UNIVERSITA' POLITECNICA DELLE MARCHE
FACOLTA' DI SCIENZE
Dottorato di Ricerca in Biologia ed Ecologia Marina
XV° ciclo

Development of innovative approaches for assessing the impact of personal care products and other micro-contaminants in the marine environment

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Anno Accademico 2013/2016

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INTRODUCTION

Human activities alter biodiversity and composition of the ecosystems worldwide, endangering their capacity to deliver ecosystem services (Mouillot et al., 2016). Coastal marine environments are disappearing rapidly (ca. 20% annual loss of coastal areas, Millennium Ecosystem Assessment, 2005), indeed about 35% of the mangroves have been lost or converted, while more than 80% of coral reefs are severely threatened (Claire et., al. 2006). In addition, it has been estimated that only less than 15% of the European coastline is still considered in good conditions (Airoldi & Beck, 2007) with a regression of seagrasses exceeding 50% (and peaks above 80% in the Mediterranean) of those present in the pristine area. In this context, coastal areas appear to be among the most threatened ecosystems by anthropogenic and global change impacts (Neumann et al., 2015; UNEP, 2015).

The reasons for this ecosystem degradation are complex, but there is evidence that demographic factors play a significant role (Creel et al., 2003). Today, about 3 billion people live within 200 km of coastline and this number is expected to increase up to 4.2 billion by 2030 (UNEP, 2005). Furthermore, coastal areas are the major destinations for tourism, which represents the fastest-growing sector of the global economy (grew by 4.4% in 2015). It has been estimated that 85% of all tourism worldwide takes place in coastal areas, generating an annual revenue of US $1,050 billion in in 2014 (UNWTO, 2015). Only the Mediterranean coastal area, hosted approximately 250 millions of visitors in 2008 and this number could increase to 312 million by 2025 (UNWTO, 2011).

It has been estimated that every year, millions of tourists travel to tropical destinations (Creel, 2003). For example, the Coral Triangle region receives an even

greater proportion of people visiting for coastal and marine areas than other parts of the world with 33.5 million international visitors only in 2014 (WWF-Pacific, 2015). In the Caribbean, for instance, official estimates indicate that 70,000 tons of waste are generated annually from tourism activities (UNWTO, 2015).

In the last decade, the study of the impact of micro-pollutants (i.e., synthetic organic and inorganic compounds) on marine ecosystems has increased considerably (Quiles & Tovar-Sánchez, 2015; Sánchez-Quiles et al., 2014; Danovaro et al. 2008) parallel with the rapid expansion of tourism (Sánchez-Quiles & Tovar-Sánchez, 2015). Pharmaceuticals and personal care products (PPCPs), defined as "emerging contaminants" (Hoyett et al., 2016), including antibiotics, industrial compounds/products, sunscreens and other micro-contaminants, are increasing in terms of quantities produced and the number of new molecules entering the market are attracting increasing interest for their potential impact on organisms and ecosystems (Lapworth et al., 2012). Pharmaceuticals and some personal care products are released directly or indirectly into marine environment by human activities (Arpin-Pont et al., 2016). Several chemicals are not necessarily new pollutants, as they may have been present in the environment since several years, but their presence and significance are only now being evaluated (Daughton, 2001). Recent studies have found a wide presence of these compounds in marine organisms (Martinez Bueno et al., 2014; Picot-Groz et al., 2014; Nakata et al., 2012; Gomez et al., 2012; Wille et al., 2011; Gatidou et al., 2010) and various environmental compartments such as sea water (Liang et al., 2013; Na et al., 2013; Wille et al., 2010; Knee et al., 2010) and sediments (Beretta at al., 2014; Liang et al., 2013; Na et al., 2013; Amine et al., 2012; Zeng et al., 2008). The main concern

is that these contaminants can act as endocrine disruptors able to interfere with the reproductive system and the normal development of living organisms (Molins-Delgado et al., 2015) and many of them also have adverse ecological effects at low concentrations (Danovaro et al., 2008; Kümmerer et al., 2004). In particular, previous studies, indeed, have shown that the sunscreen products and their ingredients (e.g., UV filters such as butylparaben, ethylhexylmethoxycinnamate, benzophenone-3, TiO_2 and ZnO nanoparticles) can harm marine plankton and coral reefs worldwide (Downs et al., 2015; Sánchez-Quiles et al., 2014; Danovaro et al., 2008; Danovaro & Corinaldesi, 2003). Sunscreen products contain organic (e.g., aminobenzoic acid, ethylhexyl triazone, cinnamates, salicylates, benzophenone, dibenzoyl-methane, Benzimidazole) and inorganic filters (e.g.TiO_2 and ZnO), preservatives, adjuvants, moisturizing and antioxidant chemicals. Different ingredients of sunscreens, particularly UV filters and preservatives, have been investigated for their potential impact on marine life, from unicellular (Hazeem et al., 2015; Sànchez-Quiles et al., 2014; Danovaro & Corinaldesi, 2003) to pluricellular organisms (Downs et al., 2015; Fent et al., 2014; Picot Groz et al., 2014; Kim et al., 2011; Kim et al., 2011a). Due to the lipophilic characteristic of these cosmetics (Tian et al., 2015) and the insolubility of many of their chemicals, sunscreen products tend to bioaccumulate in aquatic animals (Gago-Ferrero et al., 2013; Giokas et al., 2007), causing effects similar to those reported for other xenobiotic compounds (Nakata et al., 2009). A number ostudies report the presence of UV filters also in different environmental matrices as sediments (Emnet et al., 2015; Hazeem et al., 2015; Sànchez-Quiles et al., 2014), sea water (Nakata et al., 2009). Some UV filters (e.g., oxybenzone, octocrylene, benzophenone or

ethylhexyl triazone) are known to be toxic to aquatic organisms (Downs et al., 2015; Maipas et al., 2015; Paredes et al., 2014; Kim et al., 2014; Fent et al., 2010; Dìaz-Cruz et al., 2009; Kunz et al., 2006) and exhibit effects consistent as endocrine disruptors. Paredes et al. (2014) demonstrated that species from different trophic levels are more sensitive to some UV filters than to others, for example, microalgae appeared to be the most sensitive species studied, in particular to benzophenone-3 (BP3) whereas sea urchin larvae were most sensitive to ethylhexyl methoxycinnamate (EHMC). Pharmaceuticals and personal care products are typically found in the environment as complex mixtures, therefore, even if the individual compound concentrations are low, a so-called cocktail effect might be of ecotoxicological and ecological relevance (Heath et al., 2016) However, contrary to pollutants such as organochlorine pesticides, PCBs and PAHs there is a gap of knowledge about the effects of these micropollutants on marine organisms and the possible environmental implications (Nodler et al., 2016). Now, multiple studies have provided experimental evidence that the only analytical- chemical approach does not provide sufficient tools to define the environmental risk associated with single pollutants or a mixture of these. In fact, a high number of studies published since 2002, have been focussed on the investigation of these contaminants in marine environment from a toxicological rather than an ecological point of view. Therefore, it is needed acquiring more accurate data on the fate of PPCPs in the marine ecosystem and their effects on marine life and ecosystem.

The present dissertation includes both laboratory and field experiments, focusing on the impact of different categories of personal care products and other micro-contaminants, including pharmaceuticals, on marine organisms and ecosystems.

The objectives of this work are: i) to identify a series of biological tests/parameters and model marine organisms, ii) to develop new approaches for assessing the effect of different types of PPCPs, and ultimately iii) to create/validate standardized protocols for evaluating if a novel or existing products are eco-friendly with marine life. To do so, a series of experiments (in the field and in the laboratory) have been conducted to assess the compatibility of PPCPs on different Mediterranean and tropical organisms including: *Acropora sp*. (the most common tropical hermatypic hard corals), *Anemonia viridis* (a soft coral, common along the Mediterranean coasts), c) *Paracentrotus lividus* (an echinoderm, epi-grazer of the Mediterranean rocky shores), *Corallium rubrum* (a key species of the Mediterranean Sea). The organisms used for testing the biological responses (physiological and behavioral) were also selected to be compliant to the LAV restrictions and ECO-CERT indications.

Figure 1. Map of sampling areas: Mediterranean Areas (1, 2) and Tropical Areas (3, 4).

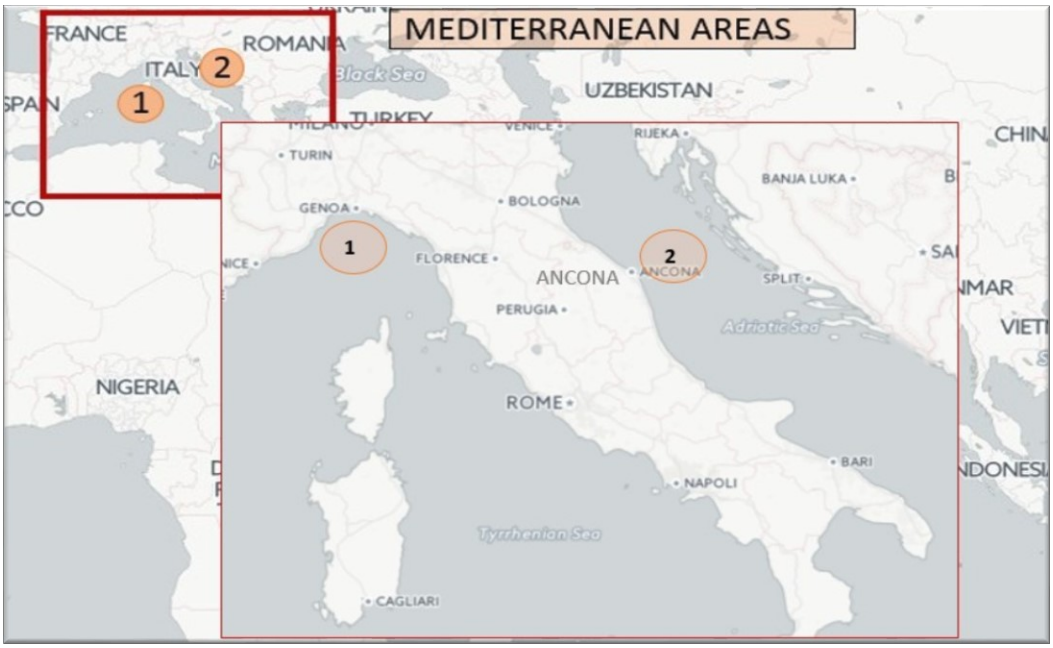

Figure 2. Map of Mediterranean sampling areas. 1: North Tyrrhenian Sea (44°18’58.77”N 9°09’28.21”E, Mediterranean Sea); 2: Central Adriatic Sea (43°37’11.29”N 13°31’52.98”E, Mediterranean Sea).

Figure 3. Map of Tropical sampling areas. 3: Vavvaru Island, Maldives (5°25’06.26”N 73°21’13.87”E, Indian Ocean); 4: Fiji (18°51’06.69”S 178°31’31.29”E, Onu Island, Indian Ocean).

I) In order to assess the potential impacts of sunscreens and their ingredients on marine life, independent laboratory and in field experiments were conducted. on the Mediterranean coastal species, *P. lividus* and *A. viridis*. The experiments were carried out in aquaria by exposing the adult organism of *A. viridis* and the embryos and larvae of *P. lividus* at different brands of sunscreens. *A. viridis* contains algae symbionts, commonly known as zooxanthellae. Such symbionts are susceptible to environmental stress, which can lead to an increase in ROS production and oxidative stress in both symbiont and host cells. This often results in the subsequent release of zooxanthellae from the host cells, known as bleaching (Venn et al., 2008). Zooxanthellae abundance is a commonly used parameter to indicate oxidative stress in corals and anemones in response to stressors such as pollution and increasing temperature (Venn et al., 2008). Therefore, studies investigating the bleaching phenomenon and symbiotic relationships in corals use *A. viridis* as a model (Leutenegger et al., 2007; Merle et al., 2007). *Paracentrotus lividus* (Lamarck, 1816), is one of the most important grazers along the Mediterranean coasts and plays key roles in determining the structure of the whole community structure in this system. The sea urchin *P. lividus*, particularly during the first stage of development, is a very sensitive model for a great variety of pollutants (Pesando et al., 2003), and is used as a target organism in a number of studies to evaluate the effects of various emerging pollutants, such as components of cosmetic products (Paredes et al., 2014). Furthermore, the sea urchin is one of the main models considered by the European Agency for Alternative models (Falugi et al., 2008).

The impact of sunscreens, and the inorganic filters contained therein, was also evaluated on tropical organisms such as Acropora sp. (Maldives and Fiji). The

experiments were conducted in aquaria to assess whether different brands of solar products and UV filters, could have negative implications on these organisms and their symbionts and consequently on the state of health and resilience of the reef ecosystems. *Acropora sp*. is an important reef-building coral, defined as a bleaching-susceptible genus (Baird et al., 2009). The release of symbiotic components (zooxanthellae), the bleaching rates and microbial contamination were determined to assess the sunscreens' impact.

II) In order to assess the potential impacts of different pharmaceuticals and other micro-contaminants, both individually and as mixture, on Mediterranean and tropical marine life, independent laboratory and in field experiments were conducted on *Corallium rubrum.* The red coral is considered endangered and included in the red list of the IUCN (EN, A2c), therefore could be vulnerable to this type of anthropogenic impact. Independent experiments were carried out to assess the responses of the coral in terms of polyps' activity and the morpho-physiological effects.

Impact of pharmaceuticals and other micro-contaminants on (Fiji)

Experiments were also conducted on tropical corals (*Acropora sp.)* to assess whether, the pharmaceutical products and other micro-contaminants, could have negative implications on *Acropora sp.* and their symbionts and consequently on the reef ecosystems. In these experiments, we evaluated the release of symbiotic components (zooxanthellae), the bleaching rates and microbial contamination.

Our findings reveal new information about the negative impacts of PPCPs on marine life and in particular provide cues for stimulating the scientific research to

evaluate if novel or existing products such as sunscreens and UV filters are compatible with marine ecosystems.

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CHAPTER 1

Impact of sunscreen products on *Paracentrotus lividus* and *Anemonia viridis*

1.1 INTRODUCTION

Coastal areas are among the most threatened marine ecosystems by anthropogenic impacts and climate changes (Sanchez-Avila et al., 2012). In the last decade, the impacts of micro-pollutants (i.e., synthetic organic and inorganic compounds) contained within personal care products have been intensively studied because there is evidence that such compounds can affect marine life (Sánchez-Quiles et al., 2015; Sánchez-Quiles et al., 2014; Danovaro et al .2008, Wilkinson, 2004). In particular, the release of sunscreens, commonly used for human skin protection against UV radiation damage (Diffey et al., 2005) is associated with the rapid expansion of tourism in marine coastal areas (Wilkinson 2004). The Mediterranean coastal areas, indeed, only in 2008 hosted approximately 250 millions of visitors and this number could increase to 312 million by 2025 (UNEP, 2009). Nowadays, sunscreens present the fastest growing sales globally and in the Western Mediterranean countries, such products occupy the largest market (Sánchez-Quiles et al., 2015).

Sunscreen products contain organic (e.g., aminobenzoic acid, ethylhexyl triazone, cinnamates, salicylates, benzophenone, dibenzoyl-methane, benzimidazole) and inorganic filters (e.g.TiO_2 and ZnO), preservatives, adjuvants, moisturizing and antioxidant chemicals. Different ingredients of sunscreens, particularly UV filters and preservatives, have been investigated for their potential impact on marine life, from unicellular (Danovaro & Corinaldesi, 2003) to pluricellular organisms (Picot Groz et al., 2014; Fent et al., 2014; Kim et al., 2011; Kim et al., 2011a; Nakata et

al., 2009; Danovaro et al., 2008). Previous studies, indeed, have shown that these compounds (e.g., butylparaben, ethylhexyl methoxycinnamate, benzophenone-3, TiO2 and ZnO nanoparticles) can harm marine plankton and coral reefs worldwide (Sánchez-Quiles et al., 2014; Danovaro et al., 2008; Danovaro & Corinaldesi, 2003). Other investigations have reported that preservatives such us parabens and some UV-filters (e.g., oxybenzone, octocrylene or ethylhexyl triazone) can be toxic to marine invertebrates and fish (Maipas et al., 2015; Kim et al., 2014; Paredes et al., 2014; Fent et al., 2010; Diaz-Cruz et al., 2009; Kunz et al., 2006). Due to the lipophilic characteristic of sunscreen products and the insolubility of many of their ingredients, such products tend to bioaccumulate in aquatic animals (Giokas et al., 2007; Santos et al., 2012), causing effects similar to those reported for other xenobiotic compounds (Danovaro et al., 2008; Balmer et al., 2005).

Despite authorization and restriction rules for the use and commercialization of cosmetic ingredients are already available in different countries of the world (e.g., the directive 76 / 768 / CEE (EC, 1976 and n.1907/2006 REACH in Europe and the FDA, US Food 2013), there is strong evidence of the negative impacts of sunscreen products on marine ecosystems.

1. 2 OBJECTIVES

In the present study, we investigated the effects of three different brands of sunscreen products (i.e. two widely used products in Europe and USA, and a product, which was defined eco-friendly), at different concentration (50, 20 and 10µl L^{-1}), on two key species of coastal ecosystems of the Mediterranean Sea and Atlantic Ocean. In particular, we assessed the effects of sunscreens on the reproduction and larval development of *Paracentrotus lividus*, which is an accredited model for environmental toxicology studies (Meseric et al., 2015; Gambardella et al., 2015). In addition, the physiological response of *Anemonia viridis* to different sunscreen products was evaluated. Being *A. viridis* a widespread organism in coastal ecosystems and easy to rear in the aquarium it could represent a potential alternative indicator of environmental contamination.

Our findings expand knowledge about the negative impacts of sunscreen products on marine life and provide cues for stimulating the scientific research in the identification of solar products safe for human skins and at the same time compatible with marine ecosystems.

1. 3 MATERIALS AND METHODS

1. 3. 1 Ethics Statement

Farming in aquaria of *A. viridis* and *P. lividus* was performed in accordance with the best practices developed for the cnidarian and echinoderm communities in order to optimize animal health. No specific permissions were required for the locations/activities because *A. viridis* and *P. lividus* are not classified as endangered or protected species. All facilities and procedures complied with the guidelines of European Union (Directive 609/86).

1. 3. 2 Sunscreen Products

We selected three different brands of sunscreens characterized by the same protection degree (SPF 40-50) and a different composition in terms of UV filters and preservatives, some of which have been demonstrated to impact marine life (Danovaro et al., 2008).

European sunscreen (SPF 50+): a sunscreen commercially available throughout Europe, containing UV filters in the following order of decreasing concentration: octocrylene, TiO_2 (nano), butylmethoxydibenzoylmethane, bis-ethyl hexyl oxyphenol methoxyphenyl triazine and preservatives (benzyl benzoate);

USA sunscreen (SPF 50): a popular sunscreen commercially available in USA containing UV filters in the following order of decreasing concentration: homosalate, benzophenone-3, octyl salicylate, butylmethoxydibenzoylmethane, octocrylene and preservatives (methylisothiazolinone, methyl dibromo glutaronitrile);

Eco-friendly sunscreen (SPF 40): a newly patented sunscreen based on ingredients that have all been tested for protecting marine organisms, including corals and all the marine species depending on them. It contains the following UV filters in order of decreasing concentration: diethylamino hydroxybenzoyl hexyl benzoate, methylene bis-benzotriazolyl tetramethylbutylphenol, ethylhexyl triazone and the preservatives sorbic acid and potassium sorbate.

1. 3. 2 Impact of sunscreens on the development of P. lividus zygote

Mature specimens of *P. lividus* were collected from a coastal area of the Central Adriatic Sea (43°37'11.29''N 13°31'52.9''E, Mediterranean Sea) and immediately transported to the laboratory in refrigerated bags (about 8 -10 °C), enveloped in wet tissues. In the laboratory, the samples were maintained in aquaria with filtered seawater (FSW 0.2µm) for at least 1 week. The spawning of gametes was obtained as described by Amemiya (1996) using an oral injection of 1mM acetylcholine chloride diluted 1:1000 in autoclaved and ultra-filtered sea water (UFSW 0.02 µm). The eggs were collected in FSW (20 ml), while the sperm directly from the genital pores and maintained in aliquots at 4°C (2 ml). Gametes from 3 different male and 3 female specimens were mixed to minimize errors due to differences among adult specimens. In particular, 130 µL of UFSW containing 3000 eggs (counted under a microscope) were mixed with 100 µl of sperms (700 ul: 7 ml, sperm: UFSW).

The experiments were performed according to the tests validated by ISO (Falugi et al. 2008) using 90 sterile tanks (110 ml) containing 100 ml of FSW and 230 µl of mixed gametes at the temperature of 18°C that is the optimal temperature for the synchronous development of urchin eggs (Falugi et al. 2008). Three sunscreens at different concentrations (10 µL/L, 20 µL/L and 50 µL/L final concentrations) were

added to the systems (n=3 for each concentration) and compared with untreated systems (without the addition of sunscreens, n=3) used as controls.

Sub-samples from treated (added with sunscreens) and untreated systems were collected after the addition of sunscreens (t_0= start of the experiment), after 3 h (corresponding to the stage of morula) and 24 h (corresponding to gastrula stage) from the start of the experiment. In order to monitor the different stages of fertilization additional tanks were used.

The sub-samples were fixed with paraformaldehyde (PFA 4% pH 7.4) and observed under a microscope (Zeiss Axioskop, 10× magnification) within 1 week in order to determine the number of anomalous embryos over a total of 100 embryos for each sample and their morphological characterization.

1. 3. 3 Impact of sunscreens on larval development

Fifteen ml of eggs were mixed with 100 µl of diluted sperms (as described above) incubated at 18°C in the thermostatic room for 48 h in order to obtain 4-arms larvae of *P. lividus* (Gambardella et al., 2013). Time-course experiments were performed by using 90 sterile tanks (110 ml) added with 10 ml of UFSW containing *P. lividus* larvae. The different sunscreen products at different concentrations were inoculated as described above. Systems without the addition of sunscreens were used as controls. Sub-samples from treated (added with sunscreens) and untreated systems were collected immediately after the addition of sunscreens (t_0= start of the experiment, 4-arms *pluteus*) and after 24 h and 48 h after the start of the experiment.

The sub-samples were fixed with paraformaldehyde (PFA 4% pH 7.4) and observed under a light microscope (DM3000B, Leica, Germany, 10× magnification) within

1 week in order to determine the number of anomalous larvae over a total of 100 larvae for each sample and their morphological characterization.

1. 3. 4 Morphological analyses of embryos and larvae

The health state of the embryos and larvae was assessed by using a light microscope **(**Leica, Germany) and classified on the basis of the morphology and synchronicity of embryonic and larval development compared with controls. In detail, embryos were separated into three categories, designated as developed (D), anomalously developed (AD) and non-developed embryos (ND), according to Gambardella et al. (2013). Developed embryos showed normal development, with well-structured archenteron and migratory cells enter into the coelom; anomalously developed embryos were characterized by defective gastrulae, with typical signs of asymmetrical migration of primary mesenchyme cells and non-developed embryos showed both an arrested development and gastrulae lacking archenteron and coelom. Cone-shaped larvae at pluteus stage with four fully developed arms, with complete skeletal rods and with a skeleton of similar size to control larvae, were considered as normal larvae. Furthermore, on the basis of this criterion based on specific abnormalities, different types of malformations were distinguished: (a) crossed tips, (b) separated tips (c) fused arms, (d) incomplete or absent skeletal rods, (e) absence of skeletal rods and folded tip, (f) fractured ectoderm, (g) developed larvae with abnormalities and larvae who show regression and/or block of development.

1. 3. 5 Impact of sunscreens on Anemonia viridis

Anemonia viridis specimens were collected on the same site and brought to the laboratory in tanks filled with *in situ* seawater. In the laboratory, the samples were maintained in aquaria at *in situ* T seawater for at least 2 weeks using a refrigerator and two recirculation pumps; the light was supplied by appropriate lamps, with a 14:10 light: dark photoperiod. Along the maintenance and experimental times, the specimens were fed two times a week with *Artemia salina* nauplii.

Time-course experiments in aquaria were used to test the effects of different brands of sunscreen products on *Anemonia viridis.* Replicate sets containing adult organisms (n = 3) were supplemented with 50 µl L^{-1} of sunscreens and compared with untreated systems (used as controls). The adult organisms of *Anemonia viridis* were incubated in 6 L of UFSW under *in situ* conditions of temperature, salinity, light exposure, and oxygen concentration.

Sub-samples from treated (added with sunscreens) and untreated systems were collected immediately after the addition of sunscreens (t_0= start of the experiment) and after 1 (t_1), 2 (t_2), 3 (t_3), 4 (t_4), 5 (t_5), 6 (t_6), 7 (t_7), 8 (t_8) and 9 days (t_9) from the start of the experiment. After 9 days, seawater (containing sunscreens) surrounding cnidarians was replaced with 6 L of UFSW (without the addition of sunscreens) to assess the potential recovery of *A. viridis.*

The sub-samples (5 ml) were fixed with 0.02 µm pre-filtered and buffered 2% formalin (pH 8) and maintained at 4°C to determine the potential enrichment of prokaryotic abundance. Additional sub-sample (10 ml) were fixed with 3%

glutaraldehyde and successively used for the analysis of the state of healthy and rate of release of zooxanthellae.

At the sampling times t_0, t_4 and t_9 unfixed tentacles (n=3 from each organism) of *A. viridis* were sampled and frozen for further analysis of cholinesterase activity. In the recovery phase, seawater and tentacles were sampled at the start of the recovery phase (i.e., t_0) and after 2 and 5 days (t_2 and t_5, respectively).

1. 3. 6 Changes in biomass

The biomass was measured at the beginning of the experiment immediately after the addition of sunscreens and after (t_0) and after 2, 4 and 9 days (t_2, t_4 and t_9, respectively). Anemones were weighted inside a beaker containing the same amount of UFSW and at temperature if 18°C. The weight was also measured at the beginning (t_0), at t_2 and t_4 during the recovery experiment. Systems without the addition of sunscreens were used as controls.

1. 3. 7 Determination of feeding rate analysis

The impact of sunscreens on the activity and vitality of *A. viridis* was measured through the determination of its feeding rate. Nauplii of *Artemia salina* (n=3000) reared in the laboratory, were introduced in treated (added with 50 µl sunscreens) and untreated systems and subsamples (10 mL; n=3) were collected immediately and after 3 hr (t_1). The experiment was carried out immediately after the addition of sunscreens (t_0= start of the experiment), after 2 (t_2), 4 (t_4) and 9 days (t_9). We repeated the same experiment also after restoring pre-impact conditions (at t_0, t_2, t_4). The number of dead nauplii settled to the tank bottom was also determined and

subtracted from the number of nauplii removed by *A. viridis*. Subsamples from each aquarium were analyzed using a microscope (Zeiss Axioskop, 10× magnification).

1. 3. 8 Analyses of health status of zooxanthellae

Zooxanthellae analyses were performed by sampling the water surrounding *Anemonia viridis* in order to evaluate the viability and amount of the released symbiotic organisms. Sub-samples from treated (added with sunscreens) and untreated systems were collected immediately after the addition of sunscreens (t_0= start of the experiment), after 1 (t_1), 4 (t_4) and 9 days (t_9) and at t_4 recovery times. Subsamples of seawater (5 ml) were filtered through 2.0-µm polycarbonate filters, which were mounted on glass slides. Zooxanthellae were counted under a Zeiss Axioplan epifluorescence microscope (Carl Zeiss Inc., Jena, Germany; ×400 and ×1,000). Based on the auto-fluorescence and gross cell structure, zooxanthellae released from *A. viridis* were classified as: a) healthy (H, brown/bright yellow colour, intact zooxanthellae); b) pale (P, pale yellow colour, vacuolated, partially degraded zooxanthellae) and c) transparent (T, lacking pigmentations, an empty zooxanthellae; Mise and Hidaka 2003; Danovaro et al., 2008).

