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Physical and cellular impact of environmentally relevant microplastic exposure on thermally challenged *Pocillopora damicornis* (Cnidaria, Scleractinia)

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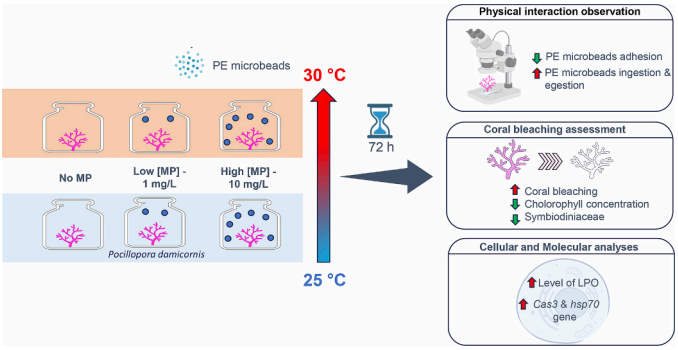
HIGHLIGHTS

- Corals are increasingly affected by microplastic (MP) pollution in addition to climate change.
- The interaction of heat stress and MPs was tested on *Pocillopora damicornis*.
- Corals ingested and egested MPs even at the lowest concentration tested.
- MPs exacerbated bleaching and cellular stress in thermally challenged corals.
- Management of MP is essential for coral reefs in the present climate change era.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastic pollution is an increasing threat to coral reefs, which are already strongly challenged by climate change-related heat stress. Although it is known that scleractinian corals can ingest microplastic, little is known about their egestion and how microplastic exposure may impair corals at physiological and cellular levels. In addition, the effects of microplastic pollution at current environmental concentration have been little investigated to date, particularly in corals already impacted by heat stress. In this study, the combined effects of these environmental threats on *Pocillopora damicornis* were investigated from a physical and cellular perspective. Colonies were exposed to three concentrations of polyethylene microplastic beads (no microplastic beads: [No MP], 1 mg/L: [Low MP]; 10 mg/L: [High MP]), and two different temperatures (25 °C and 30 °C) for 72 h. No visual signs of stress in corals, such as abnormal mucus production and polyp extroflection, were recorded. At [Low MP], beads adhered to colonies were ingested but were also egested. Moreover, thermally stressed colonies

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showed a lower adhesion and higher egestion of microplastic beads. Coral bleaching was observed with an increase in temperature and microplastic bead concentration, as indicated by a general decrease in chlorophyll concentration and Symbiodiniaceae density. An increase in lipid peroxidation was measured in colonies exposed to [Low MP] and [High MP] and an up-regulation of stress response gene *hsp70* was observed due to the synergistic interaction of both stressors. Overall, our findings showed that heat stress still represents the main threat to *P. damicornis*, while the effect of microplastics on coral health and physiology may be minor, especially at control temperature. However, microplastics could exacerbate the effect of thermal stress on cellular homeostasis, even at [Low MP]. While reducing ocean warming is critical for preserving coral reefs, effective management of emerging threats like microplastic pollution is equally essential.

1. Introduction

Corals are increasingly threatened by different anthropogenic stressors including pollution, coastal development, overfishing, ocean acidification, and rising sea temperatures (Hoegh-Guldberg et al., 2007, 2019; Hughes et al., 2018). These stressors are driving the global decline of coral reefs and impacting the ecosystem services that they provide to tens of millions of people worldwide (Moberg and Folke, 1999; Hollander et al., 2020). Among these anthropogenic stressors, marine heat waves caused by global warming are considered as the major threat to coral reefs (Leggat et al., 2019). In this context, model projections forecast that >75 % of coral reefs worldwide will be subjected to annual severe bleaching events due to thermal stress by 2070 (Van Hooidonk et al., 2016). In addition, the fate of coral reefs could be worsened by emerging stressors such as microplastic particles or fibers (<5 mm) (Ban et al., 2014) and nanoplastic (<150 nm) pollution (Huang et al., 2021; Isa et al., 2023). Microplastic (MP) reaches the marine environment mainly from land flow, but also from coastal tourism and ship transportation (Li et al., 2021). It can also derive from the fragmentation of larger plastic elements transported by currents and accumulated in certain areas of the ocean, and since the persistence in the environment ranges between 100 and 1.300 years (Turner et al., 2020), it is considered very harmful to marine organisms (Andrady, 2015). The interaction between corals and microplastics occurs through different mechanisms: ingestion (Allen et al., 2017; Axworthy and Padilla-Gamiño, 2019), egestion (Reichert et al., 2018), and surface adhesion (Martin et al., 2019). Microplastic ingestion has already been observed in scleractinian corals and alcyonaceans (Hall et al., 2015; Vencato et al., 2021). Recent studies suggested that microplastics negatively affect the health of corals. Indeed, exposure to high microplastic concentrations has been reported to adversely affect the growth rate and the physiological status of corals, changing the feeding behavior, decreasing the photosynthetic performance and skeletal calcification, leading to coral bleaching and tissue necrosis (Huang et al., 2021). At the cellular and molecular level, previous research demonstrated that high concentrations of polyethylene (PE) microplastic microbeads (200 μm in diameter) can cause cellular damage through oxidative stress in the soft coral *Coelogorgia palmosa* (Montalbetti et al., 2022). The temperate coral *Corallium rubrum* showed preferential ingestion of microplastic mixture (composed of 76.6 % polyethylene, 10.9 % polypropylene, 7.3 % polystyrene, 3.3 % polyvinylchloride and 1.8 % polyethylene terephthalate particles) compared to natural prey, leading to feeding impairment, mucus production, and altered expression of genes involved in key metabolic processes (Corinaldesi et al., 2021).

Moreover, corals are also capable of egesting microplastics, although this aspect has been less studied compared to ingestion (Alimba and Faggio, 2019). Microplastic egestion has been reported to occur within the first 24 h after ingestion, and the persistence time of microplastics within coral tissues is crucial to evaluate the effective severity of microplastic impact on coral physiology (Chen et al., 2022). Although the effect of microplastics on corals has increasingly been studied, few studies have examined the interactions of this emerging threat with other stressors. In this context, short-term co-exposure of corals to microplastics and pollutants, such as polyethylene terephthalate (PET) and polyvinylchloride (PVC), caused a decrease in chlorophyll content, an increase in oxidative stress, and a decrease in enzymes activity (such as pyruvate kinase, Na-K-ATP) in corals *Tubastrea aurea* and *Acropora* sp. (Liao et al., 2021; Xiao et al., 2021). On the other hand, Reichert et al. (2021) found that PE microplastic fibers (100–300 μm in length) and heat stress had minor cumulative effects on some coral species (*Acropora muricata*, *Montipora digitata*, *Porites lutea*, *Pocillopora verrucosa*, and *Stylophora pistillata*) suggesting species-specific effects of microplastics. Axworthy and Padilla-Gamiño (2019) also found that the combined effect of PE microbeads (150–180 μm in diameter) and heat varied between coral species depending on the affinity of different species to ingest microplastics (mouth size and microplastic size) and the differential response of corals to thermal stress. However, how the synergistic impact of microplastics and heat stress could affect the physiological and cellular processes and pathways of corals remains largely unexplored.

Microplastic concentrations used in previous laboratory studies were 10-fold higher than the concentration of microplastics occurring in marine environments (Hall et al., 2015; Jiang et al., 2020; Chen et al., 2022) and only a few studies have investigated the interaction between microplastics at current environmental concentrations on aquatic organisms (Corona et al., 2020). As argued by Lenz et al. (2016), microplastic concentrations used in laboratory stress experiments should reflect real concentrations currently found in the marine environment, to provide a plausible forecast of the actual effect of this contaminant on marine organisms.

