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Organic solvents alter photophysiological and oxidative stress profiles of the coral *Zoanthus* sp. – towards an optimization of ecotoxicological protocols

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Abstract

Coral reefs are declining, affected by climate change and escalating anthropogenic pressures, such as pollution or habitat alteration. Consequently, ecotoxicological assays with tropical corals have increased, specifically towards the study of emergent or persistent pollutants. However, standardized methodology to test for corals is non-existent, and their response to organic solvents, recurrently required in ecotoxicological appraisals, remains unknown. Therefore, we aimed to establish a threshold for the safe use of the selected solvents in ecotoxicological studies with these organisms. We assessed the oxidative stress response (antioxidant response and oxidative damage), cellular energy allocation and photophysiology of the photosynthetic coral *Zoanthus* sp. (Anthozoa, Hexacorallia) exposed to six doses of three different organic solvents (ethanol, methanol and dimethyl sulfoxide – DMSO). Our results suggest that the coral is more sensitive to methanol and DMSO than to ethanol. Methanol and DMSO LOEC were 0.01 mL L^{-1} affecting maximum quantum yield (F_v/F_m) and glutathione S-transferase (GST) activity, respectively, while for ethanol was 0.03 mL L^{-1}, influencing F_v/F_m. Despite the higher tolerance of *Zoanthus* sp. to ethanol, 2.9 mL L^{-1} of this organic solvent 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.

Keywords: photobiology; cellular energy allocation; oxidative damage; ecotoxicology; marine invertebrates; Zoantharia

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1. Introduction

Coral reefs are the mainframe for the tropical marine biome, due to unique relationships with the entire ecosystem. Despite their ecological and economic relevance, these organisms are increasingly threatened by anthropogenic factors (Hughes et al., 2017). As test models, tropical photosynthetic corals can provide valuable information concerning the ecosystem, since a plethora of organisms depend on them (Spalding et al., 2001). Therefore, there is an urgent need to comprehend how corals can respond to anthropogenic pressures, by assessing the potential risks. Generating ecotoxicological data on the effects of different contaminants on coral biological responses will help to fulfil such gaps. Nevertheless, 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 requires the use of organic solvents to be solubilized to promote their 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 contaminants or with low solubility in water (Hutchinson et al., 2006). The use of organic solvents in ecotoxicology can have a significant effect in the test organisms, acting as a clouding factor to the outcomes, impacting directly or indirectly the physiological processes without it being recognized or estimated by assessors. However, the use of organic solvents is mandatory to several ecotoxicology studies, which imposes the need for adequate standardized doses that cause no effect to the study organism. Regulatory organizations suggest the use of seven different solvents,

including those used in this study, and recommend a 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 higher doses are often used in scientific experiments due to toxicant solubility or other methodological limitations as reviewed by Green and Wheeler (2013). This approach is not specifically conducted for ecologically relevant questions, but mainly from a mechanistic standpoint.

The cosmopolitan photosynthetic soft coral *Zoanthus* sp. (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 (Leal et al., 2017). In fact, zoanthids tolerate and acclimate promptly to environmental changes (Rosa et al., 2018). This plasticity suggests an adaptation and resilience impaired by most of its relatives, giving them high relevance for the testing of pollutants, and other anthropogenic stressors. Thus, preliminary studies to identify the response of these organisms to commonly used organic solvents are paramount for good quality ecotoxicological studies.

In the present study, we evaluate the toxicity of three of the most common organic solvents used in ecotoxicology, *viz.* ethanol, methanol and DMSO, considering the holobiont oxidative stress response, and cellular energy allocation, and the endosymbiont photobiology in *Zoanthus sp.* Ultimately, we intend to contribute to the standardization and optimization of ecotoxicological protocols using mini-colonies by establishing baselines for solvent use. To our knowledge, identifying the response of photosynthetic corals to commonly used organic solvents is an entirely unaddressed issue.

