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Abundance and distribution of coral fluorescent proteins along an artificial light gradient

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Declaração de autoria de trabalho

Declaro ser o(a) autor(a) deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Abstract

Corals in nearshore marine environments are exposed to a depth-light gradient imposed by the attenuation of light with depth. To a certain extent, corals are adapted to a broad range of available irradiances by producing changes in their physiology. One solution that coral organisms have, to cope with this different light condition, is through fluorescent proteins (FPs). FPs produced by corals have many roles including photoprotection and thermal stress resilience thus being one of the mechanisms by which corals can adapt to different conditions. In this study, I investigate the existence of inter- and intra-specific variation in tissue location, emission intensity, and spectral curve of FPs. These differences suggest potential differential roles and expression of FPs inside a colony at tissue level, among colonies, light habitats and even amongst different species.

Keywords: light gradient, fluorescent proteins, emission spectra, laser scanning confocal microscopy

Abstrato

Grande parte das espécies de corais existentes atualmente são zooxanteladas, o que significa que vivem em simbiose com dinoflagelados fotossintéticos comummente designados por Zooxantelas (Filo: Dinoflagellata, Família: Symbiodiniaceae). Estas microalgas endosimbiontes são muito importantes para o coral hospedeiro, na medida em que contribuem com grande parte da matéria orgânica por si produzidas através da utilização da energia do sol. Consequentemente, a luz é um grande fator ambiental que delimita a presença e o crescimento dos recifes de coral. Recifes de coral podem ser encontrados desde águas rasas até grandes profundidades onde a luz é escassa. Ao longo deste gradiente de luz, é possível encontrar corais restritos a condições de maior luminosidade, outros especializados a habitar águas profundas, e ainda espécies generalistas que ocupam habitats distintos.

As condições de luminosidade variam muito com a profundidade, em termos de quantidade e qualidade. A intensidade de luz que chega a ecossistemas mesofóticos (30m – 150m) é uma fração mínima (~1%) da luz que atinge a superfície da água. Este valor depende de muitos fatores, como por exemplo a presença de sedimento, partículas e microrganismos em suspensão na coluna de água, ente outros, que contribuem para

turbidez, sumarizada pelo coeficiente de atenuação da luz na água. Para além disso, corais encontrados a grandes profundidades, encontram-se expostos a luz restrita a comprimentos de onda tendencialmente menores (violeta-azuis). Face a estas mudanças na quantidade e qualidade da luz, corais em ambientes mesofóticos e corais presentes em águas rasas encontram-se em condições luminosas completamente diferentes. Corais da mesma espécie, existentes nos dois extremos, possuem mecanismos de aclimatização que lhes permitem aproveitar ao máximo a luz e potenciar assim o seu crescimento e reprodução.

Sendo um organismo simbiótico, a aclimatização do coral a ambientes de baixa luminosidade pode ser manifestada a nível do simbionte e/ou do animal/hospedeiro. As zooxantelas simbiontes são também organismos dinâmicos capazes de se aclimatizar à mudança de luz. Alguns dos mecanismos de aclimatização a baixas intensidades de luz incluem, regulação da quantidade e proporção de pigmentos fotossintéticos (como por exemplo o incremento da quantidade de clorofila nos cloroplastos), regulação de antioxidantes e aumento do tamanho celular. Para além disso, mecanismos de aclimatização também são manifestados a nível do animal como regulação do número de zooxantelas nos tecidos (de modo a minimizar o auto-sombreamento), mudanças na morfologia da colónia de coral (de modo a maximizar a exposição do tecido à luz e diminuir o auto-sombreamento), características do esqueleto (modificando assim a luz interna) e finalmente, modificação na expressão e localização de proteínas fluorescentes (PFs). Consequentemente, corais em ambientes profundos possuem uma eficiência de utilização da luz mais alta, contudo, é sugerido que estes corais dependem mais da heterotrofia como a sua fonte de energia principal.

PFs de corais são grandes determinantes da diversidade de cores encontrada em recifes. Existem três cores básicas de PFs de corais (azul-ciano, verde e vermelho) sendo que existem outros morfotipos. A cor deste pigmento é determinada pela sequência de ADN de um único gene, o que proporciona uma oportunidade para o estudo da evolução da cor dos recifes de coral a nível molecular. A variação na cor entre indivíduos da mesma espécie pode dever-se a diferenças no número de cópias do gene para a PF, devido a polimorfismos no genoma, ou até mesmo devido a uma diferente regulação na expressão de PFs. Os corais podem expressar uma ou múltiplas PFs, o que resulta potencialmente numa alteração da sua cor ao longo do ciclo de vida, devido a fatores ambientais e ao longo de um gradiente de luz (por exemplo com a profundidade), relevante para este estudo. As funções das PFs na ecologia dos corais não são totalmente compreendidas, contudo, a maioria dos autores concordam que as PFs contribuem para um melhoramento de processos fotossintéticos em ambientes de luz escassa. Em águas rasas, onde a excessiva intensidade de luz é prejudicial, desempenham um papel de fotoproteção (ensombreando as zooxantelas e dissipando o excesso de energia, o que preserva os fotossistemas e previne a formação de espécies reativas de oxigénio).

Para caracterizar, identificar e estudar a variação de PFs em corais, foi usado um microscópio confocal para a obtenção de imagens de fluorescência, assim como as curvas de emissão de fluorescência de PFs. A microscopia confocal é uma técnica ótica de obtenção de imagens de alta resolução com grande contraste e seleção do ponto de focagem. Como técnicas confocais focam a luz numa zona reduzida, todo o ruido que não esteja no plano focal é eliminado o que permite observar fontes de fluorescência que passam despercebidas em métodos de microscopia convencional. Para quantificar quantidades de clorofila e proteína presentes no coral recorri a métodos de espectrofotometria.

Este estudo confirmou a existência de mecanismos de aclimatização em corais presentes no Oceanário Haus des Meeres, Viena e descreve características como localização e curva de emissão de PFs. Estas PFs são vistas por todo o tecido de coral, associadas a diferentes estruturas como a epiderme, tentáculos e tecido muscular. Foi confirmada a existência de múltiplas PFs, muitas vezes colocalizadas apesar de que em microscopia convencional podem ser confundidas como apenas uma cor devido à sua sobreposição. Isto sugere que a cor fluorescente dominante pode ser determinada através de um gradiente de PFs, visível em *Echinopora lamellosa*. Nesta espécie de coral é visível fluorescência verde em redor do disco oral dos pólipos que dá lugar a fluorescência azul mais perto do coenosarco (tecido inter-pólipo). Neste trabalho também foi possível identificar diferenças inter e intra-especificas de PFs em duas espécies de corais (*Echinopora lamellosa* e *Stylophora pistillata*) presentes no oceanário “Haus des Meeres” em Viena. Para além disso, comparando as curvas de emissão entre colónias expostas a diferentes intensidades de luz e entre espécies de coral distintas, foi possível identificar diferenças nomeadamente nas PFs ciano-azul. Foi encontrado em *Stylophora pistillata* um pico de emissão a 479nm que não foi encontrado em *Echinopora lamellosa*. O facto de a fluorescência ciano-azul ser dominante em diferentes estruturas nas duas espécies sugere que a maior componente azul em *S. pistillata* desempenha uma função específica na estrutura à qual se encontra

associada. Finalmente, aclimatização de várias colónias de coral foi confirmada também a nível dos simbiontes. Foi observado um menor número de zooxantelas e uma maior concentração de clorofila em colónias que vivem sob baixa luminosidade. Contrariamente ao que era esperado, uma maior intensidade de fluorescência foi obtida em níveis médios de luminosidade e não em altas intensidades de luz.

O trabalho científico desenvolvido nesta tese pode e deve ser melhorado em pesquisas futuras principalmente incluindo métodos otimizados de obtenção de curvas de emissão de PFs. Aumentando a diferença de luminosidade entre colónias estudadas também seria relevante para replicar e avaliar condições *in situ* com maior precisão e entender e estudar diferenças nas PFs de corais. Este estudo contribuiu para o trabalho corrente sobre fluorescência em corais produzindo imagens histológicas de grande qualidade assim como a identificação de variações nas PFs, chave para um melhor conhecimento do impacto de PFs na eco fisiologia de corais.

Palavras-chave: Proteínas fluorescentes, microscopia confocal, fotobiologia, aclimatização

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Introduction

1. Coral ecology

Coral reefs are amongst the most productive and biodiverse marine ecosystems. Corals are ecosystem engineers and the reefs they build can be considered living networks. Besides their main role as carbon storages, coral reefs are the world's most diverse marine ecosystems and provide fundamental ecological services like nursery, breeding and feeding areas for many marine species (Moberg and Folke, 1999), and support for coastal human populations (e.g. food, tourism, and coastal protection) (Hoegh-Guldberg et al., 2017). However, human actions are putting corals reefs under severe threat. In the past 20 years, five global mass bleaching events were recorded, and it is estimated that 50% of coral reefs worldwide have already been lost (Good & Bahr, 2021). Coral bleaching is a phenomenon in which the endosymbiotic dinoflagellate either leaves or is evicted from the coral host exposing the white coral skeleton. Bleaching events lead to a reduction on the translocation of photosynthetic products, which has an immediate effect on the survival of corals (McClanahan et al., 2009). The length, frequency and intensity of bleaching events is increasing due to a combined rise in sea surface temperature with ocean acidification and pollution leading to mass mortality of corals (Hoegh-Guldberg et al., 2017).

Coral reefs can be found from shallow to mesophotic depths. Along this gradient of depth, it is possible to find corals that are restricted to shallow, well-lit water, deep dark dwelling specialists, and species that can cover a great part of the gradient of light. In both lagoonal and coastal reefs, the peak of coral richness appears to be in the region of least disturbance, at 20m (Sheppard, 1980). This is the optimal depth for most species, below shallow turbulent water with major sedimentation and above dark and cooler waters, yet sufficient light reaches 20m that allows corals to thrive. Light conditions vary greatly with depth and region, both in quantity and quality. Light intensities in the mesophotic zone (30-150m) are up to 99% lower than that experienced by shallow corals (0–30 m). In the red sea, at 7m, irradiance levels are around 350 µmol photons m^{-2} s^{-1}, whereas at 30 m, irradiance is heavily reduced to 50 µmol photons m^{-2} s^{-1} (Schwarz & Hellblom, 2002). Irradiance levels differs regionally, locally, and seasonally. Additionally, mesophotic coral ecosystems (MCEs), reefs at depths of 30 m to 150 m, are also exposed to a restricted light wavelength centered around the blue region of the spectrum (Kahng et al., 2019) Since corals can photoacclimatize to their natural light conditions, mesophotic corals are most likely acclimatized to blue light, as this is the prominent wavelength at depths of 30–150 m (Ben-Zvi et al., 2021). Coral photoacclimatization to MCEs is manifested at the coral-host level with changes in colony morphology (deeper corals generally have a flatter colony morphology as this increases exposure of the tissue to downwelling light and decreases self-shading), skeletal features that modify the internal light environment (Enríquez et al., 2005; Kahng et al., 2012) and expression of FPs (Roth et al., 2010). These changes, among others, potentially assist corals in maintaining a successful symbiosis with their photoautotrophic dinoflagellate endosymbionts (family Symbiodiniaceae). Modifications in the composition of photosynthetic pigments, structure of the photosynthetic complex (Einbinder et al., 2016), modifications in the genetic composition of Symbiodiniaceae, and their photophysiological dynamics such as cell densities and size (Frade et al., 2008) are some of the strategies applied by corals in the photoacclimation.

Accordingly, deeper corals will usually present a higher efficiency in using low light levels (Einbinder et al., 2016; Ben-Zvi et al., 2021), a lower Symbiodiniaceae cell density accompanied by higher chlorophyll concentration within algal cells (Mass et al., 2007), and a reduced capacity to manage excess light (Einbinder et al., 2016; Ben-Zvi et al., 2021). Additionally, mesophotic corals are hypothesized to rely more on heterotrophy rather than autotrophy as their main strategy for acquiring energy (Mass et al., 2007; Lesser et al., 2010). With growing anthropogenic pressure and increasing sea surface temperatures on shallow water reefs, mesophotic ecosystems have been suggested as potential thermal refugia for coral communities (Smith et al., 2019). Since the light environment changes dramatically with depth (being greatly attenuated in quantity and narrowed towards the blueish part of the spectrum) and given the importance of FPs on light enhancement for symbiont photosynthesis (Bollati et al., 2021), the knowledge of how corals and their symbiotic algae can cope with low irradiances and narrow light quality spectra is crucial to understand the capability of various species to survive at greater depths.

2. Coral photoautotrophic symbionts

With the help of microscopic algae, coral polyps transform the energy of the sunlight into chemical energy and biomass and thus provide the basis for all life on the reef. Much of the great productivity of this ecosystem relies on the symbiotic relationship between the coral animal (cnidarian, in the order Scleractinia) and photosynthetic dinoflagellates (the so-called zooxanthellae, in the family Symbiodiniaceae) living inside its tissue. The unicellular dinoflagellates are lodged in the host gastrodermal cells and supply a considerable amount of the host metabolic requirements via translocation. According to Carballo-Bolaños et al. (2019), the Symbiodiniaceae supply up to 95% of the energy requirements of the host in optimal conditions. As mentioned before, with depth, incident light intensity and composition is greatly reduced, therefore, impacting productivity. Corals have the capacity to photo-acclimatize to different light regimes by inducing changes in their physiology. Some adjustments to reduced light intensity include a decline in zooxanthellae population densities, cell size, division, and degradation (Titlyanov et al., 2002). Corals contain genetically and functionally different genera of Symbiodiniaceae which can potentially confer different advantages or disadvantages according to the light corals are acclimated to. For example, some of the Symbiodiniaceae genera, like *Durusdinium*, are thermally tolerant which may confer bleaching resistance (LaJeunesse et al., 2018).

Malfunctioning of the symbiosis can result in the loss of the dinoflagellate partner and/or a reduction in their photosynthetic pigment contents (Iglesias-Prieto et al., 1992), therefore having a negative effect on the ability of the coral host to obtain food. This symbiotic relationship can be broken under conditions of stress such as excess of nutrients, high UV irradiance and, most often, high surface seawater temperatures (Lesser & Farrell, 2004). Unusually high sea temperatures and UV irradiance are both major stressors to coral reefs and, in combination with other stressors, are responsible for coral bleaching and the degradation of coral reefs around the globe (Ban et al., 2014). Seawater temperatures above a tolerance threshold cause zooxanthellae to leave the tissues of reef-building corals resulting in a reduced number of zooxanthellae in their tissues (Hoegh-Guldberg, 1999). UV radiation (UVR) can cause direct effects such as damage at cellular and molecular level leading to cell cycle arrest and cell death (Lesser & Farrell, 2004). Indirect effects include the formation of reactive oxygen species (ROS) that damage cellular components. The effects of UVR or high intensities of photosynthetically active radiation (PAR) on corals and their symbionts are diverse: changes in coral community composition, reduction in primary production, breakdown of metabolic pathways, especially those of photosynthesis, reduced growth, and organism mortality (Ben-Zvi et al., 2019). The damage to photosynthetic and mitochondrial membranes generated by ROS in corals (Higuchi et al., 2010) is highly harmful, and leads the coral to expel their symbionts. The loss of symbionts reveals the white calcium carbonate skeleton through its now-transparent tissue, a phenomenon called coral bleaching. In the recent past, mass coral bleaching has increased in frequency and intensity, thus affecting large areas of reefs in different parts of the world, due to positive thermal anomalies resulting from a rapidly warming climate (Hoegh-Guldberg, 2011). If bleached corals do not recover their symbiont populations quickly, they become subject to starvation. The resulting increased mortality rates are a major contributor to reef decline (Hughes et al., 2017). Corals have a wide array of mechanism to deal with stress and the loss of their symbionts. When bleached, some coral species can change their dominant symbiont partner, either by using background symbionts in their tissues (shuffling) or by acquiring new symbiont species from the environment (switching) (Baker, 2003). Thermally tolerant symbiont genotypes may help the coral recover faster from bleaching, even when chlorophyll levels are significantly lower than in control conditions (Carballo-Bolaños et al., 2019).

Being sessile organisms and living under high-irradiance conditions, corals have evolved the ability to accumulate UV-absorbing compounds, such as mycosporine-like amino acids (MAAs). MAAs protect corals from light-induced damage. MAAs are known to efficiently absorb light in the UVR range and also to display moderate antioxidant activity (Ben-Zvi et al., 2019). Moreover, in response to thermal stress, the host promotes changes in genes expressed including genes involved in antioxidant systems, heat-shock proteins, calcium homeostasis, cytoskeletal reorganization, and expression of fluorescent proteins (FPs) (Salih et al., 2000). These different mechanisms support further differences in the observed response across coral species and even among individuals of the same species (Dizon et al., 2021). A relatively rare but known response of corals to thermal stress is a phenomenon called colorful bleaching (Bollati et al., 2020). Colorful bleaching happens due to the upregulation of fluorescent proteins after the reduction in symbiont pigment absorption in bleached corals and the associated increase in internal light fluxes due to the highly effective multiple scattering of incident light by the coral skeleton (Enríquez et al., 2005). The increase in internal light leads to an increased expression of FPs and chromoproteins (CPs) in the coral tissue (D’Angelo et al., 2008). The high accumulation of FPs makes the coral appear very colorful. Because fluorescent proteins absorb light and thus remove some of the incident light, this process may represent an adaptive mechanism to minimize high light stress and promote recolonization with symbionts after sublethal stress events. (Bollati et al., 2020).

