Universidade de Aveiro Departamento de Biologia
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Ricardo Calado, Investigador Principal no Departamento de Biologia da Universidade de Aveiro, do Professor Doutor João Serôdio, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro, e do Doutor Newton Gomes, Investigador Principal no Departamento de Biologia da Universidade de Aveiro.

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Dedico este trabalho a Leonor, minha filha.

Em memória do meu avô Alberto Miranda.

o júri / the jury

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palavras-chave

Corais; *Sinularia*; *Sarcophyton*; *Acropora*; *Stylophora*; Zooxantela; fluorometria PAM

resumo

O potencial dos corais como espécies emergentes com interesse para a aquacultura evidencia-se pela sua possível comercialização em três segmentos de mercado: *a)* aplicações biotecnológicas para pesquisa e exploração de novos compostos bioativos, *b)* aquariofilia marinha, e *c)* restauração de recifes de coral. Deste modo, o crescente interesse na aquacultura de corais conduziu a um esforço da comunidade científica para otimizar protocolos de cultivo, nomeadamente cultivo *ex situ*. O desempenho dos corais cultivados *ex situ*, em sistemas fechados com recirculação, pode ser afetado por diversos fatores físicos, químicos e biológicos. A iluminação representa um fator chave para o cultivo de corais que se caraterizem por um modo de vida em simbiose com dinoflagelados fotossintéticos (zooxantelas), uma vez que este fator influencia diretamente o desempenho destes endossimbiontes e, consequentemente, a fisiologia e crescimento do coral hospedeiro. A viabilidade económica de uma exploração aquícola está dependente do equilíbrio entre receitas e custos de produção, sendo que no caso do cultivo *ex situ* de corais, o custo relacionado com a utilização de sistemas de iluminação artificial influencia de forma preponderante o custo total de produção. No presente trabalho foi desenvolvido um sistema modular e versátil para o cultivo experimental de corais, recorrendo unicamente a materiais e equipamentos disponíveis no mercado á escala global; a utilização generalizada deste sistema permitirá a execução de desenhos experimentais estatisticamente robustos, bem como comparar de forma direta os resultados obtidos por diferentes equipas de investigação. Posteriormente foi avaliado o efeito de diferentes níveis de radiação fotossintética ativa (PAR – "Photosynthetically Active Radiation"), bem como diferentes espetros de emissão (comprimento de onda), na atividade fotossintética dos endossimbiontes (zooxantelas) de corais, recorrendo ao métodos não invasivo e não destrutivo designado como Fluorometria de Pulso Modulado (PAM – "Pulse Amplitude Modulation fluorometry"), para avaliar a eficiência fotoquímica do fotossistema II (F_v/F_m). Avaliou-se a concentração de clorofila *a* de forma indireta, através do cálculo do índice NDVI ("Normalized Difference Vegetation Index") que resulta da reflectância espectral obtida de forma não invasiva e não destrutiva; para além do método mencionado realizaram-se análises para obtenção da concentração de pigmentos fotossintéticos e acessórios. Por fim, avaliou-se o efeito da luz na taxa de crescimento e percentagem de sobrevivência das espécies de corais estudadas. As variáveis independentes estudadas foram as diferentes fontes de iluminação artificial utilizadas para cultivo *ex situ* de corais, nomeadamente: lâmpadas de halogeneto metálico (HQI – "hydrargyrum quartz iodide") com diferentes temperaturas de cor, lâmpadas T5 fluorescentes, lâmpadas de plasma (LEP – "Light Emitting Plasma") e lâmpadas de LED ("Light Emitting Diode"). Estudaram-se duas espécies de corais moles, *Sarcophyton* cf. *glaucum* e *Sinularia flexibilis*, que representam dois dos géneros com mais espécies na família Alcyoniidae, onde se incluem numerosas espécies com interesse para a biotecnologia marinha e para o mercado da aquariofilia marinha; foram igualmente estudadas duas espécies de corais duros, *Acropora formosa* e *Stylophora pistillata*, comercialmente importantes para o mercado da aquariofilia marinha, e relevantes para ações de repovoamento nos recifes de coral. Demonstrámos através dos resultados das diferentes experiências realizadas, que a cicatrização e recuperação de fragmentos de *S. flexibilis* mantidos após fragmentação nas mesmas condições luminosas das colónias mãe dos fragmentos são aceitáveis, embora possam igualmente ser mantidos com sucesso em regimes luminosos com uma menor intensidade do valor de PAR. Ficou também demonstrado que o cultivo do coral *S.* cf. *glaucum* em regimes luminosos com uma baixa intensidade do valor de PAR é biologicamente viável, o que permite uma redução acentuada nos custos de produção associados á iluminação artificial. Finalmente, concluiu-se que a utilização de sistemas de iluminação que emitam nos comprimentos de onda na faixa de radiação azul proporciona taxas de crescimento mais elevadas aos corais *A. formosa* e *S. pistillata*, para além de promover alterações ao nível da organização das micro e macroestruturas que integram os exosqueletos destes corais, referidos como um biomaterial com potencial para aplicação biomédica em implantes ósseos ou reconstrução maxilo-facial. Ficou também provado neste estudo, que as novas tecnologias de iluminação (LED) são bastante promissoras para o cultivo *ex situ* de corais.

keywords Corals; *Sinularia*; *Sarcophyton*; *Acropora*; *Stylophora*; Zooxanthellae; PAM fluorometry

abstract

The increasing interest in coral culture for biotechnological applications, to supply the marine aquarium trade, or for reef restoration programs, has prompted researchers to optimize coral culture protocols, with emphasis to *ex situ* production. When cultured *ex situ*, the growth performance of corals can be influenced by several physical, chemical and biological parameters. For corals harbouring zooxanthellae, light is one of such key factors, as it can influence the photosynthetic performance of these endosymbionts, as well as coral physiology, survival and growth. The economic feasibility of *ex situ* coral aquaculture is strongly dependent on production costs, namely those associated with the energetic needs directly resulting from the use of artificial lighting systems. In the present study we developed a versatile modular culture system for experimental coral production *ex situ*, assembled solely using materials and equipment readily available from suppliers all over the world; this approach allows researchers from different institutions to perform truly replicated experimental set-ups, with the possibility to directly compare experimental results. Afterwards, we aimed to evaluate the effect of contrasting Photosynthetically Active Radiation (PAR) levels, and light spectra emission on zooxanthellae photochemical performance, through the evaluation of the maximum quantum yield of PSII (F_v/F_m) (monitored non-invasively and non-destructively through Pulse Amplitude Modulation fluorometry, PAM), chlorophyll *a* content (also determined non-destructively by using the spectral reflectance index Normalized Difference Vegetation Index, NDVI), photosynthetic and accessory pigments, number of zooxanthellae, coral survival and growth. We studied two soft coral species, *Sarcophyton* cf. *glaucum* and *Sinularia flexibilis*, as they are good representatives of two of the most specious genera in family Alcyoniidae, which include several species with interest for biotechnological applications, as well as for the marine aquarium trade; we also studied two commercially important scleractinian corals: *Acropora formosa* and *Stylophora pistillata*. We used different light sources: hydrargyrum quartz iodide (HQI) lamps with different light color temperatures, T5 fluorescent lamps, Light Emitting Plasma (LEP) and Light Emitting Diode (LED). The results achieved revealed that keeping *S. flexibilis* fragments under the same light conditions as their mother colonies seems to be photobiologically acceptable for a short-term husbandry, notwithstanding the fact that they can be successfully stocked at lower PAR intensities. We also proved that low PAR intensities are suitable to support the *ex situ* culture *S.* cf. *glaucum* in captivity at lower production costs, since the survival recorded during the experiment was 100%, the physiological wellness of coral fragments was evidenced, and we did not detect significant differences in coral growth. Finally, we concluded that blue light sources, such as LED lighting, allow a higher growth for *A. formosa* and *S. pistillata*, and promote significant differences on microstructure organization and macrostructure morphometry in coral skeletons; these findings may have potential applications as bone graft substitutes for veterinary and/or other medical uses. Thus, LED technology seems to be a promising option for scleractinian corals aquaculture *ex situ*.

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Chapter 1

1.1. General Introduction

1.1.1. Biology and Ecology of corals

The common designation of *coral* is applied to identify cnidarians from class Anthozoa. The majority of extant corals are colonial organisms, typically living in a compact colony of several identical individual polyps. Corals are present in a wide range of environments, from shallow warm tropical waters, to temperate seas and the deep sea (Hughes et al., 2003; Reed, 2002; Veron, 1995, 2000; Wafar et al., 2011).

Tropical coral reefs are among the most biodiverse, complex and productive marine ecosystems in the world. These ecosystems thrive under optimal physical and chemical conditions, and can be found throughout subtropical and tropical regions, mainly between the Tropic of Capricorn and Tropic of Cancer (30°S, 30°N latitude), in the Indian, Pacific and Atlantic oceans (Veron, 1995, 2000).

Corals exhibit a very limited organ development and do not have a central nervous system. Anatomically, coral polyps are composed by two epithelial cell layers (Fig. 1.1.1): 1) epidermis or ectoderm, which promotes the separation between the coral itself and the external environment; and 2) gastrodermis or endoderm, which lines the gastro-vascular cavity, also designed as coelenteron. Between these two epithelial layers (epidermis and gastrodermis) lies the mesoglea, which is a translucent substance, mostly composed by water, and other substances such as fibrous proteins like collagen, and heparan sulphate proteoglycans. The composition of mesoglea is mostly acellular, but contains nerve fibres, muscle bundles and amoebocytes, which are involved in phagocytosis processes involving debris and bacteria. The elastic properties of mesoglea help to restore the shape of corals after muscles contraction, still depending on the buoyancy of water to support it (e.g. Fosså and Nielse, 1998; Sarras Jr et al., 1991).

The gastro-vascular cavity (Fig. 1.1.1) is separated from the external environment by the mouth, which is surrounded by tentacles. Heterotrophic feeding in corals can occur in a more passive way by water intake through the mouth which fills the gastro-vascular cavity, or, in a more active way, through food capture mediated by the stinging cells (nematocysts) harboured in the tentacles surrounding the mouth. Ingested food (phytoplankton, zooplankton, bacteria, or organic particles) is decomposed by enzymes in the interior of the

gastro-vascular cavity, following by phagocytosis of cells in the gastrodermis for posterior intracellular digestion inside cell vacuoles (Ferrier-Pages et al., 2011; Fosså and Nielse, 1998; Houlbrèque and Ferrier-Pages, 2009).

Several coral species live in symbiosis with unicellular dinoflagellates of genus *Symbiodinium*, commonly known as zooxanthellae. The coral host provides a protected environment to the zooxanthellae, and also contributes with nutrients and carbon dioxide necessary for photosynthesis. Zooxanthellae provide their coral host with photosynthetically derived carbon compounds, and also with amino acids, saturated and polyunsaturated fatty acids (Hoegh-Guldberg et al., 2007; Muscatine and Porter, 1977; Papina et al., 2003). This symbiotic relation allows for an efficient nutrient recycling in the oligotrophic environment of coral reefs.

Coral species harboring zooxanthellae can be considered mixotrophic, as they complement the nutrition they derive from the photosynthates provided by their endosymbionts (the zooxanthellae) with heterotrophic feeding. However, few species present a facultative symbiotic relation with zooxanthellae, since the majority cannot thrive for long periods without these endosymbionts. Photosynthates commonly display a very high C:N ratio (i.e. low nitrogen concentration), and therefore do not support the growth of the coral host *per se*. For this reason, these photosynthetic products have been classified as "junk food" (Dubinsky and Jokiel, 1994; Falkowski et al., 1984), which highlights the importance of heterotrophic feeding to enhance coral growth.

Zooxanthellae can be directly transmitted from the parental colony to the eggs and the larvae, or either uptaked from the natural environment (Oppen, 2004). In the last case zooxanthellae enter in the gastro-vascular cavity and are phagocytised by cells in the gastrodermis (Abrego et al., 2012; Schwarz et al., 1999), after are incorporated in perialgal vacuoles within these cells and continue to photosynthesize (Schwarz et al., 2002). The mechanisms of zooxanthellae recognition, which avoid the coral host to digest these cells and allow them to remain photosynthetically active inside the cell vacuoles can varies between coral species and the clade of zooxanthellae. Nonetheless, it is worth referring that some coral species can harbor more than one clade of zooxanthellae (Cooper et al., 2011;

Oppen, 2004). Moreover, corals are capable to regulate their zooxanthellae population by digestion and extrusion of zooxanthellae remnants (Titlyanov et al., 1996; Titlyanov et al., 1998).

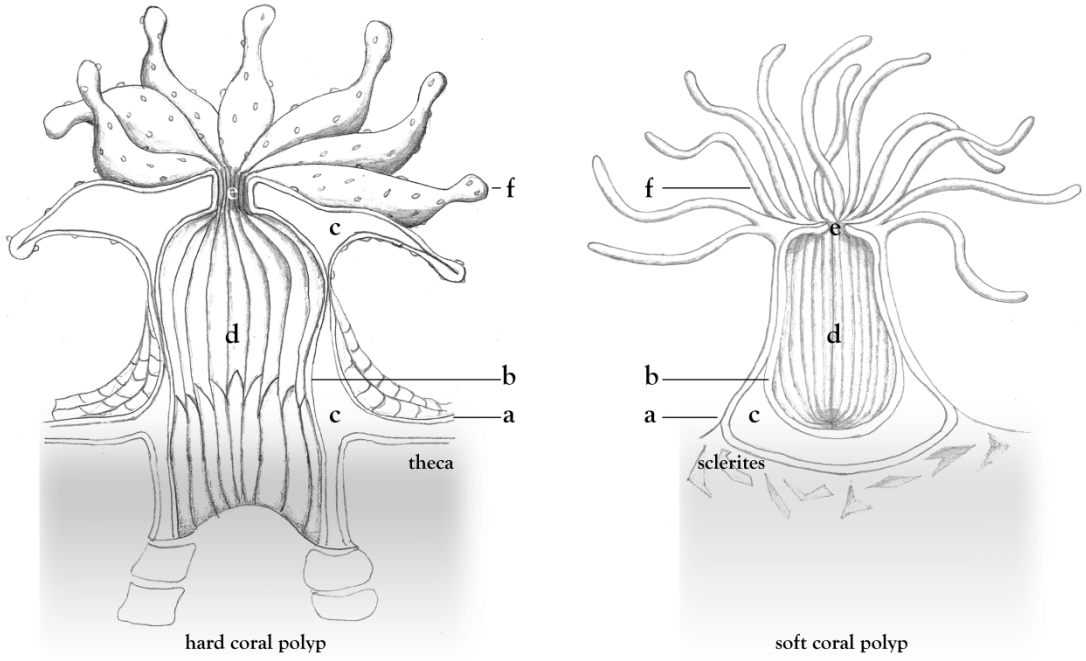

Fig. 1.1.1. Hard and soft coral polyp illustrations: a – epidermis or ectoderm, b – gastrodermis or endoderm, c – mesoglea, d – gastrovascular cavity, e – mouth, f – tentacles. Polyp illustrations were a courtesy of José Pedro Deus.

Corals have two main types of reproduction - sexual and asexual; both of these processes present a large variety of reproductive strategies.

Concerning sexual reproduction, corals can be hermaphrodites or instead have separated sexes. In the first case, corals can develop ovaries or testes in the same mesentery, or in different mesenteries within the same polyp; alternatively corals may have male and female polyps in the same colony, which can be present at different points in the time. Synchronous or simultaneous hermaphrodite corals, produce oocyte and sperm cells at the same time; sequential (protandric or protogynous) hermaphrodites corals produce oocyte and sperm cells alternately (Fosså and Nielse, 1998). Moreover, fertilization and larval development until settlement can take place in the water environment if the corals are "free spawners", or instead, fertilization and larval development can take place inside the polyp, if the corals are "brooders" (Fosså and Nielse, 1998).

Asexual reproduction also occurs naturally in corals. This reproductive process is devoid of fertilization and produces an offspring genetically identical to the mother colony (clones). We can identify three main types of asexual reproduction in corals: 1) fragmentation, which occurs naturally when a coral fragment is separated from the colony by a physical factor, the tissues do not die and continue to thrive in a new place; 2) fission, when a original colony splits in two halves, or a new individual colony appears in an original colony foot; 3) polyp bail-out, when polyps in stony corals detach themselves from the colony skeleton and then develop a new colony in a new place (Fosså and Nielse, 1998). There are a few additional reports on less common strategies of asexual reproduction in corals, which are recognized to be species-specific and/or triggered by specific environmental conditions in coral reefs.

Corals are informally divided in two main groups: 1) hard or stony corals, and 2) soft corals, which are schematically presented in figure 1.1.1. This distinction is mainly based in the presence or absence of a calcium carbonate skeleton to support the coral colony. In spite of hard corals being the major contributors for the structure and framework of coral reef ecosystems, some soft corals are also able to cement sclerites and form solid structures through the consolidation of dense sand agglomerates in their basis and thus also contribute to reef building (Jeng et al., 2011).

1.1.2. Socio-economic relevance of coral reefs

For the scientific community, particularly ecologists, a diverse ecosystem such as a coral reef is priceless. However, to get the attention of politicians and economists, attributing a monetary value to an ecosystem has become a common exercise. The economical value of an ecosystem such as a coral reef, which contributes directly and indirectly to human welfare, as they can be considered as part of Earth's life-support system, must be considered as part of the total value of the planet (Costanza et al., 1997).

In many regions coral reefs act as a protection barrier for coastal communities where wave impact and erosion may become acute problems. Moreover, coral reefs provide habitats for fish, and therefore contribute to increase the fish stocks, which have a social relevance for local populations, and can also be exploited by commercial fisheries markets, or in

recreational fisheries (Costanza et al., 1997). The touristic and recreational utilization of coral reefs (e.g. diving) also have an important economic impact (Brander et al., 2007; Laurans et al., 2013), involving local people and companies (e.g. touristic operators, flying companies).

In the last decades, coral reefs started to be looked also as a source of natural bioactive compounds, particularly those produced by corals (Blunt et al., 2007, 2008, 2009; Blunt et al., 2013; Brown and Bythell, 2005), with interest for pharmacological, biochemical and biomedical purposes. Recently, some studies and reviews point promising marine bioactive compounds, from the therapeutically point of view, isolated from cnidarians (Leal et al., 2013; Leal et al., 2012a; Leal et al., 2012b; Rocha et al., 2011). Antitumor activity has been the major area of interest of the scientific community, which focus mainly in terpenoids (monoterpenoids, diterpenoids, sesquiterpenoids) (Rocha et al., 2011).

In several cases the perception of the real and immediate value of a coral reef only takes place after an environmental disaster or catastrophic meteorological event that causes coral bleaching (the disruption of the symbiotic relation between coral and zooxanthellae, which are expelled) and/or leads to the death of significantly areas in a coral reef. These important ecosystems are still threatened by both natural and anthropogenic factors, such as global warming and ocean acidification, pollution, overfishing, destructive fishing practices (e.g. use of cyanide and blast fishing), or irresponsible dive tourism (Bellwood et al., 2004; Fox et al., 2005; Hughes et al., 2003; Leao and Kikuchi, 2005; Tissot et al., 2010). In the last decades studies have addressed restoration efforts (Rinkevich, 2005; Shafir et al., 2006) that aim to attenuate the deterioration of these threatened ecosystems (Hughes et al., 2003; Wild et al., 2011).

1.1.3. State of the art of corals aquaculture

The increasing demand for corals, either for biotechnological research on marine natural products (Blunt et al., 2008, 2009; Brown and Bythell, 2005; Leal et al., 2012a), or to supply the marine aquarium trade (Olivotto et al., 2011; Osinga et al., 2011; Wabnitz et al., 2003), has prompted an increase on their harvest (Castanaro and Lasker, 2003). The sustainability

of this approach can be negatively affected by the dependence on wild organisms. Therefore, it has been recommended that research on marine natural products (Mendola, 2003; Proksch et al., 2003) and the marine aquarium industry (Calfo, 2007; Olivotto et al., 2011) should consider the use of specimens produced in captivity, rather than wild organisms.

In this context, coral aquaculture can be a potential solution for a continuous and sustainable supply of coral biomass. Coral aquaculture can be performed either *in situ* or *ex situ*. *In situ* aquaculture benefits from natural conditions (water physical and chemical parameters, water currents, light and nutrients) and requires no adaption to artificial propagation systems. However, fragments can be exposed to potential deleterious factors present in the natural environment, such as sedimentation, meteorological conditions, predators, parasites, competitors and other natural hazards, which can reduce survival and growth (Rinkevich, 2005). In contrast, coral production *ex situ* involves technological recirculated culture systems with inherent production costs, but has the advantage of maximizing survival and growth rates of cultured corals, through the manipulation of culture conditions, such as light, water flow and food availability (Forsman et al., 2006; Khalesi, 2008; Schutter et al., 2011; Schutter et al., 2008).

Corals present two principal advantages when compared with other cultured marine organisms: a) the ability to reproduce asexually by fragmentation, which reduces the production costs associated with broodstock management and larval rearing, and allows the clonal production of a given genotype displaying interesting features (e.g., colour, metabolite…); b) coral production does not have to target one specific market, as cultured corals can be employed to supply three contarsting markets: 1) the bioprospecting of new natural products with potential pharmacological and biomedical applications (Leal et al., 2013); 2) the marine aquarium trade (Rhyne et al., 2012); and 3) coral reef restoration programs (Shafir et al., 2006).

Coral husbandry *ex situ* attracted the attention of marine biologists in the last decades. While several authors provided important contributions on the technical issues associated with recirculated aquaculture systems for coral culture, either in small or large scale, one must highlight the contributions by Delbeek and Sprung (Delbeek and Sprung, 1994, 2005;

Sprung and Delbeek, 1997) provided by their remarkable book series entitled "The Reef Aquarium". These authors have improved and standardized zootechnical practices and technological equipment for the maintenance of corals in captivity, using recirculated systems. Most of the techniques described by these authors are still efficiently used, allowing the successfully *ex situ* culture of most coral species. Nevertheless, the optimization of recirculating systems to culture corals in captivity continued to receive the attention of researchers, namely on issues related with biological filtration processes, (Seo et al., 2001; Toonen and Wee, 2005; Yuen et al., 2009), as well as in the development of computerized and automatic systems to assist on the monitoring of water parameters (Widmer et al., 2006).

Several parameters can influence coral physiology and growth performance under culture, such as temperature (Sella and Benayahu, 2010), water movement (Chappell, 1980; Riegl et al., 1996), nutrient supply (Fleury et al., 2000; Muscatine et al., 1989), heterotrophic feeding (Ferrier-Pages et al., 2003; Houlbrèque and Ferrier-Pages, 2009; Houlbrèque et al., 2004), or light (Schutter et al., 2012). Among these parameters, light is unanimously identified as playing a key role on coral aquaculture *ex situ*, due to the association of symbiotic corals with zooxanthellae. Light variation is known to affect zooxanthellae density, photosynthetic efficiency, and photosynthetic and accessory pigments concentration (Frade et al., 2008; Kühl et al., 1995; Lesser et al., 2010). This photophysiological acclimations will affect coral physiology and its response to stress (Venn et al., 2008), and consequently growth performance (Schutter et al., 2011; Schutter et al., 2008).

The economic feasibility of an *ex situ* coral production facility is also conditioned by light, since it largely affects the overall production costs (Osinga et al., 2011). Therefore, in order to improve *ex situ* coral aquaculture economical viability, it is urgent to optimize culture protocols (Osinga, 2008), namelly those with major influence in production costs.

As reviewed by Osinga et al. (2011), several studies have already focused on the effects of irradiance on corals and their photosynthetic endosymbionts. Most of these studies focused on the light intensity of wavelengths in the Photosynthetically Active Radiation (PAR) range, which designates the spectral range of solar radiation, approximately from 400 to 700 nm.

Only a few works have investigated the role of the spectral quality of light on coral photobiology, physiology and growth. This current gap of knowledge should be addressed, as the spectral quality of light is assumed to play a major role in the success of *ex situ* coral production (Schlacher et al., 2007; Wijgerde et al., 2012).

The importance of light on *ex situ* coral culture fostered the development of innovative technologies for coral illumination, as well as the optimization of culture protocols under low PAR light regimes. The application in aquaculture of new light sources, such as light emitting plasma (LEP) or light emitting diode (LED), has already started to be evaluated and is partially replacing the most frequently used illumination solutions for the culture of corals *ex situ* (the T5 fluorescent and hydrargyrum quartz iodide (HQI) lamps). Some of these new light sources allow the reduction of operation and maintenance costs. Coral culture under low light PAR regimes also contribute in an active way to reduce the costs with electrical power, since with the same power consumption of a single lamp a higher production area can be illuminated.

1.1.4. Objectives

The main objective of this thesis is to provide new data on light PAR intensity and spectral quality applied in *ex situ* coral production. Four coral species were used as models: two species of soft corals of the subclass Octocorallia, family Alcyoniidae (Lamouroux, 1812), and two species of hard corals of the subclass Hexacorallia, one of the family Acroporidae (Verrill, 1902) and other of the family Pocilloporidae (Gray, 1842).

The two species of soft corals selected, *Sinularia flexibilis* (Quoy & Gaimard, 1833) (Fig. 1.1.2-A) and *Sarcophyton* cf. *glaucum* (Quoy & Gaimard, 1833) (Fig. 1.1.2-B) are popular in the marine aquarium trade and have also been widely surveyed for new natural products (e.g., cembrane diterpenes such as sarcophytol or flexibilide) (Badria et al., 1998; Khalesi, 2008; Yang et al., 2012).

Fig. 1.1.2. Coral fragments: A - *Sinularia flexibilis* (Quoy & Gaimard, 1833), and B - *Sarcophyton* cf. *glaucum* (Quoy & Gaimard, 1833).

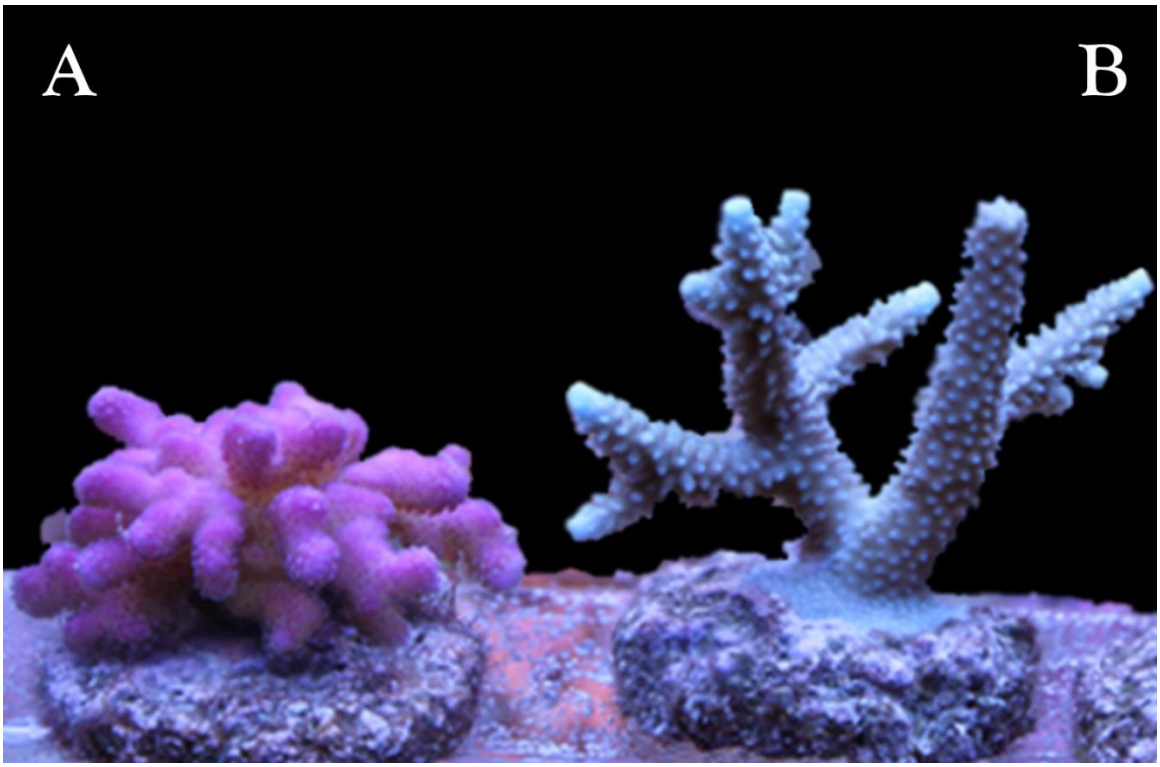

Fig. 1.1.3. Coral fragments: A - *Stylophora pistillata* (Esper, 1797), and B - *Acropora formosa* (Dana, 1846). *S. pistillata* and *A. formosa* photos were a courtesy of Jorge Machado de Sousa (Maternidade do Coral Lda., Portugal).

Selected hard corals, *Stylophora pistillata* (Esper, 1797) (Fig. 1.1.3-A) and *Acropora formosa* (Dana, 1846) (Fig. 1.1.3-B), are two of the most well known and popular species in the marine aquarium trade, attaining high commercial values. Additionally, the interconnectivity, porosity and three-dimensional structure of coral skeletons, which mimics human bone, makes them potential substitutes of bone grafts for veterinary and medical applications (Sopyan et al., 2007).

Overall, the results obtained during this 4-year project are presented in the form of chapters as follows: 2) Development of a standardized modular system for experimental coral culture; 3) Effect of light intensity and light spectra in *ex situ* culture of soft corals, 3.1. Effect of light intensity on post-fragmentation photobiological performance of the soft coral *Sinularia flexibilis*, 3.2. Photobiology and growth of leather coral *Sarcophyton* cf. *glaucum* fragments stocked under low light in a recirculated system, 3.3. Photobiology and growth of leather coral *Sarcophyton* cf. *glaucum* fragments stocked under different light spectra in a recirculated system; 4) Effect of light spectra in *ex situ* culture of hard corals, 4.1) Comparative performance of light emitting plasma (LEP) and light emitting diode (LED) in *ex situ* aquaculture of scleractinian corals, 4.2) Contrasting light spectra trigger morphological shifts in the skeleton of reef building corals.

Three of the aforementioned chapter sections are published, two are submitted, and one is in preparation for submission, all in international journals ranked on ISI. Their complete reference is provided in the beginning of each chapter or chapter section.

Chapter 2

2.1. Development of a standardized modular system for experimental coral culture

Submitted with section 3.3 as: Rui J. M. Rocha, João Serôdio, Bogdan Bontas, Paulo Cartaxana, José M. Ferreira, Rui Rosa and Ricardo Calado. Development and validation of a standardized modular system for experimental coral culture.

2.1.1. Overview

In the last decade, coral aquaculture has received a renewed interest by the scientific community, as it is currently considered to play a key role in coral reef restoration efforts (Rinkevich, 2005; Shafir et al., 2006) that aim to attenuate the deterioration of these threatened ecosystems (Hughes et al., 2003; Wild et al., 2011). The increasing interest on coral bioprospecting for new natural products (Blunt et al., 2013; Leal et al., 2012; Mayer and Gustafson, 2006; Moore et al., 2006), as well as the growing demand for corals to supply the marine aquarium trade (Olivotto et al., 2011; Osinga et al., 2011), has also contributed to the optimization of protocols to culture these organisms *ex situ* at a commercial scale.

The complexity and interaction of parameters that can influence coral physiology and growth, such as temperature (Sella and Benayahu, 2010), water movement (Chappell, 1980; Riegl et al., 1996), nutrient supply (Fleury et al., 2000; Muscatine et al., 1989), or light (Schutter et al., 2012), have set the guidelines for culture experiments being performed under controlled laboratory conditions.

While several authors provided important contributions on the technical issues associated with coral husbandry *ex situ*, one must highlight the contributions by Delbeek and Sprung (Delbeek and Sprung, 1994, 2005; Sprung and Delbeek, 1997) provided by their remarkable book series entitled "The Reef Aquarium". In these works the authors have significantly improved and somehow standardized the practices associated with the maintenance of corals in aquarium using recirculated systems with. In fact, the majority of recirculated systems currently employed to stock corals *ex situ*, including the one discussed in the present study, are still set according to the principles and practices described by the work of these authors. It is important to say that the optimization of recirculating systems to stock corals in captivity continued to receive the attention of researchers, namely on issues related with biological filtration processes, such as nitrification (Seo et al., 2001; Toonen and Wee, 2005; Yuen et al., 2009) and denitrification (Grommen et al., 2006; Kropp et al., 2009; Singer et al., 2008), as well as in the development of computerized systems (Widmer et al., 2006) to assist on the monitoring of water parameters, or in the application of technology developed for

aquariums in intensive fish rearing in Recirculating Aquaculture Systems (Fontaine et al., 1996).

The design of small scale models (commonly termed microcosms) allow researchers to simulate natural environments (Roeselers et al., 2006) under controlled physical, chemical and biological conditions. Moreover, microcosms allow testing different hypotheses with a degree of experimental control and replication that would not be possible to achieve with experimentation *in situ* (Luckett et al., 1996; Small and Adey, 2001). Nonetheless, while the basic principles employed to stock and culture corals in captivity are not too contrasting, the diversity of systems currently being employed can at times be a bottleneck to research efforts, as it impairs sound comparisons of experimental results and the true replication of culture protocols.

To overcome this constraint, we present a versatile, modular and standardized system for coral experimentation in the laboratory, which can be operated either under a recirculated or flow through regime. While this system employs well known approaches for coral husbandry and culture that are already widely employed by researchers working on this topic, it is innovative by being assembled solely with materials and equipment readily available from suppliers all over the world, by allowing researchers to perform complex experimental designs using statistical replication (not pseudo-replication) and operate either as an open, semi-open or close recirculated system. Ultimately, our goal is to provide a tool that can allow researchers from different institutions to assemble truly replicated experimental set-ups for coral culture.

2.1.2. Materials and methods

- *Basic set-up of the modular culture system*

The basic concept of our system was to provide a versatile framework for experimental manipulation of chemical, physical and biological parameters that may interfere with *ex situ* coral growth and physiology (Fig. 2.1.1). This system was developed using a modular construction concept, in order to allow researchers to work over a wide range of

configurations and address specific questions, using statistically robust experimental designs (e.g., high number of independent replicates and complex experimental designs). The system was assembled using affordable materials and equipment, which are readily available in local or online stores, allowing it to be easily replicated across the world (see Table 2.1.1 in supplementary material for a detailed list of materials and equipment employed, as well as their respective suppliers).

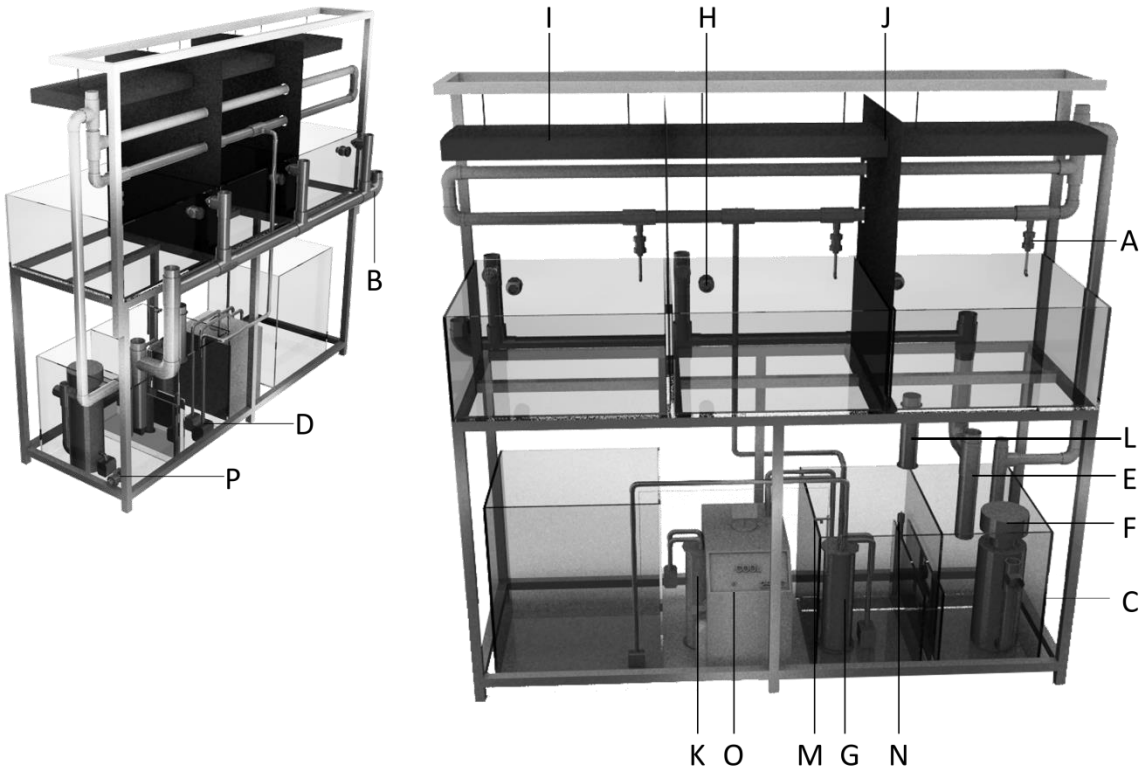

Fig. 2.1.1. Modular culture system basic set up: A) PVC valve inlet pipe system, B) outlet pipe system, C) 150-L filter tank, D) inlet pipe system submerged pump, E) 250 µm mechanical filtration bag, F) protein skimmer, G) fluidized sand-bed biological filter, H) circulation pump, I) individual lighting system, J) PVC screen, K) calcium reactor, L) calcium hydroxide reactor, M) osmoregulator, N) submergible heater, O) water chiller, P) filter tank connection valves.

