Universidade de Aveiro Departamento de Biologia

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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 em Pós-Doutoramento do Departamento de Biologia e CESAM, Universidade de Aveiro) e co-orientação científica do Doutor João Pestana (Investigador Auxiliar do Departamento de Biologia e CESAM, Universidade de Aveiro) e do Doutor Ricardo Calado (Investigador Principal do Departamento de Biologia e CESAM, Universidade de Aveiro).

Dedicada à minha avó Teresa, que apesar de já não estar presente continua sempre comigo através de todos os valores que me transmitiu.

o júri

presidente

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palavras-chave protectores solares; produtos de cuidado pessoal; Benzofenona-3; 4-MBC; ecotoxicologia marinha

resumo Os recifes de coral são ecossistemas extremamente complexos e produtivos, que fornecem habitat a milhares de espécies marinhas. Apesar da sua importância ecológica e socioeconómica, os recifes de coral estão globalmente ameaçados por impactos naturais e antropogénicos. A descarga de resíduos domésticos e industriais contribui para a introdução de xenobióticos, nomeadamente filtros UV orgânicos, nestes ecossistemas marinhos, como os recifes de corais, contribuindo para sua degradação nas últimas décadas. Estima-se que aproximadamente 40 % dos recifes de corais localizados ao longo das áreas costeiras correm o risco de exposição a filtros UV orgânicos, como a Benzophenona-3 (BP-3) e 3- (4-metilbenzilideno) cânfora (4-MBC), amplamente usados em protetores solares e produtos de cuidados pessoais. Por conseguinte, é importante avaliar os efeitos destes contaminantes emergentes nas espécies que habitam as áreas mais afetadas, nomeadamente as zonas intertidais. O género Zoanthus (Anthozoa: Hexacorallia) contém inúmeras espécies abundantes em recifes de corais e áreas intertidais de regiões tropicais e subtropicais. Estes corais fotossintéticos, que vivem em simbiose com dinoflagelados do género Symbiodinium, podem ter potencial aplicação como organismos indicadores. A pesquisa existente em ecologia de zoantídeos é, no entanto, escassa, em comparação com outros grupos de cnidários. Neste estudo, procuramos avaliar os potenciais efeitos nefastos da exposição a curto prazo de Zoanthus sp. a BP-3 e 4-MBC. Expuseram-se mini colónias (4 a 6 pólipos) de Zoanthus sp. a 4 concentrações (0.5; 1; 2 e 4 mg/L) de BP-3 e 4-MBC durante 96 h. Após exposição, as mini colónias foram fotografadas para uma avaliação da resposta comportamental dos pólipos, mediu-se in vivo a eficiência fotossintética do fotossistema II, através da fluorometria de pulso modulado (PAM) e, finalmente, as células de Symbiodinium sp. foram quantificadas e normalizadas para o peso seco de Zoanthus sp.. Os resultados sugeriram que a exposição de Zoanthus sp. a concentrações sub-letais e ambientalmente relevantes de BP-3 e 4-MBC, induziu reações comportamentais nos pólipos (aumento do número de pólipos fechados com o aumento das concentrações), diminuição da eficiência fotossintética e do número de endossimbiontes. Para além da alteração comportamental dos pólipos, os filtros UV orgânicos testados provaram ter potencial para induzir o branqueamento de corais. Estudos ecotoxicológicos adicionais devem ser realizados com outros compostos e diferentes espécies de corais, para avaliar o efeito destes contaminantes emergentes em recifes de corais e também para a identificação de filtros UV menos prejudiciais ao meio ambiente.

keywords sunscreens; personal care products; Benzophenone-3; 4-MBC; marine ecotoxicology;

abstract

Coral reefs are extremely complex and productive ecosystems, providing habitat for thousands of marine species. Despite their ecological and socio-economic importance, coral reefs are globally threatened by natural and anthropogenic impacts. The discharge of domestic and industrial wastes contributes for the introduction of xenobiotics, namely organic UV filters, in marine ecosystems such as coral reefs, contributing for their degradation over the past few decades. It is estimated that approximately 40 % of coral reefs located along coastal areas are at risk of exposure to organic UV filters such as Benzophenone-3 (BP-3) and 3-(4-methylbenzylidene) camphor (4-MBC), two widely used compounds in sunscreen lotions and personal-care products. It is therefore important to evaluate the effects of these emerging contaminants on local species inhabiting the more affected areas, namely the intertidal environments. The genus Zoanthus (Anthozoa: Hexacorallia) contains numerous species abundant in coral reefs and intertidal areas of tropical and sub-tropical regions. These photosynthetic corals, which live in symbiosis with dinoflagellates of genus Symbiodinium, might have potential application as indicator organisms. The existing research in zoanthids ecology is however scarce in comparison with other cnidarian groups. In this study, we aimed to evaluate the effects of short-term exposure of Zoanthus sp. to BP-3 and 4-MBC. Zoanthus sp. mini colonies (4 – 6 polyps) were exposed to 4 concentrations (0.5; 1, 2 and 4 mg/L) of BP-3 and 4-MBC during 96 h. After exposure, mini colonies were photographed for polyp behavioral response evaluation, the photosynthetic efficiency of photosystem II was measured in vivo, through PAM fluorometry, and finally, the Symbiodinium sp. cells were quantified and normalized to Zoanthus sp. dry weight. Results suggested that Zoanthus sp. exposure to sub-lethal and environmentally relevant concentrations of BP-3 and 4-MBC induced behavioral reactions in the polyps (increase of closed polyps with increased concentrations), decreased photosynthetic efficiency and the number of endosymbionts. Beside the polyp behavioural response, tested organic UV filters proven to have potential to induce coral bleaching. Further ecotoxicological studies should be undertaken with other compounds and with different coral species, to evaluate the effect of these emergent contaminants on coral reefs, and identify UV filter compounds less harmful to the environment.

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Chapter I

General Introduction

1. General introduction

1.1. Coral reefs

Coral reefs are known as the rainforests of the sea, for being one of the most diverse and productive marine ecosystems, providing shelter, food, spawning and nursery habitat to several species of fish, sponges, cnidarians, worms, crustaceans, molluscs, echinoderms, algae, sea snakes, sea turtles, and sea squirts (Reaka-Kudla, 1997; Ruppert et al., 2004).

Besides their ecological relevance, coral reefs also have an important socio-economic value for several countries, estimated in more than 20 trillion dollars annually (Costanza et al., 1997), through commercial fish stocks maintenance (Costanza et al., 1997) and tourism activities, providing a high impact on local employability and economies around the world (Brander et al., 2007). Despite their huge ecological and economic importance, coral reefs are suffering a serious decline. It is estimated that 30 % are already highly damaged and in 2030 close to 60 % may be lost (Hughes et al., 2003a).

Corals are affected by a number of natural stressors, such as environmental factors, predation, competition, or natural diseases, being bacteria considered the main cause of diseases in these organisms (Bourne et al., 2009; Cooney et al., 2002; Pantos et al., 2003; Sheridan et al., 2013). Among the competition effects, phase shifts processes, characterized by dominance of non-reef-building organisms (e.g. algae or soft corals) and the consequent decrease in coral abundance or cover (Done, 1992), can lead to changes in biodiversity (Done, 1999) and threaten ecosystem stability (Mumby, 2009). The natural stressors can also substantially affect corals. El Nino events and global warming contribute for ocean acidification and rising water temperature, which can induce massive bleaching events (loss of endosymbiotic dinoflagellates; figure 1) (Hoegh-Guldberg, 1999; Lesser et al., 1990).

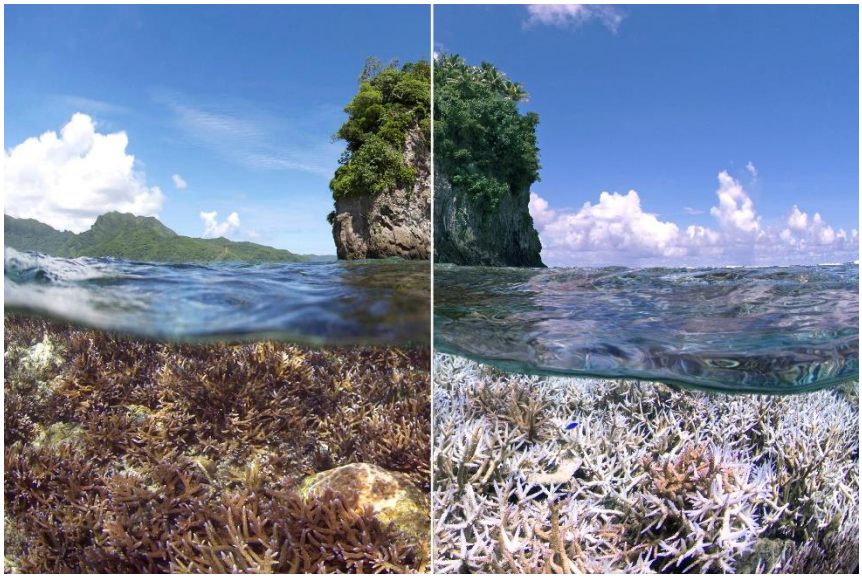

Figure 1: Massive bleaching event (posted by Courtney Mattison of Mission Blue in Ocean Views on October 29, 2015).