1. 3. 9 Acetylcholinesterase activities

Unfixed samples of *P. lividus* larvae and tentacles of *A. viridis* were used to determine acetylcholinesterase activity (AChE, EC, 3.1.1.7) by using the spectrophotometric method (Ellman 1961). Such an activity was measured in *P. lividus* larvae (n=200) collected from treated and untreated systems (n=3) immediately after sunscreen addition (50µl L^{-1}) and after 3 h and 24 h incubations. Similarly, tentacles (three from each organism) were collected from *Anemonia viridis* immediately after sunscreen addition (t_0) and after 4 (t_4) and 9 days (t_9) of

incubation. Larvae and tentacles were frozen after each time point overnight. The frozen samples were then thawed, homogenized with a minipotter (B.Braun Melsunger), passed through a syringe with a thin needle, (Ultrafin 29G, 12,7 mm length), in the presence of 1% triton X100, sonicated for 25 min (Branson, 3510) and centrifuged for 3 min at 8000 rpm. The supernatants were used to determine AChE at $\lambda = 412$ nm. The kinetic of AChE activity was obtained by measuring the velocity of substrate cleavage for 3 min compared with the linear equation of a standard curve that had been previously obtained by supplying known amounts of ChEs (Gambardella et al., 2013). The protein content in the supernatants of untreated and treated samples was measured using the method described by Lowry et al. (1951), subsequently modified by Hartree (1972). The AChE units were obtained by the ratio between the micromoles of substrates hydrolyzed/min/mg protein at room temperature.

1. 3. 10 Statistical analysis

Differences in the investigated variables (univariate tests) between controls and treatments, during the experimental time, were assessed using permutational analyses of variance (PERMANOVA; Anderson, 2005; McArdle and Anderson, 2001).

The design included three factors (time, treatment and concentration) for the *P. lividus* experiment while two factors for *A. viridis* experiment (time and treatment). When significant differences were encountered ($p < 0.05$) post-hoc pairwise tests were also carried out. Statistical analyses were performed using the routines included in the PRIMER 6+ software (Clarke and Gorley, 2006).

1. 4 RESULTS

1. 4. 1 Impact of sunscreens on the development of zygote and pluteus stages of *P. lividus*

The percentage of anomalous embryos increased significantly over time after addition of the three different concentrations of European sunscreen ($P < 0.001$, Figure 4. 1). The percentage of abnormal embryos also increased after the addition of the different concentrations of USA sunscreen but the increase was more evident (up to 100 %) when the highest concentration (50µL L^{-1}; $P = 0.0001$) was inoculated (Figures 4. 1 and 4. 2). The different concentrations of the eco-friendly sunscreen did not cause a significant increase in the percentage of embryonic anomalies over time but a significant difference with the control was observed especially after sunscreen addition (at t_0, $P < 0.001$, Figure 4. 1).

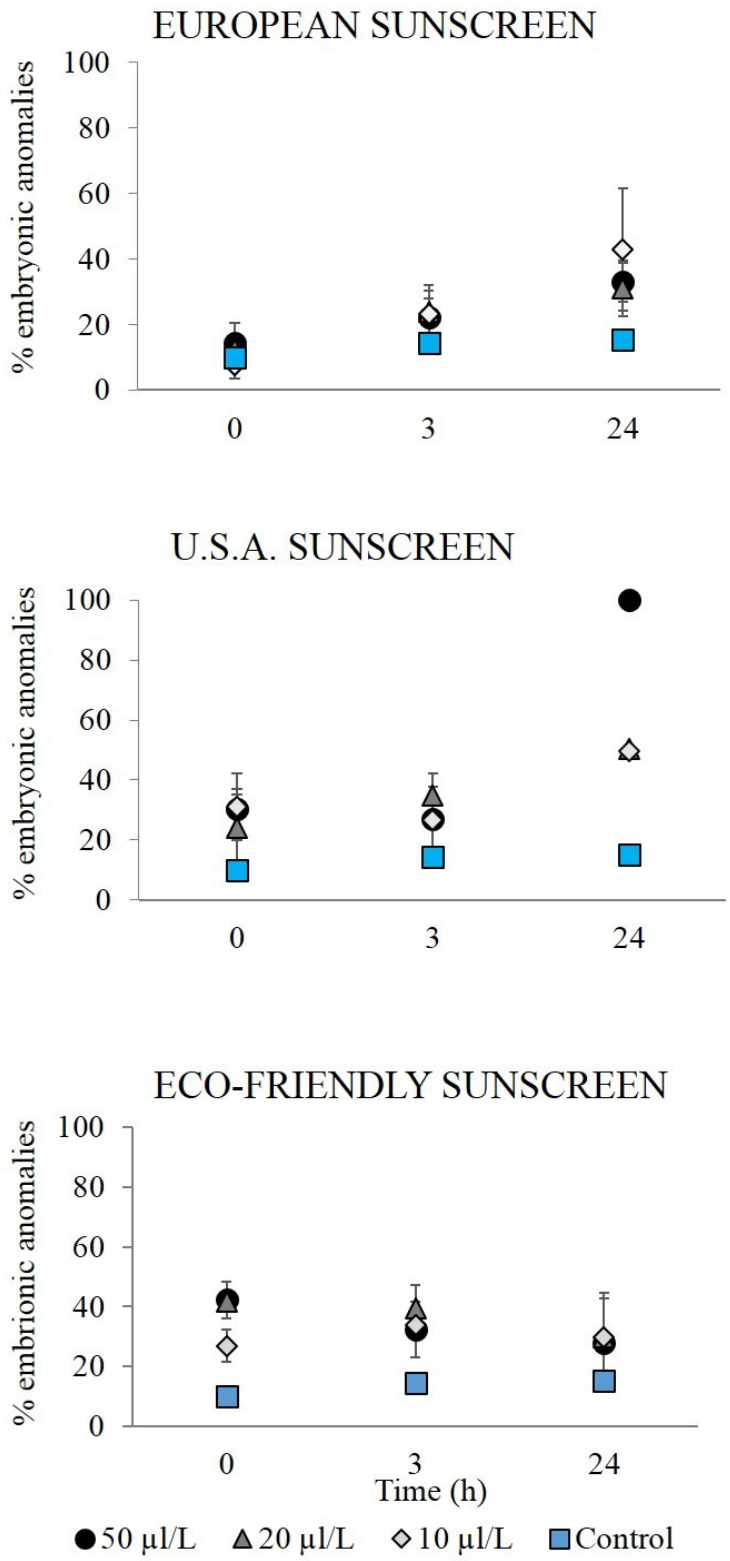

Figure 4. 1. Percentages of *P. lividus* anomalous embryos after exposure to different sunscreen products and at a different concentration over time. ± = SD.

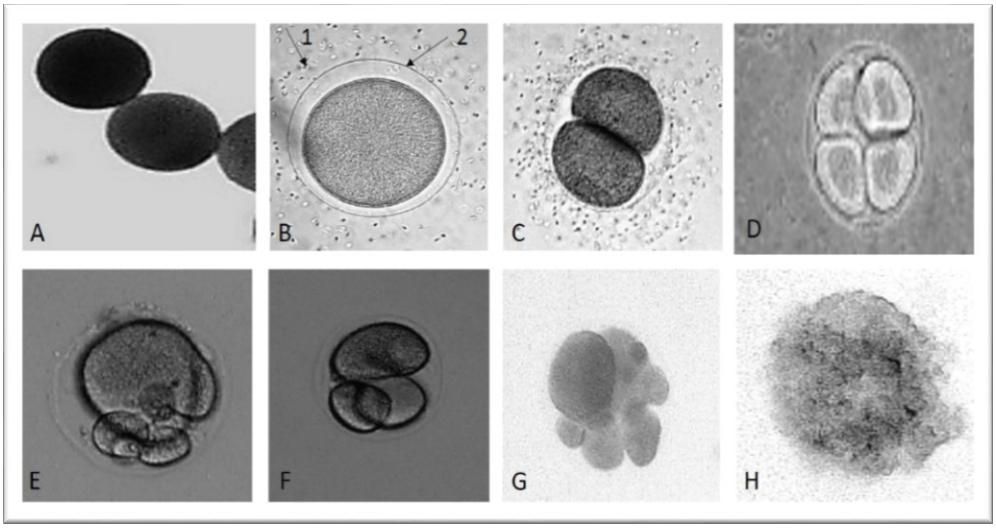

Figure 4. 2. Unexposed embryos before the experiment (A, healthy unfertilized egg cells), fertilized egg cells at the start of the experiment (B, where the elevated fertilization layer indicates a successful fertilization (2) and sperms (1) are visible), after 70 min (C) and 2h (D) from the beginning of the experiment. Main anomalies found in early embryos after fertilization (E-H) and anomalies found in early embryos after incubation with sunscreens represented by asymmetric division into blastomeres (E-G) and undeveloped embryos (F).

In all the three treatments, significant differences in the percentages of anomalous larvae were observed after addition of each concentration of sunscreen compared to the control ($P < 0.001$, Figure 4. 3). We also observed that the addition of all sunscreens, at the different concentrations, determined an immediate increase in the percentage of anomalous larvae already at the beginning of the experiment (t_0, Figure 4. 3). The percentage of abnormal larvae remained constant over time in the systems treated with all concentrations of the European and eco-friendly

sunscreens, whereas in the system added with the highest concentration of U.S.A. sunscreen such a percentage increased up to 100% ($P < 0.001$). The different classes of anomalies found after addition of U.S.A. sunscreen (50 $\mu l L^{-1}$) are showed in Figure 4. 4 B-F.

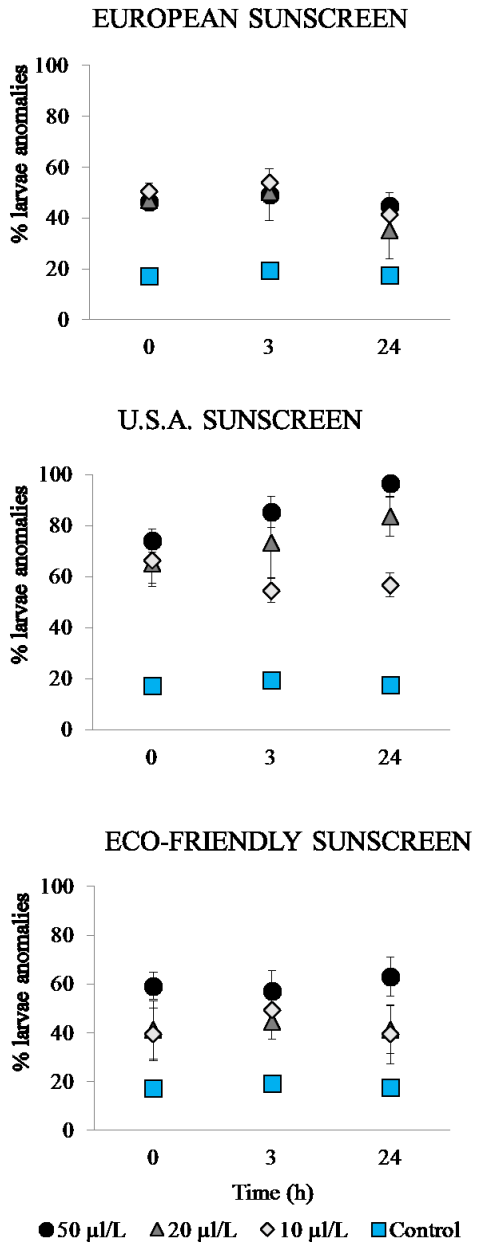

Figure 4. 3. Percentages of *P. lividus* anomalous larvae after exposure to different sunscreen products and at a different concentration over time. ± = SD.

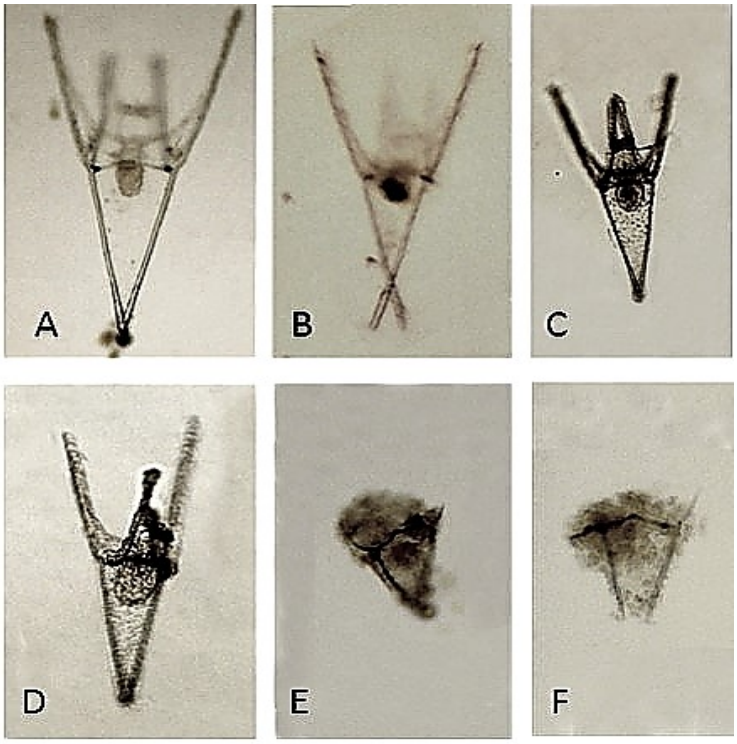

Figure 4. 4. Main anomalies found in *P. lividus* larvae after different exposure time. A: unexposed plutei, control; B: pluteus showing crossed skeletal tips at the hood apex; C, D: pluteus with joined anterior arms; E, F: incomplete or absent skeletal roots.

1. 4. 2 AChE activity

The exposure of the embryos to the different sunscreens (at the concentration of 50 µL L^{-1}) caused a general decrease over time in AChE activity in all the treatments (Figure 4. 5). AChE activity in the controls ranged from 2.40 to 4.01 x 10^{-4} µmoli per minute per mg of protein (at the beginning of the experiment and after 3 h, respectively). European sunscreen determined immediately a significant decrease in AChE activity (at t_0, $p < 0.001$), which after 3 h and 24 h continued to decrease down to 7.46 x 10^{-5} µmoli per minute per mg of protein respectively. In the treatment with USA sunscreen, AChE activity decreased significantly over time ($p < 0.001$) down to 7.01 x 10^{-6} µmoli per minute per mg of protein after 24h, whereas Eco-friendly sunscreen determined an immediate increase of the enzymatic activity (4.30 x 10^{-4} µmoli per minute per mg of protein), which then dropped to zero after 24 h of the experiment.

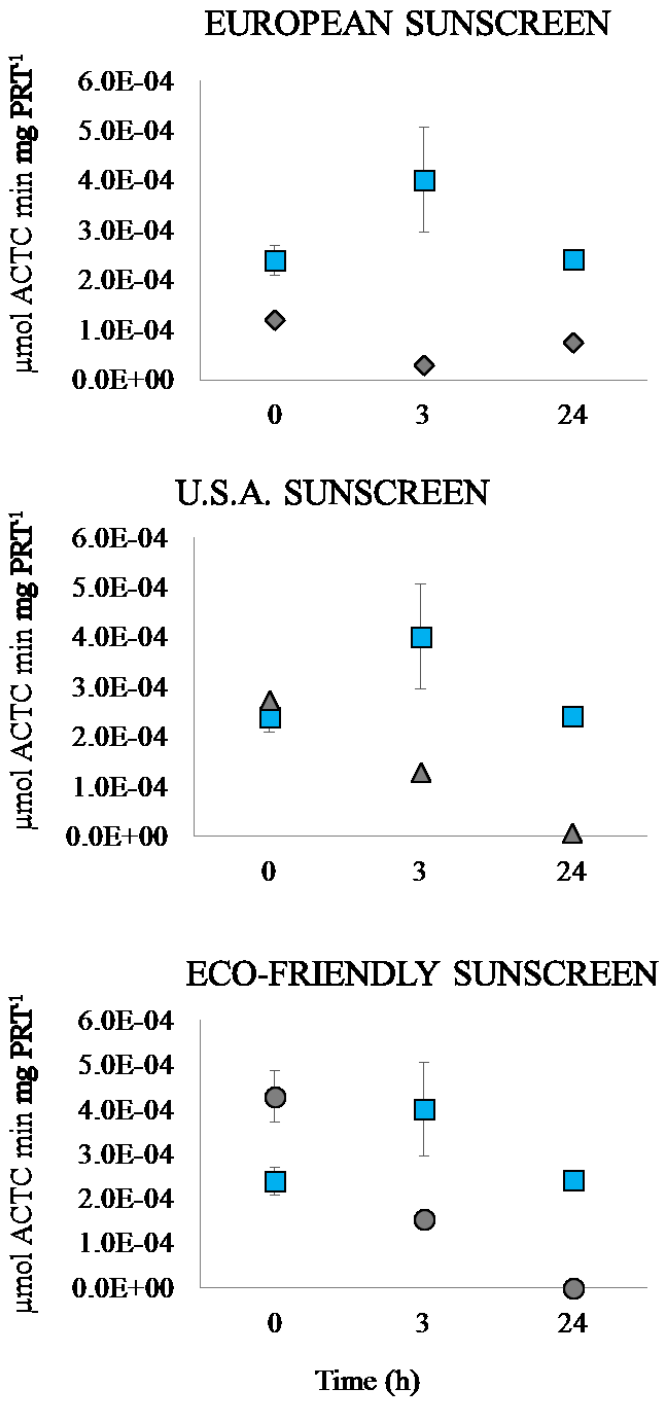

Figure 4. 5. AChE activity in control (square symbols) and exposed larvae of *P. lividus*. Sunscreens concentration = 50 µL/L; X-axis = sampling times; Y-axis = AChE Units (AChE U), where 1 AChE U= 1 micromole of ACh hydrolyzed/min/mg protein. The square dots represent control samples. ± = SD.

1. 4. 3 Impact of sunscreens on *Anemonia viridis*

1. 4. 3. 1 Changes in biomass

The biomass of *Anemonia viridis* exposed to three different treatments did not show a significant difference compared to controls.

The general trend of the control samples was towards the loss of time-progressive biomass. The *Anemonia viridis* exposed at the European sunscreen showed a slight weight loss in the first exposure time (t_0 and t_2) and then remained constant during the exposure, especially in recovery times (Fig. 4. 6). As opposed, in the U.S.A. and eco-friendly sunscreen, the weight of exposed organisms remains constant throughout the experiment but shows a slight decrease during the recovery time.

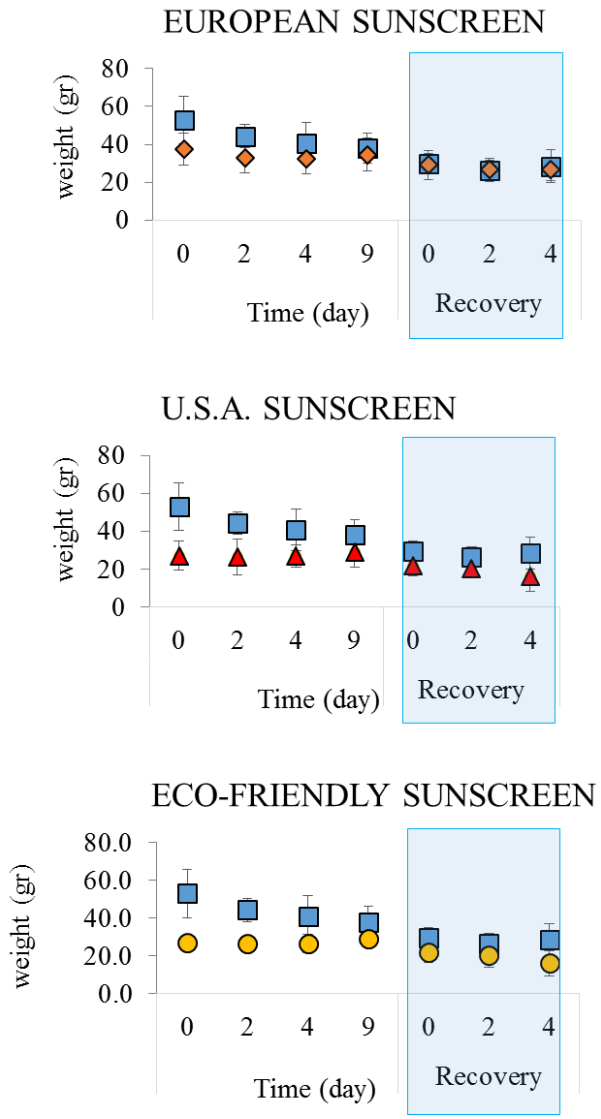

Figure4. 6. The weight variation (g) of *Anemonia viridis* in the control (square symbol) and treated. ± = SD.

1. 4. 3. 2 Feeding rate analysis

The specimens exposed to the three treatments at a concentration of 50 µl L^{-1} showed the same pattern of the feeding rate (Fig. 4. 7). During the period of exposure, the number of nauplii removed from the water was similar in the threats and in the control. Also during the recovery phase, in general, we did not observe

significant differences in the rate of feeding between the organisms of *A. viridis* exposed to the three treatments and the control (Figure 4. 7).

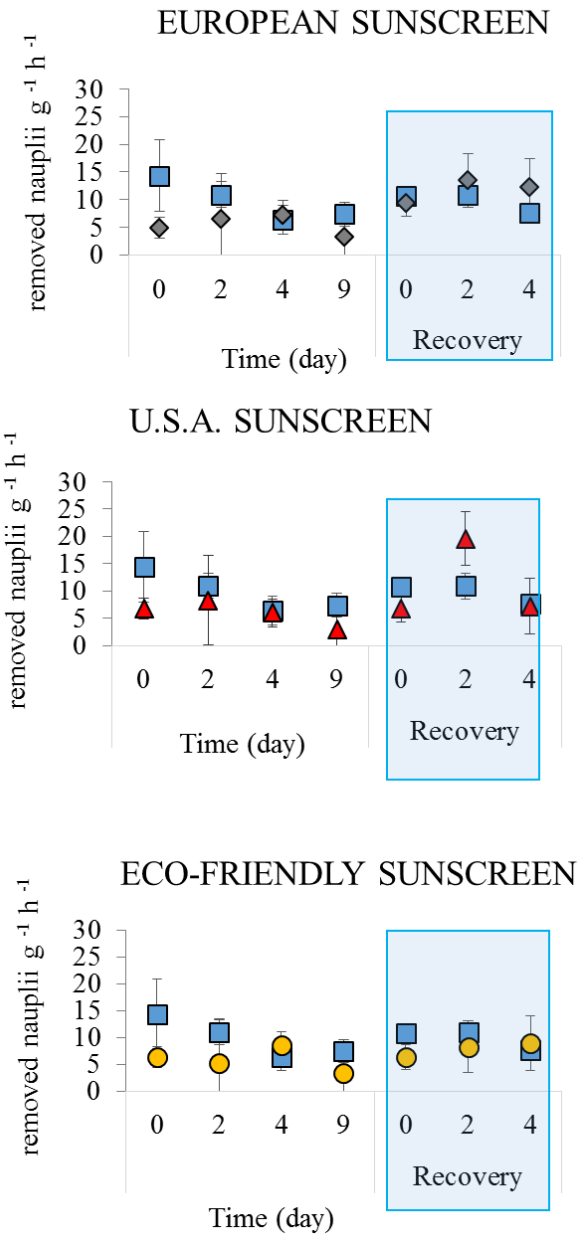

Figure 4. 7. Removed nauplii of *Artemia salina* (g $^{-1}$ h^{-1}) in the control (square symbol) and treated systems. ± = SD.

1. 4. 3. 3 Amount of released zooxanthellae

Eco-friendly and, especially USA sunscreen, determined a higher release of symbiotic organisms than in the control during the exposure phase, already after 1-4 h from the beginning of the experiment ($p < 0.001$). Such a release decreased during the recovery phase and particularly in the system added with Eco-friendly sunscreen, we observed a reduction of the fraction of released zooxanthellae compared to the control. After addition of the European sunscreen, a number of released symbionts was always lower compared to the control both during the exposure and recovery time.

The analysis of the health status of zooxanthellae showed that the fraction of healthy zooxanthellae, significantly increased in all treats (Figure 4. 10). In particular, the U.S.A. sunscreen caused an immediate increase in the percentage of healthy zooxanthellae already immediately after addition of the sunscreen (Figure 4. 10). In the recovery phase, all the treatments determined a decrease of healthy symbiotic algae when compared to the control.

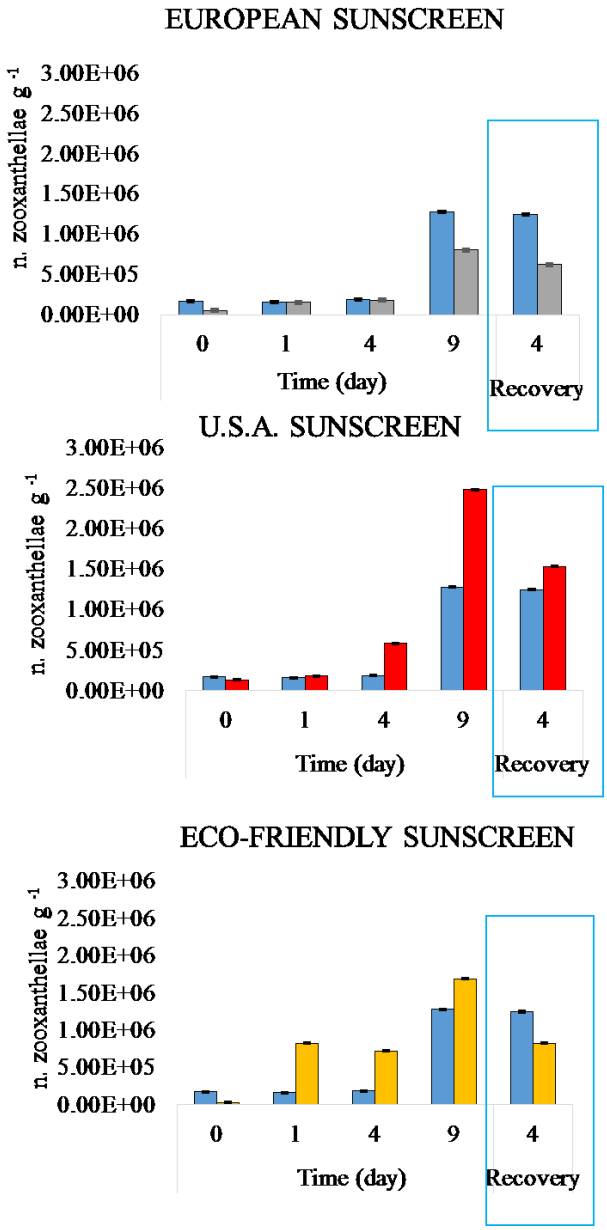

Figure 4. 9. A number of zooxanthellae released into the water boundary in experimental systems with *Anemonia viridis* treated and untreated (blue columns) at different sampling times. ± = Standard Error.

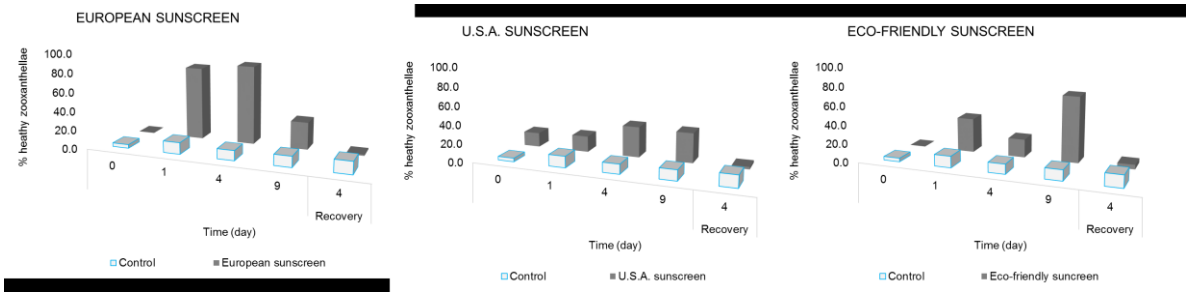

Figure 4. 10. Percentage of healthy zooxanthellae released into seawater surrounding *Anemonia viridis in* treated and untreated (blue columns) at different sampling times.

