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Prevalence and photobiology of photosynthetic endosymbionts of *Exaiptasia diaphana* in *Berghia stephanieae*

Universidade de Aveiro
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha Aplicada, realizada sob a orientação científica do Doutor Ricardo Jorge Guerra Calado – Investigador Principal com Agregação em Regime Laboral, Universidade de Aveiro e sob coorientação do Doutor Paulo Jorge Sousa Dias Cartaxana: Equiparado a Investigador Auxiliar, Universidade de Aveiro.

Ao meu irmão, pais e avós, os meus maiores exemplos de força e persistência.
Sem vocês nada disto era possível.

Metade da minha alma é feita de maresia – Sophia de Mello Breyner Andersen, Mar

o júri

Presidente   Professora Doutora Maria Marina Pais Ribeiro da Cunha, Professora
Associada, Universidade de Aveiro

Vogal - Arguente   Professor Doutor Gonçalo Jorge Pestana Calado, Professor Catedrático,
Universidade Lusófona de Humanidades e Tecnologias

Vogal - Orientador   Doutor Ricardo Jorge Guerra Calado, Investigador Principal com Agregação
em Regime Laboral, Universidade de Aveiro

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E assim acaba mais uma etapa. Aos 4 anos disse que um dia seria biólogo marinho, hoje esse sonho está um passo mais próximo. O meu muito obrigado a todos os que me ajudam nesta caminhada.

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palavras-chave   Dinoflagelados endossimbiontes fotossintéticos, espécies estenófagas, fotobiologia, branqueamento

resumo

Os nudibrânquios são moluscos gastrópodes marinhos de corpo mole, sem concha, que ocorrem numa grande variedade de ambientes. Algumas destas espécies são conhecidas pelos seus hábitos alimentares estenófagos e por reterem endossimbiontes fotossintéticos presentes nas presas das quais se alimentam. Uma dessas espécies é o aeolídeo *Berghia stephanieae*, um nudibrânquio estenófago que retém os dinoflagelados endossimbiontes fotossintéticos e os nematocistos da sua presa, a anémona de vidro *Exaiptasia diaphana*.

Berghia stephanieae é considerada uma espécie modelo para estudos ecológicos referentes à endossimbiose entre moluscos e dinoflagelados fotossintéticos. No entanto, estes estudos são frequentemente realizados com recurso a metodologias invasivas, que invariavelmente causam danos físicos aos organismos em estudo, ou podem mesmo levar ao seu sacrifício.

A anémona de vidro, *E. diaphana*, é considerada uma espécie modelo para o estudo da simbiose entre os cnidários e os seus microbiomas. Devido à sua simbiose com dinoflagelados fotossintéticos, esta espécie tem sido utilizada para estudar o fenómeno de branqueamento, fenómeno resultante da perda de simbiontes ou pigmentos fotossintéticos presentes num animal hospedeiro.

O presente trabalho estuda a prevalência da associação entre *B. stephanieae* e os dinoflagelados endossimbiontes fotossintéticos obtidos troficamente pela ingestão de *E. diaphana* através da utilização de uma metodologia não invasiva, a medição da fluorescência de pulso modelado da clorofila. Esta metodologia é utilizada de forma rotineira em plantas e algas, tendo, no entanto, já sido utilizada com sucesso para o estudo da cleptoplastia em lesmas marinhas da superordem Sacoglossa. Esta metodologia foi também já utilizada para estudar a manutenção da eficiência fotossintética de dinoflagelados endossimbiontes fotossintéticos presentes nalgumas espécies de nudibrânquios. Através da sua utilização foi possível identificar uma correlação positiva entre a abundância de dinoflagelados endossimbiontes fotossintéticos presentes em *B. stephanieae* e a fluorescência mínima de animais adaptados ao escuro (F_o).

A abundância de dinoflagelados endossimbiontes fotossintéticos presentes em *B. stephanieae* e a sua eficiência fotossintética foram estudadas em lesmas marinhas com e sem acesso a fonte de alimento e criadas a diferentes intensidades de luz (luz alta, 80 µmol m^{-2} s^{-1}; luz baixa, 10 µmol m^{-2} s^{-1}). A exposição a diferentes intensidades de luz não teve efeitos na manutenção da eficiência fotossintética ou na abundância dos endossimbiontes fotossintéticos presentes em *B. stephanieae*. No entanto, a exposição à inanição teve um impacto significativo, fazendo decrescer tanto a abundância dos dinoflagelados endossimbiontes fotossintéticos presentes em *B. stephanieae*, tal como a sua eficiência fotossintética.

Foi também testado o efeito de diferentes regimes tróficos na abundância de dinoflagelados endossimbiontes fotossintéticos presentes em *B. stephanieae* e a sua eficiência fotossintética. Este estudo permitiu evidenciar que indivíduos alimentados com anémonas branqueadas (anémonas sem dinoflagelados endossimbiontes fotossintéticos) apresentavam um impacto

semelhante ao verificado em conspecíficos sujeitos a inanição, ou seja, um decréscimo na abundância de endossimbiontes fotossintéticos, assim como da sua eficiência fotossintética. Estes resultados mostram que os dinoflagelados endossimbiontes fotossintéticos presentes em *B. stephanieae* necessitam de ser repostos regularmente por estes nudibrânquios através da predação de anémonas vidro que apresentem estes endossimbiontes fotossintéticos.

Este estudo serve de ponto de partida para trabalhos futuros nos quais o parâmetro de fluorescência da clorofila F_o poderá ser usado para inferir a abundância, manutenção e eficiência fotossintética de endossimbiontes fotossintéticos em nudibrânquios, assim como a manutenção da sua simbiose. Esta metodologia pode ser uma ferramenta valiosa para o estudo das consequências do branqueamento de organismos marinhos nos seus predadores estenófagos, consequências essas que são pouco conhecidas e, como tal, carecem de investigação.

abstract

Nudibranchs are soft bodied, shell-less marine gastropod molluscs. They are widespread over a wide variety of environments. Some of these species are known due to their stenophagous dietary habits and their ability to retain photosynthetic endosymbionts from their preys. One of such nudibranchs is the aelolid *Berghia stephanieae*, a stenophagous nudibranch that retains both the photosynthetic endosymbionts and the nematocysts of its prey, the glass anemone *Exaiptasia diaphana*.

Berghia stephanieae is known to be one of the best models for ecological studies addressing the mollusc-photosynthetic dinoflagellate endosymbiosis. However, these studies are often made with the use of invasive techniques, that commonly harm the study subject, and may even lead to their sacrifice.