Anthropogenic pressures such as destructive fishing practices (use of explosives and cyanide), irresponsible tourism activities and urban and industrial pollution might also negatively affect coral reefs (Hughes et al., 2003b). Amongst xenobiotics released to coastal areas and coral reefs, some constituents of sunscreens, the organic UV filters are of special importance due to their widespread occurrence and potential ecological effects.

1.2. Corals

"Coral" is a common designation to identify some cnidarians of class Anthozoa.

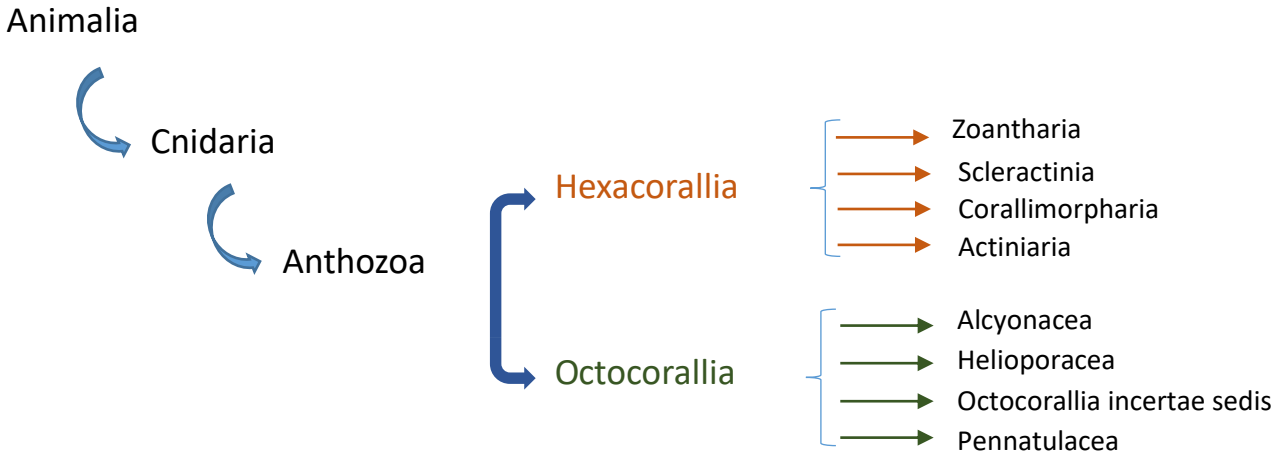

Figure 2: Corals phylogeny schematic representation (WoRMS, 2017).

Anatomically, corals do not have a central nervous system and their polyps have two epithelial cell layers, epidermis and gastrodermis. Between these two layers is the mesoglea (involved in phagocytosis processes). The mouth is surrounded by tentacles, and separates the gastro-vascular cavity from the exterior (Rocha, 2013).

Coral species that live in symbiosis with *Symbiodinium* sp. have a mixotrophic feeding, once their nutrition is both autotrophic and heterotrophic. In autotrophy, the coral provides protection, nutrients and carbon dioxide used for photosynthesis, whereas the symbiont dinoflagellate reciprocates its host with amino acids, photosynthetically derived carbon compounds and saturated and polyunsaturated fatty acids (Muscatine and Porter, 1977; Papina et al., 2003). Through heterotrophy, coral feeds on phytoplankton, zooplankton, bacteria, or organic particles in a more passive way (water intake through the mouth) or more actively capturing food with the tentacles (mediated by the stinging cells). Food intake is decomposed by enzymes in the gastro-vascular cavity with posterior intracellular digestion (Ferrier-Pagès et al., 2011; Houlbrèque and Ferrier-Pagès, 2009).

Corals are informally divided into two main groups, hard or stony corals, and soft corals. Hard corals contain a skeleton formed by calcium carbonate that gives support to the coral colony, while soft corals do not.

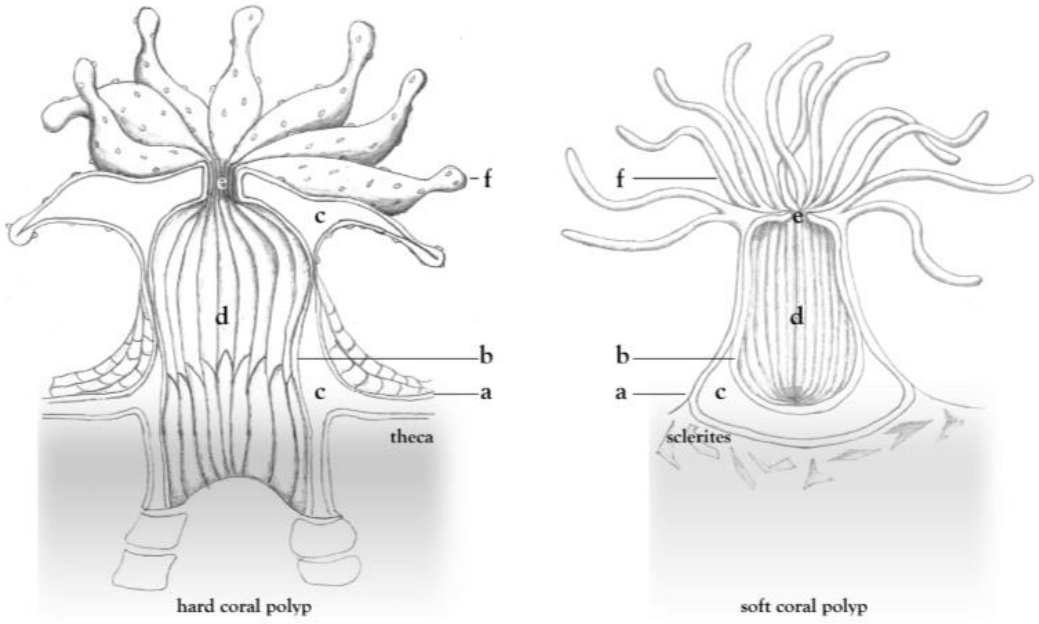

Figure 3: Hard and soft coral polyp illustrations: a – epidermis or ectoderm, b – gastrodermis or endoderm, c – mesoglea, d – gastrovascular cavity, e – mouth, f – tentacles. Image addapted from Rocha (2013).

The genus *Zoanthus* (Anthozoa: Hexacorallia) includes cosmopolitan species, commonly found in coral reefs and intertidal zones of tropical and sub-tropical regions, living in symbiosis with dinoflagellates of genus *Symbiodinium* (Reimer et al., 2006). *Zoanthus* sp. might be good models to evaluate the effect of contaminants since it forms a colony bound by a single tissue, being each individual polyp a genetic clone of the rest of the colony, allowing the assessment of effects without confounding effects arising from genetic variability. Moreover, they are easy to reproduce asexually and maintain in laboratory, having polyps with good dimensions for behavioral analysis (Acosta et al., 2005; Ryland, 1997).

1.1. Organic UV filters and the marine environment

People are becoming concerned about protecting themselves from the effects of sun exposure (sunburns, photo aging, DNA damage and potential cancerous skin lesions), thus leading to a greater use of products with organic ultraviolet filters (UV filters) in their constitution.

Organic UV filters have been developed to absorb or block ultraviolet radiation (UV-A, UV-B), protecting the skin from solar radiation through dermal application, and materials from degradation (Murphy, 1999). Sunscreens are divided into three categories: products composed with UV organic chemical absorbers, metal oxides (inorganic UV filters) or products that use of a combination of organic and inorganic agents (Dransfield, 2000; Gasparro et al., 1998; Sambandan and Ratner, 2011).

With an annual average consumption per capita of 20 mL (with a worldwide increase of 7 % per year), the economy of the sunscreens market is growing with an estimated profit of 7 billion euros in 2014 (Osterwalder et al., 2014). Organic UV filters are increasingly used in sunscreens and in other personal care products (PCPs) such as creams, lipsticks, shampoos or insect repellants, being considered important emerging pollutants due to its increasing demand and high production volume (CIR, 2005; Hauri et al., 2003).

Among organic UV filters, Benzophenone-3 (BP-3, oxybenzone, 2-hydroxy-4-methoxybenzophenone) and 4-methylbenzylidene camphor (4-MBC) are two of the most commonly used chemicals, frequently reported as pollutants of aquatic systems (Balmer et al., 2005; Gago-Ferrero et al., 2011; Kameda et al., 2011; Ramos et al., 2015). These chemicals can be indirectly transferred to the aquatic environment through wastewater treatment plant discharges (WWTP) or directly, when released from the skin into the environment during recreational activities (Eichenseher, 2006; Poiger et al., 2004). It is estimated that about 6.000 and 14.000 tons of sunscreen lotion are released into coral reefs every year, putting approximately 40 % of coral reefs located in coastal areas at risk of exposure (Danovaro et al., 2008; Shaath, 2005). These substances act as pseudo-persistent pollutants, since their half-life in seawater is of several months and

the contamination of the exposed sites can often be renewed (Vione et al., 2013). BP-3 and 4-MBC due to their lipophilic characteristics (log Kow of 3.79 and 4.95 respectively) and high stability (Gago-Ferrero et al., 2012) tend to be more accumulated in soils and particles and more concentrated in the microlayer surface (Tovar-Sánchez et al., 2013a). Also, potential for bioaccumulation and biomagnification has been suggested for organic UV filters such as BP-3 and 4-MBC (Giokas et al., 2007).