3. Fluorescent proteins

Since the original discovery of the green fluorescent protein from the *Aequorea victoria* jellyfish (class Hydrozoa) in 1961, the study of fluorescent proteins (FPs) has introduced a new era in the cell biology field, especially in the study of protein dynamics and gene transcription (Kendall & Badminton, 1998). Until this day, fluorescent proteins have been discovered in marine life belonging to a variety of different phyla, including lancelets (Chordata), copepods (Arthropoda), corals, sea anemones and hydrozoa (Cnidaria), and comb jellies (Ctenophora) (Dedecker et al., 2013). The key to the success of FPs use in life sciences reside in its ability to attain fluorescence after expression without the need for cofactors and that emission is contained with the coding gene (Dedecker et al., 2013). Fluorescence labelling, easiness in manipulation, imaging and potential use as biosensors make FPs very relevant in many scientific approaches. Unfortunately, most of the reef-derived fluorescent proteins suffer from significant disadvantages with respect to applications including aggregation complexes, slow FP maturation and toxicity, which compromises their use in biotechnology (Remington, 2011).

Coral FPs are major determinants of coral reef color diversity, accounting for practically every visible coral color other than the typical brown color provided by the photosynthetic pigments of algal symbionts (Alieva et al., 2008). Corals are extremely colorful under short-wavelength lighting conditions, and this is attributed to the phenomenon of fluorescence (Ben-Zvi et al., 2021). There are four basic colors of coral FPs: three fluorescent ones (cyan, green and red) and a non-fluorescent one (purple blue) but other morphs may exist (Alieva et al., 2008). FPs are the only known natural pigments in which the color is determined by the sequence of a single gene, which provides a unique opportunity to directly study the evolution of coral reef color at the molecular level (Alieva et al., 2008; Field et al., 2006). Variation among individuals within the same species may be due to differences in the number of FP gene copies, as well as to differences in the regulation of FP expression (Gittins et al., 2015; Dizon et al., 2021). Fluorescence polymorphism within the same coral species has been previously described as resulting either from a difference in the expression levels of a single protein (Gittins et al., 2015) or the presence of different FPs (Eyal et al., 2015; Ben-Zvi et al., 2019; Ben-Zvi et al., 2021)

Corals can either express one or multiple FPs and CPs, altering their color morph during the course of different life stages, under changing stressors, environmental conditions, or along a depth gradient (Ben-Zvi et al., 2019). The full extent of FP function is still controversial, but most authors agree that they have a role in the enhancement of photosynthesis where light is scarce (Dove et al., 2008; Wiedenmann & Nienhaus, 2006); and photoprotection under high-light conditions by means of reducing the light available to the photosymbionts inside the coral tissues (Gittins et al., 2015; Salih et al., 2000). During bleaching, light availability in the tissue increases due to reduced self-shading of the symbionts and to permanent scattering properties of the coral skeleton. High light induces strong expression of the photoprotective coral host pigments, which absorb light and contribute to mitigate light stress (Gittins et al., 2015; Salih et al., 2000). A more recent study has presented evidence that a photoconvertible red FP (pcRFP) may provide longer wavelengths to the zooxanthellae present deeper in the coral host tissue (Ben-Zvi et al., 2019), supplying light to shaded zooxanthellae by absorbing and emitting at a longer wavelength. Overall, optimization of the symbiont light environment is arguably one of FPs’ primary roles.

Thermal stress is one of the major threats to corals through the induction of ROS production (Baird et al., 2009). FPs also contribute to thermal resistance through their ROS scavenging properties, and, in addition, green fluorescence has been shown to enhance symbiont phototaxis. Thus, the presence of multiple green FP transcripts may reduce thermal stress susceptibility by providing the coral with an enhanced mechanism for recovery of symbionts in the event of bleaching (Dizon et al., 2021). In the severe 1998 coral mass bleaching event on the Great Barrier Reef, researchers established a correlation between bleaching resistance (high dinoflagellate biomass in coral tissue) and concentration of FPs in coral tissue (Salih et al., 2000). Temperature tolerant species of corals had a larger and more abundant complement of green FP transcripts, whereas temperature sensitive species had fewer or none (Dizon et al., 2021).

Another proposed adaptive function for coral FP, is to function as a beacon to attract both free living zooxanthellae and zooplankton. Symbiotic algae exhibit phototactic attraction to green fluorescence and swim towards a light source (Aihara et al., 2019). The same response was observed in zooplankton (Ben-Zvi et al., 2022). Coral prey exhibit preferential swimming towards green fluorescence. This provides an advantage in mesophotic coral ecosystems where energy produced by symbiont photosynthesis is not enough and corals have to rely on heterotrophy. FPs also have been demonstrated to play an important role in coral adaptation to greater depths (Bollati et al., 2017; Smith et al., 2017). FPs are capable of photoconversion of certain wavelengths into others, more favorable for symbiont photosynthesis. Efficient conversion of blue wavelengths, abundant at greater depths (>20 m) into orange-red light, could constitute an adaptation of corals to life in light-limited environments (Bollati et al., 2017). Many cyan, green, and red FPs are regulated at the transcriptional level by the intensity of incident light, particularly in the blue spectral range (D'Angelo et al. 2008). Additionally, two groups of FPs can be distinguished by their opposingly different light-dependent regulations. The low-light threshold group contains mainly cyan fluorescent proteins. Cyan FPs are expressed in considerable amounts at very low light intensities, and FPs tissue content increases with light to a maximum at a photon flux of 400 μmol photons m^{-2} s^{-1}. The high-threshold group includes green and red fluorescent proteins as well as non-fluorescent chromoproteins. These pigments are essentially absent in corals grown under very low light, but their tissue content increases in proportion to photon flux densities >400 μmol photons m^{-2} s^{-1} (D'Angelo et al. 2008).

4. Laser scanning confocal microscopy

Laser scanning confocal microscopy (LSCM) is an optical imaging technique for obtaining high resolution images with high contrast and depth selectivity (Paddock, 2000). Since the confocal technique focuses light from the sample on a very small pinhole, all information not coming from the focal plane is eliminated. This way, emissions from less common FPs can be visualized without being swamped by the more abundant FPs above and below the focal plane. LSCM can thus provide information on the proteins which otherwise may be undetected by conventional methods. In this thesis, LSCM was used to localize FPs distribution within the coral tissue. The imaging mode includes spectral analysis to disentangle different FPs allowing to infer on their specific photobiological and ecological role.

5. Objectives

To provide greater insight into the relationship between FPs and coral acclimation to light, I observed and studied changes in coral fluorescent emission intensity as well as coral symbiont densities and chlorophyll contents, in response to different light regimes. FPs identification and intensity measurements were conducted by LSCM techniques. These techniques combine excitation and emission properties to evaluate the presence and overall abundance of FPs in coral tissues. Differences in fluorescence intensities are indicative of distinct responses corals have to their environmental light levels by means of altering the synthesis of FPs.

Based on findings on the crucial roles of FPs for coral adaptation to climate change and their potential future use as targets for bioengineering projects, my aim is to study the variation of coral FPs along an artificial light gradient for corals kept in captivity. I will therefore study the distribution of fluorescent proteins with light intensity in two coral species to further decipher the role of FPs on the acclimative response of corals.

This study is divided in four main goals:

(1) To identify, localize and quantify FPs distribution within coral tissue;

(2) To distinguish and quantify different FPs as well as to measure changes in FP emission intensity;

(3) To measure changes in symbiont density and the relation between zooxanthellae and coral host in response to light;

(4) To discuss the ecological value that these proteins have on corals;

My main hypotheses are that corals under intense light will have more FPs that will protect the tissue and the zooxanthellae from harmful excessive energy. Furthermore, corals that have been long acclimatized to shaded areas should have a lower density of zooxanthellae containing higher chlorophyll concentration, than the corals under high irradiance.

Materials and methods

1.1 Target species

Echinopora lamellosa and *Stylophora pistillata* are two shade tolerant species that can be found in a broad array of light environments. *E. lamellosa* is an Indo-Pacific coral that occurs from 10 to 15m depth and *S. pistillata* at shallow (<10m) depths (Sheppard, 1980). *Stylophora pistillata* is a branching hermatypic coral abundant in the Red Sea and shows a wide range of dispersal. The growth rate of this coral is among the fastest of the scleractinian corals although when facing competition with other species for space, *S. pistillata* is the first to be competitively excluded unlike more competitive corals like *E. lamellosa* (Loya, 1976). *Echinopora lamellosa* is a foliaceous like hermatypic coral with a broad vertical range of distribution, which occupies reef slopes generally below 15m. Both species display tolerance to low light that includes some acclimation responses and some characteristics from symbionts that live within the coral animal.

1.2 Sample collection

The stony coral species *Stylophora pistillata* and *Echinopora lamellosa* were chosen based on their abundance at Haus des Meeres Aqua Terra Zoo (HdM), where they were growing in aquarium-habitats under different light conditions. Furthermore, these two species were selected for this study due to the presence of fluorescence, which can be easily observed with blue light. Corals from both species were obtained from Rotterdam Zoo in 2012 and have been growing in HdM tanks ever since. For both species, five fragments of around 5cm length were collected from three different colonies (all same genotype, total of 15 fragments per species) growing in the main exhibit tanks under different light levels: low light (LL: 10~30µmol photon/m² s^{-1}), medium light (ML: 70~80 µmol photon/m² s^{-1}) and high light (HL: 200~240 µmol photon/m² s^{-1}). Coral fragments were separated into two subsamples, one for LSCM of the animal fluorescent proteins (see details below), and the other to characterize the photosymbiont component (zooxanthellae densities, protein quantification and chlorophyll quantification), which was frozen in -80ºC until further processing.

1.3 Sample preparation for LSCM

Fragments collected at HdM for LSCM were fixed in PFA (4%), then transferred to Ethylenediaminetetraacetic acid (EDTA) for decalcification. Decalcification in EDTA (10%) facilitates sectioning and further imaging at the LSCM, and proceeded for a week or more, depending on the thickness and density of the skeleton. After the decalcification process, 1cm^2 coral fragments were transferred to a 30% Sucrose media overnight and stored in -20ºC until further handling. For cryosectioning, decalcified coral fragments were transferred into OCT, frozen on dry ice and cut using a section thickness of 50 µm. The sections were then rehydrated in PBS for 5 min to remove the OCT and mounted onto a slide with Möwiol, ready to be imaged at the LSCM.

1.4 Processing for symbiont photobiology

To characterize the photosymbiont component, coral fragments were wrapped in aluminum foil and stored in -80ºC. To remove the tissue from the underlying skeleton, a modified air gun connected to an air compressor was used at Natural History Museum, in Vienna (NHM). Coral tissues were first rinsed in 1x PBS and then airbrushed into a ziplock bag filled with 5 mL of 1x PBS until all tissue was removed. The resultant tissue slurry was transferred into a falcon tube. To avoid tissue loss, the bag was rinsed with 3 ml of 1x PBS which were then transferred into the collection tube. The final volume slurry was complemented with PBS to a final volume of 10 ml. The 10ml of slurry were then homogenized at 20 rpm (x100) for a minute in the vortex. 1 mL of the suspension was immediately fixed in 1 mL of 10% formalin for subsequent counting of zooxanthellae. Six aliquots of 1 ml were kept for chlorophyll and protein quantification. These aliquots were centrifuged at 16 000 rcf for 5 min and the supernatant discarded. The resulting tissue pellets were stored at -80ºC.

2. Generation of FPs emission spectra

Coral tissue sections were scanned and imaged using Zeiss LSM 780 and Zen Black processing software (2011 v14.0.16.201). Distinct lasers lines were used to excite and image the coral samples: (λ=405nm), (λ=488nm), (λ=546nm) and (λ=633nm) were used to image the coral samples. The excitation wavelength of each laser allowed for the visualization of fluorescence in the host (animal fluorescent proteins) and Symbiodiniaceae (chlorophyll *a* fluorescence) at a magnification of 20x.

To obtain an emission spectrum of the FPs present in the coral animal tissue and in the zooxanthellae symbiont, a lambda scan function was applied. This function was run with each of the 6 available lasers lines (λ=405nm, λ=458nm, λ=488nm, λ=514nm, λ=561nm and λ=633nm). The laser scan confocal microscope has a spectral detector able to detect fluorescence (every 3nm, from 412nm to 693nm) creating an emission spectrum of a selected region of interest.

Zooxanthellae size

Zooxanthellae size was calculated using a measuring tool available in Zeiss LSM 780 and Zen Black processing software. A total of 288 zooxanthellae were measured across light levels divided between the two species available. *E. lamellosa* HL (n=35), *E. lamellosa* ML (n=52), *E. lamellosa* LL (n=55), *S. pistillata* HL (n=49), *S. pistillata* ML (n=43) and *S. pistillata* LL (n=54).

3.1 Determination of zooxanthellae densities

Each of n=15 samples for each species (i.e., 5 replicates per light level) were processed in the following manner: the aliquot was shaken vigorously followed by 30 seconds in the vortex; then, using a clean pipette, 20 μL of the sample were placed onto a Neubauer (0.1 mm of depth) hemocytometer, and viewed under 20x magnification under a light microscope. To mitigate ‘edge effects’ (i.e. counting cells lying on quadrat margins more than once) only the cells which touched the top and left-hand side of each square were counted.

It was established a minimum of 8 squares, and 200 cells counted per replicate and a coefficient of variation below 15% to have a more robust and precise counting of the zooxanthellae. Zooxanthellae densities (number of cells per cm^2 of coral surface area) were calculated accordingly. The surface areas of respective branches were determined using the aluminum foil method (Marsh 1970); whereby branches were carefully wrapped with a uniform single layer of aluminum foil, which was then weighed to establish the surface area of the foil. A calibration curve of the surface area to mass ratio was constructed based on pieces of aluminum foil with known area (y＝305.18x+0.1367, r2 ＝0.9981, n＝12). This curve was then used to back- calculate the surface area of aluminum pieces wrapped around each coral sample.

3.2 Chlorophyll quantification

Frozen tissue pellets obtained from the coral tissue blastate were thawed, and 950 µl of acetone and 50 µl of DMSO were added before vortexing the sample. The sample tubes containing chlorophyll were then wrapped in aluminum foil and incubated in the dark for 24h at 4ºC to prevent light induced degradation of chlorophyll. Later, samples were centrifuged at 16000rcf for 10min and 200µl of the supernatant were pipetted into a 96-well plate in triplicate. Absorbance was measured at 630, 663 and 750nm using BioTek microplate reader and Chlorophyll *a* concentration was calculated through Jeffrey & Humphrey (1975) equation:

$$\mathit{Chl\ a}\ (\mu g/ml) = 11.43 \text{ x } A_{633} - 0.64 \text{ x } A_{630}$$

3.3 Protein quantification

Pierce BCA Protein Assay Kit (MAN0011430 Rev. A) was used in this study for coral protein quantification. Tissue pellets, from aliquots containing coral tissue blastate, were thawed and resuspended in 1ml of 1x PBS. 25µl of the homogenized resuspension were added to 200 µl of working reagent from the kit into a 96-well plate. The plate was in a shaker for 30 seconds and then incubated at 37ºC for 30min. After the plate cooled down at room temperature, measurement of the absorbance at 563nm was conducted in a Cytation 3 multi-mode microplate reader (BioTek, Winooski, USA) and analysis made using the software Gen5 (BioTek. Winooski, USA). The measurements were blank corrected to remove background absorbance. For each plate, a protein standard curve was obtained using bovine serum albumin (BSA) solution at concentrations between 25 and 2000µg/ml. Finally, for each sample, the total protein concentration was calculated using the standard curve. Ratios of zooxanthellae and chlorophyll per protein (shown in the results section) were calculated using measurements straight from the blastate volume (ml).

4. Data analysis

Statistical analyses were performed using RStudio 2022.07.0 Build 548 (http://www.rstudio.com) to compare emission peaks from different FPs between species and light levels, and study the existence of significant differences between species and light levels. Parametric ANOVA test and non-parametric Kruskal-Wallis test were conducted accordingly in RStudio to identify statistically significant differences among levels of the explanatory variables (light and species). Data was "log" transformed to be used, preferably, for parametric tests. Correlation tests between variables like zooxanthellae cell densities and total protein were performed using Past software. GraphPad Prism version 9.0.0 (www.graphpad.com) was used in the construction of graphs. Normalized data was calculated dividing the emission value detected for each wavelength for the maximum emission value of fluorescence.

Inter and intraspecific comparisons were performed to investigate potential differences that could exist between the corals selected for this study. Comparisons among light levels allow us to see if a species has adaptation capabilities and plasticity to cope with the variety of environmental conditions they are inserted in as an ecosystem. Comparison between two species allows the study of a coral's general fitness and assess how susceptible a certain species is to shifts in light environment.

