Davide Augusto Machado Silva

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Doutor Rui Miranda Rocha, investigador auxiliar do Departamento de Biologia da Universidade de Aveiro; do co-orientador Doutor Mário Guilherme Garcês Pacheco, professor auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro; e da co-orientadora Doutora Catarina Marques, investigadora assistente do Departamento de Biologia da Universidade de Aveiro.

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Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Aos meus queridos pais e insuportáveis irmãos.

o júri

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Investigadora principal em regime laboral da Universidade de Aveiro

Doutor Rui Miranda Rocha
Investigador Doutorado da Universidade de Aveiro

Prof. Doutor João António de Almeida Serôdio
Professor auxiliar com agregação da Universidade de Aveiro

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A todos, obrigado.

resumo

Os recifes de coral fornecem importantíssimos bens e serviços ecossistémicos nas regiões tropicais e subtropicais do planeta. No entanto, o aumento das pressões antropogénicas, como por exemplo a poluição marinha ou a procura crescente de biomassa de coral para diversas aplicações económicas, bem como os danos provocados pelas alterações climáticas, têm contribuído para a degradação deste ecossistema a um ritmo alarmante. Consequentemente a comunidade científica internacional tem intensificado a investigação realizada com corais, com os objetivos de estudar e conhecer os efeitos dos fatores de stress existentes e identificar possíveis estratégias de mitigação. Deste modo, a realização de estudos ecotoxicológicos, com o objetivo de estudar o efeito de poluentes emergentes ou persistentes nestes organismos, pode contribuir para o avanço do estado da arte. Contudo, não existem metodologias standardizadas para testes com corais tropicais fotossintéticos e, consequentemente, a sua resposta a solventes orgânicos, frequentemente utilizados em ensaios toxicológicos, permanece desconhecida. Neste contexto, o presente estudo teve como principal objetivo analisar os perfis de stress oxidativo e a resposta fotobiológica de corais fotossintéticos expostos a diferentes solventes orgânicos (etanol, metanol e dimetil sulfoxido - DMSO), normalmente utilizados em estudos ecotoxicológicos, procurando contribuir para a standardização e otimização de protocolos. Os resultados do primeiro ensaio, realizado com a espécie *Zoanthus sociatus* (Ellis, 1786) (Hexacorallia: Zoantharia), sugerem que esta espécie é mais sensível ao DMSO do que ao etanol ou metanol. A concentração mais baixa com efeito (LOEC) do DMSO foi 0.01 mL L^{-1}, enquanto que para o etanol e metanol, o LOEC foi de 0.1 mL L^{-1}, ainda que a concentração mais alta de etanol (2.9 mL L-1) tenha sido a única a provocar mortalidade na espécie *Z. sociatus.* Contudo, os resultados obtidos, designadamente a forma diferenciada como esta espécie respondeu aos solventes testados, evidenciou a necessidade da realização de estudos semelhantes com outras espécies de corais, dada a diversidade apresentada por estes organismos. Neste sentido, realizou-se um segundo ensaio, para avaliar o efeito dos mesmos solventes orgânicos em duas espécies distintas, *Montipora digitata* (Hexacorallia: Scleractinia) e *Sarcophyton glaucum* (Octocorallia: Alcyonaria). A espécie *M. digitata,* com capacidade de segregar o seu exosqueleto de carbonato de cálcio, cujo papel na formação de recifes é imprescindível, foi mais sensível ao etanol do que ao metanol ou DMSO. O LOEC para o etanol foi de 0.01 mL L^{-1}, para o metanol foi de 0.3 mL L^{-1} e para o DMSO foi de 0.1 mL L^{-1}. Ainda assim, a concentração mais alta de etanol e DMSO (2.9 mL L-1) causou mortalidade na espécie *M. digitata.* A espécie *S. glaucum,* sem capacidade de segregar exosqueleto, foi mais sensível ao metanol do que ao etanol ou DMSO. O LOEC para a exposição a etanol foi de 2.9 mL L^{-1}, para a exposição a metanol 0.01 mL L^{-1} e para a exposição a DMSO 0.3 mL L^{-1}. Considerando os resultados obtidos, os solventes orgânicos podem ser utilizados em ecotoxicologia de corais tropicais com uma base sólida. Não obstante, é importante referir que existe ainda uma grande falta de informação no que diz respeito à fisiologia de corais, que deve ser aprofundada.

abstract

Tropical coral reefs provide important ecosystem services and goods in the tropical and subtropical regions of the planet. However, the increase of anthropogenic pressures, such as marine pollution or the escalating coral biomass demand for several different economic applications, as well as the damage caused by climate change, have contributed to the degradation of this ecosystem at an alarming rate. Consequently, the international scientific community has been intensifying coral research, aiming to study and acknowledge the effects of existing stress factors and to identify possible mitigation strategies. Thus, ecotoxicology studies, targeting the effect of emergent or persistent pollutants in these organisms can contribute to the state of the art. However, there are no standardized methodologies to test tropical photosynthetic corals, and their response to organic solvents, recurrently required in toxicological appraisals, remains unknown. In this context, the present study aimed to assess the oxidative stress profiles and photochemical response of photosynthetic corals exposed to different organic solvents (ethanol, methanol and dimethyl sulfoxide – DMSO), commonly used in ecotoxicological studies, expecting to contribute to the standardization and optimization of protocols. The results from the first study, with the species *Zoanthus sociatus* (Hexacorallia: Zoantharia), suggested that this species is more sensitive to DMSO than to ethanol or methanol. DMSO lowest observed effect concentration (LOEC) was 0.01 mL L^{-1}, while for ethanol and methanol LOEC were 0.1 mL L^{-1}, even though the highest concentration of ethanol (2.9 mL L^{-1}) was the only treatment causing mortality to *Z. sociatus*. Nevertheless, the distinctive response of this species evidenced the need to perform further similar studies with other coral species, given the diversity of these organisms. In this sense, a second trial was performed in order to assess the effect of the same organic solvents in two different species, *Montipora digitata* (Hexacorallia: Scleractinia) and *Sarcophyton glaucum* (Octocorallia: Alcyonaria). The species *M. digitata,* able to produce its own calcium carbonate skeleton, and therefore important to reef build, was more sensitive to ethanol than to methanol or DMSO. Ethanol lowest observed effect concentration (LOEC) was 0.01 mL L^{-1}, for methanol LOEC was 0.3 mL L^{-1}, and for DMSO LOEC was 0.1 mL L^{-1}. Notwithstanding, the highest concentration of ethanol and DMSO (2.9 mL L^{-1}) caused mortality in *M. digitata*. The species *S. glaucum*, unable to segregate a calcium carbonate skeleton, was more sensitive to methanol than to ethanol or DMSO. Ethanol LOEC was 2.9 mL L^{-1}, for methanol LOEC was 0.01 mL L-1, and for DMSO LOEC was 0.3 mL L^{-1}. Considering this, a baseline for the use of organic solvents with tropical corals was set. Nevertheless, it is important to stress out that a major knowledge gap regarding coral physiology still exists.

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(LPO), energy consumption (Ec), maximum quantum yield (F_v/F_m) in the concentrations tested. C1 = 0.01 mL L^{-1}; C2 = 0.03 mL L^{-1}; C3 = 0.1 mL L^{-1}; C4 = 0.3 mL L^{-1}; C5 = 0.93 mL L^{-1}; C6 = 2.86 mL L^{-1}. Dotted line represents the threshold for solvent use with distinguished impact on oxidative stress response or photosynthetic activity, although endpoints with anecdotic evidence were disregarded. Mortality was observed in C6 concentration of ethanol and DMSO.

Chapter III

Chapter I

Introduction

1. Introduction

Tropical coral reefs are household to most tropical marine biodiversity, being one of the most productive marine ecosystems (Moberg and Folke, 1999).

Coral reefs can provide shore protection and promote fisheries, tourism or other important socio-economic activities (Costanza et al., 2014). Reefs are also a major source for prospection of natural bioactive compounds with medical or pharmacological potential (Blunt et al., 2018; Rocha et al., 2011).

Tropical corals are organisms belonging to the class Anthozoa, that can be divided in two major groups: Octocorallia or Hexacorallia, based on the number of tentacles per polyp. Corals that belong to Octocorallia are often informally called soft corals. Hexacorallia is composed of several orders including Scleractinia and Zoantharia. Scleractinia is often referred to as hard or stony corals, as they generally produce a structural calcium carbonate skeleton, essential for reef formation. Zoantharians are closely related to scleractinians despite the absence of calcium carbonate skeleton. Usually tropical photosynthetic corals have a symbiotic relation with unicellular microalgae of the genus *Symbiodinium* commonly termed zooxanthellae. The host provides protection, nutrients and carbon sources that are essential for photosynthesis and in return benefits from amino acids, fatty acids and organic carbon resultant from photosynthesis (O. Hoegh-Guldberg et al., 2007; Papina et al., 2003). This symbiotic relation is paramount for the survival of both organisms in such oligotrophic environments. Furthermore corals have an intricate holobiontic relationship with a vast array of microorganisms (Rohwer et al., 2002).

Unfortunately, the increasing scientific interest to corals and coral reefs is mainly due to increasing anthropogenic threats and climate change, such pollution, destructive fishing practices, sea surface temperature increase or acidification (Forbes et al., 2016; Hughes et al., 2017b). Therefore, the need to better comprehend corals is paramount (Truhaut, 1977), and ecotoxicology can be particularly useful to assess the risk of coral exposure to contaminants. Nevertheless, there is a vest gap of knowledge on coral physiology and basic responses are still overlooked. Regulatory guidelines for the use of organic solvents (ASTM, 1997; OECD, 2019) are based in the response of microalgae, nudibranchs or bivalves (Choi et al., 2011; Pennington and Hadfiled, 1989; Yang et al., 2008) but neglect the response of intricate species like photosynthetic corals.

The aim of this dissertation was to assess the oxidative stress and photosynthetic response of the zoanthid *Zoanthus sociatus* (Ellis, 1786) (Hexacorallia: Zoantharia), the hard coral *Montipora*

digitata (Dana, 1846) (Hexacorallia: Scleractinia) and the soft coral *Sarcophyton glaucum* (Quoy and Gaimard, 1833) (Octocorallia: Alcyonacea) to ethanol, methanol and DMSO. Therefore, this work can be a baseline for coral ecotoxicology, providing valuable information for future risk assessment studies on corals.

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Chapter II

Interference of organic solvents in the photophysiological and oxidative stress profiles of the coral *Zoanthus sociatus* – contribution to the standardization of ecotoxicological protocols

Interference of organic solvents in the photophysiological and oxidative stress profiles of the coral *Zoanthus sociatus* – contribution to the standardization of ecotoxicological protocols[1]

[1] Submited as:

Interference of organic solvents in the photophysiological and oxidative stress profiles of the coral *Zoanthus sociatus* – contribution to the standardization of ecotoxicological protocols

Davide A. M. Silva; Ana P. L. Costa; Andreia C. M. Rodrigues; Pedro Bem-Haja; Sílvia S. F. Pires; Amadeu M.V.M. Soares; Catarina R. Marques; Mário Pacheco and Rui J.M. Rocha

Abstract

Coral reef decline, promoted by climate change and escalating anthropogenic pressures, allied to increasing coral demand for the aquarium trade or biotechnological proposes, gathered interest by the scientific community. Consequently, ecotoxicological assays with tropical corals have increased, specifically the study of emergent or persistent pollutants. However, standardized methodology to test for tropical photosynthetic corals is non-existent, and their response to organic solvents exposure recurrently required in toxicological appraisals remains unknown. Here we assess the photophysiological and oxidative stress profiles of the photosynthetic coral *Zoanthus sociatus* (Anthozoa, Hexacorallia) exposed to different organic solvents (ethanol, methanol and dimethyl sulfoxide – DMSO), aiming to establish a threshold for the safe use of these solvents in ecotoxicological studies with these organisms. Results suggest that the species *Z. sociatus* is more sensitive to DMSO than to ethanol or methanol. DMSO lowest observed effect concentration (LOEC) was 0.01 mL L^{-1}, while for ethanol and methanol LOEC were 0.1 mL L^{-1}, even though the highest concentration of ethanol (2.86 mL L^{-1}) was the only treatment causing mortality. Based on these findings, thresholds for the use of organic solvents with tropical corals can now be adopted. Nevertheless, species specificities should not be overlooked, and it is advised that similar tests should be done with other tropical photosynthetic corals, due to high specificity and diversity of these organisms.

Keywords: ecotoxicology; oxidative stress; photobiology; marine invertebrates; Zoantharia.