The reefs closest to the coast, usually used for tourist purposes such as bathing areas or for recreational dives, are usually more exposed to these pollutants. Although there is no exhaustive information on this problematic, it is known that in coral reefs located from 300 to 600 m away from public swimming beaches in Okinawa, BP-3 concentrations ranged from 4×10^{-7} and 3.8×10^{-6} μg L^{-1} (Tashiro and Kameda, 2013) and in South America, also in sediments near coral reefs, concentrations between 0.054 and 0.578 μg Kg^{-1} were reported (Barón et al., 2013). The highest concentration of BP-3 found in literature was reported for nearshore U.S. Virgin Islands, ranging from 75 μg L^{-1} to 1.4 mg L^{-1} (Downs et al., 2016). Also nearshore, in Norway concentrations of 4-MBC in seawater reached 0.488 μg L^{-1} (Langford et al., 2008). Significant concentrations of organic UV filters have been also measured in lakes, swimming pools, wastewaters, sediments, rivers and sewage sludge (Barón et al., 2013; Downs et al., 2016; Fent et al., 2010, 2008).

Mostly, organic UV filters are reported to act as neurotoxicants and endocrine disruptors that induce reproductive pathologies, vitellogenin induction and reduction of the reproductive fitness (Blüthgen et al., 2012; Coronado et al., 2008; Kunz and Fent, 2006; Schlumpf et al., 2001; Schmitt et al., 2008).

In vertebrates, exposure to BP-3 led to a reduction in the number of fish eggs produced as well as egg hatchings (Coronado et al., 2008; Kunz and Fent, 2006). Also, a potential antiandrogenic activity has been observed in two different life stages of zebrafish when exposed to BP-3 (Blüthgen et al., 2012). Several benzophenones, including BP-3, have been shown to induce oxidative stress in freshwater fish, *Carassius auratus* and also histopathological lesions (Liu et al., 2015a). Exposure to 4-MBC induced muscle and neuronal defects in Zebrafish (*Danio rerio*) embryos, which can therefore cause developmental defects (Li et al., 2016).

Exposure to BP-3 and 4-MBC, has shown to harm the growth and development in invertebrates (Campos et al., 2017b; Paredes et al., 2014), to alter the activity of hormonal genes and cause a disruptive effect in the initial genomic response to ecdysteroids in the insect *Chironomus riparius* (Ozáez et al., 2014, 2013a), to inhibit feeding in *Sericostoma vittatum* larvae (Campos et al., 2017a) and decrease the somatic growth of *Daphnia magna* (Sieratowicz et al., 2011). It has been reported that 4-MBC also disrupts normal endocrine function and development in rats, sea urchins, aquatic molluscs and insects (Ozáez et al., 2013a; Schlumpf et al., 2004; Schmitt et al., 2008; Torres et al., 2016).

Although the ecotoxicological data on the ecological effects of these compounds on marine invertebrates is scarce, it is known that there is a negative effect on corals. It was demonstrated that both BP-3 and 4-MBC promote lytic viral cycle in *Symbiodinium* sp. (symbiotic dinoflagellates) in concentrations of 10 µL L^{-1} of sunscreen containing a percentage concentration of the respective UV filters allowed in sunscreen formulations, 6 and 3 % of BP-3 and 4-MBC respectively, causing viral infections, which lead to complete bleaching of hard corals (*Acropora* sp*., Stylophora pistillata and Millepora complanata*) (Danovaro et al., 2008). Another study has also shown that BP-3 causes bleaching of a scleractinian coral (*Stylophora pistillata*) in larval stage by promoting the ossification of the planula, being therefore a skeletal endocrine disruptor (Downs et al., 2016).

Taking all these into account, both pollutants have entered European Union's SIN List (Substitute It Now) as a substance of "Very High Concern" (ChemSec., 2017).

1.2. Objectives

The aim of this thesis was to evaluate the effect of two of the main compounds used in sunscreens (organic UV filters), BP-3 and 4-MBC, in the photobiology and behavioral reaction of *Zoanthus* sp. polyps, which are known to react to stressors by retracting and closing the oral disc.

We formulated as null hypothesis that organic UV filters, BP-3 and 4-MBC, do not impair the photobiology, endosymbiont density nor the behavioral reaction of *Zoanthus* sp. polyps.

Mini colonies of *Zoanthus* sp. were exposed to a gradient of concentrations of BP-3 and 4-MBC in laboratory trials. Effects were evaluated in terms of behavioral response, photosynthetic efficiency of photosystem II and density of *Symbiodinium* sp. quantification.

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Chapter II

Effects of the organic UV filters, oxybenzone and 4-methylbenzylidene champor, on the photobiology of the hexacoral *Zoanthus* sp.

2. Effects of the organic- UV filters, oxybenzone and 4-methylbenzylidene, on the photobiology of the hexacoral *Zoanthus* sp.

2.1. Abstract

Benzophenone-3 (BP-3; oxybenzone) and 4-methylbenzylidene camphor (4-MBC) are organic ultraviolet filters (UV filters), considered important emerging pollutants and used for protection against radiation in sunscreens, personal care products (PCPs) and other materials. These chemicals can be transferred to the aquatic environment indirectly from wastewater treatment plant discharges (WWTP) or directly released by the skin in contact with water. Several studies reported its presence in distinct environments and their adverse effects on different species, prevailing their capacity of endocrine disruption. However, the effect of these pollutants in marine invertebrates, namely corals, remains understudied. The detrimental effects of BP-3 and 4-MBC on zoantharians, were evaluated through exposure of *Zoanthus* sp. mini colonies to 4 concentrations (0.5; 1; 2 and 4 mg L^{-1}) of these pollutants during 96 h. After exposure, polyps were photographed for behavioral response evaluation, the photosynthetic efficiency of photosystem II was measured *in vivo*, through PAM fluorometry, and lastly, the *Symbiodinium* sp. cells were quantified and normalized to *Zoanthus* sp. dry weight. Both organic UV filters shown to induce behavioral reactions in *Zoanthus* sp. polyps and impaired both photosynthetic efficiency and endosymbionts density in comparison to control treatments. These compounds have a potential bleaching effect on these marine invertebrates and may pose a threat to coral reefs, so further ecotoxicological studies on this topic should be undertaken.

Key-words: corals; emerging contaminants organic UV filters; Benzophenone-3; 4-MBC; marine ecotoxicology

2.2. Introduction

Organic UV filters are commonly used in sunscreen lotions, personal care products (PCPs), such as lotions, lipsticks, shampoos and insect repellants protecting skin from sun exposure by absorbing ultraviolet radiation (UV-A, UV-B) and other materials from degradation (e.g. textiles) (CIR, 2005; Díaz-Cruz et al., 2008; Hauri et al., 2003).

The contents and concentrations of organic UV filters in PCPs can vary in different countries/regions, according to the legislation practiced at the place of manufacture, being able to constitute up to 20 % of the composition of the product (Salvador and Chisvert, 2007). Due to their wide production and applications organic UV filters leads to an increase in the introduction of these compounds into the environment (Danovaro et al., 2008). Organic UV filters can be transferred to many aquatic environments either directly due to the release of the compound from skin, or indirectly from wastewater treatment plant discharges (WWTP) (Eichenseher, 2006; Poiger et al., 2004). These substances can be considered as potential emerging pollutants due it's growing trend and once they might act like pseudo-persistent pollutants, since the exposure of organisms in contaminated areas can frequently be renewed (Vione et al., 2013). EU authorities have recognized organic UV filters as important organic contaminants of the aquatic environment due to its high lipophilic properties, potential leading to bioaccumulation in organisms and potential biomagnification through aquatic food chains (Kunisue et al., 2012; Díaz-Cruz et al., 2008).

The presence of organic UV filters has been detected in coastal waters, sediments, rivers, lakes, swimming pool waters and sewage sludge (Barón et al., 2013; Downs et al., 2016; Fent et al., 2010, 2008) as well as in biota (Balmer et al., 2005; Blüthgen et al., 2012; Buser et al., 2006; Fent et al., 2010).

Benzophenone-3 (BP-3) and 3-(4-methylbenzylidene) camphor (4-MBC) are among the most found organic UV filters in the environment. The presence of these compounds has already been recorded the U.S. Virgin Islands (1.4 mg L^{-1} of BP-3) (Downs et al., 2016), in Majorca island (577.5 ng L^{-1} of BP-3 and 113.4 ng L^{-1} of 4-MBC) (Tovar-Sánchez et al., 2013b) or in Norway (269 ng L^{-1} of BP-3 and 488 ng L^{-1} of 4-MBC) (Langford et al., 2008).

Regarding these two organic UV filters one of the most reported ecotoxicological effects on the aquatic ecosystem is hormonal activities in fish, including alteration in reproduction and endocrine disruption (Blüthgen et al., 2012; Christen et al., 2011; Coronado et al., 2008; Kunz and Fent, 2006; Schlumpf et al., 2001). A potential antiandrogenic activity has been observed in two different life stages of zebrafish when exposed to BP-3 (Blüthgen et al., 2012). For the same species, 4-MBC has shown to induce muscle and neuronal defects in embryos, which may cause developmental defects (Li et al., 2016). In invertebrates BP-3 and 4-MBC shown to harm their growth and development (Campos et al., 2017b; Paredes et al., 2014), and causes an inhibition of feeding in *Sericostoma vittatum* larvae (Campos et al., 2017a). It has also been shown to cause a decrease in somatic growth of *Daphnia magna* (Sieratowicz et al., 2011) and reduce the reproductive output in oligochaete *Lumbriculus variegatus* (Schmitt et al., 2008). Furthermore, in *Desmodesmus subspicatus* algae and *Isochrysis galbana* microalgae, exposures lead to reductions in the cell density (Paredes et al., 2014; Sieratowicz et al., 2011).