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2. Materials and Methods

2.1. Organisms and Culture System

Parental colonies were collected from Batam, Indonesia, and shipped to Aveiro, Portugal (Ecomare, University of Aveiro). Specimens were possible to identify to the genus. Further identification was not possible as the long-lasting state of zoanthid morphology is quite chaotic due to intraspecific variation, phenotypic plasticity or species synonyms (Burnett et al., 1997; Ong et al., 2015; Ryland and Lancaster, 2003). Specimens were preserved for future molecular identification.

Parental colonies were acclimated in aquaria for one 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 submersible pump (Eheim, universal 1200). Culture tank circulation was also performed by a submersible 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 photosynthetically active radiation (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 ºC. The 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 three polyp mini-colonies with a 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. Corals were not fed during the acclimation, or the experiment.

2.2. Experimental design

Six doses of ethanol absolute (≥99.5%), analytical standard methanol (≥99.9%) and molecular grade DMSO (≥99.9%) were set by applying a factor of 3.1 to the maximum solvent dose (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 doses were obtained by diluting a stock solution (2.9 mL L^{-1}) of each solvent in saltwater.

Mini-colonies were individually stocked in 200 mL flasks with five replicates per treatment (*i.e.*, solvent dose). During the experiment, corals were kept for 96 h in 200 mL flasks and maintained in a 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 (Fig. 1).

Water parameters were checked daily, 1 h 30 min after the beginning of the light period, and 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). Evident signs of mortality were registered at the same moment.

Before the test, five mini-colonies were sampled (t0) to validate the experiment. The oxidative stress response, cellular energy allocation and photochemical parameters were evaluated (*cf.* methodology detailed in section 2.4).

2.3. Biological responses

2.3.1. Oxidative stress response and cellular energy allocation

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 of ultra-pure water 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 (4ºC) 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) and glutathione S-transferase (GST) activity, as well as total glutathione (tGSH) were

measured in PMS. CAT was determined through the decomposition of hydrogen peroxide (H_2O_2) at 240 nm (Clairborne, 1985). GST activity was measured at 340 nm, by combining reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB)(Habig et al., 1974). tGSH 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, (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). Estimated 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). The 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. Aerobic energy production (ETS) 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).

2.3.2. 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 α 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). The 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 (RLC) were calculated based on Platt et al. (1980). The maximum

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electron transport rate (ETRmax) was obtained from RLC as well as the light-saturation coefficient (Ek) and initial slope (α). The maximum quantum yield (F_v/F_m) was calculated with the first RLC pulse, considering dark level fluorescence (F_0) and maximum fluorescence (F_m) as described by Schreiber et al. (1986).

$$\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m}$$

2.4. Statistical analysis

Because of the non-normal and multivariate nature of the data, significant differences were calculated through PERMANOVA analysis, in multivariate euclidian space (McArdle and Anderson, 2001), confirmed with the analysis of homogeneity of dispersion by PERMDISP (Anderson et al., 2006), followed by a Kruskal-Wallis test on ranks supported by Conover many-to-one posthoc test. A significant analysis of dispersion may indicate that the statistical difference in the PERMANOVA may be related to heterogeny of dispersion rather than differences in the group locations (in the euclidian space). A Tukey honestly significant differences (Tukey HSD) test was performed on the dispersion data with statistical significance to reveal the groups responsible for the difference in dispersion. Hypothesis testing between groups with heterogeneous dispersion was disregarded. Analyses were run in *R* software version 3.6.1. (R Core Team, 2020), using the packages *vegan* (Oksanen et al., 2019), *PMCMRplus* (Pohlert, 2018) and *ggplot2* (Wickham, 2016). Significance was set to ≤ 0.05, and pairwise comparisons were adjusted according to Benjamini and Hochberg (1995).

Data were standardized prior to the PERMANOVA based on *z-scores:*

$$z_i = \frac{x_i - \overline{x}}{\sigma_x}$$

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The PERMANOVA was calculated with solvents and doses nested within solvents in order to test the following hypothesis:

- H_0^1: The oxidative stress response, cellular energy allocation and photobiological response is similar between the different solvents used;
- H_0^2: The oxidative stress response, cellular energy allocation and photobiological response are similar between the different doses of each solvent used.

The LOEC was calculated as the lowest dose of solvent with significant differences between the control group. The NOEC (when possible) was calculated as the dose immediately infra the LOEC.

