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SHORT-TERM EXPOSURE OF THE MAYFLY LARVAE (*Cloeon dipterum*, EPHEMEROPTERA: BAETIDAE) TO SARS-COV-2-DERIVED PEPTIDES AND OTHER EMERGING POLLUTANTS: A NEW THREAT FOR THE AQUATIC ENVIRONMENTS

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ABSTRACT
The input of SARS-CoV-2 or its fragments into freshwater ecosystems (via domestic or hospital sewage) has raised concerns about its possible impacts on aquatic organisms. Thus, using mayfly larvae [Cloeon dipterum (L.), Ephemeroptera: Baetidae] as a model system, we aimed to evaluate the possible effects of the combined short exposure of SARS-CoV-2-derived peptides (named PSPD-2001, PSPD-2002, and PSPD-2003 – at 266.2 ng/L) with multiple emerging pollutants at ambient concentrations. After six days of exposure, we observed 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. In the “PSPD” and “Mix+PSPD” groups, the activity of superoxide dismutase, catalase, DPPH radical scavenging activity, and the total thiol levels were also lower than in the “control” group. In addition, we evidenced the induction of nitrosative stress (inferred by increased nitrite production) and reduced acetylcholinesterase activity by SARS-CoV-2-derived peptides. On the other hand, malondialdehyde levels in larvae exposed to treatments were significantly lower than in unexposed larvae. The values of the integrated biomarker response index and the principal component analysis (PCA) results confirmed the similarity between the responses of animals exposed to SARS-CoV-2-derived peptides (alone and in combination with the pollutant mix). Although viral peptides did not intensify the effects of the pollutant mix, our study sheds light on the potential ecotoxicological risk associated with the spread of the new coronavirus in aquatic environments. Therefore, we recommend exploring this topic in other organisms and experimental contexts.

Keywords: novel coronavirus, non-target organisms, freshwater ecosystems, insects, biomarkers.
1. INTRODUCTION

COVID-19 (Coronavirus Disease-2019), caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), has been considered an unprecedented pandemic in the modern era (Yan, 2020; Nkengasong, 2021). As of 07 June 2022, the WHO Coronavirus (COVID-19) Dashboard recorded 530,266,292 confirmed cases of COVID-19, including 6,299,364 deaths (WHO, 2022). Economically, as McKibbin & Fernando (2020) highlighted, the short- and long-term fiscal and budgetary effects associated with COVID-19 point to the most significant recession in contemporary history. In addition, socially, the COVID-19 pandemic has influenced the daily lives of millions of people, from the obligation to follow social isolation rules to the planning and adoption of health measures (Saladino et al., 2020; Fraser et al., 2022). However, recent studies have shown that the impacts of the COVID-19 pandemic can be even broader, especially considering the potential effect of SARS-CoV-2 on non-target organisms (Charlie-Silva & Malafaia, 2022). The identification of SARS-CoV-2 or its fragments in hospital and domestic sewage (Marlet et al., 2021; Ahmed et al., 2021; Dharmadhikari et al., 2022; Pellegrinelli et al., 2022) and in aquatic environments (e.g., rivers – De-Oliveira et al., 2021; Fongaro et al., 2021; Fonseca et al., 2022; Rocha et al., 2022), have raised concerns not only about the possible secondary transmission of SARS-CoV-2 (Liu et al., 2020; Thakur et al., 2021; Ahmed et al., 2022), as well as its ecotoxicological impacts (Charlie-Silva & Malafaia, 2022).

Since the pandemic’s beginning, only recently have some studies been dedicated to evaluating the ecotoxicological effects associated with SARS-CoV-2 on non-target organisms. In the pioneering study by Charlie-Silva et al. (2021), the authors exposed Physalaemus cuvieri tadpoles to peptide fragments of the Spike protein in SARS-CoV-2 (named PSPD-2001, PSPD-2002, and PSPD-2003), for a period of only 24 h, and reported an increase of the oxidative processes, as well as alterations in the activity of the enzyme acetylcholinesterase (AChE) of the animals. Subsequently, Mendonça-Gomes et al. (2021) and Malafaia et al. (2021) observed that the effects of exposure to SARS-CoV-2 peptide fragments are not restricted to the tadpoles evaluated in Charlie-Silva et al. (2021). On that occasion, alterations in locomotor activity and the olfactory behavior of Culex quinquefasciatus larvae, as well as a significant increase in production of reactive oxygen species (ROS) and AChE activity, were correlated with larval exposure to PSPD-2002 and PSPD-2003 peptides (to 40 µg/L) (Mendonça-Gomes et al., 2021). In addition, Poecilia reticulata juveniles exposed to peptide fragments also show behavioral changes, redox imbalance, and impaired growth/development (Malafaia et al., 2022).
More recently, it has been demonstrated the potential of SARS-CoV-2 to induce genomic instability and DNA damage in *P. reticulata* juveniles (Gonçalves et al., 2022), histopathological inflammatory reaction, and damage in different organs (Fernandes et al., 2022), as well as olfactory dysfunction in *Danio rerio* adults (Kraus et al., 2022). On the other hand, Luz et al. (2022) reported that mice exposed to SARS-CoV-2-derived peptides show behavioral changes predictive of memory deficit, which demonstrates that the effects of the novel coronavirus fragments are not restricted to aquatic organisms. Although these studies represent preliminary and incipient findings about the potential impact of SARS-CoV-2 on non-target organisms, they certainly “shed light” on the (eco)toxicological potential of peptide fragments of SARS-CoV-2 in biota.

