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Effects of nanostructure antifouling biocides towards a coral species in the context of global changes

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Abstract

Biofouling prevention is one of the biggest challenges faced by the maritime industry, but antifouling agents commonly impact marine ecosystems. Advances in antifouling technology includes the use of nanomaterials. Herein we test an antifouling nano-additive based on the encapsulation of the biocide 4,5-dichloro-2-octyl-4-isothiazolin-3-one (DCOIT) in engineered silica nanocontainers (SiNC). The work aims to assess the biochemical and physiological effects on the symbiotic coral *Sarcophyton* cf. *glaucum* caused by (1) thermal stress and (2) DCOIT exposure (free or nanoencapsulated forms), in a climate change scenario. Accordingly, the following hypotheses were addressed: (H1) ocean warming can cause toxicity on *S.* cf. *glaucum*; (H2) the nanoencapsulation process decreases DCOIT toxicity towards this species; (H3) the biocide toxicity, free or encapsulated forms, can be affected by ocean warming.

Coral fragments were exposed for seven days to DCOIT in both free and encpasulated forms, SiNC and negative controls, under two water temperature regimes (26 ºC and 30.5 °C). Coral polyp behavior and photosynthetic efficiency were determined in the holobiont, while biochemical markers were assessed individually in endosymbiont and coral host. Results showed transient coral polyp retraction and diminished photosynthetic efficiency in the presence of heat stress or free DCOIT, with effects being magnified in the presence of both stressors. The activity of catalase and glutathione-*S*-transferase were modulated by temperature in each partner of the symbiosis. The shifts in enzymatic activity were more pronounced in the presence of free DCOIT, but to a lower extent for encapsulated DCOIT. Increased levels of oxidative damage were detected under heat conditions. The findings highlight the physiological constrains elicited by increase seawater temperature to symbiotic corals and demonstrate that DCOIT toxicity can be minimized through encapsulation in SiNC. The presence of both stressors magnifies toxicity and confirmed that ocean warming enhances the vulnerability of tropical photosynthetic corals to local stressors.

Keywords: Nanotechnology; Ocean warming; Octocorals; Oxidative stress; Photosynthesis; Symbiosis

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1. Introduction

One of the major challenges faced by the maritime industry is to prevent and control biofouling, the spontaneous biological colonization of submerged surfaces. The presence of fouler organisms contributes to surface roughness, increases drag and fuel consumption (Schultz, 2007) and, consequently, the release of greenhouse gases (Champ, 2000). Besides increasing the carbon footprint, biofouling causes serious economic, social and ecological constraints, such as surface corrosion or the transport of non-native species worldwide (Hellio and Yebra 2009).

In 2008, with the global prohibition of tributyltin use in antifouling paints (IMO, 2008), biocides containing inorganic, organo-metallic or organic molecules were included in antifouling coatings (Omae, 2003). Recent advances in antifouling (AF) technology also includes the use of biocidal-free solutions such as the functional surfaces (Nurioglu et al., 2015) and natural antifouling compounds (Neves et al., 2020). However, functional surfaces present the drawback of losing AF efficiency under low-speed conditions or static structures and natural solutions fail in large-scale applicability (Lejars et al., 2011; Wang et al., 2017). Thus, biocide AF paint coatings are still commonplace given its cost effectiveness, availability and easy of application. The broad-spectrum booster biocide 4,5-dichloro-2-n-octyl-4-isothazolin-3-one (DCOIT) has been authorized by the European Biocidal Regulation No. 528/2012 (EU, 2012) and is the active ingredient of some commercially available paints. DCOIT is effective against some fouler species at low doses (EU, 2014), however it is well tolerated by other cosmopolitan target species, such as barnacles (Jacobson and Willingham, 2000) or mussels (Figueiredo et al., 2019). Additionally, acute toxicity towards non-target microalgae and invertebrate species (Fernandez-Alba et al., 2002; Jesus et al., 2021) have been reported after DCOIT exposure as well as chronic effects on fish at low doses, with effects at the neurotransmission and proteome levels and reproductive function (Chen et al., 2015, 2014). The continuous exposure to DCOIT at sublethal concentrations for four weeks also impaired cholinergic neurotransmission and reduce crustacean growth and reproductive output (Do et al., 2018). Given the maximum biocide environmental concentrations detected in marine waters (3.7 μg L^{-1}) and sediments (0.81 μg g^{-1}) (Batista-Andrade et al., 2018; Martinez et al., 2000) and the ecotoxicological data available (Chen and Lam, 2017; Figueiredo et al., 2020), concerns are raised regarding DCOIT ecological hazard to the marine compartment (Campos et al., 2021), specially for benthic species (Soroldoni et al., 2020).

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Nanotechnology-based responsive engineered materials have been successfully used to encapsulate biocides aiming at controlling biocide premature leaching and reduce environmental impacts (Maia et al., 2015). These antifouling engineered nanomaterials, particularly DCOIT encapsulated in silica mesoporous nanocapsules (DCOIT encapsulated) has been shown to display (a) biocidal controlled release over time (Figueiredo et al., 2019), (b) low toxicity and hazard towards several non-target invertebrate temperate and tropical species when compared to the commercialized biocides (Figueiredo et al., 2020; Gutner-Hoch et al., 2019; Jesus et al., 2021) and (c) high efficacy against target species (Figueiredo et al., 2020; Gutner-Hoch et al., 2018; Santos et al., 2020). However, there is no information available on this antifouling nanomaterial toxicity towards cnidarians, which are key representative species of marine areas and amongst the most endangered group of marine organisms worldwide (Hughes et al., 2018).

Coral reefs (Cnidaria, Anthozoa) are important biodiversity hotspots, providing several ecosystem services and contributing actively to carbon biogeochemical cycle (Roberts et al., 2002). In symbiotic corals, the mutualist association with the microalgae of genus *Symbiodinium* (Alveolata, Dinophyceae) is important to fulfil metabolic energetic requirements of both coral (host) and microalgae (endosymbiont) (Muscatine and Porter 1977). This symbiotic association is referred as *holobiont* and its disruption, either through abiotic or anthropogenic stressors, is triggered by a cascade of cellular events that culminate in coral bleaching – i.e. the reduction of *Symbiodinium* cells density due to apoptosis, necrosis or cell detachment (Ainsworth et al., 2008; Gates et al., 1992) and the degradation of photosynthetic pigments (Venn et al., 2006). The occurrence of bleaching events associated to increased seawater temperature (Glynn and D'Croz, 1990; Hughes et al., 2018), high irradiance (Dykens et al. 1992; Lesser and Farrell 2004) and contaminants runoff (Downs et al., 2005; Glynn et al., 1984; Markey et al., 2007) induced a rapid decline in corals' population since 1980 (Hughes et al., 2018). Unbalanced redox homeostasis is reported in thermally bleached corals (Shick and Dykens 1985; Lesser 1997; Nii and Muscatine 1997; Saragosti et al. 2010; Hawkins et al. 2015), however the cellular mechanisms underlying the symbiosis breakdown, particularly the contribution of each symbiosis partner to reactive oxygen species (ROS) levels and its implication on coral bleaching, are yet to be fully understood (Davy et al. 2012; Nielsen et al. 2018).

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Soft corals of genus *Sarcophyton* (Octocorallia) are amongst the most thermal tolerant species (Strychar et al., 2005). The species *Sarcophyton glaucum* is conspicuous in shallow and continental shelf waters of tropical and subtropical regions (Benayahu et al., 2019; Benayahu and Loya, 1986; Fabricius, 1995; Shoham and Benayahu, 2017). Previous studies showed that DCOIT exposure modulates the activity of antioxidant enzymes in this species (Cima et al., 2013). However, the mechanisms underlying the fine-tune regulation of antioxidant defences of each partner in the symbiosis is yet to be investigated. Additionally, few studies addressed the combined effects of contaminants to symbiotic corals under global climate change scenarios (Banc-Prandi and Fine, 2019; Fonseca et al., 2021, 2017; Kwok et al., 2016; Negri et al., 2011), although the photosynthetic performance of these species is known to be negatively affected by the combination of high temperature and xenobiotics (Banc-Prandi and Fine, 2019; Kwok et al., 2016; Negri et al., 2011; Negri and Hoogenboom, 2011).

The present work aims to assess the physiological and sub-cellular effects towards the coral *Sarcophyton* cf. *glaucum* caused by: (1) thermal stress, and (2) anti-fouling biocide DCOIT exposure, on both free or nanoencapsulated forms, in a climate change scenario. In this context, the following working hypotheses were addressed: H1 – seawater temperature rise can cause toxicity on the selected coral species; H2 – the nanoencapsulation process contributes to decrease the toxicity of the antifouling biocide DCOIT on the selected tropical coral species, in the current climate scenario; H3 – the biocide toxicity, either on the free or encapsulated forms, can be affected in a global warming scenario.

To achieve this, coral fragments were exposed to the free or encapsulated biocides and corresponding controls, under current ocean temperature in tropical regions and forecasted seawater warming conditions predicted for 2100 (Stocker et al., 2014). Sublethal effects were evaluated at the individual level in the holobiont and in the photosynthetic efficiency of endosymbionts, but also at the cellular level, looking at biochemical endpoints (catalase; glutathione-*S*-transferase and lipid peroxidation) in isolated fractions of the animal-algae association.

