Universidade do Algarve

Otimização da fragmentação e dos processos de fixação na reprodução assexuada do coral mole *Sarcophyton sp.*

Simão Luís Moreira dos Santos

Dissertação para obtenção do grau de Mestre em Aquacultura e Pescas

Mestrado em Aquacultura e Pescas (Ramo Aquacultura)

Trabalho efetuado sob a orientação de:

Doutor Rui Miranda Rocha, Departamento de Biologia e CESAM, Universidade de Aveiro

Prof. Doutora Elsa Cabrita, Faculdade de Ciências e Tecnologias, Universidade do Algarve

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Simão Luís Moreira dos Santos

Simão Santos

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Resumo

O género *Sarcophyton* é amplamente conhecido pelos metabolitos secundários que produz, com importantes aplicações biomédicas. Como a coleta destes organismos da natureza não é sustentável nem prática, a aquacultura apresenta-se como a mais promissora alternativa. O cultivo *ex situ* de corais, além do maior controlo da produção, permite a seleção de colónias com características de maior interesse e, a possibilidade de reprodução assexuada por fragmentação, perpetuando estas características genéticas de interesse e possivelmente os mesmos endossimbiontes associados. O controlo da fixação e cicatrização, após a fragmentação, será crucial para a otimização da produção, pela diminuição da mortalidade. O presente estudo teve como objetivo avaliar pela primeira vez, em cinco experiências consecutivas, o efeito do: i) espectro luminoso – luz azul, branca e vermelha, ii) intensidade de Radiação Fotossintética Ativa (PAR) – 60 e 120 µmol quanta/m^2/s, iii) substrato de adesão – carbonato de cálcio e plástico, iv) processo de fixação – cola de etilcianoacrilato, cola de N-butil-2-cianoacrilato e elástico, e v) posicionamento do fragmento – tecido intacto e tecido cortado em contacto com o substrato de fixação. Os fatores testados foram avaliados com base na: i) percentagem de sobrevivência, ii) eficiência fotossintética (F_v/F_m), e iii) força de adesão (Newton/mm^2). A intensidade PAR de 120 µmol quanta/m^2/s e o espetro de luz vermelha foram, no geral, os melhores tratamentos de luz. O tipo de substrato e a posição do fragmento não tiveram influência na sobrevivência e força de adesão dos fragmentos. A cola de N-butil-2-cianoacrilato foi o método de fixação mais eficaz. Este estudo realça a importância dos métodos pós-fragmentação no sucesso da aquacultura de corais, tendo contribuído para a otimização dos procedimentos a adotar no processo de reprodução assexuada de corais moles do género *Sarcophyton*.

Palavras-chave: Aquacultura; Coral; Fragmentação; *Sarcophyton sp.*; Sobrevivência; Força de adesão; Fluorometria PAM.

Otimização da fragmentação e dos processos de fixação na reprodução assexuada do coral mole *Sarcophyton sp.*

Abstract

The genus *Sarcophyton* is well known for their secondary metabolites, with important biomedical applications. Since the wild harvest of these organisms is neither sustainable nor practicable, the aquaculture presents itself as the most promising solution. *Ex situ* coral farming aside from enabling for a greater production control, allows for the selection of colonies with more desirable characteristics and, the possibility of asexual reproduction through fragmentation, preserving these same genetic characteristics and possibly the same associated endosymbionts. The control of fixation and cicatrization, post-fragmentation, is crucial for the optimization of production, by decreasing the survival. The present study aimed at evaluate for the first time, through five consecutive experiments, the effect of: i) spectra – white, blue and red light, ii) Photosynthetic Active Radiation (PAR) intensity - 60 e 120 µmol quanta/m^2/s, iii) substrate – calcium carbonate and plastic, iv) fixation process – ethyl cyanoacrylate glue, N-butyl-2-cyanoacrylate glue and rubber band, e v) fragment position – intact tissue and cutted tissue in contact with the fixation substrate. The tested factors were evaluated based on: i) survival percentage, ii) photosynthetic efficiency (F_v/F_m), and iii) fixation force (Newton/mm^2). PAR intensity of 120 µmol quanta/m^2/s and red light spectrum were the overall best light related treatments. Substrate type and fragment position were found not to affect the fragment survival and fixation force of the fragments. N-butyl-2-cyanoacrylate glue was the most effective fixation process. This study highlights the importance of the post-fragmentation techniques in determining the success of coral aquaculture.

Keywords: Aquaculture; Coral; Fragmentation; *Sarcophyton sp.*; Survival; Fixation force; PAM fluorometry.

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1. General introduction

1.1 Biology and ecology of corals

Corals, being such vital organisms of coral reefs, have long been subject to several biological studies (McFadden et al., 2006b; Osinga et al., 2011). Most coral species live in compact colonies formed by several polyps, which in turn are formed by two epithelial cell layers, the epidermis (or ectoderm) and the gastrodermis (or endoderm), and by an oral end surrounded by tentacles (Rocha, 2013). The majority of tropical coral species establish a mutualistic symbiosis with photosynthetic dinoflagellates of the genus *Symbiodinium*, commonly known as zooxanthellae (Glynn, 1996), that live inside specialized cells in the coral endoderm. Through this symbiosis, coral provides shelter and nutrients such as nitrogen and carbon dioxide, while it receives sugars, fatty acids and amino acids produced photosynthetically by the zooxanthellae (Ellis and Sharron, 1997; Glynn, 1996). In recent studies, zooxanthellae were found to belong to eight broad clades (clade A to clade H) (Coffroth and Santos, 2005; Stat et al., 2006). The symbiosis established between different corals and specific clades of zooxanthellae are mostly habitat-specific, affecting corals distribution and response to extreme environmental conditions, with corals exhibiting a depth/light relationship with their zooxanthellae (depth zonation)(Baker and Rowan, 1997; Mass et al., 2010; Robison and Warner, 2006; Rowan and Knowlton, 1995; Ziegler et al., 2015). Zooxanthellate-coral holobiont (coral host and associated endosymbionts) can regulate its photosynthetic potential according to the prevailing environmental conditions in such a way that photosynthesis is always optimal for growth (photoacclimation), under a broad range of photon flux densities. They do so by adjusting zooxanthellae density, pigment density (Titlyanov et al., 2001), or the pigment composition based on the available light spectra (Dustan, 1982).

Corals are commonly divided in hard and soft corals, being the distinction based mainly in the presence or absence of a calcium carbonate skeleton to support the colony (Rocha, 2013). Hard corals, or scleractinian corals, being the main builders of coral reefs, play an important role in the ecology of these ecosystems. Soft corals have also a leading role as structural components and contributors to the biomass of the coral reef. Soft corals often equal or exceed scleractinian corals in reef total coverage (McFadden et al., 2006b; Osinga et al., 2011). As sessile organisms, corals have developed several defense

mechanisms to ensure their survival, and from these mechanisms, chemical interactions play a vital role (Sammarco and Coll, 1992). Coral tissues, skeleton and mucus layer contains dense and diverse populations of bacteria and archaea that provide support, nutrition and protection against pathogens (Bythell and Wild, 2011; Chen et al., 2012; Rocha et al., 2011; Sharon and Rosenberg, 2008). The production of secondary metabolites acts as an obstacle to predation, competition (Hay, 1996a; Raveendran and Mol, 2009; Sammarco et al., 1985) and have displayed diverse bioactivities, promising for drug development (e.g., cytotoxicity, antibiotic and anticancer activity) (Dobretsov et al., 2015; Rocha et al., 2011).

1.2 Ecological and socio-economic importance of coral reefs

Coral reefs represent an epicenter of global marine biodiversity and are ranked among the most productive and complex ecosystems (Hoegh-Guldberg, 1999). They act as home and nursery grounds for many marine species, protect coastlines, and provide food sources and income to people living along coastlines (Burke et al., 2011; Osinga et al., 2011; Van Zanten et al., 2014). It is estimated that about 10% of the world's population depend on coral reefs, directly or indirectly, either as a source of food, natural products or as a tourism attraction (Osinga et al., 2011).

Despite their ecological and economic importance, ecological role and ecosystem services, approximately 75% of coral reefs worldwide are currently threatened by a combination of stressors that act locally or globally (Burke et al., 2011; Dudgeon et al., 2010). Threats like climate change (Burke et al., 2011; Roberts et al., 2002), pollution (Ross and Hallock, 2014), the use of destructive fishing practices (e.g. dynamite or cyanide) (Burke et al., 2011; Calado, 2006; Hodgson, 1999; McClanahan et al., 1996; Olivotto et al., 2003), the intensive recreational tourism (Lamb et al., 2014), collection of corals for the extraction of natural products (Faulkner, 2001; Leal et al., 2012; Rocha et al., 2011; Thakur et al., 2005) or for the marine aquarium trade (Tlusty, 2002; Wabnitz et al., 2003).

1.3 Economic importance of corals

1.3.1 Bioprospection of marine natural products

Corals alongside other marine invertebrates have long been arousing interest as source of prototype molecules with cosmetic and pharmaceutical value (Blunt et al., 2005; Grosso et al., 2014; Haefner, 2003; Leal et al., 2012; Rocha et al., 2011; Sipkema et al., 2005; Vignesh et al., 2011). About 40 years ago the first marine compounds were described in published reports, since then the discovery of bioactive compounds has progressed at a booming rate (Mendola, 2003). By 2011 around 3000 new natural products had been extracted exclusively from corals (Rocha et al., 2011). Nowadays, the screen for new natural products is still largely dependent on the harvest of wild specimens. This is a major constraint for the development of new marine drugs, due to two main bottlenecks, sustainability and replicability (Montaser and Luesch, 2012). Sustainability since most marine natural compounds are mainly secondary metabolites, as such, their yields are very low on a wet-weight basis, requiring impracticable amounts of harvested biomass material to produce sufficient quantities of a given compound (Mendola, 2003). Replicability is hindered in wild harvesting by the environmental variability and community level changes to the chemical ecology of the target organisms, meaning that the chemical composition of individuals of the same species may vary spatial and temporally and therefore may not assure the supply of the target metabolite (Hay, 1996b).

1.3.2 Corals in the marine ornamental trade

Corals exhibit many shapes and colors making them desirable to display in captive reef systems. Their popularity promotes an ever growing demand for reef specimens, including both hard and soft corals, the majority of which are collected, sometimes unrelenting, form the wild. (Ellis, 1999; Green and Shirley, 1999).

It is estimated that nearly 80% of all traded corals along with other reef organisms, collected from the ocean to the ornamental trade die, directly or indirectly, during capture, shipment, handling due to destructive fishing practices, poor handling and diseases (Rhyne et al., 2014; Wabnitz et al., 2003; Wood, 2001). The ornamental trade brought a

new and much needed source of income to coastal communities of the Indo-Pacific and improved access to coral reef organisms for the purpose of research and education (Tlusty, 2002; Tlusty et al., 2013) but, it also increased the use of destructive fishing practices, impacts on rare population and endemic species and the introduction of invasive species in foreign habitats (Calado, 2006; Gertzen et al., 2008; Olivotto et al., 2003; Tlusty, 2002).

1.3.3 Coral reefs restoration

In the face of severe degradation of reefs, active coral transplantation measures have become more regularly employed and recognized as a key management tool for rehabilitation of coral reefs, a practice meant to replace dead coral colonies and accelerate the reef natural recovery (Ammar, 2009; Chavanich et al., 2015; Epstein et al., 2003, 2001). This practice involves the transplantation of whole coral colonies, coral fragments or seeding by planula larvae (Okamoto et al., 2012; Rinkevich, 2005; Yap, 2004). Various methodologies have been suggested, including the construction of *in situ* or *ex situ* nurseries to grow large numbers of coral fragments. Nursery-grown fragments are then transplanted to degrade reef sites (Epstein et al., 2001; Okamoto et al., 2012; Rinkevich, 2005; Shafir et al., 2006).