Thus, this scenario raises the concern that the presence and dispersion of SARS-CoV-2 may intensify the impacts on the biota caused by the pollution already known in several river systems. Pollution by heavy metals (Muhammad & Usman, 2022), surfactants (Al-Ani et al., 2020), phenolic compounds (Ramos et al., 2021), petroleum (Edori & Edori, 2021), pharmaceutical waste (He et al., 2021), al., 2022; Quincey et al., 2022), pesticides (Kalantary et al., 2022; Jorfi et al., 2022), personal care products (Liu et al., 2021), microplastics (Talbot & Chang, 2022), among others, has been well documented in recent years. Thus, it is questioned whether the co-existence of multiple pollutants with SARS-CoV-2 in the aquatic system constitutes an additional concern for aquatic species. Assessing “if” and “how” the COVID-19 pandemic can affect wildlife, even intensifying the effects of current water pollution, is an opportunity to anticipate actions to reduce its impact on non-target organisms.

Therefore, we used mayfly larvae [*Cloeon dipterum* (L.), Ephemeroptera: Baetidae] to evaluate the possible effects of combined exposure to SARS-CoV-2-derived peptides with multiple pollutants of diverse chemical nature. Using biometric, antioxidant, nitrosative, and cholinesterasic biomarkers, we tested the hypothesis that co-exposure to viral peptides and the pollutant mix induces more intense changes in the growth/development of animals, more significant redox imbalance, increased nitrosative stress, and reduced activity of the acetylcholinesterase (AChE) when compared to isolated exposure to peptides and the mix of pollutants. According to Subramanian & Sivaramakrishnan (2007), insects are critical ecological components of freshwater ecosystems and are widely used as environmental indicators.

In particular, *C. dipterum* is part of the order Ephemeroptera, which includes hemimetabola insects that live in freshwater ecosystems and have over 3000 species distributed in 40 different families approximately (Barber-James et al., 2007; Almudi et al., 2019). Salles et al. (2014) highlight that the mayflies are found in almost all freshwater habitats worldwide and
display an amphibiotic life cycle; the larval stage is aquatic, and the alate stage is terrestrial. According to Jacobus et al. (2019), mayflies constitute a significant part of the macroinvertebrate biomass and production in freshwater habitats and are widely endorsed as bioindicators of water quality and ecological integrity (Barbour et al., 1999; Menetrey et al., 2007; Vilenica et al., 2022), and therefore included in many of the biological water quality assessment methods for streams [e.g., Hilsenhoff (1988), Hamid & Rawi (2017), and Kietzka et al. (2019)]. Therefore, we believe that our study contributes to advancing knowledge about the global impacts of the COVID-19 pandemic under an ecological/environmental optimum, going beyond what we already know about transmissibility, pathogenesis, and therapeutics of the disease.

2. MATERIAL AND METHODS

2.1. SARS-CoV-2-derived peptides

The synthesis, cleavage, purification, and characterization of the peptides of the Spike protein of SARS-CoV-2 used in our study (PSPD-2001, PSPD-2002, and PSPD-2003) were performed according to methods described in detail by Charlie-Silva et al. (2021). Briefly, the synthesis was conducted using the solid phase peptide synthesis method (SPPS) following the Fmoc strategy, based on Behrendt et al. (2016). The resins used for synthesis were Fmoc-Cys (Trt)-Wang, Fmoc-Thr (TBu)-Wang, and Fmoc-Asn (Trt)-Wang for peptides Arg-Val-Tyr-Ser-Ser-Ala-Asn-Cys-COOH (PSPD-2001); Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH (PSPD-2002) and Asn-Asn-Ala-Thr-Asn-COOH (PSPD-2003) (Figure 1). This resin made it possible to obtain peptides with a carboxylated C-terminal end at the end of the synthesis. After coupling all the amino acid residues of the peptide sequence, the chains were removed from the solid support utilizing acid cleavage via trifluoroacetic acid, similarly to Guy & Fields (1997). The crude compounds were purified by high-performance liquid chromatography [based on Klaassen et al. (2019)], using different purification methods according to the retention time obtained in a gradient program of 5 to 95% in 30 min (exploration gradient) in analytical HPLC. Only compounds with purity equal to or greater than 95% were considered for in vivo evaluation, following the National Health Surveillance Agency (ANVISA/Brazil) rules and Food and Drug Administration (FDA/USA).
2.2. Mix of emerging pollutants

The pollutant mix used to simulate the aquatic contamination by different xenobiotics was composed of 14 pollutants (in addition to those that make up the tannery effluent) in environmentally relevant concentrations (i.e., that were previously identified in water surfaces), based on Araújo et al. (2022ab). The physicochemical and inorganic characterization and the profile of organic compounds identified in this mix can be observed in Souza et al. (2018). Briefly, such pollutants more realistically represent the diversity of chemical compounds/substances that can enter freshwater ecosystems, including pesticides (glyphosate and abamectin), agro-industrial effluent (tannery effluent), pharmaceutics (amoxicillin, acetylsalicylic acid, sodium diclofenac, ibuprofen, fluoxetine, clonazepam, dipyrone monohydrate, and ranitidine), hormones (estradiol cypionate), agricultural fertilizers (nitrogen), surfactants (domestic detergent), and constituent substances of petroleum (benzene). General information about the mixed emerging pollutants used in our study is presented in Table S1.

