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Photobiology of *Anemonia viridis* under different light and temperature conditions

Fotobiologia di *Anemonia viridis* sotto condizioni differenti di luce e temperatura

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ABSTRACT

Gli invertebrati marini sono oggetto di un crescente interesse da parte degli scienziati per l'uso e il consumo umano grazie alle loro proprietà e ai loro potenziali benefici. Questo porta talvolta ad un eccessivo sfruttamento di questi organismi, riducendone la popolazione e rendendo necessarie azioni di ripopolamento. Tuttavia, gli studi per valutare la fattibilità dell'allevamento di questi organismi sono limitati.

Anemonia viridis (Forskål, 1775) è un celenterato antozoo della famiglia Actiniidae diffuso nel Mar Mediterraneo e nell'Oceano Atlantico orientale, che vive in simbiosi con le dinoflagellate appartenenti alla famiglia Symbiodiniaceae. Esso contiene numerosi composti bioattivi e nutritivi che lo rendono una promettente specie di interesse commerciale nei campi nutrizionale, cosmetico, farmaceutico e medico. Attualmente il consumo di *A. viridis* non è ampiamente diffuso, ma nelle aree in cui è considerato una fonte edibile esso potrebbe essere soggetto a raccolta illegale. La conservazione in acquacoltura potrebbe essere una soluzione per azioni di ripopolamento o per un eventuale allevamento sostenibile di questa specie.

L'obiettivo di questo studio è quello di valutare come differenti condizioni di luce e temperatura influenzano l'ospite e la relazione Cnidaria-Symbiodiniaceae al fine di un eventuale allevamento di questo organismo.

In particolare, individui di *A. viridis* sono stati raccolti e posizionati all'interno di specifici acquari illuminati da luce PAR (460-700 nm) e da luce rosso-lontano/infrarosso (730 nm) alle temperature di 20°C e 23°C per due mesi e

periodicamente sono state analizzate la morfologia dell'ospite, la densità e l'esocitosi di Symbiodiniaceae, il contenuto di clorofilla e la fotosintesi.

In generale i risultati suggeriscono che sia l'anemone che i simbionti hanno mantenuto un buono stato di salute per tutta la durata dell'esperimento e che la luce rosso-lontano/infrarossa ha alterato la densità di Symbiodiniaceae e il contenuto dei pigmenti, mentre la fotosintesi è rimasta costante e funzionale. Più precisamente, si è verificato un aumento di concentrazione dei simbionti e una riduzione del contenuto di clorofilla nei campioni sottoposti a luce rosso-lontano/infrarosso.

Attualmente pochi studi hanno indagato gli effetti di diversi spettri luminosi oltre la PAR sugli organismi simbiotici, di conseguenza questo esperimento vuole essere un punto di partenza per future indagini.

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INTRODUCTION

PHOTOPROTECTION

The subclass Hexacorallia includes anthozoan cnidarians of the order Actiniaria, commonly known as sea anemones. Among Anthozoa, the order Actiniaria is the one that shows the greatest morphological diversity (M. Daly *et al.*, 2008) and a frequent tendency to live in symbiosis with other organisms, including some unicellular dinoflagellate microalgae commonly named “zooxanthellae” (Shick, 2012).

The relationship between organisms that live in symbiosis with these algae combines an heterotroph organism, the host animal, with an autotroph organism, the symbiont algae, in a functional unit called holobiont. Symbionts live in a protected habitat, receive a continuous supply of nutrients from the host’s waste products and have unlimited availability of CO_2 produced by the host’s respiration; the host animal, on the other hand, receives O_2 for respiration and glucose for growth (Yellowlees *et al.*, 2008).

The light spectrum quality is one of the abiotic factors that most influences organisms living in shallow waters, including symbiotic sea anemones, whose distribution, growth and health are symbionts dependent and consequently spectrum quality dependent (Tamir *et al.*, 2019).

In high light intensity conditions the photosynthetic rate of the symbionts increases, generating high concentration of dissolved oxygen. This can lead to the formation of high concentrations of reactive oxygen species (ROS), oxidizing agents capable of

reacting with cellular structures causing significant damage and impairing the proper function of lipids, proteins and DNA (Hawkins *et al.*, 2015).

ROS include superoxide, hydrogen peroxide and hydroxyl radicals (Bayr, 2005).

A key factor of holobionts is to optimize the photosynthetic efficiency of the symbionts and, at the same time, to protect against excessive solar radiation. To mitigate the damage caused by high light intensity, both the host and the symbionts have therefore evolved various photoprotective mechanisms, which can be distinguished into behavioural, mechanical and molecular mechanisms.

Behavioural Photoprotection

Behavioural photoprotection refers to the behaviour adopted by organisms in order to protect themselves from light radiation. Several studies have been carried out on different sea anemone species investigating the behavioural strategies determined by light, and more specifically phototaxis, tentacle retraction and gravel attachment to the column of sea anemones.

Phototaxis is defined as the movement of a mobile organism in response to light and is distinguished into positive phototaxis, when the movement is towards the light, and negative phototaxis, when the organism moves away from it (Foo *et al.*, 2020). Symbiotic sea anemones assume this mechanism to move towards a specific light regime or away from an extremely bright and potentially harmful environment (Strumpen *et al.*, 2022). This ability guarantees sea anemones to search a favourable habitat that allows symbiont algae to best exploit environmental light conditions and it

seems to depend on sea anemone and symbiont genus and the wavelength of light (Foo *et al.*, 2020). Some tropical sea anemones show negative phototaxis when exposed to full sunlight, choosing partially shaded habitat and therefore sheltered from high light intensity (Shick, 2012). Some of the factors affecting phototaxis response are light intensity and the life history of the organism. Studies carried out on the anemone *Aiptasia* have shown that individuals located in low light environment show a greater probability of movement, which decreases as the amount of light increases (Strumpen *et al.*, 2022) and non-tropical species of *A. elegantissima* from sunny habitat show a positive response to high light intensity, while conspecifics from shaded habitats avoid this level of irradiance and perform positive phototaxis when the intensity is reduced (Shick, 2012). These observations are confirmed by Henney's studies (2021), in which *Anemonia viridis* individuals acclimatized to low light levels have shown a greater phototaxis response compared to individuals acclimatized to high light intensity.

Thus, these studies suggest that phototaxis is more likely to occur in low light intensity and that individuals exposed to high solar radiation tend to seek shadier habitats.

The first studies carried out on the behaviour of actinians in relation to light date back to 1901, where direct observation in aquaria of species such as *Edwardsia, Cerianthus, Cladactis* and *Paractis* led to evidence of the contraction and retraction of individuals in bright light and expansion and extension as the intensity decreased (Hargitt, 1907). In fact, another common photoprotection mechanism in several anemones is the retraction of the tentacles and contraction of the oral disc. The extension and retraction of anemones tentacles is determined by various factors, such as light, currents and availability of prey. Focusing on light, this behaviour can modulate in different

symbionts anemones the amount of light to which the algae are exposed. The extension of the tentacles in exposes the algae to light favouring photosynthesis, while retraction allows shading and protection from high light intensity and UV radiation.

During intervals of high irradiance, the anemone *A. elegantissima* retracts its tentacles and contracts the sphincter that narrows the oral disc, thus shading the symbionts and reducing oxygen production (Dykens *et al.*, 1984), while under moderate light intensity it extends them (Pearse, 1974). Specimens of *A. ballii* from surface waters show tentacles retraction that does not occur in conspecifics from deeper waters (12m), confirming a possible defense strategy in reducing the damaging effect of high light intensity and UV radiation (Bell *et al.*, 2006).

Similarly, the anemone *Exaiptasia pallida* retracts its tentacles when exposed to high-intensity light and moves away from the light source (Kishimoto *et al.*, 2023).

In addition to these strategies just described, *A. elegantissima* attaches gravel, calcareous debris and pieces of macroalgae to its column, which act as a protective layer reducing the direct exposure of the animal itself and the symbiotic algae to light. In fact, specimens of *A. elegantissima* kept in UV-protected tanks attach less debris to their bodies and the removal of these causes the zooxanthellae to be expelled (Shah Pinal *et al.*, 2015). Therefore, there is a possible correlation between this attitude and light intensity.

Mechanical photoprotection

Mechanical photoprotection refers to the role of sea anemones tissues in relation to light.

Actiniaria cnidarians are characterized by the absence of a skeleton and the presence of three body layers: the external ectoderm (epidermis), the intermedia mesoglea and the internal endoderm (gastrodermis) (figure 1).

The ectoderm forms a thin monostratified epithelium, made up of different cell types in terms of structure and function, including tectorial, glandular, sensory and nervous cells. Tectorial cells are those in direct contact with the external environment and supported by a basal lamina, made up of glycoproteins, proteoglycans and collagen. Mesoglea is a viscoelastic material of collagen fibers and glycoprotein polymers that separates the epidermis from the gastrodermis (Dimond *et al.*, 2012). The gastrodermis is the internal layer that covers the gastrointestinal cavity and is made up mostly by myoepithelial and glandular cells (Chapman, 1974).

There are several studies that attribute the role of photoprotection towards the symbionts to the anemone tissues, demonstrating the greater sensitivity to light of the isolated symbionts compared to the symbionts in the anemone and therefore an attenuation of light through the anemone tissues.

Dimond *et al.* (2012) demonstrated that light stress in an algal symbiont, measured as the variation in the photosynthetic efficiency, can vary depending on the thickness of the host's tissues, taking into consideration two species of anemones of the genus *Anthopleura* (*A. elegantissima* and *A. xanthogrammica*) and investigating the relationship between tissue thickness and light attenuation. Epidermis, mesoglea and

gastrodermis were thicker in *A. xanthogrammica* compared to *A. elegantissima*, and light attenuation by the epidermis and mesoglea was 1.6 times better in *A. xanthogrammica* compared to *A. elegantissima*, confirming the possible mechanical photoprotective role of tissues towards symbiotic algae. Specifically, among the different layers, the mesoglea showed the greatest difference in thickness between the two anemones (62%) and the epidermis the smallest (29%). Dimond *et al.* suggest that the gastrodermis may also participate in the photoprotective role, shading subsequent layers of symbiotic algae in the gastrodermis cells. Currently, there are no further studies investigating the role of anemone tissue thickness in the photoprotection of symbiotic algae, but it remains an aspect to take into consideration.

However, many studies have been conducted on the optical properties of corals. The optical properties of a host determine how light travels between the components that constitute the host and the symbionts and therefore how the light reaches the algae, influencing photosynthetic activity and everything that follows. In particular, studies have been conducted on the optical properties of the coral skeleton, while there is still little information on those of the tissues (Wangpraseurt *et al.*, 2016). Since Scleractinia corals are also characterized by epidermis, mesoglea and gastrodermis, followed by the calcareous skeleton, it can be hypothesized that the behavior of light incident on the coral is similar to that on the anemone, with the exception of the skeletal component absent in this last. In thick tissue corals light gradients are generated from the tissue surface, where light levels reach >200% of incident light, to the tissue-skeleton interface, where light levels are reduced to 10%, demonstrating the role of tissue in attenuating the light intensity reaching the symbionts. However, in corals with thin

tissues the scattering property of the skeleton is of fundamental importance (Ferreira *et al.*, 2023). Studies on these corals have until now hypothesized that the scattering is negligible and that the refractive index of the tissues is similar to that of water, consequently the light is absorbed by the tissues and then reaches the skeleton (Wangpraseurt *et al.*, 2016).

By relating this information to the characteristics of the anemones, it can be hypothesized that the light passes through the ectoderm, mesoglea and endoderm, and is attenuated by them depending on their respective thicknesses, then reaching the algae present in the endoderm.

The optical properties of tissues currently remain a concept to be explored further, as it would allow us to understand how light propagates in the organism and how much light reaches the symbionts, influencing photosynthetic activity.

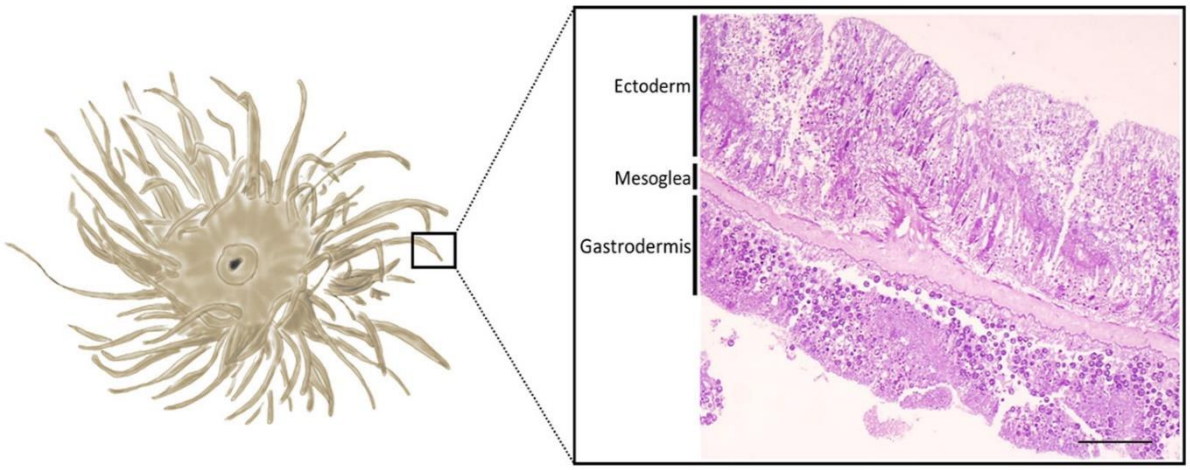

***Figure 1**: Illustration of the epithelial layers of Anemonia viridis tentacles (scale bar: 100 µm) (La Corte et al., 2023)*

Molecular photoprotection

High concentration of dissolved oxygen leads to the production of ROS. In normal condition the resulting damages are prevented by the molecular photoprotective mechanisms of the host and the symbiont.

Molecular photoprotection mechanisms adopted by both host and symbiont against stress conditions are the production of antioxidant enzymes and the mycosporine-like amino acids (MAAs).

