂NO₄        | 600 mg/L Peruzzo et al. (2008) |
| Aminocetin                | C₁₁H₁₄O₃        | 4 mg/L Vasciavieto et al. (2016) |
| Detergent                 | –                  | 740 mg/L Mortatti et al. (2012) |

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control. Between one session and another, the boxes were sanitized (with 30% alcohol) and dried. The waters were replaced entirely to avoid an animal's possible interference with the following animal's behavior. The cameras (coupled to an external computer) were positioned 1.2 m high in the boxes. The number of times the animals crossed the quadrants plotted virtually in the boxes (Fig. 2A) was used to evaluate the general locomotor activity of zebrafish. In addition, the swimming speed of the animals was calculated. To assess the possible anxiogenic effect of the treatments, the box was divided into two zones (peripheral and central) (Fig. 2B). The time that the animal remained in the peripheral zone was used to calculate the anxiety index, according to Eq. 1. In addition, the frequency of exploration was recorded in the state's central area. Higher locomotion rates in the peripheral quadrants are associated with increased behavior of thigmotaxis, which is typically considered an indicator of anxiety (Maximino et al., 2010; Schnörr et al., 2012; De-Oliveira et al., 2021). PlusMZ software was used for behavior recording.

\[
\text{Anxiety index} = \frac{\text{Time spent in the peripheral zone of the apparatus}}{\text{Total test time (300 s)}} \times 100
\]  

(1)

2.4.1.2. Social aggregation in response to a potential aquatic predator. On the 30th experimental day, the animals were submitted to the social aggregation test in the presence of a potential predator whose adopted procedures were like those described in Chagas et al. (2021). In this test, we aimed to evaluate the shoals' responses when confronted with a potential aquatic predator, represented by a juvenile male Hoplias malabaricus (known locally as the traíra) (total length: 16.26 ± 1.02 cm). H. malabaricus is a carnivorous freshwater fish of the Erythrinidae family, for which the main food items are other fish (Carvalho et al., 2002). Briefly, the test consisted of introducing the animals of each replica into a polypropylene box (40 cm length x 30 cm width x 16.5 cm height) containing 5 L of dechlorinated water (free of treatments, temperature: 28 °C) and filming them for 5 min (habituation session) (Fig. 2C). Then, the potential predator was introduced into the box, and the animals were filmed for another 5 min (test session) (Fig. 2D). Six specimens of H. malabaricus were used alternately between the replicas of the same group and between the experimental groups, avoiding reducing the possible influence of behavior predator on the behavior of their potential prey (zebrafish).

After testing, animals' social aggregation, which is also a defensive response, was evaluated based on the calculation of cluster scores. Such scores were determined from the demarcation of 30 quadrants (40 cm²/each) in virtual images displayed on the researcher's computer screen, like in Collins et al. (2011) and Parker et al. (2014). Cluster scores were generated by dividing the maximum number of zebrafish positioned in a given quadrant by the number of quadrants occupied by all the animals (total). Scores were provided at 3 s intervals every 3 s in a 5-min (300 s) test.

2.4.2. Biometrics

To evaluate the possible effects of treatments on zebrafish biometrics, at the end of the experiment, the following parameters were assessed: total length (cm), body width (cm), caudal peduncle width (mm), head length (mm), and head height (mm), as described by Chagas et al. (2021). The results were expressed in indexes, considering each animal's total length.

2.4.3. Biochemical biomarkers

2.4.3.1. Sample preparation. To evaluate the possible biochemical effects of the treatments, fragments of the brain, gills, and muscles were extracted. Then, the fragments were macerated in 1 ml of phosphate-buffered saline (PBS) solution (pH 7.2) and centrifuged at 13,000 rpm for 5 min (at 4 °C), like Araújo et al. (2022). Then, the supernatant was filtered through 0.45-μm syringe filters and subsequently stored at -80 °C until its use in evaluating the biomarkers described below.