Furthermore, hyperspectral analysis of emission curves from FPs, done in this study, consists of analyzing the derivatives of an emission curve to have a higher resolution of the differences in trends that exist on a curve. Thus, differences in the curve shape, which might be caused by variation in relative abundance of the different molecules/proteins, can be disentangled. In this study I will focus on the use of the 2nd derivative which allow the visualization of the differences between the species and light levels, subject of this study. Differences seen in the 2nd derivative high light the diversity of coral associated pigments and proteins that interact with light by absorbing and emitting light/fluorescence in distinct wavelengths. One potential application of the hyperspectral study of coral is remote sensing to check coral health and surface coverage by coral, algae, and sand (Hedley & Mumby, 2002).

Results

1. Preliminary observations

One of the first differences that were noticed in the corals present in this study was that *E. lamellosa* colonies seem to express different colors and tones depending on light intensity. Colonies exposed to higher intensities of light appeared to be paler whereas colonies grown under low intensities of light exhibited a darker-brown color with clearly visible polyps in green (Fig. 1). This scenario led to the first question that I tried to answer in this work: why are colonies exposed to higher intensities of light paler? In the case of *S. pistillata*, it was clear again that colonies under low light were much darker than those under higher light intensities (Fig. 2). In *S. pistillata* colonies, it is possible to see whitish branch tips. Colorless branch tips occurs when transparent coral tissue grows revealing the white skeleton underneath. When tissue is later colonized by zooxanthellae, the tissue regains its normal color. This phenomenon also happens on the edges of *E. lamellosa* plates and does not relate to a stress response, but simply to the fact that the animal first replicates its own cells and only then are these cells colonized by the symbionts (Jones & Yellowlees, 1997). Macro images reveal very characteristic green polyps in *E. lamellosa* (Fig. 3) which is not seen in *S. pistillata* (Fig. 4 and Fig.5)

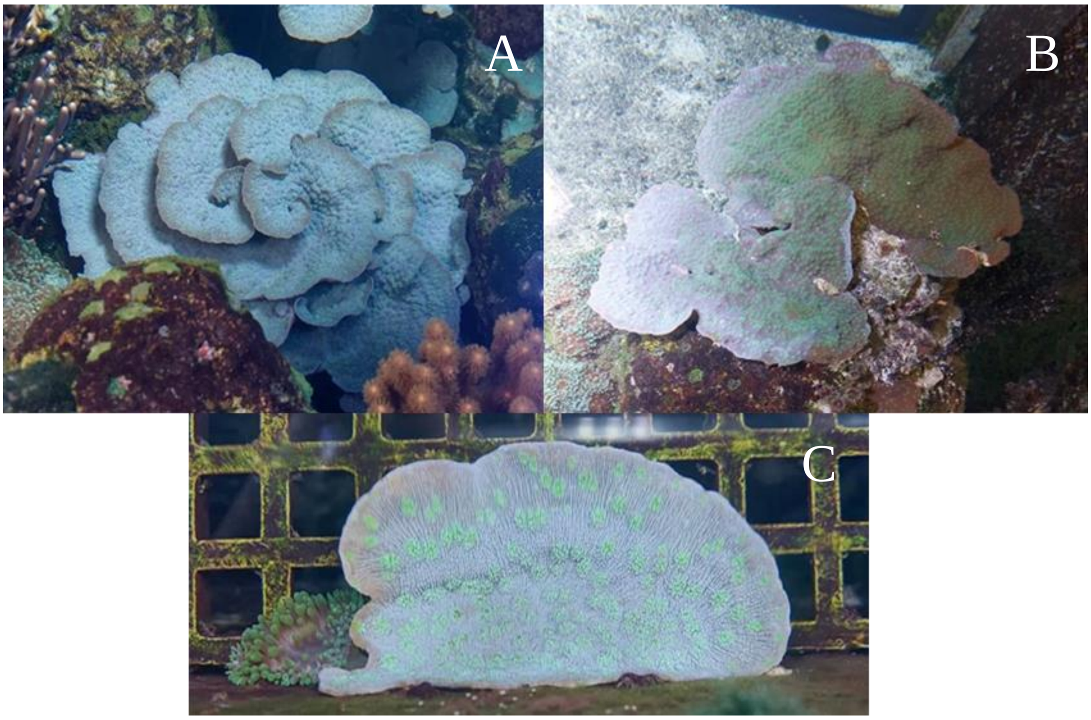


Figure 1. *E. lamellosa* corals growing at Haus des Meeres Aqua Terra Zoo aquariums. The images show the colonies from which fragments were taken for this study. (A) *E. lamellosa* under high light (~240 µmol photon.m^{-2}s^{-1}), (B) *E. lamellosa* under medium light (~70 µmol photon.m^{-2}s^{-1}), (C) *E. lamellosa* under low light (~10 µmol photon.m^{-2}s^{-1}). Photos by David Coelho.

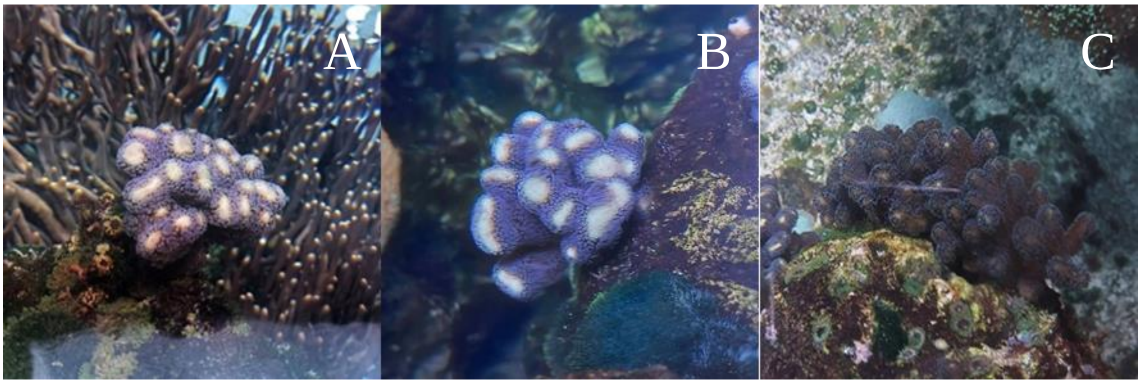


Figure 2. *S. pistillata* corals growing at Haus des Meeres Aqua Terra Zoo aquariums. The images show the colonies from which fragments were taken for this study. (A) *S. pistillata* under high light (~200 µmol photon.m^{-2}s^{-1}), (B) *S. pistillata* under medium light (~80 µmol photon.m^{-2}s^{-1}), (C) *S. pistillata* under low light (~30 µmol photon.m^{-2}s^{-1}). Photos by David Coelho.

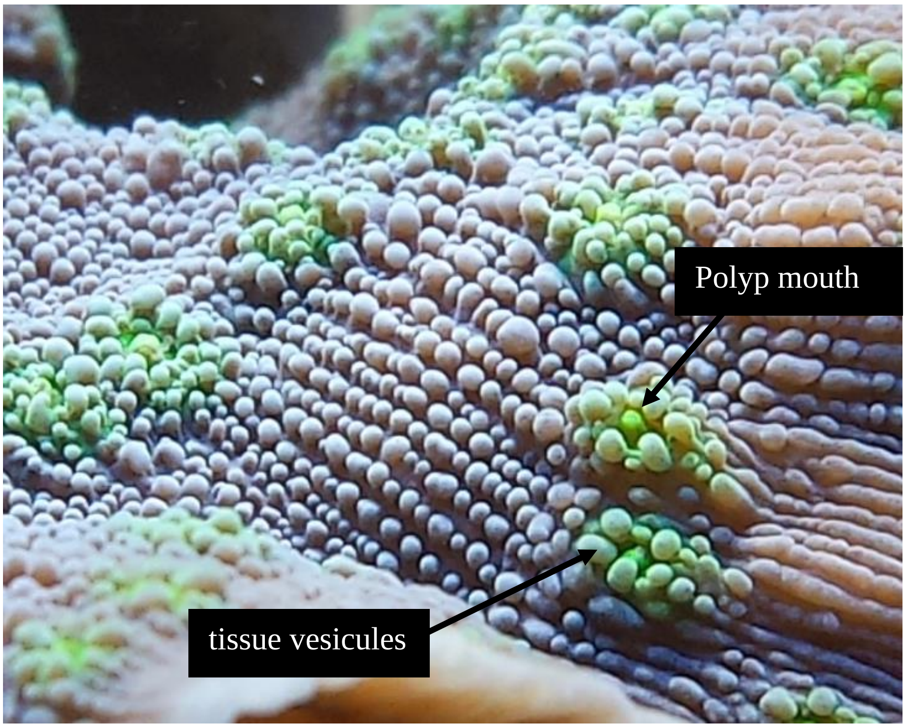


Figure 3. Macro image of the scleractinian coral *E. lamellosa*. Polyps with green fluorescence are visible. Image taken from (https://www.nano-reef.com/gallery/image/43315-echinopora-lamellosa/?browse=1).

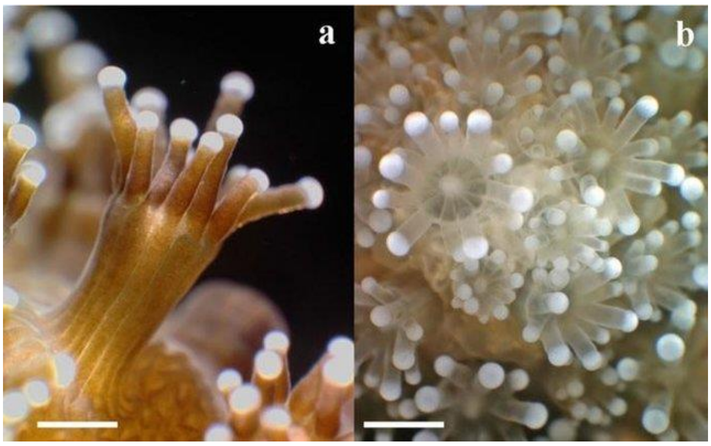


Figure 4. Macro photographs showing polyp tissue of the scleractinian coral *Stylophora pistillata*. Panel (a): lateral view of polyps with visible zooxanthellae population, panel (b): overhead view of polyps with nearly transparent tissue; scale bars: 0.25 mm (a) and 0.5 mm (b) Photography: E. Tambutté (Centre Scientifique de Monaco) (Plass-Johnson. 2015).

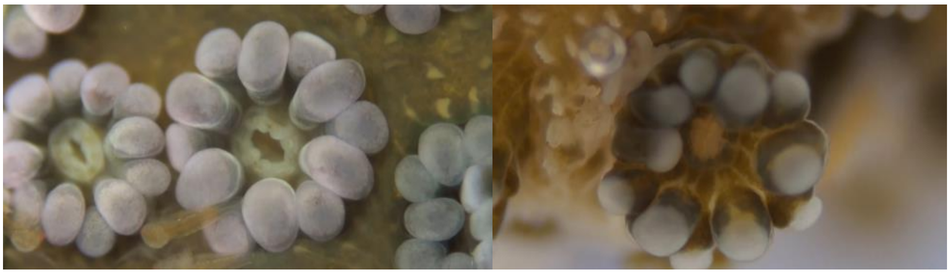


Figure 5. Close up images of partially retracted *S. pistillata* polyps. Image taken from (https://www.youtube.com/watch?v=9dloJs9LMR4).

2. Descriptive image analysis

Throughout the coral tissue, pigments with different emission spectra were colocalized. Visual comparison of images, from the same species under different light, revealed high similarity between coral colonies regarding fluorescence in both coral and symbiont. Fluorescence in these images had two main sources, from coral fluorescent proteins and from chlorophyll-*a* present in the zooxanthellae. Coral fluorescent proteins could either be seen diffused in cells or concentrated in granules scattered in the gastrodermis of the coral tissue (Fig. 6). Granules of FP can be seen in the epidermis and gastrodermis, amongst and under the layer of zooxanthellae. FP granules can also be found distant from the symbionts, deep in the tissue where it could have a specific function. In both species, the red fluorescence observed comes from the chlorophyll *a* contained within zooxanthellae cells.

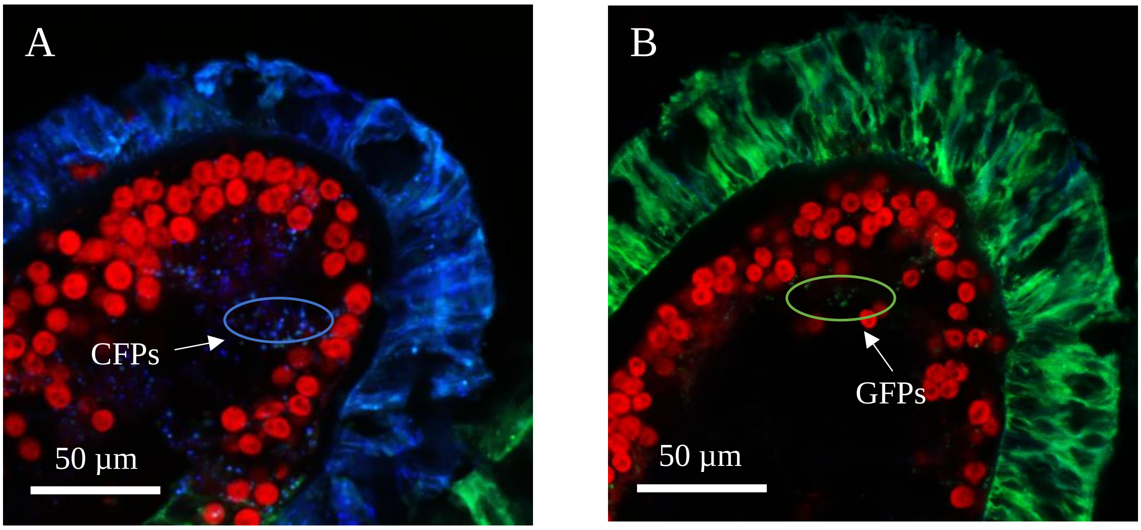


Figure 6. Different FP granules are located amongst and under the layer of zooxanthellae in *E. lamellosa* LL coral tissue. CFPs (A) and GFPs (B). Image magnification 20x.

Echinopora lamellosa

The poor quality of images from replicates under high light made the analysis and description of fluorescence in that *E. lamellosa* colony difficult. The problems can be related to cutting and mounting of the sample. Comparing replicates from high light is very difficult as there seems to be no common trends and replicates are very different from each other. In *E. lamellosa* images, three main fluorescence colors can be identified: red fluorescence form the chlorophyll *a* contained within the zooxanthellae; blue/cyan and green fluorescence in the epidermis of the coral tissue (most outer cellular layer) (Figure 7). Green fluorescence can also be found deep in the tissue associated with other structures (Fig. 8).

E. lamellosa ML (medium light) displays higher fluorescence than those from high light (under the same LSCM settings). The epidermis layer always contains green and blue fluorescence; however, the dominant color depends on the location. In all replicates, green fluorescence was mostly concentrated in the epidermis of the oral disk. Further away from the polyp mouth, blue fluorescence becomes the dominant color. Green fluorescence is visible from the pharynx to the gastrovascular cavity. Furthermore, the pharynx and the gastrovascular cavity also contain cells that display cyan/blue fluorescence.

Finally, *E. lamellosa* LL (under low light) revealed the same fluorescent patterns throughout the tissue within biological replicates. Once again, polyps displayed a more noticeable green fluorescence on the epidermis of the oral disk and blue fluorescence further from the polyp mouth (coenosarc). The biggest differences observed in images from replicates under LL, is that these corals seem to have a higher abundance of FP granules than HL and ML, amongst and under the layer of symbionts located in the gastrodermis (Fig. 6).

Overall, aside from some already mentioned differences, all *E. lamellosa* corals displayed some common characteristics of fluorescence: higher intensity of fluorescence in the epidermis of the oral disk; blue fluorescence in the tissue vesicles just outside the polyp mouth; FP granules are highly abundant within the layer of zooxanthellae.

