SCUOLA DI DOTTORATO

UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Department of Earth and Environmental Sciences DISAT

PhD program in Chemical, Geological and Environmental Sciences

Cycle XXXV

# Plastic pollution in coral reefs: interaction patterns between primary and secondary micro and nano plastic particles and tropical corals in controlled environments

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**ACADEMIC YEAR 2021/2022**

# Table of contents

## Abstract

Coral reefs provide fundamental ecosystem services such as shoreline protection, subsistence for hundreds of millions of people, and a habitat for a wide range of marine organisms. Currently, worldwide coral reefs are facing mass mortalities of reef-building corals and these phenomena are mainly driven by recurrent marine heat waves as a consequence of global climate change. Heat stress disrupts the obligate symbiosis of reef-building corals with dinoflagellate microalgae (Symbiodiniaceae) and causes coral bleaching and widespread coral mortality. Furthermore, diverse local stressors such as nutrient enrichment and pollution of coastal waters might be related to a significant loss of coral cover and diversity and have been shown to decrease the tolerance of reef-building corals to heat stress. The increasing plastic pollution in coastal waters is an emerging stressor that affects reef-building corals worldwide. Especially microplastic (MP, i.e., particles <1 mm) and nanoplastic (NP, i.e., particles<1µm) are suspected to pose an additional threat to corals. Microplastic occurs in various shapes (most often in form of particles and fibers), and polymer types (most commonly polyethylene and polypropylene). Although their main sources have been identified, concentrations and geographic distributions are highly variable and methodological challenges hamper standardized quantifications, especially regarding NPs. Previous studies already documented ingestion, egestion, and retention of plastic particles. These responses can have adverse effects on the organisms, causing decreases in energy reserves, feeding capacity, or fecundity. Besides direct physical or chemical effects, particle-associated microbial biofilms may influence reef organisms and potentially plastic particles may act as vectors of coral disease. Unfortunately, there are knowledge gaps regarding the effects of microplastic and nanoplastic impact on coral reefs. First, previous investigations have mainly been done in reef-building corals. However, little is known about the responses of soft corals, which are also fundamental coral reefs. Second, the vast majority of previous studies focused on the adverse effects of microplastics related to the ingestion of particles, but few studies analysed the egestion rate of microplastic and the combination between heat stress and plastic pollution. This research assesses and provides an overview of how plastic pollution impacts different genera of tropical corals and the combined effect of microplastic and heat stress. Firstly, the adhesion and ingestion rate of polyethylene microbeads were assessed on the tropical coral *Coelogorgia palmosa*. Secondly, the impact of secondary nanoplastics of Polypropylene (PP) has been evaluated on the tropical soft coral *Pinnigorgia flava,* monitoring the health status of coral nubbins by applying a standardized protocol for evaluating the health status of corals in response to pollutants. In

conclusion, the reef-building coral species *Pocillopora damicornis* has been selected to evaluate the combined effect of microplastic pollution and heat stress, to achieve it *P.damicornis* nubbins were exposed to two different concentrations of PE at 25 and 30°C. results showed that *C.palmosa* had plastic microbeads adhered to the surface and the microbeads were embedded within mucus filaments produced by coral nubbins, therefore in response to microplastic corals produce mucus as a microplastic tra from the surrounding water column, in fact we found statistical strong ositive correlation among the presence of mucus and the number of adhered. Furthermore, *C.palmosa* in both exposure treatments could ingest microplastic, but to a lesser extent than reef-building corals. Secondly PP NP,s caused no mortality on *Pinnigorgia flava*, but evident stress effects were registered on corals. Significant differences in abnormal production of mucus, polyps' retraction, and coral tissue bleaching were found in relation to nanoplastic concentration. Based on these results, NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect concentration) on *P.flava* fragments after 72h of exposure with secondary PP nanoplastics were 0.1mg/L and 10 mg/L respectively. Regarding the combined effects of Microplastic pollution and heat stress, here we found microplastic ingestion, adhesion, and egestion microplastic at environmentally relevant concentrations (200 microbeads/$L^{-1}$). No significant difference was observed in microplastic ingestion among treatments. However, thermally stressed corals had higher microplastic ingestion and egestion rate at high microplastic concentrations. We also found that microplastic had less impact on coral bleaching compared to heat stress. Furthermore, we found that microplastic exposure produces an increase in Lipid Peroxidase production at both temperatures. We anticipate our assay to be a starting point for more species-specific in vitro microplastic-coral biomolecular studies. Furthermore, This study highlights that mitigating ocean warming remains of uttermost importance to conserve coral reefs while managing the emergence of new threats like microplastic and nanoplastic pollution.

# CHAPTER 1

# 1. General introduction

## 1.1. Coral reefs overview

Coral reefs are biogenic structures based on a rigid calcium carbonate framework deposited over geological timescales through the calcification process (Stoddart *et al.*, 1969). The organisms directly involved in the formation of coral reefs are mainly corals belonging to the order Scleractinia also called stony or reef-building corals and coralline algae (Goreau, 1959). Coral reefs are one of the most productive and biodiverse ecosystems on earth, and they are also defined as "the rain forest of the sea" (Mulhall *et al.*, 2008). Despite the estimated coral reefs coverage is comprised between 0.25% (Spalding & Grenfell, 1997) and 0.1% (Burke *et al.*, 2011) of the oceanic bottom, they host more than 25% of the oceanic species (Strain *et al.*, 2019) accounting for the highest species diversity of any marine ecosystem (Nagelkerken *et al.*, 2002). Moreover, they provide fundamental ecosystem services to humans and many living organisms and, other interconnected ecosystems, such as seagrass meadows and mangrove forests (Bastos *et al.*, 2022). Tropical corals thrive in clear and warm waters, characterized by light availability, low turbidity, and specific chemical requirements, consequently, their distribution is circumcised between the tropic of cancer and the tropic of Capricorn (Kusumoto *et al.*, 2020). Despite corals can grow between 19 °C and 36 °C, they can generate complex reef structures only where temperature values are optimal all year long, between 26 °C and 28 °C, and salinity is between 32ppth and 40 ppth (Veron, 1995). The optimal calcification rate, suitable for coral growth, occurs at 27 °C (Fabricius *et al.*, 2011). Therefore, the reef-formation process is restricted to tropical and warm waters where temperature fluctuations throughout the year are almost absent (Goldberg, 2013). The distribution peak of tropical coral species is located between 5 m and 40 m depth (Huston *et al.*, 1985). In addition, tropical cnidarians peak their biodiversity in the so-called "Coral triangle" (Figure 1), an area in the Indo-Pacific Ocean considered the biodiversity hotspot for corals and for mangroves and many other marine species. Their biodiversity decreases further from the coral triangle area (Bellwood *et al.*, 2012).

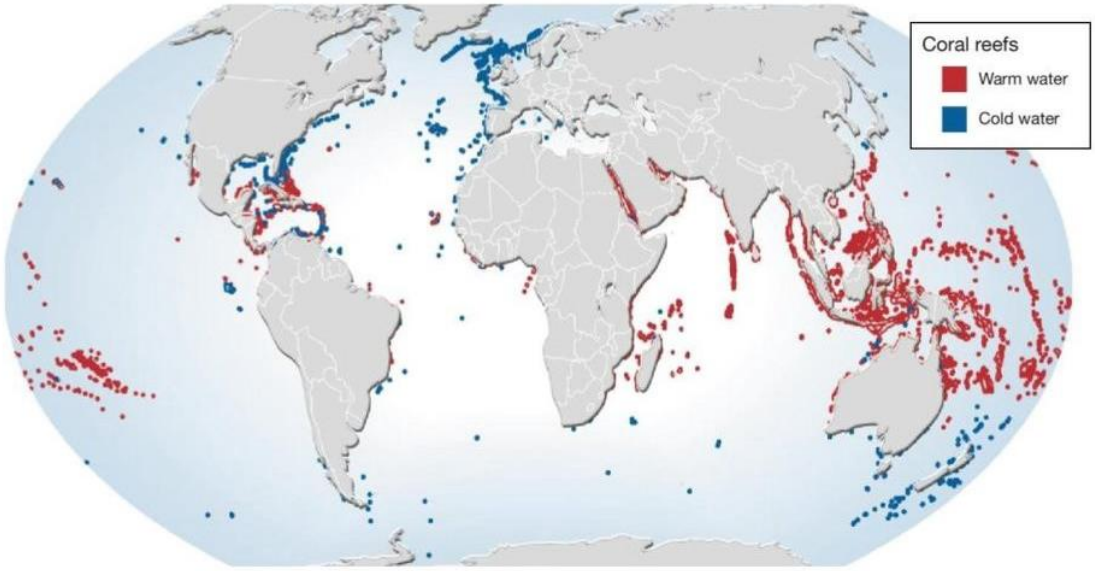


Figure. 1: Major coral reef locations (Bryant et al. 1998)

Within the tropical oceans, four major biogeographic regions could be defined, in terms of coral biodiversity (Birkeland, 1997). Overall, the Indo-West Pacific (IWP) region hosts several scleractinian coral ten times higher than the Western Atlantic (WA) region. Fish diversity follows the same pattern, as well as mangrove diversity (Bellwood *et al.*, 2012). From the anatomical point of view, the unit of coral reef growth are corallites, or rather the calcareous skeleton of a single coral polyp (Gutierrez–Heredia *et al.*, 2016). Characterized by biradial symmetry, coral polyps are composed of two-layer soft tissue (Figure 2): an outer epidermis and the gastrodermis (Goldberg *et al.*, 2002). These two soft tissues are connected by the mean of a thin gelatinous connective tissue called mesoglea, mainly composed of water (Berzins et al., 2021). Each polyp is equipped with only one opening, the mouth, which is responsible for all the exchanges with the external environment (Pacherres Reano, 2021). Coral polyps are surrounded by a crown of tentacles containing stinging cells called nematocysts (Santhanam, 2020). Nematocysts have a double function since they are used as a defensive mechanism and feeding appendages. Therefore, coral tentacles play a crucial role in heterotrophic feeding activities since corals are passive suspension feeders (Goldberg, 2018; Houlbreque *et al.*, 2009).

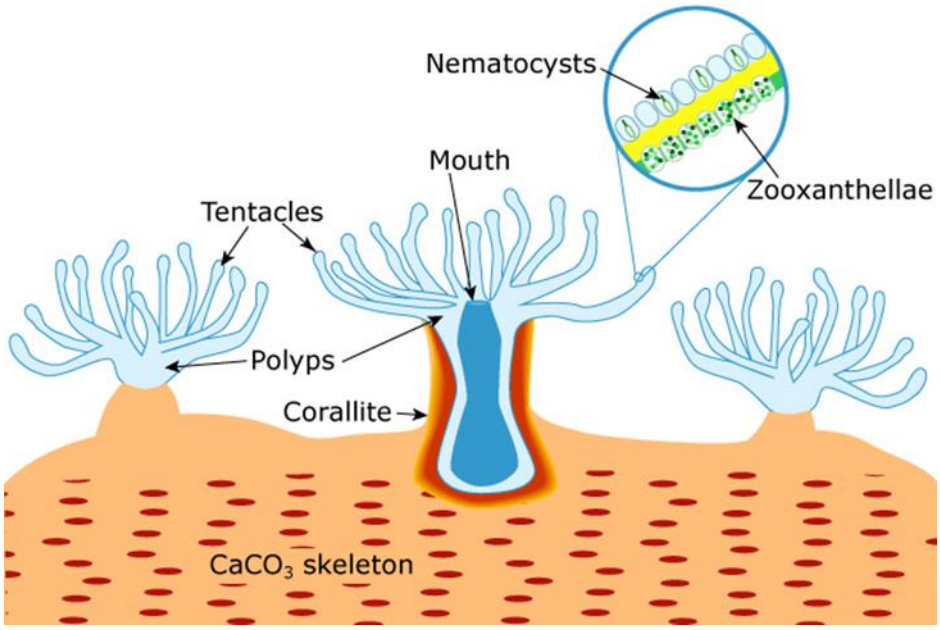


Figure 2: Anatomy of coral polyps. Source: US Geological Survey USGS website

The majority of scleractinian corals are colonial animals, meaning that they are modular organisms composed of hundreds of polyps interconnected together**.** Polyps within the same colony are composed of clonal cells and, therefore, their genomes are identical since they reproduce by cellular budding (Highsmith et al., 1982). Genetically identical polyps belonging to the same colony are connected due to the gastrovascular system that allows molecules exchanges among all the polyps (Hughes *et al.,* 2017). Within the cells of the gastrodermis, a crucial mutualistic symbiosis with unicellular dinoflagellates belonging to the genus symbiodinium occurs (Fournier, 2013). This intracellular symbiosis between scleractinian corals and unicellular photosynthesizing dinoflagellates belonging to the Symbiodiniaceae family (LaJeunesse *et al.,* 2018) commonly referred to as zooxanthellae, is fundamental for the development of complex three-dimensional structures as well as for the growth of coral colonies. Furthermore, each coral colony host several Symbiodiniaceae clades characterized by different adaptive capabilities and tolerances to environmental stress (LaJeunesse *et al.,* 2003, Hughes *et al.,* 2017). Regarding this symbiosis, tropical corals provide protection and expose the symbiotic algae to an environment with high light penetration (Trench, 1979). On the other side, the symbiont provides the host cell with nutrients obtained through photosynthesis and has a direct effect on $CaCO_3$ deposition, responsible for coral growth and influencing the carbonate chemistry within the coral cells (Muller-Parker *et al.,* 2015). Therefore, microalgal symbionts provide up to 90% of coral's energy requirements, exchanging with their host 95% of their photosynthetic products, mainly in form of glycerol, amino acids, peptides, and complex carbohydrates, in return, the coral host provides an ideal

environment for dinoflagellates growth (Stat *et al.,* 2008) and inorganic nutrients such as phosphate and ammonium. Furthermore, the oxygen produced throughout the photosynthetic process is transferred to the coral host and hence used for respiration which leads to an increase in $CO_2$ concentration. The produced $CO_2$, which if combined with $H_2O$ leads to the formation of $HCO_3$- and subsequently $CO_3$-- ions, which are involved in the calcification process described by the following chemical reaction:

$$\mathbf{CO_2 + H_20 \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^- \rightarrow 2H^+ + CO_3^= \rightarrow CaCO_3}$$

Most of the coral's energy requirements are satisfied through the Symbiodiniaceae's photosynthetic activity and not through the heterotrophic feeding activities. However, the main source of nutrients might vary substantially according to different environmental and health conditions (Rossi et al., 2020). Indeed, during the night nutrients are uptaken by the heterotrophic feeding activity, since zooplankton is captured by the extroverted polyps (Goldberg *et al.,* 2018). Recently many researchers supported the hologenome co-evolution theory: throughout their evolution, tropical corals established a symbiosis with bacteria, algae, and other invertebrates able to deeply influence the evolutive history of organisms taking part (Leggat *et al.,* 2007). Recent studies showed the importance of this associated microbiome (bacteria associated with corals) in preventing disease and mitigating heat stress (Epstein *et al.,* 2019). Moreover, the term ''holobiont'' describes an assemblage composed of coral together with the associated invertebrates (bacteria, archea, unicellular eukaryotes, fungi and viruses). These organisms are strongly interconnected with each other, exchanging nutrients, services, and benefits (Bourne et al., 2016).

## 1.2. Ecological goods and services of coral reef ecosystems

Coral reefs provide direct and indirect ecosystem services to humans, both on global and local scales Earth (Costanza et al., 1997). They provide renewable resources, in terms of bioactive compounds and proteins, physical structure services, biotic services (further divided into biotic services within and between ecosystems), biogeochemical services, sociocultural services, and tourism income (Moberg *et al.,* 1999). Services provided in each area depend firstly on the type of reef. Around the globe, 4 main reef configurations can be observed: fringing reefs, barrier reefs, atolls, and platforms reefs. Their functionality is different, as well as their interconnection with other ecosystems (Moberg *et al.,* 1999). Tropical coral reefs are hot spots of biodiversity, particularly difficult to investigate because of the wide abundance of tiny invertebrates and cryptic species (**Reaka-Kudla et al., 1997)**. Almost a third of the world's marine fish species

rely on coral reefs and colonize this ecosystem (McAllister et al., 1994), despite the reef coverage is very limited compared to the total oceanic extension. Previous studies tried to quantify the area covered by coral reefs and relate it to the global oceanic extension: Spalding and Grenfell, 1997 estimated that the surface of coral reefs range around 255000 $km^2$, while Bryant *et al.,* 1998 demonstrated that, despite occupying approximately 0.25% of the marine environment, coral reefs host more than 25% of know fish species. The complex three-dimensional structures made by calcium carbonate deposition, has the function of nursery and feeding areas for a multitude of organisms, providing shelter and food for juveniles and, meanwhile, enhancing niche diversification (Harborne *et al.,* 2006). Therefore, they host and maintain a vast biological and genetic diversity. New coral reef-associated species are described daily (Brandl *et al.,* 2018; Bouchet, 2006). Many tropical countries rely on coral reefs as a source of food **(**Souter and Linden, 2000). In remote and developing countries, the study conducted by Jennings and Polunin, 1996 estimated that 1 $km^2$ of healthy coral reefs could satisfy the protein requirements of 300 people if no other protein sources are available. In some parts of the Indo-Pacific region, reef fisheries account for 25% of the total fish catch (Cesar, 1996). Nowadays, a growing research branch is focusing on the exploitation of corals and coral reef-associated organisms to discover and extract pharmaceutical products with anticancer, antimicrobial, and anti-inflammatory properties (Cerri *et al.,* 2022). Moreover, coral reefs host many species of seaweed that, once collected, can be used in agar and carrageenan production (Birkeland, 1997). Furthermore, other products indirectly produced by coral reefs are pearls and ornaments, indeed, coral reefs host mother-of-pearl shells (*Trocus spp*.), giant clams (*Tridacna spp*.) and red coral (*Corallium rubrum*). Unfortunately, it is common to observe worldwide the overexploitation of these resources that, if well managed, could be renewable (Moberg *et al.,* 1999). Coral reefs dissipate energy, providing physical structures services, specifically, they protect the coastline from currents, waves, and storms (Guannel et al., 2016). These services are extremely valuable for human settlements displaced in the coastline proximity, as well as for other interconnected ecosystems (Maire et al., 2016). Moreover, coral reefs create sheltered lagoons and sedimentary environments, hence, favorable conditions for the growth and development of seagrasses meadows and mangrove forests (Ogden, 1988). Coral reefs are strongly interconnected with seagrass beds and mangrove forests (Ogden and Gladfelter, 1983), as further demonstrated by the inter-exchange of valuable services between these ecosystems. From a biogeochemical perspective, coral reefs act as nitrogen fixers (O'Neil & Capone, 2008) and carbon dioxide sinkers. In addition, reef-building corals allow the investigation of past planetary environmental conditions, acting as climate

records (Stirling et al., 1998). These studies are possible by assessing the isotopic composition of the successive layers of the coral skeleton. These studies provide valuable information regarding the past sea surface temperature and salinity variation (Gagan *et al.*, 1998).

Corals belonging to the order Alcyonacea (soft corals and sea fans) are commonly considered to be less functionally important in tropical coral reef ecosystems than the reef-building Scleractinia (Evans et al., 2011). However, many Alcyonacea contribute to reef growth, and many of them are zooxanthellate, thus contributing to primary productivity (Fabricius and Alderslade 2001). Furthermore, they are an important component of coral reef assemblages, providing a source of food and shelter for other organisms (Fabricius and Alderslade 2001). In addition, Alcyonacea are amongst the most conspicuous and impressive members of reef communities, making them important to the diving tourism sector (Allen and Steene 1999) and the marine aquarium trade (Wabnitz et al. 2003). Their biological properties also hold potential value for the medical research field (e.g., Duh et al. 2002) and for the development of marine antifouling agents (e.g. Cooper et al., 2014). As scleractinian corals, Alcyonacea are vulnerable to the many potential threats, including coastal development, overexploitation, destructive fishing, pollution and climate change (Burke et al. 2011).

## 1.3. Environmental Threats of coral reefs

*Disease and predators*

Corals are susceptible to diseases and predation (Montano et al., 2017). They might be affected by diseases through indirect or direct contact with vectors such as parasites or predators (Aeby amd Santavy, 2006). One of the known typology of coral disease is known as ’black band disease’, inflicted by the cyanobacterium *Phormidium corallyctum*. This invades the tissue, creating a black band around the coral and killing the tissue (Johan et al., 2012). The bacterium feeds on the organic compounds released by the dying coral cells. The band advances a few millimeters daily, leaving the coral’s bare skeleton exposed, which might be colonized by filamentous algae (Peters, 2015). Many other diseases afflict corals by means of ciliates, such as skeleton eroding band, brown band disease or white syndrome. However, their recognition and diagnosis are often difficult (Kaiser et al., 2005).

The best-known predator of corals is the Crown-of-Thorns sea star *Acanthaster planci*. Outbreaks of this sea star show an increase in density from 1-20 per km2 to around 500 per km2. This results in a decimation of the coral population, and coral reef recovery can easily take (Saponari et al., 2018) 20 to 50 years. Furthermore, there is a knowledge gap about the explanation of the occurrence of these outbreaks, but the most common is related to the reduction of natural predators due to overfishing (Saponari et al. 2018). Studies hypotize that anthropogenic factors might enhance these outbreaks, making them more dangerous for the ecosystem (Brodie et al., 2005). Many others are predators of corals, such as mollusks belonging to the genus *Drupella* (Saponari et al.2021).

*Ocean acidification*

One Environmental threat derived from the increase of atmospheric CO2 related to global warming is the acidification of the oceans(Pandolfi et al., 2011). The most vulnerable organisms to this environmental threat are the ones that deposit calcium carbonate in its two forms, calcite and aragonite, such as coralline algae and corals (Kleypas and Yates, 2009). Changes in CaCO3 deposition due to acidification were observed from 1975 to 2008, and successively cases of marked erosion of the reef were already reported (Silbiger et al., 2014). Studies reported that the calcification process of corals decreases as aragonite saturation decreases, reaching zero when the latter is equal to 1 (Broecker et al., 2001). Following this theory, studies show that with an increase in the atmospheric concentration of carbon dioxide to 560 ppm (double pre-industrial levels), the calcification of corals by aragonite deposition decreases by 40% (Silverman et al., 2007). This is due to a decrease in the concentration of carbonate ions resulting from the acidification of the sea (Kleypas et al., 2005). Corals respond to ocean acidification in different ways, the most frequent is a reduction of coral growth and skeletal density (Pandolfi et al., 2011). Up to date, there is a knowledge gap regarding the energetic cost of calcification, hence further study could certainly be useful in predicting the response of calcifying organisms to future increases in ocean acidification (Pandolfi et al., 2011).

*Global warming and heat stress*

Over the past three decades, the global temperature has been increasing rapidly on a continuous basis compared to the post-industrial period (Gao et al., 2018). For this reason, it has been predicted that the surface temperature of the oceans will increase by 1ºC to 4ºC by the end of the century (Pachauri and Meyer, 2014). Scleractinian corals live in temperature conditions about one to two degrees below the tolerance threshold (Coles et al., 1976). This increase in temperature might impair the health status of corals through a phenomenon called Coral bleaching(Downs et al., 2002). Heat stress causes the interruption of the mutualistic symbiotic relationship between symbionts and coral (Lesser, 2006). In fact, under thermal stress the symbiosis becomes dysfunctional, and the symbiotic dinoflagellates are expelled from their host corals (Rosset, 2020). The term bleaching comes from the white coloration that corals take on after the loss of their symbionts (Figure 3). It is in fact the microalgae. Once the symbionts are expelled from the tissue of the corals, they present the transparent tissue that shows off the underlying bare skeleton (Brown, 1997). Sea surface temperature anomalies are increasing and the general rise in temperatures due to climate change, leading to an increase in the frequency of mass bleaching events. more frequent (Dilworth et al., 2020). Therefore, mass-bleaching events might lead to shifts in coral composition and, in the worst cases, to an ecosystem dominated by algae instead of corals (McManus and Plsenberg, 2004). Currently, 75% of tropical coral reefs could be completely lost before the end of the current century (Hoegh-Guldberg, 1999). However, coral's susceptibility to thermal stress and, consequently, coral heat tolerance differs according to different colonies, species, reef type, and first, different Symbiodiniaceae species hosted (LaJeunesse *et al.*, 2020). An emerging research branch is focused on identifying coral-Symbiodiniaceae assemblages particularly resistant to thermal stress.

Figure 3. The Ocean Agency / XL Catlin Seaview Survey / Richard Vevers

If the symbiosis is not recovered, by a decrease in water temperature, the coral colony dies of starvation first, then the remaining bare skeleton is gradually colonized by filamentous algae and eventually (Figure 3) (Drury et al. 2019). Symbionts leave the coral tissue in several ways, for example by cell necrosis, apoptosis (Dunn et al., 2007; Tuckett et al 2018), and autophagy of the host cell (Downs et al., 2009), or detachment of the host cell, or finally by exocytosis of the symbiont (Weis, 2008). This phenomenon might occur in certain coral species in which the stress causes the symbiont cells to move from their intracellular position to the coral's gastrovascular cavity (Parrin et al., 2012). Once ideal conditions have returned, the cells return to their original position. This will certainly be a winning behavior in the natural selection of coral reef species, as it promotes the survival of both members of the mutualistic symbiosis (El Rahmany, 2019). From the molecular point of view, the molecular mechanism triggered by thermal stress from a physiological perspective, that causes the symbiosis disruption is due to the overproduction of Reactive Oxygen Species (ROS). Specifically, heat stress impairs the photosynthetic machinery of the symbiont, the photosystem II, which overproduces ROS species, causing oxidative stress (Figure 4). Oxidative stress is defined as an imbalance between prooxidant and antioxidant levels in favor of prooxidants (Nielsen, 2018). Pro-oxidant molecules leak into the coral cell, damaging the host cellular membrane through lipid peroxidation, protein oxidation, and DNA degeneration (Freeman, 1982).

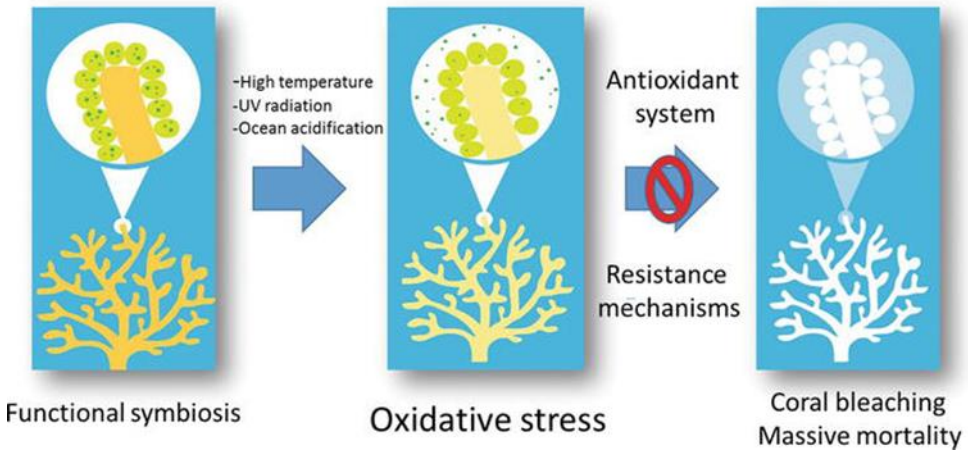


Figure 4. Coral bleaching and oxidative stress theory. Source: Norma Olguìn-Lòpez *et al.*, 2017

ROS are inevitable produce during the electron transport chain in mitochondria and chloroplast, but changes in temperature and UV rays might enhance the overproduction (Hu et al., 2008). ROS are cell signaling molecules intrinsically produced at a low and stationary level in healthy conditions. These molecules are characterized by an impaired number of electrons which means that they are extremely reactive, hence, they might bind lipids, proteins, and DNA molecules, altering their functionality (Freeman, 1982). Moreover, the fact that ROS molecules are highly mobile and water-soluble, might enhance their dangerousness (Ayala *et al.*, 2014). They are formed through the reduction of molecular oxygen ($O_2$) following many reactions involving different enzymes. Examples of ROS species are: peroxides ($H_2O_2$), superoxide ($O^-_2$), hydroxyl radical (·HO), hydroxide ion ($HO^-$), triplet oxygen ($3O_2$) and nitric oxide (NO) (Auten & Davis, 2009). Coral cells try to minimize the negative impact by increasing the production of antioxidant enzymes such as superoxide dismutase (SOD), lipid peroxidase (LPO), and glycogen phosphorylase (GP) (Montalbetti *et al.*, 2021; Seveso *et al.*, 2017). These enzymes transform ROS species into non-harmful molecules ($H_20$, $O_2$ or $H_2O_2$). Once coral cells are heavily impacted by oxidative stress they might expel symbiotic cells, leading to coral tissue bleaching, hence, to a decrease in symbiont density, loss of coloration, reduction in photosynthetic rate and efficiency, and mass mortality events (Oakley *et al.*, 2018).