1.4 State of the art of coral aquaculture

The extraction of bio-active compounds with bio-medical applications (Carlson, 1999; Leal et al., 2013; Rocha et al., 2011), the ornamental aquarium trade (Olivotto et al., 2011; Tlusty, 2002; Wabnitz et al., 2003), and the coral reef restoration (Bongiorni et al., 2011; Epstein et al., 2001; Shafir et al., 2006; Soong and Chen, 2003; Yap and Molina, 2003; Yap, 2000) have been increasing the demand for these organisms, leading to their massive harvesting (Castanaro and Lasker, 2003). Researchers, collectors and hobbyists began a worldwide effort in order to address the mitigation of the negative impacts caused by the harvest of these organisms from the wild (Castanaro and Lasker, 2003; Wood, 2001) and therefore establish a sustainable approach to supply the coral demand (Wood, 2001). They began to focus on optimizing culture techniques to

maximize coral survival and growth, and reduce the associated production costs, contributing to the economic feasibility of coral aquaculture and offset wild specimens collection (Carlson, 1999; Ellis and Ellis, 2002; Ellis and Sharron, 1997; Fox et al., 2005; Leal et al., 2013; Mendola, 2003; Parks et al., 2003; Pomeroy et al., 2006; Rocha, 2013; Sella and Benayahu, 2010; Soong and Chen, 2003).

Coral aquaculture can be performed *in situ* or *ex situ.* Despite the low associated expenses, *in situ* aquaculture can make corals vulnerable to pathogens, predators and competitors (Rinkevich, 2005; Rocha et al., 2013b). On the other hand, *ex situ* production involves higher start-up and production costs, but allows for a better production control of the biotic and abiotic parameters affecting coral growth (Borneman and Loivrie, 2001; Forsman et al., 2006; Yap and Molina, 2003). Coral biomass can be continuously produced using homogenous environmental conditions, which is especially important for the purpose of extraction of natural products (Leal et al., 2013). As coral aquaculture in closed artificial seawater systems is being more commonly implemented, the process is becoming increasingly more simple and cost effective (Borneman and Loivrie, 2001; Osinga et al., 2011).

Coral aquaculture is a practice centered on the ability of corals to reproduce asexually by fragmentation (Borneman and Loivrie, 2001; Delbeek, 2001; Ellis and Ellis, 2002; Ellis, 1999; Highsmith, 1982; Sella and Benayahu, 2010). The asexual propagation of hard and soft corals through fragmentation dates back to the 1960s (Delbeek, 2001), since then this simple and inexpensive method has been commonly used by researchers and aquarium hobbyists, for the mass production of corals, where it is possible to fragment a mother colony into several clones (Forsman et al., 2006; Rocha et al., 2013c; Sella and Benayahu, 2010). Wild-collected or captive-grown colonies are used for the production of fragments. The latter are usually attached or glued by various processes until natural attachment is achieved (Delbeek, 2001). Fragmentation enables the biomass of a genotype to increase beyond the mechanical limit of an individual colony (Hughes *et al*, 1992), and helps maintain high growth rates (Hughes and Jackson, 1985), while displaying high survival rates of the frags and reduced impact on donor colonies (e.g. Fox *et al*, 2005), presenting therefore as a crucial step in most of coral production systems. Fragmentation can also produce large sample sizes with minimal genetic variation, which can be an advantage for some experimental designs (Shafir et al., 2003).

For the success of zooxanthellate coral aquaculture it is important to consider multiple and interacting factors, such as light, water flow, genotype, temperature,

inorganic nutrients, fixation substrate or the fragmentation protocol (Ferse, 2010; Osinga et al., 2011; Rocha et al., 2013a,b,c ; Sella and Benayahu, 2010). Therefore, multifactorial culture experiments are desired, not only to maximize the productivity, but also to further unfold the interactions between potentially influential factors.

Light, undoubtedly, plays a vital role in the growth of zooxanthellate corals. The efficiency of the photoautotrophic processes in these symbionts is largely affected by the light intensity and spectral quality (Al-Horani et al., 2003; Khalesi et al., 2009; Osinga et al., 2008; Rocha et al., 2013a,c ; Wijgerde et al., 2014, 2012) and, can affect their contribution to coral growth, metabolism (Apprill et al., 2007; Fitt and Cook, 2001; Marubini et al., 2001; Reynaud et al., 2004; Schlacher et al., 2007), physiology and survival (Venn et al., 2008). Light-related growth limitations are commonly caused by low internal pH due to low photosynthesis (Schneider and Erez, 2006), insufficient production (Titlyanov et al., 2001) or translocation of photosynthates (Marubini and Davies, 1996). However, corals exposed to high irradiance levels can also experience photoinhibition, which also translates in retarded growth (Iglesias-Prieto et al., 1992).

Corals also require a solid and stable substrate for attachment and, safe and reliable "gluing" materials. In culture production, raising the corals off the bottom of the tank with small substrate blocks can reduce smothering. Several substrate types have been used as anchor material, including: plastic florist vials, scleractinian coral skeleton (aragonite), epoxy putties, ceramic tiles, fabricated blocks with sand, shell or aragonite, as well as fabricated plastic stands (Boch and Morse, 2012; Borneman and Loivrie, 2001; Ellis and Ellis, 2002; Ellis and Sharron, 1997; Forsman et al., 2006; Sella and Benayahu, 2010; Tortolero-Langarica et al., 2014; Yap, 2004). As fixation methods, farmers and hobbyists commonly use rubber bands for its cost-effectiveness (Rocha et al., 2013a), cyanoacrylate glues for the immediate and strong bond they form (Bongiorni et al., 2011; Ellis and Sharron, 1997) and, more recently, n-butyl-2-cianoacrylate, which is used in several medical procedures due to its lower toxicity (Kumar and Priyayadav, 2010).

After fragmentation, coral fragments are more prone to necrosis and bacterial infection, which can result in death. The way in which a coral is cutted can have a significant effect on the survival of the fragment (Ellis and Sharron, 1997).

1.5 *Sarcophyton sp.*

The species of the genus Sarcophyton (Cnidaria: Anthozoa: Octocorallia: Alcyonacea) are ecologically important members of shallow reef communities in the Indo-West Pacific and Red Sea region, often found in high energy areas such as surge zones and tide pools, but also occurring in deeper water. (Ellis, 1999; McFadden et al., 2006a). The incomplete knowledge regarding *Sarcophyton sp.* taxonomy, caused by the existence of few diagnostic morphological features and by the historical lack of taxonomic work done on this genus, hinders their precise identification (McFadden et al., 2006a). *Sarcophyton sp.* has long been known to be a problematic taxon, as recognized by Verseveldt (1982): ‘‘Many investigators already pointed out the great variability found in this species, especially with respect to shape and size of the colony, and shape and dimensions of the coenenchymal sclerites in the stalk”. It is however a remarkably resilient genus, characterized by a distinctive bulky stalk, a mushroom shaped top called a capitulum, and by their sclerites shape, which are found in the interior coenenchymal tissue of the colony (Aratake et al., 2012). The hardiness displayed by *Sarcophyton sp.* enhances their potential as farmed coral, because they are able to survive handling stress during fragmentation and shipping.

This genera is known to have many secondary metabolites, like sarcophytoxide (allelopatic), which are toxic and used by corals in competition for space, or as a strategy to inhibit growth and survival of their neighbors (Elahwany et al., 2013; Fleury et al., 2004; Lages et al., 2006; Maida et al., 1995; Sammarco and Coll, 1992). They have been highly surveyed for their natural products, especially cembrane diterpenes and sesquiterpenes (antitumor activities) and are among the most popular and valued organisms in the marine aquarium trade and have (Badria et al., 1998; McFadden et al., 2006a; Rocha et al., 2011; Rocha, 2013). For these reasons, *Sarcophyton* species make good candidates for commercial production.

1.6 Objectives

In this project proposed to optimize the *ex situ* coral production with emphasis on the factors affecting the success of the fragmentation process in *Sarcophyton sp.*. With that mindset several consecutive culture experiments were developed, some

multifactorial, to evaluate the effect of these factors and, the possible interactions between them. We studied three different light spectra (white, blue and red), two PAR (Photosynthetically Active Radiation) intensities (120 and 60 μmol quanta m^{-2} s^{-1}), two different substrates (plastic stands and calcium carbonate disks), three fixation techniques (rubber band, ethylcyanoacrylate (Superglue), N-butyl-2-cyanoacrylate (Histoacryl)), two fragment positions (cut and intact side facing down) and, the interactions between them. Survival, fixation ratio, fixation force and photosynthetic efficiency were evaluated at the end of each experiment.

2. Material and Methods

The species employed in this study will be termed *Sarcophyton sp.*, since recent studies have revealed a complex and incomplete taxonomy in this genus (Aratake et al., 2012).

2.1 Coral Husbandry

A total of eighteen colonies of the soft coral *Sarcophyton sp.* (Figure 1), collected in Sumbawa, Indonesia, approximately between 5 and 15 m depth, were purchased from a marine aquarium wholesaler and transported to the laboratory facilities.

The colonies were stocked in our laboratory culture system for a period of 14 days, with a photoperiod of 12L: 12D, to acclimatize to the experimental conditions (light regime, water parameters and circulation) and to detect the eventual presence of diseases or parasitical infections.

Following the 14 days of acclimatization to water parameter and without any evidence of infection or disease, the colonies were prepared for fragmentation.

2.2 Fragmentation

Fragmentation was performed with a sterilized scalpel, latex gloves and a mask, in a board containing water from the experimental system, to discard all the mucus released by fragmentation. The capitulum of each mother colonies was fractionated,

producing fragments of similar size (approximately 1-2 cm^2). After fragmentation, each coral fragment was attached to the respective labeled stand (Figure 2). The remaining colonies were kept in our laboratory system.

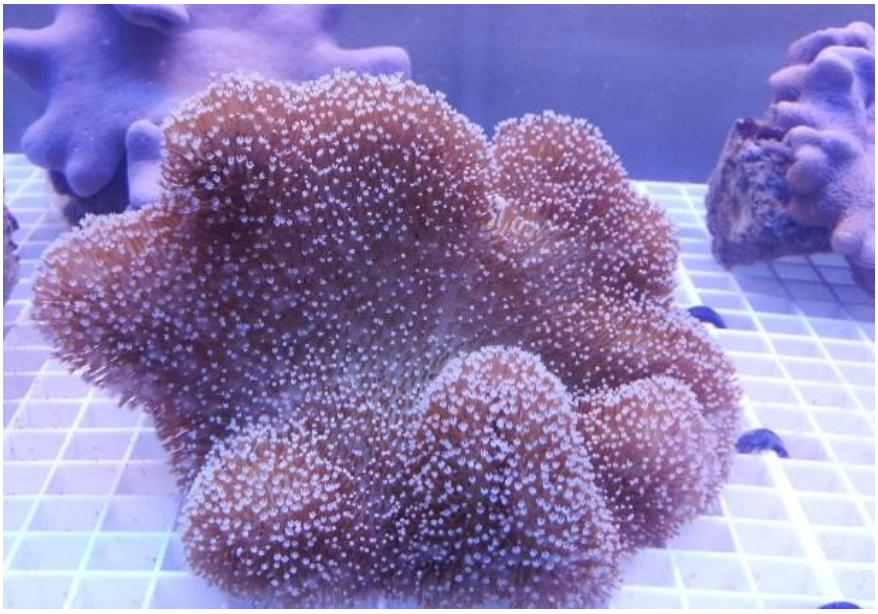

Figure 1: *Sarcophyton sp.* colony during acclimatization to experimental conditions.