2.4.3.2. Lipid peroxidation, antioxidant capacity, and nitric oxide production. The malondialdehyde (MDA) levels were evaluated according to the method described in Esterbauer and Cheeseman (1990), with detailed modifications in Freitas et al. (2022). The evaluation of this biomarker was proper to infer lipid peroxidation (LPO) levels (Grotto et al., 2009) in the different organs of the animals. The total antioxidant capacity was estimated from the evaluation of the free radical scavenging capacity (via DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method, described in Brand-Williams et al. (1995)), total thiols levels (nonenzymatic antioxidant), determined based on Ellman (1959) (with modifications described in Hu (1994)); as well as superoxide dismutase (SOD) (according to the procedures described in Deawati et al. (2017) and Deawati et al. (2018)) and catalase activities, like Hadwan and Abed (2016). On the other hand, no levels were evaluated using the Griess colorimetric reaction (Grisham et al., 1996), according to the steps adopted by Araújo et al. (2022). The results of all biochemical biomarkers were relativized with the level of total proteins of the analyzed samples, which was determined based on the Bradford (1976) method.

2.4.3.3. Acetylcholinesterase activity. Considering that acetylcholinesterase (ACHE) is responsible for the termination of impulse transmission at cholinergic synapses (Silman and Sussman, 2008), we evaluated the possible anticholinesteratic effect of treatments on the brain and muscles of animals. For this, the Ellman et al. (1961) method was used, as described by Guimarães et al. (2021).

2.4.4. Mutagenicity

To evaluate the possible mutagenicity induced by the treatments, we performed the micronucleus test and other erythrocytic abnormalities according to the methodology described in Anifowoshe et al. (2022). This test consists of evaluating the ability of compounds/substances to induce structural or numerical chromosomal damage (Hayashi et al., 2007). Immediately after being removed from the aquariums, the fish were anesthetized on ice, and a cut in the caudal region removed the blood. Subsequently, blood smears were prepared to make a slide per animal. After drying for 24 h at room temperature, the slides were flushed using the Rapid Panoptic Kit (Laborclin, Pinhais, PR, Brazil) to color the nuclei and cytoplasm of erythrocytes, like Castro et al. (2022). Cell analysis was performed blindly by a single researcher using a binocular optical microscope. The frequency of micronuclei and other nuclear abnormalities in erythrocytes was assessed using 2000 cells per sample, considering only red blood cells with intact nuclear and cytoplasmic membranes. The classification of

Fig. 2. Schematic images of the behavioral tests to which Danio rerio adults were submitted. (A-B) Open field test, with emphasis on (A) demarcations of the movement of animals in the apparatus and (B) the central and peripheral areas of the apparatus. (C–D) Social aggregation test in the presence of a potential predator, highlighting the “habituation” (i.e., without predator) and (D) “test” sessions (i.e., with predator – Hoplias malabaricus). On the computer screen, dashed lines were drawn to evaluate the behavioral biomarkers evaluated in the present study.
Fig. 4. (A) Malondialdehyde levels and (B) nitrite production in the brain, gills, and muscle of *Danio rerio* (zebrafish) adults exposed or unexposed to different treatments. Parametric data are presented by the mean ± standard deviation, whereas non-parametric are presented by the median and interquartile range. Summaries of statistics are displayed at the top of the graphs. “Mix” refers to zebrafish exposed to the mix of pollutants (see concentrations in Table 1), and “PSPD” refers to the group composed of animals exposed only to SARS-CoV-2-derived peptides (PSPD-2001 + PSPD-2002 + PSPD-2003; at 266.2 ng/L), and “Mix+PSPD” include zebrafish exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants.
micronuclei was based on Hooftman and De Raat (1982) and the nuclear abnormalities (e.g., nuclear bud, apoptotic fragments, bilobed cells, and binucleated cells) according to Baršienė et al. (2006).