1. 4. 3. 5 AChE activity

The exposure to the European and U.S.A. sunscreens did not determine any effect on the activity of AChE, which remained rather constant over time and non-significantly different from controls (Fig 4. 11).

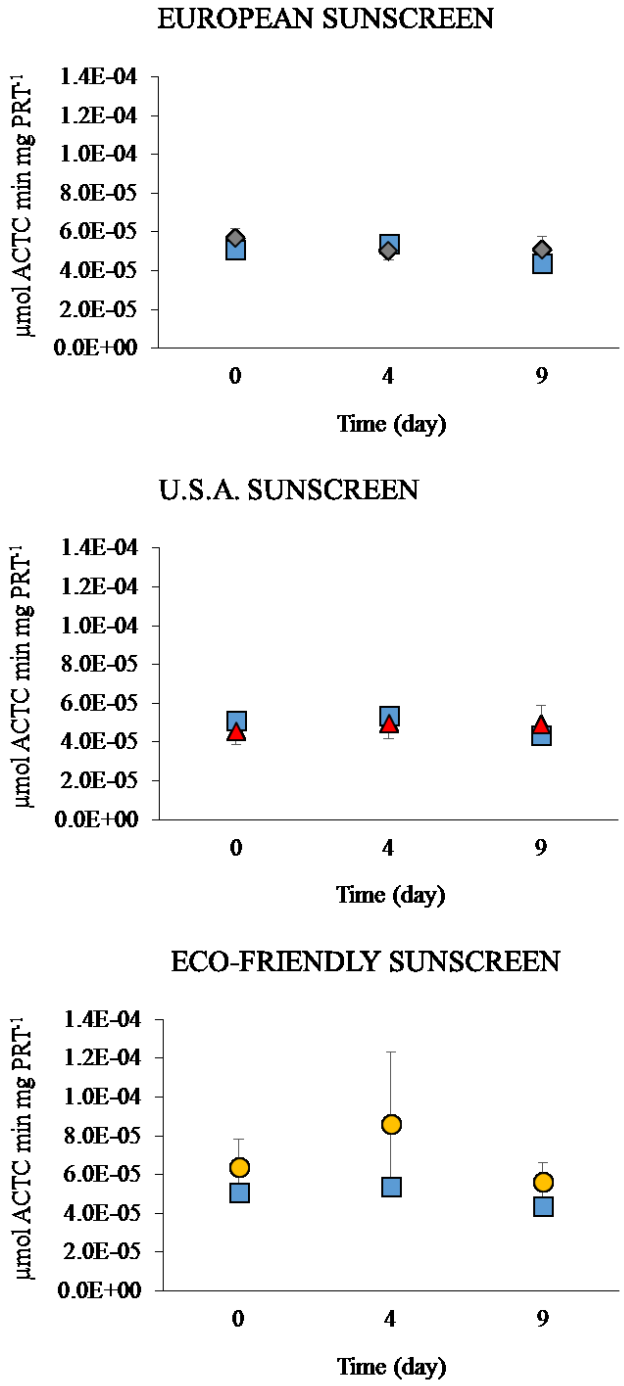

Figure 4. 11. AChE activity in control (square symbols) and *Anemonia viridis*. Sunscreens concentration = 50 µL/L; X-axis = sampling times; Y-axis = AChE Units (AChE U), where 1 AChE U= 1 micromole of ACh hydrolyzed/min/mg protein. The square dots represent control samples. ± = SD.

1. 5 DISCUSSION

1. 5. 1 Effect of sunscreen products on P. lividus

In the last decade, a number of studies have revealed that UV filters and preservatives can have a variety of negative effects on different marine organisms (Danovaro et al. 2008; Fent et al., 2010; Kim et al., 2014; Paredes et al., 2014; Maipas et al., 2015; Tovar-Sanchez et al., 2013). In particular, such effects have been investigated in coral reef ecosystems where sunscreens are generally released by tourists, including scuba divers (Ferrigno et al., 2016).

Here, we investigated the effects of three different sunscreen brands on the embryo and larval development of *Paracentrotus lividus*, a species widely diffused in Mediterranean and Atlantic coastal ecosystems (Verlaque and Boudouresque, 2001). Such ecosystems can be strongly affected by human activities and pollution (Mesaric et al., 2015) and recent studies have provided evidence that sunscreen products are a significant source of organic and inorganic chemicals reaching coastal ecosystems with potential ecological consequences on marine life (Tovar-Sanchez et al. 2013).

Since *P. lividus* is a very sensitive for a great variety of pollutants particularly during the first stages of development (Pesando et al., 2003, Carballeira et al., 2012) it is widely used as a model organism to evaluate the effects of chemicals, including components of cosmetic products (Mesaric et al., 2015; Gambardella et al., 2013; Manzo et al., 2013; Falugi et al., 2008; Pardes et al., 2014). Such information is very useful to allow comparisons with results provided in the present studies.

Our findings indicate that commonly used sunscreens can affect the embryo and larval development of *Paracentrotus lividus* depending on the product and concentration tested. In particular, we found that USA sunscreen at the highest concentration ($50ulL^{-1}$) caused anomalies in all embryos investigated after 24h of treatment, hampering the embryo development after the gastrula stage. Conversely, Eco-friendly sunscreen after 24 h of treatment produced a fraction of abnormal embryos similar to that found in the control suggesting a minor effect on their development.

Even USA sunscreen, at 10 uL^{-1} and $20ulL^{-1}$ concentrations, and European sunscreen, at all concentrations, determined a significant effect on embryo development (after 24 of exposure, ca. 30-40%) but less severe than that induced by USA sunscreen at the highest concentration.

The embryonic abnormalities observed in the different treats were represented by defective gastrulae, with typical signs of asymmetrical migration of primary mesenchyme cells and non-developed embryos, which were especially found in the systems added with USA sunscreen.

The negative effects of the sunscreens tested were even more evident in the development phase of the larvae. Indeed, all the sunscreen products caused an immediate negative effect on the larval development, with the highest percentage of abnormalities (100%) already in the systems added with $20ulL^{-1}$ of U.S.A. sunscreen. The fraction of abnormal larvae was high also when they were exposed to European and Eco-friendly sunscreens (50-60%) and remained rather constant over time. The anomalies observed included crossed and/or separated tips at the

hood apex, fused anterior arms, and incomplete or absent skeletal rod. The categories of the anomalies identified in both embryos and larvae were similar for all the systems exposed to sunscreens. These anomalies were previously identified also in other studies in which *P. lividus* larvae and gametes were exposed to nanoparticles (Gambardella et al., 2015; Mesaric et al., 2015; Gambardella et al. 2013; Manzo et al., 2013), organophosphate pesticides (Aluigi et al., 2010) and mixtures of organic wastes and contaminants of different nature (Carballeira et al. 2012). These results suggest that sunscreen products act as a wide category of pollutants that have been reported to affect the sea urchin skeleton apparatus, by modifying the location of the skeletal rods or primary mesenchymal cell migration, or by inactivating the gene regulatory system underlying the development of the embryonic skeleton (Peterson & McClay, 2003).

Despite the typologies of anomalies induced by the different sunscreens were similar, we observed that their quantitative relevance changed depending on the sunscreen product and in some cases, on the concentration tested. In particular, high concentrations of USA sunscreen determined the most severe effects on embryos and larvae since ca. 80% of them showed the block or regression of the development or death (particularly referred to larvae). Based on the criteria previously established for embryonic and larval alterations (Gambardella et al. 2013, Carballeira et al. 2012, Guillou et al., 2000), the effects due to USA sunscreen can be referred to a toxicity level 3, which represents the most severe toxicity for contaminants (Carballeira et al. 2012). The reason why such a sunscreen had a major negative effect already in the first stage of development of *P. lividus* can be explained by the presence of different ingredients contained within USA sunscreens

such as benzophenone 3 and homosalate, which have been reported to be highly toxic to marine organisms (Krause et al., 2012).

Despite the embryonic development of *P. lividus*, in terms of percentage of anomalous zygotes, was more affected by European sunscreen than Eco-friendly sunscreen, the opposite effect was observed for larval development. However, based on the morphological alterations observed, European sunscreen resulted in a higher toxicity level (resulting on average in ca. 30% of embryos and larvae with the block of their development) compared to Eco-friendly sunscreen (on average, only ca. 4% of embryos and larvae with the block of their development).

Since previous studies have reported that, being sea urchin larvae very plastic, their anomalies can be also reversible. Therefore, we suggest that the anomalous *plutei* exposed to Eco-friendly sunscreen could even survive and transform in healthy sea urchins. The higher toxicity of the European sunscreen than that of Eco-friendly, particularly on early stages of development of *P. lividus* induced by European sunscreen can be due to the presence of octocrylene, TiO_2 (nanoparticles) and preservatives such as benzyl benzoate in its composition (Petra et al., 2006). The delay of development in embryos of *Paracentrotus* appears to be primarily a consequence of changes in AChE activity, which is responsible for regulating neurotransmission and other relevant biological processes, including the correct cell migration during gastrulation (Drews, 1975, Falugi, 2008, Gambardella et al., 2015). It has been also reported that changes in AChE activity due to the exposure to cholinesterase inhibitors (i.e., neurotoxic pesticides) during the first life stages of *P. lividus* can lead to cytoskeletal alterations (Aluigi et al. 2008).

Since AChE activity is known to rapidly respond to a wide range of chemical and environmental stress (Gambardella et al., 2015; Falugi et al., 2012; Gambardella et al., 2013; Pesando et al., 2003) we analyzed the rates of AChE activity in the larvae of *P. lividus* exposed to sunscreens to investigate their neurotoxic effects. The analysis of the AChE activity revealed that sunscreens here tested, altered such an enzymatic activity compared to the control, determining its general decrease over time. These findings are in line with other studies where the exposure of *P. lividus* larvae to different micropollutants were shown to inhibit AChE activity (Gambardella et al., 2015; Mesaric et al., 2015; Aluigi et al., 2010; Pesando et al., 2003). The inhibition of AChE activity could be caused by the irreversible or reversible binding of the sunscreen ingredients to the enzyme's catalytic sites and to be involved in the skeletogenic aberrations (Falugi et al., 2008, Gambardella et al. 2012). Despite AChE activity decreased over time, also when sea urchin larvae were exposed to Eco-friendly sunscreen, at the stage of 4-arms *pluteus* we observed a significant increase compared with the control was observed. Previous studies have suggested that an increase in AChE activity in *P. lividus* might be the result of differential locomotion and feeding patterns to enhance its survival and fitness (Jennings et al. 2007, McEdward and Miner, 2007).

Overall, our findings suggest that commonly used sunscreens particularly containing chemical filters (i.e., octocrylene, homosalate and benzophenone-3) and preservative (methylisothiazolinone, benzyl benzoate) can affect the embryo-larval development of *P. lividus,* reducing the ability of larvae to survive, thus potentially affecting the abundances/biomasses of sea urchins and the trophic webs of coastal ecosystems. In addition, data reported here reveal that the Eco-friendly sunscreen,

whose ingredients have been defined compatible with marine life (especially of tropical ecosystems), impacted to a lesser extent the embryos and larvae of *P. lividus*, although stress signals due the exposure to high concentrations of such a product were still evident. Therefore, it is strongly advisable to develop eco-compatible sunscreen products, specifically tested *a priori* even on the early developmental stages of marine organisms, which might be more sensible than adults to these contaminants.

1. 5. 2 Effect on sunscreen products on Anemonia viridis

Sea anemones, as well as other Cnidarians, are models commonly used for studying the impact of contaminants on marine life (Venn et al., 2008). Several species of anemones contain symbiotic dinoflagellates (*Symbiodinium sp*.) in their tentacles known as zooxanthellae (Caparkaya et al., 2010), which are highly susceptible to a wide range of stress (Merle et al., 2007).

The experiments here conducted on *Anemonia viridis* exposed at 50 μL L^{-1} of different sunscreen products revealed that these organisms, respond with behavioral alterations in presence of sunscreens. Indeed, already after 2 days of the beginning of the exposure (especially the anemones exposed to USA sunscreen) retracted the tentacles, contracted their body and released a large amount of mucus suggesting the stress of the animals. However, during the experiment with the different sunscreens, we did not observe a significant change in biomass, feeding rates and AChE activity in the anemones over time, as well as during the recovery phase. Conversely, the rate of release of zooxanthellae in seawater increased in all systems during the exposure at the different sunscreens. This was most evident in the systems treated with USA sunscreens where after 9 days the number of

zooxanthellae released was 19 fold higher than at the start of the experiment. We observed that Eco-friendly sunscreen determined a more important effect in the release of symbionts than European sunscreen. However, after 9 days of exposure to Eco-friendly sunscreen, we observed that most of the released zooxanthellae were healthy (ca. 70%), whereas only ca. 30% of these was healthy in the systems incubated with European sunscreen. Previous studies revealed that the release of healthy zooxanthellae by their host organism can be a reversible process (Leichter et al., 1999), suggesting a potential recovery of the organism. The results obtained during the recovery phase showed a decrease of the release of zooxanthellae in seawater in both the systems added with European and Eco-friendly sunscreen. Conversely, anemones exposed to USA sunscreen continued to release their symbionts, indicating the impossibility to recover. Indeed, during the recovery phase anemones exposed to USA sunscreen died. We hypothesize that the death of these organisms was caused by the change in seawater conditions to which they were relatively adapted.

Overall, we can suggest that *A. viridis* appeared to be rather resistant to sunscreens tested, except for USA sunscreen that caused the death of the organisms when the pre-impact conditions were restored. Conversely, European and Eco-friendly sunscreens, even if promoted the release of their symbionts did not affect particularly the health of anemones.

1. 6 CONCLUSION

All these results indicate that sunscreens cause damage to early stages of development of *Paracentrotus lividus*, resulting in decreased reproductive capacity of the sea urchin, due to the alteration in both embryo development and larvae structure. The anomalies identified in this study are similar to those identified in numerous investigations in response to exposure of sea urchin gametes to neurotoxic pesticides and nanoparticles. This may mean that the mechanism of action of these pollutants on the stages of early development of sea urchin is the same. Indeed, the occurrence of abnormalities in the sea urchin development is in accordance with the decrease in the activity of acetylcholinesterase, already reported in several studies.

Otherwise, *Anemonia viridis* seems to resist better to this type of stress, not showing effects in terms of change in biomass, the rate of feeding and activity of acetylcholinesterase except for their symbiotic organisms.

We can suggest that the different effect of sunscreens on *P. lividus* and *A. viridis* is due to the different sensitivity of the organisms considered and to the different life cycle considered.

Since, we observed that the sunscreens defined eco-friendly produced a relatively slight impact on larvae of *P. lividus*, this suggests the need to test the products on different model marine organisms, before placing them on the market, to develop cosmetic products that effectively are compatible with marine life. Finally, this study provides new evidence of the direct effects of sunscreens on marine environments and paves the way for further research on different marine organism

models, both on different trophic levels and different stage of developments, to understand how sunscreens can affect the health of marine organisms and the balance of the environment in which they live.

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Chapter 2

Impact of different brands of sunscreen products on *Acropora sp*.

2.1. INTRODUCTION

Coral reefs are amongst the most diverse and productive ecosystems on Earth that support a huge biodiversity (around 830,000 multi-cellular species, Fisher et al., 2015), and provide ecosystem services to half a billion people including food security (Hughes et al., 2012), financial incomes (Teh et al., 2013) and protection against natural hazards (Ferrario et al., 2014). Approximately 60% of coral reefs are currently threatened by several natural and anthropogenic impact (Danovaro et al., 2008). Numerous environmental conditions can cause stress to corals and coral bleaching (i.e. loss of symbiotic zooxanthellae hosted within scleractinian) that in the last 20 years has increased dramatically in frequency and severity (Douglas et al., 2003). In particular, the excess of ultraviolet radiation (UV), temperature abnormalities, the presence of pathogens and pollutants such as sunscreen products have been reported to be responsible for coral bleaching (Hedouin et al., 2016; Danovaro et al., 2008; Douglas, 2003).

Previous studies have shown that the sunscreen products and their ingredients (e.g., UV filters such as butylparaben, ethylhexyl methoxycinnamate, benzophenone-3, benzophenone-2) can harm coral reefs worldwide causing bleaching and genotoxicant effects (Downs et al., 2015, 2013; Danovaro et al., 2008).

Production and consumption of personal care products including sunscreens have had a strong growth over the last decades, presenting to date the fastest growing sales globally (Sánchez-Quiles et al., 2015). In particular, the release of sunscreens

is associated with the rapid expansion of tourism in marine coastal areas (Wilkinson 2004). It has been estimated that every year, millions of tourists travel to tropical destinations (Creel, 2003) with potentially important consequences on environmental contamination (Danovaro et al., 2008).

The properties and persistence of the sunscreens once applied to the skin can be changed due to immersion, UV radiation, temperature, moisture or abrasion with beach sand (Langford & Thomas, 2008; Stokes & Diffey, 2000) leading to the release of altered products into the marine environment. For example, commonly used UV filters, such as zinc oxide and titanium dioxide, can release hydrogen peroxide into marine waters generating high levels of oxidative stress in marine organisms (Sánchez-Quiles & Tovar-Sánchez, 2014).

In recent years there has been growing interest in producing and marketing products /ingredients defined green, natural, eco-friendly, eco-compatible or biodegradable (D'Souza et al., 2007) but there isn't any scientific evidence on the effective eco-compatibility of these products with marine ecosystems.

2. 2. OBJECTIVES

Here we assessed the potential impact of different sunscreen products, defined by the producers "eco-friendly, natural and reef-safe", on hard corals (*Acropora sp.)* and their symbiotic algae from the Indian Ocean (Vavvaru Island, Maldives). Independent experiments were conducted in two different periods in 2013 and 2014.

2.3. MATERIALS AND METHODS

2.3.1. Sunscreen Products

We selected different brands of sunscreens, including sunscreen products defined "eco-friendly, natural and reef-safe sunscreens", characterized by a different composition in terms of UV filters, preservatives, emulsifiers, moisturizing and other ingredients. The names assigned to the sunscreens tested not correspond to the real name of the sunscreens, but have been assigned on the basis of the characteristics of solar products expressed by different manufacturers.

The following sunscreen products were tested during the first experiment performed in Maldives.

European sunscreen: a sunscreen containing UV filters such TiO_2 and other organic filters (i.e., octyl methoxycinnamate, octyl triazone, phenylbenzimidazole sulfonic acid) and preservatives (i.e, benzyl benzoate).

U.S.A. sunscreen: a product commercially available in USA containing different organic UV filters such as butyl methoxydibenzoylmethane, homosalate and benzophenone-3 and preservatives (i.e., methylisothiazolinone, methyldibromo glutaronitrile).

Eco-friendly sunscreen: sunscreen based on new-patented ingredients tested for protecting marine organisms, including corals and all the marine species depending on them. The composition of this product is characterized by UV filters such as methylene bis-benzotriazolyl tetramethylbutylphenol, diethylamino

hydroxybenzoyl hexyl benzoate and ethylhexyl triazone and preservatives such as sorbic acid and potassium sorbate.

Eco-friendly sunscreen 1: sunscreen containing UV filter such us TiO_2, aluminum hydroxide ($Al(OH)_3$ compound used to minimize the photocatalytic reactions and as anti-agglomerating), additive (iron oxide) and other ingredients of natural origin (i.e., silica and various seed oil).

The following sunscreen products were tested during the second experiment performed in Maldives.

Reef-Safe sunscreen: a sunscreen defined "reef safe" containing inorganic UV filters such as TiO_2 and different organic filters (i.e., oxybenzone, homosalate, octisalate and octinoxate), and preservatives such as methylparaben.

Reef-Safe sunscreen 1: a sunscreen product defined "reef safe" containing different UV filters such as Titanium dioxide and Zinc oxide (ZinClear ^{TM}), preservatives such as sodium benzoate and potassium sorbate, and other ingredients of natural origin.

Natural sunscreen: the composition of this product, defined 100% natural and biodegradable, is characterized by UV filters such as zinc oxide (uncoated), antioxidants (tocopherol) and > 87% of organic ingredients (i.e., different seed oil, sunflower).

Natural sunscreen 2: this product is characterized by physical UV filters (i.e., TiO_2 and zinc oxide) and various ingredients such as chelating agent (gluconolactone),

antioxidants (tocopherol) and 85 % of organic ingredients (i. e., aloe vera, seed oil, glycerin, glycine soja oil).

Ecoreach sunscreen 1: sunscreen containing UV filters (i.e, ethylhexyl triazone), preservatives (potassium sorbate) and other ingredients such as citrus arantium dulcis peel oil, citrus grandis peel oil, propylene glycol and caprylyl glycol.

Ecoreach sunscreen 2: sunscreen characterized by physical UV filter, TiO_2 (no-nanoparticles and it coated with alumina which acts as a photo-stabilizer) and other ingredients such as emulsifiers (i.e., sucrose stearate) and other organic compounds such as simmondsia chinensis oil, candelilla cera. Eco-friendly sunscreen and Eco-friendly sunscreen I are products developed by Ecoreach l.t.d. based on new-patented ingredients (in collaboration with Polytechnic University) selected and tested for protecting marine life (EcolCare^{TM} protocol, A+++) on different marine organisms including algae, cnidarians, and crustacean. These sunscreen products are characterized by different composition in terms of ingredients used in the final formulations.

2.3.2. Study areas and experimental design

In field experiments were conducted in Vavvaru Island (Maldives, Indian Ocean) in two consecutive years. The first experiment was performed in December 2013 while the second experiment in January 2014. Nubbins from different donor colonies belonging to the genus *Acropora* (3-6 cm) were collected and placed in aquaria for the acclimatization process (i.e., 48 hr under stable and *in situ* conditions of temperature and salinity) before the experiments. After acclimatization, only healthy species (without any signs of necrotic tissue and with polyps regularly open) were used to perform the experiments. Nubbins of *Acropora sp.* were washed with virus free seawater filtered onto 0.02 μm membranes (Anotop syringe-filters; Whatman, Springfield Mill, UK), immersed in polyethylene Whirl-pack bags (Nasco, Fort Atkinson, WI, USA) and filled with 2 L virus-free seawater. Replicate sets containing nubbins (n = 3, including more than 300 polyps each) were supplemented with aliquots of different brands of sunscreens (at final quantities of 50 μL/L seawater) and compared with untreated systems (used as controls). Corals were incubated in aquaria with seawater in a continuous flow, directly from the ocean, maintained at *in situ* conditions (temperature and salinity).

2.3.3. Analyses of zooxanthellae

Zooxanthellae analyses were performed by sampling the seawater surrounding of coral colonies in order to evaluate the viability and amount of the released symbiotic organisms.

Sub-samples from treated (added with sunscreens) and untreated systems were collected immediately after the addition of sunscreens (t_0= start of the experiment), after 24 hr (t_{24}) and at the end of the experiments: 48 hr (t_{48}) in the first experiment

and 32 hr (t_{32}) in the second experiment. Aliquots of water samples and filtered through 2.0-μm polycarbonate filters. Then, filters were mounted on glass slides. Zooxanthellae were counted under a Zeiss Axioplan epifluorescence microscope (Carl Zeiss Inc., Jena, Germany; ×400 and ×1,000). Based on the auto fluorescence and gross cell structure, zooxanthellae released from coral colonies were classified as a) healthy (H, brown/bright yellow color, intact zooxanthellae); b) pale (P, pale yellow colour, vacuolated, partially degraded zooxanthellae); transparent (T, lacking pigmentations, an empty zooxanthellae; Mise and Hidaka 2003; Danovaro et al., 2008).

2.3.4. Quantification of bleaching

To quantify the levels of coral bleaching (Siebeck et al. 2006), we performed a colorimetric analysis on digital photographs of corals taken at the beginning of the experiments and at the established sampling times. Photographs were taken under identical illumination with a Canon EOS 400D digital camera (Canon Inc., Tokyo, Japan) with a scale meter on the background. The photographs were successively analyzed with a photo-editing software for color composition cyan, magenta, yellow, black (CMYK). Levels of bleaching were measured as the difference between the coral's color at the beginning of the experiments (t_0) and after 32 and 48 h from the sunscreen addition (t_{32} and t_{48}, respectively for the first and second experiment). Inside the coral, areas were made 30 random measurements of variables CMYK. Variations in the percentage of the different color components (CMYK) were analyzed with one-way analysis of variance (ANOVA). To rank the bleaching effect due to the different sunscreen tested we obtained Bray–Curtis similarity matrix and multidimensional scaling analysis of the shifts in CMYK color composition of treated corals using Primer 5.0 software (Primer-E Ltd., Plymouth, UK). Bleaching rates were measured as the dissimilarity percentage in CMYK color composition between treated and control corals using Primer 5.0 software (Primer-E Ltd). In addition, scores of the degree of bleaching were attributed to the average values obtained by mean of a mathematical function (Tab 1) and according to a scale organized in ranks (0 to> 60), i.e., from "no visible coral bleaching" (0-10) to the "total bleaching with 100% of coral nubbins surface (> 60).

Degree of bleaching (%)	Incidence of coral bleaching
 0-10	No visible coral bleaching
10-20	Slight color variation. No visible coral bleaching
20-30	Slight bleaching (< 10 % of coral nubbins surface)
30-60	Strong bleaching (>50% of coral nubbins surface)
> 60	Total bleaching (100 % of coral nubbins surface)

Table 1. Main classes of degree of bleaching

2.3.5. Prokaryotic and viral abundance

Prokaryotic and viral abundance in the seawater surrounding corals was determined according to the protocol described by Noble and Fuhrman (1998) without previous fixation to avoid underestimations of viral abundances (Danovaro et al 2001, Wen et al 2004). Sub-samples (10 ml) from treated (added with sunscreens) and untreated systems were collected immediately after the addition of sunscreen (t_0= start of the experiment), after 24 hr (t_{24}) and at end of the experiments (48 hr, t_{48} in the first experiment and 32 hr, t_{32} in the second experiment). After collection, seawater samples (n=3) were stored at -20°C until the analysis. Sub-samples were filtered onto 0.02µm pore size filter (Whatmann Anodisc; diameter, 25 mm; Al_2O_3) and stained with 100 µL of SYBR Gold (stock solution diluted 1:5000). The filters were incubated in the dark for 20 min, washed three times with 3 mL of prefiltered Milli-Q water and mounted onto glass slides with 20µl of 50% phosphate buffer (6.7 mM phosphate, pH 7.8) and 50% glycerol (containing 0.5% ascorbic acid). Slides were stored at -20°C. Prokaryote and viruses counts were obtained by epifluorescence microscopy (Zeiss Axioskop 2). For each slide, at least 20 microscope fields were observed and at least 200 prokaryotes and viruses were counted per filter.

2.3.6. Statistical analysis

Differences in each of the investigated variables (univariate tests) between the controls and treats, during the experiment, were assessed using permutational analyses of variance (PERMANOVA; Anderson, 2005; McArdle and Anderson, 2001). The design included two factors (time and treatment). When significant differences were encountered ($p < 0.05$) post-hoc pairwise tests were also carried out. Statistical analyses were performed using the routines included in the PRIMER 6+ software (Clarke and Gorley, 2006).

2.4. RESULTS

2.4.1. Analyses of zooxanthellae

In both the experiments performed, the addition of all sunscreens (i.e., 50 µL/L) resulted in a significant release of total and damaged zooxanthellae ($p>0.001$) respect to the controls (Figure 4.1-4.4).