Validation was assured by Mann-Whitney tests between T0 and the 96 h control, with Benjamini and Hochberg adjustments.

3. Results

No mortality was observed in the control groups. During the trial, mortality occurred uniquely in the highest ethanol dose where the tissue of every fragment began to decompose at 72h of exposure.

The trial was validated, as no statistical differences were found when comparing T0 and the 96h control.

PERMANOVA results were significant for solvents [$F_{(2, 97)} = 3.78$, $p = 0.002$] and doses within each solvent [$F_{(17, 83)} = 3.63$, $p < 0.001$]. The analysis of dispersion was not significant for solvents [$F_{(2, 97)} = 2.88$, $p = 0.055$], and doses within ethanol [$F_{(5, 24)} = 1.07$, $p = 0.397$] and DMSO [$F_{(6, 28)} = 0.78$, $p = 0.595$] but was significant for doses within methanol [$F_{(6, 28)} = 3.34$, $p = 0.015$]. Methanol at 0.3 mL L^{-1} ($M = 2.50$, SD =

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1.11) showed significantly higher dispersion than Control (M = 1.54, SD = 0.64), but the remaining pairwaise comparisons of dispersion were not significant.

3.1. Oxidative stress response and cellular energy allocation

When comparing the response between different solvents, *Zoanthus* sp. exhibited significantly different responses, namely in GST [$\chi^2_{(2)}$ = 13.67, p = 0.001)], LPO [$\chi^2_{(2)}$ = 17.76, p < 0.001)] and ETS [$\chi^2_{(2)}$ = 6.03, p = 0.049)]. The global GST activity was significantly lower when exposed to methanol (*Mdn* = 16.65) and DMSO (*Mdn* = 15.73), than to ethanol (*Mdn* = 21.38). LPO was significantly lower for corals exposed to DMSO (*Mdn* = 0.52) than to ethanol (*Mdn* = 0.82) and methanol (*Mdn* = 0.73).

Kruskal-Wallis revealed that ethanol exposure affected corals GST activity [$\chi^2_{(5)}$ = 11.80, p = 0.038)], methanol affected GST [($\chi^2_{(6)}$ = 15.43, p = 0.017], and DMSO affected CAT [($\chi^2_{(5)}$ = 15.17, p = 0.019], GST [($\chi^2_{(6)}$ = 17.07, p = 0.009] and tGSH [$\chi^2_{(6)}$ = 18.53, p = 0.005]. Despite the Kruskal-Wallis results, no significant pairwise comparisons were found for the GST activity exposed to ethanol. On the other hand, GST activity significantly decreased at 0.1 mL L^{-1} (Median= 14.35) and 2.9 mL L^{-1} (*Mdn* = 14.17) methanol when compared to the control (*Mdn* = 20.91). When exposed to DMSO, GST activity significantly decreased at 0.01 mL L^{-1} (*Mdn* = 13.82) and 0.03 mL L^{-1} (*Mdn* = 10.21) when compared with the control (*Mdn* = 20.91). No statistical differences were found for CAT activity and tGSH exposed to DMSO (Fig. 1).

3.2. Photobiology

Solvents induced significantly different photobiological responses of *Zoanthus* sp., namely in ETRmax [$\chi^2_{(2)}$ = 8.05, p = 0.018)] and Ek [$\chi^2_{(2)}$ = 11.07, p = 0.004)]. ETRmax was significantly lower with ethanol (*Mdn* = 11.71) than with DMSO (*Mdn* =

16.30). Ek was significantly higher for DMSO (*Mdn* = 165.79) than ethanol (*Mdn* = 133.22) and methanol (*Mdn* = 134.46).

The Kruskal-Wallis tests revealed that ethanol affected ETRmax [$\chi^2_{(5)}$ = 13.12, p = 0.022)] and F_v/F_m [$\chi^2_{(5)}$ = 15.61, p = 0.008)], methanol affected α [$\chi^2_{(6)}$ = 20.40, p = 0.002)], ETRmax [$\chi^2_{(6)}$ = 14.83, p = 0.022)] and F_v/F_m [$\chi^2_{(6)}$ = 16.27, p = 0.012)], and DMSO affected α [$\chi^2_{(6)}$ = 14.87, p = 0.021)] and F_v/F_m [$\chi^2_{(6)}$ = 14.79, p = 0.022)].