Each system module is composed of 3 experimental glass tanks (0.3 m high, 0.6 m long and 0.6 m wide, for a maximum functional water volume of approximately 90 L). The tanks can be made assembled using any other material considered suitable, such as Polymethylmethacrylate (PMMA) instead of glass, depending on the local price and availability of the material, as well as on the experimental purpose (e.g. the use of some chemical products to be experimentally tested in ecotoxicological studies, can influence the choice of the material for tank construction). The system can be operated in a flow-through regime or, alternatively, using water recirculation; in this last option, the tanks are connected to a common filtration sump (0.5 m high, 0.7 m long and 0.5 m wide), operating with a maximum functional water volume of approximately 150 L. System modules may be connected through polyvinyl chloride (PVC) pipes and valves, in order to increase the number of tanks for coral experimentation (Fig. 2.1.1).

The modular system design described below allows researchers to perform robust experimental designs (and avoid pseudo-replication) and control fundamental factors that condition the biological and physiological aspects of corals, namely: water temperature, water chemistry, nutrition (such as the supply of live prey) and artificial illumination (with emphasis to photoperiod, light intensity, photosynthetically active radiation (PAR), light colour temperature and spectrum).

- *Filtration and water flow*

Depending on experimental design, the modular culture system can work in 4 possible configurations:

a) Flow-through system

The system can operate with 3 × *n* experimental tanks (*n* being the number of modular systems) in flow-through (Fig. 2.1.2). The flow-through system can operate either with natural or synthetic seawater, stocked in a reservoir (Fig. 2.1.2F) and pumped through the PVC inlet pipes supplying the experimental tanks (Fig. 2.1.2A). Water renewal is adjusted by a PVC valve in each experimental tank (Fig. 2.1.2B). The outlet pipe system drains the water from the experimental tanks to a collector so it can be discarded (Fig. 2.1.2C). Each tank is equipped with 1 or 2 circulation pumps (Turbelle nannostream - 6025 Tunze, Germany;

approximate flow of 2500 L h^{-1}) (Fig. 2.1.2D), depending on the water flow required for each experimental set up. This system configuration is adequate to test factors that modify water chemistry (e.g., higher or lower levels of calcium) or address heterotrophic feeding in corals (e.g., experiments requiring the supply of phytoplankton, zooplankton or processed feeds), thus making recirculation a challenge due to potential cross contamination between treatments and controls. The number of modular systems required for an experimental designs with *n* replicates × *y* experimental treatments can be calculated by multiplying *n* × *y*, dividing by 3 (number of tanks per module) and rounding up (e.g., a factorial experiment testing 3 different light regimes and 2 different water temperatures = 3×2 = 6 experimental treatments × 4 replicates = 6×4 = 24 tanks/3 = 8 culture modules).

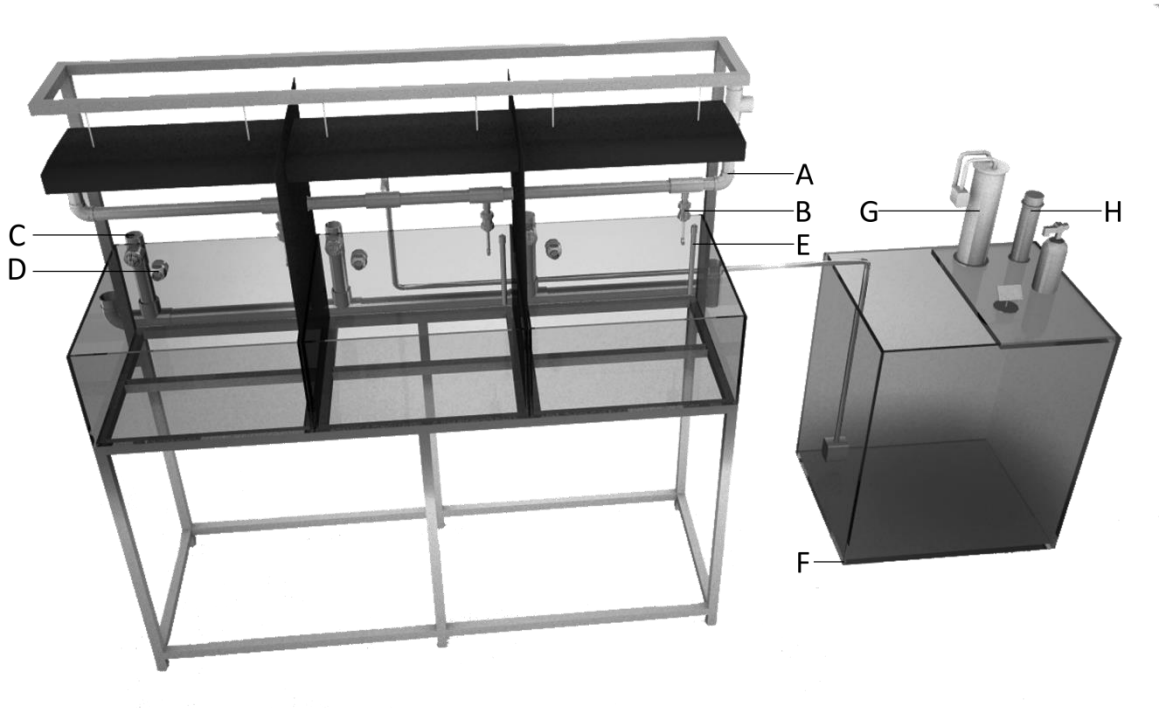

Fig. 2.1.2. Modular culture system with individual tanks operating in flow- through open system: A) inlet PVC pipe system, B) PVC valve, C) outlet pipe system, D) circulation pumps, E) submergible heater, F) water reservoir, G) calcium reactor, H) calcium hydroxide reactor.

b) Recirculated system using independent experimental tanks

To operate the system in recirculated mode with 3 × *n* (*n* being the number of modular systems) individual experimental tanks design, an individual filtration system needs to be set on each tank (Fig. 2.1.3); this filtration system is composed by an external hang-on protein skimmer (MCE 600 Deltec, Germany) (Fig. 2.1.3A) and a fluidized sand reactor (FLF100 ReefSet, Portugal) (Fig. 2.1.3B) with 0.8 kg of aragonite sand (1-2 mm of grain size) operating with a water flow of 1000 L h^{-1} (Aquabee UP 2000 water pump, Germany), which ensures biological filtration. Partial water changes are performed using the water reservoir and the PVC inlet pipe system described above. Experimental tanks are also equipped with 1 or 2 circulation pumps (Turbelle nannostream - 6025 Tunze, Germany; approximate flow of 2500 L h^{-1}) (Fig. 2.1.3C). This system configuration also enables researchers to perform short or long-term experiments that promote shifts in water chemistry or address heterotrophic feeding in corals, but the costs involved in a flow-through approach (as described above) are prohibitive (e.g., laboratories operating with synthetic seawater may not afford to operate flow-through systems, even for short term experiments). This approach avoids the bias of pseudo-replication (e.g., providing a common feed to the 3 experimental tanks set in a single culture module to avoid cross contamination), as it allows the use of true replication (see above for the calculation of the numbers of cultured modules required according to the desired experimental design).

c) Recirculated system with independent modules of experimental tanks

Each system module (described in the section *Basic set up of the modular culture system*) is connected to a 150-L filter tank (the sump) (Fig. 2.1.1C). A submerged pump (EHEIM 1262, Germany) (Fig. 2.1.1D) placed in the sump provides an approximate flow of 1000 L h^{-1} to each of the three experimental tanks on each modular system, through an inlet PVC pipe system (Fig. 2.1.1A). Mechanical filtration is ensured by a filter bag (250 µm) (Fig. 2.1.1E) placed in the collector of outlet pipes (Fig. 2.1.1B). After passing through the filter bag, the water is then filtered by the protein skimmer (ESC150 ReefSet, Portugal) (Fig. 2.1.1F). Biological filtration is ensured through a fluidized sand reactor (FLF200 ReefSet, Portugal) (Fig. 2.1.1G) operating with 3 kg of aragonite sand (1-2 mm of grain size) and at a water flow

of 2000 L h^{-1} (Aquabee UP 2000/1 water pump, Germany). To improve water flow, each experimental tank is equipped with 1 or 2 circulation pumps (Turbelle nannostream - 6025 Tunze, Germany; approximate flow of 2500 L h^{-1}) (Fig. 2.1.1H). This system configuration can be used to perform nested experimental design (e.g., culture module 1 is set for a temperature of X ºC and each of the 3 experimental tanks is exposed to a different light regime (a, b and c); culture module 2 is set for a temperature of Y ºC and each of the 3 experimental tanks are also exposed to a different light regime (a, b and c); the factor "light treatment" would be nested in the factor "temperature").

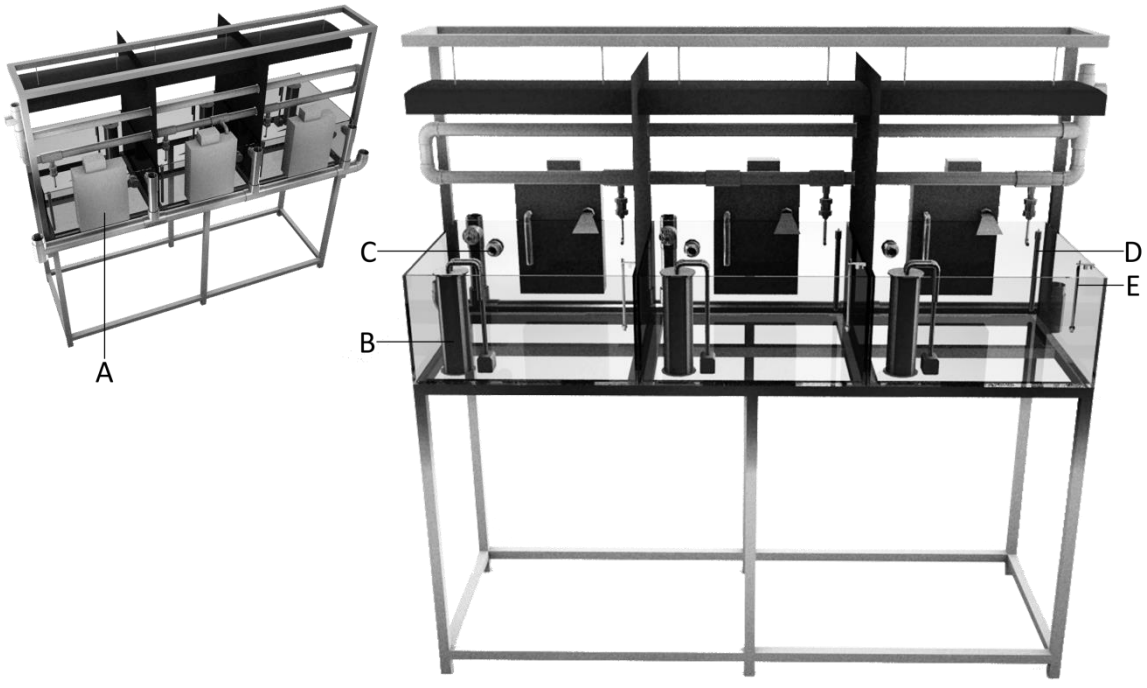

Fig. 2.1.3. Modular culture system with individual tanks operating in recirculating system: A) external hang-on protein skimmer, B) fluidized sand bed biological filter, C) circulation pump, D) submergible heater, E) osmoregulator.

d) Recirculated system with interconnected modules of experimental tanks

This configuration is similar to the one described above (*Recirculated system with independent modules of experimental tanks*), but the modules are interconnected by opening the valves placed in the bottom of the filtration tanks (Fig. 2.1.1P). To promote water circulation between filter tanks the water is pumped (Aquabee UP 2000, Germany) from the filter tank of the first module to the filter tank of the last module. This system configuration allows the randomization of *n* factors in triplicate (being *n* the number of modular systems), as light (spectra or intensity), hydrodynamic aspects (water current or flow) or zootechnical procedures (fragmentation techniques, attachment substrates or fixation methods) in a recirculated system. The use of this configuration is recommended when researchers want to ensure that no unpredictable effects related with water quality (namely chemical and microbiological shifts) can bias experimental results, as recirculating water is common to all experimental tanks (e.g., in experiments solely testing light regimes).

- *Artificial illumination*

Experimental glass tank are illuminated from above with individual lighting systems (Fig. 2.1.1I) and are separated from each other by an opaque PVC division (in order to assure that each experimental tank only receives light radiation from its own artificial lighting system) (Fig. 2.1.1J). The modular system has been designed to work with different lighting systems, with the following options having already been successfully tested: metal halide lamps (150 W Hydrargyrum quartz iodide (HQI) lamps recommended), T5 fluorescent lamps (maximum 55 cm length), Light Emitting Diode (LED) and Light Emitting Plasma (LEP). Experimental trials can address: a) the effect of light spectra by using different light sources, b) the effect of Photosynthetically Active Radiation (PAR) or Photosynthetically Usable Radiation (PUR), both by adjusting the distance between the lighting system and stocked corals (or increasing the number of lamps).

- *Water chemistry and temperature control*

Water chemistry and temperature can be adjusted to each experimental set-up, as required. The modular system is designed to operate with a calcium reactor (Deltec PF 501, Germany,

with a Tunze pH/CO_2 Controller-Set (7074/2), Germany, and a dosing pump Grotech TEC 1 NG, Germany) (Fig. 2.1.2G) and a calcium hydroxide reactor (KM500 Deltec, Germany) (Fig. 2.1.2H) in the water reservoir (Fig. 2.1.2F), namely for experiments addressing hermatypic corals and employing the set-ups of "*Flow-through system*" or "*Recirculated system using independent experimental tanks*". For experiments performed with the experimental design of "*Recirculated system with independent modules of experimental tanks*" or "*Recirculated system with interconnected modules of experimental tanks*" each filter tank has a calcium reactor (Deltec PF 501, Germany, with a Tunze pH/CO_2 Controller-Set (7074/2), Germany, and a dosing pump Grotech TEC 1 NG, Germany) (Fig. 2.1.1K). The modular system is also equipped with a calcium hydroxide reactor (KM500 Deltecc, Germany) (Fig 2.1.1L) in each filter tank, which is operated during the nocturnal period with a dosing pump (Grotech TEC 1 NG, Germany, Germany) programmed to a specific dosing volume, and controlled with a mechanical timer Omnirex (Legrand, France). The calcium hydroxide reactor supplies the water in the experimental system with a solution composed by calcium hydroxide and freshwater purified by a reverse osmosis unit (Aqua-win RO-6080).

Depending on the use of natural or synthetic seawater, water chemistry can be adjusted to fulfil different experimental requirements by supplying additives, either to the main water reservoir before use (Fig. 2.1.2F) or to the filter tank of each module (Fig. 2.1.1C), depending on the configuration of the culture system.

Salinity is maintained by employing an osmoregulator (Deltec Aquastat 1000) (Fig. 2.1.1M) that automatically compensates evaporated water by dosing freshwater purified by a reverse osmosis unit (Aqua-win RO-6080). The modular culture system is designed to operate with an osmoregulator in: a) the large sized water reservoir when the experiment is performed in "*Flow-through system*" (Fig. 2.1.2F); b) each experimental tank, when the experimental system operates with the "*Recirculated system using independent experimental tanks*" configuration (Fig. 2.1.3E); c) filter tank of each experimental module for experiments in "*Recirculated system with independent modules of experimental tanks*" configuration (Fig. 2.1.1M).

Water temperature is maintained through the use of submergible heaters, water chillers and room temperature control. When the experiment is performed in the individualized

experimental tanks in flow-through open system, or in tanks with individual recirculating system, the temperature is controlled with a submergible heater in each tank (Eheim Jäger 150W, Germany) (Fig. 2.1.2E and Fig. 2.1.3D, respectively), complemented by room acclimatization at a lower temperature than that desired for the experimental set up (as the water inside each tank can only be heated; using an individual chiller per culture tank would be a significant financial burden for setting-up and operating the system). For experiments performed with the experimental tanks connected to the filter tank, the temperature is controlled in the filter tank with a submergible heater (Eheim Jäger 300W, Germany) (Fig. 2.1.1N) and a water chiller (Teco TR10, Italy) (Fig. 2.1.1O).

- *Nutrition*

The modular system design allows the performance of a wide range of experiments focusing on nutritional requirements of different cultured coral species, such as: a) heterotrophy, by testing different phytoplankton or zooplankton organisms as live prey, as well as a range of formulated feeds; it may also be possible to manipulate the concentration of particulate organic matter (POM), as it is hypothesized that this may also be an important source of exogenous food for corals; b) heterotrophy vs. autotrophy, by combining exogenous feeding and variable light regimes (autotrophic feeding provided through endosymbiotic zooxanthellae); c) autotrophy, see subsection "*Artificial illumination*"; and d) nutrition and zootechnical conditions, by combining heterotrophic feeding with several zootechnical factors (such as the use of protein skimmers, water movement and variable physical and chemical water parameters). The possibility to work either in a flow-through or a recirculated system (with an individual life support system per culture tank) allows researchers to have independent replicates for each experimental treatment. The number of modular culture systems can be adjusted to each experiment, allowing researchers to test numerous factors simultaneously in complex experimental designs (e.g., factorial experiments).

2.1.3. Modular System implementation and operational costs

The described modular culture system can be easily reproduced all over the world due to the use of materials and equipment which are easily available in the marine aquarium trade (either in wholesale or retail aquarium shops or the World Wide Web). Several research facilities addressing coral production are not located near the coast line and/or have significant space limitations to set up their culture systems, as well as limited budgets for assembling and operating these systems. The modular culture system presented can be implemented in a relatively reduced space (an area of 6 m^2 per module is adequate) and its implementation costs are moderate (~ 9000 € per module).

Assuming 0.098 € per kilowatt/hour (kW h) to be the average base price of electricity (excluding VAT) in the European Union, we can estimate an operational cost per month of approximately 110.00 € (excluding VAT) with electric power for the 3 modular culture systems operating interconnected. To prepare the volume of synthetic seawater required for 1 month of utilisation we used approximately 1800 L of reverse osmosis purified water (produced from about 7200 L of tap water; assuming an average price of 1.5 €/m^3 for tap water, the total cost associated with the production of reverse osmosis purified water was 10.80 €) and about 60 kg of salt (costing approximately 165.00 € wholesale price, excluding VAT). Overall, the operational cost for 1 month of experiment is approximately 290 €.

2.1.4. Modular System advantages

The main advantage of the modular culture system presented here is its versatility, as it allows researchers to perform short or long term experiments with a wide range of hermatypic or ahermatypic coral species on all major topics related with coral production (e.g. growth, photobiology, physiology).

While the use of modular systems for coral culture and experimentation has already been recognized (Luckett et al., 1996), the reproducibility and comparison of results obtained from experimental studies could certainly be improved if similar systems were employed by research groups addressing similar questions. As examples, we can refer the studies

conducted by Khalesi et al. (2009) and Rocha et al. (2013b) addressing the importance of light on the optimization of the captive culture of the symbiotic soft coral *Sinularia flexibilis*. While the findings reported by both studies are important to optimize the production of *S. flexibilis ex situ*, the use of different culture systems impairs the establishment of an optimized culture protocol. It is noteworthy that such standardization of experimental set-ups, procedures and designs is even more important when addressing coral culture for either the bioprospecting or the production of bioactive compounds. Symbiotic microorganisms present in the coral host are recognized to play a fundamental role in the regulation and production of certain bioactive compounds (Dunlap et al., 2007; Newberger et al., 2006). However, coral's microbiome is prone to dramatic shifts if its host organims is stocked under different biotic or abiotic conditions. In this way, it is important that researchers employ similar culture systems, so they can easily replicate optimal conditions for culturing their target species without disrupting its mircobiome structure and richness.

Overall, by employing standardized culture conditions and performing statistically robust experiments, researchers from different groups will be able to compare collected data in a more reliable way and advance the current state of the art on this research field.

Supplementary Material

Table 2.2.1. Artificial life support system main components, their manufacturers and suppliers.

Equipment	Manufacturer/Model	Supplier
Reverse osmosis unit	Aqua-win RO-6080	www.aquawin.com.tw
Water pump	Aquabee UP 2000/1	www.aquabee-aquarientechnik.de
Water pump	Aquabee UP 2000	www.aquabee-aquarientechnik.de
Water pump	EHEIM 1262	www.eheim.com
Circulation pump	Tunze Turbelle nannostream - 6025	www.tunze.com
Heater	Eheim Jäger 150W	www.eheim.com
Heater	Eheim Jäger 300W	www.eheim.com
Refrigerator	Teco TR10	www.tecoonline.eu
Hang-on protein skimmer	MCE 600 Deltec	www.deltec-aquaristik.com
Protein skimmer	ESC150 ReefSet	www.reefset.com
Luminaire systems	ReefSET® LM HQI 150 (1 × 150 W)	www.reefset.com
Fluidized sand reactor	FLF100 ReefSet	www.reefset.com
Fluidized sand reactor	FLF200 ReefSet	www.reefset.com
Calcium reactor	Deltec PF 501, Germany	www.deltec-aquaristik.com
pH controller	Tunze Controller-Set (7074/2)	www.tunze.com
CO_2 bottle	V² Cylinder 567g (CGA320 conn.)	www.tropicalmarinecentre.co.uk
Calcium hydroxide reactor	KM500 Deltec	www.deltec-aquaristik.com
Dosing pump	Grotech TEC 1 NG	www.grotech-aquarientechnik.de
Osmoregulator	Deltec Aquastat 1000	www.deltec-aquaristik.com
Synthetic Sea Salt	Tropic Marin Pro Reef salt	www.meisalt.com
PVC valves (Solvent socket)	PVC-U ball valve (Pressure) tap 20 mm	Local store
PVC valves (Solvent socket)	PVC-U ball valve (Pressure) tap 50 mm	Local store
Union (Solvent socket)	PVC-U (Pressure) 20 mm	Local store
Union (Solvent socket)	PVC-U (Pressure) 40 mm	Local store
Union (Solvent socket)	PVC-U (Pressure) 50 mm	Local store
90º Elbow (Solvent socket)	PVC-U (Pressure) 20 mm	Local store
90º Elbow (Solvent socket)	PVC-U (Pressure) 40 mm	Local store
90º Elbow (Solvent socket)	PVC-U (Pressure) 50 mm	Local store
90º Tee (Solvent socket)	PVC-U (Pressure) 40 mm	Local store
90º Tee (Solvent socket)	PVC-U (Pressure) 50 mm	Local store
90º Tee (Solvent socket)	PVC-U (Pressure) 63 mm	Local store
90º Red. Tee (Solvent socket)	PVC-U (Pressure) 40 x 20 mm	Local store
Conical Reducer (Solvent	PVC-U (Pressure) 50 x 40 mm	Local store
Spigot Connect. (Solvent socket)	PVC-U (Pressure) 20 x 20 mm	Local store
PVC Solvent Cement	TANGIT® PVC-U	Local store
PVC Pipe	PVC-U (PN 10) 20, 40, 50 and 63 mm	Local store
Threaded Bulkhead	PVC 1 $^{1/2}$"	Local store
90º Tee (solvent socket - fem. thread)	PVC-U (Pressure) 50 mm × 1 $^{1/2}$" × 50 mm	Local store
Experimental culture tank	8 mm glass, glued with neutral silicone	Local store
Filter tank	8 mm glass, glued with neutral silicone	Local store
Saltwater Reservoir	10 mm glass, glued with neutral silicone	Local store
Structure	Stainless Steel 118 structure 40 x 2mm	Local store
PVC panel	PVC black panels (6mm)	Local store

Chapter 3

Effect of light intensity and emission spectra in *ex situ* culture of soft corals

Chapter 3

3.1. Effect of light intensity on post-fragmentation photobiological performance of the soft coral *Sinularia flexibilis*

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Abstract

The soft coral *Sinularia flexibilis* is currently considered as a suitable candidate for aquaculture. This soft coral is commonly traded for marine aquariums, is used in reef restoration efforts, as well as in the bioprospecting of marine natural products. The production of this coral under controlled laboratory conditions may be the best option for a sustainable and continuous supply of its biomass. It is known that the fragmentation of corals harboring photosymbiotic unicellular dinoflagellates of genus *Symbiodinium*, commonly termed zooxanthellae, can be influenced by light, as the photosynthetic performance of zooxanthellae can affect coral physiology and growth. This study aimed to investigate the effect of different light intensities on the photobiology of *S. flexibilis* following *ex situ* fragmentation. *S. flexibilis* mother colonies were fragmented after being acclimated for 5 months to a photoperiod of 12 hours light with an irradiance of 120 μmol quanta m^{-2} s^{-1}. Fragments were then distributed by three light treatments (50, 80 and 120 μmol quanta m^{-2} s^{-1}) for monitoring their photosynthetic performance, photosynthetic and accessory pigment concentration, zooxanthellae density, and growth. No significant differences were recorded one month post-fragmentation on the maximum quantum yield of PSII (F_v/F_m), neither on zooxanthellae density, between fragmented corals placed under tested light intensities. However, zooxanthellae density significantly increased after 5 months in fragments exposed to 50 and 80 μmol quanta m^{-2} s^{-1}, while F_v/F_m and pigment concentration decreased under the highest light intensity (120 μmol quanta m^{-2} s^{-1}). This study showed that the use of low light levels after fragmentation, in the absence of heterotrophic feeding, do not significantly affect coral growth. Moreover, light levels used after fragmentation should be adjusted according to the intended stocking time of produced coral fragments.

Keywords

Fragmentation; Sinularia; Zooxanthellae; Photosynthetic pigments; Light intensity

3.1.1. Introduction

The increasing demand for soft corals (Octocorallia), either for biotechnological research on marine natural products (Blunt et al., 2008, 2009) or to supply the marine aquarium trade (Wabnitz et al., 2003), has prompted an increase on their harvest (Castanaro and Lasker, 2003). However, the dependence on organisms collected from the wild compromises the sustainability of this approach. In this way, it has been recommended that future research on marine natural products should consider the use of specimens produced in captivity (Mendola, 2003; Proksch et al., 2003) and that the marine aquarium industry should promote the trade of cultured soft corals, rather than wild specimens (Calfo, 2007; Olivotto et al., 2011).

In this context, coral aquaculture can be a potential solution for a continuous and sustainable supply of soft coral biomass (Sella and Benayahu, 2010). Coral propagation by asexual reproduction is a relatively simple and inexpensive process, which has been commonly used for the production of new colonies, with a high survival rate of fragments and a reduced impact on mother colonies (Fox et al., 2005; Soong and Chen, 2003). Coral fragments can be produced either *in situ* or *ex situ*. *In situ* fragmentation and grow-out may benefit from natural environmental conditions and requires no adaption to artificial propagation systems. However, fragments are exposed to potential deleterious factors, such as sedimentation, pathogens, predators, competitors and other natural hazards, which can reduce survival (Rinkevich, 2005). In contrast, *ex situ* fragmentation has the advantage of maximizing survival and growth rates through the manipulation of culture conditions, such as light, flow and food availability (Forsman et al., 2006; Khalesi, 2008a).

Light is a key factor for symbiotic corals due to their association with photosymbiotic unicellular dinoflagellates from genus *Symbiodinium* (commonly termed as zooxanthellae) (Osinga et al., 2011; Schutter et al., 2012; Wijgerde et al., 2012). The photosynthates produced by the zooxanthellae are transferred to the coral host and fulfil a significant part of its energetic requirements (Falkowski et al., 1984; Hoogenboom et al., 2006). Light variation is known to affect zooxanthellae density, photosynthetic pigments concentration and photosynthetic efficiency (Frade et al., 2008b; Kühl et al., 1995; Lesser et al., 2010).

Ultimately, changes in the density of zooxanthellae can affect coral physiology and its response to stress (Venn et al., 2008). As the fragmentation process *per se* induces stress to both coral mother colony and produced fragments, it is expected that light can play an important role on the post-fragmentation photophysiological processes and, therefore, on coral recovery.

The captive culture of the soft coral *Sinularia flexibilis*, one of the dominant benthic invertebrate inhabitants of Indo-Pacific reefs (Bastidas et al., 2004; Van Ofwegen, 2002) has been recently addressed by several studies (Khalesi, 2008b, a; Khalesi et al., 2009). However, a gap of knowledge persists in some practical issues related to coral recovery post-fragmentation. The present study aimed to investigate the effect of different light intensities in the physiology and photobiology of the soft coral *S. flexibilis* following *ex situ* fragmentation, namely photosynthetic performance, zooxanthellae density, photosynthetic and accessory pigments concentration and coral fragments growth.

3.1.2. Materials and methods

- *S. flexibilis husbandry and fragmentation*

Five colonies of *Sinularia flexibilis* were kept for 5 months in a recirculating system with synthetic saltwater (prepared by mixing Tropic Marin Pro Reef salt – Tropic Marine, Germany – and freshwater purified by a reverse osmosis unit). The glass tank holding the mother colonies (90 L water volume) was connected to a 100 L filter tank. The mother colonies' tank was equipped with a circulation pump (Turbelle nannostream- 6025 Tunze, Germany), which provided an approximate water flow of 2500 L h^{-1}. The filter tank was equipped with a protein skimmer (APF-600 Deltec, Germany), a biological filter (composed by 20 kg of live rock and submerged bio-balls), two submersible heaters (Eheim Jäger 300 W, Germany) and a submerged pump (EHEIM 1260, Germany) that supplied a flow of approximately 1500 L h^{-1} to the coral stocking glass tank. The tank holding the mother colonies was illuminated with a 150 W (10000 K) metal halide lamp (BLV, Germany) delivering a Photosynthetic Active Radiation (PAR) of 120 μmol quanta m^{-2} s^{-1} at the level of

the colonies with a 12h light : 12h dark photoperiod. Salinity was maintained at 35 using an osmoregulator (Deltec Aquastat 1000) that provided automatic compensation of evaporated water with freshwater purified by a reverse osmosis unit. Other water parameters were maintained as follows: temperature 26 ± 0.5 °C, Total Ammonia Nitrogen 0.05 ± 0.01 mg L^{-1}, NO_2^-–N 0.03 ± 0.01 mgL^{-1}, NO_3^-–N 1.0 ± 0.1 mgL^{-1}, PO_4^{3-}–P 0.01 ± 0.01 mgL^{-1}, pH 8.2 ± 0.2, alkalinity 3.90 ± 0.20 mEq L^{-1}, Ca^{2+} 420 ± 20 mg L^{-1}, Mg^{2+} 1300 ± 20 mg L^{-1}.

S. flexibilis colonies were fragmented using a scalpel producing 6 similar sized fragments (about 10 cm) per colony, with each one being individually attached with a rubber band to a plastic coral stand (Coral Cradle®), labelled and randomly distributed among the different experimental treatments (see below).

- *Experimental design*

Twenty-seven fragments of the pool of 30 fragments produced (6 fragments x 5 mother colonies) were randomly selected and distributed by the stocking tanks of the 3 coral propagation modules, each with 3 stocking tanks. Each tank was stocked with 3 coral fragments. Each coral propagation module was composed by three 90 L glass tanks (0.6 m x 0.6 m x 0.25 m) connected to a 150 L filter tank equipped with a protein skimmer (ESC150 ReefSet, Portugal), a biological filter (composed by 30 kg of live rock and submerged bio-balls), two submergible heaters (Eheim Jäger 300W, Germany), a calcium hydroxide reactor (KM500 Deltec, Germany) connected to an osmoregulator (Deltec Aquastat 1000) and a submerged pump (EHEIM 1262, Germany; providing an approximate flow of 1000 $L.h^{-1}$ to each tank). Additionally, each tank was equipped with a single circulation pump (Turbelle nannostream - 6025 Tunze, Germany; approximate flow of 2500 $L.h^{-1}$). Each tank was illuminated from above with a 150 W (10000 K) metal halide lamp (BLV, Germany) with 12h light: 12h dark photoperiod. The distance between the lamps and water surface were adjusted to provide one of the following PAR intensities (±5%) at the level of coral fragments: 50, 80 and 120 μmol quanta m^{-2} s^{-1}. During the experiment, PAR values were measured once a week at the level of coral fragments using a Quantum Flux meter (Apogee, MQ-200) with a submergible sensor. The position of each coral fragment in the tank was adjusted so that all fragments in each light treatment had the same PAR value. The use of

three independent modules allowed the use of three replicates per light intensity treatment, each replicate being composed by three coral fragments. Water parameters were kept as described above for mother colonies. Partial water changes using synthetic saltwater (10% of total experimental system volume) were performed every week. The experiment was performed during 5 months.

- *In vivo chlorophyll fluorescence*

Pulse Amplitude Modulation fluorometry (PAM) was used to monitor photosynthetic activity by measuring non-intrusively variable chlorophyll fluorescence (Schreiber et al., 1986). The PAM fluorometer comprised a computer-operated PAM-Control Unit (Walz) and a WATER-EDF-Universal emitter-detector unit (Gademann Instruments, GmbH, Würzburg, Germany) (Cruz and Serôdio, 2008). Measuring, actinic and saturating lights were 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 fiber optics bundle. The fiber optic was positioned perpendicularly to the surface of the coral cutting. Measurements were carried out 2 h after the start of the daylight period, to ensure the full activation of the photosynthetic apparatus, in 9 different points in the initial coral colonies immediately before fragmentation (T0), and in the coral fragments 1 month (T1) and 5 months (T5) after fragmentation and transfer to the experimental system. Coral fragments were kept in a recirculating water bath (Frigiterm-10, Selecta, Spain) at 25 ºC during PAM measurements. At each measuring occasion, corals were dark-adapted for 15 min, after which one saturation pulse (0.8 s) was applied to determine the minimum- or dark-level fluorescence, F_o, a parameter expected to correlate with the Chl *a* content (Serôdio et al., 2001), and the maximum fluorescence, F_m. F_o and F_m were used to determine the maximum quantum yield of PSII (Schreiber et al., 1986):

$$F_v/F_m = \frac{(F_m - F_o)}{F_m} \qquad (1)$$

- *Zooxanthellae*

Following *in vivo* PAM measurements, a sample of coral tissue was removed with a scalpel to analyse zooxanthellae density and determine photosynthetic and accessory pigments (see

below). Zooxanthellae density was assessed in 9 tissue fragments of the initial coral colonies immediately before fragmentation (T0), at T1 and at T5. The time between sampling events allowed the complete cicatrisation and total recovery of surveyed coral fragments. Sampled coral tissue was homogenised in tubes containing 15 mL of filtered (0.2 μm) seawater. The homogenate was diluted and homogenized before zooxanthellae counting in a hemacytometer with improved Neubauer ruling (5 cell counts for each coral fragment). After counting the total volume of each sample was centrifuged (10 min, 5000 rpm), the supernatant water was discard and the pellet freeze-dried for 24 h to determine total dry weight. Zooxanthellae concentration was normalized to *S. flexibilis* dry weight.