In particular, we observed a higher release of (damaged) zooxanthellae after exposure to U.S.A. sunscreen and Eco-friendly sunscreen 1 than Eco-friendly (Fig. 4.2). Eco-friendly sunscreen 1 induced a very high release of (damaged) zooxanthellae after 24 and 48 hours of exposure ($p <0.001$, Fig. 4.1) whereas U.S.A. sunscreen, particularly after 48 h. In the second experiment.

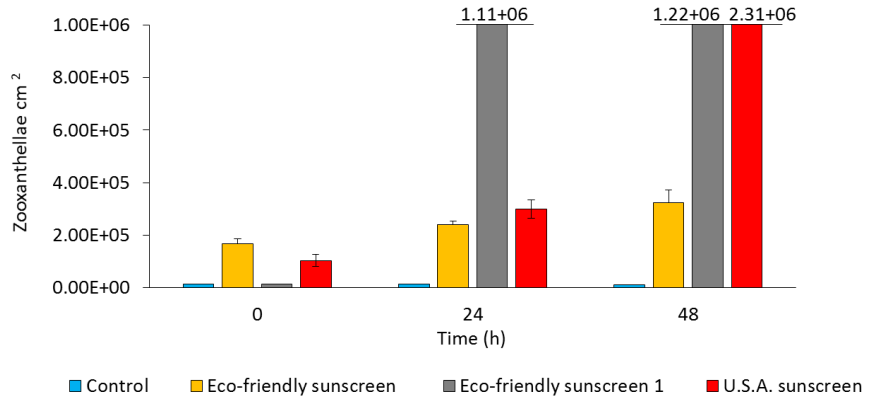

Figure 4.1. A number of zooxanthellae released into seawater surrounding coral branches treated with different brands of sunscreens and in the control, during the first experiment. ± SD.

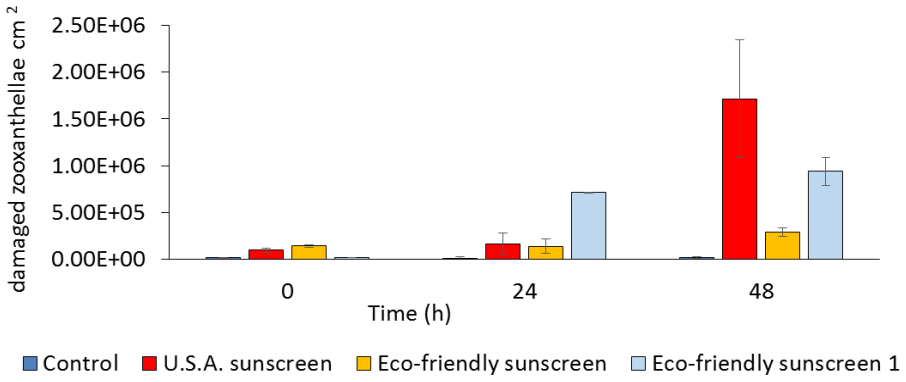

Figure 4.2. A number of damage zooxanthellae released into seawater surrounding coral branches treated with different brands of sunscreens and in the control, during the first experiment. ± SD.

During the second experiment performed in the Maldives nubbins treated with Natural sunscreen 2, Reef-safe sunscreen and Reef-safe sunscreen 1 showed the highest values of zooxanthellae released and damaged compared to the control (p>

0.001). Natural sunscreen 2 and Reef-safe sunscreen 1 caused a very high release particularly after 24 hours of exposure (Fig. 4.3 ad 4.4). Instead, European sunscreen, Reef-safe sunscreen, Natural sunscreen 1 and Ecoreach sunscreens (1 and 2) caused a significant release of zooxanthellae after 32 hours from the beginning of the experiment especially, reef-safe sunscreens. However, hard corals treated with Ecoreach sunscreens (1 and 2) showed always-lower values of released zooxanthellae compared to the other sunscreens.

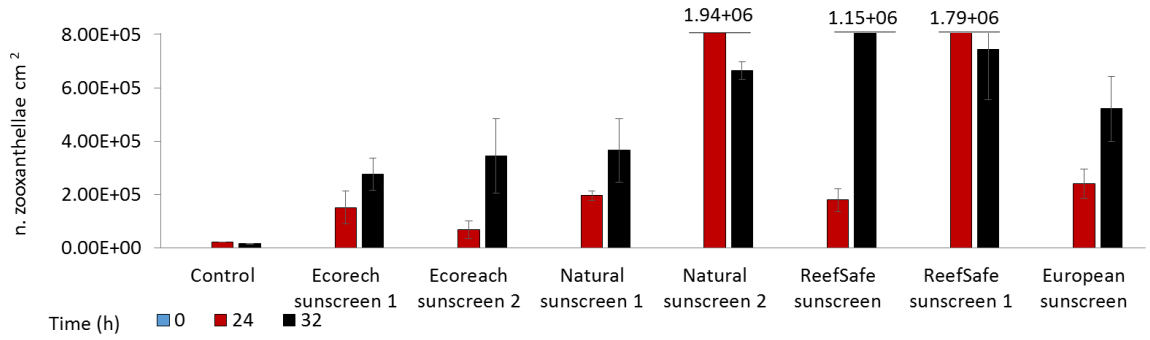

Figure 4.3. A number of zooxanthellae released into seawater surrounding coral branches treated with different brands of sunscreens and in the control, during the second experiment. ± SD.

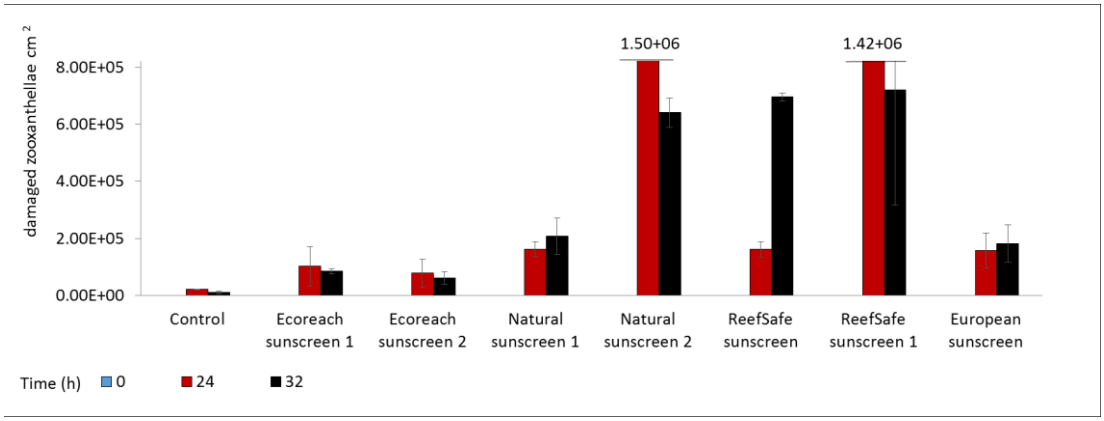

Figure 4.4. A number of zooxanthellae released into seawater surrounding coral branches treated with different brands of sunscreens and in the control, during the second experiment. ± SD.

2.4.2. Quantification of bleaching

The results of the colorimetric analysis for the quantification of bleaching in hard corals exposed to different brands of sunscreens revealed that untreated nubbins did not show any color change during the entire duration of the experiments whereas all treaties, except those exposed to Ecoreach sunscreens ($p < 0.05$), showed significant colorimetric changes (Figure 4.5 and 7.4, $p > 0.05$).

In particular, after 24h of exposure to Eco-friendly sunscreen 2 we observed the complete bleaching of the coral nubbins (Figure 4.6). A strong bleaching (> 50 % of coral surface) was also observed after addition of Reef-safe sunscreen 2 (within 24 hr of the beginning of the experiment), Natural sunscreen 2, Natural sunscreen 1 and Reef-safe sunscreen (in order of severity of bleaching degree). On the contrary, European sunscreen determined a slight bleaching of the coral nubbins (< 10 % of coral surface) during the experiment and no visible bleaching was also observed after exposure to Ecoreach sunscreens (1 and 2).

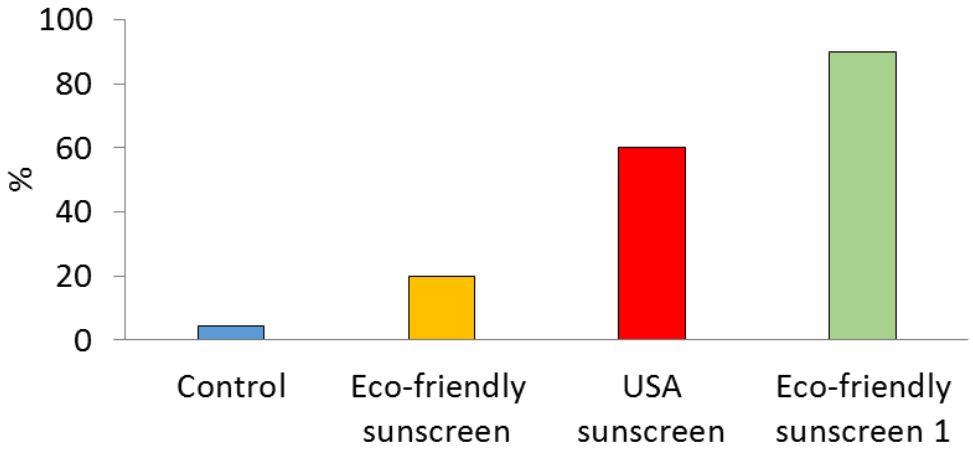

Figure 4.5. Degree of bleaching (%) of the hard corals treated with different brands of sunscreens and untreated nubbins used as a control, during the first experiment.

Figure 4.6. Effect of 50-µL Eco-friendly sunscreen 1 on *Acropora sp*. nubbins after 24-hr incubation.

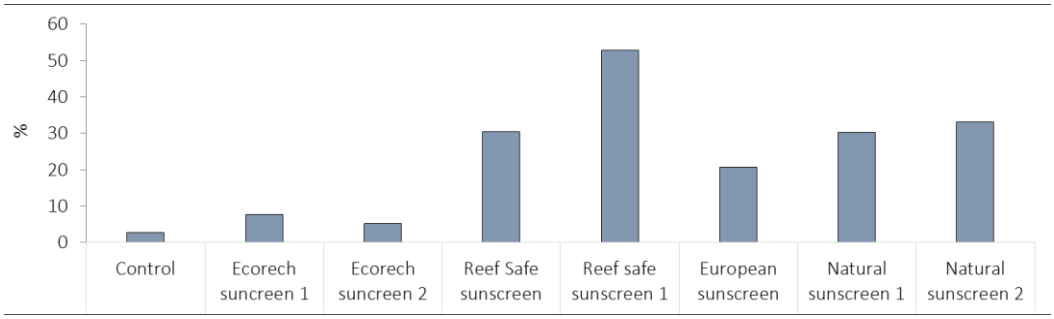

Figure 4.7. Degree of bleaching (%) of the hard corals treated with different brands of sunscreens and untreated coral nubbins used as a control, during the second experiment.

2.4.3. Prokaryotic and viral abundance

All sunscreens tested, during the experiments performed in Maldives, determined an enrichment of microbes compared to the control (Fig. 4.8-4.11).

In the first experiment after the addition of U.S.A. sunscreen, prokaryotic and viral abundance in seawater surrounding coral branches increased significantly over time compared to the control systems ($p > 0.01$; Fig. 4.8 and 4.9). Also the systems treated with Eco-friendly and Eco-friendly sunscreen 1 showed a significant ($p > 0.001$) increase overtime of the number of viruses and prokaryotes respect to the control, with the highest values after 24 h of exposure and a general decrease at the end of the experiment (t_{48}, Fig. 4.8 and 4.9). In the systems treated with Eco-friendly sunscreen, the number of viruses at the end of the experiment (t_{48}) showed values similar to those observed in the control systems (Figure 4.9).

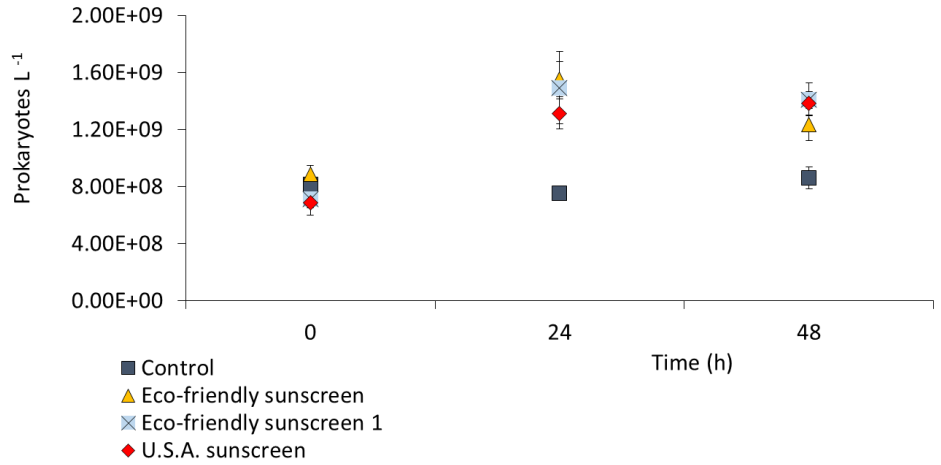

Figure 4.8. Prokaryotic abundance in the seawater containing nubbins of *Acropora sp*. exposed to the different sunscreen and in the control (square symbol), during the first experiment. ± = SD.

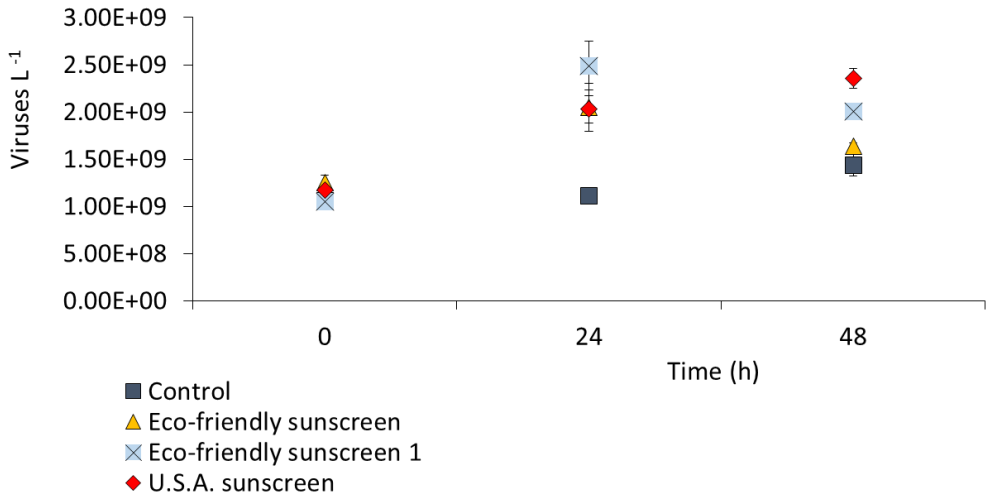

Figure 4.9. Viral abundance in the seawater containing nubbins of *Acropora sp.* exposed to the different sunscreen and in the control (square symbol), during the first experiment. ± = SD.

In the second experiment, all sunscreens tested caused a constant increase over time of the amount of prokaryotes and viruses into the coral surrounding seawater, with a significant increase ($p > 0.05$) at the end of the experiment (t_{32}). In particular, the highest values of virus and prokaryotic abundance were observed after addition of Reef-safe sunscreen and Reef-safe sunscreen 1 after 32 hr of exposure, reaching values three times higher than the controls. Despite also Ecoreach sunscreens (1and 2) determined a significant increase in microbial abundance over time compared to the control, values observed were always the lowest.

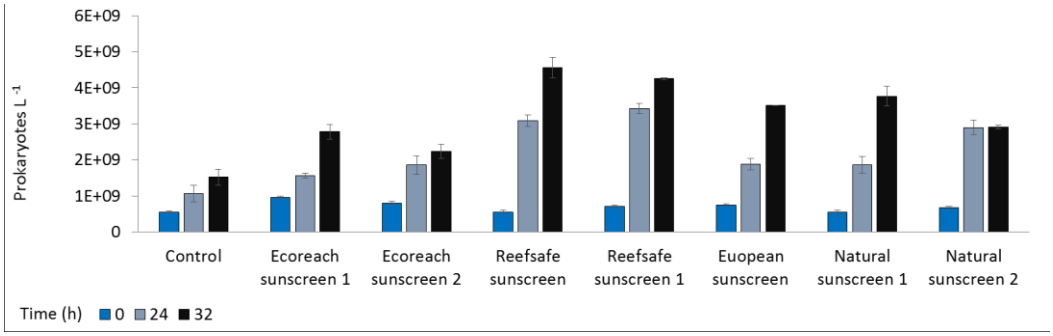

Figure 4.10. The amount of prokaryotes in the seawater containing nubbins of Acropora sp. exposed to the different sunscreen and in the control (square symbol), during the second experiment. ± = SD.

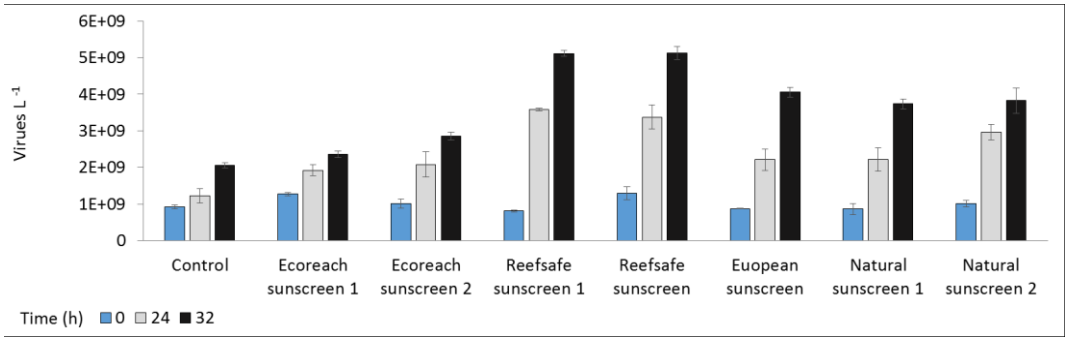

Figure 4.11. The amount of viruses in the seawater containing nubbins of *Acropora sp.* exposed to the different sunscreen and in the control (square symbol) during the second experiment. ± = SD

2. 5 DISCUSSION

The release of coral symbiotic zooxanthellae, a phenomenon known as coral bleaching, has negative impacts on biodiversity and functioning of reef ecosystems and their production of goods and services (Wild, 2004). Recently, it has been demonstrated that sunscreen products have a rapid effect on hard corals and cause bleaching by damaging the symbiotic zooxanthellae (Danovaro et al., 2008). Our results are in line with those previously obtained. In fact, in all systems, the addition of sunscreens resulted in the significant release of symbiotic algae, most of which was damaged. However, the different brands of sunscreens tested caused different responses in term of the release of zooxanthellae and damaged zooxanthellae. In particular, the most severe effects were observed after addition of Eco-friendly sunscreen 1, Reef-safe sunscreen 1 and Natural sunscreen 2 within 24 hr. We also observed that USA sunscreen caused a significant release of zooxanthellae already at the beginning of the experiment with a strong increase after 48 hr of exposure.

These findings were confirmed by the colorimetric analysis of the coral surface. Indeed, among the 10 sunscreens tested on hard corals, 5 sunscreens (U.S.A. sunscreen, natural sunscreen 1 and 2, Reef-safe sunscreen and Reef-safe sunscreen 1) caused a strong bleaching of the coral nubbins. The Eco-friendly sunscreen 1 caused even total bleaching on corals surface within 24 hr of the experiment. On the other hand, no visible bleaching of the coral nubbins was observed after exposure to Ecoreach sunscreens (1 and 2).

The results here obtained revealed that the most severe effects were caused by sunscreen products defined natural, organic, biodegradable, eco-friendly and reef-safe.

Organic products should also be biodegradable and, hence, at low environmental impact. However, not all natural substances are degradable in the short term or have a low environmental impact (Dayan et al., 2011). For example, caffeine is a natural substance, but several studies have shown that it has a negative effect on marine organisms (Schneider et al., 2016; Jiangn et al., 2014). At the same time, a cosmetic product defined eco-compatible with marine life or reef-safe should be tested on marine organisms, or at least on corals. Therefore, findings obtained in this study suggest that the claim of eco-compatibility in the commercial sunscreen products here tested appears to be unjustified.

The different impact of sunscreen products observed may be due to the different formulation in terms of ingredients used in the different brands tested.

In the present study, we have not carried out tests of the single ingredients contained in the different sunscreens, recently numerous studies have shown that UV filters

(e.g., Avobenzone, oxybenzone, homosalate,benzophenone-3, 4-methylbenzylidene camphor, ethylhexyl- methoxycinnamate, 4-methylbenzylidene camphor) and preservatives (parabens) can affect marine life, including hard corals and their symbionts (Downs et al., 2015; Krause et al., 2012; Danovaro et al. 2008; Onesios, 2008; Kunz et al., 2006).

In particular, we observed that the sunscreens, responsible of the most severe bleaching degrees, contained ingredients defined harmful for marine organisms, such as benzophenone 3 and homosalate contained within U.S.A. sunscreen, as well as oxybenzone, octisalate, homosalate and methylparaben contained in the sunscreens defined non-toxic to marine life (Reef-safe sunscreen). The high impact of Eco-friendly sunscreen 1 could be due to TiO_2 contained therein, which is commonly used as nanoparticles in sunscreen products but it has been already demonstrated to harm marine life (Hazeem et al., 2015; Miller et al., 2012; -Peng et al., 2011; Miller et al., 2010). Also zinc oxide contained within Natural sunscreen 2 could be responsible for the high bleaching effect on the corals since previous studies revealed that caused toxic effects (e.g., oxidative stress) in many marine organism such as algae (Miller et al. 2010), crustaceans (Ates et al. 2013a), molluscs (Wu et al., 2015; Trevisan et al, 2014; Buffet et al., 2012), echinoderms (Manzo et al., 2013a; Fairbairn et al., 2011) and fishes (Miao et al., 2010). Same thing for Reef-Safe sunscreen 1, whose formulation includes both zinc oxide (nanoparticles) and titanium dioxide. Previous studies have suggested that some brands of sunscreens can significantly enhance prokaryotic and viral abundance in seawater (Danovaro & Corinaldesi 2003). Here, we found that all sunscreens tested

caused a constant increase over time of prokaryotic and viral abundances in the seawater surrounding corals, in particular, Reef-safe sunscreens.

The reason of this microbial enrichment could be due to the nutrients contained in sunscreens, or to the induction of lytic cycle in latent viruses of zooxanthellae (Danovaro et al. 2008). However, we observed that Ecoreach sunscreens, based on new-patented ingredients, determined always a lower microbial enrichment than other treaties.

2. 6 CONCLUSIONS

Overall, our findings indicate that most of the sunscreen products tested can affect rapidly hard corals causing bleaching and damaging the symbiotic zooxanthellae. Such an effect depends on the products tested and the different ingredients contained in their final formulation. The most severe effects were determined by products defined reef-safe, natural and eco-friendly suggesting that such products are not actually tested on marine life or the tests carried out are not specific for marine organisms. On the basis of our results, we can conclude that a cosmetic product defined eco-friendly or biodegradable before entering the market should be evaluated with specific biodegradability tests and rigorous tests on marine organisms belonging to different levels of the food web.

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Chapter 3

Impact of inorganic UV filters on *Acropora sp.*

3. 1. INTRODUCTION

Ultraviolet (UV) filters, as the active ingredients in sunscreen products, block solar radiation (Sang et al., 2016) by absorbing UV radiation and protect human skin from direct exposure to deleterious wavelengths of sunlight (Maipas et al., 2015). Titanium dioxide (TiO_2) and zinc oxide (ZnO) are two metal oxides traditionally known for their sun-blocking properties. Sun lotion manufacturers formerly used the bulk form of these materials, but nanotechnology advances have made possible the use of their nanoscale form (Gottschalk et al., 2009). In fact, in order to be efficacious and nonwithening on the skin, the particles of these substances have to be very small, typically in the size range around 100 nm or below (Osterwalder et al., 2014). The innovative properties showed by engineered nanomaterials respect to bulk materials make nanotechnologies growing faster in many different fields (Wiesenthal et al., 2011).

The properties and persistence of TiO_2 and ZnO, contained in sunscreen formulations, applied to the skin can be compromised due to immersion, UV radiation, temperature, moisture or abrasion with beach sand (Langford & Thomas, 2008; Stokes & Diffey, 2000), leading to the release of altered products into the marine environment. Indeed the inorganic UV filters are water-dispersible but may be rendered dispersible in sunscreen/oil by adding organic coatings (Osterwalder et al., 2014). Depending on the pH and salinity of the solution and the concentration of natural organic matter ZnO and TiO_2, and more generally the solar formulations,

undergo rapid alterations in seawater, promoting the dispersion of particles into an aqueous medium, dissolving into ions, aggregating or settling (Botta et al., 2011). TiO_2 nanoparticles used in sunscreens are generally surface-modified using aluminium oxide/hydroxide and/or silicon oxide (Lee et al., 2007) to inhibit their photo-activity and consequently prevent their photo-toxicity (Johnston et al., 2009). This coating applied to the surface of these molecules act as a protective cement for the nanocomposites, slow down the alteration processes in seawater (Botta et al., 2011).

Titanium dioxide (TiO_2) and zinc oxide (ZnO) are among the most abundant compounds discharged into the environment (Miller et al., 2010) with potential ecological consequences on marine life (Hazeem et al., 2015; Miller et al., 2012; Miller et al., 2011; Peng et al., 2011; Chen et al., 2010; Miller et al. 2010). Every year a lot of inorganic UV filters can be released in reef areas, about 36-56 tons only of TiO_2 (Botta et al. 2011) by millions of tourists (Creel, 2003).

These inorganic materials are well-known to generate reactive oxygen species (ROS) and to release metal ions into aquatic environment, which can damage organisms through a variety of interrelated effects (Hazeem et al., 2015; Sánchez-Quiles & Tovar-Sánchez, 2014; Carp et al., 2012; Peng et al., 2011; Miller et al., 2010; Nel et al., 2006). Miller et al., (2010), in a recent investigation have reported different effects of ZnO and TiO_2 on the different species of marine algae depending on the algal species and the characteristics of the compounds (i.e, size, crystal form, the morphology of particles).

Further studies have suggested that the size and morphology of the particles can be an extremely important factor of direct toxicity (Peng et al., 2011; Moore et al., 2006) on the marine environment.

Despite the increased awareness of the effects of these compounds on marine organisms, scientific research on the environmental impact associated with the use of inorganic UV filters in marine ecosystems is very scarce.

3.2. OBJECTIVES

In this study. we evaluated the impact of inorganic UV filters (ZnO and TiO2) on hard corals (*Acropora sp)* and their symbiotic algae by independent experiments conducted in two different reef areas of the Indian Ocean: Vavvaru Island, Maldives and Onu Island, Fiji. Our findings provide new evidence of the direct effects of inorganic UV filters on marine environments and paves the way for further research to understand how these products can affect the health of marine organisms.

3.3. MATERIALS AND METHODS

3.3.1. Inorganic UV filters

We selected different types of inorganic UV filters characterized by different particles, size, crystal form and modifications useful to minimize or eliminate photo-reactivity. In particular, in the first experiment conducted in Maldives we tested zinc oxide characterized by uncoated particles of size ranging from 20 to 200 nm (Fig. 1) and two different types of titanium dioxide (Optisol™ and Eusolex® T2000 at the final concentration of 50 μL/L) characterized by modifications of the particles. In particular, **Optisol™** (Oxonica Ltd and UK Nanotechnology

Company; crystal form rutile) is a specially modified form of titanium dioxide, which is traditionally used in many sunscreens. A small amount of manganese is incorporated into this patented product to act as a free radical scavenger and to minimize the formation of new free radicals. **Eusolex® T2000** (Merck KGaA) is TiO_2 (crystal form rutile; particle size 20 nm) characterized by the surface coated with alumina and dimethicone. These modifications have the scope to reduce the potential reactivity of photo-activated TiO_2 particles by quenching and/or reducing the reactive species generated before they can interact with the other ingredients in a formula and with skin components itself (Tiano et al., 2010).