2.3. Model systems and experimental design

In this study, we used larvae of C. dipterum collected in a semi-natural breeding site installed outdoors on the premises of the Goiano Federal Institute- Campus Urutaí (GO, Brazil). Animals were captured by sweeping a net through the water column. After that, the animals were immediately taken to the laboratory and kept in an aquarium with dechlorinated water for seven days (for acclimatization), under temperature (21.9°C ± 0.54°C, mean ± standard deviation) and luminosity (12:12-h light: dark photoperiod) controlled. Before and during the
experiment, larvae were fed once a day with commercial fish food (composition: 45% crude protein, 14% ether extract, 5% natural fiber, 14% mineral matter, and 87% dry matter) (10 mg ration/L). After that, 320 healthy larvae [stages L4-5, according to Cianciara (1979), total length: 5.30 mm ± 0.66 mm; body biomass: 1.85 mg ± 0.48 mg - mean ± standard deviation] presenting normal swimming behavior and no morphological deformities, or apparent lesions, were distributed into four experimental groups (n = 80 larvae/each - 8 replicates composed of 10 larvae/each). While the group 'PSPD' was formed of animals kept in water containing an equitable mixture of the viral peptides PSPD-2001, PSPD-2002, and PSPD-2003, totaling 266.2 ng PSPD/L; C. dipterum larvae exposed to the mix of emerging pollutants described above (see Table S1) (without the presence of SARS-CoV-2-derived peptides), composed the “Mix” group. On the other hand, the group 'Mix+PSPD' was formed of larvae submitted to the combined exposure of SARS-CoV-2-derived peptides with the mix of pollutants at the same concentrations defined in the previous groups. In the "control" group, C. dipterum larvae were kept in dechlorinated water free of viral peptides and any component of the pollutant mix. All experimental groups were kept in polyethylene containers filled with 200 mL of dechlorinated water added with viral peptides and/or pollutants in the respective experimental groups. The exposure period was six days (to simulate brief exposure to pollutants), and the complete water renewal occurred every two days, characterizing a semi-static exposure system.

The concentration of SARS-CoV-2-derived peptides tested in our study simulates the presence of viral particles in a predicted environmental concentration, lower than the concentrations tested in Mendonça-Gomes et al. (2021), Malafaia et al. (2021) and Gonçalves et al., 2022. In addition, we are based on the study by Tampe et al. (2021), in which urinary levels of SARS-CoV-2 nucleocapsid protein (SARS-CoV-2-N) from patients with confirmed SARS-CoV-2 infection (admitted to the Department of Anesthesiology, Emergency and Intensive Care Medicine, University Medical Center Göttingen, Germany) ranged from 475 to 1484 pg/mL = 475 to 1484 ng/L. The concentration tested in our study (222.6 ng/L) corresponds to 15% of the highest concentration detected in the urine of these patients, which constitutes realistic concentrations, possibly resulting from dilution in areas close to the discharge point of hospital sewage (untreated) in a small watercourse.

2.4. Toxicity biomarkers

2.4.1. Larvicidal effect

Daily, the experimental units were monitored, and, in case of deaths, the individuals were counted and removed from the experimental container. The total percentage mortality
was corrected (TMRc) considering the natural mortality observed in the “control” group, applying the formula proposed by Abbotts (1925) (see Equation 1).

\[
TMRc(\%) = \frac{\% \text{Mortality observed in treatments} - \% \text{Mortality observed in the control group}}{100 - \% \text{Mortality observed in the control group}} \times 100
\]

Eq. 1

2.4.2. Biometry

Assuming that the treatments could induce adverse effects on the growth/development of the animals, at the end of the experiment, the developmental phase was determined, as Cianciara (1979) proposed. In addition, the total length and body biomass were measured to determine the body condition index, based on Hayes & Shonk vilc (2001).

2.4.3. Biochemical biomarkers

2.4.3.1. Sample preparation

For biochemical evaluation, samples were prepared based on Mendonça-Gomes et al. (2021). Briefly, pools of five larvae/sample (8 samples/group - total of 40 animals per group) formed the analyzed samples. These animals were weighed, individually euthanized by immersion in either an ice-water and, subsequently, 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). The supernatant was separated into aliquots to evaluate the biomarkers described below.

2.4.3.2. Lipid peroxidation

The levels of malondialdehyde (MDA) were helpful in inference and the possible consequence of increased production of reactive species induced by viral peptides (alone or in combination with the pollutant mix), considering that the MDA is an indicator of lipid peroxidation (LPO) level (Grotto et al., 2009). We adopted the procedures from Esterbauer & Cheeseman (1990) and Lushchak et al. (2005). Briefly, 100 µL of the supernatant was mixed with 200 µL of trichloroacetic acid solution (to 30% w/v) and centrifuged for 10 min (10,000 rpm, 4°C). Then, 200 µL of the supernatant formed in the centrifugation was mixed with 200 µL of thiobarbituric acid (0.67%, w/v) and hydrochloric acid (0.1 M) solution and incubated at 95°C for 15 min. Subsequently, the samples were cooled (in a freezer -80°C, for 10 min), plated on a 96-well sterile microplate (in duplicate, 180 µL/each), and read at 492 nm in an ELISA reader.
2.4.3.3. Antioxidant capacity

To estimate the possible effects of treatments on the antioxidant capacity of *C. dipterum* larvae, we evaluated the superoxide dismutase (SOD) and catalase (CAT) activities, which are considered enzymes that make up the organisms' first line of antioxidant defense (Ighodaro & Akinloye, 2018). Furthermore, the total antioxidant capacity was estimated using the DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) free radical method and by thiol groups assay. SOD activity was measured by the indirect spectrophotometric method of riboflavin photoreduction, which was previously described (Deawati et al., 2017; Deawati et al., 2018). We used the method described by Hadwan & Abed (2016) to determine CAT activity, which is based on the reaction of undecomposed hydrogen peroxide with ammonium molybdate to produce a yellowish color. DPPH free radical method was performed according to Brand-Williams & Cuvelier (1995). This method is used worldwide to predict antioxidant activities by a mechanism in which antioxidants inhibit lipid oxidation, scavenging DPPH radicals and, therefore, determining free radical scavenging capacity. Total thiol concentration or sulfhydryl groups were measured by the methods described initially by Elmman (1959) and modified by Hu (1994). Here, thiols interact with 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), forming a highly colored anion.