For these reasons, in this study, we exposed the hard coral *Pocillopora damicornis* to PE microbeads, at an environmentally relevant concentration (200 microplastic beads/L corresponding to 1 mg/L of the microplastic beads used in this study) and at a high concentration (2000 particles/L or 10 mg/L), under two temperature conditions; control temperature (25 °C) and elevated temperature (30 °C). In particular, we hypothesized that microplastic contamination and heat stress could synergically affect coral health status and physiology, by enhancing coral bleaching, increasing the cellular oxidative damage, affecting the expression of genes involved in the stress response, and on coral physical and morphological aspects. To test this hypothesis, visual signs of stress such as abnormal mucus production, tissue necrosis, and coral polyp extroflection decrease were assessed (Vencato et al., 2021). Furthermore, the adhesion, ingestion, and egestion of microplastic beads were quantified to evaluate their physical interaction with *P. damicornis* under different conditions. Coral bleaching was analyzed by measuring the chlorophyll concentration and Symbiodiniaceae density. Lipid peroxidation (LPO) was measured to assess oxidative damage in coral tissues (Lesser, 2006; Montalbetti et al., 2021, 2022). Finally, since up to date the gene expression of thermally stressed corals combined with exposure to microplastic has been poorly investigated, the expression of genes commonly used as biomarkers of cellular stress in corals, such as the gene Caspase3 (*cas3*), a key effector enzyme in inducing cell apoptosis (Salvesen, 2002; Ghavami et al., 2009), and Heat shock protein 70 (*hsp70*), a molecular chaperone involved in maintaining cellular homeostasis during stress (Downs et al., 2000; Louis et al., 2017, 2020; Seveso et al., 2020), was assessed. Indeed, since the Indian and North Pacific Oceans are reported to have the largest amount of plastic and heat stress episodes in terms of frequency and intensity (Eriksen et al.,

2014), we consider it essential to understand how these two stressors interact to impact the health status of *P. damicornis*, which is widely distributed in the Indo-Pacific region, and represent a model species commonly used in climate change studies (Torda et al., 2013).

2. Materials and methods

At the Aquarium of Genoa, 72 nubbins (8–10 cm in length each) of *Pocillopora damicornis*, were sampled from six different mother colonies. The nubbins were promptly fixed on supports using epoxy resin. Subsequently, they were transferred into two 50 L experimental tanks for 2 weeks of acclimation at the control temperature of 25 °C and light:dark period of 11:13 h. The irradiation was equal to 250 PAR (μmol photons m^{-2} s^{-1}). Technical details of the aquarium setup are reported in Supplementary Material S1.

2.1. Experimental setup

After acclimation, each nubbin was transferred to an interaction chamber (a glass beaker filled with 1.5 L of filtered seawater). Inside each interaction chamber, an air pump was used to keep microplastic beads in constant motion and mimic water turbulence occurring at a natural reef (Martin et al., 2019). Chambers were allocated in water baths to maintain a constant temperature. Nubbins (n = 6) were randomly assigned to two different treatments consisting of two different concentrations of PE microbeads. Both treatments were carried out in two replicate tanks. In the first treatment, the concentration of PE microbeads was equal to 1 mg/L, corresponding to an environmentally relevant concentration of microplastics, and it was indicated as "Low [MP]". In the second treatment, the microplastic bead concentration inside each chamber was equal to 10 mg/L, corresponding to a high concentration of microplastics (indicated as "High [MP]") (Fig. S2). The microplastic bead concentration used in the present study is based on environmentally relevant concentrations already occurring in certain oceanic areas (Doyle et al., 2011; Cunningham and Sigwart, 2019; Opitz et al., 2021). The microplastic bead exposure experiment was performed at two different temperatures, 25 °C (control) and 30 °C (heat stress), for 72 h. The increase in temperature was gradual with a 0.5 °C increment per day from 25 °C to 30 °C (Axworthy and Padilla-Gamiño, 2019; Reichert et al., 2019). The heat stress temperature of 30 °C was selected as it generally represents a threshold temperature for coral bleaching (Johnston et al., 2019). PE microbead concentrations used in the present study were chosen based on recommendations of Lenz et al. (2016) to use environmentally relevant microplastic concentrations in laboratory experiments. The PE microbeads size range was 180–212 μm and the density was equal to 0.98 g/cm^3 (Cospheric LLC). PE has been chosen since it is one of the most common types of plastic present in the marine environment (Steinberg et al., 2020).

2.2. Visual signs of stress in corals

All the nubbins were visually monitored at the end of the 72 h of exposure. In particular, the presence of abnormal mucus produced by each fragment was assessed by analyzing the occurrence of mucus filaments streaming off the polyps' mouth as previously reported (Turner et al., 2020; Vencato et al., 2021). Moreover, the polyp extroflection was quantified in relation to the total surface of the coral (and reported in %), and the presence of necrotic tissue was noted. These parameters were monitored by visual inspection following Bejarano et al. (2022). More information on the visual assessment of corals is reported in Fig. S3.

2.3. Physical interaction between microplastic and corals

The physical interaction between PE microbeads and *P. damicornis* was evaluated by counting the number of microplastic beads that were adhered to the coral surface and ingested by the nubbins after 72 h of exposure. Subsequently, the number of egested microplastic beads was counted and reported in % in relation to the total number of previously ingested ones.

2.3.1. Microplastic adhesion

Nubbins (n = 12 per treatment) were quickly removed from their chambers and carefully rinsed with salt water, to collect the adhered PE microbeads inside a petri dish (as described by Martin et al., 2019). In addition, the number of PE microbeads adhered to the coral's external surfaces was also counted under a stereomicroscope (M125C, Leica). Microplastic beads were classified as "adhered" when they were found attached to the corals' external surface, outside of the polyps' mouth (Martin et al., 2019). The value of the adhered microplastics was then calculated as the sum of microplastic beads collected in the petri dish and those observed attached to the corals' external surface and were expressed as the average number of microbeads adhered per treatment.

2.3.2. Microplastic ingestion

For each treatment, 6 of the 12 nubbins were randomly chosen and used to evaluate microplastic beads ingestion. The number of PE microbeads inside the polyps' mouths was counted under the stereomicroscope and classified as ingested (Martin et al., 2019). Nubbins were then placed in a petri dish and dissolved in sodium hypochlorite for 2 h to allow the complete digestion of the coral tissue (Martin et al., 2019). Subsequently, the coral skeleton was inspected under the stereomicroscope to count all PE microbeads ingested. The value of the ingested microplastic beads was then calculated as the sum of microplastic beads found inside the polyps' mouths and those observed inside the skeleton corallites following the complete tissue dissolution of each *P. damicornis*. This value was then expressed as the average number of microbeads ingested per treatment.

2.3.3. Microplastic egestion

The remaining 6 nubbins per treatment were thoroughly rinsed with salt water, visually inspected, and then placed in new interaction chambers free of PE microbeads. The fragments were kept in the chambers for a further 24 h to measure the egestion of microplastic beads. Subsequently, the water within the interaction chamber was filtered with a nylon sieve (50 μm mesh size) to count all the PE microbeads egested by the nubbins. Finally, the fragments were dissolved in sodium hypochlorite, to count all the PE microbeads that were previously ingested to report the egestion data as % of microbeads egested compared to the total ingested.

2.4. Coral bleaching assessment and molecular analyses

2.4.1. Sampling of the coral fragments

Before processing the nubbins for the evaluation of the physical interaction between microplastic beads and corals, three small fragments (1–2 cm each) were sampled from six random nubbins per treatment at the end of the experiment. One fragment was kept at −20 °C to measure the coral bleaching status through the quantification of the chlorophyll (*a* and *c2*), and the density of Symbiodiniaceae present in the coral tissue. The second sample was immediately frozen in liquid nitrogen and then stored at −80 °C for the LPO analysis, while the third one was placed in a tube containing RNAlater© Stabilisation Solution (ThermoFisher Scientific), kept at 4 °C for 24 h and then stored at −80 °C until the analysis of the expression of two genes of interest (GOI), Caspase3 (*cas3*) and Heat shock protein 70 (*hsp70*), as cellular stress biomarkers.

2.4.2. Quantification of chlorophyll a and c2

A stream of compressed air was used to strip tissue off coral fragments in 5 mL of ice-cold phosphate buffer saline (Voolstra et al., 2020). The tissue slurry was centrifuged at 3600 ×*g* for 4 min and homogenized

using a syringe and needle. 1 mL of each sample was stored at −20 °C and fixed with 4 % formalin for Symbiodiniaceae counts (Ladrière et al., 2014). The supernatant was removed, and the remaining pellet was incubated in 100 % acetone for 24 h in the dark at 4 °C. Following extraction, the sample was centrifuged again at 3600 ×*g* for 4 min. The supernatant was used to determine concentrations of chlorophyll *a* and *c2* from the fluorescence measured at 630 nm, 663 nm, and 750 nm, and applied to dinoflagellate-specific equations (Jeffrey and Humphrey, 1975). Chlorophyll concentrations were normalized to the coral surface area. The remaining skeletons of coral fragments were soaked in 10 % bleach and left to dry for 48 h. The surface area of fragments was measured using the paraffin wax dipping method (Veal et al., 2010). The change in weight due to wax addition was compared against a standard curve of dipped clay cylinders of known surface area to calculate the skeletal surface area of each fragment.

2.4.3. Symbiodiniaceae density

Samples previously fixed in 4 % formalin were used to count Symbiodiniaceae cells from six independent hemocytometers (Improved Newbauer) counts, under an optical microscope (Leica Company, France). Cell density was calculated from the surface area of respective fragments (Ladrière et al., 2014).