OECD solvent recommended dose for marine invertebrates: 0.01 mL L^{-1}
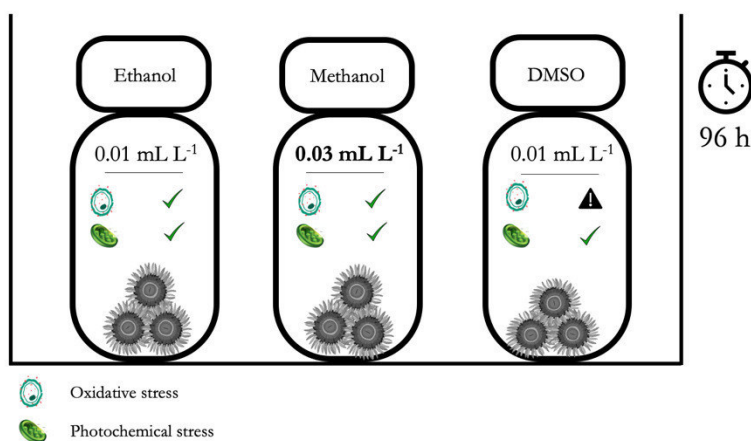
Figure 1 – Graphical abstract

1. Introduction

Coral reefs sustain most tropical marine biodiversity. Corals unique relationship with an entire ecosystem makes this group of organisms the mainframe for a whole tropical marine biome, being important socio-economic drivers, with estimated worth of US\$ $9.9(10^{12})$/year (Costanza et al., 2014). Despite their ecological relevance, these organisms are increasingly threatened by anthropogenic factors, either directly or indirectly (Hughes et al., 2017a). As test models, tropical photosynthetic corals can provide valuable information concerning the reef ecosystem, since a plethora of organisms depend directly on them (Spalding et al., 2001; Wilson et al., 2006). Consequently, there is an urgent need to comprehend how corals can respond to anthropogenic pressures, as well as to assess the potential inherent risks. Therefore, generating ecotoxicological data on the effect of different contaminants on coral biological responses will help to fulfil such gap, and will simultaneously contribute to the development of environmental management schemes and conservation of coral reef ecosystems. Even so, few tools and methodologies have been adjusted or optimized to assess coral responses to stressful conditions.

Anthropogenic pressure on ecosystems further increases the need to assess the risk of exposure to chemical contaminants. In particular, the ecotoxicological assessment of hydrophobic compounds require the use of organic solvents to be solubilized and promote bioavailability or exposure to the test organism (Weyman et al., 2012). Water-miscible solvents, such as ethanol, methanol and dimethyl sulfoxide (DMSO), are frequently used in ecotoxicological assays to evaluate the toxicity of hydrophobic substances or with low solubility in water (Hutchinson et al., 2006). Nevertheless, solvents can affect organism responses, acting as a clouding factor in toxicity tests outcomes, impacting directly or indirectly physiological processes (Hutchinson et al., 2006; Jung et al., 2017; Stange et al., 2012). Regulatory organizations suggest the use of seven different solvents, including those used in this study, and recommend conservative administration (between 0.01 mL L^{-1} and 0.05 mL L^{-1}) in tests with aquatic organisms (ASTM, 1997; OECD, 2019), even though assays often use higher concentrations due to toxicant solubility or other methodological limitations, as reviewed by Green and Wheeler (2013). Additionally, recommendations briefly contemplate sub-lethal responses of marine organisms to organic solvents, such as microalgae (Choi et al., 2011; Stratton and Corke, 1981; Stratton and Smith, 1988), nudibranchs (Pennington and Hadfiled,

1989) or bivalves (Yang et al., 2008), but disregard symbiotic marine invertebrates like photosynthetic corals.

The cosmopolitan photosynthetic soft coral *Zoanthus sociatus* (Ellis, 1786) (Hexacorallia, Zoantharia) can inhabit reefs and intertidal areas of tropical and sub-tropical regions, including tidal pools, where it can lie in stagnant water or be exposed to air for several minutes during low tide (Leal et al., 2016). Tidal pool inhabitants are frequently exposed to high water temperatures, low dissolved oxygen, high salinity, eutrophication or other harsh conditions (Helmuth et al., 2006; Leal et al., 2017). In fact, zoanthids tolerate and acclimate promptly to environmental changes (Rosa et al., 2018). This plasticity suggests that zoanthids might develop morphological or physiological mechanisms to deal with environmental change, to which most of its relatives cannot. Therefore, *Z. sociatus* can be a good biological model to study individual or combined effects of pollution (Grant et al., 2010; Leal et al., 2017) and climate change (Leal et al., 2016) in photosynthetic corals, as it lays in the resilient side of the tolerance spectrum. Thus, it is important to carry out preliminary studies to identify the response of these organisms to commonly used organic solvents.

In the present study we aim to evaluate the toxicity of three of the most common organic solvents used in ecotoxicology, *viz.* ethanol, methanol and dimethyl sulfoxide (DMSO), considering the holobiont oxidative stress and the endosymbiont photobiology in *Zoanthus sociatus*. We expect that tested doses of solvent will have different impacts on oxidative stress and coping mechanisms (antioxidant defences and oxidative damage) as well as on photosynthesis (rapid light curves), as zoanthids may have different pathways to metabolize distinctive solvents. As ultimate goal, it is intended to contribute to the standardization and optimization of ecotoxicological protocols using *Z. sociatus* mini-colonies, by establishing baselines for solvent use. To our knowledge, identifying the response of photosynthetic corals to commonly used organic solvents, is a completely unaddressed issue and the first study to do so.

2.   Materials and Methods

2.1.   Organisms and Culture System

Parental colonies (originally from Batam, Indonesia) were acclimated in aquaria for 1 month in standardized ~600 L culture systems according to Rocha et al. (2015) composed of two 250 L (150 cm length × 40 cm width × 50 cm height) culture tanks 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 500), osmoregulator to automatically compensate water evaporation (Deltec, Aquastat 1001), ~5 L of biological filter media, and ~2 L of activated charcoal. Water recirculation through chiller and UV system was performed by a submergible pump (Eheim, universal 1200). Culture tank circulation was also performed by a submergible pump (Eheim, universal 3400), providing an approximate flow of 1200 L h^{-1} to each tank. Additionally, each tank was equipped with two circulation pumps (Tunze, Turbelle nanostream-6055) with a Turbelle controller providing wave simulation with oscillation flow (200 – 4500 L h^{-1}). Each culture tank was equipped with four 80 W fluorescent lamps (Red Sea, REEF-SPEC) regulated to a 12:12 photoperiod, emitting a PAR of 100 ± 10 µmol m^{-2} s^{-1}, measured at colony level (Apogee MQ-500 PAR Meter). Salinity was kept at 35 ± 1 and temperature at 25 ± 1 °C. Culture system functioned with synthetic saltwater prepared by mixing synthetic salt (Red Sea, Coral PRO salt) and reverse osmosis water (TMC, V2 Pure 360). Partial water changes (~15 % of total system volume) were made every week. After acclimation, parental colonies were fragmented into 3 polyp mini colonies with scalpel and a small spatula. Mini colonies were fixed to plastic coral cradles with n-butyl-cyanoacrylate and subsequently acclimated during 15 days under the same conditions as parental colonies.

2.2. Test concentrations

Six concentrations of ethanol, methanol and DMSO were set by applying a factor of 3.1 to the maximum solvent concentration (0.01 mL L^{-1}) recommended by the OECD (2019) guideline for toxicity testing of chemicals: 0.01 mL L^{-1} (C1); 0.03 mL L^{-1} (C2); 0.1 mL L^{-1} (C3); 0.3 mL L^{-1} (C4); 0.9 mL L^{-1} (C5); 2.9 mL L^{-1} (C6) for each solvent. Test concentrations were obtained by diluting a stock solution of each solvent in saltwater.

2.3. Experimental design

Mini-colonies were individually stocked in 200 mL flasks with five replicates per treatment (*i.e.* solvent concentration). During the experiment, corals were kept for 96 h in 200 mL flasks and maintained in water bath with one heater (Eheim, Jager 300W). Two water pumps (100 L h^{-1}) ensured homogenous water temperature (EHEIM CompactON 300). Flasks were

maintained in a static-renewal exposure system, according to OECD (2019), following a 50 % medium renewal per day. The experimental system was illuminated with four 80 W fluorescent lamps (Red Sea, REEF-SPEC) with 12:12 photoperiod, emitting a PAR of 100 ± 10 µmol m^{-2} s^{-1}, measured at colony level (Figure 1).

Daily, 1 h 30 min after the beginning of the light period, water parameters were checked, being observed that remained stable throughout the experiment (dissolved oxygen: 8.13 ± 0.39 mg L^{-1}, pH: 8.04 ± 0.05, salinity: 36.08 ± 0.81 and temperature: 24.9 ± 0.21 °C). Before the test start, 5 mini colonies were sampled (t0) to evaluate photochemical and oxidative stress parameters (*cf.* methodology detailed in section 2.4).
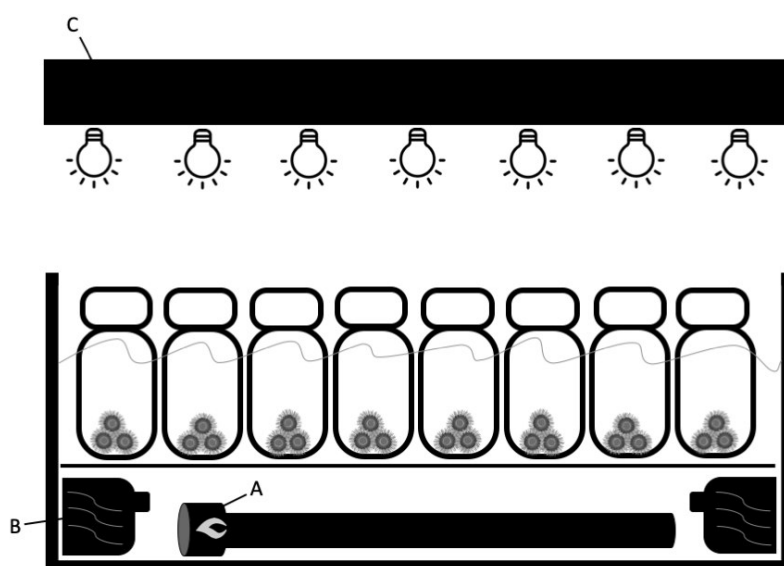
Figure 2 - Experimental system used for coral toxicity trials: A) Heater; B) Water pump; C) Lighting system

2.4.   Biological response

2.4.1.   Oxidative stress-related biomarkers

Immediately after the photobiological assessment, mini-colonies were frozen in liquid nitrogen and preserved at -80 °C until further processing. Later, samples were homogenized in 1600 µL on an ice bath for biomarker analysis. After homogenization, samples were separated into different aliquots: 300 µL for electron transport system (ETS) activity; 200 µL for lipid

peroxidation (LPO), in which 4% of butylated hydroxytoluene (BHT) was added; 600 μL of the remaining homogenate diluted with 600 μL of 0.2 M K-phosphate buffer, pH 7.4, and centrifuged at 10,000 g for 15 min (4°C) to obtain the post mitochondrial supernatant (PMS).

PMS protein content was determined according to Bradford (1976) and adapted from the Bio-Rad micro-assay, using bovine γ-globulin as standard. Catalase (CAT) activity was determined through the decomposition of hydrogen peroxide (H_2O_2) in PMS at 240 nm (Clairborne, 1985). Glutathione S-transferase (GST) activity was measured at 340 nm, by combining reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) in the PMS fraction (Habig et al., 1974). Total glutathione (TG) was quantified at 412 nm using a reaction of GSH with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) combined with glutathione reductase (GR) in excess, also in PMS (Baker et al., 1990; Rodrigues et al., 2017). LPO was obtained by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm (Bird and Draper, 1984). ETS activity was determined through the iodonitrotetrazolium (INT) reduction method, read at 490 nm (De Coen and Janssen, 1997), as modified by (Rodrigues et al., 2015). Cellular oxygen consumption rate was calculated based on the stoichiometric relationship in which 2 μmol of formazan is formed per 1 μmol of oxygen is consumed. Energy consumption (Ec) was obtained by the conversion to energetic values using the specific oxyenthalpic equivalent for the average lipid, protein and carbohydrate mixture of 480 kJ mol^{-1} O_2 (Gnaiger, 1983).

2.4.2.   Photobiology

By the end of the experiment, photobiology parameters were estimated non-intrusively through PAM fluorometry using Junior-PAM (Walz ™, Germany) as described by Rocha et al. (2013). Chlorophyll *a* fluorescence was measured through rapid light curves that resulted from increasing saturating light pulses, spaced by 10 seconds with irradiance ranging from 0 - 1500 μmol m^{-2} s^{-1} at 450 nm (half-bandwidth of 20 nm) adapted from Ralph et al., (2005). Light was delivered by a 1.5 mm plastic optical fiber, positioned perpendicularly to the coral surface. Corals were dark-acclimated for 30 min to ensure full relaxation of the photosystem II reaction centres. Rapid Light Curves were calculated based on (Platt et al., 1980). Maximum electron transport rate (ETR$_{max}$) was calculated as: $ETR = ((F'_m - F_s)/F'_m)$ considering F_s as steady-state fluorescence and F'_m as maximum fluorescence, light-saturation coefficient (E_k) and photosynthetic response (α), as well as the maximum quantum yield (F_v/F_m) considering dark level fluorescence (F_0) and maximum fluorescence (F_m) as described by Schreiber et al. (1986) ($F_v/F_m = (F_m - F_0) / F_m$).