2.4.3.4. Nitric oxide production

For the measurement of NO, we used the Griess colorimetric reaction (Grisham et al., 1996), which consisted of the detection of nitrite (NO$_2$), resulting from the oxidation of NO, like Nascimento et al. (2021).

2.4.3.5. Acetylcholinesterase activity

To assess the potential of treatments to induce changes in the cholinergic system, we also evaluated the activity of acetylcholinesterase (AChE), which is responsible for the termination of impulse transmission at cholinergic synapses by hydrolysis of the acetylcholine (ACh) (Silman & Susman, 2008). For this, we have adopted the procedures proposed by Ellman et al. (1961), modifications that are detailed in Malafaia et al. (2020).

2.4.3.6. Total protein, carbohydrate, and lipid levels

Assuming that treatments could interfere with the content of molecules important in energy metabolism, we also evaluated the total protein, carbohydrate, and triglycerides levels in the *C. dipterum* larvae. Total tissue protein levels were determined based on Lowry et al.
(1951) method. On the other hand, triglycerides and total carbohydrate levels were determined using the Folch method (Folch et al., 1957) and the methodology suggested by Dubois et al. (1956), respectively. A detailed description of these methods is presented in the previous study by Guimarães et al. (2021). It is noteworthy that the results referring to MDA levels, antioxidant capacity biomarkers, NO production, and AChE activity were expressed by the “g of proteins” of the samples.

2.5. Measurement of water physicochemical parameters

To assess the possible influence of SARS-CoV-2-derived peptides and pollutant mix (alone or in combination) on water quality conditions, different physicochemical water parameters of each experimental replica were measured daily. Water temperature (°C), electronic conductivity (µS/cm), total dissolved solids (mg/L), resistivity (MΩ·m), oxidation-reduction potential (mV), salinity (%), and pH were measured on-site with a portable multi-parameter (Instrutemp, ITPH-3000). The dissolved oxygen levels (mg/L) were measured using a dissolved oxygen meter (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), which is based on the reference deviation between a disturbed and undisturbed state. For this, we adopted the methodology proposed by Sanchez et al. (2013) and described in Malafaia et al. (2022). In our study, the deviation between biomarkers measured in larvae exposed to SARS-CoV-2-derived peptides and a mix of pollutants were compared to those in C. dipterum larvae unexposed (“control” group). The biomarker response scores were plotted as radar graphs. The area above 0 reflects biomarker induction, and the area below 0 indicates biomarker.

2.7. Statistical analysis

2.7.1. Standard curves (biochemical evaluations)

Standard curves were created by correlation analysis and linear regression obtained by absorbances versus known concentrations of MDA, NO, and total thiol levels. For MDA we used different concentrations of tetraethoxypropane (66; 33; 16.5; 8.3; 4.1; 2.1 and 1 µM) (used as the standard MDA), similarly to Mendes et al. (2009) (correlation analysis: Spearman r = 1.0; p-value = 0.0004; linear regression: equation: y = 20.9x − 0.007682; R² = 0.9996; F-value = 13579; p-value < 0.0001). For NO, different concentrations of sodium nitrite (33.3; 16.7; 8.3;
4.2; 2.1; 1.0 and 0.5 and μM) were used to create the standard curve (analysis of correlation: Spearman $r = 1.0$; p-value = 0.0001; linear regression: equation: $y = 0.01407x + 0.01808$; $R^2 = 0.9966$; F-value = 1447; p-value < 0.0001) and in the thiol groups assay we used different concentrations of reduced glutathione [used as sulfhydryl group standard; 6.9; 5.7; 4.8; 4.2; 3.7; 3.3 and 3.0 mmol/L; like Costa et al. (2006)] (correlation analysis: Spearman $r = 1.0$; p-value = 0.0001; linear regression: equation: $y = 0.05255x + 0.08234$; $R^2 = 0.9841$; F-value = 308.5; p-value < 0.0001). Concentrations of quality controls and unknown samples were estimated by applying the linear regression equation of the standard curve to the unknown sample.

### 2.7.2. Mean comparison and correlations analysis

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 data 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 the Kruskal-Wallis test (with Dunn’s post-test). Significance levels were set at Type I error (p) values lower than 0.05. Additionally, correlations were performed using Pearson's (for parametric data) or Spearman's (for non-parametric data) correlation coefficients. GraphPad Prism Software Version 9.0 software was used to perform the statistical analyzes.

### 2.7.3. Principal component analysis

The principal component analysis (PCA) was also applied to the dataset obtained in this study, a widely used statistical method and well-documented in textbooks. Briefly, the technique uses a linear mathematical algorithm to derive a new set of variables, called principal components (PCs), from the original variables so that the new set of variables are no longer correlated. PCA was applied to process the structural and compositional descriptors of the samples to remove the correlations between the descriptors and reduce dimensionality. The obtained PCs were then used for clustering and link analysis. The hypothesis was that the experimental groups with high toxicity would be distinguished as a cluster based on the structural and compositional descriptors. The descriptors underlying clustering can then be identified as those responsible for the observed toxicity. In all PCA analyses in this work, the outliers’ values (identified via the Grubbs test) were excluded from the original data, sequentially logarithmized before PCA analysis. The variables considered in the PCA were total protein (TP), total carbohydrates (TC), triglycerides (TRI), superoxide dismutase (SOD),
catalase (CAT), DPPH radical scavenging activity (DPPH), acetylcholinesterase activity (AChE), body biomass (BB), malondialdehyde (MDA), development stages (DS), nitrite (NO), total thiols (TT), and body condition index (BCI). After PCA, the rotated loading (coefficient) matrix, loading plot, PCA score plot, the proportion of variance plot, scree plot, and PCA biplot of the first two PCs were generated in GraphPad Prism Software Version 9.0.