The glass anemone, *E. diaphana*, is seen as a model species to the study of cnidarian-microbiome symbiosis. As this species establishes a symbiosis with photosynthetic dinoflagellates, it has been used to study bleaching, the phenomenon that results from the loss of photosynthetic endosymbionts or pigments from the animal host.

The present work addresses the maintenance of the symbiosis between *B. stephanieae* and its photosynthetic dinoflagellate endosymbionts acquired through the predation of *E. diaphana* using a non-invasive methodology, the measurement of pulse amplitude modulated chlorophyll fluorescence. While this methodology is commonly employed on plants and algae, it has already been successfully used to study kleptoplasty in sacoglossan sea slugs, as well as the maintenance of photosynthetic efficiency of photosynthetic dinoflagellate endosymbionts in some nudibranchs. Our experimental trials made possible to detect a positive correlation between the abundance of photosynthetic dinoflagellate endosymbionts within *B. stephanieae* and the minimum fluorescence of dark-adapted animals (F_0).

The abundance of photosynthetic dinoflagellate endosymbionts within *B. stephanieae* and their photosynthetic efficiency were studied in sea slugs reared under different light intensities (high light, 80 µmol m^{-2} s^{-1}; low light, 10 µmol m^{-2} s^{-1}) and access to food. Exposure to different light intensities had no significant impact in the maintenance of *B. stephanieae*'s photosynthetic dinoflagellate endosymbionts, nor on their photosynthetic efficiency. However, starvation was shown to have a significant impact by decreasing both the abundance of photosynthetic dinoflagellate endosymbionts within *B. stephanieae* and their photosynthetic efficiency.

The effect of different trophic regimes in the abundance of photosynthetic dinoflagellate endosymbionts within *B. stephanieae* and their photosynthetic efficiency was also tested. It was possible to show that sea slugs fed with bleached anemones (anemones lacking photosynthetic dinoflagellate endosymbionts) were impacted in a similar way to starved sea slugs, as they both exhibited a decrease in the abundance of photosynthetic dinoflagellate endosymbionts and a lower photosynthetic efficiency. This finding shows that the photosynthetic dinoflagellate endosymbionts present within *B. stephanieae* need to be replenished regularly by these nudibranchs

by preying on symbiotic glass anemones (anemones that have photosynthetic dinoflagellate endosymbionts).

Overall, the present study is the starting point to future works on which chlorophyll fluorescence parameter F_o may be used to infer the abundance, maintenance and photosynthetic efficiency of endosymbionts in nudibranchs. This methodology may be a powerful tool to study the consequences of bleaching of marine organisms on their stenophagous predators, as these are widely unknown and need to be further investigated.

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1 General Introduction

1.1 Nudibranch Biology

Nudibranch molluscs (Mollusca: Gastropoda: Nudibranchia), popularly termed as sea slugs, are marine gastropods that are part of order Nudibranchia (Figure 1) (The Editors of Encyclopaedia Britannica, 2020). There are over 3000 species of nudibranchs widespread over various environments ranging from shallow temperate and tropical reefs to Antarctica and hydrothermal vents (Cheney & Wilson, 2018).
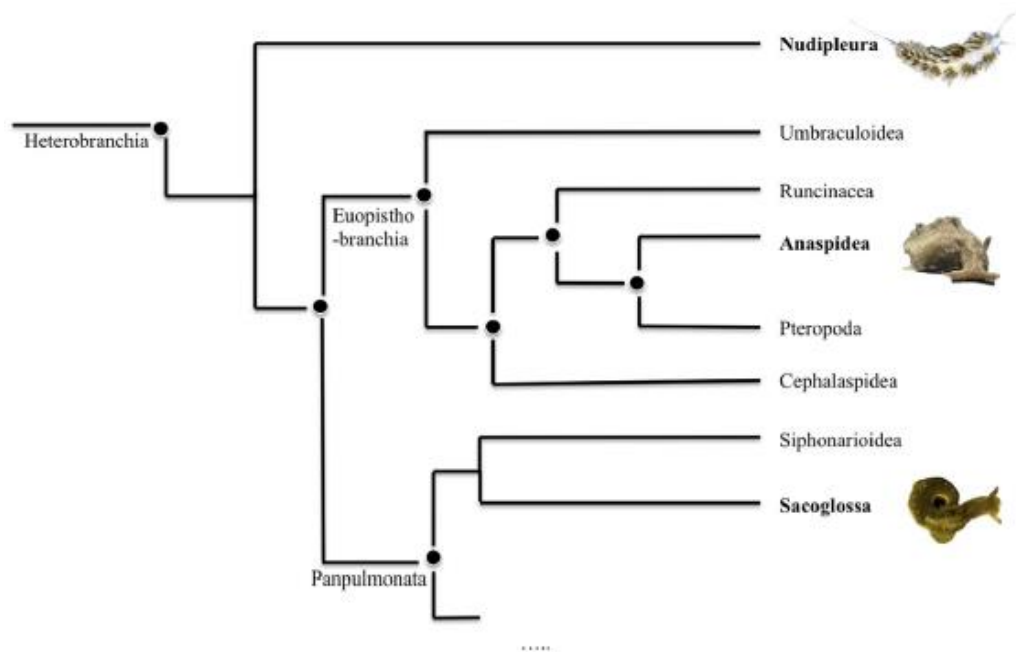
Figure 1 – Schematics of Heterobranchia phylogeny. Difference between branches is a measurement of divergence of time, with black nodes showing significant support. Nudipleura superorder is here represented by *Berghia stephanieae*; Anapsidea, that is now known as Aplysiida, is represented by *Aplysia sp.*; and Sacoglossa is represented by *Elysia viridis* (after Dionísio et al., 2013).

Nudibranchs are marine snails that lack a shell. They are often colourful organisms. These colours were found to serve as a warning to predators of their chemical defences (aposematism). However, some nudibranchs have patterns that closely resemble the subtract where they dwell or their preys (crypsis) (Cheney & Wilson, 2018). In addition to aposematism and camouflage,

nudibranchs present a great variety of defence mechanisms, such as the reinforcement of the soft tissue with spicules, the alteration of their preys' defensive compounds to enhance their biological activity and *de novo* synthesis of defensive chemical compounds (Cheney et al., 2016; Cheney & Wilson, 2018; Cimino et al., 2004; Winters et al., 2017). There are also some nudibranchs which can incorporate nematocysts, the stinging cells of cnidarians, that they obtain from preyed organisms and store in cnidosacs located in the tip of their cerata; there nematocysts are incorporated in cells and mature, with a pH shift, to be used for defence against predators (Obermann et al., 2012).