In the second experiment carried out at the Fiji Islands, we tested titanium dioxide (at the final concentration of 0.0015%) characterized by the anastase crystal form and uncoated particles of 10-20 nm.

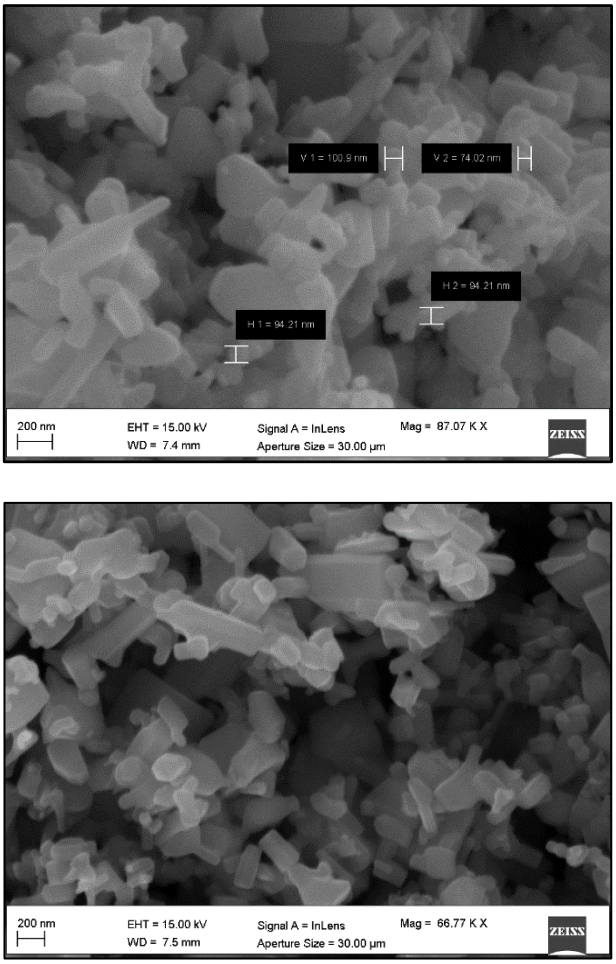

Figure 3.1. Particles of zinc oxide used for the experiment in Maldives, analyzed by a scanning electron microscope SEM.

3.3.1. Study areas and experimental design

Independent experiments were conducted in two different coral reef areas: Maldives and Fiji. Nubbins from different donor colonies belonging to the genus *Acropora* (3-6 cm) were collected and placed in aquaria for the acclimatization process for 48 h under stable and *in situ* conditions (temperature and salinity) before the experiments. After acclimatization, only healthy species (without any signs of necrotic tissue and with polyps regularly open) were used to perform the experiments. Nubbins of *Acropora sp.* were washed with virus-free seawater

filtered onto 0.02 μm membranes (Anotop syringe-filters; Whatman, Springfield Mill, UK) and immersed in polyethylene Whirl-pack bags (Nasco, Fort Atkinson, WI, USA) filled with 2 L virus-free seawater. Replicate sets containing nubbins (n = 3, including more than 300 polyps each) were supplemented with aliquots of different UV filters and compared with untreated systems (used as controls). Corals were incubated at the same depth of donor colonies in Fiji, and in aquaria maintained at *in situ* conditions (temperature and salinity), with seawater in a continuous flow directly from the ocean in Maldives.

3.3.2. Analyses of health state of zooxanthellae

Zooxanthellae analyses were performed by sampling the seawater surrounding of coral colonies in order to evaluate the amount of the released symbiotic organisms and health status.

Sub-samples (10 mL) from treated (added with filters) and untreated systems were collected immediately after the addition of filters (t_0= start of the experiment) and every 24 hours until the end of the experiment. In particular, in Maldives, the sub-samples were collected after 24 h (t_{24}) and 48 h (t_{48}) at the beginning of the experiment while in Fiji after 24 h (t_{24}) 48 h (t_{48}), 72 h (t_{72}) and 96 h (t_{96}) at the beginning of the experiment. Aliquots of seawater samples were filtered through 2.0-μm polycarbonate filters and mounted on glass slides. Zooxanthellae were counted under a Zeiss Axioplan epifluorescence microscope (Carl Zeiss Inc., Jena, Germany; ×400 and ×1,000). Based on the autofluorescence and gross cell structure, zooxanthellae released from coral colonies were classified as a) healthy (H, brown/bright yellow color, intact zooxanthellae); b) pale (P, pale yellow colour, vacuolated, partially degraded zooxanthellae); transparent (T, lacking

pigmentations, an empty zooxanthellae; Mise & Hidaka 2003; Danovaro et al., 2008).

3.3.3. Quantification of bleaching

To quantify the levels of coral bleaching (Siebeck et al. 2006), we performed a colorimetric analysis on digital photographs of corals taken at the beginning of the experiments and after various times of treatment with filters (specified above). Photographs were taken under identical illumination with a Canon EOS 400D digital camera (Canon Inc., Tokyo, Japan) with a scale meter on the background. The photographs were successively analysed with a photo-editing software for color composition cyan, magenta, yellow, black (CMYK). Levels of bleaching were measured as the difference between the coral's color at the beginning of the experiments (t_0) and after treatments. Inside the coral areas were made 30 random measurements of variables CMYK. Variations in the percentage of the different color components (CMYK) were analysed with one-way analysis of variance (ANOVA). To rank the bleaching effect due to the different sunscreen tested we obtained Bray–Curtis similarity matrix and multidimensional scaling analysis of the shifts in CMYK color composition of treated corals using Primer 5.0 software (Primer-E Ltd., Plymouth, UK). Bleaching rates were measured as the variation percentage in CMYK color composition between treated and control corals using Primer 5.0 software (Primer-E Ltd). In addition, to the average values obtained were attributed of scores of the degree of bleaching (Tab 1) by mean of a mathematical function, according to a scale organized in ranks (0 to> 60), i.e., from "no visible coral bleaching" (0-10) to the "total bleaching with 100% of coral nubbins surface (> 60).

Degree of bleaching (%)		Incidence of coral bleaching
	0-10	No visible coral bleaching
	10-20	Slight color variation. No visible coral bleaching
	20-30	Slight bleaching (< 10 % of coral nubbins surface)
	30-60	Strong bleaching (>50% of coral nubbins surface)
	> 60	Total bleaching (100 % of coral nubbins surface)

Table 1: Main classes of degree of bleaching

3.3.4. Prokaryotic and viral abundance (Maldives)

Prokaryotic and viral abundance in the seawater samples was determined according to the protocol described by Noble & Fuhrman (1998) without previous fixation to avoid underestimations of viral abundances (Danovaro et al 2001, Wen et al 2004). Sub-samples (10 ml) from treated (added with filters) and untreated systems were collected immediately after the addition of sunscreen (t_0= start of the experiment) and after 24 h (t_{24}) and 48 h (t_{48}) at the beginning of the experiment. After collection, three replicate seawater samples were stored at -20°C until the analysis. Sub-samples were filtered onto 0.02µm pore size filter (Whatmann Anodisc; diameter, 25 mm; Al_2O_3) and stained with 100 µL of SYBR Gold (stock solution diluted 1:5000). The filters were incubated in the dark for 20 min, washed three times with 3 mL of prefiltered Milli-Q water and mounted onto glass slides with 20µl of 50% phosphate buffer (6.7 mM phosphate, pH 7.8) and 50% glycerol (containing 0.5% ascorbic acid). Slides were stored at -20°C. Prokaryote and viruses counts were obtained by epifluorescence microscopy (Zeiss Axioskop 2). For each slide, at least

20 microscope fields were observed and at least 200 prokaryotes and viruses were counted per filter.

3.3.5. Prokaryotic Abundance (Fiji)

Direct counts of total prokaryotic abundance in surrounding coral branches seawater in Fiji experiment, were carried out using the method described by Noble and Fuhrman (Noble & Fuhrman, 1998) with few modifications. Seawater subsamples (10 ml) were collected from treated (added with UV filters) and untreated systems, utilized for evaluating the inorganic UV filters impact on hard corals, immediately after the addition of filters (t0= start of the experiment) and 24 h (t_{24}) 48 h (t_{48}), 72 h (t_{72}) and 96 h (t_{96}) at the beginning of the experiment. Seawater subsamples were preserved in 0.02 µm prefiltered formalin (2% final concentration) and were concentrated onto 0.02 µm pore size filters (Anodisc 25 mM, Al2O3), and stained with 20 µl SYBR Green I (stock solution diluted 1:20). Filters were incubated in the dark for 15 min and mounted on glass slides with a drop of 50% phosphate-buffered saline and 50% glycerol, containing 0.5% ascorbic acid. Slides were stored at 20°C until analysis. Counts were obtained by epifluorescence microscopy (magnification, ×1,000; Zeiss Axioplan) by examining at least 10 fields, that is, at least 200 cells or particles per replicate.

3.3.6. Statistical analysis

Differences in each of the investigated variables (univariate tests) between the controls and treatments, during the experimental time, were assessed using permutational analyses of variance (PERMANOVA; Anderson, 2005; McArdle and Anderson, 2001). The design included two factors (time and treatment). When significant differences were encountered ($p < 0.05$) post-hoc pairwise tests were

also carried out. Statistical analyses were performed using the routines included in the PRIMER 6+ software (Clarke and Gorley, 2006).

3.4. RESULTS

3.4.1 Analyses of zooxanthellae

All inorganic filters used in the experiments performed in Maldives and Fiji (50 µL/L) resulted in the release of total and damaged zooxanthellae (Figure 4.1-4.3) from the hard corals.

Figure 4.1 and 4.2 show the amount of total and damaged zooxanthellae released by hard corals in control and samples added with filters in the experiment performed in Maldives. The highest number of (damaged) zooxanthellae was released by zinc oxide causing a significant increase over time ($p= 0.0001$) and reaching values two orders of magnitude higher than the controls and other treatments, already after 24 hr of exposure. The addition of Eusolex T2000 caused an immediate and significant increase ($p> 0.001$) of a number of zooxanthellae released into the seawater surrounding coral nubbins but these values remained rather constant during the experiment. On the contrary, hard corals treated with Optisol showed a significant increase of symbionts released after 24 hours exposure ($p>0.001$) compared to the control, with a slight increase at the end of the experiment (Fig. 4.1). In addition, the amount of damaged zooxanthellae released by coral nubbins treated with the two different types of modified titanium dioxide (Eusolex T2000 and Optisol, Fig. 4.2), showed a significant variation compared to the control only after 48 hours of exposure (Eusolex T2000, $p> 0.001$; Optisol, $p > 0.05$).

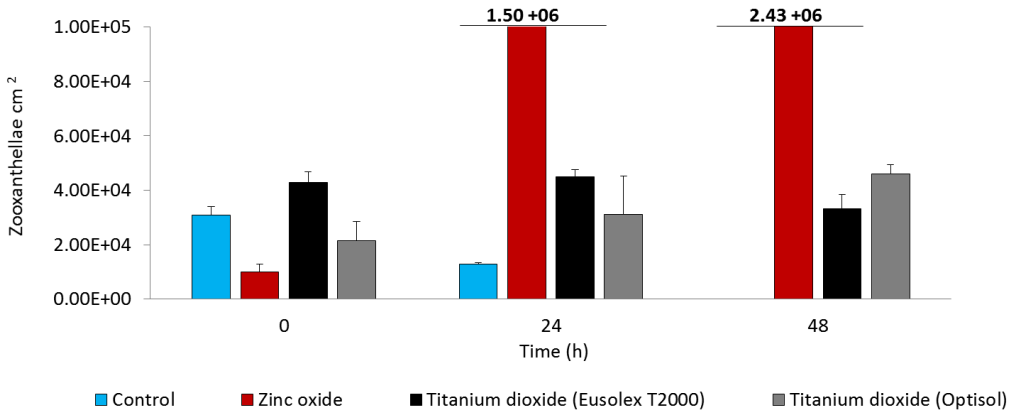

Figure 4.1. A number of zooxanthellae released into seawater surrounding coral branches treated with zinc oxide, Optisol and Eusolex T2000 during the experiment performed in Maldives. ± SD.

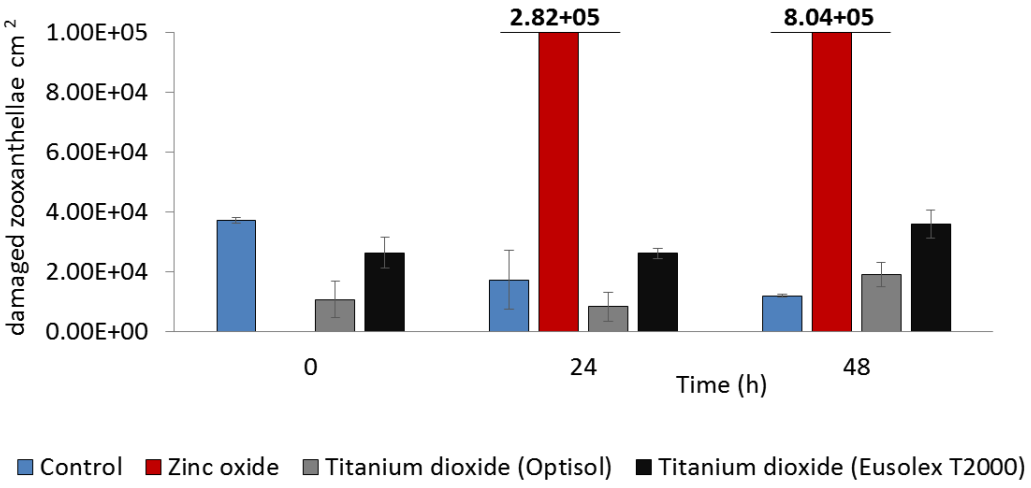

Figure 4.2. A number of damage zooxanthellae released into the seawater surrounding coral branches treated with zinc oxide, Optisol and Eusolex T2000 during the experiment performed in Maldives. ± SD.

Figure 4.3 shows the amount of total and damaged zooxanthellae released from hard corals in control and titanium dioxide addition samples, during the 96 hr of the experiment performed in Fiji. A high release of total and damaged symbionts was observed after exposure to titanium dioxide respect to the control ($p = 0.0001$). In addition, 98% of the total zooxanthellae released from hard corals treated with titanium dioxide was represented by damaged symbiotic algae (Fig. 4.3).

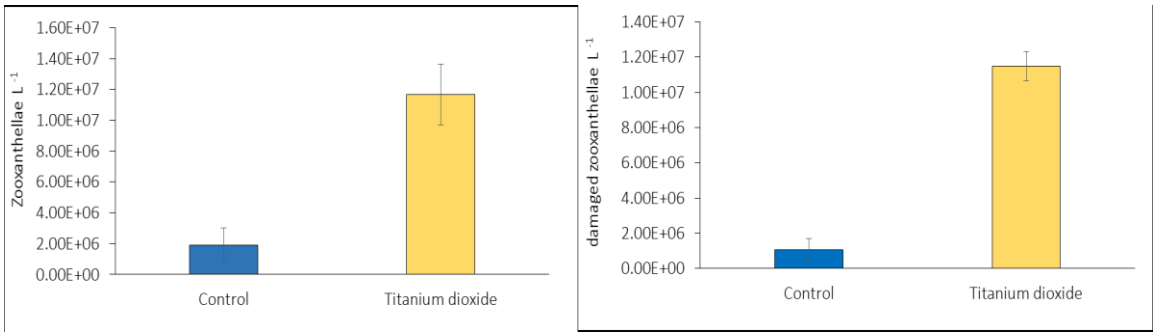

Figure 4.3. A number of zooxanthellae released into the seawater surrounding coral branches treated with titanium dioxide, during the 96hr of the experiment performed in Fiji. ± SD.

3.4 2. Quantification of bleaching

Figure 4.5 and 4.6 show the results of the colorimetric analysis for quantification of bleaching of hard corals exposed to different inorganic UV filters, during the experiments performed in Maldives and Fiji.

In Maldives, the nubbins exposed to zinc oxide and titanium dioxide with uncoated particles showed a significant change in the colorimetric variables (CMYK; zinc oxide, $p = 0.0001$; titanium dioxide, $p > 0.05$) from the beginning to the end of the

experiment. No significant changes in the colorimetric variables (CMYK) were observed in hard corals treated with Eusolex T2000 and Optisol.

A strong bleaching (> 50 % of coral nubbins surface) was observed after addition of titanium dioxide (Fiji experiment) and zinc oxide while no visible bleaching of the coral nubbins was observed after exposure to Eusolex T2000 and Optisol ($p < 0.05$; Fig. 4.5).

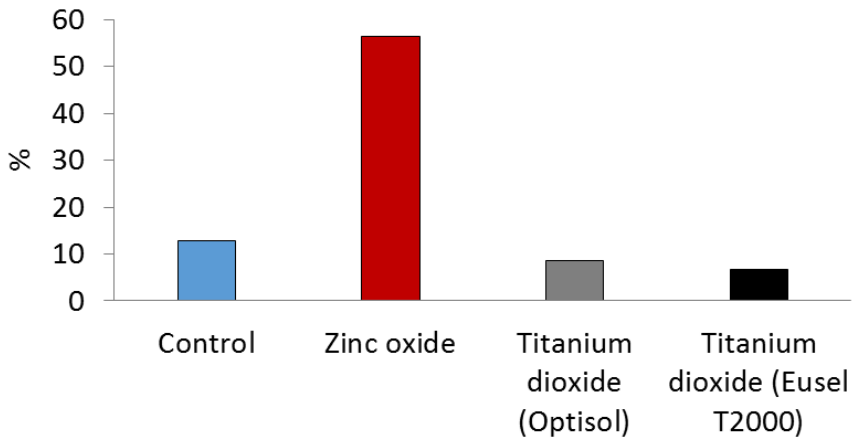

Figure 4.5. Degree of bleaching (%) of the hard corals treated with zinc oxide, Optisol and Eusolex T2000 during the experiment performed in Maldives.

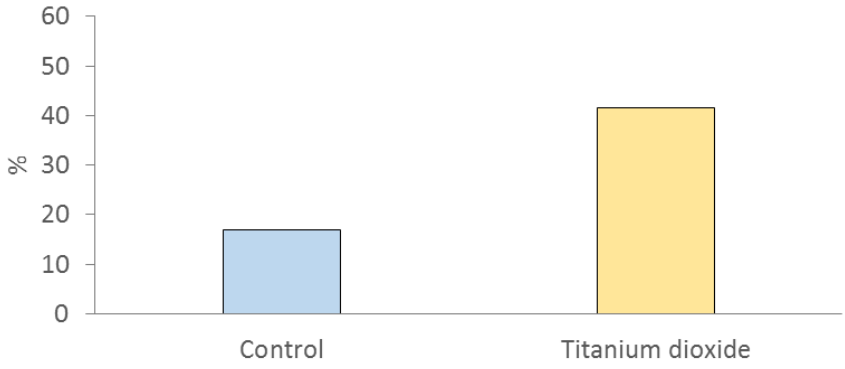

Figure 4.6. Degree of bleaching (%) of the hard corals treated with titanium dioxide, during the 96hr of the experiment performed in Fiji.

3.4.3 Prokaryotic and viral abundance

Figure 4.8 shows the prokaryotic and viral abundance into the seawater surrounding coral colonies observed during 48 hr of exposure to different inorganic UV filters in Maldives.

The addition of zinc oxide caused a significant increase in the prokaryotic and viral abundance in seawater surrounding coral branches ($p > 0.001$), reaching the highest values (about three times higher than those observed in the control and the other treaties) 48 hr from the start of the experiment. In the systems added with Eusolex T2000 and Optisol, we found the lowest abundances of prokaryotes and viruses, with values lower or similar to those of controls during the experiments. Figure 4.9 shows the prokaryotic abundance in surrounding coral colonies observed during 96 hr of exposure to titanium dioxide in Fiji. The addition of titanium dioxide caused a significant increase of the prokaryotic abundance in seawater surrounding coral

branches ($p > 0.001$) during the 96 hr of exposure, respect to the control systems, in an experiment performed in Fiji.

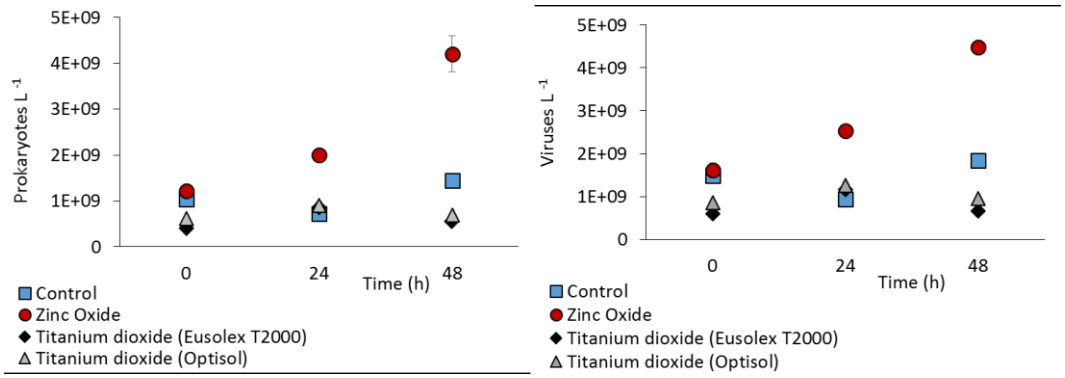

Figure 4.8. Amount of prokaryotes (right) and viruses (left) into the seawater containing nubbins of *Acropora sp.* exposed to the different inorganic UV filters and in the control (square symbol), during an experiment performed in Maldives. ± = SD.

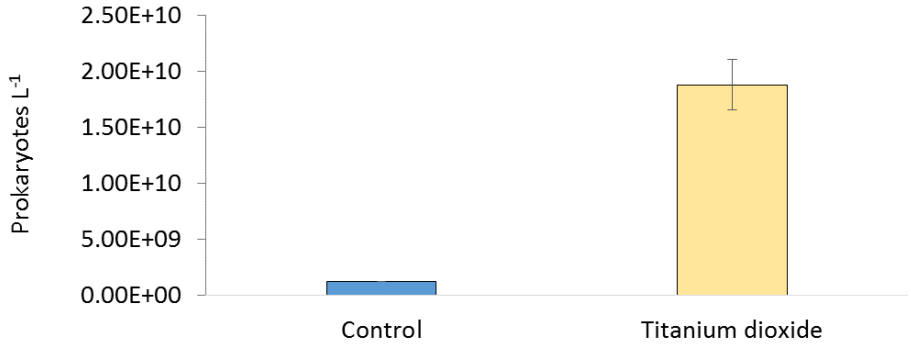

Figure 4.9. Amount of prokaryotes in the seawater containing nubbins of *Acropora sp.* exposed to titanium dioxide and in the control (square symbol), during the experiment performed in Fiji. ± = SD.

3. 5 DISCUSSION

Titanium dioxide (TiO_2) and zinc oxide (ZnO) are two metal oxides traditionally known for their sun-blocking properties (Maipas et al., 2015). In the last decade, the toxicity of inorganic UV filters (TiO_2 and ZnO) on marine organisms has been addressed in previous studies (Hazeem et al., 2015; Miller et al., 2012; Peng et al., 2011; Navarro et al., 2008) but the scientific research on the environmental effects associated with the use of these compounds in marine ecosystems is still limited.

Here, we investigated the effects of different typologies of inorganic UV filters on hard-corals of the genus *Acropora sp.* and their symbiotic organisms. In particular, we tested ZnO and TiO_2 characterized by uncoated particles, and two different typologies of TiO_2 with structurally modified (Optisol) or coated particles (Eusolex T2000) with the objective to reduce photocatalytic activity.

Our findings indicate that inorganic UV filter affects hard-corals causing the release of damaged zooxanthellae, depending on the type of UV filter used even in terms of structural modifications and coating. In particular, uncoated zinc oxide and titanium dioxide have a rapid negative effect on hard corals and causes a strong bleaching by damaging the symbiotic zooxanthellae. In fact, we found that (uncoated) zinc oxide caused the release of large amounts of damaged zooxanthellae and coral mucus over time, reaching values two orders of magnitude higher than the controls and other treatments at the end of the experiment. Similarly, we observed the release of a lot of symbiotic algae, mostly damaged, and coral mucus even after the addition of titanium dioxide. This inorganic ingredients are well-known to generate reactive oxygen species (ROS) and to release metal ions

into aquatic environment which can damage organisms through a variety of interrelated effects (Hazeem et al., 2015; Sánchez-Quiles & Tovar-Sánchez, 2014; Carp et al., 2012; Peng et al., 2011; Miller et al., 2010; Nel et al., 2006). Conversely, Optisol and Eusolex T2000 determined minor effects in the release of zooxanthellae and bleaching.

We can suggest that the modifications of the inorganic particles made to minimize the potential reactivity of photo-activated particles making them initially inert in water, caused lower stress levels to the organisms exposed. In particular, Eusolex is characterized by rutile TiO_2 particles, coated with alumina and dimethicone that retard the dissolution in seawater and minimize the phototoxicity of TiO_2. Optisol is a specially modified form of rutile titanium dioxide with small amounts of manganese incorporated into the structural lattice conferring free radical scavenging behavior. It's well known that the coating applied to the surface of these molecules acts as a protective cement for the nanocomposites, slowing down the alteration processes in seawater (Botta et al., 2011).

The analyses of microbial abundance in the seawater surrounding coral branches used as an index of stress caused by addition of UV filters revealed that also (uncoated) zinc oxide and titanium dioxide resulted in a microbial enrichment as previously reported for other organic filters (Danovaro et al., 2008; Danovaro & Corinaldesi et al., 2003). Conversely, Eusolex T2000 and Optisol did not cause an enrichment of microbial component during the experiment. The increase of prokaryotes and viruses in seawater surrounding corals, after addition of (uncoated) zinc oxide and titanium dioxide, can be a response to the stress caused by these compounds as previously reported for corals subjected to different stress as

chemicals, UV radiation, temperatures, diseases and pH (Davy et al., 2006; Wilson et al., 2006). At the same time, zinc oxide and titanium dioxide could act as agents inducing lytic cycle in latent infections of coral zooxanthellae as previously reported for organic filters (Danovaro et al. 2008). It has been suggested that microbial communities may facilitate acclimatization of the coral halobiont to changes in the environment through rapid changes in the microbial community composition (Reshef et al., 2006), and studies indicate that an intact coral microbiome is essential to coral immunity and health (Krediet et al., 2013). Therefore, the destabilization of balance host-microbial components can lead to uncontrolled proliferation of opportunistic taxa that would otherwise be suppressed (Rosenberg & Kushmaro, 2011), causing a greater sensitivity of these organisms to possible pathogens and affecting their health. Further analysis would be fundamental to understanding this mechanism and whether the addition of these pollutants can cause a shift in the microbial communities associated with coral.

3. 6 CONCLUSION

Our findings indicate that inorganic UV filters can affect hard-corals causing a significant alteration to the coral health, depending on the type of UV filter used in terms of structural modifications and presence of the coating. In particular, all results suggest that the modifications made to minimize or eliminate the potential reactivity of inorganic UV filters may decrease the toxicity of these compounds on marine organisms potentially stabilizing them. Therefore, the use of modified and

coated inorganic UV filters in sunscreen composition is preferable not only to provide more protection to human skins but also to better preserve coral reefs.

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Chapter 4

Effect of pharmaceutical products on *Corallium rubrum*

4.1 INTRODUCTION

Chemicals in marine environment affect marine life often by reducing the reproductive success or increasing vulnerability to disease (Gunderson et al., 2016). These impacts have been observed at different levels of the marine food web, and often accumulate in mammals that have a longer life span than other species (E.E.A., 2010). Every year the amount of chemicals produced and the number of new molecules entering the market increase (Lapworth et al., 2012). Most of these new products purchased by millions of consumers are discarded in the environment, causing potentially harmful effects on the marine environment. Statistics published by EURO- STAT in 2013 reveal that, between 2002 and 2011, environmentally harmful compounds represent over 60% of the total production of chemicals.