2.5. Physicochemical quality of the display waters

The potential interference of treatments (SARS-CoV-2-derived peptides and/or a mix of pollutants) on water quality conditions was evaluated using the following attributes: water temperature (°C), electronic conductivity (μS/cm²), total dissolved solids (mg/L), resistivity (MΩ·cm), potential oxidation-reduction (mV), salinity (%), and pH. Such attributes were measured with a portable multi-parameter (Instrutemp, ITPH-3000), like Freitas et al. (2022). The dissolved oxygen levels (mg/L) were recorded through a dissolved oxygen sensor (CommerceAll, AT-155).

2.6. Integrated Biomarker Response Index (IBRv2)

To evidence the toxicity of the treatments, the results of all biomarkers evaluated were applied to the “Integrated Biomarker Response Index” (IBRv2). Such an index is an effective way of combining multiple biomarkers into a single index. The second-generation IBR index (IBRv2) was calculated following the methods described in Malafaia et al. (2022). The deviation between biomarkers measured in zebrafish exposed to SARS-CoV-2-derived peptides and a mix of pollutants compared to those in unexposed animals (“control” group). The biomarker results are shown as a star plot, where the area above zero reflects biomarker induction, and the area below zero indicates biomarker inhibition.

2.7. Data analysis

2.7.1. Mean comparison

Initially, all data obtained were evaluated regarding the assumptions for using parametric models. For this, we used the Shapiro-Wilk test to assess the distribution of residual data, and the Bartlett test was used to assess the homogeneity of variances. The datasets that met the assumptions for parametric models were analyzed via the one-way ANOVA test (with Tukey post-test). The non-parametric data were compared via Kruskal-Wallis test (with Dunn’s post-test). Data on the social aggregation test in response to a potential aquatic predator were analyzed as a paired test because the zebrafish in the first (habituation) and second (test) sessions were the same. The student’s t-test was used in these cases to compare the parametric data. The differences between the experimental groups in the first and second sessions were compared through one-way ANOVA (parametric data). Significance levels were set at Type I error (p) values lower than 0.05. GraphPad Prism Software Version 9.0 software was used to perform the statistical analyses.

2.7.2. Principal component analysis

To comprehensively evaluate the data set obtained in our study, the principal component analysis (PCA) was also applied. By then focusing on these “principal components,” the data can be re-analyzed 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 the PCA analysis. The variables considered in the PCA were CAT brain (CATB), CAT gills

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**Fig. 5.** (A) Superoxide dismutase and (B) catalase activity in the brain, gills, and muscles of *Danio rerio* (zebrafish) adults exposed or unexposed to different treatments. Parametric data are presented by the mean ± standard deviation, whereas non-parametric are presented by the median and interquartile range. Summaries of statistics are displayed at the top of the graphs. “Mix” refers to zebrafish exposed to the mix of pollutants (see concentrations in Table 1), and “PSPD” refers to the group composed of animals exposed only to SARS-CoV-2-derived peptides (PSPD-2001 + PSPD-2002 + PSPD-2003; at 266.2 ng/L), and “Mix + PSPD” include zebrafish exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants.
CATG, CAT muscle (CATM), SOD brain (SODB), SOD gills (SODG), SOD muscle (SODM), DPPH brain (DPPHB), DPPH gills (DPPHG), DPPH muscle (DPPHM), thiol brain (TB), thiol gills (TG), thiol muscle (TM), MDA brain (MDAB), MDA gills (MDAG), MDA muscle (MDAM), body biomass (BB), body biomass/total length (BB/TL), swimming speed (SS), AChE brain (AChEB), AChE muscle (AChEM), nitrite brain (NB), nitrite gills (NG), nitrite muscle (NM), anxiety index (AI), total crossings (open field test; TC), frequency in the central zone (open field test) (FCZ), total length (TL), body width/total length (BW/TL), peduncle depth/total length (PD/TL), head length/total length (HL/TL), head width/total length (HW/TL), total erythrocytic nuclear abnormalities (TENA), and cluster score (test session) (CS). After PCA, we use the rotated loading (coefficient) matrix, loading plot, and PCA score plot of the first two PCs generated in GraphPad Prism Software Version 9.0. Ward’s hierarchical clustering method was also used to identify the group distributions according to the variables on the PCA results via PAST (PAlaeontology STatistic) software.

