Universidade de Aveiro

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The effect of olivine in the tropical coral *Montipora digitata*: toxicological assessment and resilience to thermal stress

O efeito da olivina no coral tropical *Montipora digitata*: avaliação toxicológica e resiliência a stresse térmico

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Rui Miranda Rocha, professor auxiliar convidado do Departamento de Biologia da Universidade de Aveiro e coorientação do Doutor Igor Cruz, professor da Universidade Federal da Bahia.

O júri

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palavras-chave

Scleractinia, branqueamento, recife de coral, mineral olivina, eficiência fotossintética, stresse oxidativo, reservas energéticas, aquecimento global, SEM

resumo

O futuro dos recifes de coral está a ser moldado pela resiliência, ou falta dela, de diferentes espécies às alterações climáticas. Os corais são organismos marinhos sob uma grande ameaça ecológica como resultado do aquecimento e acidificação dos oceanos e das atividades antropogénicas. As áreas marinhas protegidas e a redução das emissões de carbono são certamente relevantes, embora provavelmente insuficientes para a preservação deste ecossistema. Assim, estratégias adicionais de conservação são necessárias. A desagregação de minerais como a olivina, proposta como uma estratégia de remoção de CO_2, tem o potencial de aumentar a alcalinidade da água do mar enquanto consome CO_2 da atmosfera. Embora verificado o potencial do aumento de valores de pH e alcalinidade, o impacte em organismos marinhos e no seu ecossistema, ainda não foi avaliado. O presente estudo teve como objetivo avaliar, pela primeira vez, os efeitos da exposição do mineral olivina no coral tropical *Montipora digitata*, avaliando biomarcadores moleculares de stresse e dano oxidativo e perfis metabólicos, bem como a eficiência fotossintética, crescimento do coral e variações de pH. Em primeiro lugar, o nosso estudo avaliou os efeitos sub-letais de diferentes concentrações de olivina, 6,00, 9,00, 13,50, 20,25, 30,38, 45,56, 68,35 e 102,5 mg L^{-1} durante uma exposição aguda de 96 h. Os resultados inicias não revelaram tendência geral de concentração-resposta, embora a eficiência fotossintética tenha sido menor em concentrações crescentes. Os níveis de HSP70 e o teor de proteína diminuíram em concentrações mais elevadas, enquanto o teor de lípidos apresentou valores superiores, assim como o pH e o oxigénio dissolvido. Um segundo ensaio foi realizado avaliando os efeitos da exposição crónica no coral *M. digitata* a uma concentração de 20,25 mg L^{-1} de olivina, do qual podemos concluir que a exposição prolongada a olivina facilitou o crescimento relativo de *M. digitata* e a sua eficiência fotossintética, contribuindo também para a manutenção de valores de pH significativamente mais elevados. *M. digitata* demonstrou stresse oxidativo moderado, eficientemente combatido pelas defesas antioxidantes, assim como uma maior taxa de crescimento relativo por intermédio de um consumo do conteúdo lipídico do tecido do coral, apesar de não demonstrar necessidade adicional de energia para manter os mecanismos de defesa ou atividade metabólica. Adicionalmente, as macroestruturas dos corais não foram afetadas pela exposição a olivina. A etapa final deste estudo teve como objetivo avaliar as respostas biológicas do coral *M. digitata* ao aumento da temperatura (30 ºC) em 24h, quando exposto a 20,25 mg L^{-1} de olivina. Além disso, avaliamos se a exposição anterior à olivina aumenta a resiliência dos corais ao aumento da temperatura. No geral, a resposta biológica de *M. digitata*, associada às vias antioxidantes, alocação celular de energia e consumo energético não foi indicativa de indução de stresse térmico, do qual não podemos concluir que a exposição ao mineral olivina aumente os mecanismos de resiliência no coral *M. digitata* quando este experiencia temperaturas anormais.

keywords

Scleractinia, bleaching, coral reef, olivine mineral, photosynthetic efficiency, oxidative stress, cellular energy allocation, global warming, SEM

abstract

The future of coral reefs is already being shaped by the resilience, or lack of it, of different species to climate change. Corals are currently undergoing a major ecological threat as result of ocean warming and acidification and anthropogenic activities. Marine protected areas and reduced carbon emissions are certainly relevant although likely insufficient to preserve this ecosystem. Therefore, additional conservation strategies are required. Olivine weathering, proposed as a CO_2 removal strategy, is expected to increase seawater alkalinity while consuming CO_2 from the atmosphere. Although verified the potential of increase pH values and alkalinity, the impact on marine organisms and ecosystem is still to be understood. Our study aimed to evaluate olivine exposure effects on tropical coral *Montipora digitata*, assessing molecular biomarkers of oxidative stress and damage and metabolic profiles, as well as photosynthetic efficiency, coral growth, pH and dissolved oxygen variations. Firstly, our study assessed sub-lethal effects of different olivine concentrations, 6.00, 9.00, 13.50, 20.25, 30.38, 45.56, 68.35 and 102.5 mg L^{-1} under an acute experiment of 96h. Primary results did not show a general dose-response tendency, although photosynthetic efficiency was impaired for increasing concentrations. HSP70 levels and protein content decreased for higher concentrations, while lipid content showed higher values as well as pH and DO. A second experiment was performed assessing chronic exposure effects on *M. digitata* to a concentration of 20.25 mg L^{-1} of olivine sand, of which we can conclude that a long-term exposure to olivine facilitates *M. digitata* relative growth and photosynthetic efficiency as well as significantly higher pH values. *M. digitata* experienced moderate oxidative stress efficiently tackled by antioxidant enzymes and a higher relative growth rate through the expense of tissue lipid content, but no additional energy demands to maintain defence mechanisms or metabolic activity. Additionally, coral macrostructures were not affected by olivine exposure. The final stage of this study aimed to assess biological responses of the coral *M. digitata* to increase temperature (30 ºC) for 24h, while exposed to 20.25 mg L^{-1} of olivine sand. Besides, we evaluated if previous olivine exposure enhances coral resilience to rising temperatures. Overall, biological response of *M. digitata,* associated with antioxidant pathways and cellular energy allocation and consumption was not indicative of induced thermal stress. Photosynthetic efficiency exhibited significantly higher values when exposed to olivine at 30 ºC, although being impaired in both treatments at the higher temperature. Therefore, we cannot conclude that olivine exposure enhances resilience mechanisms in the coral *M. digitata* whilst abnormal temperatures.

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1. Introduction

Reef-building corals (phylum Cnidaria, class Anthozoa, order Scleractinia) such as Hexacorallia, live in symbiosis with unicellular dinoflagellate algae, belonging to the genus *Symbiodinium* (Figueiredo et al., 2017; Muscatine & Porter, 1977; Roth, 2014) and known as zooxanthellae. These tropical corals, commonly referred as stony or hard corals, produce a calcium carbonate skeleton which provides a complex 3D structure that shapes the building of the reef (Kennedy et al., 2013). It is thought the symbiosis between coral and these photosynthetic endosymbionts has originated in the mid-Triassic (Trench, 1997) and accounts for the development of coral reefs in oligotrophic environments (Muscatine & Porter, 1977). These *Symbiodinium* spp. live in coral tissues and contribute to their energetic requirements, providing the coral's nutritional demands and photosynthates, whilst helping with the calcification process and growth (Muscatine & Porter, 1977; Roth, 2014). The coral, in return, provides essential inorganic nutrients, including ammonium and phosphate, and a safe, luminous harbour to their symbiont (Roth, 2014; Stat et al., 2006). Considered one of the most biodiverse and complex ecosystem, coral reefs provide refugia for several species and habitat for different life stages organisms (Fisher et al., 2015; Graham & Nash, 2013; Ortiz & Tissot, 2012), even though they represent less than 0.1% of the total surface of the oceans (Ove Hoegh-Guldberg et al., 2019). When found along coastline countries, coral reefs constitute a source of sustenance and crucial economic relevance, providing services such as food, tourism and cultural benefits (Albert et al., 2015; Grafeld et al., 2017). Apart from supporting biodiversity and species habitat, coral reefs regulate water quality, biogeochemical cycles (Woodhead et al., 2019) and coastal protection, enhancing wave attenuation and preventing land erosion (Ferrario et al., 2014; Moberg & Folke, 1999). In the recent years, corals have received increasing attention as providers of marine bioactive compounds with a wide range of biological properties, such as antimicrobial, antifouling and anti-inflammatory activities with a variety of potential applications in the pharmaceutical fields (Rocha et al., 2011), as well as biomimetic processes as drug carriers or applications in human bone regeneration (Demers et al., 2002; Green et al., 2017).

1.1. A changing planet

Coral reefs survived multiple climate shifts over long time scales since the Triassic. However, when compared with the shorter time scales of human activities and rapid environmental changes, the ecological variability and implications could be much different (Stat et al., 2006). From anthropogenic pressures responsible for the abnormal input of CO_2 and other greenhouse gases into the atmosphere, pollution, and overfishing, to natural phenomena, such as El Niño-Southern Oscillation, marine ecosystems are suffering unprecedented shifts in temperature, stratification, nutrient input, oxygen loss and ocean acidification, with major biological impacts. Accordingly, to the IPCC Fifth Assessment Report (IPCC, 2013), ocean warming estimates for sea surface temperature are between 0.6 to 2.0 ºC by the end of the 21st century. As an outcome of the extensive time scales of heat transfer from sea surface to depth, ocean warming will continue even if greenhouse gas emissions decrease or are kept constant, contributing for the continuing sea level rise and deep ocean acidification.

As a consequence of increasing concentrations of CO_2, surface ocean pH is estimated to fall, by the end of the century, 0.06 units, for the most optimistic pathway, and 0.31 for the worst-case climate scenario (IPCC, 2013). While influencing the global temperature of the ocean, carbon dioxide may, as well, be absorbed by seawater and form carbonic acid (Dubinsky & Stambler, 2011), changing the carbonate chemistry of surface waters (Lam et al., 2019). Once reacting with seawater, carbonic acid dissociates to form bicarbonate ions and protons, leading to pH reduction. The released protons can also react with carbonate ions and generate additional bicarbonate ions (Hoegh-Guldberg et al., 2007). Since coral skeleton composition is directly associated with the surrounding seawater, water chemistry acts as an important factor in coral growth (Gagnon et al., 2012). Corals transport seawater to an internal calcification site, supplying calcium (Ca^{2+}) and carbonate (CO_3^{2-}) ions required for the calcium carbonate skeleton formation (Barnes, 1970; Gagnon et al., 2012). With the decreasing availability of carbonate to biological systems, calcification rates of marine organisms, such as coral reefs, are impaired, favouring its erosion (Kleypas et al., 1999) and lowering aragonite saturation state (Ω_a) (Anthony &

Kleypas, et al., 2011; Suzuki et al., 1995). Calcium carbonate precipitates from the corals' calcifying fluid (CF), which maintains both pH and dissolved inorganic carbon with higher levels than the surrounding seawater (Comeau et al., 2018; McCulloch et al., 2017; Venn et al., 2011). Consequently, the saturation state within the CF is maintained above seawater, which favours the precipitation of calcium carbonate (Comeau et al., 2019) and allows near-optimal rates of calcification. Once seawater pH decreases, the coral capacity to maintain elevated pH in the calcifying fluid could be reduced or impaired, requiring higher energy costs to preserve constant elevated levels (McCulloch et al., 2012; Venn et al., 2013). In spite of the high capacity of corals to modulate the chemistry at their site calcification (Anthony & Kleypas, 2011; Comeau et al., 2018), no evidence of acclimatization was found in calcifying corals exposed to elevated P_{CO_2} (Comeau et al., 2019; Crook et al., 2013). Corals can retain both skeletal growth and density under carbonate unsaturation by spending additional energy in the process of calcification. This strategy may, on the other hand, imply limited resources for essential processes such as reproduction, ultimately reducing the potential for recolonization (Hoegh-Guldberg et al., 2007; Szmant & Gassman, 1990).

A direct implication of unusually high sea temperatures is mass coral bleaching events, described since the eighteenth decade (Brown, 1997; Glynn, 1993; Hughes et al., 2017). These events are not uniform, showing irregular impacts over micro (µm) to meso scales (km) and varying in the duration and periodicity, which results from shifts in environmental conditions, genetic differences in hosts and symbionts, spatial location of reef surfaces and bleaching events history (Anthony et al., 2007; Baker et al., 2008; Pratchett et al., 2013). The rise of ocean temperature and the synergistic effect of thermal anomalies and high irradiation levels, triggers the disruption of the symbiotic relationship between the coral host and the algal symbiont, which is followed by loss of colour, physiological damage and eventual coral mortality (Baker et al., 2008; Higuchi et al., 2013; Hughes et al., 2017). The basic mechanism of bleaching begins with the direct or indirect damage of zooxanthellae' photosynthetic system, resulting in the overproduction of oxygen radical species (ROS), such as superoxide anion radical ($\bullet O_2^-$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\bullet$) (Richier et

al., 2006). ROS oxidize the thylakoid membranes' lipids and induce cellular damage, impairing the coral integrity, which can lead to the expulsion of the symbionts and subsequent disruption of the symbiosis (Lesser, 2006; Tchernov et al., 2004). Following bleaching events, the prevalence of coral disease is higher, resulting from shifts in the coral-associated microbial community (Bourne et al., 2008; Muller et al., 2008). Ocean warming and acidification are thus critical factors for reducing coral resilience, growth and survival (Anthony & Maynard, 2011) and can have a synergetic interaction, where higher temperatures can accelerate and worsen ocean acidification impacts (Anlauf et al., 2011; Anthony et al., 2008; Anthony & Maynard, 2011; Pandolfi et al., 2011).