## 1.3.1. Plastic pollution

*Microplastic*

Plastics are a diverse group of synthetic polymers that have their origins in the late 19th century, but which really started to be deeply used in the mid-twentieth century (Ryan, 2015). Their low density, durability and relatively low cost make plastics ideal materials for a wide range of manufacturing and packaging applications. Global plastics production in 2019 almost reached 370 million tons (PlasticsEurope,2020). The intense consumption and rapid disposal of plastic products are leading to a visible accumulation of plastic debris in the marine environment. At least 8 million tons of plastic end up in our oceans every year, from land-based sources, ships and other installations at sea, from point and diffuse sources (Koisor and Crescenzi, 2020). Microplastic abundance in the surface water of coral reefs generally ranges from zero to tens of thousands of items/$m^3$, while in sediments and corals it is difficult to quantify due to lack of a relatively standardized unit or enough available data (Sharma et al., 2020). It is also important to take in mind that, sources, distribution patterns and composition of microplastics vary from reef to reef, because complex environmental factors, such as plastic fragmentation and transport mechanisms are highly variable (Huang et al., 2021). The properties that make plastics so useful make them a significant environmental threat, because, due to their durability they persist in the environment for many years, and their low density allows them to be readily dispersed by currents and winds, traveling thousands of kilometers from source areas (Ryan et al., 2009) and reaching also the most remote places of the world. Plastics are exposed to different environmental conditions that lead to their physical, chemical, and biological degradation (Fotopoulou and Karapanagioti, 2017). Microplastics are plastic debris smaller than 5 mm in diameter and originate from a variety of sources, but these can be broadly categorized as primary which is the direct release of small particles, for example from cosmetics and cleaning agents, also known as microbeads, or secondary, which results from the fragmentation of larger items (Rillig, 2012). In photic environments such as the sea surface and on beaches, degradation of larger plastic items occurs primarily through UV radiation inducing photo-oxidation reactions or also through erosion provided by currents (Andrady, 2015). Below the photic zone in the water column, plastics degrade very slowly resulting in the high persistence of plastic litter, especially on the seafloor. The density of microplastics also affects the distribution of microplastics in the water column: polypropylene (PP) and polyethylene (PE) are characterized by low density and float in the water, while polystyrene (PS), polyvinyl chloride (PVC), polyamide (PA), and polyethylene terephthalate (PET) with

higher density deposit by inclination through the water column (Guo and Wang, 2019). It has been noticed that the global surface load of plastic is well below that expected from production and input rates; there is an important gap in floating plastic debris size smaller than 5 mm, suggesting that the surface waters are not the final destination for buoyant plastic debris in the ocean; indeed, shore deposition, nano-fragmentation, biofouling, or ingestion could be possible sinks of microplastic (Cózar et al., 2014). Indeed, beaches across the globe are covered with plastic debris derived either from inland sources transported to the coast by rivers, wind, man-made drainage systems or human activity or directly from the oceans where low-density floating particles are transported across great distances (Cooper et al., 2010). The colonization of the plastic material by fouling organisms increases the density of the particles and enhances the sink to the bottom of the ocean, allowing their deposition in the benthic environment (Bergmann et al., 2015). Finally, the small size of microplastics makes them available for interaction with marine biota at different trophic levels(Lusher, 2015). Ingestion of microplastics by amphipods, copepods and zooplankton is a potential concern for coral reef health since these planktonic organisms are the prey of corals and other coral reef filter feeders organisms (Ferrier-Pages et al., 2003). Scleractinian corals could be important players in the removal of microplastics, since they screen huge volumes of water, through suspension feeding. However, microplastic debris could be considered a sink but also a source for chemical contaminants (Palmer and Herat, 2021), since they apparently seem to be very similar to zooplankton and, deceiving marine organisms, they enter in the food web **(**Setälä et al., 2014**)**. Of great concern is the trophic transfer of MPs and associated pollutants from lower-trophic, keystone organisms, including zooplankton to the upper level of the food chain, with the potential for bioaccumulation and thus adverse health effects in higher trophic level organisms (e.g. small fish and sharks), which may ultimately lead to contaminated seafood for humans (Miller et al., 2020)**.**

*Microplastic pollution and coral reefs*

Microplastics are ubiquitous in the marine environment, and they have been detected also in coral reef areas (John et al., 2021. Regarding the detection of microplastic inside scleractinian corals the analytical methods for the evaluation of the microplastic pollution mainly consist in sampling (e.g., trawl, in-situ sampling), extraction (e.g., removal of organic matter, flotation, filtration), microscope inspection, and polymer identification (e.g., Raman or FTIR spectroscopy) (Ding et al., 2019). In general, identification consists of the morphological analysis (abundance, size, shape, and color) and the chemical analysis (polymeric composition)

(Frere` et al., 2016). Microplastic concentration in surface water of coral reefs generally ranges from zero to tens of thousands of items/$m^3$ (Lamb et al., 2018), while in sediments and corals it is difficult to quantify due to lack of a relatively standardized unit or enough available data (Harris, 2020). It is also important to take in mind that, sources, distribution patterns, and composition of microplastics vary from reef to reef, because complex environmental factors, such as plastic fragmentation and transport mechanisms are highly variable (Huang et al., 2021). Microplastics in coral reefs are mainly represented by fibers, foam, pellets, fragments, films, and granules, of different sizes and colors. The common plastic polymers mainly include Polypropylene (PP), Polyethylene terephthalate (PET), Polyamide (PA), Polyvinyl chloride (PVC), Polyethylene (PE), Polystyrene (PS), Polyurethane (PU) and Polyacrylonitrile (PAN), which predominantly originated from waste emissions from coastal cities (Huang et al., 2021). A work conducted in four wild-captured coral colonies from Rhode Island demonstrated a microplastic abundance of 112 items/per polyp among which fibers were the most abundant, averaging 73.4% of the total particles (Rotjan et al., 2019). Another investigation conducted, by Ding et al., 2019 in three atolls of Xisha Islands in the South China Sea, identified microplastics (24-4729 μm) in corals with a concentration of 0.02–1.3 items/g. However, the distribution characteristics of microplastics on coral skeleton surfaces and inside are still poorly understood (Tang et al., 2021). The microplastic adhesion to the coral skeletal surface might play a role significantly more relevant with respect to microplastic ingestion by corals (Martin et al., 2019). Furthermore, in coral reef ecosystems microplastic particles, transported by currents may act as a vector of coral disease, since they might be colonized by marine pathogens (Bowley et al., 2021)

*Nanoplastic*

In respect to microplastics, nanofraction of the marine litter, represent a new infant field of investigation as suggested by the number of papers published in 2019 which doubled the studies of the previous year and that the first 2 weeks of 2020 have recorded almost the entire 2018 scientific production on this topic (Piccardo et al., 2020).

Some authors adopted the definition taken from the nanomaterial including particles smaller than 100 nm, others prefer to raise the upper size limit to 1 μm, (Alimi et al., 2018; Cole and Galloway, 2015; Gigault et al., 2018). Furthermore, most researchers include in the definition, either primary (manufactured) and secondary (originated from degradation) nanoplastics (Goncalves and Bebianno, 2021). Personal care products, industrial abrasives, paints, and particles used in drug delivery can be considered primary nanoplastics (Alimi et al., 2018; Hernandez et al., 2017). Also, recent technologies. Despite nanomaterials productions is ever-expanding (Inshakova and Inshakov, 2017), nanoplastics represents only a thin slide of the market (Vance et al., 2015). Mostly because of technological limitations up to date, it is laborious to extract and quantify nanoplastics in marine environments (Piccardo et al., 2020). Despite the impetus in the research which took place in recent years, there are still many lights and shadows on the subject which, this review aims to highlight (Koelmans et al., 2015). The majority of the observations proposed in this study focus the attention on polystyrene (PS) because most of research knowledge (about 97%) is based on this polymer(Gagne` et al., 2019). Regarding the nanoplastics occurrence in marine samples Ter Halle et al. (2017) first reported the occurrence of the nanofraction (1‑999 nm) of the marine litter in environmental samples. collected in the North Atlantic Subtropical Gyre, were ultra-filtrated and analyzed under a dynamic light scattering (DLS). In order to obtain a chemical fingerprint of colloidal samples and confirm their plastic nature, they performed a pyrolysis coupled to gas chromatography‑mass spectrometry. PE, PS, PVC and PET have been detected in the sample.

*Biological effects in aquatic organisms*

Given the lack of protocols of extraction and detection of nanoplastics in wild organisms, the only possible effects of nanoplastic interaction with biological systems are confined to laboratory contexts (Ivleva, 2021). Due to their small size<1 μm, lower than the average dimension of vegetal and animal's cellular mean diameter (10‑30 μm), they are potentially able to cross the contact surfaces (gills, gastrointestinal tract, cellular wall) translocate to inner organs and directly interact at a cellular level (Rossi et al., 2014; Forte et al., 2016). As regards

to microplastics, PS nanoparticles can exert intrinsic toxicity or act as vectors for other pollutants (Chen et al., 2017a, 2017b; Cui et al., 2017; Shen et al., 2019). Further, they can interact with organisms at the base of trophic chain and be transferred to top consumers (Cedervall et al., 2012; Chae et al., 2018). More than half of the experiments (54.8%) deployed invertebrates followed by fish (16.9%), algae (12.7%), microbes (9.4%), cell lines (5.2%) and rat (Indrajit et al., 2022). Within of the huge, and biologically speaking extremely different category of invertebrate, crustaceans have been tested in almost half of the cases (43.1%) followed by worms (23.5%), molluscs (21.6%), rotifers (7.8%), and sea urchin (3.9%). Rotifers are major components of zooplankton in freshwater and coastal marine ecosystems throughout the world and could be useful indicator species. Furthermore, handful of studies have been conducted on rotifers.

For example, Jeong et al. (2016) evaluated the accumulation and adverse effects of PS micro- and nanoplastic (6 μm, 500 nm, 50 nm) in the rotifer *Brachionus koreanus*. Using different concentration (0.1-1-10 -20 mg/L), all sizes led to significant size- dependent effects, including reduced growth rate, reduced fecundity, decreased lifespan and longer reproduction time. Manfra et al. (2017) reported an increase of mortality rate of *Brachionus plicatilis* after 24 h and 48 h of exposure to cationic (−NH2) PS. Furthermore, PMMA-NPs were capable to induce mortality in rotifers at concentrations higher than 4.69 mg/L with an estimated 48 h median lethal concentration of 13.27 mg/L (Venâncio et al., 2019). Della Torre et al. (2014) investigated the disposition and toxicity of two surface modified polystyrene nanoparticles (-COOH and -NH2, 40 and 50 nm respectively) in early development of sea urchin embryos (*Paracentrotus lividus*). PS-COOH accumulated inside embryo's digestive tract but no embryotoxicity was observed up to 50 mg/L. PS-NH2 were more dispersed and caused severe developmental defects in addition to induce cas8 gene at 24 h post fertilization (at the concentration of 3 mg/L). Copepods are widely used as model species in ecotoxicity and nanotoxicity test (Ockenden et al., 2021; Bergami et al., 2017). A size-dependent effect of micro- and nano polystyrene particles in the marine copepod *Tigriopus japonicas* has been investigated by Lee et al. (2013). The study reported some effects on survival and development of first and second generation, after administration of 50 nm PS particles at concentration higher than 1 mg/L. Further, the 500 nm PS treated individuals, reported a decrease in fecundity, which was not recorded for the smaller 50 nm particles. Several studies demonstrated the accumulation of nanoplastics in different organs and developmental stages of Daphnia spp. (Brun et al., 2017, Rist et al., 2017; Cui et al., 2017;Liu et al., 2020). A wide

range of effects have been recorded: the decrease in survival, reproduction and body size (Besseling et al., 2014; Cui et al., 2017). Finally, regarding longer-term exposure, during a 14-days experiment, the presence of 50 nm NPs significantly enhanced the bioaccumulation of phenanthrene-derived residues in daphnid body (NPs: 5 mg/L; Phe: 0.1 mg/L) (Ma et al., 2016). *Artemia spp*. has been also deployed in ecotoxicological studies highlighting the ability to bioaccumulate nanoplastics and several sub-lethal effects (Bergami et al., 2016; Bergami et al., 2017;). Bivalve molluscs are abundant from freshwater to marine ecosystems, where they are extensively used in biomonitoring programs but also in studies of nanoparticles toxicity (Canesi et al., 2012; Rocha et al., 2020). Reef-building corals are increasingly threatened by global and regional stresses, which affect the stability of the coral-Symbiodiniaceae association. Among them, plastic pollution has been an ongoing and growing concern. Whereas several studies have highlighted the detrimental impact of microplastics (0.1 μm - 5mm). However, there is a knowledge gap about the health status changes induced by NPs on scleractinian corals. Up to date there is only one study about the effect of polystyrene NPs at the concentration of 0.5 mg/L on the tropical coral *Stilophora pistillata* (Marangoni et al., 2021). Results showed an increase in oxidative stress due to NPs exposure. Since the impact of both microplastic and nanoplastic on tropical corals is poorly known and given the importance of coral reef ecosystems more studies should be conducted non only regarding plastic pollution in itself but also about the combination between plastic pollution and heat stress induced by climate change.

## 1.4 Research objective

The plastic and global warming threats on coral reefs, need to be addressed urgently, using the full set of knowledge tools available. This includes in vitro studies to address species-specific responses of the most ecologically important both reef-building and soft corals when exposed to these environmental challenges. This research aims to contribute to this challenging undertaking by providing new insights into contemporary approaches, both physical (in terms of adhesion, ingestion and retention time of micro and nano-sized plastic particles, associated with a general change of the health status of corals, i.e. coral mucus production, bleaching and necrosis), molecular (analyses of biomarkers associated with oxidative stress) and genetic (Gene expressions analyses).

Thus, the objectives of this research are:

- Developing a methodology to assess the interaction patterns between soft corals and microplastic using the alcyonacean soft coral *Coelogorgia palmosa* as model species.
- Assessing for the first time the impact of secondary nanoplastic on tropical corals, by applying standardized protocols to assess how weathered nanosized plastic particles impact the health status of corals, as potentially occurring in marine ecosystems impacted by plastic pollution. Secondly, this research aims to evaluate the impact of plastic pollution coming from the single-use plastic health protection items (Surgical face masks) used for the Covid-19 pandemic.
- Evaluating how the combination of microplastic pollution with thermal stress impact reef-building corals. To achieve it, coral nubbins have been exposed to different concentrations of microplastic (Polyethylene microbeads) at two different temperatures (25 and 30°C). The main aim of this section is to evaluate the corals' response at the physical level ( ingestion, adhesion, and egestion), molecular level (quantification of coral bleaching and the oxidative stress) and genetical level (Gene expression analyses), in areas impacted by plastic pollution and heat stress as a consequence of global warming. To achieve this goal, we exposed coral nubbins to environmentally relevant concentrations of microplastic in order to simulate the real impact of these pollutants on coral reef ecosystems already impacted by global warming.

## 1.5. References


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# CHAPTER 2

## 2.1. Patterns of interaction between alcyonaceans and microplastic

## 2.2 Introduction

Coral reefs are complex three-dimensional structures and represent the marine regions with the highest complexity, biodiversity, and productivity (Norström et al., 2009). They provide habitat and trophic support for one-third of all marine organisms and are economically essential for many human societies (Huang et al., 2020). However, coral reefs are nowadays exposed to numerous pressures, such as climate change, ocean acidification, marine pollution, coral diseases and the combination between natural perturbations and human activities (Allen et al., 2017; Hughes et al., 2017).

Plastic is estimated to account for 80% of all ocean waste accumulated, with 8 million tonnes reach the sea each year (Geyer et al., 2017). Lamb et al. (2018) reported that 11.1 billion plastic items were entangled on coral reefs across the Asia-Pacific, estimating that this number probably will increase of 40% by 2025. Plastic pollution threats marine life by direct physical interaction (entanglement, blocking of the digestive tract following ingestion) (Gall et al., 2015), by acting as a vector for alien species and diseases (Lamb et al., 2018) and by transporting and leaching toxic substances (Koelmans et al., 2016; Saliu et al., 2019). Plastic ingestion by scleractinian corals has recently been demonstrated and several studies have documented microplastic negative effects on coral health (Hall et al., 2015; Allen et al., 2017; Reichert et al., 2018). The microplastics-coral interaction involves ingestion (Allen et al., 2017; Axworthy et al., 2019), egestion (Reichert et al., 2018) and surface adhesion (Corona et al., 2020; Martin et al., 2019). Also, laboratory studies have demonstrated that microplastic exposure can adversely influence the energetics, the growth rate, the health status and physiology of hard corals, with consequences for feeding behaviour, photosynthetic performance, energy expenditure, skeletal calcification, and even tissue bleaching and necrosis (reviewed in Huang et al., 2020).In this context, corals differently respond to microplastic stress, depending on the species (Reichert et al., 2018), the size (Syakti et al; 2019) and the presence of microbial biofilm on the plastic (Allen et al., 2017).

## 2.3 Interaction among soft corals and microplastics: adhesion and ingestion.

Microplastic adhesion to the coral surface is an important mechanism of microplastic and induced stress corals (Allen et al., 2017; Hankins et al., 2018; Martin et al., 2019; Procter et al., 2019).

Adhesion was shown to be 40 times more effective in removing microplastics from the water than ingestion in three species of coral in the Red Sea: *Acropora hemprichii*, *Pocillopora verrucosa* and *Goniastrea retiformis* (Martin et al., 2019). The first two coral species are branching species with small sized polyps, while *G. retiformis* is a massive coral with large polyps (i.e. 2–3 mm). These findings indicate that despite growth, coral form, distinct distribution of species, and polyp size, passive adhesion may be an important mechanism (Soraes et al., 2022). This also suggests that corals are efficient sinks of microplastics in the oceans owing to their rugose and complex skeletons that trap microplastics transported by sea currents (Martin et al., 2019; Corona et al., 2020). Furthermore, microplastics may interfere with the coral cleaning mechanisms (direct interaction, overgrowth, mucus production) and interact with feeding mechanisms (i.e., interaction with mesenterial filaments, ingestion, and egestion) depending on the species and the size of coral polyps (Tang e al., 2021). Suspension-feeding organisms will have different procedures and clearance rates depending on their feeding (passive or active) strategy (Arossa et al., 2019; Hall et al., 2015). Corals probably lack a selection mechanism to allow the polyps to discern between food items (e.g., plankton) and microplastics when they occur simultaneously in the marine environment (Savinelli et al., 2020).

However, previous studies on this topic focused mainly on scleractinian species, while to date non-scleractinian anthozoans have been neglected. Currently, studies on the interaction between non-scleractinian anthozoans and microplastics are circumscribed to the Zoanthids, commonly known as “button polyps” (Anthozoa: Hexacorallia: Zoantharia). In Rocha et al. (2020), *Zoanthus sociatus* showed a high sensibility to PVC microplastics that caused a high adhesion to its epidermis, increased photosynthetic efficiency, lipid peroxidation, and antioxidant defences. Microplastics adhered to the coral surface are shown also in Jiang et al. (2020). The microplastics entered in the Protopalythoa sp. body, caused coral to secrete mucus and lose symbiotic zooxanthellae.

Anthozoans represent a diverse component of coral reef communities that occurs worldwide from the intertidal to abyssal depths and are considered the second most common group of

benthic animals on many shallow reefs, after the reef-building stony corals (Fabricius & Alderslade 2001; Norström et al., 2009; Benayahu et al. 2019). They play a crucial role in coral reef communities since they create 3-dimensional structures providing food, suitable habitat and shelter for other reef dwellers and other services that underpin ecosystem biodiversity (Steinberg et al., 2020). Moreover Anthozoans are widely used in the global aquarium trade (Ellis et al., 1999). The current morphology-based taxonomic classification of non-scleractinian anthozoans corals (belonging to the subclasses Octocorallia) recognizes three orders, with most families and species belonging to the order Alcyonacea (Lamouroux, 1816).

corals belonging to this species have been used for its importance in research, especially for the extraction of substances such as sesquiterpenes, used in the medical field (Wiemer et al., 1990). This study aimed to explore for the first time the main interaction mechanisms between microplastics and the soft coral *Coelogorgia palmosa* Milne-Edwards & Haime 1857 (Order: Alcyonacea). Plastic ingestion and adhesion on the external coral tissue were measured with 2 different microplastic concentration treatments. In addition, the health status of corals was evaluated by analyzing the mucus production and the polyp's extension.

## 2.4. Development of a methodology for the exposure of soft corals to microplastics

Relying on previous studies regarding corals-microplastic exposure (hall et al., 2015; martin et al., 2019), at the Acquario di Genova a total of 12 fragments of ~10 cm in length of the branching soft coral *Coelogorgia palmosa* were collected with pliers from 6 different random colonies. The fragments were promptly fixed on supports made by two-component epoxy resin, built to keep straight each fragment and prevent polyps from being attached to the feeding chamber glass. Subsequently, they were transported in the experimental tank for an acclimation period of 48 hours. After the first 24 h of acclimation, each fragment was transferred in single feeding chambers 2 L-capacity glass beakers, filled with 1.5 L of filtered seawater collected from the aquarium water system. Each feeding chamber was equipped with an air pump, to allow the circulation of microplastics in the chamber and imitate the turbulent motion of particles that occurs in nature. Chambers were allocated in a water bath aquarium's tank to maintain the constant optimal temperature of 25 °C.

Fragments were assigned to 2 treatments with different concentration of polyethylene (PE) fluorescent microbeads 0.98 g/cc, size range of 180-212 µm (Cospheric LLC). PE has been chosen since it is one of the most common kind of MP present worldwide (Steinberg et al., 2020) and one of the most used type of Microplastics used in same kind of studies (Corona et al., 2020; Martin et al., 2019). Specifically, in the first treatment (T1) 0.1 g of microplastics were added in each chamber, corresponding to the concentration of 0.07 g/L or 18.421 microbeads/L. In the second treatment (T2) 0.013 g of microplastics were added in each chamber, corresponding to the concentration of 0.01 g/L or 2.632 microbeads/L. The concentrations of PE were chosen based on previous experiments on scleractinian corals (Allen et al., 2017; Hall et al., 2015; Martin et al., 2019; Reichert et al., 2018; Tang et al., 2018).

For each treatment, five fragments of *C. palmosa*, were exposed in single chamber to microplastics for 48 h. In addition, one chamber with a coral fragment but without the Microplastics (Blank) and one chamber with the support but without the fragment (Control) were also used during each treatment experiment. In particular, the Control chamber was set up to evaluate the loss of microplastics in the system (presence of air pump and support, Microplastics attached to the chamber glass) and the Blank chamber to check the coral health status at experimental conditions.

Three 2 ml water aliquots were collected from each chamber at the beginning of the microplastic treatments (0) and after 2, 4, 6, 12, and 24 h, to evaluate the variation of MPs's concentration through time. Subsequently, they were filtered using a 100 µm nylon mesh and the microbeads were counted under Paralux Stereomicroscope, equipped with Stereo Microscope Fluorescence Adapter with UV light head (NIGHTSEA) kit. During the collection of the aliquots, the presence of mucus produced by the coral and the degree of extension of the polyps were noted and classified as normal production and abnormal production.

The degree of polyps' extension was classified as completely introflected (State 1), extroflected with closed tentacles (State 2) and extroflected with open tentacles (State 3)

After 48 h of treatment, the adhesion and ingestion of microbeads by corals were assessed. Specifically, fragments were removed from the feeding chambers and accurately rinsed with aquarium system seawater, to count the number of microbeads adhered to the coral surface. Moreover, fragments were inspected under stereomicroscope and UV light to ensure the absence of beads attached to the coral surface. Finally, each coral fragment was placed in a petri dish and dissolved in sodium hypochlorite for 1 h, to allow the complete digestion of the

coral tissue. The solution was then observed under a stereomicroscope equipped with UV light and a yellow filter in order to count all microplastics ingested.

For each aliquot, the amount of microbeads was used to evaluate both the microplastics interaction rate and loss rate through experimental time. Interaction rate refers to the amount of missing plastic from the system due to adhesion and/or ingestion by each coral fragment. The loss rate describes the missing plastic due to the adhesion to the chambers' walls and/or other loss of microbeads not coral related.

The Kruskal-Wallis test was used to evaluate significant differences in microplastics ingestion, adhesion, mucus presence and polyps' status (degree of polyp's extension) between the 3 treatments. Spearman's correlation test was performed to investigate the correlation between mucus presence and microplastics adhesion, as well as that between the degree of polyps' extension and the aliquots of Microplastics present in the system. Kendall's tau-b test was used to determine the correlations between the degree of polyps' status and the different Microplastics concentrations. All statistical analyses were performed in IBM SPSS 26.0 software (IBM Corp, Armonk, NY).

## 2.5. Results and discussion

In the feeding chambers, the concentration of PE microbeads decreased linearly through time (Figure1a). The combination of interaction and loss rate resulted in a decrease of microplastics by 19 % in T1 and 38% in T2 compared to the initial number of beads/feeding chamber for each treatment. In all the treatments, *Coelogorgia palmosa* fragments showed evidence of stress by an abnormal mucus production and the shrinkage of tentacles. After 48h of exposure 57% of the treated fragments were in State 2(Figure 1b), with extroflected but closed polyps, while untreated fragments (blank) mainly occurred in the healthier State 3 (64% of fragments) and never presented polyps in State 1. Jiang et al. (2020) reported similar responses for the button coral *Protopalythoa sp.* interacting with microplastics at 0.05 mg/L. Regarding the production of mucus, *Coleogorgia palmosa* untreated fragments did not exhibit signs of stress, while fragments that interacted with microplastics showed a quick abnormal mucus production that generally persisted for the total duration of the exposure in all treatments (Figure 1c).

However, no statistically significant differences in the abnormal mucus occurrence between treatments ($U$ = 19.5, z = 0.257, $p$ = 0.818) and in the polyp state according to the diverse microplastic concentrations ($U$ = 8.5, z = - 0.949, $p$ = 0.421) were observed.

At the end of the treatments, all *Coleogorgia palmosa* fragments showed PE microbeads stuck to their surface and trapped by the produced mucus. The highest adhesion value of PE beads per coral fragment was observed in T1 (320.9 (± 313.1) compared to T2 (35.4 (± 18.6) microbeads per fragment). However, the differences in microplastic adhesion between diverse PE bead concentrations were not statistically significant ($U$ = 10, z = 0.584, $p$ = 0.686). By contrast, both T1 and T2 showed a statistically significant strong positive correlation between abnormal mucus presence and adhered microplastic number (Rho-Spearman test of correlation, and $\rho = 0.949$, $p = 0.05$ for T1, $\rho = 0.975$, $p < 0.005$ for T2).

Coral polyps ingested and retained microplastics in both treatments. *Coleogorgia palmosa* in T1 reported the highest values of ingested PE microbeads per fragment (5.4 ± 3.7) but no statistically significant differences in microplastic ingestion between the treatments were detected ($U$ = 4.5, z = - 1.433, $p$ = 0.190). Under the fluorescent stereomicroscope, most of the ingested microplastics were found inside polyps' mouth, while others entered the coral tissue.

