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Original Article

Time-dependent Biochemical Responses of the Zoanthid *Zoanthus* sp. Exposed to Polyvinyl Chloride Microplastics

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Abstract

The present study investigates if the polyvinyl chloride microplastics (79-149 µm) could cause any time-dependent responses of the microplastics absorption and biochemical responses (GST, TBARs, DNA strand breaks) for *Zoanthus* sp. during and acute (7 days) and chronic (14 days) exposures. For the role experiments mini colonies of *Zoanthus* sp. were exposed to 10 mg/L of PVC microplastics for 7 and 14 days. Exposure time had a direct influence under the PVC microplastics absorption, what consequently contributed for alterations on GST activity, TBARS concentration and DNA damage. These results provide evidence of biochemical damages caused by microplastics in corals, and insights for future perspectives of coral reefs health once plastic debris have been recorded at the ocean for at least 40 years.

Keywords: DNA Strand Break; GST activity; *Hexacorallia Zoantharia*; TBARS

INTRODUCTION

Since the beginning of the 1970s plastic waste has been reported in the ocean (Colton *et al.* 1974, Carpenter & Smith 1972). As a cheap, lightweight, and durable product these synthetic polymers have been piling up in the oceans' waters, sediment, and organisms. In 2018, global annual plastic production reached 359 million metric tons (PlasticEurope, 2019). Currently, plastics can be found from the polar seas (Lusher *et al*. 2015) to the deep oceanic sediment (Woodall *et al.* 2014). Between the main kinds of plastics consumed worldwide are, polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyethylene terephthalate (PET) and polyurethane (PU) (Bond *et al.* 2018).

Once in the sea, plastic debris slowly breaks down, generating smaller particles through physical, chemical and biological processes. Particles up to 5 mm in diameter are called microplastics, or MP (Wagner & Lambert 2018). MP ingestion has been confirmed by all sorts of different taxonomic groups such as seabirds (van Franeker *et al.* 2011), mussels (Li *et al.* 2021), fish (Zhu *et al.* 2019), and cnidarians (Albano *et al*. 2021, Devereux *et al.* 2021), possibly due to accidental ingestion (Andrady 2011). MP have been proved to serve as carriers for various associated organic pollutants like polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), heavy metals such as lead, cadmium, zinc, and

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nickel (Rochman *et al.* 2014), as well as colonized microbial communities called "Plastiphere"(Amaral-Zettler *et al.* 2020, Wang *et al.* 2021), from the environment becoming even more toxic and bioaccumulative when ingested by organisms (Eerkes-Medrano *et al.* 2015, Paul-Pont *et al.* 2016). When internalized, MP can cause mechanical problems, decrease fecundity, immunity, retard growth, cause oxidative damage, induce the development of pathologies, and even induce death in aquatic organisms (de Sá *et al*. 2018, Jeyavani *et al.* 2021).

Plastic pollution can be considered an emerging threat to coral reefs due to their complex interactions (Hung *et al*. 2022). Coral reefs are crucial ecosystems for continued marine biodiversity, providing habitats to a variety of organisms through their calcification process and hosting a vast ecosystem. 275 million coastal people benefit from the fishing stock and coastal protection areas adjacent to these areas (Moberg & Folke 1999, Wild *et al*. 2011, Spalding & Brown 2015). Coral reefs are also one of the most threatened ecosystems of the last decades, being exposed to stressors like climate changes, marine debris, ocean acidification, pathogens diseases, and microplastics (Hughes *et al.* 2018).

MP in coral reefs is mainly composed of fibers, pellets, fragments, films, and granules, of a variety of sizes and colors (Huang *et al.* 2021). A study conducted by Cheang *et al.* (2018) on coral seabed sediments of four coral reef islands in the South China Sea showed that the concentration of MP ranged from 171.7 ± 57.6 to 223 ± 51.4 items/kg. Huang e*t al.* (2019) investigated the abundance of MP in the Zhubi Reef surface seawater and results ranged from 1400 to 8100 items/m^3. In the surface water of the Nanxum Reef, Nie *et al.* (2019) recorded a concentration of 1250–3200 items/m^3 MP. In the Great Barrier Reef, the presence of MP was detected in 57 sampling sites from the waters around Australia with an average concentration of 4256.46 ± 757.79 items/km^2 (Reisser *et al*. 2013).