In pairwise comparisons, ETRmax decreased in corals exposed to 0.1 mL L^{-1} (*Mdn* = 11.01), 0.3 mL L^{-1} (*Mdn* = 8.19) and 0.9 mL L^{-1} (*Mdn* = 10.54) ethanol, when compared to the control (*Mdn* = 17.08) and F_v/F_m was compromised at 0.03 mL L^{-1} (*Mdn* = 0.635), 0.1 mL L^{-1} (*Mdn* = 0.625), 0.3 mL L^{-1} (*Mdn* = 0.523) and 0.9 mL L-1 (*Mdn* = 0.564) ethanol when compared to the control (*Mdn* = 0.663). No significant pairwise comparisons were found for α when exposed to methanol. ETRmax decreased at 0.1 mL L^{-1} (*Mdn* = 5.91) methanol when compared to the control (*Mdn* = 17.08) and F_v/F_m was negatively affected at 0.01 mL L^{-1} (*Mdn* = 0.643), 0.03 mL L^{-1} (*Mdn* = 0.632), 0.1 mL L^{-1} (*Mdn* = 0.653), 0.3 mL L^{-1} (*Mdn* = 0.612) and 2.9 mL L^{-1} (*Mdn* = 0.611) methanol, comparing to the control (*Mdn* = 0.663). DMSO negatively affected α at 0.1 mL L^{-1} (*Mdn* = 0.083) and 0.3 mL L^{-1} (*Mdn* = 0.084), when comparing to the control (*Mdn* = 0.226), as well as F_v/F_m at 0.3 mL L^{-1} (*Mdn* = 0.639) and 0.9 mL L^{-1} (*Mdn* = 0.611) DMSO, comparing to the control (*Mdn* = 0.663).

In summary, the zoanthid LOEC was 0.01 mL L^{-1}, affecting F_v/F_m and GST activity, when exposed to methanol and DMSO, respectively, while for ethanol was 0.03 mL L^{-1}, influencing F_v/F_m. The no observed effect concentration (NOEC) for ethanol exposure was 0.01 ml L^{-1}. NOEC could not be calculated for methanol and DMSO, as the lowest dose tested showed significant differences from the control (Fig. 2 and 3).

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4. Discussion

Our work enabled us to assess the response to three of the most used solvents in ecotoxicology in important physiological parameters of *Zoanthus* sp. Previous works assessing the effects of organic solvents in marine organisms are insufficient for the solvents hereby tested. The closest comparison to our study is the work of Okumura et al. (2001) that tested solvents with different species of marine algae in 96 h experiments and reported several NOECs for ethanol (1.78 x 10^{-5} to 0.013 mL L^{-1}), methanol (3.05 x 10^{-5} to 0.018 mL L^{-1}) and DMSO (3.81 x 10^{-4} to 0.011 mL L^{-1}). But experiments with daphnids revealed an EC_{50} of 0.03 mL L^{-1} for ethanol, 0.04 mL L^{-1} for methanol and 0.07 mL L^{-1} for DMSO in 24 h tests (Kalčíková et al., 2012) and tests with grass shrimp (*Palaemonetes pugio*) during 4 days showed an LC_{50} of 0.02 mL L^{-1} for ethanol and DMSO. Other studies are coherent with the abovementioned studies (Kaviraj et al., 2004; Young et al., 2019). These results demonstrate lower sensitivity of *Zoanthus* sp. to organic solvents. Nonetheless, our results are not directly comparable to previous works, as corals show a completely different complexity when compared to shrimp, daphnids or algae. Data shining a light on the effect of organic solvents in marine organisms, especially in invertebrates, is rather scarce. The comparability between our work, with the work of others is, therefore, very difficult, affirming the grave requirement for more, and more detailed data on the effects of solvents on marine invertebrates, particularly, in photosynthetic corals.