In particular, pharmaceuticals including their metabolites and conjugates (Carlsson et al., 2006) are a class of emerging environmental contaminants that are extensively and increasingly being used in human and veterinary medicine. Daughton and Ternes (1999) define the pharmaceuticals drugs those chemicals used for diagnosis, treatment (cure/mitigation), alteration, or prevention of disease, health condition, or structure/function of the human body. The definition is extended to veterinary pharmaceuticals and can also be applied to illicit (recreational) drugs. These substances have different chemical structure, behavior, applications and metabolism in the human and animal body and hence in the environment (Jiang et al. 2013; Fawell and Ong 2012). Most pharmaceuticals are

hydrophilic in nature, soluble in water, easily break down and have a short life span (Tijiani et al., 2016), while some pharmaceuticals (e.g., naproxen, sulfamethoxazole) can remain in the environment without degradation for more than a year (Fent 2008; Fent et al. 2006). Recent studies have found a wide presence of these compounds in marine organisms (Capolupo et al., 2016; Pires et al., 2016; Ericson et al., 2010;) and various environmental compartments such as seawater (Loose et al., 2013; Zhang et al., 2013) and sediments (Beretta at al., 2014; Liang et al., 2013; Na et al., 2013). The negative and specific effects that the pharmaceuticals have on marine organisms include alterations of physiological functions (Ericson et al., 2010), inhibition of the cholinergic system (Luis et al., 2015), induction of oxidative stressor (Pires et al., 2016) and estrogenic responses, (Richardson, 2008).

Aquatic organisms are particularly important targets because they are continuously at risk of exposure and will probably be exposed to a variety of substances simultaneously, over long periods or even over their entire life cycle (Cleuvers, 2003). Although their environmental concentrations are generally very low (μg, ng/L), these levels would be sufficient to affect marine life. Therefore, long-term exposure to low doses of these compounds could cause adverse effects in the ecosystems (Fent et al., 2006). To date information about synergistic, additive and antagonistic effects of complex compound mixtures and their *in vivo* effects in the ecosystems is still limited (Cleuvers, 2003). The current lack of knowledge holds in particular for chronic effects that have only rarely been investigated. Intense human activities, in regions surrounding enclosed and semi-enclosed seas, near important harbors, big cities and industrial areas, such as the Mediterranean Sea

always produce, in the long term, a strong environmental impact (Arpin-Pont et al., 2016). In this regard, the European Environment Agency in a 2006 report has identified the chemical pollution, as the main critical aspect affecting the Mediterranean coasts (E.E.A., 2006). The environmental characteristics of the Mediterranean make it vulnerable to anthropogenic impacts so that it has been predicted that, the point of saturation of the contaminants discharged in the Mediterranean will be more quickly achieved than in the oceans (Turley, 1999, Richir, 2016). The Mediterranean is also one of the richest regions of Europe in terms of diversity of marine species with a high rate of endemic species (Cerrano et al., 2000) such as *Corallium rubrum* (Linnaeus, 1758). The red coral *Corallium rubrum* is a habitat-forming species with a prominent and structural role in mesophotic habitats, which sustains biodiversity hotspots (Ballesteros 2006, Cau et al., 2015, Cerrano et al., 2013). This species has been long threatened by different impacts such as trawling, illegal harvesting for commercial purposes, (Cerrano et al., 2013) and *C. rubrum* is included in the red list of the IUCN (EN, A2c). However, despite the attempts of protection, chemicals such as pharmaceuticals and drugs can anyway affect this unique species with relevant consequences on the biodiversity and functioning of the whole Mediterranean Sea. Studies conducted to evaluate the impact of pharmaceuticals on *Corallium rubrum* are absent but could be of great importance in order to understand its vulnerability to such a kind of chemical pollution.

4.2 OBJECTIVES

Here we investigated the responses of *Corallium rubrum* to different PPCPs including lifestyle compounds as nicotine end caffeine tested individually or as mixtures at different concentrations. In particular, we investigated the effects on the coral polyps' activity and the morpho-physiological responses (morphological alterations of the coral sclerites). In addition, the measure of cholinesterase activity was used as an index of the stress of *Corallium rubrum* and prokaryotic and viral abundances, as an index of microbial contamination induced by the pharmaceuticals.

4.3 MATERIAL AND METHODS

4.3.1 Specimen collection

On the 24th June 2013 *C. rubrum* apical fragments (n = 60, about 4 cm long with ca 200 polyps each, collected from ca. 60 different coral colonies) were collected at 30 ± 0.5 m depth in the Marine Protected Area of Portofino, North Tyrrhenian Sea (44°18'58.77"N 9°09'28.21"E, Mediterranean Sea). Fragments were cut from the primary branch of each specimen sample (one fragment per specimen) (Previati et al 2010). All coral fragments were placed in a several 50 L plastic tank containing continuously filtered maintained at *in situ* temperature (~13.6 °C). Samples were transferred to the laboratory within 12 h to reduce thermal stress. Colonies were immediately cleaned from possible sediment particles and placed in aquaria. All coral colonies were placed in aquaria for the acclimatization process for one month under stable conditions (i.e., temperature ~13.6 ± 0.5 °C, salinity ~37.5 ± 0.7) before the experiment. After acclimatization, only healthy specimens (without any

signs of necrotic coenenchyme and with polyps regularly open) were used to perform the experiment. Coral colonies were fed three times a week at 2 pm with 2 ml of a concentrated solution of Artemia salina nauplii in 20 µm filtered seawater.

4.3.2 Experimental set-up

Two independent time-course experiments were conducted on colonies of *C. rubrum*. Coral colonies were maintained in 6 L plastic aquaria filled with 20-µm filtered seawater collected in the Adriatic Sea during the experimental run. Three coral branches of *C. rubrum* were placed in each mesocosm. All systems were compared with untreated systems (without the addition of chemicals) used as controls. The first experiment, of the 33-day duration, included 3 control mesocosms and 6 mesocosms (n = 3 replicates for each treatment) exposed to caffeine (17.842 ng L^{-1}) and paracetamol (21.6 ng L^{-1}). Instead, the second experiment, of the 35-day duration, included 3 control mesocosms and 12 mesocosms (n = 3 replicates for each treatment/concentration) exposed to ibuprofen (3.490 µg L^{-1}), nicotine (11.815 µg L^{-1}) and a mixture of these substances at 2 different concentrations (low, Mix L and high, Mix H). In Table 1 were showed the individual contaminant concentrations used in the mixture both the low and the high concentrations. All concentrations of the substances added have been defined on the basis of a bibliographic survey of average concentrations found in the aquatic environment (Tab. 2). All aquaria were placed in a large tank to maintain *in situ* temperature of 13.6 ± 0.4 °C using TECO SeaChill Chiller TR5 during the experiment. Water flow in the large aquarium was generated by three pumps: the first pump recirculated the water through the chiller (1200 L h^{-1}), and the other two (480 L h^{-1}, located within the water bath) created a constant, turbulent water

movement, to avoid temperature gradients amongst the aquaria. Mesocosms were shaded to reproduce *in situ* light conditions using an opaque cloth to reduce normal indoor irradiance (by 80%) out of direct sunlight. Water within each mesocosm was continuously re-circulated and mechanically filtered at the rate of 190 L h^{-1}. Chemical-physical variables were measured daily with an YSI TDS conductivity meter. Sub-samples from treated (added with pharmaceuticals) and untreated systems were collected immediately after the addition of chemicals (t_0= start of the experiment) and within specific times in the course of the experiments (see onward).

Two independent time-course laboratory experiments were conducted on red coral colonies. In particular, were tested: ibuprofen, paracetamol, caffeine and nicotine, both individually and as a mixture (i.e. combined mixture of the abovementioned substances). In the specific, the mixture was tested at two different concentrations, low (i.e., similar to those found in the aquatic environment, in the order of ng) and high (i.e., in the order of μg), to assess whether the potential effects on *C. rubrum* depended on the concentration.

Table 1. Quantities of the individual contaminants within the mixture, in the two different concentrations.

Contaminants	**Concentration**	
	Low	**High**
Paracetamol	21.6 ng L^{-1}	43.2 ng L^{-1}
Ibuprofen	3.490 µg L^{-1}	6.98 µg L^{-1}
Caffeine	17.842 ng L^{-1}	35.684 ng L^{-1}
Nicotine	11.815 µg L^{-1}	23.63 µg L^{-1}

Table 2. Average concentrations found in the aquatic environment extrapolated by the bibliographic survey.

Contaminants	**Concentrations**	**References**
Paracetamol	200 µg/l	Klaas Wille 2011
	6 µg/l	Carlsson et al. 2006
	76-94 ng/l	Bound et al. 2006
	231-347 ng/l	Bound et al 2006
	< 20 ng/l	Thomas et al. 2004

	0.6 µg/l	Wu et al 2012
	0.16 µg/l	Wu et al 2012
	1.9 µg/l	Wu et al 2012
	4.3 µg/l	Wu et al 2012
	5.99 µg/l	Wu et al 2012
	0.418 µg/l	Wu et al 2012
	0.22 µg/l	Wu et al 2012
Ibuprofen	7.7 ng/l	Weigel et al. 2004
	0.6 ng/l	Weigel 2003
	0.87-85 µg/l	Heberer 2002
	2.7µg/l	Heberer 2002
	0.12-0.23 µg/l	Ericson et al. 2010
	339-423 ng/l	Bound et al 2006
	2016-2184 ng/l	Bound et al 2006
	57.1 ng/l	Fang et al. 2012
	928-<8 ng/l	Thomas et al. 2004
	<5-41 ng/l	Stumpf et al. 1996

	0.07-0.7 ng/l	Weigel et al. 2004
	17.4 ng/l	Zuccato et al. 2008
Caffeine	7-87 ng/l	Weigel et al. 2004
	2-5.4 ng/l	Weigel 2003
	4.9-16.1 ng/l	Weigel 2003
	15 ng/l	Weigel 2003
	8 ng/l	Weigel 2003
	9.7 ng/l	Weigel 2003
	10-52 µg/l	Teijon et al. 2010
Nicotine	0.17-0.09 µg/l	Martinez Bueno et al. 2011
	13-34 µg/l	Teijon et al. 2010

4.3 Polyps' activity

Polyps' activity was measured twice a day (at 8 am and 5 pm) for the entire duration of the experiment following Torrents et al. (2008). Polyps' activity (number of open vs. closed vs. partly open polyps) was assessed by determining the state of activity of its polyps and expressed as the prevailing state of the polyps' expansion (Torrents et al. 2008).

Briefly, each colony was assigned to three different expansion states of the polyp's body as follow: a prevailing number of totally expanded polyp and tentacles (value 2), tentacles or polyps emerging from the gastric cavity (value 1) and totally retracted polyps (value 0) (Fig 3.1).

These data were averaged on a daily basis and reported as a percentage of the maximum body expansion state. In addition, the percentage of open polyps per each colony was also determined.

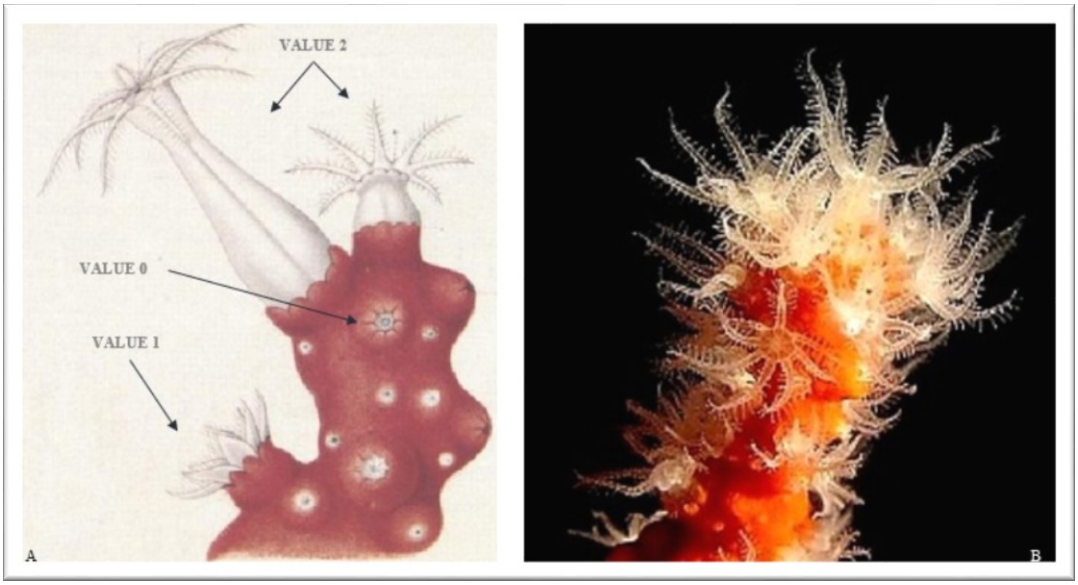

Fig. 3.1 (A) Three different states of polyps' activity determined according to Torrents et al. (2008). Particular of the *C. rubrum* polyps (B)

4.4. Sclerites dimensional analysis

To detect the possible effects of pharmaceuticals on sclerites of *C. rubrum* dimensional analysis of these was conducted (Cerrano et al. 2013). One red coral colony from each mesocosm (treated and untreated) was taken after 14° day (t_{14}), 28° day (t_{28}) and at the end of the experiments (33° day (t_{33}) and 35° day (t_{35}), respectively for the first experiment and second experiment). The samples were stored at -20 ° C for one week before being analyzed. Only their apical parts were soaked in 12% solution of sodium hypochlorite for 24 h until all organic material was removed (Manzello 2010) (i.e., only the axial skeleton and sclerites remained). *C. rubrum* calcifies more rapidly in its apical regions where the sclerites are directly incorporated to form the medullary part of the axial skeleton (Debreuil et al. 2011). The sclerites were then rinsed several times with reagent grade water (Milli-Q), mounted on slides and analyzed under the optical microscope (Zeiss Axioskop) with a 40x magnification (Cerrano et al. 2013). Length and width of sclerites were carried out through a micrometer reticle. The ratio between these two measurements was also calculated.

4.5. Prokaryotic and viral abundance

Prokaryotic and viral abundance for the seawater samples was determined according to the protocol described by Noble and Fuhrman (1998) without previous fixation to avoid underestimations of viral abundances (Danovaro et al 2001, Wen et al 2004). Sub-samples (5 ml) from treated (added with pharmaceuticals) and untreated systems were collected immediately after the addition of pharmaceuticals (t_0= start of the experiment) after 12 h (t_{12}), 24 h (t_{24}) and 48 h (t_{48}) incubation for

the red coral colonies. After collection, three replicate seawater samples were stored at -20°C until the analysis. Subsamples were filtered onto 0.02μm pore size filter (Whatmann Anodisc; diameter, 25 mm; Al_2O_3) and stained with 100 μL of SYBR Gold (stock solution diluted 1:5000). The filters were incubated in the dark for 20 min, washed three times with 3 mL of prefiltered Milli-Q water and mounted onto glass slides with 20μl of 50% phosphate buffer (6.7 mM phosphate, pH 7.8) and 50% glycerol (containing 0.5% ascorbic acid). Slides were stored at -20°C. Prokaryote and viruses counts were obtained by epifluorescence microscopy (Zeiss Axioskop 2). For each slide, at least 20 microscope fields were observed and at least 200 prokaryotes and viruses were counted per filter.

4.6 Scanning Electron Microscopy

Coral colonies were prepared for inspection under Scanning Electron Microscope (SEM) to visualize the potential effects of pharmaceuticals analyzing the *C. rubrum* tissue. At the 28° day (t_{28}) of the experiment, one colony was withdrawn from each mesocosm and apical branches (1 cm) of the colonies were cut. The apical branches of the colonies were subjected to progressive dehydration by immersing the samples in ethanol and distilled water solutions and finally overnight in 100% ethanol. Subsamples were mounted on aluminium stubs using carbon adhesive tabs and first subjected to "critical point dry", then coated with gold/palladium (Au/Pb) for five minutes using a Polaron Range sputter coater. SEM observations were conducted with a Philips® XL 20 microscope.

4.7 Acetylcholinesterase activities

The enzyme activity was measured in polyps within coenenchyme of *C. rubrum*. Unfixed samples of red coral colonies were used for the test. The activity of acetylcholinesterase (AChE, EC, 3.1.1.7) was determined by using the spectrophotometric method according to the Ellman's (1961). Such an activity was measured in controls and in the samples (n=3) of tissue red coral colonies collected immediately after addition of pharmaceuticals (t_0) and 7° day (t_7) incubation for *C. rubrum*. Subsamples from controls and treatments were frozen after each time point overnight. The frozen samples were then thawed, through a scalpel was cut the coenenchyme of red coral, with inside the polyps, homogenized with a minipotter (B.Braun Melsunger), passed through a syringe with thin needle, (Ultrafin 29G, 12,7 mm length), in the presence of 1% triton X100, sonicated for 25 min (Branson, 3510) and centrifuged for 3 min at 8000 rpm. The supernatants were used to determine AChE at λ = 412 nm. The kinetic of AChE activity was obtained by measuring the velocity of substrate cleavage for 3 min compared with the linear equation of a standard curve that had been previously obtained by supplying known amounts of ChEs.

The protein content in the supernatants of the controls and treatments was measured using the method described by Lowry et al. (1951) (Lowry et al., 1951), subsequently modified by Hartree (1972) (Hartree, 1972). The AChE units were obtained by the ratio between the micromoles of substrates hydrolyzed/min/mg protein at room temperature.

4.8. Statistical analyses

Differences in each of the investigated variables (univariate tests) between the controls and treatments, during the experimental time, were assessed using permutational analyses of variance (PERMANOVA; Anderson, 2005; McArdle and Anderson, 2001) after testing for the homogeneity of variances using the Cochrans‟ test.

The design included two factors: time and treatment. When significant differences were encountered ($p < 0.05$) post-hoc pairwise tests were also carried out. Statistical analyses were performed using the routines included in the PRIMER 6+ software (Clarke and Gorley, 2006).

4.4. RESULTS

4.4.1. Polyps' activity

The coral's polyps activity and the percentage of open polyps overall did not exceed 70%, even in the control mesocosms (Figure 4.1, A-B and figure 4.2, A-B). Polyps' activity recorded in this experiment fluctuated around values reported for a healthy red coral population collected at similar or higher depths (Torrents et al. 2008) and recent mesocosm experiments (Cerrano et al. 2013). To provide additional information on the health status of the red coral colonies, we also measured the percentage of open polyps according to Cerrano et al. 2013.

Figure 4.1, A and B show the polyp's activity and the percentage of open polyps of red coral colonies exposed to caffeine and paracetamol during the 33 days of the first experiment. Red coral colonies exposed to paracetamol and caffeine compared to the control showed significant variations in the percentage of open polyps ($p < 0.05$ and $p < 0.01$, respectively), during the last 23 days of the experiment.

The polyps' activity showed a significant increase compared to the control only in mesocosms treated with paracetamol ($p < 0.01$), during the first period (0-10 days) of the experiment, while showed no significant variation during the last 23 days of exposure.

On the other hand, in the systems treated with paracetamol both polyps' activity ($p > 0.05$) and the number of open polyps ($p > 0.001$) decreased significantly during the last 23-days of the exposure respect to the first period of exposure (10 days, fig. 4.1 A- B). On the contrary, in the systems treated with caffeine, the coral's polyps'

activity did not show significant changes in the time and compared to the controls, during the experiment.

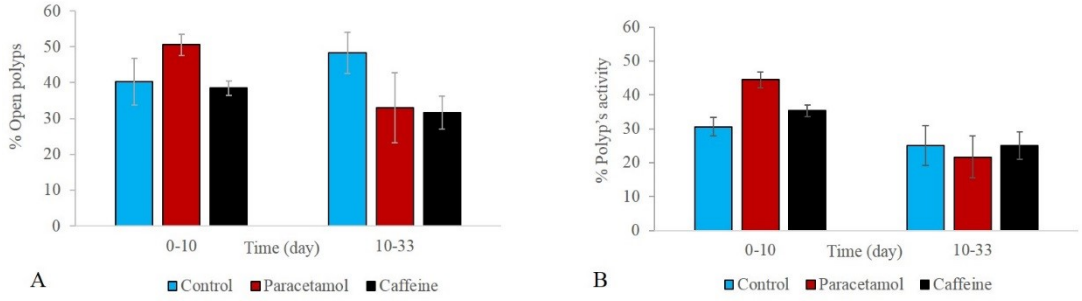

Figure 4.1. Percentage of corals open polyps (A) and polyps' activity (B) in the control and treatment mesocosms for different time intervals (0-10, 10-33 days) during the first experiment. ± = ES

Furthermore, both polyps' activity and the number of open polyps of the coral colonies, after addition of nicotine and ibuprofen showed significant decrease respect to the control (Fig 4.2 A - B) but did not show significant variations over time. Regard to the mix used in the experiment, red coral colonies treated with the Mix L and Mix H showed an increased over time in the polyp's activity and in the percentage of open polyps, similar to the control trend. In particular in the systems treated with Mix L, were observed a significant variation in the percentage of open polyps and polyps' activities only the early 16 days of the exposure, compared to the control but we did not observe any change in the last period of exposure. On the contrary, the percentage of open polyps and polyps' activities in the Mix H did not show significant difference respect to the control, during the experiment.

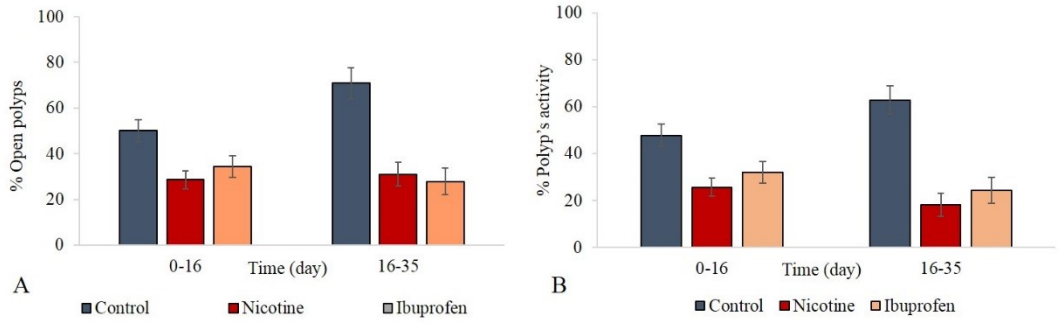

Figure 4.2. Percentage of corals open polyps (A) and polyps' activity (B) in the control and treatment mesocosms for different time intervals (0-16, 16-35 days) during the second experiment. ± = ES

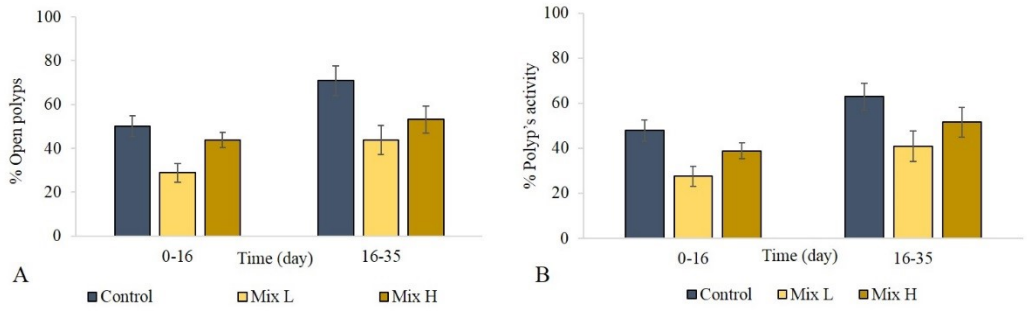

Figure 4.3. Percentage of corals open polyps (A) and polyps' activity (B) in the control and treatment mesocosms for different time intervals (0-16, 16-35 days) during the second experiment. ± = ES

4.4.2. Sclerites dimensional analysis

Red coral colonies exposed to all substances used in the experiments, including the two mixtures did not show significant differences in the length and width of sclerites compared to control and over time (Fig. 4.4-4.6).

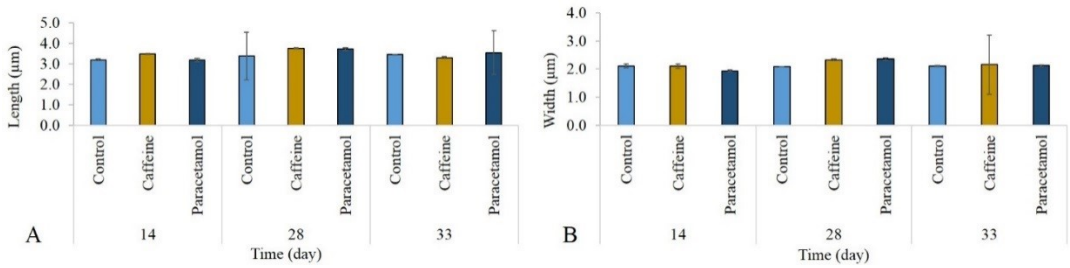

Figure 4.4. Length (A) and width (B) sclerites of *Corallium rubrum* colonies exposed to paracetamol and caffeine and in the control mesocosms, during the first experiment. ± SD.

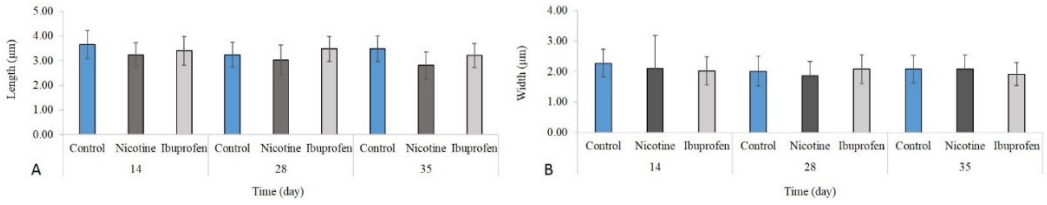

Figure 4.5. Length (A) and width (B) sclerites of *Corallium rubrum* colonies exposed to nicotine, ibuprofen and in the control mesocosms, during the second experiment. ± SD.

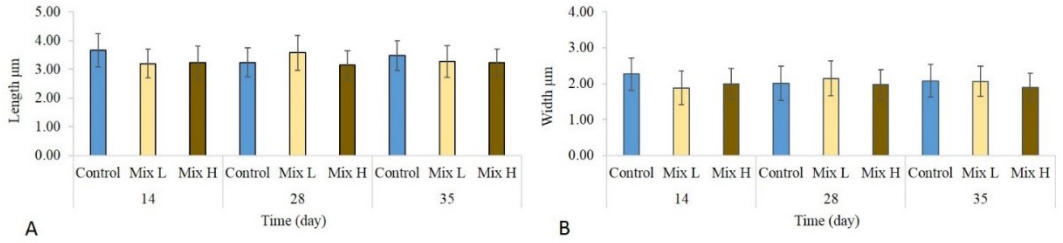

Figure 4.6. Length (A) and width (B) sclerites of *Corallium rubrum* colonies exposed to the mixture at low and high concentration and in the control mesocosms, during the second experiment. ± SD.

4.4.3 Prokaryotic and viral abundance

Prokaryotic and viral abundances were monitored during the entire duration of the experiment to ensure that changes in polyps'activity or eventual mortality were not ascribed to the outbreak of pathogenic infections (Danovaro et al. 2008 b; Cerrano et al. 2013). Previous studies confirm that stressing environmental conditions can promote coral diseases (Vezzuli et al. 2010).