Lamb *et al.* (2018) showed that in 124,000 reef-building corals from 159 reefs in the Asian-Pacific region the likelihood of disease increases from 4% to 89% when the corals were in contact with plastic. This way, plastic debris such as MP become a physical barrier in the acquisition of food and cause alterations to photochemical efficiency, oxidative stress, and physiology (e.g., lipid peroxidation, antioxidant defense) (Rocha *et al.* 2020, Chen *et al*. 2022, Tang *et al.* 2018, Jiang *et al*. 2021). Tissue necrosis and bleaching responses which demand more energy consumption from the coral have also been noted (Reichert *et al.* 2018, Syakti *et al*. 2019). These consequences have been shown to be species-specific for corals (Tang *et al.* 2018, Chen *et al.* 2022). Moreover, Li *et al*. (2022), demonstrates through a meta-analysis, that MP can induce time-dependent oxidative stress responses in invertebrates, such as bivalves. Besides the existing literature about invertebrates, few studies regarding biological responses due to MP exposure have been carried out with respect to cnidaria phylum (Patra *et al.* 2022), emphasizing the growing need to better understand the toxic effects of environmentally relevant concentrations of MP for these organisms.

Interactions between *Hexacorallia zoantharia* and MP are understudied (Rocha *et al.* 2020), most of these studies focus on to Scleractinian corals (Syakti *et al.* 2019, Reichert *et al.* 2018, Tang *et al.* 2018). Marine cnidarian (Anthozoa: Hexacorallia: Zoantharia) belong to the third largest order of Hexacorallia and can be found all around the world, from temperate to tropical locations, from shallow reefs to deep seas of 500 m deep (Kumari *et al.* 2016, Rabelo *et al.* 2015). Just like other Hexacorallia, zoanthids host symbiont dinoflagellates (Symbiodiniaceae) inside their tissue, developing essential ecological roles for a reef function (Kumari *et al.* 2016, Santos *et al*. 2016). Zoanthids are also known for their phenotypic plasticity, adaptive capabilities, and resistance to extreme stressors, making them valuable scientific resources for studies about anthropic actions on corals (Leal *et al.* 2016, Rosa *et al.* 2016). Beyond that, *Zoanthus* sp. is an easily captive bred coral, which allows experimental research without any negative impact for coral reefs, which are already a threatened ecosystem.

Therefore, in the present work, we exposed *Zoanthus* sp. mini colonies to a 10 mg/L of PVC MP for 7 and 14 days. Then, the absorption of MP and their related biochemical damages were measured. The hypothesis of this study was that (I) the absorption of PVC MP by *Zoanthus* sp. could cause biochemical alterations such as DNA, proteins, lipids, and activity alterations to GST; and (II) chronic exposure to PVC MP would increase the absorption of the PVC particles by *Zoanthus* sp., consequently influencing the biomarkers responses.

MATERIAL AND METHODS

Test Species: culture conditions and fragmentation

The experimental and holding procedures were approved by the Universidade Santa Cecília, Santos, São Paulo ethics board (01/2022). A local coral seller donated the colonies of *Zoanthus* sp. ("Reef do Leandro", São Paulo, SP, Brazil). The colonies all came from the same tank system and were captive bred. Corals were kept in a tank (58.5 cm x 36.5 cm x 23 cm) for 2 weeks for acclimation before being fragmented. The tank was filled with 30 L of reconstituted seawater. For water flow, two Sarlo Better1000® (1000 L/h) pumps were used on opposite sides of the tank. Monitored parameters were temperature (26), salinity (33), pH (8.2), lightning intensity (70 ± 90 μmol quanta/m^2/s), and photoperiod (10:14).