Ethanol did not seem to affect the oxidative stress response or the cellular energy allocation in pairwise comparisons, even though Kruskal-Wallis reported significant differences in the distributions of the GST activity within the various doses. CAT and GST activities, as well as tGSH remained mostly stable, without significant activation

or inhibition, and no noticeable change in ETS activity (despite tendentially greater in higher doses). The same was true for the lipidic damage throughout the experiment (see Fig. 1). These findings are quite impressive, given the fact that ethanol was the only solvent causing mortality. This can be partially explained by the ethanol metabolism that results in acetaldehyde, which is known to bind to the reduced glutathione (GSH) and to reduce the synthesis of glutathione (Anni et al., 2003). In this scenario, the GSH production is hindered even in the presence of ROS. Nonetheless, tGSH response showed a non-significant downward trend, but mostly remained stable where it should have taken a toll. Additionally, F_v/F_m and ETR_{max} were affected at 0.03 and 0.1 mL L^{-1}, respectively, contrasting with the almost absent response of the antioxidant defences (see Fig. 2 and 3). The worsened photobiologic parameters, without significant alterations to the coral antioxidant response may be related to the upregulation of bacteria (de Bruyn et al., 2020) and the alteration of the energy flux of the holobiont by ethanol degradation and metabolization.

Methanol, at 0.1 mL L^{-1} caused GST activity decrease without significant differences in other metabolic parameters or LPO. The photosynthetic apparatus was affected at 0.01 mL L^{-1} where F_v/F_m had a significant decrease. The zoanthid exhibited a response to the presence of methanol, mainly in the lower doses, but the sift in the response in the higher doses is intriguing (see Fig. 3). What appears to be the primary cause for methanol toxicity is the accumulation of formate, through the oxidation of methanol, which seems to damper the electron transport chain (Tephly, 1991). On the other hand, much alike ethanol, methanol can be used as a carbon source for an array of microorganisms (Dinasquet et al., 2018; Dixon et al., 2011a; Murrell et al., 1993) that are likely present in the coral holobiont. Methanol is also reported as stimulatory to microalgae (Kotzabasis et al., 1999), which can in some extent hamper more severe

toxicity in the higher doses, as the balance between the toxicity and the zooxanthellae or bacterial stimulation, could be advantageous to the coral.

Overall DMSO seemed to be the least toxic of the three solvents. Comparisons between the three solvents revealed that GST activity and LPO was significantly lower for DMSO than for the other solvents, and ETRmax and Ek were significantly higher which are signs of a weaker effect on coral oxidative stress response and also on photophysiology. Comparing doses, DMSO caused a GST activity decrease at 0.01 and 0.03 mL L^{-1}, without significant increase of other metabolic parameters or LPO. The photosynthetic apparatus was affected at 0.1 mL L^{-1}, where α significantly decreased, showing a slower initial slope, which can mean lower efficiency to harvest lower intensity radiation. Still, the trend shift in the photobiological response to DMSO was interesting, particularly in the highest dose as F_v/F_m was non-significant, and similar to the lower doses, if not for one outlier (see Fig. 3). In the same extent, the higher dose caused higher ETR_{max}, showing the ability to utilize higher light intensity in the photosynthesis (see Fig. 2). DMSO is no alien to corals, as it is part of the sulfur cycle, and ubiquitous in the oceans. Tropical corals are exposed to DMSO directly, or indirectly as dimethylsulfoniopropionate (DMSP) or dimethylsulfide (DMS), one of the most abundant organic compounds in the marine environment (Nightingale and Liss, 2003). DMSP occurs naturally in corals, or rather in its dinoflagellate symbionts (Ishida, 1996) and is readily degraded into DMS (Raina et al., 2009), which can be subsequently metabolized into DMSO as reviewed by Schäfer et al. (2010). Therefore, when compared to the abovementioned solvents, DMSO is the least foreign to corals. Nevertheless, DMSO is known to inhibit antioxidant enzymes, such as CAT, as shown by Finkelstein and Benevenga (1986), but that was not the case in our study, as there are no significant pairwise comparisons in CAT. Still, DMSO clearly showed impaired

GST activity in the lower tested dose (0.01 mL L^{-1}), and the pattern of CAT was similar to GST. DMSO and its sulfuric relatives can be metabolized by DMS monooxygenase into formaldehyde (De Bont et al., 1981). Subsequently, formaldehyde can be converted into CO_2, which may feed the Calvin cycle, and therefore, induce similar effects as those of ethanol or methanol (Schmitz et al., 2000), in zooxanthellae or bacteria.