Usually nudibranchs are short-lived simultaneous hermaphrodites, that reproduce following a semelparous model, which is defined by a single spawning event (Todd et al., 2001). Even though these sea slugs are semelparous, it does not mean they can only reproduce once in a lifetime, usually nudibranchs produce several spawn masses per reproductive season (Todd et al., 2001). The larvae within the nudibranchia order usually behave in the same way disregarding their species. For the first few hours, up to days, veliger larvae swim upwards. They will then swim in a random pattern over the bottom, this phase is known as the searching phase of Thompson (Hadfield, 1963). The swimming stage of the larvae is usually short, with metamorphosis occurring one to two days after hatching (Thompson, 1967). Most nudibranch larvae are planktotrophic, meaning that larvae must feed on plankton. However, some nudibranchs display direct development, or emerge from the egg as lecithotrophic (non-feeding) larvae, which subsist until metamorphosis through the catabolism of maternal yolk reserves (Thompson, 1967). Planktotrophic larvae usually hatch from smaller eggs (mode of ~75 µm diameter), pelagic lecithotrophic larvae hatch from intermediate eggs (mode of ~135 µm diameter) and non-pelagic lecithotrophic larvae hatch from the largest nudibranch eggs (mode of ~205 µm diameter) (Todd et al., 2001). Sometimes, it is possible to notice some shifts on the ontogenetic stages displayed by larvae within the same spawn that is caused by environmental factors; this phenom is termed poecilogony (Hadfield, 1963; McEdward & Chia, 1991; Picton & Morrow, 1994).

Nudibranchs feed with the use of a radula (Figure 2), an organ found in molluscs, that consists of many rows of chitinous teeth. The radula usually differs between species within order Nudibranchia, and the shape and number of radular teeth is an important character used in taxonomic studies (Picton & Morrow, 1994).
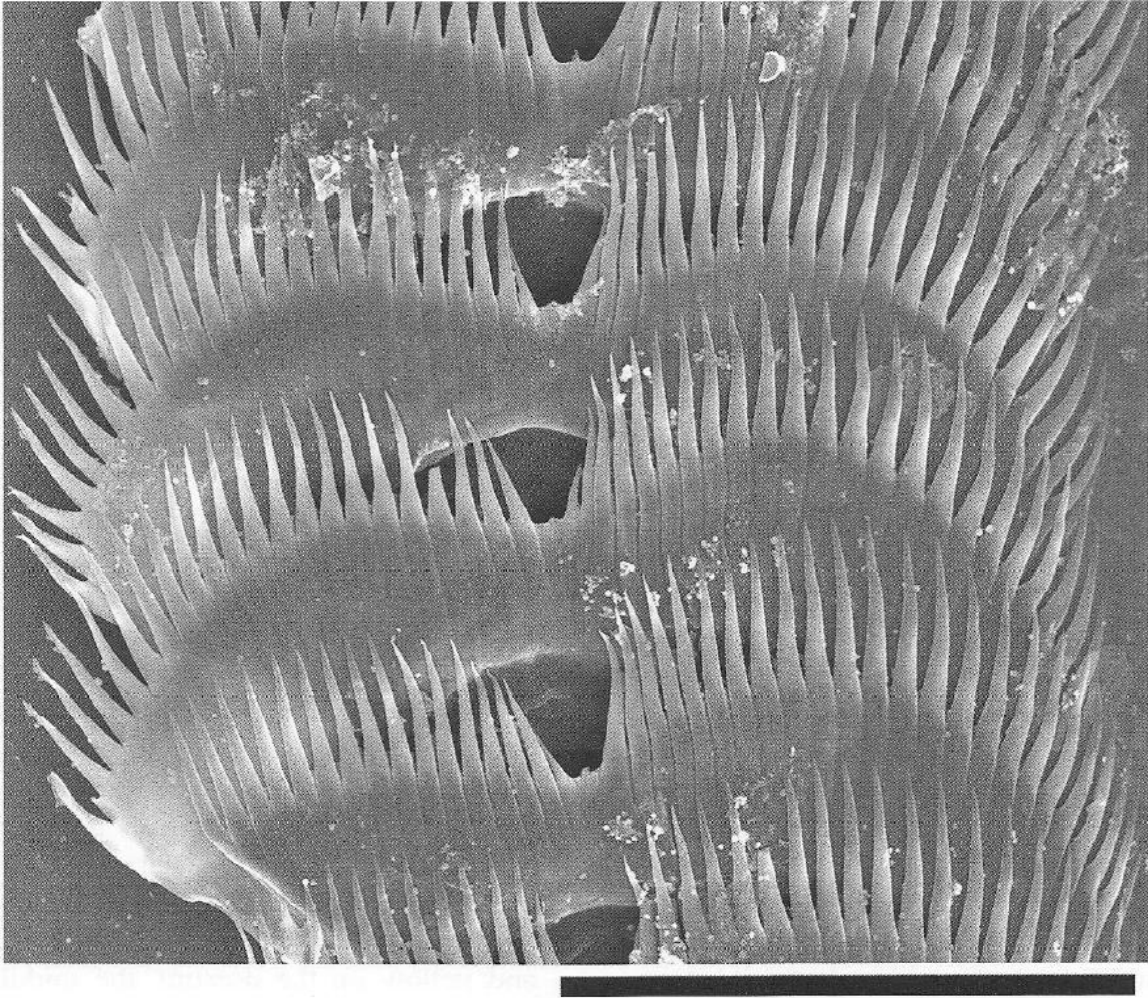
Figure 2 – Scanning electron microscope view of the radula teeth of *Berghia stephanieae*. Scale bar = 100 μm (after Valdés, 2005).

Nudibranchs commonly display a specialised diet, with some species being monophagous, species that prey upon a single species, and most of the species being stenophagous, species that prey upon two or three different prey species (Todd et al., 2001). There are also reports of polyphagous species, (Todd et al., 2001). There are some species of nudibranchs, the so called solar-powered nudibranchs that may benefit from nutrients derived from photosynthesis (Cheney & Wilson, 2018). While sacoglossan sea slugs harvest functional chloroplasts from algae (kleptoplasty), nudibranchs establish symbiosis with photosynthetic dinoflagellates (zooxanthellae) retrieved from their preys (Angelard & Bever, 2013; Baker, 2011; Burghardt et al., 2008; Cruz et al., 2013; Wägele & Johnsen, 2001). This association is yet to be completely understood, while it has been suggested that it allows the sea slug to survive through periods of food shortage while searching for and testing other food sources (Wägele et al., 2010); it has

also been suggested that the incorporation of symbionts serves solely a purpose of crypsis (Burghardt et al., 2005).