After the acclimation period, colonies were fragmented into 72 mini colonies with a minimum of 4 polyps each. Fragmented polyps were put into an iodine prophylactic bath with Reef Dip Seachem® (2 ml/L) to help healing and avoid possible infections. Polyps were affixed (Super Bonder® cyanoacrylate) to a ceramic substrate (4 cm x 4 cm). After, mini colonies went through a healing period of three weeks in the acclimation tank. During this time, corals were carefully monitored for any sign of diseases or infection.

Experimental setup

Until now, MP concentration in ocean water has not been accurately reported, therefore, we compared MP exposure concentrations documented by coral-related references presented at (Jiang *et al.* 2021) and chose a relatively low exposure concentration (10 mg/l) and sizes ranging from 79-149 µm (Figure 1). Virgin PVC MP and the polymer composition was confirmed via RAMAN spectroscopy (Figure 2) (Lenz *et al.* 2015, Marulanda *et al*. 2014).

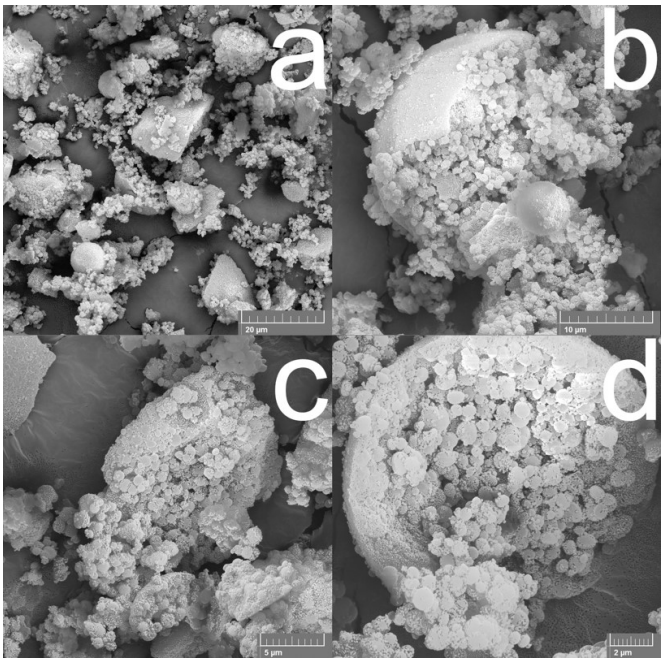

Figure 1. Microphotographs taken with a scanning electron microscope (SEM) of the microplastic used in this study. a) magnification of 2.6 kx; b) magnification of 5.8 kx; c) magnification of 8 kx, View d) magnification of 15.0 Kx.

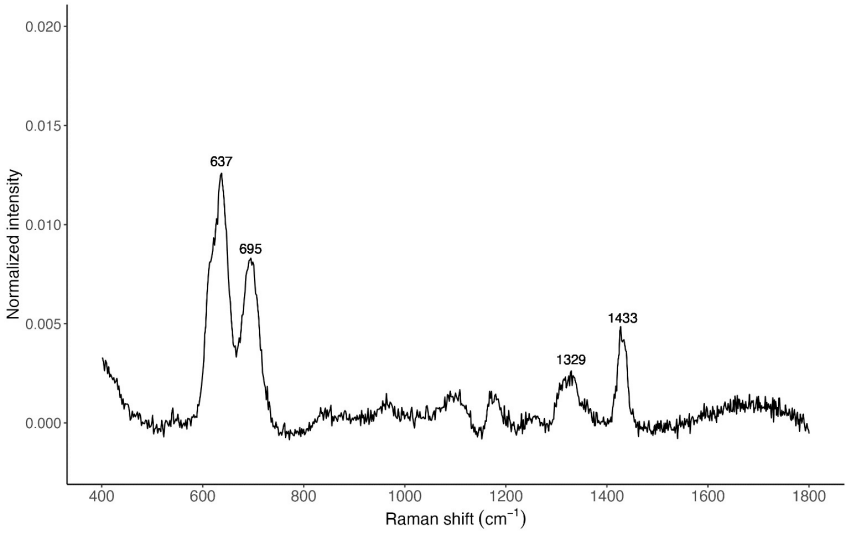

Figure 2. Raman spectra of the virgin PVC (polyvinyl chloride).