3. Results

Our study did not register any deaths during the experimental period in the different groups. The analysis of the animals in the open field test did not reveal locomotor alterations, as inferred by the total crossings of the quadrants of the apparatus (Fig. 3A) and the swimming speed (Fig. 3B). We also did not report an anxiolytic or anxiogenic effect in the animals exposed to the treatments, as suggested by the anxiety index (Fig. 3) and the frequency of visits to the central zone of the open field (Fig. 3D). Also, animals in all groups reacted to predator potential, reducing the cluster score in the social aggregation test (Fig. 3E).

In biometric terms, we also did not show differences between the groups regarding the biomarkers evaluated (total length, body biomass, and the indices “body biomass/total length”, “body width/total length”, “peduncle depth/total length”, “head length/total length”, and “head width/total length”) (Fig. S1). The total number of erythrocyte nuclear abnormalities recorded in the “Mix”, “PSPD” and “Mix + PSPD” groups did not differ from the “control” group (Fig. S2), suggesting that the treatments did not induce a mutagenic effect from the test of the micronucleus and other abnormalities.

On the other hand, the biochemical response of the animals to the treatments was organ-dependent. While in the gills, we observed a significant increase in MDA levels in the “PSPD” and “PSPD + Mix” groups (compared to the “control” group); in the brain and muscles, the production of this metabolite in animals exposed to treatments did not differ from unexposed zebrafish (Fig. 4A). The increase in nitrite production was observed only in the brains of the animals in the “PSPD + Mix” group (Fig. 4B). A 65.7 % increase was observed in this group compared to that reported in unexposed animals, which suggests that co-exposure to PSPD and the pollutant mix induced nitrosative brain stress. In addition, we noticed that SOD activity was drastically affected by exposure to treatments in all organs evaluated (Fig. 5A). In contrast, catalase activity was superior to the “control” group only in the brains of the animals in the “Mix”, “PSPD” and “Mix + PSPD” groups (Fig. 5B).

On the other hand, the suppression of DPPH radical scavenging activity was observed only in the brain (with an average reduction of 58.7 %, about

![Fig. 6.](image-url)
the “control” group) of these same animals (Fig. 6A), similarly to the total thiol levels reported in the gills (mean reduction of 19.5 %) (Fig. 6B). In addition, we observed an anticholinesterase effect in the muscles of the animals in the “PSPD-Mix” group, when compared to the “control” group, marked by a significant reduction in AChE activity (Fig. 7).

Regarding the physicochemical attributes of the exposure waters throughout the experimental period, we observed that the pollutant mix (alone (“Mix” group) or in combination with the SARS-CoV-2-derived peptides (“Mix + PSPD”) group) increased the electrical conductivity, the total dissolved solids (mg/L) and salinity, in addition to reducing resistivity (p-value < 0.0001). Table 2 shows that the treatments did not change the other attributes (oxidation-reduction potential, pH, temperature, and dissolved oxygen).

The results obtained were applied to PCA to reduce the dimensionality of the data and determine the similarity between the experimental groups considering the zebrafish’ responses to SARS-CoV-2-derived peptides and a mix of pollutants (alone or in combination). We observed that the first two principal components (PC1 and PC2) cumulatively explained 80.94 % of the total variation, whose eigenvalues for PC1 and PC2 were 16.03 and 10.68, respectively. The loadings plot (Fig. 8A) and Table 3 demonstrated that most biomarkers were negatively associated with PC1 and separation of groups into three subgroups, which was also confirmed by the hierarchical clustering analysis (Fig. 8C). Furthermore, in Fig. 9A, it is possible to notice a similarity between the IBRv2 values and star graph (polygon) obtained for the “Mix” and “PSPD” groups. Despite this, on average, the “Mix + PSPD” group presented an IBRv2 value 41.68 % higher than the other groups. Increased brain nitrite levels, reduced muscle AChE activity, and radical DPPH scavenging activity in the brain and muscle were the most discerning factors for this group (Fig. 9B).