2.4.3.   Behavior

Polyp contraction/relaxation was recorded daily to validate data with behaviour. Three classification levels were considered: i) relaxed - mini-colonies with all polyps open (open oral disc and distended tentacles); ii) contracted - mini-colonies that had polyps with open oral disk but retracted tentacles; and iii) closed - mini-colonies with every polyp closed. Evident signs of mortality were also recorded.

2.5.   Statistical Analysis

Significant differences were calculated through the Kruskal-Wallis test on ranks, followed by a Dunnett's test (Dunnett, 1955), given the non-normal distribution of most variables. Significance was set to *p*-value ≤ 0.05. Bayesian Independent-samples T-tests were run on endpoints significantly different within the Dunnett tests to estimate the effect probability, independently of the sample size. Bayesian evidence was based in the Bayesian Factor (0-3 Anecdotal; 3-10 Moderate; 10-30 Strong; 30-100 Very strong; >100 Extreme). Outliers were calculated as ± 1.5 Interquartile Range and trimmed before analysis. Descriptive data were presented as mean ± standard error. Analyses were run in R software version 3.6.1. (R Core Team, 2019), using the package PMCMRplus (Pohlert, 2018) and ggplot2 (Wickham, 2016), except for the Bayesian analysis, which was performed using JASP software version 0.10.2 (JASP Team, 2019).

The statistical approach herein undertaken provides an exploratory interpretation of our data, at particular situations in which Dunnett's tests were taken into consideration regardless of the Kruskal-Wallis result. This can incur in a false positive outcome (*i.e.* type I errors). False positive results in ecotoxicological reports are of least concern and more conservative than false negatives (*i.e.* type II errors). Nevertheless, Bayesian analysis pretends to assess the robustness of the abovementioned, diminishing the impact of such effect.

3.   Results

In our tests, mortality was observed only at the highest ethanol concentration (2.9 mL L^{-1}), where every replica began to decompose at 72 h exposure.

3.1. Biological response

3.1.1. Oxidative stress profile

Statistical results for the biochemical biomarkers are reported in the table I. When exposed to ethanol, *Z. sociatus* GST activity significantly decreased at 0.3 mL L^{-1} with moderate bayesian evidence, when compared to control. GST activity decreased at 0.1 mL L^{-1} methanol although with anecdotical evidence, and at 2.9 mL L^{-1} methanol with strong evidence, when compared to control. LPO was significantly lower when corals were exposed to 0.1 mL L^{-1} methanol, with moderate evidence, when compared to control. Exposure to 2.9 mL L^{-1} methanol caused an increased Ec with moderate evidence, when compared to control. CAT activity significantly decreased when *Z. sociatus* was exposed to 0.03 mL L^{-1} DMSO, with moderate bayesian evidence, and increased at 2.9 mL L^{-1} DMSO, with very strong evidence, when compared to control. GST activity significantly decreased at 0.01 mL L^{-1} DMSO, with moderate evidence, at 0.03 mL L^{-1} DMSO, with strong evidence and at 0.3 mL L^{-1}, with anecdotal evidence, when compared to control. TG was significantly higher at 2.9 mL L^{-1} DMSO, but with anecdotal bayesian evidence.

Table I - Oxidative stress response for *Z. sociatus* exposed to ethanol, methanol and DMSO expressed as mean ± standard error: catalase activity (CAT); glutathione-S-transferase activity (GST); total glutathione (TG); lipid peroxidation (LPO); energy consumption (Ec). Statistical differences between the control group and the treatment are marked (p<.1[+]; p<.05*; p<.01**; p<.001***).

		CAT μmol min^{-1} mg prot^{-1}	GST nmol min^{-1} mg prot^{-1}	TG nmol tnb^{-2} min^{-1} mg prot^{-1}	LPO tbars nmol g ww^{-1}	Ec mj h^{-1} mg^{-1}
	Control	12.80 ± 1.33	21.24 ± 1.49	71.34 ± 12.02	0.63 ± 0.08	1.01 ± 0.20
Ethanol mL L^{-1}	**0.01**	14.38 ± 1.56	23.54 ± 0.88	54.23 ± 7.68	1.01 ± 0.28	1.19 ± 0.22
	0.03	13.45 ± 0.57	23.57 ± 1.14	108.2 ± 23.07	1.62 ± 0.61	0.84 ± 0.05
	0.1	20.23 ± 4.18	23.87 ± 2.72	82.40 ± 17.64	1.15 ± 0.36	1.16 ± 0.25
	0.3	12.62 ± 1.16	14.39 ± 1.46 [+]	55.19 ± 7.69	0.88 ± 0.13	1.34 ± 0.35
	0.9	13.07 ± 0.84	17.42 ± 1.40	64.70 ± 10.81	0.82 ± 0.151	1.70 ± 0.24
Methanol mL L^{-1}	**0.01**	13.47 ± 0.66	17.50 ± 0.85	71.52 ± 10.08	0.77 ± 0.16	1.48 ± 0.38
	0.03	14.27 ± 0.74	17.72 ± 2.46	51.18 ± 1.27	0.84 ± 0.05	1.34 ± 0.30
	0.1	14.55 ± 1.70	15.16 ± 0.75 [+]	39.00 ± 4.27	1.05 ± 0.07 [+]	1.38 ± 0.19
	0.3	17.19 ± 2.60	21.47 ± 2.49	98.49 ± 18.98	0.73 ± 0.05	1.78 ± 0.43
	0.9	13.42 ± 0.36	16.29 ± 1.42	59.32 ± 5.13	0.56 ± 0.05	1.49 ± 0.10
	2.9	10.40 ± 0.69	14.54 ± 0.53 *	91.85 ± 6.87	0.58 ± 0.11	2.11 ± 0.26 [+]
DMSO mL L^{-1}	**0.01**	9.44 ± 2.04	12.33 ± 1.49 **	73.73 ± 2.13	0.50 ± 0.10	1.44 ± 0.45
	0.03	6.74 ± 0.98 *	10.53 ± 0.37 **	51.28 ± 1.32	0.43 ± 0.02	1.49 ± 0.28
	0.1	13.05 ± 1.08	18.17 ± 2.40	46.36 ± 3.39	0.47 ± 0.02	1.52 ± 0.15
	0.3	13.75 ± 0.90	15.60 ± 1.42 [+]	74.43 ± 9.76	0.61 ± 0.02	1.16 ± 0.15
	0.9	14.43 ± 1.32	16.25 ± 1.45	62.08 ± 2.81	0.47 ± 0.04	1.79 ± 0.31
	2.9	24.38 ± 0.51 ***	21.30 ± 1.51	100.4 ± 9.94 [+]	0.81 ± 0.20	1.47 ± 0.13

3.1.2. Photobiology

Statistical results for the photobiology data are reported in the table II. When exposed to ethanol, *Z. sociatus* F_v/F_m proved to be significantly lower at 0.3 mL L^{-1} ethanol with moderate evidence, and at 0.9 mL L^{-1} with extreme evidence, when compared to control. ETR$_{max}$ significantly decreased at 0.03 mL L^{-1} ethanol, although with anecdotal evidence. At 0.1 mL L^{-1}, 0.3 mL L^{-1} and 0.9 mL L^{-1} ethanol the ETR$_{max}$ significantly decreased, supported with strong evidence. E_k was significantly lower at 0.1 mL L^{-1} ethanol, with moderate and at 0.3 mL L^{-1} ethanol, with strong evidence. F_v/F_m and ETR$_{max}$ were significantly lower at 0.1 mL L^{-1} methanol, with moderate evidence, when compared to control. DMSO exposure affected negatively F_v/F_m at 0.9 mL L^{-1}, supported by moderate evidence. α was significantly lower at 0.1

mL L^{-1}, 0.3 mL L^{-1} and at 0.9 mL L^{-1} DMSO, with strong evidence and at 2.9 mL L^{-1}, with anecdotal evidence.

Table II – Photobiology response for *Z. sociatus* exposed to ethanol, methanol and DMSO expressed as mean ± standard error: photosynthetic response (α); maximum electron transport rate (ETR$_{max}$); light-saturation coefficient (E$_k$) and maximum quantum yield (F$_v$/F$_m$). Statistical differences between the control group and the treatment are marked (p<.1$^+$; p<.05*; p<.01**; p<.001***).

		F_v/F_m	ETR$_{max}$	α	E$_K$
				electrons photons^{-1}	µmol photons m^{-2} s^{-1}
Control		0.658 ± 0.003	18.41 ± 1.51	0.116 ± 0.003	150.8 ± 5.60
Ethanol mL L^{-1}	**0.01**	0.635 ± 0.016	16.76 ± 1.04	0.115 ± 0.003	142.1 ± 3.12
	0.03	0.645 ± 0.005	12.98 ± 2.49$^+$	0.111 ± 0.010	116.3 ± 18.06
	0.1	0.627 ± 0.001	9.85 ± 1.12 **	0.092 ± 0.005	99.79 ± 14.39 $^+$
	0.3	0.564 ± 0.027 **	8.50 ± 0.56 **	0.101 ± 0.011	77.21 ± 10.29 *
	0.9	0.555 ± 0.011***	10.64 ± 0.87 **	0.098 ± 0.003	115.7 ± 13.34
Methanol mL L^{-1}	**0.01**	0.643 ± 0.002	10.37 ± 1.61	0.099 ± 0.009	95.24 ± 0.35
	0.03	0.607 ± 0.026	10.66 ± 1.98	0.094 ± 0.008	124.7 ± 8.33
	0.1	0.569 ± 0.023 *	8.58 ± 2.40 *	0.090 ± 0.011	104.9 ± 33.60
	0.3	0.593 ± 0.021	10.81 ± 2.01	0.103 ± 0.008	105.4 ± 14.68
	0.9	0.656 ± 0.005	23.06 ± 1.02	0.129 ± 0.006	186.5 ± 6.00
	2.9	0.612 ± 0.019	15.33 ± 4.52	0.138 ± 0.005	114.6 ± 35.93
DMSO mL L^{-1}	**0.01**	0.654 ± 0.004	14.16 ± 2.68	0.112 ± 0.009	126.2 ± 20.50
	0.03	0.655 ± 0.006	16.24 ± 1.07	0.119 ± 0.011	153.6 ± 5.53
	0.1	0.640 ± 0.003	16.09 ± 0.11	0.086 ± 0.004 $^+$	193.2 ± 9.41
	0.3	0.639 ± 0.002	14.60 ± 0.41	0.087 ± 0.004 $^+$	158.3 ± 25.05
	0.9	0.603 ± 0.016 ***	15.09 ± 1.45	0.085 ± 0.006 *	177.9 ± 15.48
	2.9	0.646 ± 0.003	21.13 ± 3.89	0.088 ± 0.011 $^+$	217.6 ± 38.72

3.1.3. Behavior

Z. sociatus was mostly open in the control with sporadic contracted polyps. In general, concentrations below 0.3 mL L^{-1} did not incite polyp closure, except one sample exposed to 0.03 mL L^{-1}, at 96 h, that was completely closed. Above 0.3 mL L^{-1} ethanol polyps were more frequently closed. At 2.9 mL L^{-1} ethanol polyps were completely closed at every given time, except for one mini-colony, at 48 h. At 72 h, mini-colonies exposed to 2.9 mL L$^-$1 ethanol

perished but were hereby registered as closed. Methanol exposure behaviour follows a trend with more polyps relaxed at lower concentrations, but also more polyps contracted or completely closed at higher concentrations, apart from one mini-colony that remained closed from 48 h to 96 h at the lowest concentration of methanol. 0.03 mL L^{-1}, 0.3 mL L^{-1} and 0.9 mL L^{-1} methanol did not incite polyp closure. At 0.1 mL L^{-1} another amok was observed and registered as closed at 48 h and 96 h. At 2.9 mL L^{-1} methanol colonies were more frequently closed. Regarding DMSO, mini-colonies were always relaxed or contracted throughout the experiment, except for 2.9 mL L^{-1} DMSO, where polyps were more frequently closed (Figure 2).
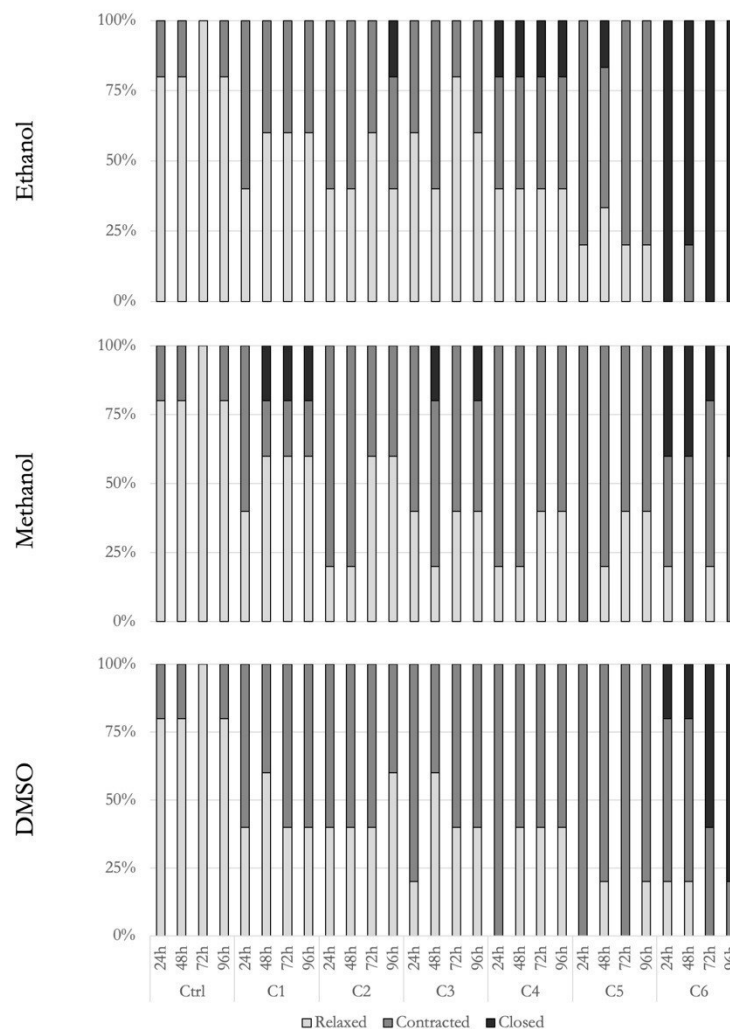
Figure 3 - Behavior of *Z. sociatus* exposed to increasing concentrations of ethanol, methanol and DMSO throughout the experiment, assessed every 24h. Ctrl – control; C1 = 0.01 mL L^{-1}; C2 = 0.03 mL L^{-1}; C3 = 0.1 mL L^{-1}; C4 = 0.3 mL L^{-1}; C5 = 0.9 mL L^{-1}; C6 = 2.9 mL L^{-1}.