2. Materials and methods

2.1 Coral husbandry and fragmentation

The octocoral *Sarcophyton glaucum* (Quoy & Gaimard, 1833) was chosen as the ecotoxicological model species to study symbiotic cnidarians given its wide geographic

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distribution, easy propagation in captivity (Sella and Benayahu, 2010) and lack of conservation status (IUCN, 2021). To the best of our knowledge, *Sarcophyton* taxonomy is not consensual, supporting the use of gene barcoding for molecular phylogeny (Aratake et al., 2012; McFadden et al., 2010). Hereby, the present work refers to *Sarcophyton glaucum* as *Sarcophyton* cf. *glaucum*, and coral samples were stored at -80 °C for future molecular validation of its taxonomy.

Two mother colonies of *S.* cf. *glaucum* were provided by a certified supplier (TMC Iberia, Portugal) and stocked in a recirculated life support system (LSS) (Rocha et al., 2015) composed by three 90 L glass tanks, connected to a filtering reservoir of 150 L, equipped with a protein skimmer (ESC—150 ReefSet, Portugal), biological filter and a submergible heater (Eheim Jäger 300 W, Germany). Water recirculation (flow of ≈ 2000 L h^{-1}) was provided by a submerged pump (Eheim 1262, Germany) assembled to the filter tank. Additionally, each glass tank was equipped with a circulation pump (Turbelle nanostream-6025 Tunze, Germany), which promoted an approximated water flow of 2500 L h^{-1}. The illumination system consisted in a white 150 W hydrargyrum quartz iodide (HQI) 10 000 K lamp (BLV, Germany) under a 12h light:12h dark photoperiod. The lamps position was adjusted to obtain a homogenous photosynthetically active radiation (PAR) of 50 μmol $quantam^{-2}$ s^{-1} measured with a quantum flux meter (Apogee, MQ-200) with a submergible sensor. This PAR level was chosen based on previous studies showing good physiological performance of this coral species under this light regime (Costa et al., 2016; Pimentel et al., 2016). Artificial seawater (ASW) was prepared by dissolving Tropic Marin Pro Reef salt (Tropic Marin®, Germany) in tap water filtered by reverse osmosis (Aqua-win RO—6080, Thailand) to achieve 35 ± 1 salinity and used one day after preparation. Reverse osmosis water was used to adjust ASW salinity due to water evaporation. Water parameters were checked twice a week with probes and colorimetric tests and, on average, were as follows: 26 °C ± 0.5 °C; pH 8; 0 mg L^{-1} $NH4^{+}$, 0 mg L^{-1} NO_2^{-}; <5 mg L^{-1} NO_3^{-}; 0 mg L^{-1} PO_4^{3-} ; 400 ± 10 mg L^{-1} Ca^{2+} and 10-12 carbonate hardness (KH). During the acclimation period (15 days), water parameters, polyp retraction, mucus production and external signs of infection were monitored to assess the coral health status. Thereafter, the capitulum of each mother colony was fragmented with a sterilized scalpel, generating 25 similar sized clonal coral fragments (≈30 mm diameter) that were attached to a coral stand (Coral Cradle®) with a rubber band and identified. On a weekly basis, 10% of total water volume of the system was renewed. Coral fragments

(n=50) were kept in the LSS for one month to allow for full tissue regeneration, adhesion to the cradle (**Figure S1**) and acclimation to each of the temperature scenarios being employed in the ecotoxicological assays: 26 ± 0.5 °C (current ocean mean temperature in tropic regions) or 30.5 ± 0.5 °C (likely range of temperature increment interval as forecasted by RCP 8.5 emission scenario until 2100) (Schwalm et al., 2020; Stocker et al., 2014). A ramping rate of 1 °C day^{-1} was used for adaptation to the warming scenario during the acclimation phase (Middlebrook et al., 2010).

2.2 Chemicals

The antifouling biocide solution SEA-NINE™ 211 N, containing 30% of 4,5-dichloro-2-octyl-4-isothiazolin-3-one (DCOIT) in mixed xylenes was obtained from Rohm and Haas Ltd. (Philadelphia, USA). Tropic Marin Pro Reef Salt was purchased from Tropic Marin®. All other chemicals used were obtained from Sigma-Aldrich Europe, except the Bradford reagent which was purchased from Bio Rad (Germany) and the 35% (v/v) hydrogen peroxide (H_2O_2) that was obtained from Merck (Germany).

2.3 Test solutions and dispersions

Mesoporous silica nanocontainers (SiNC) were synthesized according to Chen *et al.* (2008). The encapsulation of SEA-NINE™ 211N in SiNC (DCOIT encapsulated) was performed as described by Maia *et al.* (2015). Briefly, the formation of silica nanocapsules and biocide encapsulation occurs in one step, as a result of an oil-in-water microemulsion polymerization process in the presence of cetyltrimethylammonium bromide (CTAB), ammonium hydroxide, tetraethoxysilane (TEOS) and solvents mixture. Mesoporous capsules with differentiated porosity from core to outer shell regions is a result of the gasification of solvents (oil phase), arising from the exothermic polymerization of TEOS. The obtained nanomaterials were characterized by Maia et al. (Maia et al., 2015) and Figueiredo et al. (2019) in terms of particle size and morphology, textural properties, surface area, biocide loading efficiency and dissolution.

After synthesized, both nanomaterials (SiNC and DCOIT encapsulated) were filtered, washed, and dried (140 °C) to remove solvent residues. The SEA-NINE™ 211 N solution was also dried for 30 min at 140 °C to evaporate xylene.

Stock solutions of DCOIT (3 mg L^{-1}), SiNC (10 mg L^{-1}) and DCOIT encapsulated (10 mg DCOIT L^{-1}) were prepared using ultra-pure water (UP, Millipore® Academic Milli-

Q system) and placed for 30 min in an ultra-bath sonicator (Selecta; 550W; 40 KHz of frequency). Then, stocks of both DCOIT and DCOIT encapsulated were diluted in filtered ASW to obtain a nominal concentration of 50 µg DCOIT L^{-1} . This test concentration were chosen based on previous ecotoxicological trials using *Sarcophyton* cf. *glaucum* (Cima et al., 2013) and lack of more recent data regarding DCOIT effects to cnidaria species (Figueiredo et al., 2020; Martins et al., 2017). To discriminate the toxicity solely associated to silica nanoparticles, 196 µg SiNC L^{-1} in ASW were also prepared, corresponding to the same amount of SiNC present in DCOIT encapsulated (nanocontainer control). Negative controls (ASW) were also added, one for each tested temperature (26 and 30.5 °C).

2.4 Exposure to the engineered nanomaterials and free biocide

Coral fragments were individually placed in 1-L glass beakers. Five fragments (n = 5) were exposed for seven days to each of the tested conditions: DCOIT, DCOIT encapsulated and controls (ASW, SiNC) at the selected temperatures (26 and 30.5 °C). The beakers were filled with 800 mL of the test solutions and randomly distributed in the water-bath setup in the LSS, with constant temperature and air flow adjusted to ≈ 1 bubble s^{-1}. Lighting PAR levels, salinity and photoperiod were identical to those employed at the acclimation phase. Dissolved oxygen, pH and conductivity levels were daily checked with a portable sensor. No media renewal was carried out in the exposure period, as no significant DCOIT degradation was previously recorded for a time frame matching the test period (Figueiredo et al., 2019).

2.5 Endpoints' assessment

2.5.1 Coral polyps´ behavior

Retraction of polyps has been reported in coral species under exposure to several chemical substances known to cause bleaching and/or mortality (Grant et al., 2010; Markey et al., 2007; White et al., 2012). Herein, all clonal coral fragments were daily photographed two hours after the lighting was switched on and scored based on the percentage (%) of expanded polyps: fully retracted (0%); fully expanded (100%) and intermediate stages (25, 50 or 75%) of polyp expansion (**Figure 1**).

2.5.2 In vivo chlorophyll a fluorescence of endosymbionts

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The effects of biocidal exposure to the microalgae *Symbiodinium* spp. were monitored *in vivo* by measuring the maximum quantum yield of the photosystem II (*Fv/Fm*), a proxy of photosynthetic function (Schreiber et al., 1986), using pulse amplitude modulation (PAM) fluorometry. Measurements were performed daily, three hours after the lighting system was switched on to ensure full-functioning of the photosynthetic apparatus, using a Junior-PAM instrument equipped with a blue-LED lamp, a plastic fiber optic bundle and the WinControl-3 software (Walz, Germany) (Rocha et al., 2013). Each coral fragment was placed in a water bath set to 26 or 30.5 °C, to avoid temperature fluctuations, and dark-adapted for 15 min prior to *Fv/Fm* quantification. The fiberoptic was positioned at a 1-mm distance from the coral surface. The minimum fluorescence (F0) was measured by applying a weak pulse of light and maximum fluorescence (Fm), was obtained through a saturation pulse of actinic light for 0.8 s. Three measurements were recorded for each fragment in distinct regions, with *Fv/Fm* being calculated according to Schreiber *et al.* (1986) as follows and presented as an average value:

$$Fv/Fm = \frac{(Fm - F0)}{Fm}$$

2.5.3 Biochemical markers

At the end of the exposure period, coral fragments were transferred individually to falcon tubes, flash frozen in liquid nitrogen and stored at -80 °C until analysis of the levels of LPO and the enzymatic activities of CAT and GST. An additional group of control organisms (n = 5) were flash frozen and used for chlorophyll a (chl *a*) determinations, a proxy of the presence of *Symbiodinium* cells and indirect estimation of coral fraction contamination by intracellular symbionts. All the biochemical markers were determined in both microalgae (endosymbionts) and coral (host) cell fractions, separately.