Antioxidant enzymes are enzymes able to react and neutralize ROS. Among them, there are superoxide dismutases (SOD), catalases (CAT) and glutathione peroxidase (GPX).

SOD is a class of antioxidant enzymes that convert superoxide anion ($\cdot O_2^-$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2); catalases is the enzyme catalyzing the conversion of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) and therefore contributing to the detoxification of ROS; GPX transforms hydrogen peroxide into water using oxidizing glutathione (Lesser, 2006).

SOD and catalase activities increase with increasing irradiance, both with and without UV (Lesser, 1989) and act both for visible sunlight and UV radiation (Dykens & Shick, 1984).

Symbionts exposed to UV in culture and in the host showed a 30-40% increase in SOD activities compared to those exposed to similar radiation without UV. Furthermore, SOD and catalase activities doubled in zooxanthellae exposed in culture compared to those exposed in the host, which suggests a protective role of the host towards the symbionts compared to light (Shick, 2012).

A study conducted on specimens of *Anthopleura elegantissima* highlighted that SOD and catalase activity reflect the distribution of chlorophyll: greater chlorophyll and consequently greater oxygen production were found in the tentacles and oral disc, which also contained greater activity of protective enzymes, SOD and catalase (Dykens & Shick, 1984).

Three families of the SOD enzyme have been identified, based on the metals they contain: copper and zinc (CuZnSOD), manganese (MnSOD) and iron (FeSOD). Symbiotic algae possess all three families, while hosts only CuZnSOD and MnSOD (Lesser, 2006).

Another important photoprotective response of both the host and the symbiont is the presence of mycosporine-like amino acids (MAAs), ultraviolet absorbing compounds (λmax= 309-360 nm) which act as a filter against harmful wavelengths. These compounds are widespread in a wide variety of organisms, such as fungi, microalgae and invertebrates, including anemones (Arbeloa *et al.*, 2010).

Thanks to their chemical characteristics, in particular their absorption spectra and their high molar absorptivity, they have the important ability to efficiently absorb most wavelengths of UV light, providing a protective role from the harmful effects of UV radiation. Light, temperature, salinity and nutrients are some of the environmental factors that influence the concentration of MAAs, but exposure to UV rays has been seen as the main driver (Geraldes & Pinto, 2002). The concentration of MAAs is directly related to the level of UV exposure (Shick *et al.*, 2002). An increase in the concentration of MAAs in zooxanthellae was seen in specimens of the zooxanthellate anemone

Phyllodiscus semoni acclimated to natural levels of UV light compared to zooxanthellae of conspecifics sheltered from UV light (Shick, 2012).

Sea anemone *A. artemisia*, which lives in sheltered microhabitats, as well as *A. sola* and *A. xanthogrammica*, low intertidal species subjected to low solar irradiance, present lower MAAs concentrations than *A. elegantissima*, typically exposed to direct sunlight and characterized by high concentrations of MAAs (Shick *et al.*, 2002). The same situation occurs in the anemones *A. chilensis* and *O. mucosa*, present in sheltered habitats less exposed to sunlight and characterized by low levels of MAAs, while *A. marplatensis*, exposed to direct sunlight, shows a high concentration of MAAs (Arbeloa *et al.*, 2010). MAAs are present both in the host and in the symbiont, and since non symbiotic species also present these compounds it demonstrates that the symbiosis with algae is not a necessary factor for the presence of MAAs in the anemone. The presence of MAAs in the host is therefore not linked to the presence of zooxanthellae (Stochaj *et al.*, 1994). Studies on different species of organisms have reported the presence of different types of MAAs, both in the host and in the symbionts. Among the various MAAs, mycosporine-glycine is the most frequent in different species of cnidarians and it has not just the protective role against UV, but also it acts as antioxidant (Banaszak *et al.*, 2006; Geraldes & Pinto, 2021). There does not appear to be a marked relationship between the number, identity and concentration of MAAs in the symbiont and in the host (Banaszak *et al.*, 2006), but the finding of the same MAAs in symbiont and in the animal tissue from which they were isolated suggests a possible exchange of these compounds between host and symbionts (Stochaj *et al.*, 1994). MAAs can be acquired through the diet or the symbionts (Geraldes & Pinto, 2021). If the host has a lower

concentration of MAAs than the symbiont, it's possible that not all the MAAs produced by the symbiont are translocated to the host; if the host has a higher concentration of MAAs than the symbiont, it's possible that the host acquires them through the diet and doesn't transfer them to the symbiont (Banaszak *et al.*, 2006).

In conclusion, the correlation between exposure to UV radiation and the concentration of MAAs and the presence of MAAs in both the host and the symbiont highlight the importance of these compounds in the attenuation of harmful light energy.

Other host components playing an important photoprotective role are the Green Fluorescent Proteins (GFPs), proteins present in the epithelium of many anthozoans, including anemones (Smith, 2012), and capable of absorbing potentially harmful high energy photons of light and emitting lower energy green light when excited (Govenar, 2021). These proteins have a dual function: they can act both as photoprotectors and as photoenhancers. In high light intensity condition, GFPs act as a screen blocking part of the light through absorption and thus exposing the algae to lower light intensity, preventing them from being photodegraded. This function is therefore useful in shallow water environment, where photoinhibition is more likely to occur. On the contrary, in low light intensity condition GFPs are able to increase photosynthetic efficiency, favouring organisms living in more sheltered environment (Kyle-Henney, 2021). In fact, exposing the anemone *Anthopleura sola* to different levels of light intensity, the lowest one, corresponding to 22% of the environmental light intensity, led to an increase in the concentration of GFPs, confirming the potential role of photoenhancement in the individual (Govenar, 2021).

Photoprotective mechanisms adopted by symbiont algae occur through the presence of carotenoid pigments and the regulation of the chlorophylls content.

Carotenoids are pigments that not only have a role in capturing light energy, but also in quenching excess energy due to high light intensity. When the absorption of light by microalgae exceeds the photosynthetic capacity, the dissipation of excess energy is necessary to avoid damage to the photosynthetic systems and therefore photoinhibition (Simkin *et al.*, 2022). In fact, excess energy can be transferred to the oxygen present in the chloroplasts, generating ROS and consequent cellular damage. The production of carotenoids increases in corals subjected to high levels of solar radiations (Ambarsari *et al.* 1997).

The regulation of the chlorophyll content is another photoprotective mechanism modulated by solar and UV radiations. The amount of this pigment for algae is inversely proportional to irradiance, so greater irradiance leads to a decrease in the chlorophyll content with consequent reduction in photosynthetic activity and oxygen production. Furthermore, the addition of UV radiation causes a decrease in the chlorophyll content, both at low and high irradiance (Lesser, 1989).

The holobiont has also the capacity to regulate the number of symbiont algae according to light intensity. In shallow waters and in laboratory condition it has been seen how individuals subjected to high light intensity exhibit downregulation of symbiont density, reducing division rate and so photosynthesis and oxygen production as a defense response (Titlyanovl *et al.*, 1999; Leutenegger *et al.*, 2007; Kyle-Henney, 2021).

Anemonia viridis

The temperate sea anemone *Anemonia viridis* (Forskål, 1775) (figure 2) is an anthozoan cnidarian of the Actiniidae family belonging to the genus *Anemonia* (Risso, 1826); it is widespread in the Mediterranean Sea and in the eastern Atlantic Ocean. Its population is often dense and can form aggregations covering the shallow rocky bottoms of the infralittoral zone (Chintiroglou & Koukouras, 1992).

A. viridis lives in association with the symbiotic dinoflagellates of the Symbiodiniaceae family from which it receives oxygen and photosynthetic products for respiration and nutrition. These dinoflagellates are unicellular algae and are commonly called zooxanthellae. They are localized in the host's gastrodermis, inside vacuoles called symbiosomes (Stambler, 2011). This symbiotic relationship limits zooxanthellate organisms to the water surface layers and makes light the driver of their distribution. In addition to the photosynthetic compounds produced by zooxanthellae, anemones are active predators and feed on organisms, such as small crustaceans and invertebrates, thanks to the movements of the tentacles and column and feed on particulates, thanks to suspension feeding. They can absorb dissolved organic material, such as amino acids and glucose, from their environment (Harland, 1992).

A. viridis shows five color morphs, depending on the pigment content, of which the *rustica*, *smaragdina* and *rufescens* variants are the most frequent (Porro *et al.*, 2020).

As Anthozoa has no medusoid stage and reproduces both sexually, through the fertilization of oocytes and sperm internally to the gastrovascular cavity, and asexually, through the formation of a new individual from the body of the adult organism (Utrilla *et al.*, 2019).

A. viridis is considered a model species to study the Cnidarian-Symbiodiniaceae relationship and climate change (Arossa *et al.*, 2021) and it adopts complex behaviour in response to

abiotic variations as a mechanism to maintain symbiotic balance in variable environmental condition (Kyle-Henney, 2021).

***Figure 2**: Anemonia viridis from the Adriatic Sea*

Photoprotection in *A. viridis*

Light plays a decisive role in Actiniaria symbionts which, to maintain a correct balance between host and symbionts, adopt mechanisms that allow them to survive even in light stress and non-optimal conditions.

A study conducted in England in 2021 demonstrates that *A. viridis* exhibits phototaxis in response to different light intensities, in order to achieve an ideal environment and optimize photosynthetic efficiency. In particular, it has been observed that phototaxic behaviour is stimulated more in individuals acclimated to low light intensity compared to those acclimated to high light intensity. However, the former show positive phototaxis, the latter negative phototaxis, thus moving away from the light source (Henney, 2021).

Bell *et al.* (2006) from the direct observation of nine species of temperate anemones, including *A. viridis*, compared the diurnal and nocturnal behaviour of expansion and contraction of tentacles and polyps. Among these anemones, the only symbiont containing species besides *A. viridis* was *Anthopleura ballii,* living in shallow waters. While *A. ballii* retracted its tentacles during the hottest hours, *A. viridis* extended them towards the light source. In fact, almost 100% of the specimens observed from 12.00pm to 16.00pm was active and extended, while at sunset and during the night hours, when the light intensity was lower or absent, they were inactive and collapsed (Bell *et al.*, 2006). The collapse response of the polyp during hours of low light may be associated both with a lower probability of being preyed and with an energy conservation mechanism during the hours in which photosynthesis of the zooxanthellae is not possible due to the darkness. The extension and expansion of the tentacles and polyps during the most intense light hours suggest instead a tolerance to high light intensity of *A. viridis* specimens. In fact, *A. viridis* does not seem to adopt the tentacle retraction mechanism (Bell *et al.*, 2006) and their extension during the hottest hours of sunlight may suggest that the tentacles are exposed to capture all the light to protect the column and the components inside, such as mesenteries and eggs.

However, few studies have been conducted on the responses of *A. viridis* and the *Anemonia* genus to light intensity, and consequently more investigations are needed to have a clearer vision both on phototaxis and on the behaviour of extension/retraction of the tentacles and expansion/contraction of the polyp.

Information on the mechanical role of tissues is limited. *A. viridis* is composed of three layers: external epidermis, internal gastrodermis and between these the intermediate mesoglea. Being thin layers it is assumed that the light is absorbed by them and reaches the zooxanthellae present in the gastrodermis. How the thickness of the respective layers affects the absorption

of light has not yet been studied, probably the greater the thickness, the greater the attenuated light, and vice versa. If in specimens of Scleractinia it is the skeleton that has the property of harvesting and scattering light, this component is missing in *Anemonia*. The great thickness of *A. viridis* column may suggest that it can somehow compensate for the absence of the skeleton. It's therefore assumed that tissues have a light screening function.

The chemical photoprotection mechanisms seen in sea anemones have also been seen in *Anemonia* spp. and therefore in *A. viridis*, on which there are studies that demonstrate the presence of SOD and catalase activity, MAAs, GFPs, mechanisms regulating the number of zooxanthellae and carotenoid and chlorophyll contents, but do not deep the effects of light on these chemical defenses in *Anemonia viridis* or *Anemonia* spp.

Superoxide desmutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) have been identified as antioxidant enzymatic defenses in *A. viridis* (Hawkridge *et al.*, 2000). The study conducted by Furla et al (2011) on *A. viridis* showed the presence of all three SOD families (FeSOD, MnSOD and CuZnSOD) and seven isoforms, present both in the endoderm and ectoderm of the host, and in zooxanthellae, with the exception of CuZnSOD isoforms, present only in the host (Furla et al, 2011). According to the studies by Richier *et al.* (2003) on the tolerance of *A. viridis* to conditions of anoxia and hyperoxia to which it is daily exposed, SOD activity is lower in the ectoderm and higher in the endoderm and in zooxanthellae; furthermore CuZnSOD appears to be the most abundant SOD family (Richier *et al.*, 2003). Hawkridge *et al.* (1996) investigated the localization of antioxidant enzymes in specimens of *A. viridis* and confirmed a greater abundance of CuZnSOD, both in the host and in zooxhantellae, which, together with MnSOD, was localized in cnidia and algae symbiotic, as well as the catalase and GPX enzymes.

Chiplunkar (2014) instead demonstrated that individuals of *A. viridis* exposed gradually to increasing temperatures maintained the symbiotic relationship and contained an increase in the activity of the catalase enzyme, unlike individuals subjected to a non-gradual increase in temperature, in which bleaching events have occurred. This suggests the potential role of catalase to thermal stress. Furthermore, Merle *et al.* (2006) found a lower catalase activity in zooxanthellae compared to the host and a greater concentration in the ectodermal layer. So, catalase activity is lower in the endoderm and in zooxanthellae, where SODs are more active. This distribution has not yet been explained, highlighting the need for more studies related to catalase characterization in this species (Merle *et al.*, 2007). However the high diversity of SOD present both in the host and in the symbionts and the importance of catalase enzyme suggest a high defense of *A. viridis* against the oxidative stress to which it is subjected.

MAAs compounds have been found in tissues of *Anemonia* spp. and *A. viridis* (Nahon *et al.*, 2012; Leutenegger *et al.*, 2007) acting as both photoprotectors and antioxidants.