Figure 4.7 and 4.8 shows a number of viruses and prokaryotes in the coral colonies surrounding seawater along 48 h exposure to paracetamol, caffeine and in the control systems. After addition of paracetamol and ibuprofen did not show a significant difference in the prokaryotic and viral abundance, as compared to the controls. On the contrary, caffeine caused a significant increase in the number of viruses ($p < 0.001$) and prokaryotes ($p < 0.001$) overtime in the surrounding seawater coral colonies, respect to the controls (Fig. 4.7 A and B).

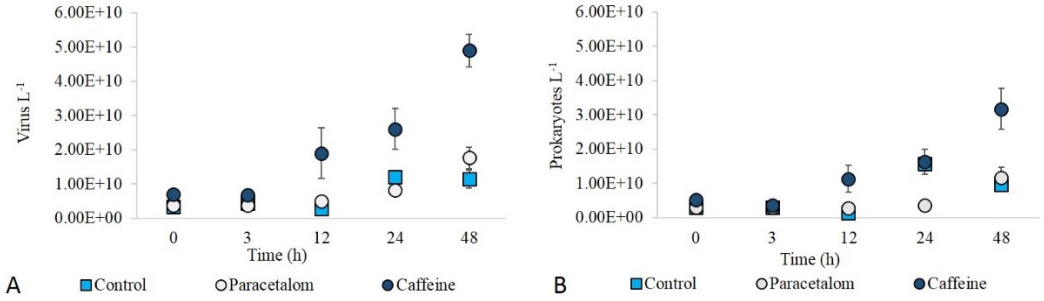

Figure 4.7. Amount of viruses (A) and prokaryotes (B) in the surrounding seawater in mesocosms treated with paracetamol and caffeine, during the experimental time. The X-axis shows the sampling times after exposure at time 0; Y-axis shows the average number of virus and prokaryotes cells /L. ± = SD.

Similarly, the addition of nicotine caused an immediate increase of viruses in the surrounding seawater of coral fragments, since the beginning of the exposure (t_0) and showed the highest values (Fig 4.8 A, $p < 0.001$). On the contrary, the prokaryotic abundance showed a significant increase only after 48 h (t_{48}) from the beginning of the experiment, compared to the control (Fig. 4.8 B, $p < 0.01$).

The two mixtures had a different response in terms of microbial abundances. In fact, systems treated with Mix L (Fig. 4.9, A, B) showed a significant increase ($p > 0.01$) of microbial abundance after 12 hours (t_{12}) from the beginning of the experiment and then decreased over time. Otherwise, systems treated with Mix H showed a significant increase of the prokaryotic and virus abundance during the last days (t_{24} and t_{48}) of exposure ($p > 0.01$ and $p > 0.05$ viruses and prokaryotes respectively).

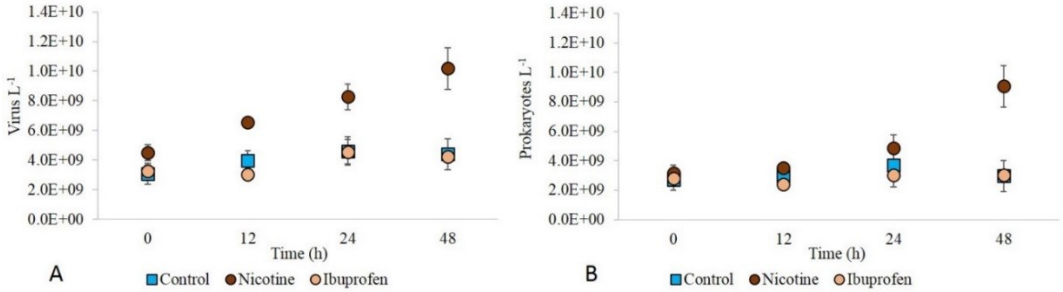

Figure 4.8. Amount of viruses (A) and prokaryotes (B) in the surrounding seawater in mesocosms treated with nicotine and ibuprofen, during the experimental time. The X-axis shows the sampling times after exposure at time 0; Y-axis shows the average number of virus and prokaryotes cells /L. ± = SD.

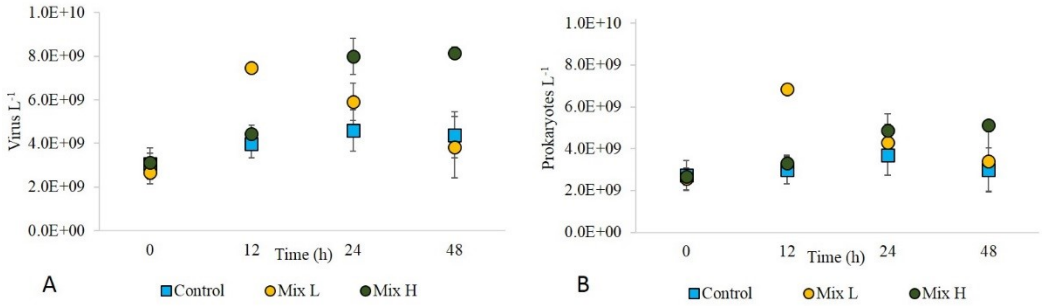

Figure 4.9. Amount of viruses (A) and prokaryotes (B) in the surrounding seawater in mesocosms treated with low (Mix L) and high (Mix H) mixture, during the experimental time. The X-axis shows the sampling times after exposure at time 0; Y-axis shows the average number of virus and prokaryotes cells /L.± = SD.

4.4.4 Scanning Electron Microscopy

Figure 4.10 shows two possible prokaryotes morphotypes present in the tissue of the red coral colonies, photographed by SEM. In particular, figure 4.13 A, shows the tissue of the coral in the control systems with prokaryotic rod-shaped cells, while figure 4.13 B shows the tissue of corals exposed to caffeine with prokaryotic morpho-types similar to cocci. Both morphotypes were also found during the analysis of contour of the corals to epifluorescence microscope (Figure 4.11).

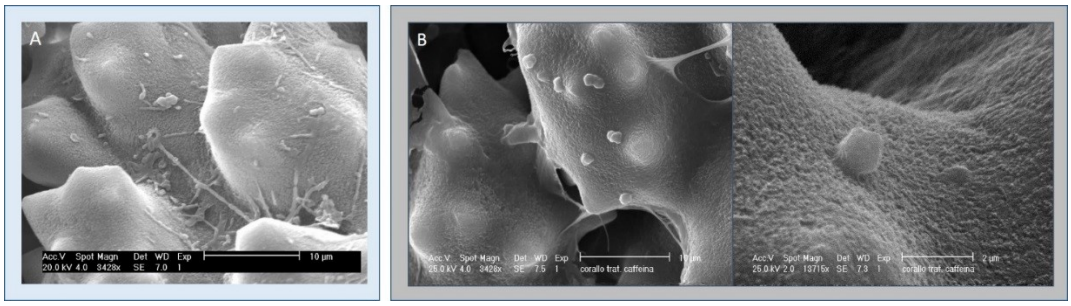

Figure 4.10. The tissue of the coral colonies unexposed (A) and exposed (B) at caffeine, photographed by SEM.

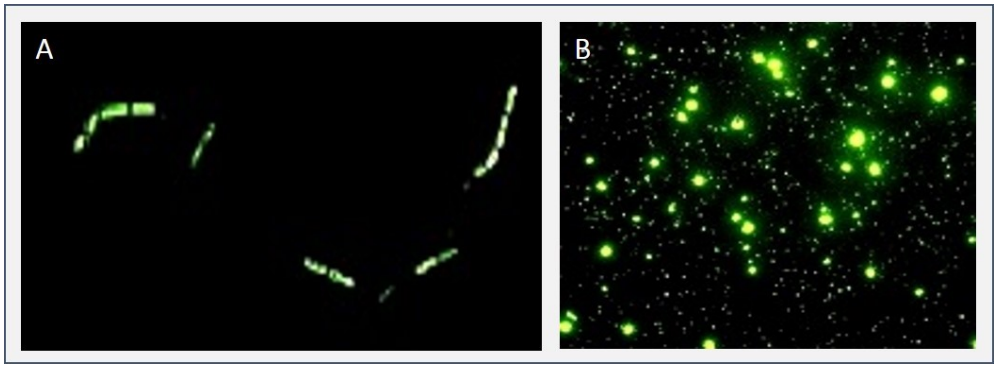

Figure 4.11. Different prokaryotes morphotypes found in surrounding seawater mesocosms unexposed (A) and exposed (B) to pharmaceuticals, photographed by epifluorescence microscope.

4.4.5 Acetylcholinesterase activities

The exposure of the coral colonies to nicotine, ibuprofen and two mixture (low and high concentration) caused a significant increase over time in AChE activity in all the treatments (Figure 4.12 and 4.13). In particular, nicotine and ibuprofen determined a significant increase in AChE activity ($p < 0.001$) at 7 days to the beginning of the experiment. Similarly, in the systems treated with the mix at low and high concentrations, AChE activity increased significantly over time ($p < 0.01$).

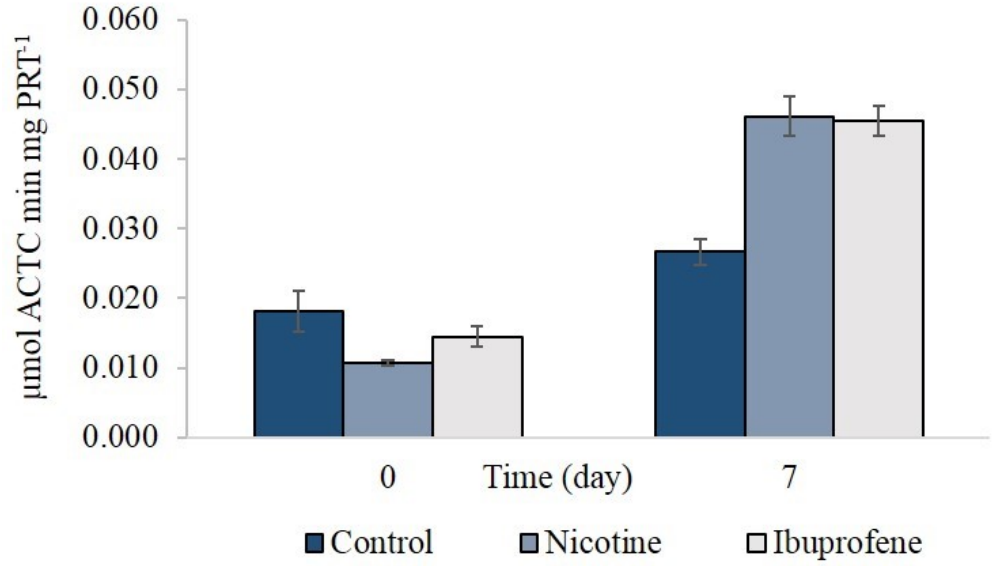

Figure 4.12. AChE activity speed in control and exposed *C rubrum* colonies to nicotine and ibuprofen; X-axis = sampling times; Y-axis = AChE Units (AChE U), where 1 AChE U= 1 micromole of ACh hydrolyzed/min/mg protein.

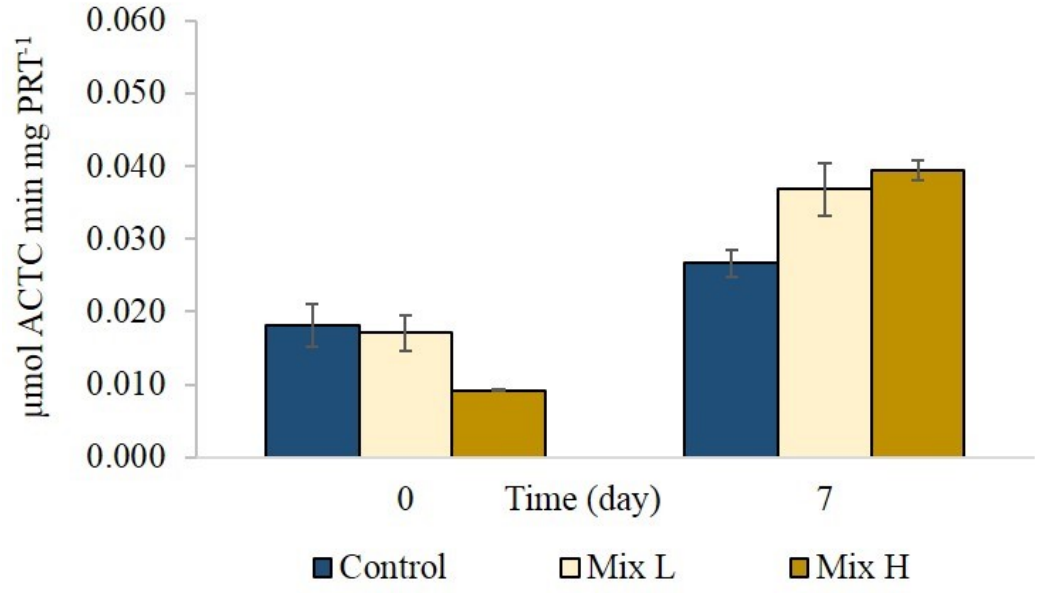

Figure 4.13. AChE activity speed in control and exposed *C rubrum* colonies to the mixture at low and high concentrations; X-axis = sampling times; Y-axis = AChE Units (AChE U), where 1 AChE U= 1 micromole of ACh hydrolyzed/min/mg protein.

4.5 DISCUSSION

C. rubrum is an endemic species of Mediterranean and Eastern Atlantic rocky habitats where it is an important engineering species of the *Coralligenous* (Ballesteros 2006) and semi-dark cave communities (Angiolillo et al., 2016). This long-lived and slow-growing species (Cerrano et al., 2013) is characterized by a solid, red, magnesium calcite axial skeleton produced by fused sclerites (skeletal structures), that is coated with a coenenchyme layer in which polyps and associated gastrodermal canals are embedded (Marschal et al., 2004). Sclerites are suggested to provide structural support and may have a potential role of physical defense against predation (Debreuil et al., 2012). Many studies have already shown that the effect of environmental stress such as ocean acidification can reduce calcification rate (Bramanti et al., 2013; Cerrano et al., 2013) but studies about the potential effects of pharmaceutical products on the red corals are absent.

The dimensional analysis of sclerites performed in this study did not show variations between controls and treated during the experiment suggesting that pharmaceutical products considered in this study, including nicotine and caffeine, do not alter the process of formation and growth of coral skeleton. However, it is also possible that the exposure time of the coral colonies to these contaminants were too short to see any significant effects on the coral colonies growth. Indeed, is well known that *C. rubrum* is a very slow-growing species (in the order of mm / y^{-1}; Pichon 2004).

Despite we did not observe effects on the growth of sclerites, pharmaceutical products had a negative impact on polyps' activity. In particular, we report that

polyps' activity significantly decreased after paracetamol, nicotine and ibuprofen exposure. The expansion of polyps by Anthozoa is energetically highly expensive (Cerrano et al., 2013). Therefore, in natural environment polyps are expanded in presence of high water flows and food, and in healthy condition of the colonies. A decreasing activity of the coral polyps under stress will imply also reduced feeding and respiration efficiencies (Previati et al., 2010).

Previous studies have demonstrated that micropollutants, such as sunscreens significantly enhance prokaryotic and viral abundance in seawater (Danovaro & Corinaldesi 2003). Here, we show that a similar phenomenon occurred also in the red coral after addition of nicotine and caffeine. Indeed, in this case, prokaryotic and viral abundance in seawater surrounding coral branches increased significantly over time reaching values about three times higher than those found in the control. On the contrary, the exposure to paracetamol and ibuprofen did no show significant changes in the number of prokaryotes over time and compared to control.

Regarding the mixture effects, we observed a different behavior of the two concentrations of prokaryotic component over time. In fact, the mix L seemed to cause an increase in the prokaryotic abundance in the short term, conversely, in the systems treated with mix H, an increase in the number of prokaryotes was observed in the long term. However, the effect observed in the systems treated with the mixture, at low and high concentration, showed effects on prokaryotic abundance, lower than those observed in the exposure to individual compounds. These results are in line with recent studies in which multicomponent mixtures showed antagonistic effects (Watanabe et al., 2016; Vasquez et al., 2016). In fact, the behavior of each substance in multi-component mixtures can vary depending on the

composition, concentration and biological assay applied to evaluate the effects (Cleuvers et al., 2004). As a consequence, mixtures may have different effects on different tissues and organisms (Yang et al., 2008).

The reason for the increase of the prokaryotic component due to the addition of nicotine and caffeine is difficult to identify. From one hand, these compounds could stimulate the microbial replication in seawater and in particular of some specific taxa. From the other hand, the red coral, which generally feeds microorganisms (Picciano et al. 2007) can decrease the removal rate (feeding) of prokaryotes in the seawater due to the stress induced by these contaminants.

The investigation of the microbiota associated with *C. rubrum* through electron microscopy revealed that, although it was not possible to identify the composition of prokaryotic assemblages, different morphotypes of prokaryotes were present in the control and in corals treated with caffeine and nicotine. The prokaryotic community constitutes a fundamental component to the health of corals, providing an immune response against the pathogens and environmental stress. When this component changes the coral becomes more susceptible to viral infections (Brown et al. 2007; Reshef et al 2006). As observed for the prokaryotic component, viral abundance increased during the experiment in the presence of both caffeine and nicotine, compared with the control. On the contrary, ibuprofen and paracetamol did not show an effect in terms of viral abundance variation with respect to the control.

These results allow us to hypothesize that the increased viral abundance observed after addition of caffeine and nicotine might be also due to the increase of their main hosts.

To evaluate the effect of the pharmaceutical products on *C. rubrum*, the analysis of the acetylcholinesterase activity was carried out for the first time on the red coral polyps.

Acetylcholinesterase (AChE) is a key role in terminating neurotransmission at cholinergic synapse (Mezzelani et al., 2016) and is used as a biomarker of response to stress conditions such as pollutants (i.e. neurotoxic pesticides, sunscreen products, nanomaterials, Luis et al., 2015; Carballeira et al., 2012; Falugi et al., 2008; Harrison et al., 2002) and environmental stress (Matranga et al., 2006).

Acetylcholinesterase and the cholinergic pathway have also been detected in various life forms, such as bacteria, plants, fungi and various other metazoans such as cnidarian (Denker et al., 2008; Cousin et al., 2005; Horiuchi et al., 2003). In fact, many studies have confirmed the presence of acetylcholinesterase (AChE) in planula larvae of hydromedusae (Pierobon et al., 2012; Falugi et al., 1994), in nematocytes and hypostomal ganglion cells of Hydra (Chapman et al., 2010), in ephyrae of jellyfish (Costa et al., 2015)

The phylum Cnidaria is the most primitive phylogenetic group that possesses a nervous system. Despite the lack of direct evidence of the existence of ACh or AChE in cnidarians, it is known that these animals are sensitive to cholinergic drugs (Takahashi et al., 2010). Our results confirmed a significant increase in acetylcholinesterase activity in all treated, after 7-day of the exposure respect to the

control, suggesting the presence and sensitivity of the cholinergic system in the red coral polyps to pharmaceutical compounds tested.

4.6 CONCLUSION

The investigation of the responses of *Corallium rubrum* to different PPCPs including lifestyle compounds as nicotine end caffeine tested individually or as mixtures revealed that *Corallium rubrum* can be vulnerable to this type of anthropogenic impact. The individual impact of the PCPs tested was higher than when they were combined, and in particular, paracetamol, nicotine, and ibuprofen affected polyp's activity whereas nicotine and caffeine potentially enhanced microbial contamination and shifts in assemblage composition. In addition, our results showed a significant change of acetylcholinesterase activity in all treats, suggesting the sensitivity of the cholinergic system of the red coral's polyps to pharmaceutical compounds tested. Concluding, the release of the pharmaceuticals tested here in seawater, although in very small concentrations (order of ng / mL^{-1}), could represent an important source of chronic pollution altering the functions of ecosystem engineers, such as *C. rubrum*, and potentially the functioning of the marine ecosystem.

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Chapter 5

Impact of pharmaceuticals and other micro-contaminants on *Acropora sp.*

5.1. INTRODUCTION

Coral reefs are in decline worldwide (Wear & Thurber, 2015). Reef ecosystems are exposed to a wide multitude of stressors due to human activities (Spalding et al., 2001) and as a result have experienced drastic declines in spatial coverage and diversity over the past 50 years (Burke et al., 2011). Recent work on the status of reefs has estimated that at least 25% of coral reefs has been functionally lost globally, and one-third of all coral species are threatened by extinction (Carpenter et al., 2008).

The reasons for this environmental degradation are complex, but there is evidence that demographic factors play a significant role (Creel et al., 2003). Currently, 275 million people worldwide reside within 30 km of coral reefs (Wenger et al., 2015). It has been also estimated that every year, millions of tourists travel to tropical destinations (Creel, 2003). For instance, the Coral Triangle region receives an even greater proportion of people visiting for coastal and marine areas than other parts of the world with 33.5 million international visitors only in 2014. In the Caribbean, for instance, official estimates indicate that 70,000 tons of waste are generated annually from tourism activities (UNWTO, 2015). Micro-contaminants, including pharmaceutical products (analgesics, antiseptics, anti-inflammatory drugs) and many other “lifestyle compounds” (i.e., nicotine, caffeine), are receiving increased attention as their prevalence in the marine environment becomes more apparent

(Hoyett et al., 2016; Arpin-Pont et al., 2016; Wenger et al., 2015; Fawell and Ong 2012). They can enter the marine environment via sewage outflows, wastewater treatment plants and land run-off (Daughton & Ternes, 1999). Often wastewater treatment plants do not remove pharmaceuticals, due to both detection and removal difficulties (Wenger et al., 2015).

A number of studies have measured the presence of pharmaceutical products in marine water and sediments (Prichard & Granek, 2016) with concentrations of ng/µg (Gaw et al., 2014; Munaron et al., 2012; Fent et al., 2006). Although their environmental concentrations are generally very low (µg, ng/L), these levels would be sufficient to affect marine life. Indeed, the chronic exposure to levels of pharmaceuticals currently detected in the environment can result in sublethal effects (Daughton & Ternes, 1999) on marine organisms, such as alterations of physiological functions (Ericson et al., 2010), inhibition of the cholinergic system (Luis et al., 2015), induction of oxidative stressor (Pires et al., 2016) and estrogenic responses, (Richardson, 2008).

Coral reef is among the most diverse and complex ecosystems on Earth and among the most heavily exploited habitat by humankind in the past 25 years (Downs et al., 2015; Spalding et al., 2001). Therefore, the impact of pharmaceuticals could be an additional stress for these already degraded ecosystems. Despite this, information on the effects of these compounds on a coral reef is very limited.

5.2 OBJECTIVES

We evaluated the potential impact of pharmaceuticals and other micro-contaminants on hard corals and their symbiotic algae by field experiments in Onu Island, Fiji (Indian Ocean). We tested the effects of paracetamol, ibuprofen, sodium dodecyl sulfate (SDS) and nicotine on *Acropora sp*.

5.3 MATERIALS AND METHODS

5.3.1 Study area and experimental design

In a field experiment was conducted in Onu Island (Fiji). Nubbins from different donor colonies belonging to the genus *Acropora* (3-6 cm), were collected and placed in aquaria for the acclimatization process for 48 h under stable and *in situ* condition (temperature and salinity) before the experiments. After acclimation, only healthy species (without any signs of necrotic tissue and with polyps regularly open) were used to perform the experiments. Nubbins of *Acropora sp.* were washed with virus free seawater filtered onto 0.02 μm membranes (Anotop syringe-filters; Whatman, Springfield Mill, UK), immersed in polyethylene Whirl-pack bags (Nasco, Fort Atkinson, WI, USA) and filled with 2 L virus-free seawater. Replicate sets containing nubbins (n = 3, including more than 300 polyps each) were supplemented with aliquots of pharmaceuticals and compared with untreated systems (used as controls). Corals were incubated at the same depth of donor colonies at *in situ* temperature (Fiji). In particular were tested: nicotine (final concentration 10 $\mu g\ ml^{-1}$), paracetamol (final concentration 0.8 $\mu g\ ml^{-1}$), sodium dodecyl sulphate (SDS, final concentration 1.5 $\mu g\ ml^{-1}$) and ibuprofen (final concentration 1.5 $\mu g\ ml^{-1}$).

5.3.2 Analyses of health state of zooxanthellae

Zooxanthellae analyses were performed by sampling the seawater surrounding of coral colonies in order to evaluate the viability and amount of the released symbiotic organisms.

Sub-samples (10 mL) from treated (added with pharmaceuticals) and untreated systems were collected immediately after the addition of chemicals (t_0= start of the experiment) and after 24 h (t_{24}) 48 h (t_{48}), 72 h (t_{72}) and 96 h (t_{96}) at the beginning of the experiment. Aliquots of seawater samples were filtered through 2.0-µm polycarbonate filters and mounted on glass slides. Zooxanthellae were counted under a Zeiss Axioplan epifluorescence microscope (Carl Zeiss Inc., Jena, Germany; ×400 and ×1,000). Based on the autofluorescence and gross cell structure, zooxanthellae released from coral colonies were classified as a) healthy (H, brown/bright yellow color, intact zooxanthellae); b) pale (P, pale yellow colour, vacuolated, partially degraded zooxanthellae); transparent (T, lacking pigmentations, an empty zooxanthellae; Mise & Hidaka 2003; Danovaro et al., 2008).

5.3.3 Quantification of bleaching

To quantify the levels of coral bleaching (Siebeck et al. 2006), we performed a colorimetric analysis on digital photographs of corals taken at the beginning of the experiments and after various times of treatment with filters (specified above). Photographs were taken under identical illumination with a Canon EOS 400D digital camera (Canon Inc., Tokyo, Japan) with a scale meter on the background. The photographs were successively analyzed with a photo-editing software for color composition cyan, magenta, yellow, black (CMYK). Levels of bleaching were

measured as the difference between the coral's color at the beginning of the experiments (t_0) and after treatments (t_{96}). Inside the coral, areas were made 30 random measurements of variables CMYK. Variations in the percentage of the different color components (CMYK) were analyzed with one-way analysis of variance (ANOVA). To rank the bleaching effect due to the different sunscreen tested we obtained Bray–Curtis similarity matrix and multidimensional scaling analysis of the shifts in CMYK color composition of treated corals using Primer 5.0 software (Primer-E Ltd., Plymouth, UK). Bleaching rates were measured as the dissimilarity percentage in CMYK color composition between treated and control corals using the SIMPER tool of Primer 5.0 software (Primer-E Ltd). In addition, to the average values obtained were attributed of scores of the degree of bleaching (Tab 1) according to a scale organized in ranks (0 to > 60), i.e., from "no visible coral bleaching" (0-10) to the "total bleaching with 100% of coral nubbins surface (> 60).

MAIN CLASSES OF DEGREE OF BLEACHING

DEGREE OF BLEACHING (%)	INCIDENCE OF CORAL BLEACHING
0-10	No visible coral bleaching
10-20	Slight color variation. No visible coral bleaching
20-25	Slight bleaching (< 10 % of coral nubbins surface)
25-60	Strong bleaching (>50% of coral nubbins surface)
> 60	Total bleaching (100 % of coral nubbins surface)

Table 1: Main classes of degree of bleaching

5.3.4 Prokaryotic Abundance

Direct counts of total prokaryotic abundance in surrounding coral branches seawater in Fiji experiment, were carried out using the method described by Noble and Fuhrman (Noble & Fuhrman, 1998) with few modifications. Seawater subsamples (10 ml) were collected from treated (added with pharmaceuticals) and untreated systems, utilized for evaluating the pharmaceutical products impact on hard corals, immediately after the addition of filters (t_0= start of the experiment) and 24 h (t_{24}) 48 h (t_{48}), 72 h (t_{72}) and 96 h (t_{96}) at the beginning of the experiment. Seawater subsamples were preserved in 0.02 μm prefiltered formalin (2% final concentration) and were concentrated onto 0.02 μm pore size filters (Anodisc 25 mM, Al2O3), and stained with 20 μl SYBR Green I (stock solution diluted 1:20). Filters were incubated in the dark for 15 min and mounted on glass slides with a drop of 50% phosphate-buffered saline and 50% glycerol, containing 0.5% ascorbic acid. Slides were stored at 20°C until analysis. Counts were obtained by epifluorescence microscopy (magnification, ×1,000; Zeiss Axioplan) by examining at least 10 fields, that is, at least 200 cells or particles per replicate.