### 4. Discussion

Undoubtedly, the COVID-19 pandemic has been a milestone in the recent history of humanity, given its impacts on the health of populations, its indirect and direct effects on the economy, social issues, and the environment (Panakaje et al., 2022; Kiran, 2020; Park et al., 2022; Wang et al., 2022; Banna et al., 2022; Aburto et al., 2022; Ozili and Arun, 2023). In our study, using a design that simulates the exposure of freshwater fish to a mix of emerging pollutants in combination with SARS-CoV-2-derived peptides, we demonstrate that the impacts of the COVID-19 pandemic can be even more significant and comprehensive. Even though the treatments did not cause any changes in behavior (Fig. 3), we noticed a biochemical response in different organs when we evaluated biomarker endpoints (Fig. S1) or mutagenic biomarkers (Fig. S2), which can affect the fitness of individuals and pose a risk to their health.

Regarding the behavior of animals exposed to SARS-CoV-2 (alone) peptides, our data differ from those evidenced in recent studies by Mendonça-Gomes et al. (2021) and Malafaia et al. (2022). While in Malafaia et al. (2022), exposure of P. reticulata juveniles to PSPD-2002 and PSPD-2003 peptides induced anxiety-like behavior in the open field test and increased the “control” group of these same animals (Fig. 6A), similarly to the total thiol levels reported in the gills (mean reduction of 19.5 %) (Fig. 6B). In addition, we observed an anticholinesterase effect in the muscles of the animals in the “PSPD-Mix” group, when compared to the “control” group, marked by a significant reduction in AChE activity (Fig. 7).

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AChe activity, Mendonça-Gomes et al. (2021) reported changes in the locomotor and olfactory-driven behavior of the C. quinquefasciatus larvae (exposed to these same peptides), which were associated with increased production of ROS and cholinesterasic effect. Furthermore, we did not report alterations suggestive of mutagenicity by the micronucleus test and other erythrocyte abnormalities performed in zebrafish subjected to the peptides (Fig. S2), unlike what was evidenced in P. reticulata juveniles exposed to PSPD-2022 peptides (Gonçalves et al., 2022). In particular in this study, a ten days exposure to peptides was enough to significantly increase the frequency of erythrocytic nuclear alteration and all parameters assessed in the comet assay (length tail, %DNA in tail, and Olive tail moment), suggesting that PSPD-2002 peptides were able to cause genomic instability and erythrocyte DNA damage. In general, it can be assumed that the biological differences between the evaluated models (i.e., different species), exposure times, age (or phase development), and peptide concentrations are reasons which might explain the discrepancy between the results.

Another plausible reason for these differences refers to how the animals were exposed. While in our study the peptides were introduced in the aquarium together (PSPD-2002 + PSPD-2003 + PSPD-2003), in the other studies the animals were exposed to the peptides alone and free from any combination with other pollutants. In this case, although there are no studies involving the combined toxicity of SARS-CoV-2-derived peptides (to each other), previous investigations into the combined toxicity of emerging pollutants have suggested that their interactions may induce effects different from those reported when isolated. This is the case, for example, of the work by Estrada et al. (2021), in which the authors did not observe an increase in lipid peroxidation processes in the brain of Swiss mice exposed to the combination of zinc oxide nanoparticles and polystyrene nanoplastics, whereas the increase in the production of TBARs was observed when the animals were exposed to pollutants alone. In Araújo et al. (2022), the exposure of zebrafish to polyethylene microplastics induced a significant reduction in the locomotor activity of the animals; but when combined with a pollutant mix, a hyperactive-like behavior was observed in the animals.