1.2. Saving the reefs

It is known that many coral species live close to their upper thermal tolerance limits and as they inhabit shallow waters of the photic zone (Baker et al., 2008), their thermal tolerance is highly variable, depending on the spatial scales of bleaching events (Baker et al., 2008), heating rates (Middlebrook et al., 2010), holobiont genetic variability (Rohwer et al., 2002), acclimatory mechanisms and adaptive processes (Krueger et al., 2015a). Therefore, it is crucial to understand if corals can respond to accelerated increasing temperatures and levels of CO_2 as a result of anthropogenic pressures (Baker et al., 2008). If not, what strategies can we approach to mitigate or minimize coral bleaching and the impacts of ocean acidification?

Strategies of coral reef restoration have been receiving increasing attention, using asexual and sexual reproduction, where corals are first grown in *in situ* or *ex situ* nurseries, followed by transplanting them onto damaged reefs (Rinkevich, 2014). However, in current climate change scenarios, where habitats are experiencing unprecedented shifts, using original colonies may be inadequate for successful restoration (Jones & Robins, 2011). Alternatives include conservation engineering and resilient restoration taking advantage of natural variation in heat tolerance among coral species (Camp et al., 2018; Morikawa & Palumbi, 2019), or even proposals of enhanced corals via assisted evolution, which include stress exposure of natural colonies to induce acclimatization, active modification of community composition of coral-associated microbiome or selective breeding to

generate desirable genotypes with resilient phenotypic traits (Van Oppen et al., 2015).

Additionally, several CO_2 removal techniques have been suggested to restore normal ocean chemistry, such as phytoremediation and enhanced weathering (Albright & Cooley, 2019). Chemical remediation, such as olivine weathering, is an intervention inspired by mineral weathering reactions that naturally neutralize acids and remove CO_2 from the ocean-atmosphere system. Besides consuming CO_2, these reactions increase alkalinity and aragonite saturation state (Albright & Cooley, 2019; Montserrat et al., 2017), which can potentially benefit coral skeleton growth, and even enhance its resilience to changing habitats.

1.3. Olivine enhanced weathering

Enhanced silicate weathering (ESW) is a carbon dioxide removal (CDR) strategy or negative emission technology (NET), with the goal of removing CO_2 from the surface ocean and reducing acidification (Köhler et al., 2013), mimicking, in an artificially stimulated approach, the natural process of silicate weathering (Hartmann et al., 2013; Schuiling & Krijgsman, 2006). Enhanced weathering techniques have already been applied, adjusting the soil pH and nutrient supplies through the use of phosphorous, potassium, silica and carbonate minerals (Hamilton et al., 2007; Haque et al., 2019; Hindar et al., 2003; Kreutzer, 1995).

Olivine ($Mg_2{-}xFe_xSiO_4$) occurs with more than one composition and its name is determined by the identity of the dominant divalent metal cation: Mg-rich olivine is known as forsterite (Mg_2SiO_4), while Fe-rich is referred as fayalite (Fe_2SiO_4) (Griffioen, 2017; Oelkers et al., 2018). This ultramafic silicate mineral has been considered as a key candidate for ESW application due to its abundance and fast-weathering properties (Schuiling & Krijgsman, 2006). Natural weathering is surface-reaction-limited, optimized by the abrasion of the grains and the removal of coatings (Hay, 1959; Olsson et al., 2012). For this reason, high energetic marine environments which experience continuous tidal currents and heavy wave action are suitable for the breakdown of olivine (Schuiling & de Boer, 2011), such as reef-flat zones. Accordingly to Meysman and Montserrat 2017, there are two possible ESW applications: deposition of olivine gravel onto high-energetic environments, characteristic of the seafloor, where olivine suffers natural grinding

process under the action of currents and waves; beach application involving the distribution of fine olivine sand over beaches and shallow subtidal areas (Hangx & Spiers, 2009a), whose dissolution can be accelerated through wave action and other forms of biotic processes in the seabed.

The dissociation reaction of Mg-rich olivine is given as the following (Hartmann et al., 2013):

$$\mathrm{Mg_2SiO_4 + 4CO_2 + 4H_2O \rightarrow 2Mg^{2+} + 4HCO_3^- + H_4SiO_4} \quad (1)$$

The dissolution of silicate minerals releases cations such as Ca^{2+} and Mg^{2+}, while consuming CO_2 and increasing total alkalinity (Wolf-Gladrow et al., 2007). The following equation describes total alkalinity (TA) (Zeebe & Wolf-Gladrow, 2001) and aragonite saturation state (Jiang et al., 2015):

$$\mathrm{TA} = [\mathrm{HCO_3^-}] + 2[\mathrm{CO_3^{2-}}] + [\mathrm{B(OH)_4^-}] + [\mathrm{OH^-}] - [\mathrm{H^+}] + \mathrm{minor\ compouds} \quad (2)$$

$$\Omega_{\mathrm{arag}} = \frac{[\mathrm{Ca^{2+}}] \times [\mathrm{CO_3^{2-}}]}{\mathrm{K'_{sp}}} \quad (3)$$

Where Ω is the aragonite saturation state and K'_{sp} is the apparent solubility product. The shifts in the acid-base equilibrium from dissolved CO_2 to carbonate CO_3^{2-} and bicarbonate HCO_3^- ions (Hartmann et al., 2013), can increase alkalinity and pH levels maintaining high aragonite saturation state which is crucial to coral calcification (Ries et al., 2009; Wall et al., 2016). These shifts in seawater chemistry could represent a potential mitigation strategy in the decline of coral skeletal densities under ocean warming and acidification (Chan & Connolly, 2013; Mollica et al., 2018), and allow calcifying organisms to expend additional energy in metabolic processes and growth (Bach et al., 2015). Therefore, exposure to olivine weathering could be potentially used in assisted evolution, if the enhancement of resilience characteristic in corals, is proved.

This strategy can, however, represent ecological disadvantages regarding potential secondary effects of olivine dissolution. Some downsides include the increase in suspended particulate matter and turbidity, accumulation of toxic levels of dissolution products, particularly nickel, which can pose ecotoxicological effects in marine organisms (Meysman & Montserrat, 2017; Montserrat et al., 2017).

1.4. Goals

While the majority of the published studies covers enhanced weathering as a CO_2 removal strategy, focused on its application onto terrestrial soils (Dietzen et al., 2018; Lefebvre et al., 2019; Renforth et al., 2015), the surface mixed layer of the ocean (Köhler et al., 2013) or by spreading the minerals across coastal zones (Hangx & Spiers, 2009b; Schuiling & de Boer, 2011), it is still to be understood how the exposure to olivine may affect marine organisms.

The present study aims to assess sub-lethal effects of olivine sand in the tropical coral *Montipora digitata*, evaluating photobiology, oxidative stress response and metabolic profiles following acute and chronic exposures. The potential of increased thermal tolerance once *M. digitata* is exposed to olivine, will as well be evaluated. Ultimately, it is intended to estimate at, laboratorial scale, the implications of using olivine as an enhanced weathering strategy in coastal environments, mostly regarding potential shifts in pH values.

2. Materials and methods

2.1. Study species

Montipora digitata, is a fast-growing branching coral, that occurs in tropical and subtropical shallow Indopacific's reef-flat zones (Veron & Wallace, 1984; Veron J.E.N., Stafford-Smith M.G., 2016). These species rely predominantly on autotrophy (Anthony, 1999) and both sexual and asexual reproduction (Haynes & Johnson, 2000; Heyward & Collins, 1985). It is an ecologically relevant species that forms large clonal stands and fragments naturally, while simple to cultivate *ex situ* (Leuzinger et al., 2012).

2.2. Culture conditions and fragmentation

Montipora digitata mother colonies, donated by Oceanário de Lisboa, were fragmented into 3-5 cm tall fragments. The resulting fragments were fixed to a sand-concrete mixed base using ethyl 2-cyanoacrylate (super glue). Subsequently, *M. digitata* fragments were acclimated and left to cicatrization for 3 months to be used in the pilot experiment, and for 2 weeks to be used in the chronic experiment. The aquaria was made of standardized 600 L culture systems, composed of two 250 L (150 cm length x 40 cm width x 50 cm height) culture tanks (Rocha et al. 2015), connected to a filtration sump equipped with two heaters (Eheim, Jager 300 W), chiller (Hailea, HC-300A), UV disinfection system (TMC, P1-55 W), protein skimmer (Eheim, Skimmarine 800), kalkwasser reactor (Deltec, KM 500S), osmoregulator (Deltec, Aquastat 1001), ~5L of biological filter media and ~2L activated charcoal. Culture tank circulation was performed by a submergible pump (Eheim, universal 3400), providing an approximate flow of 1200 Lh^{-1} to each tank. A different submergible pump (Eheim, universal 1200) was used to perform water recirculation through chiller and UV system. Illumination was provided by four 80 W fluorescent lamps (Red Sea, REEF-SPEC) with 12:12 photoperiod (light:dark), emitting a PAR of 90 ± 10 µmol m^{2-} s^{-1}. Temperature was kept at 25 ± 1ºC and salinity at 35 ± 1. Synthetic saltwater was prepared by mixing synthetic salt (Red Sea, Coral PRO salt) with reverse osmosis water (TMC, V2 Pure 360). Approximately 2 times a week, partial water changes (~15% of total system volume) were carried.

2.3. Olivine characterization

Commercially available olivine sand ($Mg_{2-x}Fe_xSiO_4$) was purchased at QUIMIALMEL, which provided the chemical analysis presented in **table 1** and particle size quantification, **table 2.** Given the chemical characterization, olivine can be characterized as Mg-rich olivine, known as fosterite.

Table 1. Concentration (%) of different chemical constituents in olivine sand. L.O.I = Loss on ignition.

Chemical Constituents	Concentration (%)
MgO	42.35
SiO_2	44.15
Al_2O_3	1.30
$FeO+Fe_2O_3$	7.25
CaO	2.35
L.O.I	1.25

Table 2. Particle size (mm) of olivine sand.

Particle size (mm)	%
0.315	0.03
0.200	0.10
0.160	0.15
0.100	2.20
0.080	6.60
0.063	8.45

2.4. Experimental design

2.4.1. Acute experiment

An acute experiment was performed to estimate possible lethal or sub-lethal effects of increasing concentrations of olivine sand on the coral *Montipora digitata*, when compared to the control treatment.

Fragments of the mother colony were individually stocked in 200 mL flasks, each one with 170 mL of saltwater and 10 mL of osmosis water. The added osmosis

water prevented the rise of water salinity resulted by saltwater evaporation. Five nubbins were used as replicates per treatment (i.e. olivine sand concentration) while kept for 96 h. During the experiment, the flasks were maintained in a water bath with one heater (Eheim, Jager 300W) and one water pump (Eheim, CompactON 300), ensuring homogenous water temperature. The experimental system was illuminated with four 54 W fluorescent lamps (Red Sea, REEF-SPEC) with 12:12 photoperiod, emitting a PAR of 70 ± 10 µmol m^{2-} s^{-1}. An aeration system was set for each flask using an air compressor (Hailea, VB-290G) connected to 45 hoses, one for each flask. 50% of the medium was renewed per day. Eight concentrations of olivine sand were set by applying a factor of 1.5 between them: 6.00, 9.00,13.50, 20.25, 30.38, 45.56, 68.35 and 102.52 mg L^{-1}. The chosen olivine concentrations allow a wide range of different concentrations to be tested, since no previous experiment has tested olivine sand in marine organisms. The test concentrations were obtained weighing the olivine sand using a microbalance (RADWAG MYA 2.3Y).

Photosynthetic efficiency, oxidative stress and damage biomarkers and cellular energy allocation were measured at the beginning of the experiment, sampling 5 fragments from the culture system (0 h), and at the end (96 h) in 5 fragments from each treatment. Each day, water parameters were checked to ensure they remained stable throughout the experiment: salinity: 35.50 ± 1.64 and temperature: 24.92 ± 0.85 ºC. Dissolved oxygen and pH were also measured every day but were used as comparative parameters between the control group and each olivine concentration and are reported in section 3.1.1.

2.4.2. Chronic experiment

A chronic experiment was conducted exposing *M. digitata* to olivine sand for seventy days, to assess biological responses, in particular, photosynthetic efficiency and relative coral growth rate, after a long-term exposure. The chosen olivine concentration was 20.25 mg L^{-1}, since it was the lowest to exhibit a significant increase in pH values. Olivine sand was weighed with a precision balance (FX-5000i).

Fragments of the mother colony were evenly distributed between 2 systems, for seventy days. Each treatment (i.e. olivine concentration) was composed by 35

replicates, between two ~150 L culture systems composed of five ~10 L (28 cm length x 18 cm height x 20 cm width) culture tanks each, connected to a filtration sump, prepared with two heaters (Eheim, Jager 300 W), chiller (Hailea, HC-300A), UV disinfection system (TMC, P1-55 W), protein skimmer (Eheim, Skimmarine 800), osmoregulator (Deltec, Aquastat 1001) and ~5L of biological filter media. Each tank was equipped with four 54 W fluorescent lamps (Red Sea, REEF-SPEC) with 12:12 photoperiod, emitting a PAR of 50 ± 10 µmol m^{2-} s^{-1}. Water parameters were measured weekly, at the same period, to ensure they remained stable throughout the experiment: salinity 35.1 ± 0.7 and temperature 25.29 ± 0.20 ºC. Dissolved oxygen and pH were also measured weekly and are reported in section 3.5.2. Photosynthetic efficiency, growth and cellular energy allocation were measured at the beginning of the experiment, sampling 7 fragments from the culture system (0 h), and again at the end of the experiment (70 d)

2.4.3. Acute thermal stress experiment

By subjecting *M. digitata* to thermal stress while under previous and current olivine exposure, we aim to assess if this mineral enables corals with resistance mechanisms to cope with climate change scenarios, and its consequences specially, bleaching or ocean acidification. The same concentration of 20.25 mg L^{-1} of olivine sand was used for the acute stress test, as well as the equivalent procedure previously stated.