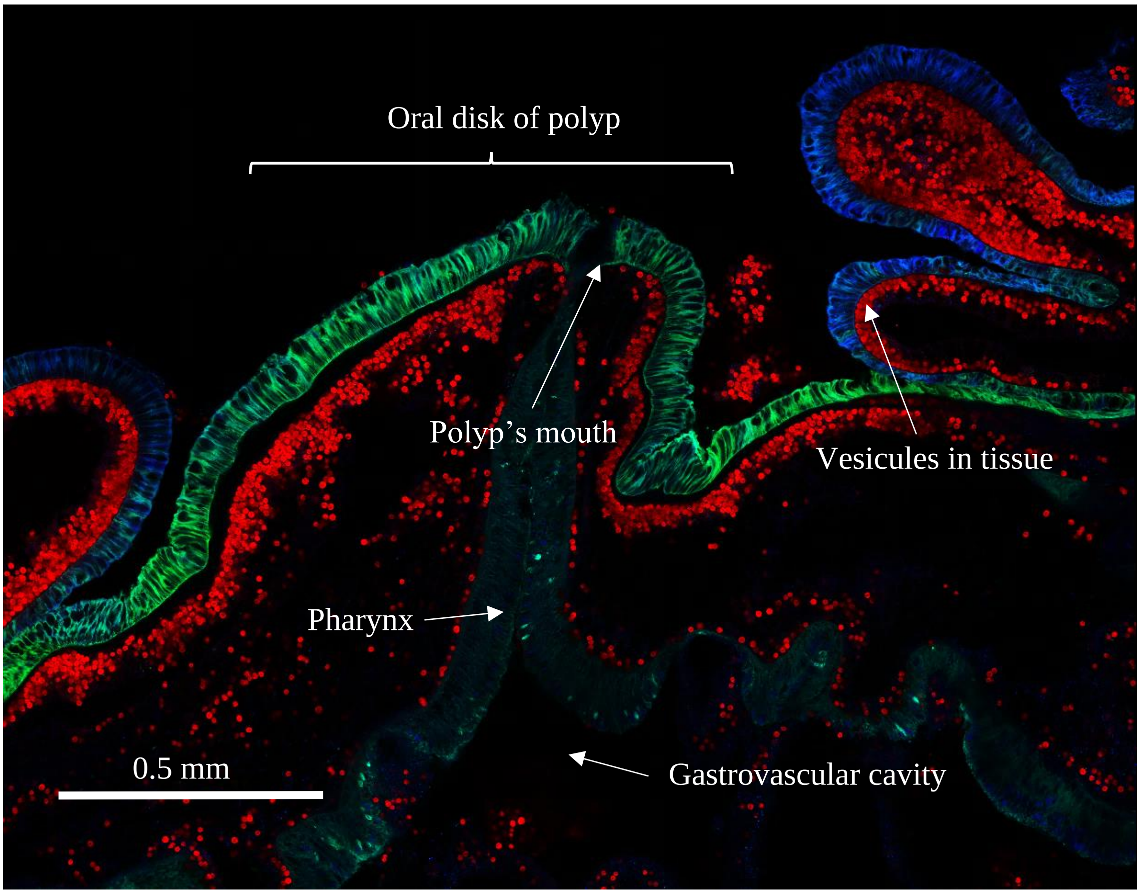


Figure 7. LSCM image obtained from a 50 µm thick tissue section of *E. lamellosa* ML. In the presented image is visible red fluorescence from the chlorophyll *a* contained within the zooxanthellae, green and blue fluorescence from coral FPs. Magnification of 20x.

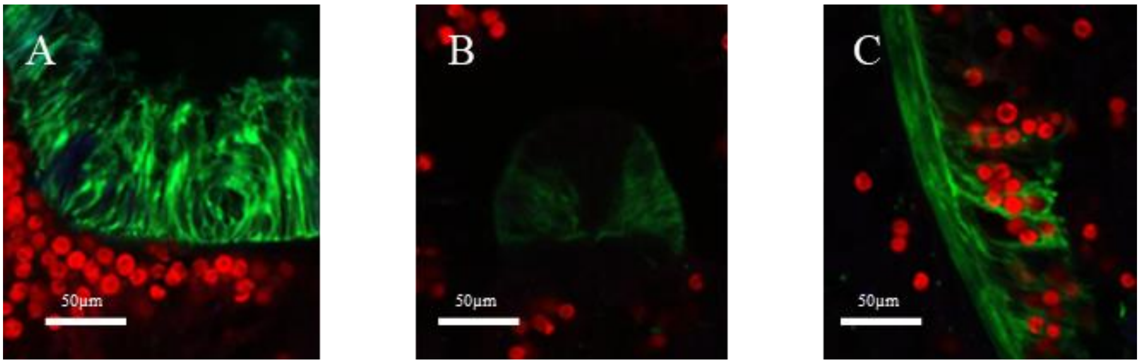


Figure 8. 20x Longitudinal cut of *E. lamellosa* HL (under high light) coral tissue exhibiting different sources and locations of fluorescence. A (epidermis), B and C (muscle band) high light structures where GFPs were detected.

Stylophora pistillata

S. pistillata, like *E. lamellosa*, has three main sources of fluorescence: red fluorescence coming from the chlorophyll, green fluorescence in tissue muscles and blue fluorescence in the acrosphere (tip of the tentacle) (Fig.9). Unlike zooxanthellae densities, patterns and location of FPs, fluorescence remained unchanged in the three light treatments (HL, ML, LL). Green fluorescence in *S. pistillata* is located in the epidermis and in long muscle bands vital for the coral polyp. Green fluorescence in the epidermis, surrounding the polyp, is dim compared with fluorescence in the muscles. Based on the images, blue fluorescence, from CFPs, is only present on the sides of the tentacle tip. Furthermore, when images from both species were compared, it was noticed that FP granules were almost absent in all of *S. pistillata* treatments. Green and blue FP granules were mostly absent and were not detected in images from corals under LL.

There was one feature that was seen exclusively on *S. pistillata* HL, the presence of red fluorescing microorganisms underneath the coral tissue. These endolithic microorganisms are known in coral biology although their fluorescence properties are not very well described in literature.

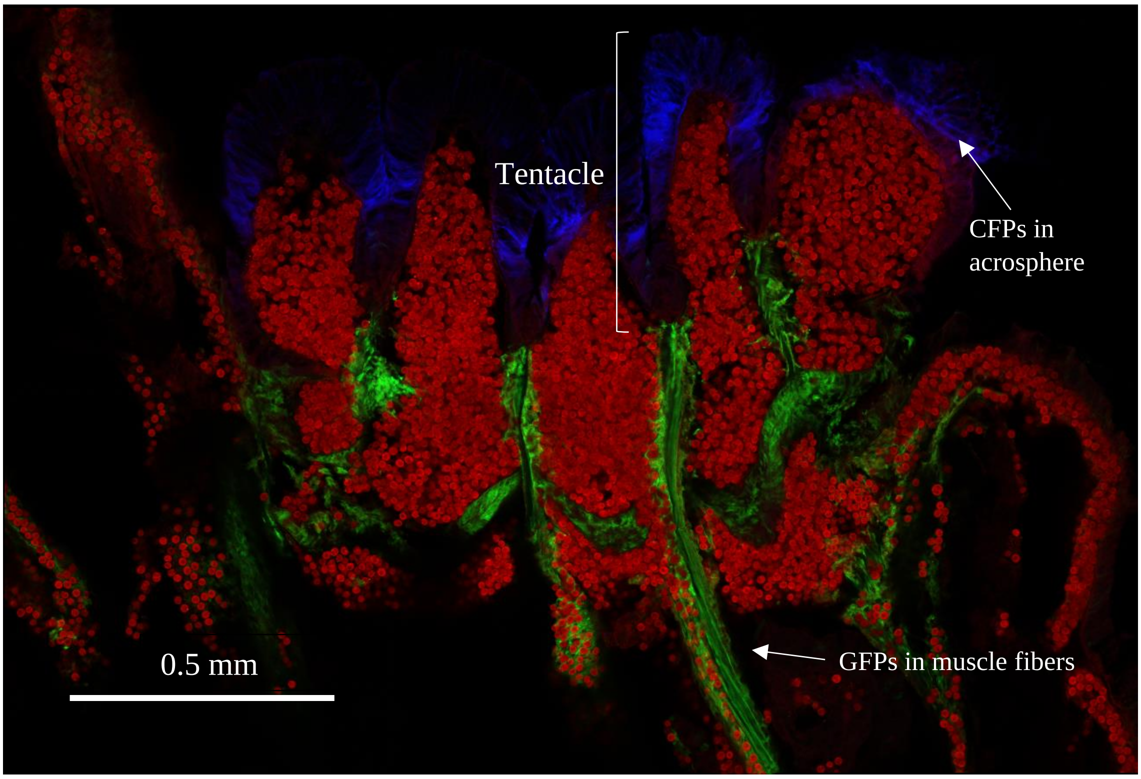


Figure 9. Image obtained through imaging in the LSCM of 50 µm tissue section of *S. pistillata* HL (A). In the presented image is visible red fluorescence from the chlorophyll *a* contained within the zooxanthellae, green and blue fluorescence form coral FPs. Magnification of 20x.

2.1 Identification of FPs

FP identification was based on their typical emission maximum. Using the LSCM, four FPs (CFP, GFP, YFP, RFP) could be detected as well as the fluorescent photosynthetic pigment present in the zooxanthellae, chlorophyll *a*. After image analysis, four regions of interest were selected, where FPs' signal could be detected (Fig. 10). The first region selected was a single cell symbiont which contains chlorophyll (fluoresces in the far-red wavelengths; ~679nm). The second region, associated with GFPs, differed between species. On one hand, *E. lamellosa* displayed green fluorescence in the epidermis directly on the polyp's oral disc. On the other hand, in *S. pistillata*, green fluorescence and consequent GFPs were concentrated in muscle bands that stretched from the oral disk down to the basis of the polyp. The third region was selected based on its predominant blue color characteristic of CFPs. Again, there were differences between species: *E. lamellosa* presented blue fluorescence in tissue villosities just outside the polyp mouth, whereas in *S. pistillata*, blue fluorescence was mainly concentrated on the tentacle tips. Finally, region 4 was selected mainly because of an absence of fluorescence indicative of the present of FPs but we later found out using confocal lambda scans that there were FPs in the gastrodermis (inner tissue) namely YFPs and RFPs that remained unnoticed until then. Both these FPs were present equally in both species of coral and with no apparent positional differences, unlike those detected in GFPs and CFPs. Table 1 combines the information regarding peaks of emission and excitation of some FPs identified and studied in many corals (Alieva et al. 2008; Kogure et al. 2006).

Table 1. Identified coral fluorescent proteins and chlorophyll *a* pigment in *E. lamellosa* and *S. pistillata*. Presented in the table are the wavelength at which the emission (Em) peak was obtained and the excitation (Ex) laser that yielded the highest emission of fluorescence.

Fluorescence source	Ex max in this study	Em peak in this study	Em peak in literature
CFP	488 nm	~501 nm	~497 nm
GFP	488 nm	~510 nm	~515 nm
YFP	561 nm	~577 nm (*E. lamellosa*)	~586 nm
YFP	561 nm	~608 nm (*S. pistillata*)	~586 nm
RFP	561 nm and 633 nm	~648 nm	~608 nm
Chlorophyll *a*	561 nm and 633 nm	~679 nm	~679 nm

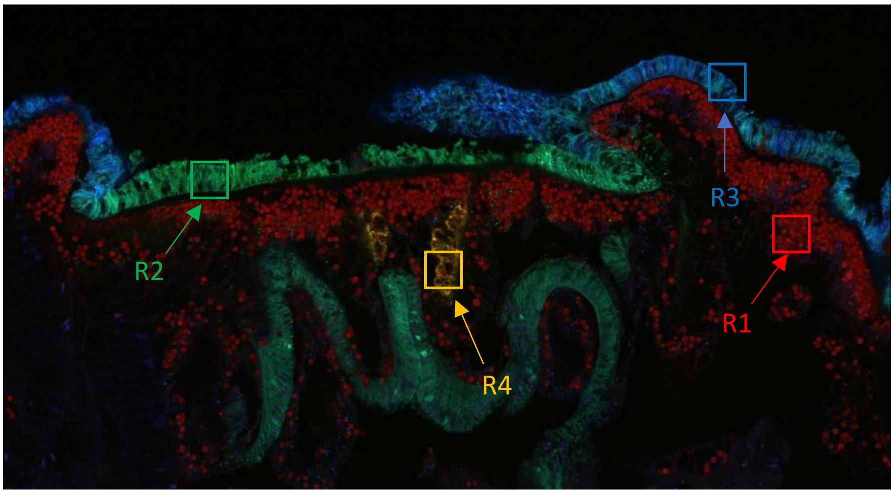


Figure 10. Selection of regions of interest in coral tissue to study the fluorescence emission of FPs. In R1, only one zooxanthella was selected to obtain the emission spectra of chlorophyll *a*. For R2 and R3, an area containing GFPs and CFPs (respectively) was selected. R4 contained the emission spectra of two FPs, YFP and RFP. Image magnification 20x of *E. lamellosa* LL.

3. Study of the emission spectra of coral FPs

3.1 Chlorophyll

Chlorophyll is characterized by a fluorescence emission peak around 679nm, an apparent shoulder at around 650nm and a spectral width of 20 to 30nm (measured between the points on either side of the peak at which the intensity is half of its peak). Fluorescence intensity differed among light levels. *E. lamellosa* yielded the highest fluorescence intensity under medium light (Fig 11A). This was almost twice as much as the intensity measured at high and low light. The same observation was made for *S. pistillata*, which also achieved the highest intensity of fluorescence in medium light, 1.5x higher than measured at low light (Figure 11B). When comparing fluorescence yields between species (Fig. 11C), *S. pistillata* had a slightly higher intensity of fluorescence than the one measured in *E. lamellosa*.

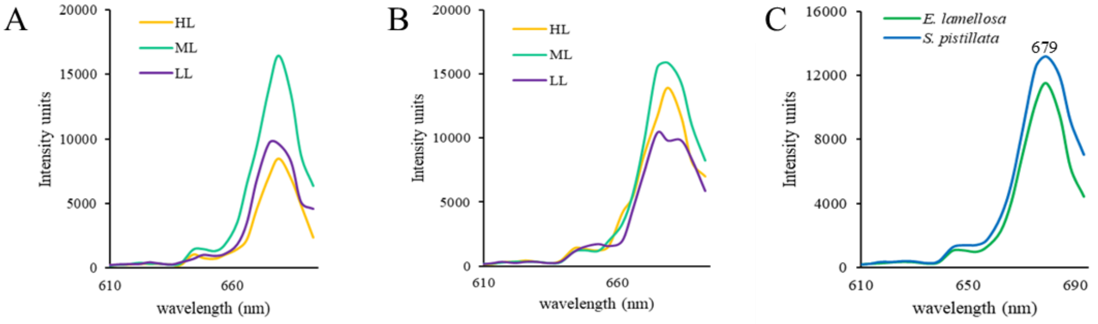


Figure 11. Fluorescence emission spectra for the zooxanthellae region showing the emission curve of photosynthetic pigment chlorophyll when excited with the 458nm laser in *E. lamellosa* (A) and *S. pistillata* (B), across light levels (HL: high light, ML: medium light and LL: low light). Averaged species including the distinct light levels (C).

Although *E. lamellosa* under medium light has a higher intensity (average 16444 intensity units (IU) ± 9056 SD) of fluorescence, this difference was not statistically significantly different from other light levels (two-way ANOVA test, F value=2.912, Df=2, n=29, p value=0.073). *S. pistillata* seems to have a slightly higher average of fluorescence when compared with *E. lamellosa*, yet again, no statistically significant differences were found between the two species ($p>0.05$) (Fig. 12).

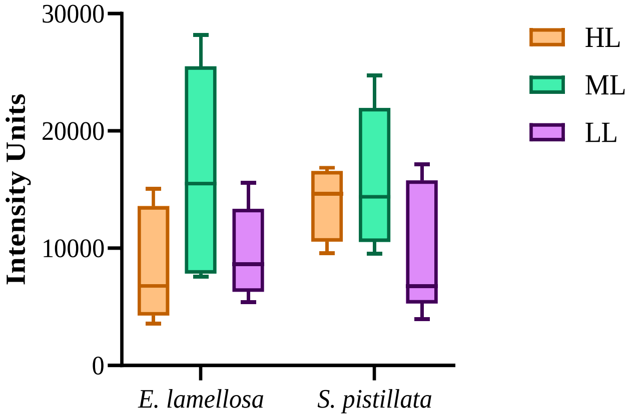


Figure 12. Intensity of fluorescence emission peak of chlorophyll at the peak of the curve, 679nm. No statistically significant differences were found between species nor among light levels (p>0.05 two-way ANOVAs test).

Normalized emission curves overlapped in both species and in the three light levels of this study (Fig. 13A-C). The derivatives of the three light levels in both species overlap except for some narrow regions of the spectral range (Figure 13D-F). Between 657-675 nm, it can be seen some differences in the 2nd derivative amongst the light levels of *S. pistillata*. This coincides with a small shoulder present in the curve of emission of chlorophyll suggesting different optical properties that might come from other pigments that interfere with light in those wavelengths. The high variability amongst some replicates also contribute to these differences in the derivative curves.