The fact that nudibranchs establish the aforementioned association with that of most species being highly sensitive to shifts in seawater temperature, has sparked the interest of the scientific community addressing climate change (California Office of Environmental Health Hazard Assessment, 2019; Dionísio et al., 2013; Goddard et al., 2011).

1.2 Climate change

According to IPCC special report (Masson-Delmotte, et al., 2018), a global warming of one degree Celsius relative to pre-industrial levels would result in severe and widespread impacts or risks for marine organisms. Nowadays, human-induced warming has already surpassed the one degree Celsius above pre-industrial levels and, at the current rate, it will reach one and a half degrees Celsius by 2040 (Table 1) (Masson-Delmotte, et al., 2018).

Table 1 – projected global mean surface temperature change for two time periods under four representative concentration pathways (RCPs) (after Abram et al., 2019).

Scenario	Near-term: 2031–2050		End-of-century: 2081–2100	
	Mean (°C)	*Likely* range (°C)	Mean (°C)	*Likely* range (°C)
RCP2.6	1.6	1.1 to 2.0	1.6	0.9 to 2.4
RCP4.5	1.7	1.3 to 2.2	2.5	1.7 to 3.3
RCP6.0	1.6	1.2 to 2.0	2.9	2.0 to 3.8
RCP8.5	2.0	1.5 to 2.4	4.3	3.2 to 5.4

Climate warming has particularly affected coral reef ecosystems, which have entered into a continuous state of decline, with one of the worst coral bleaching events having been recorded in 2017 (Mcleod et al., 2019). Bleaching is the process in which there is a loss of photosynthetic endosymbionts or photosynthetic pigments from host tissue (Glynn, 1993; Mies et al., 2018). These bleaching effects prompted severe declines in coral mass worldwide that have ecological, economic and social impacts, namely the decrease in fish production and tourism activity (Hussain & Ingole, 2020). Climate change, in particular the increase in seawater temperature, is the main cause of bleaching (Fitt et al., 2001; Glynn, 1993; Iglesias-Prieto et al., 1992). The exposure to thermal stress damages the PSII in the photosynthetic endosymbiont (Lesser, 2006). There is also the formation of reactive oxygen species that damage cells in both host tissues and the endosymbiont, which ultimately leads to the expulsion from the host (Baird et al., 2009; Lesser, 2006; Weis, 2008).

It is assumed that the ocean will increase its atmospheric CO_2 uptake. It is probable that the ocean uptake of anthropogenic emitted CO_2 in the last two decades was between 20-30% (Abram et al., 2019). When CO_2 is present in seawater, it reacts and forms carbonic acid, that dissociates into the ions bicarbonate and carbonate, while releasing hydrogen ions (Figure 3). The free hydrogen ions will then raise the partial pressure of CO_2, this is known as hypercapnia, a process that will ultimately lead to the reduction of oceanic pH (Caldeira & Wickett, 2003).
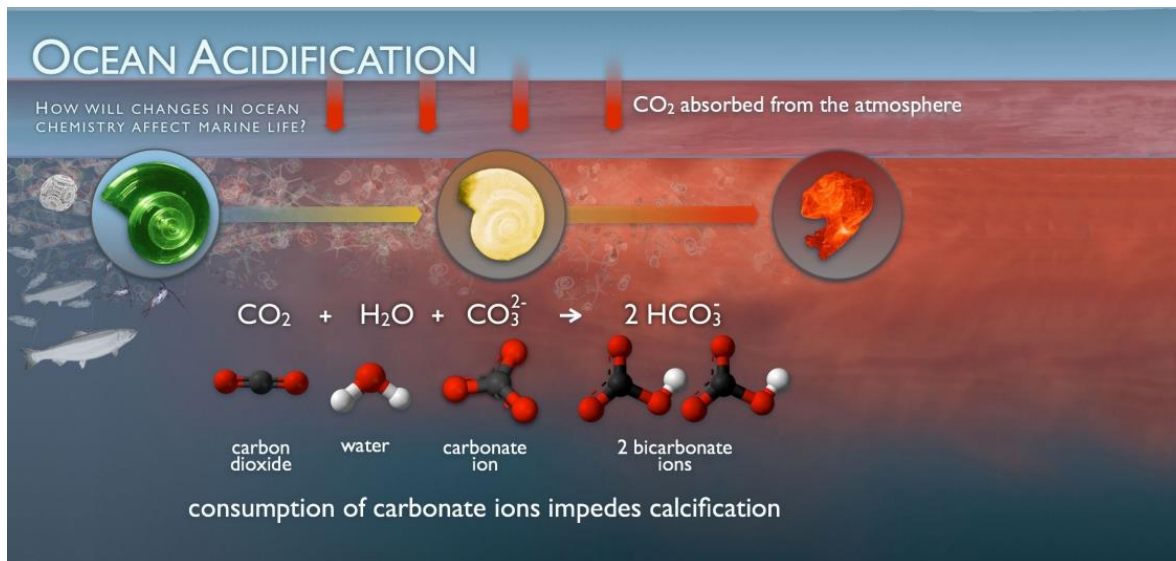
Figure 3 – Process by which ocean acidification leads to the formation of bicarbonate ions, and consequently the stop of calcification (after NOAA, 2020).

Calcium carbonate ($CaCO_3$) skeletal structures are widespread across marine animals, such as within corals, crustaceans, echinoderms and molluscs (Fabry et al., 2008). The evidence points to the fact that the calcifying rate in these organisms is controlled by the $CaCO_3$ saturation (Fabry et al., 2008). With climate change and ocean acidification, $CaCO_3$ saturation will decrease and, consequently, animals that depend from $CaCO_3$ to create defensive or supportive structures will have their early development stages hindered (Fabry et al., 2008).

When studying climate change, researchers tend to use domesticated animals to infer about their wild counterparts (Morgan et al., 2019). These animals that are used to examine a particular hypothesis are called model or study organisms (Koivula, 2011; Morgan et al., 2019). *Berghia stephanieae* is one of such organisms, being considered by some as the best model for ecological studies in the mollusc-dinoflagellate association (see below section 1.5) (Monteiro et al., 2019).

1.3 **Berghia stephanieae**

Berghia stephanieae (Mollusca, Gastropoda, Nudibranchia, Aeolidiidae) was first described as *Aeolidiella stephanieae* by Valdés, 2005. It has since been transferred from the genus *Aeolidiella* to *Berghia* by Carmona et al. (2013) supported on molecular phylogeny evidence using COI and 16S mitochondrial gene and H3 nuclear gene.