Following the seventy days of chronic exposure, thirty-six fragments previously subjected to the exposure or absence of olivine, were evenly distributed between four tanks (nine fragments/tank) with distinct temperatures (i.e. thermal stress). For each treatment, fragments early exposed to olivine sand were subjected to the same previous concentration of 20.25 mg L^{-1}, while corals that were not previously exposed to olivine, remained with the control treatment of 0.00 mg L^{-1}.Two tanks, equipped with four 80 W fluorescent lamps (Red Sea, REEF-SPEC) with 12:12 photoperiod, emitting a PAR of 70± 10 µmol m^{2-} s^{-1} , were placed in a water bath with one heater (Eheim, Jager 300 W) and one water pump (100L h^{-1}) each, to secure homogenous water temperature. The other two tanks were placed in identical conditions except for the water temperature which was

increased from 25 ºC to 30 ºC, applying a near-instantaneous heating rate, identically to previous studies (Griffin et al., 2006; Richier et al., 2005; Yakovleva et al., 2009). Water parameters remained stable throughout the experiment: salinity: 35.47 ± 0.56; temperature: 25.22 ± 0.21 ºC and 30.15 ± 0.20 ºC. Dissolved oxygen and pH were also measured and are reported in section 3.6.2. Photosynthetic efficiency, oxidative stress and damage biomarkers and cellular energy allocation were measured at the beginning of the experiment, sampling 7 fragments from each culture system (i.e. olivine concentration/temperature) and again after 24 h (i.e. end of the experiment).

2.5. Relative growth rate

Coral fragments were weighted in the first and last day of the chronic experiment to calculate the growth rate for each coral fragment. The relative growth rate, as daily biomass increase per day, was calculated using the following formula (Casabianca & Laugier, 1997) :

$$\mathrm{RGR} = [\ln(\mathrm{wf}/\mathrm{wi})]/[(\mathrm{tf} - \mathrm{ti})] \times 100 \qquad (2)$$

Where wf = final weight; wi = initial weight; tf -ti = total of days from the beginning until the end of the experiment.

2.6. SEM evaluation and EDX analysis

Six samples from the chronic experiment (three per treatment) were immersed in a 2% sodium hypochlorite solution for 24 h to remove all the organic matter from the skeleton and rinsed with osmosis water. After the procedure, the coral fragments were dried and placed on aluminium supports which were covered with a conductive thin film of carbon deposition. Scanning Electron Microscopy (SEM) was carried in a HITACHI TM4000PLUS equipped with a Bruker EDS (Energy Dispersive System) detector at an acceleration voltage of 15 kV (at TEMA, University of Aveiro, Portugal). Images were processed using ImageJ 1.46r, for both number of corallites per area and corallite area. The measurement of corallite area is exemplified in **figure 17 (appendix).**

2.7. Biological response

By the end of every experiment and immediately after photobiology assessment, fragments were snap frozen in liquid nitrogen and preserved at -80 ºC until further manipulation. Later, each coral fragment was individually homogenized on ice using 1200 µL of ultra-pure water and the sonicator (pulsed mode of 10% for 30 s, 250 Sonifier, Branson Ultrasonics). From each sample, 3 aliquots were taken for the analysis of lipid, sugar, and protein contents, and ETS activity. One aliquot containing 4 % butylated hydroxytoluene (BHT) in methanol was used for the determination of LPO. The remaining homogenate was diluted 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 15 min at 10 000 g (4 ºC). The post-mitochondrial supernatant (PMS) was divided into microtubes and kept in -80 ºC until further analyses of oxidative stress related biomarkers.

All of the biomarker determinations were performed spectrophotometrically, in micro-assays set up in 96-well flat-bottom plates at 25 °C, with the Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA) (A. C. M. Rodrigues et al., 2015; Rodrigues et al., 2017).

2.7.1. Photobiology

Photobiology parameters were estimated non-intrusively through pulse amplitude modulation (PAM) fluorometry, using Junior-PAM and WinControl3 software (Walz ™) at the beginning and end of the experiments. Coral fragments were dark-adapted for approximately 30 min before measured the maximum quantum yield of photosystem II (PSII), ensuring the full relaxation of PSII reaction centres. Saturating light pulses (with a peak at 450 nm, half-bandwidth of 20 nm) were delivered by a 1.5 mm plastic optical fiber, and measurements were taken perpendicularly to the surface of the coral, in three different points in each fragment as described by Cruz et al. 2015. When applied, the saturation pulse allows the determination of the dark-level fluorescence (F_o) and maximum fluorescence (F_m), both used to determinate the maximum quantum yield of PSII (Schreiber et al., 1986):

$$F_v/F_m = \left(\frac{F_m - F_o}{F_m}\right) \quad (3)$$

2.7.2. Oxidative stress related biomarkers

Coral samples were submitted to maceration with liquid nitrogen. Coral samples were individually homogenized on ice using 1200 µl of ultra-pure water, using a sonicator (pulsed mode of 10% for 30 s, 250 Sonifier, Branson Ultrasonics). 300 µl of homogenate were diluted in 0.2 M K-phosphate buffer, pH 7.4 (1:1), and centrifuged for 10 min at 10 000 g (4 ºC), the post-mitochondiral supernatant (PMS) used for protein, CAT, GST and tGSH determinations. From each sample, 3 aliquots were taken for the analysis of lipid, sugar and protein contents, and ETS activity. One aliquot containing 4 % butylated hydroxytoluene (BHT) in methanol is used for the determination of LPO. Another aliquot was used for HSP70 determination. All biomarkers determinations were adapted to micro-assays set up in 96 well flat bottom plates (Rocha et al., 2020; Rodrigues et al., 2017; Vieira et al., 2021), and read spectrophotometrically with the Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA).

Protein concentration of PMS was determined according to the Bradford method (Bradford, 1976), using bovine γ-globulin as a standard. Catalase (CAT) activity was determined in PMS by measuring decomposition of H_2O_2 at 240 nm (Clairborne, 1985). Glutathione-S-transferase (GST) activity was determined in PMS following the conjugation of GSH with 1-chloro-2,4- dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974). Endogenous lipid peroxidation (LPO) was determined by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm (Bird & Draper, 1984). HSP70/HSC70 content was assessed by ELISA, adapted from (Rosa et al., 2014). 50 µL of the sample was added to a 96 well microplate and allowed to incubate overnight at 4°C. The next day, the microplates were washed (3×) in 0.05% PBS-Tween-20. A 100 µL sample of blocking solution (1% BSA, Sigma-Aldrich) was added to each well and left to incubate at room temperature for 2 h. Microplates were washed and 5 µg mL^{-1} primary antibody (1º Anti-HSP70 mouse mAB (C92F3A-5) Millipore), detecting 72 and 73 kDa proteins corresponding to the molecular mass of inducible HSP and HSC70, was added to each well and then incubate overnight at 4 °C. The non-linked antibodies were removed by washing the microplates again, which were then incubate overnight at 4 °C with 1 µg mL^{-1} of the secondary antibody,

anti-mouse IgC (2º Anti-mouse IgC (fab specific) Sigma). After another wash, 100 µL of substrate p-nitrophenyl phosphate was added to each well and incubated for 30 min at room temperature. Then, 50 µL of stop solution (3 mol L^{-1} NaOH) was added to each well and the absorbance was read at 405 nm in a 96 well microplate reader (Benchmark, Bio-Rad, Hercules, CA, USA). The amount of HSP70/HSC70 in the samples was calculated from a curve of absorbance based on serial dilutions of purified HSP70 active protein standard (HSP70 protein Millipore) to a range from 0 to 2000 ng m L^{-1}.

2.7.3. Cellular energy allocation

Energy available (Ea – as sum of sugars, lipids and proteins) and energy consumption (Ec – estimated as ETS activity) were assessed using the methods described by De Coen & Janssen (1997) with slight modifications for microplate (Rodrigues et al., 2015). The final CEA value was calculated as: CEA = Ea/ Ec (Verslycke et al., 2004).

Total lipid content of each organism was determined adding chloroform, methanol, and ultra-pure water in a 2:2:1 proportion. After centrifugation, the organic phase of each sample was transferred to clean glass tubes and H_2SO_4 was added prior to incubation during 15 min at 200 ºC. Absorbance was measured at 375 nm and tripalmitin was used as a lipid standard. Carbohydrates quantification was performed by adding 5% phenol and H_2SO_4 to the samples, with glucose as a standard, the absorbance read at 492 nm. Bradford's method (Bradford, 1976) was used for total protein content quantification using bovine serum albumin as a standard and absorbance measured at 520 nm. Fractions of energy available were converted into energetic equivalent values using the corresponding energy of combustion: 39500 mJ/ g lipid, 17500 mJ/ g glycogen, 24000 mJ/ g protein (Gnaiger, 1983).

Electron transport system (ETS) activity was measured using the INT (Iodonitrotetrazolium) reduction assay, in which ETS was measured as the rate of INT reduction in the presence of the nonionic detergent Triton X-100, with the absorbance read at 490 nm. Cellular oxygen consumption rate was calculated based on the stoichiometrical relationship in which for 2 µmol of formazan formed, 1 µmol of oxygen is consumed. Ec value was obtained by the conversion to

energetic values using the specific oxyenthalpic equivalent for an average lipid, protein and carbohydrate mixture of 480 kJ mol^{-1} O_2 (Gnaiger, 1983).

2.8. Statistical analysis

All data were reported as mean ± standard deviation Outliers were excluded before statistical analysis and calculated as the mean ± 2*standard error. Normality was tested based on Shapiro-Wilk method, $\alpha=0.05$. For non-normal distribution, data was transformed. For pH comparisons in the acute experiment, one-way analysis of variance (ANOVA) was used, followed by posthoc Dunnett's test ($p<0.05$). For DO comparisons in the acute experiment, Kruskal-Wallis test was performed due to non-normal distribution, followed by posthoc Dunn's test ($p<0.05$). To test the effect of olivine in different concentration on coral physiology in the acute experiment linear regression model was used ($p<0.05$). Comparisons in the long-term experiment were performed using unpaired t-test ($p<0.05$), and Wilcoxon for non-normal distributions ($p<0.05$). Relative growth rate results were performed using an unpaired t-test one-tailed ($p<0.05$), rather than two-tailed, once our alternative hypothesis is that the olivine's effect is to increase coral growth. Comparisons in the thermal challenge experiment were performed using two-way analysis of variance (ANOVA), followed by posthoc Sidak's test ($p<0.05$). Analysis were run in GraphPad Prism version 8.4.3 and regressions were performed using linear regression function in Statsoft Statistica v. 8.0.

3. Results

3.1. Acute olivine exposure

3.1.1. Chemical water parameters

I. pH

pH values per olivine concentration tested are reported in **figure 1**. One-way ANOVA revealed that higher olivine concentrations significantly affected water pH (ONE-WAY ANOVA [$F_{(8,36)}$=0.444], p=0.001). Dunnett's multiple comparisons test detected significant differences between the control group and olivine concentrations of 20.25, 30.38, 68.35 and 102.5 mg L^{-1}, reporting a significant increase in pH values, **table 3 (appendix).**

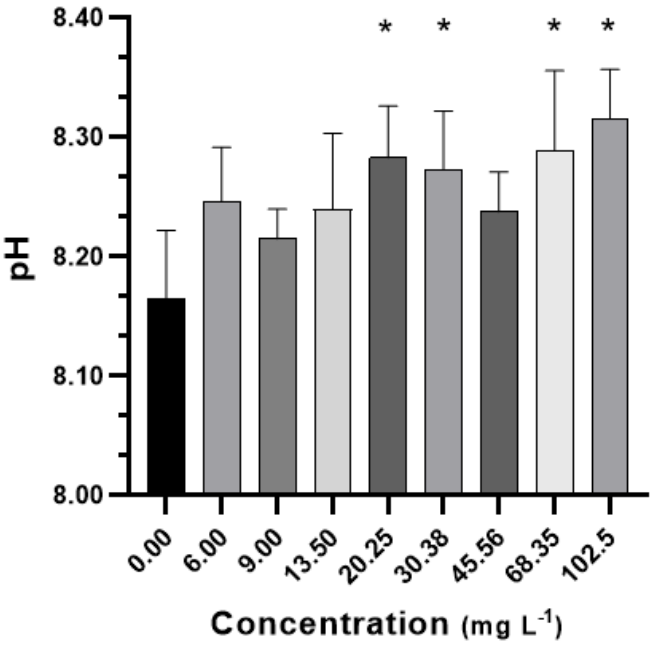

Figure 1. Visual summary of pH values after the acute exposure (96 h) to increasing concentrations of olivine. Data presented as mean ± standard deviation. (*) denotes significant differences in comparison to the control group, once performed Dunnett's test, (p<0.05).