3. RESULTS

Initially, our data showed that larval mortality in the “PSPD” and “Mix+PSPD” groups was, on average, 22.5% higher than that observed in the “control” and “Mix” groups (Table 1). Furthermore, we noted that body biomass and larval developmental stage did not differ between experimental groups at the end of the experiment (Figure 2A-B). However, larvae exposed to SARS-CoV-2-derived peptides (alone or in combination with the pollutant mix) had a lower body condition index when compared to unexposed larvae (Figure 2C).

Table 1. The mortality rate of Cloeon dipterum larvae recorded in the different experimental groups.

| Experimental groups | C          | Mix        | PSPD       | Mix+PSPD   |
|---------------------|------------|------------|------------|------------|
| Number of replicas/group | 8         | 8          | 8          | 8          |
| Number of larvae/group | 80        | 80         | 80         | 80         |
| Mortality (%)*      | 25.00 ± 17.73 | 25.00 ± 15.12 | 31.25 ± 6.40 | 30.0 ± 15.12 |
| Summary statistical analysis | One-way ANOVA test - F-value = 0.4256; p-value = 0.7361 |
| Corrected Mortality (%)* | -         | 0          | 8.33       | 6.66       |

*Values represent mean ± standard deviation. **The values indicate the average of corrected mortality. “C” refers to the control group, “Mix” refers to those exposed to the mix of pollutants (see concentrations in Table S1), 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 animals exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants.
Figure 2. (A) Body biomass, (B) development stages, and (C) body condition index of Cloeon dipterum larvae (mayfly) 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 statistical analyzes are displayed at the top of the graphs. Distinct lowercase letters indicate significant differences between the experimental groups. “C” refers to the control group, “Mix” refers to those exposed to the mix of pollutants (see concentrations in Table S1), 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 animals exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants. n=20 (“C” group); n=20 (“Mix” group); n=15 (“PSPD” group), and n=18 (“Mix+PSPD” group).

We also evidenced suppression of antioxidant activity in exposed larvae, especially to SARS-CoV-2-derived peptides (alone or in combination with mixed pollutants) (“PSPD” and “Mix+PSPD” groups). SOD and CAT activity, DPPH radical scavenging activity in these groups, and total thiol levels were statistically significantly lower than in the “control” group (Figure 3A-D, respectively). On the other hand, surprisingly, MDA levels in larvae exposed to treatments were significantly lower than in unexposed larvae (Figure 4A). On average, the MDA levels in the “Mix”, “PSPD” and “Mix+PSPD” groups were 58.5% lower compared to the “control” group.
Figure 4B demonstrates a significant increase in nitrite levels in larvae exposed to SARS-CoV-2-derived peptides (alone and in combination with the pollutant mix). On average, the increase in NO in the “PSPD” and “Mix+PSPD” groups was 480% higher than in the “control” group. However, AChE activity in these groups was significantly reduced compared to unexposed larvae (Figure 4C). In addition, our data showed that exposure to treatments induced changes in the content of molecules important in energy metabolism in animals. C. dipterum larvae exposed to the pollutant mix and SARS-CoV-2-derived peptides (alone or in combination) showed a significant reduction in total carbohydrate and triglyceride levels (Figure 5A-B, respectively) compared to unexposed larvae. On the other hand, total protein levels were increased only in the “Mix+PSPD” group (Figure 5C).

Figure 3. (A) Superoxide dismutase activity, (B) catalase activity, (C) DPPH radical scavenging activity, and (D) total thiol levels in Cloeon dipterum larvae (mayfly) exposed or unexposed to different treatments. Parametric data are presented by the mean $\pm$ standard deviation, whereas non-parametric are presented by the median and interquartile range. Summaries of statistical analyzes are displayed at the top of the graphs. Distinct lowercase letters indicate significant differences between the experimental groups. “C” refers to the control group, “Mix” refers to those exposed to the mix of pollutants (see concentrations in Table S1), 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 animals exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants. To evaluate biochemical
biomarkers, pools of five larvae/sample (8 samples/group) formed the analyzed samples, totaling 40 animals/group. All statistical comparisons were based on the averages of each replicate (i.e., n=8 replicates).

**Figure 4.** (A) Malondialdehyde, (B) nitrite levels, and (C) acetylcholinesterase activity in *Cloeon dipterum* larvae (mayfly) 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 statistical analyzes are displayed at the top of the graphs. Distinct lowercase letters indicate significant differences between the experimental groups. “C” refers to the control group, “Mix” refers to those exposed to the mix of pollutants (see concentrations in Table S1), 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 animals exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants. To evaluate biochemical biomarkers, pools of five larvae/sample (8 samples/group) formed the analyzed samples, totaling 40 animals/group. All statistical comparisons were based on the averages of each replicate (i.e., n=8 replicates).
Figure 5. (A) Total carbohydrates, (B) triglycerides, and (C) total proteins in *Cloeon dipterum* larvae (mayfly) 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 statistical analyses are displayed at the top of the graphs. Distinct lowercase letters indicate significant differences between the experimental groups. “C” refers to the control group, “Mix” refers to those exposed to the mix of pollutants (see concentrations in Table S1), and “1STP” 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 animals exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants. To evaluate biochemical biomarkers, pools of five larvae/sample (8 samples/group) formed the analyzed samples, totaling 40 animals/group. All statistical comparisons were based on the averages of each replicate (i.e., n=8 replicates).