Samples were placed in the experimental tanks (six glass aquaria with 7 L each of reconstituted seawater: Mg^{2+}: 1.250-1.340 mg/L; Ca^{2+}: 410 – 440 mg/L; K^{+}: 380 – 400 mg/L; Na^{+}: 10.300 – 10.700 mg/L; Cl^{-}: 19.100 – 19.800 mg/L; Alkalinity: 8.0 – 8.5; Strontium: 7.0 – 9.0 mg/L) and one Maxspect Bio Sphere® each for biological filtration. Glass aquaria were put into a water bath, with an AQUAEL platinum heater® (100 w). In two plastic tanks to facilitate water temperature control. Water flow (400 L/h) and the water parameters were constantly monitored and kept the same as in the acclimation tank. Salinity (33 ± 0.6) and temperature (26 ± 0.4ºC) were measured every day and pH once every two days (8.2 ± 0.2).

Each experimental group consisted of six mini colonies. Controls for time 1 (7 days) and 2 (14 days) and treatments for time 1 (7 days) and 2 (14 days) were placed in the same tanks, in triplicates (3 tanks for controls, with 12 mini colonies; and 3 tanks for treatments, with 12 mini colonies). After 7 (acute) and 14 (chronic) days of exposure, mini colonies from Control1 and MP1 Control2 and MP2, respectively, were removed and frozen in ultrafreezer (-80ºC).

MP and coral food Coral Sprint Fauna Marin® were added at the same concentration (10 mg/L). Food was provided to stimulate coral heterotrophic behavior. Every 72 h, 100% of tank water was exchanged. During water changes, mini colonies were placed into 1 L containers with the same tank water, then the mini colonies were returned to the experimental tanks. These water exchanges were performed to attempt a constant water quality and keep the MP concentration during the exposure periods. The same protocol was followed for the controls, except for the MP insertion.

MP quantification

The method for MP extraction was performed according to previous studies (Karami *et al.* 2017, Nuelle *et al.* 2014, Prata *et al.* 2019, Zhao *et al.* 2017). To evaluate the particles of MP absorbed by *Zoanthus* sp., samples were separately placed in beakers with 20 ml of H_2O_2 (30%) each into a stove at 50°C until they were completely dissolved (24 h). After this, all the samples were vacuum filtered with inox filters of 70 µm. Filters were analyzed in a stereomicroscope and MP particles were counted. The number of MP was expressed as particles/mg fresh tissue.

GST, TBARS, DNA Strand Break and Total protein determination analysis

To measure GST activity, TBARS concentration, DNA damage, and total protein assays, the frozen coral samples were weighed, then homogenized on ice in 5 volumes of ice-cold phosphate buffer (100 mM KH_2PO_4, 5 mM disodium EDTA, pH 7.5 at 25ºC), using an ultrasonic homogenizer (Eco-Sonics® Ultronic QR300). Homogenates were centrifuged at 13000 rpm for 15 min at 4ºC and the supernatants were used for the TBARS, GST, and total protein assays. Aliquots for DNA damage were separated before centrifugation.

GST activity was determined as described by Keen et al. (1976) using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The change in absorbance was recorded at 340 nm in a spectrophotometer (Molecular Devices® SpectraMax M2e), and the enzyme activity was calculated as nmol CDNB conjugate/min/mg protein, using a molar extinction coefficient of 9.6 mM/cm.

Concentrations of TBARS was determined as described by (Kei 1978). This indicator of lipid peroxidation was measured through the reaction of malonaldehyde (MDA) and thiobarbituric acid (TBA) with the MDA-TBA adduct formation under high temperature (100°C) and acidic conditions. The MDA-TBA adduct formation was measured colorimetrically at 530 nm. The concentrations of TBARS were expressed as µmol TEP equivalents/mg protein.