II. Dissolved oxygen

Dissolved oxygen values per treatment are presented in **figure 2**. Kruskal-Wallis test revealed that higher olivine concentrations significantly affected water DO (KRUSKAL-WALLIS [$X^2_{(9)}$=38.78], p<0.001). Dunn's multiple comparisons test detected significant differences between the control group and olivine concentrations of 20.25, 30.38, 68.35 and 102.5 mg L^{-1}, reporting a significant increase in DO values, **table 3 (appendix).**

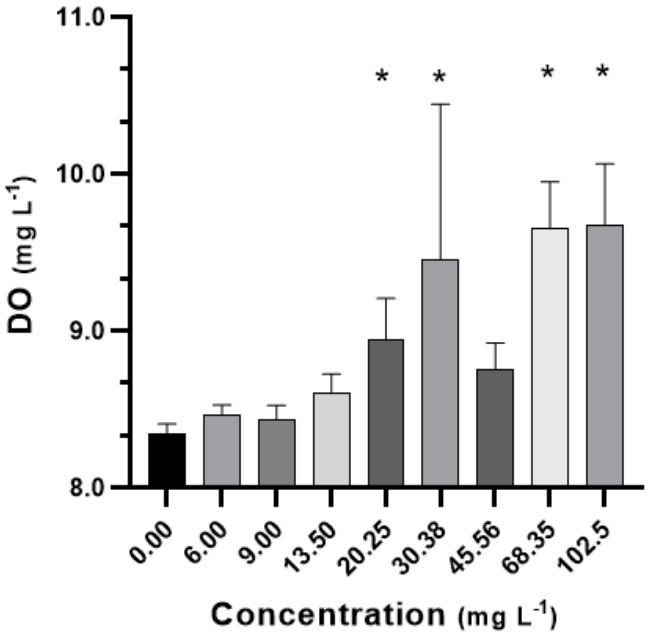

Figure 2. Visual summary of pH values after the acute exposure (96h) to increasing concentrations of olivine. Data presented as mean ± standard deviation. (*) denotes significant differences in comparison to the control group, once performed Dunn' test, ($p<0.05$).

3.1.2. Photosynthetic efficiency

Photobiology results are reported in **figure 3**. *M. digitata* underwent significant decreases in F_v/F_m when exposed to increasing concentrations of olivine (R^2=0.142, [$F_{(1,133)}$=22.068], $p<0.001$).

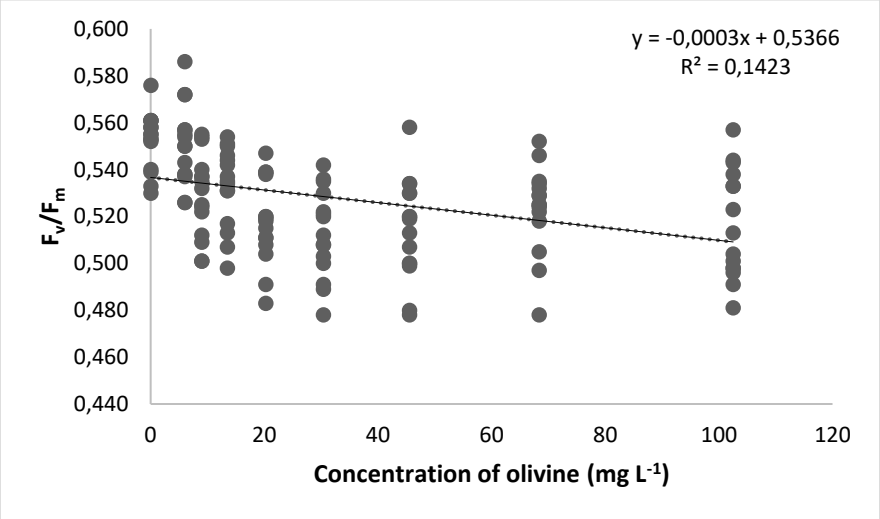

Figure 3. Relation between the maximum photochemical quantum yield (F_v/F_m) of *M. digitata* and increasing concentrations of olivine, after a short-term exposure (96 h).

3.1.3. Oxidative stress related biomarkers

Results for oxidative stress and damage biomarkers are reported in **figure 4.** *M. digitata* exhibited no correlation between CAT activity, GST activity and LPO levels and increasing olivine concentrations. For HSP70 activity, a negative correlation was reported (R^2=0.176, [$F_{(1,43)}$=9.151], p=0.004), as values significantly decrease with higher olivine concentrations.

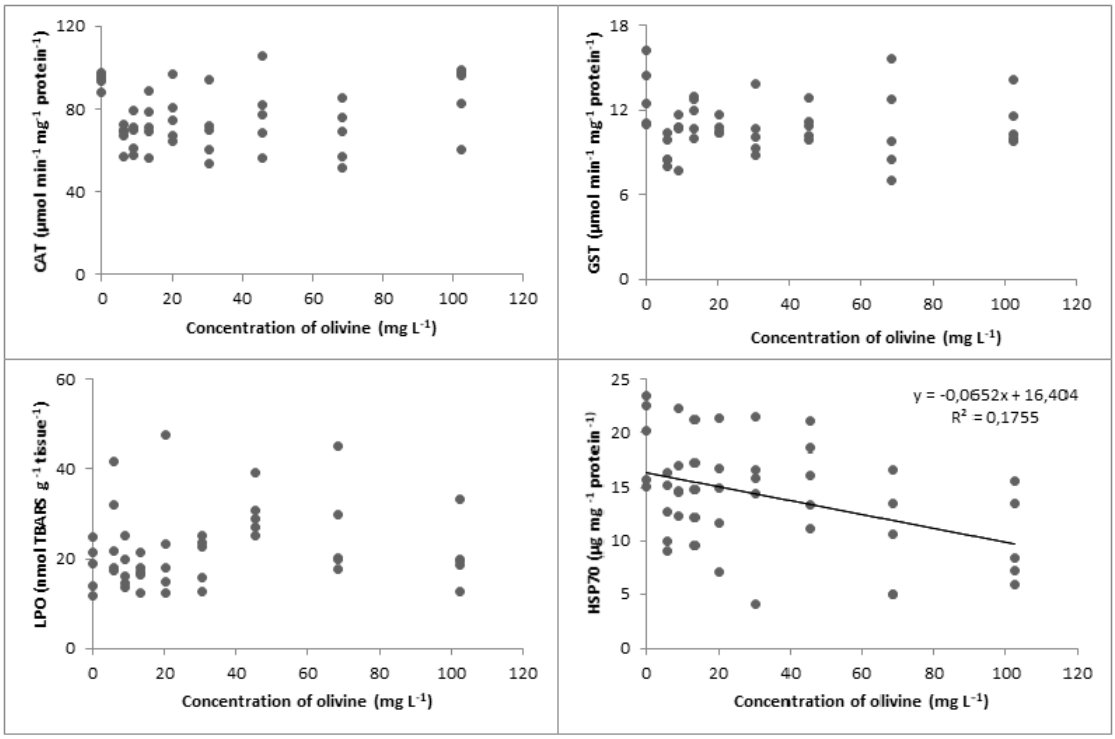

Figure 4. Relation between oxidative stress and damage response and increasing olivine concentrations, in *M. digitata* after an acute exposure (96 h).

3.1.4. Cellular energy allocation

Results for cellular energy allocation and electron transport (ETS) activity are reported in **figure 5.** *M. digitata* exhibited no correlation between sugar content and increasing olivine concentrations. The same results were reported for energy consumption (ETS). Lipid content exhibited a positive correlation, demonstrating higher values of lipids for increasing olivine concentrations (R^2=0.222, [$F_{(1,43)}$=12.26], p=0.001). As for protein content, a negative correlation was reported with lower values of proteins for increasing olivine concentrations (R^2=0.349, [$F_{(1,43)}$=23.00], p<0.001).

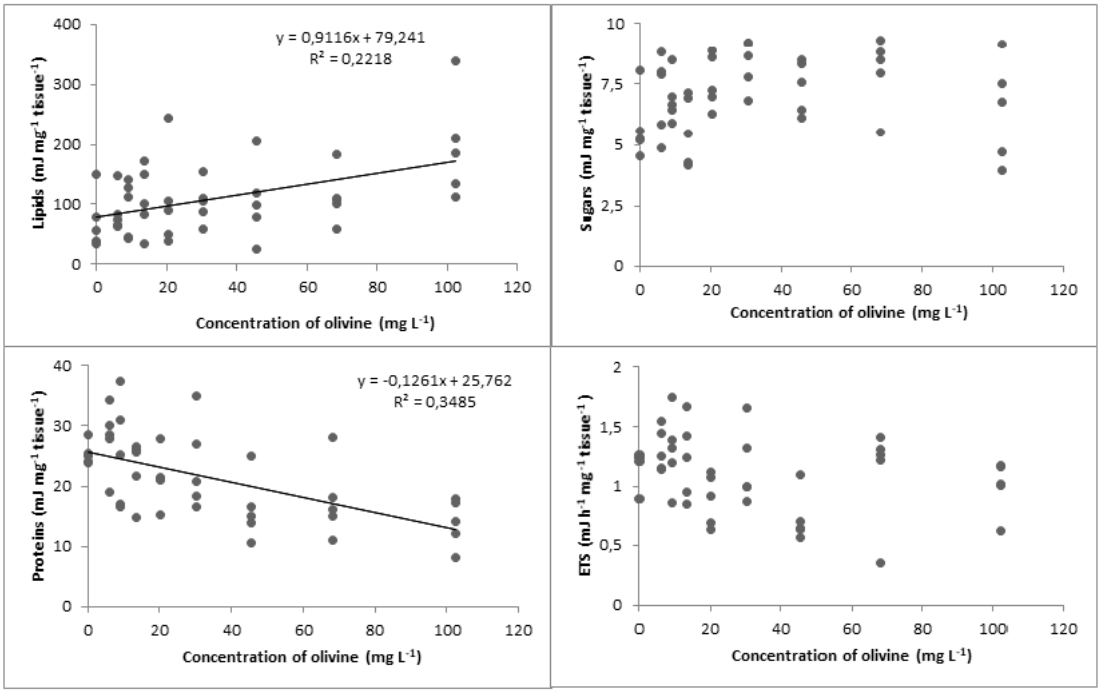

Figure 5. Relation between cellular energy allocation response and electron transport (ETS) and increasing olivine concentrations in *M. digitata* after an acute exposure (96 h).

3.2. Chronic exposure experiment

3.2.1. Chemical water parameters

I. pH and dissolved oxygen

Values of pH and dissolved oxygen are represented in **figure 6.** pH was significantly affected throughout the long-exposure experiment (WILCOXON TEST, W=66.00 p=0.001), exhibiting higher mean in the olivine treatment. DO values did not underwent significant changes when exposed to olivine (UNPAIRED t-TEST, $t_{(11)}$=1.921, p=0.081).

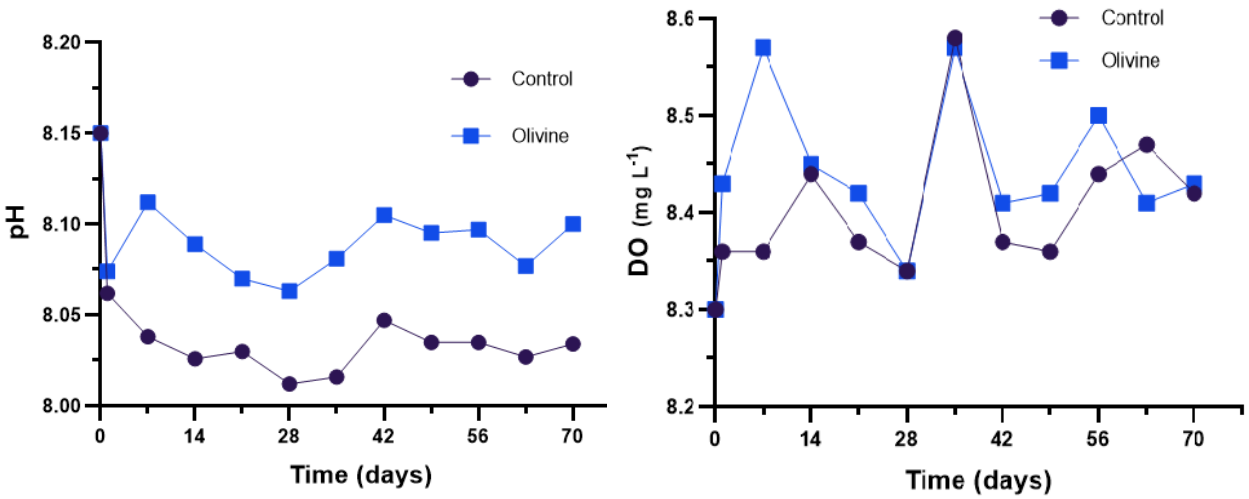

Figure 6. Visual representation of pH and dissolved oxygen (mg L^{-1}) values during a chronic exposure (70 days) to olivine 20.25 mg L^{-1}. Data presented as a single measure.

3.2.2. Photosynthetic efficiency

Photobiology results are reported in the **figure 7**. When long-term exposed to 20.25 mg L^{-1} olivine, *M. digitata* F_v/F_m proved to be significantly different (UNPAIRED t-TEST $t_{(208)}$=4.909, p<0.001) from the control, with an increased photosynthetic efficiency.

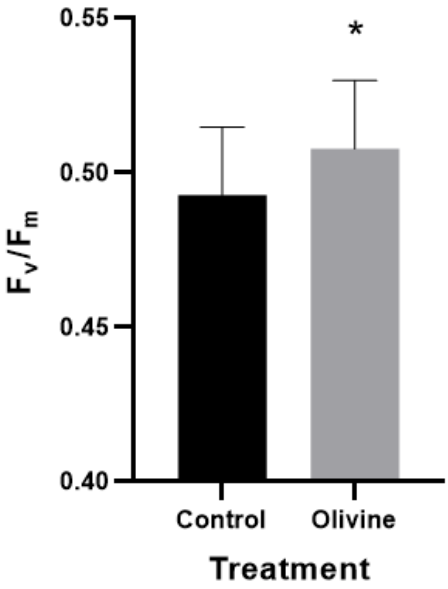

Figure 7. Effects on the maximum photochemical quantum yield (F_v/F_m) of *M. digitata* after the chronic exposure (70 days) to 20.25 mg L^{-1} of olivine. Data presented as mean ± standard deviation. (*) denote statistical differences between the control group and olivine treatment ($p<0.05$), once performed a UNPAIRED T-TEST.