These sea slugs are hermaphroditic with a diaulic reproductive system, that is reproductive system with the gonoduct divided into two (Figura 4 – C), they lay eggs in spiral clusters (Figure 5) of up to 350 eggs each, that hatch into a short lecithotrophic larvae, a larvae that feeds from its yolk, or the larvae undergo metamorphosis within the egg and hatch as juveniles (Carroll & Kempf, 1990; Ghiselin, 1996; Monteiro et al., 2019; Valdés, 2005). After oviposition the eggs take 11 to 12 days to hatch. One to three days after hatching the larvae undergoes through metamorphosis and becomes a crawling juvenile (Carroll & Kempf, 1990).

Berghia stephanieae has an elongated translucent grey body that's wider on its anterior part. This sea slug's cerata, singular ceras, are arranged in equidistant straight rows and divided into four groups. The first group has five rows of cerata, the second and third groups have four rows of cerata, and the last seven rows of cerata are clustered together in a fourth and final group. The cerata increase in size from the first to the last group (Figure 4 - E), with those from this species being greyish with the distal third being opaque white; some specimens may present a slight orange tinge. Each ceras is composed of a digestive gland and a cnidosac, located in the apex of the ceras, where the sea slug stores the nematocysts incorporated from its prey (Figure 4 – B) (Obermann et al., 2012; Valdés, 2005).

Berghia stephanieae bears two rhinophores, which are chemossensorial organs, located atop the head, with six to 10 inconspicuous lamellae that cover the entire surface of the distal half of the rhinophore (Figure 4 – D) (Valdés, 2005).

This sea slug has oral tentacles that are twice as long as its rhinophores, being located adjacent to the mouth (Figure 4 – A). *Berghia stephanieae*, has a radula, with pectinate teeth, a concave base and a central cusp that is reduced to a small, triangular denticle. Each side of the cusp displays 20 - 25 long and pointed denticles, with the innermost and outermost denticles being shorter than those in the laterals (Valdés, 2005).
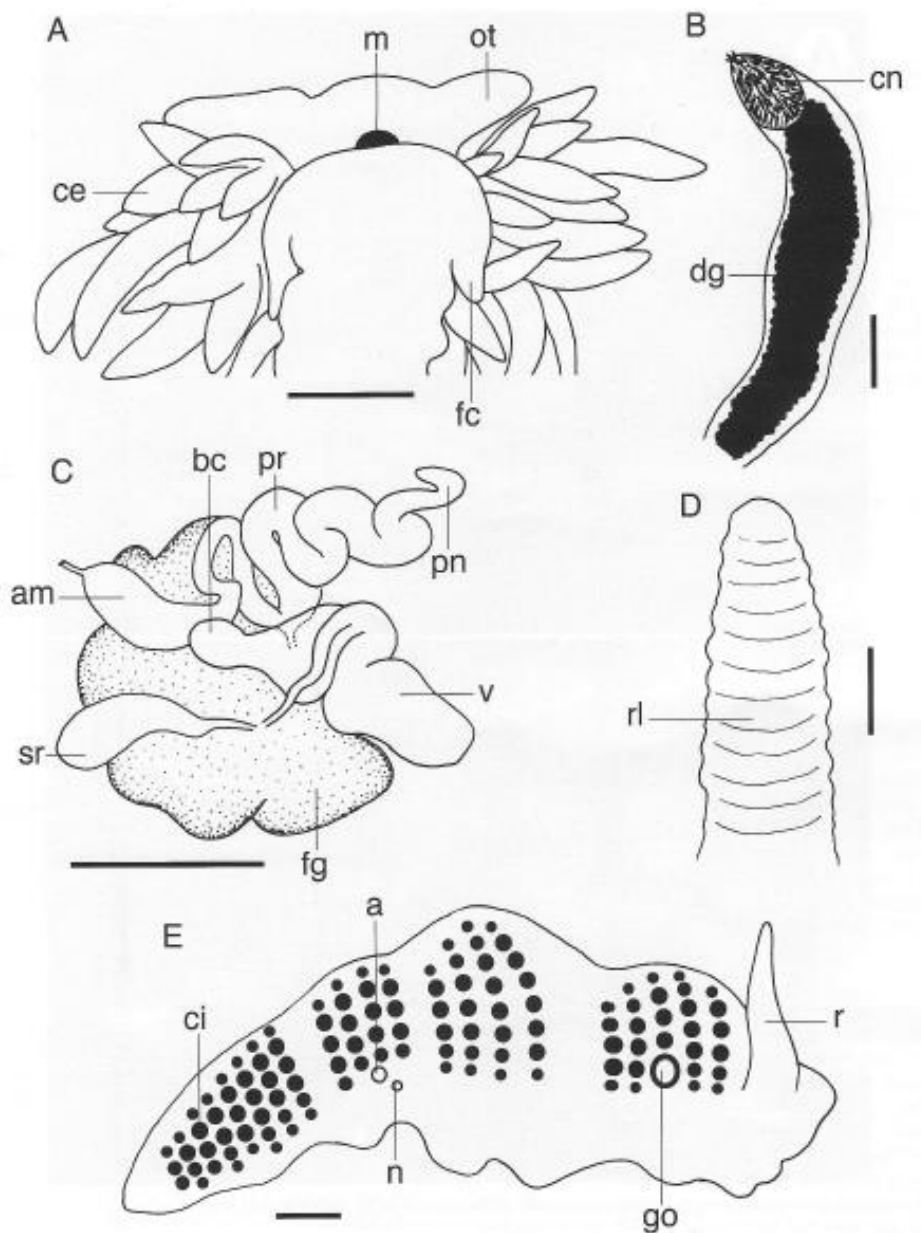
Figure 4 – Anatomy of a paratype of *Berghia stephanieae*. A – ventral view of the anterior portion of the animal (Scale bar = 1 mm). B – Ceras (Scale bar = 1 mm). C – *Berghia stephanieae* reproductive system (Scale bar = 1 mm). D – Rhinophore (Scale bar = 1 mm). E – Distribution of cerata in an animal, it is visible the genital aperture, the anus and the nephroproct (Scale bar = 2mm). Abbreviations – a) anus; am) ampulla; bc) bursa copulatrix; ce) ceras; ci) ceras insertion; cn) cnidosac; dg) digestive gland; fc) foot corner; fg) female glands; go) gonopore; m) mouth opening; n) nephroproct; ot) oral tentacle; pn) penis; pr) prostate; r) rhinophore; r1) rhinophoral lamella; sr) seminal receptacle; v) vagina (after Valdés, 2005).
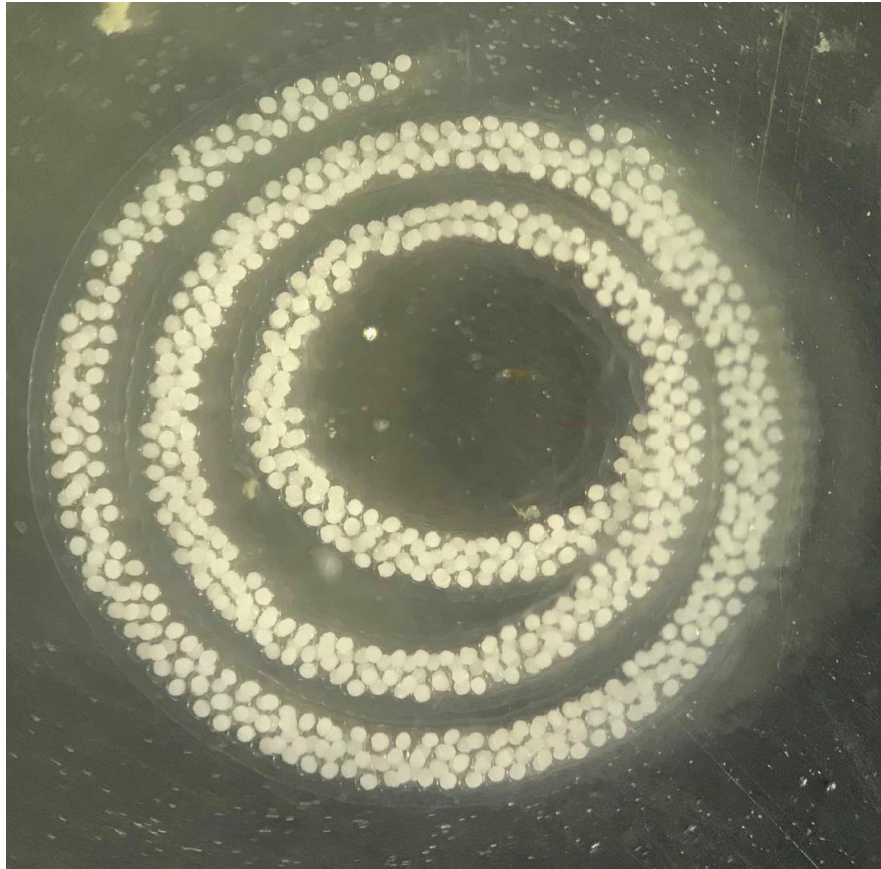
Figure 5- *Berghia stephanieae* egg cluster under a dissection microscope.