The genotype of the *Symbiodinium sp.* (commonly termed as zooxanthellae) harboured by *S. flexibilis* mother colonies was identified by sequencing of the entire ITS1-5.8S-ITS2 region of the ribosomal gene according with Santos et al. (2001).

- *Photosynthetic and accessory pigments*

The concentration of the following photosynthetic and accessory pigments was determined in coral colonies before fragmentation (T0) and in coral fragments at T1 and at T5: chlorophyll *a* (Chl *a*), chlorophyll c_2 (Chl c_2), diadinoxanthin (DD), diatoxanthin (DT), peridinin (Per) and β-carotene (β -Car). Freeze-dried samples of 0.04 to 0.12 g were extracted with 3-5 mL of 95% cold buffered methanol (2% ammonium acetate) for 30 min at –20 °C in total darkness. Samples were sonicated (Bransonic, model 1210) for 30 s at the beginning of the extraction period. Extracts were filtered (Fluoropore PTFE filter membranes, 0.2 μm pore size) and immediately injected in a Shimadzu HPLC system with photodiode array (SPD-M10AVP) and fluorescence (RF-10AXL) detectors. Chromatographic separation was carried out using a C18 column for reverse phase chromatography (Supelcosil; 25 cm long; 4.6 mm in diameter; 5 μm particles) and a 35 min elution programme. The solvent gradient followed (Kraay et al., 1992) with a flow rate of 0.6 mL min^{-1} and an injection volume of 100 μL. Pigments were identified from absorbance spectra and retention times and concentrations calculated from signals in the photodiode array detector. Calibration of the HPLC peaks was performed using commercial standards from DHI (Institute for Water and Environment, Hørsholm, Denmark).

- *Coral fragments growth*

Buoyant weight measurements (Spencer Davies, 1989) were made using a Kern Emb 200-3 balance (Kern & Sohn GmbH) to determine the growth of coral fragments between T0 and T1, and between T1 and T5. The buoyant weights of each coral cradle and rubber bands used to attach each coral fragment was also determined prior to fragmentation. The buoyant weight of all coral fragments was determined and corrected with the weight of the respective cradle and rubber band to obtain net and total weights. Coral cradles were cleaned thoroughly with seawater and a tooth-brush the day before each measurement, in order to minimize any potential bias promoted by the development of biofouling. To ensure reproducibility, each coral fragment was weighted 3 times at T0-5. Water temperature and salinity were kept stable during all buoyant weight measurements. To calculate the percentage of daily coral growth (% CG day^{-1}) for each coral fragment, the following formula was used:

$$\% \, CG \; day^{-1} = \left(\left(\frac{w_f - w_i}{w_i} \right) / \; \Delta t \right) \times 100 \quad (2)$$

where w_f and w_i are the final and initial coral net weights expressed in grams (g), and Δt is the time interval in days. CG is expressed in percentage of coral weight increase per day.

- *Statistical analysis*

Statistical analyses were carried out using the software Statistica version 8.0 (StatSoft Inc.). Repeated measurements ANOVAs was used to evaluate the existence of significant differences in the maximum quantum yield of PSII (F_v/F_m), zooxanthellae density, photosynthetic pigment concentrations, relation between zooxanthellae and photosynthetic pigments, and coral growth recorded for fragments of *S. flexibilis* kept with different light regimes. Mauchly's test of sphericity was used to determine if the variances of the differences between all combinations of related groups were equal. Post hoc Tukey HSD test was used to determine differences between light treatments and between sampling points of each treatment. When the assumptions of homogeneity of variances and homoscedasticity were

not met, data transformation methods were used. Particularly, zooxanthellae counting and photosynthetic pigment data were square root and log transformed, respectively.

3.1.3. Results

- *In vivo chlorophyll fluorescence*

Maximum quantum yields of PSII (F_v/F_m) measured on *S. flexibilis* exposed to different light intensities are presented in figure 3.1.1. At the end of the experiment (T5) *S. flexibilis* fragments reared under the lowest PAR treatment (50 μmol quanta m^{-2} s^{-1}) showed significantly higher F_v/F_m values ($P < 0.001$) when compared to the fragments reared under the highest PAR (120 μmol quanta m^{-2} s^{-1}). During the experimental period the mean values of F_v/F_m decreased significantly ($P < 0.001$) in the corals under the highest PAR light treatment (120 μmol quanta m^{-2} s^{-1}).

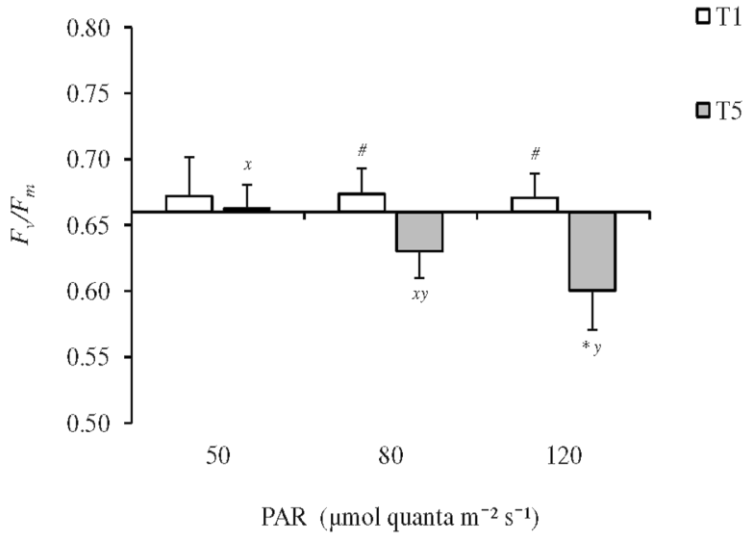

Fig. 3.1.1. Average values of maximum quantum yield of PSII (F_v/F_m) measured on 9 *S. flexibilis* fragments (one month – T1 and 5 months – T5 after the beginning of the experiment) exposed to three different light treatments (50, 80 and 120 μmol quanta m^{-2} s^{-1}). Horizontal axis cross vertical axis in the mean value obtained before fragmentation –T0 (standard deviation = 0.025). n = 9 coral fragments per light treatment. Vertical lines represent standard deviation. Significant different from T0 are marked (*); # represents significant differences between T1 and T5 in the same light PAR treatment; different superscript letters represents significant differences within the same time, ($P < 0.001$ for all comparisons; Tukey HSD post-hoc comparisons).

- *Zooxanthellae*

The reduction of photosynthetic active radiation (PAR) increased zooxanthellae density (Fig. 3.1.2). Zooxanthellae concentration in *S. flexibilis* fragments reared with a PAR of 50 μmol quanta m^{-2} s^{-1} was significantly higher ($P < 0.01$) at the end of the experiment (T5) ($3.09\times10^8 \pm 0.80\times10^8$ cell g^{-1} DW; average ± standard deviation) when compared to cell concentration at T0 ($2.46\times10^8 \pm 0.16\times10^8$ cell g^{-1} DW). At the end of the experiment the fragments reared under a PAR of 80 μmol quanta m^{-2} s^{-1} also showed significantly higher ($P < 0.001$) zooxanthellae density ($3.20\times10^8 \pm 0.65\times10^8$ cell.g^{-1} DW) when compared to results at T0 ($2.46\times10^8 \pm 0.16\times10^8$ cell g^{-1}). No significant differences were registered throughout the experiment for fragments reared under the highest PAR (120 μmol quanta m^{-2} s^{-1}). At T5 corals reared under 50 and 80 μmol quanta m^{-2} s^{-1} PAR had significantly higher zooxanthellae density ($P < 0.01$) when compared to corals reared under the highest PAR (120 μmol quanta m^{-2} s^{-1}), as shown in figure 3.2.

Symbiodinium sp. present in the *S. flexibilis* were identified as type B_1 after their successful genotyping.

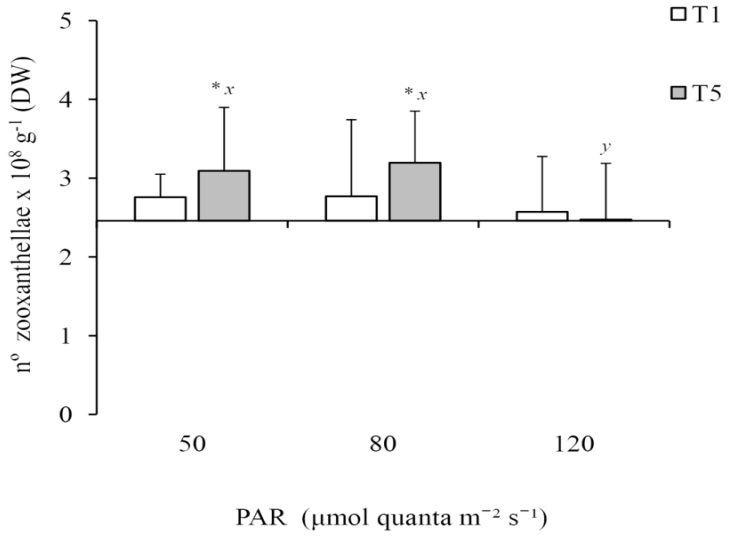

Fig. 3.1.2. Zooxanthellae density (per gram of coral dry weight). Average measurements made on 9 *S. flexibilis* fragments per light treatment (50, 80 and 120 μmol quanta m^{-2} s^{-1}). Vertical lines represent one standard deviation. Horizontal axis cross vertical axis in the mean value obtained before fragmentation –T0 (standard deviation = 0.155). Significant different from T0 are marked (*); # represents significant differences between T1

and T5 in the same light PAR treatment; different superscript letters represents significant differences within the same time, ($P < 0.01$; Tukey HSD post-hoc comparisons) in and between light treatments during the experimental period (T0– beginning , T1 – one month and T5 – 5 months after the beginning of the experiment).

- *Photosynthetic and accessory pigments*

The results of photosynthetic pigments analysis are displayed in figure 3.3. Overall, each pigment concentration ($\mu g.g^{-1}$ DW) increased in all light treatments one month after fragmentation and decreased after 5 months. The concentration of Chl *a* recorded at T5 under the lowest light intensity treatment (50 μmol quanta m^{-2} s^{-1}) was significantly higher ($P < 0.001$) than the mean value obtained for *S. flexibilis* fragments reared with the highest PAR value (120 μmol quanta m^{-2} s^{-1}). Under 80 and 120 μmol quanta m^{-2} s^{-1}, Chl *a* concentration was significantly lower at T5 than at T1 ($P < 0.001$). The mean value of Chl c_2 concentration (Fig. 3.1.3-B) in the light treatment with the lowest PAR value (50 μmol quanta m^{-2} s^{-1}) did not change significantly during the experimental period. The mean value of Chl c_2 (Fig. 3.1.3-B) concentration in the light treatment with the highest PAR (120 μmol quanta m^{-2} s^{-1}) was significantly higher at T1 than in the mother colonies, T0 ($P < 0.05$). At the end of the experiment, Chl c_2 concentration was significantly lower ($P < 0.001$) than results observed at T0 and T1. βCar concentration (Fig. 3.1.3-C) in coral fragments reared under a PAR of 80 and 120 μmol quanta m^{-2} s^{-1} were significantly lower at T5 than at T1 ($P < 0.01$) and T0 ($P < 0.05$). The mean values of DD (Fig. 3.1.3-D) and peridinin concentration (Fig. 3-E) did not change significantly for *S. flexibilis* fragments reared under the lowest light PAR treatment (50 μmol quanta m^{-2} s^{-1}). However, in fragments reared under a PAR of 80 μmol quanta m^{-2} s^{-1}, the mean values of both these two photosynthetic pigments were significantly higher at T1 than at T5 ($P < 0.001$ and $P < 0.01$ for peridinin and DD, respectively), as well as those determined for mother colonies (T0) ($P < 0.001$ and $P < 0.05$ for peridinin and DD, respectively). *S. flexibilis* fragments reared under 80 and 120 μmol quanta m^{-2} s^{-1} showed higher DD and peridinin concentration at T1 than at T0 and T5 ($P < 0.05$ and $P < 0.01$) for DD and peridinin, respectively.

- *Photosynthetic pigments/Zooxanthellae*

The results of the ratio of photosynthetic pigment (µg) per zooxanthellae (both normalized to *S. flexibilis* DW) were similar to those described above for photosynthetic pigment alone and are represented in figure 3.1.4. No significant differences were recorded throughout the experiment for any of the tested ratios in coral fragments cultured under a PAR of 50 µmol quanta m^{-2} s^{-1}. Significantly lower values ($P < 0.05$) of µg Chl *a* per zooxanthellae (Fig. 3.1.4-A), µg Chl c_2 per zooxanthellae (Fig. 3.1.4-B), µg DD per zooxanthellae (Fig. 3.1.4-D) and µg peridinin per zooxanthellae (Fig. 3.1.4-E) were found in coral fragments reared under the PAR values of 80 and 120 µmol quanta m^{-2} s^{-1} at the end of the experiment, when compared to the values recorded at T1 results.

- *Coral fragments growth*

The mean values of coral growth registered in the coral fragments (% CG mean ± standard deviation, n = 9 coral fragments per treatment) in the first time interval (between T0 and T1) were 0.042 ± 0.012% day^{-1}, 0.041 ± 0.009%.day^{-1} and 0.043 ± 0.014%.day^{-1} for coral fragments from the light PAR treatments of 50, 80 and 120 µmol quanta m^{-2} s^{-1}, respectively. In the second time interval (between T1 and T5) the mean values of coral growth were 0.040 ± 0.010%.day^{-1}, 0.039 ± 0.012%.day^{-1} and 0.039 ± 0.009%.day^{-1} for coral cuttings from the light PAR treatments of 50, 80 and 120 µmol quanta m^{-2} s^{-1}, respectively. No significant differences were found in the growth of coral fragments between light PAR treatments in both time intervals (T0 to T1 and T1 to T5).

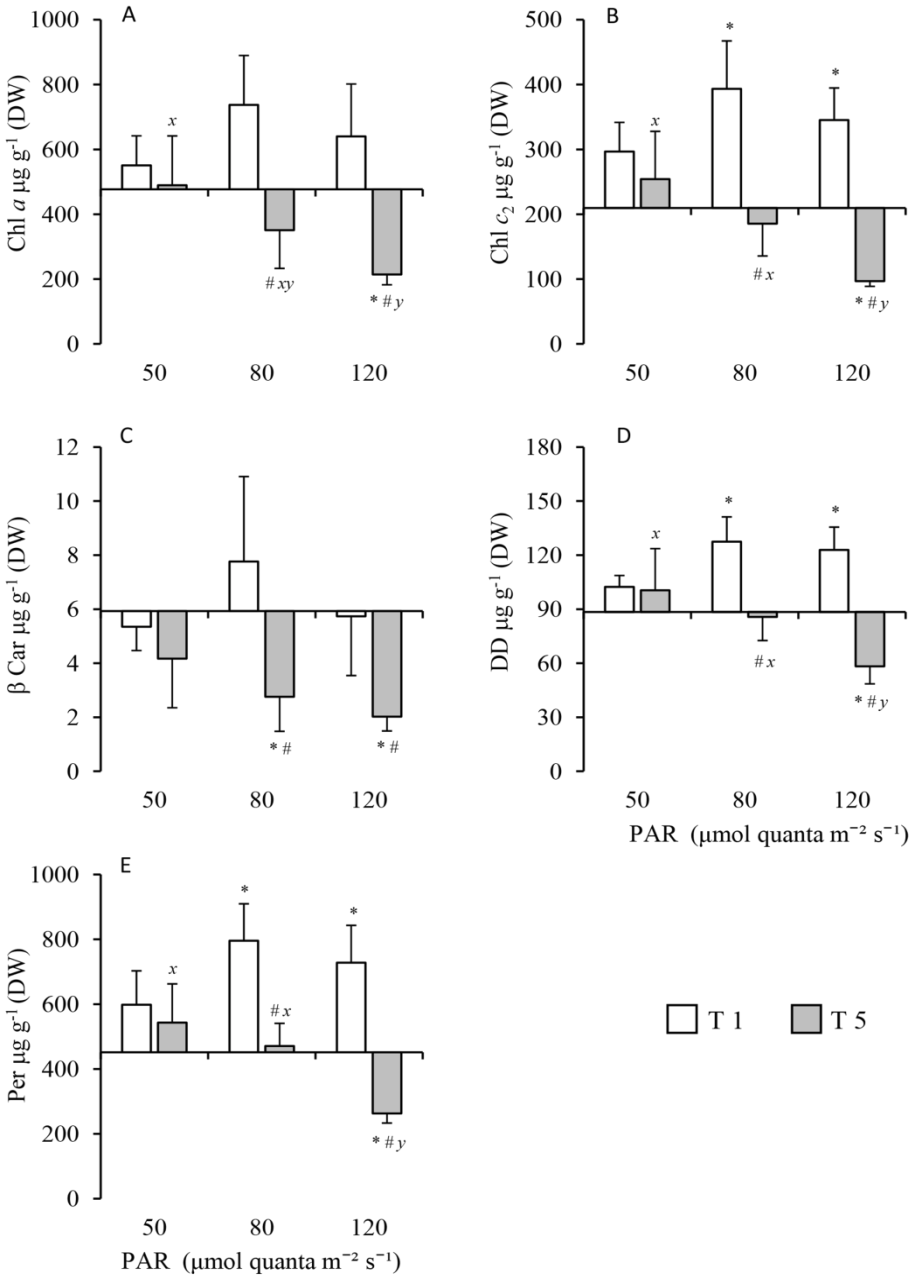

Fig. 3.1.3. Average concentration of photosynthetic pigments (µg.g^{-1} soft coral dry weight) measured at T0 (beginning of the experiment, before fragmentation) , T1 (one month) and T5 (5 months) after fragmentation in 9 *S. flexibilis* fragments from each light treatment (A) chlorophyll *a* (Chl *a*), (B) chlorophyll c_2 (Chl c_2), (C) *β*-carotene (*β*-Car), (D) diadinoxanthin (DD) and (E) peridinin (Per.). Horizontal axis cross vertical axis in the mean value obtained before fragmentation –T0 (standard deviation was 45.05, 29.84, 0.76, 13.11 and 83.10 for Chl *a*, Chl c_2, *β*-Car, DD and Per., respectively). Vertical lines represent one standard deviation. Significant different from T0 are marked (*); # represents significant differences between T1 and T5 in the same light PAR treatment; different superscript letters represents significant differences within the same time, ($P < 0.05$; Tukey HSD post-hoc comparisons).

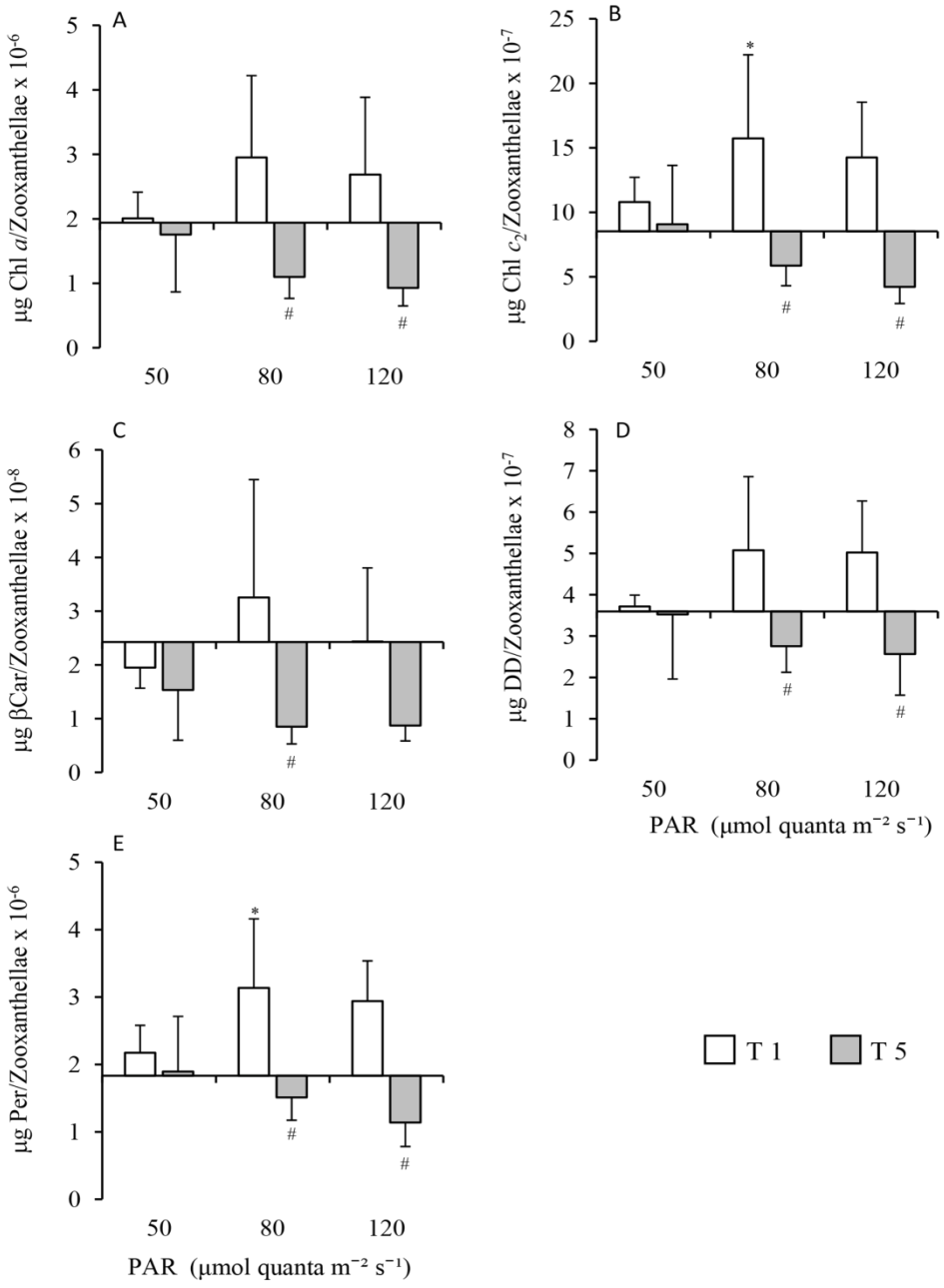

Fig. 3.1.4. Average ratios of photosynthetic pigment (µg) per zooxanthellae (both normalized to *S. flexibilis* dry weight in the three sampling points (T0, T1 and T5) in the 9 fragments in each light treatment (50, 80 and 120 µmol quanta m^{-2} s^{-1}). (A) chlorophyll *a* (Chl *a*), (B) chlorophyll c_2 (Chl c_2), (C) *β*-carotene (*β*-Car), (D) diadinoxanthin (DD) and (E) peridinin (Per.). Horizontal axis cross vertical axis in the mean value obtained before fragmentation –T0 (standard deviation was 0.17×10^{-6}, 1.06×10^{-7}, 0.39×10^{-8}, 0.45×10^{-7} and 0.29×10^{-6} for Chl *a*, Chl c_2, *β*-Car, DD and Per., respectively). Vertical lines represent one standard deviation. Significant different from T0 are marked (*); # represents significant differences between T1 and T5 in the same light PAR treatment; different superscript letters represents significant differences within the same time, ($P < 0.05$; Tukey HSD post-hoc comparisons).

3.1.4. Discussion

An excessive increase in light levels is known to commonly damage the photosynthetic apparatus of zooxanthellae, while an increase in zooxanthellae density is usually recorded when corals are exposed to suboptimal light intensities (Frade et al., 2008a; Hoegh-Guldberg and Jones, 1999). High and low light levels will ultimately lead to an adaptive response of the coral holobiont, either through the action of photoprotective mechanisms (such as the increase of photoprotective pigments) or adapting the photosynthetic apparatus to maximize light capture (Titlyanov and Titlyanova, 2002a). According to the previous authors, acclimation to low light involves the maximization of the light harvesting capacity through: 1) the increase of photosynthetic pigment concentration in zooxanthellae; and 2) the multiplication of zooxanthellae (increased density). However, while changes in pigment concentrations present in zooxanthellae usually occur within 2–4 days, changes in the number of zooxanthellae (thus zooxanthellae densities) only commonly occur within 40 days (Titlyanov et al., 2001).

In the present study, the soft coral *S. flexibilis* was initially adapted to a light intensity of 120 μmol quanta m^{-2} s^{-1}. Overall, results of photosynthetic efficiency of zooxanthellae and zooxanthellae density one month after fragmentation were similar to those recorded prior to fragmentation. This suggests a recovery from fragmentation stress to initial photophysiological conditions within a one month period. However, five months after fragmentation, significantly lower photosynthetic efficiencies were observed in corals reared on 120 μmol quanta m^{-2} s^{-1}. While no significant changes on zooxanthellae concentration were observed, an increasing concentration of photosynthetic and accessory pigments was observed at T1, even for corals from 120 μmol quanta m^{-2} s^{-1} PAR treatment. Despite the same PAR level being provided before and after fragmentation, the overshadow effect after fragmentation is expected to be lower in coral fragments than in large mother colonies, thus maximizing the light intensity reaching the coral tissue of fragments. The increasing trend observed for pigment concentration per zooxanthellae might have been promoted by the fragmentation process, as a result of the reduction of the overshadow effect (thus shifting the light environment affecting the coral tissue of produced fragments). The use of a lower PAR

after fragmentation induced a contrasting effect on the density of zooxanthellae as it significantly increased after 5 months in corals reared at 50 and 80 μmol quanta m^{-2} s^{-1}. These results are in accordance with previous studies that report an increase in zooxanthellae density to maximize light absorption under low PAR values (Titlyanov et al., 2001). In contrast, results for corals reared under the highest PAR level show decreasing pigment concentrations per zooxanthellae after 5 months, suggesting an adaptive response to high light levels (Titlyanov and Titlyanova, 2002b). Although the mother colonies of *S. flexibilis* were acclimated to a light intensity of 120 μmol quanta m^{-2} s^{-1}, this light level may be inadequately high to coral fragments and can potentially induce light stress. As part of light-protecting reactions it would be expected to observe an increase on *β*-Car concentration (Bandaranayake, 2006; Mobley and Gleason, 2003). Curiously, the concentration of this pigment decreased, which may be associated with a potential high light stress that may have prompted the zooxanthellae to reach a point of no return. As no fragment mortality was observed and, in general, photobiological performance decreased over time in higher light PAR treatments, we can hypothesize that fragmentation recovery processes took over light-stress photoprotective mechanisms. This scenario resulted in a decrease of photosynthetic efficiency and photosynthetic pigments concentration, which may ultimately culminate in a decrease of photosynthates translocation from endosymbiotic zooxanthellae to their cnidarians host (Levy et al., 2003; Titlyanov and Titlyanova, 2002a). The same authors reported that fragmented corals allowed to regenerate under lower light levels were able to acclimate and maximize light absorption. This acclimation probably promoted an increase on the production and translocation of photosynthetates and a larger contribution of autotrophy to the coral's mixotrophic nutrition (Levy et al., 2003).

No significant effects were recorded on coral growth between the different light treatments. Therefore, the present results suggest that the use of lower light levels can be a suitable option following fragmentation. Additionally, this option can contribute to decrease the costs associated with coral culture, as Osinga et al. (2011) have already reported that the use of artificial light is one of the factors influencing the economic viability of *ex situ* coral aquaculture. It must also be stressed that the trend recorded in the present work may be species specific, may shift for the same coral species harboring different zooxanthellae clades

or for corals recovering *in situ*. Kuguru et al. (2008) performed an experimental study using different light levels in the field and in the laboratory and found different photoacclimation results, particularly in terms of pigment concentration per zooxanthellae. Furthermore, as the morphology and physiology of symbiotic invertebrates can vary widely among species (Gates and Edmunds, 1999), a given *Symbiodinium* type may experience very dissimilar environments, depending on the symbiotic invertebrate species that it inhabits (Goulet et al., 2005).

3.1.5. Conclusion

Our work showed that keeping *S. flexibilis* fragments under the same light conditions as their mother colonies seems to be photobiologically acceptable for a short-term husbandry (e.g. when producing a large number of small sized fragments for research studies), lower light intensities than those used for mother colonies may favor the photobiological performance of coral fragments intended to be stocked for longer periods and contribute to a reduction of production costs (e.g. when producing large sized colonies that can yield a larger biomass production for biotechnological applications and need to be stocked in captivity for several months).

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Chapter 3

3.2. Photobiology and growth of leather coral *Sarcophyton* cf. *glaucum* fragments stocked under low light in a recirculated system

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Abstract

Corals are considered promising sources of new natural products and their culture, under controlled conditions, may be the solution for a sustainable and continuous supply of their biomass. Light is of utmost importance for *ex situ* production of corals harboring zooxanthellae, as their photosynthetic performance can significantly affect coral physiology and growth. The present study aimed to evaluate the effect of three light Photosynthetically Active Radiation (PAR) treatments (50, 80 and 120 μmol quanta m^{-2} s^{-1}) promoted by 150 W (10000 K) Hydrargyrum Quartz Iodide (HQI) lamps on the photobiology, survival and growth of the soft coral *Sarcophyton* cf. *glaucum* produced *ex situ* in recirculated systems. After 60 days of experiment coral fragments exposed to 50 μmol quanta m^{-2} s^{-1}presented significantly higher values of F_v/F_m and Normalized Difference Vegetation Index, zooxanthellae density and most photosynthetic pigment concentrations, when compared with corals under higher PAR values. No significant differences were found on coral fragment survival and growth. S. cf. *glaucum* production under low PAR intensities can effectively reduce the production costs with electrical power, and simultaneously maximize the production of photosynthetic pigments or bioactive compounds mediated by the zooxanthellae.

Keywords

Chlorophyll fluorescence; Coral propagation; Marine ornamentals; Photobiology; Photosynthetic pigments; Zooxanthellae

3.2.1. Introduction

The optimization of coral husbandry and production techniques has deserved the attention of the scientific community in the last decades. In opposition to other groups of marine organisms (e.g. fish, crustaceans or mollusks), corals can be easily reproduced asexually by fragmentation. This simple and inexpensive process has been commonly used by researchers, traders and marine aquarium hobbyists, for the mass production of corals, as it displays a high survival rate of produced fragments and a reduced impact on donor colonies (e.g. Calfo, 2007; Olivotto et al., 2011). Cultured corals are known to be highly prized for three main markets: a) the bioprospecting of new natural products with potential pharmacological and biomedical applications (Blunt et al., 2012; Blunt et al., 2013; Leal et al., 2013; Rocha et al., 2011); b) the marine aquarium trade (Olivotto et al., 2011; Rhyne et al., 2012); and c) coral reef restoration efforts (Rinkevich, 2005; Shafir et al., 2006).

Successful coral culture is influenced by numerous factors, such as water movement (Riegl et al., 1996), temperature (Sella and Benayahu, 2010), nutrients and heterotrophic feeding (Ferrier-Pages et al., 2003; Houlbrèque and Ferrier-Pages, 2009; Orejas et al., 2011), and light (Rocha et al., 2013a, 2013b; Schlacher et al., 2007; Schutter et al., 2012, 2008).

While the *ex situ* culture of corals involves higher production costs, it has the advantage of maximizing survival and growth rates through the optimization of culture conditions (Forsman et al., 2006). Light is considered as one of the most important factors influencing coral production *ex situ*. The costs associated with the implementation of lighting systems, maintenance, and electrical power consumption play a key role on the economic viability of coral aquaculture (Osinga et al., 2011). Additionally, the intensity and spectral quality of light largely affect the efficiency of photoautotrophic processes (Khalesi et al., 2009). Shifts in light regimes are known to condition the density of symbiotic zooxanthellae, the concentration of photosynthetic pigments and their photosynthetic efficiency (Frade et al., 2008a, 2008b; Lesser et al., 2010). Additionally, light regimes also affect the contribution of zooxanthellae for coral growth, metabolism (Apprill et al., 2007; Fitt and Cook, 2001; Iglesias-Prieto and Trench, 1994), physiology and survival (Venn et al., 2008).

All the issues referred above may play a decisive role on coral development and energy budget, but are of utmost importance for coral production. The relevance of light is even greater when coral production is performed with specific goals, such as the exploitation of photosynthetic pigments for applications in sun-screens and bio-cosmetics (e.g. by adapting technologies developed for plant extracts (Hupel et al., 2011)). The same rationale can be applied for the production of bioactive compounds (Khalesi et al., 2009; Michalek-Wagner et al., 2001), as zooxanthellae can be directly involved in their production (Boehnlein et al., 2005; Mydlarz et al., 2003; Newberger et al., 2006).

A recent study published by Rocha et al. (2013b) stressed the importance of light intensity on the photobiological performance of the soft coral *Sinularia flexibilis* after its propagation through asexual fragmentation. However, the same authors argue that coral response to light intensity can be species specific, and may even shift for the same coral species harboring different clades of zooxanthellae. In this way, it can be relevant to investigate if species phyllogenetically close to *Sinularia* present similar photobiological performances post-fragmentation, and a trend for alcyonid corals may somehow be suggested. According to the World Register of Marine Species (WoRMS, 2013), genus *Sarcophyton* and *Sinularia* are two of the most speciose genera in this family (with 46 and 168 valid species, respectively). In this way, a species within genus *Sarcophyton* would certainly be a suitable option to evaluate the similarity of photobiological responses of alcyonids during the post-fragmentation period. The zooxanthellate soft coral *Sarcophyton glaucum* (Quoy &Gaimard, 1833) (Family Alcyoniidae) is one of the most well-known and popular soft corals in the marine aquarium trade and has also been widely surveyed for new natural products (e.g., cembranoid diterpenes such as Sarcophytol) (Badria et al., 1998). Considering the biotechnological potential of this soft coral, as well as its current demand for the marine aquarium trade, the present study aimed to evaluate how different Photosynthetically Active Radiation (PAR) intensities (which result in different costs with electrical power per m^2 of *ex situ* production area) can affect the photosynthetic performance, survival and growth of *S. cf. glaucum* fragments, experimentally produced in a recirculating aquaculture system. The rationale for our experimental approach was based on the major influence that light regimes are known

to play in both zooxanthellae and coral physiology (Beer et al., 2000; Chalker et al., 1983; Kühl et al., 1995).

3.2.2. Materials and methods

- *Corals husbandry and fragmentation*

The taxonomic status of *Sarcophyton glaucum* is far from being consensual among the scientific community (Aratake et al., 2012; McFadden et al., 2006). In this way, we decided to classify the corals used on this experiment as *Sarcophyton* cf. glaucum, and preserve some material for a future identification of the species, after current taxonomic issues are solved (Aratake et al., 2012).

Five colonies of *S. cf. glaucum* with a capitulum (the polyp-bearing part of the colony) diameter of 18 – 20 cm, harvested in depths ranging from 5 to 15 m in Sumbawa, Indonesia, were shipped by a wholesaler aquarium company, and about 48 h post collection they were stocked in a recirculating system for quarantine in our laboratory. During this period coral colonies acclimated to captive conditions (water parameters, water flow and light) and were daily observed to detect any disease or parasite infection. The maximum quantum yield of photosystem II (PSII) (see bellow in *In vivo Chl fluorescence* for more details) was monitored none intrusively every other week in all coral colonies to evaluate if the photosynthetic performance (mediated by the zooxanthellae) was similar in all the colonies to be used in the experiment.