Prior to the determinations, endosymbionts located within coral fragments were quickly and carefully isolated on ice as described by Richier *et al.* (2003), with some modifications. Briefly, endosymbionts were first detached from coral tissues by placing each fragment in a weighing glass containing 500 µL of cold 0.2 M potassium (K)-phosphate buffer (pH 7.4) and microalgae were dragged out from coral tissues with a round scalpel. The brown-looking microaglae suspension was transferred to an eppendorf and the remaining algae cells were washed out with additional 500 µL K-phosphate buffer and collected to the same vial. This suspension was centrifuged at 500

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g for 5 min at 4 °C to remove debris and washed twice in K-phosphate buffer (pH 7.4). At last, cells were ressuspended in 1 mL of K-phosphate buffer (pH 7.4) and sonicated on ice (3x10 s) (Sonifier, Branson Ultrasonics Corp, USA) to obtain the microalgae homogenate. The host tissues were then reduced to powder using mortar and pestle in liquid nitrogen (Downs et al., 2002). The frozen tissue was ressuspend in k-phosphate buffer (pH 7.4) (100 µL per mg of wet weight) and sonicated on ice (3x10 s) to obtain the coral homogenate.

Aliquots of each homogenate were transferred to microtubes to determine LPO (added 4 µL of 4% butylated hydroxytoluene, BHT, in methanol) and protein content. The remaining sample was centrifugated at 10 000 *g* for 20 min at 4 °C to obtain a post-mitochondrial supernatant (PMS) which was aliquoted separately to determine protein concentration, CAT and GST activities. All aliquots were kept at -80 ºC until analysis and the spectrophotometric quantifications were performed using a microplate reader (MultiSkan Spectrum, Thermo Scientific, USA).

Protein concentration in crude and PMS samples were assayed according to the Bradford method (1976), adapted from Bio-Rad's Bradford microassay, using bovine γ-globulin as a standard at 595 nm wavelength.

Levels of LPO were determined by measuring the production of thiobarbituric acid-reactive substances (TBARS), as described by Ohkawa et al. (1979) and modified to microplate reading by Bird and Draper (1984). The absorbance was immediately read at 535 nm and the results were expressed as nmol TBARS hydrolysed per min per mg protein, using the molar extinction coefficient (ε = 1.56 x 105 M^{-1} cm^{-1}).

The activity of CAT (EC 1.11.1.6) was determined acording to Clairborne (1985), with adaptations to microplate reading (Ferreira et al., 2015), monitoring H_2O_2 consumption for 2 min at 240 nm wavelength. The enzymatic activity was expressed as units (U) per mg of protein, corresponding U to 1 µmol of substrate hydrolysed per min, given the extinction coefficient (ε = 40 M^{-1} cm^{-1}).

The activity of GST (EC 2.5.1.18) was determined based on Habig *et al.* (1974) following adaptations to microplate (Frasco and Guillhermino, 2002). The conjugation of L-glutathione reduced (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by GST was measured at 340 nm for 5 min. The enzymatic activity was expressed as units (U) per mg of protein, corresponding U to 1 nmol of substrate hydrolysed per min, given the extinction coefficient (ε = 9.6 x 10^{-3} M^{-1} cm^{-1}).

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Levels of chl *a* were determined according to Lorenzen (1967) with some modifications. Briefly, both microalgae and host fractions homogenates were obtained as previously described. Samples were centrifugated at 4000 rpm, for 10 min at 4 °C and ressuspended in 2 mL of cold acetone 90% (v/v) for pigment extraction. Eppendorfs were incubated in the dark for 24 h at 4 °C and then centrifugated again at 4000 rpm for 10 min at 4 °C. The supernatant absorbance was determined spectrophotometrically (Genesys 6, Thermo Spectronic, Whalthman, USA) at 664 and 750 nm wavelenghts before and after acidification with 18 µL of 1N hydrochloric acid (HCl), respectively. The chl *a* content was calculated as below and normalized against corresponding homogenate protein content (mg).

Chl *a* ($\mu g\ L^{-1}$) = $\frac{A\ x\ K\ [(A664-A750)-(A664a-A750a)]\ x\ v}{V\ x\ L}$, where

A = absorbance coefficient of chlorophyll *a* (11.4)

v = acetone volume (mL)

V = sample volume (L)

L = path length of cuvette (cm)

R = maximum ratio of $664_{initial}:664_{acidified}$ in the absence of pheophytin pigments (1.7)

K = correction factor to equate the reduction in absorbance to initial chl *a* concentration, K = R/(R-1) (2.43)

2.6 Statistical analysis

Data normality and homoscedasticity were determined using Shapiro-Wilk and Levene´s tests respectively, with a significance level of 0.05. Statistical differences due to the combination of antifouling treatment and seawater temperature were analyzed using a two-way analysis of variance (ANOVA) for the biochemical markers or repeated measurements two-way ANOVA for photosynthetic activity. Whenever significant differences were attained ($p < 0.05$) a post-hoc Holm-Šidák multiple comparison test was performed. Since normality and homoscedasticity of data were no achieved, alterations in holobiont polyps´ behavior were determined through an ANOVA on ranks (Kruskal-Wallis), to test differences between treatments in each temperature scenario, and Mann-Whitney rank test to compare the temperature effect for each combination of treatment and day. All statistical analyses were performed using the software SigmaPlot v.12.5 (Systat Software, UK).

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The biochemical responses in the holobiont isolated fractions were integrated using multivariate statistics (PRIMER & PERMANOVA v.6). A dummy variable was added to the dataset whenever outliers were detected (Rudin et al., 2012). Biomarkers' data matrix (endpoints x replicates) was square root transformed, normalized and the euclidean distance matrix calculated between samples (treatments x treatments). The distance among centroids was calculated among the combination of all independent variables (treatment, temperature, tissue fraction) and the resulting matrix was analysed using the principal coordinates analysis (PCO). The relationship between experimental conditions and the biomarkers' levels was estimated by Pearson correlation coefficient (r), considering a correlation level > 0.75.

3. Results

3.1 Effects on coral polyps´ behavior

Changes in holobiont behavior are presented as percentage (%) of coral open polyps through seven days (D) of exposure to the different treatments at the two tested temperatures (**Figure 2**: A - 26 °C and B - 30.5 °C).

No behavioral alterations in the holobiont were detected in the negative control group (CTL) at 26 ºC, throughout the experiment. Under heat conditions (30.5 °C) polyp retraction were observed in the CTL at days three and five when comparing to the initial stage, D0 (**Figure 2** - A, $p < 0.05$), but those differences were transient and not significant by the end of the assay (D7).

To study shifts in holobiont behavior due to temperature increment (26 ºC vs 30.5 ºC), comparisons were also performed within each treatment and timepoint. Physiological acclimation to thermal stress was responsible for a strong polyp retraction effect observed in the CTL (day 3 and 5) and free DCOIT (day 5) groups (**Figure 2** - B), $p < 0.05$). In all remaining treatments, besides an overall decrease on the % of open polyps within each timepoint under heat conditions, differences were not statistically significant.

At current ocean temperature (**Figure 2** - A) the percentage of open polyps in corals exposed to free DCOIT significantly decreased from 100% to 20 ± 27% after 24 h, being statistically distinct of the corresponding CTL ($p < 0.05$). No significant morphological changes were detected in the same period for SiNC and encapsulated DCOIT treatments comparatively to the CTL. Additionally, corals exposed to the encapsulated DCOIT exhibited a higher percentage of open polyps comparatively to

those subjected to the commercial form (free DCOIT, ($p < 0.05$). The time-course variations in polyp morphology, considering the initial level (D0) as reference, showed significant differences in free or encapsulated DCOIT treatments in the first 24 h of exposure at 26 ºC ($p < 0.05$). However, differences were no longer significant after 72 h and a complete reversion of the polyp expansion behavior was observed in the last day of exposure.

Under heat conditions (**Figure 2** - B) the percentage of expanded polyps decreases from 100% to 10 ± 22% within the first 24 h in the free DCOIT treatment, relatively to the CTL ($p < 0.05$). When compared the two antifouling biocides, the corals exposed to free DCOIT presented more retracted polyps than those exposed to the encapsulated DCOIT in distinct timepoints ($p < 0.05$). During the seven days exposure, the effects generated by the co-exposure to heat conditions and experimental treatments were noticed in the DCOIT treatment from day one to day five ($p < 0.05$). Furthermore, an overall closure of coral´s polyps was observed in all other treatments from day three ($p < 0.05$) to day five, except in encapsulated DCOIT. On the last day of exposure (D7), an increment on the % of open polyps was registered in all treatments, but only the encapsulated DCOIT treatment showed a similar polyp expansion percentage to that recorded at initial stages (D0) ($p > 0.05$) and remained statistically different from DCOIT. The empty silica nanocontainers showed similarities to CTL and encapsulated DCOIT throughout the assay.