3.2. Dunnett's heatmap

Given the large volume of data, for a better understanding of the study findings, we present below an overview of results, through a Dunnett's heatmap by solvent and by endpoint measured (Figure 3).
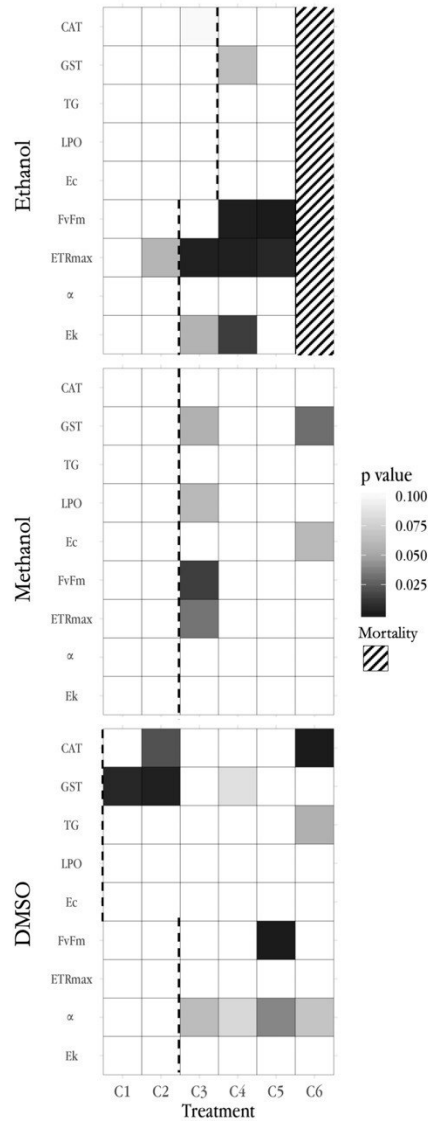
Figure 4 – Dunnett's heatmap as a visual representation of the p-values observed in Dunnett's test for catalase activity (CAT), glutathione S-transferase (GST), total glutathione (TG), lipid peroxidase (LPO), energy consumption (Ec), photosynthetic response (α), maximum electron transport rate (ETR$_{max}$), light saturation coefficient (E$_k$) and maximum quantum yield (F$_v$/F$_m$), in every concentrations tested. C1 = 0.01 mL L^{-1}; C2 = 0.03 mL L^{-1}; C3 = 0.1 mL L^{-1}; C4 = 0.3 mL L^{-1}; C5 = 0.9 mL L^{-1}; C6 = 2.9 mL L^{-1}. Dotted line represents the threshold of solvent concentration with significant impact on oxidative stress responses or photosynthetic activity. Mortality was observed at 2.9 mL L^{-1} (C6) concentration of ethanol.

Dunnett's heatmap is a visual representation of the lowest p-values observed in Dunnett's test, which is useful for decision-making regarding recommended solvent concentrations for toxicity tests. Taking into consideration a p-value $\leq$ .05 ethanol triggered oxidative stress response at 0.3 mL L^{-1} but seemed to affect the photosynthetic apparatus at 0.1 mL L^{-1} with a LOEC of 0.1 mL L^{-1}. Methanol seemed to affect both oxidative stress response and photosynthetic apparatus at 0.1 mL L^{-1} with a corresponding LOEC of 0.1 mL L^{-1}. DMSO triggered oxidative stress at the first concentration used (0.01 mL L^{-1}), but photosynthetic activity was affected at 0.93 mL L^{-1} with a LOEC of 0.01 mL L^{-1}.

4.   Discussion

Coral symbiosis is often referred to coral-algae associations (unicellular dinoflagellate symbionts, genus *Symbiodinium*), but prokaryotic diversity associated with corals is considerably higher (Rohwer et al., 2002). As a holobiont, a vast array of microorganisms can take part in metabolic mechanisms. Many bacteria associated with corals are related to the nutrient cycle in oligotrophic environments (Wegley et al., 2007), and are far more efficient at assimilating limiting nutrients (Cavender-Bares et al., 2001). Solvent degradation may take part in various organisms of the coral, bacteria, archaea, fungi, algae or viruses (Rohwer et al., 2002) blurring what could have been a clearer process (Downs and Downs, 2007). More so, coral holobiont is complex and often depends on environmental conditions leading to substantial biotic and abiotic variation (Ainsworth et al., 2011) probably affecting the endpoints we assessed.

Photosynthetic symbionts (usually termed as zooxanthellae), however, play a very important role in coral metabolism, acting as a key source of energy. As zooxanthellae need carbon for the photosynthetic process to occur, and the diffusion of CO_2 in aquatic systems is much slower when compared to that in the atmosphere, zooxanthellae often need carbon sources to be delivered by the coral host, such as dissolved inorganic carbon (DIC) (Goiran et al., 1996), or even adopt similar heterotrophic feeding strategies (Jeong et al., 2012). Some algae are also able to utilize organic solvents, such as ethanol and methanol as a source of carbon (Kotzabasis et al., 1999; Matsudo et al., 2017), which could be a possible mechanism for zooxanthellae to cope with naturally oligotrophic environments (Dixon et al., 2011). However, the toxicity of organic solvents to algae (El Jay, 1996; Ma and Chen, 2005) as well as invertebrates (Kaviraj et al., 2004),

has been frequently reported. Therefore, we hypothesized that similar effects may occur in corals exposed to organic solvents.

Ethanol can be converted intracellularly into acetyl-CoA, and used subsequently for NADH production by almost every living organism (Riveros-Rosas et al., 2003). However, it is also known to affect cytochrome P450 reactions (Rubin et al., 1971), highly conserved enzymes throughout most life forms, including invertebrates (Nebert and Gonzalez, 1987; Rewitz et al., 2006) and usually related to xenobiotic metabolism (Kamataki and Fujita, 2002). Ethanol can also affect neurotransmission systems, membrane channelling, and other metabolic pathways (Scholz and A. Mustard, 2011). Okumura et al. (2001) exposed nine species of marine algae to ethanol and determined the no observed effect concentration (NOEC) from 14 to 10,000 mg L^{-1}, and Rayburn and Fisher (1997) suggested the use of <1 g L^{-1} ethanol in studies with the freshwater grass shrimp *Palaemonetes pugio*. Furthermore, previous studies commonly suggest that ethanol can modulate microbial growth in test vessels (Hutchinson et al., 2006; OECD, 2019), with possible implications in ethanol degradation by the holobiont. Our results showed that ethanol-induced oxidative stress was translated into GST activity inhibition at 0.3 mL L^{-1}. The highest ethanol concentration seemed overwhelming for this species, as far as it induced 100% mortality.

Methanol, on the other hand, can be converted into CO_2 (Colas Des Francs-Small et al., 1993) and used in the Calvin cycle by photosynthetic organisms, thereby leading to a stimulatory effect on the growth of several different microalgae (Kotzabasis et al., 1999; Navakoudis et al., 2007). Regardless, through methanol oxidation formate is accumulated, which seems to be the main cause for methanol toxicity, through the inhibition of the electron transport chain in the mitochondria (Nicholls, 1976; Tephly, 1991). To our knowledge Okumura et al. (2001) is the only study with marine algae toxicity to methanol and no studies regarding marine invertebrates NOEC were found. Okumura et al. (2001) is the only study focused on the toxicity of methanol to marine algae and reported the NOEC values ranging from 24 to 14000 mg L^{-1}. Our data showed that methanol inhibited GST activity at 0.1 ml L^{-1}, causing oxidative damage in exposed corals at this exposure level. At the highest tested concentration (2.9 mg L^{-1}), GST activity was impaired and Ec increased.

As part of the sulfur cycle, DMSO is ubiquitous in the oceans, either directly, or indirectly as dimethylsulfoniopropionate (DMSP), or one of the most abundant organic compounds in the marine environment, dimethylsulfide (DMS) (Nightingale and Liss, 2006). DMSP occurs naturally in corals, or rather in its dinoflagellate symbionts (Broadbent and Jones, 2004; Ishida,

1996) and is readily degraded into DMS (Kiene, 1990; Raina et al., 2009), which can be subsequently metabolized into DMSO (Schäfer et al., 2010; Zhang et al., 1991). Therefore, when compared to the abovementioned solvents, DMSO is the least alien for corals. DMSO or its sulfuric relatives can be metabolized by DMS monooxygenase into formaldehyde and methanethiol (De Bont et al., 1981). Formaldehyde can be sequentially converted into CO_2 that may feed the Calvin cycle, and therefore induce similar effects as those of methanol (Schmitz et al., 2000). Methanethiol, on the other hand, is known to bind easily to a great variety of proteins (Quarforth et al., 1976; Waller, 1977). It can for instance inhibit antioxidant enzymes, namely catalase, as shown by Finkelstein and Benevenga (1986). Once again, Okumura et al. (2001) reported NOEC for marine microalgae ranging from 420 to 11000 mg L^{-1} and (Rayburn and Fisher, 1997) suggested the use of <6 g L^{-1} DMSO in studies with *Palaemonetes pugio*. To our knowledge, no further work was developed briefing DMSO NOEC to marine invertebrates. We report catalase inhibition in corals exposed to 0.03 mL L^{-1} DMSO but an increased activity at 2.9 ml L^{-1} DMSO. GST activity decreased at 0.01 mL L^{-1} and 0.03 ml L^{-1} DMSO. These antioxidant mechanisms proved to be effective as no oxidative damage (LPO) was verified.

Rapid light curves are good indicators of photophysiological health, as has been used as an endpoint for symbiotic algae toxicity (Leal et al., 2016; Maxwell and Johnson, 2000; Rosa et al., 2018). Photochemical inhibition is not necessarily correlated with photochemical damage, once these mechanisms have been reported to be reversible and often embody temporary responses to environmental change (Brown et al., 1999), but are nonetheless good proxies for photosynthetic symbionts health (Baker, 2008). Our results showed that all tested organic solvents can interfere with coral photosynthesis, as F_v/F_m and ETR_{max} were significantly affected, with the exception of DMSO that seemed to only affect F_v/F_m.

In sum, *Z. sociatus* seemed more sensitive to DMSO, exhibiting oxidative stress at the lowest tested concentration, although with no signs of cellular damage. Nevertheless, photochemical activity was affected at the same concentrations as by the other solvents, with a wider gap between oxidative stress and photochemical stress. Ethanol and methanol induced similar effects, although the oxidative stress response for ethanol seemed numb or non-responsive, as differences were noted only for GST activity, but mortality was observed at the highest concentration. Behaviour data supported oxidative and photochemical stress response and reinforced the natural variability inherent to corals.

The present study allowed us to point out a possible baseline for solvent use in ecotoxicology, crucial for the viability of future studies with photosynthetic corals. Ethanol

triggered photosynthetical decay at 0.1 mL L^{-1} and oxidative stress at 0.3 mL L^{-1}, but was the only solvent causing mortality at the highest used concentration. Methanol showed oxidative stress and photochemical decay at 0.1 mL L^{-1}. DMSO showed oxidative stress at the lowest tested concentration (0.01 mL L^{-1}) and photosynthetical decay at 0.1 mL L^{-1}. Ethanol and methanol were therefore the most tolerated tested solvents for *Z. sociatus* with no effect at concentrations lower than 0.1 mL L^{-1}. Furthermore, *Z. sociatus* response should be addressed carefully when extrapolating to other photosynthetic corals, as this species is expected to be less sensitive to variations. Nevertheless, as recommended by OECD (2019) solvent concentration should be at least one order of magnitude below the NOEC and therefore, the use of organic solvent in studies with photosynthetic tropical corals at concentrations ≥ 0.001 mL L^{-1} should be appropriately weighted.