3.2.3. Relative growth rate

Results for the relative growth rate are showed in the **figure 8**. *M. digitata* growth rate was significantly (UNPAIRED t-TEST $t_{(68)}=1.798$, $p=0.038$) affected by the exposure to olivine, being higher than the control treatment.

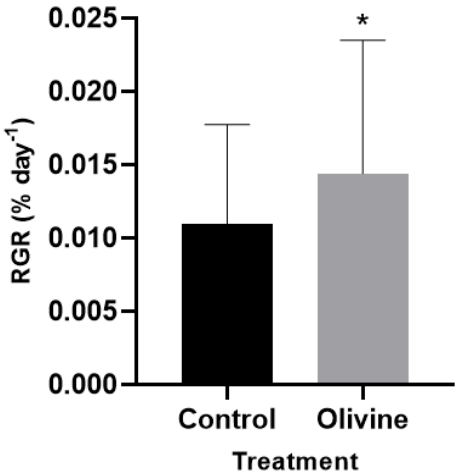

Figure 8. Visual summary of relative growth rate (RGR) of the coral *M. digitata* after a long-exposure (70 days) to 20.25 mg L^{-1}. Data presented as mean ± standard deviation (*) denotes significant differences, ($p<0.05$) once performed a UNPAIRED T-TEST.

3.2.4. SEM and EDX evaluation

At the end of the chronic exposure, skeletons from the control treatment, evidenced corallites with larger areas (98956.6 ± 25382.5 μm^2), although not significantly different (UNPAIRED t-TEST $t_{(6)}$=0.421, p=0.689) from the olivine treatment (93485.1 ± 5633.1 μm^2), **figure 10**. The number of corallites per area was also measured and although control treatment exhibited a higher number (0.685 ± 0.261 corallite mm^{-2}), the difference was not significant (UNPAIRED t-TEST $t_{(4)}$=0.345, p=0.747) in comparison to the olivine treatment (0.624 ± 0.156 corallite mm^{-2}), **figure 11**.

Results for Ca^{2+} and Mg^{2+} elemental analysis are reported in **figure 9**. At the end of the chronic exposure, coral skeletons from the control treatment exhibited significant higher values of Mg^{2+} (UNPAIRED t-TEST $t_{(4)}$=3.155, p=0.034) than olivine exposed fragments. As for Ca^{2+}, skeletons exposed to olivine treatment reported significant higher values (UNPAIRED t-TEST $t_{(4)}$=9.922, p<0.001) in comparison to the control treatment.

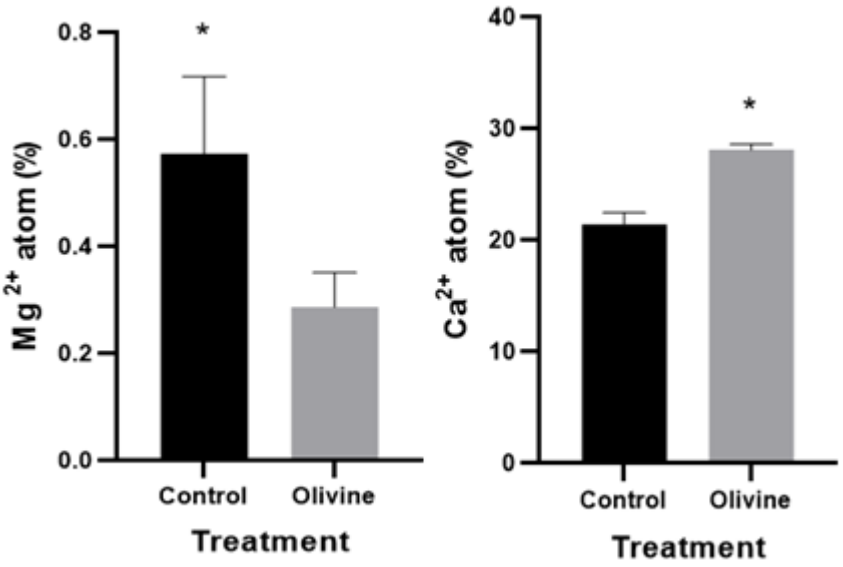

Figure 9. Mg $^{2+}$ and Ca $^{2+}$ elemental analysis of coral skeleton in control and olivine treatment, after chronic exposure. Data presented as mean ± standard deviation. (*) denotes significant differences, (p<0.05) once performed a UNPAIRED T-TEST.

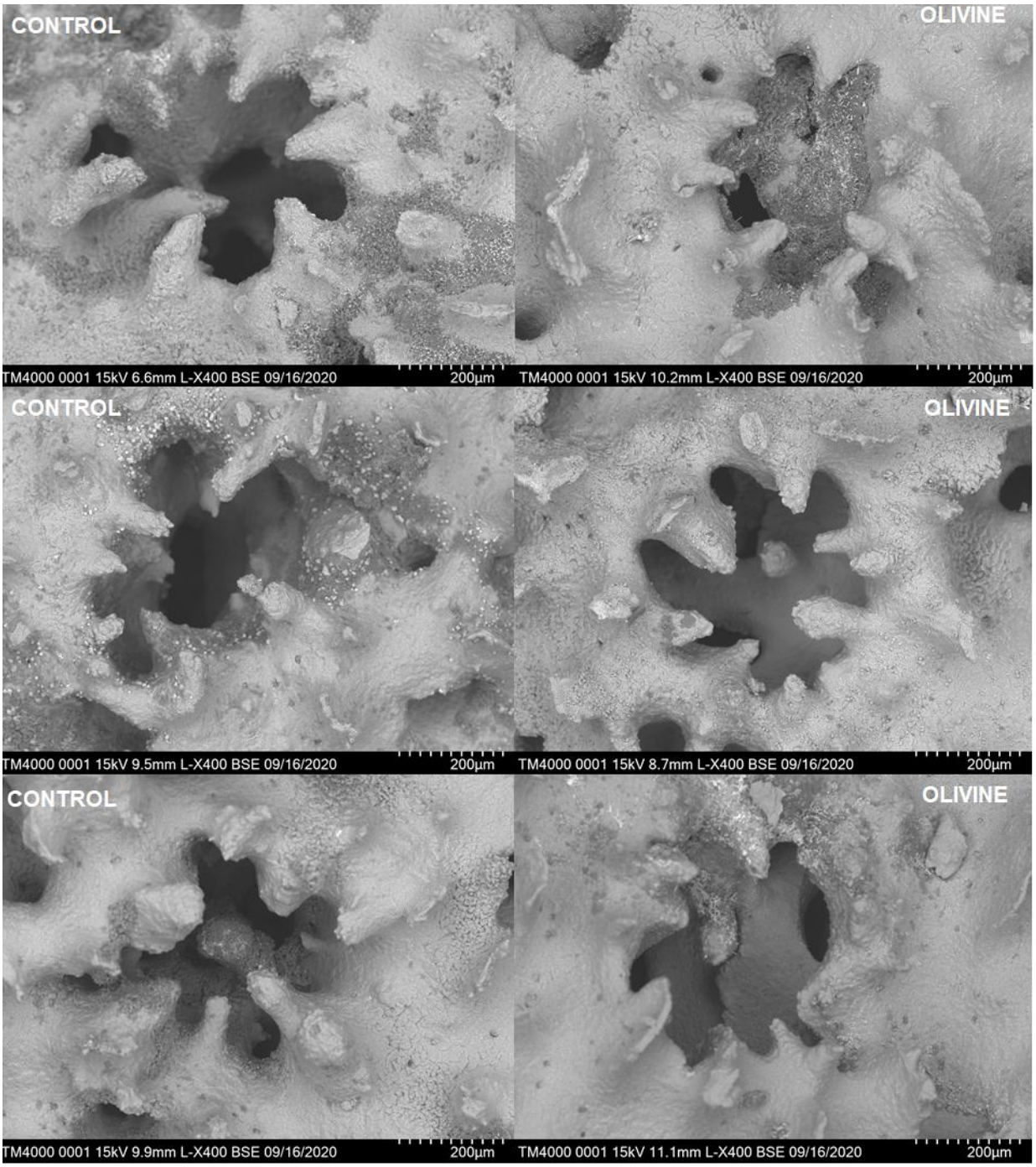

Figure 10. Scanning electron microphotographs (magnification of 400x) of corallite area from six selected fragments of *M. digitata*, developed under different treatments, control and olivine. For control treatment, fragments exhibited higher corallite areas, although not significantly different ($p<0.05$).

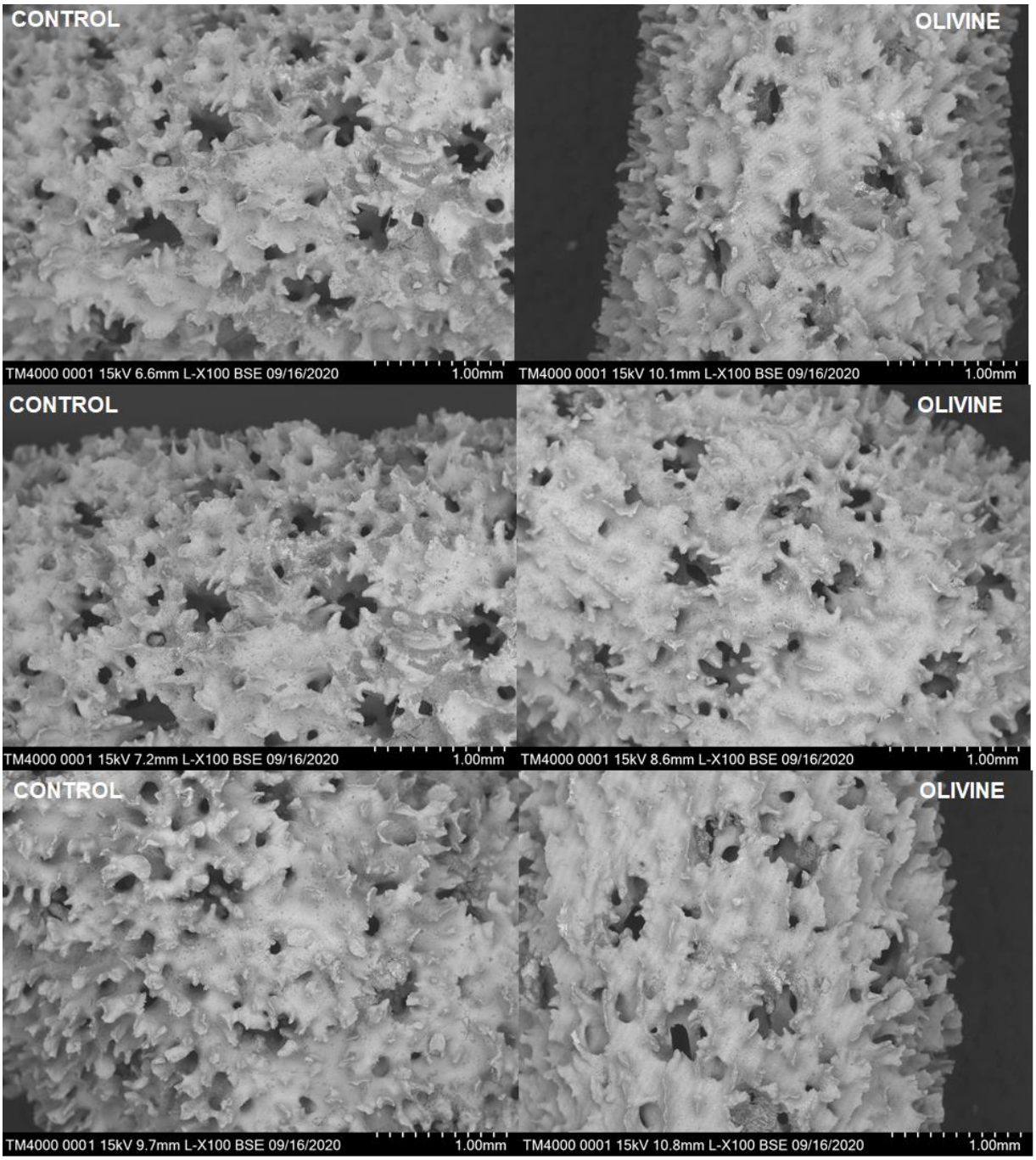

Figure 11. Scanning electron microphotographs (magnification of 100x), of corallites per coral area from six selected fragments of *M. digitata*, developed under different treatments, control and olivine. Control treatment exhibited higher number of corallites per area, although not significantly different ($p<0.05$).

3.2.5. Oxidative stress related biomarkers

Oxidative stress and damage results are presented in **figure 12.** The chronic exposure to olivine significantly (UNPAIRED t-TEST $t_{(24)}$=4.634, p<0.001) affected CAT activity, increasing it when compared to the control treatment. Both GST activity (UNPAIRED t-TEST $t_{(24)}$=1.170, p=0.254) and HSP70 (UNPAIRED t-TEST $t_{(24)}$=1.672, p=0.107) were not significantly impacted by the presence of olivine, although reporting a higher mean when compared to the control treatment. LPO was significantly (UNPAIRED t-TEST $t_{(26)}$=3.392, p=0.002) higher in corals from the control treatment.