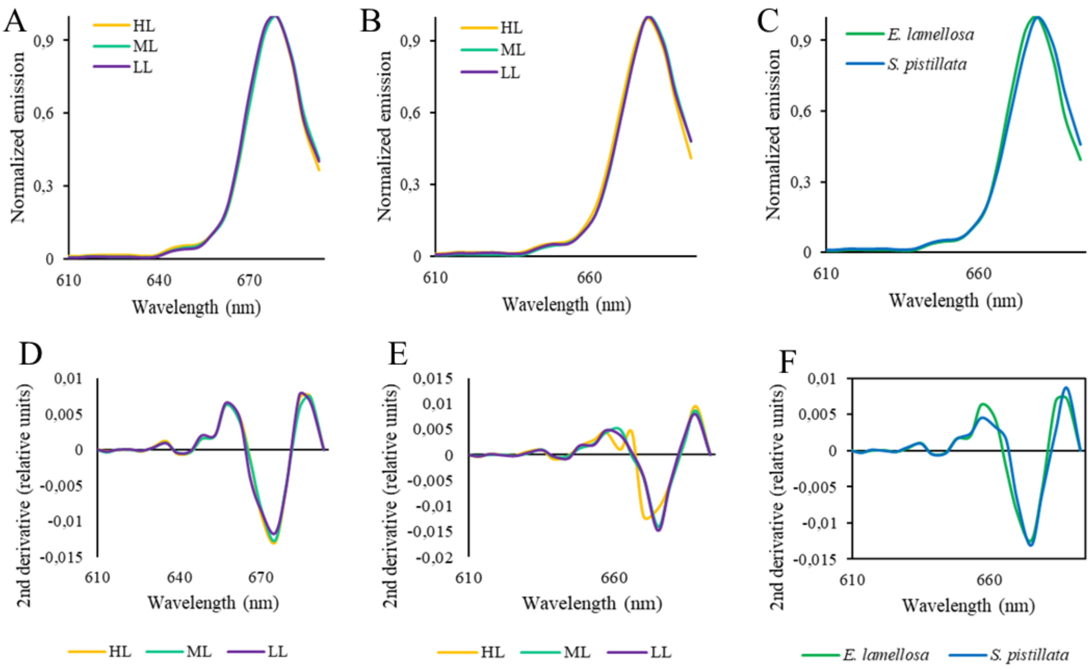


Figure 13. Normalized emission curves for each light level, as measured from each studied species, *E. lamellosa* (A) and *S. pistillata* (B). Light levels averaged (C). The 2nd derivative of each treatment curve show minimal intra and inter-specific variation of the spectral features. *E. lamellosa* (D) *S. pistillata* (E) and light level averaged for both species (F). Light levels: HL – High light; ML – medium light; LL – low light.

3.2 GFPs

Both species displayed similar curves of emission throughout the spectra (from 410 nm to 693 nm). GFPs are characterized by an emission peak at 510-515nm and two shoulders, one at 479nm and the other at 577nm (Fig. 14A-B). The spectra width is 50 ± 5 nm. In *E. lamellosa* there were no differences in terms of intensity between the light treatments. However, in medium light, the GFPs exhibit a more cyan-shifted peak (506 nm). In *S. pistillata*, alike seen for chlorophyll before, GFPs have higher intensity of fluorescence under medium light as compared with the other light levels. When comparing species (Fig. 14C) there was difference, not only in intensity but also in the position of the peak: In *E. lamellosa* the peak emission of fluorescence is of 59035 IUs and is located at 510nm but in *S. pistillata* the peak comes slightly earlier, at 506nm, and with less intensity of fluorescence (53983 IUs).

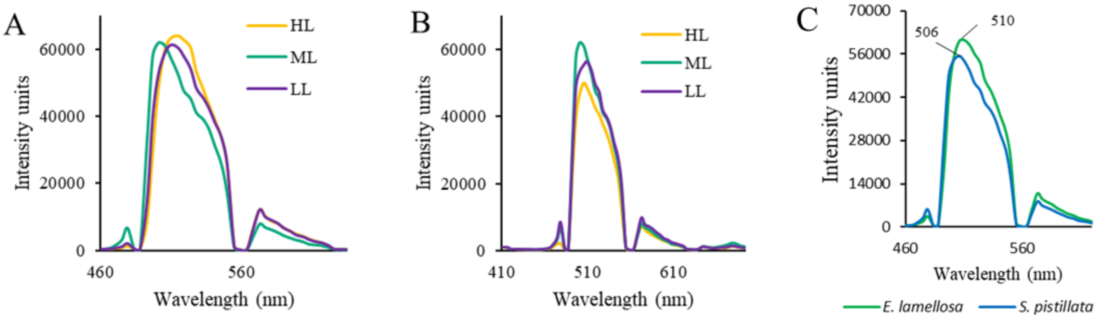


Figure 14 Fluorescence emission spectra for the second region showing the emission curve of GFPs when excited with the 488nm laser of *E. lamellosa* (A) and *S. pistillata* (B) and light levels (high, medium and low light). Averaged species including the distinct light levels (C).

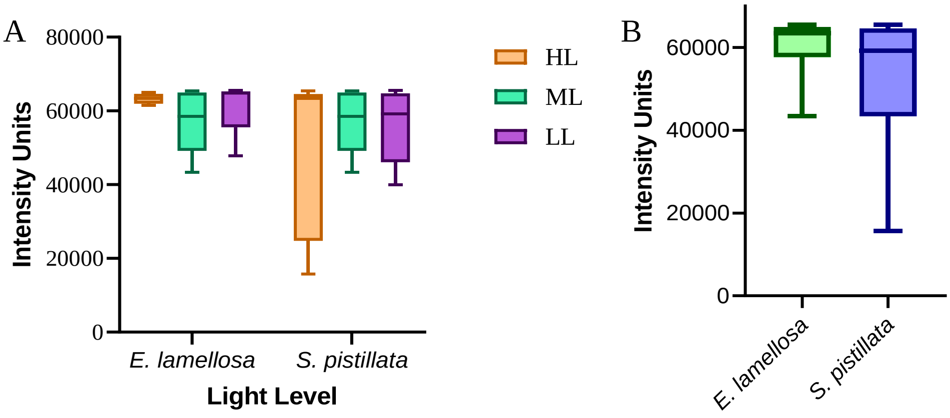


Figure 15. Emission intensity of fluorescence at the peak of the curve, 510nm. No statistically significant differences were found neither amongst treatments (KW test Chi^2= 0.559, Df= 2, n=29, p value=0.7562) nor between species (KW test Chi^2=0.965, Df=1, n=29, p value=0.325). Values of peak intensity of fluorescence captured at 510nm.

In line with observations made in the fluorescence emission curves, comparing the peaks of emission resulted in similar conclusions (Fig. 15A-B). There are no statistically significant differences among light levels in both species. *E. lamellosa* shows slightly higher yield of green fluorescence but not statistically different from *S. pistillata.*

The hyperspectral analysis revealed some differences in the normalized spectra of emission and on the 2nd derivative in both species and their respective light levels (Figure 16A-C). The distinct light levels in *E. lamellosa* exhibited different derivative curve behaviors between 490nm and 510 nm possibly due to the presence of CFPs in that region (Fig. 16D-F)). In *S. pistillata* the different FPs are more spatially separated in the tissue, which explains why these differences in the 2nd derivative do not exist in *S. pistillata* from 490nm to 510nm. *E. lamellosa* under high light has a dip in the 2nd derivative at 675nm, that other light levels do not have. This is probably the influence of chlorophyll that has an emission peak at 679nm. It is also evident some differences in the peaks, not only between both species, but also among the light levels. Both species differ regarding peak wavelength, some being more in the cyan, other more in the green wavelengths.

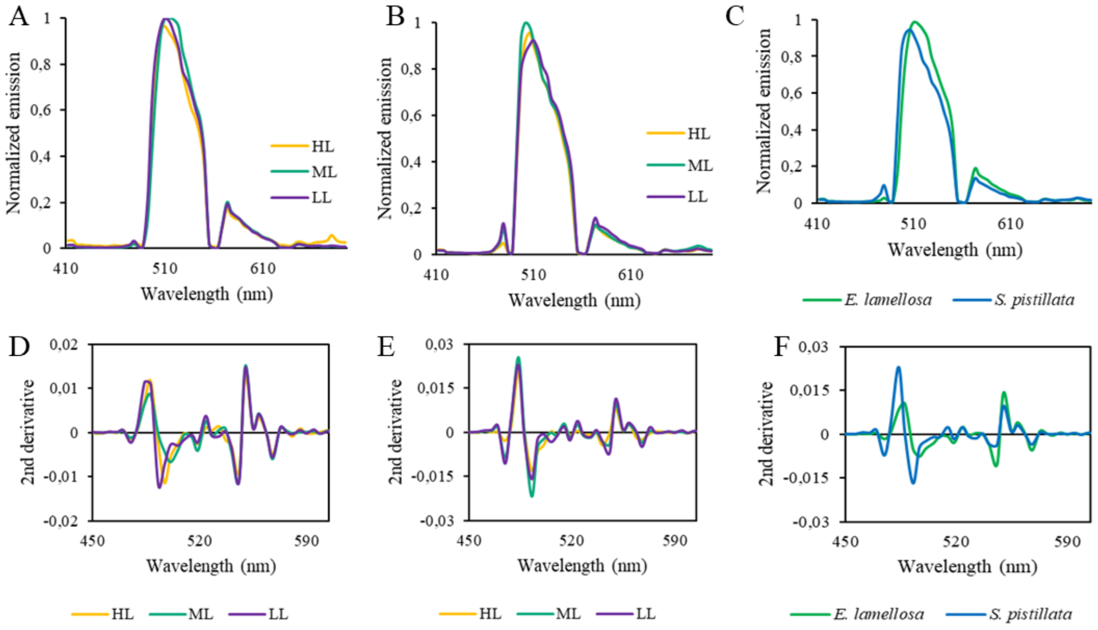


Figure 16. Emission curves of GFPs normalized to their maximum from each studied species, *E. lamellosa* (A), *S. pistillata* (B) and for both species, averaging light levels (C). The 2nd derivative of each treatment curve show intra and inter-specific variation in trends of the emission spectra. *E. lamellosa* (D) *S. pistillata* (E). Light levels: HL – High light; ML – medium light; LL – low light.

3.3 CFPs

CFPs were found to differ between species in this study (Fig. 17). CFPs from *E. lamellosa* have a curve similar to GFPs', with two shoulders at 479nm and 581 nm and a spectra width of about 40nm, shorter than the closely related GFPs. The emission peak of CFPs curve is located at 501nm although other studies consider the emission peak of CFPs to be situated earlier in the spectra (485nm-497nm). The co-location and similar curves of CFPs and GFPs in *E. lamellosa* makes them hard to distinguish. CFPs in *S. pistillata* have a much stronger component of blue fluorescence, resembling a second peak at 479nm, having slightly less intensity of fluorescence than the peak of emission at 501nm. The first peak at 479 has a spectral width of 18nm as for the second peak, it has a width of 40nm. When comparing the intensity of fluorescence of CFPs between species, statistically significant differences were found in the 479nm peak, the blue component of the CFPs in *S. pistillata* is much higher when compared with *E. lamellosa* corals (Fig. 18).

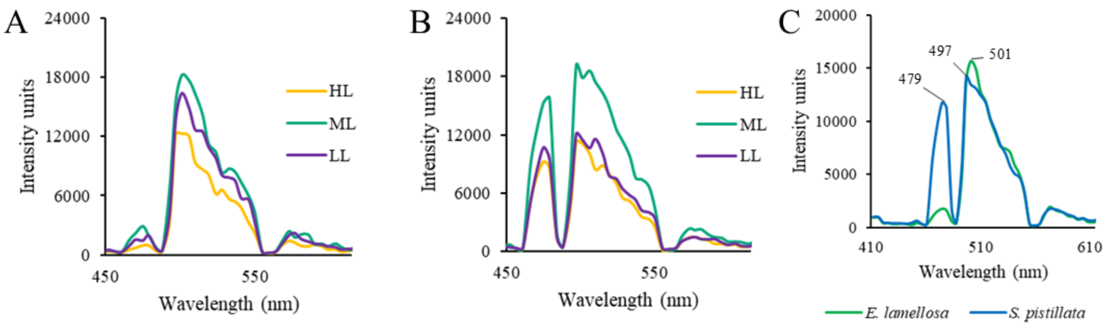


Figure 17. Fluorescence emission spectra for the third region showing the emission curve of CFPs when excited with the 458nm laser of *E. lamellosa* (A) and *S. pistillata* (B) and light levels (high light, medium light and low light). Averaged species including the distinct light levels (C).

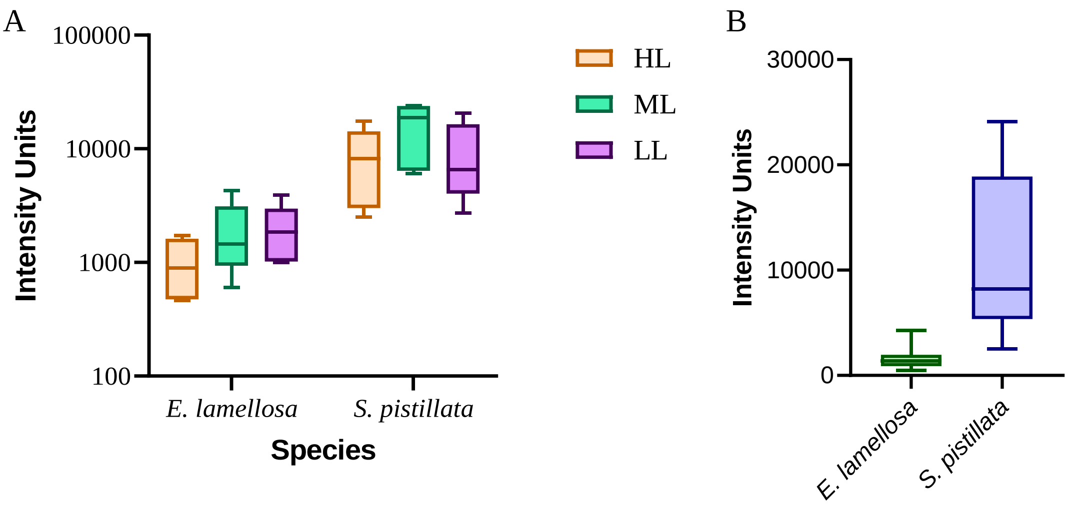


Figure 18. Emission intensity of fluorescence of CFPs at the peak of the curve, 479nm. Statistically significant differences were found between species ($p<0.05$ two-way ANOVA: F value=52.118, dF=1, P value=1.45×10^{-7}). Values of peak intensity of fluorescence captured at 479nm. Light levels: High light (HL), medium light (ML), low light (LL). Log scale of intensity units in (A).

KW test revealed inter and intra-specific statistically significant differences between treatments. Two-way ANOVA comparison among treatments resulted in the discovery of differences between species (F value=52.118, p=1.45e^{-7}).

Normalized curves are very irregular as we can see for both species (Fig. 19). That could be due to the presence of other sources of fluorescence that interfere with the signal, producing "noise" that makes it difficult to analyze the fluorescence of CFPs. The biggest differences in light levels are visible after the peak.

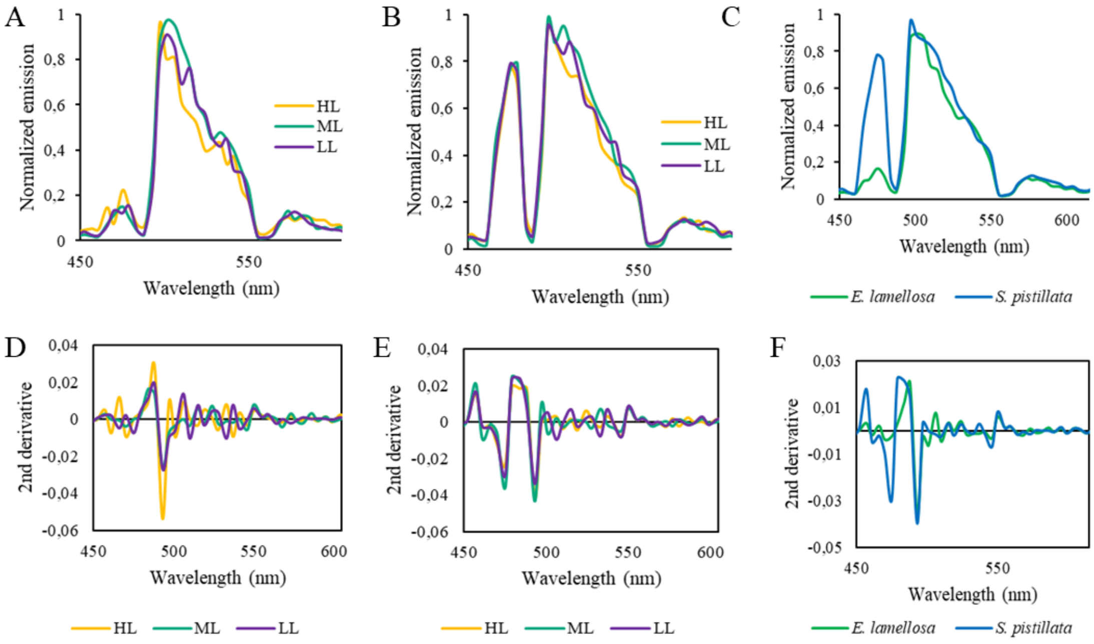


Figure 19. Emission curves of CFPs normalized to the maximum from each studied species, *E. lamellosa* (A), *S. pistillata* (B) and light levels averaged for both species (C). The 2nd derivative of each treatment curve show intra and inter-specific variation in trends of the emission spectra of *E. lamellosa* (D) and *S. pistillata* (E). Light levels: HL – High light; ML – medium light; LL – low light.

3.4 YFPs

YFPs' emission curve differed from other FPs. There was no evident peak of emission and not only that, the highest values of intensity of fluorescence were quite different in both species (Fig. 20). YFPs in *E. lamellosa* has a maximum of emission (16585 IUs) at 577nm and a width of 56nm that goes from 573nm (yellow) to 626nm (orange red). In *S. pistillata*, YFPs' curve has the opposite behavior. There is a maximum of emission (13912 IUs) at 608 nm and a peak width of 56nm. In the emission curve, it is seen emission of fluorescence before the excitation wavelength of 561nm which can be artifacts and are not taken into consideration in the analysis and interpretation of data. Two-way ANOVA test revealed statistically significant differences in the intensity of fluorescence between the two species, *E. lamellosa* (16585 ± 10348 SD) and *S. pistillata* (10611 ± 9923 SD), emitted at 577nm (Fig. 21).