Future studies should focus on using other coral species and solvents in order to enlighten the tolerance of various species to organic solvents.

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Annexes

Table A. I - Top-Down statistical analysis for ethanol. Bayesian analysis was performed when Kruskal-Wallis test on ranks followed by Dunnett's test were significant, or marginally significant (*).

Ethanol	K-W [p-value]	DOSE [mL L^{-1}]	DUNNETT'S [p-value]	BAYES FACTOR [BF$_{10}$]	EVIDENCE
CAT	.75				
GST	.01225	0.3	.064*	3.051	Moderate
TG	.2974				
LPO	.7386				
Ec	.2936				
F_v/F_m	.005553	0.3	.00127	4.184	Moderate
		0.9	.00048	137.722	Extreme
ETR$_{max}$	.01138	0.03	.0585*	1.288	Anecdotal
		0.1	.0018	20.942	Strong
		0.3	.0019	14.843	Strong
		2.9	.0045	19.675	Strong
α	.1401				
E$_k$	.05804*	0.1	.057*	3.290	Moderate
		0.3	.013	23.341	Strong

Table A. II - Top-Down statistical analysis for methanol. Bayesian analysis was performed when Kruskal-Wallis test on ranks followed by Dunnett's test were significant, or marginally significant (*).

Methanol	K-W [p-value]	DOSE [mL L^{-1}]	DUNNETT'S [p-value]	BAYES FACTOR [BF$_{10}$]	EVIDENCE
CAT	.1981				
GST	.0172	0.1	.056*	0.493	Anecdotal
		2.9	.031	12.707	Strong
TG	.02171	0.1	.23	1.762	Anecdotal
LPO	.05553*	0.1	.061*	4.104	Moderate
Ec	.2974	2.9	.061*	5.160	Moderate
F_v/F_m	.01549	0.1	.014	4.616	Moderate
ETR$_{max}$	.01008	0.1	.033	6.256	Moderate
α	.002348				
E$_k$	.1158				

Table A. III - Top-Down statistical analysis for methanol. Bayesian analysis was performed when Kruskal-Wallis test on ranks followed by Dunnett's test were significant, or marginally significant (*).

DMSO	K-W [p-value]	DOSE [mL L^{-1}]	DUNNETT'S [p-value]	BAYES FACTOR [BF$_{10}$]	EVIDENCE
CAT	.008276	0.03	.021	5.168	Moderate
		2.9	< .0001	33.136	Very Strong
GST	.005335	0.01	.0049	9.516	Moderate
		0.03	0.019	16.943	Strong
		0.3	0.0853*	2.830	Anecdotal
TG	.002484	2.9	.055*	1.251	Anecdotal
LPO	.1975				
Ec	.4894				
F_v/F_m	.001885	0.9	.0003	3.715	Moderate
ETR$_{max}$	.3532				
α	.0152	0.1	.064*	35.768	Very Strong
		0.3	.080*	36.111	Very Strong
		0.9	.039	19.065	Strong
		2.9	.068*	2.166	Anecdotal
E$_k$	.1761				

Chapter III

How organic solvents affect the photophysiology and oxidative stress response of the scleractinian *Montipora digitata* and the alcyonacean *Sarcophyton glaucum*

How organic solvents affect the photophysiology and oxidative stress response of the scleractinian *Montipora digitata* and the alcyonacean *Sarcophyton glaucum*[2]

Davide A. M. Silva; Ana P. L. Costa; Andreia C. M. Rodrigues; Pedro Bem-Haja; Sílvia S. F. Pires; Jamen J. A. Mussa; Amadeu M.V.M. Soares; Catarina R. Marques; Mário Pacheco and Rui J.M. Rocha

Abstract

Climate change and the increasing anthropogenic pressures have been determinant on tropical coral reef decline worldwide. The increasing demand of corals for the aquarium trade or biotechnological proposes, aligned with the imperative need to protect and conserve these threatened species, have been attracting the scientific community interest. In recent years, particularly due to the increasing awareness to marine pollution, studies focusing on the emergent or persistence of pollutants get in the agenda of the scientific community. Notwithstanding, there is still a huge gap regarding standardized methodology on ecotoxicological assays for tropical photosynthetic corals and their response to organic solvents. Our goal was to establish a threshold for the safe use of different organic solvents (ethanol, methanol and dimethyl sulfoxide – DMSO) in order to assess the photophysiological and oxidative stress profiles of the photosynthetic corals *Montipora digitata* (Anthozoa: Scleractinia) and *Sarcophyton glaucum* (Anthozoa: Alcyonaria) exposed to these solvents in ecotoxicological studies. Our results suggest that the species *M. digitata* is more sensitive to ethanol than to methanol or DMSO. Ethanol lowest observed effect concentration (LOEC) was 0.01 mL L^{-1}, for methanol LOEC was 0.3 mL L^{-1}, and for DMSO LOEC was 0.1 mL L^{-1}. Nevertheless, the highest concentration of ethanol and DMSO (2.86 mL L^{-1}) caused mortality. *S. glaucum* is more sensitive to methanol than to ethanol or DMSO. Ethanol LOEC was 2.9 mL L^{-1}, for methanol LOEC was 0.01 mL L^{-1}, and for DMSO LOEC was 0.3 mL L^{-1}. These findings allow us to establish thresholds for the use of organic solvents with tropical corals. Nonetheless, further insights in coral physiology are vital to better comprehend coral response to stress.

Keywords: ecotoxicology, oxidative stress, photobiology, marine invertebrates, Scleractinia, Alcyonaria.

1. Introduction

Tropical coral reefs are among the most productive ecosystems on Earth (Hatcher, 1988). Reefs ecosystem services and goods are critical to tropical and subtropical countries (Moberg and Folke, 1999) providing important cultural and recreational benefits, but also food supply and coastal protection (Spurgeon, 1992). Yet, coral reefs suffered from major degradation in the past decades, jeopardizing the equilibrium of this complex ecosystem. Photosynthetic corals have tight symbiotic bonds with microalgae of the genus *Symbiodinium*, commonly known as zooxanthellae. The host provides protection and nutrients for the photosynthetic process, vital in the oligotrophic environment they inhabit (Goiran et al., 1996), and zooxanthellae provide energy harvested from solar radiation through photosynthesis (Ove Hoegh-Guldberg et al., 2007). Yet, this bond can be disrupted by climate change, *e.g.* rising sea surface temperature (Lesser, 1997; Spalding and Brown, 2015), but also, increasing coastal anthropogenic pressures, such as agricultural runoffs (Forbes et al., 2016), or various other pollutants (Downs et al., 2014; Haman et al., 2015; Haynes and Johnson, 2000).

Hermatypic scleractinian corals (Anthozoa, Scleractinia), commonly named as hard corals, are reef forming organisms with the ability to assemble calcium carbonate structures, cornerstone for the coral reef. Scleractinian corals usually dominate healthy reefs (Gardner et al., 2003) and their degradation may lead to phase shift events(Hughes, 1994) with severe biodiversity loss in coral reef ecosystems (Richmond, 1993). As so, hermatypic corals are referred as keystone species (Peters et al., 1997) with urgent need for research (Vijayavel and Richmond, 2012). Additionally, alcyonacean corals (Anthozoa, Alcyonacea), frequently termed as soft corals lack an underlying limestone skeleton but hold several small structural sclerites composed of calcium carbonate. Although not as important as scleractinians for reef building, alcyonacean corals play an essential role when it comes trophodynamics and reef equilibrium (Karlson, 2002). Soft corals are increasingly explored for its high biotechnological potential (Blunt et al., 2018; Rocha et al., 2011), or for marine aquarium trade (Wabnitz et al., 2003). However, these organisms have received much less attention from the scientific community than scleractinian corals, and as such, knowledge about some physiological aspects remain scarce. Therefore, there is an urgent need to better study these organisms.

In the current context, with the increase of anthropogenic pressures and pollution, ecotoxicological studies can be useful to assess the effects of natural and synthetic pollutants on corals. However, ecotoxicological assays with marine organisms often test hydrophobic or

poorly soluble xenobiotics. Organic solvents are usually the most used resolution to create mixtures that can be administered to the test organism (Kahl et al., 1999; OECD, 2019; Rufli et al., 1998; Weyman et al., 2012). For tests in aquatic organisms, regulatory organizations advised the use of seven different solvents, which includes ethanol, methanol and dimethyl sulfoxide (DMSO), following a conservative administration (between 0.02 and 0.05 mL L^{-1}) (ASTM, 1997; OECD, 2019). Despite that, Green and Wheeler (2013) reviewed that higher concentrations are normally used in several assays, due to toxicant solubility or other methodological limitations. Notwithstanding, coral photosynthetic responses to organic solvents are exceedingly neglected and recommendations only contemplate microalgae ((Choi et al., 2011; Stratton and Corke, 1981; Stratton and Smith, 1988), nudibranchs (Pennington and Hadfiled, 1989) or bivalves (Yang et al., 2008).

The hermatypic photosynthetic hard coral *Montipora digitata* (Dana, 1846) (Hexacorallia: Scleractinia) is a fast growing branching coral with predominant asexual reproduction (Heyward and Collins, 1985) that occurs commonly in tropical and subtropical shallow reef flats, where it is usually a dominating species (Devantier et al., 2008). *M. digitata* is an ecologically relevant species, simple to maintain *ex situ,* alike most scleractinian corals (Becker and Mueller, 2001; Borneman and Lowrie, 2001; Rinkevich, 2005), easy to reproduce asexually, and sensitive to environmental change, therefore a good candidate for ecotoxicological studies.

The photosynthetic soft coral *Sarcophyton glaucum* (Quoy and Gaimard, 1833) (Octocorallia: Alcyonacea) is one of the most abundant species in the Indo-Pacific as well as in the Red Sea. This species is frequently used for its biotechnological potential *e.g.* (Sawant et al., 2006) or in the aquarium trade (Wabnitz et al., 2003), leading to an optimization of its culture (Costa et al., 2016; Rocha et al., 2013a; Sella and Benayahu, 2010). However, when considering ecotoxicology there is very limited information (see Cima et al., 2013).

In this study our aim was to establish a threshold for the three organic solvents previously mentioned (ethanol, methanol and DMSO), in order to estimate their toxicity, assessing the holobiont oxidative stress and the endosymbiont photobiology in *M. digitata* and *S. glaucum*. We expect that tested doses will impact differently oxidative stress coping mechanisms (antioxidant defenses and oxidative damage) and on photosynthesis (maximum quantum yield), since corals can have different mechanisms to metabolize distinctive solvents. Our hypothesis relays on the assumption that the exposure to the three organic solvents will not cause any toxic effect to *M. digitata* or *S. glaucum* fragments. To our best knowledge, this is the first study to identify the tolerance of these organisms to commonly used organic solvents.

2. Materials and Methods

2.1. Organisms and Culture System

Parental *M. digitata* colonies (gently conceded by Oceanário de Lisboa) and *S. glaucum* (originally form Sumbawa, Indonesia) were acclimated in aquaria for 1 month in standardized ~600 L culture systems according to Rocha et al. (2015) composed of two 250 L (150 cm length × 40 cm width × 50 cm height) culture tanks 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 500), osmoregulator to automatically compensate water evaporation (Deltec, Aquastat 1001), ~5 L of biological filter media, and ~2 L activated charcoal. Water recirculation through chiller and UV system was performed by a submergible pump (Eheim, universal 1200). Culture tank circulation was performed by a submergible pump (Eheim, universal 3400), providing an approximate flow of 1200 L h^{-1} to each tank. Additionally, each tank was equipped with a two circulation pumps (Tunze, Turbelle nanostream-6055) with a Turbelle controller providing wave simulation with oscillation flow (200 – 4500 L h^{-1}). Regarding illumination, each culture tank was equipped with four 80 W fluorescent lamps (Red Sea, REEF-SPEC) with 12:12 photoperiod, emitting a PAR of 100 ± 10 μmol m^{-2} s^{-1}, measured at colony level (Apogee MQ-500 PAR Meter). Salinity was kept at 35 ± 1 and temperature at 25 ± 1 ℃. Culture system functioned with synthetic saltwater prepared by mixing synthetic salt (Red Sea, Coral PRO salt) and reverse osmosis water (TMC, V2 Pure 360). Partial water changes (~15 % of total system volume) were made every week. After acclimation, *M. digitata* parental colonies were fragmented into 3-5 cm tall fragments and *S. glaucum* parental colonies fragmented into 1 cm^2 fragments. *M. digitata* fragments were fixed to 1 cm^2 ceramic tiles and *S. glaucum* fragments fixed to a plastic stand (Coral Cradle ®), both with n-butyl-cyanoacrylate. After cicatrization for 15 days for *M. digitata* fragments and 60 days for *S. glaucum* fragments in the same conditions as parental colonies, specimens were used to evaluate the effect of organic solvents.