Although organic UV filters presence in the environment and their effects on the biota have already been proven, there are still few studies that report the potential ecotoxicological impact of these pollutants on invertebrates from marine ecosystems, as corals.

Coral reefs are the most diverse and productive marine ecosystems, providing shelter, food, spawning and nursery places for fish, algae, mollusks, cnidarians and crustaceans, being estimated that between 172.000 and 9 million species can be found in this ecosystem (Reaka-Kudla, 1997; Ruppert et al., 2004). Additionally, coral reefs also have a high socio-economic value being evaluated in more than 20 trillion dollars annually (Costanza et al., 1997). Despite their ecological and socio-economic value, coral reefs around the world are increasingly threatened both by environmental disasters and anthropogenic threats (Hughes et al., 2003b). Studies of organic UV filters effect on corals are scarce. However, it is known that organic UV filters can promote viral infections on scleractinian corals that lead to bleaching (Danovaro et al., 2008), induce ossification of coral planules and promote genotoxicity (Downs et al., 2016). Due to the

scarcity of studies and the importance of corals in such a relevant ecosystem, further studies are needed to explore the effects of organic UV filters.

Zoanthids (sub-class hexacorallia and family Zoanthidae) may be good models to evaluate the effects of organic UV filters in photosynthetic corals. This coral genus includes reef and intertidal species from tropical and sub-tropical areas (Burnett et al., 1995), thus inhabiting coastal areas which are more susceptible to a higher concentration of these substances by direct transmissions to water. Moreover, zoanthids form colonies with several polyps/replicates without genetic variability, are easy to reproduce asexually and maintain in laboratory, having polyps with good dimensions for behavioral observations.

The objective of this study was to test the potential deleterious effects of BP-3 and 4-MBC in *Zoanthus* sp. polyps. After 96 h exposure to different concentrations behavioral reaction of *Zoanthus* sp. polyps was analyzed, the photosynthetic efficiency of photosystem II was assessed *in vivo* and *Symbiodinium* sp. cell density quantified.

2.3. Materials and methods

2.3.1. *Zoanthus* sp. husbandry and fragmentation

Two colonies of *Zoanthus* sp. were kept in the laboratory for a 3 weeks quarantine in a recirculating system, with artificial salt water (ASW) prepared by mixing Red Sea Coral Pro Salt (Red Sea, Germany) with freshwater purified by reverse osmosis (Aqua-win RO-6080, Taiwan). Colonies were individually stabulated in a system composed of two 90L tanks (60 × 60 × 30 cm) equipped with a circulation pump (Turbelle nanostream - 6025; Tunze, Germany), providing a water flow rate of approximately 2.500 L/h. Quarantine tanks were illuminated with 54 W T5 luminaires (2 × T5 Reef-Spec Actinic 22.000 K and 2 × T5 Reef-Spec Pink; Red Sea, Germany), with a PAR intensity of 120 ± 10 µmol quanta/m^2/s, and a photoperiod of 12 h of light and 12 h of darkness. The main tank used for the assays, was connected, through polyvinyl chloride (PVC) pipes, to a 100 L filter tank, equipped with a protein skimmer (ESC150 ReefSet, Portugal), a biological

filter (composed of live rock and submersed bioballs), a 300 W submersible heater (EHEIM Jäger, Germany), activated carbon (placed at the water outlet of the skimmer) and a pump (Universal 2.400, EHEIM, Germany) to return the water back to the main aquarium with a flow rate of approximately 1.500 L/h. The system was also equipped with a UV filter (Vecton V2 600, UV-C 25 Watt; TMC, U.K.), allowing the disinfection of microorganisms present in water.

The TAN (Total Ammonia Nitrogen), alkalinity, PO_4^{3-}, NO_2^-, NO_3^-, Ca^{2+} and pH concentrations in water were regularly monitored. Temperature was maintained at 26 ± 0.5 ºC. Salinity was maintained at 35 through an osmoregulator which compensated the evaporation with reverse osmosis water (Aquastat 1000, Deltec, Germany).

After quarantine, colonies were fragmented using a scalpel, and mini colonies with four to six polyps were produced, as represented in figure 4. Fragments were fixed to the top of Eppendorf tubes with veterinary surgical glue (Surgibond®, UK). Eppendorf tubes, used as substrate, were filled with coral rock to provide weight so that it remained submerged and stable on the support. After bonding, mini colonies were placed in the same tank of original colonies and allowed to heal for three weeks.

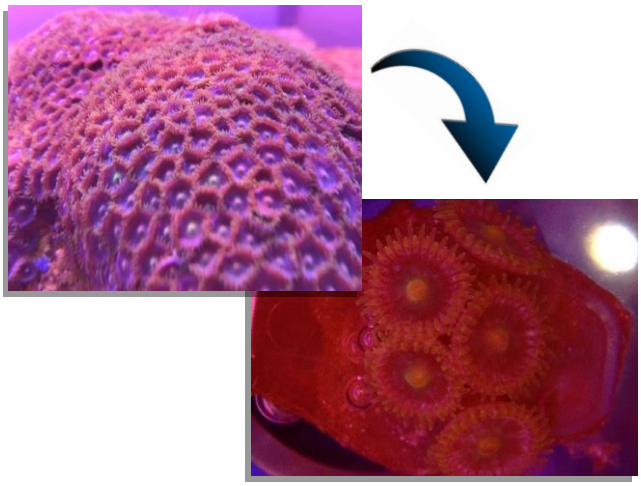


Figure 4: *Zoanthus* sp. colony and detail of post-fragmentation mini colonies used in assays.

2.3.2. Experimental design

2-hydroxy-4-methoxybenzophenone (BP- 3; CAS No. 131-57-7; purity ≥98 %) and 3-(4-methylbenzylidene) camphor (4-MBC; CAS No. 36861-47-9, purity ≥98 %) were obtained from Sigma-Aldrich (Portugal).

Stock solutions were prepared with 20 g L^{-1} and 4 g L^{-1} of BP-3 and 4-MBC, respectively, dissolved in dimethyl sulfoxide (DMSO). To prepare the experimental solutions (0.5; 1; 2 and 4 mg L^{-1}), different volumes of stock solutions were diluted in artificial salt water (salinity of 35). ASW control (containing only artificial salt water) and solvent control (DMSO) (0.002 % and 0.01 % for BP-3 and 4-MBC tests, respectively) were also prepared. Seven replicates were used in each treatment. Each mini colony was placed in a 300 mL flask for individual exposure to organic UV filters and control experimental solutions. Flasks were placed inside a 90 L water-bath tank and the temperature was maintained at 26 ± 0.5 ºC through a refrigerator (HC 500A, Hailea, China) and a 300 W submergible heater (EHEIM Jäger, Germany). Artificial illumination PAR and photoperiod were maintained under the same conditions as previously described for quarantine system. Experimental flaks were randomly distributed under the illumination system, in order to ensure that PAR values were similar for all mini colonies. Mini colonies were exposed for 96 h. Every twenty-four h 50 % of the medium was renewed and replaced with a new solution with the same concentration.

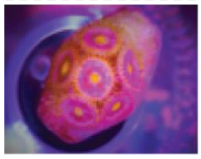
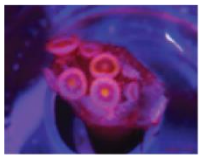
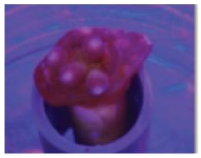
Figure 5: Experimental design of assays for determination of BP-3 and 4-MBC exposure effects, in *Zoanthus* sp. mini colonies.

2.3.3. Polyp behavioral response

To evaluate the polyp reaction to the organic UV filters exposure, a photographic record was performed at the end, after 96 h exposure.

The data were treated by analyzing each mini colony image, registering the state of each polyp, according to the criteria defined in table 1, obtaining a percentage for each state in each replicate (mini colony).

Table 1: Brief description of behavioral states of *Zoanthus* sp. polyps, after 96 h exposure to different concentrations of BP-3 and 4-MBC.