2.4.4. Lipid peroxidation

The oxidative damage at the cellular level produced by microplastic bead exposure and heat stress was assessed by the quantification of lipid peroxidation (LPO). LPO was measured by quantifying the malondialdehyde (MDA) contents using an MDA assay kit (Bioxytech LPO-586, Oxis International, United States). Frozen coral samples (approximately 1 g each) were ground with a pre-chilled mortar and pestle and homogenized in 1 mL of 20 mM phosphate buffer, pH 7.4. Subsequently, 10 μL of 0.5 M butylated hydroxytoluene in acetonitrile was added to 1 mL of tissue homogenate. Following centrifugation (3000*g* at 4 °C for 10 min), an aliquot of the supernatant was used for protein determination using the Bradford assay. The subsequent assay procedure (hydrochloric acid solvent procedure) was performed according to the manufacturer's instructions. We measured the absorbance at 586 nm of the resulting supernatant (Gérard-Monnier et al., 1998). Results are presented in μmol of MDA per μg of proteins.

2.4.5. Gene expression analysis

RNA isolation was performed using the Qiagen RNA Mini kit (Qiagen) following the manufacturer's instructions with minor adjustments. Briefly, coral tissue was blasted off 0.3–0.5 g of coral fragments, in a pre-cooled mortar, using filtered compressed air for a maximum of 3 min (Voolstra et al., 2020). Tissues were immediately disrupted by grinding under liquid nitrogen. Without allowing the disrupted tissue to thaw, 600 μL of lysis buffer (RLT Buffer containing β-mercaptoethanol) was immediately added. The resulting tissue lysate was passed through a 20-gauge (0.9 mm) needle attached to a sterile plastic syringe until a homogeneous lysate was achieved. RNA extractions were then continued according to the manufacturer's instructions for the purification of total RNA from animal tissues. DNA contamination was removed using the DNase I Set (Zymo Research) in combination with the RNA Clean & Concentrator-25 kit (Zymo Research) according to the manufacturer's protocol. RNA quality was checked by examining with gel electrophoresis for clear, sharp bands of ribosomal RNAs. RNA concentration was estimated using Qubit (RNA Broad Range Assay Kit, Thermo Fisher Scientific).

One-step qPCR was performed using QuantiNova SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's instructions, in triplicate reactions. Reactions were performed on a CFX Connect Real-Time PCR System (Biorad) using the following protocol: reverse transcription for 10 min at 50 °C, PCR initial heat activation for 2 min, 40 cycles of 95 °C for 5 s (denaturation) then 60 °C for 10 s (combined annealing/extension). At the end of the cycling, a melt curve was performed from 60 °C to 95 °C to ensure a single peak was observed indicating no non-specific PCR products.

The gene elongation factor (*ef*) was used as an internal control to normalize all samples. The *ef* gene has been used as an internal control gene in numerous studies as it displays a constant expression level under different experimental stress conditions, including heat stress (Yu et al., 2017; Zhang et al., 2018), increased partial pressure of carbon dioxide (Hoadley et al., 2015), and microplastics (Limonta et al., 2019). The stability of the *ef* gene as an internal control in the present study was tested following a normalization strategy previously used and the geNorm software (Vandesompele et al., 2002). The details of the qPCR primers used are shown in Table 1. The efficiency of each primer set was determined using a serial dilution (between 1 in 10) of a mixed RNA sample, in all cases the efficiency was between 0.94 and 1.10. Relative change in the expression of GOIs was calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001). Calculations were performed using the R-based program qRAT (Flatschacher et al., 2022), and results were reported as log2 fold-change in expression compared to samples kept at control temperature (25 °C) and not subjected to any microbeads treatment (control group).

2.5. Statistical analysis

The Shapiro-Wilk test was used to assess the normal distribution of data and where assumptions were violated, the data were corrected by transformations. The chi-square test of homogeneity was performed to evaluate significant differences in the abnormal coral mucus production among treatments since the dependent variable was considered categorical (0 = no abnormal production; 1 = abnormal production).

For all other investigated parameters, except the microplastic adhesion, significant differences between the distinct treatments were analyzed with a two-way ANOVA followed by Tukey's HSD post hoc tests for multiple pairwise comparisons of means, using the temperature (control, 25 °C and heat stress, 30 °C) and the microplastic bead concentration (low [MP] and high [MP]) as factors, and the values obtained from the different parameters as dependent variables. For the microplastic adhesion, since transformed data still did not satisfy the assumptions of ANOVA, significant differences between treatments were assessed by using a non-parametric Kruskal-Wallis test followed by multiple pairwise comparisons. All the analyses were performed using SPSS ver. 28 (IBM, New York).

For gene expression analysis, a statistically significant difference in gene expression between (1) the treatment groups and the control group, as well as (2) among the different treatment groups was calculated using qRAT. The program calculations were based on the R package limma (Ritchie et al., 2015) and used moderated t-statistics for multiple comparisons. The *p*-value was adjusted by Benjamini and Hochberg's method to control the false discovery rate.

In cases where two-way ANOVA indicated significant effects in both factors (Temperature and MP), or a significant interaction between the stressors, we calculated the expected additive inhibition from each parameter according to a standard ecotoxicological model (Bliss, 1939) to compare observed effects with expected effects (Schmidt et al., 2014). We determined combined effects from fraction changes of the treatments compared with the control (25 °C and no MP). Additivity can be presumed when both individual factors (A: Temperature; B: MP) are significant, but their interaction (C) is not (total effect C = A + B). In cases where a significant interaction term is present, a comparison of the predicted with the observed effect of the combined stressors can reveal if antagonism (C < A + B), or synergism is indicated (C > A + B), (Crain et al., 2008; Schmidt et al., 2014).

Table 1
Primer sequences of GOI and internal control gene used for qPCR analyses. In the primer sequence column, F and R indicate the orientation (F: forward; R: reverse). T_m (°C): melting temperature.

Gene	Primer sequence 5′ to 3′	T_m (°C)	NCBI n°	Reference
cas3	F: TGTTGCTGATAGCGCCCAGA R: TCGCTGTGTCTTTCTCCGTTCA	60	KU882098	Yu et al., 2017
hsp70	F: ATCCAGGCAGCGGTCTTGT R: TCGAGCAGCAGGATATCACTGA	60	AB201749	Mayfield et al., 2014 Putnam et al., 2013
Elongation factor (*ef*)	F: CGCTGGCAAAGTGACAAAGG R: CAGACTTGCGATGAAATAGATAGGA	60	_	Yu et al., 2017 Zhang et al., 2018

3. Results

3.1. Visual signs of stress

In general, a higher abnormal mucus production was observed in nubbins kept at control temperature of 25 °C and subjected to microplastic beads addition, compared to those subjected to heat stress (30 °C) in combination with microplastic beads. In particular, no colony showed mucus production at 25 °C without microplastic bead exposure, while 16.7 % and 25 % of the nubbins showed abnormal mucus production when subjected to low [MP] and high [MP] treatments at 25 °C, respectively. At 30 °C, nubbins showed abnormal mucus production (8.3 % of the analyzed corals) only when subjected to the high [MP] treatment. However, the chi-square test of homogeneity did not show statistically significant differences in the percentage of nubbins producing mucus among treatments.

Polyp extroflection analysis showed no statistically significant differences among treatments (Tables 2A, 3; Fig. 1). Furthermore, no tissue necrosis was observed in all the conditions tested.

3.2. Physical interaction between microplastic and corals

3.2.1. Microplastic adhesion

At the end of the different treatments, 56 % of *P. damicornis* nubbins showed at least one microplastic bead adhered to their surface. A statistically significant difference in the average number of microplastic beads adhered to nubbins was observed between the different treatments (Kruskal-Wallis test, $p < 0.001$). Microplastic bead adhesion was higher in corals in the control treatment (25 °C) compared to those subjected to heat stress (30 °C), (Fig. 2A). Within each temperature treatment, corals subjected to high [MP] had higher microplastic bead adhesion compared to those subjected to low [MP]. In particular, no microplastic bead adhesion was observed on nubbins subjected to heat stress and low [MP], (Fig. 2A).

3.2.2. Microplastic ingestion

P. damicornis ingested microplastic beads in all treatments. Most of the ingested microplastic beads were found inside polyps' mouths, while others entered the coral tissue and were observed after the dissolution of tissue. We observed a statistically significant difference in the average number of microplastic beads ingested by corals only among the various microplastic treatments, with no statistically significant differences observed between temperatures of the interaction of the two factors (Table 2B). Therefore, the concentration of the MP in the water alone had a significant effect on microplastic ingestion (Table 3). In detail, a similar trend in microplastic bead ingestion and adhesion was observed, with higher values in corals subjected to high [MP] compared to those subjected to low [MP] within each temperature, although this difference was not always statistically significant (Fig. 2B).