2.2. Test concentrations

Six concentrations of ethanol, methanol and DMSO were set by applying a factor of 3.1 to the maximum solvent concentration (0.01 mL L^{-1}) recommended by the OECD (2019) guideline

for toxicity testing of difficult test chemicals: 0.01 mL L^{-1} (C1); 0.03 mL L^{-1} (C2); 0.1 mL L^{-1} (C3); 0.3 mL L^{-1} (C4); 0.9 mL L^{-1} (C5); 2.9 mL L^{-1} (C6) for each solvent. Test concentrations were obtained by diluting a stock solution of each solvent in saltwater.

2.3. Experimental design

Fragments were individually stocked in 200 mL flasks with five replicates per treatment (*i.e.* solvent concentration) in static-renewal exposure system (OECD, 2019), with 50 % medium renewal per day. During the experiment, corals were kept for 96 h in 200 mL flasks and maintained in water bath with one heater (Eheim, Jager 300W). Two water pumps (100 L h^{-1}) ensured homogenous water temperature (EHEIM CompactON 300). Experimental system was illuminated with four 80 W fluorescent lamps (Red Sea, REEF-SPEC) with 12:12 photoperiod, emitting a PAR of 100 ± 10 µmol m^{-2} s^{-1}, measured at coral fragment level (Figure 1).

Daily, 1 h 30 min after the beginning of the light period, water parameters were checked, being observed that it remained stable throughout the experiment (dissolved oxygen 8.15 ± 0.06 mg L^{-1}, pH 7.93 ± 0.01, salinity 35.37 ± 0.04 and temperature 24.84 ± 0.01 °C). Before the test, 5 fragments were sampled from the culture system (t0) to evaluate photochemical parameters and oxidative stress biomarkers (*cf.* methodology detailed in section 2.4).
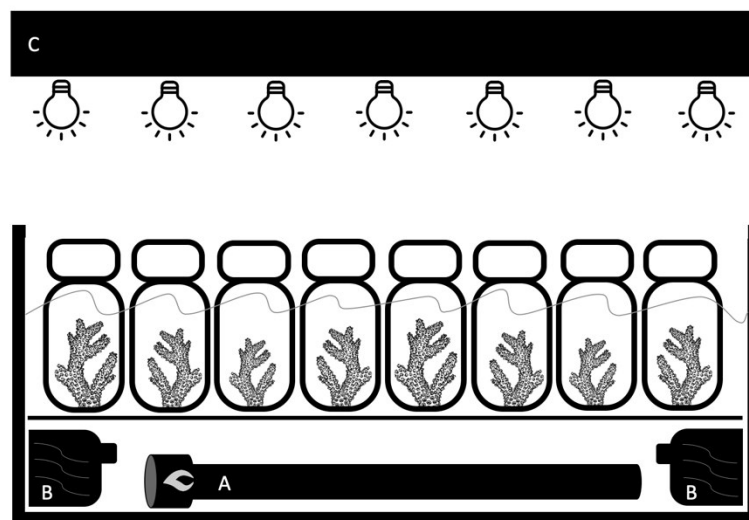

Figure 1 – Experimental system used for coral toxicity trials. A) Heater; B) Water pump; C) Lighting system.

2.4. Biological response

2.4.1. Biochemical biomarkers

Immediately after the photobiological assessment, fragments were frozen in liquid nitrogen and preserved at -80 ºC. Later, samples were homogenized in 1600 µL on ice bath for biomarker analysis. After homogenization, samples were separated into different aliquots: 300 µL for electron transport system activity (ETS); 200 µL for lipid peroxidation (LPO), in which 4% of butylated hydroxytoluene (BHT) was added; 600 µL of the remaining homogenate diluted with 600 µL of 0.2 M K-phosphate buffer, pH 7.4, and centrifuged at 10000 g for 15 min (4ºC) to obtain the post mitochondrial supernatant (PMS).

PMS protein content was determined according to Bradford (1976) adapted from the Bio-Rad micro-assay, using bovine γ-globulin as standard. Catalase (CAT) was determined through the decomposition of hydrogen peroxide (H_2O_2) in PMS at 240 nm (Clairborne, 1985). Glutathione S-transferase (GST) was measured combining reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) in the PMS fraction at 340 nm (Habig et al., 1974). Total glutathione (TG) was quantified at 412 nm using a reaction of GSH with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) combined with glutathione reductase (GR) in excess, also in PMS (Baker et al., 1990; Rodrigues et al., 2017). LPO was obtained measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm (Bird and Draper, 1984). ETS activity was measured using iodonitrotetrazolium (INT) reduction method, read at 490 nm (De Coen and Janssen, 1997), modified by (Rodrigues et al., 2015). Cellular oxygen consumption rate was calculated based on the stoichiometric relationship in which 2 µmol of formazan formed and 1 µmol of oxygen is consumed. Energy consumption (Ec) was obtained by the conversion to energetic values using the specific oxyenthalpic equivalent for the average lipid, protein and carbohydrate mixture of 480 kJ mol^{-1} O_2 (Gnaiger, 1983).

2.4.2. Photobiology

By the end of the experiment, F_v/F_m was estimated non-intrusively through PAM fluorometry using Junior-PAM (Walz ™, Germany) based on Rocha et al. (2013). Light was delivered by a 1.5 mm plastic optical fiber, positioned perpendicularly to the coral surface. Corals were dark-acclimated for 30 min to ensure full relaxation of the photosystem II reaction

centers. Maximum photochemical quantum yield (F_v/F_m) is estimated considering dark level fluorescence (F_0) and maximum fluorescence (F_m) as described by Schreiber et al. (1986).

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

2.4.3. Behavior

Polyp contraction/relaxation was recorded daily to validate data with behavior. Three classification levels were considered: i) relaxed - fragments with all polyps open (open oral disc and distended tentacles); ii) contracted - fragments that had polyps with retracted tentacles; and iii) closed - fragments with every polyp closed. Evident signs of mortality were also recorded.

2.5. Statistical Analysis

Differences between acclimated organisms, prior to the experiment and the control were assessed with a Mann-Whitney test. Regarding the experiment *per se,* significant differences were estimated through Kruskal-Wallis test on ranks, followed by a Dunnett's test (Dunnett, 1955), given the non-normal distribution of most variables. Significance was set to *p*-value ≤ 0.05, although marginal significance was tolerated. Bayesian Independent-samples T-tests were run on endpoints significantly different within the Dunnett tests to corroborate the trend with probability, independently of the sample size. Bayesian evidence was based in the Bayesian Factor (0-3 Anecdotal; 3-10 Moderate; 10-30 Strong; 30-100 Very strong; >100 Extreme). Significant Outliers were calculated as ± 1.5 Interquartile Range and trimmed before analysis. Descriptive data was presented as mean ± standard error. Analysis was run in R software version 3.6.1. (R Core Team, 2019), using the package PMCMRplus (Pohlert, 2018) and ggplot2 (Wickham, 2016) except for the Bayesian analysis, which was performed using JASP software version 0.10.2 (JASP Team, 2019).

The statistical approach herein undertaken provides an exploratory interpretation of our data, at particular situations in which Dunnett's tests were taken into consideration regardless of the Kruskal-Wallis result. This can incur in a false positive outcome (*i.e.* type I errors). False positive results in ecotoxicological reports are of least concern and more conservative than false negatives (*i.e.* type II errors). Nevertheless, Bayesian analysis pretends to assess the robustness of the abovementioned, diminishing the impact of such effect.

3. Results

In our tests, *M. digitata* mortality was observed at the highest (2.9 mL L^{-1}) ethanol and DMSO concentrations. Fragments exposed to ethanol began to show signs of bleaching and mortality at 24h and DMSO exposed fragments at 72h. *S. glaucum* remained alive in every treatment.

3.1. Biological response

3.1.1. Biochemical biomarkers

Statistical results of the biochemical biomarkers are reported in the table I for *M. digitata* and table II for *S. glaucum*. Regarding *M. digitata*, CAT activity was significantly higher at 0.9 mL L^{-1} ethanol with moderate evidence. GST activity was higher at 0.01 mL L^{-1} ethanol, although with anecdotal evidence. Ec was significantly lower at 0.01 mL L^{-1}, 0.3 mL L^{-1} and 0.9 mL L^{-1} ethanol, with strong evidence. CAT activity was significantly higher at 0.3 mL L^{-1} and 0.9 mL L^{-1} methanol, with moderate evidence. GST activity was higher at 0.3 mL L^{-1} methanol, with moderate evidence, and 0.9 mL L^{-1} methanol with strong evidence. LPO was significantly lower at 0.03 mL L^{-1} and 2.9 mL L^{-1} methanol, with anecdotal evidence, but significantly higher at 0.3 mL L^{-1} methanol, with moderate evidence. Exposure to 0.9 mL L^{-1} DMSO caused increased CAT activity, with anecdotal evidence. GST activity was significantly higher at 0.3 mL L^{-1} DMSO, with strong evidence and at 0.9 mL L^{-1} DMSO, with very strong evidence.

S. glaucum CAT activity was lower at 2.9 mL L^{-1} ethanol, with strong evidence. Ec was lower also at 2.9 mL L^{-1} ethanol, with moderate evidence. When exposed to methanol, *S. glaucum* CAT activity was inhibited at 0.01 mL L^{-1}, with moderate evidence, at 0.9 mL L^{-1}, with very strong evidence and 2.9 mL L^{-1}, with anecdotal evidence. LPO was significantly higher at 2.9 mL L^{-1} methanol, with anecdotal evidence. Ec was lower at 0.01 mL L^{-1} methanol, with anecdotal evidence, at 0.1 mL L^{-1} methanol, with moderate evidence and 2.9 mL L^{-1} methanol, with moderate evidence. DMSO exposure caused less Ec at 0.3 mL L^{-1}, with moderate evidence, at 0.9 mL L^{-1}, with anecdotal evidence and at 2.9 mL L^{-1}, with moderate evidence.

Table I - Oxidative stress response for *M. digitata* exposed to ethanol, methanol and DMSO expressed as mean ± standard error: catalase activity (CAT); glutathione-S-transferase activity (GST); total glutathione (TG); lipid peroxidation (LPO); energy consumption (Ec). Statistical differences between the control group and the treatment are marked (p<.1[+]; p<.05*; p<.01**; p<.001***).

| | | CAT | GST | LPO | EC |
		μmol min^{-1} mg prot^{-1}	nmol min^{-1} mg prot^{-1}	TBARS nmol g ww^{-1}	mJ h^{-1} mg^{-1}
	Control	9.70 ± 1.83	9.27 ± 0.64	72.84 ± 9.15	0.88 ± 0.06
Ethanol mL L^{-1}	**0.01**	12.79 ± 2.64	59.68 ± 24.06 *	58.40 ± 12.58	0.40 ± 0.08 ***
	0.03	12.84 ± 1.85	30.80 ± 3.91	59.10 ± 7.45	0.68 ± 0.11
	0.1	9.12 ± 0.17	19.27 ± 1.85	56.66 ± 4.60	0.74 ± 0.06
	0.3	8.47 ± 1.06	17.96 ± 0.48	38.53 ± 0. 50	0.42 ± 0.06 **
	0.9	29.55 ± 4.65 ***	27.15 ± 1.89	83.17 ± 12.27	0.47 ± 0.04 **
Methanol mL L^{-1}	**0.01**	11.54 ± 0.70	27.74 ± 3.33	69.71 ± 9.11	0.72 ± 0.09
	0.03	13.84 ± 3.28	27.23 ± 2.76	44.32 ± 4.15 *	0.87 ± 0.10
	0.1	17.27 ± 2.06	23.64 ± 6.65	50.90 ± 4.15	0.93 ± 0.13
	0.3	18.40 ± 3.14 *	42.29 ± 11.26 **	107.15 ± 5.21 **	0.73 ± 0.06
	0.9	18.04 ± 1.41 [+]	32.99 ± 8.45 [+]	52.41 ± 6.41	0.79 ± 0.09
	2.9	14.37 ± 1.79	25.34 ± 3.21	48.05 ± 2.91 [+]	0.64 ± 0.08
DMSO mL L^{-1}	**0.01**	8.11 ± 1.33	10.84 ± 1.03	69.29 ± 9.62	0.89 ± 0.08
	0.03	8.32 ± 0.38	9.86 ± 0.76	58.04 ± 1.48	0.57 ± 0.15
	0.1	7.85 ± 0.88	13.89 ± 1.45	54.72 ± 0.52	0.68 ± 0.02
	0.3	7.24 ± 0.82	22.36 ± 3.12 **	60.45 ± 3.29	1.14 ± 0.08
	0.9	17.44 ± 2.09 **	33.00 ± 3.98 ***	75.14 ± 11.18	0.73 ± 0.18

Table II - Oxidative stress response for *S. glaucum* exposed to ethanol, methanol and DMSO expressed as mean ± standard error: catalase activity (CAT); glutathione-S-transferase activity (GST); lipid peroxidation (LPO); energy consumption (Ec). Statistical differences between the control group and the treatment are marked (p<.1[+]; p<.05*; p<.01**; p<.001***).