Kyle-Hennel (2021) demonstrated the presence of GFPs in *A. viridis* confirming the photoprotective role against high intensity light and UV radiation but suggesting the need for more insights into the primary role of GFPs in temperate environments.

Finally, *Anemonia* spp. have demonstrated regulation mechanisms of symbionts density in response to light variation and the consequent increase in the concentration of carotenoid pigments and decrease in the concentration of chlorophyll in conditions of greater light (Fox & Pantin, 1944).

Socioeconomical interest

Anemonia viridis potential lies in the nutritional, cosmetical, pharmaceutical and medical fields making it a promising commercial interest species.

Edible food interest

Anemonia viridis is an edible species, whose consumption is limited in some regions of Spain, Greece and Italy (Thorpe *et al.*, 2000; Del Pazo *et al.*, 2014). In Italy it's mainly eaten in Sardinia (Pais *et al.*, 2008). It is well known that marine invertebrates are an excellent source of high quality proteins, polyunsaturated fatty acids, vitamins and minerals, representing an important resource for human nutrition: Gonzàlez *et al*. (2001) investigated the nutritional value of *A. viridis* and confirmed that the anemone is a resource rich in proteins and lipids. *A. viridis* contains a high amount of polyunsaturated fatty acids, whose functions are fundamental for human health. Among these functions there is the containing of cholesterol levels. Studying the effect of a diet based on *A. viridis* on rats, a decrease in total cholesterol was observed in rats feeding on *A. viridis* compared to control groups, likely to the high concentration of polyunsaturated fatty acids. Furthermore, comparing the water, protein and lipid content between fresh *A. viridis* and fried *A. viridis*, a higher lipid and protein content and a lower water content were observed in fried *A. viridis*. Among the amino acids analyzed in this study, *A. viridis* appears to have a high concentration of glycine, arginine and cysteine, important amino acids for example for the synthesis of proteins, the transmission of nervous impulses, the organism growth and defense.

A. viridis can therefore be considered a food resource with an excellent nutritional profile for humans and with a role in reducing total cholesterol levels, which needs to be explored further.

Pharmaceutical and nutraceutical interest

Marine organisms contain several bioactive compounds used in antimicrobial, anticancer and antitumoral drug discovery research (Thangaraj *et al.*, 2019). In recent years, attention has also been placed on sea anemones, which include a vast range of compounds responsible for neurotoxic, cytolytic, hemolytic, analgesic, anti-inflammatory, antimicrobial, anti-hyperglycemic and antidiabetic activities. In fact, they are an important source of active ingredients with low molecular weight (<5000 Da) (Jain & Tailor, 2020). Anemones venom is made up of peptides and small proteins (Loret *et al.*, 2021; Menezes *et al.*, 2022), which act as neurotoxins and cytotoxins and can therefore be used to develop new drugs against various disorders. Neurotoxins can have analgesic and anesthetic effects, as they block potassium channels facilitating the release of acetylcholine and can also be used in the treatment of autoimmune diseases; cytotoxins, on the other hand, kill cells and therefore have antimicrobial potential and can be used in the treatment of cancer.

Among the order Actiniaria, the family Actiniidae appears to include the largest number of species that produce compounds that benefit human health (Thangaraj *et al.*, 2019).

Anemonia viridis has been demonstrated to be a source of low molecular weight proteins of pharmaceutical interest (Loret *et al.* 2021). This species was used in therapeutic and medical practices already during ancient Greece and the Byzantine period. Ancient texts report that it was used in the form of broth for laxative uses, cooked meat for diuretic purposes and to relieve swelling and abdominal pain, and that it could be applied to the skin for depilatory uses (Voultsiadou, 2010).

Recent studies on the bioactive compounds of *A. viridis* have revealed the contribution of this species in several medical fields. Among the seven proteins that bind to ion channels that have

been characterized in *A. viridis*, there is the blood depressing protein BDS 1 which is of great importance, as it has anti-hypertensive properties and is therefore used to treat heart disease (Loret *et al.*, 2021).

A. viridis extracts show antiproliferative effects on cancer cells, capable of altering the growth of certain tumor cell lines (Bulati *et al.*, 2016), antioxidant enzymes activity (Thangaraj *et al.*, 2019) and antiangiogenic activity (Loret *et al.*, 2021).

Choresh *et al.* (2001) identified for the first time the Heat Shock Protein 60 (HSP60) in *Anemonia viridis*, the synthesis of which represents a defense mechanism of the organism from damage at the protein level due to numerous stressors such as high temperatures, oxidative stress, exposure to UV radiation (Coresh *et al.*, 2001). HSP60 is also involved in numerous biological processes, such as inflammation, apoptosis, pathogen infections, neurodegeneration and cancer (Singh *et al.*, 2024). Research is currently investing energy to exploit HSP60 as a biomarker for diagnostic and prognostic purposes for various conditions, including cancer, inflammatory disorders and neurodegenerative diseases (Singh *et al.*, 2024) (Tang *et al.*, 2022).

Another interesting aspect is the presence of the peptide toxin Av3 in the venom of *A. viridis*. This toxin has been shown to be selective and active on crustaceans and inactive on mammals. It has been seen that Av3 also has a strong effect on insects, probably due to the phylogenetic relationship present between crustaceans and insects, and due to this property the toxin can be considered a compound for the synthesis of selective anti-insect molecules, i.e. insecticides (Bosmans & Tytgat, 2007; Moran *et al.*, 2007; Trapani *et al.*, 2014; Zhu *et al.*, 2021).

Furthermore, further studies on *Anemonia sulcata* have demonstrated anti-inflammatory and Alzheimer's disease prevention activities thanks to the neuroprotective role of the BDS 1 toxin (Peña *et al.*, 2023).

Cosmetics interest

The marine environment has been considered a promising source of cosmetic ingredients. It is currently a field that is still little explored and only a small percentage of the potential of marine compounds has been investigated, but interest in their use as cosmetics is continually growing (Fonseca *et al.*, 2023). Some of the properties researched in a cosmetic can be distinguished into antiaging, photoprotective and moisturizer properties. The skin is influenced by intrinsic factors, such as genetics, ethnicity and sex, and extrinsic factors, such as UV radiation, smoking, pollution, to which it is exposed on a daily basis. All these agents lead to oxidative stress which is responsible for skin aging. To counteract this stress, it is possible to add compounds that act as antioxidants into cosmetics (Fonseca *et al.* 2023).

The antioxidant properties of carotenoids are well known and make them an ideal potential antioxidant and antiaging cosmetic ingredient. They are currently still little marketed, as their absorption by the skin is yet to be defined, so further studies are needed to understand their potential in the cosmetics field (Fonseca *et al.* 2023; Alparslan *et al.* 2018).

Photoprotection is another increasingly requested feature in cosmetics, probably due to the greater awareness of the damage caused by solar radiation on the skin. MAAs, acting as protectors against UV, represent a potential ingredient for sunscreens and cosmetics and their low molecular weight together with the fact that they are water soluble and stable molecules when exposed to light and heat make them more suitable (Alparslan *et al.* 2018).

Hydration is another widely required and important factor for skin care. Collagen is an excellent hydrating and humectant ingredient for the formulation of cosmetics. It is one of the proteins most present in the extracellular matrix of the animal body and marine invertebrates constitute its most abundant and promising source (Rahman, 2019). Collagen has hydrating

benefits as it cannot be absorbed by the stratum corneum of the skin, so it remains on the surface and binds water, thus hydrating the skin and acting as a humectant (Prajaputra *et al.*, 2024). Furthermore, according to Chattopadhyay and Raines (2014), it also protects the skin from possible microbes in cases of wounded tissue (Chattopadhyay & Raines, 2014).

The phylum Cnidaria is still little studied in this field. Fonseca *et al.* (2023) carried out an extensive bibliographic search on marine bioactive compounds that have been applied in cosmetics so far, distinguishing the compounds analyzed, their bioactivity and the phylum to which they belong. From this research it appears that currently from the phylum Cnidaria only collagen has been applied in cosmetics, which has been shown to improve skin regeneration in wounds, acting as wound healing, to reduce skin pigmentation, acting as whitening, and to modulate oxidative stress induced by UV rays, acting as an antioxidant, anti-aging and photo-aging (Fonseca *et al.* 2023; Prajaputra *et al.* 2024). We know that in addition to collagen, Actiniaria and more precisely *Anemonia* spp. also contain MAAs (Nahon *et al.*, 2012; Leutenegger, 2007), carotenoids (Czeczuga, 1972) and alkaloids (Loret *et al.*, 2018).

MAAs have been applied in cosmetics for a photoprotective, anti-aging, antioxidant and anti-inflammatory role; more precisely, mycosporine-glycine, considered the most abundant form of MAAs in several cnidarians (Banaszak *et al.* 2006), has been shown to be responsible for the reduction of oxidative stress induced by UV radiation in vitro, for the healing of wounds in keratinocytes and of the modulation of age and inflammation related genes in vitro. Carotenoids and more precisely beta-carotene have been used for an antiaging, antioxidant and photoprotective function, while astaxanthin as a strong antioxidant, photoaging and whitening. Finally, alkaloids such as antioxidants and photoprotectors, present in sunscreens, facial moisturizers and creams.

Consequently, although studies are needed to investigate the affinity of the bioactive compounds of *Anemonia* spp. and *A. viridis*, *A. viridis* could also have great potential in the field of cosmetics.

Biomedical engineering interest

Collagen is the main structural component of the extracellular matrix of invertebrate and vertebrate tissues. Thanks to its properties it is of interest to humans in the pharmaceutical, nutraceutical, cosmetic and biomedical fields. In recent years, attention has shifted to the marine environment, where collagen can be extracted from marine vertebrates, algae and marine invertebrates, including sea anemones (Lim *et al.*, 2019;Rigogliuso *et al.*, 2023).

The characteristics that make marine collagen an ideal candidate for biomedical engineering are its biocompatibility, biodegradability, easy extractability, water solubility, safety, low production costs and low immunogenicity, which decreases the probability of rejection within a different body (Lim *et al.* 2019; Coppola *et al.*, 2020; Rigogliuso *et al.*, 2023). In fact, marine collagen is used for the engineering and regeneration of bone, cartilaginous, skin, vascular, dental and corneal tissues and for wound healing, wound dressing and skin repair (Lim *et al.*, 2019; Coppola *et al.*, 2020; Rigogliuso *et al.*, 2023; Imran & Sapuan, 2023; Barzkar *et al.*, 2024).

Currently the marine collagen used derives mainly from fish, sharks, jellyfish, starfish, molluscs, sponges and there seems to be no specific studies on the use of collagen derived from specimens of the Actiniaria order in this field, but it is well known that they have different types of collagen (Parisi *et al.*, 2021) and consequently they can also represent an important source of collagen that can be used in biomedical engineering.

Issue

Despite the numerous potential uses of *Anemonia viridis*, human interest is currently mostly focused on its being an edible source. Its consumption is not yet widespread, but in the areas in which *A. viridis* is normally considered as a food source it is harvested in large quantities. This may alter sea anemone populations reducing individual number and putting them at risk in the future. In addition, sea anemone population recovery after overharvesting can be very slow (Frisch *et al.*, 2019). To address these situations, repopulation actions, harvesting normative and guidelines may be necessary. Another possible solution could be the aquaculture conservation, with the aim to refill the harvested population or to develop sustainable farm of this species.

SYMBIODINIACEAE

The term symbiosis refers to the association established between individuals of different species which may be closely related or distantly related taxa. The latter is the case of dinoflagellates and cnidarians, which form a mutualistic relationship through which both species benefit (Ravindran *et al.*, 2022). The host provides the microalgae a safe environment from predators and optimal for growth, necessary nutrients such as nitrogen, phosphorus and sulphate, and CO_2; dinoflagellates transfer photosynthetic products to the host, such as organic carbon in various forms, like glycerol, fatty acids and glucose, important in structural, metabolic, reproductive, reserve terms (Goffredo & Dubinsky, 2016). Furthermore, they also contribute to the host's cellular respiration, producing oxygen (Ravindran *et al.*, 2022).

The dinoflagellates involved in this dinoflagellate-cnidaria relationship belong to the family Symbiodiniaceae, and are commonly called zooxanthellae (Howe, 2013). They are photosynthetic unicellular marine algae which in symbiosis are localized in endodermal cells, within host cell vacuoles (González-Pech *et al.*, 2024) and can be acquired from the host via vertical transmission, from the parents to the embryo, or horizontally, from the surrounding environment (Lewis *et al.*, 2024). Dinoflagellates proliferate by mitosis inside the host and are present in extremely high densities, which however can vary depending on external environmental abiotic factors, such as temperature, salinity, solar radiation. In fact, conditions of prolonged stress or extreme stress can lead to the breakdown of the symbiotic relationship with consequent loss of zooxanthellae from the host cells. This can occur via in *situ* degradation of the symbionts, exocytosis, detachment, apoptosis or necrosis of the host cell (Howe, 2013)(Figure 3). The causes leading to the loss of zooxanthellae are related to environmental perturbations, like lowered salinity, extremely high or low water temperature,

UV irradiation, etc. (Palka, 2010). For example Steen and Muscatine (1987) demonstrated that brief exposure to low temperature (4°C for 4 hours) of the sea anemone *Aiptasia pulchella* lead to the exocytosis of 98% of symbionts, moved towards the apex of the host cells and released to the coelenteron; while Fujise *et al.* (2004) highlighted the expulsion of symbionts in response to moderate thermal stress (30°C) in Scleractinia corals *Acropora* spp. In addition, densities of symbionts and so the expulsion of them have been shown to variate also under normal and seasonal conditions (Palka, 2010). Fagoonee *et al.* (1998) conducted a 6 years monitoring of zooxanthellae populations dynamics within the coral *Acropora formosa* in Mauritius and demonstrated a seasonal abundance of zooxanthellae density and highlighted that in addition to temperature, solar radiation and other environmental fluctuations there may be other factors related to seasonal cycle that are significant. Fitt *et al.* (2000) established a 4 years monitoring of zooxanthellae density within five species of Caribbean reef corals and confirmed clear seasonal cycles of symbionts density and suggested a possible correlation with changes in host tissue biomass due to temperature and consequently respiratory metabolism variations.