3.2.3. Microplastic egestion

Microplastic bead egestion was observed in *P. damicornis* nubbins subjected to all treatments. A statistically significant difference in the microplastic bead egestion was observed only between the different temperatures (Table 2C), with an increase in the MP egestion as the temperature increased (Table 3). Indeed, coral nubbins egested more microplastic beads during the 30 °C treatments compared to the 25 °C treatment (Fig. 3). The concentration of the MP in the water alone and the interaction of temperature and [MP] had no statistically significant effects on the egestion of microplastic beads by corals (Tables 2C, 3). Notably, coral exposed to the 25 °C + High [MP] treatment exhibited lower microplastic bead egestion compared to corals subjected to both microplastic bead treatments at 30 °C ($p < 0.05$), (Fig. 3).

Table 2
Summary of the two-way analysis of variance (ANOVA) for the parameters analyzed in *P. damicornis* nubbins subjected to temperature change, microplastic bead addition (MP), and the interaction of both factors. Significant values ($p < 0.05$) are in bold.

	Source of variation	Df	MS	F	P
A.	**Polyp extroflection**				
	Temperature	1	1196.591	1.922	0.171
	MP	2	1289.175	2.071	0.135
	Temp × MP	2	801.212	1.287	0.284
	Residual	59			
B.	**Ingestion**				
	Temperature	1	0.625	0.21	0.649
	MP	1	27.225	9.151	**0.005**
	Temp × MP	1	0.025	0.008	0.927
	Residual	20			
C.	**Egestion**				
	Temperature	1	1012.201	1.926	**0.033**
	MP	1	1554.644	2.959	0.104
	Temp × MP	1	1869.343	3.557	0.086
	Residual	20			
D.	**Chl a**				
	Temperature	1	12.597	16.246	**<0.001**
	MP	2	1.174	1.515	0.24
	Temp × MP	2	0.067	0.086	0.918
	Residual	30	0.775		
E.	**Chl c2**				
	Temperature	1	3.108	10.194	**0.004**
	MP	2	0.644	2.112	0.141
	Temp × MP	2	0.089	0.292	0.749
	Residual	30	0.305		
F.	**Symbiodiniaceae**				
	Temperature	1	1.011	9.841	**0.005**
	MP	2	0.42	4.092	**0.032**
	Temp × MP	2	0.337	3.28	0.059
	Residual	30	0.103		
G.	**LPO**				
	Temperature	1	0.032	2.112	0.167
	MP	2	0.09	5.976	**0.012**
	Temp × MP	2	0.003	0.225	0.801
	Residual	30	0.015		
H.	***cas3***				
	Temperature	1	0.264	21.148	**<0.001**
	MP	2	0.003	0.277	0.662
	Temp × MP	2	0.017	1.378	0.202
	Residual	30	0.012		
I.	***hsp70***				
	Temperature	1	0.382	83.078	**<0.001**
	MP	2	0.005	1.025	0.283
	Temp × MP	2	0.019	4.029	**0.033**
	Residual	30	0.005		

Table 3
Summary of the significance of individual effects and their interaction based on the two-way ANOVA given in Table 2 (x: non-significant; arrows: significant, $p < 0.05$) and its interpretation based on calculations of predicted additive inhibition and fraction changes compared with the controls. The up arrow indicates an increase in the parameter as the factors (Temp, MP, or interaction) increase, while the down arrow indicates a decrease in the parameter as the factor increases. N.a. denotes cases where only a single factor and not the interaction term were significant. For the factor MP, the Low [MP] has been considered in the analysis.

Parameter	Summary of 2-way ANOVA			Observed inhibition compared with the control treatment			Predicted inhibition (Additive)	Combined effect
	Temp	MP	Interaction	A: Temp	B: MP	C: Interaction	A + B-(A*B)	
Polyp extroflection	x	x	x	n.a	n.a	n.a	n.a	No sign. effect
Ingestion	x	↑	x	n.a	1.545	n.a	n.a	Only MP effect
Egestion	↑	x	x	0.483	n.a	n.a	n.a	Only temp effect
Chl a	↓	x	x	−0.780	n.a	n.a	n.a	Only temp effect
Chl c2	↓	x	x	−0.614	n.a	n.a	n.a	Only temp effect
Symbiodiniaceae	↓	↓	x	−0.688	−0.691	−0.681	−1.854	Additive
LPO	x	↑	x	n.a	3237	n.a	n.a	Only MP effect
cas3	↑	x	x	1.204	n.a	n.a	n.a	Only temp effect
hsp70	↑	x	↑	1.356	−0.154	2.257	1.411	Synergistic

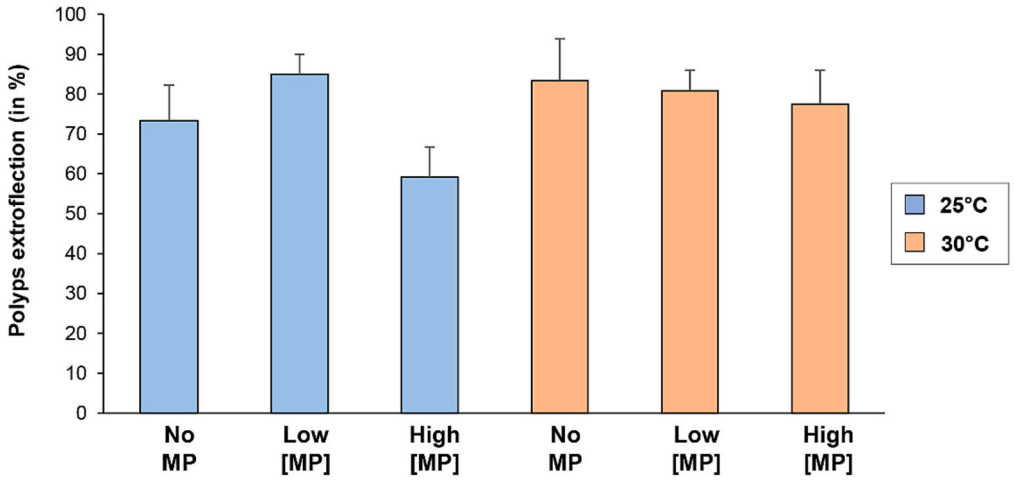

Fig. 1. Percentage of polyps extroflection observed in *P. damicornis* nubbins following 72 h of exposure to different microplastic treatments (No MP: absence of microplastics; low [MP]: 1 mg/L of PE microbeads; high [MP]: 10 mg/L of PE microbeads) and temperatures (control temperature: 25 °C; elevated temperature: 30 °C). Data are reported as mean % ± SEM ($n = 12$ for each treatment). Two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons was performed. No statistically significant differences were observed between treatments ($p > 0.05$).

3.3. Coral bleaching assessment and molecular analyses

3.3.1. Chlorophyll a and c2 concentration

The concentration of chlorophyll *a* and *c2* exhibited a similar pattern across the different treatments, showing a general reduction in nubbins subjected to heat stress and in those exposed to microplastic beads (Fig. 4A and B). However, for both chlorophyll *a* and *c2* statistically significant differences in their concentration were observed only between the different temperatures, while the [MP] in the water alone and the interaction of the two factors had no statistically significant effects on chlorophyll level in corals (Tables 2D and E, 3). Particularly, chlorophyll concentration was highest in corals kept at 25 °C and without microplastic beads, but this mean concentration was not statistically different from chlorophyll concentrations measured in nubbins subjected to microplastic bead exposure (both low and high concentrations), (Fig. 4A and B). The heat stress alone (30 °C – No MP) led to a decrease in chlorophyll concentration, although the change was not statistically significant. However, when microplastic beads were added to heat-stressed corals, a statistically significant reduction in the concentration of both chlorophyll *a* (1.8-fold decrease for both Low [MP] and High [MP]) and *c2* (1.4-fold decrease for Low [MP] and 1.9-fold decrease for High [MP]) occurred compared to corals maintained at 25 °C without microplastic beads (Fig. 4A and B).

3.3.2. Symbiodiniaceae density

The density of Symbiodiniaceae showed significant changes as a result of the different temperatures and microplastic bead treatments (Table 2F). In particular, high temperature and the presence of microplastics had a significant negative effect on algal cell density in *P. damicornis* nubbins (Table 3, Fig. 4C). However, there was no statistically significant change in Symbiodiniaceae density following the interaction of these two stress factors (Tables 2F, 3). Nonetheless, the addition of microplastic beads to corals at control temperature caused a statistically significant decrease in Symbiodiniaceae density (1.9-fold decrease for Low [MP] and 2.1-fold decrease for High [MP]). Corals maintained at 25 °C without microplastic beads had higher Symbiodiniaceae density compared to coral nubbins subjected to all other treatments, including thermal stress in combination with microplastic bead enrichment (Fig. 4C).