We also showed that the SARS-CoV-2-derived peptides or the pollutant mix did not change the oxidation-reduction potential (68.49 mV ± 6.00 mV), pH (8.13 ± 0.1), temperature (21, 92 °C ± 0.54°C), and dissolved oxygen (8.53 mg/L ± 0.24 mg/L) of the exposure waters of the respective experimental groups (Figure S1). On the other hand, we observed that the pollutant mix significantly increased the electrical conductivity, total dissolved solids, and salinity (Figure 6A-C, respectively), as well as reduced the water resistivity of the “Mix” and “Mix+PSPD” groups’ exposure waters. (Figure 6D). However, our statistical analyzes showed...
no significant correlation (or weak correlations) between most of the evaluated biomarkers and the altered physicochemical parameters in the exposure waters of these groups (Figure S2).

![Figure 6](image)

**Figure 6.** (A) Electric conductivity, (B) total dissolved solids, (C) salinity, and (D) resistivity of the exposure waters of the different experimental groups. Parametric data are presented by the mean ± standard deviation, whereas non-parametric are presented by the median and interquartile range. Summaries of statistical analyzes are displayed at the top of the graphs. Distinct lowercase letters indicate significant differences between the experimental groups. “C” refers to the control group, “Mix” refers to those exposed to the mix of pollutants (see concentrations in Table S1), 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 animals exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants.

Considering the set of responses of the *C. dipterum* larvae when exposed to SARS-CoV-2-derived peptides and a mix of pollutants (alone or in combination), the results obtained were applied to the IBRv2. In Figure 7, it is possible to notice a high similarity between the IBRv2 values and star graph (polygon) obtained for the “PSPD” and “Mix+PSPD” groups. Regarding PCA, we observed that the first two principal components (PC1 and PC2) cumulatively explained 92.09% of the total variation (Figure 8A), whose eigenvalues for PC1 and PC2 were superior à 2.5 (Figure 8B). The loadings plot (Figure 8C) and Table 1 demonstrate that most biomarkers were negatively associated with PC1 and PC2. Furthermore, we observed that the
Experimental groups were clearly separated by PC1, with “PSPD” and “Mix+PSPD” groups positioned in positive quadrants (PC score: 1.977 and 2.382, respectively) and opposite to the “control” group (PS score: -4.332). The “Mix” group showed intermediate positioning in PC1 (PC score: -0.026) (Figure 10C-D). Therefore, these data confirm the similarity between the responses of the “PSPD” and “Mix+PSPD” groups.

Figure 7. (A) Integrated biomarker responses index (IBRv2) values and (B-D) star graph (polygon) obtained with the IBRv2 method for the (B) “Mix”, (C) “PSPD”, and (D) “Mix+PSPD” groups. Total protein (TP), total carbohydrates (TC), triglycerides (TRI), superoxide dismutase (SOD), catalase (CAT), DPPH radical scavenging activity (DPPH), acetylcholinesterase activity (AChE), body biomass (BB), malondialdehyde (MDA), development stages (DS), nitrite (NO), total thiols (TT), and body condition index (BCI). “C” refers to the control group, “Mix” refers to those exposed to the mix of pollutants (see concentrations in Table S1), 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 animals exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants.
Figure 8. (A) proportion of variance plot (PC1, PC2, and PC3), (B) scree plot (eigenvalue), (C) loadings plot of the investigated variables, (D) PC score plot, and (E) PCA biplot of the first two principal components that simultaneously shows scores of experimental groups (gray points) and loadings of explanatory variables (vectors – arrows). Total protein (TP), total carbohydrates (TC), triglycerides (TRI), superoxide dismutase (SOD), catalase (CAT), DPPH radical scavenging activity (DPPH), acetylcholinesterase activity (AChE), body biomass (BB), malondialdehyde (MDA), development stages (DS), nitrite (NO), total thiols (TT), and body condition index (BCI). “C” refers to the control group, “Mix” refers to those exposed to the mix of pollutants (see concentrations in Table S1), 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 animals exposed to SARS-CoV-2-derived peptides in association with the mix of pollutants.
Table 1. Rotated loading (coefficient) matrix provided by the multivariate analysis to define factors or principal components PC1 and PC2.

| Biomarkers                        | Abbreviation | Principal components |          |          |
|-----------------------------------|--------------|----------------------|----------|----------|
|                                   |              | PC1                  | PC2      |          |
| Total protein                     | TP           | 0.879                | -0.415   |          |
| Total carbohydrates               | TC           | -0.865               | -0.145   |          |
| Triglycerides                     | TRI          | -0.997               | -0.059   |          |
| Superoxide dismutase              | SOD          | -0.833               | 0.535    |          |
| Catalase                          | CAT          | -0.730               | -0.681   |          |
| DPPH radical scavenging activity  | DPPH         | -0.973               | 0.163    |          |
| Acetylcholinesterase              | AChE         | -0.742               | 0.669    |          |
| Body biomass                      | BB           | 0.840                | 0.124    |          |
| Malondialdehyde                   | MDA          | -0.932               | -0.264   |          |
| Development stages                | DS           | 0.757                | 0.623    |          |
| Nitrite                           | NO           | 0.929                | -0.330   |          |
| Total thiols                      | TT           | -0.981               | 0.082    |          |
| Body condition index              | BCI          | -0.488               | -0.715   |          |

Variables with a high loading coefficient are highlighted in bold.