The recirculated system employed operates with synthetic saltwater (prepared by mixing Tropic Marin Pro Reef salt – Tropic Marine, Wartenberg, Germany – with freshwater purified by reverse osmosis). *S. cf. glaucum* colonies were stocked in a glass tank (90 L water volume, 0.6 m × 0.6 m × 0.25 m) connected to a 100 L filter tank. The colonies tank was equipped with a circulation pump (Turbelle nanostream- 6025 Tunze, Penzberg, Germany), which provided an approximate water flow of 2500 L h^{-1}. The filter tank was equipped with a protein skimmer (APF-600 Deltec, Delmenhorst, Germany), a biological filter (composed by approximately 20 kg of live rock and submerged bio-balls), two submersible heaters (Eheim

Jäger 300 W, Deizisau, Germany) and a submerged pump (Eheim 1260, Deizisau, Germany) that supplied a flow of approximately 1500 L h^{-1} to the coral stocking glass tank. The tank holding the mother colonies was illuminated from above with a 150 W Hydrargyrum quartz iodide (HQI) lamp (BLV, Steinhöring, Germany) delivering a Photosynthetic Active Radiation (PAR) of 120 µmol quanta m^{-2} s^{-1} at the level of the colonies with a 12 h light : 12 h dark photoperiod. PAR value was measured with a Quantum Flux meter (ApogeeMQ-200, Logan, Utah, USA) with a submergible sensor. Salinity was maintained at 35 using an osmoregulator (Deltec Aquastat 1000, Delmenhorst,Germany) that provided automatic compensation of evaporated water with freshwater purified by a reverse osmosis unit. Other water parameters were maintained as follows: temperature 26 ± 0.5 ºC, Total Ammonia Nitrogen 0.05 ± 0.02 mgL^{-1}, NO_2^-–N 0.03 ± 0.02 mgL^{-1}, NO_3^-–N 1.0 ± 0.2 mgL^{-1}, PO_4^{3-}–P 0.01 ± 0.01 mgL^{-1}, pH 8.2 ± 0.2, alkalinity 3.90 ± 0.30 mEq L^{-1}, Ca^{2+} 420 ± 30 mg L^{-1}, Mg^{2+} 1300 ± 40 mg L^{-1}.

After two months of acclimation, the five colonies of S. cf. *glaucum* presented similar values of F_v/F_m (please see Section 2.4.1. for more details) and were free of any evidence suggesting either disease or parasitic infections. The five colonies were fragmented using a scalpel producing 6 similar sized fragments (about 40 mm diameter) per colony, with each one being individually attached with a rubber band to a labelled plastic coral stand (TMC Coral Cradle®, Bristol, UK). All produced coral fragments were stocked during 6 weeks (to allow the cicatrisation of the fragment and its attachment to the coral stand) in an experimental aquarium system identical to the one described above for the mother colonies.

- *Experimental design*

Twenty-seven fragments of the pool of 30 fragments produced (6 fragments × 5 mother colonies) were randomly selected and distributed by the stocking tanks of the 3 coral propagation modules, each with 3 stocking tanks. Each tank was stocked with 3 coral fragments. Each coral propagation module was composed of three 90-L glass tanks (similar to the tank used for the mother colonies) connected to a 150-L filter tank equipped with a protein skimmer (ESC150 ReefSet, São Mamede Negrelos, Portugal), a biological filter (composed by approximately 30 kg of live rock and submerged bio-balls), two submergible

heaters (Eheim Jäger 300W, Deizisau, Germany), a calcium hydroxide reactor (KM500 Deltec, Delmenhorst, Germany) connected to an osmoregulator (Deltec Aquastat 1000, Delmenhorst,Germany) and a submerged pump (Eheim 1262, Deizisau, Germany; providing an approximate flow of 1000 Lh^{-1} to each tank). Additionally, each tank was equipped with a single circulation pump (Turbelle nanostream - 6025 Tunze, Penzberg, Germany; approximate flow of 2500 Lh^{-1}). Each tank was illuminated from above with a 150 W HQI lamp (BLV, Steinhöring, Germany) installed in a ReefSet lighting system (with an Alanod Miro 4 reflector), operating with 150W electronic ballast (ReefSet, São Mamede Negrelos, Portugal). During the experiment photoperiod was 12 h light: 12 h dark.

Reflectance spectra of lights used in the experimental treatments (Fig. 3.2.1) were measured at Ti (at the beginning of the experiment) and at Tf (at the end of the experiment) over a 340-840 nm bandwidth, with a spectral resolution of 0.33 nm, using a USB2000 spectrometer (USB2000-VIS-NIR, grating #3, Ocean Optics, Dunedin, Florida,USA) connected to 400 µm diameter fiberoptic (QP400-2-VIS/NIR-BX, Ocean Optics, Dunedin, Florida, USA). A reference white panel (WS-1-SL White Reflectance Standard with Spectralon, Ocean Optics, Dunedin, Florida, USA) was placed under the light source at a constant distance. The fiberoptics was maintained perpendicular to the reference panel surface in order to measure the reflected light spectra. The distance between the lamps and coral fragments was adjusted to provide one of the following PAR intensities (±10%) at the level of coral fragments: 50, 80 and 120 µmol quanta m^{-2} s^{-1}. PAR values were measured during the experiment with a Quantum Flux meter (Apogee, MQ-200, Logan, Utah, USA) with a submergible sensor at the level of coral fragments and also to evaluate the light (as PAR) distribution pattern in the experimental tanks. The position of each coral fragment in the tank was adjusted so that all fragments in each light treatment had the same PAR value. The use of three independent modules allowed the use of three independent replicates per light intensity treatment, being each replicate composed by three coral fragments. Water parameters were kept as described above for mother colonies. Partial water changes using synthetic saltwater (10% of total experimental system volume) were performed every week. The experiment was performed during a period of 60 days.

- *Spectral reflectance*

Diffuse reflectance spectra were measured at the beginning and at the end of the experimental period over a 330 - 1000 nm bandwidth, with a spectral resolution of 0.33 nm, using the same spectrometer and fiberoptic described above. In order to carry out the radiance measurements, each coral fragment was transported in a 300 mL container filled with water from the experimental tank and placed on a recirculating water bath (Frigiterm-10, JP Selecta, Abrera, Spain) maintained at 26 ºC. The fiberoptics was maintained perpendicular to the coral surface, at a fixed distance, in order to match a view field covering a circular area of approximately 8 mm diameter on the surface of each coral fragment. The light spectrum reflected from each coral fragment was normalized to the spectrum reflected from a reference white panel (WS-1-SL White Reflectance Standard with Spectralon, Ocean Optics, Dunedin, Florida, USA), both measured under a constant irradiance of 200 µmol m^{-2} s^{-1}, provided by a halogen lamp (Volpi Intralux 5000-1 Volpi, Schlieren, Switzerland). A spectrum measured in the dark was subtracted to both spectra to account for the dark current noise of the spectrometer. Coral fragments were measured in 3 different, non-overlapping areas, and the mean spectrum was smoothed using a 10-point moving average filter before used for the subsequent calculations.

The biomass index NDVI (Normalized Difference Vegetation Index; (Rouse et al., 1973) was calculated as:

$$NDVI = \left(\frac{R_{750} - R_{675}}{R_{750} + R_{675}}\right) \qquad (1)$$

where R_{750} and R_{675} represent the average diffusive reflectance in the intervals of 749.73–750.39 nm and 674.87–675.55 nm, respectively.

- *In vivo Chl fluorescence*

Photosynthetic activity was estimated by measuring non intrusively variable chlorophyll fluorescence through PAM fluorometry (Schreiber et al., 1986), using a fluorometer comprising a computer-operated PAM-Control Unit (Walz, Effeltrich,

Germany) and a WATER-EDF-Universal emitter-detector unit (Gademann Instruments, GmbH, Würzburg, Germany) (Cruz and Serôdio, 2008). Measuring, actinic and saturating light were 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, and following the same protocol described above to spectral reflectance and to maintain water temperature.

a) 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- or dark-level fluorescence, F_o, a parameter expected to correlate with the Chl *a* content (Serôdio et al., 2001) and the maximum fluorescence, F_m. F_o and F_m were used to determine the maximum quantum yield of PSII (Schreiber et al., 1986):

$$F_v/F_m = \left(\frac{F_m - F_o}{F_m}\right) \qquad (2)$$

b) Rapid light-response curves (RLC)

The photosynthetic activity of coral fragments was assessed by generating rapid light-response curves (RLCs) of relative electron transport rate on PSII (ETR). Coral fragments were exposed to eight incremental 10 second steps of irradiance ranging from 1 to 1149 µmol quanta m^{-2} s^{-1}. The protocol for the construction of RLCs was set to match the most commonly used procedures for corals (Ralph et al., 2002). ETR was calculated as:

$$ETR = E \times \left(\frac{F'_m - F_s}{F'_m}\right) \qquad (3)$$

where F_s and F'_m are steady-state and maximum fluorescence emitted by a light-adapted sample (arbitrary units), respectively. RLCs were characterized by fitting the model of Platt et al. (1980) ETR versus *E* curves estimating E_k (light-saturation coefficient), and by estimating

the parameters α (initial slope), ETR_{max} (maximum ETR) and β (photoinhibition parameter), as described by Cruz and Serôdio (2008).

- *Zooxanthellae extraction and quantification*

One day after the non intrusive measurements (spectral reflectance and PAM fluorometry), two samples of coral tissue were removed from each fragment with a scalpel to analyze the density of zooxanthellae and to determine photosynthetic and accessory pigments (see below). For zooxanthellae quantification, one sample of coral tissue from each fragment was homogenized in a tube containing 50 mL of filtered (0.2 μm) saltwater. The zooxanthellae were counted in a hemacytometer with improved Neubauer ruling (5 cell counts for each coral fragment). After counting, the samples were centrifuged (10 min, 5000 rpm), the supernatant water was discarded and the pellet was freeze-dried for 24 h to determine total dry weight. After this process zooxanthellae concentration was normalized to *S. cf. glaucum* dry weight.

- *Photosynthetic and accessory pigments*

The sample of coral tissue removed from each coral fragment was immediately frozen in liquid Nitrogen after sampling and freeze-dried. Sampling was performed in the light period (approximately 6 h after the lights turned ON). The concentration of the following photosynthetic and accessory pigments was determined in each coral fragment: chlorophyll *a* (Chl *a*), chlorophyll c_2 (Chl c_2), diadinoxanthin (DD), diatoxanthin (DT), peridinin (Per) and β-carotene (β-Car). Freeze-dried samples of 0.04–0.12 g were extracted with 3-5 mL of 95% cold buffered methanol (2% ammonium acetate) for 30 min at –20 °C, in the dark. Samples were sonicated (Bransonic, model 1210, Danbury, Connecticut, USA) for 30 s at the beginning of the extraction period. Extracts were filtered (Fluoropore PTFE filter membranes, 0.2 μm pore size) and immediately injected in a Shimadzu HPLC system with photodiode array (SPD-M10AVP) and fluorescence (RF-10AXL) detectors. Chromatographic separation was carried out using a C18 column for reverse phase HPLC chromatography (Supelcosil; 25 cm long; 4.6 mm in diameter; 5 μm particles) and a 35 min elution program. The solvent gradient followed Kraay et al. (1992) with a flow rate of 0.6 mL min^{-1} and an injection volume of 100 μL. Pigments were identified from absorbance spectra and retention

times and concentrations were calculated from signals in the photodiode array detector. Calibration of the HPLC peaks was performed using commercial standards from Sigma-Aldrich (St. Louis, MO, USA) and DHI (Institute for Water and Environment, Hørsholm, Denmark).

- *Coral fragments growth*

To determine the growth of coral fragments, buoyant weight measurements (Davies, 1989) were made at the start and the end of the experiment using a Kern Emb 200-3 balance (Kern & Sohn GmbH, Balingen, Germany). The buoyant weights of each coral cradle and rubber bands used to attach each coral fragment was also determined prior to fragmentation. The buoyant weight of all coral fragments was determined and corrected with the weight of the respective cradle and rubber band to obtain net and total weights. Coral cradles were cleaned thoroughly with seawater and a tooth-brush the day before each measurement, in order to minimize any potential bias promoted by the development of biofouling. Buoyant weight of each coral fragment was determined 3 times at the beginning and at the end of the experiment to guarantee reproducibility. Water temperature and salinity were kept stable during all buoyant weight measurements. To calculate the daily percentage of specific growth rate (SGR) of coral fragments (% day^{-1}), the following formula was used:

$$SGR(\%\, day^{-1}) = \left(\frac{\ln(w_f) - \ln(w_i)}{\Delta t}\right) \times 100 \quad (4)$$

where $\ln(w_f)$ and $\ln(w_i)$ are the ln of final and initial coral net weights expressed in grams (g), and Δt is the growth interval in days. SGR is expressed as a percentage of coral weight increase per day.

- *Statistical analysis*

The existence of significant differences among the maximum quantum yield of PSII (F_v/F_m), α, ETR_{max}, E_k, zooxanthellae density, photosynthetic and accessory pigment concentration, relation between zooxanthellae and pigments, and coral growth, recorded for *S.cf. glaucum* fragments cultured under the different light treatments, was tested using one-way ANOVA and post-hoc Tukey HSD test. Repeated measures ANOVA was used to evaluate the

existence of significant differences in the NDVI of S.cf *glaucum* fragments cultured under the different light treatments. Assumptions of normality and homogeneity of variance were checked prior to the analysis through the Shapiro-Wilks and Levene tests, respectively. Post hoc Tukey HSD test was used to determine differences between light treatments and between sampling points of each treatment. Light treatment was used as a categorical factor to all the performed analysis. Statistical analyses were carried out using the Statistica 8.0 software.

3.2.3. Results

- *Light PAR treatments*

The average reflectance of the HQI lamps used in the 9 experimental tanks is represented in Fig. 3.2.1. As evidenced in the graphic, reflectance at Ti and at Tf was similar for all experimental treatments.

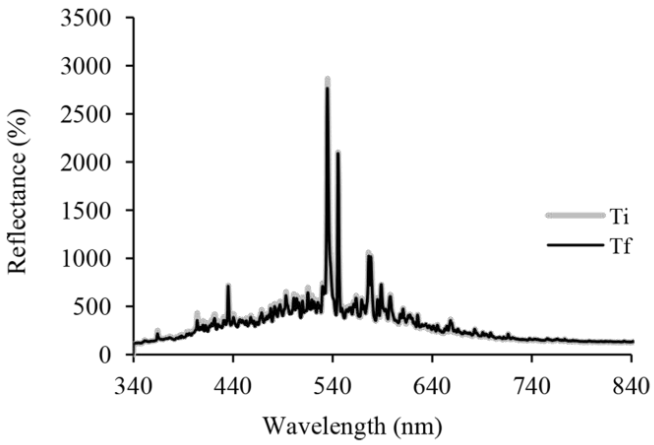

Fig. 3.2.1. Average values of reflectance spectra of lights used in the experimental treatments at Ti (in the beginning of the experiment) and at Tf (in the end of the experiment).

According to our calculations, each 150W HQI lamp was suitable to illuminate a production area of approximately 0.60 m^2 operating with a minimum PAR value of 50 μmol quanta m^{-2} s^{-1}, and a production area of approximately 0.20 m^2 operating with minimum PAR value of

120 μmol quanta m^{-2} s^{-1}.In both scenarios the production area should have a maximum water column of 0.25 m.

The operation of the experimental system with a photoperiod of 12 h light : 12 h dark leads to an electrical power consumption of 1.8 kW day^{-1} for each 150 W HQI lamp.

- *Spectral reflectance*

The reflectance spectra measured on coral fragments at the end of the experiment (Fig. 3.2.2-A) were characterized by relatively low reflectance levels in the blue (400 – 500 nm) and red (650 – 700 nm) wavelength range, denoting the high absorption of light by photosynthetic pigments. The lowest reflectance values were registered on the coral fragments exposed to the lowest PAR values. In all cases, a steep increase in the reflectance values was observed in the 700 – 750 nm region. Higher reflectance levels were recorded for the near-infrared region (> 750 nm, data not shown), which was compatible with the low absorption by photosynthetic pigments on this spectral region. In the visible wavelength range, reflectance spectra showed a wide, well-defined inverted peak in the red region (650 – 700 nm), with a minimum at 672 – 675 nm, corresponding to the absorption peak of Chl *a*. Regardless of the light treatment, all corals exhibited a pattern of reflectance features with three peaks near 575, 600 and 650 nm. In the wavelength range between 500 – 700 nm, coral fragments exposed to 80 and 120 μmol quanta m^{-2} s^{-1} treatments exhibited higher values than those from the 50 μmol quanta m^{-2} s^{-1} treatment.

Fig. 3.2.2-B shows means of the NDVI values measured on corals from the three light treatments at the beginning (Ti) and at the end (Tf) of the experiment. No significant differences were registered in the coral fragments at the beginning of the experiment, between light treatments. At the end of the experiment NDVI values were significantly different in all treatments when compared with the values measured in the same coral fragments at the beginning of the experiment. An increase in NDVI values was registered in coral fragments from 50 μmol quanta m^{-2} s^{-1} treatment ($P < 0.05$), whereas coral fragments from 80 and 120 μmol quanta m^{-2} s^{-1} treatments displayed a decrease on NDVI values ($P < 0.001$).

At the end of the experiment coral fragments from the 50 µmol quanta m^{-2} s^{-1} treatment presented NDVI values significantly higher than those recorded for fragments grown under 80 and 120 µmol quanta m^{-2} s^{-1} ($P < 0.001$).

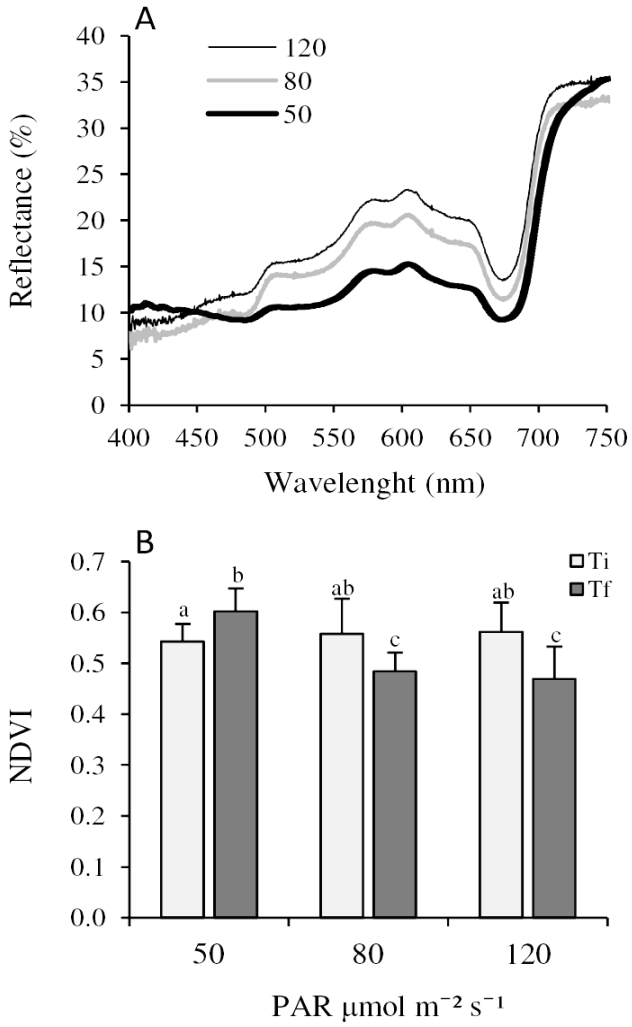

Fig. 3.2.2. A - values of reflectance spectra measured in *S. cf glaucum* fragments at the end of the experiment, on the three (PAR) treatments, 50, 80 and 120 µmol quanta m^{-2} s^{-1}, respectively; **B** - values of NDVI index measure at the beginning (Ti) and at the end (Tf) of the experiment in 3 different and non-overlapping areas of 9 coral fragments per light PAR treatment. Vertical lines represent one standard deviation. Statistically significant differences are marked with different letters, ($P < 0.05$ for all comparisons; Tukey HSD post-hoc comparisons).

- *Chl fluorescence*

The mean values of F_v/F_m measured on coral fragments exposed to the three light treatments are presented in Fig. 3.2.3-A. Coral fragments reared under the PAR of 50 μmol quanta m^{-2} s^{-1} presented significantly higher values of F_v/F_m when compared with the coral fragments from the light treatment with a PAR of 120 μmol quanta m^{-2} s^{-1} (ANOVA, F(2, 72) = 3.760, P = 0.028). The values of α (Fig. 3.2.3-B) were significantly higher in coral fragments from 50 μmol quanta m^{-2} s^{-1} PAR light treatment (ANOVA, F(2, 12) = 9.509, P = 0.003). No significant differences were recorded for ETR_{max} (Fig. 3.2.3-C) (ANOVA, F(2, 12) = 0.660, P = 0.535) and E_k (Fig. 3.2.3d) (ANOVA, F(2, 12) = 1.301, P = 0.308).

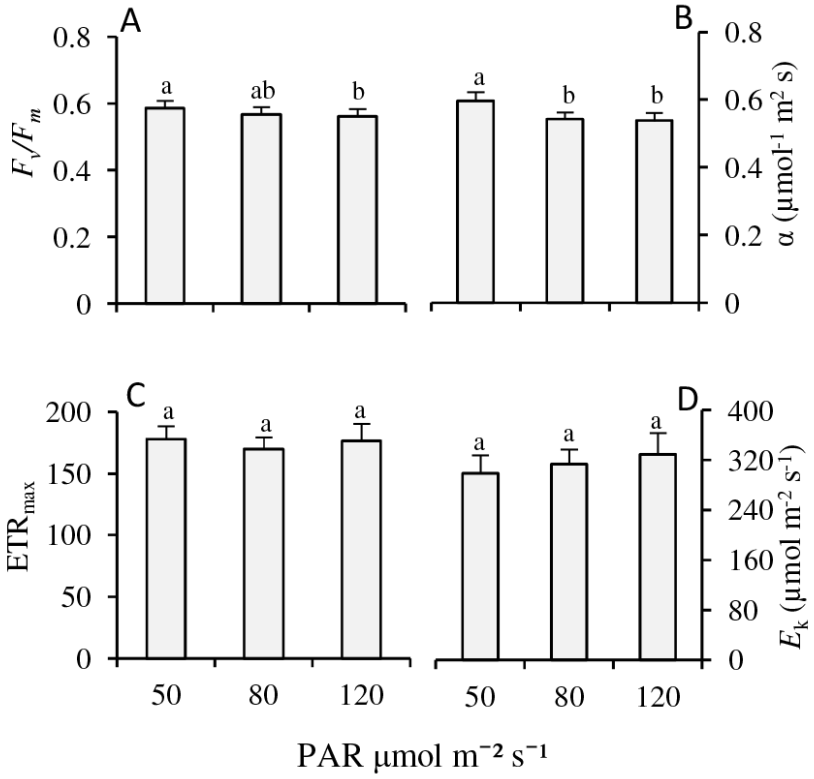

Fig. 3.2.3. A - Values of F_v/F_m, **B -** values of α, **C -** values of ETR_{max} and **D -** values of E_k, measured at the end of the experiment in 9 coral fragments per PAR treatment. Vertical lines represent one standard deviation. Statistically significant differences are marked with different letters, ($P < 0.05$ for all comparisons; Tukey HSD post-hoc comparisons).

- *Zooxanthellae*

A significant effect of light PAR on the concentration of zooxanthellae was found (ANOVA, $F(2, 51) = 182.467$, $P < 0.001$). The concentration of zooxanthellae per gram of coral tissue dry weight (DW) decreased with increasing PAR values (Fig. 3.2.4). The concentrations of zooxanthellae (mean value × 10^7 ± S.D. value × 10^7) recorded were 8.138 ± 0.573; 13.016 ± 0.266 and 21.388 ± 2.572 for the light PAR treatments of 120, 80 and 50 µmol quanta m^{-2} s^{-1}, respectively.

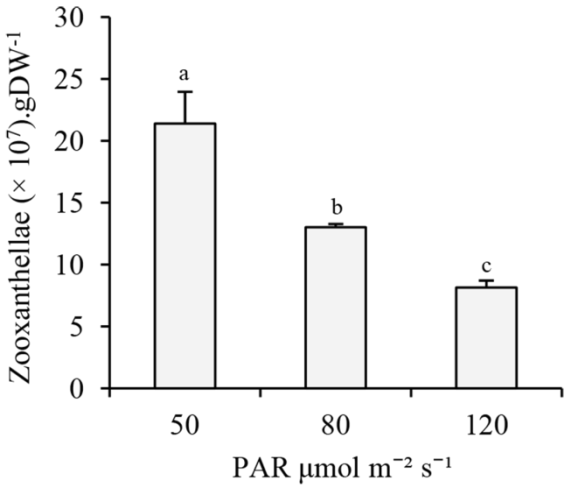

Fig. 3.2.4. Zooxanthellae density (per gram of coral dry weight). Average of measurements made on 9 coral fragments per light treatment. Vertical lines represent one standard deviation. Different letters indicate the existence of statistically significant differences ($P < 0.05$ for all comparisons; Tukey HSD post-hoc comparisons).

- *Photosynthetic and accessory pigments*

The results of photosynthetic and accessory pigments analysis are displayed in figure 3.2.5. Coral fragments reared under the light treatment with lowest PAR value (50 µmol quanta m^{-2} s^{-1}), presented higher concentration of Chl *a* ($F(2, 6) = 21.517$, $P=0.002$), Chl c_2 ($F(2, 6) = 21.822$, $P = 0.002$), DD ($F(2, 6) = 26.253$, $P = 0.001$), Per ($F(2, 6) = 31.696$, $P < 0.001$) and β-Car ($F(2, 6) = 9.248$, $P = 0.015$), when compared with coral fragments from other light treatments. No significant differences were found regarding DT content ($F(2, 6) = 1.340$, $P = 0.330$). Concerning the values of photosynthetic pigments per zooxanthellae (Fig. 3.2.6), coral fragments reared

under a PAR of 80 μmol quanta m^{-2} s^{-1} presented a significantly lower concentrations of Chl*a*, Chl c_2, DD, Per and β-Car per zooxanthellae, when compared with coral fragments from other light treatments ($P < 0.05$). The concentration of DT per zooxanthellae (Fig. 3.2.6D) was significantly different in all treatments ($P < 0.05$), increasing with light PAR intensity.

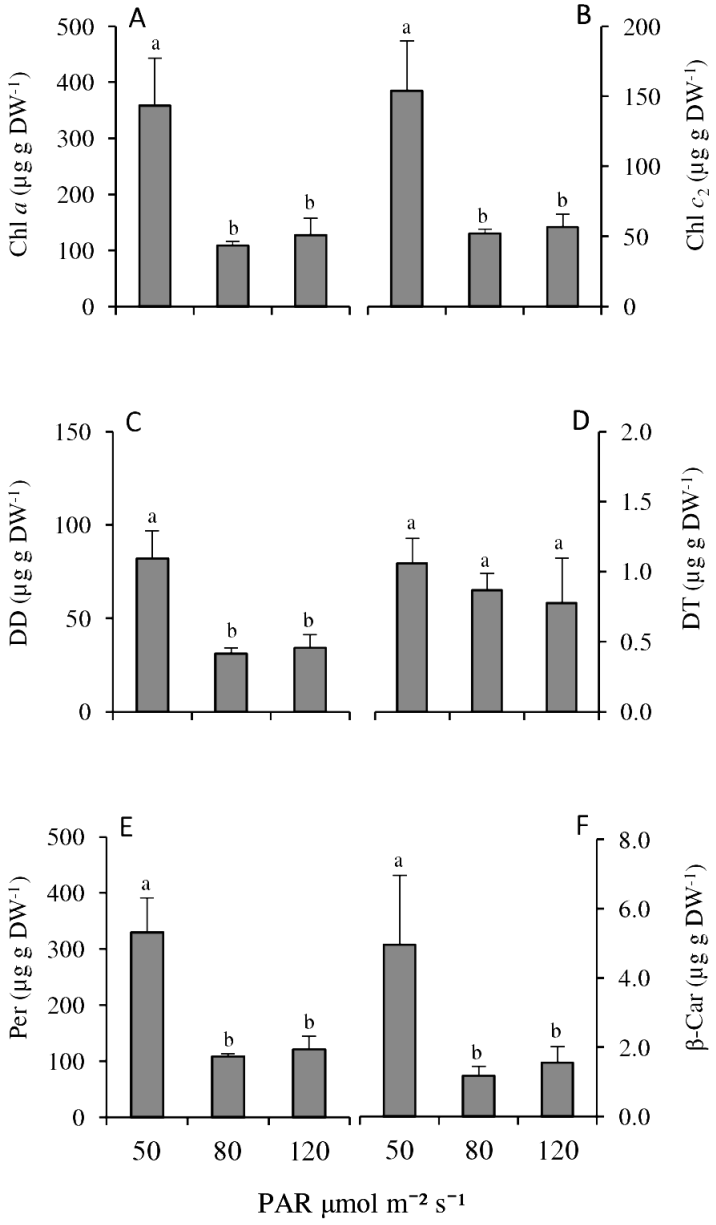

Fig. 3.2.5. Photosynthetic pigments concentration measured at the end of the experiment in 9 coral fragments from each light treatment **A** - chlorophyll *a* (Chl *a*), **B** - chlorophyll c_2 (Chl c_2), **C** - diadinoxanthin (DD), **D** - diatoxanthin (DT), **E** - peridinin (Per) and **F** - β-carotene (β-Car). Vertical lines represent one standard

deviation. Statistically significant differences are identified with different letters ($P < 0.05$ for all comparisons; Tukey HSD post-hoc comparisons).

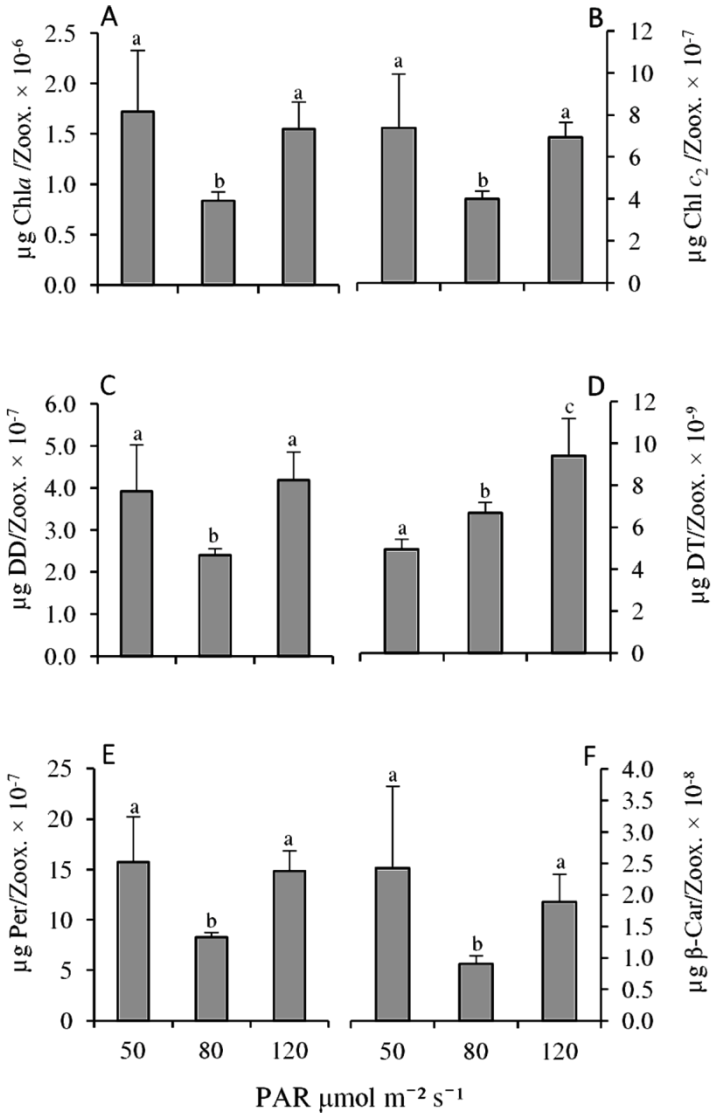

Fig. 3.2.6. Photosynthetic pigments concentration per zooxanthellae, measured at the end of the experiment in 9 coral fragments from each light treatment **A** - chlorophyll *a* (Chl *a*), **B** - chlorophyll c_2 (Chl c_2), **C** - diadinoxanthin (DD), **D** - diatoxanthin (DT), **E** - peridinin (Per) and **F** - β-carotene (β-Car). Vertical lines represent one standard deviation. Statistically significant differences are identified with different letters ($P < 0.05$ for all comparisons; Tukey HSD post-hoc comparisons).

- *Coral fragments growth*

The mean values (± SD, n= 9 coral fragments per treatment) of specific growth rate registered were 0.040 ± 0.010 % day^{-1}, 0.038 ± 0.007 % day^{-1}, and 0.035 ± 0.009 %day^{-1}, for coral fragments from the light PAR treatments of 50, 80 and 120 μmol quanta m^{-2} s^{-1}, respectively. No significant differences were recorded in the growth of coral fragments under different PAR treatments (ANOVA, $F(2, 24) = 0.867$, $P = 0.433$).

3.2.4. Discussion

The light intensity exhibited by the different treatments did not change during the whole experimental period (60 days), with the PAR levels used in this study (120, 80 and 50 μmol quanta m^{-2} s^{-1}) being within the range of values that had previously promoted higher survival for *Sarcophyton glaucum* cultured *ex situ* (Sella and Benayahu, 2010).

The reflectance spectra exhibited by corals in all treatments, displaying three peaks around 575, 600 and 650 nm, rank them as brown corals according to the classification proposed by Hochberg et al. (2004). The pattern of reflectance spectra is related with pigment composition displayed by the zooxanthellae, whose major pigments are Chl *a*, Chl c_2, β-Car, DD, and peridinin (Gil-Turnes and Corredor, 1981), with peridinin being unique to the Dinophyta (Prezelin, 1987). A very pronounced inverted peak was observed in the range 672-675 nm in all treatments, corresponding to the absorption peak of Chl *a*. The lowest values of reflectance and the higher values of NDVI index shown by coral fragments from the 50 μmol quanta m^{-2} s^{-1} light treatment were likely due to an increase in the photosynthetic pigments concentration. This increase reveals a photoacclimation process, as all coral fragments were stocked prior to the experiment under a PAR of 120 μmol quanta m^{-2} s^{-1} (identical to that of mother colonies). Moreover the acclimation to low light intensity is known to promote an increase in Chl *a* concentration present in the photosynthetic endosymbionts of corals (Dubinsky et al., 1984; Falkowski and Dubinsky, 1981), causing low light-acclimated corals to absorb more light than corals adapted to high-light conditions (Dubinsky et al., 1990). This aspect is reflected on NDVI values, where corals from the

lowest PAR treatment (50 μmol quanta m^{-2} s^{-1}) have shown significantly higher values than those stocked under a higher PAR (80 and 120 μmol quanta m^{-2} s^{-1}).

The comparison between F_v/F_m values revealed significant differences between corals from the 50 μmol quanta m^{-2} s^{-1} and 120 μmol quanta m^{-2} s^{-1} PAR treatments. A similar result was recorded by Rocha et al. (2013b). F_v/F_m is used as an accurate measure of the maximum photochemical efficiency of PSII (Dove, 2004) and the decrease of this index values is usually interpreted as an indication of photodamage or photoinhibitory processes of photosynthesis (Franklin et al., 1992). Despite the differences found among treatments, F_v/F_m data were always high and close to the maximum values commonly reported in the literature for corals (Levy et al., 2003; Rodrigues et al., 2008; Winters et al., 2009). These results indicate that stocked corals were physiologically healthy under all light treatments that were tested.

Notwithstanding the fact that no significant differences were observed between treatments on ETR_{max} and E_k (light-saturation coefficient), corals under the 50 μmol quanta m^{-2} s^{-1} PAR treatment presented significantly higher values for α. Rapid light curves can be considered as a reliable indicator of the potential ability of corals to respond to rapid light fluctuation (Ralph and Gademann, 2005). Therefore, this difference on α values can indicate a higher efficiency on light utilization in lower PAR due to an increase in zooxanthellae density in fragments stocked under 50 μmol quanta m^{-2} s^{-1}. This shift is known to be a photoacclimation mechanism associated with the adaptation to low light environments (Khalesi et al., 2009). In this way, the significant increase in the number of zooxanthellae.g^{-1} (DW) with step decreasing PAR values was somehow expected to occur in the present study. This photoacclimation process is a way to optimize light utilization and decrease possible light-induced damages to the photosynthetic apparatus (Titlyanov and Titlyanova, 2002). However, Rocha et al. (2013b) have not recorded any significant differences in the concentration of zooxanthellae between coral fragments of *S. flexibilis* stocked under PAR values of 50 and 80 μmol quanta m^{-2} s^{-1}.The occurrence of photoacclimation is further supported by the fact that during the experimental period no organic food had been supplied, as the existence of an exogenous feeding source may affect the dynamics of this physiological process (Titlyanov et al., 2001). Furthermore, this photoacclimation evidences

species specificity, as unlike *S. flexibilis*, *S. cf. glaucum* exhibited significant differences between coral fragments stocked under the three light PARs tested.