		CAT μmol min^{-1} mg prot^{-1}	GST nmol min^{-1} mg prot^{-1}	LPO TBARS nmol g ww^{-1}	Ec mJ h^{-1} mg^{-1}
Control		8.90 ± 0.24	10.21 ± 0.47	3.47 ± 0.40	0.76 ± 0.08
Ethanol mL L^{-1}	0.01	6.58 ± 0.92	11.08 ± 2.97	4.13 ± 0.17	0.51 ± 0.07
	0.03	8.75 ± 0.61	8.51 ± 0.68	4.26 ± 0.50	0.50 ± 0.04
	0.1	8.11 ± 0.65	9.99 ± 2.15	4.70 ± 0.72	0.70 ± 0.18
	0.3	7.25 ± 0.69	9.89 ± 1.32	4.18 ± 0.71	0.67 ± 0.12
	0.9	8.79 ± 0.55	7.11 ± 1.04	3.34 ± 0.17	0.47 ± 0.04
	2.9	6.15 ± 0.31 [+]	7.80 ± 2.09	3.88 ± 0.40	0.37 ± 0.11 [+]
Methanol mL L^{-1}	0.01	6.07 ± 0.37 *	9.17 ± 1.95	3.24 ± 0.58	0.47 ± 0.11 [+]
	0.03	6.85 ± 0.62	8.39 ± 1.86	4.60 ± 0.20	0.71 ± 0.11
	0.1	6.90 ± 0.61	12.64 ± 2.18	3.80 ± 0.28	0.43 ± 0.03 *
	0.3	6.93 ± 0.53	10.65 ± 2.50	3.49 ± 0.21	0.48 ± 0.01
	0.9	5.71 ± 0.29 *	7.78 ± 1.36	3.96 ± 0.57	0.59 ± 0.05
	2.9	6.04 ± 0.92 *	8.19 ± 2.94	6.61 ± 1.20 **	0.30 ± 0.02 **
DMSO mL L^{-1}	0.01	10.22 ± 2.28	8.80 ± 1.53	2.84 ± 0.28	0.66 ± 0.10
	0.03	5.59 ± 0.23	8.43 ± 1.26	2.96 ± 0.58	0.60 ± 0.03
	0.1	4.44 ± 0.31	8,56 ± 0.28	2.33 ± 0.10	0.56 ± 0.02
	0.3	7.01 ± 0.46	10.81 ± 1.16	2.71 ± 0.59	0.46 ± 0.04 *
	0.9	5.59 ± 1.33	6.46 ± 0.64	1.42 ± 0.14	0.54 ± 0.06 [+]
	2.9	4.24 ± 0.26	10.44 ± 1.82	4.01 ± 1.10	0.40 ± 0.05 **

3.1.2. Photobiology

Results for photobiology are reported in the figure 2 for *M. digitata* and *S. glaucum*. When exposed to 0.01 mL L^{-1} ethanol *M. digitata* F_v/F_m was significantly lower, with moderate evidence. F_v/F_m was also significantly lower at 0.03 mL L^{-1} DMSO, with anecdotal evidence, at 0.1 mL L^{-1} DMSO, with moderate evidence and 0.9 mL L^{-1} DMSO, with very strong evidence. Regarding *S. glaucum*, F_v/F_m was significantly lower at 0.9 mL L^{-1} ethanol, with anecdotal evidence.
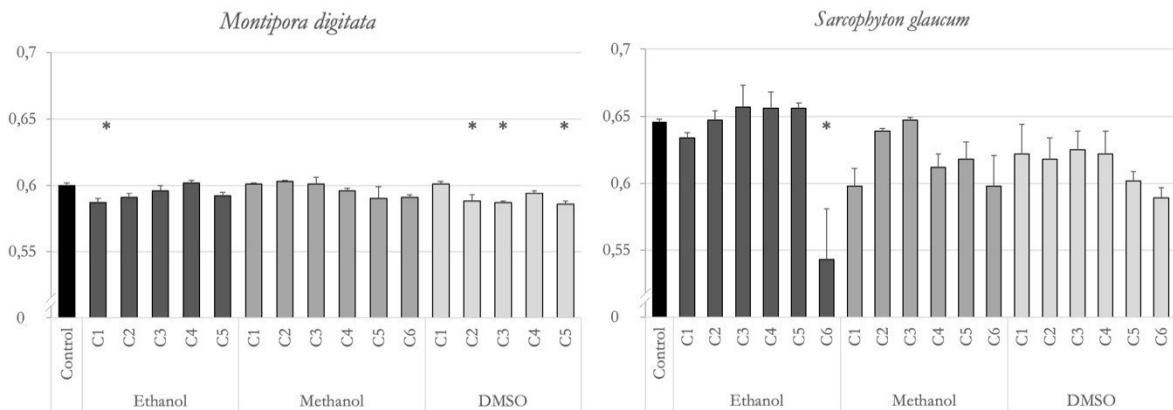
Figure 2 - Maximum photochemical quantum yield for *M. digitata* and S. glaucum exposed to increasing concentrations of ethanol, methanol and DMSO. Ctrl – control; C1 = 0.01 mL L^{-1}; C2 = 0.03 mL L^{-1}; C3 = 0.1 mL L^{-1}; C4 = 0.3 mL L^{-1}; C5 = 0.9 mL L^{-1}; C6 = 2.9 mL L^{-1}. Statistical differences between the control group and the treatment are marked (p<.05*).

3.1.3. Behavior

Ethanol and DMSO only affected *M. digitata* behavior above 0.9 mL L^{-1} with polyps starting to contract at the first 24 h in ethanol and only at 48h in DMSO. At 2.9 mL L^{-1} ethanol every fragment had all polyps completely closed. Nevertheless, polyps began to gradually close from 48 exposure at 0.9 mL L^{-1} ethanol. In the other solvents, closure occurs only at 2.9 mL L^{-1} (Figure 2).

S. glaucum behavior was very inconsistent throughout the experiment. Nevertheless, exposure to 0.3 mL L^{-1} ethanol or higher ethanol concentrations resulted in polyp closure. *S. glaucum* colonies also remained closed at 2.9 mL L^{-1} DMSO exposure in every record. *S. glaucum* was recorded relaxed at 0.01 mL L^{-1} ethanol, every methanol concentration, despite only one colony stayed relaxed in the first 24 h at 2.9 mL L^{-1}, and every DMSO concentration except for 2.9 mL L^{-1} DMSO.
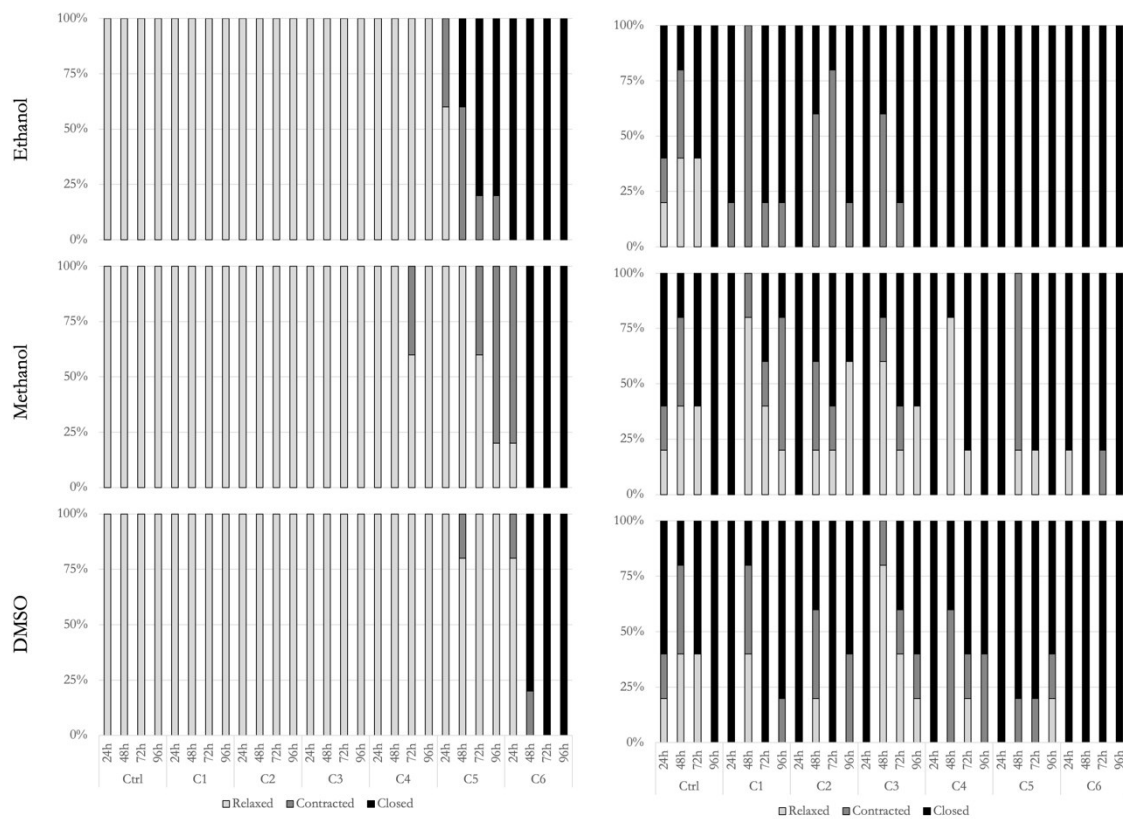
Figure 3 - Behavior of *M. digitata* (left) and *S. glaucum* (right) exposed to increasing concentrations of ethanol, methanol and DMSO throughout the experiment, assessed every 24h. Ctrl – control; C1 = 0.01 mL L^{-1}; C2 = 0.03 mL L^{-1}; C3 = 0.1 mL L^{-1}; C4 = 0.3 mL L^{-1}; C5 = 0.9 mL L^{-1}; C6 = 2.9 mL L^{-1}.

3.2. Dunnett's heatmap

Given the large volume of data, for a better understanding of the study results, we present below (Figure 3) an overview of results, through a Dunnett's heatmap by solvent and by endpoint.
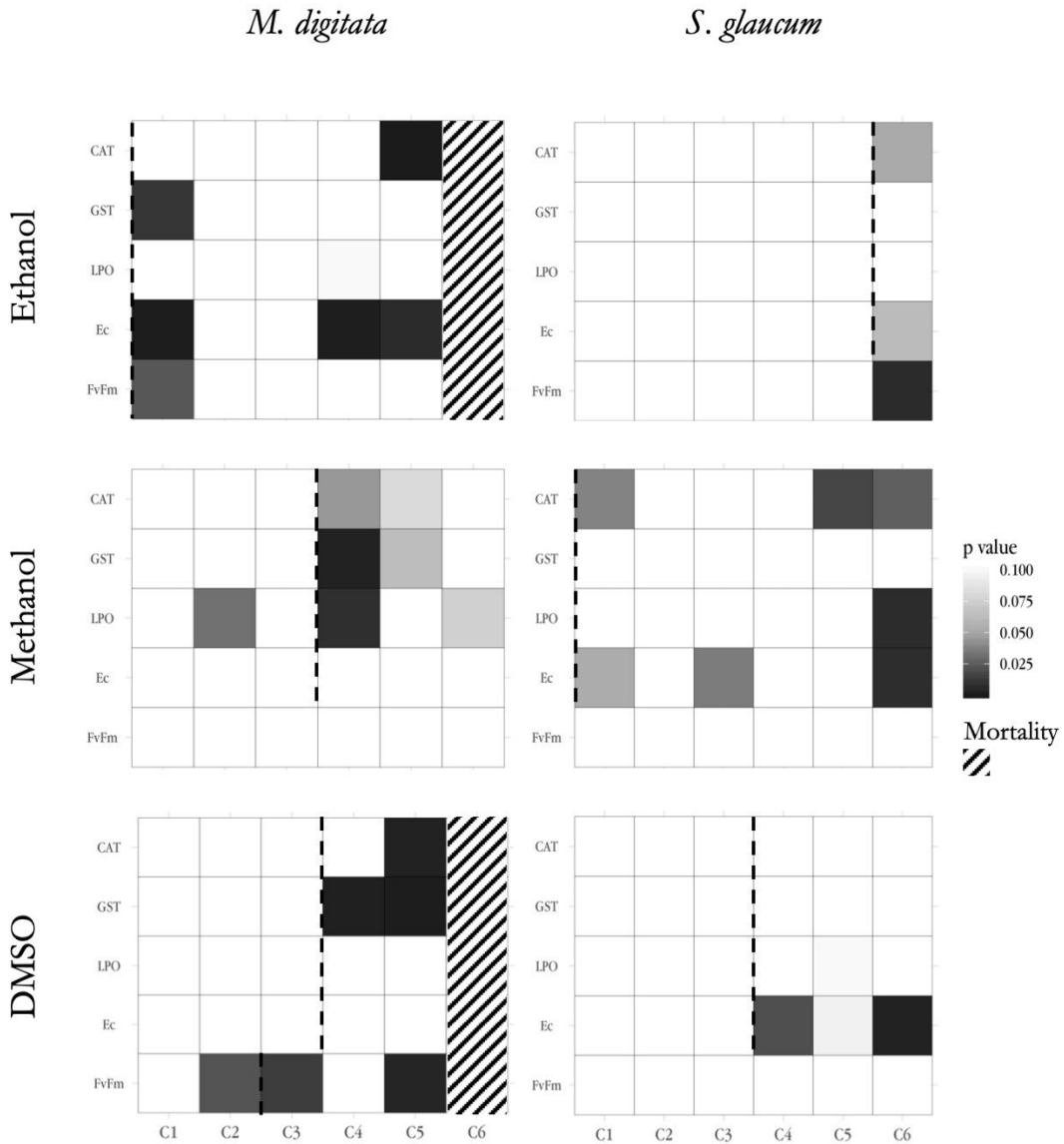
Figure 4 - Dunnett's heatmap as a visual representation of the lowest p-values observed in Dunnett's test for catalase activity (CAT), glutathione S-transferase (GST), lipid peroxidase (LPO), energy consumption (Ec), maximum quantum yield (F_v/F_m) in the concentrations tested. C1 = 0.01 mL L^{-1}; C2 = 0.03 mL L^{-1}; C3 = 0.1 mL L^{-1}; C4 = 0.3 mL L^{-1}; C5 = 0.93 mL L^{-1}; C6 = 2.86 mL L^{-1}. Dotted line represents the threshold for solvent use with distinguished impact on oxidative stress response or photosynthetic activity, although endpoints with anecdotic evidence were disregarded. Mortality was observed in C6 concentration of ethanol and DMSO.