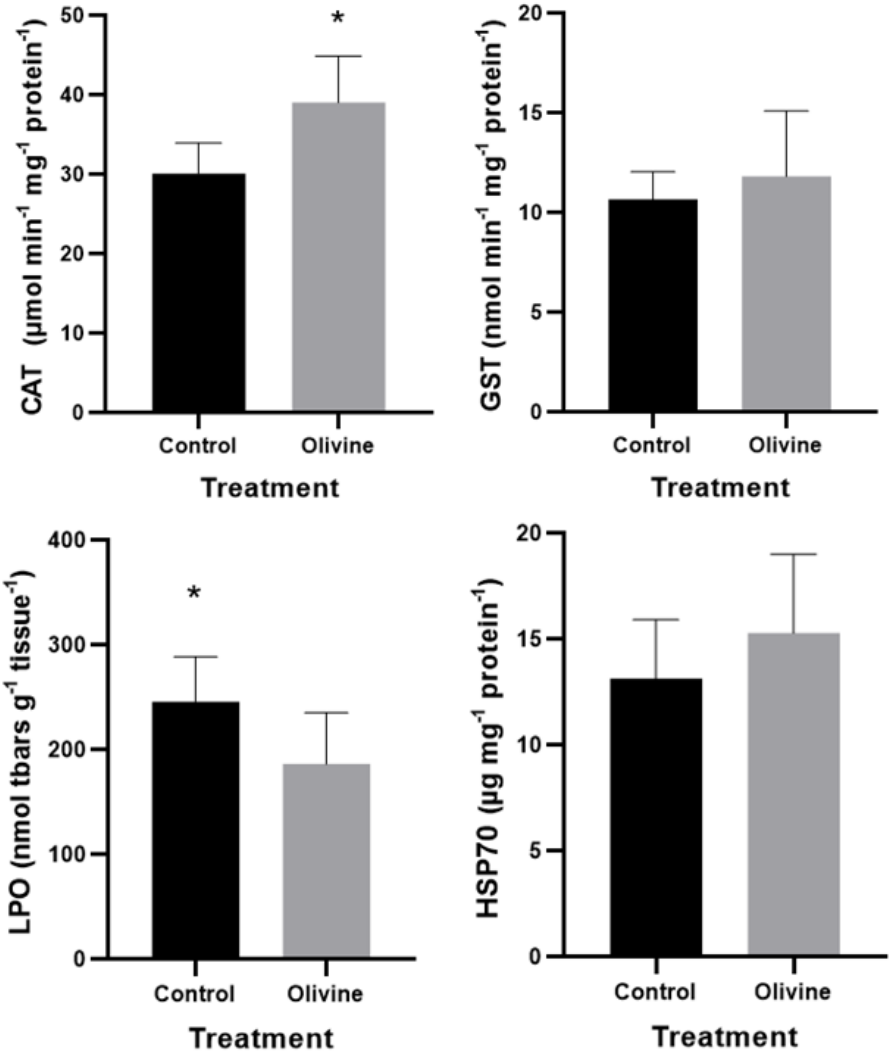

Figure 12. Visual summary of oxidative stress and damage biomarkers related to *M. digitata* after 70 days of exposure to 20.25 mg L^{-1} of olivine. Catalase activity (CAT), Gluathione S- transferase levels (GST), lipid peroxidation (LPO) and heat-shock proteins (HSP70). Data presented as mean ± standard deviation. (*) denote significant statistical differences between the control group and olivine treatment (p<0.05), once performed an UNPAIRED t-TEST.

3.2.6. Cellular energy allocation

Results regarding cellular energy allocation are reported in **figure 13**. The unpaired t-test revealed that lipid content was significantly (UNPAIRED t-TEST $T_{(25)}$=2.579, p=0.017) higher in the control group. However, no significant differences were reported in sugar (UNPAIRED t-TEST $t_{(25)}$=1.454, p=0.158) and protein (UNPAIRED t-TEST $t_{(25)}$=0.687, p=0.498) content under the exposure to olivine. The unpaired t-test reported no significant effects in the electron transport system (ETS) activity (UNPAIRED t-TEST $t_{(25)}$=0.513, p=0.613) after the long-term exposure to olivine.

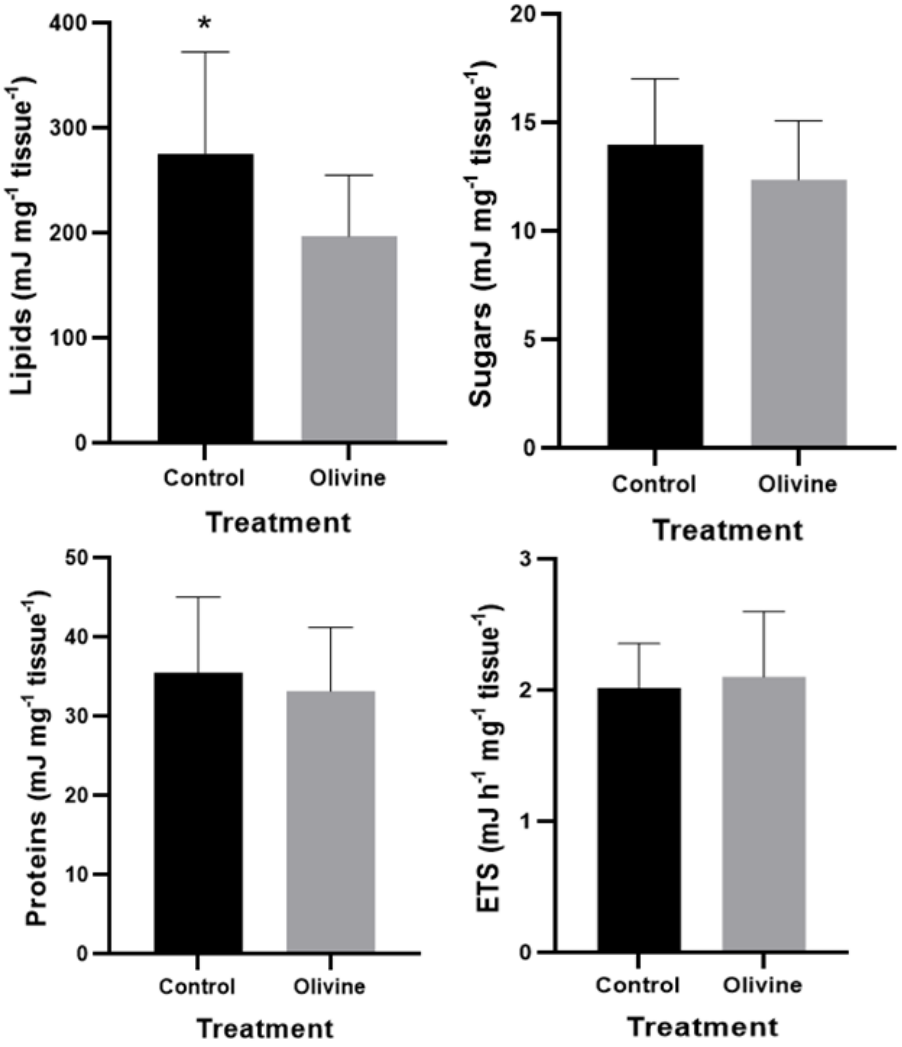

Figure 13. Visual summary of cellular allocation biomarkers and electron transport (ETS) activity related to *M.digitata* after 70 days exposed to 20.25 mg L^{-1} of olivine. Data presented as mean ± standard deviation. (*) denote significant statistical differences between the control group and olivine treatment (p<0.05), once performed UNPAIRED t-TEST.

3.3. Acute thermal stress experiment

Mortality was observed in the control treatment (i.e. absence of olivine) subjected to high temperature of 30 ºC (n=2), and in the olivine treatment subjected to high temperature of 30ºC (n=1). The total of bleached fragments was discarded for photobiologic response, oxidative stress, and metabolic profiles.

3.3.1. Chemical water parameters

I. pH

For the tanks at 25 ºC: in absence of olivine pH=7.776 ± 0.147; exposed to olivine pH=7.967 ± 0.028. For tanks at 30 ºC: in absence of olivine pH=7.748 ± 0.158; exposed to olivine pH=7.890 ± 0.063.

II. Dissolved oxygen

For the tanks at 25 ºC: in absence of olivine DO=8.03 ± 0.14 mg L^{-1}; exposed to olivine DO=8.08 ± 0.04 mg L^{-1}. For tanks at 30 ºC: in absence of olivine DO=7.65 ± 0.28 mg L^{-1}; exposed to olivine DO=7.81 ± 0.16 mg L^{-1}.

3.3.2. Photosynthetic efficiency

Photosynthetic efficiency results are presented in **figure 14**. Two-way ANOVA revealed significant differences in *M. digitata* between treatments (TWO-WAY ANOVA [$F_{(1,95)}$=12.78], p<0.001). Sidak's multiple comparisons test reported that significant differences were found at 30 ºC, as olivine treatment exhibited higher F_v/F_m levels, **table 5 (appendix).**

3.3.3. Oxidative stress related biomarkers

Oxidative stress and damage results are presented in the **figure 15**. Two-way ANOVA revealed that temperature and treatment, as well their interaction, did not significantly affected the activity of CAT, GST and HSP70, neither induced lipid peroxidation (LPO), **table 6 (appendix)**

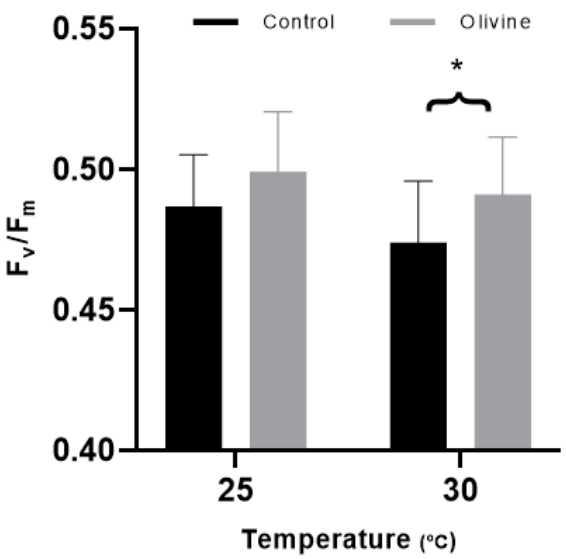

Figure 14. Maximum photochemical quantum yield (F_v/F_m) of *M. digitata* after 24 h of acute thermal stress (30ºC), in the presence and absence of olivine. Data presented as mean ± standard deviation. (*) denotes significant statistical differences between the control group and olivine treatment at 30ºC ($p<0.05$), once performed TWO-WAY ANOVA ($p<0.05$), followed by Sidak's multiple comparisons ($p<0.05$).

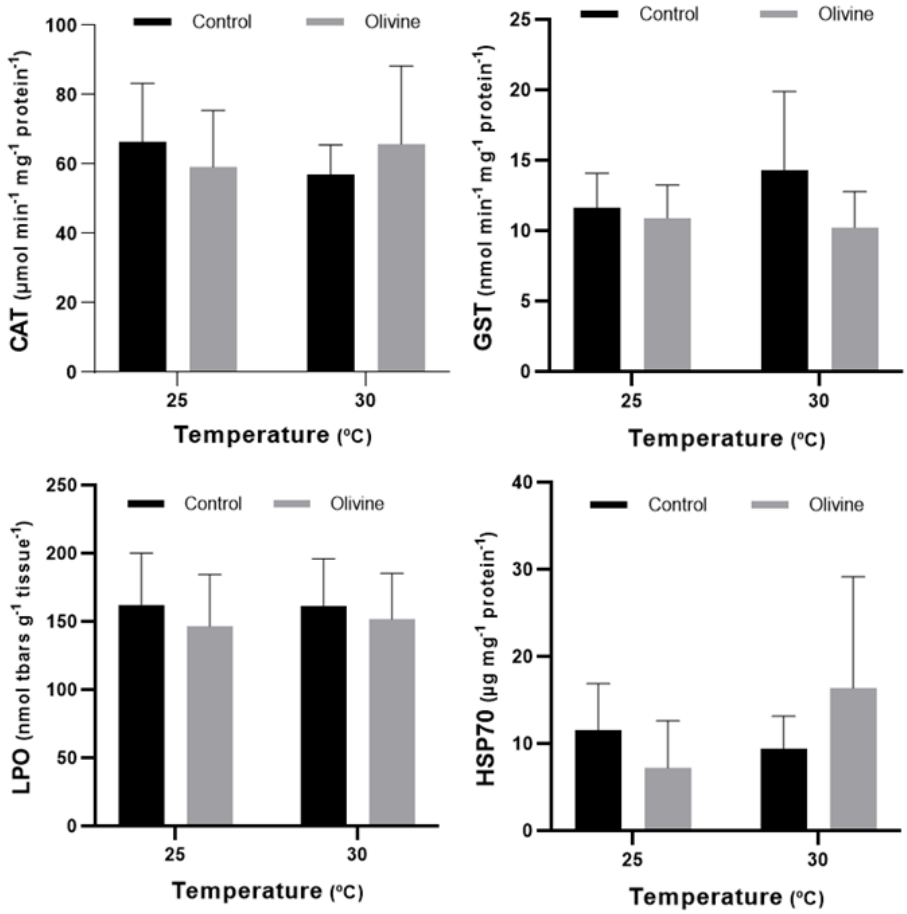

Figure 15. Visual summary of oxidative stress and damage biomarkers related to *M. digitata* after 24 h of thermal stress. Catalase activity (CAT), Gluathione S- transferase levels (GST), lipid peroxidation (LPO) and heat-shock proteins (HSP70). Data presented as mean ± standard deviation.