With the exception of DT, the concentration of all photosynthetic pigments significantly increased in the lowest PAR treatment (50 μmol quanta m^{-2} s^{-1}). In corals, pigment concentrations can be influenced by environmental conditions (Kuguru et al., 2010; Myers et al., 1999; Winters et al., 2009) and may result from changes in zooxanthellae density and/or in pigment concentration per zooxanthellae.

According to Falkowski and Raven (1997), an inverse relationship between light levels and Chl *a* concentration is often observed in aquatic photosynthetic organisms, so that organisms living under high light environments typically have reduced concentrations of pigments.

In the present study, Chl *a* concentration was significantly higher in coral fragments stocked at the lowest PAR (50 μmol quanta m^{-2} s^{-1}), when compared with fragments stocked at a PAR of 80 and 120 μmol quanta m^{-2} s^{-1}), agreeing with the studies cited above. Nonetheless, Rocha et al. (2013b) recorded different responses on fragments of *S. flexibilis* produced under similar experimental conditions; Chl *a* concentration in fragments stocked at a PAR of 80 μmol quanta m^{-2} s^{-1} was not significantly different from that recorded in fragments stocked at 50 and 120 μmol quanta m^{-2} s^{-1}, respectively. The concentrations of other photosynthetic and accessory pigments, namely Chl c_2, DD and Per, recorded for *S. cf. glaucum* fragments socked at 50 μmol quanta m^{-2} s^{-1}, were significantly higher from those displayed by fragments stocked under a PAR of 80 μmol quanta m^{-2} s^{-1}. Once more, these results differ from those described by Rocha et al. (2013b) for *S. flexibilis*, where no significant differences were found between coral fragments stocked under these two PAR values. Concerning the concentration of β-Car, Rocha et al. (2013b) has not observed any significant differences among the three light treatments tested for *S. flexibilis*, while in the present study *S. cf. glaucum* stocked at 50 μmol quanta m^{-2} s^{-1} presented significantly higher concentration of this pigment. Overall, these results support the hypothesis that light acclimation post-fragmentation can indeed be species specific; moreover they evidence that further studies addressing other species within the same genus and other genera within the family Alcyoniidae are required to clarify any

potential trends on the photobiological performance of alcyonid corals fragmented in captivity.

In this study, corals stocked under the lowest PAR (50 μmol quanta m^{-2} s^{-1}) were found to have the highest values of zooxanthellae g^{-1} (DW) and the highest content of photosynthetic pigments. However, the concentration of pigments per zooxanthellae did not vary significantly between the lowest and the highest PAR treatments.

The different response pattern of DT concentration per zooxanthellae, increasing in corals exposed to highest PAR levels, can be related with its photoprotective role under high light. This pigment is involved in the xanthophyll cycle, through which DD is converted in DT, allowing the dissipation of excessive absorbed light energy. Both DD and DT concentrations are known to be influenced by light conditions, and their concentrations typically decrease in low growth light (Brown et al., 1999).

The present study confirmed the importance of PAR intensity used in the captive production of *S. cf. glaucum* under controlled conditions in recirculated systems. Relatively small differences in PAR intensities can have a significant impact on electrical costs with illumination. The electrical power consumption of a 150W HQI lamp working with a photoperiod of 12 h light : 12 h dark is 1.8 kW day^{-1}. The production area illuminated by a single lamp increases if lower PAR values are applied. The 150 HQI lamps used in this study where enough to illuminate a production area of approximately 0.60 m^2 operating with minimum PAR value of 50 μmol quanta m^{-2} s^{-1}, whereas will only be suitable to illuminate a production are of approximately 0.20 m^2 if operating with a minimum PAR value of 120 μmol quanta m^{-2} s^{-1}. As light is a key factor influencing the economic viability of coral production *ex situ* (Osinga et al., 2011), it is important to optimize the PAR value to be employed for each produced species. Assuming 0.098 € per kilowatt/hour (kW h) to be the average base price of electricity (excluding VAT) in the European Union (Eurostat 2012), we can estimate an operational cost (excluding VAT) with artificial illumination of approximately 0.294 € m^{-2} $day^{-1}$and 0.882 € m^{-2} day^{-1},when using a PAR of 50 or 120 μmol quanta m^{-2} s^{-1}, respectively.

The goal driving coral production is of paramount importance to select the PAR value to being used. As highlighted in our experiment, low light environments promote an increase in the density of symbiotic zooxanthellae and it can be possible to take advantage of this feature for biotechnological applications. Zooxanthellae can promote significant shifts in the chemistry of their cnidarian host (Michalek-Wagner and Bowden, 2000) and can be directly involved in the production of some bioactive compounds (Boehnlein et al., 2005; Mydlarz et al., 2003; Newberger et al., 2006). In this way, researchers targeting the production of biomass of *S. cf. glaucum* with a specific compound produced by its zooxanthellae may modulate PAR values to maximize the production of corals with a higher or lower number of endosymbionts. Coral producers supplying specimens for the aquarium trade may also modulate coral shade by inducing a higher or lower production of zooxanthellae and photosynthetic pigments.

Overall, the survival recorded during the present experiment (100%), the physiological wellness of coral fragments, and the absence of significant differences in coral growth suggest that low PAR intensities are suitable to support the *ex situ* culture *S. cf. glaucum* in captivity at lower production costs.

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Chapter 3

3.3. Photobiology and growth of leather coral *Sarcophyton* cf. *glaucum* fragments stocked under different light spectra in a recirculated system

Submitted with section 2.1 as: Rui J. M. Rocha, João Serôdio, Bogdan Bontas, Paulo Cartaxana, José M. Ferreira, Rui Rosa and Ricardo Calado. Development and validation of a standardized modular system for experimental coral culture.

Abstract

The increasing interest in coral culture for reef restoration, biotechnological applications or to supply the marine aquarium trade has prompted researchers to optimize coral culture protocols, with emphasis to *ex situ* production. When cultured *ex situ*, the growth performance of corals can be influenced by several physical, chemical and biological parameters. For corals harbouring zooxanthellae, light is one of such key factors as it can influence the photosynthetic performance of these endosymbionts, as well as coral physiology. Hydrargyrum quartz iodide (HQI) lamps are still widely used as artificial illumination for corals harbouring zooxanthellae. The price of these lamps depends on the emitted spectra, which is usually presented as light colour temperature (expressed in Kelvins – K). The higher the colour temperature, the higher the commercial price. In the present experimental trial, we aimed to evaluate how light with a low colour temperature, but delivering an identical PAR, can affect the photosynthetic performance, zooxanthellae density, photosynthetic and accessory pigments concentration, survival and growth of *Sarcophyton* cf. *glaucum* fragments. No significant differences in the photobiological and physiological parameters determined were recorded between coral fragments from the different light treatments (3000, 5000 and 10000 K). Therefore, production costs associated with the artificial illumination employed for growing *S.* cf. *glaucum* can be reduced by using HQI lamps emitting a lower light colour temperature (3000 K) than the one commonly termed as optimal for growing this species (10000 K).

Keywords

Light colour temperature; Photobiology; Photosynthetic pigments; *Sarcophyton*; Zooxanthellae

3.3.1. Introduction

The soft coral *Sarcophyton glaucum* (Family Alcyoniidae), popularly known as leather coral, is recognized as a species with high potential for aquaculture due to its popularity in the marine aquarium trade and as a source of important marine natural products (e.g., cembranoid diterpenes such as sarcophytol (Badria et al., 1998)).

Light is known to be a key factor for *ex situ* production of symbiotic corals (Schlacher et al., 2007), due to its major influence in both zooxanthellae and coral physiology (Beer et al., 2000; Chalker et al., 1983; Kühl et al., 1995; Venn et al., 2008). Moreover, artificial illumination is recognized as being one of the biggest costs associated with the *ex situ* production of corals (Osinga, 2008). While the effect of irradiance on coral and its algal endosymbionts has already been well investigated (as reviewed by Osinga et al., 2011), only a few works have addressed the role of different light spectra on coral photobiology and growth. This current gap in knowledge is somehow puzzling, as the spectral quality of light is assumed to play a major role in the success of *ex situ* coral production (Rocha et al., 2013a; Schlacher et al., 2007; Wijgerde et al., 2012).

Hydrargyrum quartz iodide (HQI) lamps are still widely used as artificial illumination in systems stocking corals harbouring zooxanthellae. The price of HQI lamps depends on the emitted spectra, which is usually presented as light colour temperature (expressed in Kelvins - K) in the technical details specified by manufacturers. The higher the colour temperature, the lower the wavelength of emitted light spectra. The price of a 150W HQI lamp in Europe (from the same manufacturer), can range between an average of 12 € to 60 € for light colour temperatures from 3000 K to 20000 K, respectively. In general, traders recommend higher colour temperatures (from 10000 K to 20000 K) to keep healthy symbiotic corals in captivity.

The present experimental trial aimed to evaluate how light with a low colour temperature, but delivering an identical PAR, can affect the photosynthetic performance, zooxanthellae density, photosynthetic and accessory pigments concentration, survival and growth of *S. cf. glaucum* fragments. The experiment was performed using 3 modular culture systems connected (for a total of 9 culture tanks), to avoid the unpredictable effects associated with

water chemical or microbiological variations that could somehow bias the evaluation of light spectra effects *per se*.

3.3.2. Materials and methods

- *Corals husbandry and fragmentation*

As the taxonomic status of *Sarcophyton glaucum* is still far from being consensual (Aratake et al., 2012; McFadden et al., 2006), we decided to classify the corals used on this experiment as *Sarcophyton* cf. *glaucum*. Coral fragments have been preserved for future species identification once the taxonomic issues highlighted by Aratake et al. (2012) are clarified.

Five colonies of *S. cf. glaucum* with a capitulum (the polyp-bearing part of the colony) diameter of approximately 150 mm were stocked during two months in a glass tank (90 L water volume, 0.6 m × 0.6 m × 0.25 m) connected to a 100 L filter tank. The glass tank was equipped with a circulation pump (Turbelle nannostream- 6025 Tunze, Penzberg,Germany), which provided an approximate water flow of 2500 L h-1. Illumination was provided by a 150 W Hydrargyrum quartz iodide (HQI) lamp (BLV, Steinhöring,Germany) delivering a Photosynthetic Active Radiation (PAR) of 120 µmol quanta m^{-2} s^{-1} at the level of the colonies with a 12 h light : 12 h dark photoperiod. PAR value was measured with a Quantum Flux meter (Apogee, MQ-200) with a submergible sensor. The filter tank was equipped with a protein skimmer (APF-600 Deltec, Delmenhorst,Germany), a biological filter (composed by approximately 20 kg of live rock and submerged bio-balls), two submersible heaters (Eheim Jäger 300 W, Deizisau, Germany) and a submerged pump (Eheim 1260, Deizisau, Germany) that supplied a flow of approximately 1500 L h^{-1} to the coral stocking glass tank.

The experiment was performed using synthetic saltwater prepared by mixing a commercially available salt mixture (Tropic Marin Pro Reef salt – Tropic Marine, Germany) with freshwater purified by a four stage reverse osmosis unit (Aqua-win RO-6080). The water chemistry is described in the section bellow.

After this period of acclimation, *S. cf. glaucum* colonies were fragmented using a scalpel

producing 10 similar sized fragments (about 30 mm diameter) per colony, with each one being individually attached with a rubber band to a labelled plastic coral stand (Coral Cradle®).

- *Experimental design*

Forty-five fragments from the initial pool of 50 fragments produced (10 fragments × 5 mother colonies) were randomly selected and distributed by the 9 experimental tanks (3 tanks × 3 modular culture systems). As each experimental tank can be considered as a true experimental replicate, the experiment was performed with 3 independent replicates per light treatment, with each replicate being composed by 5 coral fragments. Each experimental tank was illuminated from above with a 150 W HQI lamp (BLV, Germany) with one of the following light colour temperatures: a) 10000 K, b) 5500 K, and c) 3000 K. The 3 light treatments were randomized within each system module (so each module held an experimental tank illuminated with 1 of each colour temperatures tested). A PVC screen was placed between tanks to prevent light from adjacent tanks to illuminate the coral fragments stocked under each light treatment. The photoperiod employed during the whole experiment was 12 h light: 12 h dark.

The distance between the lamps delivering the light with the different colour temperatures and the water surface was adjusted to provide a PAR intensity of 200 ± 20 μmol quanta m^{-2} s^{-1} at the level of the coral fragments in all treatments. The PAR value was measured at the level of coral fragments with a Quantum Flux meter (Apogee, MQ-200) with a submergible sensor.

Reflectance spectra of lights used in the experimental treatments were measured at Ti (at the beginning of the experiment) and at Tf (at the end of the experiment) over a 340-840 nm bandwidth, with a spectral resolution of 0.33 nm, using a USB2000 spectrometer (USB2000-VIS-NIR, grating #3, Ocean Optics, USA) connected to 400 μm diameter fiberoptic (QP400-2-VIS/NIR-BX, Ocean Optics). A reference white panel (WS-1-SL Spectralon Reference Standart, Ocean Optics) was placed under the light source at a constant distance. The fiberoptics was maintained perpendicular to the reference panel surface in order to measure the reflected light spectra.

During the experiment partial water changes using synthetic saltwater (prepared as described above) were performed every week (10% of total experimental system volume per week). Water flow in each tank was complemented by a single circulation pump (Turbelle nannostream - 6025 Tunze, Germany; approximate flow of 2500 L h^{-1}). The experiment was performed during a period of 60 days.

- *In vivo Chl fluorescence*

Photosynthetic activity was estimated by measuring non intrusively the variable chlorophyll (Chl) fluorescence through PAM fluorometry (Schreiber et al., 1986), using a fluorometer comprising a computer-operated PAM-Control Unit (Walz) and a WATER-EDF-Universal emitter-detector unit (Gademann Instruments, GmbH, Würzburg, Germany) (Cruz and Serôdio, 2008). Measuring actinic and saturating light were 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 performed 2 h after the start of the daylight period, to ensure the full activation of the photosynthetic apparatus. Each coral fragment was placed in a 300 mL container filled with water from the experimental tank and placed on a recirculating water bath (Frigiterm-10, Selecta, Spain) maintained at 26 ºC.

The following parameters were monitored:

a) Maximum quantum yield of PSII

Measurements were carried out in 4 sampling times: 1) at the beginning of the experimentation (the day corals were fragmented); 2) 1 week after fragmentation; 3) 2 weeks after fragmentation; 4) at the end of the experiment (2 months after fragmentation). Coral fragments were dark-adapted for 15 min, after which one saturation pulse (0.8 s) was applied to determine the minimum- or dark-level fluorescence, F_o, a parameter expected to correlate with the Chl *a* content (Serôdio et al., 2001) and the maximum fluorescence, F_m. F_o and F_m were used to determine the maximum quantum yield of photosystem II (PSII) (Schreiber et al., 1986):

$$F_v/F_m = \left(\frac{F_m - F_o}{F_m}\right) \qquad (1)$$

b) Rapid light-response curves (RLC)

The photosynthetic activity of coral fragments was assessed by generating rapid light-response curves (RLCs) of relative electron transport rate on PSII (ETR). RLCs were performed at the beginning and at the end of the experiment in each coral fragment, after F_v/F_m measurements. Coral fragments were exposed to 8 incremental 10 second steps of irradiance ranging from 1 to 1149 μmol quanta m^{-2} s^{-1}. The protocol for the construction of RLCs was set to match the most commonly used procedures for corals (Ralph et al., 2005; Ralph et al., 2002).

ETR was calculated as:

$$ETR = E \times \left(\frac{F'_m - F_s}{F'_m}\right) \qquad (2)$$

where F_s and F'_m are steady-state and maximum fluorescence emitted by a light-adapted sample (arbitrary units), respectively. RLCs were characterised by fitting the model of Platt et al. (1980) to ETR versus E curves estimating E_k (light-saturation coefficient), and by estimating the parameters α (initial slope), ETR_{max} (maximum ETR) and β (photoinhibition parameter), as described by Cruz and Serôdio (2008).

- *Spectral reflectance*

Diffuse reflectance spectra were measured after *in vivo* Chl fluorescence measurements in the same sampling times of maximum quantum yield of PSII measurements. Measurements were carried out over a 330-1000 nm bandwidth, with a spectral resolution of 0.33 nm, using a USB2000 spectrometer (USB2000-VIS-NIR, grating #3, Ocean Optics, USA) connected to 400 μm diameter fiberoptic (QP400-2-VIS/NIR-BX, Ocean Optics) (Serôdio et al., 2009). Water temperature was maintained following the same protocol described above.

The fiberoptics was maintained perpendicular to the coral surface, at a fixed distance, in order to match a view field covering a circular area of approximately 8 mm diameter on the

surface of each coral fragment. The light spectrum reflected from each coral fragment was normalised to the spectrum reflected from a reference white panel (WS-1-SL Spectralon Reference Standard, Ocean Optics), both measured under a constant irradiance of 200 µmol m^{-2} s^{-1}, provided by a halogen lamp (Volpi Intraluz 5000-1 Volpi, Switzerland). A spectrum measured in the dark was subtracted to both spectra to account for the dark current noise of the spectrometer. Coral fragments were measured in 3 different, non-overlapping areas, and the mean spectrum was smoothed using a 10-point moving average filter before used for the subsequent calculations.

The biomass index NDVI (Normalized Difference Vegetation Index; (Rouse et al., 1973) was calculated as:

$$NDVI = \left(\frac{R_{750} - R_{675}}{R_{750} + R_{675}}\right) \qquad (3)$$

where R_{750} and R_{675} represent the average diffusive reflectance in the intervals of 749.73 – 750.39 nm and 674.87 – 675.55 nm, respectively.

- *Zooxanthellae extraction and quantification*

At the end of the experiment, after the non-intrusive measurements (PAM fluorometry and spectral reflectance), two samples of coral tissue were removed from each fragment with a scalpel to analyze the density of zooxanthellae and to determine photosynthetic and accessory pigments (see below). For zooxanthellae quantification, one sample of coral tissue from each fragment was homogenized in a tube containing 20 mL of filtered (0.2 µm) saltwater. The zooxanthellae were counted in a hemacytometer with improved Neubauer ruling (3 cell counts for each coral fragment). After counting, the samples were centrifuged (10 min, 5000 rpm), the supernatant discarded and the pellet flash freeze in liquid Nitrogen followed by freeze-drying during 24 h to determine total dry weight. After this process zooxanthellae concentration was normalized to S. cf. *glaucum* dry weight.

- *Photosynthetic and accessory pigments*

Coral tissue samples were flash frozen in liquid nitrogen immediately after sampling and freeze-dried during 24 h. Sampling was performed in the light period (approximately 6 hours after the tanks were lit). The concentration of the following photosynthetic and accessory pigments was determined in 3 coral fragments from each experimental tank (9 samples per light treatment): Chlorophyll *a* (Chl *a*), Chlorophyll c_2 (Chl c_2), Peridinin (Per), Dinoxanthin (Dino), β-Carotene (β-Car), Pheophytin *a* (Pheo *a*), Diadinoxanthin (DD), and Diatoxanthin (DT). Freeze-dried samples of 0.06 – 0.16 g were extracted with 2-5 mL of 95% cold buffered methanol (2% ammonium acetate) for 30 min at –20 °C, in the dark. Samples were sonicated (Bransonic, model 1210) for 30 s at the beginning of the extraction period. Extracts were filtered (Fluoropore PTFE filter membranes, 0.2 μm pore size) and immediately injected in a Shimadzu HPLC system with photodiode array (SPD-M10AVP) and fluorescence (RF-10AXL) detectors. Chromatographic separation was carried out using a C18 column for reverse phase HPLC chromatography (Supelcosil; 25 cm lenght; 4.6 mm diameter; 5 μm particles) and a 35 min elution programme. The solvent gradient followed Kraay et al. (1992) with a flow rate of 0.6 mL min^{-1} and an injection volume of 100 μL. Pigments were identified from absorbance spectra and retention times and concentrations calculated from signals in the photodiode array detector. Calibration of the HPLC peaks was performed using commercial standards from DHI (Institute for Water and Environment, Hørsholm, Denmark).

- *Coral fragments growth*

To determine the growth of coral fragments, buoyant weight measurements were performed at the beginning and at the end of the experiment using a Kern Emb 200-3 balance (Kern & Sohn GmbH), according to the modifications proposed by Rocha et al. (2013b) to the protocol described by Davies (1989). Water temperature and salinity were kept stable during all buoyant weight measurements. To calculate the specific growth rate (% day^{-1}) for each coral fragment, the following formula was used:

$$SGR\ (\%\ day^{-1}) = \left(\frac{\ln\left(w_f\right) - \ln(w_i)}{\Delta t}\right) \times 100 \quad (4)$$

where $\ln(w_f)$ and $\ln(w_i)$ are the ln of final and initial coral buoyant weights expressed in grams

(g), and Δt is the growth interval in days. SGR is expressed as a percentage of coral weight increase per day.

- *Statistical analysis*

The existence of significant differences among the α, ETR_{max}, E_k, zooxanthellae density, photosynthetic and accessory pigments concentration, and coral growth, recorded for S. cf. *glaucum* fragments cultured under the different light treatments, was tested using one-way ANOVA and post-hoc Tukey HSD test. Repeated measures ANOVA was used to evaluate the existence of significant differences in the maximum quantum yield of PSII (F_v/F_m) and in the NDVI of S.cf. *glaucum* fragments cultured under the different light treatments. Mauchly's test of sphericity was used to determine if the variances of the differences between all combinations of related groups were equal. Assumptions of normality and homogeneity of variance were checked prior to the analysis through the Shapiro-Wilks and Levene tests, respectively. Post-hoc Tukey HSD test was used to determine differences between light treatments and between sampling points for photosynthetic and accessory pigments concentrations. Unequal-N HSD post-hoc comparisons were used to determine the existence of significant differences between light treatments and between species under the same light treatment for the other analyses. Light treatment was used as the categorical factor to all the performed analysis. Statistical analyses were carried out using the Statistica 8.0 software.

3.3.3. Results

- *Water parameters*

During the whole experiment, salinity was maintained at 35 using an osmoregulator as already described. Other relevant water parameters were monitored every other day, presenting the following average (± S.D.) values: temperature 26 ± 0.4 °C, Total Ammonia Nitrogen 0.04 ± 0.01 mg L^{-1}, NO_2^-–N 0.03 ± 0.01 mgL^{-1}, $NO3^-$–N 0.9 ± 0.2 mgL^{-1}, PO_4^{3-}–P 0.01 ± 0.01 mgL^{-1}, pH 8.2 ± 0.15, alkalinity 3.90 ± 0.35 mEq L^{-1}, Ca^{2+} 410 ± 25 mg L^{-1}, Mg^{2+} 1350 ± 40 mg L^{-1}.

- *Light reflectance spectra*

The mean reflectance spectra of lights used in the experimental treatments are presented as supplementary data Fig. 3.3.1. No significant differences were detected in reflectance spectra between lamps of the same colour temperature treatment or between measurements performed at the beginning and at the end of the experiment.

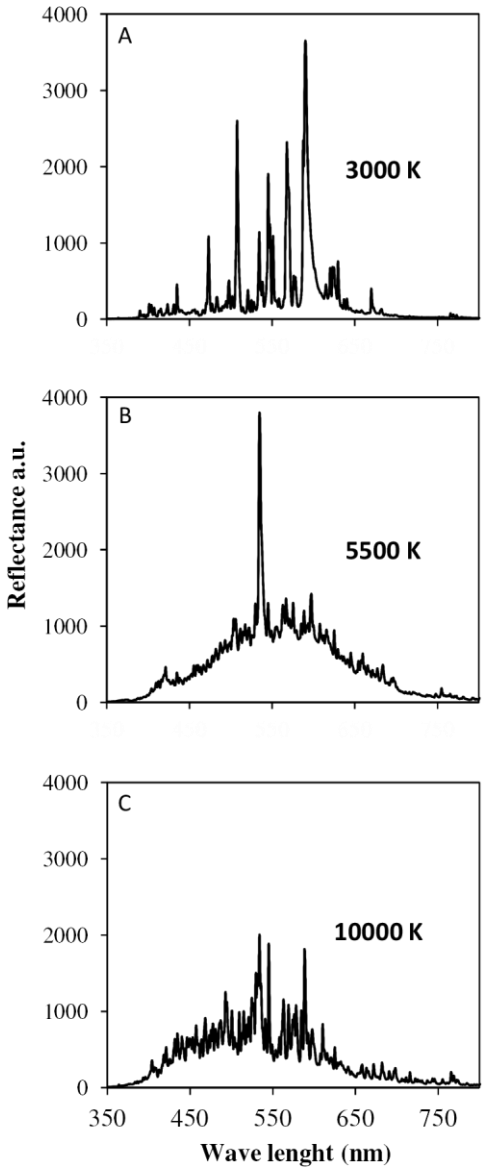

Fig. 3.3.1. Average values of reflectance spectra of 150 W Hydrargyrum quartz iodide (HQI) lamps used in the experimental treatments: A) 3000 K, B) 5500 K, and C) 10000 K.

- *In vivo Chl fluorescence*

The mean values of F_v/F_m measured on coral fragments exposed to the three light treatments are presented in figure 3.3.2. No significant differences were recorded between light treatments at each sampling time. At the end of the experiment (2 months after fragmentation) coral fragments presented significantly lower F_v/F_m values ($P < 0.05$) when compared with the values measured in the same coral fragments at other times.

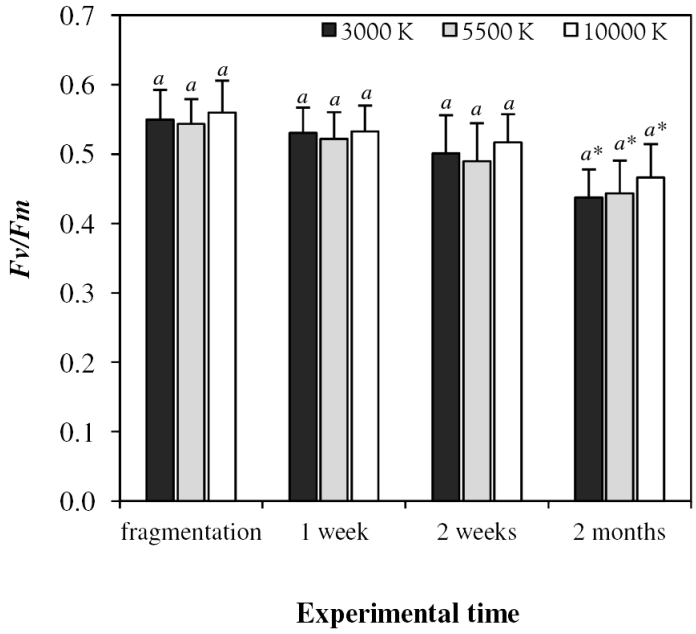

Fig. 3.3.2. Mean ± standard deviation of F_v/F_m, measured at fragmentation and 1 week, 2 weeks and 2 months after fragmentation, in 3 different and non-overlapping areas of each coral fragment stocked under the 3 different light treatments tested. At the end of the experiment (2 months) n = 13 for 3000 K treatment, and n = 15 for 55000 K and 10000 K treatments, respectively. Statistically significant differences between light treatments in each sampling point are marked with different superscript letters, significant differences in each light treatment during the experimental period are marked (*asterisk*), ($P < 0.05$ for all comparisons; Unequal N HSD post-hoc comparisons).

The values of α (Fig. 3.3.3-A) were significantly higher in coral fragments stocked in the 10 K light treatment ($P < 0.05$) at the end of the experiment (Tf), when compared to the values recorded for the same specimens at the beginning of the experiment (Ti - fragmentation).

Coral fragments reared in 5.5 K light presented significantly lower values of ETR_{max} ($P < 0.05$) at Tf, when compared with values obtained in the RLC performed in the same coral fragments at Ti (Fig. 5.3-B). The values of E_k (Fig. 3.3.3-C) were significantly higher at the

end of the experiment (Tf) in coral fragments from all light treatment ($P < 0.05$), when compared with E_k values obtained in the RLC performed in the same coral fragments at Ti. No significant differences were observed between light treatments on α, ETR_{max} and E_k (Fig. 5) in the 2 measurement times (Ti and Tf).

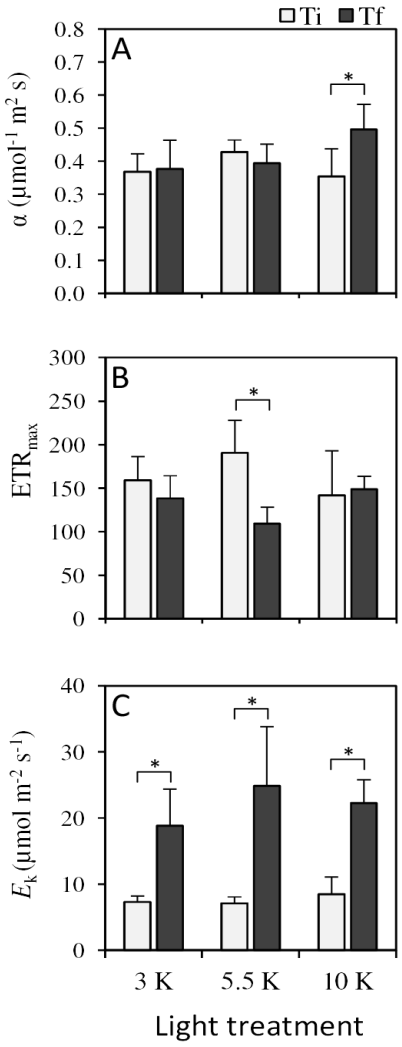

Fig 3.3.3. Mean ± standard deviation of α (A), ETR_{max} (B) and E_k (C), measured at the beginning and at the end of the experiment on *S. cf. glaucum* fragments stocked under the 3 different light treatments tested. In Ti, n = 15 for all light treatments; in Tf, n = 13 for 3.000 K (3 K), n = 15 for 5.500 K (5.5 K) and n = 15 for 10.000 K (10 K). Statistically significant differences between Ti and Tf in each light treatment are marked (*asterisk*) ($P < 0.05$ for all comparisons; Unequal N HSD post-hoc comparisons). No significant differences were obtained on coral fragments between light treatments in each sampling point (Ti and Tf) ($P > 0.05$ for all comparisons; Unequal N HSD post-hoc comparisons).

- *Spectral reflectance*

The reflectance spectra in the visible wavelength range measured on coral fragments at Tf (presented as supplementary data Fig. 3.3.4-A, 3.3.4-B and 3.3.4-C for light treatments 3000 K, 5500 K and 10000 K, respectively) was similar in all replicates from the different light treatments. Reflectance was characterized by relatively low levels in the wavelengths between 475 – 500 nm and 650 – 700 nm, which corresponds to the blue and red regions, respectively. Reflectance values increased in all cases between 700 – 750 nm, being the higher reflectance levels recorded for the near-infrared region (> 750 nm, data not shown).

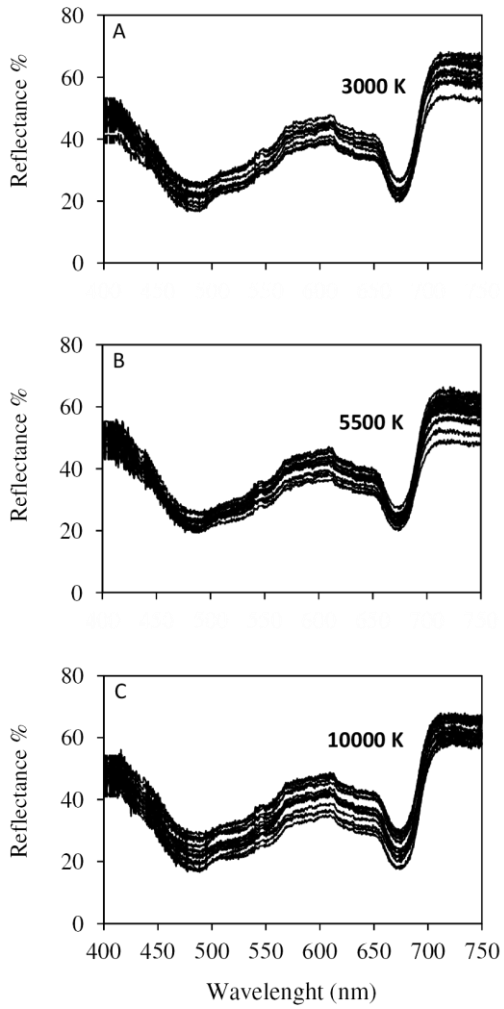

Fig. 3.3.4. Reflectance spectra measured in S. cf. *glaucum* fragments at the end of the experiment (2 months), on the three light treatments: A) 3000 K, n = 13 coral fragments; B) 5500 K, n = 15 coral fragments, and C) 10000 K, n = 15 coral fragments. Each line represents a mean spectrum from each coral fragment, resulting from a smoothed 10-point moving average filter reflectance.

Figure 3.3.5 shows mean values of NDVI measured on coral fragments from the three light treatments at the beginning of the experiment, 1 week, 2 weeks and 2 months after fragmentation. One week after fragmentation, coral fragments from the 10 K treatment presented significantly higher NDVI values than coral fragments from the 3 K treatment ($P < 0.05$). However at the end of the experiment no differences were found between coral fragments stocked under the different light treatments tested. NDVI values measured on coral fragments just after fragmentation (at the beginning of the experiment) were significantly lower ($P < 0.001$) in all fragments when compared with those determined in the same corals 1 or 2 weeks after, as well as in the end of the experiment (2 months after fragmentation).

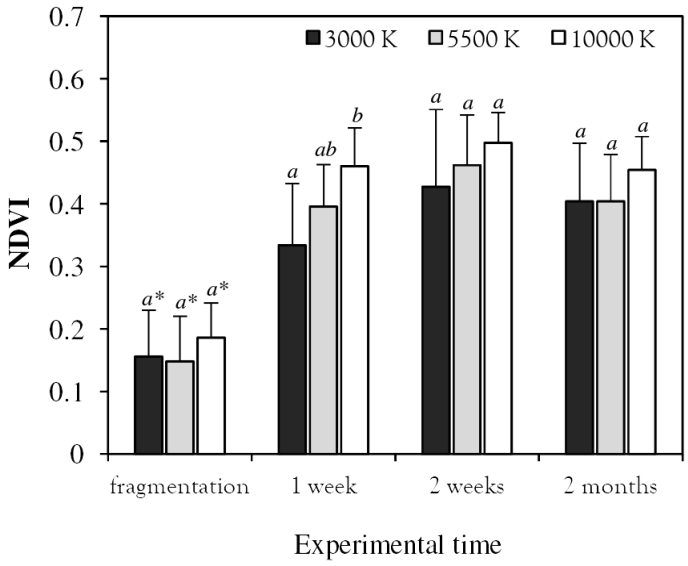

Fig. 3.3.5. Mean ± standard deviation of NDVI, measured at fragmentation and 1 week, 2 weeks and 2 months after fragmentation, in 3 different and non-overlapping areas of each coral fragment stocked under the 3 different light treatments tested. At the end of the experiment (2 months) n = 13 for 3000 K treatment, and n = 15 for 55000 K and 10000 K treatments, respectively. Statistically significant differences between light treatments in each sampling point are marked with different superscript letters, significant differences in each light treatment during the experimental period are marked (*asterisk*), ($P < 0.05$ for all comparisons; Unequal N HSD post-hoc comparisons).

- *Zooxanthellae quantification*

While the concentration of zooxanthellae slightly increased with colour temperature (Fig. 3.3.6-A), no significant differences were recorded ($P > 0.05$). The concentration of

zooxanthellae (mean value ± S.D. x 10^8) per gram of coral tissue dry weight (DW) recorded was 2.19 ± 0.54, 2.43 ± 0.50 and 2.72 ± 0.67 for the light treatments of 3 K, 5.5 K and 10 K, respectively.