Coral symbiosis is often referred to as coral-algae associations (unicellular dinoflagellate symbionts of the genus *Symbiodinium*), but the prokaryotic diversity associated with corals is considerably higher. The photosynthetic symbionts (usually termed as zooxanthellae), however, play a fundamental role in coral metabolism, acting as a vital source of energy. In turn, the coral host delivers carbon sources, like dissolved inorganic carbon (DIC) to the zooxanthellae (Goiran et al., 1996), as diffusion of CO_2 in aquatic systems is much slower when compared to that in the atmosphere. Some algae are also able to exploit organic solvents, such as ethanol and methanol and even DMSO as a source of carbon (De Bont et al., 1981; Kotzabasis et al., 1999; Matsudo et al., 2017; Schmitz et al., 2000), which could be a possible mechanism for zooxanthellae to cope with naturally oligotrophic environments (Dixon et al., 2011b). As a holobiont, a vast array of microorganisms can take part in the metabolic mechanisms. Many bacteria associated with corals are related to the nutrient cycle in oligotrophic environments (Wegley et al., 2007), and are exceptionally efficient at assimilating limiting nutrients (Cavender-Bares et al., 2001). Therefore, solvent degradation may take part in various coral symbionts, such as bacteria, archaea, fungi, algae or viruses (Rohwer et al., 2002; Toledo-Hernández et al., 2008; Wilson et al., 2005) blurring what could have been a more transparent process. More so, coral holobiont is complex and depends on environmental conditions leading to substantial biotic and abiotic variation (Ainsworth

et al., 2011). Despite that, our data are adequate to propose a baseline for the use of solvents in coral ecotoxicology.

5. Conclusion

Our results showed that the LOEC for ethanol was 0.03 mL L^{-1}, while for methanol and DMSO was 0.01 mL L^{-1}. Despite a lower LOEC, comparisons between solvents revealed lower oxidative stress response and damage with DMSO and a more robust photophysiological response. Solvent toxicity can be ordered as methanol ≈ DMSO > ethanol . *Zoanthus* sp. seemed more sensitive to methanol and DMSO, with photobiologic distress at the lowest tested dose of methanol, as well as an antioxidant response at the lowest tested dose of DMSO. Ethanol, despite having higher LOEC, was the only solvent causing mortality. Nevertheless, despite the evidence that the solvents used can affect the oxidant/antioxidant status and photobiologic response, no signs of increased cellular damage (measured as LPO) or changes on aerobic energy production (as ETS activity) were evident. The present study allowed us to point out ethanol as the preferable solvent to use in coral ecotoxicology, as no effects were found in the lowest dose. A crucial baseline for the three solvents was assessed, pivotal for the viability of future studies. Furthermore, *Zoanthus* sp. responses should be carefully addressed when extrapolating to other photosynthetic corals, as this genus is expected to be less sensitive to stressors.

Future studies should explore other coral taxa and solvents to enlighten the tolerance of various taxonomic groups (*e.g.* Scleractinia and Alcyonacea) to organic solvents.

6. Acknowledgments

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CRediT author statement

DAMS, ACMR, PBH, CRM, MP, RJMR: Conceptualization, Methodology, Data curation; DAMS, PBH: Formal analysis; DAMS, APLC, ACMR, SFSP, CRM, RJMR: Investigation; AMVMS, CRM, MP, RJMR: Resources, Supervision. All authors: Writing, Reviewing.