For DNA Strand Break an alkaline precipitation technique, proposed by Olive (1998), was used to quantify the strands in the DNA. For the calibration curve, a salmon sperm (SIGMA 31149; CAS 100403-24-5) solution was prepared with a buffer solution (Trizma base-HCl (10 mM) and EDTA (1mM) diluted in 100 mL of Mili-Q water - pH=8.0). The reading was performed at 360 nm of excitation and 450 nm of emission. Results were expressed as µg DNA/mg protein.

Total protein content was measured according to Bradford (1976) at 595 nm and expressed as mg proteins using bovine albumin serum as standard.

Data Analysis

All data are reported as means ± SEM. Statistical significance was accepted at $p < 0.05$. To assess whether significant differences in MP particles/mg of fresh coral tissue between different exposure times occurred (MP1 and MP2), we conducted a Mann–Whitney U test. This choice was made because the residuals did not meet the assumptions of normality. To compare GST activity among the experimental groups, a Kruskal–Wallis test was carried out. Subsequently, we performed a Dunn's Multiple Comparison test with Bonferroni adjustments. The decision to use the Kruskal-Wallis test and the post-hoc Dunn's test was based on the violation of normality assumptions in the model residuals. To analyze the TBARS values between the experimental groups, we employed a one-way Analysis of Variance (ANOVA). Post-hoc comparisons were conducted using Tukey's HSD test. To determine if there were any statistical differences in MP particles per milligram, GST activity, TBARS, and DNA strand break, values between the two Control groups (C1 and C2), we conducted both a Student's t-test and a Mann–Whitney U test. No significant differences were found, leading us to consider them as a single group (C) for statistical analysis. All statistical analyses were performed using R (R Core Team, 2022).

RESULTS

All corals survived, and were no apparent differences (e.g., polyp extension, opening, coloration, or downsizing) were observed.

Microplastics concentration on *Zoanthus* sp. fresh tissue

For the chronic exposure (MP2) we found an average of 4.04 ± 0.5 MP particles/mg of fresh tissue, 3 times higher than the concentration found in MP1 (1.34 ± 0.18 MP particles/mg of fresh tissue), differing significantly (W = 23, $p < 0.01$). For the control, we found an average of 0.74 ± 0.1 MP particles/mg of fresh tissue, as shown in Figure 3. Presence of MP in the control can be justified due to external air contamination such as the air conditioner.

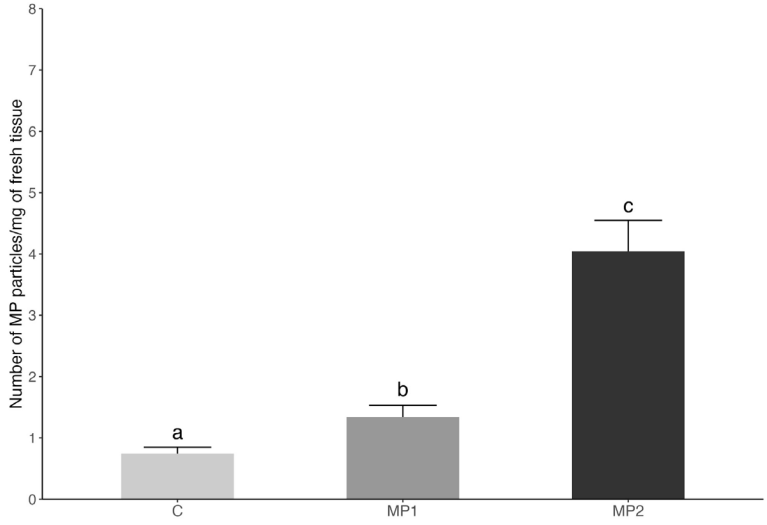

Figure 3. Number of microplastics (MP) particles absorbed by *Zoanthus spp*. after acute (MP1, 7 days) and chronic (MP2, 14 days) exposure to 10mg/L of polyvinyl chloride (PVC MP). Data are presented as mean ± standard error (N=36). Different letters denote significant differences between compared groups (Mann–Whitney, $p < 0.05$).