These findings show that alcyonacean corals are able to ingest microplastic, as observed in scleractinian corals (Allen et al., 2017; Corona et al., 2020; Hall et al., 2015; Martin et al., 2019; Reichert et al., 2018) and button corals (Jiang et al., 2020; Rocha et al., 2020). Conversely to other studies (Martin et al., 2019; Jiang et al., 2020), in this work we found a small number of microplastics ingested and not correlated to the microplastic concentration in the water. About the number of ingested microplastics, a similar report was described by Rocha et al. (2020), where the average ingestion was equal to 1.0 ± 0.8 microbeads/coral, at PE concentration 10 mg $*L^{-1}$. The authors proposed that the low levels of microplastics observed in *Zoanthus sociatus* gut were due to low ingestion or retention of these particles caused by a potential low heterotrophy need of *Z. sociatus* in short-term exposure. This hypothesis could be valid also for our observations, as *C. palmosa* is a zooxanthellate alcyonacean and it relies for energy and carbon source from zooxanthellae photosynthesis. However, it is even possible that, as already reported in previous studies (Martin et al., 2019; Reichert et al., 2018; van Cauwenberghe et al., 2015), the occurrence of mucus here acted like a microplastic trap. In this case, microplastics on the coral surface may produce an involucre that might bury the coral polyps. This situation could stress the coral and block polyps to open and catch external particles, natural or not (Corona et al., 2020; Reichert et al., 2018). Since abnormal mucus production and polyp status resulted to be similar among treatments, this may suggest that the occurrence and intensity of these coral responses do not depend, as expected, on the microplastic concentration, while they could be an expression dependant on the time of

interaction between *C. palmosa* and microplastics. Nowadays, microplastic pollution is spreading worldwide, reaching even the most remote places and depths (Moore, 2008;Willis et al., 2017). This implies that an increasing number of marine organisms, soft corals included, will interact with microplastics, even at the lowest concentration. Since the only presence of microplastics seems to stress *C. palmosa* , additional studies on more realistic environmental microplastics concentration and long-term exposure are needed to get a clearer picture of the microplastic effects on soft corals.

Recently, adhesion has been recognized as one of the dominant interaction mechanisms between microplastics and scleractinian corals, responsible for removing microplastics from the water column (Corona et al., 2020; Martin et al., 2019). Our results could sustain this hypothesis and extend it to soft corals. At 48 h of exposure, all *C. palmosa* fragments had the same number of PE particles adhered to the surface, regardless of the microplastic concentration tested. This suggests that the adhesion may not depend only on the microplastic concentration, but on the coral mucus production, as observed during our experiments. Indeed, microplastic beads attached to *C. palmosa* surface were mostly glued to the mucus filaments produced by the stressed polyps. The positive correlation between mucus production and the number of microplastic beads adhered highlights the concept that the more *C. palmosa* creates mucus, the more microplastics will stick on its surface. Since corals produce mucus if subjected to stress (Brown et al., 2005), consequently stress factors that induce soft coral to produce mucus may enhance the adhesion of random plastic present in the water column, promoting the adhesion and adding plastic pollution to every other coral stressing factors.

## 2.6. Conclusions

Generally, even though alcyonacean are often considered "non-primary" habitat forming species and may be overlooked, their ecological role provides fundamental services to the coral reef ecosystems (Steinberg et al., 2020), acquiring even greater importance in the reefs of the future, since transitions from scleractinian-dominated to non-scleractinian dominated reefs have been already suggested (Bradbury & Mundy 1983; Norström et al., 2009; Bryce et al., 2018). This study reports the applicability of a methodology that consists of the use of soft corals as amodel organismsfor studies on exposure to microplastics. Findings showed the firevidenceces of the interaction between alcyonacean soft coral and microplastic, highlighting the soft coral capacity of ingestion and adhesion of microplastic particles. Our results can be considered as a pilot study that ccallsfor further investigations on the effects of the realistic microplastic concentrations found in the marine environment and the time of interaction on soft corals. This might lead to a better understanding of resilience capacities in coral reef ecosystems affected by marine plastic pollution.

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## 2.8. Illustrations

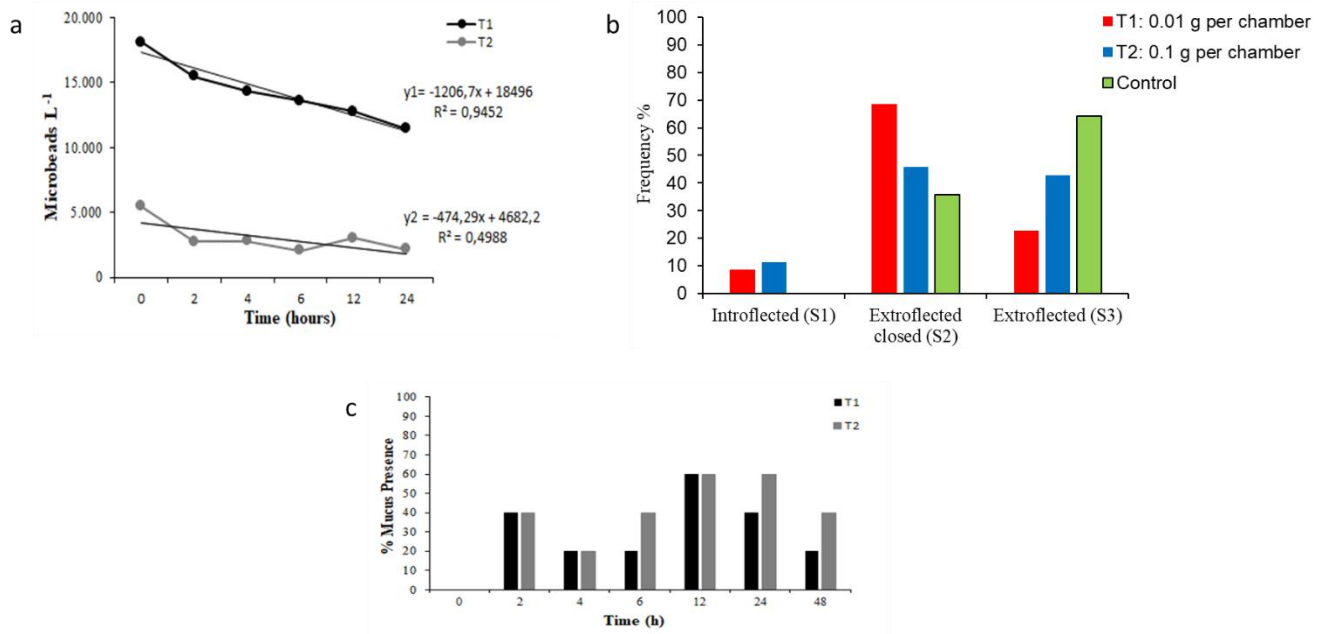


Figure 1. a) Variation of microplastic concentration inside the feeding chambers through time. b) State of the polyps' frequencies for T1, T2. c) percentage of abnormal mucus occurrence during the experiment at different microplastic's concentrations, no abnormal mucus production was observed in control fragments.

# CHAPTER 3

## 3.1. Soft corals and polypropylene secondary nanofibers short terms-interaction: evidence of health status changes in the alcyonacean *Pinnigorgia flava*

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Keywords: Nanoplastics, Textiles, Alcyonaceans, Marine pollution, Coral bleaching, Pinnigorgia flava, Toxicity, nonwoven

*This section is inserted as under review in Chemosphere (Second round)*

## 3.2. Abstract

Up to date, the available information regarding the effects of both micro and nano plastic debris on coral reefs is scarce. Especially, the toxicity onto corals of secondary nanosized fibers originating from the photodegradation of synthetic textiles is not addressed. To provide new insights, we exposed, therefore, the alcyonacean coral *Pinnigorgia flava* to different concentrations of polypropylene secondary nanofibers (0.1, 1.0 and 10 mg/L) and we assayed mortality, mucus production, polyps retraction and coral tissue bleaching. The material for the assays was obtained by artificially weathering non-woven filter fabrics retrieved from commercially available personal protective equipment. Specifically, nanofibers displaying a hydrodynamic size of 114.7± 8.1 nm and a polydispersity index (PDI) of 0.431 were obtained after 180 h exposition to 340 nm; 0.76 $W \cdot m^{-2} \cdot nm^{-1}$. Tests showed no mortality but evident stress after 72h of exposure. Moreover, the difference in the effects registered at the various concentration tested resulted in statistically significant (ANOVA, p=0.014, p=0.001 and p=0.008 for mucus production, polyps retraction and coral tissue bleaching, respectively). NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect concentration) on *P.flava* fragments after 72h of exposure with secondary PP nanofibers resulted 0.1 mg/L and 10 mg/L respectively. Overall, the study indicates that nanofibers may display direct adverse effects onto corals and thus act as potential stress factor in coral reefs. The application of accelerated aging to produce secondary nanofibers from synthetic textiles and to assay their toxicity is also discussed in the paper.

## 3.3 introduction

According to recent estimation, the amount of primary microplastic (particles with diameters < 5 mm, intentionally manufactured at this size scale by the producer to fulfill specific applications in consumer products) released into marine environments accounts from 0.8 to 2.5 million tonnes every year and, unfortunately, it is expected to grow in the next year if no action will be taken by regulatory bodies (Boucher and Friot 2017; Vighi et al., 2021). In addition, an amount between 4.8 to 12.7 million metric tons of plastic waste of different sizes it is estimated to enter the ocean each year (Jambeck et al., 2015). These debris are expected to be converted into secondary plastic particles of micro and nano size (diameters < 1 µm) due to a combined effect of photo-oxidative degradation and mechanical stress (Huang et al. 2021; Chubarenko et al., 2020; Gonçalves and Bebianno, 2021), and thus to largely contribute to the micro and nano debris pollution the marine environment. In fact, cruise surveys indicates that up to 5.25 trillion plastic particles are floating in the world's oceans (Eriksen et al., 2014; Alfaro-Nunez et al., 2021), corresponding to 93 to 268 ktons of dispersed material (Van Sebille et al., 2015) and displaying concentration ranging from 8 to 9180 particles/$m^3$ (Desforges et al., 2014) with fibers often accounting for 80–90% of microplastic counts and detected even in remote areas, although the confirmed synthetic polymers only account for a small portion of the fibers extracted, while the most results composed by cellulose (Suaria et al., 2020). Data regarding the occurrence of nanoplastic, due to a lack of suitable analytical technologies for their determination in the complex environmental matrices, are mostly obtained by estimation, considering a power law fragmentation from microplastic counts (Bouwmeester et al., 2015; da Costa, 2018; Saliu et al., 2020).In the two last year, the outbreak of the Covid-19 pandemic the need of material for health protection on a larger scale has driven an additional and relevant increase in the production of plastic worldwide (Silva et al., 2021). For instance, the production of non-woven polypropylene fabrics, that are mostly used in the production of the filter layers applied in the personal protection equipment for the respiratory tract, increased by 300% between 2019 and 2020 (Uddin et al., 2022). According to a report by Shams et al., 2021 the delivery of personal protective equipment has increased 50.4 million pieces from 5.5 million just between June and July 2020, and more than 200 million pieces in store for delivery to 138 countries (Shams et al., 2021). Unfortunately, this has been associated also to an increase in the plastic waste discharged in the environment (Shruti et al., 2020), *e.g.* it has been estimated that 1.56 million face masks entered the oceans in 2020 (Peng et al., 2021).

At the same time, it has already been elucidated that once dispersed in the marine environment, this material may be partially degraded and fragmented and then spread in the form of secondary micro and nanoplastic (Saliu et al., 2021).

Among the various marine habitat that might be impacted by mucro and nanoplastics, coral reefs deserve a careful consideration due to the presence of key and sensitive species together with the largest marine biodiversity of the planet (Axworthy et al., 2019; Reichert et al., 2019; John et al., 2021; Montano et al., 2020; Isa et al., 2022). In coral reefs surface water concentrations of microplastics were found to span from 2.4 to 15.9 items/L (Ding et al., 2019; Rotjan et al., 2019; Tang et al., 2021; Huang et al., 2021) and microplastics were found also attached to the mucus and within the tissues of different wild collected coral species (Raguso et al., 2022). In the context of the global warming, that leads of the current massive global coral bleaching event (the process in which stressed corals expel their symbionts, causing them to turn white), it is questioned whether the presence of micro and nanoplastic may act as additional stressors on coral health and may impair their function by possible synergy with other environmental stressors (Huang et al., 2021).

Up to date, the few exposure studies carried out in laboratories have mostly highlighted the detrimental impact of MPs (fiber and/or beads) (0.1 μm - 5mm) on corals and their symbiotic dinoflagellate algae with effects onto coral physiology such as reduced growth, and change in photosynthetic performance (Reichert et al., 2019; Corinaldesi et al., 2021). Their effects were shown to be dependent of particle size, shape and chemical makeup (Lanctôt et al., 2020, Okubo et al., 2018). Regarding nanoplastic, the only study available for coral was performed by Marangoni et al. 2021, (Marangoni et al., 2021) and showed significant increase of the oxidative stress in scleractinian *Stilophora pistillata* after exposition of polystyrene nanoplastic (NPs) but no mortality.

Starting from this basis we aimed to evaluate the effects on the alcyonacean *P.flava* caused by secondary particles at the nanosize scale. Specifically, the experiments were carried out by employing polypropylene nanofibers obtained by the artificial weathering of the non-woven polypropylene fabrics used in the fabrication of the filtering layers commonly applied in protective equipment. Mortality and stress indicators were assayed for 72h of exposition.

## 3.4 Materials and methods

### 3.4.1. Nanofiber preparation and characterization

Weathered (secondary) nanofibers were prepared by submitting polypropylene fibers from nonwoven fabrics to accelerated weathering, following the procedure described in Saliu et al. 2021. Briefly, melt blown nonwoven polypropylene fabrics were recovered from the filtering layer placed in the middle of commercial surgical masks. The fabric was cut into approximately 5 × 5 mm pieces (290-270 mg) avoiding the point of ultrasonic sewing. The morphological observation of the fibers and the confirmation of their chemical identity was obtained by microFTIR analysis by employing a PerkinElmer Spotlight 200 Spectrum Two apparatus with MCT detector operating in transmission mode as described in Saliu et al 2021. The material was then exposed to UV light by using a UV-A lamps (340 nm; 0,76 $W \cdot m^{-2} \cdot nm^{-1}$) at 65°C for a total time of 180 hours. For uniformity, the procedure was applied to five different pieces of nonwoven fabric retrieved from five different surgical masks (different vendors) and the nanofibers obtained from each batch were collected and placed in the same glass vial. Morphological characterization of both the pristine and UV-A treated fibers was carried out by SEM (Fig.1.), employing and HITACHI TM3030 Plus instrument equipped with a backscattering detector. The samples were mounted on aluminium stubs using carbon tape. The weathered surfaces were analysed directly (without coating) at 15 kV at a magnification of 500x.

### 3.4.2. Preparation and characterization of the nanofiber stock solution

The weathered polypropylene nanofibers obtained as described in the previous section, were than used to prepare stock solution for the toxicological assay. Specifically, 30 mg of nanofibers was weighted with an analytical balance (Kern ABS ABJ) and then dispersed in 20 mL of artificial seawater (36.7 salinity and 8.12 pH) by a probe sonicator (Vibra-Cell VC 505 PULSER), at 20 ± 0.1 kHz for 30 s. From the initial suspension by repeated sonication and the subsequent dilution (to 50 mL and then to100 mL) a cloudy solution was obtained. In order to remove any floating fiber aggregates, the surnatant was removed by decantation and the solution was filtered (1 μm pore size Puradisc 25 TF) then diluted to a final volume of 1.0 L (corresponding to a concentration of 30 mg/L). Subsequently, by dilution of the stock solution the three-test solution at 0.1, 1.0 and 10.0 mg/L were obtained. Each solution was characterized by using dynamic light scattering (DLS) to determine the hydrodynamic size of the particles and to ensure the stability of the colloidal dispersion (by checking every 12 hours any change

in the particle distribution). The DLS experiments were conducted using a Malvern Zetasizer (Malvern Instruments, Malvern, UK),), with a HeNe laser at a wavelength of 633 nm and a fixed scattering angle of 90º. Measurements of the size of the nano-PS were collected every 15 s at 25 ºC for a total time of DLS measurement of 25 min. Data were calculated considering the obtained autocorrelation function using the cumulants method of three independent experiments (Mean ± SD). An aliquot of 100 mL of the 10 mg/L solution was concentrated in vacuum oven at 80 ◦C, dissolved in xylene, and reprecipitated to obtain a suitable surface for micro-FTIR analysis.

### 3.4.3. Sample preparation

Toxicological assays were carried out by employing specimens of *Pinnigorgia flava* raised at the Acquario di Genova tanks dedicated to a joint research project between University of Milano-Bicocca and Acquario di Genova. 24 *P. flava* fragments of about 3 cm, 2.5 g, displaying an average of 32 (±6.8 SD) polyps for fragment, were collected with pliers from six different random colonies. The fragments were promptly fixed on supports made of two-component epoxy resin and transferred inside the acclimatization tank for 1 week. In this tank (3x1x0,7 m, 3100 L, composed by acrylic and glass resin), the water was uptaken by a pump (Astralpool, Victoria Plus) with a 24-hour flow rate of 8 $m^3 h^{-1}$(to ensure complete water change every about 30 minutes) and reinserted into the tank after passing through the filtration system. The filtration system was composed of a sand filter (Astralpool Artic, filtering particles from 0.4 to 2mm), a UV filter (Panaque 750 s AB 4 lamps of 40W). A solution of 2 L of water containing the algae *Tetra selmis* and zooplankton belonging to the Phylum Rotifera (the average concentration of zooplankton is 250 individuals/mL and the average dimension is 0.5 mm) were added daily inside the tanks in order to feed the corals. Inside the tanks, the temperature was maintained at 25°C with an irradiance of about 200 μmol photons $m^{-2} s^{-1}$ (12 h:12 h light: dark cycle). Chemical parameters such as salinity, pH, ammonium, nitrite, and nitrate concentration were monitored before and after the treatment to assure absence of significant variation during the assays (Further details are reported in Table S1 of supplementary).

### 3.4.4. Study design and assessment of the endpoints

After one week of acclimatization, single fragments (N=1 for beaker ) were transferred in 0.6 L-capacity glass beakers (N=6) filled with 0.5 L of artificial seawater, in this case providing the control experiment (N=6 replicates) or 0.5 L of the three test concentrations (0.1, 1.0 and 10.0 mg/L), providing the toxicological assays test (N=6 replicates for each exposure concentrations for a total of N=18 runs). Each beaker was equipped with an air pump, to allow water motion and oxygenation. Beakers were allocated in a water bath aquarium's tank (400 L) to maintain the temperature of 25 °C. Fragments were randomly assigned to exposure and control treatments. At 0, 2, 4, 6, 12, 48, and 72 h, the following endpoints were assessed: 1) mortality, 2) percentage of retracted polyps, 3) quantification of the polyps' retraction state, 4) percentage of colonies displaying the presence of mucus, 5) classification of the amount of mucus production, 6) percentage of colonies presenting bleaching, 7) quantification of bleaching amounts by semi-quantitative classification, 8) percentage of colonies presenting tissue swelling and 9) quantification of swelling amounts by semi-quantitative classification. Details of the classification adopted for each endpoint are reported in sections 2.4.1 - 2.4.5. Health parameters, requiring semi-quantitative classification, were assessed visually using a four-level scoring system. Parameters were scored on a scale of 0 (normal limits) to 3 (severely affected), with an intermediate condition evaluated by applying a 0.5 unit (thus half-scores are permitted). This scoring system was adapted from a histologically verified stress index developed for a coral health assessment that has been previously used for evaluating hydrocarbon effects on corals (Renegar et al., 2015; Renegar and Turner, 2021; Renegar et al., 2017b). Assessments were performed by two research staff members independently; all results are reported with Standard Error. More details regarding the protocol are reported in Section 2 of Supplementary.

### *3.4.5. Assessment of polyps retraction*

The percentage of retracted polyps was assessed by considering the number of completely closed polyps of each fragment at 0, 2, 4, 6, 24, 48 and 72h. The polyps' retraction state was defined by considering a classification including 4 main classes (with the 0.5 unit for the classification of intermediate condition) and by applying the scoring protocol described in the supplementary file associated to this paper. Briefly, polyps that were normally extended or slightly retracted received a score of 0. Polyps that were retracted and partly closed received a score of 1. Fully closed polyps received a score of 2, and those with very tightly retracted

polyps received a score of 3. This last classification state was used for establishing the effect of NPs on *P.flava* since an extreme polyp retraction represents a significant disruption in normal coral behaviour (Turner et al., 2021).

### 3.4.6. Occurrence and quantification of mucus production

The presence of mucus was monitored in all the tested coral and at every time point. Specifically. the production of mucus was noted if present, for data reporting we considered the percentage of specimens in each treatment displaying mucus production. Secondly, the amount of mucus production was quantified according to the scoring system protocol applied by Renegar et al., 2017b. Since all corals continually produce a thin layer of mucus (Brown and Bythell, 2005) this condition is evaluated with a score equal to 0; corals showing an amount of mucus greater than this normal level but localized receive a score of 1. A score of 2 is given to corals producing more significant amounts of mucus, with more visible mucus strings or thin sheets extending upward from the coral. Corals producing copious amounts of mucus, in thick sheets or with pools of mucus around the base of the coral receives a score of 3. Mesenterial filament extrusion is considered possible, but we did not see it in these exposure experiments.

### 3.4.7. Presence and quantification of coral bleaching

The presence of coral tissue bleaching was evaluated by checking each coral at every time point compared to the color present at the beginning of exposure (t=0), subsequently, the occurrence of tissue bleaching was noted as present or absent, and then the values were reported as a percentage in each treatment. A score of 0 was assigned to corals displaying their original coloration. Corals with slight lightening on color received a score of 1. A score of 2 represents a coral that is moderately bleached, and a score of 3 indicates significant bleaching/loss of color. To quantify the tissue bleaching correctly, corals were photographed under the same lighting conditions at each time point and reviewed by all the research staff members. Moreover, in order to have further assessment of the bleaching process, corals were monitored also with the Coral Health Chart, created by the University of Queensland in 2002 (Fig. 6. of Supplementary).

### 3.4.8. Tissue swelling

The occurrence of swelling of tissue was evaluated by checking each coral at every time point, noting the presence of absence of the process. Subsequently for each treatment data were reported in percentage, considering the total of individuals examined. The tissue swelling was evaluated also by a semi-quantitative classification, that attribute score of 0 when no swelling is observed, score 1 for a slight localized swelling usually in the coenenchymal tissue, score 2 for extensive coenenchymal swelling, and score 3 for extreme swelling.

### 3.4.9. General health status

To evaluate the overall impact caused by nanoplastics on the general health status in *P. flava,* the values of the semi-quantitative classification of polyps retraction, mucus production, tissue bleaching, and tissue swelling were considered all together to provide to each fragment a score ranging from 0 (healthy corals) to 12 (heavily stressed corals) as described in Renegade and Turner 2021.

### 3.4.10. Statistical analysis

The One-way ANOVA test was performed in order to evaluate significant differences in the percentage of open tentacles, percentage of corals showing the production of mucus, and percentage of corals showing tissue bleaching and swelling at the various nanoplastic concentration tested. The test was replaced with Kruskal-Wallis test when the conditions of homogeneity of variances was not met. The Independent-sample t-test was performed in order to evaluate differences in the score of each evaluated parameter, considering the initial condition and the condition at the end of the 72h exposure time, accordingly to the assay protocol described in section 2.4. The Mann-Whitney U test was performed to evaluate significant differences among treatments through time if the conditions of homogeneity of variances (required independent-sample t-test) was not met All Statistical analyses were performed by IBM SPSS 28.

## 3.5. Results

### 3.5.1 Characterization of the nanofibers

SEM imagines of the microfiber after application of the accelerated aging treatment displayed the presence of fractured fibers (micro and nanosized) together with the presence of fiber aggregates (Figure 1). DLS analysis of the solution obtained after sonication and filtration of the photodegraded fibers provided an hydrodynamic size of 114.7± 8.1 nm, and a polydispersity index (PDI) of 0.431. These results are similar to those previously reported by Ren and coauthors (Ren et al., 2018) for secondary nanoparticles of PP. Analysis of the fibers aggregates by micro-FTIR displayed the characteristics features of PP, namely the peaks at 2920, 2846, 1465 and 719 cm- 1 and signs of the chemical degradation related to the the presence of hydroxyl (HO), carbonyl (C=O), and carbon-oxygen bonds (C–O).

## 3.5.2. Mortality assessment

No mortality of *P.flava* specimens was observed during the test. All corals survived at the 72 h. exposure of nanofiber event at the higher concentration tested and also the control benches showed no mortality too.

## 3.5.3. Polyps retraction evaluation

The polyps retraction assays showed that the increase in the concentration of nanofiber do cause a reduction in the average percentage of open polyps. However, statistical test indicates that this reduction is not statistically significant (One-way ANOVA, p= 0.48) (Figure 2a). Also the difference in the polyps retraction score at the end of the exposure time (72h) among the various treatments (Fig.2b.) resulted not significant (One-way ANOVA, p= 0.56). On the other hand, data showed that within the same treatment, the polyp retraction score increased significantly between 4h and 72h in the control and at 10 mg/L (Mann-Whitney U test, p=0.015 and p= 0.020). These data are reported in Figure 2b.

### 3.5.4. Mucus production evaluation

The mucus production evaluation showed that at the highest concentration of nanofibers (10 mg/L) coral fragments started to show evidence of stress such as an abnormal production of mucus already after 2 h of treatment. This production of mucus persisted and increased throughout the exposure time: after 4h of treatment, more than the 80% of fragments showed the presence of this stress factor (Figure 3a). No production of mucus was reported in control fragments instead, assuring the good health status of the specimens in the chamber and the relationship between mucus production and polypropylene nanofiber exposition. The test showed also no production of mucus at the lowest concentration of nanofibers (0.1 mg/L). At the concentration of 1 mg/L fragments started to produce mucus after 4h of treatment, reaching the maximum percentage of 50% of fragments producing mucus after 24h of exposure. Statistical test confirmed that the increase in nanofibers produced in *P.flava* a significant increase in abnormal mucus production (One-way ANOVA, p=0.001) (Figure 3b). After 72 h significant differences were found at 1 mg/L and 10 mg/L compared to control fragments (One-way ANOVA, p= 0.031, p= 0.001), and among 10 g/L with 0.1 mg/L (One-way ANOVA p=0.006). Starting (4h) and final values (72h) of the mucus production at 1 mg/L and 10 mg/L were statistically significant (Indipent-sample t-test, p=0.035, and p=0.003), on the contrary, this comparison was not statistically significant regarding control fragments and the 0.1 mg/L concentration (Mann-Whitney U test, p=0.99, p=0.99).

### 3.5.5. Tissue bleaching evaluation

The evaluation of tissue bleaching showed evidence of the process only at the end of the exposure (72h) and with more individual affected by increasing the concentration of nanofibers. Specifically, the treatment at 10 mg/L of nanofiber caused evidence of tissue bleaching on the highest percentage of *P .flava fragments*, with 60% of coral colonies classified as bleached (Figure 4a). No bleached fragments were found in the control treatment, and intermediate bleaching percentages were observed in 0.1 and 1.0 mg/L concentrations (Figure 4b). Significant difference was highlighted considering the percentage of bleached specimens at 4 and 72 h of exposure for both 1 and 10 mg/L concentration tests (Kruskal-Wallis, p=0.022, and independent-sample t-test , p=0.026), while no significant differences were highlighted by considering the 0.1 mg/L test concentration and the control treatment (Mann-Whitney U test, = 0.2, p=0.39, Fig.3b ).