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Declaration of interests

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

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

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Fig. 1 – Oxidative stress and cellular energy allocation of *Zoanthus* sp. Mean ± SD of: **A -** CAT activity (μmol min^{-1} mg $protein^{-1}$); **B** - GST activity (nmol min^{-1} mg $protein^{-1}$); **C** - tGSH (μM mg $protein^{-1}$); **D** - LPO (TBARS $nmol^{-1}$ g ww^{-1}); **E** - estimated ETS (mJ h^{-1} mg $tissue^{-1}$) for the three tested solvents (E – ethanol, M – methanol and D – DMSO), with the six doses (0, 0.01, 0.03, 0.1, 0.3, 0.9, 2.9). Statistical differences are marked with asterisks (*) between the respective dose and the control ($p < 0.05$).

Fig. 2 – Dark-acclimated rapid light curves with mean ETR in every pulse, grouped by solvent and dose. Curves resulted from increasing saturating light pulses, spaced by 10 seconds with irradiance ranging from 0 - 1500 μmol m^{-2} s^{-1}. From RLC we can calculate ETR_{max}, E_k and α.

Fig. 3 – Boxplots with maximum quantum yield (F_vF_m), grouped by solvent and dose. Lower and upper boundaries are the 25^{th} and 75^{th} percentile, the horizontal line represents the median, the mean is denoted by the dark dot, and the light grey dots represent the outliers (± 1.5 x IQR). Statistical differences are marked with asterisks (*) between the respective dose and the control ($p < 0.05$).

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Graphical abstract

Highlights

The effect of organic solvents in photosynthetic corals was unassessed until now;
Zoanthus sp. was exposed to ethanol, methanol and dimethyl sulfoxide;
Oxidative stress, energetic budget and photophysiological profile were measured;
LOEC for ethanol was 0.03 mL L^{-1}, for methanol 0.01 mL L^{-1} and DMSO 0.01 mL L^{-1};
Ethanol did not affect zoanthids three times above the general recommendations

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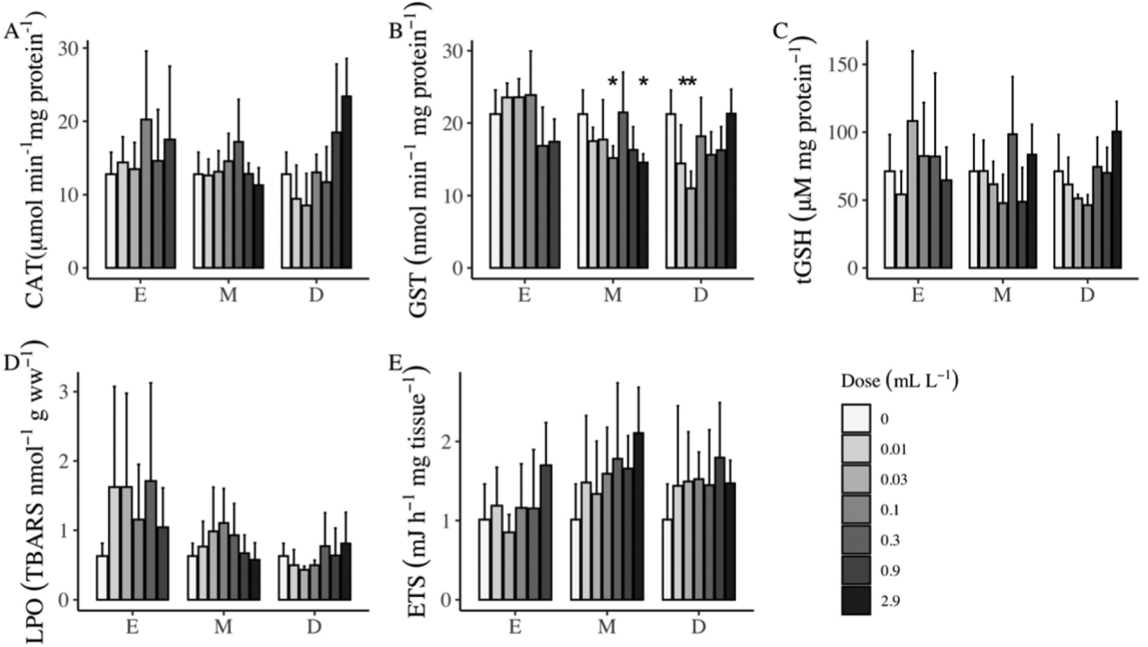
A
CAT(μmol min-1mg protein-1)
B
GST (nmol min-1 mg protein-1)
C
tGSH (μM mg protein-1)
D
LPO (TBARS nmol-1 g ww-1)
E
ETS (mJ h-1 mg tissue-1)
E
M
D
*
*
**
Dose (mL L-1)
0
0.01
0.03
0.1
0.3
0.9
2.9