The family Symbiodiniaceae initially included a single genus, *Symbiodinium* (Freudenthal 1962), later distinguished in nine lineages, referred to as Clades A-I (Howe, 2013), to which new lineages continue to be discovered and added. Recently there has been a systematic revision that split these clades into 15 genus-equivalent lineages, five of them lacking material for species designation and so are yet to be formally described (LaJeunesse *et al.*, 2018). Among these genus *Philozoon* is the one to which *Anemonia viridis* belongs.

Philozoon genus was formerly referred to in the literature as “temperate Clade A” and it is most closely related to *Symbiodinium*, previously referred to as “Clade A” (LaJeunesse *et al.*, 2022) considered its sister group (Nitschke *et al.*, 2022). This genus has been found in

Actiniaria, Octocorallia and Scleractinia orders and particularly associated with cnidaria hosts in coastal habitats. It is distributed in temperate environments in the northern and southern hemispheres, including the Mediterranean Sea (LaJeunesse *et al.*, 2022) (figure 4). Its distribution in temperate environments suggests an adaptation to cold and seasonably environments and so a greater tolerance to variability of temperature, oxygen, solar radiation and other abiotic factors.

LaJeunesse *et al.* (2022) collected *Anemonia viridis* individuals from different sites in Italy, Spain, France, Croatia, United Kingdom and so belonging to Western Mediterranean, Adriatic Sea and North Atlantic Ocean and identified *Philozoon actiniarum* as the *Philozoon* species harboured by *A. viridis*.

In Symbiodiniaceae the main light harvesting pigments are chlorophyll a, chlorophyll c_2 and peridinin (Nietschke *et al.*, 2022). When light is absorbed by the light harvesting complex present in the photosystems I and II, energy is passed from pigment to pigment until it reaches the reaction center (RC), where initiates the electron transfer process of photosynthesis. In Symbiodiniaceae chlorophyll a is located both in the photosynthetic reaction centers and in the light harvesting complexes and is considered the main collector of light energy between λ 400–450 nm and λ 650–700 nm transferred to the RC (Simkin *et al.* 2022). Chlorophyll c_2 is present in light harvesting complex and its absorption spectra reaches its maximum between λ 440-460 nm and λ 580-630 nm (Myśliwa-Kurdziel *et al.*, 2019; Büchel, 2020), while the carotenoid peridinin between λ 400-550 nm (Jiang *et al.*, 2012).

Accessory pigments in Symbiodiniaceae are mostly diadinoxanthin, diatoxanthin and β-carotene (Nietschke *et al.*, 2022). These three accessory pigments are carotenoids playing an important role both in absorbing and transferring energy to chlorophylls in the blue-green zone (λ 460-550 nm) and in quenching of excess energy (Simkin *et al.*, 2022). When light

intensity is too high the excess of energy is transferred from chlorophylls to carotenoids acting as photoprotector and protecting cells from damaging ROS (Consalvey *et al.*, 2005) (figure 5).

As light increases, the algal growth, photosynthesis, respiration and β-carotene increase, while chlorophyll a, chlorophyll c_2 and peridinin concentrations decrease (Stambler & Dubinsky, 2004). Furthermore, the pigment content is typically inversely related to cell size, due to the fact that the absorption efficiency decreases as cell volume increased and pigments become gradually self shading (Smith, 2012) (Nietschke *et al.*, 2022).

All these mechanisms adopted by both the host and the symbionts act together to maintain the symbiotic relationship and benefit both.

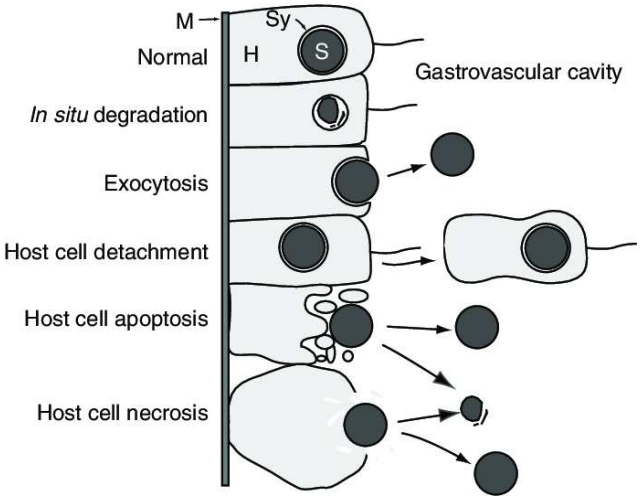

***Figure 3**: Mechanisms of zooxanthellae loss. (Weis, 2008)*

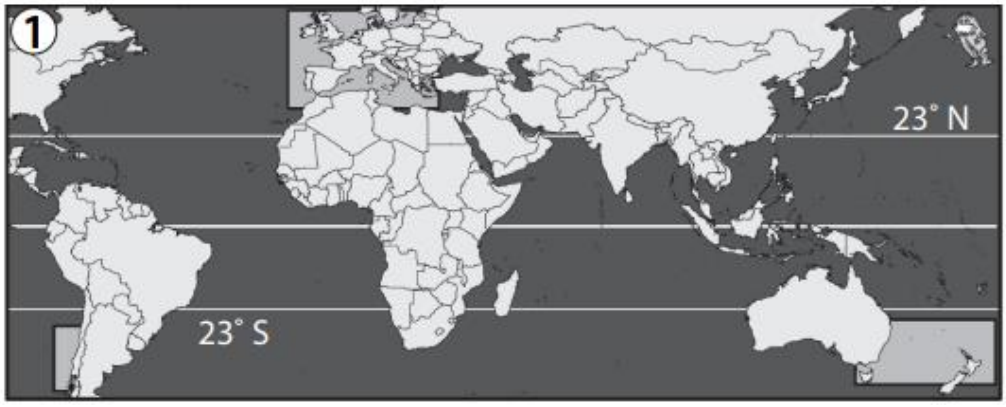

Figure 4*: The distribution of Philozoon in northern and southern temperate zones. (LaJeunesse et al., 2022)*

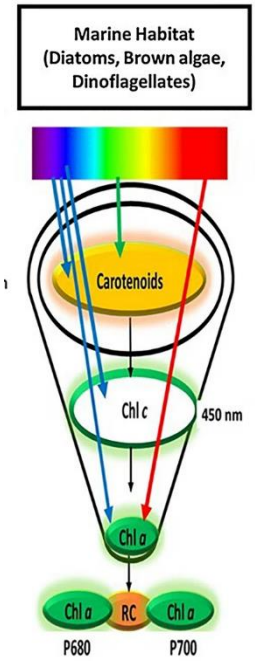

Figure 5*: Photosynthetic pigments in Symbiodiniaceae. (Simkin et al., 2022)*

OBJECTIVE OF THE STUDY

Considering the growing interest in novel food and bioactive compounds from marine organisms, this study aims to assess how spectrum quality and temperature regulate the relationship Cnidaria-Symbiodiniaceae in the model species *A. viridis*. In particular, the objective is to evaluate the responses of the holobiont to different light spectra and temperature values (table 1) and to find optimal conditions for rearing this species. Despite the global interest in the production and cultivation of seaweeds and aquatic plants for consumption and conservation purposes (Buschmann *et al.*, 2017), the knowledge on the effects related to the application of different spectrum composition on symbiotic corals seems to be scant. The evidence that horticultural plants may benefit from the application of light spectra beyond PAR (i.e. shorter or longer wavelengths), stimulates research in the effects of non-PAR light in marine plants and corals of conservation and/or commercial interest. Some studies reported the application of far red/infrared radiation in vegetable crops and demonstrated different results: some of them found out that this portion of light spectrum can promote plant growth and increase photochemical efficiency in tomato and lettuce yields (Lin *et al.*, 2013; Li *et al.*, 2023); others that according to the amount of red and far red light, it could promote the germination of lettuce seeds, the induction of flowering of the herbaceous plant *Hyoscyamus*, the expansion of *Sinapis*, but also the inhibition of flowering in *Xanthium*, of mesocotyl elongation in the shoots of grasses, lettuce and *Sinapis* (Carfagna *et al.*, 2021). Few studies focused on the effects in the utilization of PAR in the hydroponics field and mostly researched the optimal combination of white, red and blue light, able to modulate the growth and the edible quality of the reared species (Lin *et al.*, 2013; Talukder *et al.*, 2018;

Metallo *et al.*, 2018). Finally, other studies investigated the effects of different spectrum quality, from blue (430-480 nm) to red (660-700nm) and far red/infrared (735 nm) on Scleractinia corals (Hamley, 2016; Izumi *et al.*, 2023; Kinzie *et al.*, 1984; Squire, 2000; Wang *et al.*, 2008) with different results in relation to the considered species. Consequently, this is a pioneer study which aim to contribute to the knowledge on the effects of far red/infrared radiation on the marine invertebrate *A. viridis* and its symbionts dinoflagellates.

***Table 1**: Scheme of the Issues, Objectives, Research questions, Hypothesis and Methods of the study.*

Issues	**Objectives**	**Research questions**	**Hypothesis**	**N.**	**Methods**
Marine invertebrates may represent alternative sources of proteins/nutraceutical compounds for human use and consumption. They are subject to an increased interest by scientists thanks to their properties and potential benefits. This sometimes leads to the overexploitation of these organisms, reducing the population and making repopulation or breeding actions necessary. Studies to assess feasibility of rearing these organisms are still scant.	To assess the parameters that optimize the health of the sea anemone *A. viridis*, potentially used as a source of novel food, cosmetic and pharmaceutical products and in biomedical applications	How variations in the spectrum quality and in temperature affect *A. viridis* health status	*A. viridis* health status do not change as a function of two factors: factor "light spectrum" (two levels: PAR; PAR + IR) and factor "temperature" (two levels: 20°C; 23°C)	1	Sampling of *A. viridis* individuals
				2	Maintenance of individuals in controlled tanks, at different temperatures and light spectrum
				3	Periodical analyses on *A. viridis* individuals in terms of colour and behaviour, Chl a and Chl c_2 contents, photosynthetic capacity, Symbiodiniaceae concentration, mitotic index and exocytosis
	Contribute to understanding the relationship Cnidaria-Symbiodiniaceae using the model species *A. viridis*	How variations in the spectrum quality and in temperature affect symbionts concentration, pigments content (Chl a - Chl c_2), photosyntethic efficiency, mitotic index and exocytosis	Symbionts concentration, pigments content, photosynthetic capacity, mitotic index and exocytosis do not change as a function of two factors: factor "light spectrum" (two levels: PAR; PAR + IR) and factor "temperature" (two levels: 20°C; 23°C)	4	Comparison of data obtained from three different analyses periods, one month apart each

MATERIALS AND METHODS

Study site, sampling and aquarium system

The sampling area is located near the port of Pescara and sampling was made the 24th of March 2024. The area is characterized by low depth and moderate currents, and the individuals of *Anemonia viridis* were collected from shallow artificial rocks at a depth between 30 cm and 5 meters, transported in the laboratory through tanks filled with sea water and placed in aquaria the 26th of March 2024.

The aquarium system consisted of 8 tanks of 40 L each (42x31x35 cm) with a single filter composed by mechanical, chemical and biological filter and seawater was changed every two weeks. Specimens were regularly fed with frozen Artemia spp., in particular two Artemia per individual were given once a week. During the experiment two individuals reproduced asexually and to those new individuals only one Artemia was given in order to not create differences among individuals.

An irregular substratum was placed in each aquarium in order to create three height levels allowing specimens to place themselves in their preferable position.

The illumination system consisted of two different LEDs: the Cosmorrow LED, composed by light from 500 nm to 730 nm, with 33 % infrared light (730 nm) (figure 6) and the SilverMoon Marine LED, composed of PAR (57%) and 43% blue light (460 nm). Control samples were illuminated by SilverMoon Marine LED, henceforward “PAR”, while treatment samples by both illumination systems, henceforward “PAR+IR”.

Since the study aims to study the effect of different spectrum quality and different temperatures, two tanks were kept at 20°C and illuminated by PAR; two tanks were kept at

23°C and illuminated by PAR; two tanks were kept at 20°C and illuminated by PAR+IR; two tanks were kept at 23°C and illuminated by PAR+IR. The first four tanks represented the control samples named C20.1; C20.2; C23.1; C23.2 and the other four tanks represented the treatment samples named TIR20.1; TIR20.2; TIR23.1; TIR23.2 (figure 7).

Treatment samples were illuminated by infrared system for 10h a day.