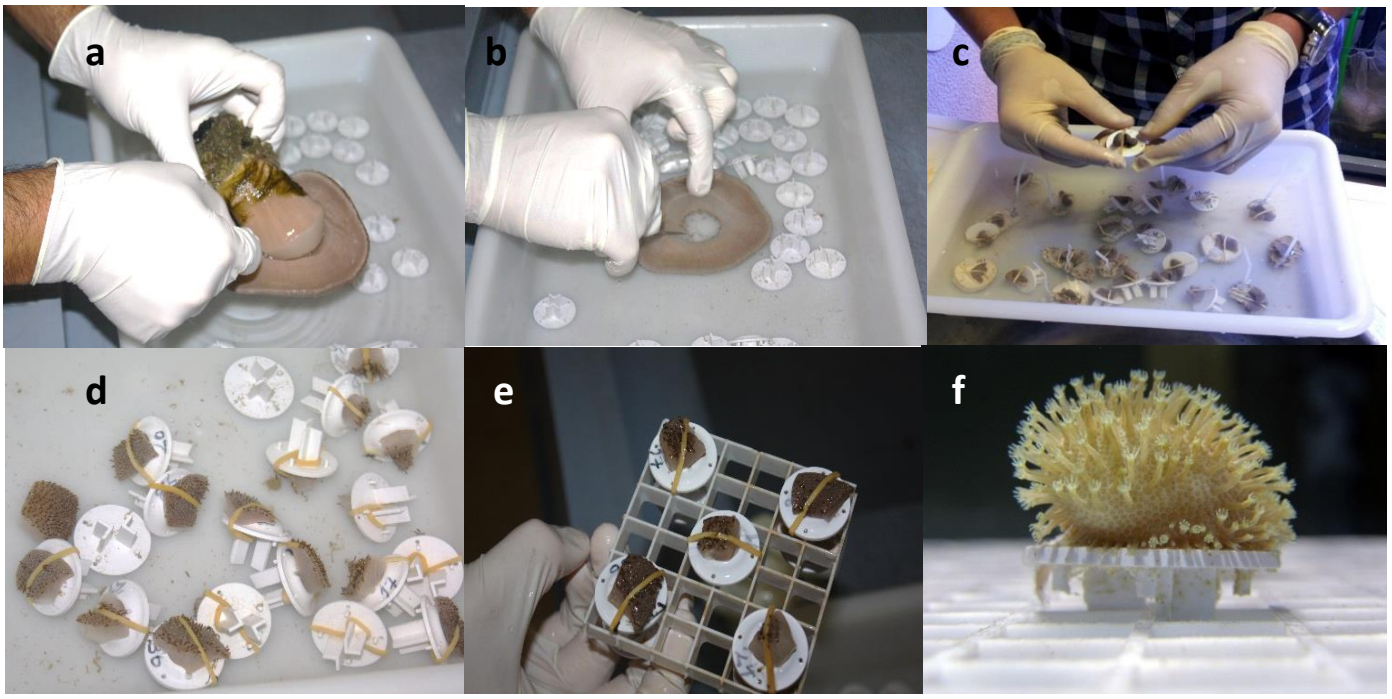

Figure 2: Step-by-step of the fragmentation process. a) Cutting the mother colony's capitulum; b) Fragmentation of the capitulum into several fragments (1-2cm);

c) and d) attachment of the fragments to their respective labeled stand; e) placement of the stands onto the platform, and f) Coral fragment in the culture system after cicatrization.

2.3 Experiment 1 (Light spectra)

The spectral quality of light is assumed to be a parameter of outmost importance in any *ex situ* coral production, nonetheless, few studies have ever addressed this question (Rocha et al., 2013b; Wijgerde et al., 2014, 2012). Therefore in this experiment, the effect of three different light spectra (blue, white and red) on the post-fragmentation success of *Sarcophyton sp.* was studied.

2.3.1 Experimental culture system (1)

The system (Figure 3) was composed by three glass tanks (65L of water volume, 0.50m L x 0.45 W x 0.31m H), both connected to a sump tank (145L of water volume, 1m L x 0.45m W x 0.37m H). Each tank was equipped with a circulation pump (SICCE voyager nano, SICCE, Vicenza, Italy), which provided a water flow of, approximately, 2000 L h^{-1}. The sump was equipped with one protein skimmer (TMC V2Skim 1000, Bristol, UK), a biologic filter (composed of about 10L bio-balls), two submersible heaters (one Eheim Jäger 300W, Deizisau, Germany, and a SERA 200W, Heinsberg Germany), a UV filter (SUNSUN CUV-207 7W, Zhejiang, China) and one recirculation pump (Eheim 1262, Deizisau, Germany) to provide a water flow of, approximately, 1200 L h^{-1} to the coral tanks. The white light tank was illuminated by two T5 fluorescent lamps Arcadia 24 W Marine White (Red Hill, UK), the red light tank by two T5 fluorescent lamps Aqualine 24 W Plant Grow (Aqua Medic, Bissendorf, Germany) and the blue light tank by two T5 fluorescent lamps REEF-SPEC™ 24 W Actinic (Red Sea, France) of 24 W (Figure 4), with a photoperiod of 12L: 12D. The experimental system operated with synthetic saltwater (prepared by mixing Tropic Marin Pro Reef salt – Tropic Marine, Wartenberg, Germany – with water purified by reverse osmosis). Salinity was maintained at 35 through the daily addition of fresh water purified by a reverse osmosis system (Aqua-win RO-6080, Kaohsiung, Thailand).

2.3.2 Experimental design

After the 14 days of acclimatization three mother colonies were fragmented (see 2.2 Fragmentation) to produce 20 fragments each, in a total of 60 fragments. The fragments were illuminated by a PAR intensity of 120 μmol quanta m^{-2} s^{-1} and were secured with rubber bands to labeled plastic stands (TMC Coral Cradle®, Bristol, UK) (Figure 6.b), as described by Rocha (2013). The fragments were distributed among the 3 tanks of the experimental culture system, with each tank having a total of 20 fragments. Each mother colony contributed approximately equally to the number of fragments in each tank. This experiment ran for 60 days. PAR value was measured with a Quantum Flux meter (Apogee MQ-200, Logan, Utah, USA) with a submergible sensor.

2.3.3 Laboratorial analysis

Several parameters were recorded: the survival and fixation ratio (fragments fixed: fragments unfixed), the fixation force (see 2.8.1 Fixation force) at the end of the experiment.

2.3.4 Data analysis

The existence of significant differences among the survival and fixation ratio for *Sarcophyton sp.* fragments cultured under the different light spectra, was tested using a χ^2 test.

The existence of significant differences among the fixation force recorded was tested using a one way ANOVA and a Tukey's HSD post-hoc test. Assumptions of normality and homogeneity of variance were checked prior to the analysis through the Shapiro-Wilks and Levene tests, respectively. Tukey's HSD test was used to determine differences between light treatments. Light spectra was used as the categorical factor to the performed analysis. Statistical analyses were carried out using the Statistica 12.0 software.

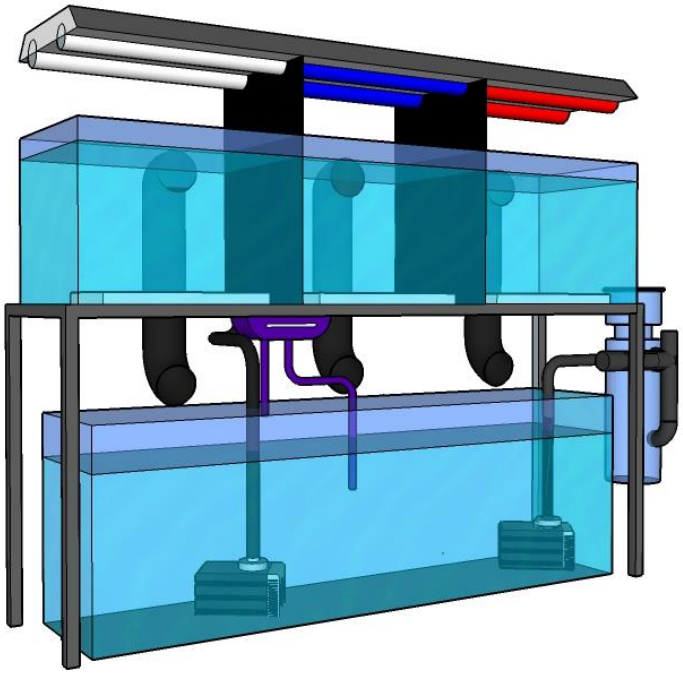

Figure 3: Three-dimensional model of the experimental culture system (1), depicting all the major features.

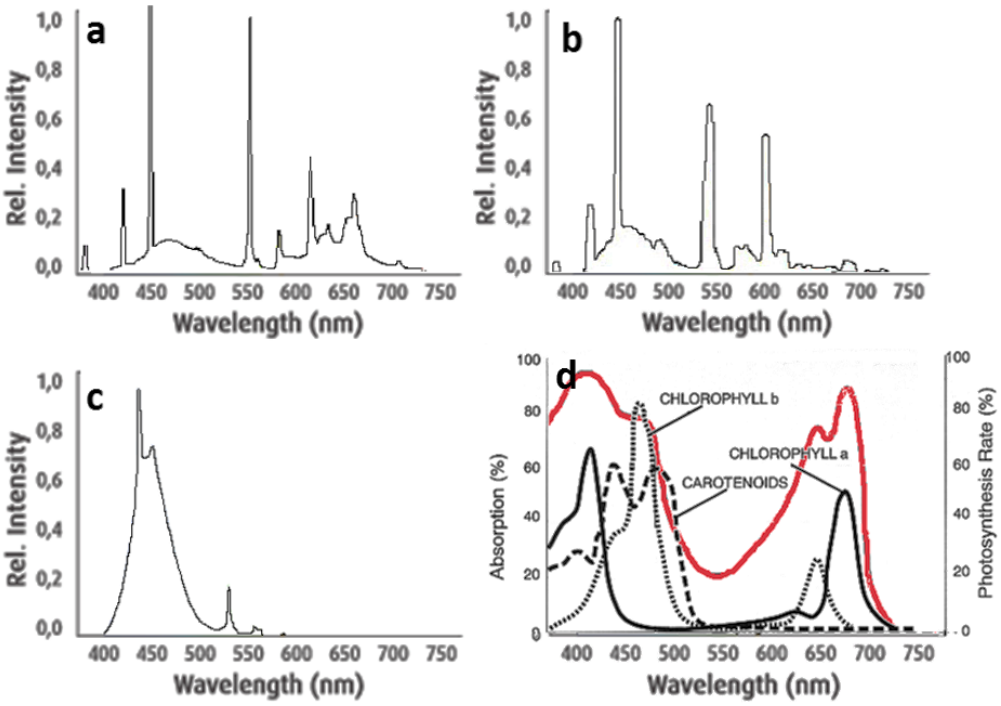

Figure 4: Wavelength emission of: a) Red light spectra: Aqualine Plant Grow (Aqua Medic, Bissendorf, Germany); b) White light spectra: Arcadia Marine White (Red Hill, UK); c) Blue light spectra: REEF-SPEC™ Actinic 22000K (Red Sea, France); and d) Action spectra of photosynthesis (in red), shown together with the estimated absorption spectra of chlorophyll a, chlorophyll b and carotenoids (adapted from Lodish et al., 2000).

2.4 Experiment 2 (Light spectra x PAR intensity)

Light PAR intensity as a vital effect on coral photobiology, physiology and growth, as reviewed by Osinga et al. (2011). Furthermore, it represents one of the major expenses in any *ex situ* coral production and, for that reason, we decided to use two light PAR intensities in our next experiment, a low light PAR of 60 μmol quanta m^{-2} s^{-1} and a high light PAR of 120 μmol quanta m^{-2} s^{-1}, similar to the study of Rocha et al. (2013a). Spectral quality was also included (blue and red spectra). A multifactorial experiment was set to study the effect of PAR intensity, spectra and their combined effect on the post-fragmentation success of *Sarcophyton sp.*.