State	Polyp representation	Polyp picture	State Description
I			Open oral disc and distended tentacles
II			Semi-closed oral disc and retracted tentacles
III			Closed oral disc and closed tentacles

2.3.4. *In vivo* chlorophyll fluorescence

Chlorophyll fluorescence was measured *in vivo* using a Pulse Amplitude Modulation fluorometer (Junior PAM, Walz, Germany). Saturating light was provided by a blue LED-lamp (peaking at 450 nm) located inside the fluorometer. The fiber optic was placed perpendicularly to the top of each polyp next to the oral disc. Measurements were

performed 2 h after the illumination system turn on and mini colonies were dark adapted for 30 minutes before the application of the saturating pulse (0.8 s). Each mini colony was measured in 5 non-overlapping points (different polyps) F_o (minimum- or dark-level fluorescence) and F_m (maximum fluorescence after a saturation pulse), were used to quantify the maximum quantum yield of PSII according to (Schreiber et al., 1986): $F_v/F_m = (F_m - F_o)/F_m$

2.3.5. *Symbiodinium* sp. quantification

After the chlorophyll fluorescence analyses, all the mini colonies were preserved in 15 mL falcons with 2 mL of Lugol solution and 2 mL of ASW for later counting of *Symbiodinium* sp. cell density. Mini colonies were chopped with a scalpel until a homogeneous mixture was obtained and transferred back to the falcon filled with 14 mL of ASW. Falcon tubes were shaken for approximately 1 minute to aid in the homogenization of the sample. The counting was performed using an improved Neubauer chamber, with 6 counts being made for each replicate. Samples were then centrifuged for 10 minutes at 4000 rpm, the supernatant was discarded and the resulting pellet dried in a 60-degree oven for about 48 h. After cooldown, the dry pellet was weighed and the concentration of *Symbiodinium* sp. cell normalized to the total dry weight of *Zoanthus* sp.

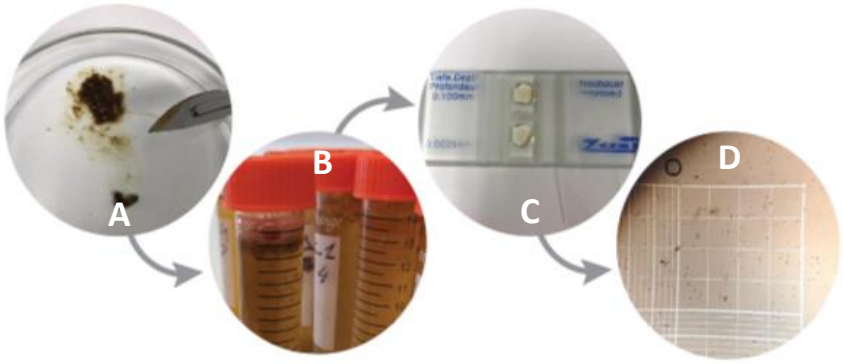


Figure 6: Resume of *Symbiodinium* sp. quantification procedure. A) Chopped samples; B) Resuspension with ASW; C) Neubauer chamber; D) Symbiodinium sp. cell counting.

2.3.6. Statistical analysis

T-tests were performed to access differences between control and solvent control in both assays. As no significant differences were found between control and solvent control, multiple comparisons were conducted between all treatments and the solvent control. To assess effects of UV filters all endpoints were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons post-hoc test. All variables were previously assessed for normality using Bartlett's test while Brown-Forsythe test verified the homoscedasticity of data. Differences in all statistical tests were considered significant at $p \leq 0.05$. All statistical analyses were performed using GraphPad Prism (version 7.00, GraphPad Software, La Jolla California, USA).

2.4. Results

2.4.1. Polyp behavioral response

Image analysis suggested that BP-3 and 4-MBC induced a behavior response in *Zoanthus* sp. mini colony polyps. Overall, there were behavioral changes induced by the compounds through retraction of the polyps as well as in a visible diminution of size, becoming more noticeable with the increase of the concentrations. Most polyps used in ASW and DMSO control treatments, in both assays, were in state I, having the oral disc and tentacles completely open. ASW control revealed 92 % and 67 % of polyps completely open (state I) for exposures with BP-3 and 4-MBC, respectively. The solvent control also demonstrated a high number of polyps in the state I, 97 % in BP-3 and 71 % in 4-MBC exposure.

BP-3 induced a strong behavioral response since all polyps were in state II or state III in the two highest concentration tested, whereas in the two lower concentrations it was still possible to verify the existence of some polyps in the state I. Additionally, an

increase from 13 to 49 % of polyps in stage III was observed, comparing the results obtained in 2 mg L^{-1} and 4 mg L^{-1} treatments, respectively.

Effects of 4-MBC were also observed with about half of the polyps already in state III at the concentration of 1 mg L^{-1} and this percentage increased considerably in the two highest concentrations to 64 % and 79 %, respectively. It was also possible to observe polyps in state I up to the concentration of 2 mg L^{-1}.

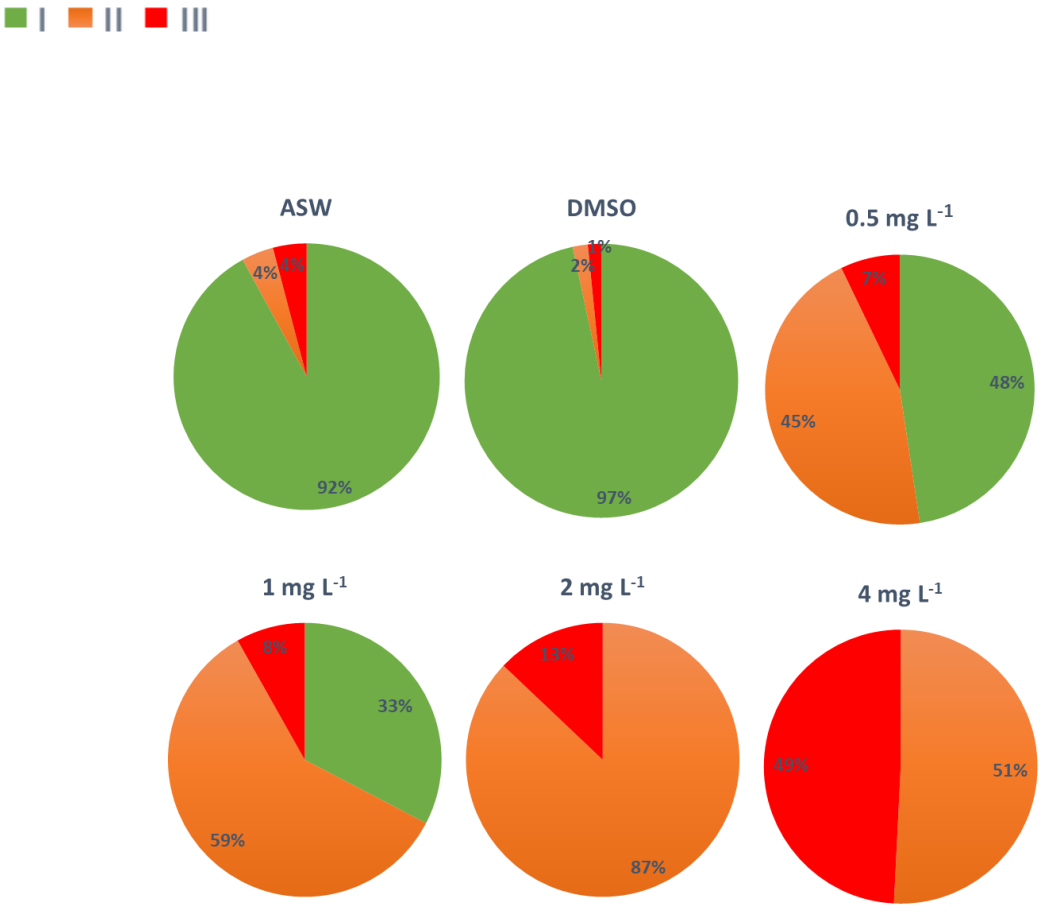


Figure 7: Polyp behavioral response of *Zoanthus* sp. after 96 h of exposure to sub-lethal concentrations of BP-3. State I: Polyps with the oral disc open and distended tentacles; State II: polyps with the oral disc semi-closed and the tentacles retracted; State III: completely closed polyps.

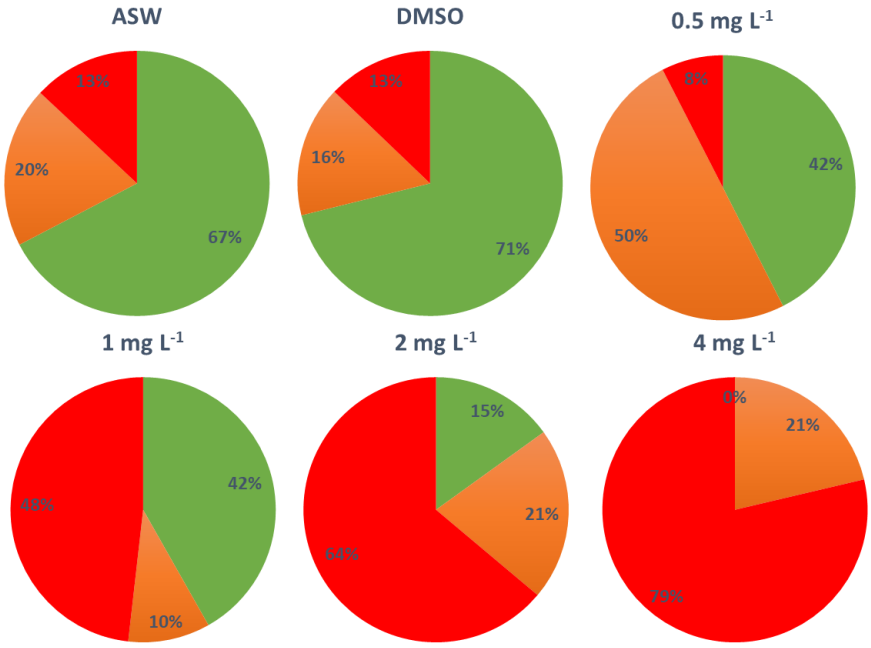


Figure 8: Polyp behavioral response of *Zoanthus* sp. after 96 h of exposure to sub-lethal concentrations of 4-MBC. State I: Polyps with the oral disc open and distended tentacles; State II: polyps with the oral disc semi-closed and the tentacles retracted; State III: completely closed polyps.