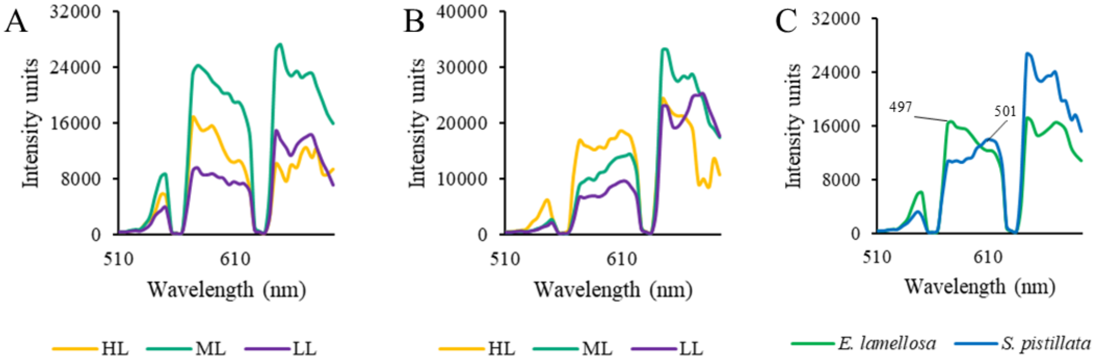


Figure 20. Fluorescence emission spectra for the fourth region showing the emission curve of YFPs when excited with the 561nm laser for *E. lamellosa* (A) and *S. pistillata* (B) acclimated to different light levels (high light, medium light and low light). Averaged species replicates in (C).

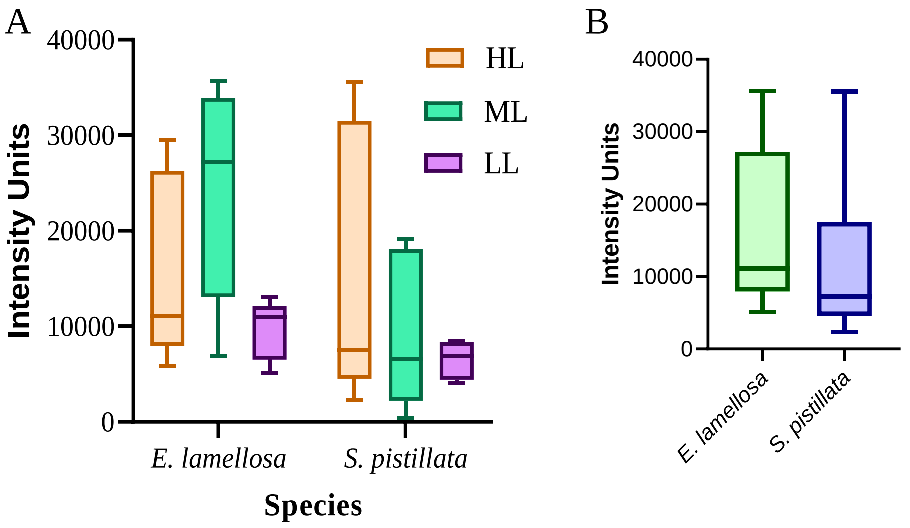


Figure 21. Emission intensity of fluorescence of YFPs in the distinct light levels (A) and comparison of both species (B). No statistically significant differences were found between treatments. Statistically significant differences were found between species (two-way ANOVA test: F value=4.433, Df = 1, p-value = 0.0451, n=30). The peak intensity of fluorescence was captured at 577nm. “Log” transformation applied in data.

The most evident difference between FPs profile in both species is how the curve behaves: in *E. lamellosa* there is a better-defined peak, whereas in *S. pistillata*, FPs do not seem to have an emission peak (Fig. 22A-C). The presence of red fluorescence might also contribute to the unclear peak of YFPs, one possible solution could be restriction of detection of fluorescence only until the ~630nm. Chromoproteins interact with light between 560nm and 610nm, being one possible cause for the differences detected between species in that range. This irregularity in the curves profile is also reflected on the 2nd derivative (Figure 22D-F). Differences are more pronounced in the range of the YFPs and RFPs.

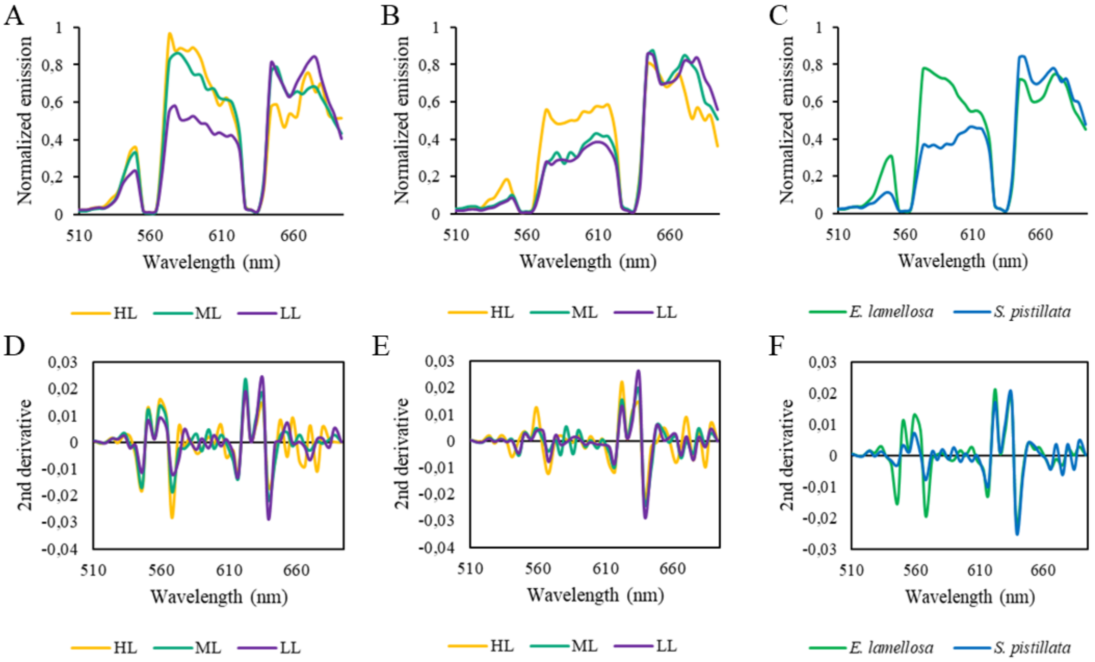


Figure 22. Emission curves of YFPs normalized to the maximum from each studied species, *E. lamellosa* (A), *S. pistillata* (B) and light levels averaged for both species (C). The 2nd derivative of each treatment curve show high variation in trends of the emission spectra between 577nm and 608nm (D-F). *E. lamellosa* (D) and *S. pistillata* (E). Light levels: HL – High light; ML – medium light; LL – low light.

3.5 RFPs

Although in the literature RFPs have emission maximums at 600-610 nm, the one detected in *E. lamellosa* and *S. pistillata* have an emission peak at 648 nm and an approximate spectra width of 20nm (figure 23A-C). The peak in *E. lamellosa* reaches a maximum intensity of 23789 IUs while *S.* pistillata reaches 34474 IUs. In both species the highest intensity of fluorescence was achieved in medium light treatments. In *E. lamellosa* medium light, the intensity (at the peak) reached two-fold than the one obtained in low light, and almost 5x the emission intensity in high light. In *S. pistillata*, the difference observed among light levels was not as evident. The emission curve, shown in Fig. 23A-C, displays a second peak at 679 nm, which coincides with the emission peak of chlorophyll which is also excited by the 633nm laser. In the emission curve, emission of fluorescence before the excitation wavelength of 633nm can is most likely artifacts and are not taken into consideration in the analysis and interpretation of data.

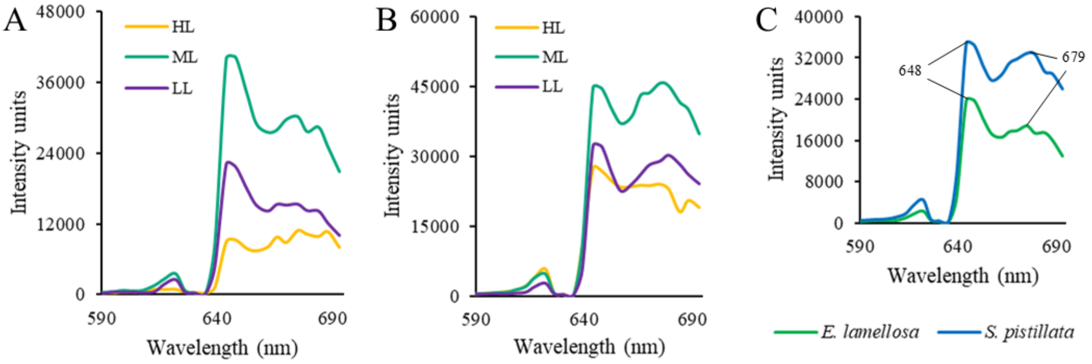


Figure 23. Fluorescence emission spectra for the fourth region showing the emission curve of RFPs when excited with the 633nm laser of *E. lamellosa* (A) and *S. pistillata* (B) and light levels (high light, medium light and low light). Averaged species replicates in (C).

In *S. pistillata* it is also clear the medium light yields the highest values of fluorescence from RFPs and the lowest being from high light, a fact also observed in *E. lamellosa* (Fig 24). Finally, it was also revealed that *S. pistillata* has a higher peak of emission at 648nm when compared with the species *E. lamellosa.*

The comparison of intensity peaks of fluorescence among light levels and between species revealed differences. In *E. lamellosa*, medium light is about twice as much, the intensity value of red fluorescence of other light levels. Comparisons of the intensity of fluorescence among the distinct light levels and species revealed statistically significant differences (Fig. 24).

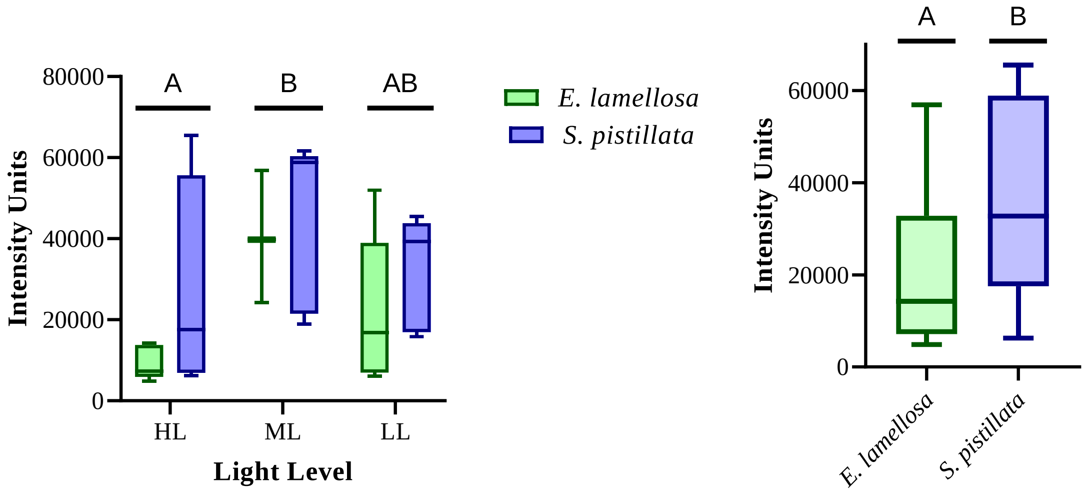


Figure 24. Emission intensity of fluorescence at the peak of the curve, 648 nm. Intensity values averaged for each species (on the right). Statistically significant differences were found between species (two-way ANOVA: Df=1, F value=5.163, p value=0.0337, n=27) and amongst light levels (two-way ANOVA: Df=2, F value=5.368, p value=0.0131, n=27). "A" and "B" represent groups where statistically significant differences were found. "Log" transformation was applied in the data.

The curve of fluorescence obtained with the 633nm laser shows the emission RFPs as well as some fluorescence from chlorophyll which corresponds to the 679nm peak (Fig.25). Until 610nm, *E. lamellosa* has more ups and downs than *S. pistillata* which has a more stable profile. Regarding RFPs in both species, there seems to be no difference between the light levels as the tendency curve of the derivative is quite similar. The biggest differences observed were in the later part of the spectra mainly due to the presence of other pigments that interact with the light possibly.

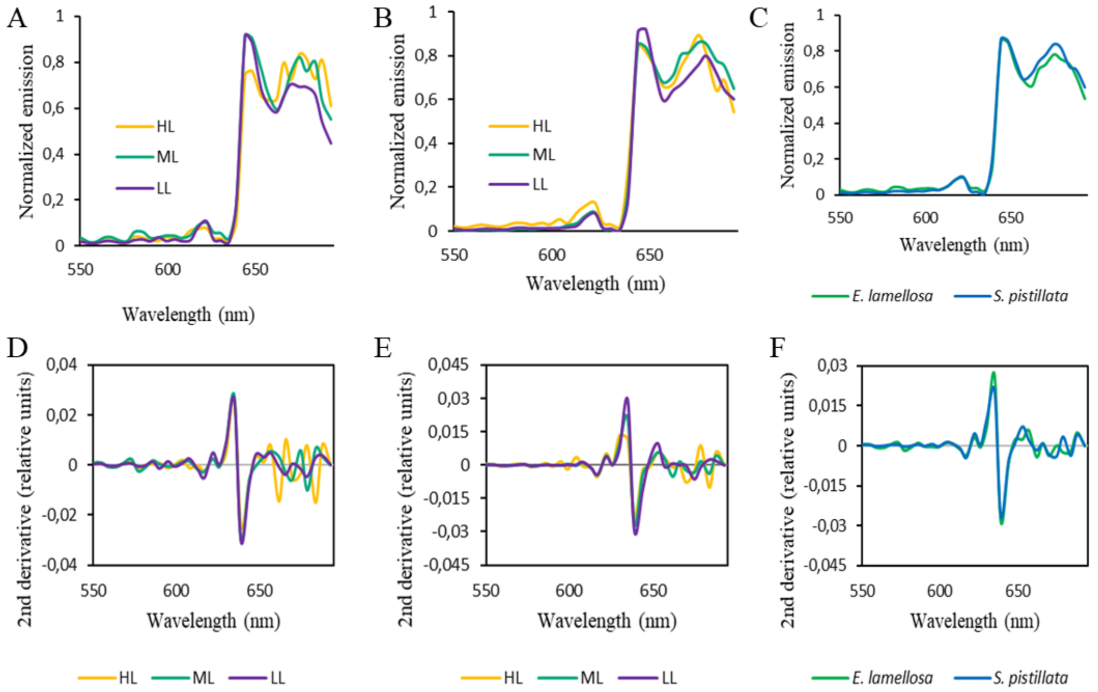


Figure 25. Emission curves of RFPs normalized to the maximum from each studied species, *E. lamellosa* and *S. pistillata* (A-C) The 2nd derivative of each treatment curve show variation in trends of the emission spectra. (D-F). *E. lamellosa* (D) *S. pistillata* (E) Light levels: HL – High light; ML – medium light; LL – low light.

4. Symbiont photobiology

4.1 Zooxanthellae densities

Zooxanthellae densities per surface area were similar in both species with an average of 2.1x10^6 ± 4.1x10^5 cells/cm^2 in *E. lamellosa* and 2.0x10^6 ± 2.3x10^5 cells/cm^2 in *S. pistillata* (Fig. 26). In both species the highest zooxanthellae densities were obtained in high light and the lowest in low light. *E. lamellosa* corals under low light intensity have about half the density of zooxanthellae than corals exposed to high light intensity (1.3x10^6 ± 1.1x10^5 cells/cm^2 and 2.7x10^6 ± 9.9x10^5 cells/cm^2 respectively). In *S. pistillata*, the difference from the high light to medium and low light was relatively smaller, with 2.4x10^6 ± 1.9x10^5 cells/cm^2 in high light and 2.3x10^6 ± 2.2x10^5 cells/cm^2 and 1.8x10^6 ± 2.7x10^5 cells/cm^2 in medium and low light, respectively. Statistical tests revealed significant differences in the zooxanthellae densities of corals under the distinct light levels (two-way ANOVA: F value=

0.37333, Df = 2, p-value = 0.0003). Tukey's multiple comparisons of means test showed statistically significant differences between LL and each of the other two light levels. No statistically significant differences were found between both species (p value > 0.05).