### 3.5.6. Tissue swelling evaluation

No tissue swelling was observed in control and 0.1 mg/L treatment. Differently, at 72h the both 1 mg/L and 10 mg/L showed a significant increase of tissue swelling (Figure 5a). Specifically, pairwise comparisons (Kruskal-Wallis) showed a significative difference between 10 mg/L and control, 0.1 mg/L and 1 mg/L concentration (p= 0.008, p= 0.030, p=0.042 respectively). Considering exposition time and scores of tissue swelling, a statistically significant difference between t=0 and the 72h treatment was highlighted only at 10 mg/L (Indipendent-sample T-test, p=0.049, Figure 5b).

### 3.5.7. General health status evaluation

The general health status was evaluated by considering the score of all the parameters described in the previous sections. Results relative to the exposure at 72h showed that nanofibers had a significant negative impact on the health status of *P. flava*, (One-way ANOVA, p=0.014), furthermore Games Howell post-hoc test showed a significant difference between control and 10 mg/L treatment (p=0.044) (Figure 6). Furthermore, and at 10 mg/L values after 72 h, are significantly higher than the ones at 1 mg/L (One-way ANOVA, p=0.011).

## 3.6. Discussion

Alcyonaceans (Anthozoa: Octocorallia: Alcyonacea) represent a diverse component of coral reef communities and the second most common group of benthic animals on shallow reefs (Norström et al., 2009). They are fundamental in coral reef communities since they provide food, suitable habitat, shelter for reef dwellers, and other services that underpin ecosystem biodiversity (Steinberg et al., 2020). *Pinnigorgia flava* belongs to the order Alcyionacea (Nutting 1910) and is considered a model species for the assessment of the impacts related to anthropogenic stressors (e.g. temperature raise and acidification) and to provide insights into the octocoral resilience mechanisms (Vargas et al., 2021).

In this study, we focused onto plastic nanoparticles as pollutants in coral reefs, and specifically onto the possible effects driven by secondary nanofibers onto the coral *Pinnigorgia flava*. The tested material was made of polypropylene, the polymer that constitutes up to the 24% of the marine plastic debris (Chapron et al., 2018).

To the best of our knowledge, this is the first research dealing with the potential toxic effects onto corals of secondary nanosized fiber, while much of the efforts till now has been focused onto primary NPs with the exposure studies carried out by employing particles directly provided by plastic vendors and already manufactured at the nanoscale (Shen et al., 2019). Specifically for corals and weathered nanoplastics, only one papers is available reporting the effects of different concentrations of weathered polypropylene particles, with a size of about 0.22 µm on gamete fertilization, embryo development, and larval settlement of the reef-building coral *Acropora tenuis* (Berry et al., 2019). Another previous study was carried out by Marangoni et al. 2022 by exposing the coral *Stilophora pistillata* at the concentration of 0.5 mg/L of PS nanoplastic (20 nm size) for 4 weeks. These authors observed that PS-NPs impaired to oxidative stress in the coral host tissue and significant bleaching. In both the cases no mortality was observed as in our trials.

Overall, our results indicates that *P. flava* displays signs of negative impact at the physiological level already after 2h of exposure to secondary polypropylene nanofibers, and all the considered endpoints shows a direct correlation with concentration. This is in line with previous studies with Alcyionacea displaying increases in mucus production with increasing exposure to organic pollutants, both in terms of concentration and time (Turner et al., 2016). Furthermore, it was frequently observed that low concentrations of toxic substances caused a slower increase of mucus production, while higher concentrations resulted in copious mucus production already in the early stage of exposure (Turner et al., 2021).

Noteworthy, we observed a continuous secretion during the 72h of exposure time while this previous studies with organic molecules showed an initial peak of mucus production and then a decrease, that may be interpreted by the activation of a detoxification mechanism. It must be pointed out that, differently from several organic pollutants that have known receptors and well explained toxicity mechanism, for nanoplastics the elucidation at the molecular level of the mechanisms of interaction with biological targets must be considered underway. Moreover, conventional contaminants are present in aquatic environments in the form of dissolved molecules or ions, that are described in deep by thermodynamics, using well known molecular descriptors (*e.g.* partition coefficients) while nanoparticles are colloids and aggregation and stability play a pivotal role in determining their transport and fate.

Since in our experiments the increase of exposure time led to a clear increase in the stress but no mortality, it can be hypothesized that experiments with exposure time longer than 72h might

highlight effect to the survivorship of corals. The absence of mortality observed in our experiment is in line with the results reported in most of the previous short-term exposure assays with nanoplastics conducted on marine organisms of different phyla where mortality was not a predominant effect (Gonçalves and Bebianno, 2021). On the other hand, literature reports that some organisms may be very sensitive to nanoplastics exposition. This underlines how the response may vary among different phyla and different species and the importance of specie-specific studies (Piccardo et al., 2020). For instance, a mortality up to 83% of all the individuals after 2 of days of exposure was observed in *Daphnia Galeata* exposed to PS nanoplastic at the concentration of 5 mg/L (Cui et al., 2017). For cnidarians, it is reported that these organisms can ingest nanoplastics and absorb them within their tissues (Gagné et al., 2019). Mortality was reported with the freshwater cnidarian *Hydra viridissima* at the concentration of 40 mg/L of Poly(methyl-methacrylate) NPs reached 60% after 69h of exposure (Venancio et al., 2021)

Other previous research focused with microparticles and confirmed that PP might be found inside the mesenterial tissue within the coral gut cavity and have a negative impact on the health status of coral (Krishnakumar et al., 2021; Hall et al., 2015). More specifically, Corinaldesi et al., 2021 showed that fragments of the octocoral *Corallium rubrum* after a long-term (14 days) exposure of a mixture of MPs (mixture of particles with dimensions ranging from 20 to 100 µm, including PP) presented more than 50% of necrotic tissue and significant reduction of the feeding activity. No mortality was also assured by a five month aquarium-based experiment carried out with specimens of four coral species (*Acropora valida, Montipora capricornis, Pocillopora damicornis and Seriatopora hystrix*) exposed to high concentrations (ca. 0.5 g L-1) of polyethylene terephthalate (PET) microplastic particles (< 500 µm) carried out by Hierl et al., 2021.

In summary, our results are in line with previous results obtained by exposition of corals and other cnidarians to nano and microplastic, with toxic effect displayed at concentration that that are more than 3 orders of magnitude greater than the concentration estimated in the marine environment (Lenz et al., 2016). Under this light, future research should focus on synergistic effects and examining if nanoplastics can affect the energetic status of the coral in the long term, especially during bleaching events when energy reserves are critical for the coral's survival, since it is advised that in future, corals will have to endure increasingly prolonged and intense thermal stress, and any amount of energy wasted could be significant (Van Hooidonk et al., 2016).

As microplastic pollution is predicted to increase by 50 times by 2100 (Everaert et al., 2018), a parallel concentration increase is expected for nanoplastics as well, and especially secondary nanoplastics derived by the breakdown and weathering of microplastics. Consequently, given the ecological importance of coral reefs globally further studies should focus on the effect of secondary nanoplastics combined with other environmental stressors, such as the increase in water temperature

In more general terms, it should be considered that if for primary particles ecotoxicological information are general more readily available because international regulatory bodies require testing before being placed on the market (Allan et al., 2021), however for secondary particles, a set of new toxicological information is required. It is in fact expected that secondary particles do not retain the physicochemical characteristics of the original material (e.g. they change in shape, size and surface properties) and therefore they may also display a different potential toxic effect (Boucher and Friot, 2017; Alimi et al., 2018). For instance, the photo-aged nanofibers used in our test are expected to be relatively polar and more prone to interact with the organism compared to their non-weathered and larger counterparts. Moreover, their higher mobility as colloidal dispersion it is expected to facilitate the nanofiber migration through the media and their internalization in tissues because small enough to cross epithelial barriers. Under this view, current research should aim to provide toxicological assessment of secondary micro and nanoplastic, *e.g.* by employing in the assays materials submitted to accelerated weathering that properly mimic the processes encountered by the plastic material in the environment (Jahnke et al 2017). In case of knowledge gaps and limitations it is envisaged that regulatory body should work according to the Precautionary Principle (Lanzarote Declaration, Micro2022).

## 3.7. Conclusion

In conclusion, the study showed that 72h exposure of *P. flava* fragments to polypropylene nanofibers do not cause mortality, but the intermediate concentration of 1.0 mg/L was sufficient to produce significant adverse effects onto the behaviour and physiology of the coral as retraction of polyps, production of mucus and tissue bleaching. Concentration-dependent effects were observed with the highest concentration (10 mg/L) showing the most severe effects. Considering all the behavioural and physiological parameters surveyed in the study, NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect

concentration) on *P.flava* fragments after 72h of exposure resulted 0.1mg/L and 1 mg/L, respectively. These concentrations are relatively higher than those currently found in coral reefs but underline how nanofibers may act as potential stress factor in these habitats. Basing on precautionary principle and considering the predicted increase of plastic pollution in the next years, this study highlights once more that mitigating plastic release is of uttermost importance to conserve marine biodiversity. The strategies for managing the potential impact of current and future sources of secondary nanoplastics in marine environments requires a proper knowledge of photooxidative stability and fragmentation pathways of plastic material. Under this view, the accelerated weathering set up employed in this study for the preparation of the secondary nanofibers and their testing, may represent a reference model for future ecotoxicity assays onto different marine organism.

## 3.8. Acknowledgements

We would like to sincerely thank Dr. Fabio Mattioli and the tropical department of Acquario di Genova for their useful technical and logistical support.

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## 3.10. Illustrations

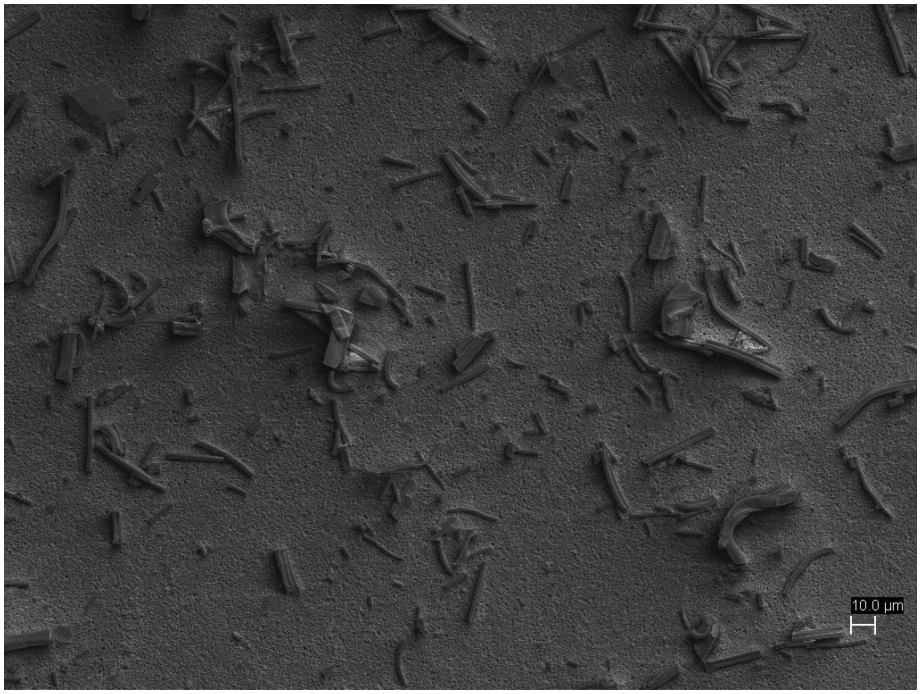



Figure 1. SEM image of the photo-degraded polypropylene microfibers before preparation of the nanofibers stock solution.

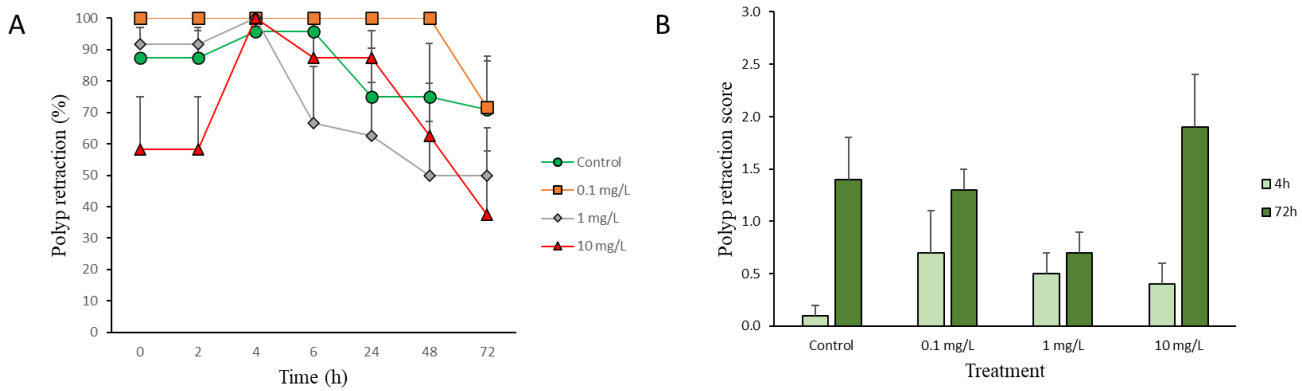



Figure 2. Polyps' retraction in function of secondary nanoplastic concentration in *P. flava*. (a) Average value of polyps' retraction expressed in terms of percentage, through time. (b) Semi quantitative score of polyps' retraction in control and exposure batches after 4 and 72 h.

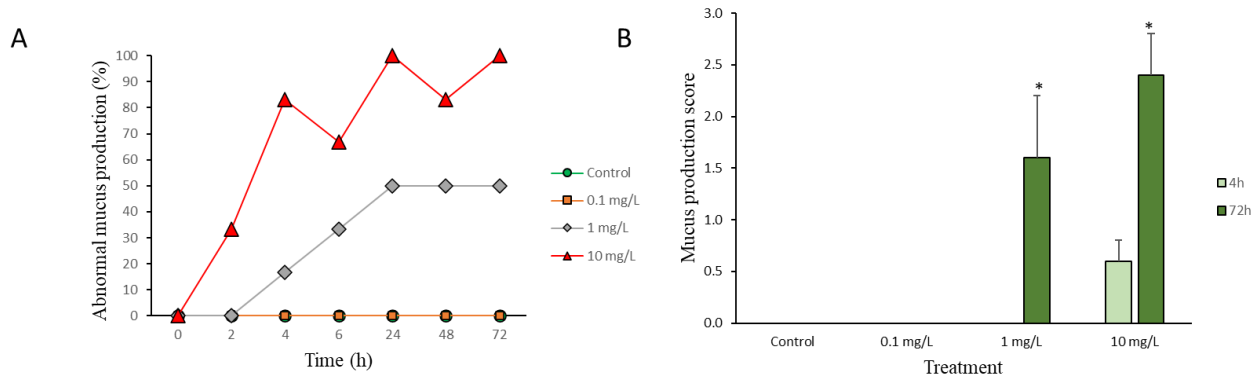



Figure 3. Abnormal mucus production in *P.flava*. (a) Percentage of fragments showing mucus production. (b) Average values of the semi-quantitative score of mucus production according to the protocol adopted by Renegar et al. 2017 (Asterisk indicates a significant difference in respect to the control).

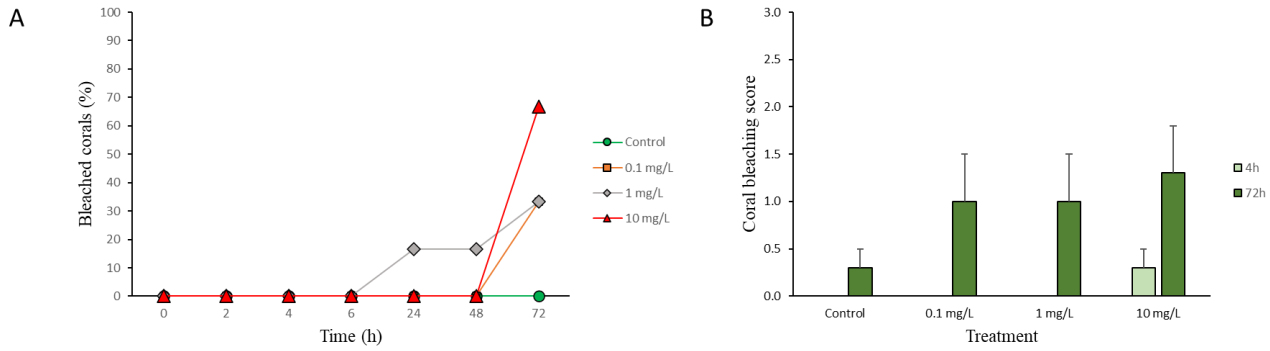



Figure 4. Occurrence and quantification of tissue bleaching in *P..flava*. (a) Percentage of fragments showing evidence of bleaching. (b) Semi-quantitative score of coral bleaching according to the protocol adopted by Renegar et al. 2017.

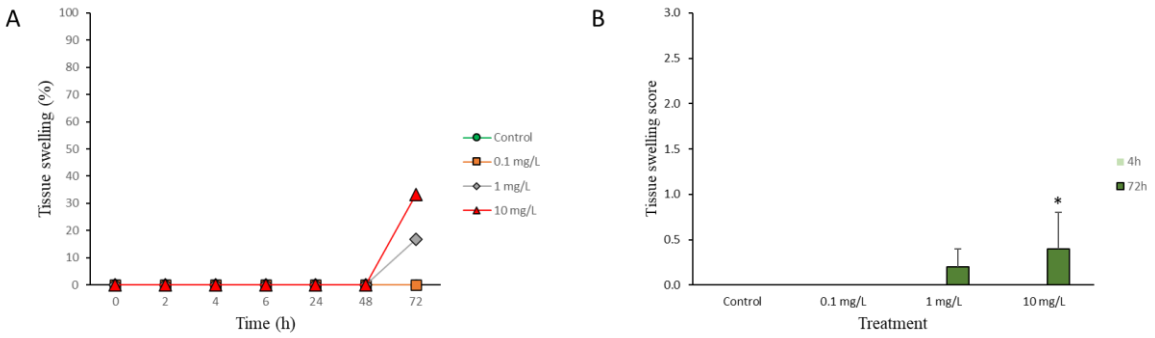



Figure 5. Polyps' tissue swelling. (a) Percentage of polyps showing tissue swelling in the function of the time and treatment. (b) Semi-quantitative score of tissue swelling in the function of the time and treatment asterisks show significance differences in respect to the control.

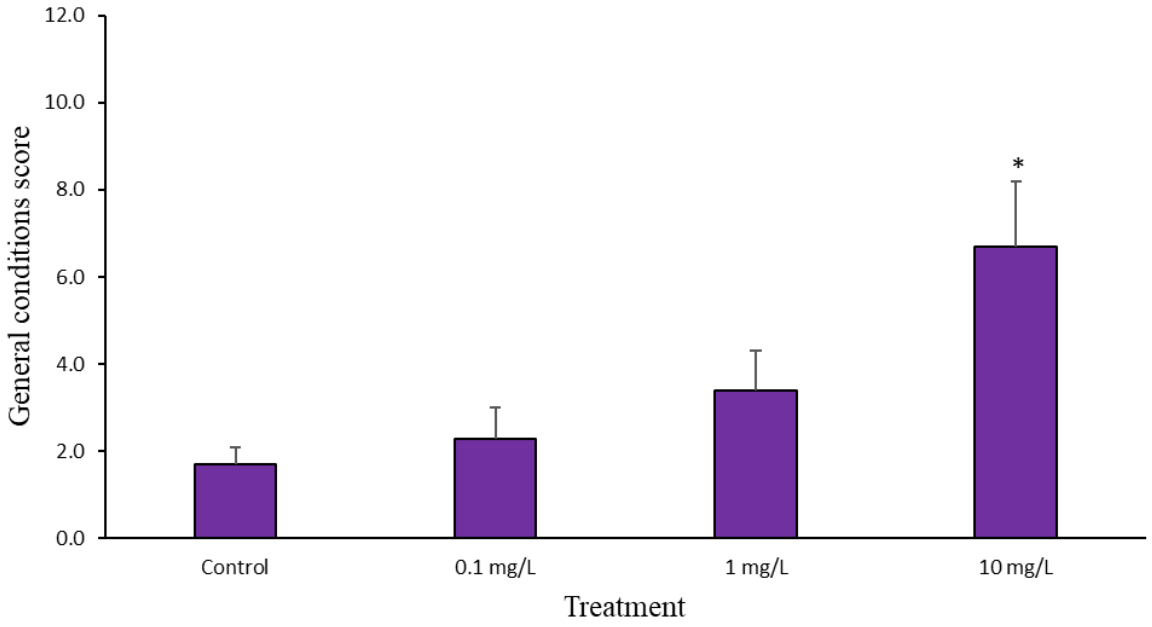



Figure 6. General health status of *P.flava* fragments after 72h of exposure. For each coral fragment, general health condition was calculated by adding the semi-quantitative score of the individual parameters (polyps' retraction, mucus production, tissue bleaching and swelling). The asterisk indicates significant differences with respect to the control.

## 3.11. Supplementary

SUPPLEMENTARY

S1 Description of the Genoa Aquarium water uptaking and maintenance of coral colonies

The coral colonies sampled come from the tanks of the Genoa Aquarium, where the water system collects seawater from 200m outside the Foranea dam of the port of Genoa at 50m depth. The collected water is pumped through the filtration system made of 2 sand filters and one UV filter, used for disinfection. After the filtration, the seawater is stored inside 4 accumulation tanks (200m^3 each). If the results of the analyses show that the chemical-physical parameters (Ph, salinity, ammonia, nitrites, nitrates, and phosphates) are optimal for the aquarium, the seawater of one accumulation tank is pumped into a mixing basin, where the water is kept in constant motion. After further UV filtration, the water is pumped from the mixing basin to all the tanks of the aquarium. During the day from 8:00 to 16:00, the water is pumped from the mixing basin to the aquarium tanks with a flow of 1 liter every 30 seconds, so the tanks are considered as a semi-open system (the tanks are considered as a closed system from 16:00 to 8:00). In the tanks, used for the sampling (3x1x0,7 m, 3100 l, composed by acrylic and glass resin), the water is uptaken by a pump (Astralpool, Victoria Plus) with a 24-hour flow rate of 8m^3/h (to ensure complete water change every about 30 minutes) and reinserted into the tank after passing through the filtration system. The filtration system is composed of a sand filter (Astralpool Artic, filtering particles from 0.4 to 2mm) and a UV filter (Panaque 750 s AB 4 lamps of 40W). The water passage through the UV filter is instantaneous since water passes with a flow equal to $8m^3$ per hour. 2 l of water containing a solution of the algae *Tetra selmis* and zooplankton belonging to the Phylum *Rotifera* (the average concentration of zooplankton is 250 individuals / mL and the average dimension is 0,5mm) are placed daily inside the tanks in order to feed the corals. Both Algae and zooplankton are farmed inside 80L cylindrical tanks made of plexiglass. Furthermore, twice a week 20g of food mixture are daily in the tanks to feed the corals, this mixture is composed of 70% of silverside fishes (5cm in length) and 30% carrots, while the next day the mixture is composed by 70% of mussels and 30% of courgettes. In order to facilitate the calcification of the coral skeleton 50l of water containing 500g of calcium hydroxide.

Table S1 Water parameters analysis in each exposure chamber before and after each treatment

| **10 mg/L** | **Time [h]** | **pH** | **Salinity** | **$NH_4^+$** | **$NO_2^-$** | **$NO_3^{2-}$** |
|---|---|---|---|---|---|---|
| 1 | 0 | 7,9 | 35,3 | 0 | 0 | 0 |
| | 72 | 7,7 | 35,9 | 0 | 0 | 0 |
| 2 | 0 | 7,9 | 35,3 | 0 | 0 | 0 |
| | 72 | 7,7 | 36,2 | 0 | 0 | 0 |
| 3 | 0 | 7,9 | 35,3 | 0 | 0 | 0 |
| | 72 | 7,7 | 36,0 | 0 | 0 | 0 |
| 4 | 0 | 7,8 | 36,0 | 0 | 0 | 0 |
| | 72 | 7,8 | 35,9 | 0 | 0 | 0 |
| 5 | 0 | 7,9 | 35,7 | 0 | 0 | 0 |
| | 72 | 7,9 | 35,6 | 0 | 0 | 0 |
| 6 | 0 | 7,7 | 35,4 | 0 | 0 | 0 |
| | 72 | 7,8 | 35.5 | 0 | 0 | 0 |

| **1 mg/L** | **Time [h]** | **pH** | **Salinity** | **$NH_4^+$** | **$NO_2^-$** | **$NO_3^{2-}$** |
|---|---|---|---|---|---|---|
| 1 | 0 | 8,12 | 36,6 | 0,03 | 0,00 | 0 |
| | 72 | 7,96 | 37,2 | 0,04 | 0,01 | 0 |
| 2 | 0 | 8,12 | 36,6 | IIS | 0 | 0 |
| | 72 | 7,9 | 37,1 | IIS | 0 | 0 |
| 3 | 0 | 8,08 | 36,5 | 0,03 | 0,00 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 72 | 7,96 | 37 | 0,04 | 0,01 | 0 |
| 4 | 0 | 8,12 | 36,5 | 0,03 | 0 | 0 |
| | 72 | 7,8 | 37,2 | IIS | 0 | 0 |
| 5 | 0 | 7,9 | 35,3 | 0 | 0 | 0 |
| | 72 | 7,9 | 36,3 | 0 | 0 | 0 |
| 6 | 0 | 7,9 | 35,3 | 0 | 0 | 0 |
| | 72 | 7,8 | 36,2 | 0 | 0 | 0 |

| 0.1 mg/L | Time [h] | pH | Salinity | $NH_4^+$ | $NO_2^-$ | $NO_3^{2-}$ |
|---|---|---|---|---|---|---|
| 1 | 0 | 8,17 | 36,9 | 0,013 | 0 | 0 |
| | 72 | 8,03 | 37,4 | 0,01 | 0 | 0 |
| 2 | 0 | 8,19 | 36,6 | 0,014 | 0 | 0 |
| | 72 | 8,07 | 36,9 | 0,01 | 0 | 0 |
| 3 | 0 | 8,22 | 36.3 | 0,01 | 0 | 0 |
| | 72 | 8,02 | 36,9 | 0,012 | 0 | 0 |
| 4 | 0 | 8,11 | 36.5 | 0,01 | 0 | 0 |
| | 72 | 8,03 | 36,7 | 0,01 | 0 | 0 |
| 5 | 0 | 8,16 | 36,5 | 0,01 | 0 | 0 |
| | 72 | 8,08 | 36,7 | 0,01 | 0 | 0 |
| 6 | 0 | 8,15 | 36,0 | 0,15 | 0 | 0 |
| | 72 | 8,07 | 36,6 | 0,015 | 0 | 0 |

| Control | Time [h] | pH | Salinity | $NH_4^+$ | $NO_2^-$ | $NO_3^{2-}$ |
|---|---|---|---|---|---|---|
| 1 | 0 | 8,23 | 36,6 | 0,01 | 0 | 0 |
| | 72 | 8,04 | 37,1 | 0,016 | 0 | |
| 2 | 0 | 8,23 | 36,6 | 0,01 | 0 | 0 |
| | 72 | 8,04 | 36,7 | 0,014 | 0 | 0 |
| 3 | 0 | 8,23 | 36,6 | 0,01 | 0 | 0 |
| | 72 | 8,08 | 36,8 | 0,013 | 0 | 0 |
| 4 | 0 | 8,09 | 36.7 | 0,01 | 0 | 0 |
| | 72 | 8,02 | 36,5 | 0,01 | 0 | 0 |
| 5 | 0 | 8,13 | 36,4 | 0,01 | 0 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 72 | 8,05 | 36,7 | 0,01 | 0 | 0 |
| 6 | 0 | 8,15 | 36,3 | 0,02 | 0 | 0 |
| | 72 | 8,12 | 36,6 | 0,02 | 0 | 0 |

Table S2 Criteria for scoring coral condition characteristics**.** Scores for color, polyps, mucus, and tissue are assigned to each coral. From Renegar et al. (2017).

| Diagnostic Criteria | Range |
|---|---|
| Color | • 0 (normal): color appears normal<br>• 1 (mild): slight lightening of coloration<br>• 2 (moderate): moderate lightening of coloration<br>• 3 (severe): significant lightening of coloration, evident bleaching |
| Polyps | • 0 (normal): fully extended or loosely retracted<br>• 1 (mild): retracted and slightly closed<br>• 2 (moderate): evident polyp retraction with full polyp closure<br>• 3 (severe): polyps tightly retracted |
| Tissue swelling | • 0 (normal): no swelling<br>• 1 (mild): slight coenenchyme swelling and/or polyp distension<br>• 2 (moderate): moderate coenenchyme swelling and/or polyp distension<br>• 3 (severe): severe swelling of coenenchyme and/or polyp distension |
| Tissue attenuation | • 0 (normal): no attenuation<br>• 1 (mild): slight thinning of coenenchyme, flattening of polyps |

| | |
|---|---|
| | • 2 (moderate): moderate thinning of coenenchyme and polyp flattening<br>• 3 (severe): severe tissue thinning, skeletal ridges exposed |
| Mucus production | • 0 (normal): normal mucus production; no mesenterial filaments apparent<br>• 1 (mild): slightly elevated mucus production, no mesenterial filaments apparent<br>• 2 (moderate): moderately elevated mucus production; mesenterial filament extrusion possible<br>• 3 (severe): mucus sheets evident; possible mesenterial filament extrusion |

Figure S.1 Different conditions of Polyps retraction

Figure S2. Different conditions of abnormal mucus production.