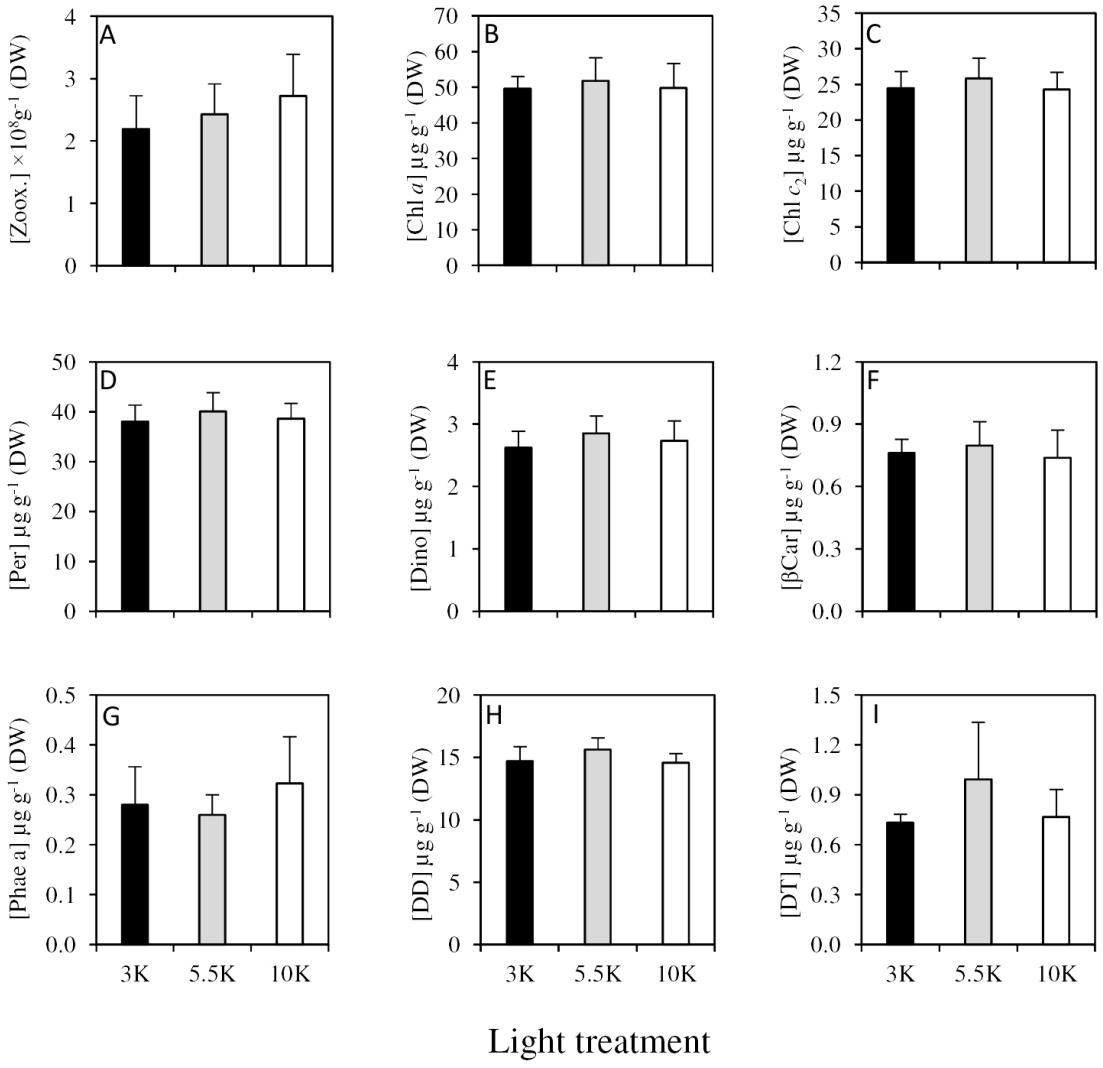

Fig. 3.3.6. Mean ± standard deviation of zooxanthellae (A) per gram of coral dry weight at the end of 2 months of experiment (Tf) measured on *S. cf. glaucum* fragments stocked under the 3 different light treatments tested. In Tf, n = 13 for 3000 K (3 K), n = 15 for 5500 K (5.5 K), and n = 15 for 10000 K (10 K). Mean ± standard deviation of Chlorophyll *a* (B), Chlorophyll c_2 (C), Peridinin (D), Dinoxanthin (E), β-Carotene (F), Pheophytin *a* (G), Diadinoxanthin (H), and Diatoxanthin (I) concentrations (µg pigment g^{-1} coral dry weight) measured at the end of the experiment in 9 *S. cf. glaucum* fragments from each light treatments tested (3000 K (3 K), 5500 K (5.5 K) and 10000 K (10K)). No significant differences were observed between light treatments ($P > 0.05$ for all comparisons; Unequal N HSD or Tukey HSD post-hoc).

- *Photosynthetic and accessory pigments*

The results of photosynthetic and accessory pigments analysis are displayed as supplementary data (Fig. 3.3.6-B-I). No significant differences were found between light treatments in the concentrations of any of the pigments measured in the coral fragments ($P > 0.05$).

- *Coral fragments growth*

The mean values of growth registered in coral fragments (% CG mean ± standard deviation) were 0.027 ± 0.008 % day^{-1}, 0.027 ± 0.007% day^{-1} and 0.028 ± 0.006 0% day^{-1} for coral fragments from the light treatments of 3000 K, 5500 K and 10000 K, respectively. No significant differences were recorded in the growth of coral fragments under different light treatments ($P > 0.40$).

- *Survival*

During the whole experiment, only 2 (out of 15) coral fragments from the 3000 K treatment died. No mortality was recorded in the other two light treatments (5500 K and 10000 K).

3.3.4. Discussion

At the end of the experiment coral fragments from all treatments presented significantly lower F_v/F_m values than those recorded at the beginning of the experiment, as well as 1 and 2 weeks after the beginning of the experiment. F_v/F_m is used as an accurate measure of the maximum photochemical efficiency of PSII (Dove, 2004) and the decrease of this index is usually interpreted as an indication of photodamage or photoinhibitory processes of photosynthesis (Franklin et al., 1992). F_v/F_m values recorded for coral fragments during the first two weeks were always close to the maximum values commonly reported in the literature for corals (Levy et al., 2003; Rodrigues et al., 2008; Winters et al., 2009). These results indicate that, at least during the first two weeks, stocked corals were physiologically healthy under all light treatments and that colour temperature played no role on photosynthetic efficiency. On the other hand, the lower F_v/F_m values observed at the end of the experiment can be related to a photoinhibitory process triggered by the use of a high

light intensity (200 ± 20 μmol quanta m^{-2} s^{-1}), as it has already been documented that such intensities may promote a decrease in photosynthetic performance (Rocha et al., 2013b; Titlyanov and Titlyanova, 2002).

Rapid light response curves can be considered as a reliable indicator of the potential ability of corals to respond to rapid light fluctuation (Ralph and Gademann, 2005), as well as a reliable method to asses mechanisms of short- and long-term photoacclimation processes in photosynthetic organisms (Serôdio et al., 2005). Notwithstanding the fact that no significant differences were observed between treatments on α, ETR_{max}, E_k and NPQ_{max} at the beginning and at the end of the experiment, light saturation (E_k) was significantly higher in coral fragments from all light treatments at the end of the experiment. The higher value of E_k in the present experiment might be related to the acclimation to high light conditions (Ralph and Gademann, 2005), as small sized coral fragments are more exposed to light than the larger mother colonies. The capitulum surface in *S. cf. glaucum* fragments is relatively flat, which reduce self-shading and allows a more uniform light exposition, since light distribution can be affected by coral morphology (Enriquez et al., 2005), due to multiple light scattering in larger colonies.

The reflectance spectra exhibited by coral fragments was similar for all light treatments, displaying three peaks around 575, 600 and 650 nm. These results rank them as brown corals according to the classification proposed by Hochberg et al (2004). The pattern of reflectance spectra is related with the pigment composition of the zooxanthellae, which are known to contain as major pigments Chl *a*, Chl c_2, β-Car, DD, and peridinin (Gil-Turnes and Corredor, 1981) (the last one being unique to the Dinophyta (Prezelin, 1987)). A pronounced inverted peak was observed in all treatments in the range of 672-675 nm, which corresponds to the absorption peak of Chl *a*. The low values of NDVI index exhibited by coral fragments in the beginning of the experiment can be directly related with fragmentation, as this invasive process always promotes the loss of zooxanthellae and the consequent decrease in Chl *a* concentrations.

One week after fragmentation coral fragments from the 10000 K treatment presented significantly higher NDVI values, in comparison to those stocked at 3000 K. As mother

colonies were stoked at 10000 K light prior to fragmentations, we can hypothesize that recovery from this traumatic process could be faster for fragments grown under this light treatment. Nonetheless, 2 weeks after fragmentation no significant differences are found in NDVI values displayed by coral fragments stocked under different light treatments, indicating a post fragmentation stabilization/acclimation.

The analysis of zooxanthellae density and concentration of photosynthetic pigments revealed no significant differences between coral fragments stocked under different light treatments. It is known that symbiotic corals can achieve a suitable protection from photooxidative damage through the reduction of the number of their endosymbionts or the decrease of their Chl content; additionally, symbiotic corals can also adjust their light absorption efficiency according to irradiance levels (Kuguru et al., 2010). This feature is particularly important in corals growing in shallow depths in the wild, such as *Sarcophyton*, as they are known to modulate the content of Chl and number of zooxanthellae according to seasonal fluctuations in light levels (Winters et al., 2009).

The xanthophyll cycle, characterized by the conversion of DD in DT, is a process which allows the dissipation of excessive absorbed light energy (Kramer et al., 2013). Both DD and DT concentrations are known to be influenced by light conditions, and their concentrations typically decrease under low light (Brown et al., 1999). As no significant differences were obtained in DD and DT concentrations between light treatments at the end of the experiment, PAR levels seem to be more important to photoacclimation mechanisms than light colour temperature (at least within the studied range: 3000 K to 10000 K).

The effects of irradiance on coral species harboring zooxanthellae have been highlighted by several studies, as reviewed by Osinga et al (2011). However, only a few studies have investigated the role of light spectra (a feature related with emitted light colour temperature) on coral photobiology, physiology and growth. It is known that corals display higher growth and photosynthetic rates when exposed to blue light (Kinzie et al., 1984), a feature that has been investigated for the successful production of corals *ex situ* (Schlacher et al., 2007; Wijgerde et al., 2012).The results obtained in the present experiment, indicate that the range of light colour temperatures tested (3000 K to 10000 K) does not significantly affect

the photosynthetic performance, survival or growth of *Sarcophyton* cf. *glaucum*. However, the performance of lamps displaying higher light colour temperature and delivering higher levels of blue light (e.g., 14000K – 20000K) was not evaluated in the present study. In this way, we can only state that the growth of this coral species *ex situ* is not enhanced by using more expensive 10000 K lamps over more affordable 3000 K or 5500 K lamps.

While no significant differences were obtained in coral growth between different light treatments, it is important to stress that no exogenous food was supplied through the whole experiment. Several studies have already highlighted the importance of heterotrophic feeding in the growth of symbiotic corals in the wild (Anthony and Fabricius, 2000; Borell et al., 2008; Ferrier-Pages et al., 2003; Houlbrèque and Ferrier-Pages, 2009; Houlbrèque et al., 2004). Therefore, it is important to investigate the added-value of providing exogenous food to speed up the growth of corals propagated *ex situ*, as well as if positive synergies arise from supplying exogenous feed and different light spectra.

While no mortality was recorded among fragments on 5500 K and 10000 K, 2 out of 15 coral fragments were lost in the 3000 K treatment. It is known that coral survival post-fragmentation can be influenced by several factors, such as the initial size of the fragment and the substrate to which it is attached to (Calfo, 2007; Lirman, 2000). To avoid any bias promoted by the fragmentation process, all coral fragments employed in the present experiment were approximately the same size and were attach to identical substrates with a rubber band. As coral fragments died during the first week of the experiment, it is possible that the traumatic process of fragmentation was responsible for the losses rather than the different light spectra.

3.3.5. Conclusions

The present study shown that production costs associated with the artificial illumination employed for growing *S. cf. glaucum* can be reduced by using HQI lamps emitting a lower light colour temperature (3000 K) than the one commonly termed as optimal for growing this species (10000 K). No significant differences were recorded between coral fragments

from the different light treatments in the photobiological and physiological parameters determined.

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Chapter 4

Effect of light spectra in *ex situ* culture of hard corals

Chapter 4

4.1. Comparative performance of light emitting plasma (LEP) and light emitting diode (LED) in ex situ aquaculture of scleractinian corals

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Abstract

Ex situ aquaculture of scleractinian corals is considered as a sustainable solution to fulfill the growing demand of these highly priced organisms by the marine aquarium industry. The economic feasibility of *ex situ* coral aquaculture is strongly dependent on the trade-offs achieved between coral production and energy costs, namely those directly resulting from the need to employ artificial lighting systems. In the present study, we hypothesize that light spectrum can influence the growth performance of cultured scleractinian corals when these are exposed to identical photosynthetically active radiation (PAR) intensities. To test our hypothesis we evaluated the effect of contrasting light spectra delivering an identical PAR of 250 ± 10 μmol quanta m^{-2} s^{-1}emitted by T5 fluorescent lamps (used as a control treatment), Light Emitting Plasma (LEP) and Light Emitting Diode (LED) on the photobiology, growth, and protein concentration of two commercially important scleractinian corals: *Acropora formosa* and *Stylophora pistillata*. Replicated coral fragments of both species were exposed to the three types of illumination for a period of 5 months after which the following features were determined: maximum quantum yield of PSII (F_v/F_m) (monitored non-intrusively through Pulse Amplitude Modulation fluorometry, PAM), chlorophyll *a* content (also determined non-destructively by using the spectral reflectance index Normalized Difference Vegetation Index, NDVI), growth and protein content. Coral fragments of both species cultured under LEP lighting, presented significantly higher values of F_v/F_m when compared intraspecifically with coral fragments cultured with T5 or LED lighting. Fragments of *S. pistillata* cultured under LEP illumination presented significantly higher NDVI values than fragments grown with T5 or LED. No significant differences were found on the protein concentration of *A. formosa* under different light treatments, while fragments of *S. pistillata* grown with LED illumination presented a significantly higher concentration of protein than those cultured with T5 or LEP illumination. Both LED and LEP technologies have shown to be interesting alternatives to provide artificial lighting for coral aquaculture *ex situ*, with the blue light spectrum of LED promoting high growth performances for both coral species and a lower energetic cost per m^2 of production area (0.71 € m^{-2} day^{-1} for T5, 0.38 € m^{-2} day^{-1} for LEP, and 0.28 € m^{-2} day^{-1} for LED).

Keywords

Acropora; *Stylophora*; Coral propagation; Marine aquariums; Marine ornamentals

4.1.1. Introduction

The global trade of marine ornamental species is recognized to be a multi-million dollar industry that still heavily relies on the collection of organisms from the wild to fulfill an ever growing demand (Thornhill, 2012; Tissot et al., 2010; Tlusty, 2002). About 140 different species of scleractinian corals are traded for marine aquariums (Wabnitz et al., 2003), with over 500000 coral colonies being imported by the U.S. alone in 2010 (Rhyne et al., 2012). While most traded species can already be propagated through fragmentation in captivity (Calfo, 2007; Olivotto et al., 2011), namely those popularly termed as small polyp scleractinian (SPS), only fast-growing corals appear to be economically profitable (Bruckner and Borneman, 2010; Rhyne et al., 2012). Coral propagation can either be performed *in situ* or *ex situ*, with *in situ* propagation significantly reducing production costs. Unlike *ex situ* propagation, corals propagated in their natural environment do not require any adaption to captive stocking conditions. However, fragments produced *in situ* are exposed to sedimentation, pathogens, predators, competitors and other natural hazards, which can reduce survival (Rinkevich, 2005). In contrast, coral production *ex situ* has the advantage of maximizing survival and growth rates through the optimization of culture conditions, namely lighting, water flow and food availability (Forsman et al., 2006; Khalesi, 2008).

Production costs associated with *ex situ* propagation can be considerable and be a potential constraint to the economic viability of this practice (Osinga et al., 2011). In order to make coral aquaculture economically feasible, it is urgent to optimize culture protocols (Osinga, 2008). This optimization requires a more in-depth knowledge of coral growth and physiology, namely the role played by coral photosynthetic endosymbionts – the zooxanthellae (dinoflagellates from genus *Symbiodinium*). The relevance of this symbiotic association in coral growth has long been recognized (Schutter et al., 2008, 2012; Wijgerde et al., 2012), with light being a key factor for *ex situ* production of symbiotic corals (Schlacher et al., 2007). Shifts in light regimes are known to affect the density of zooxanthellae, the concentration of photosynthetic pigments and their photosynthetic efficiency (Frade et al., 2008a; Frade et al., 2008b; Kühl et al., 1995; Lesser et al., 2010), as well as coral host physiology and survival (Venn et al., 2008).

Several studies have already focused on the effects of irradiance on coral and its algal symbionts (see review in Osinga et al., 2011). Curiously, only a few works have investigated the role of the spectral quality of light on coral photobiology, physiology and growth. This current gap of knowledge is somehow puzzling, as the spectral quality of light is assumed to play a major role in the success of *ex situ* coral production (Schlacher et al., 2007; Wijgerde et al., 2012).

The development of innovative technologies for coral illumination and their energetic efficiency are of paramount importance for coral production *ex situ*, as one of the most determinant aspects on the economic viability of this activity are the costs associated with coral illumination (Osinga, 2008). New light sources, such as the light emitting plasma (LEP) or light emitting diode (LED), have already started to be applied in marine aquariums, partially replacing the most frequently used illumination solutions - the T5 fluorescent and metal halide lamps (also known as hydrargyrum quartz iodide (HQI) lamps). LEP emission is characterized by a highly efficient and broad light spectrum, making this technology an interesting alternative for coral illumination in *ex situ* propagation facilities. Altought LED illumination is also a promising technology for several applications (Pimputkar et al., 2009), its narrower range of bandwidths at which light is emitted may negatively affect coral growth. In the present study, we hypothesize that light spectrum can influence the growth performance of cultured scleractinian corals when these are exposed to identical photosynthetically active radiation (PAR) intensities. The rationale for this hypothesis is that photosynthesis plays a leading role in coral calcification (Chalker and Taylor, 1975) and that symbiotic corals display higher growth and photosynthetic rates under blue light (Kinzie et al., 1984). Within this context, we aimed to evaluate the effects of different light spectra, emitted by T5 fluorescent lamps, LEP and blue LEDs, on the photobiology and growth performance of two commercially important scleractinian corals in the marine aquarium trade: *Acropora formosa* and *Stylophora pistillata*.

4.1.2. Materials and methods

- *Coral husbandry and fragmentation*

Five mother colonies of *Acropora formosa* and five mother colonies of *Stylophora pistillata* were kept for 5 months in a 750 L glass tank (2 m × 0.8 m × 0.5 m), integrated in a 9000 L recirculating aquaculture system operated with filtered natural seawater. The system was composed by 9 glass tanks (identical to the one described above) with a total water volume of about 6750 L and an approximate production area of 14.5 m^2. Culture tanks were connected in parallel to a 2250 L filtration tank. Water filtration was performed by four protein skimmers, two AP-903 Deltec (Germany) and two 400-3×F5000 H&S (Germany), with approximately 150 Kg of live rock and 60 Kg of aragonite sand (forming a deep sand bed with 10 cm depth) assuring biological filtration. Water temperature was maintained by a Profilux II GHL (Germany) controlling both water heating (through titanium heaters) and cooling (through an Eco Cooler – Deltec, Germany). The filtration tank was also equipped with a calcium reactor PF-1001 Deltec (Germany). Salinity was maintained at 35 ± 0.5 using an osmoregulator (Deltec Aquastat 1000, Germany) that provided automatic compensation of evaporated water with freshwater purified by a reverse osmosis unit. Other water parameters were maintained as follows through the experimental period: temperature 26 ± 0.5 °C, TAN 0.05 ± 0.01 mg L^{-1}, NO_2^-–N 0.03 ± 0.01 mg L^{-1}, NO_3^-–N 0.1 ± 0.1 mg L^{-1}, PO_4^{3-}–P 0.01 ± 0.001 mg L^{-1}, pH 8.2 ± 0.2, alkalinity 3.90 ± 0.20 mEq L^{-1}, Ca^{2+} 430 ± 20 mg L^{-1}, Mg^{2+} 1300 ± 20 mg L^{-1}.

Water turnover in culture tanks (including the tank holding the mother colonies) through the filtration tank was approximately 10 times the culture tank volume per hour (≈7500 L h^{-1}). Additionally, each culture tank was also equipped with four circulation pumps (Turbelle Stream 6205, Tunze, Germany).

The illumination of the tank where mother colonies were stocked was provided by T5 fluorescent lamps (lighting system Sfiligoi Stealth, Italy - with 12 × T5 fluorescent lamps ATI T5 Aquablue Special 80W, 15000 K, Germany), with a photoperiod of 12 h light : 12 h dark, delivering a PAR of 250 ± 10 μmol quanta m^{-2} s^{-1} at the level of the coral colonies. PAR

values were measured with a Quantum Flux meter (Apogee MQ-200, USA) by placing a submergible sensor at the level of coral colonies.

Coral mother colonies were fragmented using sterilized cutting pliers, producing 6 similar sized fragments per mother colony of each species (approximately 40 mm long and 4 mm in diameter, with an average weight of approximately 0.60 g, for *A. formosa*; and 10 mm long and 7 mm in diameter, with an average weight of approximately 1.70 g, for *S. pistillata*) per colony. All fragments (30 per species) were produced from terminal branches of coral mother colonies, containing both radial and axial polyps. Each coral fragment was individually attached with epoxy resin (Aqua Medic GmbH, Bissendorf, Germany) to a plastic coral stand (Coral Cradle®, UK). Each coral stand was labelled and stocked in the same tank of mother colonies during one week, before being randomly distributed among the different experimental treatments (see below).

- *Experimental design*

Coral fragments of both species were stocked in 750 L culture tanks (identical to the ones described above for mother colonies) connected to the same 9000 L culture system where mother colonies were stocked, in order to avoid any potential artifacts promoted by differences in water quality. Experimental treatments were performed using 3 different light sources (T5 fluorescent lamps 15000 K, LEP and LED) emitting light with different spectral characteristics (Fig. 4.1.1). The spectrum of each light source was measured at the beginning (T_i) and at the end of the 5 month experiment (T_f). Light spectrum was measured over a 340-840 nm bandwidth, with a spectral resolution of 0.33 nm, using a USB2000 spectrometer (USB2000-VIS-NIR, grating #3, Ocean Optics, USA) connected to 400 μm diameter fiberoptic (QP400-2-VIS/NIR-BX, Ocean Optics). The light spectrum of each light source was obtained by measuring, in ten different points, the spectrum of the light reflected from a reference white panel (WS-1-SL White Reflectance Standard with Spectralon, Ocean Optics) placed under the light source at a constant distance (25 cm). The fiberoptics was maintained perpendicular to the reference panel surface in order to measure the reflected light spectra.

Each 750 L experimental tank was illuminated from above with the same PAR light intensity (250 ± 10 μmol quanta m^{-2} s^{-1}). PAR values were measured every week during the experiment

at the level of coral fragments. The distance between each light system and water surface was adjusted in order to produce the same light PAR at the coral fragments level in all experimental treatments. Lighting systems were operated with a photoperiod of 12 h light : 12 h dark. The control treatment was performed employing T5 fluorescent lamps 15000 K (as described before in "*Coral husbandry and fragmentation*" section), mimicking the illumination employed in the tank where mother colonies were stocked. The LEP treatment was performed under a Sfiligoi Vision Dual system, Italy (2 x 260W), while the LED treatment was performed using an 8×48 W NEPTUNE LED Reef Lighting systems (Spain).

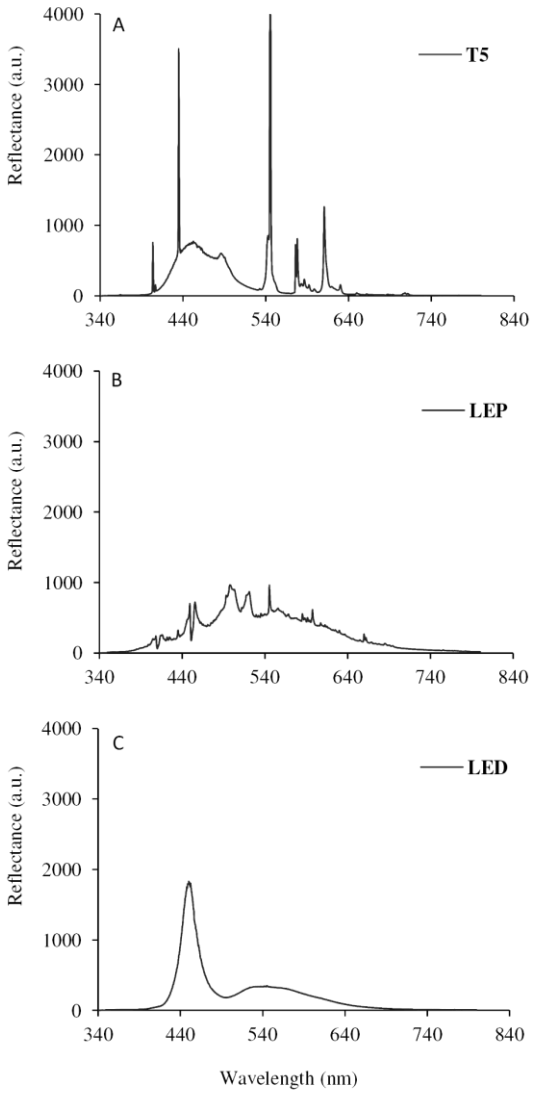

Fig. 4.1.1. Average of emission spectra of the illumination systems used to provide artificial lighting, (a) T5 - fluorescent lamps, (b) LEP – light emitting plasma and (c) LED – led emitting diode. Photosynthetically active radiation (PAR) was identical to all tested light spectra: 250 ± 10 μmol quanta m^{-2} s^{-1}.

In order to operate with a PAR of 250 ± 10 μmol quanta m^{-2} s^{-1} in a production area of 1.6 m^2, the total power consumed by the lighting systems was 960 W for T5, 520 W for LEP and 384 W for LED, representing an emitted PAR per consumed power (PAR W^{-1}) of 0.26 ± 0.02, 0.48 ± 0.02 and 0.65 ± 0.02 PAR W^{-1} for T5, LEP and LED, respectively. Consequently, the power consumption per production area was 600 W h^{-1} m^{-2} for T5, 325 W h^{-1} m^{-2} for LEP, and 240 W h^{-1} m^{-2} for LED.

Twenty-seven fragments from each species were randomly selected from the initial pool of 30 fragments (6 fragments per mother colony x 5 mother colonies) and distributed in the stocking tanks employed for each lighting treatment. Each tank was stocked with 3 groups of 3 coral fragments from each species (n = 9) placed in different locations within the experimental tank (but always exposed to the same PAR). Coral stands were fixed on white egg-crate, to allow all coral fragments to be placed at the same water depth (≈ 0.3 m). At the beginning of the experiment, the average net weight (see section *2.5. Coral fragments growth*) (± standard deviation) of *A. formosa* and *S. pistillata* fragments was 0.61 ± 0.15 g and 0.95 ± 0.17 g, respectively.

Water parameters were kept as described above for mother colonies. Partial water changes using filtered seawater (10% of total experimental system volume) were performed every other week. The experiment was ended after 5 months.

- *In vivo chlorophyll fluorescence*

Pulse Amplitude Modulation (PAM) fluorometry was used to non-intrusively monitor the photosynthetic activity of zooxanthellae (Schreiber et al., 1986). We used a PAM fluorometer comprising a computer-operated PAM-Control Unit (Walz) and a WATER-EDF-Universal emitter-detector unit (Gademann Instruments, GmbH, Würzburg, Germany) (Cruz and Serôdio, 2008). Measuring, actinic and saturating lights were provided by a blue LED-lamp (peaking at 450 nm, half-bandwidth of 20 nm) and delivered to the sample by a 1.5 mm-diameter plastic fiber optics. The fiber optic was positioned perpendicularly to the surface of the coral fragment. Measurements were carried out 2 h after the start of the daylight period, to ensure the full activation of the photosynthetic apparatus of zooxanthellae, in 3 different points in each coral fragment. Corals were dark-adapted for 20 min prior to any

measurement, after which one saturation pulse (0.8 s) was applied to determine: the minimum- or dark-level fluorescence (F_o), a parameter known to be correlated with the chlorophyll (Chl) *a* content (Serôdio et al., 2001); and maximum fluorescence (F_m). F_o and F_m were used to determine the maximum quantum yield of PSII (Schreiber et al., 1986):

$$F_v/F_m = \left(\frac{F_m - F_o}{F_m}\right) \quad (1)$$

- *Coral spectral reflectance*

Diffusion reflectance spectra were measured at the end of the experiment over a 330-1000 nm bandwidth, with a spectral resolution of 0.33 nm, using a USB2000 spectrometer (USB2000-VIS-NIR, grating #3, Ocean Optics, USA) connected to 400 µm diameter fiberoptic (QP400-2-VIS/NIR-BX, Ocean Optics). Each coral fragment was removed from the aquarium and placed in a 50 mL container, filled with water from the experimental tank. The fiberoptics was maintained perpendicular to the coral surface, at a fixed distance, defined to match a view field covering a circular area of approximately 3 mm diameter on the surface of each coral fragment. During measurements, the coral fragments and the reference white panel (see below) were measured under a constant irradiance of 200 µmol m^{-2} s^{-1}, provided by a halogen lamp (Volpi Intraluz 5000-1 Volpi, Switzerland). The light spectrum reflected from each coral nubbin was normalized to the spectrum reflected from a reference white panel (WS-1-SL White Reflectance Standard with Spectralon, Ocean Optics). The reflectance spectrum measured in the dark was subtracted to both spectra to account for the dark current noise of the spectrometer. Coral fragments were measured in the middle section of *S. pistillata* and in the radial polyps of *A. formosa*, in 3 different and non-overlapping areas. The mean spectrum was smoothed using a 10-point moving average filter before being used for subsequent calculations. In order to have 3 measurements in each coral fragment, the spectral reflectance in *A. formosa* was performed only in radial polyps.

The Normalized Difference Vegetation Index (NDVI) (Rouse et al., 1973) was calculated as:

$$NDVI = \left(\frac{R_{750} - R_{675}}{R_{750} + R_{675}}\right) \quad (2)$$

where R_{750} and R_{675} represent the average diffusive reflectance in the intervals of 749.73–750.39 nm and 674.87–675.55 nm, respectively.

- *Coral fragments growth*

Drip-dry weights of all coral fragments were determined using a Kern Emb 200-3 balance (Kern & Sohn GmbH, Germany) allowing the calculation of coral fragments growth rates between T_i and T_f. Before fragmentation the weight of each numbered plastic coral stand (Coral Cradle®) and the respective epoxy resin used to fix the coral fragment, were recorded. Total weights were corrected for the combined weights of plastic coral stands and epoxy resin, in order to obtain the net weights for each coral fragment. Coral cradles were cleaned thoroughly with seawater and a tooth-brush before each measurement, in order to minimize any potential bias caused by the development of fouling. To ensure reproducibility, each coral fragment was weighted 3 times at T_i and T_f.

To calculate the percentage of daily coral growth for each coral fragment (% CG day^{-1}), the following formula was used:

$$SGR\ (\%\ day^{-1}) = \left(\frac{\ln(w_f) - \ln(w_i)}{\Delta t}\right) \times 100 \quad (3)$$

where $\ln w_f$ and $\ln w_i$ is the ln of final and initial coral net weights, respectively, expressed in grams (g), and Δt is the time interval in days. CG is expressed in percentage of coral weight increase per day.

- *Protein*

Protein concentration was determined for both species in the crude extracts of samples from all light treatments (Bradford, 1976). The method was adapted from Bio-Rad's Bradford micro-assay set up in 96-well plates with flat bottoms, with bovine g-globulin as the standard. Protein concentration was normalized to coral surface area, which was assessed using the aluminium foil method (Marsh, 1970).

- *Statistical analysis*

Statistical analyses were performed using the software Statistica version 8.0 (StatSoft Inc.). Factorial ANOVAs were used to evaluate the existence of significant differences in the maximum quantum yield of PSII (F_v/F_m), NDVI, coral growth and protein content on fragments of *A. formosa* and *S. pistillata* kept under different light spectra. Coral species and lighting systems (T5, LED and LEP illumination) were used as the categorical factors. Assumptions of normality and homogeneity of variance were checked prior to the analysis through the Shapiro-Wilk W and Levene tests, respectively. Unequal-N HSD post-hoc comparisons were used to determine the existence of significant differences between light spectra treatments and between species under the same light treatment. The results from Factorial ANOVAs (including interactions, degrees of freedom, F and P values) are presented in table 4.1.1.

4.1.3. Results

- *Operational costs with lighting and light emission spectra*

Assuming 0.098 € per kilowatt/hour (kW h) as the average base price of electricity (excluding VAT) for the industry in the European Union, according to the statistics of European Commission (Eurostat, 2012), we can estimate that under a photoperiod of 12 hours and operating with a PAR of 250 ± 10 μmol quanta m^{-2} s^{-1}, the cost with illumination per production area will be of 0.71 € m^{-2} day^{-1} for T5, 0.38 € m^{-2} day^{-1} for LEP, and 0.28 € m^{-2} day^{-1} for LED.

Figure 1 shows the average of emission spectra of the lighting systems used on each experimental treatment (T5 (control), LEP and LED). In the T5 treatment, the irradiance covered the visible spectrum between 398 and 630nm, with two very pronounced peaks at 435 and 545 nm. LEP showed the most balanced spectrum over the entire visible range (370 – 730 nm), with the maximum irradiance value being recorded in the range 480-520 nm. The LED spectrum showed a very strong and narrow peak centered on 450 nm. The light spectra of all treatments did not vary during the experimental period.

- *Fv/Fm*

Maximum quantum yields of PSII (F_v/F_m) measured on *A. formosa and S. pistillata* fragments exposed to the different light spectra sources are presented in figure 4.1.2. At the end of the experiment coral fragments of both species cultured under LEP lighting presented significantly higher values ($P < 0.05$) of F_v/F_m (with mean values (± SD) of 0.69 ± 0.02 and 0.75 ± 0.02, for *A. formosa* and *S. pistillata*, respectively) than those cultured under T5 (0.63 ± 0.02 and 0.72 ± 0.03, for *A. formosa* and *S. pistillata*, respectively) and LED lighting (0.61 ± 0.05 and 0.71 ± 0.03, for *A. formosa* and *S. pistillata*, respectively). Moreover, F_v/F_m recorded under all tested lightings was always significantly higher ($P < 0.001$) in *S. pistillata* than in *A. formosa*.

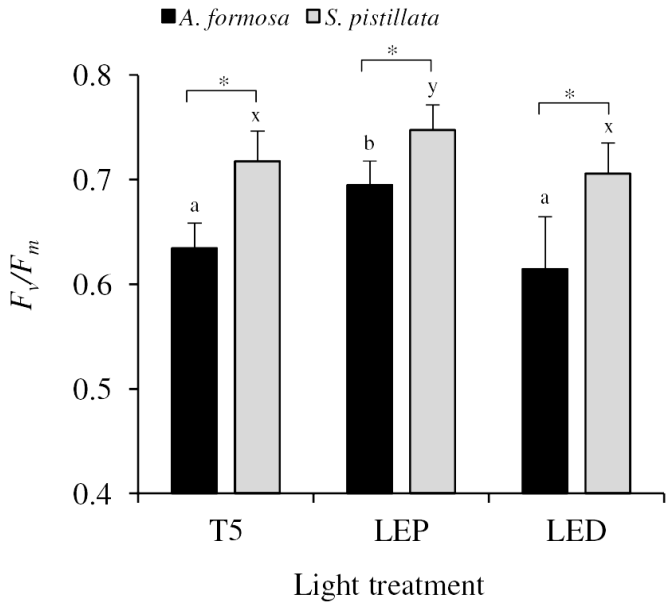

Fig. 4.1.2. Mean values (± standard deviation) of maximum quantum yield of PSII (F_v/F_m) measured on *A. formosa* (n = 9 in all light treatments) and *S. pistillata* fragments (n = 6 for T5 and LEP, n = 7 for LED) after 5 months of exposure to the three different light treatments (T5, LEP and LED). Different superscript letters represent significant differences between light treatments (*a*, *b* for *A. formosa* and *x*, *y* for *S. pistillata*); asterisk represents significant differences between both coral species under the same light treatment at $P < 0.05$.