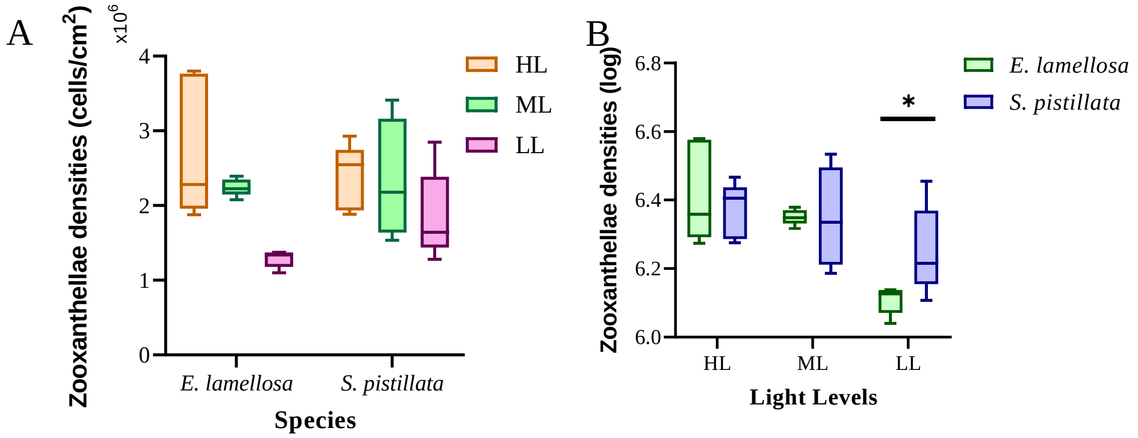


Figure 26. (A) Zooxanthellae densities under different light levels (HL-high light; ML-medium light; LL-low light) in *E. lamellosa* and *S. pistillata*. (B) Differences in zooxanthellae densities amongst the distinct light levels. "*" represent groups with statistically significant differences. "Log" transformation was applied in the data.

4.2 Chlorophyll *a* quantification

Cellular chlorophyll *a* was higher in *E. lamellosa* corals exposed to lower light intensities (Fig.27A) with 5.8 ± 1.4 pg Chl *a*/cell and corals exposed to high light had the lowest chlorophyll *a* per zooxanthella (4.3 ± 2.4 pg Chl *a*/cell), however, no statistically significant differences in cellular chlorophyll *a*, were found neither between species (two-way ANOVA: Df=1, F value=2.314, p value= 0.142) nor among light levels (two-way ANOVA: Df=2, F value=1.495, p value= 0.246). Additionally, the relationship between light levels and cellular chlorophyll is not dependent on species (two-way ANOVA with interaction: Df=2, F value=0.301, p value=0.743).

Contrary results arose when chlorophyll *a* was analyzed by surface area. Although *E. lamellosa* corals under low light had the highest contents of chlorophyll *a* per cell, the concentration of chlorophyll *a* per surface area was the highest in *E. lamellosa* under high light (1.2 µg Chl a/cm^2 ± 1.1 µg Chl a/cm^2) (Fig. 27B). A two-way

ANOVA with interaction effect revealed no statistically significant differences neither among light levels (Df=2, F value=0.481, p value=0.624) nor between species (Df=1, F value=2.068, p value= 0.164).

Contrary to the results of *E. lamellosa*, normalization of chlorophyll *a* to both zooxanthellae and surface area generated similar results in *S. pistillata*. Corals under low light had the highest amounts of chlorophyll *a* per cell (8.1 ± 3.9 pg chl *a*/cell) as high and medium light exhibited similarly lower values (6.1± 2.6 pg chl *a*/cell and 5.6 ± 2.3 pg chl *a*/cell, respectively). Chlorophyll normalized to surface area also revealed high similarity between high and low light intensities (1.5 ± 0.2 pg chl *a*/cell and 1.5 ± 0.9 pg chl *a*/cell, respectively) and the lowest value in medium light (1.1 ± 0.4 pg chl *a*/cell).

Finally, when all the replicates were averaged to compare the results between species, it was found that *S. pistillata* has both the highest contents of chlorophyll *a* per cell (6.6 pg chl *a*/cell) and the highest quantity of chlorophyll *a* per surface area (1.4 µg chl *a*/cm^2) showing some potential differences on the photobiology of these species and how they cope with the environmental factors such as light in this case.

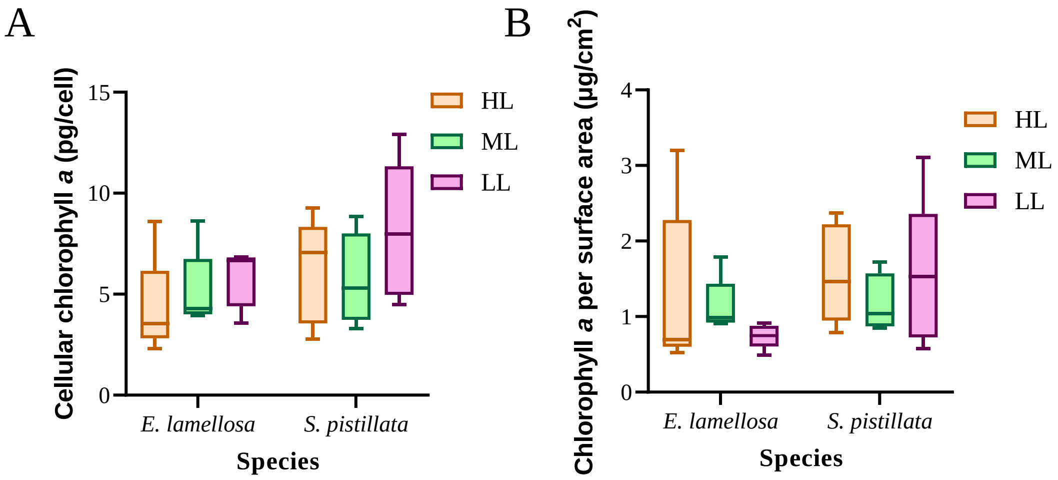


Figure 27. (A) Cellular chlorophyll *a* under different light levels (HL-high light; ML-medium light; LL-low light) in *E. lamellosa* and *S.* (B) Chlorophyll *a* per surface area under different light levels (HL-high light; ML-medium light; LL-low light) in *E. lamellosa* and *S. pistillata*. “Log” transformation was applied on cellular chlorophyll *a* data.

4.3 Protein quantification

Protein quantification normalized to surface area in *E. lamellosa* showed that corals under medium light have the highest biomass (1.9 ± 0.6 mg/cm^2) among the three light levels, with the lowest total coral protein found in low light (1.0 ± 0.47 mg/cm^2) (Fig. 28). In *S. pistillata* we found the highest concentration of protein per surface area in corals exposed to high light intensities (1.2 ± 0.2 mg/cm^2) and only 0.6 ± 0.5 mg/cm^2 in low light, about half. Finally, when comparing both species, *E. lamellosa* displays the highest coral protein biomass (1.4 ± 0.4 mg/cm^2) of both species (*S. pistillata* with 0.9 ± 0.3 µg/cm^2). Statistical tests revealed significant differences among light levels (two-way ANOVA: Df=2, F value=3.858, p value= 0.036) and between species (two-way ANOVA: Df=1, F value=6.417, p value= 0.019). The p value for the interaction between species and light level (Df=2, F=1.442, p-value=0.2570) revealed that the relationship between total protein per area and light level, is not dependent on species. Moreover, performing a Tukey's test revealed that the significant differences among treatments reside between *E. lamellosa's* ML and *S. pistillata's* LL (Tukey HSD test, p adj=0.04). No other pairwise comparisons were significant.

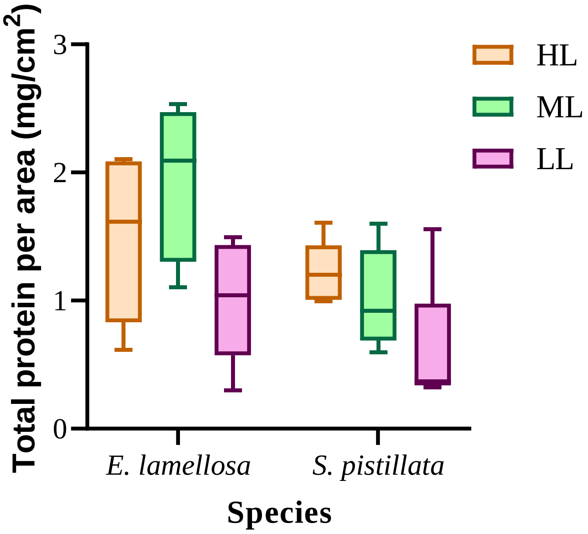


Figure 28. Coral protein per surface area under different light levels (HL-high light; ML-medium light; LL-low light) in *E. lamellosa* (n=14) and *S. pistillata* (n=15).

Comparing both species, it is clear that *E. lamellosa* has a much lower ratio of zoox/prot (number of zooxanthellae per µg of coral protein) with an average of 534 ± 106 cells/µg of coral protein, opposing to *S. pistillata* that has more than double the number (1279 ± 733 cells/µg) of zooxanthellae per coral protein (Fig. 29). Statistical comparisons, revealed significant differences between species (Kruskal-Wallis chi-squared = 15.773, df = 1, p-value = 7.14×10^{-5}). Considering intra-specific variation, *E. lamellosa* had little variation between light levels, the lowest value of the ratio being obtained in medium light. In *S. pistillata* there was a greater variation, and contrary to *E. lamellosa*, medium light had the highest number of zooxanthellae per µg of total protein.

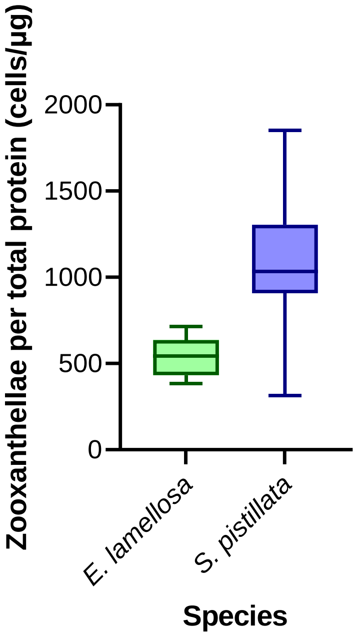


Figure 29. Zooxanthellae densities normalized to coral total protein contents of *E. lamellosa* (n=14) and *S. pistillata* (n=15). Statistically significant differences were found between species. "Log" function was applied to data for statistical analysis.

Additionally, a spearman correlation test was performed to verify a possible link between zooxanthellae densities and coral biomass (total proteins) (Fig. 30). The test revealed a positive correlation between the two factors in both species. In *E. lamellosa*, it seems to exist a strong positive correlation between the number of zooxanthellae hosted and the total biomass of the coral, and a weak positive correlation in *S. pistillata* (*E. lamellosa:* Spearmen's test: Rho=0.897; p=1.398×10^{-5}; *S. pistillata*: Spearmen's test: Rho=0.529; p=0.0426).

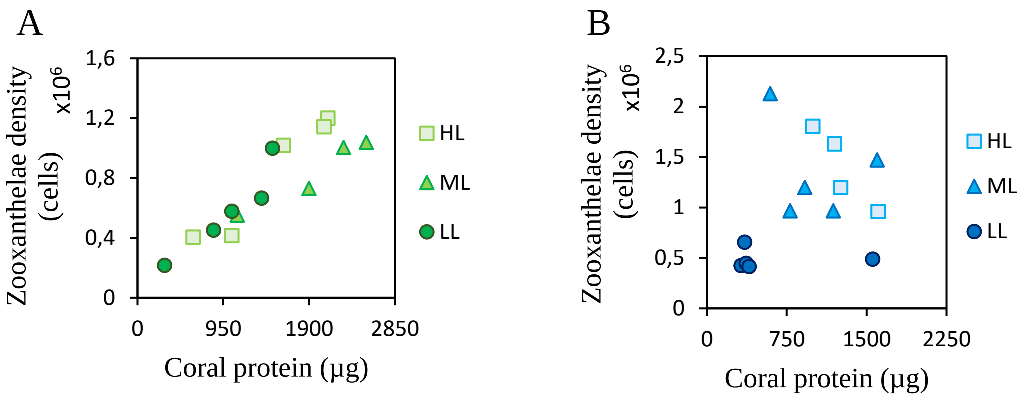


Figure 30. Inter and intra-specific correlation between zooxanthellae densities and total coral protein. *E. lamellosa* (A) and *S. pistillata* (B) (HL-square; MD-triangle; LL-circles).

The comparison between species revealed that *E. lamellosa* has a lower ratio of chlorophyll *a* to protein (0.003 µg Chl *a*/ µg prot) than *S. pistillata* (0.007 µg Chl *a*/ µg prot) (Fig. 31). Intra-specific comparisons showed that in *E. lamellosa*, the highest ratio between Chl *a*/protein is achieved under high light, coherent to what is seen in the other species, *S. pistillata*. Statistical analysis revealed that species differed on the ratio of chlorophyll per total protein (two-way ANOVA: Df=1, F value=23.379, p value=5.71x10^{-5}), unveiling a potentially increased dependency of *S. pistillata* corals on symbionts and light.

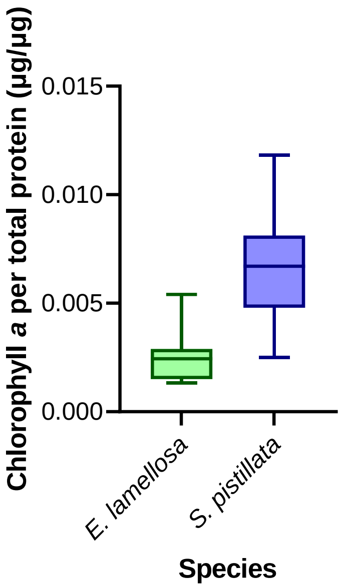


Figure 31. Chlorophyll (ug) per host coral protein (ug) in *E. lamellosa*, and *S. pistillata*. Statistically significant differences were found between species (two-way ANOVA: p value= $5.71\text{x}10^{-5}$). "Log" function was applied in the data for statistical analysis.

A correlation test was made between chlorophyll *a* and total coral protein (Fig. 32), but it did not yield any strong correlation in any of the species (correlation test < 0.60) which does not give any definitive answers whether chlorophyll *a* and coral protein contents are connected.

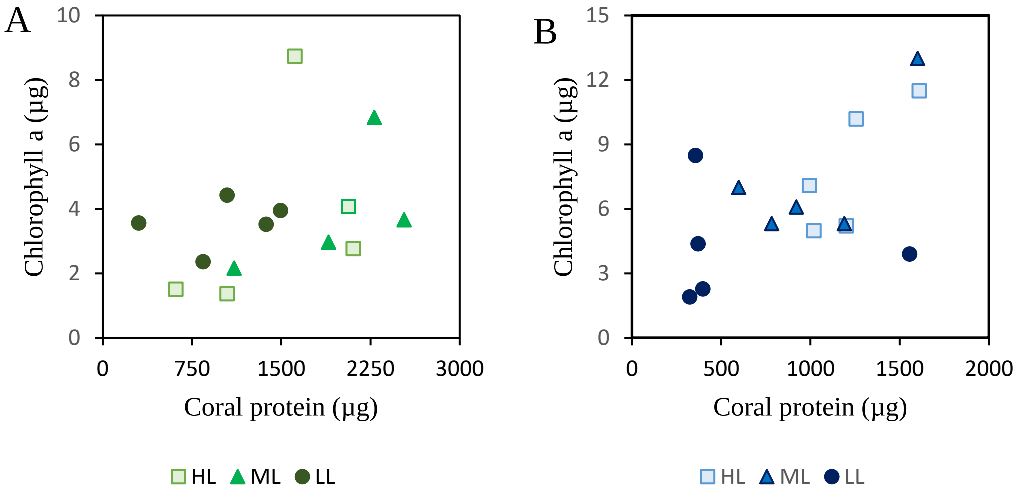


Figure 32. Inter and intra-specific correlation between chlorophyll *a* and total coral protein. *E. lamellosa* in green (HL-square; MD-triangle; LL-circles) and *S. pistillata* in blue (HL-square; MD-triangle; LL-circles).

Discussion

Incident irradiance on the surface of coral colonies is highly dependent on many factors such as, time of the day and season, attenuation of light by water, interaction with microorganisms (Kirk, 1975), suspended particles (Downing et al., 2012) and even the agitation at the surface of the water. It is known that light has a profound effect on the physiology and growth of corals and that many species are adapted to reduced light intensities and many others can tolerate excessive irradiance (Titlyanov, et al. 2002). Corals maintained and grown in large ecosystem-aquaria (mesocosms), like the ones at HdM, experience different light intensities, depending on the location of the colony itself and the light source. Other factors include intra and interspecific competition, seen in the natural environment, which influences growth and development of these organisms and their response mechanisms to light.

As mentioned before, light irradiance found *in situ* depends on many factors. Sites in close proximity can often display different irradiance at the same depth. In the Indo-pacific, where *E. lamellosa* is found, the highest intensity of light (200-240 µmol photons m^{-2} s^{-1}) corresponds to shallow depths (5m) and lower light intensities (10-30 µmol photons m^{-2} s^{-1}) correspond to the beginning of mesophotic ecosystems (>20m) (Campbell et al. 2007). Furthermore, the photosynthetic yields from corals under low light are higher than corals under high light, for both species (Iluz & Dubinsky, 2015).