2.4.1 Experimental culture system (2)

The system (Figure 5) was composed by two glass tanks (218L of water volume, 1.18m L x 0.43m W x 0.43m H), both connected to a sump tank (145L of water volume, 1.18m L x 0.35m W x 0.55m H). Each tank was equipped with a circulation pump (Turbelle nanostream-6025 Tunze, Penzberg, Germany), which provide a water flow of, approximately, 2500 L h^{-1}. The sump was equipped with two protein skimmers (APF-600 Deltec, Delmenhorst, Germany), a biologic filter (composed of about 10 kg of live rock and bio-balls), two chemical filter bag with activated carbon, two submersible heaters (Eheim Jäger 150W, Deizisau, Germany), a UV filter (V^2ecton 400, TMC, Bristol, UK) and two recirculation pumps (Eheim 1260, Deizisau, Germany) to provide a water flow of, approximately, 1500 L h^{-1} to the coral tanks. The upper tank was illuminated by four T5 lamps Aqualine Plant Grow (Aqua Medic, Bissendorf, Germany) of 54 W and, the lower tank by four T5 REEF-SPEC™ Actinic 22000K (Red Sea, France) of 54 W, providing a PAR of 120 μmol quanta m^{-2} s^{-1} at the fragment level, under a photoperiod of 12L:12D. PAR value was measured with a Quantum Flux meter (Apogee MQ-200, Logan, Utah, USA) with a submergible sensor. The experimental system operates with artificial saltwater (Tropic Marine, Wartenberg, Germany)). Salinity was maintained at 35 ‰ through the use of an osmoregulator (Deltec Aquastat 1000, Delmenhorst, Germany) which automatically compensates the evaporated water, with fresh water purified by a reverse osmosis system (Aqua-win RO-6080, Kaohsiung, Thailand).

2.4.2 Experimental design

After the 14 days of acclimatization, six mother colonies were fragmented (see 2.2 Fragmentation), producing 24 fragments each. The fragments were secured with rubber bands to labeled plastic stands (TMC Coral Cradle®, Bristol, UK) (Figure 6 b) and then divided equally by the 4 treatments, for a total of 144 fragments (6 frags x 6 mother colonies x 4 treatments).

Each experimental tank had 72 fragments and, in each tank, half of the 72 fragments (n=36) were placed on a 10 cm high platform (depth at which they received a PAR of 120 μmol quanta m^{-2} s^{-1}), and the other half were placed on the bottom of the tank (depth at which they received a PAR of 60 μmol quanta m−2 s−1). PAR value was measured with a Quantum Flux meter (Apogee MQ-200, Logan, Utah, USA) with a submergible sensor. This stage proceeded for 60 days.

2.4.3 Laboratorial analysis

The survival and fixation ratio and the fixation force (see 2.2 Fixation force) and photosynthetic efficiency (see 2.8.2 Photosynthetic efficiency) were recorded for each fragment at the end of the experiment.

2.4.4 Data Analysis

The existence of significant differences among the survival and fixation ratio for *Sarcophyton sp.* fragments cultured under the different treatments, was tested using a χ^2 test.

The existence of significant differences among the fixation force and maximum photosynthetic efficiency of photosystem II (PS II)(F_v/F_m) recorded was tested using a factorial ANOVA and an Unequal N HSD post-hoc test. Assumptions of normality and homogeneity of variance were checked prior to the analysis through the Shapiro-Wilks and Levene tests, respectively. Unequal N HSD test was used to determine differences between light spectra and PAR intensities. Light spectra and PAR intensity were used as

the categorical factor to the performed analysis. Statistical analyses were carried out using the Statistica 12.0 software.

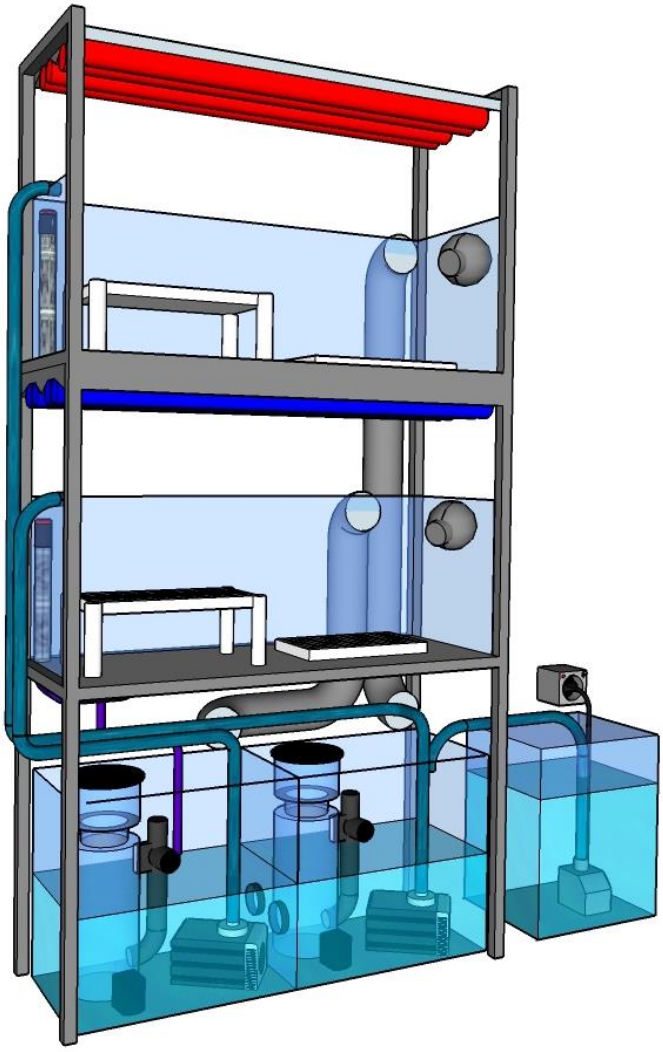

Figure 5: Three-dimensional model of the experimental culture system (2), depicting all the major features.

2.5 Experiment 3 (Light spectra x Substrate)

Having found little research focusing on the response of coral fragments to different substrate types (Ellis and Ellis, 2002; Ferse, 2010) and, none carried out in *ex situ* systems, an experiment was set to address this question. The effect of a natural and rough substrate (calcium carbonate disks made from coral skeleton) versus a more flat and smooth artificial one (plastic stands, TMC Coral Cradle®, Bristol, UK), was tested, as described by Ellis & Ellis (2002) and Rocha et al. (2013a) respectively.

For this experiment the spectra variable was kept, for which previous results were inconclusive and, therefore, stablished a multifactorial experiment to study the effect of substrate, spectra and their combined effect on post-fragmentation success of *Sarcophyton sp.*.

2.5.1 Experimental design

For this part of the experiment the same experimental conditions were used as in Experimental culture system 2, with the same light features and photoperiod.

After the 14 days of acclimatization three mother colonies were fragmented as described before, producing 24 fragments each, which were then divided equally by the 4 treatments (Red light x Plastic stand, Red light calcium carbonate stand, Blue light x Plastic stand, Blue light calcium carbonate stand), for a total of 72 fragments (6 frags x 3 mother colonies x 4 treatments).

Each experimental tank had 72 fragments placed on a 10 cm high platform (depth at which they received a PAR of 120 μmol quanta m^{-2} s^{-1}) and, half of those 72 fragments (n=36) were attached with a rubber band to labeled calcium carbonate stands (Figure 6 a), and the other half (n=36) fragments were attached to labeled plastic stands (TMC Coral Cradle®, Bristol, UK) (Figure 6b). PAR value was measured with a Quantum Flux meter (Apogee MQ-200, Logan, Utah, USA) with a submergible sensor. This stage proceeded for 60 days.

2.5.2 Laboratorial Analysis

The survival and fixation ratio were recorded and, the fixation force (see 2.8.1 Fixation force) and maximum photosynthetic efficiency of photosystem II (see 2.8.2 Photosynthetic efficiency) of the fragments were analyzed at the end of the experiment.

2.5.3 Data Analysis

The existence of significant differences among the survival and fixation ratio for *Sarcophyton sp.* fragments cultured under the different treatments, was tested using a χ^2 test.

The existence of significant differences among the fixation force and maximum photosynthetic efficiency of photosystem II (F_v/F_m) recorded was tested using a factorial ANOVA and an Unequal N HSD post-hoc test. Assumptions of normality and homogeneity of variance were checked prior to the analysis through the Shapiro-Wilks and Levene tests, respectively. Unequal N HSD test was used to determine differences between light spectra and substrate. Light spectra and substrate were used as the categorical factor to the performed analysis. Statistical analyses were carried out using the Statistica 12.0 software.

2.6 Experiment 4 (Fixation process x Light spectra)

Several fixation processes have been employed in coral production studies, as reviewed by Ellis (1999). These methods however, have not been considered as to have an influence on post-fragmentation success. This experiment aimed to study the effect of three different fixation processes (ethylcyanoacrylate (Superglue), Rubber band, N-butyl-2-cyanoacrylate (Histoacryl)) and two light spectra (red and blue), and the possible interaction between them on the post-fragmentation success.

2.6.1 Experimental design

For this part of the experiment the experimental system applied was the same as for the previous experiment (Experimental culture system 2 (Figure 5)), with the same light features and photoperiod.

After the 14 days of acclimatization four mother colonies were fragmented (see 2.2 Fragmentation technical description), producing 12 fragments each, which were then divided equally by the 6 treatments, for a total of 72 fragments (3 frags x 4 mother colonies x 6 treatments). In each tank, we had 12 fragments fixed with rubber bands, 12

fixed with ethylcyanoacrylate (Superglue (Henkel Ibérica, Bobadela, Portugal)) and 12 fixed with n-butyl cyanoacrylate glue (B. Braun Histoacryl, Rio de Janeiro, Brasil). All the fragments were secured to plastic stands (TMC Coral Cradle®, Bristol, UK) (Figure 6.b) and placed on 10 cm high platform (depth at which they received a PAR of 120 μmol quanta m^{-2} s^{-1}). This experiment lasted for 60 days.

2.6.2 Laboratorial analysis

The survival and fixation ratio were recorded and, the fixation force (see 2.8.1 Fixation force) and photosynthetic efficiency (see 2.8.2 Photosynthetic efficiency) of the fragments at the end of the experiment was analyzed.

2.6.3 Data Analysis

The existence of significant differences among the survival and fixation ratio for *Sarcophyton sp.* fragments cultured under the different treatments, was tested using a χ^2 test.

The existence of significant differences among the fixation force and photosynthetic efficiency (F_v/F_m) recorded was tested using a factorial ANOVA and a Tukey's HSD post-hoc test. Assumptions of normality and homogeneity of variance were checked prior to the analysis through the Shapiro-Wilks and Levene tests, respectively. Tukeys's HSD test was used to determine differences between light spectra and fixation process. Light spectra and fixation process were used as the categorical factor to the performed analysis. Statistical analyses were carried out using the Statistica 12.0 software.

2.7 Experiment 5 (Fragment position)

With no studies found addressing the influence of fragment position (cut side or intact side facing down) a final experiment was performed to address this question.