3.3.3. Lipid peroxidation

The presence and concentration of microplastic beads in the water caused a statistically significant effect on MDA concentration in coral cells and therefore on lipid peroxidation (Table 2G). In particular, a

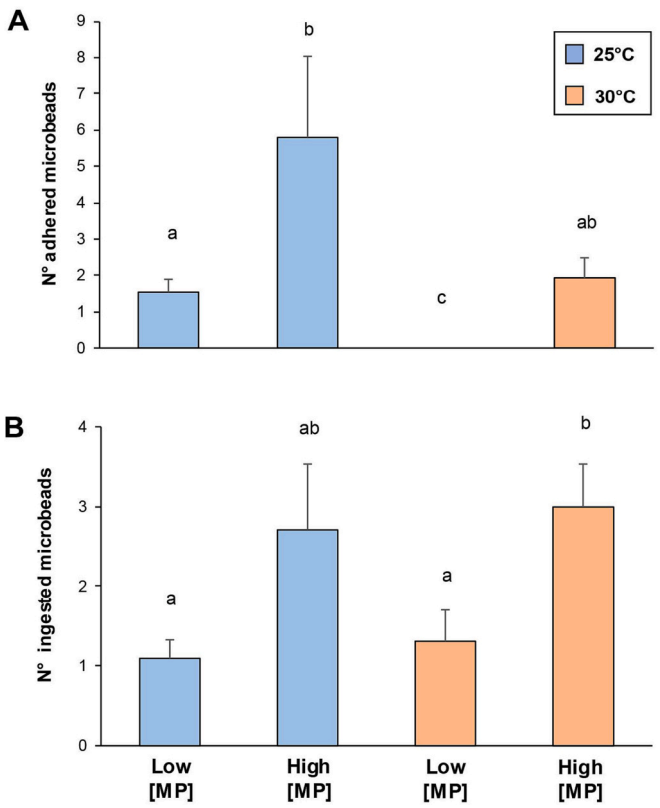

Fig. 2. Number of microplastic beads adhered to *P. damicornis* surface (A) and ingested by *P. damicornis* nubbins (B) following 72 h of exposure to different microplastic bead concentrations and temperatures (control: 25 °C; heat stress: 30 °C). Data are expressed as mean ± SEM ($n = 12$ per treatment for A; $n = 6$ per treatment for B). Kruskal-Wallis test followed by multiple pairwise comparisons (for A) and two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons (for B) were performed. Letters denote statistically significant differences between the different treatments ($p < 0.05$). The same letter indicates no significant difference ($p \geq 0.05$). Low [MP]: 1 mg/L of PE microbeads; High [MP]: 10 mg/L of PE microbeads.

significant increase in LPO was observed in relation only to the change in [MP] in the water (Table 3). On the other hand, the only increase in temperature and the combined effect of high temperature and microplastics did not affect the MDA concentration in coral cells (Table 2G). At 25 °C, the MDA concentration was 1.85-fold higher in coral nubbins exposed to low [MP] and 1.75-fold higher in corals exposed to high [MP] compared to the control treatment (Fig. 5). The highest concentration of MDA (0.5 μmol/μg of protein) was observed in coral nubbins co-exposed to heat stress and environmentally relevant microplastic concentration (2.4-fold higher compared to control treatment). However, this level was not statistically different from that measured in corals subjected to low [MP] at control temperature, corals subjected to heat stress only, as well as nubbins co-exposed to heat stress and high [MP], (Fig. 5).

3.3.4. *Cas3 and hsp70 expression*

A statistically significant modulation of the *cas3* and *hsp70* genes was observed following the different temperature treatments, with a significant increase in their expression at 30 °C (Tables 2H and I, 3). Microplastic bead concentration alone and the interaction of temperature and microplastics did not cause a statistically significant modulation in the *cas3* expression (Tables 2H, 3). On the other hand, different concentrations of microplastic beads within the same temperature treatments caused a statistically significant modulation of *hsp70* expression (Tables 2I, 3).

In particular, at 25 °C, *cas3* expression was down-regulated in coral nubbins exposed to the two different concentrations of microplastic beads (Fig. 6A), however, this decrease was not statistically significant. On the other hand, *cas3* expression was significantly up-regulated when corals were co-exposed to elevated temperature (30 °C) and microplastic beads, and a progressive up-regulation was observed with increasing concentrations of microplastic beads (Fig. 6A). Overall, the highest statistically significant modulation of *cas3* was observed at 30 °C with high [MP].

Similarly, *hsp70* was down-regulated in corals exposed to microplastic bead treatments at control temperature and was significantly up-regulated in all the treatments at 30 °C (Fig. 6B).

At 25 °C, the down-regulation of *hsp70* compared to the control condition was significantly higher only when corals were exposed to the low [MP]. The highest up-regulation was observed when corals were co-exposed to elevated temperature and low [MP] (Fig. 6B).

Overall, the combined effects of both stressors synergistically influenced the expression of *hsp70* in *Pocillopora damicornis* nubbins. The

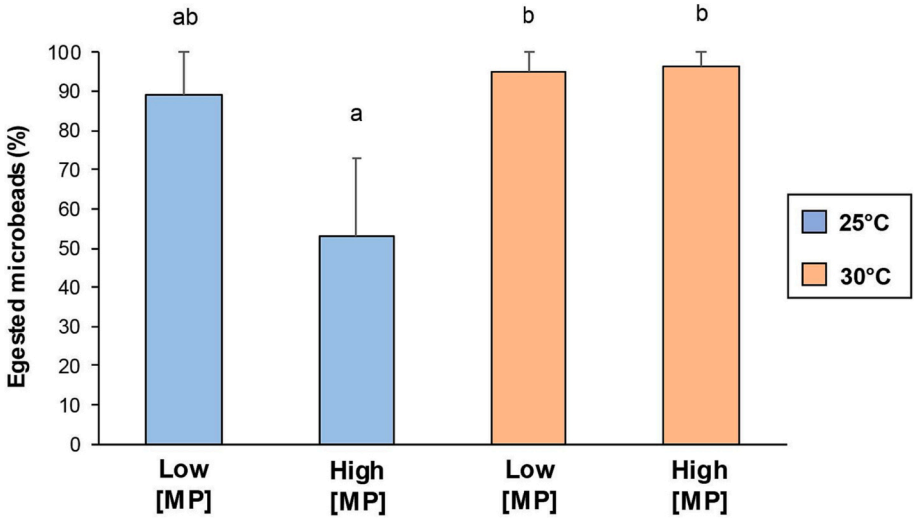

Fig. 3. Percentage of microplastic beads egested after 24 h by *P. damicornis* nubbins following 72 h of exposure to different microplastic bead concentrations and temperatures. Data are expressed as mean ± SEM (n = 6 per treatment). Two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons was performed. Letters denote statistically significant differences between the different treatments ($p < 0.05$). The same letter indicates no significant difference ($p \geq 0.05$). Low [MP]: 1 mg/L of PE microbeads; High [MP]: 10 mg/L of PE microbeads.