3.3.5 Statistical analysis

Differences in each of the investigated variables (univariate tests) between the controls and treatments, during the experimental time, were assessed using permutational analyses of variance (PERMANOVA; Anderson, 2005; McArdle and Anderson, 2001). The design included two factors (time and treatment). When significant differences were encountered ($p < 0.05$) post-hoc pairwise tests were

also carried out. Statistical analyses were performed using the routines included in the PRIMER 6+ software (Clarke and Gorley, 2006).

5.4 RESULTS

5.4.1 Analyses of zooxanthellae

All pharmaceutical products caused the release of damaged of zooxanthellae (Fig. 4.1) by hard corals treated during the 96 hr of exposure. In particular, the highest values of the release of zooxanthellae were presented by paracetamol in which was observed a significant increase ($p= 0.0001$) respect to the control and other treaties, during the experiment. In addition, about 95% of symbionts released in the systems treated with paracetamol were damaged. Moreover, the systems treated with ibuprofen and SDS showed significant variation ($p > 0.05$), respect to the control in the release both of zooxanthellae and damaged zooxanthellae, during the experiment, where about more of 80% of zooxanthellae released by corals treated with this substances were damaged. On the contrary, the addition of nicotine caused a significant release of symbiotic algae ($p> 0.05$) but not significant variations were observed in the release of damaged zooxanthellae, compared to the control, during the exposure ($p< 0.05$).

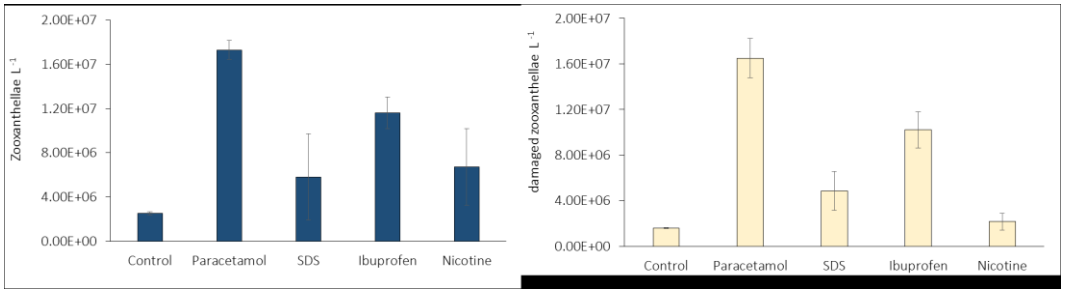

Figure 4.1. A number of zooxanthellae (right) and damaged zooxanthellae (left) released into seawater surrounding coral colonies treated with different pharmaceutical products and in the control systems ± SD.

5.4.2 Quantification of bleaching

Figure 4.2 show the results of the colorimetric analysis for quantification of bleaching of hard corals exposed to paracetamol, ibuprofen, SDS and nicotine, during the 96 hr of the experiment.

All treatments caused visible bleaching especially nicotine and paracetamol (Fig. 4.3). In particular, after addition of paracetamol, nicotine and SDS was observed a strong bleaching of treated nubbins (> 50 % of coral nubbins surface) while the exposure to ibuprofen caused a slight bleaching (< 10% of coral nubbins surface) of the hard corals.

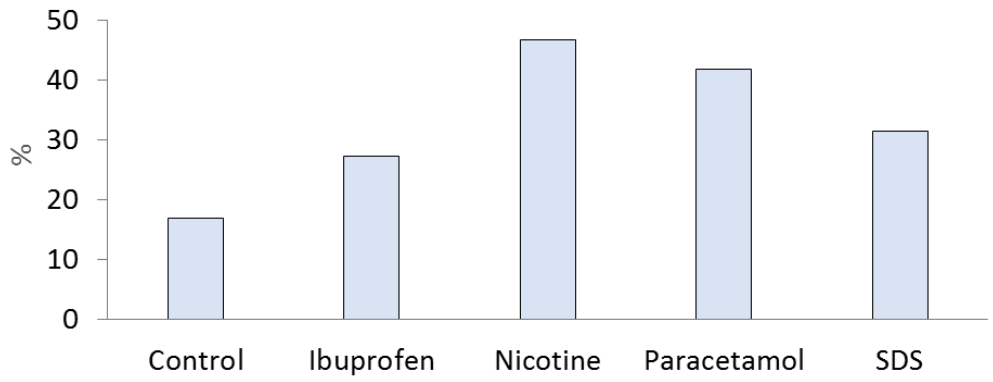

Figure 4.2. Degree of bleaching (%) of the hard corals treated with different pharmaceuticals products and in the control systems.

5.4.3 Prokaryotic and viral abundance

Figure 4.4 shows the prokaryotic abundance into the seawater surrounding coral colonies observed during 96 hr of exposure to paracetamol, nicotine, ibuprofen and SDS.

All treatments showed significant variations ($p > 0.01$) in the prokaryotic abundance in the surrounding seawater compared to the control, during the experiment.

The highest values respect to the control and the other treaties in the prokaryotic abundance were observed after the addition of paracetamol and ibuprofen ($p > 0.001$), during the 96 hr of exposure while the lowest values were detected in the systems exposed to SDS.

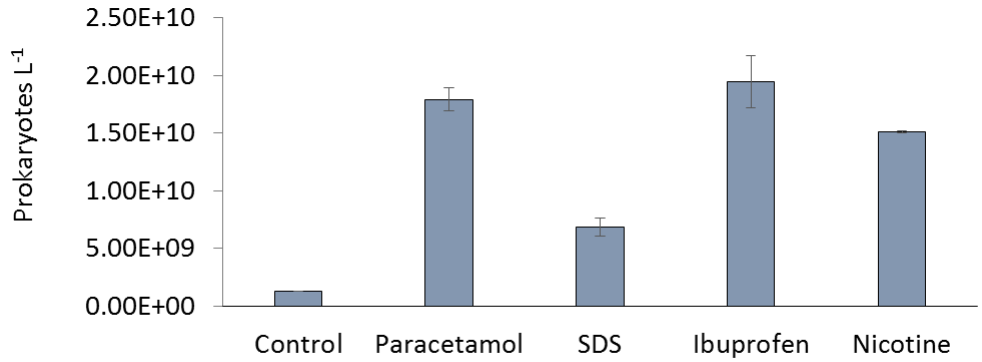

Figure 4.4. The amount of prokaryotes in the seawater containing nubbins of *Acropora sp.* exposed to the different pharmaceutical products and in the control. ± = SD.

5. 5 DISCUSSION

Here we tested the potential effect of pharmaceuticals and other micro-contaminants (i.e., ibuprofen, paracetamol, nicotine, and SDS) on hard corals. All results showed that exposure of hard corals (*Acropora sp*) to these substances caused negative effects on hard corals and their symbionts.

It is known that different pollutants such as sunscreens, cyanide, caffeine, insecticides and heavy metals can have extremely harmful effect on reef-building corals (Pollack et al., 2009; Danovaro et al., 2008;Markey et al., 2007; Jones et al., 2005; Cervino et al., 2004). However, so far little attention has been dedicated to the study of the possible effect of pharmaceuticals and other micro-pollutants on a coral reef. Since more than a century, pharmaceuticals have been released freely in marine environment without any restrictions and are still found in the coastal environment and inland waters (Benotti & Brownawell, 2007; Matamoros et al.,

2009), representing an important form of pollution in marine ecosystems (Stumpf et al., 1999).

The exposure of *Acropora sp*. to the different substances caused bleaching in all colonies within 96 hours from the start of the experiment, showing that these substances caused a significant alteration of the coral health. The effect of these substances was already evident at 24 hours exposure. In particular, paracetamol and nicotine caused a strong bleaching on the surface of nubbins. The ability of chemical pollutants to induce bleaching has already been reported in other studies (Danovaro et al., 2008; Danovaro & Corinaldesi, 2003; Cervino et al., 2003). In our work, the bleaching observed in response to exposure of hard corals to pharmaceuticals and other micro-contaminants was clearly linked to the expulsion of zooxanthellae by the corals. This is reflected in the greater amount of symbionts released by corals exposed to paracetamol, ibuprofen, nicotine and SDS, compared to that released by corals unexposed. Furthermore, damaged zooxanthellae released by treated corals were found to be greater, than those found in control systems. In particular, hard-corals treated with paracetamol showed the highest values in the number of symbionts released, with about 95% of released damaged zooxanthellae. Similarly, after addition of ibuprofen, and SDS a lot of damaged zooxanthellae release was observed. On the contrary, the systems treated with nicotine showed a lot of zooxanthellae release at the end of an experiment but only a small fraction of these was damaged. This suggests that nicotine causes coral bleaching in corals by releasing symbiotic algae but not affecting their health. In this regard, some studies have highlighted that the exposure of coral fragments to various types of stress can

induce bleaching through different processes (e.g, tissue necrosis or programmed cell death of the animal's cells or expulsion of the symbionts (Dunn et al., 2007). Many studies have shown the negative effect that substances such as paracetamol and ibuprofen have on different marine organisms (Tijani et al., 2016; Ericson et al., 2010; Cleuvers, 2004), while knowledge about the effects of nicotine and SDS on marine life is still very limited.

We found that the exposure of corals to ibuprofen, paracetamol, nicotine, and SDS has also caused a significant increase in the prokaryotic abundance into the surrounding seawater branches of the exposed corals. In particular, paracetamol, ibuprofen, and nicotine caused a strong increase of a number of prokaryotes compared to the control systems. The lowest values of the prokaryotic abundance compared to the other treatments were observed in the systems treated with nicotine but significant compared to the control. Microbial response to the addition of these substances can be due either to the presence of these compounds or as a response to the stress. In particular, it has reported that mucus produced by the coral as a response to the stress immediately dissolves in the seawater, providing a food source for planktonic bacteria (Wild et al., 2004) and stimulating prokaryotes proliferation. All results obtained in this study suggest that pharmaceuticals and other micro-pollutants can have a direct harmful effect on the corals and their symbiotic organisms.

5.6 CONCLUSIONS

These results for the first time provide new information on the potential ecological impact associated to release of some pharmaceuticals on tropical corals. Our findings indicate that pharmaceutical products including nicotine and SDS are important factors of vulnerability for tropical corals, as they can exert a negative effect directly on the host and could affect the balance between microbial communities and coral, making the latter most vulnerable to any infectious diseases.

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Chapter 6

EcolCare™ Protocol (Ecoreach Ltd)

6. 1 ECOREACH LTD: A LINK BETWEEN SCIENCE AND SOCIETY

Ecoreach Ltd is a Spin-Off of the Polytechnic University of Marche, consisting of a pool of professors, researchers and PhDs with experience in the field of applied research in the marine environment. The Core Team of Ecoreach Ltd has extensive experience in the field of marine biology and applied ecology as revealed from the numerous articles published in prestigious scientific journals (e.g., *Nature, Science, Current Biology, PLoS Biology, Trends in Ecology and Evolution, Environmental Health Perspectives*). The development of procedures for the validation of the level of eco-sustainability of commercial products, with particular reference to the possible effects on the health of marine ecosystems, represents one of the main objectives of Ecoreach Ltd.

6. 2 RATIONAL

Production and consumption of personal care and cosmetic sun products are increasing worldwide, reaching unexpected levels, with potentially important consequences on environmental contamination (Danovaro et al., 2008). The release of these products is also linked to the rapid expansion of tourism in the marine coastal area, which represents the fastest-growing sector of the global economy (UNWTO, 2015). Scientific evaluation of the impacts of the compounds released into the marine environments thus represents a key issue for the conservation of marine life.

Chemical compounds contained in sunscreen and other personal care products are known to have a negative impact on different marine organisms (Downs et al., 2015; Maipas et al., 2015; Kim et al., 2014; Fent et al., 2010; Diaz-Cruz et al., 2009; Kunz et al., 2006; Danovaro & Corinaldesi, 2003). A Recent study has proved that, even at low concentrations, sunscreens are able to induce coral bleaching (Danovaro et al., 2008). Just one drop of the most commonly- sold sunscreen, such as the ones containing parabens, can bleach a square meter of coral reef.

In this context, it becomes increasingly important to assess the scientific approach for the possible biological and ecological damage caused by the release of chemical and natural compounds, in the marine environment. From this scientific awareness comes the concrete idea of developing procedures for the validation of the level of eco-sustainability of commercial products (with particular reference to the possible effects on the health of marine ecosystems) and create a real eco-friendly sunscreen product, which guarantees total protection of life and marine biodiversity.

6. 3 ECOLCARE™ PROTOCOL

On the basis of tests performed during the doctoral thesis, I collaborated with the Ecoreach team to the development of a new protocol (EcolCare™, trademark) to evaluate the degree of compatibility of different chemicals used in sunscreen and personal care products on marine biological components. The protocol does not rely on the measurement of the model organisms survival rates (such as in the common tests of acute toxicity, which imply the sacrifice of organisms), but only the estimate of the behavioral stress level of the organisms exposed to different chemicals tested. The biological models used in the protocol include photosynthetic organisms (e.g., uni- or multicellular algae), herbivore or filter feeders (e.g., pelagic crustacean) and benthic invertebrates (i.e., cnidarian). The protocol is based on a statistical validation of the effects of the tested products, in standard concentrations (concentrations estimated from the amount of product release in the sea) on some behavioural traits of model organisms. In particular, model organisms are exposed to the product to be tested in aquarium, under standard conditions of temperature, salinity, light and oxygen concentration. At regular time intervals (variables from organism to organism) we evaluate quantitatively some physiological and behavioural traits that do not involve the sacrifice of the organisms. For instance, in the experimental tests carried out on algae the growth rate is evaluated, while in the case of cnidarians the percentage of polyp's activity and open polyps of the colony or the release of symbiont organisms are assessed. At the end of the experiment, the algae are placed in a laboratory culture system, while the animals are released into the sea or kept in the aquarium. Results produced during this thesis can be used to evaluate on the basis of responses obtained from the model organisms

a score of eco-friendliness of the different tested products, according to a scale organized in ranks of the EcolCare^{TM} protocol (from A +++ to H), i.e. from "total eco-friendly" (A+++) to "very high impact on marine life" (H) (Tab. 1).

RANK	VALUE	ENVIROMENTAL COMPATIBILITY JUDGEMENT
A +++	10	Top eco-friendly
A++	9.8 - 9.9	Eco-friendly double plus
A+	9.7 - 9.8	Eco-friendly plus
A	9.5 - 9.7	Eco-friendly
B	9.1 - 9.5	Fully eco-compatible
C	8.6 - 9.1	Eco-compatible
D	8.0 -8.6	Compatible with marine life
E	7.7 - 8.0	Minor impact on marine life
F	7.2 - 7.7	Moderate impact on marine life
G	6.7 - 7.2	High impact on marine life
H	< 6.7	Very high impact on marine life

Table 1. A score of eco-friendliness organized in ranks (EcolCare^{TM} protocol).

6. 4 ECOREACH SUNSCREEN PRODUCTS

MarineCare and SunBioCare (Fig.6.1) are eco-compatible sunscreens carried out at the Polytechnic University of Marche. They have been tested on different marine systems (tropical reefs and Mediterranean organisms) by marine biologists to guarantee the full protection of marine life and biodiversity. The patent formulation excludes all the components that might be harmful to the marine environment (such as ethylhexyl methoxycinnamate, benzophenone-3, parabens, and nanoparticles).

Several experiments in the laboratory and in the field were carried out to assess the potential effect of individual ingredients and the whole formulations of Ecoreach sunscreens (MarineCare and SunBioCare). Replicate sets of model organisms, placed in separate systems were exposed to aliquots of the single ingredients and final formulation of Ecoreach sunscreens, and compared with untreated systems (used as controls). In particular, the tests used to assess the eco-compatibility of sunscreen SunBioCare were conducted on *Nannochloropsis salina*, *Artemia salina* nauplii, and *Anemonia viridis*. Some results about the effects of Ecoreach sunscreens on tropical organisms are reported in chapter 2.

Figure 6.1. Ecoreach sunscreen products

6. 5 RESULTS

The final formulation of SunBioCare and its ingredients did not have significant effects on adult organisms of *A. viridis* during 10 days of exposure both in terms of release of symbiotic organisms (Fig. 6.2) and in changes in prokaryotic abundance in the surrounding seawater (Fig. 6.3). The response of the microbial component, in terms of variation in abundance over time was used as an index of stress caused by the addition of different tested components. Similar effects were observed on the algal component (*N. salina*), whose growth rate did not change over time compared to control (Fig. 6.4). Also, the fraction of living nauplii of *A. salina* remained constant overtime and did not change compared to the control (Fig 6.5). Similarly,

A. salina nauplii with positive response to phototropism did not show significant difference compared to the control, for the entire duration of the experiment.

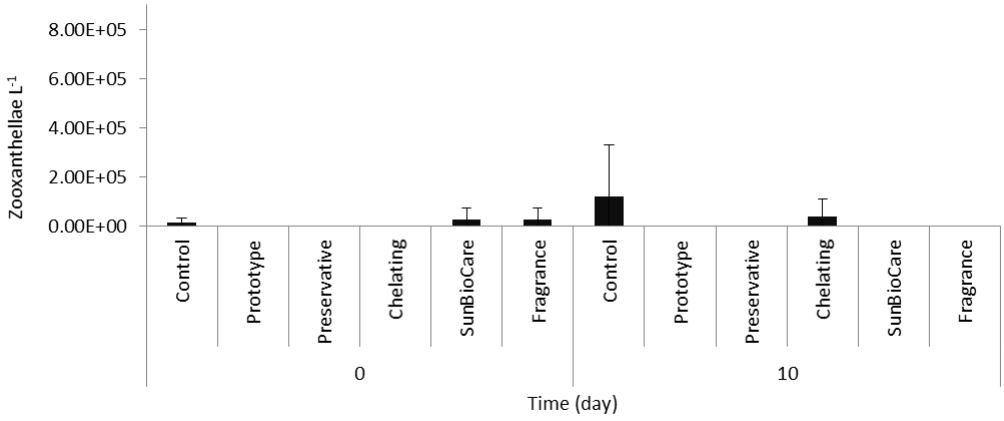

Figure 6.2. Amount of zooxanthellae release in surrounding seawater of *Anemonia viridis* during the exposure to individual ingredients and the final formulation of SunBioCare sunscreen. ± SD.

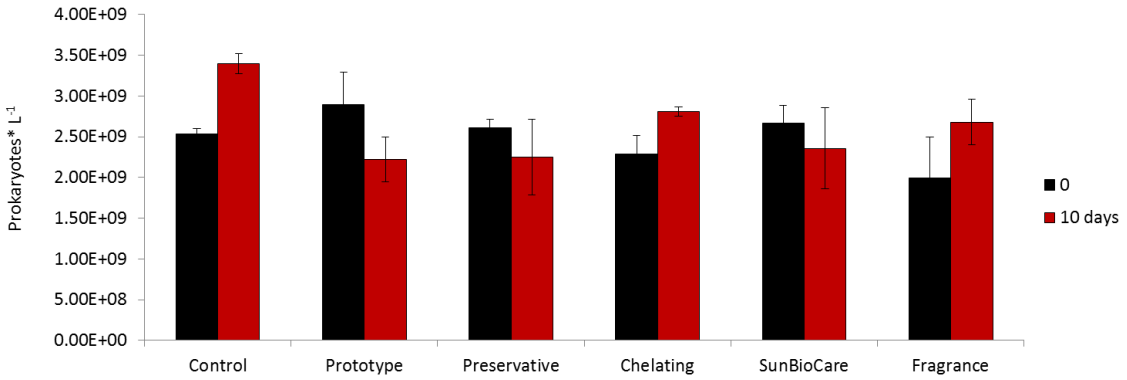

Figure 6.3. Prokaryotic abundance in surrounding seawater of *Anemonia viridis* during the exposure to individual ingredients and the final formulation of SunBioCare sunscreen. ± SD

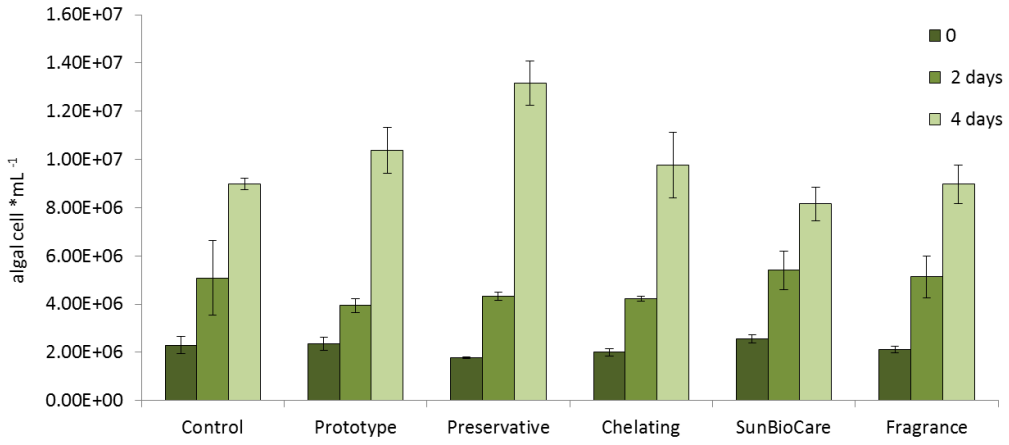

Figure 6.4. Algal cells abundance during the exposure to individual ingredients and the final formulation of SunBioCare sunscreen. ± SD

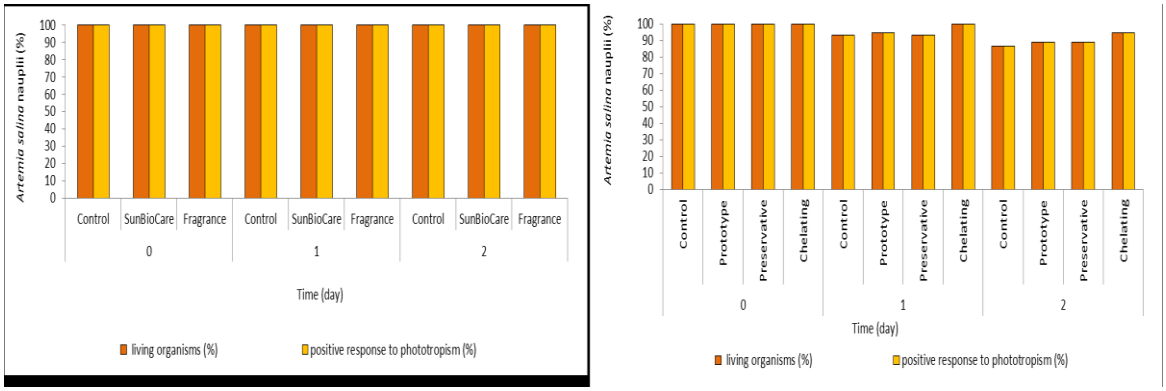

Figure 6.5. Percentage of alive *Artemia salina* nauplii and percentage of individuals that respond positively to phototropism during the exposure to individual ingredients and the final formulation of SunBioCare sunscreen.

6. 6 CONCLUSION

Based on the analysis performed on all living marine components used it is possible to conclude that the sunscreen SunBioMare is completely devoid of any biological effect and environmental impact in both the short term and more prolonged periods. Therefore, these findings allow to classify the sunscreen as totally eco-compatible with level A +++ of the EcolCare brand.

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CHAPTER 7

7.1 CONCLUSION

Our results indicate that sunscreen products, inorganic UV filters and other micro-contaminants (i.e., pharmaceutical products) can alter significantly, even at low concentrations, the functions of marine organisms causing also damage to their early stages of development such as observed for *P. lividus*.

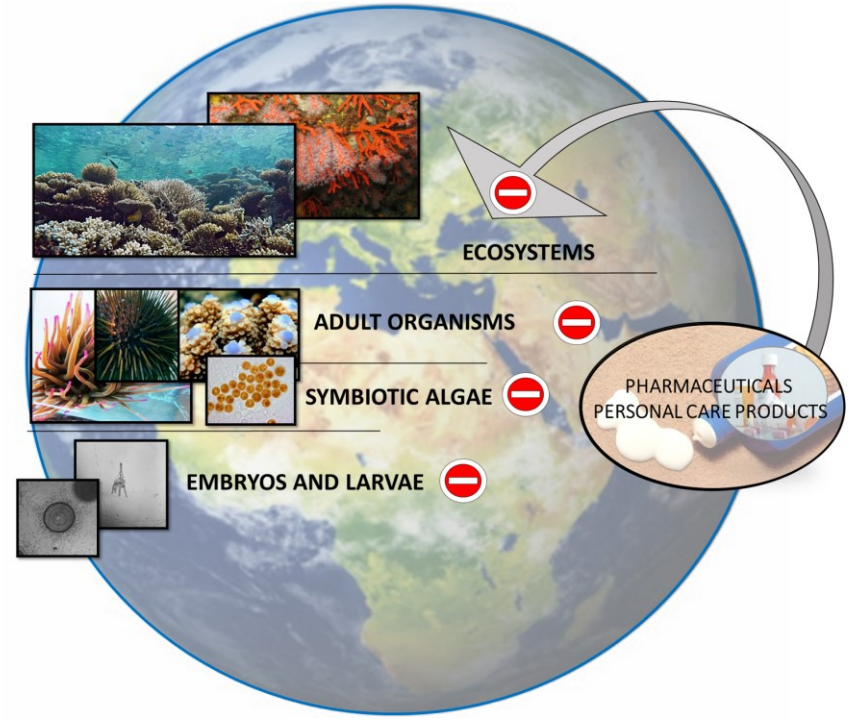
These substances can also damage zooxanthellae both through cell lysis or other degradation mechanisms and through their expulsion, causing rapidly coral bleaching. The effects of sunscreens and their ingredients, on marine organisms, can vary depending on the organism considered and its developmental stage. In addition, the level of impact on the organisms investigated can change in relation to the diverse formulation (in term of ingredients used) of the different brands of sunscreen tested, including sunscreens defined eco-compatible.

The investigation conducted to evaluate the impact of the inorganic UV filters indicate that the modifications made to minimize or eliminate the potential reactivity of inorganic UV filters (ZnO and TiO_2) may influence the toxicity of these compounds on marine organisms. In fact, uncoated inorganic UV filters, extremely reactive by nature, released in marine environment undergo a rapid transformation, promoting the dispersion of these particles in seawater and causing serious ecological consequences on marine organisms (Botta et al., 2011). Our findings suggest for the first time that inorganic filters with modified particles (e.g. Optisol and Eusolex T2000), used in many solar formulations, making these

substances more resistant when released into the aquatic environment (Maipas et al., 2015) are potentially less harmful to marine organisms.

We also observed that sunscreens defined eco-friendly, natural or reef safe are not always eco-compatible with marine life. This suggests that the claim of eco-compatibility in most of the commercial sunscreen products is unjustified. Indeed, the sunscreens tested contained several ingredients (filters and preservatives) already reported to be toxic from marine organisms. Most of the products defined "eco-friendly" and/or "reef-safe", indeed, are not actually tested on marine organisms. Therefore, rigorous procedures are needed for testing the effective eco-compatibility of the products, before placing them on the market.

The results obtained during this PhD work provide new evidence of the effects of sunscreens products and other micro- contaminants on marine environments, and paves the way for further research on different marine organism models, both on different trophic levels and different stage of developments, to understand how these products can affect the health of marine organisms and ecosystems.


ECOSYSTEMS
ADULT ORGANISMS
SYMBIOTIC ALGAE
EMBRYOS AND LARVAE
PHARMACEUTICALS
PERSONAL CARE PRODUCTS

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