4. DISCUSSION

It is a consensus that the early identification of impacts caused by compounds, molecules, or substances dispersed in aquatic environments on organisms can favor the proposition of mitigation remediation measures, in addition to contributing to the conservation of species. Thus, using *C. dipterum* larvae as an experimental model, we demonstrated that the dispersion of SARS-CoV-2-derived peptides in freshwater ecosystems constitutes an imminent threat to aquatic organisms. Although we did not observe a synergistic or additive effect of the exposure of the larvae to the viral fragments in combination with the pollutant mix, the IBRv2 value observed in the “PSPD” group demonstrates that the ecotoxicological effects induced by the SARS-CoV-2-derived peptides were superior to those caused by a combination of emerging pollutants (“Mix” group). Therefore, our data reinforce recent studies that show the impacts of the COVID-19 pandemic on non-target organisms of SARS-CoV-2 infection (Charlie-Silva & Malafaia, 2022) and, mainly, demonstrate that the effects of exposure to viral fragments are not restricted to fish (Malafaia et al., 2021; Gonçalves et al., 2021; Kraus et al., 2022; Fernandes et al., 2022), amphibians (Charlie-Silva et al., 2021) or mammals (Luz et al., 2022).
Particularly in our study, we noticed that the suppression of the antioxidant system, the induction of nitrosative stress, the reduction of the content of molecules important in energy metabolism, and AChE activity constituted the main mechanisms of action of the SARS-CoV-2-derived peptides (alone or in combination with the pollutant mix) on *C. dipterum* larvae. In Figure 3, we can see that the equitable suppression of SOD, CAT, and DPPH radical scavenging activities and the content of total thiols in the “PSPD” and “Mix+PSPD” groups suggest that the SARS-CoV-2-derived peptides negatively interfered on the enzymatic and non-enzymatic antioxidant system of the experimental model. Such results are intriguing, as they show adverse effects in an animal model (*C. dipterum*) tolerant to different adverse conditions in its habitat (e.g., Cianciara, 1979; Lee et al., 2013), but also because they differ from previous reports in which other organisms were exposed to the viral peptides tested in our study. Contrary to what we observed, Charlie-Silva et al. (2021), Mendonça-Gomes et al. (2021), and Malafaià et al. (2022) showed increased antioxidant activity (inferred by SOD and CAT activity) when *Physalaemus cuvieri* tadpole, *Culex quinquefasciatus* larvae, and juvenile guppy (*Poecilia reticulata*), respectively, were exposed to PSPD-2002 and PSPD-2003 peptides. In these studies, in particular, such increases were associated with an adaptive response against increased production of reactive species (ROS), as well as a possible interaction between the SARS-CoV-2-derived peptides and antioxidant enzymes evaluated (confirmed by molecular docking).

On the one hand, we acknowledge that the physiological differences of the animals evaluated, associated with the different concentrations of viral peptides and exposure periods in these studies, are factors that may explain that divergence with our research; however, the induction of a redox imbalance appears to be a common mechanism of SARS-CoV-2-derived peptides on these models, either because of the inefficiency of increased SOD and CAT activity in controlling ROS production (observed in the studies mentioned above) or by the suppression of antioxidant activity, as demonstrated in our research. In this case, it is tempting to speculate that this suppression is also related to the interactions of viral peptides with the enzymatic and non-enzymatic components of *C. dipterum* larvae, as suggested by Luz et al. (2022) and Gonçalves et al. (2022) when evaluating the effect of exposure of Swiss mice and juvenile guppy to PSPD-2002, respectively.

On the other hand, the nitrosative stress observed in the “PSPD” and “Mix+PSPD” groups - similar to that observed in *P. cuvieri* tadpoles exposed to PSPD-2002 and PSPD-2003 (Charlie-Silva et al., 2021) - suggests participation influence of the increase in NO production (Figure 4B) on the animals' response. It is known that when produced at low/moderate levels, NO participates in signaling events that regulate a series of physiological processes, such as the
maintenance of vascular tone, the control of ventilation and erythropoietin production, and signal transduction from membrane receptors in different processes that regulate the response of cells to pro-inflammatory stimuli (Patel et al., 2000; Bruckdorfer, 2005; Khazan & Hedayati, 2015; Ghimire et al., 2017). However, at high concentrations, NO reacts with ROS producing reactive nitrogen species (RNS) that are known to have harmful implications for biological systems. As discussed by Paakkari & Lindsberg (1995) and Lee et al. (2016), there is strong evidence indicating that NO itself serves as a cytotoxic 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. Therefore, it is possible that the generation of peroxynitrite in C. dipterum larvae affected essential macromolecules and, consequently, contributed to the impairment of antioxidant defenses, which would explain the low activity of SOD and CAT, as well as the reduction of DPPH radical scavenging activity, and the total thiols levels observed in the larvae of the “PSPD” and “Mix+PSPD” groups (Figure 3). This hypothesis is supported especially by the studies by Asahi et al. (1995) and Dobashi et al. (1997), involving the inactivation of glutathione peroxidase by nitric oxide and the modulation of endogenous antioxidant enzymes by nitric oxide in Rat C6 Glial cells, respectively. Furthermore, the increase in energy demand to reestablish redox homeostasis may be the cause of the reduced levels of triglycerides and total carbohydrates (Figure 5) and the lower indices of body condition observed in C. dipterum larvae of “PSPD” and “Mix+PSPD” groups (Figure 2C). As discussed by Arrese & Soulages (2010), the pre-metamorphic phase of insects consists of a phase of high energy demand. Therefore, the reallocation of energy to maintain physiological homeostasis can compromise the growth and development of animals.

On the other hand, it is possible that the nitrosative stress observed in larvae exposed to SARS-CoV-2-derived peptides (alone or in combination with the mix of pollutants) plays a role in the reduction of MDA levels (Figure 4A), as corroborated by Rubbo et al. (2000). At the time, these authors demonstrated that NO also serves as a more potent inhibitor of lipid peroxidation propagation reactions than α-tocopherol (αTH) and protects αTH from oxidation. Thus, the induction of high NO production by SARS-CoV-2-derived peptides might have played a paradoxical role in C. dipterum larvae, which requires further research focused on viral peptides and their role in regulating membrane and lipoprotein lipid oxidation reactions.