Dunnett's heatmap is a visual representation of the lowest p-values observed in Dunnett's test and can be useful for decision-making regarding recommended solvent concentrations for toxicity tests. *M. digitata* exposed to ethanol triggered oxidative stress response and influenced the photosynthetic activity at 0.01 mL L^{-1}. Methanol seemed to affect *M. digitata* oxidative stress response at 0.3 mL L^{-1}, as evidence for LPO at 0.03 mL L^{-1} was anecdotal without apparent effect on photosynthetic activity, with a LOEC of 0.3 mL L^{-1} methanol. DMSO triggered *M. digitata* oxidative stress at 0.3 mL L^{-1}, but photosynthetic activity was affected at 0.1 mL L^{-1}, as the effect at 0.03 mL L^{-1} was not supported by bayesian evidence, with a corresponding LOEC of 0.1 mL L^{-1} DMSO. Ethanol only seemed to affect *S. glaucum* oxidative stress response and photosynthetic activity at 2.9 mL L^{-1}, with a LOEC of 2.9 mL L^{-1} ethanol. *S. glaucum* oxidative stress response was affected at 0.01 mL L^{-1} methanol with no apparent effect on photosynthesis. DMSO only seemed to affect *S. glaucum* energy consumption at 0.1 mL L^{-1} with no significant difference in any other endpoint.

4.   Discussion

The present study provides a complemental work to Silva et al. (unpublished data) (*c.f* chapter II) to construct a baseline for coral ecotoxicology, specifically the effect of organic solvents, frequently used to increase solubility of hydrophobic or low soluble toxicants in photosynthetic corals.

Our tests showed that *M. digitata* oxidative stress due to ethanol exposure translated into increased GST activity at the first tested concentration (0.01 mL L^{-1}), while CAT activity increased only at 0.9 mL L^{-1}. Despite exhibiting a clear oxidative stress response, *M. digitata* consumed less energy. F_v/F_m was affected at the first concentration, but the effect of ethanol seems to dissipate in higher ethanol concentrations. F_v/F_m is widely used as a fast and non-intrusive proxy for photochemical stress (*e.g.* Serôdio et al., 2011). Generally, F_v/F_m reduction is a sign of stress as efficiency loss can be related to photoinhibition (Maxwell and Johnson, 2000). The metabolism of *M. digitata* to ethanol can possibly have more intervenients than those hereby tested, *i.e.* ethanol can promote proliferation of microbiota capable of using it as a source of carbon and therefore shifting patterns in the coral microbiota are probable (*e.g.* Sulphur reducing bacteria) (Hao, 2003). Methanol exposure with *M. digitata* showed a consistent stress response

at 0.3 mL L^{-1} making it a reliable threshold for methanol. DMSO exposure with *M. digitata* showed activation of CAT and GST activity, although without lipidic damage. Photochemical stress was observed at earlier concentrations than oxidative stress.

Regarding *S. glaucum,* ethanol exposure inhibited CAT activity, but caused lower Ec at 2.9 mL L^{-1}, which is not a very reliable oxidative stress response. Still, ethanol exposure to *Z. sociatus* had what seems to be a numbing effect to the oxidative stress response, and *M. digitata* was affected at the lowest ethanol concentration used. As so, the response of *S. glaucum* to ethanol should be considered carefully. Regardless, ethanol was the only tested solvent to cause lower F_v/F_m, although with anecdotal evidence. *S. glaucum* CAT activity was generally lower throughout methanol exposure, but only 0.01 mL L^{-1} and 0.9 mL L^{-1} were statistically significant and supported by bayesian analysis. This response was clearly inhibited by methanol. CAT inhibition is distressing as no other oxidative stress response showed significant differences and LPO was higher at 2.9 mL L^{-1}. DMSO exposure with *S. glaucum* triggered no oxidative stress response and Ec was significantly lower, probably meaning that, although DMSO caused effect on *S. glaucum,* the coral was not suffering from oxidative stress. DMSO is resultant from the degradation of the dimethyl sulfoniopropionate (DMSP) (Schäfer et al., 2010; Zhang et al., 1991) produced naturally by zooxanthellae (Broadbent and Jones, 2004; Ishida, 1996), and therefore the low sensitivity of *S. glaucum* to DMSO is somewhat expected. Notwithstanding, coral cells still need to metabolize DMSO, but the pathway is unknown.

Interestingly, the most consistent result was that tested organic solvents reduced Ec in *S. glaucum.* As measured oxidative stress considers the holobiont, the average response can be shortsighted at the expense of few organisms with distinct ways to metabolize organic solvents.

This study endorsed the baseline for solvent use in ecotoxicology, central for future studies with photosynthetic corals. *M. digitata* proved to be less sensitive to methanol and DMSO, comparing with ethanol. Ethanol triggered *M. digitata* oxidative stress and photosynthetical decay at 0.01 mL L^{-1} and caused mortality at the highest used concentration. Methanol caused *M. digitata* oxidative stress at 0.3 mL L^{-1}. Regarding DMSO, *M. digitata* showed oxidative stress at 0.3 mL L^{-1} and photosynthetical decay at 0.1 mL L^{-1} and caused mortality at the highest used concentration. Ethanol triggered *S. glaucum* oxidative stress and photosynthetical decay at 2.9 mL L^{-1}. Methanol caused *S. glaucum* oxidative stress at 0.01 mL L^{-1}. Regarding DMSO, *S. glaucum* had Ec affected at 0.3 mL L^{-1}.

As recommended by OECD (2019) solvent concentration should be at least one order of magnitude below the NOEC and therefore, the use of organic solvent in studies with

photosynthetic tropical corals at concentrations $\geq$ 0.001 mL L^{-1} should be appropriately weighted. Nevertheless, *S. glaucum* did not showed clear oxidative stress responses to ethanol and DMSO, thus the use of these solvents with this species is most likely safe.

5.   References

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6. Annexes

Table A.I - Top-Down statistical analysis for *M. digitata* exposure to ethanol Bayesian analysis was performed when Kruskal-Wallis test on ranks followed by Dunnett's test were significant, or marginally significant (*).

	K-W [*p*-value]	DOSE [mL L^{-1}]	DUNNETT'S [*p*-value]	BAYES FACTOR [BF$_{10}$]	EVIDENCE
CAT	.025	0.9	.0001	9.648	Moderate
GST	.004765	0.01	.011	1.532	Anecdotal
LPO	.06441*		.10		
		0.01	.00083	12.894	Strong
Ec	.009607	0.3	.00127	22.305	Strong
		0.9	.00686	29.650	Strong
F_v/F_m	.01603	0.01	.023	6.667	Moderate

Table A.II - Top-Down statistical analysis for *M. digitata* exposure to methanol. Bayesian analysis was performed when Kruskal-Wallis test on ranks followed by Dunnett's test were significant, or marginally significant (*).

	K-W [*p*-value]	DOSE [mL L^{-1}]	DUNNETT'S [*p*-value]	BAYES FACTOR [BF$_{10}$]	EVIDENCE
CAT	.08223*	0.3	.045	2.013	Anecdotal
		0.9	.081*	4.913	Moderate
GST	.05017*	0.3	.0033	3.401	Moderate
		0.9	.0636*	23.970	Strong
LPO	.005307	0.03	.033	2.302	Anecdotal
		0.3	.0081	3.388	Moderate
		2.9	.0751*	1.812	Anecdotal
Ec	.325				
F_v/F_m	.06957*				

Table A.III - Top-Down statistical analysis for *M. digitata* exposure to DMSO. Bayesian analysis was performed when Kruskal-Wallis test on ranks followed by Dunnett's test were significant, or marginally significant (*).

	K-W [*P*-VALUE]	DOSE [ML L^{-1}]	DUNNETT'S [*P*-VALUE]	BAYES FACTOR [BF$_{10}$]	EVIDENCE
CAT	.06893*	0.9	.0034	2.735	Anecdotal
GST	.0005943	0.3	.0019	11.156	Strong
		0.9	> .0001	58.708	Very strong
LPO	.4849				
Ec	.06952*				
F_V/F_M	.002007	0.03	.0220	1.677	Anecdotal
		0.1	.0137	3.347	Moderate
		0.9	.0042	58.708	Very strong

Table A.IV - Top-Down statistical analysis for *S. glaucum* exposure to ethanol. Bayesian analysis was performed when Kruskal-Wallis test on ranks followed by Dunnett's test were significant, or marginally significant (*).

	K-W [*P*-VALUE]	DOSE [ML L^{-1}]	DUNNETT'S [*P*-VALUE]	BAYES FACTOR [BF$_{10}$]	EVIDENCE
CAT	.04725	2.9	.051	19.979	Strong
GST	.7428				
LPO	.5633				
Ec	.1601	2.9	.062*	3.150	Moderate
F_V/F_M	.02234	2.9	.0066	7.153	Moderate

Table A.V - Top-Down statistical analysis for *S. glaucum* exposure to methanol. Bayesian analysis was performed when Kruskal-Wallis test on ranks followed by Dunnett's test were significant, or marginally significant (*).

	K-W [*P*-VALUE]	DOSE [ML L^{-1}]	DUNNETT'S [*P*-VALUE]	BAYES FACTOR [BF$_{10}$]	EVIDENCE
		0.01	.038	15.167	Strong
CAT	.123	0.9	.017	41.695	Very Strong
		2.9	.027	1.697	Anecdotal
GST	.738				
LPO	.1464	2.9	.007	2.182	Anecdotal
		0.01	.0555*	1.617	Anecdotal
Ec	.01319	0.1	.0382	4.712	Moderate
		2.9	.0068	6.432	Moderate
F_V/F_M	.0465				

Table A.VI - Top-Down statistical analysis for *S. glaucum* exposure to DMSO. Bayesian analysis was performed when Kruskal-Wallis test on ranks followed by Dunnett's test were significant, or marginally significant (*).

	K-W [*P*-VALUE]	DOSE [ML L^{-1}]	DUNNETT'S [*P*-VALUE]	BAYES FACTOR [BF$_{10}$]	EVIDENCE
CAT	.003913				
GST	.1048				
LPO	.07322*				
		0.3	.0201	3.169	Moderate
Ec	.02675	0.9	.0952*	1.598	Anecdotal
		2.9	.0028	7.945	Moderate
F_V/F_M	.002007	0.03	.0220	1.677	Anecdotal

Chapter IV

Final Considerations

1. Final considerations

The baseline set in this study represents an important step towards better ecotoxicology with photosynthetic corals, allowing researchers to have a better foundation for organic solvent use. Nevertheless, the knowledge on physiology of photosynthetic corals is scarce, hampering the comprehension of coral response to stressors.

Future studies should take into consideration:

- Other biochemical parameters, like superoxidase dismutase (SOD), as this enzyme plays a big role in photosynthetical organisms in the chloroplast oxidative stress response. DNA damage (comet assay) could also be an interesting approach, as the response of the coral is independent of the symbionts, as zooxanthellae cells are easily distinguished at fluorescence microscopy.

- Flow-through systems should be better suited for tests with these organisms, minimizing human error and avoiding coral manipulation. One cannot naively disregard the human error inherent to the solution renewal that can be avoided by flow-through systems. The continuous solution intake, even at very low volume, could also overcome the evaporation of volatile organic solvents. More so, manipulation may likely cause stress to the test organisms, interfering with the results. Despite being a better system for this type of study, flow-through systems are costly opposing to the relatively inexpensive used system.

- Bigger sample size could also mean a more consistent response, accounting for the natural variation we observed. Nevertheless, corals are not short-lived organisms, like most biological models for ecotoxicology and bigger sample sizes would imply a very large coral husbandry facility to satisfy the need for coral biomass.

- Holobiont characterization could also be useful to correlate response patterns and the organisms that have a relevant role in solvent metabolism. Even so, it could be interesting to observe the response of the different microorganisms, independently. This procedure can have several methodological limitations, mainly due to over-manipulation of the samples, but could be the key to better understand the response patterns of photosynthetic corals.