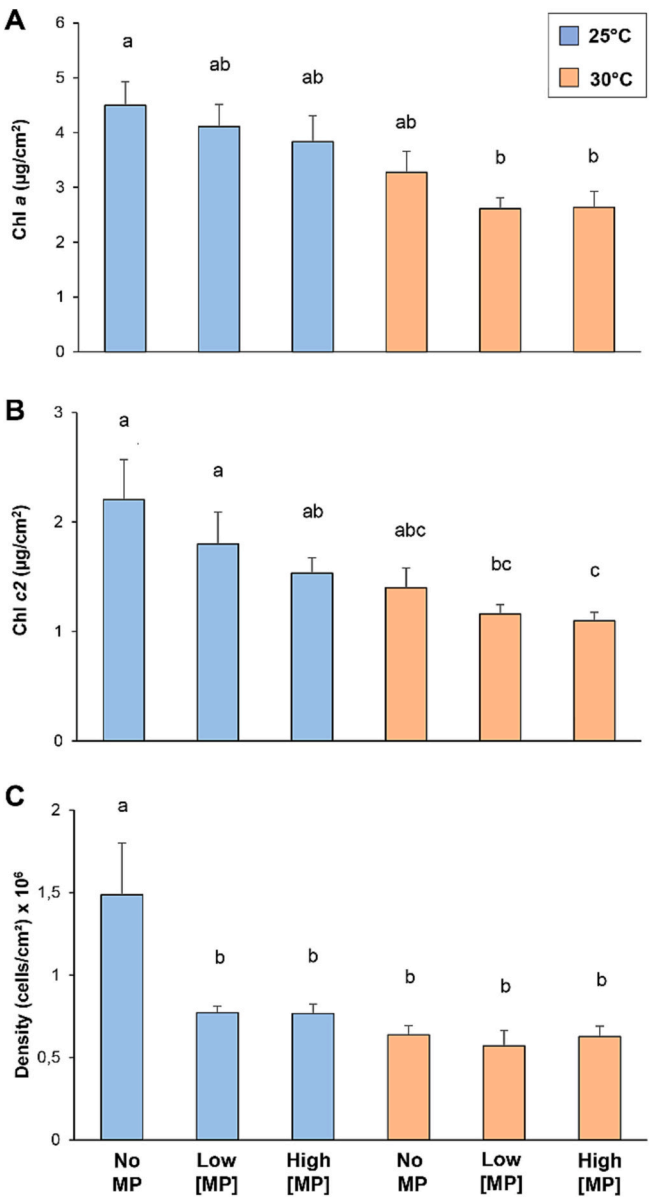

Fig. 4. Concentration of chlorophyll *a* (A) and *c2* (B) and Symbiodiniaceae density (C) in *P. damicornis* nubbins following 72 h of exposure to different microplastic bead concentrations and temperatures (control: 25 °C; heat stress: 30 °C). Data are expressed as mean ± SEM ($n = 6$ per treatment). Two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons was performed. Letters denote statistically significant differences between the different treatments ($p < 0.05$). The same letter indicates no significant difference ($p \geq 0.05$). Low [MP]: 1 mg/L of PE microbeads; High [MP]: 10 mg/L of PE microbeads.

proportional change in comparison to the control group was approximately 60 % higher than the predicted additive effect (Table 3). However, it is noteworthy that only temperature had an impact on the expression of *cas3*.

4. Discussion

Coral reefs are increasingly threatened by marine heatwave episodes and microplastic pollution (Van Woesik et al., 2022; Rahman et al., 2023), and, consequently, it is crucial to investigate the interaction of these factors on the coral health status and physiology. In this study, the reef-building coral *Pocillopora damicornis* was exposed for 72 h to different concentrations of microplastic beads both at control water temperature and in combination with heat stress. The lowest concentration of microplastic used in the present study corresponded to an environmentally relevant concentration of 1 mg/mL and it was relatively lower compared to microplastic used in previous similar studies on *Dipsastrea pallida*, *Astrangia poculata* and *Montipora capitata* (i.e., Hall et al., 2015; Allen et al., 2017; Axworthy and Padilla-Gamiño, 2019).

Visual observations showed that there were no visual signs of stress in corals following the different treatments. Indeed, neither the microplastic bead addition, the temperature increase, nor the combination of these two factors led to a statistically significant increase in mucus production and polyp extroflection in *P. damicornis*. An increase in mucus production is normally observed when corals are stressed (Nguyen-Kim et al., 2015; Wright et al., 2019). However, the production of abnormal mucus has also been reported to be a species-specific stress response, with some species producing copious amounts of mucus as their primary mechanism to remove particles from their surface, whereas other corals producing mucus more sparingly but then use additional clearing mechanisms such as ciliary action (reviewed in Erftemeijer et al., 2012). We, therefore, suggest that the abnormal production of mucus may be a species-specific response also following microplastic exposure. Notably, in a prior investigation, the same PE microbeads induced the production of abnormal mucus and an increase in polyp extroflection in the alcyonacean coral *Coelogorgia palmosa* (Vencato et al., 2021), while in the present study, no production of abnormal mucus and polyp extroflection was observed. The differences in experimental parameters should however be considered and may have influenced these disparate outcomes. Specifically, in the present study, a lower PE microplastic bead concentration was used, and the size of the polyp mouth was different for the two species. For instance, the mouth of *C. palmosa* polyp is about 1 mm in diameter (Vencato et al., 2021), twice the size of the mouth of *P. damicornis* polyp (300–400 µm; Jaffe et al., 2022). Our findings also showed that microplastic beads adhered to *P. damicornis* surface even at the lowest microplastic concentration used in this study (1 mg/L), which is the environmentally relevant concentration and lower than previous experimental microplastic exposure studies focused on microplastic adhesion to scleractinan (*Acropora hemprichii*, *Goniastrea retiformis* and *P. verrucosa*) and alcyonacean (*C. palmosa*), (Martin et al., 2019; Vencato et al., 2021). This observation is therefore ecologically significant as it suggests that current microplastic concentration in oceans may already be adversely impacting corals. Furthermore, our results showed that, in addition to the different concentrations of microplastics in the water, the temperature could also have played a role in influencing the adhesion of microplastic beads to the surface of the coral. In particular, lower microplastic bead adhesion was observed on heat-stressed coral nubbins compared to non-thermally stressed coral nubbins, especially when the concentration of microplastic beads was environmentally relevant.

Ingestion observation showed that *P. damicornis* was able to ingest microplastic beads even when present at a lower concentration compared to concentrations used in previous studies on other cnidarians (Hall et al., 2015; Martin et al., 2019) such as *A. poculata* and *Tubastrea aurea* (Rotjan et al., 2019; Liao et al., 2021). However, the amount of microplastic beads ingested by *P. damicornis* increased at high microplastic concentrations, probably due to the increased likelihood of contact between the coral and the microplastic beads, which consequently increased their possible ingestion by coral polyps. The trend of microplastic bead ingestion was similar to that of adhesion in the different treatments. The amount of microplastic bead ingested by corals was unaffected by temperature, although a previous study reported that *P. damicornis* fragments exposed to higher temperatures ingested fewer PE microbeads (150–180 µm diameter), (Axworthy and Padilla-Gamiño, 2019).

Currently, little is known about microplastic egestion by coral polyps (Martin et al., 2019; Hankins et al., 2021). Our findings showed that *P. damicornis* was able to egest a high amount of microplastic beads, close to 90 %, in most of the experimental conditions tested within 24 h

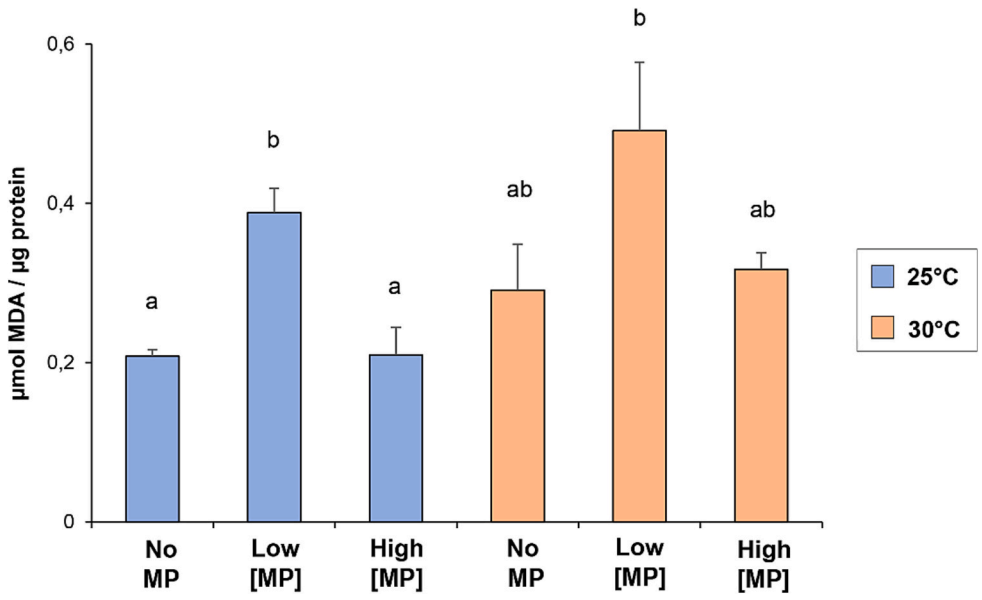

Fig. 5. Levels of lipid peroxidation (LPO) in *P. damicornis* nubbins following 72 h of exposure to different microplastic beads concentrations and temperatures (control: 25 °C; heat stress: 30 °C). Data are expressed as µmol of MDA per mg of proteins and as mean ± SEM ($n = 6$ per treatment). Two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons was performed. Letters denote statistically significant differences between the different treatments ($p < 0.05$). The same letter indicates no significant difference ($p \geq 0.05$). Low [MP]: 1 mg/L of PE microbeads; High [MP]: 10 mg/L of PE microbeads.

of ingestion. Similarly, previous studies reported that *Acropora formosa* egested 92 % of microplastic ingested (Allen et al., 2017), and *P. verrucosa* and *Acropora irregularis* egested 91 % of the ingested microplastic fibers after 18 h (Boodraj and Glassom, 2022). Furthermore, Hierl et al. (2021), studied the microplastic egestion process in other coral species (*Acropora valida*, *Montipora capricornis*, *P. damicornis* and *Seriatopora hystrix*) and concluded that this process was time and energy-consuming to corals. Currently, no data are available on the egestion of microplastics by scleractinian corals subjected to thermal stress, even if this topic has been suggested as a priority for future research (Axworthy and Padilla-Gamiño, 2019). Our results therefore showed that there was a statistically significant difference in the microplastic bead egestion between the different temperature treatments. Moreover, thermally challenged corals egested more microplastic beads at both microplastic bead concentrations in the water, compared to nubbins at control temperature in combination with elevated microplastic bead concentration. This could be because thermally stressed corals undergo different cellular events to get rid of their photosynthetic symbionts, such as exocytosis of Symbiodiniaceae, as well as, host cells detachment, which are then released in the gastrovascular cavity and ultimately ejected out of the polyp (Weis, 2008). Possibly these events within the polyp may also cause the egestion of microplastic beads during thermal stress.