Figure 1

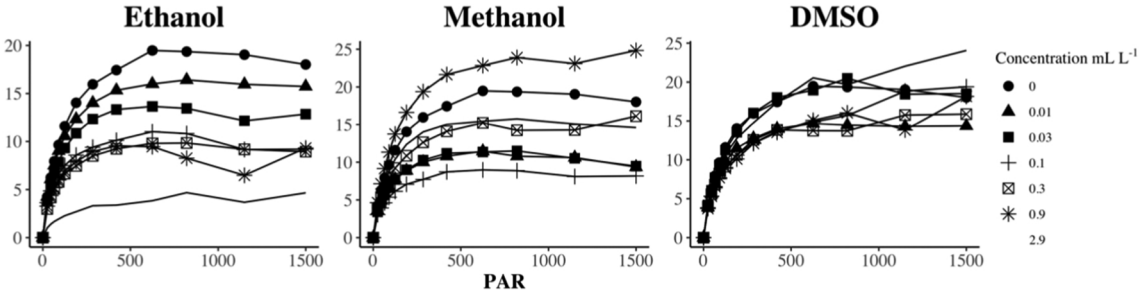
Ethanol
Methanol
DMSO
PAR
Concentration mL L-1
0
0.01
0.03
0.1
0.3
0.9
2.9

Figure 2

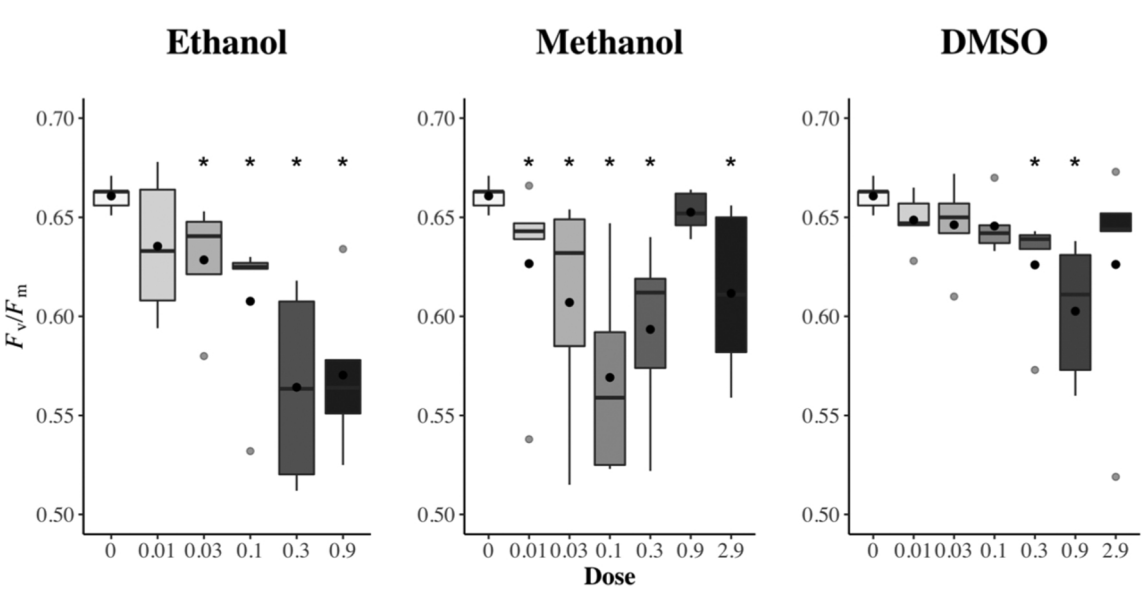
Ethanol
Methanol
DMSO
F_v/F_m
0.70
0.65
0.60
0.55
0.50
0
0.01
0.03
0.1
0.3
0.9
2.9
Dose
*

Figure 3