On the other hand, the combined exposure of newly emerged bees (Apis cerana cerana) to the pesticides acetamiprid and propiconazole significantly reduced glutathione-S-transferase activity in the midguts, which was not observed in isolated exposures to pesticides (Han et al., 2019). Furthermore, Caceres et al. (2007) first reported that the combined exposure of Daphnia carinata to chlorpyrifos and 3,5,6-trichloropyridinol did not induce toxicity in animals when present together in concentrations up to 0.12 μg/L. In humans, however, Iyyadurai et al. (2014) found that patients with mixed poisoning (chlorpyrifos + cypermethrin) appear to have fewer ventilator-free days than patients poisoned by either of the pesticides alone. Although these studies are not directly related to our investigation, they support the hypothesis that chemical interactions between compounds, substances, or molecules can interfere with their mechanisms of action, environmental availability, and, therefore, their toxicity. Consequently, it is plausible to suppose that the interaction between the peptides (whether in the aquatic environment or animals) has interfered with the mechanisms of action that culminated in the behavioral, biometric, and mutagenic changes reported in other animal models exposed to the peptides in a non-combined way (Mendonça-Gomes et al., 2021; Gonçalves et al., 2022; Malafaia et al., 2022). This would also explain why the behavioral, biometric, and mutagenic biomarkers evaluated in zebrafish exposed in our study ("PSPD" group) did not show similar effects.

We also observed divergent results on the toxicity of the pollutant mix (assessed in isolation) and reported in previous investigations in which the same mixture of pollutants was tested. While the increase in MDA production and the induction of nitrosative stress by the mix was not verified in our study; in Araújo et al. (2022), zebrafish adults exposed for 15 days showed increased production of nitrite in the brain and muscle, as well as a reduction of this metabolite in the gills. In Araújo et al. (2023), tadpoles exposed to the mix for 30 days significantly increased the production of...
which showed a 21.7% increase in the activity of this enzyme after association with the mix of pollutants. See the meanings of the abbreviations in "groups."

**Fig. 9.** Loading matrix provided by the multivariate analysis to define factors or principal components PC1 and PC2.

| Biomarkers                        | Abbreviation | Principal components |
|-----------------------------------|--------------|----------------------|
|                                   |              | PC1                  | PC2                  |
| Catalase activity in the brain    | CATB         | 0.971                | –0.172              |
| Catalase activity in gills        | CATG         | –0.846               | 0.247               |
| Catalase activity in muscle       | CATM         | 0.222                | –0.965              |
| Superoxide dismutase activity in the brain | SODB         | –0.801               | –0.096              |
| Superoxide dismutase activity in gills | SODG         | –0.982               | –0.179              |
| Superoxide dismutase activity in muscle | SODM         | –0.997               | –0.059              |
| DPPH radical scavenging activity in the brain | DPPHB         | –0.904               | 0.253               |
| DPPH radical scavenging activity in gills | DPPHG         | 0.278                | –0.228              |
| DPPH radical scavenging activity in muscle | DPPHM         | –0.437               | 0.344               |
| Total thiol in the brain          | TB           | –0.900               | 0.426               |
| Total thiol in gills              | TG           | –0.963               | –0.189              |
| Total thiol in muscle             | TM           | –0.533               | 0.805               |
| Malondialdehyde levels in the brain | MDAB         | 0.179                | 0.879               |
| Malondialdehyde levels in gills   | MDAG         | 0.889                | 0.017               |
| Malondialdehyde levels in muscle  | MDM          | –0.009               | 0.894               |
| Body biomass                      | BB           | 0.697                | 0.610               |
| body biomass/total length         | BB/TL        | –0.085               | 0.851               |
| swimming speed                    | SS           | 0.917                | –0.328              |
| Acetylcholinesterase activity in the brain | AChEB         | 0.095                | –0.925              |
| Acetylcholinesterase activity in muscle | AChEM         | –0.429               | 0.903               |
| Nitrite levels in the brain       | NB           | 0.437                | 0.707               |
| Nitrite levels in gills           | NG           | –0.699               | 0.466               |
| Nitrite levels in muscle          | NM           | 0.823                | –0.543              |
| Anxiety index                     | AI           | –0.734               | 0.173               |
| Total crossings (open field test) | TC           | 0.917                | 0.329               |
| Frequency in the central zone (open field test) | FZC          | 0.595                | 0.522               |
| Total length                      | TL           | 0.591                | –0.671              |
| Body width/total length           | BW/TL        | –0.490               | 0.763               |
| Peduncle depth/total length       | PD/TL        | 0.924                | –0.153              |
| Head length/total length          | HL/TL        | 0.895                | 0.410               |
| Head width/total length           | HW/TL        | –0.351               | 0.720               |
| Total erythrocytic nuclear abnormalities | TENA         | 0.659                | 0.587               |
| Cluster score                     | CS           | –0.607               | 0.751               |

Large loadings are highlighted in boldface to emphasize the variables contributing to each principal component.