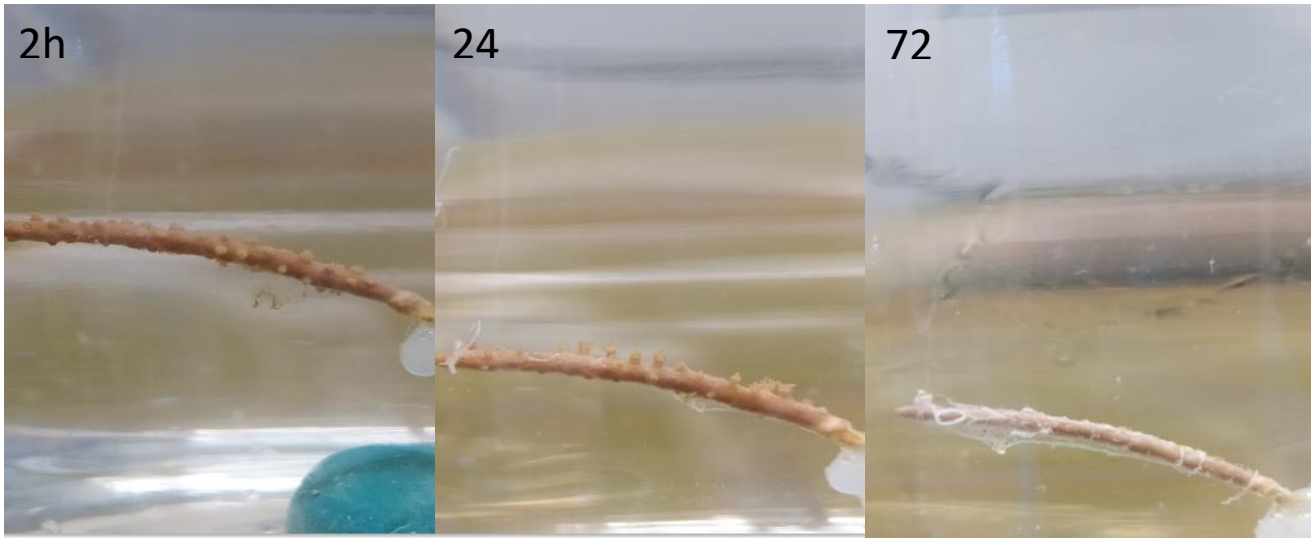



Figure S3. Different conditions of tissue bleaching

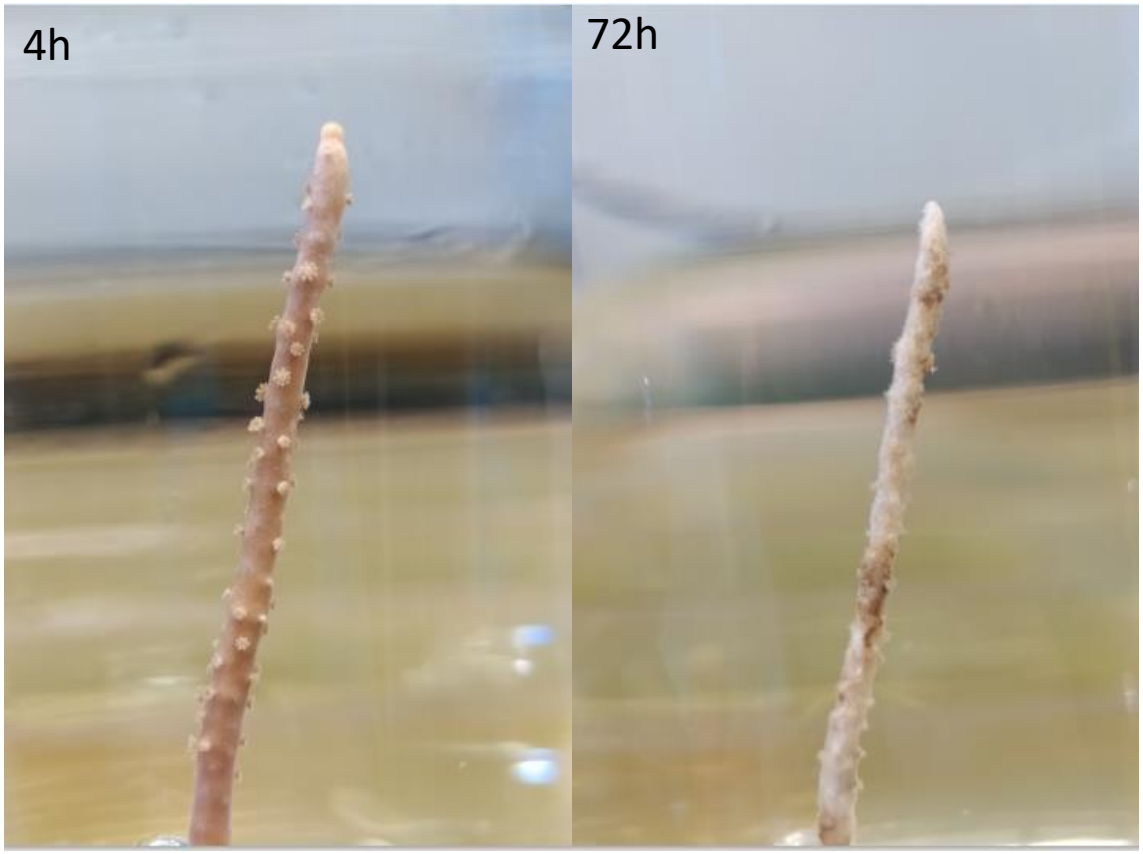


Figure S4. Boxplots of the assessed parameters

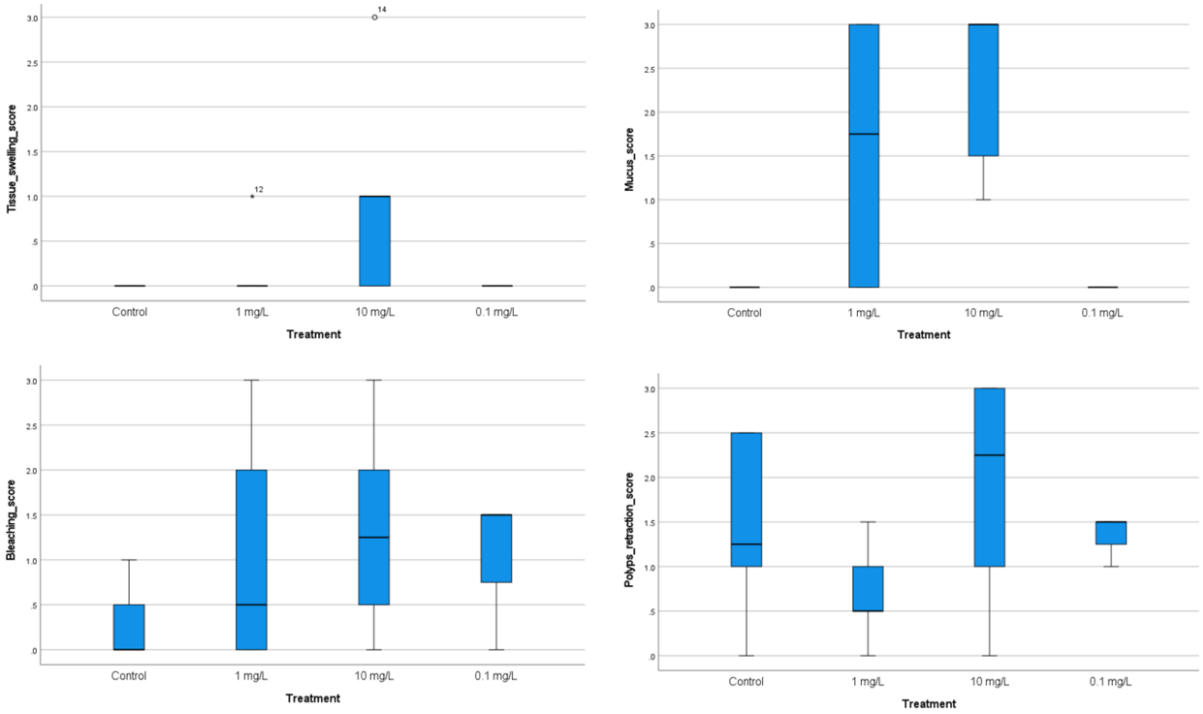


Figure S5. Boxplot of the general health status according to the scoring system standardized by Renegar et al. 2017.

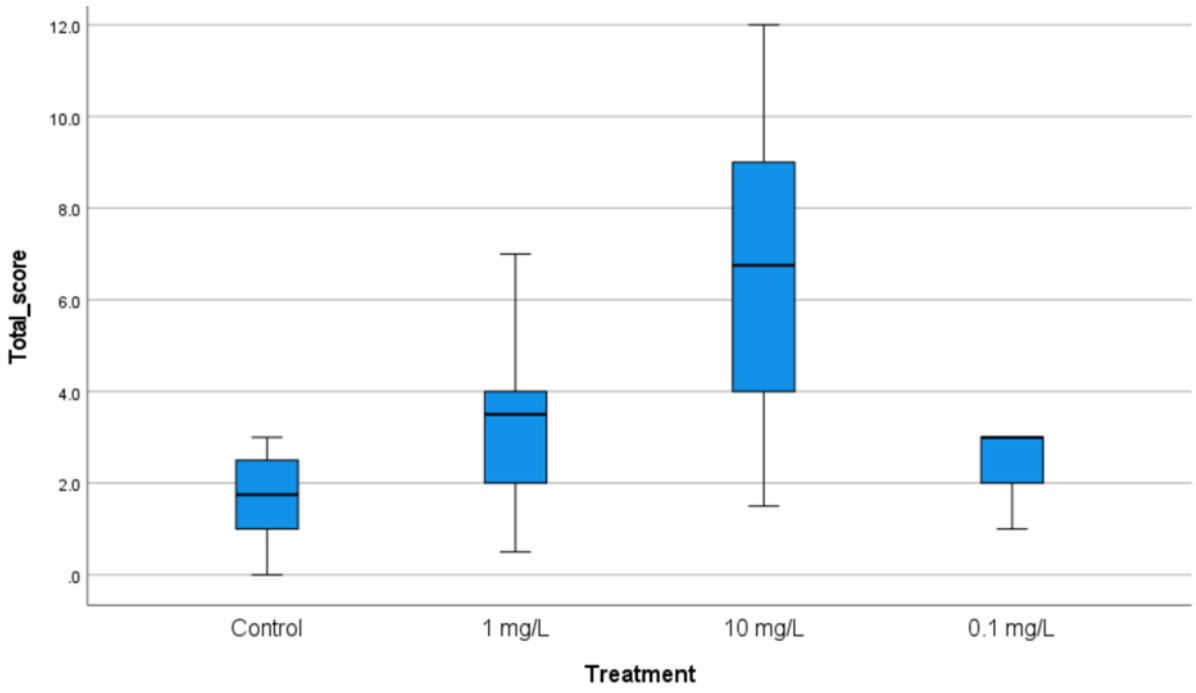
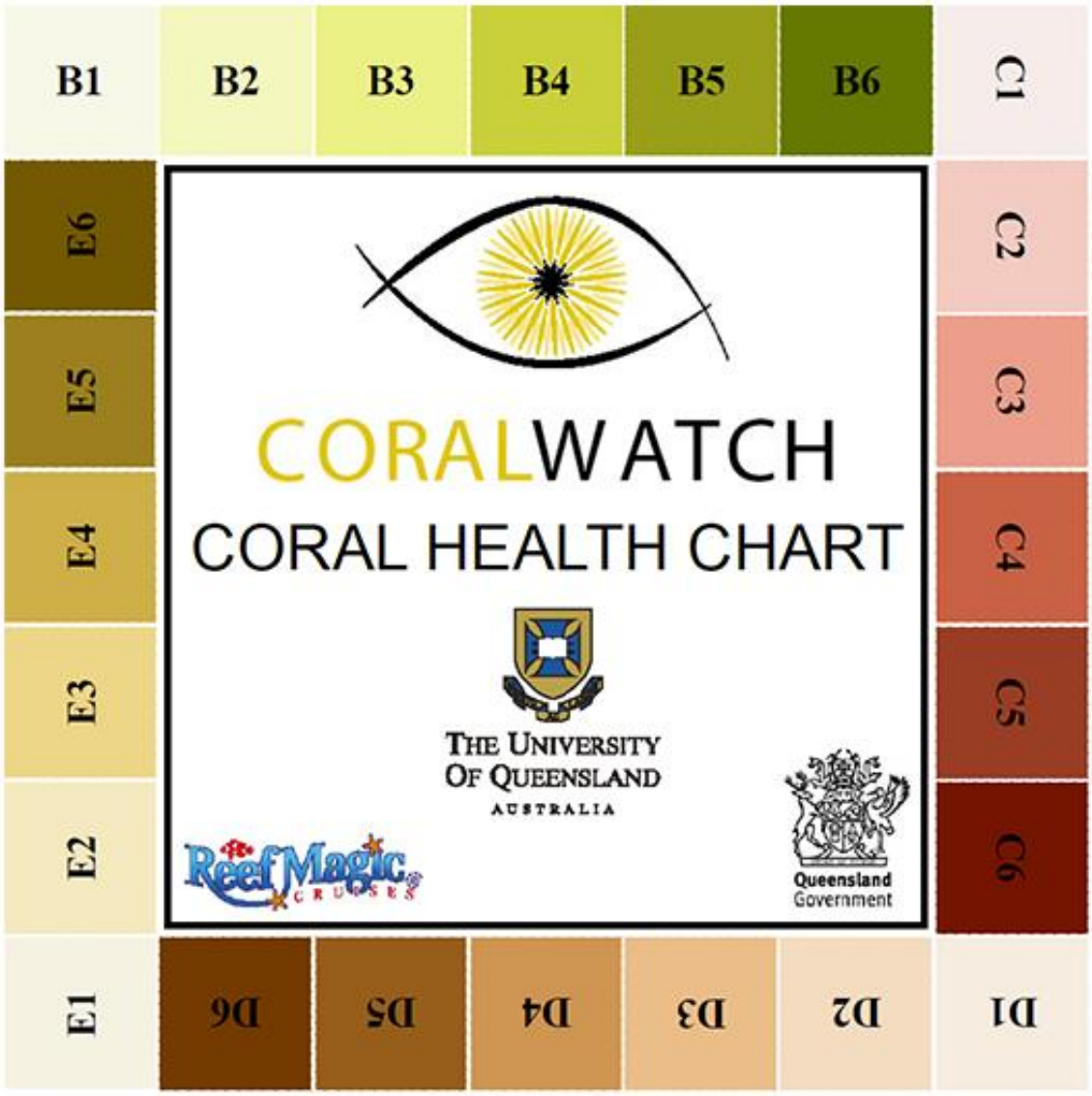


Figure S6: Coral colour chart used for quantifying the changes in colour arising from changes in the density of zooxanthellae and photosynthetic pigments. Developed university of Queensland (2002)



# CHAPTER 4

## 4.1. Combined effect of environmentally relevant microplastic concentration and thermal stress on the tropical coral *Pocillopora damicornis*

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Keywords: Microplastic, thermal stress, coral bleaching, , oxidative stress, gene expression, *Pocillopora damicornis*

*This section is inserted as submitted in Aquatic Toxicology (Second round)*

## 4.2. Abstract

Microplastic pollution is an emerging threat to coral reefs, which are already challenged by global warming. Currently, the effects of microplastic pollution combined with global warming are poorly studied. It has been demonstrated that scleractinian corals ingest microplastic such as Polyethylene. However, little is known about how these microparticles impair scleractinian corals at the physiological and molecular levels at environmentally relevant concentrations. In this study, we studied the cumulative effects of these two environmental threats, on the ecologically important reef-building species*: Pocillopora damicornis*. Therefore, fragments of *P.damicornis* were exposed to different concentrations of polyethylene microbeads, at an e environmentally relevant level (200 microbeads/L) and at a high concentration (2000 microbeads/L) at ambient temperature (25°C) and in combination with thermal stress (30°C). We found microplastic adhered to P. *damicornis*, and was the coral ingested and also egested microplastics at the environmentally relevant concentration. No significant difference was observed in microplastic ingestion among treatments. However, thermally stressed corals had higher microplastic egestion rates at high microplastic concentrations. We also found that with increasing microplastic concentration and temperature stress, coral bleaching increases, as indicated by a general decrease in chlorophyll concentration and Symbiodinaceae density. At cellular and molecular level, we found that microplastic exposure in combination to high temperature led to an increase in oxidative stress and expression of the stress response gene*shsp70* and *cas3*. Furthermore, this study highlights that mitigating ocean warming remains of uttermost importance to conserve coral reefs while managing the emergence of new threats like microplastic pollution.

## 4.3. 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). These stressors are driving the global decline of coral reefs and impacting the ecosystem services that coral reefs provide to tens of millions of people worldwide (Moberg and Folke, 1999). Among these anthropogenic stressors, global warming is viewed as the dominant threat to coral reefs. Model projections forecast that more than 75% of coral reefs worldwide will be subjected to annual severe bleaching events by 2070 (Van Hooidonk et al., 2016). The fate of coral reefs might be worsened by emerging stressors such as microplastic pollution (Ban et al., 2014). The interaction between corals and microplastic occurs in different ways; ingestion (Axworthy and Padilla-Gamiño, 2019, Allen et al., 2017), egestion (Reichert et al., 2018), and surface adhesion (Martin et al., 2019). Microplastic ingestion has been observed in scleractinian corals and alcyionacean (Vencato et al., 2021;). Recent studies suggest that microplastics (plastic particles or fibers <5 mm), negatively affect coral health (Huang et al., 2020). Exposure to high microplastic concentrations has been reported to adversely impact the growth rate and the physiological status of corals, change the feeding behavior, decrease in photosynthetic performance, and skeletal calcification, leading to coral bleaching, and necrosis (Huang et al., 2020). At the cellular and molecular level, high concentrations of microplastic cause oxidative stress, apoptosis, and heat shock response in tropical corals (Montalbetti *et al.*, 2022). The temperate corals *Corallium rubrum* showed preferential ingestion of microplastic compared to natural prey, leading to feeding impairment, mucus production, and altered expression of genes involved in key metabolic processes (Corinaldesi et al., 2021).

Though the effect of microplastic on corals is increasingly being studied, yet few studies have examined the interactions of this emerging threat and other anthropogenic stressors. 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 (Xiao et al., 2021) and a decrease in enzyme activities (such as pyruvate kinase, Na, K-ATP, glutathion) in the coral *Tubastrea aurea* (Liao et al, 2021). Concerning the combined effect of microplastic and thermal stress, Reichert et al., (2021) found that microplastic has minor cumulative effects on thermally stressed *Acropora muricata, Montipora digitata, Porites lutea, Pocillopora verrucosa*, and *Stylophora pistillata*. The authors also reported that in comparison to heat stress alone, microplastic exposure had only a minor negative effect on

coral thermal tolerance regardless of particle concentration type and shape.They further highlight that the observed effects of microplastic were species-specific. Axworthy *et al.*, (2019) also found that the combined effect of microplastic and heat can vary between coral species depending on the affinity of different species to ingest microplastic (mouth size & microplastic size) and the differential response of corals to thermal stress.

Moreover, corals are also capable of egesting microplastic, but this process has been less studied compared to microplastic ingestion. There is currently a knowledge gap on the egestion of microplastics by reef-building corals (Alimba and Faggio, 2019). In order to obtain a more complete overview of how corals respond to the exposure of microplastics, it is also necessary to study, in addition to ingestion also the egestion of microplastics, which usually occurs within the first 24 hours (Chen et al., 2022). Furthermore, to date, there are no studies that evaluate the egestion of microplastics by thermal stressed corals.

In general, microplastic concentrations used in previous laboratory studies are usually two orders of magnitude higher than the environmental ones (Hall et al., 2015; Jiang et al; 2020, Chen et al., 2022). To fulfil this lack, interaction studies between microplastics and aquatic organisms should be carried out at more realistic concentrations at the environmental level (Corona et al., 2020). For instance, a recent study by Sun et al. 2021 demonstrated that the microplastic concentration threshold to have irrelevant effects at the environmental level is 1 mg / L.

Therefore, in this study, we exposed *Pocillopora damicornis* to polyethylene microbeads, at an environmentally relevant level (200 microbeads/L corresponding to 1 mg/L of the microbeads used for this study) and at a high concentration (2000 particles/L or 10 mg/L), at ambient temperature (25°C) and at elevated temperature (30°C). We visually assessed the production of mucus and the extroflection of the coral polyps.

Furthermore, we quantified the adhesion, ingestion, and egestion of microbeads to evaluate the physical interaction of microbeads with *P.damicornis* under different conditions. We also measured coral bleaching by measuring the concentration of chlorophyll and Symbiodiniaceae density. Furthermore, in order to improve our understanding of the physiological and cellular response of coral we measured Lipid Peroxidation (LPO) to assess oxidative damage in coral tissues. We also measured the expression of *caspase 3* (*cas3*) gene a biomarker of apoptosis (Yu *et al.* 2017) and *heat shock protein 70* (*hsp70*), a widely used stress response biomarker (Louis *et al.*, 2017).

With the Indian Ocean and North Pacific Ocean reported to have the largest amount of plastic (Eriksen et al, 2014) and heat stress episodes increasing in frequency and intensity, it is essential to understand how these two stressors interact to impact the reef-building scleractinian coral *P.damicornis*, a widely distributed species in the Indo-Pacific region, and model species commonly used in climate change studies. Such information is crucial for the effective management and conservation of tropical corals.

## 4.4 Materials and methods

At the Aquarium of Genoa, 72 *P. damicornis* nubbins, with an average surface of 46,7 ±21.1 $cm^2$ were sampled from six different colonies. The nubbins were promptly fixed on supports made of two-component epoxy resin. Subsequently, they were transferred to two 50 L experimental tanks for 2 weeks of acclimatization at 25◦C and a light period 0f 11h:13h. The irradiation was equal to 250 PAR (µmol photons* $m^2$/s).

### 4.4.1 Experimental set up

Each nubbin was transferred inside L-capacity glass beakers (interaction chamber), filled with 1.5 L of filtered seawater. Inside each interaction chamber, an air pump was used to keep microplastic beads in constant motion (Martin et al. 2019). Nubbins (n =12) were randomly assigned to the two different treatments consisting of two different concentrations of polyethylene (PE) microbeads. The microplastic beads size range was 180-212 µm and the density was equal to 0.98 g/$cm^3$(Cospheric LLC). PE has been chosen since is one of the most common types of plastic present in the marine environment (Steinberg et al. 2020). In the first treatment, (Figure 1) the concentration of microplastic was equal to 200 microbeads/L-1 (Low [MP]) (environmentally relevant concentration). In the second treatment, inside each chamber, the microplastic concentration corresponded to 2000 microbeads L-1 (high concentration, High [MP]). Experiments were performed at 25 ◦C and at 30 ◦C for 72 hours. Moreover, a control treatment without microplastic beads was carried out at 25 ◦C and 30 ◦C. PE concentrations were chosen based on previous experiments on scleractinian and button corals (Hall et al. 2015, Jiang et al. 2020).

### 4.4.2. Visual signs of stress: mucus production, polyps' extroflection and tissue necrosis

Nubbins were constantly monitored at 0, 2, 4, 6, 12, 48, and 72h of exposure. We assessed the presence of abnormal mucus produced by each fragment. Abnormal mucus production is defined as the occurrence of mucus filaments streaming off the polyps' mouth as reported in Turner *et al.* (2020) and in Vencato *et al.* (2021).

Moreover, the degree of polyps' extension was evaluated according to the surface of the coral, and we assessed the presence of necrotic tissue. All these parameters were monitored by visual inspection following the procedure described by Renegar and Turner. (2021).

### 4.4.3. Sampling for laboratory analysis

After 72 hours of exposure, two fragments (1-2 cm) were sampled from six random nubbins. One fragment was placed in a tube containing RNA-later (Qiagen) and kept at room temperature for 24 hours and then stored at -80 °C. The second sample was immediately stored at -20 °C. Sample placed inside RNA-later were used to for the gene expression analysis. The other sample was used to measure the density of Symbiodiniaceae and the concentration of chlorophyll. The rest of the nubbin was used to quantify microplastic bead adhesion, ingestion, and egestion.

### 4.4.4. Microplastic bead adhesion, ingestion and egestion quantification

The physical interaction between microplastic and *P.damicornis* has been evaluated by counting the number of PE microbeads that were adhered to the coral surface, ingested by the nubbins, and subsequently, the number of egested microbeads has been counted and reported in percentage.

*Adhesion to the coral surface*

All twelve nubbins were used for the quantification of microplastic beads adhered. The nubbins were removed from their chambers and carefully rinsed with saltwater. The number of microbeads adhered to the coral exterior surfaces was counted under a stereomicroscope (M125

C, Leica). Microplastic beads were classified as “adhered” when they were found attached to the corals’ exterior surface, outside of the polyps’ mouth.

*Ingestion*

Six nubbins per treatment were used to evaluate microplastic beads ingestion. Microbeads found inside the polyps’ mouths were counted 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 a stereomicroscope to count all microplastics ingested. ingested” microbeads are hence the sum of microbeads found inside the polyps’ mouths and microplastic beads observed inside the corallites of the bare skeleton following the complete dissolution of each *P. damicornis* living tissue.

*Egestion*

The remaining 6 nubbins, once they were thoroughly rinsed and checked, were placed in new interaction chambers where there were no microbeads. the fragments remained in the chambers for a further 24 hours with the aim of measuring the egestion of microbeads. Subsequently, the water of the feeding chamber was filtered with a nylon sieve (50-micron mesh size) to count all the microbeads released by the nubbins. Finally, the fragments were treated according to the same procedure used for ingestion to count any microbeads not egested, therefore the sum of the microbeads found in the water and those still found inside the fragment correspond to those ingested before (in this way we evaluated the number of ingested microbeads for all 12 fragments) the change of water then at the end of 72h.

## 4.4.5. Coral bleaching measurement

The combination effect of microplastic and heat stress has been assessed by the quantification of chlorophyll a, chlorophyll c2, and the density of symbiodinaceae present inside the coral tissue. In order to perform this analysis one gram of sample has been collected, and then stored at -20°C, from each nubbin.

*Quantification of chlorophyll a and c2*

Chlorophyll concentration was determined by stripping off coral fragments using a stream of compressed air 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 hours in the dark at 4 °C. Following extraction, the sample was re-centrifuged at 3600 g for 4 min. The supernatant was used to determine concentrations of chlorophyll *a* and *c2* from the fluorescence measured at 630, 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 (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.

*Symbiodinaceae density*

Subsequently, symbiodinaceae cells were enumerated from six independent hemocytometer (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).