- *Spectral reflectance*

Reflectance spectra measured on coral fragments at the end of the experimental period (Fig. 4.1.3) were characterized by low reflectance levels throughout the 400-700 nm wavelength range, denoting the absorption of PAR light by photosynthetic pigments. In all light treatments, *A. formosa* fragments presented a triple-peaked pattern at 514, 566, and 650 nm, while *S. pistillata* exhibited a plateau-shaped pattern between 600 and 650 nm. Fragments of *A. formosa* and *S. pistillata* cultured under LEP illumination presented the highest and the lowest reflectance values, respectively. In all treatments a steep increase in reflectance values was recorded in the 700-750 nm region. Higher reflectance levels were also recorded for the near-infrared region (> 750 nm) (data not shown).

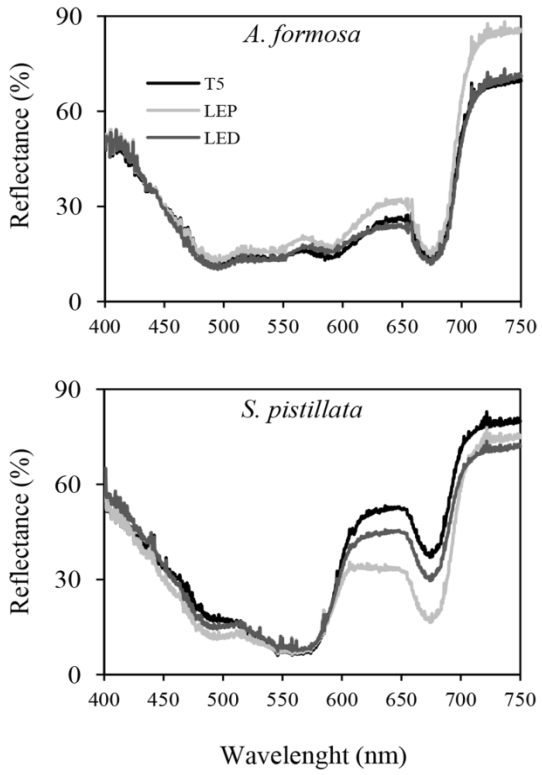

Fig. 4.1.3. Average values of reflectance spectra measured in fragments of *A. formosa* (n = 9 in all light treatments) and *S. pistillata* fragments (n = 6 for T5 and LEP, n = 7 for LED) stocked under the three light treatments (T5, LEP and LED) at the end of the experiment.

In the visible wavelength range, reflectance spectra showed a wide, well-defined inverted peak in the red region (650-700 nm), with a minimum at 672-675 nm, corresponding to the absorption peak of Chlorophyll *a* (Chl *a*). Regardless of the light treatment, the two coral species evidenced different reflectance patterns.

Figure 4.1.4 shows averages of NDVI values measured on corals from each light treatment at the end of the experiment. While no differences were found for *A. formosa*, fragments of *S. pistillata* cultured under LEP illumination presented significantly higher NDVI values than those recorded for conspecific fragments grown under T5 or LED illumination ($P < 0.001$). Concerning interspecific differences, fragments of *A. formosa* presented significantly higher NDVI values than *S. pistillata* when cultured under T5 and LED illumination ($P < 0.001$).

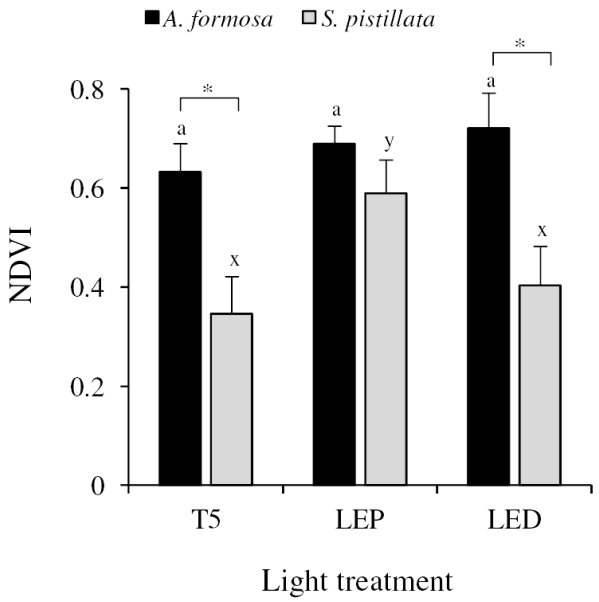

Fig. 4.1.4. Mean values (± standard deviation) of NDVI measured at the end of the experiment, in the middle section of *S. pistillata* and in the radial polyps of *A. formosa*, in 3 different and non-overlapping areas of each coral fragment stocked under the 3 different light treatments tested. For *A. formosa*, n = 9 in all light treatments. For *S. pistillata*, n = 6 for T5 and LEP, n = 7 for LED. Different superscript letters represent significant differences on coral fragments between light treatments (*a*, *b* for *A. formosa* and *x*, *y* for *S. pistillata*); asterisk represents significant differences between both coral species in the same light treatment at $P < 0.05$.

- *Coral growth and survival*

At the beginning of the experiment the average net weight (± SD) of *A. formosa* fragments was 0.41 ± 0.22 g, 0.61 ± 0.15 g and 0.71 ± 0.21 g for T5, LEP and LED lighting, respectively. For *S. pistillata* the average weight (± SD) at the end of the experiment was 1.02 ± 0.53 g, 0.95 ± 0.37 g and 1.25 ± 0.55 g for T5, LEP and LED lighting, respectively. At the end of the experiment, the average net weight (± SD) of *A. formosa* fragments was 1.48 ± 0.29 g, 1.19 ± 0.15 g and 2.82 ± 0.57 g for T5, LEP and LED lighting, respectively. For *S. pistillata* the average weight (± SD) at the end of the experiment was 2.42 ± 0.44 g, 1.78 ± 0.38 g and 2.90 ± 0.67 g for T5, LEP and LED lighting, respectively. The mean values of the specific growth rates of coral fragments (% day^{-1}) are presented in figure 4.1.5. *A. formosa* exhibited a significantly higher growth rate under LED illumination, when compared with fragments grown under T5 and LEP ($P < 0.001$). *S. pistillata* fragments grown under LEP presented significantly lower growth values when compared with fragments grown under T5 ($P < 0.01$) and LED ($P < 0.001$). The inter-specific comparison of growth performance only revealed significant differences in the LED treatment, with *A. formosa* fragments presenting significantly higher specific growth rate when compared with *S. pistillata* fragments ($P < 0.05$).

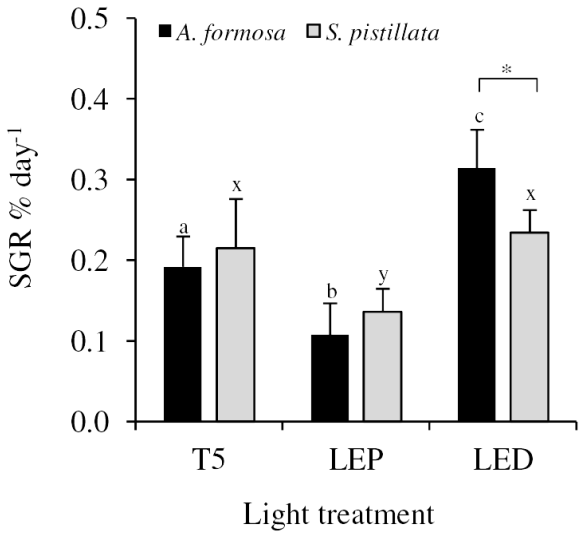

Fig. 4.1.5. Mean values (± standard deviation) growth rate (% day^{-1}) in fragments of *A. formosa* and *S. pistillata* cultured under different light treatments (T5, LEP and LED) at the end of the experiment. For *A. formosa*, n = 9 in all light treatments. For *S. pistillata*, n = 6 for T5 and LEP, n = 7 for LED. Different superscript letters

represents significant differences between light treatments (*a, b* for *A. formosa* and *x, y* for *S. pistillata*); asterisk represents significant differences between the 2 coral species in the same light treatment at $P < 0.05$.

Regarding survival, during the first month of experiment, 8 (out of 27) fragments of *A. formosa* died (3 under T5, 3 under LEP and 2 under LED lighting). No mortality was recorded through the whole experiment for *S. pistillata*, regardless of the light treatment.

- *Protein*

Average protein content (wet weight) per coral fragment surface area was significantly higher ($P < 0.001$) in *S. pistillata*, when compared with fragments of *A. formosa* cultured under the same light treatment (Fig. 4.1.6). No significant differences were recorded among *A. formosa* fragments grown under different light treatments ($P > 0.05$), whereas fragments of *S. pistillata* grown with LED illumination presented a significantly higher average concentration (± SD) of protein per coral fragment surface area (1.67 ± 0.36 mg cm^{-2}) than those cultured under T5 (1.17 ± 0.25 mg cm^{-2}) and LEP illumination (1.39 ± 0.19 mg cm^{-2}) ($P < 0.001$).

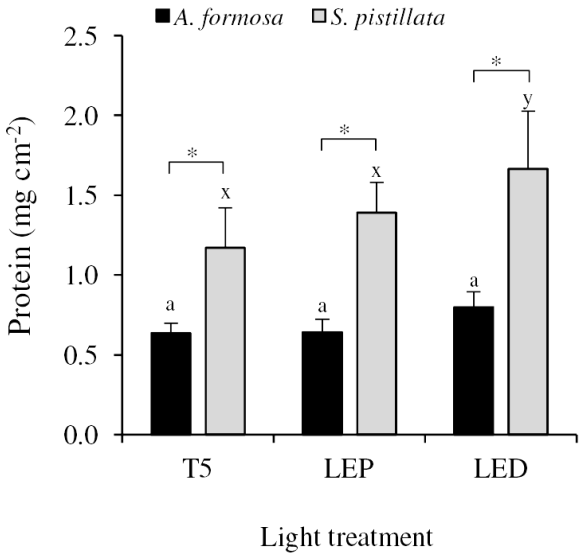

Fig. 4.1.6. Mean (± standard deviation) protein concentration (mg cm^{-2}) in fragments of *A. formosa* (n = 9 in all light treatments) and *S. pistillata* (n = 6 for T5 and LEP, n = 7 for LED) cultured under different light treatments (T5, LEP and LED) at the end of the experiment. Different superscript letters represents significant differences between light treatments (*a, b* for *A. formosa* and *x, y* for *S. pistillata*); asterisk represents significant differences between both coral species in the same light treatment at $P < 0.05$.

Table 4.1.1. Main and interactive effects of different light spectrum and species of a two-way factorial ANOVA, on the Specific Growth Rate, protein content, NDVI and F_v/F_m, over a 5 months interval.

Variable - SGR % day^{-1}			
Factor	*F*	*df*	*P*
Species	0.561	1	0.458
Light	48.699	2	0.000
Species * Light	7.939	2	0.001
Variable - Protein			
Factor	*F*	*df*	*P*
Species	220.689	1	0.000
Light	16.026	2	0.000
Species * Light	4.039	2	0.022
Variable - NDVI			
Factor	*F*	*df*	*P*
Species	76.169	1	0.000
Time	6.576	1	0.012
Light	8.202	2	0.001
Species * Time	45.033	1	0.000
Species * Light	8.364	2	0.000
Time * Light	7.876	2	0.001
Species * Time * Light	2.954	2	0.058
Variable - F_v/F_m			
Factor	*F*	*df*	*P*
Species	195.985	1	0.000
Light	45.476	2	0.000
Species * Light	4.594	2	0.012

4.1.4. Discussion

The economic feasibility of *ex situ* production of ornamental scleractinian corals in Recirculating Aquaculture Systems (RAS) can be significantly conditioned by production costs. As artificial illumination is known to be one of the highest costs associated with this type of coral production (Osinga, 2008), it is important to find alternative light sources with high energetic efficiency, which may contribute to minimize production costs. In this study, we tested three different light spectra (Fig. 4.1.1) and distinct PAR:Watt ratios. According to our estimations, the average annual cost with artificial illumination per production area (m^2) of an *ex situ* coral aquaculture located in the European Union, operating under a photoperiod of 12 hours light with a PAR of 250 ± 10 μmol quanta m^{-2} s^{-1} will be 254, 138, and 102 € $year^{-1}$ when employing T5 fluorescent lamps, LEP, or LED illumination, respectively. However, the initial investment in lighting systems for coral RAS can significantly change with the illumination type (e.g., T5, LED or LEP), as well as with the manufacturer. In general, LEP and LED illumination represent higher initial investments than T5 fluorescent lamps. Nevertheless, power consumption and maintenance costs are higher in T5 lighting systems (e.g. fluorescent lamps need to be changed at least yearly due to their progressive decrease in spectral quality).

Understanding the light requirements of the coral species being cultured is also of utmost importance for the success of *ex situ* culture, as variations in the spectral quality of light can significantly affect coral photobiology (Mass et al., 2010). The present study revealed that both *A. formosa* and *S. pistillata* have significantly higher values of F_v/F_m when cultured under LEP illumination and that F_v/F_m values displayed by *S. pistillata* were always significantly higher than those of *A. formosa* under all light treatments (Fig. 4.1.2). The index F_v/F_m is commonly used as an accurate measure of the maximum photochemical efficiency of PSII (Dove, 2004). A decrease on this index is commonly interpreted as an indicator of photodamage or photoinhibitory processes of photosynthesis (Franklin et al., 1992). However, despite the differences found among the experimental treatments, the values recorded for F_v/F_m were always within the range reported in the literature for healthy scleractinian corals in the wild (Levy et al., 2003; Rodrigues et al., 2008; Winters et al.,

2009). Therefore, our results indicate the suitability of all lighting systems tested to maintain scleractinian corals in captivity.

The reflectance spectra exhibited by coral fragments in all treatments (Fig. 4.1.3) evidence the existence of species-specific patterns. Fragments of *S. pistillata* displayed a plateau-shaped pattern between 600 and 650 nm, while those of *A. formosa* evidenced a triple-peaked pattern. According to the classification proposed by Hochberg et al. (2004), these patterns rank *S. pistillata* as a "blue coral" and *A. formosa* as a "brown coral". The pattern of reflectance spectra is determined by the spectral absorption and fluorescence properties of multiple pigments harbored by the zooxanthellae within the coral colony (Hochberg et al., 2004), namely Chl *a*, Chlorophyll c_2, β-carotene, diadinoxanthin, and peridinin (Gil-Turnes and Corredor, 1981). While in the present study *S. pistillata* presented a blue mode reflectance spectrum, Mass et al. (2010) reported a triple-peaked pattern for the same species, which is commonly exhibited by corals presenting a brown mode reflectance spectrum. In fact, Hochberg et al. (2004) had already recognized that some coral species may exhibit both types of reflectance spectra - the blue and the brown. Additionally, the same authors support the observations by Veron (2000), as corals displaying the brown and blue mode of reflectance spectra can exhibit some degree of variation on their coloration (e.g., the coloration of "brown corals" can range from brown to red, orange, yellow or green; while "blue corals" may display purple, blue, pink, or gray tones). The issues related to coral color are relevant for their aquaculture, as some color morphs are preferred over others and reach higher market values in the marine aquarium trade. Nonetheless, the different light regimes tested in the present study have not promoted any significant diference in the color or reflectance pattern exhibited by fragments produced from the same species.

The Normalized Difference Vegetation Index (NDVI) is known to be a reliable proxy for Chl *a* concentration (Rouse et al., 1973). At the end of the experimental period, the fragments of *A. formosa* did not present any significant differences in NDVI values among different light treatments (Fig. 4.1.4), whereas those of *S. pistillata* cultured under LEP illumination presented significantly higher NDVI values. The very pronounced inverted peak observed in the range 672-675 nm for both species cultured under all light treatments (Fig. 4.1.3), corresponds to the absorption peak of Chl *a*. Therefore, the lower values of reflectance

observed in *S. pistillata* cultivated in LEP light can be related with an increase in the Chl *a* concentration. In general, differences recorded in pigment concentration in the coral tissue can be associated with: 1) changes in zooxanthellae density; or 2) shifts in pigment concentration within the zooxanthellae. In both cases, these differences can be influenced by environmental conditions (e.g., salinity, temperature, and nutrients availability), as well as coral taxa (Hedley and Mumby, 2002; Myers et al., 1999). In the present study the contrasting differences in light spectra were likely the cause for the changes observed in the reflectance properties of cultured coral fragments, as water parameters and PAR intensity was identical among treatments.

The present study also revealed a significant effect of light spectrum on coral growth for both *A. formosa* and *S. pistillata*. *A. formosa* cultured under LED presented a specific growth rate 99% higher than conspecifics grown under T5 illumination (used as control). Notwithstanding the fact that no significant differences on growth were recorded for *S. pistillata* fragments grown under LED and T5, those stocked under LED presented an average growth 18% higher than conspecifics stocked under T5 illumination. Coral growth is known to be influenced by three major physiological processes: photosynthesis, heterotrophic feeding and calcification (Osinga et al., 2011). Several hypotheses have been proposed to explain the relationship between zooxanthellae and coral calcification (Allemand et al., 2004), being suggested that higher calcification rates in hermatypic corals could be strongly related with the role played by autotrophic symbionts. Higher photosynthetic performance has already been documented in scleractinian corals exposed to blue light (Kühl et al., 1995; Levy et al., 2006; Levy et al., 2003). The highest growth rates achieved in our study were indeed recorded on coral fragments grown under LED illumination, thus supporting the relevance of blue light claimed by the previous authors. Nonetheless, it was under LEP lighting that *A. formosa* and *S. pistillata* evidenced the highest photosynthetic performance (higher F_v/F_m values). It is therefore important to stress that corals may control the microenvironment of their symbiotic algae (Kühl et al., 1995), thus being able to modulate the light spectrum reaching the zooxanthellae in different coral species. In this way, given the scarcity of scientific works addressing these topics, our results should be interpreted with caution. Making any generalizations on which lighting system

favors the photosynthetic performance of corals cultured *ex situ* at this point would be highly speculative and possibly misleading.

The study performed by Wijgerde et al. (2012) comparing LED and LEP illumination for the *ex situ* culture of the coral *Galaxea fascicularis* recorded a significantly higher specific growth rate for corals cultured under LEP than LED. Additionally, the same study revealed that corals exposed to medium light intensity (125 – 150 μmol quanta m^{-2} s^{-1}) outperformed those grown under a higher light intensity (275 – 325 μmol quanta m^{-2} s^{-1}). In the present study a higher light PAR intensity (250 ± 10 μmol quanta m^{-2} s^{-1}), promoted satisfactory growth results, agreeing with previous data by Juillet-Leclerc and Reynaud (2010) for *Acropora* sp. grown under a PAR intensity of 260 μmol quanta m^{-2} s^{-1}. Concerning *S. pistillata*, while PAR intensity employed in our study was lower than the one previously referred by Ferrier-Pages et al. (2003) (300 ± 20 μmol quanta m^{-2} s^{-1}), a similar growth was recorded. Overall, all contrasting results referred above reinforce the idea that lighting requirements for scleractinian corals are species-specific, may be influenced intraspecifically by genotypic plasticity and culture system-specific differences (Osinga et al., 2011). Additionally, *ex situ* growth performances can be somehow optimized through the modulation of PAR intensity (Forsman et al., 2012). Understandably, one of the key aspects that will determine the economic suitability of such modulation will be the trade-off achieved between: 1) increased coral growth, 2) PAR intensity, and 3) production costs (e.g., higher PAR = higher costs with electric power = higher production costs; therefore, only being a feasible option if promoting superior coral growth).

The mean protein content displayed by *S. pistillata* was always higher than that of *A. formosa* grown under the same light treatment (Fig. 4.1.6). While no significant differences were recorded for mean protein content of *A. formosa* between the different light regimes, *S. pistillata* fragments grown under LED presented significantly higher protein content compared to those cultured in T5 and LEP. These results indicate that while LED promoted growth in *A. formosa* (significantly higher than growth promoted by T5 and LEP), mostly through an increase in skeletal weight, the effect on *S. pistillata* occurs in both skeletal growth (significantly higher than growth promoted by LEP) and in an increase on live coral

tissues (as evidenced by its higher protein content in comparison to T5 and LEP light treatments).

It is important to highlight that although no exogenous food was supplied through the whole experiment, the protein concentration recorded for fragments of *S. pistillata* grown under LED illumination was higher than that recorded for conspecifics in the wild and fragments of *S. pistillata* grown in captivity that were provided with a daily supply of newly hatched *Artemia* nauplii (Borell et al., 2008). While the importance of heterotrophic feeding in the growth of symbiotic scleractinian corals in the wild is now unanimously recognized among researchers (Anthony and Fabricius, 2000; Borell et al., 2008; Ferrier-Pages et al., 2003; Houlbrèque and Ferrier-Pages, 2009; Houlbrèque et al., 2004), our results reveal the need to perform further testing to evaluate the added-value of providing exogenous food to enhance the growth of scleractinian corals propagated *ex situ*.

While no mortality was recorded among fragments of *S. pistillata* during our experiment, 8 out of 27 fragments of *A. formosa* were lost. Coral survival post-fragmentation is known to be influenced by several factors, such as the initial size of the fragment and the substrate to which it is attached (Calfo, 2007; Lirman, 2000). In the present study, all coral fragments from both species were produced following the same procedures, namely using the epoxy resin and substrate to attach each fragment of *A. formosa* and *S. pistillata*. In this way, it is legitimate to assume that *S. pistillata* is probably more resistant to manipulation and fragmentation than *A. formosa*. Additionally, as all fragments of *A. formosa* were lost during the first month of the experimental trial, it is likely that mortality was rather prompted by the stress induced through the fragmentation procedure than by the contrasting illumination systems employed for coral grow-out.

4.1.5. Conclusion

We conclude that blue light sources, such as LED lighting, promote higher growth for *A. formosa* and *S. pistillata*. Thus, LED technology seems a promising option for scleractinian ornamental corals aquaculture *ex situ*. The initial investment for artificial lighting in RAS for

coral production, as well as power and maintenance costs, are decisive when analyzing the economic feasibility of *ex situ* coral aquaculture. Given the results recorded in the present study, it is urgent to fill the current gap of knowledge on the suitability of LEP and LED lighting to culture coral ex situ, by performing similar studies covering a wider range of coral genus and species. Only after further experimental testing will it be possible to confirm if these new lighting systems are a suitable alternative to "conventional" lighting solutions (e.g, T5 fluorescent and HQI lamps) for coral production *ex situ*.

Acknowledgements

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Chapter 4

4.2. Contrasting light spectra trigger morphological shifts in the skeleton of reef building corals

In preparation: Rui J. M. Rocha, Ana M. B. Silva, M. Helena Vaz Fernandes, Igor C. S. Cruz, Rui Rosa, Ricardo Calado. Contrasting light spectra trigger morphological shifts in the skeleton of reef building corals.

Abstract

Scleractinian corals variability can be evidenced in the shape of colonies during growth, skeleton macrostructure or microstructure architecture. The morphological plasticity of scleractinian corals can be influenced by numerous factors in their natural environment, although it is difficult to identify the relative influence of a single biotic or abiotic factor due to potential interactions. Due to the symbiotic relation between scleractinian corals and photosynthetic endosymbionts (the zooxanthellae), light is considered a major factor affecting coral morphology. Nonetheless, most studies addressing the importance of light on corals morphological plasticity have focused on Photosynthetically Active Radiation (PAR) intensity, while the effect of light spectra remains largely unknown. The present study evaluated how different light spectra affect the skeleton, macro- and microstructures of the skeleton of two hermatypic coral species (*Acropora formosa* and *Stylophora pistillata*) maintained under controlled laboratory conditions. We tested the effect of three light treatments with the same PAR but with distinct spectral emission: 1) T5 fluorescent lamps with blue emission (T5); 2) Light Emitting Diodes (LED) with predominantly blue emission; and 3) Light Emitting Plasma (LEP) with full spectrum color. To exclude the potential bias generated by genetic variability, the experiment was performed with clonal fragments for both species. After 6 months of exposure to the experimental light spectra, it was possible to detect for both studied species significant differences on the organization of coral skeleton microstructure, as well as in macrostructure morphometry (distance among corallites, corallite diameter, and theca thickness). The present study opens a new research field with potential applications on reef restoration efforts, as well as biotechnological applications, namely the use of coral skeletons as biomaterial for bone graft applications or biomass production for the prospecting of bioactive compounds.

Keywords

Acropora; *Stylophora*; microstructure; SEM; phenotypic plasticity

4.2.1. Introduction

Scleractinian corals morphological variability is well documented in the literature, with numerous descriptions on general shifts in colonies growth shapes (Graus and Macintyre, 1976; Muko et al., 2000; Padilla-Gamino et al., 2012; Veron, 1995, 2000), as well as in more specific features, namely corallite structure (e.g. septal length, columellar diameter, number of septa, theca thickness) or distance among corallites (Menezes et al., 2013; Veron, 1995).

This variability of scleractianian corals is reflected in their complex taxonomy, commonly classified as sibling species, sub-species, ecomorphs and morphotypes (Stobart, 2000). To support taxonomic identifications, researchers advocate the analysis of interpopulational, intrapopulational and intracolonial levels of variation (Veron, 1995).

Morphometric analyses, at distinct levels of morphologic variation, can provide a tool of utmost importance for diverse disciplines, such as physiology, ecology, biology, taxonomy, or phylogeny, which will help to understand the mechanisms of adaptation, gene connectivity and habitat selection of reef building corals (Menezes et al., 2013).

The aragonite ($CaCO_3$) macrostructures forming the exoskeleton of scleractinian corals are formed under a layer of organic material secreted by cells from basal ectoderm of coral polyps (Sorauf 1972). Aragonite crystals precipitate in a hydro-organic gel to form microstructural units, recognized as crystallites (which form the centers of calcification) and fibers (a composite of biocrystals in which organic compounds and mineral ions interact) (Dullo, 1987; Nothdurft and Webb, 2007; Sorauf, 1972; Stolarski and Roniewicz, 2001; Stolarski and Russo, 2002). While several models of biomineralization have been proposed in the last years, the remarkable diversity of corals has impaired the acceptance of a single model of skeletal growth (Nothdurft and Webb, 2007).

The morphology of scleractinian corals can be influenced by numerous factors in the natural environment (Bruno and Edmunds, 1997; Veron, 1995). Intraspecific morphological variations among scleractinian corals has been associated with genetic variability (Cuif et al., 2003; Potts, 1978), competition for space (Potts, 1976; Raymundo, 2001), concentration of nutrients in the water (Bongiorni et al., 2003b; Bongiorni et al., 2003a), and with the influence of environmental factors, such as light (Nir et al., 2011; Todd, 2008; Todd et al.,

2004a), depth and pressure (Nir et al., 2011), water movement (Chappell, 1980; Riegl et al., 1996) and sedimentation rates (Riegl et al., 1996; Stafford-Smith, 1993; Todd, 2008). However, in most cases, it is difficult to identify the relative influence of a single factor, especially for environmental parameters, due to potential interactions (e.g. water current and light intensity decrease with increasing depth (Oliver et al., 1983), sedimentation is affected by water current (Riegl et al., 1996) and can influence zooxanthellae photosynthesis and coral respiration (Telesnicki and Goldberg, 1995)).

Due to these complex interactions, only a few experiments have so far successfully identified parameters affecting phenotypic plasticity in scleractinian corals. The common procedure on these experiments consists in moving colonies to new environments and register morphological shifts over time (Foster, 1980; Graus and Macintyre, 1989). This procedure is also used in experiments that aim to identify plasticity and variation among genotypes, namely by using clonal organisms to eliminate genetic variability (Bruno and Edmunds, 1997; Raymundo, 2001; Todd, 2008; Todd et al., 2004b; Todd et al., 2001). Therefore, it becomes evident that the only way to separately control those variables is to perform experiments under controlled conditions *ex situ* (Todd et al., 2004a).

The identification of parameters that may influence skeletal macro and microstructures organization may substantially improve coral production, not only for supplying the marine aquarium trade but also for a range of biotechnological/biomedical applications. The interconnectivity, porosity and three-dimensional structure of coral skeletons, which mimics human bone (Sopyan et al., 2007), has captured the interest of the scientific community for its potential as bone graft substitutes in veterinary and medical applications (Demers et al., 2002; Moore et al., 2001; Nishikawa et al., 2009), with emphasis to low load bearing situations, such as maxillofacial repairs (Shabana et al., 1991; Viitala et al., 2009) or even as scaffold for tissue engineering purposes (Liu et al., 2013). However, the structural variability of the skeletons displayed by wild corals, as well as the large dependence of supply from harvests in coral reefs have been pointed as major constraints for a broader application of coral skeletons for biomedical purposes (Sopyan et al., 2007). Moreover, in a different scope, the identification of modulation trends in coral morphology and skeletal micro-architecture,

can allow marine biologists to "design" coral specimens able to thrive in different environments, which can improve the success of reef restoration efforts.

Due to the symbiotic relation of several hermatypic corals with dinoflagellates of genus *Symbiodinium*, commonly termed zooxanthellae, several studies have addressed the importance of light in coral morphology, macrostructure organization and microstructure architecture. For example, a study performed by Todd et al. (2004a) suggested a relationship between *Favia speciosa* and *Diploastrea heliopora* corallite morphology and light, as corallites expanded, extended and deepened under high light conditions. Additionally, a modelling study with *Galaxea fascicularis* showed that corallite width and distance among corallites decreased with the amount of incident light, while corallite height increased with the amount of light (Crabbe and Smith, 2006). These results suggest an optimization in corallite size and distribution to promote heterotrophic nutrition or zooxanthellae photosynthesis under low or high light conditions, respectively (Crabbe and Smith, 2006). Most studies focusing on light, either performed *in situ* or *ex situ,* address Photosynthetically Active Radiation (PAR) intensity. However, a few studies using artificial illumination emitting in different wavelengths of visible light, but with the same PAR, have already evidenced how light spectra can affect coral growth (Rocha et al., 2013a; Wijgerde et al., 2012).

By knowing that light spectra influence the growth rate of corals, the protein content of their soft tissues and the photochemical performance of endosymbiotic zooxanthellae (Rocha et al., 2013a), this study aimed to evaluate the effect of different light spectra, emitting the same PAR, in the skeletal morphology, at macro- and microstructural levels, in two symbiotic hermatypic coral species (*Acropora formosa* and *Stylophora pistillata*) maintained in controlled laboratory conditions.

4.2.2. Material and Methods

- *Coral husbandry and fragmentation*

Two coral colonies, one of *Acropora formosa* and one of *Stylophora pistillata,* respectively, were kept for 1 month in a 750 L tank (2 m × 0.8 m × 0.5 m), integrated in a 8.000 L

recirculating system operated with filtered natural seawater. The filtration system was composed by four protein skimmers (two AP-903 Deltec (Germany) and two 400-3×F5000 H&S (Germany)), with biological filtration being promoted by approximately 150 Kg of live rock and 60 Kg of aragonite sand (forming a deep sand bed with 10 cm depth). Water temperature was maintained by a Profilux II GHL (Germany) that controls both water heating (through titanium heaters) and cooling (through an Eco Cooler – Deltec, Germany). The filtration tank was also equipped with a calcium reactor PF-1001 Deltec (Germany). Water turnover in the tank holding the mother colonies through the filtration system was approximately 10 times the tank volume per hour (≈7500 L h^{-1}). Additionally, the tank was also equipped with four circulation pumps (Turbelle Stream 6205, Tunze, Germany).

Water parameters were maintained as follows: salinity 35 ± 0.5, temperature 26 ± 0.5 °C, TAN 0.05 ± 0.01 mg L^{-1}, NO_2^-–N 0.03 ± 0.01 mgL^{-1}, NO_3^-–N 0.1 ± 0.1 mgL^{-1}, PO_4^{3-}–P 0.01 ± 0.001 mgL^{-1}, pH 8.2 ± 0.2, alkalinity 3.90 ± 0.20 mEq L^{-1}, Ca^{2+} 430 ± 20 mg L^{-1}, Mg^{2+} 1300 ± 20 mg L^{-1}. The illumination in coral tank was provided by T5 fluorescent lamps (Sfiligoi Stealth 12 × 80W), delivering a Photosynthetic Active Radiation (PAR) of 250 ± 20 µmol quanta m^{-2} s^{-1} at the level of the colonies, with a photoperiod of 12 hours light. PAR values were measured with a Quantum Flux meter (Apogee MQ-200, USA) by placing a submergible sensor at the level of coral colonies.

After 1 month acclimation both coral colonies were fragmented using sterilized cutting pliers, producing 30 similar sized fragments (approximately 4 cm length × 0.4 cm diameter for *A. formosa* and 1cm lenght × 0.7 cm diameter for *S. pistillata*) per colony. Coral fragments, produced from the terminal branches of mother colonies, were individually attached to a labelled plastic coral stand (Coral Cradle®, UK) with epoxy resin (Aqua Medic GmbH, Bissendorf, Germany). Coral fragments of both species were stocked in the same tank of the mother colonies during one week, before the beginning of the experimental treatments (see below).

- *Experimental design*

Experimental treatments were performed during 6 months, using 3 different light sources with distinct spectra in the visible light wave lengths (Fig. 4.2.1): 1) T5 fluorescent lamps

with blue emission (T5); 2) Light Emitting Diodes (LED) with predominantly blue emission; and 3) Light Emitting Plasma (LEP) with full spectrum color. Reflectance spectra of lights used in the experimental treatments were measured at Ti (in the beginning of the experiment) and at Tf (in the end of the experiment) over a 340-840 nm bandwidth, with a spectral resolution of 0.33 nm, using a USB2000 spectrometer (USB2000-VIS-NIR, grating #3, Ocean Optics, USA) connected to 400 μm diameter fiberoptic (QP400-2-VIS/NIR-BX, Ocean Optics). The fiberoptic was maintained perpendicular to a reference white panel surface (WS-1-SL Spectralon Reference Standart, Ocean Optics) positioned under the light source, at a constant distance, to measure the reflected light spectra.

Light treatments were tested in 750 L experimental glass tanks, similar to the tank described above for the coral colonies and connected to the same 8.000 L culture system where mother colonies were stocked, in order to avoid any potential artefacts promoted by differences in water chemistry.

Each experimental tank was illuminated from above with the same PAR light intensity (250 ± 20 μmol quanta m^{-2} s^{-1}). PAR values were measured every week during the experiment with a Quantum Flux meter (Apogee MQ-200, USA) with a submergible sensor at the level of coral fragments. The distance between each light system and water surface was adjusted to have the same light PAR at the coral fragments level in all treatments. Lighting systems were operated with a photoperiod of 12 h light : 12 h dark. The control treatment (T5) was performed employing T5 fluorescent lamps (Sfiligoi Stealth 12 × 80 W, Italy), mimicking the illumination employed in the tank where mother colonies were stocked. The LEP treatment was performed under a Sfiligoi Vision Dual system, Italy (2 x 260W), while the LED treatment was performed using an 8×48 W NEPTUNE LED Reef Lighting systems (Spain).

Twenty-seven fragments from each species were randomly selected from the initial pool of 30 fragments and distributed by the stocking tanks employed for each light spectrum treatment (n = 9 for each light treatment). Coral stands were fixed on white egg-crate, to allow all coral fragments to be placed at the same water depth (≈ 0.3 m).

Water parameters were kept as described above for mother colonies. Partial water changes using filtered seawater (10% of total experimental system volume) were performed every other week.