2.4.2. *In vivo* chlorophyll fluorescence

The photosynthetic efficiency of *Zoanthus* sp. endosymbionts, evaluated through the measurement of the maximum quantum yields of PSII (F_v/F_m), presented on figures 9 and 10, was influenced by the concentration gradient of the contaminants in the water. No significant differences were observed between negative (ASW) and solvent (DMSO) control treatments in both assays.

It was possible to observe that values of F_v/F_m decrease with the increasing of BP-3 concentration in water (fig. 3) (one-way ANOVA, $F_{4,\ 30} = 28.518$; $p < 0.001$). There were significant statistical differences between each concentration and the solvent control. For the concentrations of 0.5; 1; 2 and 4 mg L^{-1}, there were decreases of 12.7 %, 13.3 %,

17.8 % and 32.6 % of F_v/F_m values, respectively, in comparison with the values obtained for the solvent control.

Concerning the exposure to 4-MBC, it was possible to observe a slightly decrease in F_v/F_m values with the increasing of contaminant concentrations (one-way ANOVA, $F_{4,\ 30} = 12.34$; $p < 0.001$). However, significant statistical differences were registered only in 4 mg L^{-1} concentration treatment, where F_v/F_m values decreased 10.4 %, when compared to the value obtained in the solvent control.

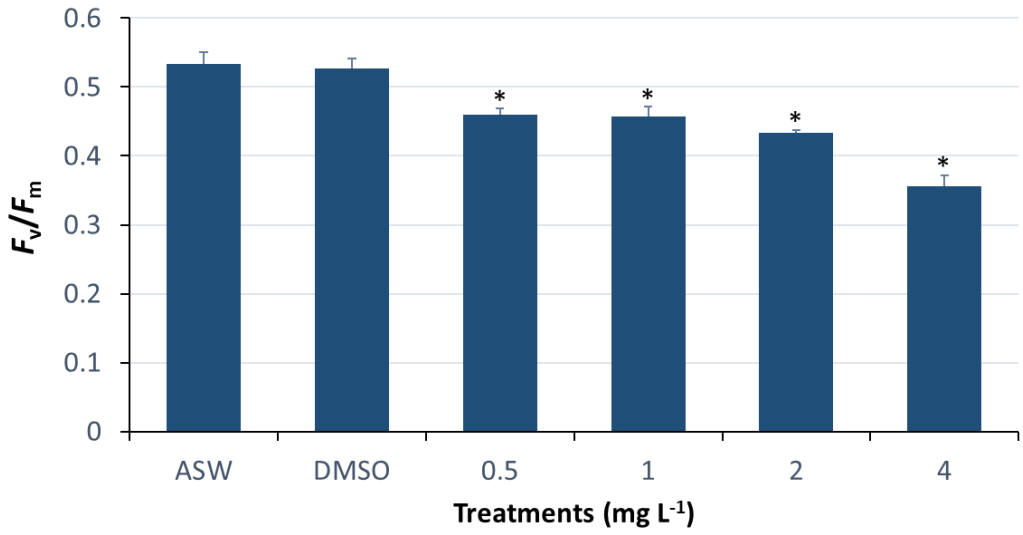


Figure 9: Maximum quantum yield of PSII (F_v/F_m) measured on *Zoanthus* sp. polyps after 96 h of exposure to BP-3 sub-lethal concentrations. All values are presented as mean + SEM, n =7. Asterisks (*) denote significant differences compared to the solvent control treatment (Dunnett's test, $p < 0.05$).

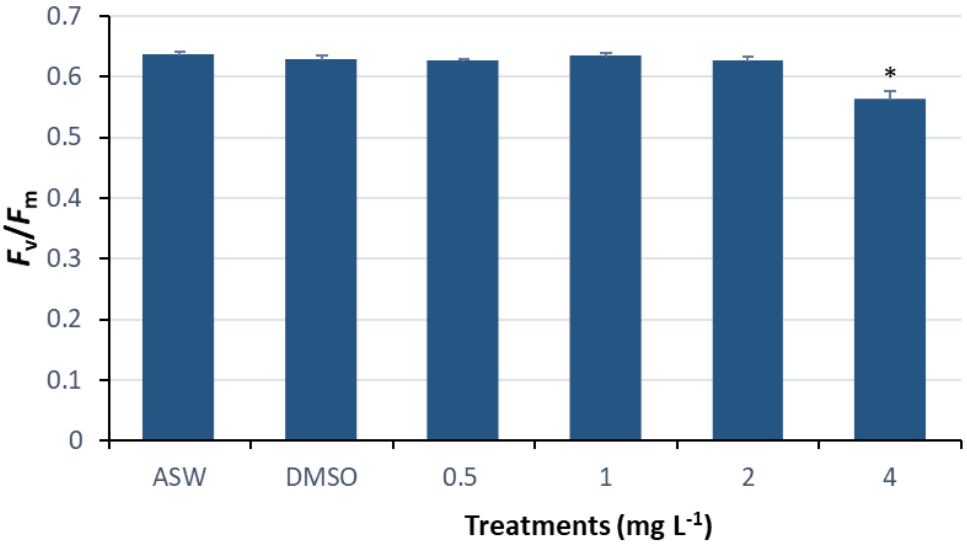


Figure 10: Maximum quantum yield of PSII (F_v/F_m) measured on *Zoanthus* sp. polyps after 96 h of exposure to 4-MBC sub-lethal concentrations. All values are presented as mean + SEM, n = 7. Asterisks (*) denote significant differences compared to the solvent control treatment (Dunnett's test, $p < 0.05$).

2.4.3. *Symbiodinium* sp. quantification

It was possible to observe a general decrease of the endosymbionts density with the increasing concentrations (one-way ANOVA, $F_{4,\ 30} = 5.363$, $p < 0.001$ and $F_{4,\ 30} = 2.427$, $p < 0.05$ for BP-3 and 4-MBC assays, respectively). Statistically significant differences were observed between the solvent control and the 4 mg L^{-1} concentration in both assays. The effect of the highest concentration of BP-3 was severe, causing a huge reduction of 73 % in the endosymbiont cell density, when compared to mini colonies from solvent control. It was possible to observe that exposure to BP-3 have induced a reduction of the number of *Symbiodinium* sp. cells immediately at the lowest concentration, with a cell density 22.3 % lower than the registered in solvent control. 4-MBC effects were not as accentuated, being noticed a decrease of 34 % in cell density in the highest concentration.

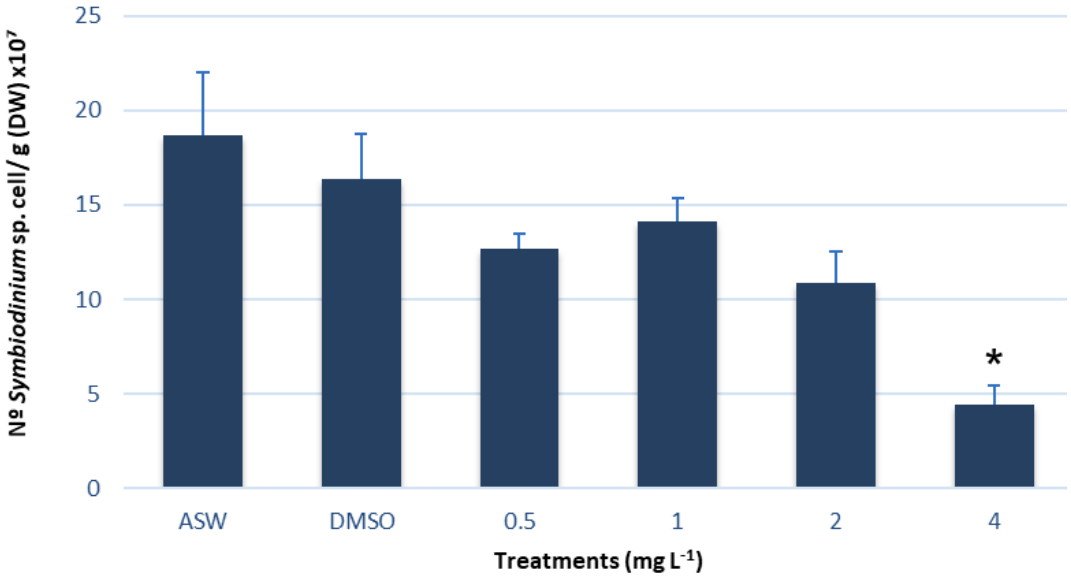


Figure 11: *Symbiodinium* sp. density (nº of cells per gram of *Zoanthus* sp. dry weight) in polyps exposed to sub-lethal concentrations of BP-3. All values are presented as mean + SEM, n = 7. Asterisks (*) denote a significant difference compared to the solvent control treatment (Dunnett's test, $p < 0.05$).