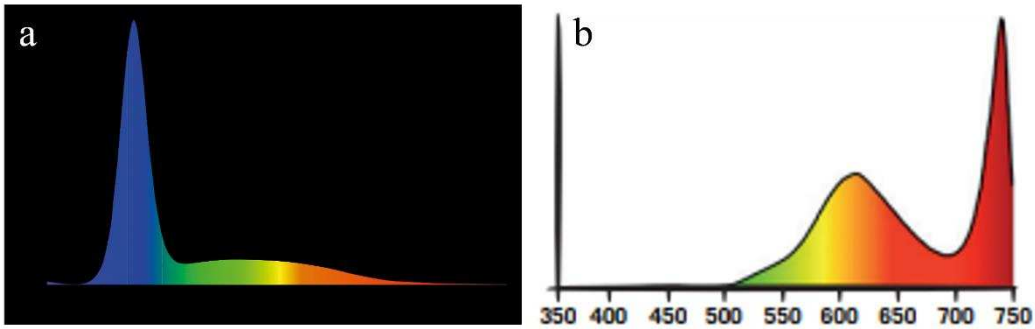

***Figure 6**: a) Light spectrum of SilverMoon Marine LED; b) Light spectrum of Cosmorrow LED.*

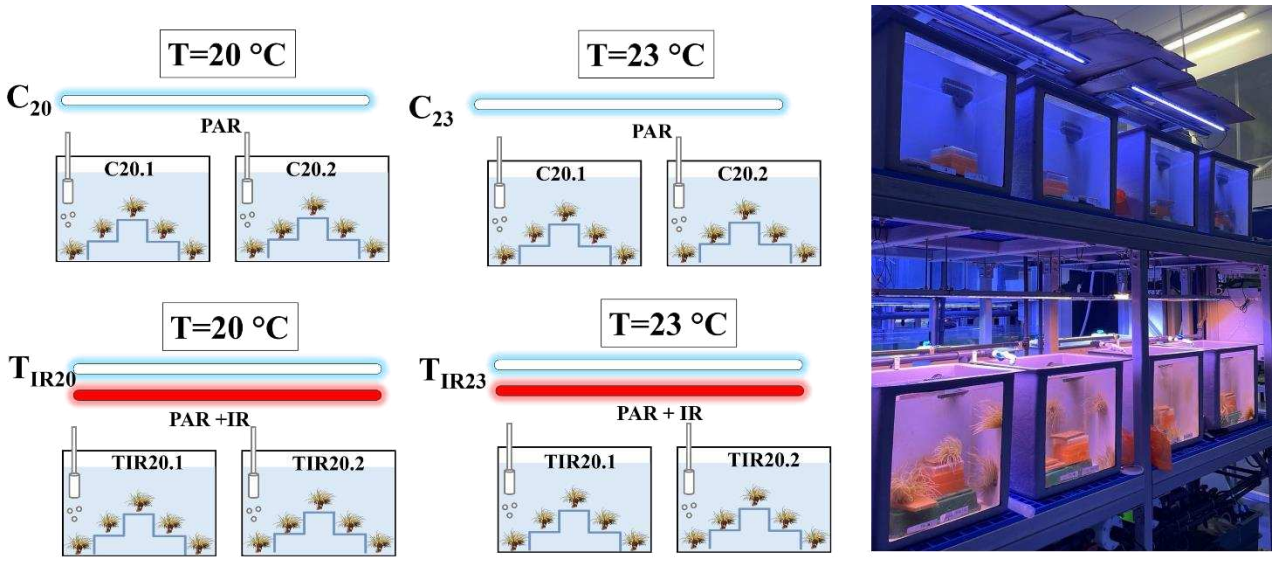

***Figure 7**: Aquarium system: the upper tanks are the control conditions, illuminated by PAR at 20°C and 23°C; the lower ones are the treatment conditions, illuminated by PAR+IR at 20°C and 23°C.*

Sampling design

Five *A. viridis* specimens were placed in each tank and acclimatized two weeks (from the 26th of March to the 8th of April) under PAR light at 20°C. In four of these tanks the temperature was gradually increased up to 23°C.

The dates of the analyses were the 9th of April (T1), the 6th of May (T2) and the 3rd of June (T3) 2024. The PAR+IR treatment started the 10th of April, so T1 refers to the initial condition pretreatment, T2 and T3 to one and two months respectively of IR treatment.

To each individual was given a number in order to identify them during the experiment period. The recognition of *A. viridis* individuals was possible by taking photographs frequently and thanks to the tendency of the specimens not to move significantly. In each analysis dates morphological, chlorophylls, Pulse Amplitude Modulation (PAM), histological analyses and zooxanthellae count were done.

Morphological analyses consisted of the monitoring of anemones colour as an indicator of its health status using the Coral Health Chart. These analyses were done to all individuals. PAM and chlorophylls analyses and zooxanthellae count were made to individual 2,3 and 4 and histological analysis to individual 2 (Table 2).

***Table 2**: Temporal sequence of the experiment design*

24/03/24	Collection
26/03/24	Placement in the aquarium
8/04/24	End of acclimatation
9/04/24 (T1)	Analyses
10/04/24	IR treatment beginning
6/05/24 (T2)	Analyses
3/06/24 (T3)	Analyses

Morphological analysis

Morphological analysis consisted in *A. viridis* health status evaluation among the two months experiment. In particular, the colour of all individuals was monitored according to the Coral Health Chart, which provide indication on the coral health using colour as an indicator of it (figure 8).

Other parameters used were the anemone's tendency to assume a collapsed or active shape, to have the tentacles extended or downwards, to be well attached to the substrates or detached from them and to place themselves in any particular position which may suggest a negative effect of far red/infrared illumination.

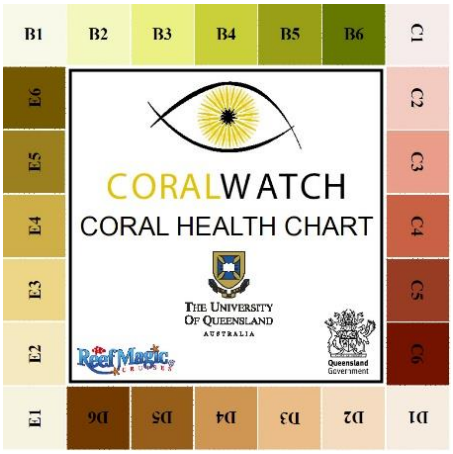

***Figure 8**: Coral Health Chart*

Zooxanthellae and histological analyses

The number of zooxanthellae was determined in T1, T2 and T3.

A tentacle from individuals 2,3,4 from each tank was taken with a biology tweezer, placed in a 1 ml Eppendorf and kept in dark at -20°C. At the moment of the count the tentacle was homogenized in the Eppendorf using a pestle with the addition of 1 ml of artificial seawater.

Then, the homogenate was centrifuged at 5000 *rpm* for 5 minutes, the supernatant removed, and the resulting pellet was resuspended in 1 ml of artificial seawater. Zooxanthellae were counted using an Improved Neubauer haemocytometer grid (bright line BRAND Tiefe Depth Profondeur 0.100 mm 0.0025) in a volume of 10 µl under a light microscope (Nikon model C-PS N). During the counting singlets, doublets and broken zooxanthellae were classified and reported. Doublets number represented the number of dividing cells and was used to calculate the mitotic index (%) through the formula:

$$mitotic\ index = \frac{number\ doublet\ cells}{total\ number\ of\ cells}\ x\ 100$$

while the concentration of zooxanthellae was calculated through the formula:

$$zooxanthellae\ concentration = \frac{total\ number\ of\ cells}{0{,}0001}$$

and then standardized to the average weight of a tentacle.

After the count 90% acetone was added to the Eppendorf for the subsequent chlorophylls analysis.

Histological analysis was carried out in T1, T2 and T3 and consisted of fixation, graded ethanol series and inclusion.

A tentacle was taken from individual 2 and placed in 5 ml Eppendorf filled with seawater, then replaced with glutaraldehyde 2,5% pH 7.8 for 24 hours. The graded ethanol series included:

- 50% ethanol for 6 hours;

- 70% ethanol for 24 hours;
- 80% ethanol for 24 hours;
- 90% ethanol for 24 hours;
- 95% ethanol for 24 hours;
- 100% ethanol for 24 hours;
- 100% ethanol + pure resin (Technovit 8100) for 24 hours;
- Pure resin (Technovit 8100) for 24 hours;
- Pure resin (Technovit 8100) for 24 hours.

Infiltration resin was prepared mixing 100 cc pure resin (Technovit 8100) + 1 bag of Hardner I in a becher with a magnet stirrer for 15 minutes. A polymerization mixture was created mixing 15 cc of infiltration resin + 0.5 cc of Hardner II in a backer with a magnet stirrer for 30 seconds -1 minute and a thin layer of it was poured into the forms of the Teflon mould. The samples were later placed inside the forms and the forms were filled with further mixture and then covered with cover films. The Teflon mould was kept in refrigerator for one night. Finally the glue was prepared mixing 9 ml of universal liquid and 18 cc of powder (Technovit 3040) in a glass jar and a thin layer of glue was poured into the forms of the Teflon mould after removing the cover films. A histoblock was putted on each form and further glue was added. After 12-24 hours it was possible to make sections of 7-9 μm using a microtome and place them on slides later stained with Toulidine blue and distilled water in a Hellendhal staining jar and dried with a plate. Eukitt mounting medium was used to glue the slides with coverglass to obtain a permanent slide, now available for the observation through the microscope.

Ectoderm, mesoglea and gastrodermis were easily recognized and so the zooxanthellae, which

showed in some sections of the tentacle a scondition in which zooxanthellae were compacted in the gastrodermis and in other sections showed exocytosis conditions in which some zooxanthellae were outside the gastrodermis (figure 9).

72 histological sections of each condition were observed under the microscope in order to report how many times exocytosis was present.

The % of exocytosis was determined through the formula:

$$\%\ exocytosis = \frac{number\ of\ exocytosis\ observations}{total\ number\ of\ observations} x\ 100$$

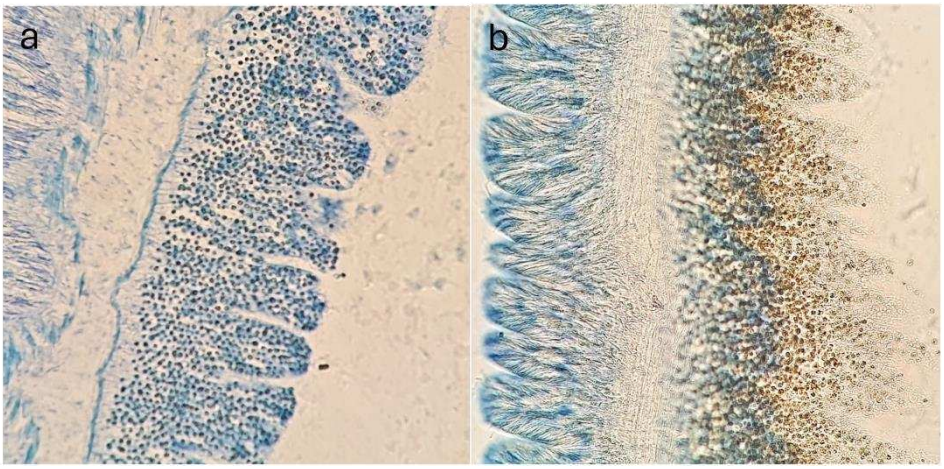

Figure 9*: a) absence of exocytosis; b) presence of exocytosis.*

Chlorophylls analysis

Chlorophylls content analysis were conducted in T1, T2 and T3.

The Eppendorf containing the homogenized tentacle with 90% acetone were centrifugated (1000 *rpm* for 5 min) and 600 µl of the supernatant were inserted in specific cuvette placed inside the spectrophotometer. Before doing that, calibration was done using a cuvette filled with acetone. The absorption spectrum of chlorophylls a and c_2 were so visible.

PAM analyses

PAM analyses were conducted in T1, T2 and T3 in order to get information about the maximum photosynthetic efficiency of PSII at dark (F_v/F_m), the maximum photosynthetic efficiency of PSII (Y(II)) and the non-photochemical quenching (NPQ). For these analyses the instrument DIVING-PAM-II underwater chlorophyll fluorometer was used, which allows a saturation kinetic of photosynthesis. This saturation kinetic consists of 11 steps of 20 seconds each, in which actinic light to which the samples were subject was gradually increased and through a saturating flash the parameters related to photosynthetic activity could be calculated.

In particular Y(II) gives indication on how much of the supplied light is used for photosynthesis, while NPQ indicates the ability to activate the photoprotective quenching mechanism, whose values measure the degree of excess energy dissipated by the photosystem and it generally increases with light intensity until it reaches a plateau. Fv/Fm represents the equivalent value of Y(II) at dark.

A tentacle for each individual was taken with a biology tweezer, placed in specific clip with sliding shutter for dark acclimation and kept in dark condition for 15 minutes. After this period of dark adaptation, the minimum fluorescence value (F_0) and the maximal fluorescence yield (F_m) can be measured through a saturation pulse without actinic light. In a second moment actinic light is turned on and fluorescence of saturating light (F'_m) and fluorescence after saturating flash in actinic light (F) can be measured. These parameters allow the measurement of F_v/F_m, Y(II) and NPQ through the formula:

$$Fv/Fm = \frac{Fm - Fo}{Fm}$$

$$Y(II) = \frac{F'm - F}{F'm}$$

$$NPQ = \frac{Fm - F'm}{F'm}$$

Experimental design and data analyses

The experimental design aiming at testing differences in the symbionts variables (i.e., living and broken cells density, mitotic index, chlorophylls a and c_2 concentration) in time under different light and temperature conditions included three crossed factors: light (fixed, 2 levels: PAR, PAR+IR), temperature (fixed, 2 levels: 20°C, 23°C) and time (fixed, 3 levels: T1, T2, T3). All data were analysed with a permutational multivariate analysis of variance (PERMANOVA), using a dissimilarity matrix based on the Euclidean distance and setting 9999 permutations. If required data were firstly square-root transformed. A posteriori pairwise comparisons were conducted in case of significant differences, especially for significance in the interactions of factors. All analyses were carried out using the software PRIMER 7.0.24 with PERMANOVA +.

RESULTS

Morphological results

According to the Coral Watch Chart, sea anemones maintained bright colors during all the experiment. Thanks to the photos frequently taken during the experiment, the tendency of the individuals to make little movement and to place themselves far from each other, from a minimum of 4 cm to a maximum of 10 cm, was observed (figure 10).

All individuals appeared active, never detached from the substrates and with well open-distributed tentacles, suggesting that all individuals were in a good health status.