Berghia stephanieae is found in coral rubble in the Gulf of Mexico, in the shallow waters of the Florida Keys (Leal et al., 2012a; Mies et al., 2017; Valdés, 2005).

As aforementioned, this sea slug is an important study subject, as it is both a good ecological model for the study of evolution of mutualistic symbiosis and it is commercially relevant for aquarium trade, as explained in the section 1.5 (Monteiro et al., 2019).

1.4 Feeding strategy

Berghia stephanieae is a stenophagous animal. As mentioned above, stenophagous animals are the ones who have a highly specialized diet composed by a small number of prey species. In the case of *B. stephanieae* it feeds exclusively on the glass anemone, *Exaiptasia* sp (Figure 6). Some authors go as far as saying that this sea slug is a monophagous species that feeds exclusively on the species *E. diaphana*, described in detail in the section 1.6, a common plague to saltwater aquariums. This sea slug is known to be nocturnal. It utilize its oral tentacles to tap the base of

the anemone to elicit the closure of the anemone tentacles, to eat without being stung (Dionísio et al., 2013; Hediger, 1950; Leal et al., 2012a; Monteiro et al., 2019; Valdés, 2005).

The fact that this animal is stenophagous may represent an hinderance to its captive large-scale production due to the need of collection or culture of their prey in large quantities (Dionísio et al., 2013). However, that same fact makes *B. stephanieae* a good scientifical model because its prey, *E. diaphana*, is simple to culture under laboratory conditions, while being itself a model organism already established in the literature (Dungan et al., 2020; Monteiro et al., 2019; Weis et al., 2008).

Figure 6 – Close up of *Berghia stephanieae* and its prey the glass anemone *Exaiptasia diaphana*.

1.5 Economic and scientific interest

Berghia stephanieae is commonly traded in the marine aquarium industry under the name *B. verrucicornis*. This animal is popular amidst aquarium owners due to its specific feeding regime, which makes it one of the best natural ways to deal with *E. diaphana* infestations in home aquariums (Figure 7) (Monteiro et al., 2019; Valdés, 2005). Nowadays a single individual with a length ranging from 5 to 20 mm can retail to nearly 41€ (Blue Zoo Aquatics).

Figure 7– *Berghia stephanieae* individuals feeding on an *Exaiptasia diaphana.*

Berghia stephanieae is seen as particularly interesting among researchers as a model organism for laboratory research, due to its hardiness, regular oviposition, short embryonic and larval period, short generation time and ease of culture of its prey organism (glass anemones from genus *Exaiptasia*). These characteristics make this animal a good model to study larval ecology, neurodevelopment and energetics (Monteiro et al., 2019). Furthermore, as it retains zooxanthellae from its prey, this animal is regarded as likely the best model for ecological studies addressing the mollusc-dinoflagellate endosymbiosis, as well as the impact that climate change may have in stenophagous organisms and its transgenerational effects (Carroll & Kempf, 1990; Leal et al., 2012a; Mies et al., 2018; Monteiro et al., 2019)

1.6 **Exaiptasia diaphana**

Exaiptasia diaphana (Rapp, 1829) (Cnidaria, Anthozoa, Actiniaria, Aiptasiidae), commonly known as glass anemone, is a symbiotic anemone that establishes symbiosis with *Symbiodinium* (Clark & Jensen, 1982).

Exaiptasia diaphana has a pedal disc of up to 10 mm diameter that is wider than the column of a living specimen. Its column is smooth and can reach up to 60 mm height. The oral disc can reach up to 10 mm diameter in preserved specimens and is surrounded by up to 96, smooth and long tentacles that taper towards the tips and measure up to 20 mm in length (Grajales & Rodríguez, 2014).

This anemone has a greyish-brownish translucent column with scattered spots distally. Oral disc and tentacles are greyish in colour, the tentacles present white transversal stripes. Its mouth is whitish and the actinopharynx is surrounded by a yellowish circle (Grajales & Rodríguez, 2014).

Exaiptasia diaphana thrives in many environments due to its ability to reproduce asexually, by pedal laceration, but also due to its trophic plasticity, being able to obtain energy both heterotrophically and autotrophically (Grajales & Rodríguez, 2014; Leal et al., 2012b). These characteristics coupled with the fact that this anemone differs from corals in the lack of carbonate skeleton, the preference for low light (<100 µmol.photon.m^{-2}.s^{-1}) and great ability to survive to bleaching events makes this anemone a popular model for cnidarian-microbiome symbiosis studies (Dungan et al., 2020).