Nitrite and MDA compared to the control group. Furthermore, the suppression of brain, gill, and muscle SOD activity observed in zebrafish in our study (Fig. 5A) was different from that observed in *P. cuvieri* tadpole, which showed a 21.7% increase in the activity of this enzyme after exposure to the mix of pollutants (Araújo et al., 2023). On the other hand, the increase in catalase activity in the brains of the animals in our study (Fig. 5B) was like that observed in the brain and muscle of zebrafish (Araújo et al., 2022), as well as in *P. cuvieri* tadpole (Araújo et al., 2023). The effects of the pollutant mix in these studies have been attributed to the toxicity of several of its chemical components. As demonstrated in a previous study by our group (Souza et al., 2018), >350 organic compounds and high concentrations of heavy metals (e.g., Pb, Ni, Zn, Cr, and Co) were reported in the composition of the pollutant mix used. In any case, such studies suggest that the toxicity of the pollutant mix depends not only on the period of exposure but also on the organ being evaluated, the stage of development (adults vs. juveniles), and the species assessed.

On the one hand, we observed a significant impact of the animals’ exposure to the SARS-CoV-2 peptides in combination with the pollutant mix, particularly in the production of nitrite in the brain (Fig. 4B), muscle AChE activity (Fig. 7), and in the DPPH radical scavenging activity in the brain and muscle of the zebrafish evaluated (Fig. 6A), which were the most discriminant factors for the "Mix-PSPD" group (Fig. 9B). Regarding nitrite levels in the brains of the animals in this group, we observed an increase of >60% compared to the “control” group (Fig. 4B), suggesting a synergistic effect of the combination of pollutants/PSPD on NO production. As already demonstrated in the literature, at high concentrations, NO reacts with ROS producing reactive nitrogen species (RNS) that are known to have harmful implications for biological systems. Evidence reported by Paakkari and Lindsberg (1995) and Lee et al. (2016), for example, suggest that NO itself serves as a cytoxic mediator by reacting with superoxide anions or hydrogen peroxide to produce peroxynitrite, which is much more reactive and toxic than NO or superoxide anions alone. Thus, it is reasonable to assume that the generation of peroxynitrite in zebrafish of the "Mix + PSPD" group — induced by the combined action of PSPDs and the different pollutants in the mix — affected essential macromolecules, impairing the impairment of SOD and DPPH radical scavenging activities in the brain of the animals in this group (Fig. 5A and 6A, respectively).

On the other hand, this effect does not seem to have affected the behavior of the animals in the tests performed, which can be explained by the increased activity of other antioxidant components not evaluated in our study (e.g., glutathione peroxidase, DT-diaphorase, vitamin E, vitamin C, carotenoid, ferritin, ceruloplasmin, selenium, reduced glutathione, manganese, ubiquinone, zinc, flavonoids, coenzyme Q, melatonin, bilirubin, taurine, cysteine, etc.) individuals. Alternatively, it is possible that such alterations were not sufficient to impact the neural circuits that regulate the locomotor activity or aspects related to fear/anxiety in the animals or that such circuits

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**Fig. 9.** (A) Integrated biomarker responses index (IBRv2) values and (B) star graph (polygon) obtained with the IBRv2 method for the "Mix", "PSPD", and "Mix + PSPD" groups. “Mix” refers to zebrafish exposed to the mix of pollutants (see concentrations in Table 1), and “PSPD” refers to the group composed of animals exposed only to SARS-CoV-2-derived peptides (PSPD-2001 + PSPD-2002 + PSPD-2003; at 266.2 ng/L), and “Mix + PSPD” include zebrafish exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants. See the meanings of the abbreviations in “B” in Section 2.7.2 (“Materials and Methods”) or Table 3.
were not affected. In this case, digging deeper into these questions could lead to interesting ideas that could be studied in the future.