3.2 Effects on endosymbionts photosynthetic activity

The photosynthetic efficiency (*Fv/Fm*) of endosymbionts under the two temperature regimes, through seven days, are presented in **Figure 3**.

The increment of 4.5 °C in temperature caused a significant reduction of 15% in the initial *Fv/Fm* value (Day 0) in the two CTL groups ($p < 0.001$). The differences were sustained in the initial 24 hours (Day 1, $p < 0.01$), but were not significant afterwards ($p > 0.05$).

In both DCOIT containing treatments (free or encapsulated), the reduction on the *Fv/Fm* value observed initially (day 0) was associated to thermal stress, with a decrease of about 11% and 8% in free and encapsulated DCOIT, respectively ($p < 0.05$) and remained significant until day three in both treatments.

When comparisons were performed considering the four distinct treatments and the two temperatures tested, no differences were detected in the corals exposed to 26 °C.

However, at 30.5 °C significant changes were observed at day 1 with a higher *Fv/Fm* being recorded for the SiNC treatment comparatively to its CTL ($p < 0.05$). At the end of the assay no differences recorded in the distinct treatments or temperatures.

3.3. Effects on biochemical markers of isolated fractions of coral symbiosis

The results indicate levels of 16.7 and 1.4 µg chlorophyll *a*/mg protein in microalgae and coral host fractions, respectively (**Table S1**). The pigment levels in the two fractions were statistically distinct (*t*-test, $p < 0.01$) showing residual contamination of host fraction from the presence of endosymbionts (< 10%). This level was higher than what was previously described by Richier *et al.* (2003), but still low enough to consider the contamination levels negligible.

The enzymatic biomarkers activity (CAT and GST) and the LPO levels in each holobiont isolated fractions are presented in **Figure 4**, while the protein content is provided as supplemental material (**Table S2**). Overall, heat treatment caused a significant inhibition ($p < 0.001$) of catalase activity (CAT) on microalgae (**Figure 4** - A). In opposition, a stimulatory effect was detected in the cnidaria host (Figure 4 - D), particularly in organisms exposed to free DCOIT ($p < 0.001$). To note that distinct CAT activities were detected in the two fractions at baseline conditions (26 °C). For instance, the enzyme levels in CTL treatment on microalgae was 13 ± 1.5 U/mg protein and 5 ± 1.8 U/mg protein in the cnidaria host. The exposure to the different treatments, within each temperature regime and fraction, caused no changes in CAT activity at 26 °C, for both fractions ($p < 0.05$). Contrary, changes in the enzyme activity were detected at 30.5 °C in microalgae, between encapsulated DCOIT (the lowest value) and free DCOIT (the highest) ($p < 0.05$). Furthermore, CAT activity in the cnidaria host was significantly enhanced in the free biocide treatment, relatively to the CTL ($p < 0.05$).

A significant rise of GST activity was recorded by microalgae ($p < 0.05$) and cnidaria host ($p < 0.001$) (**Figure 4** - B and E) after exposure to heat conditions, except for SiNC treatment in microalgae. Distinct GST baseline levels were detected in the two fractions under control conditions (CTL, 26ºC), reporting enzyme activity of 11 ± 4.6 U/mg protein in the microalgae and of 7 ± 4.8 U/mg protein in the cnidarian host. Despite having a lower baseline GST level, the rise in temperature induced a more pronounced stimulation on GST in the host ($CTL_{30.5\,°C}$ 16 ± 1.0 U/mg protein) when compared to the microalgae ($CTL_{30.5\,°C}$ 20 ± 5.8 U/mg protein). When assessing the differences within each fraction, for each tested temperature, the presence of free DCOIT and SiNC

promoted a two-fold increase on GST activity in microalgae at 26 ºC ($p < 0.05$), but no changes were detected in the coral host. The GST activity at 30.5 ºC was similar between treatments, but DCOIT containing treatments displayed the highest enzymatic levels of activity in both fractions, although not statistically distinct from CTL.

The LPO levels are represented in **Figure 4** (C and F). Exposure at a higher temperature increased LPO levels in both fractions and a significant increment ($p < 0.05$) was detected in the microalgae CTL or in cnidaria DCOIT treatments (free or encapsulated). Within each tested temperature, no significant LPO variation was detectable in none of the fractions when distinct treatments were applied. The baseline LPO values obtained in the CTL groups of both fractions at 26 °C were, approximately, 1 nmol TBARS/mg protein. However, when organisms were stocked at 30.5 °C, the microalgae duplicated the level of LPO (≈ 2 nmol TBARS/mg protein, $p < 0.01$), a trend not detected in cnidarian host isolates.

3.4 Multivariate analysis

In **Figure 5**, a PCO plot is presented with the centroids of all treatments considering biochemical markers, with axis 1 explaining 70.6% of total variation and axis 2 explaining 22.6 % of total variation. Axis 1 splits the biochemical profile of coral host due to temperature regime (under distinct chemical treatments). The positive values of axis 1 correspond to the biochemical endpoints in the cnidaria host exposed to heat conditions, as well as microalgae samples (both temperatures). Host samples at current day temperatures are represented on the negative side of axis 1. Axis 2 discriminates the changes on the biochemical profile of microalgae when exposed to 26 °C (positive values) and to 30.5 °C (negative values).

Both GST and LPO are highly correlated with the positive values of axis 1 (r = -0.95 and r=-0.88, respectively) highlighting that both biomarkers are enhanced under thermal stress. On the other side, CAT is correlated to positive values of both axis (r = -0.60 and r = -0.80 of axis 1 and 2, respectively), being the enzyme activity high in microalgae under present day ocean temperature, but also in cnidaria host cells when thermal stress is combined with free DCOIT biocide.

4. Discussion

Coral reef populations have been depleted by ocean warming (Hughes et al., 2018), but also due to the exposure to local contaminants (Negri and Hoogenboom, 2011).

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However, literature on the effects associated to the combination of both stressors to symbiotic corals is scarce and contradictory (Banc-Prandi and Fine, 2019; Fonseca et al., 2021, 2017; Kwok et al., 2016; Negri et al., 2011), making difficult the implementation of environmental risk assessment under climate change scenarios (Negri et al., 2020). The current work corresponds to the first ecotoxicological assessment on the impacts of the innovative antifouling engineered nanomaterial - silica encapsulated with the booster biocide DCOIT - to a symbiotic soft coral from tropical regions in the context of ocean global changes.

4.1 Effects of temperature on Sarcophyton **cf.** *glaucum physiology*

4.1.1 Effects on the coral polyps´ behavior

During the exposure period, polyp retraction was detected in the thermally stressed CTL group, but not in the CTL at baseline temperatures. These results may reflect a strategy to acclimate to new thermal conditions, as polyp retraction is considered a behavioral photoprotective strategy to avoid overexcitation of photosynthetic apparatus (Brown et al., 2002). In agreement to our results, polyp retraction was previously reported in cnidarians under thermal stress (Ezzat et al., 2013; Schlöder and D'Croz, 2004) and high irradiances (Brown et al., 2002; Dykens and Shick, 1984). To be noted that under heat conditions and overall coral paling and enhanced mucus production was detected (**Figure S2**), which highlight the physiologic distress.

Higher than usual seawater temperatures are known to enhance coral metabolic rates, reduce photosynthesis (Jones et al., 1998; Lesser, 1997) and activate antioxidative defences and repair mechanisms (Black et al., 1995; Krueger et al., 2015; Lesser, 2012), thus increasing the energetic demands to ensure basal metabolism (Fitt et al., 2001). In line, the coral polyp retraction observed herein might act as a strategy to decrease energetic costs as expanded polyps consume more energy that contracted ones (Levy et al., 2006). Given the transient effects on polyp retraction, it can be hypothesized that this coral species was able to acclimate to the new temperature conditions.

4.1.2 Effects on endosymbionts' photosynthetic activity

The exposure to heat conditions decreased photosynthetic capacity of coral endosymbionts as confirmed by the initial differences in the *Fv/Fm* values recorded in the CTL treatments, for both temperatures. Several authors reported similar results in symbiotic hard corals (Fonseca et al., 2017; Glynn and D'Croz, 1990; Jones et al., 1998;

Lesser, 1997; Warner et al., 2002), soft corals (Ezzat et al., 2013) as well as in cultured endosymbionts (Iglesias-Prieto et al., 1992).