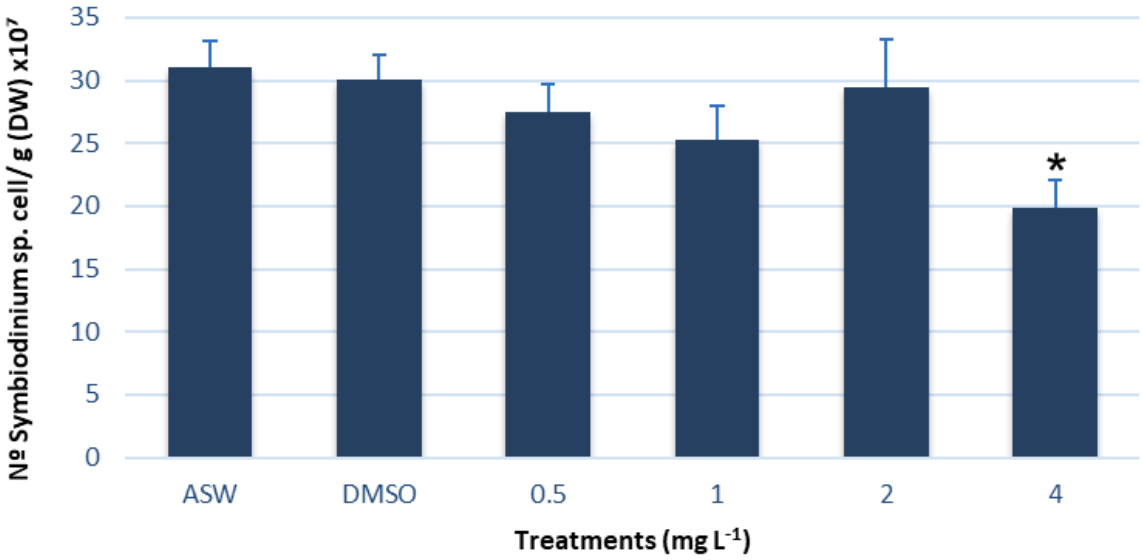


Figure 12: *Symbiodinium* sp. density (nº of cells per gram of *Zoanthus* sp. dry weight) in polyps exposed to sub-lethal concentrations of 4-MBC. All values are presented as mean + SEM, n = 7. Asterisks (*) denote a significant difference compared to the solvent control treatment (Dunnett's test, $p < 0.05$).

2.5. Discussion

The present study demonstrates that the organic UV filters, BP-3 and 4-MBC, induced effects in the polyps behavior, photobiology and endosymbiont cell density of *Zoanthus* sp..

Through the characterization of the behavioral response over the 96 h of exposure there seems to exist a pattern between concentration, induced stress and behavioral response (polyps closure). It was possible to conclude that in the highest concentrations the organisms manifested a behavioral change, once they retracted their polyps being almost all closed (with increasing concentrations the number of closed polyps raised) but also showing a visible size reduction in the retracted polyps.

The damage in the photosynthetic apparatus of the coral is one of the first symptoms of possible occurrence of bleaching, which impacts include increased susceptibility to disease, reduced growth and reproduction and sometimes leads to coral death (Baird and Marshall, 2002; Szmant and Gassman, 1990). Such damage can be measured through changes in the photochemical efficiency of PSII (Warner et al., 1999). The maximum quantum yield of PSII (F_v/F_m), is one of the most used parameter to quantify the photochemical efficiency and therefore the photo physiological health of the coral (Maxwell and Johnson, 2000). Significant differences in analysis of chlorophyll fluorescence were obtained immediately in the first tested concentration of BP-3 (0,5 mg L^{-1}), therefore it is expectable that negative impacts at lower concentrations can occur. The values determined in the controls on the evaluation of chlorophyll fluorescence (F_v/F_m) for ASW and solvent control are within the range of reported values for zoanthids in their natural habitat (ranging from 0.5 to 0.7) (eg Leal et al., 2016; Rosa et al., 2016). Some little discrepancy in values between organisms in their natural habitat and those acclimatized in laboratory can be related to PAR influence on F_v/F_m values. It is known that F_v/F_m tend to decrease with the increasing of PAR values, and usually high light acclimated corals tend to present lower F_v/F_m values than low light conspecifics (Rocha et al., 2013a, 2013b). PAR values used for both assays were similar for all replicates and mini colonies were clones from the same original *Zoanthus* sp. colony in

each assay, therefore the diminution of F_v/F_m values can be directly related with the contaminant exposure.

Both compounds appear to induce expulsion or loss of *Symbiodinium* sp. as the mean cell density decreased after 96 h of exposure. A decrease in F_v/F_m does not necessarily predict coral bleaching (Fisher et al., 2012), but both factors combined indicate a decline in the general health of the organism.

Healthy cnidarian hosts are considered mixotrophic, as their nutrition is complemented with both autotrophy, obtaining photosynthates provided by *Symbiodinium* sp., and heterotrophic feeding. Endosymbionts are the main carbon suppliers, used as energy by these organisms (Falkowski et al., 1993; Muscatine et al., 1981). Most species cannot thrive for long periods without endosymbionts, since autotrophy is the main nutritional source of zoanthids (Leal et al., 2017). The decrease in *Symbiodinium* sp. concentration as well as photochemical efficiency suggest that autotrophic nutrition in *Zoanthus* sp. may be impaired by the presence of organic UV filters. Heterotrophy is another carbon source especially important during bleaching events (Houlbrèque and Ferrier-Pagès, 2009) so, it is also important to note that the fact that the behavioral analysis demonstrate a closure of the polyps throughout the concentrations, the ability to feed on phytoplankton or zooplankton can be also compromised.

The results obtained in the present study show that BP-3 caused a greater behavioral response as well as greater damage in the photobiology of the organisms, appearing to be more toxic than 4-MBC, although further studies (e.g. using the same colony of organisms for the substances studied) are needed in order to make a comparison of toxicities more accurate. The reported results of the highest toxicity of one or other compound as the most toxic are not consensual, since depending on the species under study the results diverge (Paredes et al., 2014). It was also reported through a probabilistic risk assessment that BP-3 is more likely to pose a risk to fishes and of bleaching in hard corals and 4-MBC poses greater risk to algae (Tsui et al., 2014).

Our results are in agreement with two previous studies showing that exposure to organic UV filters can lead to an occurrence of bleaching, although both studies used species of sceleterian corals, zoantharians are phylogenetically close to sceleterian

corals. There are evidences that the occurrence of bleaching is potentially due to sunscreens capacity to promote lytic viral cycle in the endosymbionts, causing viral infections and leading to a rapid and complete bleaching of hard corals (Danovaro et al., 2008). The loss of photosynthetic pigments and membrane integrity in the endosymbionts released was also verified when exposed to a commercial sunscreen lotion which included a mixture of organic UV filters (Danovaro et al., 2008). In line with our study, a relationship between exposure to increasing concentrations and diminution of chlorophyll fluorescence values was also observed in planulae of the hard coral *Stylophora pistillata* (Downs et al., 2016). It was shown that BP-3 promoted the ossification of the planula under study, being therefore a skeletal endocrine disruptor. The author interpreted the results by stating that BP-3 probably "induces photo-oxidative stress to the molecular structures that form the thylakoid membranes (Downs et al., 2014)."

Regarding the sensitivity of the parameters used, the measurement of chlorophyll fluorescence is the most objective parameter since it is not dependent on the personal observation or counting, therefore, it is less susceptible to errors and it is also possible to perform *in vivo* and even *in situ*. Despite this, the parameters used are complementary and should be used together to obtain a perception of the photosynthetic efficiency of the endosymbionts, but also of behavior responses and endosymbiosis itself, so that it is possible to obtain the greatest response number of the organism used at all levels.

The concentrations used are environmentally relevant since values for BP-3 of 1.4 mg L^{-1} in nearshore waters were reported (Downs et al., 2016). Although the values found in nature for 4-MBC are considerably lower, in coastal areas or in tide pools (at low tide), environments inhabited by this species, the concentrations may be highly superior to those recorded nearshore by earlier studies, especially during the summer season when the tourist influx is considerably higher. This hypothesis should be analyzed with seasonal surveys so it would be possible to obtain data about long-term effects.

The model organism of this study, *Zoanthus* sp., has proved to be a good organism to be used in ecotoxicological trials to evaluate the effects of organic UV filters. These organisms allow us to evaluate the effects at three different levels, at the host level (for

having polyps with good dimensions for behavioral evaluation), at the level of the endosymbiont (measurements of changes in the photochemical efficiency) and symbiosis relationship (*Symbiodinium* sp. cell density evaluation). *Zoanthus* sp. was sensitive to the presence of organic UV filters according to the parameters used, and in following works it could be used to test different organic UV filters present in the environment.

In addition, studies indicate that the reproductive period of *Zoanthus* sp., e.g. in Great Barrier Reef during November (Ryland and Babcock, 1991) and in tropical North Atlantic-Caribbean occur between June-July (Karlson, 1983), coincides with the greater influx of tourists which is expected to be associated with a greater input of this compound into the ecosystem (Balmer et al., 2005; Torres et al., 2016). Additionally, knowing that the half-life of these UV filters can reach several months and since effects have already been reported in planulae (Downs et al., 2016), the growing presence of organic UV filters in aquatic systems can have effects even at the level of species life cycle and ecosystem balance.