## 4.4.6. Molecular analyses

The oxidative stress induced by microplastic exposure and heat stress was assessed by the quantification of lipid peroxidation (LPO) level, which is an indicator of oxidative stress (Montalbetti et al., 2022), furthermore the expression of two genes of interest (GOI) *cas3* and *hsp70* has been evaluated to assess the response of *P.damicornis* at molecular level(Louis *et al.*, 2017).

*Lipid peroxidation*

Lipid peroxidation was measured by quantifying the malondialdehyde (MDA) contents using the MDA assay kit (Bioxytech LPO-586, Oxis International, United States). Frozen coral samples (approximately 1 g each) were ground with pre-chilled mortar and pestle and homogenised 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 (3,000 × 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. The blue product was quantified by measuring absorbance at 586 nm (Gérard-Monnier et al., 1998). Results are presented in µmol of MDA per µ g of proteins.

*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 frozen fragments, in a pre-cooled mortar, using filtered compressed air for a maximum of 3 minutes (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 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 presence of 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 10 min at 50 °C, PCR initial heat activation 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 expression of two GOI *cas3* and *hsp70* were quantified and the gene *elongation factor* (*ef*) was used as an internal control to normalise all samples. The PCR primers are given 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 gene expression was calculated using the the $2^{-\Delta\Delta Ct}$ formula (Livak & Schmittgen, 2001). Calculations were performed using the R based program qRAT (Flatschacher *et al.*, 2022), and results were reported as $\log_2$ fold-change in expression.

## 4.4.7. Statistical analysis

The chi-square test of homogeneity was used to assess significant differences on the occurrence of mucus production. The Shapiro-Wilk test was used to assess normality distribution of data. One-way ANOVA statistical test was performed to evaluate statistically significant differences. If the assumption of homogeneity of variances required by the One-way ANOVA was not met, the Kruskal-Wallis non-parametric statistical test was performed. All tests were performed using SPSS 28 (IBM).

For gene expression analysis, the significance of differences in Cq values between samples was calculated using qRAT (Flatschacher *et al.*, 2022). The program calculations were based on the R package limma (Ritchie *et al.*, 2015) and used moderated t-statistic for multiple comparisons. The p-value was adjusted by Benjamini and Hochberg's method to control the false discovery rate.

## 4.5. Results

### 4.5.1. Observation of mucus production, polyps' extroflection and tissue necrosis

Visual observation reported that heat *P.damicornis* nubbins produce more mucus when they are not thermally stressed, secondly, on average thermally stressed corals had more open polyps at 30 °C than at 25 °C, even though differences were not statistically significant.

*Mucus production*

Regarding the production of mucus, the highest values of occurrence were found at 25 °C at an environmentally relevant concentration of microplastic, with 33.3% of nubbins presenting mucus filaments with embedded microplastic beads (Figure 2). At 30 °C after 72 h, control fragments did not present mucus production. Moreover at 30°C results showed that at both microplastic concentrations only 8.3% of nubbins produced mucus filaments trapping PE microbeads. Fisher's test of homogeneity did not report statistically significant differences.

*Polyps' extrotlection*

After 7h hours, at 25°C on average, the highest percentage value of open polyps was reported in the treatment corresponding to the lowest concentration of microplastic, while at 30°C the highest value was found in the control treatment (Figure 3). The one-wayANOVA test did not report statistically significant differences.

*Tissue necrosis*

Tissue necrosis did not occur in all treatments performed at both 25 and 30°C.

### 4.5.2. Microplastic bead adhesion, ingestion and egestion quantification

Results show that after 72h of exposure at both concentrations, *P. damicornis* presented microbeads on the surface, and ingested PE microbeads, and the differences wew statistically significant (Kruskal-Wallis, p=0.009 for adhesion; Kruskal-Wallis, p=0.022 for ingestion). Furthermore *P. damicornis* egested microbeads at all the tested concentrations and temperatures.

*Adhesion*

At the end of the treatments, 56% of *P. damicornis* nubbins showed microbeads stuck to their surface. The highest adhesion value of polyethylene beads per coral fragment was observed in the treatment with the high concentration of microplastic at 25°C, with an average value 2.1 times higher than the one with the low concentration (Figure 4). At 30°C the highest number of adhered microbeads was found at high microplastic concentration, with an average value 5.8 times higher than low concentration. Differences between treatments at the same temperature were statistically significant only at 30°C (Kriskal-Wallis, p=0.018). By contrast between 25°C and 30°C, the difference of adhered microbeads regarding low concentration was statistically significant (Kruskal-Wallis, p=0.002), on the contrary for the high concentration of microplastic there was not a statistically significant difference in adhered microbeads.

*Ingestion*

*P. damicornis* ingested microbeads in all treatments. Under the stereomicroscope, most of the ingested microplastics were found inside polyps' mouths, while others entered the coral tissue and were detected after the dissolution with sodium hypochlorite. The highest microplastic concentration at 30°C reported the highest values of ingested PE beads per coral nubbin (Figure 5). Between high and low concentration performed at 25 and 30°C, the only statistically significant difference was found between low microplastic concentration at 25°C and the high concentration at 30°C (Kruskal-Wallis, p=0.007). Furthermore, the difference in ingestion between the same temperature but with different concentrations was statistically significant only at 30°C (Kruskal-Wallis, p=0.013).

*Egestion*

Results showed that *P. damicornis* was able to egest microplastic beads, the treatment with the highest value of egestion was the one performed at 30°C, With the highest microplastic concentration, which nubbins egested almost all the ingested microbeads (Figure 6). The difference in terms of egestion rate was statistically significant only for the highest microplastic concentration, in which at 30°C the egestion rate was significantly higher than the one at 25°C (Kruskal-Wallis, p=0.034).

### 4.5.3. Coral bleaching measurement

The combined effects of microplastic and heat stress caused tissue bleaching in *P. damicornis*, in fact our results show that an increase in microplastic concentration led to a decrease in chlorophyll concentration, and this decrease was more evident when corals were thermally stressed.

*Chlorophyll a concentration*

The combined effects of microbeads and temperature led to a decrease in the concentration of Chlorophyll *a* in *P. damicornis*, and this decrease was statistically significant (Kruskal-Wallis, p=0.02). Pairwise comparisons showed statistically significant differences between the Control and both exposure treatment performed at 30°C (p=0.004 and p=0.013). Furthermore, exposure to the low microplastic concentration, performed at 25°C was significantly different between both exposure treatments performed at 30°C (p=0.018 with low microplastic concentration, and p=0.044 with high microplastic concentration). Finally, the last important result was given by the significantly different concentration in chlorophyll a, between low microplastic concertation at 30°C and the highest microplastic concentration at 25°C (p=0.034) (Figure 6).

*Chlorophyll c2 concentration*

Values of chlorophyll c2 linearly decrease in line with the increase of PE microbeads concentration at 25°C, but the differences are not statistically significant. Our results show a similar trend for the treatments performed at 30°C (Figure 8). Overall, the decrease in chlorophyll c2 was statistically significant (One-Way ANOVA, p=0.023). Significant differences were found when comparing treatments with the same concentration of microplastic but performed at both 25°C and 30°C (One-Way ANOVA, p=0.03, p=0.042, p=0.028). The Tuckey post-hoc test showed significant differences between the control treatment with both low and high concentrations performed at 30°C (p=0.045 with the low concentration and p=0.03 with the high concentration.).

*Symbiodinaceae density*

The density of symbiodinaceae decreased with the increase of temperature and microbeads concentration, and this decrease was statistically significant (One-Way ANOVA, p=0.021). Surprisingly between the lowest and the highest concentration of microbeads the density of symbiodinaceae showed a slight increase but was not statistically significant (Figure 9). Tukey HSD post hoc test reported statically significant differences between the control with low and high microplastic concentration. (p=0.17 and p=0.046 respectively).

## 4.5.4. Molecular analyses

Results reported that higher levels of LPO and heat-shock protein 70 were reported in both temperatures when corals were exposed to the lowest concentration of PE microbeads. On the contrary, regarding Cas3 the highest up-regulation was observed when corals were exposed at 30°C and high the highest PE microbeads concentration.

*Lipid Peroxidation analysis*

At 25$^{o}$C, the MDA level was highest in coral nubbins exposed to low concentrations of microplastic beads. MDA level in these nubbins was statistically significant compared to controls (p = 0.007) and nubbins exposed to the high concentration of microbeads (p= 0.014). The highest level of MDA (0.49 μmol/μg of protein) was observed in corals nubbins co-exposed to elevated temperature and low microplastic concentration. MDA level was statistically significant compared to controls at 25$^{o}$C. No significant differences were however observed between treatments at 30 $^{o}$C (Figure 10.).

*Gene expression analysis: Caspase 3*

At 25$^{o}$C, Cas3 was down-regulated when coral nubbins were exposed to both low and high concentrations of microplastics. The highest down-regulation (0.7-fold) was observed at high microplastic exposure. However, the down-regulations observed in Cas3 were not statistically significant from controls. On the other hand, Cas3 was significantly up-regulated when corals were co-exposed to elevated temperature and microplastic. Change in Cas3 expression was statistically significant between microplastic treatments at 25 $^{o}$C and microplastic treatments at 30 $^{o}$C.

A progressive up-regulation in Cas3 was observed between the three treatments at 30 $^{o}$C. The highest up-regulation (0.6-fold, p = 0.0005) was observed when corals were co-exposed to elevated temperature and high microplastic concentration (Figure 11). Cas3 up-regulation was significant between corals co-exposed to elevated temperature and microplastic compared to corals exposed to elevated temperature only. Overall, the highest statistically significant modulation of Cas3 was observed at high microplastic concentration whether at 30 $^{o}$C.

*Gene expression analysis: Heat shock protein 70*

Similar to the expression pattern of *cas3*, *hsp70* was down-regulated in corals exposed to microplastics at 25 $^{o}$C and up-regulated in response to microplastic exposure at higher temperatures. The modulation in *hsp70* was statistically significant between treatments at 25 $^{o}$C and treatments at 30 $^{o}$C. The highest up-regulation (1.1 fold, p = 0.0002) was observed when corals were co-exposed to elevated temperature and low microplastic concentration (Figure 12)

Overall, results show that *hsp70* and *Cas3* up-regulation was higher under combined stress.

## 4.6. Discussion

Since coral reefs are increasingly threatened by global warming and microplastic pollution (Hughes et al 2018), therefore is important to study how reef-building corals interact with these environmental stressors combined together, as occurring in determined reef areas (Reichert et al., 2019).

In this study, we found that heat stressed corals did not show abnormal production of mucus, which normally occurred if corals are under environmental stress (Wright et al., 2019; Nguyen-Kim et al., 2015). Th combined effects of microplastic exposure and heat stress in this study did not significantly increase the production of mucus in *P. damicornis*, this fact could be due to the fact that the production of mucus in response to stress is species-specific (Erftemeijer et al., 2012).

There was no statistically significant difference in polyp retraction between corals exposed at 25 and 30°C, also the concentration of PE microbeads did not have any effect on the polyps extroflection in *P. damicornis.*

These findings are in discordance to results reported by Vencato et al., 2021, in which microplastic concentrations caused a significative reduction of the polyps' opening, in the

alcyonacean *Coelogorgia palmosa* exposed to PE microbeads (200µm). Therefore, since in both studies, nubbins were exposed to the same typology of microplastic particles, in terms of polymer and dimensions, the retraction of the coral polyps in response to stresses might depend on the species and on the dimension of polyps (Bejarano et al., 2022). Moreover, our findings show that microplastic beads adhered to *P.damicornis* surface even at the lowest concentration used in this study. This environmentally relevant concentration of 200 microbeads/L was lower compared to previous experimental micro-plastic feeding studies (Reichert et al., 2018; Liao et al 2021). To the authors' knowledge, there are only two papers that addressed the adhesion of microplastic particles on corals, but the microbeads concentration was two orders of magnitude higher than current environment concentrations (Vencato et al., 2021; Martin et al., 2019). This new finding is ecologically significant as it suggests that current microplastic levels in oceans maybe already be adversely impacting corals. Microplastic adhesion is an important factor that is gaining more scientific attention since plastic particles may act as coral disease vectors (Baptista Neto et al., 2020). Moreover, results show that with an increase in water temperature, the adhesion of microplastic beads significantly decreased. Such a phenomenon might be due to the fact that corals if exposed to higher temperatures, they may be less able to capture external particles (Reichert et al., 2021). These findings might demonstrate that thermally stressed corals may be less impacted by microplastic pollution, and consequently, their primary source of stress is the increasing of water temperature.

Results of our ingestion assay showed that *P. damicornis* is able to ingest microplastic particles even at lower concentrations than the ones used in previous exposure studies on cnidarians (Hall et al., 2015; Martin et al., 2019; Reichert et al., 2018; Lia et al., 2021). The amount of microplastic beads ingested by *P. damicornis* increased at high microplastic concentrations, at both temperatures. This response could be due to the fact that an increase in microplastic concentration in the water might enhance coral to enter in contact with more microbeads, enhancing the ingestion. Moreover, our results report that at the same microplastic concentration the average number of ingested microbeads at 30°C was higher than the one at 25°C. This finding is in discordance with Axworthy et al. 2019, in which *P.damicornis* fragments exposed to higher temperatures decreased their heterotrophic activity because of the reduction in symbiodiniaceae photosynthetic efficiency. Hence more MPs exposure studies on *P.damicornis* should be carried out on the effect generated by microplastic exposure and heat stress.

Currently, there is a knowledge gap on microplastic egestion rates in coral polyps constantly exposed to microplastic particles (Reichert et al. 2018) and research on this topic has been suggested to be a priority for future research (Axworthy and Padilla-Gamiño, 2019). Additionally, responses may vary substantially among species (Stafford-Smith, 1993; Stafford-Smith and Ormond, 1992). Up to date, there are only a few studies regarding the egestion of microplastic by reef-building corals (Martin et al., 2019; Rotjan et al., 2019; Hankins et al., 2021). Currently, there are few publications on microplastic egestion in corals, but to date, there are no publications on microplastic egestion in thermally stressed scleractinian corals. Therefore, this study could be considered as a starting point for future research on the egestion rate in other ecologically important reef-building species.

Our findings demonstrated that *P.damicornis* is able to egest microplastic within 24h, in fact at 25°C corals exposed to the lowest concentration of microbeads egested 89% of ingested microbeads, compared to 53% at the highest concentration. On the other hand, at 30°C the egestion values were 95% and 96% for environmentally relevant and high microbead concentrations respectively. These results are in accordance with previous related publications, for instance, Allen *et al.* 2017, reported that *Acropora Formosa* egested 92% of the ingested microplastic, and furthermore Boodraj and Glassom, 2022 found that *Pocillopra verrucosa* and *Acropora irregularis* egested the 91% of microplastic fibers within 18h. Hierl et al., 2021 reported that *Acropora valida, Montipora capricornis, Pocillopora damicornis* and *Seriatopora hystrix* are able to egest microplastic particles, even if this process is time and energy-consuming for corals. Moreover, results show that corals exposed to higher temperatures were able to egest more microplastic particles than the ones at a lower temperature. This process might be due to the fact that heated corals are more stressed, by the process of coral bleaching, and consequently, they interact less with external particles, such as microplastic. Hence, this result is environmentally interesting because might represent the future environmental trend related to the increase of SST and microplastic pollution (Horton et al., 2020).

Our results showed that fragments exposed at 30°C have been impacted by coral bleaching since they showed lower values of chlorophyll concentration and Symbiodinaceae density compared to fragments exposed at 25°C. Moreover, both as regards chlorophyll *a* and chlorophyll *c2*, the differences in density were significant between treatments corresponding to the same microplastic concentration (environmentally relevant and high) but performed at different temperature (25 and 30°C respectively). Therefore, the lack of significant differences

among values of chlorophyll *a* and *c2* between fragments of *P.damicornis* exposed at the same temperature might imply that the bleaching is mainly due to the increase in temperature rather than to the presence of microplastics. Furthermore, values of Symbiodinium density have the same trend of the chlorophyll values. However, Xiao *et al*. (2021) found that Polyethylene terephthalate microbeads caused a significant decrease in symbiodinium in fragments of *Acropora sp.* at the concentration of 50 mg/L, but this could be explained since this concentration was 5 times higher than the highest concentration used in our study (1 and 10 mg/L respectively). On the other hand, our results are in line with previous related studies, for example, Lanctôt et al. 2020 reported that *Stylophora pistillata* show a general decrease in the content of symbiodinaceae and chlorophyll A, after 28 days of microplastic exposure (PE microbeads at the concentrations of 5,000 and 50,000 particles/L), but this reduction was not statistically significant. Furthermore, Plafcan and Stallings 2022 reported that the bleaching in the coral *Acropora cervicornis* was due only to heat stress and not related with microplastic exposure. In this context, given the variability of the results from different species, that the responses of corals to the combination of thermal stress and microplastic pollution could hence be species-specific (Lanctot et al. 2020).

At the molecular level, MDA level is commonly known as a marker of oxidative stress, particularly lipid peroxidation (Gustaw-Rothenberg et al., 2010). In the present study, we found that at ambient temperatures, exposure to the environmentally relevant concentration of microplastic caused an increase in MDA levels in coral nubbins after 72 hours. However, there were no significant increases in MDA level when nubbins were exposed to the higher microplastic beads concentration. Results imply that current levels of microplastic in oceans are sufficient to elicit oxidative stress in corals at ambient temperatures. These findings are in line with Chen et al., 2022, who reported that after 72 h of exposure compared to control fragments PE microplastic (at 10 mg/L) increased the concentration of MDA in *Goniopora Columna*, and the amount of MDA was higher than in corals exposed to a higher concentration of PE microplastic ( 100 and 300 mg/L).

In general, higher levels of MDA were observed in coral nubbins exposed to 30 $^{o}$C treatments compared to nubbins exposed to the 25$^{o}$C treatments. Similarly, previous studies found that LPO in corals increased with an increase in temperature of the water (Dias et al. 2019). Similar to observations at 25 $^{o}$C, at the elevated temperature, the highest level of MDA was recorded in coral nubbins co-exposed to low concentrations of microplastic beads. Overall, the results of this study show that contemporary levels of microplastic in oceans can cause oxidative stress

in corals and secondly thermal stress interacts with microplastics (a current environment level) to cause further oxidative stress in corals.

Gene expression biomarkers have the capacity to detect sublethal stress prior to the onset of signs at the organismal level (reviewed in Louis et al., 2017). *Hsp70* is a molecular chaperone involved in maintaining cellular homeostasis during stress. It is an early responder to general stress and its up-regulation is commonly associated with a response to increasing stress (Seveso et al., 2020; Louis et al, 2017). Here, the highest up-regulation of *hsp70* was observed when coral nubbins were co-exposed to elevated temperature and low microplastic concentration. Results, therefore, imply that this co-exposition treatment was the most stressful to the coral nubbins. Previous studies found that individually heat stress (Leggat et *al.*,2011) and microplastic (Fadare *et al.*, 2019; Abarghouei et al., 2021) cause an increase in *hsp70* but no studies so far had studied the combined effect of both stressors on *hsp70* expression in a tropical hard coral. Our results demonstrate that heat stress and microplastic exposure interact to cause increased cellular stress in corals.

On the other hand, Caspase3, the protein encoded by *caspase 3* gene, is considered a key effector enzyme in inducing cell apoptosis (Salvesen, 2002; Ghavami et al., 2009). *Cas3* gene has been used as a simple, quantitative measure of ongoing apoptosis. Therefore, an increase in *cas3* gene transcripts implies an increase the apoptotic response and, in turn, more stressful conditions. Previous studies reported that *cas3* gene expression in stony corals *Seriatopora hystrix*, *S. pistillata* and *P. damicornis* increase significantly after heat stress (Tchernov *et al.*, 2011; Kvitt *et al.*, 2015; Ros *et al.*, 2016). Microplastics have also been reported to stimulate *cas*3 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). Our study shows that the highest up-regulation of cas3 was observed in coral nubbins co-exposed to elevated temperature and high microplastic beads concentration, implying that this treatment induced the highest apoptotic response in nubbins. The difference in the gene expression profile observed between *hsp70* and *cas3* could be due to their involvement at different level of the stress response mechanism. Hsp70 is an early responder to general stress (Louis *et al.*, 2017) and is therefore triggered as soon as homeostasis starts to be disrupted, to prevent further damage and ensure 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 more effective

against oxidative stress and the stressed cell need to undergo apoptosis for removal of highly compromised symbionts, thereby maintaining tissue homeostasis (Dunn et al., 2007). For instance, Kvit *et al*. (2016) reported that in the coral *S.pistillata*, following heat stress, *hsp70* gene expression peaked after 6 hours and the studied caspase gene (*StyCasp*) expression peaked after 24 hours. However, at ambient temperature, though statistically insignificant, the same gene expression profile has been observed for *hsp70* and *cas3*, where a down-regulation has been observed for both genes. Results suggest that at ambient temperature, microplastic cause a general inhibition in genes investigated but at elevated temperature, microplastic combine with heat stress to cause increased cellular stress.

## 4.7. Conclusions

Finally, this study reports that an increase in microplastic concentration leads to higher physical interaction as the number of adhered and ingested microbeads increases. Furthermore, findings reported that with increasing temperature and microplastic concentrations, corals might reduce the retention time of these particles, enhancing their egestion. Overall, results show that the interaction between heat stress and microplastic lead to an increase in coral bleaching. The combined effect of these two stressors was more apparat at the cellular and molecular level; at elevated temperature, signs of cellular distress was observed at a low microplastic concentration such as oxidative stress and expression of *hsp70*, and at high concentration for cas3 expression. These findings suggest, in accordance with previous studies (Reichert et al., 2019; Lanctôt et al., 2020, Reichert et al., 2021) that microplastic and heat stress interact to increase cellular stress in corals. With the Indian Ocean and North Pacific Ocean reported to have the largest amount of plastic, microplastic pollution represents an imminent danger to Indo-Pacific corals (Eriksen et al, 2014) and furthermore, the interaction of the combined effect of microplastic and heat on *P. damicornis*, which is a ubiquitous and a model coral (Torda et al., 2013) on Indo-pacific reef, has not been studied, especially regarding gene expression analyses. Our results highlight that microplastic pollution is already impacting corals and it detrimental effect increases under episodes of high sea surface temperatures.

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## 4.9 Illustrations

| Gene | Orientation | Primer sequence 5' to 3' | Reference | $T_m$/°C |
|---|---|---|---|---|
| *cas3* | Forward | TGTTGCTGATAGCGCCCAGA | Yu *et al.* (2017) | 60 |
| | Reverse | TCGCTGTGTCTTTCTCCGTTCA | | |
| *hsp70* | Forward | ATCCAGGCAGCGGTCTTGT | Mayfield *et al.* (2014)<br>Putnam *et al.* 2013 | 60 |
| | Reverse | TCGAGCAGCAGGATATCACTGA | | |
| *ef* | Forward | CGCTGGCAAAGTGACAAAGG | Yu *et al.* 2021<br>Yu et al. 2017 | 60 |
| | Reverse | CAGACTTGCGATGAAATAGATAGGA | | |

**Table 1. -** Primer sequences of GOI and internal control gene used for qPCR analyses

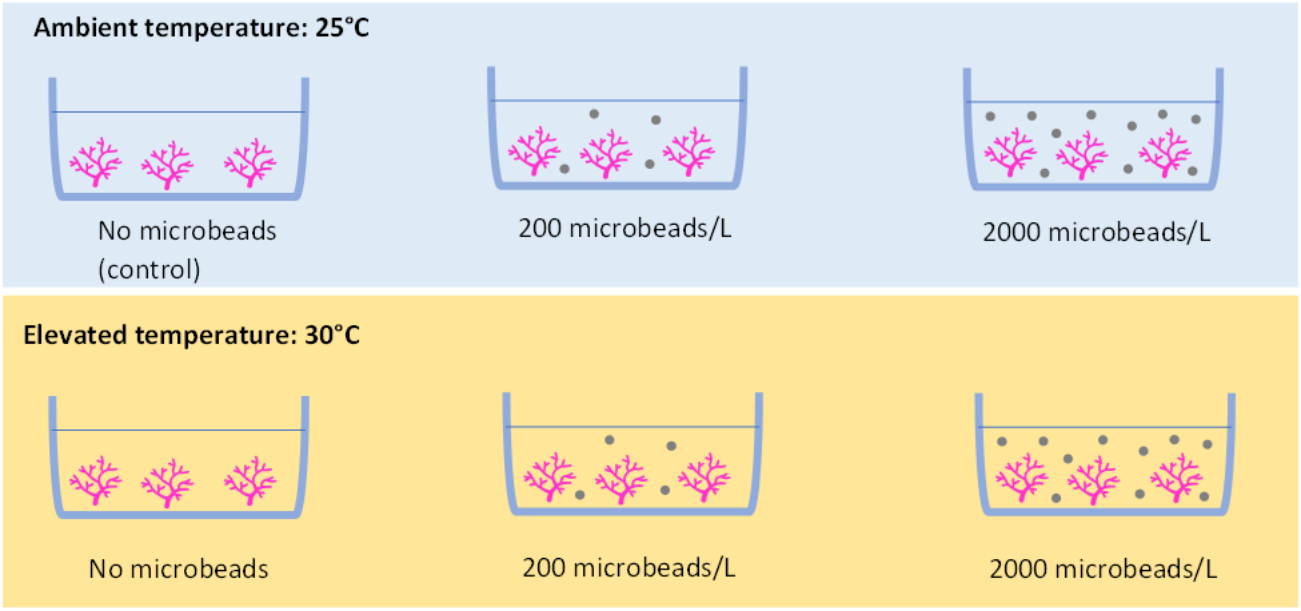


**Figure 1.** Schematic representation of the experimental design

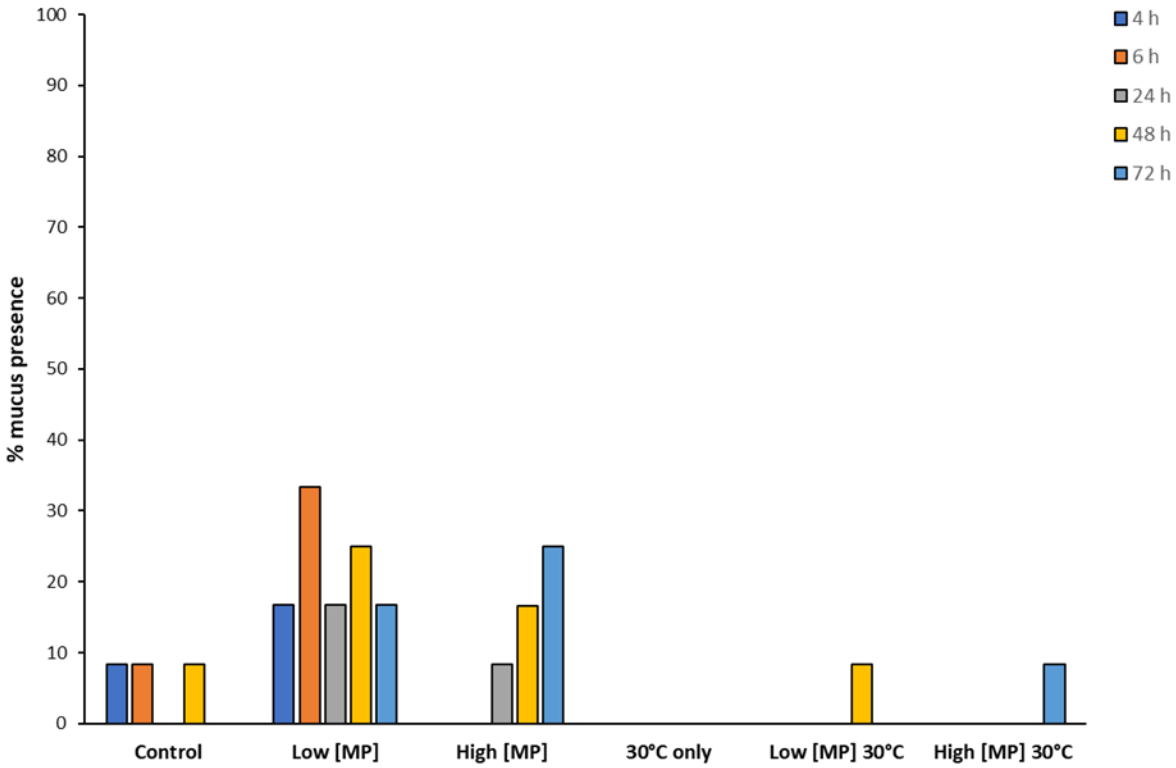


**Figure 2.** Abnormal mucus production due to polyethylene (PE) microbeads exposure. Results are reported in terms of the percentage of *P. damicornis* nubbins showing mucus at different time points (4, 6, 24, 48, 72 hours) in each treatment.