Heat stress is known to primarily damage enzymes implicated in the Calvin cycle (Crafts-Brandner and Salvucci, 2000) and inhibit photosystem repair mechanisms (Warner et al., 1999), leading to the accumulation of ROS, which in turn damage photosynthetic machinery (Campbell and Tyystjärvi, 2012) and cause a decrease on photosynthetic performance (i.e. photoinhibition) (Jones et al., 1998). The reduction on photosynthesis observed at 30.5 ºC can be related to the activation of photoinhibition mechanisms as previously reported by Smith *et al.* (2005) in endosymbionts under non-saturated light conditions and elevated temperature. In our dataset, by the end of the exposure period, corals exposed to both temperatures exhibited similar photosynthetic performances, suggesting the occurrence of transient effects. The *Fv/Fm* fluctuations recorded in our study, along with an enhanced polyp retraction (reducing surface area – enables the migration of endosymbionts to deeper locations), can suggest photodamage and photoacclimation, thus explaining the changes observed in the CTL. Takahashi *et al.* (2004) also reported temporary PSII photoinhibition driven by high temperature and irradiation. Together, the results suggest some phenotypic plasticity of cnidarians harbouring dinoflagellates to cope with variable light and abiotic conditions, a well-known biological process named photoacclimation (Falkowski and Dubinsky, 1981; Falkowski and Julie, 1991) that includes behavioral, physiological and sub cellular modifications (Durnford and Falkowski, 1997; Ezequiel et al., 2015; Kuguru et al., 2010).

4.1.3 Biochemical markers of isolated fractions of coral symbiosis

Temperature was the variable that contributed the most to the differences observed in the levels of biochemical markers, with strong positive correlations being noticed between GST, LPO and thermal stress. On the other side, CAT activity in the coral holobiont was species-specific, with temperature contributing to discriminate its catalytic activity. Increase in the levels of LPO and antioxidative enzymes was previously reported in symbiotic hard corals under heat stress and validated as sub-cellular signatures to assess coral health (Downs et al., 2000; Marangoni et al., 2019).

The overall decrease in CAT activity in microalgae under heat, will possibly have implications in the elimination of ROS, as this enzyme is proficient in converting H_2O_2 to non-oxidizing molecules. It is hypothesized that endosymbiont lost was responsible for reducing the enzyme activity. The endosymbiont density was not determined in the

present assay, but the low photosynthetic efficiency detected under heat conditions might be linked to endosymbionts expulsion. Previously Merle et al. (2007) observed reduce chlorophyll content and bleaching onset when symbiotic anemones were incubated with catalase inhibitors. Furthermore, the coral paling herein observed support our hypothesis, as coral coloration and pigment content are positively correlated (Amid et al., 2018; Winters et al., 2009).

The results of this study confirmed the recruitment of GST phase II biotransformation enzyme under heat stress, with the enzyme activity being magnified at 30.5 ºC. Besides its implication in the conjugation of xenobiotics to electrophilic compounds, GST has been recognized to detoxify DNA hydroperoxides, lipid peroxides and to repair the oxidative damage of macromolecules induced by stressors (Cummins et al., 1999; Kim et al., 2009; Limón-pacheco and Gonsebatt, 2009). In agreement with our results, Dias *et al.* (2019b) reported enhanced GST activity in 85% of Indo-Pacific coral species surveyed, when incubated for two months at 30 ºC. Transcriptomic analysis in thermally bleached hard corals also revealed up-regulation of some GST isoforms (Desalvo et al., 2008) and decrease in the glutathione pool, the scavenger molecule that conjugates to xenobiotics in the presence of GST (Downs et al., 2000).

The trend for increased levels of LPO in the two fractions under the warming scenario, revealed that antioxidative defences were not able to prevent the attack of ROS species to unsaturated fatty acids present in the cellular membranes (Bindoli, 1988). Our results are in accordance to previous studies showing increased oxidative damage in tropical corals exposed to warming seawater (Dias et al., 2019b; Downs et al., 2002; Fonseca et al., 2017). Moreover, the highest concentration of oxidized lipids detected in the microalgae CTL group can be related to the high content of unsaturated fatty acids present in chloroplast membranes (Tardy and Havaux, 1997). The absence of significant changes in LPO in the remaining microalgae treatments suggest that antioxidant defences were activated, although it could be related to a decrease in ROS production due to photoinhibition or endosymbiont expulsion.

4.2 Effects of distinct antifouling strategy exposures in the context of climate change scenarios

4.2.1 Effects on coral polyps´ behavior

The holobiont polyp retraction was induced by the two antifouling solutions (DCOIT free and encapsulated), as well as by the unloaded silica nanocontainers (SiNC) at both

temperature scenarios, although differently. The sharp retraction observed in the first twenty-four hours after the exposure to 50 µg L^{-1} of free DCOIT in both temperatures, supports the hypothesis that polyp retraction can be interpreted as an avoidance behavior. To date, few studies addressed polyp retraction in soft corals (Fabricius and Marine, 1995; Goddard, 2006). However, within hexacorallia (e.g. hard corals, zoanthids), polyp retraction was already reported as an early sign of physiological disturbance due to xenobiotics presence, often preceding bleaching or sub-cellular modifications (Markey et al., 2007; Neff and Anderson, 1981; Reimer, 1975). For instance, the exposure to copper induced retraction in zoanthids (Grant et al., 2010) and anemones, with this behavior being coincident with increased levels of bioaccumulated metal in the anemone (Main et al., 2010). In addition, temporary changes in polyps' expansion were detected in corals when transferred from contaminate media to clean seawater and thus linked with the concentration of dissolved chemicals (Turner and Renegar, 2017).

The controlled release of DCOIT from nanocontainers may justify the less pronounced effects comparatively to the free DCOIT group, by reducing biocide concentration. In accordance, Maia et al (2015) observed faster inactivation of bacteria when using free DCOIT comparatively to the encapsulated DCOIT and correlated those effects with diminished concentration of dissolved biocide in the antifouling nanomaterial treatment. The rapid DCOIT biodegradation advocated by Jacobson and Willingham (2000) may justify the time-course changes in coral morphology, due to a decrease on the concentration of the biocide. However, previous DCOIT quantifications in artificial seawater showed no significant DCOIT degradation over a week (Figueiredo et al., 2019). Nonetheless, those analyses were performed without the inclusion of test organisms and not accounted for the biocide biodegradation (Jacobson and Willingham, 2000; Negroni et al., 2010) and therefore, must be interpreted with caution.

Under the warming scenario, a slight colour loss and generalized polyp retraction was evident, which commonly indicates physiological distress and poor general condition (Dias et al., 2019a). The total closure of coral polyps was first observed in the free DCOIT treatment and thereafter registered in the CTL. The polyps opening to the initial levels were only significant in the encapsulated DCOIT, suggesting a better physiological condition of corals exposed to the novel antifouling nanomaterial comparatively to free DCOIT. These results are in line to those reported by Figueiredo *et al.* (2019), where free DCOIT exhibited a higher toxicity on target and non-target

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species, when compared to the nano-form. Additionally, in all treatments, corals exhibited an increased number of open polyps by day seven, suggesting that recovery under high temperatures may eventually occur, although at a slower rate.

4.2.2 Coral holobiont photosynthetic performance

Under current average values of temperature in tropical regions, the photosynthetic capacity of coral endosymbionts was independent of the type of antifouling strategy used or the presence of SiNC. Thus, neither DCOIT biocide nor silica nanocontainers affect the photosynthetic performance of *S.* cf. *glaucum*. Cima *et al.* (2013) described similar results when testing the acute effects induced by 100 μg L^{-1} of DCOIT in this coral species and recorded *Fv/Fm* values within the range of those reported for physiological heathy corals (Jones et al., 1998). Together both datasets support that endosymbionts *in hospite* (within host coral tissues) display a lower sensitivity to the biocide when compared to natural communities of marine phytoplankton (Devilla et al., 2005). This higher tolerance towards DCOIT by intracellular *Symbiodinium* is likely due to the protection granted by the coral host to biocide exposure. Additionally, it could also represent differences in the biochemistry composition of the distinct autotrophs species (Wang and Wang, 2009), as DCOIT shows high affinity towards thiol-containing enzymes, dehydrogenase enzymes and ATP synthetases (Collier et al., 1990; Williams, 2007).

The tested biocide is not a photosystem II inhibitor, but a disruptor of the metabolic pathways of proteins (EU, 2014). However, some authors reported photosynthesis inhibition induced by DCOIT in several autotrophs (Arrhenius et al., 2006; Johansson et al., 2012; Larsen et al., 2003), justifying the utility of photosynthetic related endpoints in symbiotic cnidarian toxicological assessments.

When future temperature scenarios are considered, our results clearly show that temperature caused a significant reduction on photosynthesis efficiency. The superior photosynthetic performance of corals exposed to SiNC when compared to the CTL might be related a shading effect associated to nanoparticle deposition on coral tissues, as observed in marine microalgae covered by silicon dioxide (SiO_2) nanoparticle aggregates (Manzo et al., 2015). This deposition reduces direct irradiation and prevents light-induced damage under warming scenarios. Low toxicity was also reported in unicellular cells when covered with silica nanoforms (Hoecke et al., 2008; Katsumiti et al., 2016), although some authors also described negative effects of nanoparticle

deposition due to cell clogging and mechanical damage (Navarro et al., 2008; Wei et al., 2010). The possibility of *Symbiodinium* displaying a better photosynthetic performance due to the supplementation of inorganic nutrients was excluded, as dinoflagellates do not require SiO_2 (Canfield et al., 2005) and low dissolution of silica nanoforms in seawater was reported (Hoecke et al., 2008; Manzo et al., 2015). The lack of differences between DCOIT containing treatments (free or encapsulated) and the corresponding CTL, reinforced the low toxicity of this biocide and silica nanomaterials to coral endosymbionts. These results corroborates the previous risk assessment survey on this antifouling nanomaterial showing that target species were more affected than non-target species (Figueiredo et al., 2020). Globally, the photosynthetic performance of coral holobiont decreased under heat conditions, but the absence of differences after a week of exposure suggests that individuals were able to acclimate to the new environmental conditions. Still, the presence of DCOIT induced an additional constrain to organisms kept at 30.5 ºC, as they exhibited lower quantum yield on days three and five comparative to organisms kept at 26 ºC and co-exposed to the biocide.