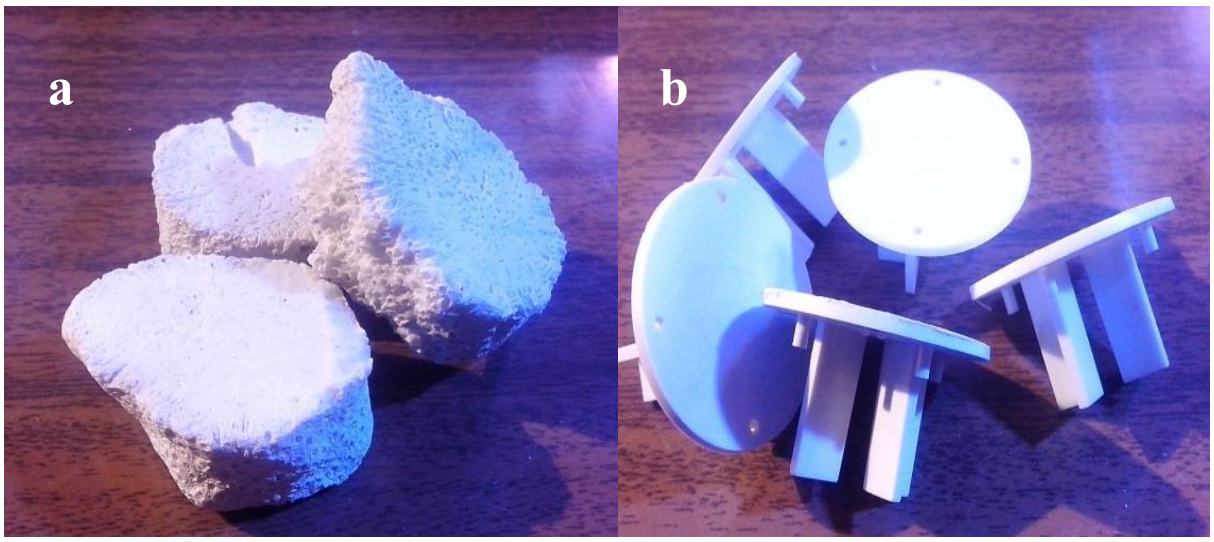

Figure 6: Types of substrates for attachment of the coral fragments used in the experiment. a) Aragonite (calcium carbonate) stands, made from scleractinian coral skeleton; b) plastic stands (TMC Coral Cradle®, Bristol, UK).

2.7.1 Experimental design

For this part of the experiment the experimental system applied was the same as for the previous experiment (Experimental culture system 2 (Figure 5)), with the same light features and photoperiod.

Using the last 2 colonies 40 fragments were produced, which were then fixed with N-butyl-2-cyanoacrylate (Histoacryl) to the plastic stands (TMC Coral Cradle®, Bristol, UK)(Figure 6.b) and placed in the experimental tank, on 10 cm high platform (depth at which they received a PAR of 120 μmol quanta m^{-2} s^{-1}). Half of the fragments (n=20) were fixed by their intact side and half by their cutted side.

Although the experimental system was the same, it was only used the red spectra tank, because this spectra yielded the best results in previous experiments. The tank was equipped with 4 T5 fluorescent lamps Aqualine T5 Plant Grow (Aqua Medic, Bissendorf, Germany) providing a PAR of 120 μmol quanta m^{-2} s^{-1}. A photoperiod of 12L: 12D was used. This stage lasted for yet another 60 days.

2.7.2 Laboratorial Analysis

The survival and fixation ratio were recorded and, the fixation force (see 2.8.1 Fixation force) and photosynthetic efficiency (see 2.8.2 Photosynthetic efficiency) of the fragments at the end of the experiment was analyzed.

2.7.3 Data Analysis

The existence of significant differences among the survival and fixation ratio for *Sarcophyton sp.* fragments cultured under the different treatments, was tested using a χ^2 test.

The existence of significant differences among the fixation force and photosynthetic efficiency (*Fv/Fm*) recorded was tested using a one way ANOVA and a Tukey's HSD post-hoc test. Assumptions of normality and homogeneity of variance were checked prior to the analysis through the Shapiro-Wilks and Levene tests, respectively. Tukeys's HSD test was used to determine differences between fragment positions. Fragment position used as the categorical factor to the performed analysis. Statistical analyses were carried out using the Statistica 12.0 software.

2.8 Water Parameters

The water parameters measured throughout the experiments on both systems, were as follows: temperature at 25-27°C, pH at 8-8.4, salinity at 35, $NH3 \leq 0.1$mg/L, $NO2 \leq 0.1$ mg/L, $NO3 \leq 10$ mg/L, Ca ≅ 400-420mg/L and KH at 7-10 dKH. Partial water changes of approximately 10% of the total system volume were performed on weekly basis.

2.9 Laboratorial analysis

2.9.1 Fixation force

The fixation force (Newtons) was measured using a designed system (Figure 7) equipped with a digital dynamometer Kern FK10 (resolution of 0.005 N; bottom detection limit of 0.01 N; upper detection limit of 10 N). The system works as a scale: a water dripping system fills the cup on the right arm, which steadily increases the pressure exerted on the fragment, once the fragment is dislodged from its substrate the dynamometer on the left arm records the fixation force.

In order to calculate the value of the fixation force it was necessary to record the attaching area of each fragment, through the use of milimetric paper. After displacement of the fragment was, it was immediately placed over the milimetric paper, and the fixation area was outlined. Based on this boundary it was calculated de fixation area that was used to calculate de fixation force, through the following formula (Santos et al., 2005):

$$\textbf{Fixation Force}\left(\frac{\textbf{N}}{\textbf{cm}^2}\right) = \frac{\textbf{Measured Fixation Force (N)}}{\textbf{Fixation Area (cm}^2\textbf{)}}$$

Figure 7: The system used to measure the fixation force of the fragments.

2.9.2 Photosynthetic efficiency

Through Pulse amplitude modulated (PAM) fluorometry ((Schreiber et al., 1986) the photosynthetic efficiency of photosystem II (PS II) was measured within the endosymbiotic *Symbiodinium* spp., by the end of each experiment. For that a Walz Junior Pam (Heinz Walz, Effeltrich, Germany) (Rocha et al., 2013a) was used. Measuring actinic and saturating light was provided by a blue LED-lamp (peaking at 450 nm, half-bandwidth of 20 nm), that was delivered to the sample by a 1.5 mm-diameter plastic fiberoptics bundle. The fiberoptic was positioned perpendicularly to the surface of the coral fragment, and all measurements were made at a fixed distance of 1 mm. Measurements were carried out at the end of the experience, 2 h after the start of the daylight period, to ensure the full activation of the photosynthetic apparatus.

To determine the maximum quantum yield of PSII, coral fragments were dark-adapted for 15 min, after which one saturation pulse (0.8 s) was applied to determine the minimum fluorescence, F_o, and the maximum fluorescence, F_m. F_o and F_m are then used to determine the maximum quantum yield of PSII (Schreiber et al., 1986), by the formula:

$$\boldsymbol{F_v/F_m = (\frac{F_m - F_0}{F_m})}$$

3. Results

3.1 General observations

During the course of the experiments, mainly 2 and 3, episodic and synchronized releases of mucus by the fragments were observed.

3.2 Experiment 1 (Light spectra)

3.2.1 Survival

Fragment survival did not show significant differences among the different light treatments (χ^2 0.05, d.f.1, P > 0.05) except, between red and white spectra (χ^2 0.05, d.f.1, P = 0.001).

Table 1: Fixation force observed among the different light treatments in the Experiment 1. (0) represents unfixed fragments and (-) survival.

Fragment	White spectra Fixation Force (N/cm^2)	Blue spectra Fixation Force (N/cm^2)	Red spectra Fixation Force (N/cm^2)
1	-	0	0
2	0	0	0
3	0	0	0.297
4	0	0	1.837
5	0	0	0.108
6	0	0	0.276
7	0	0	0.306
8	0	0	0.663
9	0	0.024	0.483
10	0	0.065	0.903
11	0	0.062	0.86
12	0.159	0.049	0.536
13	1.18	0.137	0.314
14	0.254	0.293	1.053
15	2.726	0.877	4.164
16	0.139	2.019	2.296
17	2.5	0.702	1.963
18	2.703	0.034	2.085
19	0.108	0.927	1.385
20	0.035	0.273	2.791

3.2.2 Fixation ratio

Fixation ratio was significantly different among treatments (χ^2 0.05, d.f.2, P = 0.036), being higher in the red spectra than in the blue and in the white spectra, (χ^2 0.05, d.f.1, P = 0.029) and (χ^2 0.05, d.f.1, P = 0.046), respectively. However, no differences were found in fixation force between white and blue treatments (χ^2 0.05, d.f.1, P > 0.05) (Table 1).

3.2.3 Fixation force

Regarding the fixation force, significant differences among the different spectra treatments (one way ANOVA, F (2, 56) = 4.551, p = 0.015) were found. The post hoc analysis revealed that the fixation force was significantly higher in the red spectra than in the blue spectra (p = 0.014), and white spectra was no significantly different between blue and red spectra, (p = 0.109) and (p = 0.706) respectively.

3.3 Experiment 2 (Light spectra x PAR intensity)

3.3.1 Survival

Overall differences between all the treatments related to survival (χ^2 0.05, d.f.3, P = 0.015) were found. Significant differences between the tested spectra (χ^2 0.05, d.f.1, P = 0.015) were found, observing a higher survival under the blue spectra, but no significant differences between PAR intensities (χ^2 0.05, d.f.1, P > 0.05). The multiple χ^2 test comparison revealed that the Red spectra/60 μmol quanta m^{-2} s^{-1} PAR treatment had significantly higher fragments survival than all the other treatments (Figure 8).

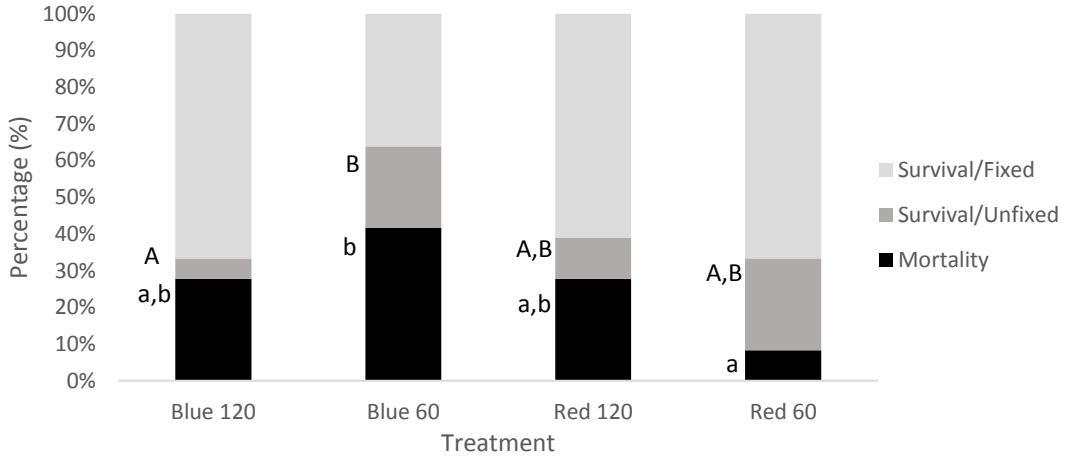

Figure 8: Survival and fixation percentages registered in *Sarcophyton sp.* fragments reared among the different treatments (Experiment 2): Blue 120: Blue Light spectra/ 120 μmol quanta m^{-2} s^{-1}; Blue 60: Blue Light spectra/ 60 μmol quanta m^{-2} s^{-1}; Red 120: Red Light spectra/ 120 μmol quanta m^{-2} s^{-1}; Red 60: Red Light spectra/ 60 μmol quanta m^{-2} s^{-1}. Different letters represent significant differences. Lower case letters refer to mortality and upper case letters to fixation ratio.

3.3.2 Fixation ratio

There were no significant differences between all the treatments related with fixation ratio (χ^2 0.05, d.f.3, P > 0.05) as well as between spectra (χ^2 0.05, d.f.1, P > 0.05). However, in the case of PAR a significantly higher fixation ratio under the PAR of 120 μmol quanta m^{-2} s^{-1} (χ^2 0.05, d.f.1, P = 0.012) was found. The multiple χ^2 test comparison revealed only differences between PAR of 120 and PAR of 60 μmol quanta m^{-2} s^{-1}, under blue light spectra (Figure 8).