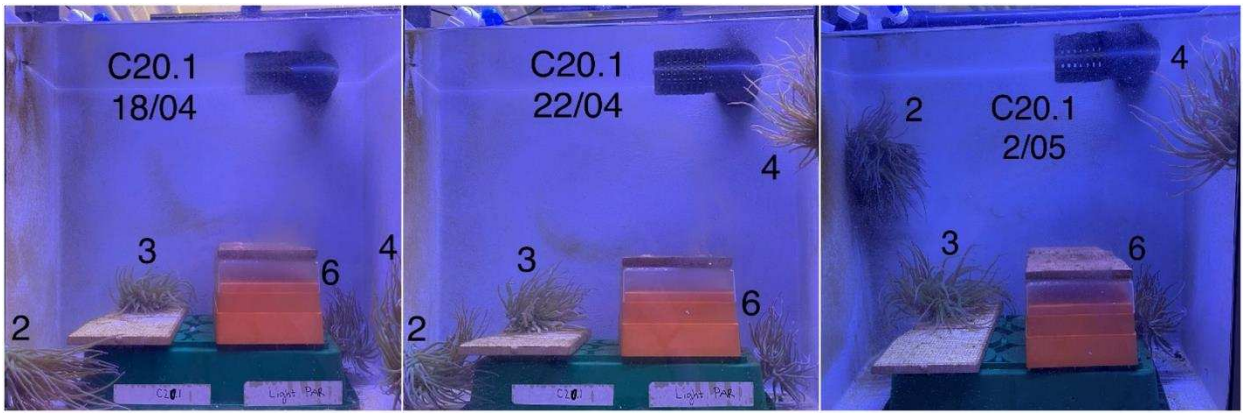

***Figure 10**: Sequence of three photos taken on April 18th, April 22th and May 2nd, showing A. viridis tendency to make little movement over time and to place themselves far from each other.*

Zooxanthellae and histological results

The values obtained from the count of the zooxanthellae extracted from the individuals of each treatment at each monitoring step (T1, T2 and T3) were used to evaluate how the concentration of zooxanthellae varied over time and between the different temperature and light conditions.

Overall, a decreasing trend of zooxanthellae density in time was observed in the individuals exposed to 20°C, while an increasing trend was instead observed in the *Anemonia viridis* exposed to 23°C and PAR+IR (PERMANOVA, $p<0.05$; table 3A), with values varying from $1.07x10^8 \pm 2.81x10^7$ n. cells g^{-1} to $5.34 x10^7 \pm 3.89 x10^7$ n. cells g^{-1} in C20 and from $5.58 x10^7 \pm 3.58 x10^7$ n. cells g^{-1} to $1.68 x10^8 \pm 9.76 x10^7$ n. cells g^{-1} in TIR23 (pairwise test, $p<0.05$; table 4B).

Furthermore, the *A. viridis* exposed to infrareds displayed a higher zooxanthellae concentration ($8.73 x10^7 \pm 3.6 x10^7$ n. cells g^{-1} in TIR20, and $1.68 x10^8 \pm 9.76 x10^7$ n. cells g^{-1} in TIR23) compared to the control light treatment, independently from the temperature to which they were subjected (figure 11) (PERMANOVA, $p<0.05$; table 3A), especially at T3 (pairwise test, $p<0.05$; table 4A).

The zooxanthellae were differentiated into singlets (healthy cells), doublets (dividing cells) and broken (degraded cells) (figure 12).

The fraction of degraded zooxanthellae was minimal and almost similar in the different conditions and at different times (PERMANOVA, $p>0.05$). In almost all samples, the fraction of dividing cells was higher in T1 ($1.02x10^7 \pm 5.20x10^6$ n. cells g^{-1} in C20; $1.43x10^7 \pm 8.61x10^6$ n. cells g^{-1} in TIR20; $4.32X10^6 \pm 1.92x10^6$ n. cells g^{-1} in C23; $3.74x10^6 \pm 3.04x10^6$ n. cells g^{-1} in TIR23) and decreased until T3, in which a small increase compared to T2 was observed (T2: $3.80x10^6 \pm 2.76x10^6$ n. cells g^{-1} in C20; $4.14x10^6 \pm 4.20x10^6$ n. cells g^{-1} in TIR20; $3.30X10^6 \pm 2.39x10^6$ n. cells g^{-1} in C23; $3.00x10^6 \pm 1.73x10^6$ n. cells g^{-1} in TIR23)(T3: $2.57x10^6 \pm 2.77x10^6$ n. cells g^{-1} in C20; $5.00X10^6 \pm 4.49x10^6$ n. cells g^{-1} in TIR20; $5.26X10^6 \pm 3.66x10^6$ n. cells g^{-1} in C23; $1.07x10^7 \pm 1.10x10^7$ n. cells g^{-1} in TIR23). Similar results were also obtained calculating the MI, with the PERMANOVA revealing significant differences between temperature

and time ($p<0.05$; table 3B). The pairwise comparison highlighted significant differences in the MI between 20°C and 23°C in T1 and T3 ($p<0.05$) (table 5).

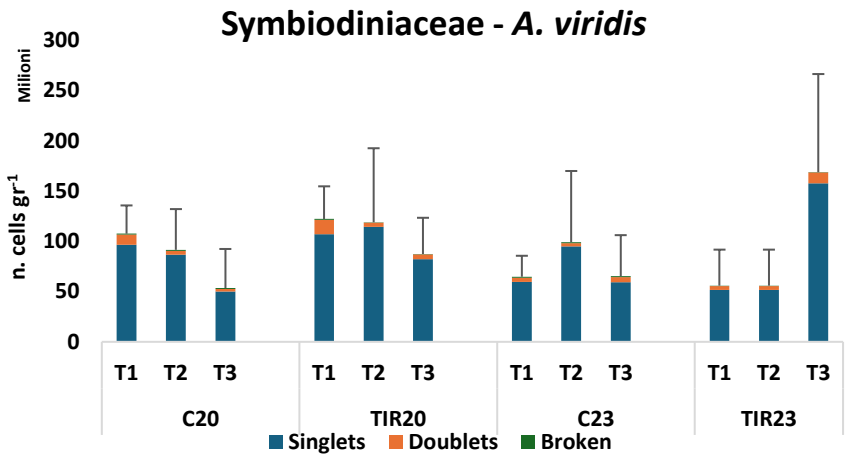

***Figure 11**: Symbiodiniaceae concentration over time.*

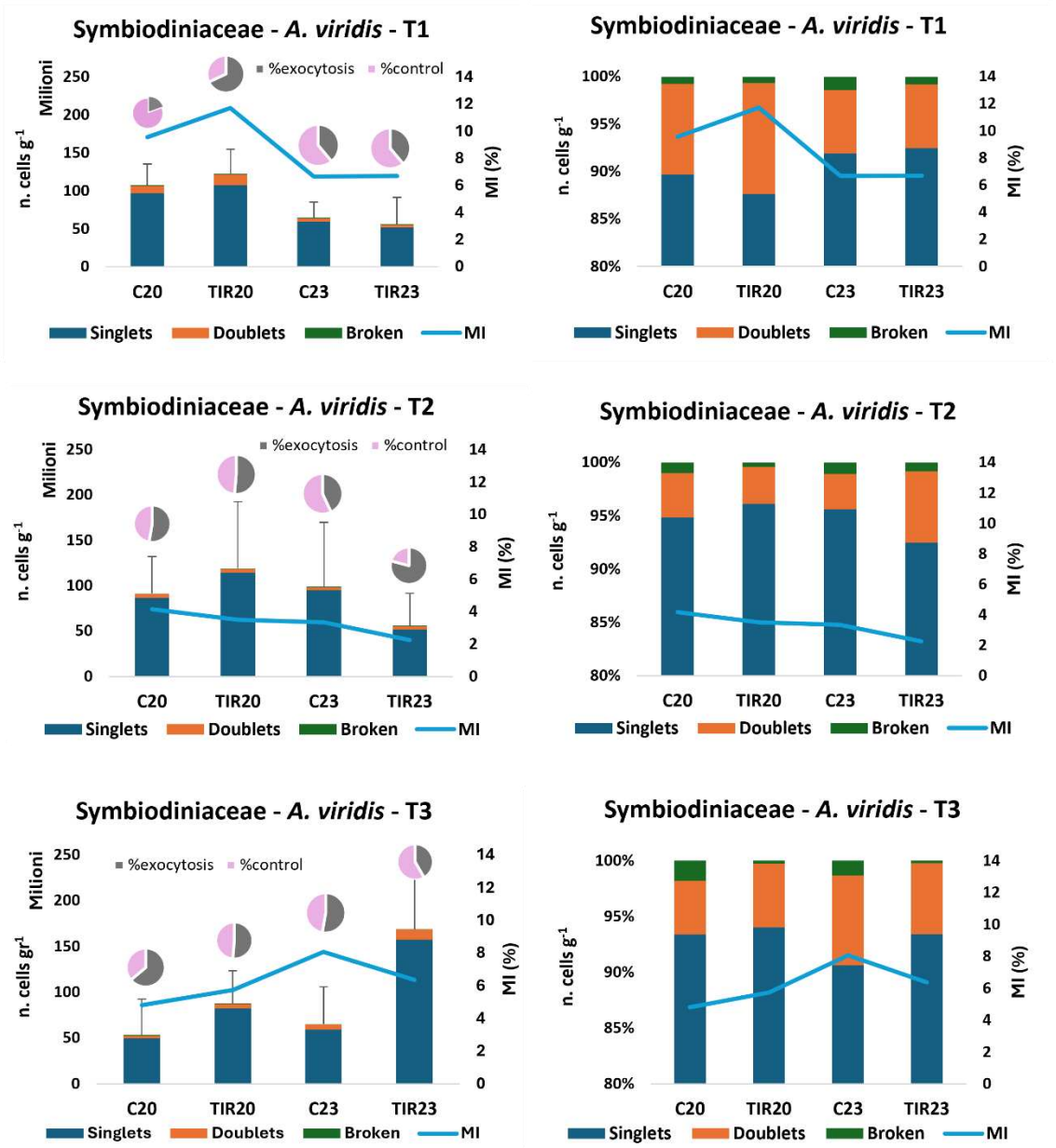

***Figure 12**:On the left: Symbiodiniaceae concentration at each monitoring step (T1, T2 and T3) expressed in terms of n. cells g-1; mitotic index (MI) and % of exocytosis in each treatment. On the right: Symbiodiniaceae concentration differentiated into singlets, doublets and broken cells and expressed in terms of %.*

***Table 3**: Results of the three-way PERMANOVA testing differences between light (PAR,PAR+IR), temperature (20°C, 23°C) and time steps (T1,T2,T3) considering: (A) the living zooxanthellae concentration, and (B) the mitotic index (MI). **Df**, degrees of freedom; **SS**, sum of squares; **MS**, mean squares; **Psuedo-F**, F-ratio; **P(perm)**, probability; **Up**, unique permutations; **P(MC)**, probability with the Monte Carlo method; **Sq.root**, square root; **Li**, light; **Te**, temperature; **Ti**, time. Significant p-values (p<0.05) are given in bold.*

A) Source	df	SS	MS	Pseudo-F	P(perm)	Up	P(MC)
Li	1	5.42090	5.42090	7.5095	**0.0089**	9830	0.009
Te	1	0.32624	0.32624	0.45194	0.493	9838	0.5057
Ti	2	2.25070	2.25070	1.559	0.2195	9949	0.2214
LixTe	1	0.040381	0.040381	0.055939	0.8128	9846	0.8065
LixTi	2	4.90570	4.90570	3.3979	**0.0397**	9947	0.0436
TexTi	2	8.5194	8.5194	5.9009	**0.0051**	9956	0.0057
LixTexTi	2	1.03360	1.03360	0.71591	0.4895	9959	0.4952
Res	60	43.312					

	Total	71	65.80900					
B)	Source	df	SS	MS	Pseudo-F	P(perm)	Up	P(MC)
	Li	1	0.06339	0.06339	0.29285	0.5963	9827	0.5982
	Te	1	0.37696	0.37696	1.7415	0.1879	9852	0.1943
	Ti	2	14.12500	7.06260	32.627	**0.0001**	9958	0.0001
	LixTe	1	0.6527	0.6527	3.0153	0.0892	9854	0.092
	LixTi	2	0.40881	0.20440	0.94429	0.3976	9963	0.3984
	TexTi	2	4.2517	2.1258	9.8209	**0.0004**	9951	0.0005
	LixTexTi	2	0.10804	0.05402	0.24957	0.7749	9948	0.7832
	Res	60	12.988	0.21646				
	Total	71	32.97400					

***Table 4**: Results of posteriori pairwise comparisons considering the living zooxanthellae concentration within time (T1,T2,T3) depending on: (A) light (PAR vs PAR+IR); (B) temperature (23°C, 23°C). **t**, t-test; **P(perm)**, probability; **Up**, unique permutations; **P(MC)**, probability with the Monte Carlo method; **Li**, light; **Te**, temperature; **Ti**, time. Significant p-values (p<0.05) are given in bold.*

A)					
	T1				
		t	P(perm)	Up	P(MC)
	PAR, PAR+IR	0.36207	0.7252	9837	0.7263
	T2				
		t	P(perm)	Up	P(MC)
	PAR, PAR+IR	1.4356	0.1632	9842	0.1681
	T3				
		t	P(perm)	Up	P(MC)
	PAR, PAR+IR	2.9481	**0.0099**	9838	0.0071
B)					
	T1				
		t	P(perm)	Up	P(MC)
	20°C, 23°C	4.493	**0.0004**	9822	0.0002
	T2				
		t	P(perm)	Up	P(MC)
	20°C, 23°C	0.21877	0.8272	9828	0.8268
	T3				
		t	P(perm)	Up	P(MC)
	20°C, 23°C	1.4294	0.167	9833	0.1669

***Table 5**: Results of posteriori pairwise comparisons considering MI within time (T1,T2,T3) depending on temperature (23°C, 23°C). **t**, t-test; **P(perm)**, probability; **Up**, unique permutations; **P(MC)**, probability with the Monte Carlo method; **Li**, light; **Te**, temperature; **Ti**, time. Significant p-values (p<0.05) are given in bold.*

T1

	t	P(perm)	Up	P(MC)
20°C, 23°C	3.1242	**0.0059**	9843	0.0055

T2

	t	P(perm)	Up	P(MC)
20°C, 23°C	1.445	0.1654	9848	0.1743

T3

	t	P(perm)	Up	P(MC)
20°C, 23°C	2.26	**0.0387**	9814	0.0381

Considering the histological analysis performed on *A. viridis*, no specific pattern in the exocytosis was observed (figure 13). As a point of fact, the control conditions showed a similar behaviour, with 19% of exocytosis in C20 and 38% of exocytosis in C23 in T1, gradually increasing during the experiment and reaching 63% of exocytosis in C20 and 52% of exocytosis in C23 in T3. Conversely, the TIR20 sample was characterized by higher values in T1 (68%), which decreased in T2 and remained constant in T3 (51%), while the TIR23 sample from an initial value of 38% of exocytosis reaches the value of 79% of exocytosis in T2 followed by a decrease in T3 (41%).