In only 15 years, documented losses in coral diversity ranging between 30 – 60 % in reefs degraded due to anthropogenic activities (Edinger et al., 1998) and it was estimated that 10 % of the coral reefs in the world are threatened by sunscreen pollution (Danovaro et al., 2008). The present study highlights the need for implementation of different UV filter substances or the development of new ones that do not have a toxic activity against aquatic organisms. Since in formulations of commercial sun lotions there is a mixture of different substances it is also important to evaluate the effects of these combinations and their possible synergistic effects (Ozáez et al., 2016). *In vivo* Chlorophyll fluorescence measurements can be good indicators of photo physiological health of symbiotic and photosynthetic organisms. The utilization of this noninvasive parameter allows the development of ecotoxicological studies *in situ*.

With the increase both populational and tourist it is difficult to be optimistic about the future of this valuable ecosystem (Wilkinson, 1999). It is, therefore, necessary to continue the development of new studies leading to a deep understanding of the ecotoxicological effects of most commonly used organic UV filters, in aquatic systems using relevant species.

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Chapter III

Final considerations and future perspectives

3. Final considerations and future perspectives

BP-3 and 4-MBC induced responses in zoanthids, leading to the reduction of their photosynthetic capacity, its number of symbionts and behavior responses. The results obtained demonstrate the toxicity of these substances used worldwide, in *Zoanthus* sp.. Thus, the null hypothesis "organic UV filters, BP-3 and 4-MBC, do not impair the photobiology, photo symbiont density nor the behavioral reaction of *Zoanthus* sp. polyps" was rejected.

One of the objectives of the work was also to test if *Zoanthus* sp. would be a good indicator to demonstrate the potential effects of organic UV filters and this was achieved since it shown to be sensitive through the parameters analyzed.

From all parameters analyzed, it is important to note that the behavioral analysis is the one most prone to display a high variability. This is due to the variation in interpretation of photographic data that may vary depending on the perspective of the observer. The organism can also resent, at the moment of the photograph, and retract its oral disc. Overall, the survey of this parameter still needs improvement, although a clear pattern between the toxic concentration and zoanthid reaction can be perceived. On the other hand, the measurement of chlorophyll fluorescence is the most objective parameter once do not dependent on this personal observation, being less susceptible to errors. Despite this, the parameters used are complementary and should be used together.

This work should have a follow-up study, where the same colony of organisms is used in all assays for the possibility of toxicological comparison of the pollutants. It's also important the execution of chronic tests, taking into account other factors to which organisms may be subject (such as tides, tourism seasonality, fluctuations in temperature, salinity, solar radiation, etc). Since measurements of chlorophyll fluorescence are performed *in vivo, in situ* investigations can also be carried out at contaminated sites. Since sunscreen formulas are made with a mixture of different UV filters, it would also be interesting to study the effects of their interactions (synergistic effects) as well as with other compounds that can be found in the area under study. Taking into account that both 4-MBC and BP-3 were found in muscle tissues of several different species of fish reaching concentrations of some μg/g (Balmer et al., 2005; Buser

et al., 2006; Gago-Ferrero et al., 2015; Subedi et al., 2011), and due to their high lipophilic properties, a possible occurrence of bioaccumulation and biomagnification should also be approached.

For coral speciesBP-3 appears to induce a higher toxicity, although there is no clear trend line when looking at available data from all animal species previously studied (e.g. Gao et al., 2013; Ozáez et al., 2013; Paredes et al., 2014; Sieratowicz et al., 2011). It is therefore necessary to keep developing further studies using, with these and other organic UV filters that may be harmful in different aquatic species for a comprehensive toxicological evaluation.

Another issue of major concern is that, although the concentrations in treated wastewater (WWTP effluent) are considerably lower (Balmer et al., 2005), these compounds are not degraded completely by common methods in WWTP. It is reported a degradation method of BP-3 by UV / H_2O_2 in aqueous solution (Gong et al., 2015) and of 4-MBC through photo-Fenton process (Ji et al., 2017). Despite the existence of effective methods for the removal of these substances, it is necessary to develop new methods of remediation that can be applied on a large scale and used in WWTP, enhancing the biodegradation of emerging contaminants.

Finally, it is of great importance to investigate organic UV filters that do not cause damage to any living being, so that they are implemented by regulatory agencies and in the PCP's industry, as an alternative to those that proven to be harmful to the environment. It is also important to keep a close monitoring of these pollutants, especially in places subject to a higher presence of tourists and where endangered species occur. Thus, future investigations should consider the persistence of these and others organic UV filters, and the possibility of bioaccumulation and biomagnification through the food chain in long-term ecotoxicological tests.

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4. Annexes

4.1. Preliminary tests with BP-3

Preliminary tests were initially conducted to refine the defined protocol and to reach optimal test conditions where no disturbances were observed in the control organisms.

• In the first preliminary test performed, the stock solution (BP-3 in 100% DMSO) had a concentration of 200 g L^{-1}. The concentrations of 20 mg L^{-1}; 10 mg L^{-1}; 2 mg L^{-1} were tested and also negative and DMSO control. Three replicates were used for each concentration. It was observed that all polyps were closed. Some of the test conditions were changed for the second preliminary test, such as the installation of a cooler to keep the temperature constant and the luminaires were moved to a larger distance from the organisms so that the corals did not have too much light exposure and also a change of water was performed.

• In the second preliminary test, only four replicates were used, with no contaminant to evaluate the new test conditions, and the four jars were placed at different points in the aquarium. The 72-hour test was successful with all polyps open.

• The test concentrations were then changed to a minimum of 0.2 mg L^{-1} and a maximum of 2 mg L^{-1}. In this test, the stock solution was 20 g L^{-1} (since the concentrations to be used were smaller than in the first test). Three replicates were used for each concentration as for the controls. In this test, it was noticed in the first 24 hours that the corals were reacting to the highest concentration. But at 72 hours and 96 hours it was also observed that the lower concentration as well as the control of DMSO were reacting with semi-open and closed polyps. It was concluded that the problem could be the amount of DMSO used to perform SS (stock solution) once that control also had effects, so the following test was only using different concentrations of DMSO.

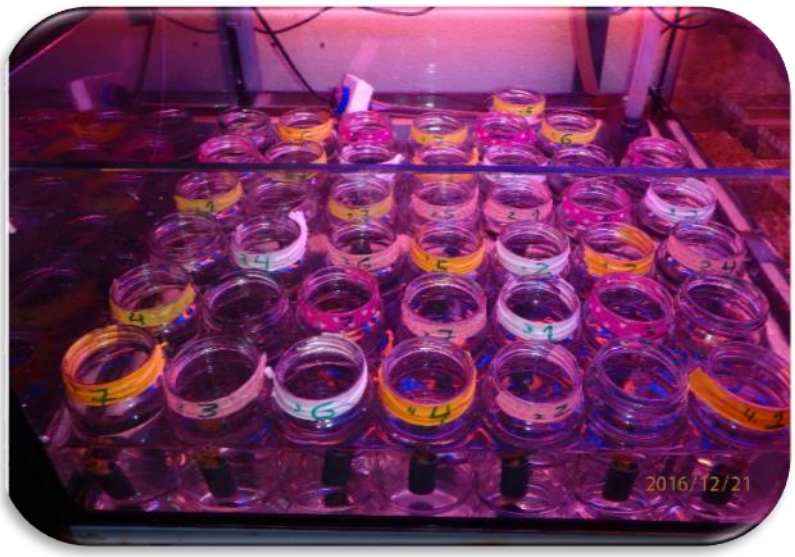
DMSO concentrations of 5 µL, 15 µL and 30 µL in 300 mL of ASW (30 µL corresponding to the concentration used in the previous assay: 100 microliters in 1L of water) + negative

control was tested. The replicates of the highest concentration reacted by closing the polyps, so it was decided to change the concentration of the stock solution again to 200 g L^{-1} so that the volume to be used of SS and therefore DMSO was smaller.

• In this last test, the luminaires were also changed so it would be possible to have a better distribution of light throughout the aquarium since for the final test 42 jars would be used. It was also decided to change half of the medium of each replica every 24 hours. This previous test was performed in order to analyse the change of all these variables. Concentrations of 0.2 mg L^{-1} and 4 mg L^{-1} (lowest and highest) were used as also the controls, having 3 replicates each. The medium was exchanged at 24, 48 and 72 h using syringes and a volume of half the medium was withdrawn.

• Results: at the first 24 h it was possible to observe closed and semi-closed polyps in the highest concentration and the same was verified at the end of the test for all their replicates. Since there were no changes in the controls, the final test was conducted.

For the final assay, briefly, concentrations of 4 mg L^{-1}; 2 mg L^{-1}; 1 mg L^{-1} and 0.5 mg L^{-1} of BP-3 and 4-MBC were used, as also both controls. Seven replicates were used per concentration, in a total of 42 jars, identified with a color code for easier identification. The jars were placed randomly in the aquarium, being performed a change of medium every 24 h and a photographic record at the beginning and end of the assay. Water was collected every 24 h after the change of medium for further analysis.



4.2. Dissemination of Results

The first results of this study were presented at SETAC Europe 27th Annual meeting, held this year in Brussels. My participation took place through the exposure of a poster, which is presented in the following figure. The results presented are only relative to the BP-3 assay.