3.3.3 Fixation force

In relation to the fragments fixation force a significantly influence of PAR intensity (Figure 9), with the factorial ANOVA test (factorial ANOVA, F (1, 102) = 9.226, p = 0.003) was detected. Posterior Unequal N HSD post hoc comparison showed no significant differences between treatments.

The one way ANOVA performed to analyze the differences between PAR 60 and PAR 120 under red light spectra, and PAR 60 and PAR 120 under blue light spectra showed that PAR treatment produced significant differences under both blue and red spectra, (F (1. 45) = 4.360, p = 0.042) and (F (1. 57) = 5.188, p = 0.027), respectively. However Unequal N HSD post hoc comparison only revealed significant difference under red light spectra (p = 0.035).

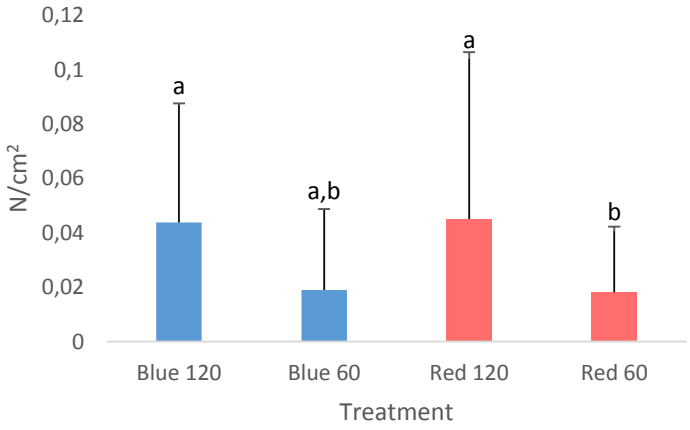

Figure 9: Mean fixation force (± standard deviation) measured in *Sarcophyton sp.* fragments reared in the different treatments: Blue 120: Blue Light spectra/ 120 µmol quanta m^{-2} s^{-1} (n=26); Blue 60: Blue Light spectra/ 60 µmol quanta m^{-2} s^{-1}(n=22); Red 120: Red Light spectra/ 120 µmol quanta m^{-2} s^{-1}(n=26); Red 60: Red Light spectra/ 60 µmol quanta m^{-2} s^{-1}(n=33). Different letters represent significant differences. Lower case letters refer to mortality and upper case letters to fixation ratio.

3.3.4 Photosynthetic efficiency (F_v/F_m)

Factorial ANOVA showed that, overall, photosynthetic efficiency had no significant differences among the different treatments, neither between treatments with the Unequal N HSD post hoc comparison. Mean F_v/F_m for the different treatments are shown in Figure 10.

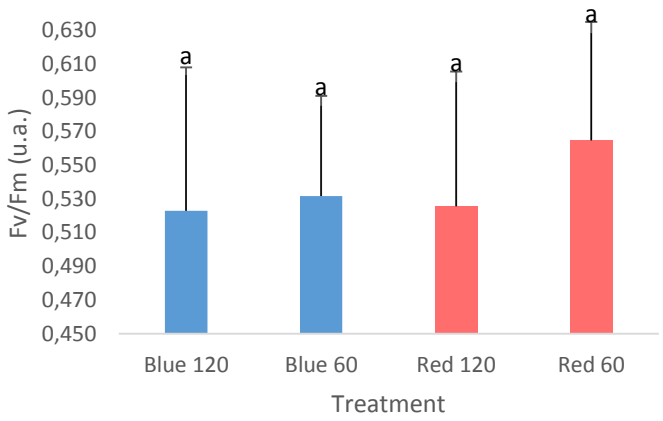

Figure 10: Mean photosynthetic efficiency (± standard deviation) measured in *Sarcophyton sp.* fragments reared in the different treatments: Blue 120: Blue Light spectra/ 120 µmol quanta m^{-2} s^{-1} (n=26); Blue 60: Blue Light spectra/ 60 µmol quanta m^{-2} s^{-1} (n=22); Red 120: Red Light spectra/ 120 µmol quanta m^{-2} s^{-1}(n=26); Red 60: Red Light spectra/ 60 µmol quanta m^{-2} s^{-1}(n=33). Different letters represent significant differences.

3.4 Experiment 3 (Light spectra x Substrate)

3.4.1 Survival

No overall differences were found for survival among the different treatments (χ^2 0.05, d.f.3, P > 0.05), nor between spectra (χ^2 0.05, d.f.1, P > 0.05) or substrate (χ^2 0.05, d.f.1, P > 0.05). Multiple comparisons between all the treatments did not retrieve any significant differences as well (Figure 11)

3.4.2 Fixation ratio

No overall differences were found for survival among the different treatments (χ^2 0.05, d.f.3, P > 0.05), nor between spectra (χ^2 0.05, d.f.1, P > 0.05) or substrate (χ^2 0.05, d.f.1, P > 0.05). Multiple comparisons between all the treatments did not retrieve any significant differences as well.

3.4.3 Fixation force

Overall no significant differences were found among treatments using a factorial ANOVA, neither between treatments with the Unequal N HSD post hoc comparison (Figure 12)

3.4.4 Photosynthetic efficiency (F_v/F_m)

Factorial ANOVA showed that, overall, photosynthetic efficiency had no significant differences among the different treatments, neither between treatments with the Unequal N HSD post hoc comparison. Mean F_v/F_m for the different treatments are shown in Figure 13.

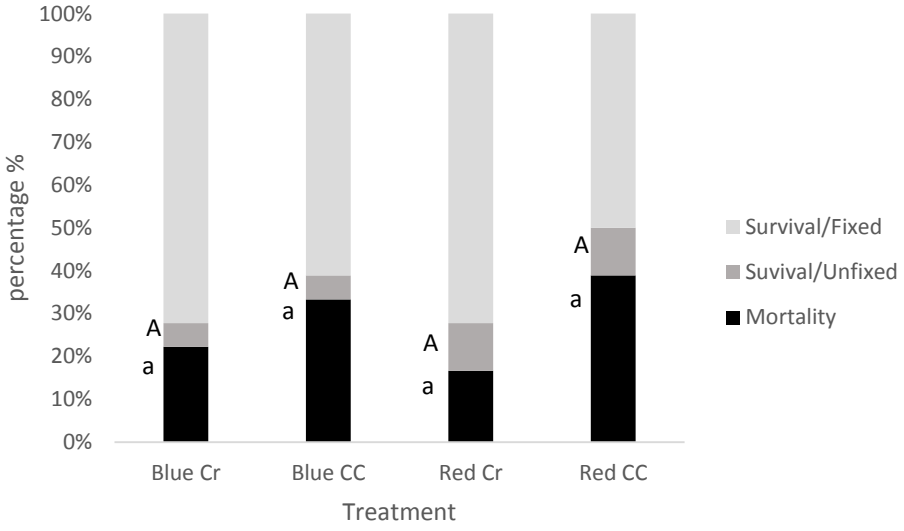

Figure 11: Survival and fixation percentages registered in *Sarcophyton sp.* fragments reared among the different treatments (Experiment 3): Blue Cr: Blue Light spectra/ Coral Cradle; Blue CC: Blue Light spectra/ Calcium carbonate disks; Red Cr: Red Light spectra/ Coral Cradle; Red CC: Red Light spectra/ Calcium carbonate disks. Different letters represent significant differences. Lower case letters refer to mortality and upper case letters to fixation ratio.

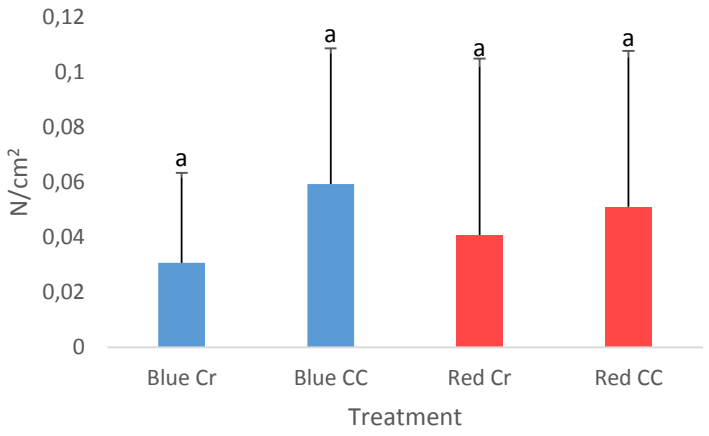

Figure 12: Mean fixation force (± standard deviation) measured in the *Sarcophyton sp.* fragments reared in the different treatments: Blue Cr: Blue Light spectra/ Coral Cradle (n=14); Blue CC: Blue Light spectra/ Calcium carbonate disks (n=12); Red Cr: Red Light spectra/ Coral Cradle (n=15); Red CC: Red Light spectra/ Calcium carbonate disks (n=11). Different letters represent significant differences.

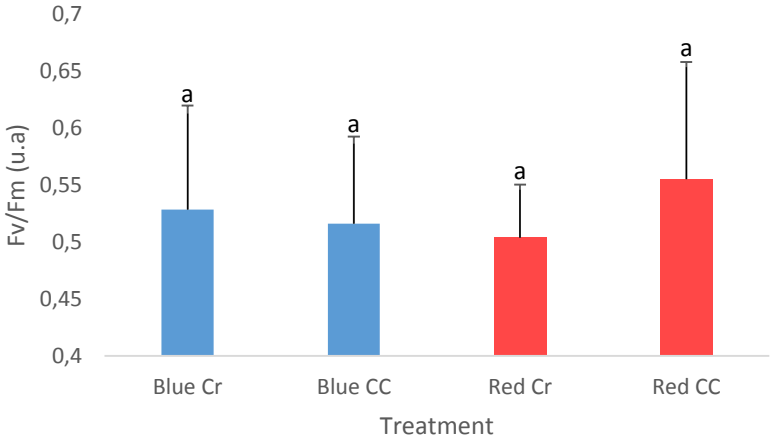

Figure 13: Mean photosynthetic efficiency (± standard deviation) measured in *Sarcophyton sp.* fragments reared in the different treatments: Blue Cr: Blue Light spectra/ Coral Cradle; Blue CC: Blue Light spectra/ Calcium carbonate disk; Red Cr: Red Light spectra/ Coral Cradle; Red CC: Red Light spectra/ Calcium carbonate disk. Different letters represent significant differences.

3.5 Experiment 4 (Light spectra x Fixation process)

3.5.1 Survival

No significant differences were found in fragment survival in any of the experimental treatments.

3.5.2 Fixation ratio

Overall, there are significant differences among treatments (χ^2 0.05, d.f.5, P = 0.003). Differences between spectra (χ^2 0.05, d.f.1, P = 0.001) were found, but not between fixation process (χ^2 0.05, d.f.1, P > 0.05). The multiple χ^2 comparison between all the treatments revealed significant differences between Blue light/ethylcyanoacrylate (Superglue) and all of the treatments under the red light spectra (Figure 14).

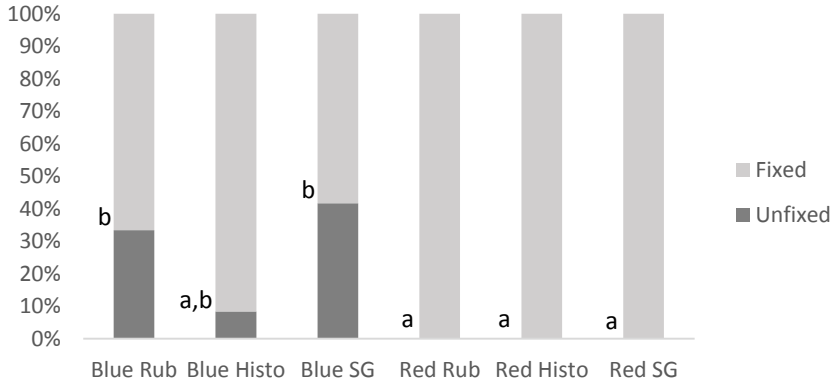

Figure 14: Fixation percentages registered in the *Sarcophyton sp.* fragments reared among the different treatments (Experiment 2): Blue Rub: Blue Light spectra/ Rubber band; Blue Histo: Blue Light spectra/ N-butyl-2-cyanoacrylate (Histoacryl); Blue SG: Blue light spectra/Ethylcyanoacrylate (Superglue); Red Rub: Red Light spectra/ Rubber band; Red Histo: Red Light spectra/ N-butyl-2-cyanoacrylate (Histoacryl); Red SG: Red light spectra/Ethylcyanoacrylate (Superglue). Different letters represent significant differences.