Glutationa S-transferase activity (GST)

Significant difference was detected between treatment groups (Chi-squared = 10.57 df = 2 $p < 0.01$). For GST activity, only chronic exposure (MP2) (56.46 ± 11.46 nmol/mg protein/min) had lower values in relation to the control group (97.23 ± 8.11 nmol/mg protein/min) ($p < 0.01$) (Figure 4).

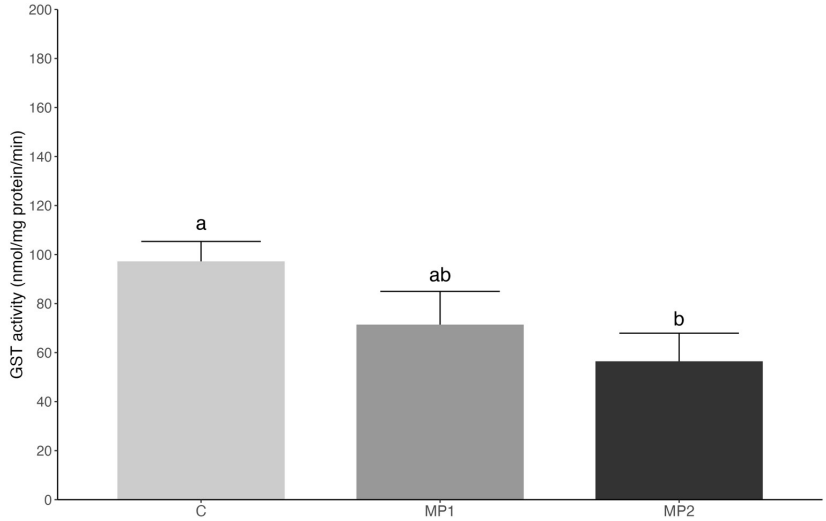

Figure 4. Effect of acute (MP1, 7 days) and chronic (MP2, 14 days) exposure to 10 mg/l of polyvinyl chloride (PVC MPs) and control (C), on Glutathione S-transferase activity of *Zoanthus spp*. (N=72). Data are presented as mean ± standard error. Different letters denote significant differences between compared groups. (Dunn post-hoc, $p < 0.05$).

Thiobarbituric acid reactive substances concentration (TBARS)

Significant difference was found between experimental groups ($F_{2,19}$ = 6.08, p < 0.01). The TBARS concentration was ~ 2 times higher for MP1 (5.99 ± 0.85 nmol TEP equivalents/mg protein) in relation to the control (3.35 ± 0.46 nmol TEP equivalents/mg protein) and in relation to MP2 (2.95 ± 0.61 nmol TEP equivalents/mg protein). TBARS concentrations was significantly different between C and MP1 (p = 0.01) as well as MP1 and MP2 (p = 0.02), although there was no significant difference between C and MP2 (p = 0.89) (Figure 5).

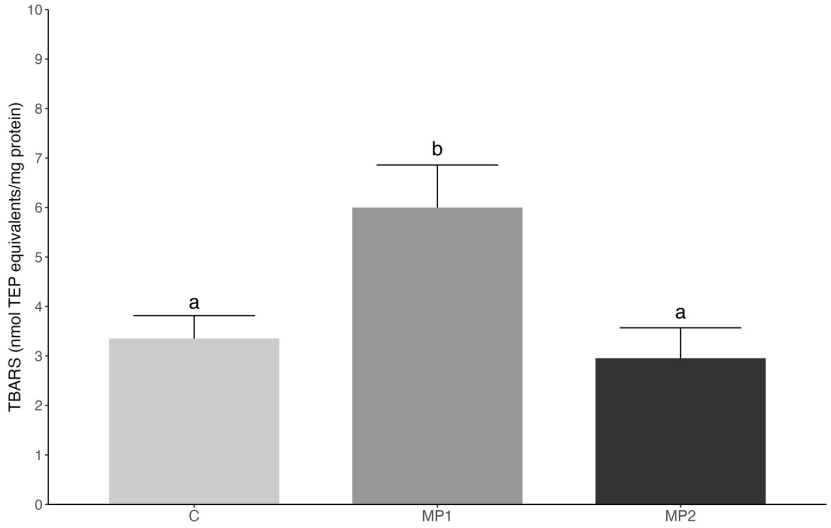

Figure 5. Effect of acute (MP1, 7 days) and chronic (MP2, 14 days) exposure to 10 mg/l of polyvinyl chloride (PVC MPs) and control (C), on Thiobarbituric acid reactive substances (TBARS) on *Zoanthus spp*. (N=24). Data are presented as mean ± standard error. Different letters denote significant differences between compared groups (Tuckey's HSD test, p < 0.05).