4.2.3 Biochemical markers of isolated fractions of coral symbiosis

Under standard temperature conditions for tropical cnidarians, the CAT and GST activities were distinct in the two cellular fractions - the microalgae and the host - with the highest enzyme levels being detected in the microalgae (CTL groups at 26 ºC). Those variations might be related to the distinct antioxidant defence profile of each symbiotic partner, as reported for symbiotic cnidarians under non-stressful conditions (Merle et al., 2007; Ramos and García, 2007; Richier et al., 2003; Shick et al., 1995) or stress conditions (Dykens et al., 1992; Fonseca et al., 2021; Hawkridge et al., 2000; Krueger et al., 2015; Richier, 2005). Thus, the distinct enzymatic profiles of both CTL groups can be interpreted as differences in cellular metabolism of animal (host) and vegetal (endosymbiont) cells and might be related with the high oxidative environment that characterize endosymbionts (Saragosti et al., 2010). Given the positive correlation between chlorophyll levels and the activity of antioxidant defence enzymes (Shick and Dykens, 1985), it is worth highlighting the importance of endosymbionts in generating oxy-radicals (Dykens et al., 1992). The lack of differences in LPO quantification between both fractions are indicative of equivalent baseline oxidative damage levels in both cell types.

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The enzymatic activities determined for CAT and GST were in the same order of magnitude of that previously reported by Cima (2013) for the same coral species and stocking conditions. However, we were not able to compare our LPO results to that reported by previous authors, as different normalization units were applied.

Under current ocean temperature, CAT activity of both the coral host and the endosymbiont were not influenced by the presence of antifouling treatments and/or unloaded silica nanomaterials. However, contradictory results showed CAT inhibition in the presence of DCOIT in *Sarcophyton* (Cima et al., 2013). Those differences can be related to the determination of the enzymatic activity in the coral holobiont instead of endosymbiont isolated fractions, but also to the rapid biocide biodegradation (Jacobson and Willingham 2000; Chen et al. 2015b), with implications on the effective biocide exposure concentration. The previous authors renewed exposure media daily with freshly made DCOIT solutions, while in our assay media were spiked only at the beginning of the exposure. The absence of changes in CAT supported by our results could also reflect that holobiont cell machinery overwhelmed the biocide toxic effects under optimal physiological conditions. On the other hand, heat stress significantly increased CAT activity on DCOIT treatments of both isolated fractions (coral and microalgae) and enhanced the enzyme activity in the coral SiNC-containing groups. The same pattern was observed at the molecular level with the overexpression of catalase gene in coral larvae when exposed to a combination of an insecticide with high temperatures (Ross et al., 2015), while no differences in gene expression was detected when each treatment was applied individually. Similarly, the combined exposure of corals to xenobiotics and high temperatures resulted in interactive effects, while reduced or no effects were observed when stressors acted solely (Negri et al., 2011; Negri and Hoogenboom, 2011; Nyström et al., 2001).

No differences in GST activity at 26 ºC was reported for the different treatments in the coral host, however enzymatic activity was significantly enhanced in microalgae submitted to free DCOIT and SiNC. The highest GST levels detected in the SiNC treatment may be related to the presence of residues of the surfactant CTAB used during synthesis of this unloaded engineered nanomaterial, as recently demonstrated by Figueiredo and co-authors (2019). Stimulation of GST in the presence of DCOIT was previously observed in this coral species (Cima et al., 2013). Based on our results, it is hypothesized that DCOIT may first target endosymbionts and trigger the activation of biotransformation processes. In agreement to this observation, marine autotrophs have

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been described to be among the most sensitive organisms to DCOIT biocide (Figueiredo et al., 2020; Martins et al., 2017). The lack of significant variation in GST in the encapsulated DCOIT treatment is possibly related to the biocide load. Stimulation of GST has occurred under warmer waters, although no changes were registered between DCOIT treatments when compared to the CTL.

No oxidative damage was induced by the antifouling treatments, in microalgae and host tissues at 26 ºC. Previously, Cima *et al.* (2013) also reported no significant changes in LPO levels when *S.* cf. *glaucum* was short-term exposed to DCOIT. Overall, it can be stated that under normal physiological conditions coral adjusted their cellular machinery to cope with stress induced by DCOIT. The absence of changes in LPO levels between experimental groups at 30.5 ºC, suggests that cellular machinery of symbiotic corals was able to cope with the short-term stress posed by antifouling compounds and no cellular damage occurred, as previously observed in other cnidarians (Fonseca et al., 2021, 2017). These results are in line with our previous results, showing lack of differences among treatments regarding photosynthetic performance and corroborate the transient effects reported in the behavioral assay.

5. Conclusion

Based on the results of our study we can conclude that shifts in seawater temperature driven by global climate change affect the tested coral species in a much more severe degree than the exposure to the anti-fouling biocides, promoting behavioral, physiological and sub-cellular changes and an overall increment on cellular oxidative damage.

Nonetheless, DCOIT prove to be toxic to non-target species but its toxicity can be minimized through encapsulation in silica nanocontainers (SiNC) as suggested by the resulted obtained in the endpoints monitored. Thus, this novel nano-engineered antifouling biocide may be suitable for marine biofouling prevention.

Overall, our results highlight that both endosymbionts and cnidarian host cells differ in their cellular mechanisms to cope with oxidative stress. Considering ocean warming, as predicted in the worst-case scenario presented by the IPCC, it is hypothesized that near-shore reef communities will become more vulnerable to local contaminants due to an overload of antioxidant defences. As such, the deleterious effect promoted by the presence of dissolved antifouling chemicals in seawater is predicted to be magnified by thermal stress. Our dataset highlights the need to evaluate the synergistic effects of

temperature and biocide exposure in environmental risk assessment of antifouling solutions.To enhance our understanding on this efficiency of this new technology, future work should include long-term filed studies using coating formulations with DCOIT-encapsulated and evaluate antifouling ability and DCOIT release profile.

6. Declarations of interest

None

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9. References

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Figures

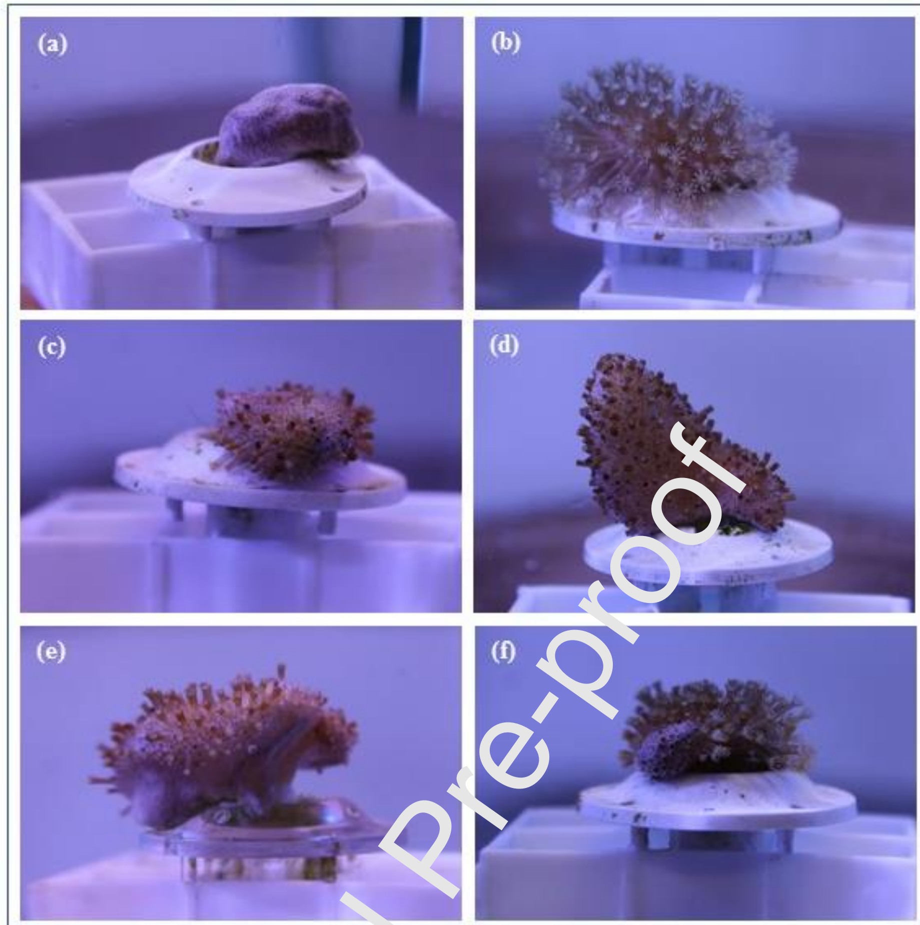

Figure 1 – Representation of the score used for assessing coral polyps' behavior, according to the percentage (%) of expanded polyps: (a) fully retracted, 0%; (b) fully expanded, 100%; (c), (d) intermediate, 25% of polyps expanded; (e) intermediate, 50% of polyps expanded and (f) intermediate, 75% of polyps expanded.