3.5.3 Fixation force

Through a factorial analysis test significant influence of spectra (factorial ANOVA, F (1, 66) = 51.604, p = 0) and fixation process (factorial ANOVA, F (1, 66) = 7.390, p = 0.001) was found. Tukey's post hoc comparisons between treatments revealed that the Red light/N-butyl-2-cyanoacrylate (Histoacryl) and the Red light/Ethylcyanoacrylate (Superglue) were the treatments yielding best results in terms of fixation force.

One way ANOVA's comparisons between treatments revealed significant differences between spectra under all the three fixation processes used, N-butyl-2-cyanoacrylate (Histoacryl) (one way ANOVA, F (1, 22) = 16.598, p = 0.001), Ethylcyanoacrylate (Superglue) (F (1. 22) = 23.253, p = 0) and Rubber band (F (1. 22) = 16.838, p = 0). It also revealed significant differences between N-butyl-2-cyanoacrylate (Histoacryl) and Rubbers bands (F (1. 22) = 11.260, p = 0.03) and, Ethylcyanoacrylate (Superglue) and Rubbers bands, F (1. 22) = 9.072, p = 0.006) (Figure 15).

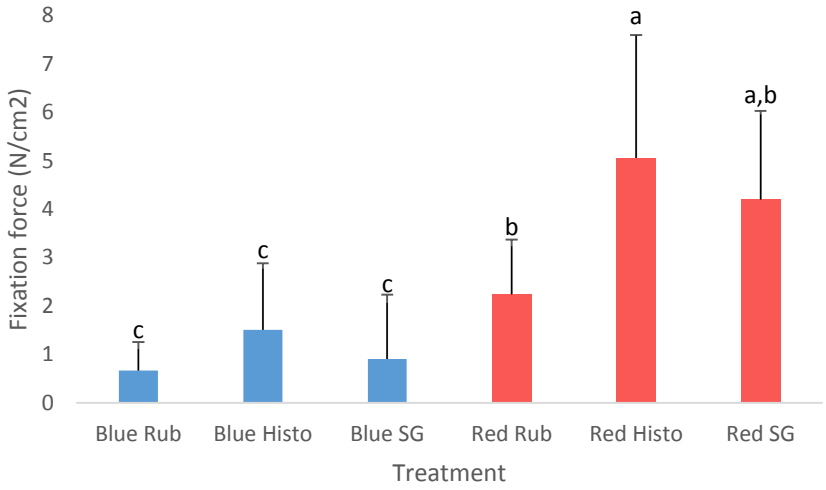

Figure 15: Mean fixation force (± standard deviation) measured in *Sarcophyton sp.* fragments reared in the different treatments: Blue Rub: Blue Light spectra/ Rubber band (n=12); Blue Histo: Blue Light spectra/ N-butyl-2-cyanoacrylate (Histoacryl) (n=12); Blue SG: Blue light/Ethylcyanoacrylate (Superglue) (n=12); Red Rub: Red Light spectra/ Rubber band (n=12); Red Histo: Red Light spectra/ N-butyl-2-cyanoacrylate

(Histoacryl) (n=12); Red SG: Red light/Ethylcyanoacrylate (Superglue) (n=12). Different letters represent significant differences.

3.5.4 Photosynthetic efficiency

Through a factorial ANOVA test a significant influence of spectra on photosynthetic efficiency (factorial ANOVA, $F(1,66) = 7.517$, $p = 0.008$) was discover. Post hoc Tukey's test revealed a significant difference between Blue light/N-butyl-2-cyanoacrylate (Histoacryl) treatment and Red light/Ethylcyanoacrylate (Superglue) ($p = 0.018$).

One way ANOVA's comparisons between treatments only revealed significant differences between spectra under N-butyl-2-cyanoacrylate (Histoacryl) fixation process ($F(1.22) = 4.169$, $p = 0.043$) (Figure 16).

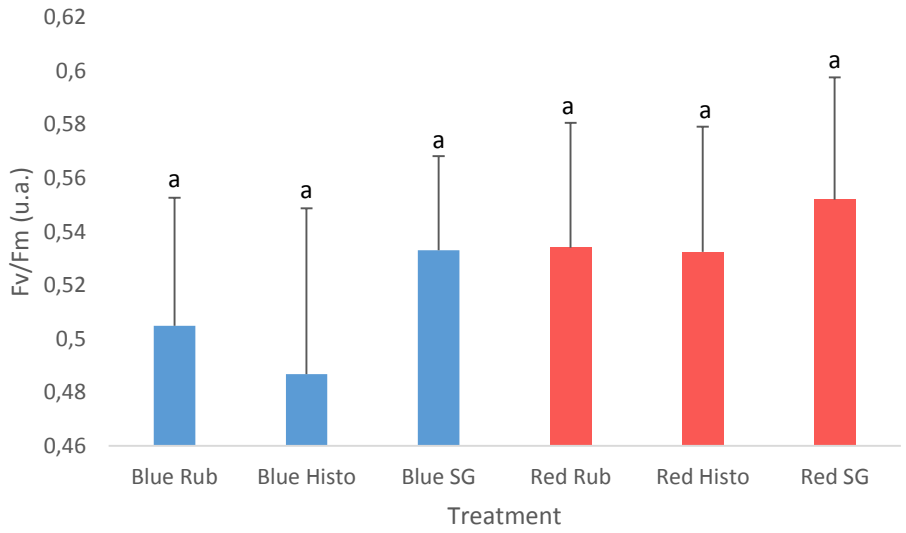

Figure 16: Mean photosynthetic efficiency (± standard deviation) measured in *Sarcophyton sp.* fragments reared in the different treatments: Blue Rub: Blue Light spectra/ Rubber band; Blue Histo: Blue Light spectra/ N-butyl-2-cyanoacrylate (Histoacryl); Blue SG: Blue light/Ethylcyanoacrylate (Superglue); Red Rub: Red Light spectra/ Rubber band; Red Histo: Red Light spectra/ N-butyl-2-cyanoacrylate (Histoacryl); Red SG: Red light/Ethylcyanoacrylate (Superglue). Different letters represent significant differences.

3.6 Experiment 5 (Fragment position)

In this final experiment, no survival was observed and all the fragments (n=20) were fixed by the end of the experiment. No significant differences were found between fixation force and photosynthetic efficiency of fragment position treatments, ($F(1.\ 18) = 0.110$, $p = 0.744$) and ($F(1.18) = 0.001$, $p = 0.983$).

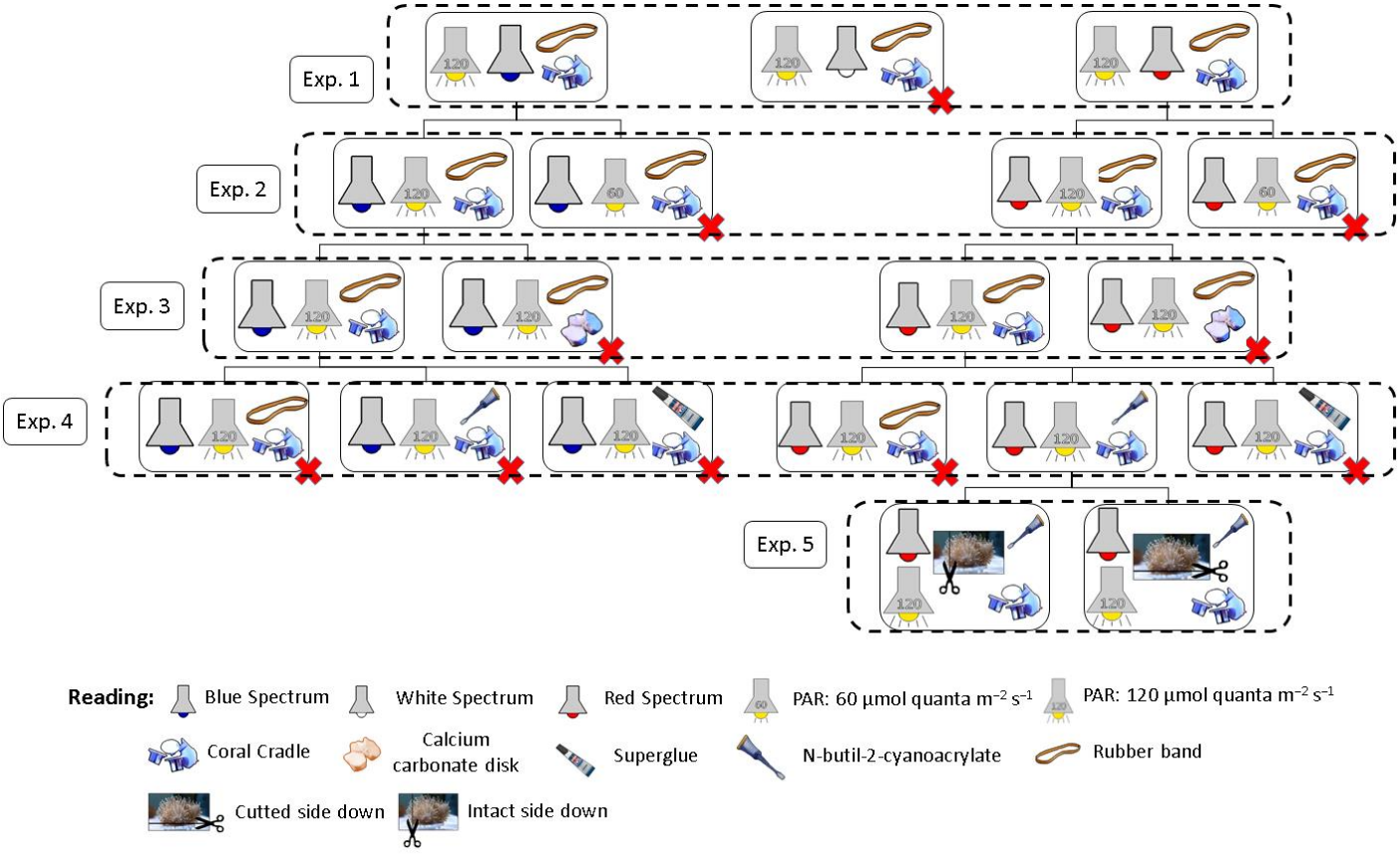

Figure 17: Flowchart depicting all the experiments employed and respective conclusions. Red crosses represent treatments that were excluded.

4. Discussion

4.1 Post-fragmentation recommendations

The experiments with the higher stocking densities (2 and 3) were the experiments were higher survival was observed and also the greater number of episodic releases of

mucus (data not shown). Mucus is proposed to be a defense mechanism of corals against environmental stressors, acting as a physical barrier and sloughing to avoid colonization by invasive microbes (Brown and Bythell, 2005; Bythell and Wild, 2011). These stocking high densities, can carry risks related to pathogen communicability, and virulence (Sheridan et al., 2013), increasing the incidence of diseases, thus, leading corals to increasing the exudation of carbon assimilated by their zooxanthellae in the form of mucus (Davies, 1984). These episodic releases of mucus can, to some extent, be related to the higher survival registered, possibly serving as an indicator of coral stress in response to environment.

Fragments at this stage of post-fragmentation are more vulnerable to suffer necrosis, possibly by the microbiological contamination of the damaged tissues. Maintenance of high water quality, adequate inorganic nutrient supply and the use of UV filters are good practices to minimize coral survival in production.