DNA strand break

For DNA damage, differences were only observed between C (326.30 ± 40.95 µg DNA/mg protein) and MP2 (1087 ± 355.40 µg DNA/mg protein) (p = 0.01) (Figure 6).

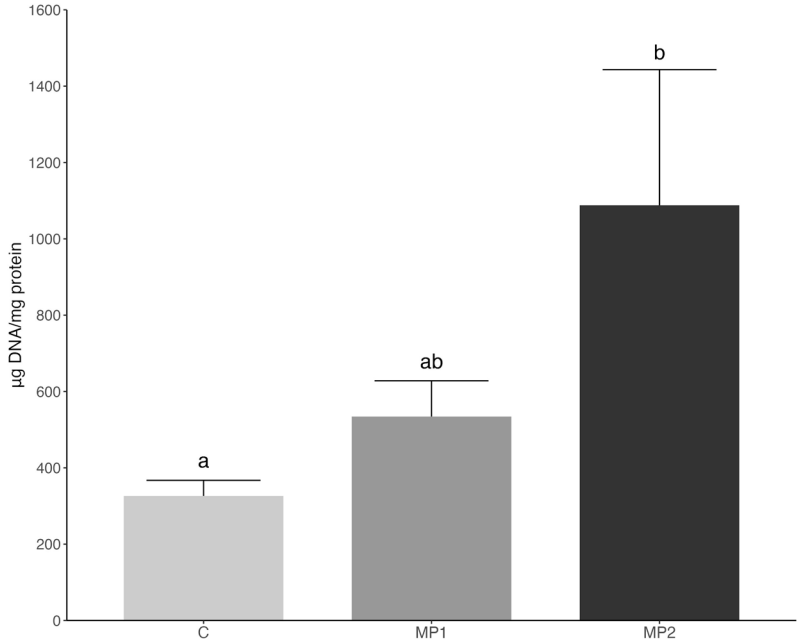

Figure 6. Effect of acute (MP1, 7 days) and chronic (MP2, 14 days) exposure to 10 mg/l of polyvinyl chloride (PVC MPs) and control (C), on DNA Strand Break on *Zoanthus spp*. (N=72). Data are presented as mean ± standard error. Different letters denote significant differences between compared groups (Tuckey's HSD test, p < 0.05).

DISCUSSION

In the present study, our hypothesis was supported. The chronic exposure increased the absorption of MP PVC in *Zoanthus* sp. and had a direct influence on the alterations of GST activity, TBARS concentration, and DNA damage.

The increases of the PVC MP particles per mg of fresh coral tissue in the chronic exposure demonstrates that the particles absorption is time-dependent for *Zoanthus* sp.highlighting how impactful MP contamination has already been to corals in the wild as plastics have been recorded in ocean water for decades (Colton *et al.* 1974, Carpenter & Smith 1972). The same way, Rocha *et al*. (2020) found that for *Zoanthus sociatus* exposed to 10 mg/L of PVC MP for 96 h, the number of particles adhered to coral epidermis increases in relation to low PVC MP concentration (1 mg/L) exposure. However, for *Acropora formosa* exposed for 2; 4; 7; 10; and 14 days to 0.15 mg/L of LDPE MP, ingestion reached its maximum at day 7 (Syakti *et al.* 2019), showing that for some coral species there may be a saturation point when it comes to MP absorption. The adhered and/or internalized MP have the potential to induce time-dependent interactions with the biological membranes in invertebrates (Li *et al.* 2022).