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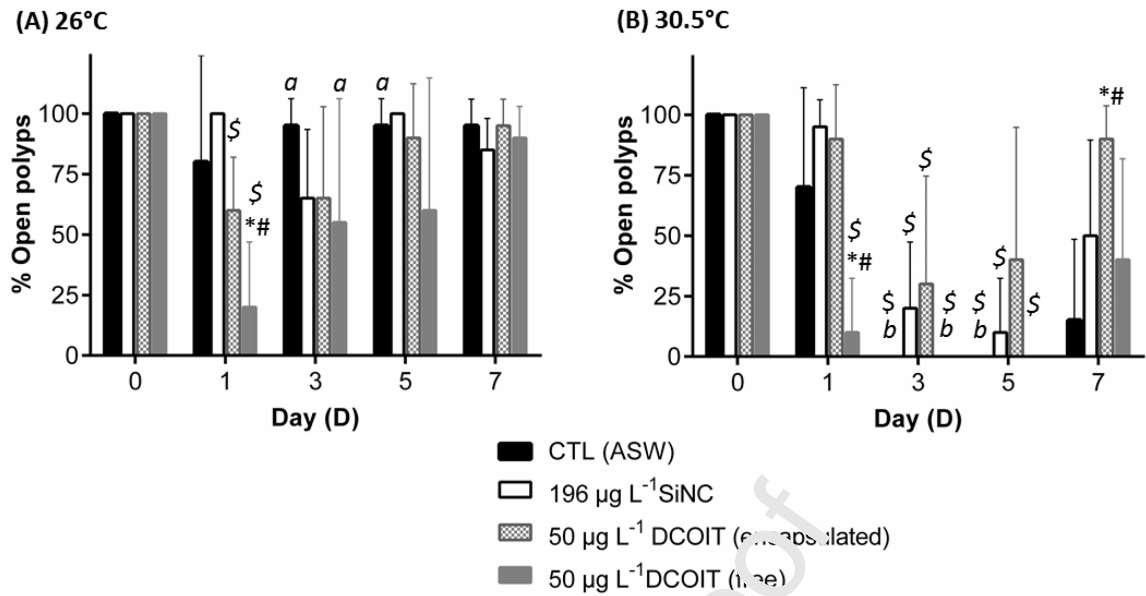

Figure 2: Percentage (%) of open polyps in *Sarcophyton cf. glaucum* holobiont during seven days (D) of exposure to clean artificial seawater (negative control, CTL), empty silica nanocontainers (SiNC), DCOIT encapsulated in SiNC (DCOIT encapsulated) and free biocide DCOIT (DCOIT free) under thermal conditions: (A) 26 ºC and (B) 30.5 ºC. Bars exhibit the average value ± standard deviation (n = 5). Letters (*a, b*) indicates differences in each treatment and timepoint due to temperature shift. The asterisks (*) denote statistical differences between treatments to the corresponding CTL group (same day), while cardinals (#) indicates significant differences between the treatments containing DCOIT (encapsulated in SiNC or free). The dollar ($) represents differences regarding the initial stage (D0) of each treatment, when the comparisons were performed along seven days. All statistical comparisons were performed considering $p = 0.05$.

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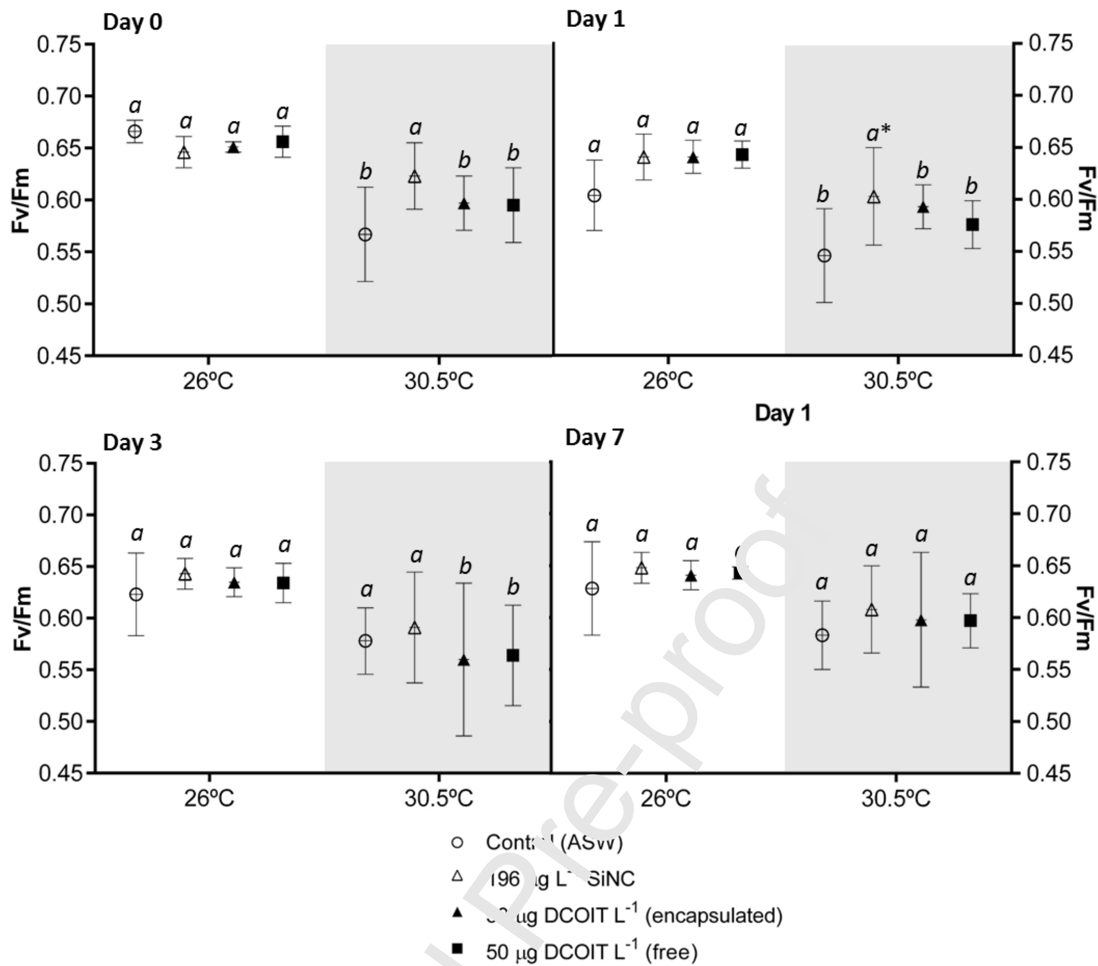

Figure 3: Maximum quantum yield of photosystem II (*Fv/Fm*) (average ± SD; n = 5) in the *Sarcophyton* cf. *glaucum* holobiont after exposure to clean artificial seawater (negative control, CTL); empty silica nanocontainers (SiNC); DCOIT encapsulated in SiNC and free DCOIT biocide, under seawater thermal conditions: 26 ºC and 30.5 ºC: day 0, day 1, day 3 or day 7. The letters (*a, b*) denote statistical differences in the *Fv/Fm* values due to temperature increment in each experimental group, while asterisks (*) indicates differences among treatments within each temperature when compared to the CTL ($p < 0.05$).