Glass anemones can be found in ecosystems ranging from temperate to tropical (Grajales & Rodríguez, 2014). These anemones are usually found in shallow, calm and protected waters, up to five meters depth. Its geographical distribution includes places like the Gulf of Mexico, the Caribbean sea, the coast of Brazil, Galapagos Islands, Australia and also the Mediterranean sea, western Africa, eastern and western Pacific coasts, Saint Helena Island and Panama (Grajales & Rodríguez, 2014; Leal et al., 2012b).

Exaiptasia diaphana was first proposed as a suitable model for cnidarian-dinoflagellate symbiosis studies by Weis (2008). The glass anemone, when compared to corals, has advantages that make it a good study model, namely having individual polyps of variable sizes, having a fast growth rate, and, due to its hardiness and method of reproduction, is easy to create a monoclonal community (Figure 8) in the laboratory; moreover specimens of *E. diaphana* can be completely cleared of its symbionts and kept in a symbiont-free state to be latter re-infected with new symbiont strains (Weis et al., 2008).

Figure 8 – *Exaiptasia diaphana* monoclonal community.

1.7 Chlorophyll fluorescence

Chlorophylls are bioactive compounds which are widely present in nature (Dharna et al., 2017). These pigments have an important role in photosynthesis. The two main types of chlorophylls are chlorophyll a (Chl a) and chlorophyll b (Chl b), being that Chl a is a light harvesting pigment, converting light energy into chemical energy, and Chl b absorbs light which it transfers to Chl a (Dharma et al., 2017).

Chlorophylls are characteristic pigments in photosynthetic plants and algae that can be found as a pigment-protein complex in the photosystem I (PSI), photosystem II (PSII), and light-harvesting complexes (LHCs). The light energy absorbed by the chlorophylls can be used to drive photosynthesis (photochemistry) or it can be re-emitted as heat or as light (fluorescence). All these three processes occur at the same time in competition with each other (Murchie & Lawson, 2013; Roy et al., 2011).

Chlorophyll fluorescence can be used as a non-invasive measurement of PSII activity (Murchie & Lawson, 2013). However, these measurements, based on the analysis of the fluorescence of the PSII, can be affected by ambient light (Schreiber et al., 1986). To overcome this problem, modulating systems were invented (Schreiber et al., 1986). These systems apply the measuring light at a known modulated frequency, which the detector is set to measure, that way the detector will only measure the fluorescence resultant from the excitation by the measuring beam (Murchie & Larson, 2013). The method of pulse amplitude modulated (PAM) fluorometry was introduced by Schreiber in 1986 to study photosynthesis in plants and has since then become a universal technique to study corals (Leal et al., 2015), seagrasses (Beer et al., 1998), macroalgae (Cruz et al., 2015), microphytophenthos (Serôdio et al., 2009, Vieira et al., 2013) and sea slugs (Cartaxana et al., 2017, 2019; Cruz et al., 2013, 2015).

PAM fluorometry measurements are used to gather fluorescence parameters, from which some of the most used are the minimum chlorophyll fluorescence (F_0) (Figure 7), that is measured in dark-adapted samples, the maximum fluorescence (Fm), recorded by closing all the samples reaction centres with a saturating pulse and the maximum quantum yield of PSII (Fv/Fm) with the variable fluorescence (Fv) being the difference between F_0 and Fm (Murchie & Lawson, 2013).

PAM fluorometry measurements present some drawbacks when applied to living motile organisms (Cruz et al., 2012). For instance, when calculating Fv/Fm, if the organisms moves between the measurement of F_0 and Fm there will be slight changes in the fluorescence recorded by the PAM fluorometer. However, PAM allows to perform highly specific and sensitive, non-destructive measurements, as well as real-time and rapid measurements (Cruz et al., 2013; Vieira et al., 2013).

1.8 Objectives

The present work aimed to: 1) validate a non-invasive methodology to determine the abundance of photosynthetic dinoflagellate endosymbionts in *B. stephanieae*; 2) evaluate the effect of different light and trophic regimes in the abundance and photosynthetic efficiency of photosynthetic dinoflagellate endosymbionts present in *B. stephanieae*; and 3) compare the profiles of photosynthetic pigments displayed by photosynthetic dinoflagellate endosymbionts present in *B. stephanieae* and *E. diaphana*.

2 Materials and methods

2.1 Species identification through DNA Barcoding

2.1.1 DNA extraction

One juvenile specimen of the batch of captive cultured sea slugs acquired from TMC Iberia (Lisbon, Portugal), and traded as *Berghia stephanieae*, and one specimen of glass anemone stocked in the laboratory and assumed to be *Exaiptasia diaphana* were preserved in ethanol (96%) until their taxonomic identity could be confirmed using DNA Barcoding. Total extraction of the genomic DNA was performed using DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions.

2.1.2 Polymerase Chain Reaction amplification of 16 S/COI genes for *Berghia stephanieae*

The amplification of partial regions of mitochondrial 16S rDNA and cytochrome c oxidase subunit I (COI) was carried out using the following primers: 16SAR (5'-CGCCTGTTTATCAAAAACAT-3') and 16SBR (5'- CCGGTCTGAACTCAGATCACGT-3') for 16S rDNA (Palumbi et al., 1991) and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') for COI (Folmer et al., 1994).

The reactions of the Polymerase Chain Reaction (PCR) were performed with a final volume of 25µL: 12.5µL of DreamTaq PCR Master Mix, which contains Dream Taq DNA polymerase, optimized Dream Taq buffer, MgCl2 and dNTPs (Fermentas, Vilnus, Lithuania) (Fischer Scientific), 0.2µM of each primer, and one µL of template DNA.

DNA amplification was performed using a Veriti 96-well Thermal Cycler (Applied Biosystems) with the following parameters for COI: an initial denaturation of five min at 95 °C followed by 35 cycles of one min at 95 °C, denaturation stage, 45 s at 45 °C (the primer-specific annealing temperature) and one min 30 s at 72 °C, for extension. After these 35 cycles there was a final extension at 72 °C for seven min. For 16S an initial denaturation stage at 95 °C, five min, followed by 40 thermal cycles of a denaturation phase at 95 °C, 45 s, a stage at 52 °C (the primer specific annealing temperature), 45 s and an extension stage of one min at 72°C were used, with a final extension phase of seven min at 72 °C being employed after these cycles.

To ensure that DNA had been successfully amplified, an agarose gel electrophoresis stained with Gel red (Biotium) was performed with the amplification products and later visualized under UV light.