Chlorophylls quantification results

To assess the photosynthetic capacity and changes in the photosynthetic apparatus of the algal symbionts, the chlorophylls content was measured. Overall, a higher concentration of chlorophyll c_2 compared to the one of chlorophyll a was recorded in almost all samples, with few exceptions in which the two concentrations were very similar.

In terms of variation in the chlorophyll a concentration over time, we observed very different conditions among treatments over time: (i) C20 was characterized by a general increase over time (from $1.73x10^{-3} \pm 3.25x10^{-4}$ pg/g to $1.32x10^{-2} \pm 9.72x10^{-5}$ pg/g); (ii) TIR23 was subjected to a gradual decrease (from $1.29x10^{-2} \pm 4.16x10^{-3}$ pg/g to $2.04x10^{-3} \pm 4.26x10^{-4}$ pg/g); (iii) TIR20 and C23 increased their chlorophyll a concentrations from T1 ($1.82x10^{-3} \pm 3.25x10^{-4}$ pg/g in TIR20; $1.60x10^{-2} \pm 1.34x10^{-3}$ pg/g in C23) to T2 ($5.00x10^{-3} \pm 2.62x10^{-3}$ pg/g in TIR20; $1.80x10^{-2} \pm 1.57x10^{-3}$ pg/g in C23) followed by a decrease in T3 ($7.14x10^{-4} \pm 4.50x10^{-4}$ pg/g in TIR20; $1.11x10^{-2} \pm 1.28\ x10^{-3}$ pg/g in C23). Differences in patterns were also recorded by the PERMANOVA considering both light and temperature over time ($p<0.05$; table 6A). In

particular, the IR treatment showed lower values than the CTR conditions in both T2 and T3, independently from the temperature (pairwise test, $p<0.05$; table 7A); lower values were also found at 20°C compared to 23°C in T1, independently from the light (pairwise test, $p<0.05$; table 7B).

A similar but less evident trend occurred for the chlorophyll c_2 concentrations: (i) C20 was characterized by a general increase over time (from 2.42x10-3 ± 1.05x10-3 pg/g to 1.35x10-2 ± 5.77x10-3 pg/g); (ii) TIR23 was subjected to a gradual decrease (from 7.73x10-3 ± 2.87x10-3 pg/g to); (iii) TIR20 and C23 showed almost constant values along the entire period (from 3.26x10-3 ± 1.49x10-3 pg/g to 3.42x10-3 ± 1.25x10-3 pg/g and from 6.69x10-3 ± 2.26x10-3 pg/g to 6.69x10-3 ± 1.18x10-3 pg/g, respectively). Also in this case, the PERMANOVA revealed significant differences in light and temperature over time ($p<0.05$; table 6B), with higher values in the PAR compared to PAR+IR in T3 (pairwise test, $p<0.05$; table 8A) and in lower values 20°C compared to 23°C in T1 (pairwise test, $p<0.05$; table 8B).

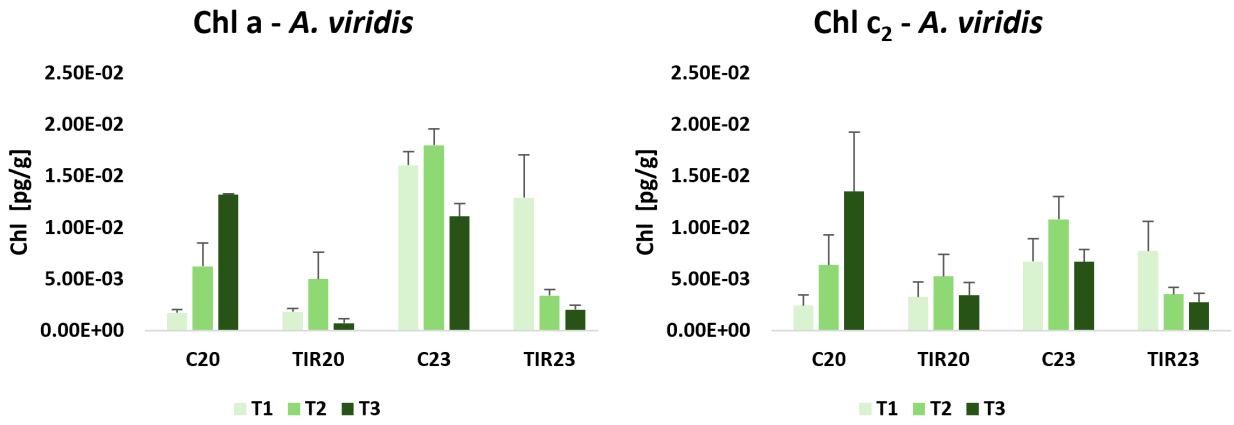

***Figure 13**: Chlorophyll a and c2 concentration over time.*

***Table 6**: Results of the three-way PERMANOVA to test differences among light (PAR,PAR+IR), temperature (20°C, 23°C) and time (T1,T2,T3) in the: (A) chlorophyll-a and (B) chlorophyll-c_2 concentrations. **Df**, degrees of freedom; **SS**, sum of squares; **MS**, mean squares; **Psuedo-F**, F-ratio; **P(perm)**, probability; **Up**, unique permutations; **P(MC)**, probability with the Monte Carlo method; **Sq.root**, square root; **Li**, light; **Te**, temperature; **Ti**, time. Significant p-values (p<0.05) are given in bold.*

A)	Source	df	SS	MS	Pseudo-F	P(perm)	Up	P(MC)
	Li	1	9.771E-09	9.771E-09	10.496	**0.002**	9835	0.0025
	Te	1	2.586E-08	2.586E-08	27.775	**0.0001**	9839	0.0001
	Ti	2	8.873E-09	4.437E-09	4.7656	**0.0102**	9944	0.0143
	LixTe	1	1.035E-09	1.035E-09	1.1113	0.3068	9854	0.2968
	LixTi	2	9.182E-09	4.591E-09	4.9313	**0.0093**	9954	0.0125
	TexTi	2	1.385E-08	6.926E-09	7.4395	**0.0018**	9953	0.0018
	LixTexTi	2	5.582E-09	2.791E-09	2.9981	0.058	9959	0.0611
	Res	43	4.003E-08	9.310E-10				
	Total	54	1.212E-07					
B)	Source	df	SS	MS	Pseudo-F	P(perm)	Up	P(MC)
	Li	1	2.0657E-09	2.0657E-09	4.6488	**0.037**	9812	0.0367
	Te	1	3.3143E-09	3.3143E-09	7.4585	**0.0085**	9833	0.0093
	Ti	2	7.9434E-10	3.9717E-10	0.8938	0.4216	9954	0.4101
	LixTe	1	2.3536E-11	2.3536E-11	0.052966	0.8266	9826	0.8164
	LixTi	2	6.2238E-09	3.1119E-09	7.0032	**0.0022**	9958	0.0021
	TexTi	2	4.1639E-09	2.0820E-09	4.6853	**0.013**	9953	0.0156
	LixTexTi	2	1.6248E-09	8.1240E-10	1.8283	0.1652	9942	0.1703
	Res	43	1.9107E-08	4.4436E-10				
	Total	54	3.8067E-08					

***Table 7**: Results of posteriori pairwise comparison related to chlorophyll a concentration within time (T1,T2,T3) depending on: (A) light (PAR vs PAR+IR); (B) temperature (23°C, 23°C). t, t-test; P(perm), probability; Up, unique permutations; P(MC), probability with the Monte Carlo method; Li, light; Te, temperature; Ti, time. Significant p-values (p<0.05) are given in bold.*

A)					
	T1				
		t	P(perm)	Up	P(MC)
	PAR, PAR+IR	0.59387	0.6962	9916	0.5663
	T2				
		t	P(perm)	Up	P(MC)
	PAR, PAR+IR	2.1338	**0.0394**	9857	0.05
	T3				
		t	P(perm)	Up	P(MC)
	PAR, PAR+IR	4.9924	**0.0002**	9861	0.0003
B)					
	T1				
		t	P(perm)	Up	P(MC)
	20°C, 23°C	6.1028	**0.0001**	9846	0.0002
	T2				
		t	P(perm)	Up	P(MC)
	20°C, 23°C	1.2425	0.2383	9880	0.2395
	T3				
		t	P(perm)	Up	P(MC)
	20°C, 23°C	1.9849	0.063	9858	0.0675

***Table 8**: Results of posteriori pairwise comparison related to chlorophyll c_2 concentration within time (T1,T2,T3) depending on: (A) light (PAR vs PAR+IR); (B) temperature (23°C, 23°C). **t**, t-test; **P(perm)**, probability; **Up**, unique permutations; **P(MC)**, probability with the Monte Carlo method; **Li**, light; **Te**, temperature; **Ti**, time. Significant p-values (p<0.05) are given in bold.*

A)					
	T1				
		t	P(perm)	Up	P(MC)
	PAR, PAR+IR	1.557	0.1297	9861	0.1445
	T2				
		t	P(perm)	Up	P(MC)
	PAR, PAR+IR	1.682	0.1146	9832	0.1182
	T3				
		t	P(perm)	Up	P(MC)
	PAR, PAR+IR	4.769	**0.0004**	9850	0.0004
B)					
	T1				
		t	P(perm)	Unique perms	P(MC)
	20°C, 23°C	3.6974	**0.0008**	9839	0.0015
	T2				
		t	P(perm)	Unique perms	P(MC)
	20°C, 23°C	0.89024	0.3982	9874	0.39
	T3				
		t	P(perm)	Unique perms	P(MC)
	20°C, 23°C	0.3398	0.7571	9862	0.7355

PAM results

To assess the photosynthetic capacity of the algal symbionts the maximum photosynthetic efficiency and the non-photochemical quenching were measured.

Data relating to the maximum photosynthetic efficiency of PSII at dark (Fv/Fm) showed an increase over time in all conditions, as well as higher values in samples at 23°C compared to the ones at 20°C (figure 14). Overall, Y(II) remained constant, independently of conditions through the experiment (figure 15). The NPQ values increased with light intensity showing a correct functioning of the photosystem. However, a different trend between the two light conditions was observed comparing T1 and T3: control samples showed lower values, while treatment samples showed higher values (figures 16).

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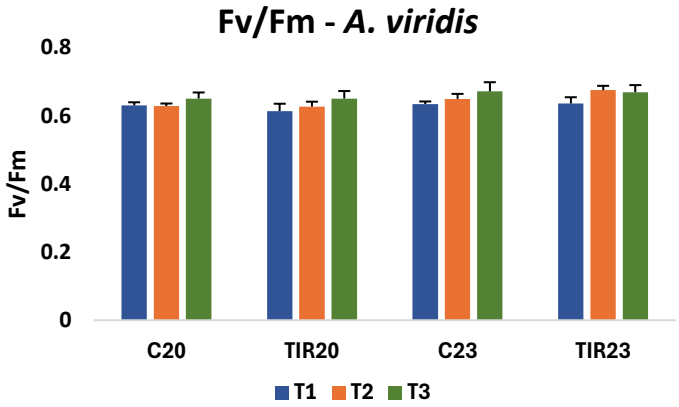