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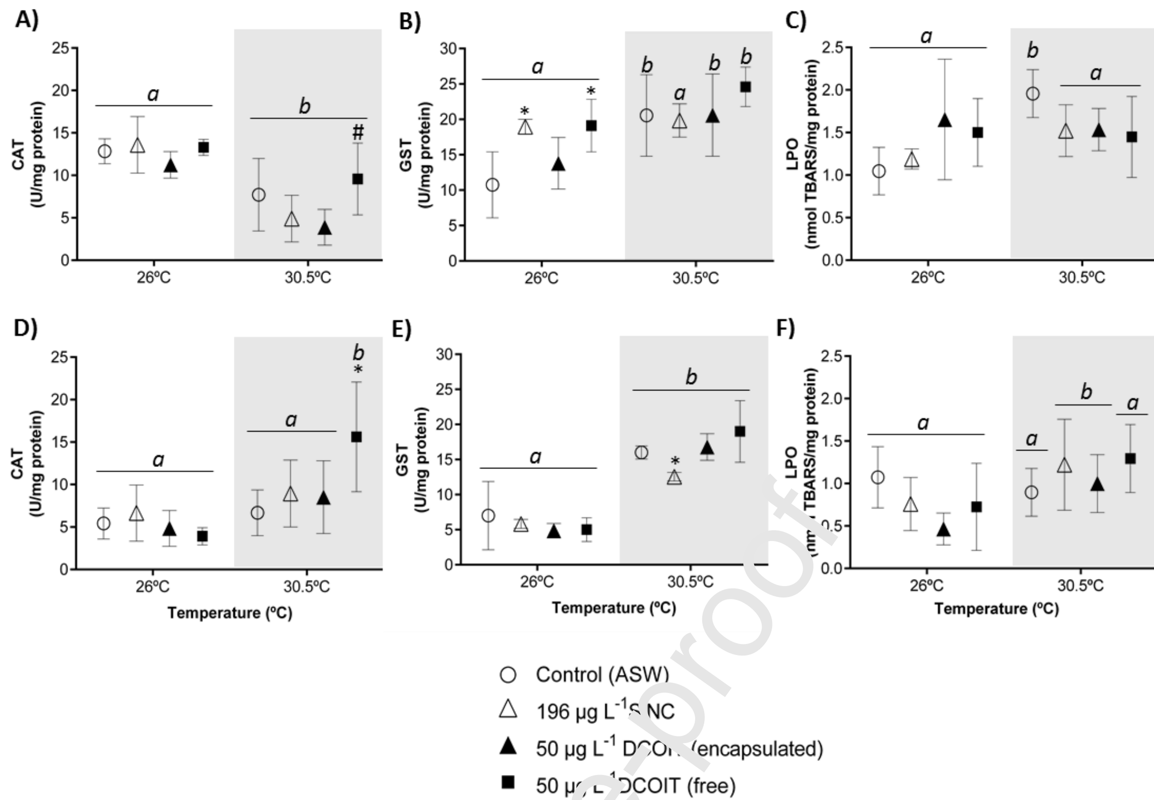

Figure 4: Activity of oxidative stress enzymes and lipid oxidative damage in *Sarcophyton* cf. *glaucum* fractions: (A-C) endosymbiotic microalgae and (D-F) cnidaria host after 7 days exposure to seawater (control), empty silica nanocontainers (SiNC), SiNC loaded with DCOIT (DCOIT encapsulated) and free DCOIT at temperatures 26 °C and 30.5 °C. Data is expressed as average values ± standard deviation (n = 5). The letters (*a, b*) denotes statistical differences (p <0.05) within each fraction due to temperature increment, while asterisks (*) indicates differences between treatments to the corresponding control group (within the same temperature) and cardinal (#) states for differences between DCOIT and SiNC-DCOIT (p < 0.05). Abbreviations: CAT – catalase; GST – glutathione-*S*-transferase; LPO – lipid peroxidation.

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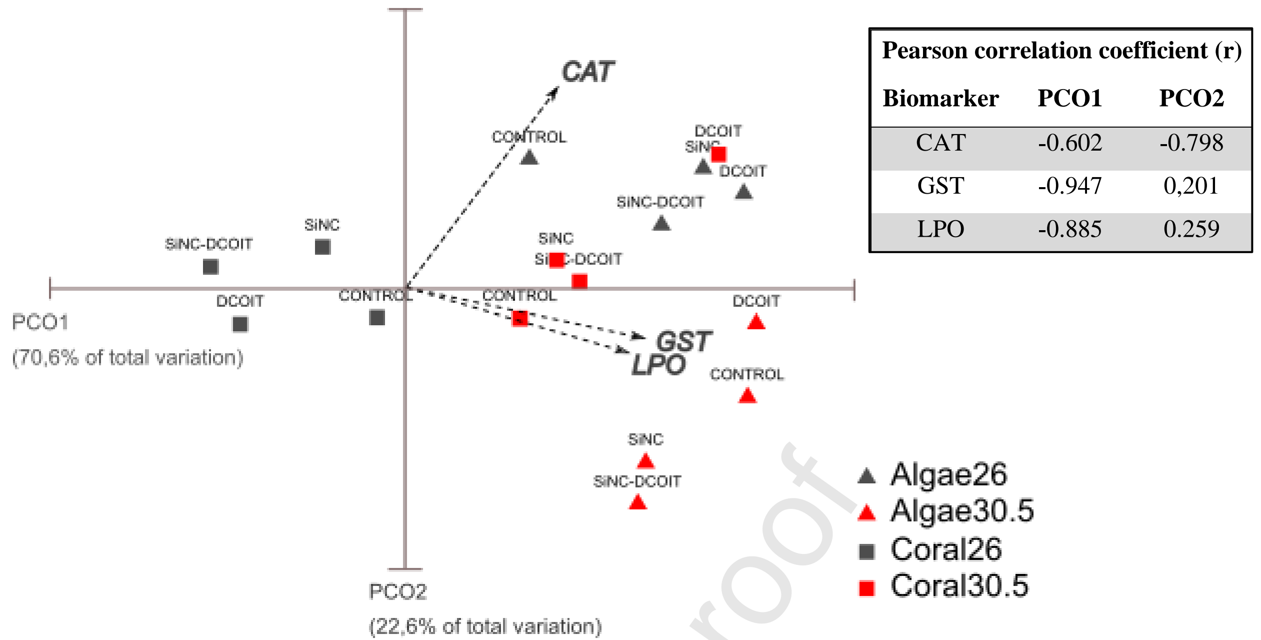

Pearson correlation coefficient (r)		
Biomarker	**PCO1**	**PCO2**
CAT	-0.602	-0.798
GST	-0.947	0,201
LPO	-0.885	0.259

Figure 5: Centroids ordination diagram (PCO) based on biochemical biomarkers, in *Sarcophyton* cf. *glaucum* isolated fractions: endosymbiotic microalgae (triangles) and cnidaria host (squares), when exposed to artificial seawater (CTL); empty silica nanocontainers (SiNC); DCOIT encapsulated in SiNC (SiNC-DCOIT) and free biocide (DCOIT) at seawater temperatures 26 °C (grey symbols) and 30.5 °C (red symbols). Inserts correspond to the Pearson correlation vectors of biochemical data and are provided as superimposed supplementary variables (black vectors). Abbreviations: catalase (CAT); glutathione-S transferase (GST); lipid peroxidation (LPO).

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CRediT author statement

Violeta Ferreira: Conceptualization, Methodology, Investigation, Data Curation, Formal analysis, Visualization, Writing - Original Draft

Maria Pavlaki: Methodology, Investigation, Data Curation, Writing – review & editing

Roberto Martins: Data Curation, Formal analysis, Writing – review & editing

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Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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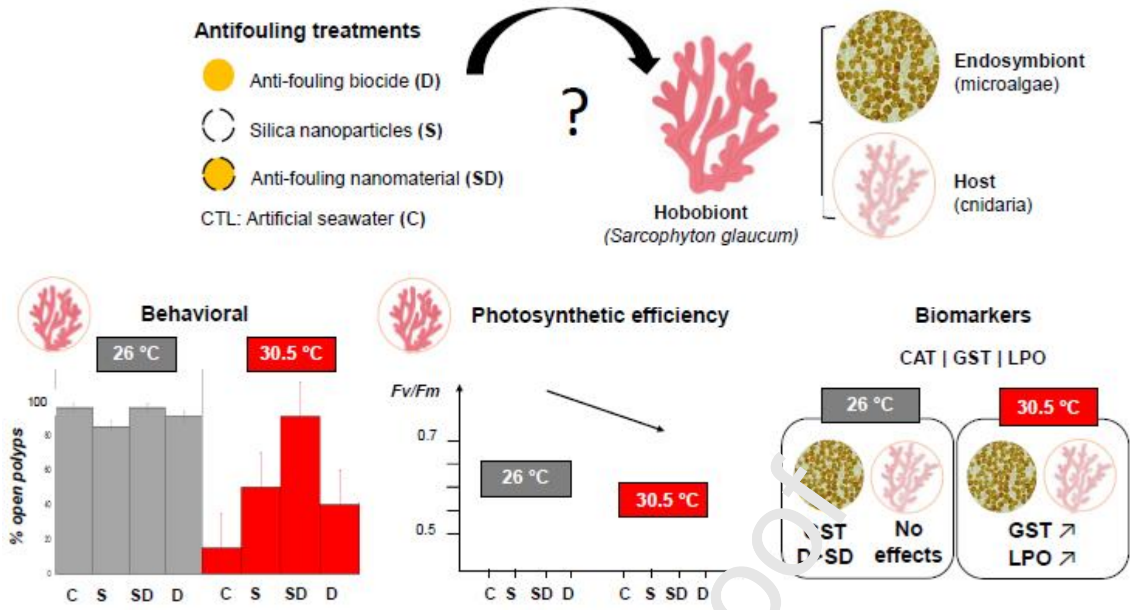

Graphical abstract

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Highlights

- Encapsulation of DCOIT in nanomaterials reduces its toxicity to non-target species.
- Behavior, photosynthesis and enzyme activity were assessed in *Sarcophyton glaucum.*Heat stress is the variable that most affects *Sarcophyton* cf. *glaucum* physiology.
- DCOIT toxicity to symbiotic corals is reversible at current ocean temperatures.
- Heat stress magnifies antifouling biocide toxicity with risks to tropical species.

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