4.2 PAR intensity

The survival recorded was not significantly different between the PAR intensities studied (Experiment 2). Similar to our work, Sella and Benayahu (2010) found no significant differences in survival between 35 and 130 μmol quanta m^{-2} s^{-1} in *Sarcophyton sp.* fragments cultured *ex situ*, but observed that extreme light PAR intensities (20 and 250 μmol quanta m^{-2} s^{-1}) triggered higher survival rates. All these evidences suggest that *Sarcophyton sp.* has an interval of tolerance to PAR intensity somewhere between these PAR values. Furthermore, we found that PAR intensity positively influenced fixation ratio and fixation force, with PAR of 120 μmol quanta m^{-2} s^{-1} yielding the best results. Our conclusion is that *Sarcophyton sp.* fragments have a better post-fragmentation performance under higher PAR intensities, within their tolerance range. An analogue phenomenon, known as light enhanced calcification, is well documented in scleractinian corals, for which an increase in light intensity within the tolerance values of the coral leads to an increase in growth/calcification (Al-Horani et al., 2003; Huston, 1985; Meesters et al., 1994; Muller-Parker and D'Elia, 1997; Schutter et al., 2008).

In the face of these results, the PAR 120 μmol quanta m^{-2} s^{-1} was stablished as the best PAR intensity value to improve the performance of *Sarcophyton sp*. fragments post-fragmentation.

A gradual light acclimatization should be performed in order to increase survival (Sella and Benayahu, 2010).

4.3 Spectra

Not all wavelengths are used equally by the different zooxanthellate coral species, which is related with ecophysiological differences among coral and symbiont species (Iglesiasprieto and Trench, 1994), and with selective absorption of visible light by seawater (Mass et al., 2010). By comparing the action spectra of zooxanthellae photosynthetic pigments with the wavelength emission of the white light treatment, it was observed an overlap in the 420-480nm range, but little in the wavelengths of 550 and 600, correspondent to green and yellow respectively, where the white light treatment has peaks of emission. Zooxanthella photosynthetic pigments can perform photosynthesis in the spectral range of light between 400 and 700 nm, the so called Photosynthetically Active Radiation (PAR) (Lalli and Parsons, 1997). However, maximum rates of photosynthesis are observed in the wavelength ranges of 400-550 nm (violet, blue) and 620-700 nm (red) (see Figure 5d, (Lalli and Parsons, 1997), as observed by Rocha et al., (2013) for the photosynthetic pigments of *Sarcophyton* cf. *glaucum* zooxanthella. Possibly this previous evidence can explain the high survival of 60% (although no statistical significant) observed under the white spectra treatment. A similar finding was reported in one study that found that *Sarcophyton sp*. exhibits lower growth rates under T8 fluorescent lamps (control group), which have a similar wavelength emission as our white light treatment (Fernandes et al., 2014).

In this study, red light spectra compared to blue and white spectra yielded the overall best results regarding survival, fixation ratio and fixation force. Through the analysis of the wavelength emission of red and blue light treatment (Figure 4) it was observed that all light treatments had emissions on the blue wavelength range, but only the red light treatment exhibited emissions of low energy red light. We can therefore

theorize that red light may be an important sensory cue (Kinzie III et al., 1984) with a photophysiological enhancement effect on *Sarcophyton sp.* performance, which is plausible considering that these are shallow water corals (McFadden et al., 2006a), normally exposed to full PAR spectra in their natural habitats (Dustan, 1982). Due to the presence of some high energy blue light in the red treatments it is not clear whether red light has a photophysiological enhancement effect *per se* or if acts in combination with blue light. Antagonistic effect was found by Wijgerde et al. (2014), who found that red light represses photophysiologically the scleractinian coral *Stylophora pistillata*, a coral with a deeper vertical range of distribution. Similarly, Rocha et al. (2013b) found that blue light promotes high growth performances in the *Acropora formosa* and *Stylophora pistillata*. Our analogue studies suggest that corals are chromatically adapted to their surrounding light environment, which can be explained by the minimum light requirements related the spectral quality of the light exhibited by the zooxanthellae (Mass et al., 2010). Furthermore they show how highly species-specific the effects of spectra can be, and highlights the need for species-specific light optimization (Wijgerde and Laterveer, 2013).

The overall Fv/Fm results did not exhibit significant between treatments in any of the experiment employed, being generally high and close to the maximum values reported in the literature for corals (Kuguru et al., 2010; Levy et al., 2003; Rocha et al., 2013c; Winters et al., 2009, 2006). These results indicate that our fragments physiologically healthy under all light treatments tested, depicting the remarkable flexibility and adaptability of these symbionts (Apprill et al., 2007; Gorbunov et al., 2001; Iluz and Dubinsky, 2015; Robison and Warner, 2006; Titlyanov et al., 2001).

Scleractinian corals are known to undergo a dynamic resource management to repair damaged tissues, maintaining the integral structure of the colony (Nagelkerken et al., 1999; Van Woesik and Jordán-Garza, 2011). This healing process happens at the expense of growth and reproduction (Meesters et al., 1994; Ward et al., 2002; Weil et al., 2009). These energy trade-offs are even more complex considering that corals are clonal organisms that rely on zooxanthellae for energy (Van Woesik and Jordán-Garza, 2011). Although this phenomenon is not well understood in octocoralles, it is possible that the fragments had to allocate resources to repair the damaged tissues after fragmentation, relying on zooxanthellae to keep a positive energy balance for colony maintenance. We speculate this light related post-fragmentation performance, after observing that the main

factors affecting survival and fragment fixation identified during the course of the study were light related parameters (spectra and PAR intensity).

4.4 Substrate

Substrate type had no influence on survival, fixation ratio, fixation force or photosynthetic efficiency in neither of the experiments undertaken. A similar study corroborate these results (Schlacher et al., 2007). It is suggested that the performance of fragments post-fragmentation do not depend on the substrate type but rather on the substrate durability (Ferse, 2010). Therefore, the plastic stands were chosen as standard substrate type for further coral fragmentation cultures, due to its higher cost-effectiveness. Calcium carbonate stands are, on the other hand, more time consuming and require specialized machinery to be prepared.

4.5 Fixation process

Regarding the fixation process, N-butyl-2-cyanoacrylate (Histoacryl) and Ethylcyanoacrylate (Superglue), under red light, both promoted the highest fixation force of all treatments. However, since N-butyl-2-cyanoacrylate (Histoacryl) performed better than Ethylcyanoacrylate (Superglue) in terms of fixation ratio, it was chosen as standard method for further fragmentation procedures.

4.6 Fragment position

Fragment position had no influence in any of the parameters evaluated. As such, fragments can be secured to the substrate with no special considerations regarding their position, increasing the efficiency of the procedure, thus reducing the costs of manpower.

5. Conclusions

The designed experimental approach was important to identify optimum post-fragmentation conditions in *Sarcophyton sp*.. It was concluded that the post-fragmentation performance of *Sarcophyton sp.* fragments in *ex situ* culture systems was mainly dependent on the artificial lighting regimes used. Therefore, the light spectral characteristics represents a vital component of photoacclimation that should be considered especially during this critical period. This study provided evidence that:

(1) Spectra plays an important role on the survival, fixation ratio and fixation force of *Sarcophyton sp.* fragments post-fragmentation, with red light spectra achieving better results;

(2) Under the tolerance levels of *Sarcophyton sp.* holobiont, higher PAR intensities yield the best results for fixation ratio and fixation force, is this case PAR of 120 μmol quanta m^{-2} s^{-1}; (3) The performance of the fragments was similar for fragments grafted onto calcium carbonate disks or plastic stands;

(4) Fragment position had no influence in the success of fragmentation. Logically, these conditions have to be coupled with aquaculture best practices, such as the maintenance of top water quality throughout the growth out procedure.

Given the continued popularity of corals and the negative impacts caused by their harvest from the wild, aquaculture assumes a leading role towards the sustainable use of marine resources by optimizing the current employed techniques for coral production.

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Annexes

Table 2: χ^2 test statistical comparison of survival and fixation ratio between all the treatments, with the respective *p value* (d.f 1; $\alpha = 0.05$), for Experiment 2. Bold values represent significant differences between treatments.

Survival				
Treatment	Blue 120	Red 120	Blue 60	Red 60
Blue 120		1	0,216	**0,032**
Red 120	1		0,216	**0,032**
Blue 60	0,216	0,216		**0,011**
Red 60	**0,032**	**0,032**	**0,011**	
Fixation ratio				
Treatment	Blue 120	Red 120	Blue 60	Red 60
Blue 120		0,385	**0,011**	0,055
Red 120	0,385		0,076	0,274
Blue 60	**0,011**	0,076		0,404
Red 60	0,055	0,274	0,404	

Table 3: χ^2 test statistical comparison of survival and fixation ratio between all the treatments, with the respective *p value* (d.f 1; $\alpha = 0.05$), for Experiment 3. Bold values represent significant differences between treatments.

Survival				
Treatment	Blue Cr	Red Cr	Blue CC	Red CC
Blue Cr		0.674	0.457	0.277
Red Cr	0.674		0.248	0.137
Blue CC	0.457	0.248		0.729
Red CC	0.277	0.137	0.729	
Fixation ratio				
Treament	Blue Cr	Red Cr	Blue CC	Red CC
Blue Cr		0.584	0.910	0.399
Red Cr	0.584		0.681	0.735
Blue CC	0.910	0.681		0.484
Red CC	0.399	0.735	0.484	

Table 4: χ^2 test statistical comparison of survival and fixation ratio between all the treatments, with the respective *p value* (d.f 1; $\alpha = 0.05$), for Experiment 3. Bold values represent significant differences between treatments. Blue Rub: Blue Light spectra/ Rubber band; Blue Histo: Blue Light spectra/ N-butyl-2-cyanoacrylate (Histoacryl); Blue

SG: Blue light/Ethylcyanoacrylate (Superglue); Red Rub: Red Light spectra/ Rubber band; Red Histo: Red Light spectra/ N-butyl-2-cyanoacrylate (Histoacryl); Red SG: Red light/Ethylcyanoacrylate (Superglue).

Fixation ratio						
Treatment	Blue Rub	Blue Histo	Blue SG	Red Rub	Red Histo	Red SG
Blue Rub		0,132	0,673	**0,029**	**0,029**	**0,029**
Blue Histo	0,132		0,059	0,307	0,307	0,307
Blue SG	0,673	0,059		**0,012**	**0,012**	**0,012**
Red Rub	**0,029**	0,307	**0,012**		1	1
Red Histo	**0,029**	0,307	**0,012**	1		1
Red SG	**0,029**	0,307	**0,012**	1	1	

Table 5: Tukey's post hoc comparisons between treatments (d.f. 66). Blue Rub: Blue Light spectra/ Rubber band; Blue Histo: Blue Light spectra/ N-butyl-2-cyanoacrylate (N-butyl-2-cyanoacrylate (Histoacryl)); Blue SG: Blue light/Ethylcyanoacrylate (Superglue); Red Rub: Red Light spectra/ Rubber band; Red Histo: Red Light spectra/ N-butyl-2-cyanoacrylate (N-butyl-2-cyanoacrylate (Histoacryl)); Red SG: Red light/Ethylcyanoacrylate (Superglue).

Treatment	Red Histo	Red SG	Red Rub	Blue Histo	Blue SG	Blue Rub
Red Histo		0,799	**0,001**	**0**	**0**	**0**
Red SG	0,799		0,056	**0,02**	**0**	**0**
Red Rub	**0,001**	0,0563		0,844	0,367	**0**
Blue Histo	**0**	**0,002**	0,884		0,948	0,198
Blue SG	**0**	**0**	0,367	0,948		0,999
Blue Rub	**0**	**0**	0,198	0,816	0,999	