Figure 14: *Fv/Fm values over time of C20, TIR20, C23 and TIR23.*

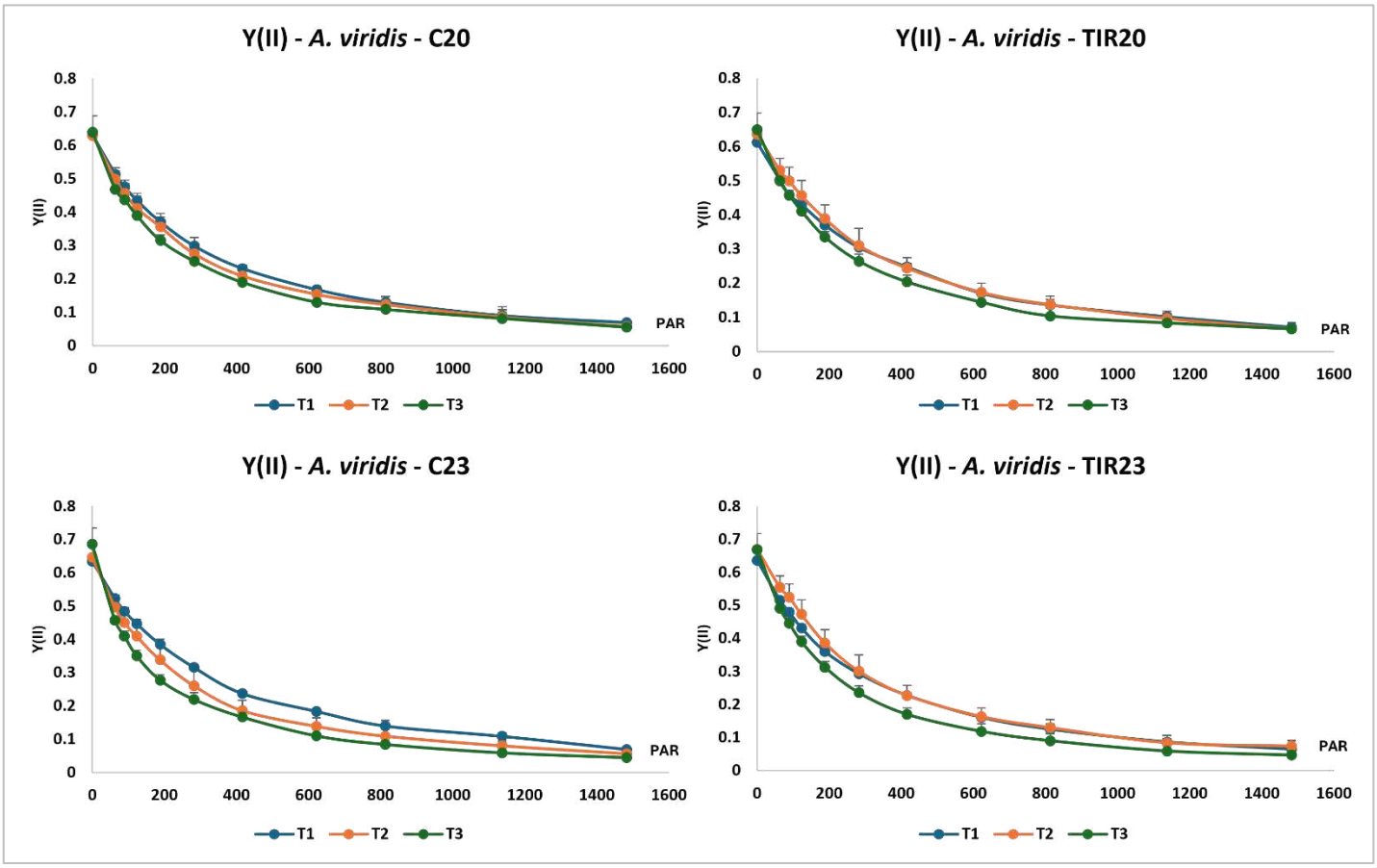

Figure 15*: Y(II) values over time of C20, TIR20, C23 and TIR23.*

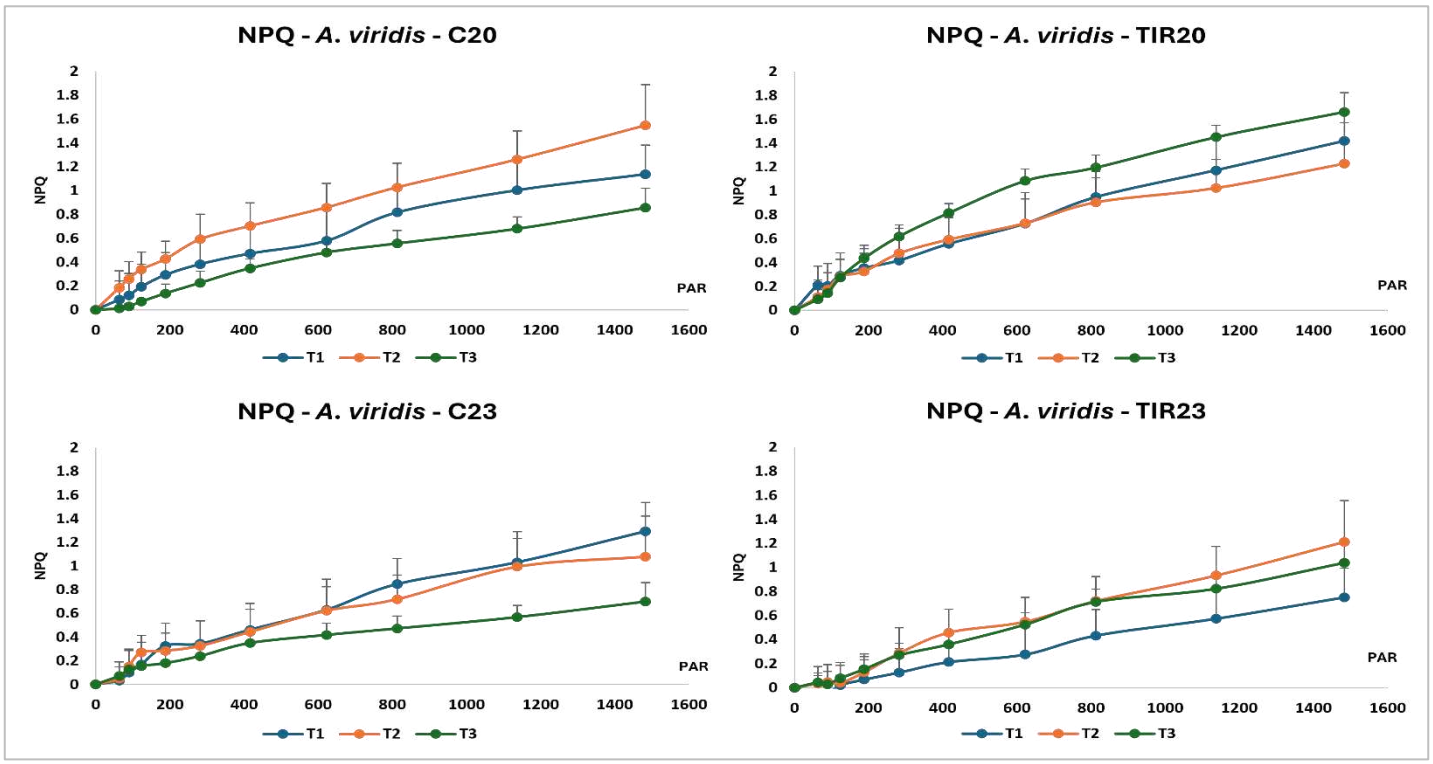

***Figure 16**: NPQ values over time of C20, TIR20, C23 and TIR23.*

DISCUSSION

The aim of this study was to evaluate the effects of different light and temperature conditions on the sea anemone *Anemonia viridis* and its symbiotic relationship with Symbiodiniaceae zooxanthellae.

For this purpose, PAR light (control condition) and PAR+IR light (treatment condition) were used to rear the actinians at 20°C and 23°C for two months. Morphological data, together with chlorophylls and symbiont concentration, and photosynthetic efficiency were collected in three different periods: at the beginning of the experiment (T1) and after one (T2) and two months (T3).

Independently of the treatment, *A. viridis* maintained good health conditions for the entire duration of the experiment (i. e., bright colours, and active movements of tentacles). This *A. viridis* ability to positively respond to environmental variations has been previously recorded in other studies, demonstrating high tolerance and resistance to different light, CO_2 and oxygen regimes (Richier *et al.*, 2003; Bell *et al.*, 2006; Urbarova *et al.*, 2019).

Notwithstanding the sea anemones were collected in an area of a few squared metres, quite different values in Symbiodiniaceae and chlorophyll concentrations were found in the samples at T1 (i.e., before starting the treatments), likely due to individual variability. While there are little variations in Symbiodiniaceae concentration after one month (T2), an increase in symbiont density was observed in the IR-trial at 23°C after two months (T3), together with a reduction in the other trials. Considering that exocytosis of the symbionts was observed in histological sections of tentacles, it is likely that cyclic expulsion of the symbionts occurs in *A. viridis*, as observed in other anthozoans (Yamashita *et al.* 2011, Fujise *et al.* 2014). While

viable algal cells in the coral's gastroderm were stained the darker due to the bind with toluidine blue dye, the expelled cells (found outside the gastroderm) appeared lighter and likely deteriorated, suggesting that exceeding symbionts are periodically eliminated to control their density *in hospite* (Koike *et al.*, 2007; Dimond & Carrington, 2008; Fujise *et al.*, 2013; Fujise *et al.*, 2018).

Several experiments conducted to assess the effects of different wavelengths (blue 430-480 nm, red 660-700 nm, and green 500 nm) on the symbiont density both in *A. viridis* (Squire, 2000) and in hard-bodied corals (Kinzie *et al.*, 1984; Wijgerde *et al.*, 2014; Izumi *et al.*, 2023), reported that symbionts density was blue enhanced compared to other lights, even if the considered species responded in different ways to the treatments.

The chlorophyll concentration in the analysed sea anemones varied from the initials values and resulted inversely correlated to the algal density; moreover, a higher concentration of chlorophyll c_2 than chlorophyll a was observed in almost samples. Changing in stoichiometry between accessory and primary pigments related to different light and temperature conditions were documented in several studies, in which endosymbiotic dinoflagellates presented higher chlorophyll c_2 content than chlorophyll a (Iglesias-Prieto & Trench, 1994; Hennige *et al.*, 2009; Roth *et al.*, 2010; Gierz *et al.*, 2016). An increase in the accessory pigments in our samples may be related to the ability of the chlorophyll c_2 of absorbing light at 460 nm (Niedzwiedzki *et al.* 2014); however, considering that the ratio chl a/chl c_2 is lower in samples under the IR lamps, we could speculate that other receptors could be involved in the regulation of chlorophyll biosynthesis (Huq *et al.* 2004, Liu *et al.* 2013).

Despite the reduction in pigment content, the PAM analysis revealed that the photosynthetic efficiency was maintained throughout the experiment, as was the energy quenching mechanism. Fv/Fm increased during the two-month experiment, showing that the organisms

acclimated to the experimental conditions tested. Y(II) remained constant, independently of conditions through the experiment, while IR-treated anemones showed a slight increase in NPQ at the end of the experiment. Overall, infrareds treatment seemed to cause an alteration in the photosynthetic apparatus, represented by the reduction in chlorophylls content, but the cells however maintained a functional photosynthesis without any apparent damage.

Scleractinia corals placed under blue (400-535 nm), red (600-700 nm) and UV (>400 nm) light treatments revealed a higher decrease in photosynthetic efficiency under UV light compared to red and blue light, while NPQ was not significant different across light treatments (Hamley, 2016).

Furthermore, from this study the mitotic index resulted more affected by temperature (MI higher at 23°C) rather than infrareds. A decrease in the mitotic index was documented in cultured symbiotic dinoflagellates isolated from the coral *Euphyllia glabrescens* due to infrared (735 ± 10 nm) light (Wang *et al.*, 2008) and in symbiotic dinoflagellates in *A. viridis* due to red light (Squire, 2000).

Overall, from this study emerged that two months infrareds treatment led to an increase in zooxanthellae density and a decrease in chlorophylls content.

Anemonia viridis collected *in situ* in the central Adriatic Sea showed an increase in symbiodiniaceae density and a reduction in chlorophyll content between spring and summer, and the opposite in winter, suggesting that the experimental conditions were more similar to the natural ones occurring in late spring (figure 17) (Arossa *et al.*, in prep.).

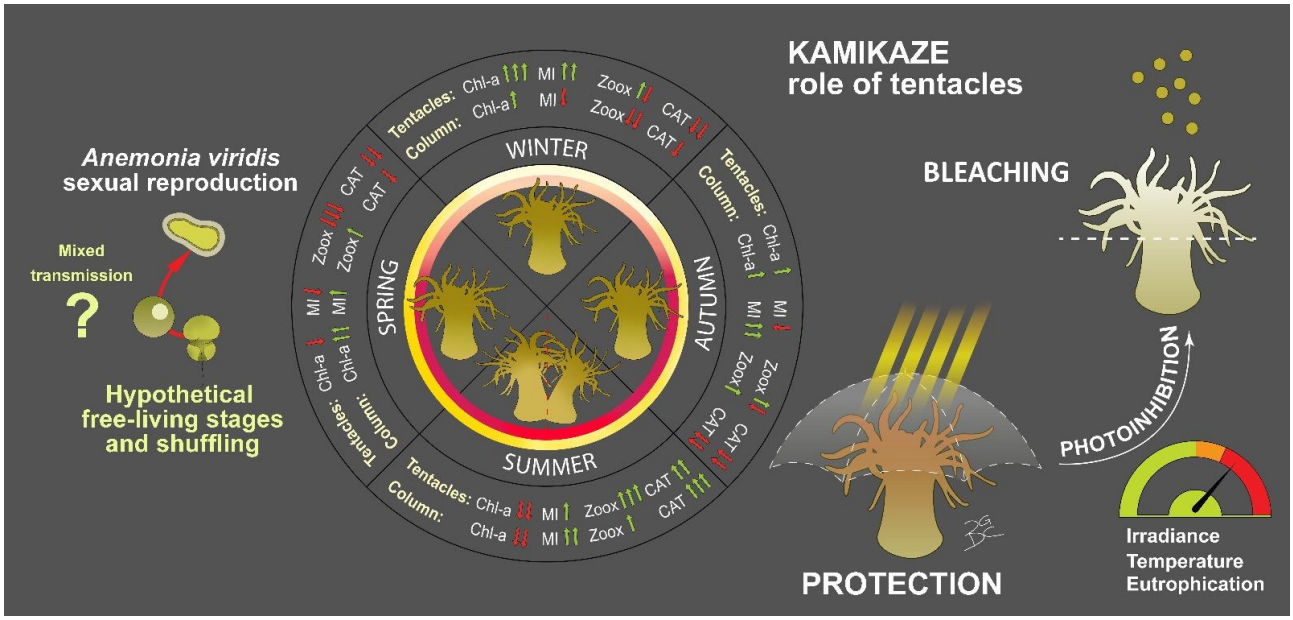

***Figure 17**: Natural cycle observed in Anemonia viridis: in summer there is an increase in zooxanthellae density and a decrease in chlorophylls content, preceding the division of sea anemones (Arossa et al., in prep.).*

CONCLUSIONS

Studies focusing on the effects of different light spectra within and beyond the PAR on symbiotic organisms are still limited. This experiment aims to evaluate how PAR and far red/infrared radiation regulate the relationship Cnidaria-Symbiodiniaceae in the model species *A. viridis* to find optimal conditions for rearing this species.

The results emerged from these analyses suggest that *A. viridis* and its dinoflagellates were not negatively affected by far red/infrared radiation and that both the symbionts and the host maintained a good health status during all the two months' experiment.

Far red/infrared radiation seemed to influence symbionts density and the pigment content, without compromising the photosynthetic functioning.

Furthermore, a cyclic expulsion of symbionts appears to occur in *A. viridis*, suggesting cycling mechanisms of regulation of the symbiont density *in hospite*, as observed in other anthozoans.

This study contributed to understanding the relationship Cnidaria-Symbiodiniaceae under different light and temperature conditions, providing a starting point in the investigation of the effects of far red/infrared radiation in a potential sea anemone rearing.

For future investigation new combinations of light wavelengths would help to better discriminate the effects of non-PAR light on symbiotic corals.

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