3.5.6. Cellular energy allocation

Results regarding cellular allocation and energy consumption are reported in **figure 16**. A Two-way ANOVA was performed, revealing no significant differences in temperature and treatment, as well as their interaction, for sugar, and protein content and ETS activity **table 7 (appendix)**. Lipids results (TWO-WAY ANOVA [$F_{(1,27)}$=7.419], p=0.011), reported significant differences between treatments. SIdak's multiple comparisons test reported that significant differences were found between treatments at 25 ºC, exhibiting higher values for olivine treatment, **table 8 (appendix).**

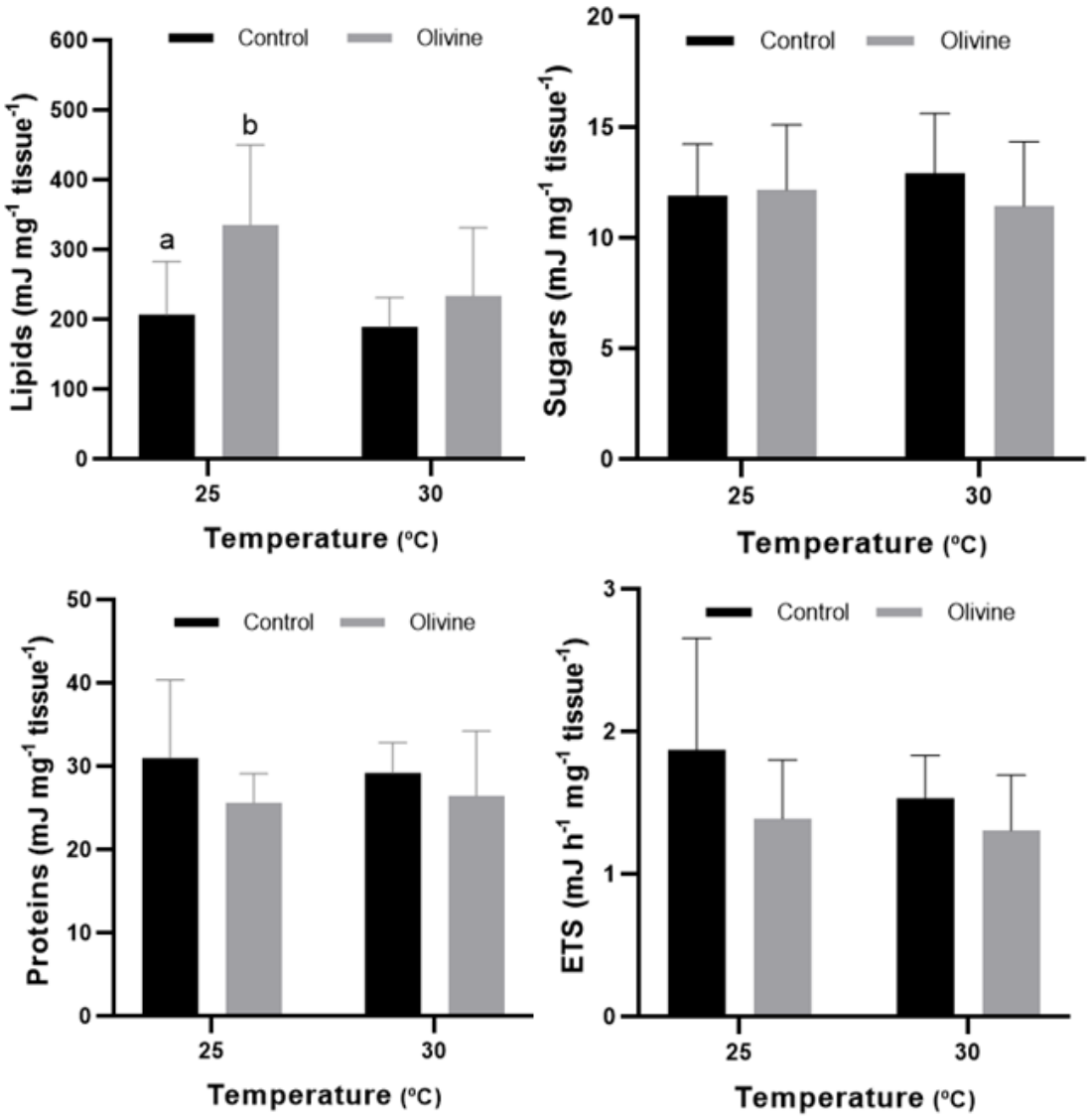

Figure 16. Visual summary of cellular energy allocation and electron transport (ETS) activity related to *M. digitata* after 24 h of thermal stress. Data presented as mean ± standard deviation. (a, b) denotes significant differences between control and olivine treatment at 25ºC by Sidak's multiple comparisons (p<0.05).

4. Discussion

4.1. Acute olivine exposure

Olivine weathering has been proposed as a large-scale climate engineering approach, expecting to increase seawater alkalinity, and resulting in additional CO_2 uptake from the atmosphere (Montserrat et al., 2017). Despite being recognized as a promising strategy, ecological implications and secondary effects on the marine ecosystem have not been addressed. The first part of this study aimed to access the biological response of the coral *M. digitata* to a range of olivine sand concentrations, integrating a complementary analysis of water parameters such as pH and dissolved oxygen (DO) to biological markers such as photobiologic response, oxidative stress response and cellular energy allocation.

The pH and dissolved oxygen exhibited higher values when sampled from mediums with higher olivine concentrations. Under ocean acidification scenarios, a significant increase in alkalinity is greatly desirable as it could shift the acid-base equilibrium from dissolved CO_2 to carbonate (Montserrat et al., 2017). Thereby, not only potentially restraining ocean acidification, but also favouring skeletal growth in reef-building corals (Mollica et al., 2018), enabling the calcium carbonate precipitation from the coral calcifying fluid. For pH, the lowest olivine value to induce significant changes was 20.25 mg L^{-1}, establishing the olivine concentration used in the chronic exposure.

Once exposed to increasing concentrations of olivine, *M. digitata* underwent a significant decrease in F_v/F_m. Photoinhibition is associated with lower efficiency of photosynthetic energy which may be due to a dynamic and reversible process, where absorbed light is dissipated as heat or a chronic photoinhibition, resulting from the damage of photosynthetic units and requiring *de novo* synthesis of repairing proteins (Jones & Hoegh-Guldberg, 1999). The decline protein content could thus be explained by the degradation of the PSII reaction center protein, D1, which is usually repaired in few hours (Gorbunov et al., 2001; Kyle et al., 1984). However, photoinhibition does not necessarily correspond to lower net photosynthetic rates or is associated with higher energetic costs (Gorbunov et al., 2001; Hoogenboom et al., 2006). It can also manifest through different mechanisms, while not always indicative of damage but rather of downregulation

(Hoegh-Guldberg & Jones, 1999). Leal et al. (2016) experiment, reported that *Zoanthus. sociatus* shows temporary downregulation of photochemical mechanisms during low tide but exhibits no signs of photodamage or bleaching. The decrease in F_v/F_m could also be due to sediment turbidity, limiting light availability. However, signs of turbidity were not visually observed during the experiment. Under turbid waters, corals require extra energetic costs in order to remove the sediments from their tissues (Erftemeijer et al., 2012; Sean & Connolly, 2004). Interestingly, dissolved oxygen levels increased, which might suggest that P/R rates (photosynthesis/respiration) were kept elevated, which contrasts other studies findings that report elevated rates of respiration due to increased metabolism for damage repair (Hoogenboom et al., 2006; Riegl & Branch, 1995). Besides, the increase in lipid content could be linked to healthy photosynthetic rates since *M. digitata*, although a mixotrophic species, is predominantly autotrophic and experienced no heterotrophic feeding during the experiment. The water used in the flasks was prepared with synthetic saltwater and reverse osmosis water, while renewed every day, reducing the possibility of particulate organic matter to be ingested by the coral.

If under oxidative stress, organisms produce reactive oxygen species (ROS) which are cytotoxic components that cause lipid peroxidation and DNA damage. Although they are produced within the cell as a result of mitochondrial electron transfer processes, ROS can also be generated due to exposure to foreign compounds or environmental factors such as thermal stress (Hayes & McLellan, 1999), impairing antioxidant defences while causing oxidative damage (Downs et al., 2002). Catalase (CAT) provides one of the first lines of defence against ROS, converting $2H_2O_2$ into $2H_2O$ and O_2. However, when exposed to olivine concentrations, *M. digitata* did not exhibited significant different CAT levels in comparison to the control group. The second line of defence against oxidative stress, is provided by enzymes like glutathione S-transferase (GST) that besides detoxifying secondary oxidative products, removes them from the cell to maintain cellular homeostasis and prevent oxidative damage such as lipid peroxidation (LPO) (Hayes & McLellan, 1999). In this experiment neither GST nor LPO levels significantly differ with the increase of olivine concentrations, suggesting a non-oxidative stress response. However, HSP70 levels reported a linear decrease as

a response to higher olivine concentrations. Heat-shock proteins, despite the name, are susceptible to different sources of stress (Ikwegbue et al., 2018) and play an important role through helping misfolded proteins regain native conformation and recover biological activity (Chiappori et al., 2016; Swain et al., 2006). The lower levels of HSP70 could be related to the linear decrease of protein content, as well reported in *M. digitata* during the acute exposure, as heat-shock proteins could be allocated to prevent irreversible aggregation of denatured proteins, while refolding them into native and functional shapes (Mizrahi et al., 2016)

Organisms energetic budgets provide important trade-offs for physiological performance, i.e. growth, reproduction and overall survival and fitness (Maltby, 1999). Lipid content, as previously said, increased while protein levels were negatively correlated with olivine concentrations. Energy reserves are metabolized and synthesised differentially by different species. Rodrigues and Grottoli (2007) reported that in *Porites compressa*, autotrophic coral, carbohydrates would be translocated, while proteins and lipids must be synthesized. Furthermore, lipid levels, a primary coral tissue constituent, were the first to recover after a bleaching episode, indicating that carbohydrates could be quickly stored as lipids in the coral (Muscatine & Cernichiari, 1969; Patton et al., 1977), which could explain the lipid increase in our experiment. Experimental results did not show any significant altered sugar content and ETS activity on *M. digitata*. Therefore, *M. digitata* did not show additional energy demands to maintain defence mechanisms or metabolic activity.

Overall, these results, support the hypothesis that olivine does not induce lethal effects in the coral *M. digitata*, and sub-lethal effects were reflected by moderate differences in antioxidant enzymes activities as well as in energetic storage. Photosynthetic efficiency was also impaired, reporting signs of photoinhibition, although values remained within healthy levels. We decided to choose an olivine concentration of 20.25 mg L^{-1} to be used in further experiments since it was the lowest concentration to exhibit a significant increase in pH levels, a result expected under olivine dissolution.

4.2. Chronic olivine exposure

Large-scale olivine distribution in coastal ecosystems is a critical issue since geophysical consequences such as an increase in suspended particulate matter, sedimentation and turbidity can lead to adverse impacts on the marine environment, especially sensitive habitats such as coral reefs (Erftemeijer et al., 2012; Montserrat et al., 2017; Sister & Dy, 1999). Chronic exposures to conditions leading to oxidative stress may decrease the overall fitness of the coral, impairing stress-protein response or enzymatic activity required for homeostasis (Downs et al., 2002), while shading might temporarily reduce photosynthetic rates, leading to slower coral calcification and thinner tissues (Fabricius, 2005). The second chapter of this experiment aims to study the biological response of *M. digitata* to a long-term exposure to 20.25 mg L^{-1} of olivine sand, including photobiologic response, relative growth rate, oxidative stress response and cellular energy allocation.

The pH values were constantly higher in olivine treatment, validating the tendency observed in the acute experiment. Given the significant difference between treatments, it is expected that relative growth rate of *M. digitata* under olivine exposure would be as well, significantly higher, since saturation state of calcium carbonate is mainly related to pH, shifting the equilibrium composition of inorganic carbon in favor of carbonate and bicarbonate ions (Falini et al., 2015). The results for RGR validate the previous hypothesis since growth was higher in the olivine treatment.

At the end of seventy days, photosynthetic efficiency of coral *M. digitata* reported significantly higher values than the control group, contrasting with the results obtained in the pilot experiment. These results can support the hypothesis of reduced photosynthetic efficiency not necessarily indicating damage in photobiological mechanisms. Therefore, *M. digitata* photoinhibition while exposed to olivine for 96h may result from downregulated mechanisms, which are normal and reversable characteristics of symbiotic dinoflagellates (Hoegh-Guldberg & Jones, 1999), followed by an adaptive response from the coral holobiont. This can occur either through photoprotective pigments or acclimation processes (Fisher et al., 2012; Hoegh-Guldberg & Jones, 1999).

Interestingly, *M. digitata* had significantly higher levels of CAT activity under olivine treatment, while showing significantly higher levels of LPO under control treatment. This could mean that no increase in LPO, under olivine exposure, was observed because antioxidant enzymes (CAT) compensated ROS production, limiting cellular damage. CAT activity might also result from increased metabolic activity, rather than stressful conditions (Krueger et al., 2015b). Another hypothesis could be that throughout growing, coral membranes' cells are continually renewed, removing peroxidised lipids from the membranes, and decreasing lipid peroxidation levels, which can be observed in the olivine treatment with higher relative growth rate. The opposite is true for the control group, where antioxidant first line defence (CAT) was inhibited or overwhelmed, unable to detoxify ROS and leading to peroxidation of membranes' lipids. Additionally, as coral growth was significant lower in the control treatment, the removal of peroxidised lipids and renewal of membrane compounds could be impaired, favouring the accumulation of cellular damage.

Previous studies (Anthony et al., 2002; Leuzinger et al., 2012) indicate that tissue growth is more sensitive to environmental conditions and resources shortage than skeletal growth, resulting in the expense of tissue lipids necessary for skeletal growth, while decreasing tissue energy content. This goes in line with our results since relative growth rate in *M. digitata* increased when exposed to olivine, while lipid content decreased. Overall, sugar and protein content, as well as energy consumption showed no significant differences under olivine exposure.

Coral macro structures, such as corallite area, columella area and septal length are mainly affected by light levels and flow patterns, holding a key role in the scattering and absorption of light by the coral symbionts (Enríquez et al., 2017; Studivan et al., 2018). In this experiment, olivine exposure, although exhibiting higher growth rates, did not impact corals' macrostructures, namely corallite area and number corallites per coral area.