Coral bleaching analyses showed a general decrease in chlorophyll concentration and Symbiodiniaceae density in *P. damicornis* nubbins with an increase in temperature and microplastic bead concentration. However, the temperature was the main factor causing bleaching according to statistical analysis, especially for chlorophyll. Furthermore, the addition of microplastic beads to heat-stressed corals caused a statistically significant reduction in chlorophyll concentration compared to coral nubbins under control conditions. In addition, our results also showed that the decrease in the density of Symbiodiniaceae was due to the different temperatures as well as to the different concentrations of microplastic beads. Moreover, the addition of microplastic beads caused a significant decrease in Symbiodiniaceae concentration in coral nubbins under control temperature. Previous studies on the anthozoans *Aipstasia* sp. and *Stylophora pistillata* suggested that high levels of microplastics disrupted the host-symbiont signaling pathways leading to the breakdown of the symbiosis (Okubo et al., 2018; Lanctôt et al., 2020). Xiao et al. (2021) reported a decrease in Symbiodiniaceae density in fragments of *Acropora* sp. exposed to polyethylene terephthalate microplastic beads at a concentration of 50 mg/L, a concentration 5-fold higher than the highest concentration used in the present study. However, no decrease in Symbiodiniaceae density was observed by Plafcan and Stallings (2022) when the coral *Acropora cervicornis* was exposed to microplastic beads (0 and 11.8 particles/L) both at control (28 °C) and elevated (32 °C) temperatures. Reichert et al. (2021) observed that in comparison to heat stress, microplastic constituted only a minor coral bleaching-inducing stressor in different coral species, including *P. verrucosa*, even if it appeared to be more likely impacted by microplastic pollution than other coral species.

Therefore, considering the variability in results from different coral species, the bleaching response of corals to the combination of thermal stress and microplastic pollution could hence be species-specific, as previously suggested with the species *Lophelia pertusa*, *Madrepora oculata*, *Acropora muricata*, *P. verrucosa*, *Pories lutea*, *Heliopora Coerulea* and *Stylophora pistillata* (Mouchi et al., 2019; Reichert et al., 2019; Lanctôt et al., 2020), and it could also depend on the exposure time as well as on the concentration of microplastic beads. Overall, our bleaching results suggested that, within 72 h of exposition, the main cause of bleaching in *P. damicornis* was the temperature increase, but the presence of microplastics seemed to play a role in exacerbating thermal stress-induced coral bleaching.

The effects of temperature and microplastic stress in *P. damicornis* were also investigated at the cellular and molecular levels. As sessile organisms, corals cannot avoid changes in the surrounding environmental conditions and therefore, they can rely only on cellular and molecular mechanisms as a first line of defense against environmental stressors (Kurtz, 2005; Rosic et al., 2014; Seveso et al., 2016; Montalbetti et al., 2021). We, therefore, analyzed the cellular level of MDA, a commonly used biomarker of ROS production and cell oxidative damage, particularly of lipid peroxidation (Gustaw-Rothenberg et al., 2010). When ROS production is higher than the antioxidant system reductive potential, ROS may generate a cascade of events with several critical outputs, among which lipid peroxidation (LPO) is one of the most evident (Gaschler and Stockwell, 2017). LPO is an indicator of the structural integrity of cell membranes. An increase in LPO indicates that ROS levels are overwhelming the antioxidant defense pathways,

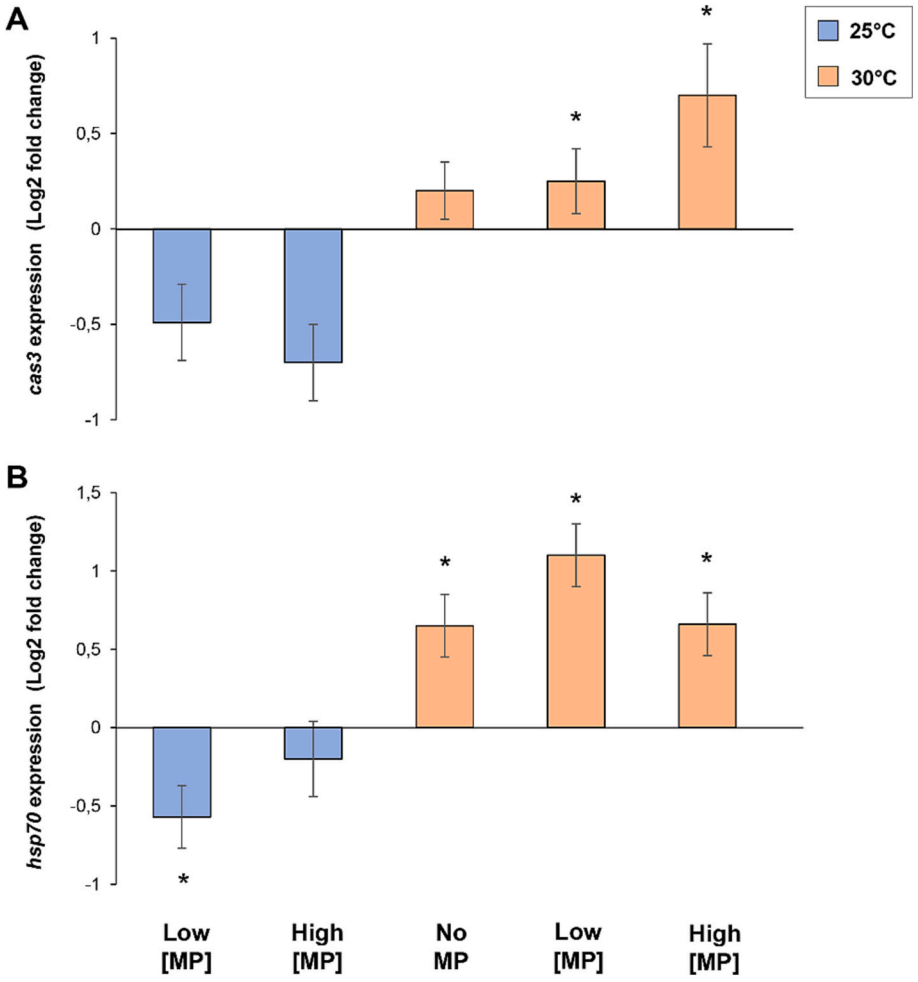

Fig. 6. Change in the expression of *cas3* (A) and *hsp70* (B) genes in *P. damicornis* nubbins following 72 h of exposure to different microplastic beads concentrations and temperatures (control: 25 °C; heat stress: 30 °C). Data are expressed as log2 fold change with respect to the control group (25 °C and no microplastic beads) and as mean ± SEM (n = 6 per treatment). Comparison between groups was done using an independent sample *t*-test. Asterisks denote statistically significant differences with respect to the control group. Low [MP]: 1 mg/L of PE microbeads; High [MP]: 10 mg/L of PE microbeads.

accumulating and damaging cellular membrane lipids, thus signifying ongoing oxidative stress (Lesser, 2006; Weis, 2008). In general, higher levels of MDA were observed in coral nubbins exposed to 30 °C treatments compared to those kept at control temperature, even if this difference was not statistically significant. Several previous studies showed an increase in LPO in response to heat stress-inducing bleaching (Downs et al., 2000, 2002; Flores-Ramírez and Liñán-Cabello, 2007; Dias et al., 2019; Marangoni et al., 2019; Montalbetti et al., 2021). However, there is also evidence that in several coral species, LPO increased following short-term thermal stress, similar to the present study (Dias et al., 2020; Fonseca et al., 2017; Marangoni et al., 2021). Our results further highlighted that the presence of microplastic increased LPO levels and oxidative stress in *P. damicornis*. Similarly, in other coral species and cnidarians, a general increase in ROS production, LPO levels, and antioxidant/detoxifying enzyme activity was observed following microplastic exposure (Tang et al., 2018; Rocha et al., 2020; Jiang et al., 2020; Xiao et al., 2021; Chen et al., 2022). ROS production could be induced by the microbead ingestion, which induced an inflammatory/immune response with the potential abrasions in mesenteric tissues within the coral gut cavity and could have altered the digestion/egestion processes, in addition to the reduction in cellular gas exchange and photosynthetic capacity due to the high number of microplastics adhered to the coral epidermis (Andrady, 2017; Tang et al., 2018; Rocha et al., 2020). Overall, our results suggest that current levels of microplastics in oceans are sufficient, even at control temperatures, to elicit oxidative damage in corals, which could be also exacerbated in combination with thermal stress.