Interestingly, we also observed that the anticholinesterase effect observed in the muscle of the animals of the “Mix + PSPD” group (Fig. 7) was not associated with the locomotor ability of the zebrafish (Figs. 3A-B). These data are intriguing not only because reduced AChE activity has already been related to locomotor disorders in different fish species (Tierney, 2011; Sarasamma et al., 2018; De-Farias et al., 2019; Pullaguri et al., 2020; Mishra et al., 2022; Chen et al., 2022; Wan et al., 2022) but also because they diverge from the cholinesterase stimulation observed in P. c. tetraodon (Charlie-Silva et al., 2021), C. quinquefasciatus larvae (Mendonça-Gomes et al., 2021), and P. reticulata juveniles (Malafaia et al., 2022) exposed to PSPD-2002 and PSPD-2003 peptides. These studies have suggested that this increase reflects a compensatory mechanism in response to the catalytic deficit induced by the peptides or a more efficient response of the AChE to the increase in the release of ACh in the synaptic clefts. However, in the zebrafish evaluated in our study, such assumptions are not plausible, which can be explained by the physiological and biochemical characteristics of the animal models investigated, the organs/tissues where AChE activity was measured, as well as by the concentrations and periods of exposure to the viral peptides. In this sense, further investigations are necessary to understand the mechanisms responsible for the anticholinesterase effect observed in our study. In this case, it might be a good idea to look into how viral peptides (alone or with the pollutant mix) affect association and catalysis mechanisms, the affinity of substrates for the AChE active site, and/or the cholinergic anti-inflammatory pathway.

5. Conclusion

We concluded in our study that despite more intense effects for some biomarkers in some organs/tissues were not observed in the “Mix + PSPD” group, PCA and IBRv2 values indicate that viral fragments are associated with the pollutant mix-induced increased toxicity in zebrafish adults. Therefore, our study reinforces the hypothesis that the spread of the new coronavirus in aquatic environments, especially in those already polluted, represents an imminent risk to the health of aquatic organisms. However, it should be borne in mind that our study is the first to assess the possible toxicological effects of combining SARS-CoV-2-derived peptides with a mix of emerging pollutants in an aquatic vertebrate model. Thus, several questions can and should be continuously explored, including conditions that allow us to evaluate the effects of the factors “exposure time”, “age”, “sex”, “species”, and “biomarkers of toxicity” on the responses of animals. In addition, studies that seek to elucidate whether the adverse effects observed in our study are reversible in an aquatic depollution scenario, or even if they are transgenerational, will be essential to support strategies and actions for mitigation and remediation of aquatic pollution.

Data availability

Data will be made available on request.

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.

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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.

CRediT authorship contribution statement

Ítalo Nascimento Freitas: designed and performed experiments, analyzed data, and co-wrote the paper. Amanda Vieira Dourado: performed experiments. Amanda Pereira da Costa Araújo: performed experiments. Sindoval Silva de Souza: performed experiments. Thiarien Marinho da Luz: performed experiments. Abraão Tiago Batista Guimarães: performed experiments. Alex Rodrigues Gomes: performed experiments. Abu Reza Md. Towfiqul Islam: revised the paper critically for important intellectual content. Md. Mostafizur Rahman: revised the paper critically for important intellectual content. Andrés Hugo Arias: revised the paper critically for important intellectual content. Nabisab Mujawar Mubarak: revised the paper critically for important intellectual content. Chinnaperumal Kamaraj: revised the paper critically for important intellectual content. Guillerme Malafaia: designed and performed experiments, analyzed data, co-wrote the paper, supervised the research, provided funding acquisition, project administration, and resources.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159838.

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