Elemental analysis showed that corals exposed to olivine exhibited higher values of Ca^{2+} in the corallite area, while control treatment showed higher values of Mg^{2+}. Coral calcification occurs through the following reaction:

$$Ca^{2+} + CO_2 + H_2O \rightarrow CaCO_3 + 2H^+ \qquad (4)$$

Through this process, calcium cation (Ca^{2+}), fundamental to the calcification mechanism, are transported from seawater to the skeleton site by passive and active transport.

Some coral species display a strong capacity to control pH in the calcification site, which may increase its resilience to ocean acidification (Ries, 2011; Ries et al., 2009; Wall et al., 2016). Alternatively and accordingly to Decarlo et al., 2018 experiment, resistant species such as *Pocillopora damicornis* can upregulate $[Ca^{2+}]_{cf}$, to resist the potential effects of ocean acidification and preserve healthy calcification rates when chronic exposed to low pH values. Thus, the increase in $[Ca^{2+}]$ in olivine treatment, could potentially enhance the calcification process of coral skeleton, reflected by higher relative growth rates obtained in the same experiment. On the other hand, high intracellular concentrations of Ca^{2+} can affect the integrity of lipid membranes duo to aggregation of proteins and nucleic acids (Case et al., 2007). Thus, maintaining a large Ca^{2+} concentration gradient can be used as a cellular regulation mechanism, but could impose an additional metabolic cost to keep intracellular Ca^{2+} at low concentrations (Davis Hovorka et al., 2019).

Mg^{2+} besides being an essential element to many enzymatic mechanisms and biochemical reactions in organisms (Tremel, 1994), can possibly play a role in stabilizing the amorphous calcium carbonate in the centers of calcification (Mass et al., 2017; Von Euw et al., 2017). Marine calcifying organisms can produce skeletons composed of either aragonite or calcite, accordingly to the molar Mg/Ca ratio of seawater (Stanley & Hardie, 1998). While present corals live in a high Mg/Ca seawater which favours aragonite precipitation, if this ratio is disrupted and corals are exposed to lower Mg/Ca, calcifying rates may decrease and a mixture of calcite and aragonite skeleton may be formed (Higuchi et al., 2014). However, seawater chemistry was not assessed in this experiment, so values of sweater Mg/Ca are not available, as well as differentiation between calcite and aragonite skeleton formation.

From this experiment results, we can conclude that chronic exposure to 20.25 mg L^{-1} of olivine sand facilitates *M. digitata* relative growth and photosynthetic efficiency. *M. digitata* experienced an increase in its enzymatic antioxidant capacity and a higher relative growth rate through the expense of tissue lipid

content, but no additional energy demands to maintain defence mechanisms or metabolic activity and no significant skeleton macrostructure modification.

4.3. Acute thermal stress

Thermal stress is one of the primary leaders to coral bleaching, a phenomenon whereby the symbiosis between the coral host and symbiotic algae is damaged, triggering the loss of zooxanthellae (Gates et al., 1992; Glynn, 1991; Hoegh-Guldberg & Smith, 1989) and leading to coral mortality, reduced growth or reproduction (Goreau & Macfarlane, 1990; Ward et al., 2000), and increase susceptibility to diseases (Harvell et al., 2001). The third experiment of this work aimed to assess biological responses of the coral *M. digitata* to increase temperature (30 ºC), while exposed to 20.25 mg L^{-1} of olivine sand. Besides, we can as well, evaluate if previous olivine exposure enhances *M. digitata* resilience to rising temperatures, expected by climate change scenarios. Photosynthetic efficiency, oxidative stress and damage response and metabolic profiles were analysed.

At the end of the experiment, treatments at 25 ºC remained non-bleached with no mortality. Control at 30 ºC showed signs of visible bleaching (pale to white colour) in two fragments. Olivine at 30 ºC also showed signs of visible bleaching in one fragment. This mortality rate could be due to instant heating rate used in the experiment, as slower heating rates delay the physiological response of the coral host and symbiont (Middlebrook et al., 2010).

The pH values followed the increasing tendency with olivine presence reported by the previous experiments. As for the dissolved oxygen levels, a depletion is noted with the increase of temperature which could be related to an initial photosynthetic impairment resulting from lower photosynthetic efficiencies triggered by higher temperatures. Additionally, dissolved oxygen has an inverse relationship with temperature, decreasing its levels with the increase of temperature (Phull & Abdullahi, 2017).

Photosynthetic efficiency, as expected, decreased when corals were subjected to thermal stress, however, significant differences were just reported between treatments at 30 ºC. Olivine treatment, although exhibiting a decrease in F_v/F_m at

30 ºC, exhibited higher values than control treatment at the same temperature, which could indicate a potential resilience mechanism enhanced by olivine exposure. Overall, the moderate differences reported in F_v/F_m between temperatures can be related to protective processes, such as photoprotective pigments or acclimatation mechanisms, maximizing light capture (Titlyanov & Titlyanova, 2002).

When under thermal stress, an increase in corals' antioxidant enzyme activities is expected since higher temperatures induce higher metabolic rates and ROS overproduction (Burdon et al., 1990; Richier et al., 2006). Overall, *M. digitata* whilst exposed to 30 ºC did not show increased antioxidant activity, neither intracellular damage generated by lipid peroxidation. Additionally, HSP70 is commonly used as thermal stress biomarker, since it activates ROS removal and maintains the mitochondrial membrane integrity (Driedonks et al., 2015). When analysed after 24 h of thermal stress, HSP70 values, although higher at 30 ºC, had no significant difference in comparison with 25 ºC treatments. Low variation in HSP70 levels could be related to constantly elevated basal levels, ensuring protection mechanisms in environments under temperature fluctuations (Madeira et al., 2014; Mizrahi et al., 2016), in *M. digitata* case, reef-flat zones.

Lipid, protein and carbohydrate content are considered to be short-term energy sources, when photosynthates are not sufficient (Kavousi et al., 2015), which can happen under thermal stress. After 24 h of increasing temperature, *M. digitata* did not exhibit significant disparities in cellular energy allocation and energy consumption. However, under higher temperature, the depletion of energy stores can happen gradually and over longer time scales than 24 h (Anthony et al., 2009). The only difference observed was in lipid content at 25 ºC, between treatments, where olivine exposed corals had higher lipid values than the control. These results were also observed in the acute experiment, where lipid content was positively correlated with olivine concentration.

Overall, the mortality of three fragments and decrease in photosynthetic efficiency, although not significant, can be markers of thermal stress or at least, the beginning of bleaching process. However, biological response of *M. digitata,* associated with antioxidant pathways and energetic storage and consumption was not yet indicative of induced thermal stress. A few hypotheses can be taken

into consideration. Thermal variations could have been not high enough to induce thermal stress in *M. digitata* since it has been characterized by some authors with lower bleaching susceptibility (Fisher et al., 2012; Imbs & Yakovleva, 2012; Krueger et al., 2015a) and/or the exposure period could also be considered short-term. Moreover, while varying between species hosting different symbiont clades, the response to temperature also differs in species hosting the same symbiont type (Fisher et al., 2012; Winters et al., 2009). Therefore, with this experiment we cannot conclude that olivine exposure enhances resilience mechanisms in the coral *M. digitata* whilst abnormal temperatures.

5. Final considerations and future perspectives

Our study concluded that olivine sand does not pose lethal effects on *Montipora digitata,* although posing moderate sub-lethal effects whilst acute and chronic exposure. Moreover, extended exposure of *M. digitata* to olivine sand highlighted additional coral growth as well as increased photosynthetic efficiency. Although potential effective as a carbon dioxide removal strategy, olivine weathering could also represent a prospective strategy to enhance corals thermal resilience. Future studies are undoubtedly required and should have some considerations:

- Chronic exposure to olivine could be extended in order to verify an effective acclimatization.
- Extended acute stress or higher temperature variability.
- Choosing a species with a higher bleaching susceptibility.
- Symbiont identification, since to different strains might correspond different thermal tolerance.
- Study of additional biological parameters such as zooxanthellae density and chlorophyll content.
- Chemical water analysis including total alkalinity, dissolved inorganic carbon and concentration of dissolution products, such as Mg^{2+}, Si, Fe^{2+} and Ni^{2+}.

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7. Appendix

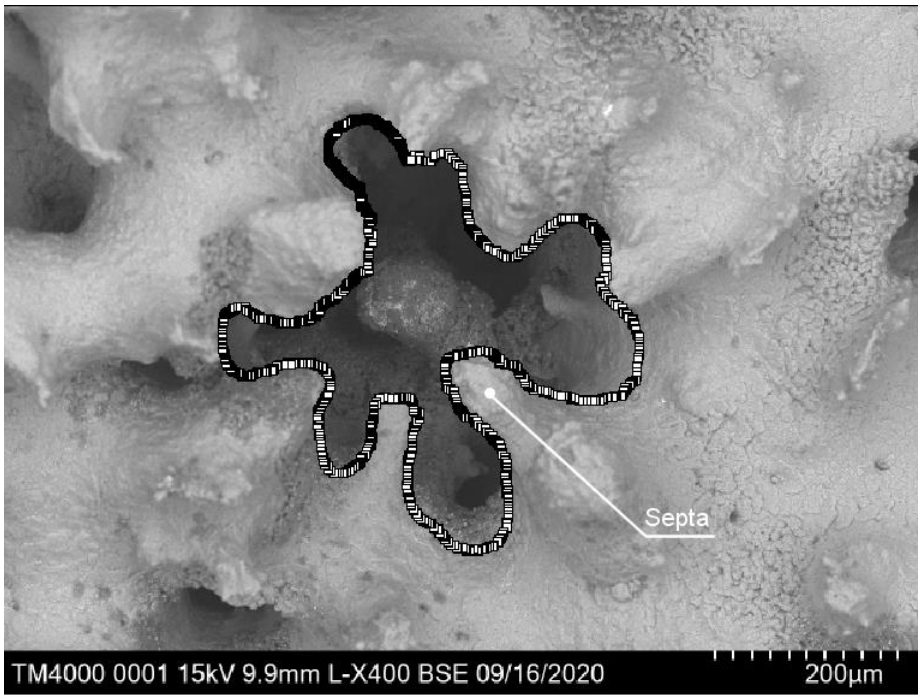

Figure 17. Scanning electron microphotograph (magnification of 400x) of corallite from the control treatment. Area selected as corallite area and measured using the program ImageJ 1.46r.

Table 3. Statistical analysis for pH. Dunnett's test was performed when one-way ANOVA was significantly different ($p<0.05$), comparing multiple olivine concentrations at 96 h with the control group.

	Olivine concentration (mg L^{-1})	**Dunnett's [p-value]**
pH	20.25	0.004
	30.38	0.009
	68.35	0.002
	102.5	<0.001

Table 4. Statistical analysis for DO. Dunn's test was performed when one-way ANOVA was significantly different ($p<0.05$), comparing multiple olivine concentrations at 96 h with the control group.

	Olivine concentration (mg L^{-1})	Dunn's [p-value]
DO	20.25	0.039
	30.38	0.003
	68.35	<0.001
	102.5	<0.001

Table 5. Sidak's multiple comparisons statistical results for photosynthetic efficiency (F_v/F_m), after 24 h of acute thermal stress at 30 ºC, ($p<0.05$). Performed after two-way ANOVA significant result ($p<0.05$).

	p-value	t	df
Control - Olivine	0.012	2.824	95

Table 6 Two-way ANOVA statistical results relative to oxidative stress and damage in *M. digitata* after acute thermal challenge ($p<0.05$). Catalase activity (CAT), Gluathione S- transferase levels (GST), lipid peroxidation (LPO) and heat-shock proteins (HSP70).

	Source of variation	p-value	F(DFn, DFd)
CAT	Interaction	0.207	F(1,28)=1.668
	Temperature	0.818	F(1,28)=0.0538
	Treatment	0.905	F(1,28)=0.0145
GST	Interaction	0.193	F(1,27)=1.784
	Temperature	0.438	F(1,27)=0.619
	Treatment	0.065	F(1,27)=3.690
LPO	Interaction	0.829	F(1,28)=0.0473
	Temperature	0.860	F(1,28)=0.0316
	Treatment	0.344	F(1,28)=0.9286
HSP70	Interaction	0.103	F(1,22)=2.888
	Temperature	0.296	F(1,22)=1.144
	Treatment	0.689	F(1,22)=0.1648

Table 7 . Two-way ANOVA statistical results relative to cellular energy allocation and electron transport activity (ETS) in *M. digitata* after acute thermal challenge ($p<0.05$).

	Source of variation	p-value	F(DFn, DFd)
Lipid	Interaction	0.196	F(1,27)=1.758
	Temperature	0.072	F(1,27)=3.516
	Treatment	0.011	F(1,27)=7.419
Sugar	Interaction	0.390	F(1,27)=0.7627
	Temperature	0.887	F(1,27)=0.0206
	Treatment	0.547	F(1,27)=0.3803
Protein	Interaction	0.618	F(1,26)=0.2546
	Temperature	0.849	F(1,26)=0.0369
	Treatment	0.127	F(1,26)=2.490
ETS	Interaction	0.520	F(1,27)=0.4255
	Temperature	0.291	F(1,27)=1.161
	Treatment	0.084	F(1,27)=3.216

Table 8 Sidak's multiple comparisons statistical results for lipid content after 24 h of acute thermal stress at 30 ºC, between control and olivine treatment, ($p<0.05$). Performed after two-way ANOVA significant result ($p<0.05$).

Lipid	p-value	t	DF
25ºC	0.014	2.914	27
30ºC	0.564	0.9718	27