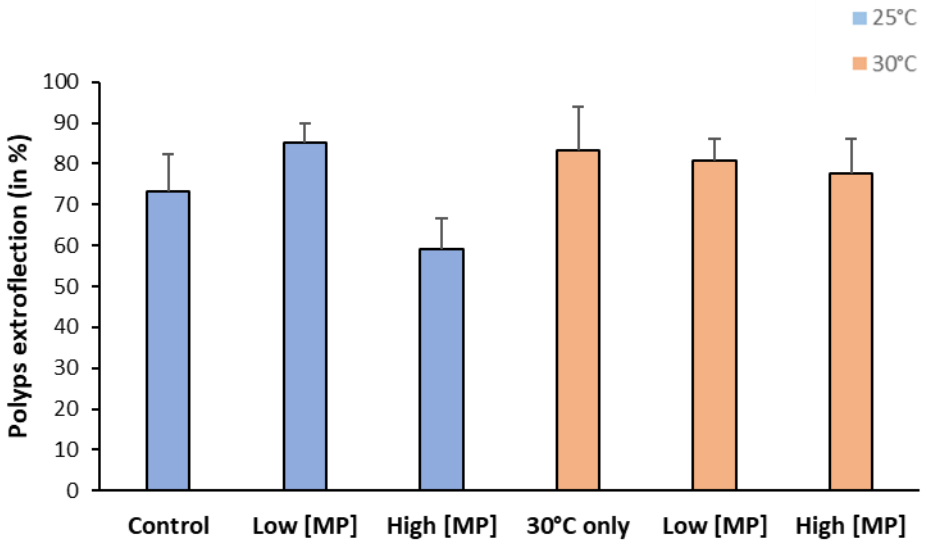


**Figure 3.** Polyps extroflection was monitored throughout the entire experiment. Results are reported as the mean percentage value of polyps extroflection observed at every time points considered in Control (absence of MPs), Low [MP] (200 microbeads/L), and High [MP] (2000 microbeads/L) at both 25 °C and 30 °C.

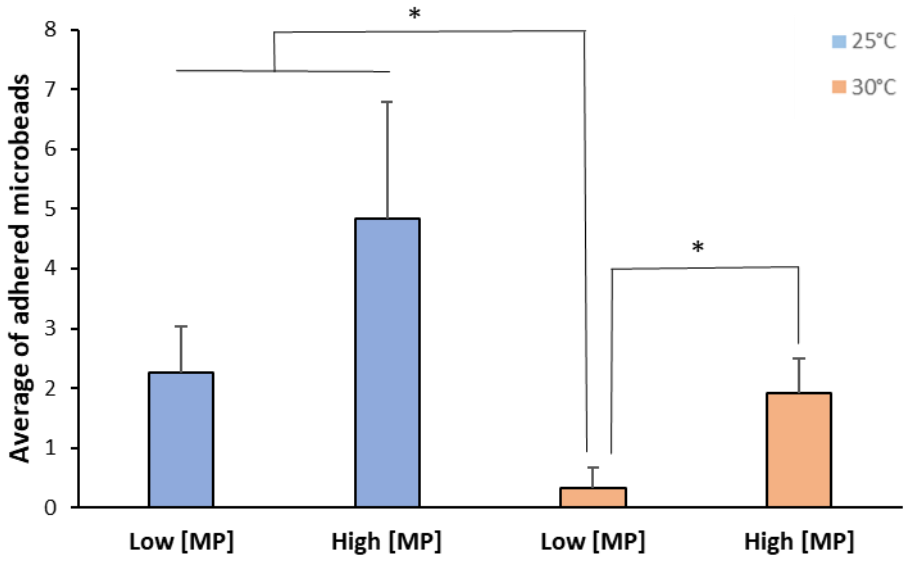


**Figure 4.** Microbeads adhesion in Low [MP] (200 microbeads/L) and High [MP] (2000 microbeads/L) at different temperatures. Results are reported in terms of the average number of adhered microbeads per fragment.

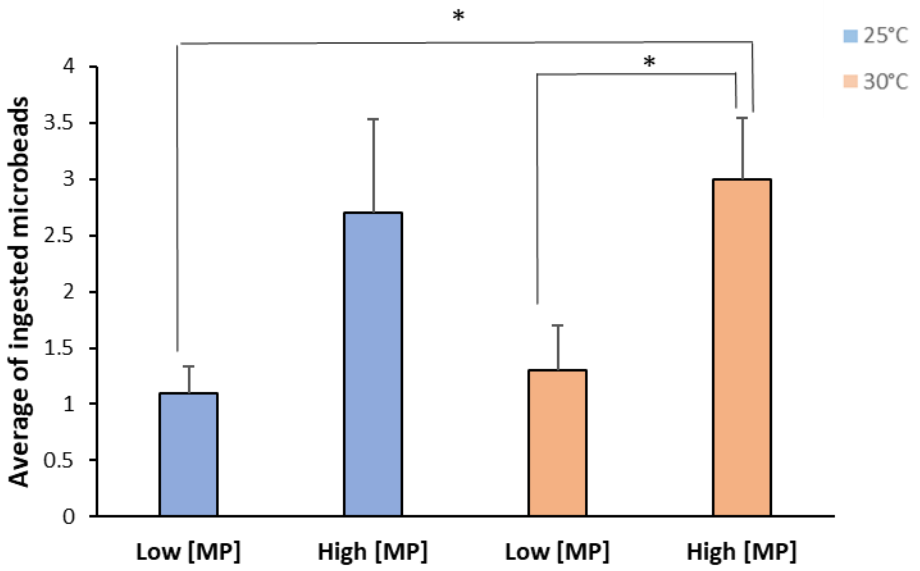


**Figure 5.** Microbeads ingestion in Low [MP] (200 microbeads/L) and High [MP] (2000 microbeads/L) at different temperatures. Results are reported in terms of the average number of ingested microbeads per fragment.

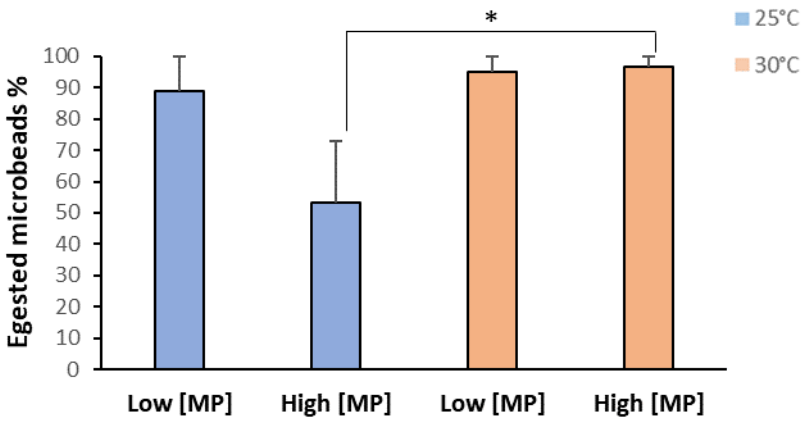


**Figure 6.** Microbeads egestion in Low [MP] (200 microbeads/L) and High [MP] (2000 microbeads/L) at different temperatures. Results are reported in terms of the percentage of microbeads egested after the ingestion

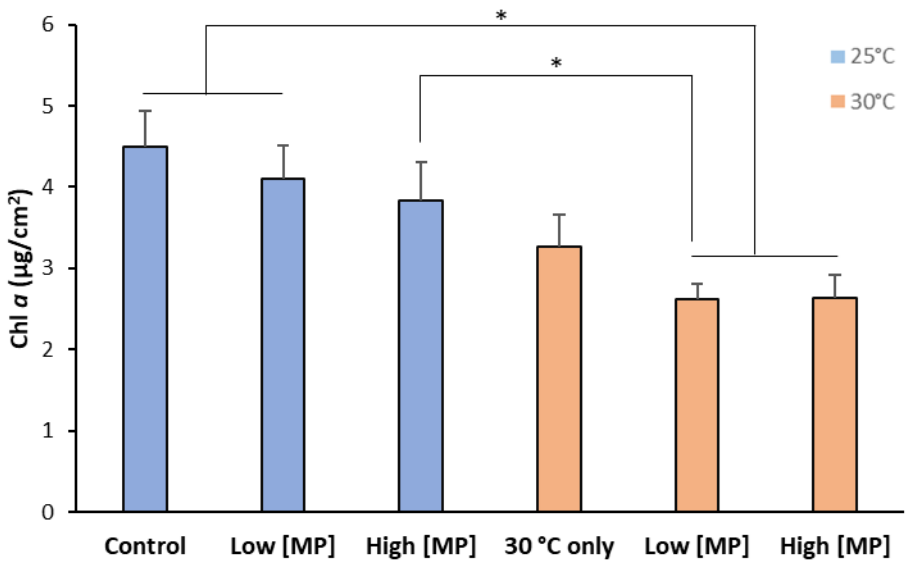


**Figure 7.** Chlorophyll *a* concentration per surface area unit (expressed as chl/cm2) in C, Low [MP] and High [MP] at 25 °C and 30 °C.

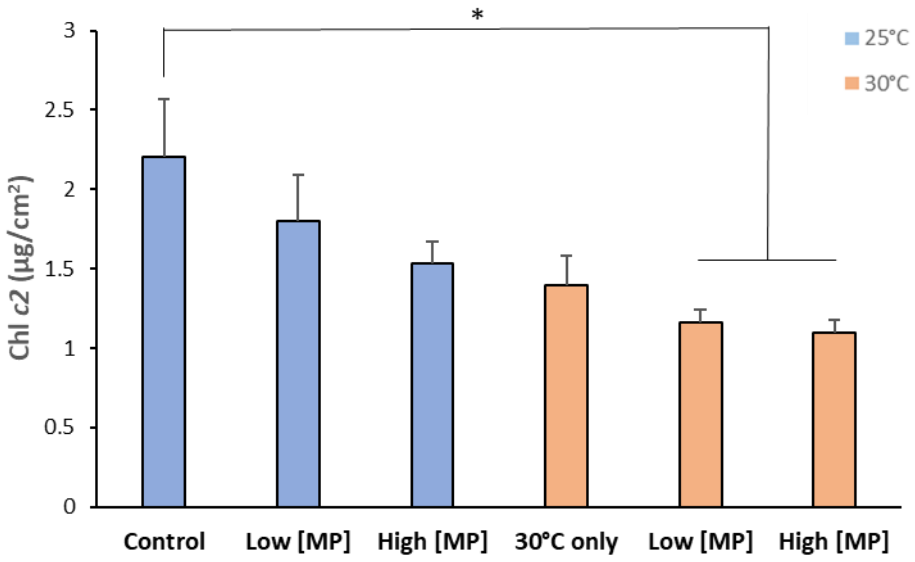


**Figure 8.** Chlorophyll *c2* concentration per surface area unit (expressed as chl/cm2) in C, LC and HC at 25°C and 30°C.

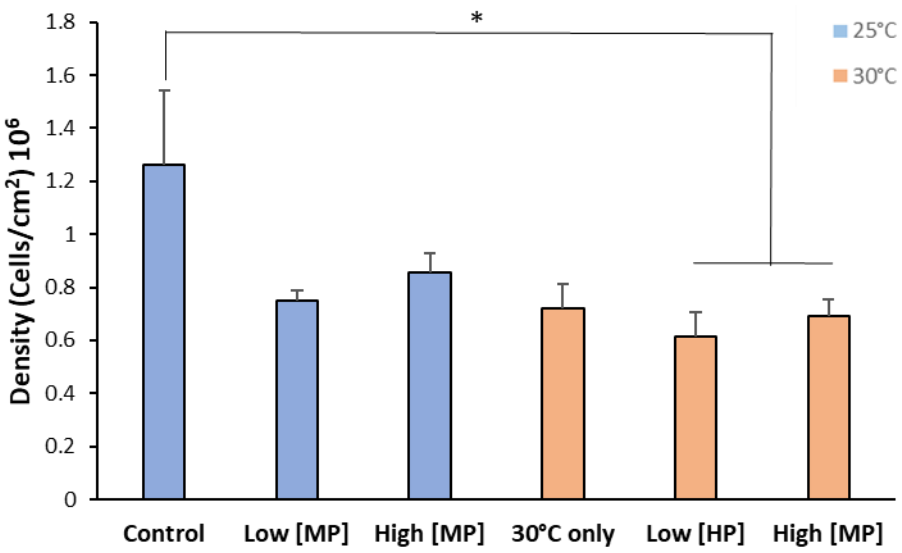


**Figure 9.** Symbiodiniaceae density in C (absence of MPs), Low [MP] (200 microbeads/L ), and High [MP] (2000 microbeads/L) at 25 °C and 30 °C. Density is reported in terms of cell/cm2 x 106.

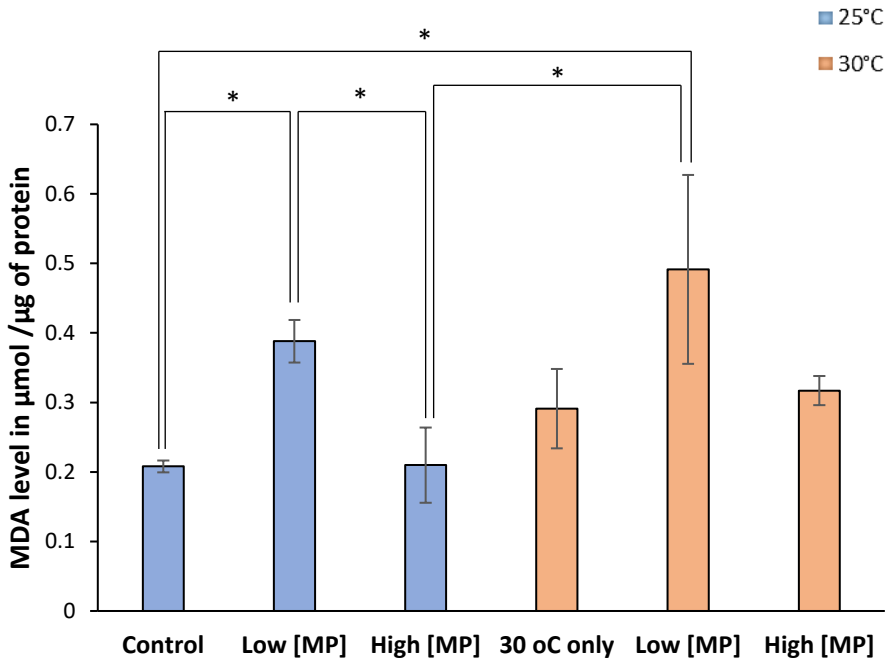


**Figure 10**. MDA level in *P.damicornis* nubbins after controls and exposure treatments at 25°C and 30°C.

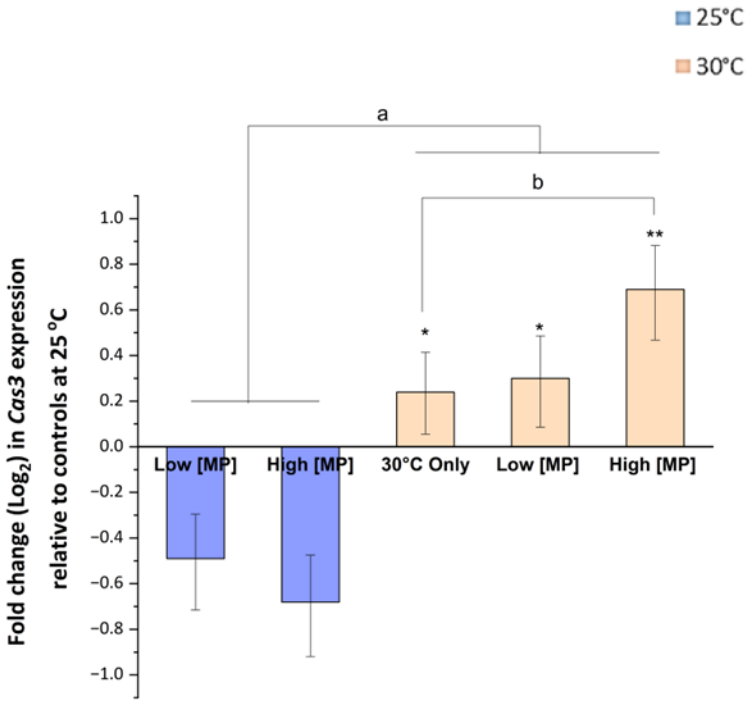


**Figure 11.** Change in the expression of cas3 expression. Fold changes in the different treatments were calculated with respect to control treatment at 25 $^{o}$C and were $log_2$-transformed. Mean ± SE is represented by bars. Asterisks represent significant differences in C*q* values between treatments and control (* represents p = 0.05, ** represents p = 0.01, *** represents p < 0.001). Letters above bars (a, b) represent significant differences in C*q* values among treatments (p < 0.05).

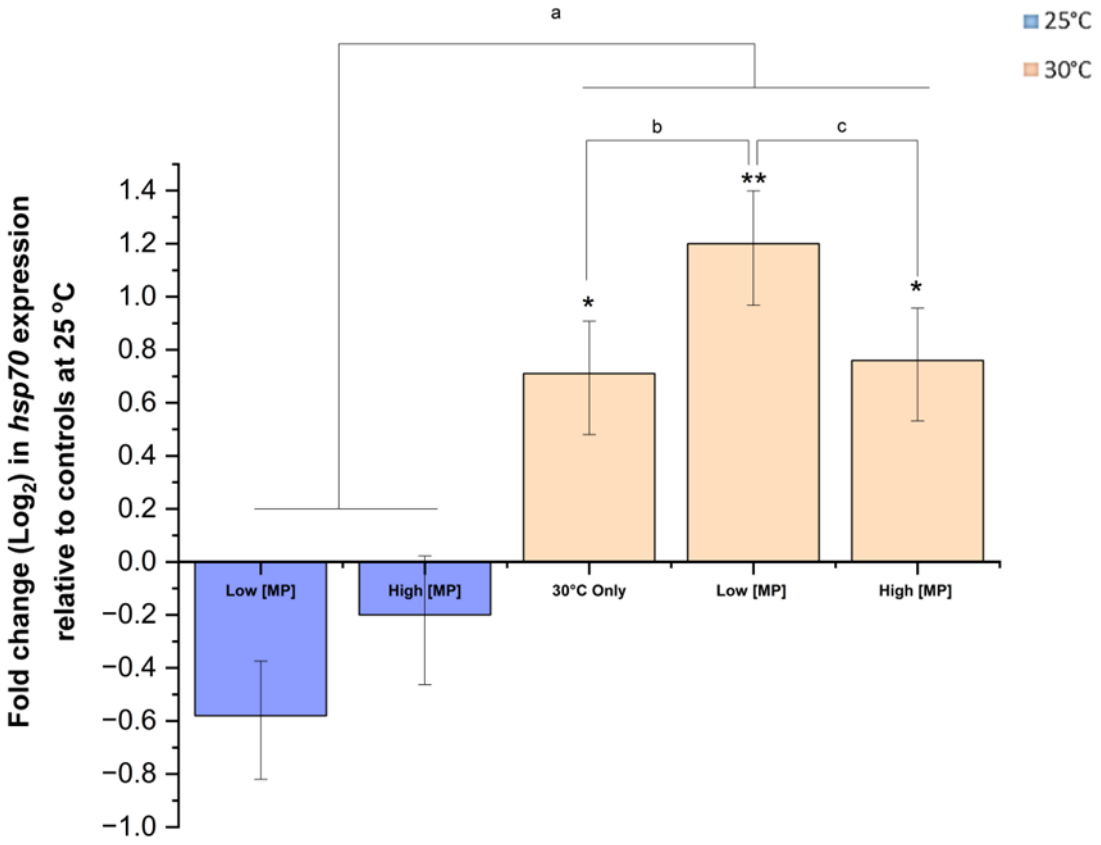


**Figure 12**. Change in the expression of hsp70 expression. Fold changes in the different treatments were calculated with respect to control treatment at 25 $^{o}$C and were $log_2$-transformed. Mean ± SE is represented by bars. Asterisks represent significant differences in C*q* values between treatments and control (* represents p = 0.05, ** represents p = 0.01, *** represents p < 0.001). Letters above bars (a, b) represent differences in C*q* values among treatments (p < 0.05).

## 4.10. Supplementary

S1 Description of the Genoa Aquarium facility

The coral colonies sampled come from the tanks of the Genoa Aquarium, where the water system collects seawater from 200m outside the Foranea dam of the port of Genoa at 50m depth. The collected water is pumped through the filtration system made of 2 sand filters and one UV filter, used for disinfection. After the filtration, the seawater is stored inside 4 accumulation tanks (200m^3 each). If the results of the analyses show that the chemical-physical parameters (Ph, salinity, ammonia, nitrites, nitrates, and phosphates) are optimal for the aquarium, the seawater of one accumulation tank is pumped into a mixing basin, where the water is kept in constant motion. After further UV filtration, the water is pumped from the mixing basin to all the tanks of the aquarium. During the day from 8:00 to 16:00, the water is pumped from the mixing basin to the aquarium tanks with a flow of 1 liter every 30 seconds, so the tanks are considered as a semi-open system (the tanks are considered as a closed system from 16:00 to 8:00). In the tanks, used for the sampling (3x1x0,7 m, 3100 l, composed by acrylic and glass resin) in which the experiment was carried out, the water is uptaken by a pump (Astralpool, Victoria Plus) with a 24-hour flow rate of 8m^3/h (to ensure complete water change every about 30 minutes) and reinserted into the tank after passing through the filtration system. The filtration system is composed of a sand filter (Astralpool Artic, filtering particles from 0.4 to 2mm) and a UV filter (Panaque 750 s AB 4 lamps of 40W). The water passage through the UV filter is instantaneous since water passes with a flow equal to $8m^3$ per hour. 2 l of water containing a solution of the algae *Tetra selmis* and zooplankton belonging to the Phylum *Rotifera* (the average concentration of zooplankton is 250 individuals / mL and the average dimension is 0,5mm) are placed daily inside the tanks in order to feed the corals. Both Algae and zooplankton are farmed inside 80L cylindrical tanks made of plexiglass. Furthermore, twice a week 20g of food mixture are daily in the tanks to feed the corals, this mixture is composed of 70% of silverside fishes (5cm in length) and 30% carrots, while the next day the mixture is composed by 70% of mussels and 30% of courgettes. In order to facilitate the calcification of the coral skeleton 50l of water containing 500g of calcium hydroxide.

## ANOVA

extroflection

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 5273.578 | 5 | 1054.716 | 1.694 | 0.150 |
| Within Groups | 36732.576 | 59 | 622.586 | | |
| Total | 42006.154 | 64 | | | |

Table S1. statistical test regarding the abnormal production of mucus in *P.damicornis*

## Multiple Comparisons

Dependent Variable:

Tukey HSD

| (I) Treatment | | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Control | lc 25 | -14.091 | 10.415 | 0.754 | -44.77 | 16.59 |
| | hc 25 | 11.742 | 10.415 | 0.868 | -18.94 | 42.42 |
| | 30 degree only | -12.424 | 12.663 | 0.922 | -49.72 | 24.88 |
| | lc 30 | -9.924 | 10.415 | 0.931 | -40.60 | 20.75 |
| | hc 30 | -6.591 | 10.415 | 0.988 | -37.27 | 24.09 |
| lc 25 | Control | 14.091 | 10.415 | 0.754 | -16.59 | 44.77 |
| | hc 25 | 25.833 | 10.186 | 0.130 | -4.17 | 55.84 |
| | 30 degree only | 1.667 | 12.476 | 1.000 | -35.08 | 38.41 |
| | lc 30 | 4.167 | 10.186 | 0.998 | -25.84 | 34.17 |
| | hc 30 | 7.500 | 10.186 | 0.977 | -22.50 | 37.50 |
| hc 25 | Control | -11.742 | 10.415 | 0.868 | -42.42 | 18.94 |
| | lc 25 | -25.833 | 10.186 | 0.130 | -55.84 | 4.17 |
| | 30 degree only | -24.167 | 12.476 | 0.391 | -60.91 | 12.58 |
| | lc 30 | -21.667 | 10.186 | 0.288 | -51.67 | 8.34 |
| | hc 30 | -18.333 | 10.186 | 0.474 | -48.34 | 11.67 |
| 30 degree only | Control | 12.424 | 12.663 | 0.922 | -24.88 | 49.72 |
| | lc 25 | -1.667 | 12.476 | 1.000 | -38.41 | 35.08 |
| | hc 25 | 24.167 | 12.476 | 0.391 | -12.58 | 60.91 |
| | lc 30 | 2.500 | 12.476 | 1.000 | -34.25 | 39.25 |
| | hc 30 | 5.833 | 12.476 | 0.997 | -30.91 | 42.58 |
| lc 30 | Control | 9.924 | 10.415 | 0.931 | -20.75 | 40.60 |
| | lc 25 | -4.167 | 10.186 | 0.998 | -34.17 | 25.84 |
| | hc 25 | 21.667 | 10.186 | 0.288 | -8.34 | 51.67 |
| | 30 degree only | -2.500 | 12.476 | 1.000 | -39.25 | 34.25 |
| | hc 30 | 3.333 | 10.186 | 0.999 | -26.67 | 33.34 |

| | | | | | | |
|---|---|---|---|---|---|---|
| hc 30 | Control | 6.591 | 10.415 | 0.988 | -24.09 | 37.27 |
| | lc 25 | -7.500 | 10.186 | 0.977 | -37.50 | 22.50 |
| | hc 25 | 18.333 | 10.186 | 0.474 | -11.67 | 48.34 |
| | 30 degree only | -5.833 | 12.476 | 0.997 | -42.58 | 30.91 |
| | lc 30 | -3.333 | 10.186 | 0.999 | -33.34 | 26.67 |

Table S2. multiple comparison on the abnormal mucus production in *P.damicornis*

**Hypothesis Test Summary**

| | Null Hypothesis | Test | Sig.[a,b] | Decision |
|---|---|---|---|---|
| 1 | The distribution of Adhered_microbeads is the same across categories of Treatment. | Independent-Samples Kruskal-Wallis Test | 0.009 | Reject the null hypothesis. |

a. The significance level is .050.

b. Asymptotic significance is displayed.

Table S3. Statistical test about the adhesion in all the exposure treatments

**Pairwise Comparisons of Treatment**

| Sample 1-Sample 2 | Test Statistic | Std. Error | Std. Test Statistic | Sig. | Adj. Sig.[a] |
|---|---|---|---|---|---|
| lc 30-hc 30 | -12.917 | 5.453 | -2.369 | 0.018 | 0.107 |
| lc 30-lc 25 | 14.083 | 5.453 | 2.583 | 0.010 | 0.059 |
| lc 30-hc 25 | 17.167 | 5.453 | 3.148 | 0.002 | 0.010 |
| hc 30-lc 25 | 1.167 | 5.453 | 0.214 | 0.831 | 1.000 |
| hc 30-hc 25 | 4.250 | 5.453 | 0.779 | 0.436 | 1.000 |
| lc 25-hc 25 | -3.083 | 5.453 | -0.565 | 0.572 | 1.000 |

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.

Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table S4. Multiple comparisons regarding the adhered microbeads among exposure treatments

**Hypothesis Test Summary**

| | Null Hypothesis | Test | Sig.[a,b] | Decision |
|---|---|---|---|---|
| 1 | The distribution of Ingested microbeads is the same across categories of Treatment. | Independent-Samples Kruskal-Wallis Test | 0.022 | Reject the null hypothesis. |

a. The significance level is .050.

b. Asymptotic significance is displayed.

Table S5 Statistical test about the ingestion in all the exposure treatments

**Pairwise Comparisons of Treatment**

| Sample 1-Sample 2 | Test Statistic | Std. Error | Std. Test Statistic | Sig. | Adj. Sig.[a] |
|---|---|---|---|---|---|
| lc 25-lc 30 | -1.250 | 5.037 | -0.248 | 0.804 | 1.000 |
| lc 25-hc 25 | -8.050 | 5.037 | -1.598 | 0.110 | 0.660 |
| lc 25-hc 30 | -13.700 | 5.037 | -2.720 | 0.007 | 0.039 |
| lc 30-hc 25 | 6.800 | 5.037 | 1.350 | 0.177 | 1.000 |
| lc 30-hc 30 | -12.450 | 5.037 | -2.472 | 0.013 | 0.081 |
| hc 25-hc 30 | -5.650 | 5.037 | -1.122 | 0.262 | 1.000 |

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.

Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table S6: Multiple comparisons regarding the adhered microbeads among exposure treatments

**Hypothesis Test Summary**

| | Null Hypothesis | Test | Sig.[a,b] | Decision |
|---|---|---|---|---|
| 1 | The distribution of % of egestion is the same across categories of Temperature. | Independent-Samples Kruskal-Wallis Test | .034 | Reject the null hypothesis. |

a. The significance level is .050.

b. Asymptotic significance is displayed.

Table S7.The significant difference in the percentage values of egested microbeads in *P.damicornis* fragments exposed at 25 and 30 °C.

**Hypothesis Test Summary**

| | Null Hypothesis | Test | Sig.[a,b] | Decision |
|---|---|---|---|---|
| 1 | The distribution of Concentration is the same across categories of Treatment. | Independent-Samples Kruskal-Wallis Test | 0.020 | Reject the null hypothesis. |

a. The significance level is .050.

b. Asymptotic significance is displayed.

Table S8. Statistical tests about the concentration of chlorophyll a in all the exposure treatments

**Pairwise Comparisons of Treatment**

| Sample 1-Sample 2 | Test Statistic | Std. Error | Std. Test Statistic | Sig. | Adj. Sig.[a] |
|---|---|---|---|---|---|
| lc 30-hc 30 | -1.300 | 5.906 | -0.220 | 0.826 | 1.000 |
| lc 30-Control 30 | 6.000 | 5.568 | 1.078 | 0.281 | 1.000 |
| lc 30-hc 25 | 11.300 | 5.331 | 2.120 | 0.034 | 0.510 |
| lc 30-lc 25 | 13.200 | 5.568 | 2.371 | 0.018 | 0.266 |
| lc 30-**Control 25** | 16.000 | 5.568 | 2.874 | 0.004 | 0.061 |
| hc 30-Control 30 | 4.700 | 5.906 | 0.796 | 0.426 | 1.000 |
| hc 30-hc 25 | 10.000 | 5.683 | 1.760 | 0.078 | 1.000 |
| hc 30-lc 25 | 11.900 | 5.906 | 2.015 | 0.044 | 0.658 |
| hc 30-**Control 25** | 14.700 | 5.906 | 2.489 | 0.013 | 0.192 |
| Control 30-hc 25 | 5.300 | 5.331 | 0.994 | 0.320 | 1.000 |
| Control 30-lc 25 | 7.200 | 5.568 | 1.293 | 0.196 | 1.000 |
| Control 30-Control 25 | 10.000 | 5.568 | 1.796 | 0.072 | 1.000 |
| hc 25-lc 25 | 1.900 | 5.331 | 0.356 | 0.722 | 1.000 |
| hc 25-Control 25 | 4.700 | 5.331 | 0.882 | 0.378 | 1.000 |

| | | | | | |
|---|---|---|---|---|---|
| lc 25-Control 25 | 2.800 | 5.568 | 0.503 | 0.615 | 1.000 |

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .050.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table S9. . Multiple comparisons regarding the density of chlorophyll a among exposure treatments

**ANOVA**

Chl / cm2

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 4.820 | 5 | 0.964 | 3.162 | 0.023 |
| Within Groups | 7.926 | 26 | 0.305 | | |
| Total | 12.746 | 31 | | | |

Table S10 Statistical tests about the concentration of chlorophyll c2 in all the exposure treatments

**Multiple Comparisons**

Dependent Variable: Chl / cm2

| | (I) Treatment | (J) Treatment | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | control 25 | lc 25 | .4043911708 | .3187767689 | .799 | -.575036112 | 1.383818454 |
| | | hc 25 | .6699394454 | .3343358959 | .367 | -.357292555 | 1.697171446 |
| | | control 30 | .8032538131 | .3343358959 | .192 | -.223978187 | 1.830485814 |
| | | lc 30 | 1.04389780* | .3343358959 | .045 | .0166658005 | 2.071129801 |
| | | hc 30 | 1.10394048* | .3343358959 | .030 | .0767084842 | 2.131172485 |
| | lc 25 | control 25 | -.404391171 | .3187767689 | .799 | -1.38381845 | .5750361120 |
| | | hc 25 | .2655482746 | .3343358959 | .966 | -.761683726 | 1.292780275 |
| | | control 30 | .3988626423 | .3343358959 | .836 | -.628369358 | 1.426094643 |
| | | lc 30 | .6395066301 | .3343358959 | .417 | -.387725370 | 1.666738630 |
| | | hc 30 | .6995493137 | .3343358959 | .322 | -.327682687 | 1.726781314 |
| | hc 25 | control 25 | -.669939445 | .3343358959 | .367 | -1.69717145 | .3572925550 |
| | | lc 25 | -.265548275 | .3343358959 | .966 | -1.29278027 | .7616837258 |
| | | control 30 | .1333143677 | .3492024543 | .999 | -.939594465 | 1.206223200 |
| | | lc 30 | .3739583555 | .3492024543 | .888 | -.698950477 | 1.446867188 |
| | | hc 30 | .4340010392 | .3492024543 | .812 | -.638907793 | 1.506909872 |
| | control 30 | control 25 | -.803253813 | .3343358959 | .192 | -1.83048581 | .2239781873 |
| | | lc 25 | -.398862642 | .3343358959 | .836 | -1.42609464 | .6283693581 |
| | | hc 25 | -.133314368 | .3492024543 | .999 | -1.20622320 | .9395944648 |
| | | lc 30 | .2406439878 | .3492024543 | .982 | -.832264845 | 1.313552820 |
| | | hc 30 | .3006866714 | .3492024543 | .952 | -.772222161 | 1.373595504 |
| | lc 30 | control 25 | -1.04389780* | .3343358959 | .045 | -2.07112980 | -.016665801 |
| | | lc 25 | -.639506630 | .3343358959 | .417 | -1.66673863 | .3877253703 |
| | | hc 25 | -.373958356 | .3492024543 | .888 | -1.44686719 | .6989504770 |
| | | control 30 | -.240643988 | .3492024543 | .982 | -1.31355282 | .8322648447 |
| | | hc 30 | .0600426836 | .3492024543 | 1.000 | -1.01286615 | 1.132951516 |
| | hc 30 | control 25 | -1.10394048* | .3343358959 | .030 | -2.13117248 | -.076708484 |
| | | lc 25 | -.699549314 | .3343358959 | .322 | -1.72678131 | .3276826867 |
| | | hc 25 | -.434001039 | .3492024543 | .812 | -1.50690987 | .6389077933 |
| | | control 30 | -.300686671 | .3492024543 | .952 | -1.37359550 | .7722221611 |
| | | lc 30 | -.060042684 | .3492024543 | 1.000 | -1.13295152 | 1.012866149 |
| Games-Howell | control 25 | lc 25 | .4043911708 | .4690678535 | .947 | -1.24234087 | 2.051123207 |
| | | hc 25 | .6699394454 | .3941895924 | .573 | -.864312205 | 2.204191096 |
| | | control 30 | .8032538131 | .4097538240 | .441 | -.738358823 | 2.344866449 |
| | | lc 30 | 1.043897801 | .3781065490 | .200 | -.502882136 | 2.590677737 |
| | | hc 30 | 1.103940485 | .3759798997 | .168 | -.446453222 | 2.654334191 |
| | lc 25 | control 25 | -.404391171 | .4690678535 | .947 | -2.05112321 | 1.242340866 |
| | | hc 25 | .2655482746 | .3236408252 | .954 | -.953503785 | 1.484600334 |
| | | control 30 | .3988626423 | .3424268466 | .842 | -.848761845 | 1.646487130 |
| | | lc 30 | .6395066301 | .3038461969 | .392 | -.578156108 | 1.857169369 |
| | | hc 30 | .6995493137 | .3011956739 | .315 | -.521079454 | 1.920178082 |
| | hc 25 | control 25 | -.669939445 | .3941895924 | .573 | -2.20419110 | .8643122053 |
| | | lc 25 | -.265548275 | .3236408252 | .954 | -1.48460033 | .9535037852 |
| | | control 30 | .1333143677 | .2293838026 | .989 | -.716953962 | .9835826971 |
| | | lc 30 | .3739583555 | .1663829769 | .327 | -.266066514 | 1.013983225 |
| | | hc 30 | .4340010392 | .1614918498 | .204 | -.201424028 | 1.069426106 |
| | control 30 | control 25 | -.803253813 | .4097538240 | .441 | -2.34486645 | .7383588226 |
| | | lc 25 | -.398862642 | .3424268466 | .842 | -1.64648713 | .8487618449 |
| | | hc 25 | -.133314368 | .2293838026 | .989 | -.983582697 | .7169539616 |
| | | lc 30 | .2406439878 | .2004895422 | .823 | -.567971709 | 1.049259684 |
| | | hc 30 | .3006866714 | .1964494314 | .663 | -.509971198 | 1.111344541 |
| | lc 30 | control 25 | -1.04389780 | .3781065490 | .200 | -2.59067774 | .5028821356 |
| | | lc 25 | -.639506630 | .3038461969 | .392 | -1.85716937 | .5781561084 |
| | | hc 25 | -.373958356 | .1663829769 | .327 | -1.01398322 | .2660665137 |
| | | control 30 | -.240643988 | .2004895422 | .823 | -1.04925968 | .5679717088 |
| | | hc 30 | .0600426836 | .1168706345 | .994 | -.368471571 | .4885569382 |
| | hc 30 | control 25 | -1.10394048 | .3759798997 | .168 | -2.65433419 | .4464532224 |
| | | lc 25 | -.699549314 | .3011956739 | .315 | -1.92017808 | .5210794544 |
| | | hc 25 | -.434001039 | .1614918498 | .204 | -1.06942611 | .2014240275 |
| | | control 30 | -.300686671 | .1964494314 | .663 | -1.11134454 | .5099711977 |
| | | lc 30 | -.060042684 | .1168706345 | .994 | -.488556938 | .3684715709 |

*. The mean difference is significant at the 0.05 level.

Table S12. Multiple comparisons regarding the density of chlorophyll c2 among exposure treatments

## ANOVA

SYMB

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 1.620 | 5 | 0.324 | 3.158 | 0.021 |
| Within Groups | 3.077 | 30 | 0.103 | | |
| Total | 4.697 | 35 | | | |

Table S13. Statistical tests about the dsity of symbiodinaceae in all the exposure treatments

## Multiple Comparisons

Dependent Variable:

Tukey HSD

| (I) Treatment | | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| control 25 | lc 25 | 0.51094695 | 0.184902732 | 0.0919301 | -0.051452136 | 1.073346037 |
| | hc 25 | 0.402715997 | 0.184902732 | 0.277319 | -0.159683089 | 0.965115083 |
| | control 30 | 0.541311771 | 0.184902732 | **0.0645309** | -0.021087315 | 1.103710857 |
| | lc 30 | .647923155428007* | 0.184902732 | **0.0166333** | 0.085524069 | 1.210322242 |
| | hc 30 | .568749457273231* | 0.184902732 | **0.0462378** | 0.006350371 | 1.131148543 |
| lc 25 | control 25 | 0.51094695 | 0.184902732 | 0.0919301 | -1.073346037 | 0.051452136 |
| | hc 25 | 0.108230953 | 0.184902732 | 0.9912743 | -0.670630039 | 0.454168133 |
| | control 30 | 0.030364821 | 0.184902732 | 0.9999815 | -0.532034265 | 0.592763907 |
| | lc 30 | 0.136976205 | 0.184902732 | 0.9750377 | -0.425422881 | 0.699375291 |
| | hc 30 | 0.057802507 | 0.184902732 | 0.9995575 | -0.504596579 | 0.620201593 |
| hc 25 | control 25 | 0.402715997 | 0.184902732 | 0.277319 | -0.965115083 | 0.159683089 |
| | lc 25 | 0.108230953 | 0.184902732 | 0.9912743 | -0.454168133 | 0.670630039 |
| | control 30 | 0.138595774 | 0.184902732 | 0.9737353 | -0.423803312 | 0.70099486 |
| | lc 30 | 0.245207158 | 0.184902732 | 0.7685006 | -0.317191928 | 0.807606244 |
| | hc 30 | 0.16603346 | 0.184902732 | 0.9439954 | -0.396365626 | 0.728432546 |
| control 30 | control 25 | 0.541311771 | 0.184902732 | 0.0645309 | -1.103710857 | 0.021087315 |
| | lc 25 | 0.030364821 | 0.184902732 | 0.9999815 | -0.592763907 | 0.532034265 |
| | hc 25 | 0.138595774 | 0.184902732 | 0.9737353 | -0.70099486 | 0.423803312 |
| | lc 30 | 0.106611384 | 0.184902732 | 0.9918565 | -0.455787702 | 0.66901047 |
| | hc 30 | 0.027437686 | 0.184902732 | 0.9999888 | -0.5349614 | 0.589836772 |
| lc 30 | control 25 | -.647923155428007* | 0.184902732 | 0.0166333 | -1.210322242 | -0.085524069 |
| | lc 25 | -0.136976205 | 0.184902732 | 0.9750377 | -0.699375291 | 0.425422881 |
| | hc 25 | -0.245207158 | 0.184902732 | 0.7685006 | -0.807606244 | 0.317191928 |
| | control 30 | -0.106611384 | 0.184902732 | 0.9918565 | -0.66901047 | 0.455787702 |
| | hc 30 | -0.079173698 | 0.184902732 | 0.9979799 | -0.641572784 | 0.483225388 |

| | | | | | | |
|---|---|---|---|---|---|---|
| hc 30 | control 25 | -.568749457273231* | 0.184902732 | 0.0462378 | -1.131148543 | -0.006350371 |
| | lc 25 | -0.057802507 | 0.184902732 | 0.9995575 | -0.620201593 | 0.504596579 |
| | hc 25 | -0.16603346 | 0.184902732 | 0.9439954 | -0.728432546 | 0.396365626 |
| | control 30 | -0.027437686 | 0.184902732 | 0.9999888 | -0.589836772 | 0.5349614 |
| | lc 30 | 0.079173698 | 0.184902732 | 0.9979799 | -0.483225388 | 0.641572784 |

*. The mean difference is significant at the 0.05 level.

Table S14. Multiple comparisons regarding the density of symbiodinaceae among exposure treatments

| | Sample | t.test | P.value | adj. p.value | significance |
|---|---|---|---|---|---|
| Control 25 v.s | Control 30 | -2.6324 | 0.0138 | 0.0276 | * |
| | High Conc 25 | 0.2593 | 0.7974 | >0.9999 | |
| | High Conc 30 | -3.9271 | 0.0005 | 0.0011 | ** |
| | Low Conc 25 | -0.561 | 0.5794 | >0.9999 | |
| | Low Conc 30 | -2.7284 | 0.011 | 0.022 | * |
| | | | | | |
| | hsp | | | | |
| | Sample | t.test | P.value | adj. p.value | significance |
| Control 25 v.s | Control 30 | -2.9586 | 0.0062 | 0.0124 | * |
| | High Conc 25 | 1.4503 | 0.1503 | 0.316 | |
| | High Conc 30 | -3.5168 | 0.0015 | 0.003 | ** |
| | Low Conc 25 | 1.8629 | 0.0729 | 0.1458 | |
| | Low Conc 30 | -4.5144 | 0.0001 | 0.002 | *** |

Table S15 Statistical significance of gene expression analyses compared to control treatment.

# CHAPTER 5

## 5.1 Conclusions

Oceans became giant collection sites for plastic waste. Production trends, usage patterns and changing demographics have resulted in an increase in the incidence of plastics debris and microplastics, in the marine environment (Andrady, 2011). The key problem is that a major portion of plastic produced each year is used to make disposable packaging items or other short-lived products that are permanently discarded within a year of manufacture (Hopewell et al., 2009). Well over a billion single-use plastic items are given out for free every day (Wabnitz and Nichols, 2010). As a result of its lightweight, plastic can be easily carried by ocean currents and transported across ocean basins, stretching the contamination from the shorelines to the deepest parts of each marine environment. Considering the ecological and economic value of coral reef systems, microplastic pollution in reef regions is gradually receiving attention (Thushari and Senevirathna, 2020). Coral reefs not only serve as one of the most charismatic and biodiverse ecosystems on our planet but also sustain the human harvesting of natural resources and the livelihoods for millions of people (Morrison et al., 2020). The impacts of microplastics and nanoplastics on coral health and its potential mechanisms remain further studied (Jacob et al., 2020). There is an urgent need to quantify the magnitude of the potential effects and assess the future impact of increasing microplastic and nanoplastic levels on the world's oceans' reefs, as well as to take severe measures to address the problem at international, national and local levels (Sharma & Chatterjee, 2017).

This research is set out to address the knowledge gaps by the examination of various effects of plastic pollution on coral reefs. Previous studies already demonstrated the negative effect of plastic pollution if corals (Li et al., 2021; Vered at al., 2022), but on the other hand the impact of microplastic in alcyonacean corals was not assessed yet (Vencato et al., 2021), secondly there was only one study about the impact of nanoplastic, which is an emerging environmental threat (Marangoni et al., 2021), and finally there is a knowledge gap regarding the interaction (in terms of adhesion, ingestion, egestion, quantification of coral bleaching, oxidative stress and gene expression analyses) of microplastic in corals exposed at the environmental concentrations and the association among microplastic exposure with the thermal stress (Reichert et al., 2019).

In Chapter one it has been assessed a methodology to test the suitability of soft corals as a potential reference organism in microplastic exposure studies.

Results demonstrated for the first time the patterns of interaction between microplastic and alcyonacean corals. These findings provided ecologically important results because they showed that in alcyonacean corals the physiological tress stress might not be directly related to the microplastic concentration but by the production of mucus that it may act as a sink of microplastic in the surrounding water column. Secondly compared with scleractinian corals, it has been proved that alcyonaceans ingest less microplastic particles compared to reef building corals. Finally, this study calls for further investigations on the effects of the real environmental microplastic concentrations found in the coral reefs ecosystems and the time of interaction on soft corals.

Chapter two was able to demonstrate that the accelerated weathering set up employed for the preparation of the secondary nanofibers and their testing, could be used as a reference model for future ecotoxicity assays onto different species of cnidarians. To assess this nubbins of *P. flava* were exposed to polypropylene nanofibers for 72 h. This study was able to demonstrate that nanofiber caused no mortality, but the exposure at the intermediate concentration was sufficient to report significant adverse effects on the physiology of corals *e.g.* retraction of polyps, production of mucus and tissue bleaching. Therefore, the accelerated weathering set up employed for the preparation of the secondary nanofibers and their testing, could be used as a reference model for future ecotoxicity assays onto different species of cnidarians. In conclusion, these findings highlight once more that mitigating plastic release is of crucial importance to conserving coral reefs. Furthermore, new strategies to manage the potential impact of current and future sources of nano-plastics in marine environments requires a proper knowledge of photooxidative stability and fragmentation pathways of different typologies of plastic material.

Regarding nanoplastics, it is very difficult to determine the concentrations in the natural marine environment, in addition the distribution could be very different from site to site. All this makes it very complex to carry out ecotoxicological studies at natural concentrations, therefore currently it is necessary to use high concentrations to evaluate their molecular effects. However, the concentration of nanoplastics in the marine environment tends to increase

therefore it is necessary to carry out studies that predict the future effect of these substances in coral reef ecosystems.

The last chapter completes the assessment of the potential mechanistic responses of scleractinian corals affected by the combination of global warming and plastic pollution, as occurring in determinate reef areas. Furthermore, microplastic in non thermally stressed nubbins increases oxidative stress which is further increased by in corals exposed to higher temperatures. This study also provides evidence that corals exposed to microplastic have lower adhesion values, but on the contrary, temperature increases the feeding capacity, since thermally stressed corals presented higher microbead ingestion values. On the other hand, this study reported an ecologically important result, showing that heat stress in corals significantly increased the egestion capacity of microplastic. Furthermore, our findings add to a small but rising branch of research on the effects of microplastics on corals, combined with global ocean warming. Hence, the mitigation of ocean warming remains of uttermost importance to preserve the health of coral reefs ecosystems while managing the emergence of new threats like plastic pollution.

Looking forward it is clear that, despite the many recent advances made in the discipline of microplastic pollution and corals, it is important to acknowledge that further research must be carried out to fill the gap of the impact of new threat such as nanoplastic pollution. In conclusion all the kind of environmental threats that impair ecosystems must be studied in combination with thermal stress, simulating the global warming of the oceans.

## 5.2 References


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# APPENDIX

Abstracts of articles published during the PhD programme.

## I. Soft corals and microplastics interaction: first evidence in the alcyonacean species *Coelogorgia palmosa*

*Aquatic Biology, DOI: https://doi.org/10.3354/ab00747*

Microplastics pollution differentially impacts coral reef systems, by threatening corals physically, through physiological distress and by increasing diseases. However, most of the studies to date have focused on scleractinian corals. The present work reports for the first time the patterns of microplastic ingestion and adhesion by the alcyonacean Coelogorgia palmosa. Feeding and adhesion tests were carried out with various concentrations of polyethylene microbeads. Results showed a wide range of surface adhesion, ranging from 3 to 1573 microbeads per coral fragment, suggesting that adhesion driven by mucus is the main mechanism of microplastic trapping. Polyethylene was ingested by 60% of coral fragments, and the average number of ingested microbeads was much lower compared to scleractinian corals. Considering the ecological importance of soft corals in coral reef ecosystems, specific attention regarding microplastic pollution effects on this taxon is recommended.

## II. Exploring the performance of mid-water lagoonnurseries for coral restoration in the Maldives

Inga Dehnert, Luca Saponari, Valerio Isa, Davide Seveso, Paolo Galli, Simone Montano

*Restoration Ecology (2021), doi: 10.1111/rec.13600*

Small island nations like the Maldives are highly dependent on healthy coral reefs and the ecosystem services they provide. Lately, Maldivian reefs have experienced considerable degradation as a result of severe mass bleaching events and accumulating threats posed by pollution, human development, coral diseases, and outbreaks of corallivores. Coral restoration can be a useful mitigation tool in assisting natural recovery, especially when economically important reef areas such as resort reefs are in poor health with slow natural recovery. This study assesses the performance efficiency of lagoon mid-water rope nurseries for coral gardening in two different atolls in the Maldives for the first time. Three different coral genera, namely *Acropora*, *Pocillopora*, and *Porites*, were assessed applying a common monitoring protocol. Fragment survival was generally very high, exceeding 90% survivorship for the genus *Acropora* and *Pocillopora*, while nursing success for Porites was significantly lower(66%). We further report benchmark growth rates for these genera in mid-water rope nurseries in the Maldives. The study also identifies potential threats to coral nursing success, namely disease occurrence and predation, as we report the corallivorous nudibranch *Phestilla* on in situ nursing stock for the first time. Overall, our results suggest that the use of mid-water rope nurseries in lagoons is an efficient and widely applicable technique for rearing corals in the Maldives. We aim to provide useful insight into best practices for applying this coral gardening technique on a wider scale in the archipelago and highlight future research requirements.

## III. Phthalates bioconcentration in the soft corals: Inter- and intra- species differences and ecological aspects

Valerio Isa, Francesco Saliu, Chiara Bises, Sara Vencato, Clarissa Raguso, Simone Montano, Marina Lasagni, Silvia Lavorano, Massimiliano Clemenza, Paolo Galli

*Chemosphere (2022), https://doi.org/10.1016/j.chemosphere.2022.134247*

The bioconcentration of dimethyl phthalate (DMP) diethyl phthalate (DEP) dibutyl phthalate (DBP) butyl benzyl phthalate (BBzP), di-(2-ethy hexyl) phthalates (DEHP), mono-butyl phthalate (MBP), mono-benzyl phthalate (MBzP), mono-(2-ethy hexyl) phthalate (MEHP) in the soft corals *Coelogorgia palmosa*, *Sinularia sp*., *Sarcophyton glaucum*, and *Lobophytum sp.* was investigated. Specimens were cultured in a microcosm environment built-up at the Genova Aquarium and analyses were carried out by in vivo SPME-LC-MS/MS. The distributions of the phthalates among the four surveyed species resulted significantly different. Calculated bioconcentration factors (BCFs) showed values spanning over two orders of magnitude, from a minimum of log10 BCFDEP = 1.0 in *Sarcophyton glaucum* to a maximum of log10 BCFDBP = 3,9 calculated for *Coelogorgia palmosa*. Moreover, the calculated BCFs of the long chain phthalates resulted up to three orders of magnitude lower than theoretically predicted (from logKow), whereas BCF of short chain phthalates resulted higher. This, together with the detection of phthalic acid monoesters, suggests the presence of species-specific different metabolic transformation among the surveyed soft coral species that involve DEHP.

## IV. Short-term microplastic exposure triggers cellular damage through oxidative stress in the soft coral *Coelogorgia palmosa*

Enrico Montalbetti, Valerio Isa, Sara Vencato, Yohan Louis, Simone Montano, Silvia Lavorano, Davide Maggioni, Paolo Galli, Davide Seveso

*Marine Biology Research (2022), https://doi.org/10.1080/17451000.2022.2137199*

Microplastics are a persistent and ubiquitous source of pollution in the marine environment, representing a severe threat to tropical coral reefs. The effects of microplastics on reef building (hard) corals have been documented (interference with normal digestion process, polyp retraction, oxidative stress, impairment of the photosynthetic machinery, bleaching). However, the impact of microplastics on soft corals, the second most abundant benthos of tropical reefs, remains to be thoroughly studied. In this work, we analysed the effects of a short-term microplastic exposure on the cellular physiology of the soft coral *Coelogorgia palmosa*. We found that samples exposed to >50 mg l−1 of microplastic showed significant increase in the activities of the antioxidant enzymes glutathione reductase, catalase, and superoxide dismutase, suggesting a rise in oxidative stress. Furthermore, exposure to microplastics increased lipid peroxidation, indicating oxidative damage. Overall, our results show that similar to hard corals, microplastic ingestion causes oxidative stress and cellular damage in soft corals. Our study provides a first assessment of physiological effects of microplastic exposure on the soft coral, *Coelogorgia palmosa*, highlighting the need for further investigations about these contaminants and their influence on marine benthic fauna. Such information is crucial to understand how different reef organisms respond to microplastic pollution and who the ecological winners or losers will be in an increasingly polluted marine environment.

# Acknowledgments

I would like to thank first of all Paolo Galli for this professional opportunity, which I really appreciated, and I hope to continue in the future. I have a special thanks to my colleague Sara Vencato, since we started together this adventure, and I am really proud to have worked with her in the Maldives and in the Genoa Aquarium.

For the professional support, I must Thank Davide Seveso and Simone Montano, Yohan Louis, Enrico Montalbetti, and a special thanks is dedicated to Francesco Saliu for his invaluable help during my journey.

A special thanks to Silvia Lavorano for believing in me and for all the occasions at the Acquario di Genova. I thank all the tropical team of the aquarium but a special thanks is dedicated to Fabio Mattioli. Last but not least I thank my family for all the emotional support.