In addition to oxidative damage, the analysis of the expression of *cas3* and *hsp70* genes provided further insights into cellular homeostasis following stress treatments. Gene expression biomarkers can detect sublethal stress before the onset of visual signs of stress (reviewed in Louis et al., 2017). Overall, our results showed that at control temperature, the microplastic bead addition caused a general down-regulation in the expression genes of interest, but in most cases, the change was not statistically significant. In contrast, at elevated temperatures the microplastic bead enrichment strongly increased the expression of the genes of interest. In particular, Caspase3, the protein encoded by the *cas3* gene, is considered a key effector enzyme in inducing cell apoptosis, and therefore *cas3* has been used as a simple, quantitative measure of ongoing apoptosis (Nicholson, 1999; Salvesen, 2002; Ghavami et al., 2009). In general, an increase in *cas3* gene transcripts implies an increase in the apoptotic response and, in turn, indicates more stressful conditions (Pernice et al., 2011; Tchernov et al., 2011; Moya et al., 2016). Several previous studies reported that caspase3 increased after heat stress in different stony coral species *Montipora capitata*, *Stylophora pistillata*, *Acropora millepora*, and *P. damicornis* (Tchernov et al., 2011; Kvitt et al., 2015; Ros et al., 2016; Thummasan et al., 2021). Similarly, in our study *cas3* expression was significantly up-regulated when coral nubbins were exposed to 30 °C. Microplastics have been reported to modulate caspase3 activity in the coral symbiont *Cladocopium goreaui*

(Su et al., 2020) and to increase *cas3* gene expression in the fish *Cyprinodon variegatus* (Choi et al., 2018). Nevertheless, in corals only one study analyzed the effect of microplastic on caspase level, reporting an insignificant correlation between the accumulated microplastics and caspase3 activities in both the coral host and algal symbiont, and demonstrating that the accumulated microplastics did not induce apoptosis in the coral *Porites pukoensis* (Zhou et al., 2023). These observations are partially in line with our results since statistically significant modulation of *cas3* expression was observed following different temperature treatments, with an up-regulation in corals exposed to heat stress. However, the addition of microplastics increased the expression of *cas3* in thermally stressed corals, with a progressive up-regulation with increasing the microplastic bead concentration. The highest upregulation of *cas3* was observed when coral nubbins were co-exposed to thermal stress and the high concentration of microplastic beads, implying the highest apoptotic response and possibly suggesting that microplastics could exacerbate the cellular stress caused primarily by high temperature. This evidence was also confirmed by the modulation of the *hsp70* expression. The 70-KDa Heat shock protein (Hsp70) is a cytosolic chaperonin involved in maintaining cellular protein homeostasis during stress. It is engaged in the assembly of newly synthesized proteins and the refolding of misfolded or aggregated proteins, contributing to the transfer of proteins to different cellular compartments or the proteolytic machinery (Balchin et al., 2016). As an early responder to a general cellular stress status, its up-regulation in corals represents a common response to environmental conditions that may affect their cellular protein structure (Chow et al., 2009, 2012; Seneca et al., 2010; Seveso et al., 2017, 2018; Louis et al., 2020). Previous studies showed that heat stress (Leggat et al., 2011; Poli et al., 2017; Traylor-Knowles et al., 2017; Zhang et al., 2018; Louis et al., 2020) and microplastics (Fadare and Okoffo, 2020; Abarghouei et al., 2021) individually can cause an increase in *hsp70* in different organisms, but no studies so far have analyzed the effect of microplastic alone and the combined effect of both stressors on *hsp70* expression in corals. Our results suggested that *hsp70* expression increased both due to the rise in temperature and due to the synergistic interaction of thermal stress and the addition of microplastic. Notably, the highest up-regulation of *hsp70* was observed when thermally stressed coral nubbins were co-exposed to the environmentally relevant microplastic concentration, in turn indicating that this treatment had the highest effect on cellular protein homeostasis. The difference in the *hsp70* and *cas3* expression profiles in coral nubbins subjected to the different microplastic concentrations in combination with heat stress could be due to the involvement of these genes at different levels of the stress response mechanism. Hsp70 is an early responder to general stress and is therefore triggered as soon as homeostasis starts to be disrupted, to prevent further damage and ensure the survival of the stressed cells. Cas3, on the other hand, is triggered in the ultimate stage of stress response in coral where the cellular defense system is no longer effective against oxidative stress and the stressed cell needs to undergo apoptosis for removal of highly compromised symbionts, thereby maintaining tissue homeostasis (Dunn et al., 2007). In line with this, Kvitt et al. (2016) reported that in the coral *Stylophora pistillata*, following heat stress, *hsp70* gene expression peaked after 6 h and the studied caspase gene (*StyCasp*) expression peaked after 24 h.

In summary, 70 % of the analyzed parameters (including the microplastic adhesion) in *P. damicornis* were significantly affected by the temperature increase compared with the controls, while microplastic concentration alone had a significant negative effect on 40 % of the parameters. However, the combination of the two factors produced significant negative effects only on the *hsp70* expression, showing a synergistic interaction of the two stressors. These results only partially corroborate our hypothesis that microplastics exacerbate stress on corals under heat-stress conditions. The observed minor impact of microplastics at a physical level under control temperatures was unexpected but could be explained by the low concentration of microplastic used in the present studies compared to previous ones. However, at the molecular level, the finding that microplastics could intensify the negative effects of thermal stress, even at low concentrations, provides evidence in support of our hypothesis. Furthermore, it is important to consider that plastic particles, in addition to being able to cause direct physical damage to polyps and cellular stress, could also act indirectly as a vector for diseases (Lamb et al., 2018; Baptista Neto et al., 2020), and by transporting and leaching toxic substances and plasticizer additives (Koelmans et al., 2013; Montano et al., 2020). These effects should be taken into consideration by future research starting from the data coming from the present study, to have a detailed description of the processes associated with plastic contamination that may take place in the environment.

5. Conclusions

Overall, our findings showed that increasing ocean temperature represents the main threat to scleractinian corals such as *Pocillopora damicornis*, while the effect of microplastics on the coral's health and physiology is comparatively minor, especially at control temperature. The temperature increase has significantly affected almost all the morphological, physical, and molecular/cellular parameters investigated, while the effects of microplastics were less significant. However, our results also suggest that microplastics could exacerbate the effect of thermal stress on cellular homeostasis, even at the concentrations of microplastics currently present in the environment. Compared to the control condition, the combined effect of these two stress factors appeared more evident at the cellular and molecular level, as indicated by the generally higher levels of cellular distress in heat-challenged coral colonies, such as higher bleaching, oxidative damage, and expression of *hsp70* and *cas3* expression. In particular, the synergistic interaction of high temperature and microplastics significantly affected the coral cellular protein homeostasis. No evidence of microplastics mitigating the negative effects of increased temperature was observed, unlike what has been suggested by previous research.

In general, our study highlights that rising sea temperatures are still the main challenge corals face, but also that microplastic pollution is already impacting corals, and its detrimental effect may increase under episodes of high sea surface temperatures. While reducing ocean warming is critical for coral reef preservation, effective management of emerging threats like microplastic pollution results are equally essential.

CRediT authorship contribution statement

Valerio Isa: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Writing – original draft. **Davide Seveso:** Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Validation, Writing – review & editing. **Luca Diamante:** Data curation, Formal analysis, Methodology, Visualization. **Enrico Montalbetti:** Conceptualization, Data curation, Investigation, Methodology, Resources, Supervision. **Simone Montano:** Methodology, Software, Validation, Writing – review & editing. **Jacopo Gobbato:** Data curation, Resources, Visualization. **Silvia Lavorano:** Funding acquisition, Visualization. **Paolo Galli:** Funding acquisition, Validation. **Yohan Didier Louis:** Conceptualization, Formal analysis, Investigation, Project administration, Software, Supervision, Writing – original draft.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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