In general, MP are expected to be rubbery (Rochman *et al.* 2014, Eerkes-Medrano *et al.* 2015, Paul-Pont *et al.* 2016). The higher the rubber content of MP, the greater the interaction with biological membranes and absorption capacity for organic compounds (Zuo *et al.* 2019). This is mainly due to the disordered arrangement of molecular chain segments in the rubbery MP and the presence of a large amount of free volume between molecular chains, which facilitates the interactions (Guo *et al*. 2012). In fact, the MP PVC particles in the present study, present irregular surfaces (Figure 1). Such characteristics may be related to the biochemical alterations observed in the present study, as discussed above.

GST activity and lipidic damages (TBARs) only increased for *Zoanthus* sp. acutely exposed to MP PVC. This could be attributed to to the degradation of the GST enzyme during ROS neutralization generated during the chronic PVC exposure since no lipid peroxidation (TBARS) was observed at this condition. The GST activity and TBARs concentrations for corals can varies with the type of xenobiotic, concentration and time of exposure. For *Zoanthus sociatus*, Rocha *et al.* (2020) did not find any alterations in GST activity after 96 h of exposure at 1 and 10 mg/L of LDPE and PVC, but increase in TBARs concentrations was observed during the 96 h of exposure to 10 mg/L of PVC, which corroborates our results. On the other hand, *Pocillopora damicornis* had a significant decrease in GST after 24 h of exposure to 50 mg/L of polystyrene (PS) (Tang *et al.* 2018) which could be explained by a higher concentration of particles, a different kind of plastic used at the experiment, or even for being a different coral species. *Goniopora columna* exposed for 1; 3; 5 and 7 days to 10 mg/L of PE presented increasing GST activity from the 5th day of exposure (Chen *et al.* 2022). On the other hand, *Protopalythoa* sp. showed no difference on GST activity for 7 and 14 days of exposure to PVC at 50 mg/L (Jiang *et al.* 2021), but found higher levels of lipid peroxidation compared after chronic exposure (21 days), establishing how these effects tend to be dose and time dependents and species-specific.

DNA damage was only observed for *Zoanthus* sp. for chronic exposure. To our knowledge, this is the first study analyzing and identifying DNA damage in Hexacorallia exposed to MP. Similar to lipid peroxidation, the DNA damage is a result of the ROS action (Sahlmann *et al*. 2017). Intracellular production of free radicals may overcome antioxidant defenses, resulting in oxidative damage to macromolecules as evidenced by lipid peroxidation, DNA strand breaks, and alterations in critical cellular processes (Livingstone 2003). This oxidative damage may be time-dependent exacerbated if the stress overwhelmed. For mussel *Mytilus* sp. chronically exposed (10 days) to 10 and 100 ug/l of PE and PP MP, there was a significant increase in DNA damages after 10 days exposure (Revel *et al.* 2019). In the same way, *Mytilus galloprovincialis* only exhibited higher values of DNA damage after chronic exposure (26 days) when compared with acute exposure (7 days) and the control (González-Soto *et al.* 2019). This response was similar to *Zoanthus* sp. in the present study, where only chronic exposure (14 days) was able to increase the damage to DNA molecules. Therefore, it is possible that the higher number of MP particles that were incorporated in the chronic exposure caused tissue damage and affected the DNA molecules.

CONCLUSION

The study concludes that the absorption of the PVC MP by *Zoanthus* sp. Is time dependent. Acute exposure lead to lipid peroxidation, not observed for chronic exposure, while the DNA damage occurs only in the chronic exposure. This study demonstrates the harmful impact of short- and long-term exposures of *Zoanthus* sp. to PVC-MP which consequently affects the biochemical.

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AUTHORS CONTRIBUTIONS

LFBA*: conceptualization, methodology, formal analysis,

investigation, project administration and writing – original draft. GDP: investigation, formal analysis, data curation, validation and writing – review and editing. LRSB: validation, resources, and data curation. AO: funding acquisition, resources and data curation. JHAC: methodology, writing – original draft and formal analysis. HSH : conceptualization, methodology, validation, formal analysis, writing – review and editing, supervision, project administration.

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