All this environmental heterogeneity found at HdM partially mimics *in situ* conditions and gives rise to differences that can be seen in corals of the same species, namely color wise. The color of corals does not have taxonomic significance but is rather a result of the many components responsible for absorptance, transmission and reflectance of light. In HdM, there are corals of the same genotype living in the same tank but exhibiting different tones and color. *E. lamellosa* under high light intensity displays brighter-white tones compared to its counterparts that reside under dimmer light. The same characteristic can be seen in another species such as *S. pistillata* that displays lighter tonalities under more intense light and darker coloration under low light intensities. One explanation is that simply because corals under more intense light reflect more light, therefore, they appear whitter to our eyes. Shaded corals appear darker indicating that very few photons are lost by reflectance, which was also observed by other authors. (Mass et al. 1981). One of the hypotheses that arose was that differences in densities of zooxanthellae and abundance of fluorescent proteins were responsible for the distinct color phenotypes between colonies within the same genotype. This study examined differences in emission intensity of fluorescence found in the coral tissue, and the photobiology characteristics of the symbionts as an indicator of coral acclimation to the light environment they are inserted in. Understanding the drivers for FPs variation and their roles in corals is crucial to better predict the effects of climate change on corals and identify potentially vulnerable species.

1. Fluorescence in corals

This study confirms some shade acclimation mechanisms adopted by corals to low light environments and describe new features namely location and emission intensity of FPs. In both species, FPs are seen widely spread on the coral tissue being associated to different structures at the same time. GFPs in *E. lamellosa* can be seen in multiple structures, such as in the epidermis and deep in the tissue like in the pharynx or in muscles. FPs are ubiquitous which enhances their importance in the growth and survival of corals, as given the many roles that these FPs have. Being associated with structures other than the epidermis and tentacles, suggest these FPs have support and structural roles in muscles for example. However, the longitudinal muscle fibers found in corals, are formed from ectoderm cells (Brittanica E., 1993), which have a genetic pre-disposition for fluorescence, and might transfer those characteristics to the muscle fibers, early on the polyp development. Furthermore, multiple FPs are colocalized, although in microscopy is perceived as only one color. This suggests that the dominant color of fluorescence can either be determined by the ratio between FPs or by the incident wavelength. Besides FPs associated with specific structures, there were also small granules, containing fluorescence, scattered on the coral tissue. These FP granules are much more visible in *E. lamellosa* specially in the colony living under low light, found mostly amongst and under the layer of zooxanthellae. This strategic position is consistent with the proposed function of light enhancement for photosynthesis by way of wavelength transformation and back-scattering (Salih et al. 2000). Surprisingly, FP granules in *S. pistillata* were very rare, even in corals under low light. Instead, in this species, it was found a layer of endolithic microorganisms between the coral tissue and the skeleton. Endolithic microbes include microalgae, fungi and prokaryotes which contribute to the ecological success of corals, providing functions such as nutrient cycling and metabolite transfer (Pernice et al., 2020). In terms of high light tolerance, colonization of endolithic algae within the coral skeleton can provide beneficial effects to coral photosynthesis by providing photoprotection to the symbionts from excessive radiation by reducing the reflectivity of the white coral skeleton (Yamazaki et al., 2008).

My hypothesis that FPs emission would be higher in corals under high light (indicating more FPs in the coral tissue supporting the photoprotective role), was not supported by the findings in the present work. It was expected that corals under high light intensities would express more FPs, such as GFPs (Roth et al. 2010), and therefore, have a higher emission intensity of fluorescence. However, in most cases, FPs emission revealed that corals under medium light yield the highest value of fluorescence. Coral FPs can constitute up to 14% of the total coral protein content, so the greater fluorescence found in corals under medium light, could be explained by a high protein content. This in fact happens in *E. lamellosa* corals under medium light exhibiting higher intensity of fluorescence than other light levels, also possess the highest contents of protein per surface area (mg/cm^2). However, this was not verified in *S. pistillata* where, instead, the highest biomass was observed in corals under high light. This higher concentration of protein under high light goes along with hypotheses that support FPs as photoprotective pigments. As there is more environmental light there is the need for protecting zooxanthellae photosystems from damage and vice versa. This hypothesis is further supported by the fact that *S. pistillata* corals under low light have about half the proteins per surface area than their high light living counterparts. However, it was quantified total protein and not only FPs, therefore, the assumption that higher intensity is related with higher biomass must be taken with caution. Protein content is also unlikely to explain why CFPs in *S. pistillata* have a stronger component of blue fluorescence detected in the emission spectra. At 479nm, the intensity of emission captured in CFPs from *S. pistillata* is almost ten times higher than the one capture in *E. lamellosa*. The emission curve from CFPs in *E. lamellosa* resembled more the GFP’s. Like GFPs, CFPs were selected from the epidermis, which could explain the high similarity between GFP and CFP curves. CFP possess the same chromophore as GFPs which confers many similarities between the two, however, it is recognized as a separated color class (Henderson & Remington, 2005). The emission spectra of CFPs were captured from distinct anatomical regions in both species. The CFPs in *E. lamellosa* were located on the most outer tissue layer of the coral just next to the oral disk of the polyp, an area which also has GFPs but at a lower intensity of fluorescence due to the lower concentration. In the case of *S. pistillata*, CFPs were concentrated on the tentacles of the polyp which could lead to a more specific role like attraction of prey (Ben-Zvi, O. et al. 2022) therefore, displaying a unique curve of emission, unlike the one found in *E. lamellosa* corals. Explanations for the gradient of FPs found in the epidermis of *E. lamellosa*, from green fluorescence around the oral disk of a polyp to blue fluorescence in tissue vesicles, remain elusive. Green fluorescence has been shown to enhance symbiont phototaxis (Dizon et al., 2021) therefore, the presence of GFPs around the polyps' mouth, may provide the coral with an enhanced mechanism for capture of free-swimming zooxanthellae (essential for recovering after an event of bleaching). YFPs also produced different unique curves of emission for both species. In both species there was not an evident peak of emission in the yellow-orange range of wavelength, contrary to what was seen for other FPs. In *E. lamellosa* it was found a pseudo-peak at 577nm and in *S. pistillata* it was found, almost like a plateau of fluorescence that stretched from yellows (577nm) until the "peak" at 613nm (orange). The absence of a well-defined peak could be explained by the presence of other sources of fluorescence that contribute to noise and influence the behavior of the YFP's curve. The distinct behaviors of YFP's curves in both species could indicate that they are two morphotypes of the same FP with small variations, or they are two completely distinct FPs. Flow cytometry linked with fluorescence sorting complemented with molecular and genetic studies could provide the answer to this question (Randall et al. 2020).

Overall, fluorescence in corals is influenced by light, producing some acclimative responses that can be seen through changes at inter and intraspecific levels. Additionally, environmental plasticity, seen in each colony, could be demonstrated by hyperspectral analysis in the present study.

2. Photo dynamics of symbionts

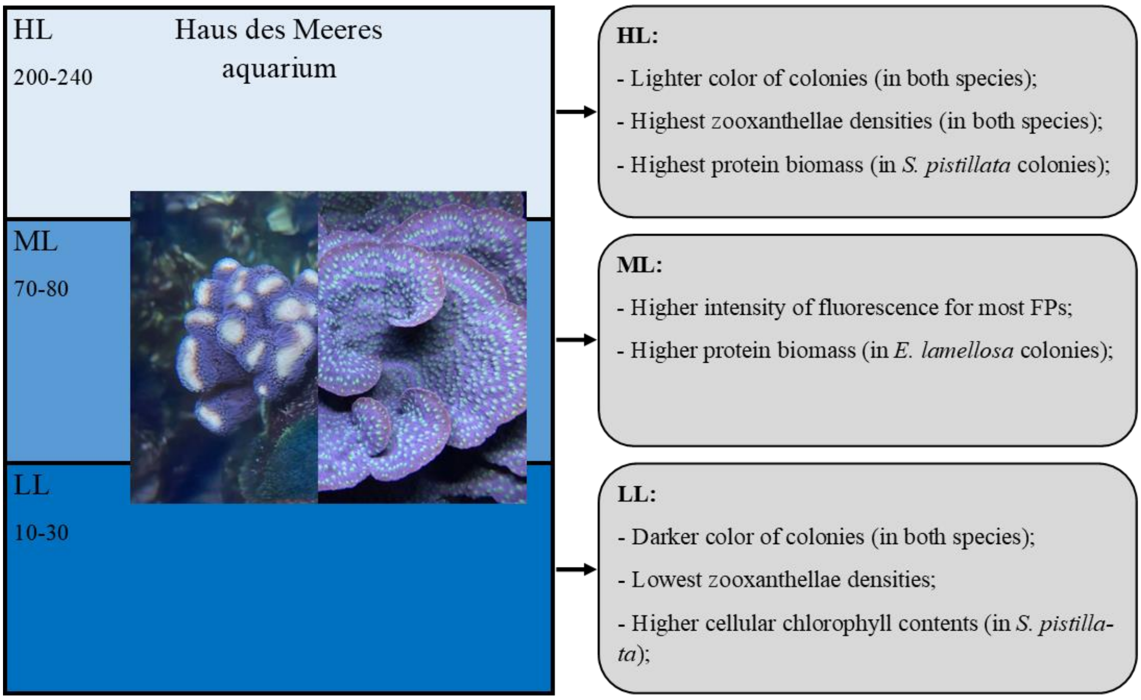
At the symbiont level, it was also possible to see differences in treatments depending on incident light. As reported by previous studies, I hypothesized that mechanisms to acclimatize to shade include reduction in zooxanthellae densities and increase in cellular chlorophyll *a* content which is confirmed by results. In both species, the highest density of zooxanthellae (cells/cm^2) was achieved in corals under high light and the lowest density in corals under low light. Furthermore, I observed that the vesicles in *E. lamellosa* were packed with zooxanthellae. One hypothesis that arose was that these vesicles were a mechanism present in *E. lamellosa* to increase the space available to harbor more symbionts. Yet, when compared both species, *S. pistillata* still displays the highest densities of zooxanthellae per surface area. Additionally, it was expected that zooxanthellae under low light would have more cellular chlorophyll *a* than their high lit counterparts. This was observed in both species, in which cellular chlorophyll in corals under low light was around 1.5x higher than in corals under high light. The decrease in zooxanthellae numbers comes as an adaptation to minimize self-shading by symbionts when irradiance is minimal. Moreover, increasing chlorophyll concentration in dinoflagellates and most algae appears to be a universal response to low light levels (shade) and the need to harvest more light (McCloskey & Muscatine, 1984). The main difference observed between species was the higher cellular content of chlorophyll in zooxanthellae from *S. pistillata*. Small polyp stony corals such as in *S. pistillata* may rely more on autotrophy than big polyp corals like *E. lamellosa* that are better equipped with long extendable tentacles to catch prey. The ratio of zooxanthellae and chlorophyll to coral protein (cell/µg and Chl *a/*µg protein, respectively) was found to be higher in *S. pistillata* which supports the hypothesis that this species is highly adapted to autotrophy making it more depended on light. Furthermore, the diameter of the zooxanthellae was measured, and it was seen that the size of the symbionts of *E. lamellosa* (10.0 ± 1.1 µm) were smaller than the ones found in *S. pistillata* (8.7 ± 0.9 µm) (personal observation). The zooxanthellae sizes analyzed across light levels revealed contrary results. In *S. pistillata*, zooxanthellae exposed to lower light levels (8.8 ± 1.0 µm) were larger than the ones under high light (8.6 ± 0.8 µm), which was expected. However, in *E. lamellosa*, zooxanthellae were found to be larger in high light (10.6 ± 1.2 µm) and smaller in low light (9.5 ± 1.0 µm), which goes against many studies already made regarding the photobiology of the symbionts. Although the fluorescent yields of chlorophyll *a* in *S. pistillata* is slightly higher than the one found in *E. lamellosa*, my data did not allow to detect any significant differences among the distinct light levels. The fact that the zooxanthellae cells in *S. pistillata* are smaller and yet have a higher cellular content of chlorophyll, and consequent higher fluorescence, gives further support to the hypothesis that this species of coral possesses zooxanthellae better adapted to capture light (Frade et al., 2008).

3. Limitations and recommendations for future studies

Throughout this study, I have faced problems in my work that I hereby expose with the intent to improve future research on this topic. I have realized that five replicates were not sufficient as there is great heterogeneity between coral colonies and even among branches in the same colony. This led to some differences in some replicates in terms of capturing the spectra behavior and difficulties in image generation. Precise light meter instruments would also allow for a more defined selection of coral fragments. In the microscopy work, subjectivity in the selection of regions of interest, unevenness in fluorescence in focal plane, unprecise area selection for fluorescence, suboptimal laser settings and some irregularity in coral tissue sectioning were factors that contributed to deficiencies in the generation of high-quality images and emission spectra of FPs. Future work on this topic could be improved by doing more replicates (for defined regions) and thinner sections, excluding problems related to the focal plane and the presence of other sources of fluorescence. Further studies should address other regions, like the coenosarc, quantification of FP granules and respective intensity of fluorescence. A more in depth, exploration of endolithic microorganisms could also provide a broader understanding of the complex interaction of corals with light.

Conclusion

Corals found at HdM under distinct light intensities display differences in the intensity and curve of FPs (Fig. 33). FPs are a highly diverse group in terms of biochemical and ecological functions which is reflected on the distinct emission curves obtained in fluorescence analysis of corals in response to environmental conditions such as light. Coral colonies exposed to different light intensities led them to develop acclimative mechanisms observed in this study such as higher zooxanthellae densities in higher light intensities and increased cellular chlorophyll *a* content in zooxanthellae under low light. Regarding fluorescence, the present study demonstrated that there is inter and intra-specific variation of intensity and emission curve of FPs (Fig. 34). This has further contributed to the ongoing research being developed on fluorescence in corals describing and characterizing different FPs on two scleractinian corals, *E. lamellosa* and *S. pistillata*. Understanding the diversity and roles of coral FPs is key for greater insight of coral reef ecosystems and this will allow prompt action with conservation efforts. Furthermore, this work further high light the use of FPs as easy to use field biomarkers to assess overall coral health during these times of climate change.



HL:

- Lighter color of colonies (in both species);
- Highest zooxanthellae densities (in both species);
- Highest protein biomass (in *S. pistillata* colonies);

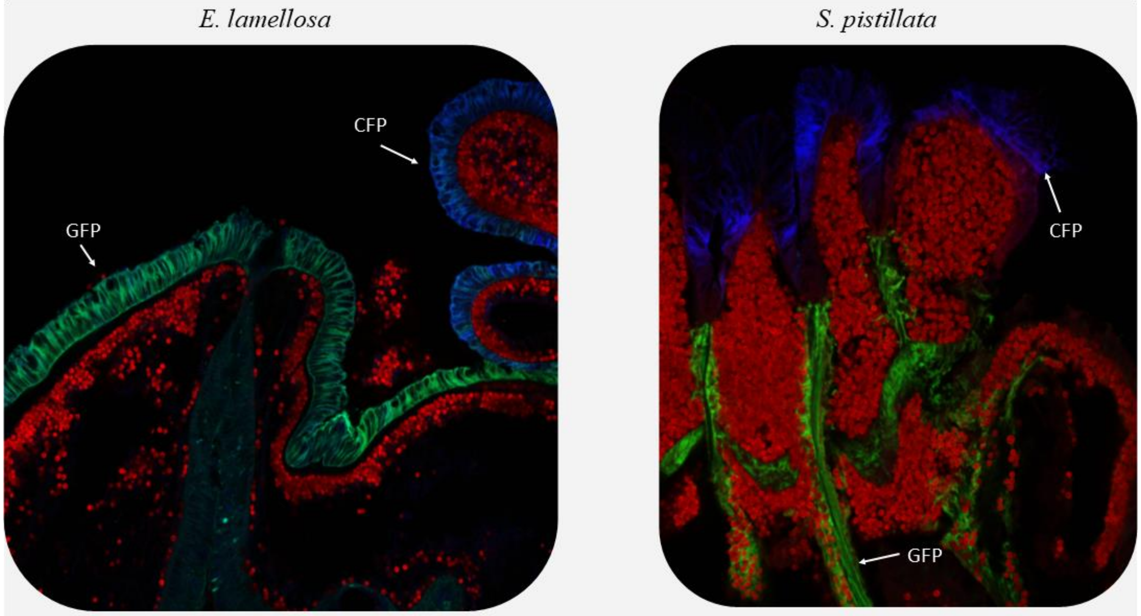
ML:

- Higher intensity of fluorescence for most FPs;
- Higher protein biomass (in *E. lamellosa* colonies);

LL:

- Darker color of colonies (in both species);
- Lowest zooxanthellae densities;
- Higher cellular chlorophyll contents (in *S. pistillata*);

Fig. 33. Two species of scleractinian corals grown under distinct light conditions are studied to identify possible inter and intra-specific differences in their fluorescence patterns. The colonies under different light environments were compared and a short list of results is shown above. Light intensity is given in µmol photon.m^{-2}s^{-1}.



E. lamellosa

S. pistillata

- GFPs present in oral disk to attract zooxanthellae;
- CFPs present in vesicles outside the polyp mouth;
- Presence of FP granules;

- GFPs present in the epidermis and longitudinal muscle bands;
- CFPs present in the acrosphere of the tentacle (10x more fluorescence than the ones found in (*E. lamellosa*);
- Presence of endolithic microorganisms;

Fig. 34. Polyp images from both species under laser scanning confocal microscopy. Differences in patterns of fluorescence found between both species are annotated as well as the presence of GFPs and CFPs. No major differences in patterns of fluorescence were found amongst light levels.

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