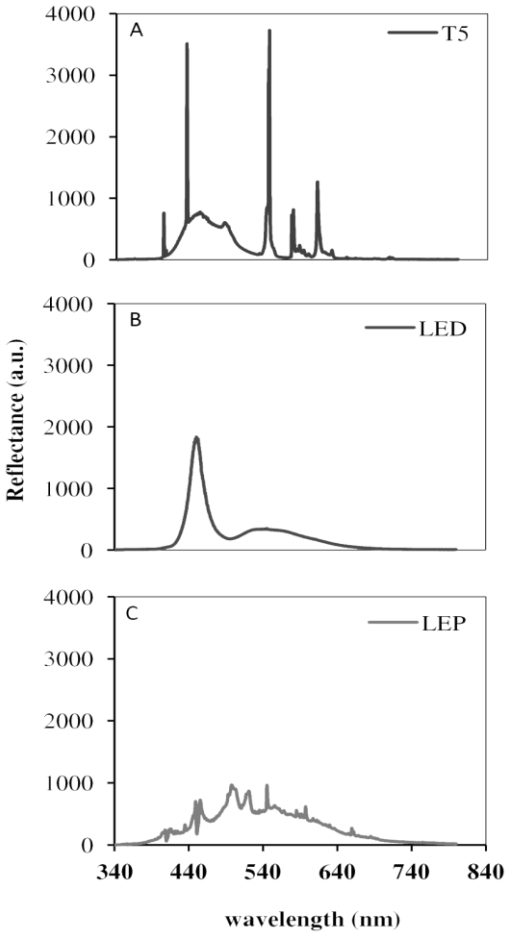

Fig. 4.2.1. Emission spectra of ligh treatments: T5 - fluorescent lamps (A), LED – led emitting diode (B) and LEP – light emitting plasma (C). Photosynthetically active radiation (PAR) was identical to all tested light spectra: 250 ± 20 μmol quanta m^{-2} s^{-1}.

- *Samples preparation and porosity measurement*

After 6 months of experiment the terminal branches of coral fragments were removed with a sterilized cutting plier to guarantee the utilization of coral skeleton grown after the beginning of light treatments. The fragments were identified and immersed in a 2% sodium hypochlorite solution for 12 – 18 h (depending on the size) to remove the organic matter from the skeleton, and rinsed thoroughly with deionised water. After this process, coral fragment skeletons were dried and porosity was determined applying the "Archimedes"-method (Bruckschen et al., 2005). Porosity was calculated as:

$$x\ (\%) = \frac{w_w - d_w}{w_w - s_w} \times 100 \qquad (1)$$

with w_w, d_w and s_w representing the wet weight, dry weight and submerged weight, respectively.

- *Samples evaluation by SEM*

After porosity determination, samples were dried and placed on aluminium support and covered with a carbon conductive thin film of carbon deposition. Samples surface and morphology modification were followed by high resolution Scanning Electron Microscopy (SEM) in a HITACHI SU-70 equipped with a Bruker EDS (Energy Dispersive System) detector at an acceleration voltage of 15 keV. (at RNME Pole of University of Aveiro, Portugal).

- *Morphometric analyses*

Morphometrics of both species were performed using the software CPCe 3.6 (Coral Point Count with Excel extensions) to analyze the images obtained with the SEM. The measures of distance among corallites (DAC), corallite diameter (CD), theca thickness (TT), and septal length (SL) were registered in coral skeleton fragments from both species in the 3 light treatments. For *A. formosa*, only the radial corallites were used. The skeletal structures used for morphometry are exemplified in figure 4.2.2.

- *Statistical analyses*

Statistical analyses were performed using the software Statistica version 8.0 (StatSoft Inc.) to evaluate the existence of significant differences (One-way ANOVA) in the morphometrics of coral fragment skeletons (DAC, CD, TT and SL) grown in the different light treatments (T5, LEP and LED, used as categorical factor) for each coral species. Assumptions of normality and homogeneity of variance were checked prior to the analysis through the Shapiro-Wilk W and Leven tests, respectively. Unequal-N HSD post-hoc comparisons were used to determine the existence of significant differences between each species coral skeletons morphometry in the different light treatments.

Morphometric data of both species was also analyzed using principal coordinates ordination (PCO). The PCO was used to describe overall relationship among the *A. Formosa* and *S. pistillata* grown in the different light treatments, respectively. The raw data matrix of morphometric data was first log (x+1) transformed, as this procedure places more emphasis on compositional differences among samples rather than on quantitative differences. After this transformation, a similarity/difference matrix was constructed using the Euclidean distance. The obtained plots (1 for each coral species) represented the distribution of specimens from the 3 light treatments according to their DAC, CD, TT and SL, together with the eigenvectors with a multiple correlation higher than 0.2. The displayed eigenvectors correspond to the obtained eigenvalues, which reflect the amount of variance explained by the PCO. Similarity percentages (SIMPER) were also explored to examine the similarity within each light treatment for each coral species. All multivariate analyses were performed using PRIMER v6 with PERMANOVA add-on (Primer-E, Ltd., Plymouth, UK).

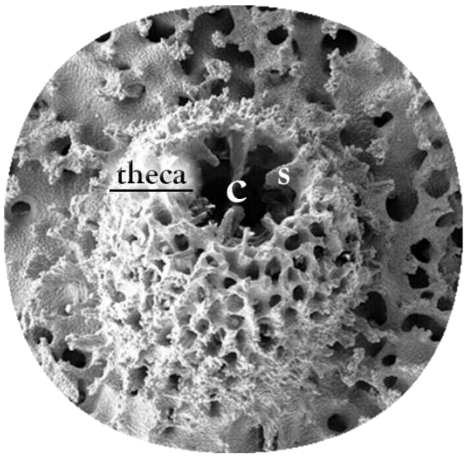

Fig. 4.2.2. Scanning electron microphotograph (magnification of 30×) of *A. formosa* radial corallite: c – columella, s – septa.

4.2.3. Results

- *Porosity*

No significant differences were registered in exoskeleton porosity of *A. formosa* fragments (45.32 ± 7.59 %, 53.63 ± 5.34 % and 52.45 ± 2.41 % for T5, LED and LEP, respectively) neither in *S. pistillata* fragments (27.52 ± 1.58 %, 25.61 ± 0.68 % and 27.06 ± 3.82 %, for

T5, LED and LEP respectively) between light treatments. However, the porosity of *A. formosa* skeletons was significantly higher when compared with porosity of *S. pistillata* in all light treatments ($P < 0.005$).

- *Evaluation by SEM*

At the end of the experiment, *A. formosa* fragments displayed an aborescent growth form, with original primary branch projecting new branches containing one axial corallite, surrounded by radial corallites. Skeletons from LED treatment (Fig. 4.2.3) evidenced corallites with larger diameter and depth. Costae of those radial corallites evidenced a ridge-shaped structure running up the outside corallites wall. Corallites from skeletons of coral fragments stocked under T5 and LEP lighting presented a structure with lower size, and not as salient as corallites from coral fragments stocked in the LED treatment. The costae of corallites from T5 and LEP lack the ridge-shaped structure found in LED treatment, and are mostly composed by spinules (Fig. 4.2.3).

The corallites present in *S. pistillata* skeleton presented a dissimilar morphologic aspect in the three light treatments tested. Corallites from T5 and LEP treatments presented the costae in a vertical position, contrarily to corallites from LED treatment whose costae was almost in a horizontal position in the majority of corallites surveyed. Columella present in corallites from LEP treatment is close to the surface of corallite calice, and its presence is more evident, when compared with columella from corallites grown in the other light treatments.

The scanning electron microphotographs of corallite edge septal surface (magnification 5000×) from both species kept in the different light treatments are depicted in figure 4.2.4. We selected one image for each species for each light treatment, but the patterns of septal microstructures were similar inside each light treatment for both species. *A. formosa* septa form corallite of specimens stocked under T5 fluorescent lamps presented a microstructure mostly composed by crystallites with spherical form and homogeneous size distribution, whereas septa observed in LED and LEP treatments presented a microstructure with the presence of fibers. Those fibers observed in septa from LED treatment presented a homogeneous growth orientation in the horizontal plan and are smaller and more compact

than fibers observed in septa from LEP treatment. Additionally, fibers observed in septa from LEP presented a growth pattern oriented to all directions in the horizontal plan. The scanning electron microphotographs of *S. pistillata* corallite septal surface from T5 light treatment presented a distinct microstructure, composed by spherical crystallites with homogeneous size distribution.

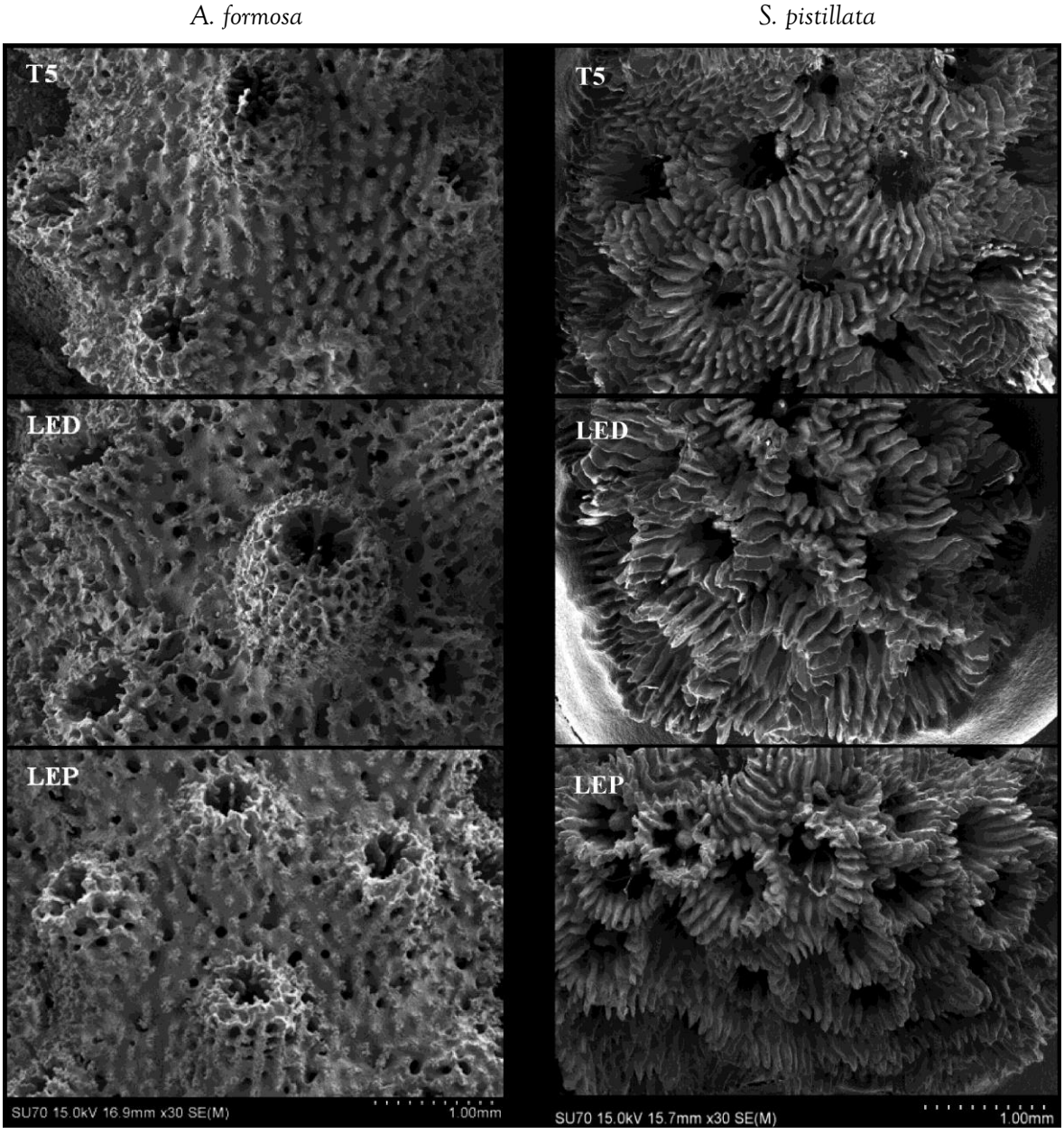

Fig. 4.2.3. Scanning electron microphotographs (magnification of 30×) of three selected images to exemplify the structure of *A. formosa* radial corallites and *S. pistillata* corallites, developed under the different light spectrum treatments: T5 fluorescent lamps (T5), light emitting diode (LED) and light emitting plasma (LEP).

The septal microstructure of corallites from the LED treatment presented a compacted aspect, where the spherical configuration of crystallites is not evidenced, whereas the LEP septa microstructure evidenced crystallites with a larger size, when compared with those from T5 light treatment.

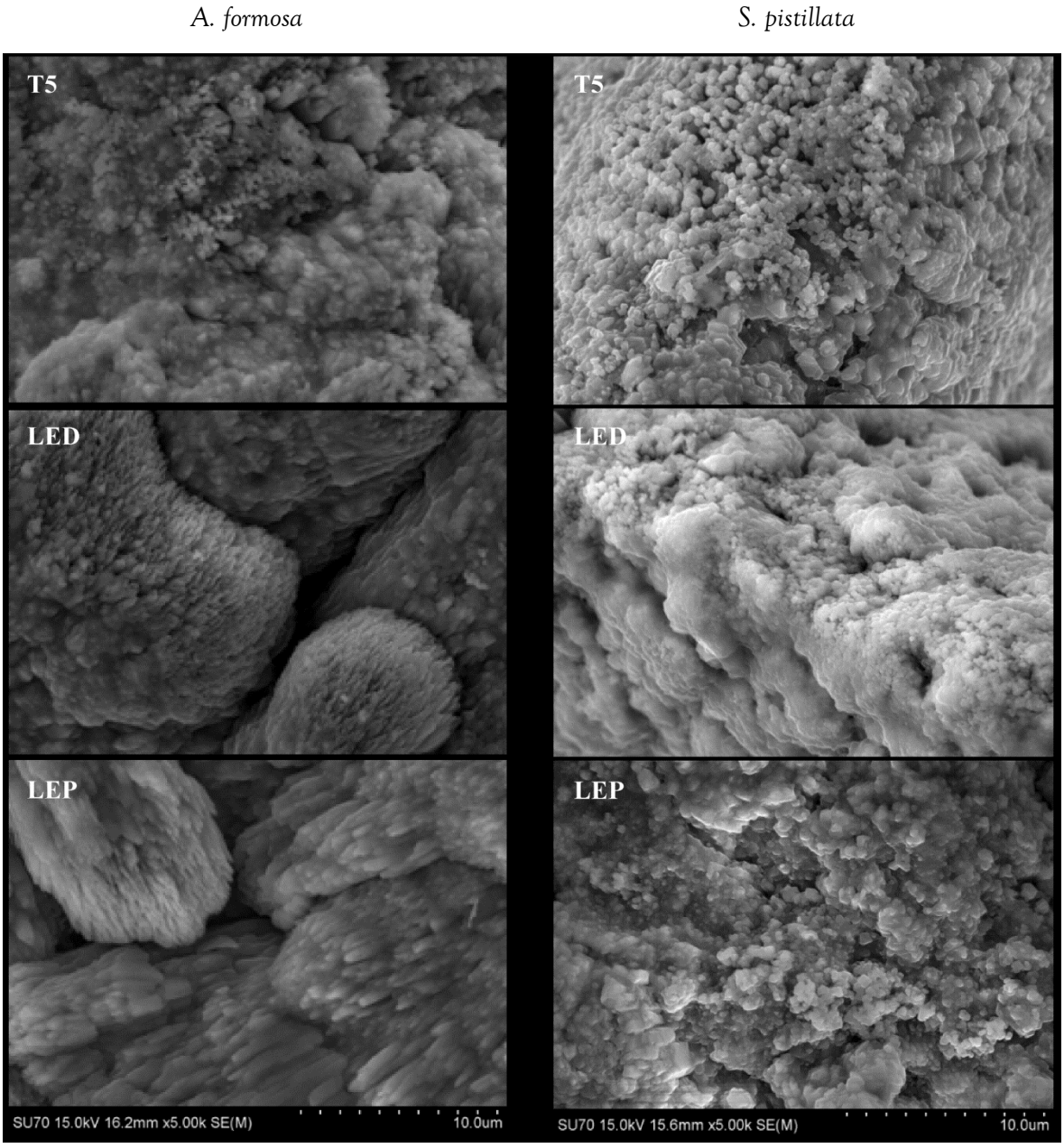

Fig. 4.2.4. Scanning electron microphotographs of three selected images to exemplify the structure of *A. formosa* and *S. pistillata* corallites septa, developed under the different light spectrum treatments: T5 fluorescent lamps (T5), light emitting diode (LED) and light emitting plasma (LEP). View with a magnification of 5000×.

- *Morphometric analyses*

Distance among corallites (DAC), corallite diameter (CD), theca thickness (TT), and septal length (SL) registered in coral skeleton fragments from both species in the 3 light treatments are presented in figure 4.2.5.

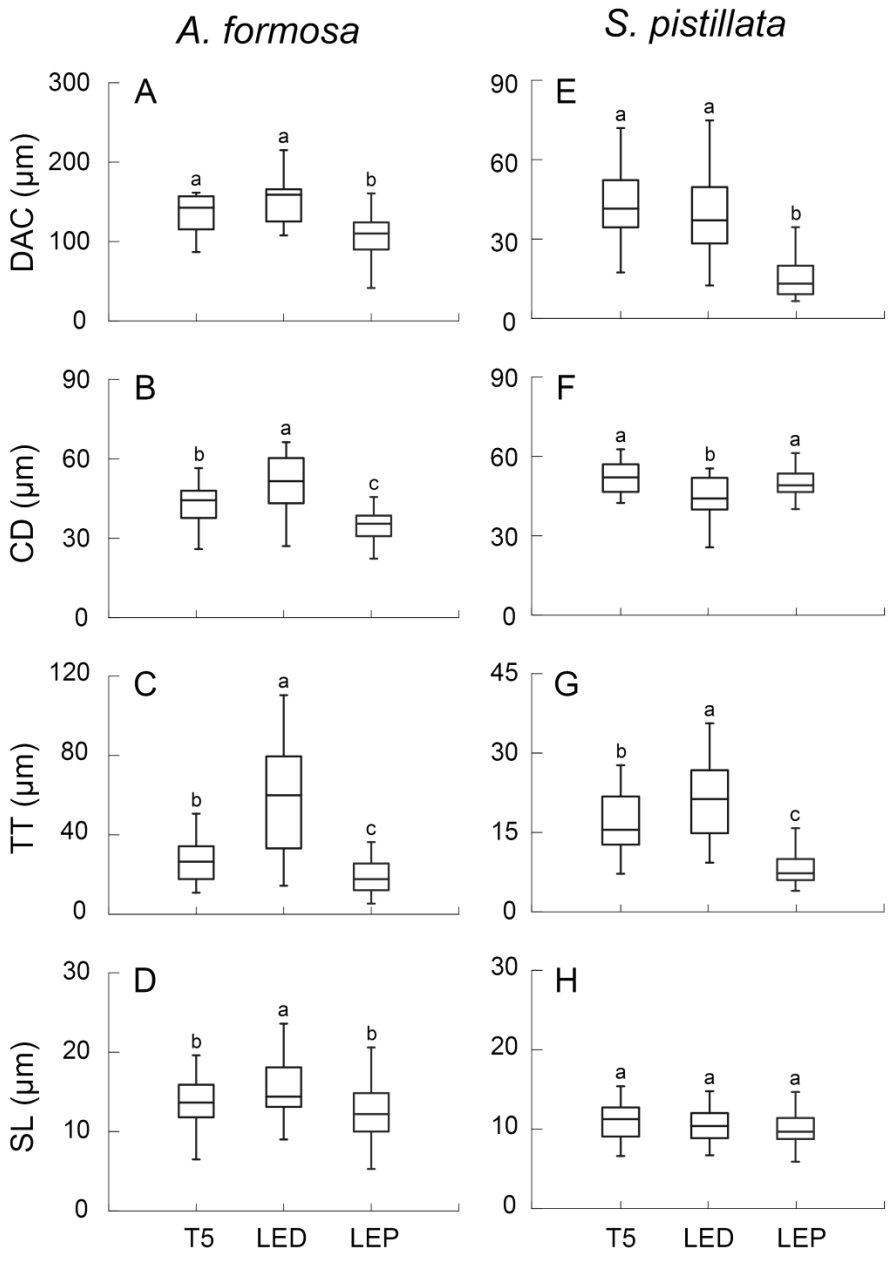

Fig. 4.2.5. Morphometric parameters of skeletal macrostructures obtained after SEM image analyses for *A. formosa* and *S. pistillata* exoskeletons from the three light treatments: T5 fluorescent lamps (T5), light emitting diode (LED) and light emitting plasma (LEP). The line in each box represents the median for the distance among corallites (DAC), corallite diameter (CD), theca thickness (TT), and septal length (SL). 50% of the

ratings have values within the box. Vertical lines extend to the minimum and maximum values, respectively. In measurements on *A. formosa* n=13, 12 and 16 for T5, LED and LEP treatments, respectively. For *S. pistillata* measurements, n=28, 25 and 38 for T5, LED and LEP treatments, respectively.

The mean distance among corallites (± standard deviation, for all results presented) in *A. formosa* was significantly lower ($P < 0.05$) in LEP treatment (108.26 ± 31.79 µm) when compared with values obtained for T5 (145.84 ± 44.71 µm) and LED (147.42 ± 40.05 µm) treatments. The corallite diameter and theca thickness were significantly different in all light treatments ($P < 0.05$ for all comparisons). Coral fragments stocked in LED presented the highest mean value of corallite diameter (48.64 ± 11.19 µm), followed by fragments from T5 (40.47 ± 9.50 µm) and LEP (33.36 ± 6.47 µm). As for corallite diameter, the highest theca thickness mean value was registered for corals from LED treatment (56.24 ± 28.46 µm), followed by fragments from T5 (29.28 ± 15.12 µm) and LEP (19.21 ± 8.26 µm). The length of septa mean value was significantly higher for corals from LED treatment (15.51 ± 3.76 µm), when compared with those from T5 (13.43 ± 3.17 µm) and LEP (12.51 ± 3.94 µm) treatments.

The mean distance among corallites (± standard deviation, for all results presented) measured in *S. pistillata*, were significantly higher ($P < 0.05$ for all comparisons) in coral fragments from T5 (43.56 ± 14.85 µm) and LED (38.14 ± 16.23 µm), when compared with those from LEP treatment (16.25 ± 9.75 µm). The mean corallite diameter in *S. pistillata* fragments from LED treatment (45.69 ± 9.77 µm) was significantly lower ($P < 0.05$) when compared with values obtained in T5 (52.35 ± 6.29 µm) and LEP (49.96 ± 7.37 µm) treatments.

The theca thickness mean values were statistically different in all light treatments, the highest value was registered on corals from LED treatment (21.52 ± 7.79 µm), followed by fragments from T5 (17.10 ± 5.70 µm) and finally the by fragments from LEP (8.12 ± 2.83 µm), which presented the lowest mean value. No significant differences were found in the length of septa in *S. pistillata*.

Figure 4.2.6 shows a principal component ordination (PCO) based in morphometric characteristics of the studied coral species. The first two axes of *A. formosa* PCOs represents

together approximately 95% of total variation. Both ordinations evidenced the differences in morphometric parameters between light treatments. The horizontal axis of variation separated specimens stocked in LED and T5 light treatment from those stocked in LEP, with major influence of distance among corallites. The vertical axis maximized the differences between exoskeletons from LED, T5 and LEP, mainly based on theca thickness.

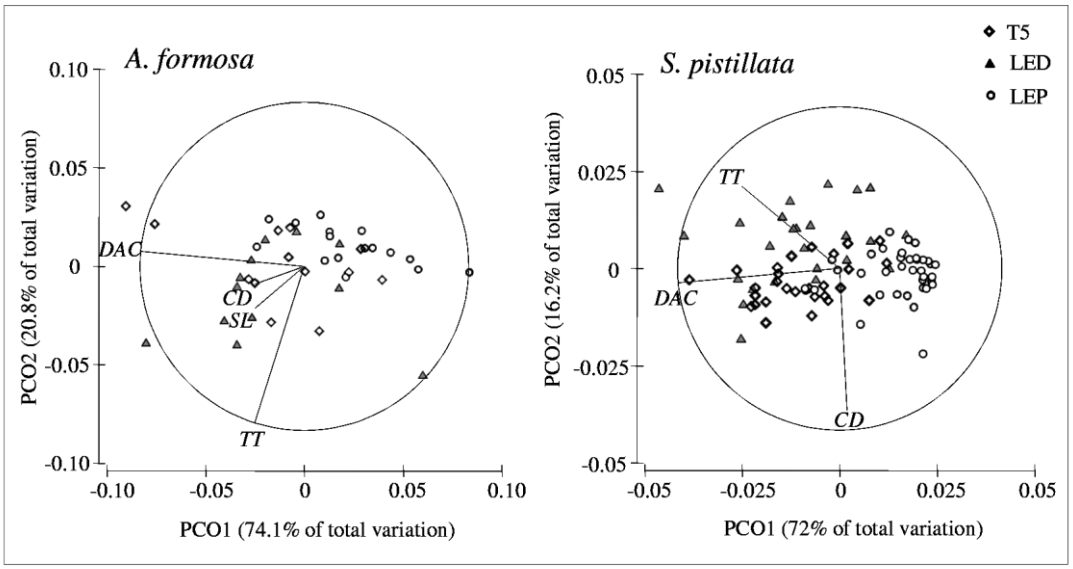

Fig. 4.2.6. Principal component ordination based on *A. formosa* and *S. pistillata* morphometry: distance among corallites (DAC), corallite diameter (CD), theca thickness (TT), and septal length (SL). Eigen vectors of multiple correlations (> 0.2) are also represented.

The PCOs of *S. pistillata* represents together approximately 80% of total variation. The distance among corallites contributed for the differentiation between corals stocked in blue light (LED and T5) from those stocked with a full spectra in visible wave lengths (LEP), while theca thickness and corallite diameter contributed essentially to differentiate corals from LED treatment.

4.2.4. Discussion

Understanding the light requirements of corals, especially for those species being cultured, is fundamental to achieve optimal production. The growth of scleractinian corals can be influenced by three physiological processes: 1) photosynthesis, 2) heterotrophic feeding, and

3) calcification (Osinga et al., 2011). The use of artificial illumination emitting in different wavelengths of visible light, but with the same PAR, has already been shown to affect coral growth (Rocha et al., 2013a; Wijgerde et al., 2012). In this topic, it is already documented the importance of blue light to the zooxanthellae photosynthetic performance (Kühl et al., 1995; Levy et al., 2006; Levy et al., 2003). It has been suggested that higher calcification rates in hermatypic corals could be strongly related with autotrophy and endosymbionts activity (Allemand et al., 2004). The effect of light in hermatypic corals is widely described in literature. Studies relying in morphological aspects suggested that corals might perform plastic adaptations depending on the surrounding environment. The aforementioned study performed by Todd et al. (2004a) suggests a relationship between corallite morphology and light, detecting that corallites expand, extend and deepen in high light conditions; another study performed *by* (Crabbe and Smith, 2006) with G*alaxea fascicularis* showed that corallite width and distance among corallites decreased with the amount of incident light, while corallite height increased with the amount of light. The increase of corallite depth with increasing light can be related with a strategy to achieve optimal internal irradiances for the photosynthetic activity of dinoflagellates harbored within coral tissues (Kaniewska et al., 2011).

It is widely known that the amount of energy in light depends on the frequency of wavelengths. Blue light has a higher frequency than red light for example, and a photon of blue light has more energy than a photon of red light (Crowell, 2013; OpenStax College, 2012). Consequently it is expected that in spite of the utilization of the same PAR, blue light treatments such as LED presents more energy than LEP. The differences in morphometric parameters evaluated for both species in LED treatment, and in some parameters (e.g. DAC or TT) also in T5 treatment (which contain a higher percentage of emission in blue spectra than LEP), can be promoted by corals to achieve the internal optimal radiances, and evidenced the importance of blue light effect in coral exoskeleton macrostructure. The general microstructure of the coral skeleton has been established for many years (Sorauf, 1972); however, the arrangement of fibbers and centres of calcification can result in a wide variety of tri-dimensional microstructural patterns, and no single model available so far is satisfactory to describe coral skeletogenesis (Nothdurft and Webb, 2007; Stolarski and

Russo, 2002). Recently, skeletal microstructure has been linked to molecular phylogenetic techniques (Cuif et al., 2003) to partially support phylogenetic relationships based on microstructural patterns. However, as recognized by Cuif and Perrin (1999), the exact microstructural patterns for scleractinian corals remain uncertain. The present study contributes with data that support this doubt on the utilization of microstructural patterns for phylogenetic approaches or taxonomic applications, as clonal specimens displayed distinct patterns under contrasting light spectra.

The effect of light spectrum in the shaping of coral skeletons can actively contribute to improve the utilization of these matrices as bone graft substitutes for medical applications (Demers et al., 2002; Moore et al., 2001; Nishikawa et al., 2009). The porosity and three-dimensional structure of coral skeletons, mimics human bone (Sopyan et al., 2007), and allow ingrowths of osteoblasts and fibrovascular tissues when used as bone graft substitutes (Moore et al., 2001). The average pore size and the observed pore interconnectivity are important requirements for the application of these coral skeletons as scaffolds for Tissue Engineering purposes. Our results show that light does not promote any shift in the porosity of coral skeleton. However, results evidenced the variability in porosity between coral species. The variability of coral skeleton structures and the dependence on wild organisms has been pointed as the major constraints to the study those applications for coral skeletons (Sopyan et al., 2007). Therefore, coral aquaculture under controlled conditions for this specific purpose, can improve the sustainability and reproducibility of studies aiming to test the feasibility of employing coral skeletons as graft substitutes. The culture of corals can also offer a wide range of scleractinian species, and in this way contribute to increase the range of options available for experimental trials.

The possibility to perform the culture of corals and mold the skeleton structure, attending the final purpose of its application, can also optimize reef restoration efforts, which has a fundamental importance in coral reef preservation (Rinkevich, 2005; Shafir et al., 2006). The manipulation of factors *ex situ*, such as light color simulating light extinction with ocean depth, or light intensity, can promote de development of structures which will enable corals to thrive under their new natural environment, depending on depth, light conditions, sedimentation or heterotrophic food availability.

Overall, results from the present experiment evidenced the major importance of light color, resulting from the emission wave length in both coral exoskeleton macro- and microstructure. It is shown that experimentation *ex situ* under controlled conditions and relying on clonal coral fragments opens the opportunity to evaluate individual parameters affecting the skeleton structure of scleactinian corals.

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Chapter 5

5.1. Final considerations and future directions

The standardized modular culture system developed represents an important tool for experimental *ex situ* coral aquaculture. The possibility to perform statistically robust experiments in standardized culture conditions, allows researchers all over the world to compare collected data in a more reliable way and advance the current state of the art of coral aquaculture.

Moreover, we achieved some relevant results on light PAR and spectral radiation applied *in ex* situ coral culture. In both experiments performed with different light PAR intensities, we obtained positive results, either in post fragmentation recovery or in coral culture, using the lowest PAR intensity studied - 50 µmol quanta m^{-2} s^{-1}. At the end of the experiment performed with *S. flexibilis,* fragments exposed to the lowest PAR intensity (50 µmol quanta m^{-2} s^{-1}) presented significantly higher values of the maximum quantum yield of PSII (F_v/F_m) as well as zooxanthellae concentration, when compared with fragments stocked in the highest PAR intensity (120 µmol quanta m^{-2} s^{-1}); moreover, the F_v/F_m values in fragments exposed to the lowest PAR treatment were similar to the values recorded for mother colonies before fragmentation, which were stocked at a PAR of 120 µmol quanta m^{-2} s^{-1}. Additionally, no differences were registered in growth and survival rates. These results confirm that *S. flexibilis* fragments do not need high PAR intensities during the recovery period after fragmentation, even those that result from mother colonies adapted to higher light PAR intensities. This result can be related to the lower size and reduced number of ramifications of coral fragments, which reduce the self-shading effect affecting bigger colonies.

In the experiment performed with *S. cf. glaucum* fragments, we did not find significant differences in survival, neither in growth of fragments under the three different PAR intensities tested (50, 80 and 120 µmol quanta m^{-2} s^{-1}). Moreover coral fragments socked in the lowest PAR treatment (50 µmol quanta m^{-2} s^{-1}) presented higher concentration of photosynthetic and most accessory pigments and zooxanthellae per dry weight of coral, when compared to fragments stocked under the other PAR treatments. Overall, results obtained in this experiment support the suitability of low PAR intensities for *ex situ* culture of soft corals.

We estimated for a 150W HQI lamp, operating with a photoperiod of 12 h light : 12 h dark, an operational cost (excluding VAT) of approximately 2.94 € m^{-2} day^{-1} and 8.82 € m^{-2} day^{-1},

when using a PAR of 50 or 120 μmol quanta m^{-2} s^{-1}, respectively. Therefore, the reduction of PAR in *ex situ* coral productions can actively contribute to reduce production costs, which can account for the economic viability of a production facility.

Additionally, in the third experiment with soft corals, we have shown that production costs associated with the artificial illumination employed for growing *S. cf. glaucum* can be reduced by using HQI lamps emitting a lower light colour temperature (3000 K) than the one commonly termed as optimal for growing this species (10000 K). However, these results just confirm that lamps with lower light colour temperature, do not negatively affect coral growth.

In the experiments performed with stony corals, we tested two recent light technologies (light emitting diode - LED and light emitting plasma - LEP) with potential for aquaculture, both emitting different light spectra and operating with distinct PAR:Watt ratios.

In the first experiment performed with stony corals, we concluded that blue light sources, such as LED lighting, promote higher growth for *A. formosa* and *S. pistillata*. Moreover, we estimated contrasting annual cost with artificial illumination per production area (m^2), by simulating an *ex situ* production of corals in the European Union, operating under a photoperiod of 12 hours light with a PAR of 250 ± 10 μmol quanta m^{-2} s^{-1} of 254 and 102 € $year^{-1}$, when employing T5 fluorescent lamps (a traditional illumination source applied to the culture of corals) or LED illumination, respectively. Therefore, in spite of higher initial costs of implementation, when compared with the traditional light sources employed to sock corals in captivity (e.g.T5 fluorescent or HQI lamps), LED seems a promising option for scleractinian ornamental corals aquaculture *ex situ*.

In the second experiment performed with stony corals, we detected that a blue light spectrum triggers morphological shifts in corals skeleton, either at a micro or macro scale. As we performed the experiment with clones from the same original colony, in tanks equipped with the same technological equipments, connected to the same life support system, we may assure that the differences recorded were exclusively associated with light wave length emission. This result may have taxonomic implications, as stony corals are still commonly classified mostly relying on the morphological features of their skeletons. Additionally, from

an ecological point of view, these findings may also be relevant, as polyps with larger corallite diameter can be more efficient in heterotrophic feeding.

Overall, the results achieved in the present thesis contributed to reduce the current gap of knowledge on *ex situ* coral aquaculture. In most published studies so far, artificial illumination was mostly been addressed from a "PAR perspective"; we have also shown that the relevance of light spectra is certainly higher that initially assumed.

Results from the experiments addressing light spectra revealed species specific patterns and in the future it will be important to identify potential common trends at genus and family levels.

The new light technologies addressed in this thesis must still be tested for different coral species. While blue light spectra evidenced positive results for the two species of hard corals tested, it is still speculative to claim that they would also favour other species of hard and soft corals. It is also important to study the cumulative effect of light spectra and heterotrophic feeding, as existing studies only focus the interaction between these heterotrophic feeding and light PAR intensity.

The suitability of the filtration systems employed in coral culture must also be revised for different coral groups (at family, genera, or in extreme situations to species level). As an example we can question the relevance of employing protein skimmers in *ex situ* culture of soft corals, as these organisms apparently resist better to suspended solids and organic matter, which can also have a positive effect on heterotrophic feeding and growth.

Finally, coral aquaculture may foster the development of various scientific fields, from marine biotechnology and natural products prospection, to the development of innovative biomedicine approaches and biomaterials. Most importantly, coral aquaculture can actively contribute to species preservation through reef restoration efforts, by allowing researchers to "tailor-make" corals *ex situ* and maximize their chances of survival once deployed in the wild.

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