Intriguing data also refers to the reduction of AChE activity in the larvae of the “PSPD” and “Mix-PSPD” groups, suggesting a divergent anticholinesterase effect from that cholinesterase stimulation observed in P. cuvieri tadpole (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. In those studies, the authors suggest that the increase in AChE activity is related to a compensatory mechanism in response to the catalytic deficit induced by the peptides or to a more efficient response of the AChE to the increase in the release of ACh in the synaptic clefts via activation of the cholinergic anti-inflammatory pathway. However, in *C. dipiterum* larvae exposed to SARS-CoV-2-derived peptides (alone or in combination with the pollutant mix), such hypotheses do not seem to be valid, and this could be due to not only the physiological differences between the evaluated models but also the locations (organs/tissues) where AChE activity was measured, as well as concentrations and exposure periods to viral peptides. In this sense, future investigations will be helpful to understand better the mechanisms that led to the anticholinesterase effect observed in our study. It is questioned whether the reduction in AChE activity in the larvae would be related to alterations in the association and catalysis mechanisms or the reduction of substrate affinity for the enzyme’s active site induced by SARS-CoV-2-derived peptides. In parallel, studies on the influence of viral peptides on the cholinergic anti-inflammatory pathway will be essential to clarify whether the reduction in AChE activity is related to the decrease in acetylcholine in the synaptic clefts or the downregulation of the AChE gene by SARS-CoV-2-derived peptides.

Finally, 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. Therefore, several issues still need to be explored. The fact that the effects observed in our study were not more intense in *C. dipiterum* larvae exposed to viral fragments in association with the pollutant mix (see IBRv2 and PCA – Figure 7 and 8, respectively) may reflect the tolerance of these organisms to the concentrations of pollutants in the mixture and to the exposure time evaluated (six days). Therefore, it is possible that longer exposures to treatments induce differentiated effects or that other organisms are more susceptible and/or sensitive to the impact of SARS-CoV-2-derived peptides (alone or in combination with the pollutant mix). On the other hand, evaluating other toxicity biomarkers (e.g., histopathological, molecular, genetic, etc.) in *C. dipiterum* larvae may indicate alterations that have not been observed in our study.

5. CONCLUSIONS

From the results obtained in our study, we conclude that the exposure of *C. dipiterum* larvae to SARS-CoV-2-derived peptides (alone or in combination with a mix of pollutants) induces changes in body condition, changes suggestive of redox imbalance and negative effect
on the cholinesterase system of these animals. However, different from what we assumed, we did not evidence a synergistic or additive action between the viral peptides and the pollutants that composed the mix, considering the set of biomarkers evaluated in this study. In any case, it is crucial to consider that our research is not exhaustive and that the continuity of investigations in this area is essential for understanding the real impact of SARS-CoV-2-derived peptides on aquatic organisms, both when exposed to these peptides alone and in contexts where the novel coronavirus fragments coexist with various environmental pollutants.

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7. DECLARATION OF COMPETING INTEREST

We confirm 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 our manuscript. Due care has been taken to ensure the integrity of the work.

8. ETHICAL ASPECTS

All experimental procedures were performed according to the ethical standards for animal experimentation, and meticulous efforts were made to ensure that the animals suffered as little as possible and 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. AUTHORS' CONTRIBUTIONS
Ítalo Nascimento Freitas: designed and performed experiments, analyzed data, and co-wrote the paper. Amanda Vieira Dourado: performed experiments. Stênio Gonçalves da Silva Matos: performed experiments. Sindoal Silva de Souza: performed experiments. Thiaren Marinho da Luz: performed experiments. Abraão Tiago Batista Guimarães: performed experiments. Aline Sueli de Lima Rodrigues: revised the article critically for important intellectual content. Nabisab Mujawar Mubarak: revised the article critically for important intellectual content. Md. Mostafizur Rahman: revised the article critically for important intellectual content. Andrés Hugo Arias: revised the article critically for important intellectual content. Guilherme Malafaia: designed and performed experiments, analyzed data, co-wrote the paper, supervised the research, and provided funding acquisition, project administration, and resources.

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SHORT-TERM EXPOSURE OF THE MAYFLY (*Cloeon dipterum*, EPHEMEROPTERA: BAETIDAE) TO SARS-COV-2-DERIVED PEPTIDES AND OTHER EMERGING POLLUTANTS: A NEW THREAT FOR THE AQUATIC ENVIRONMENTS

GRAPHICAL ABSTRACT

For this I did not expect!!! Our lifespan is only getting shorter...

Mix of emerging pollutants

SARS-CoV-2-derived peptides

Mayfly larvae

*Cloeon dipterum* (Ephemeroptera)

$\downarrow$ antioxidant defense, $\uparrow$ mortality rate, $\downarrow$ CO activity, $\uparrow$ nitrosative stress and $\downarrow$ body condition index
SHORT-TERM EXPOSURE OF THE MAYFLY (*Cloeon dipterum*, EPHEMEROPTERA: BAETIDAE) TO SARS-COV-2-DERIVED PEPTIDES AND OTHER EMERGING POLLUTANTS: A NEW THREAT FOR THE AQUATIC ENVIRONMENTS

HIGHLIGHTS

- First-time assessment of SARS-CoV-2-derived peptides toxicity, alone or in combination with pollutant mix
- PSPD (alone or in combination with pollutant mix) induces an antioxidant deficit
- Mayfly larvae exposed to SARS-CoV-2-derived peptides exhibit nitrosative stress
- SARS-CoV-2-derived peptides (alone or in combination with pollutant mix) induce anticholinesterase effect in mayfly larvae
- Exposure to PSPD poses an emerging threat to the aquatic environment biota