2.1.3 PCR amplification of 16 S/COI genes for *Exaiptasia diaphana*

The amplification was performed using a similar method to that described above for *B. stephanieae* (including the use of the same primers).

The reactions of the PCR were performed with a final volume of 25µL: 12.5µL of DreamTaq PCR Master Mix, which contains Dream Taq DNA polymerase, optimized Dream Taq buffer, MgCl2 and dNTPs (Fermentas, Vilnus, Lithuania) (Fischer Scientific), 0.2µM of each primer, and one µL of template DNA.

DNA amplification was performed using a Veriti 96-well Thermal Cycler (Applied Biosystems) with similar parameters as those used for *B. stephanieae* with the following alterations: for COI the primer-specific annealing temperature used was 52°C. For 16S the extension stage used during the cycles was of two min instead of one.

To ensure that DNA had been successfully amplified, an agarose gel electrophoresis stained with Gel red (Biotium) was performed with the amplification products and later visualized under UV light.

2.1.4 DNA sequencing and analysis

The PCR-amplified fragments (16S/COI) were acquired from two independent reactions, with nucleotide sequencing being performed by Stab Vida (Portugal).
MEGA version 6 was used to reach the consensus sequence, which was then blasted ((Basic Local Alignment Search Tool, BLAST, National Center for Biotechnology Information, NCBI) on GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) to accurately identify the species.

2.2 Monoclonal culture of glass anemones Exaiptasia diaphana

One specimen of *Exaiptasia diaphana* was placed in one of the two 260-L glass (350 mm x 500 mm x 1500 mm) tank that was part of a 640-L life support system filled with artificial sea water, prepared with fresh water previously purified by reverse osmosis, with a V2 Pure 360 Reverse Osmosis System (Tropical Marine Centre, United Kingdom), and Red Sea Coral Pro Salt according to the manufacturer's instructions. Egg crate plates were placed in the bottom of the tank, as well as hanging from a PVC bar that crossed the tank lengthwise (Figure 9), to increase the surface area available for the fixation of new glass anemones. The anemone was let to asexually reproduce through pedal laceration of its basal peduncle and, after a month, one egg crate plate that had already been colonized with small clone glass anemones was placed in the second 260-L tank to increase the production of monoclonal glass anemones. Anemones were fed daily with newly hatched *Artemia* sp. nauplii (Artemia Koral, GMBH, Germany) and kept under a 12h Light: 12 h Dark photoperiod using two Hailea sunshine T5 80 W fluorescent lamps (80 µmol m^{-2} s^{-1}).

The 640-L life support system employed was equipped with a 120-L sump housing a Deltec SC 1455 internal protein skimmer, 0,006 m³ of 20 mm diameter bioballs, a TMC UV steriliser P1 of 55 Watts, two 300 W Eheim Jäger heaters and connected to a Hailea 500AC chiller with an Eheim universal 1200 pump. Water is returned to the tanks with an Eheim universal 2400 pump that creates a flow of 380 L/h to each tank. Water temperature was kept stable at 26 °C, as well as salinity that was kept stable at 35 by using an osmoregulator connected to a reverse osmosis water reservoir to compensate for water evaporation. To ensure the quality of the water, 70-L water changes were done twice a week. The ammonia, nitrite and nitrate compounds were always below the 0.10 ppm level, this was checked biweekly with API and Salifert colorimetric tests.
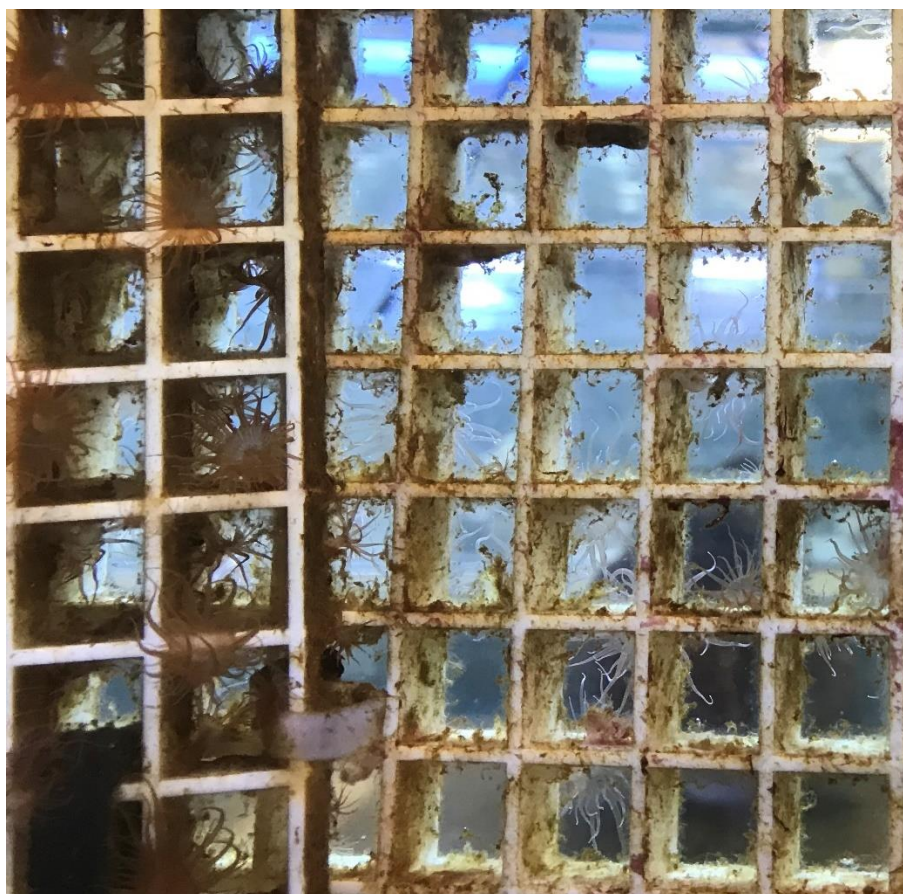
Figure 9– *Exaiptasia diaphana* growing in egg crate.

2.3 Captive culture of sea slugs **Berghia stephanieae**

Thirty captive cultured *B. stephanieae* juveniles were acquired from TMC Iberia (Lisbon, Portugal). Specimens were acclimated and housed in an identical life support system to that already detailed above for glass anemones. The sole distinct feature of this system was that one of the two 260-L (350 mm x 500 mm x 1500 mm) glass tanks was equipped with four 21-L (380 mm x 200 mm x 280 mm) plastic boxes with a sandy bottom, composed by a layer of 1-mm sand, followed by a layer of 2-mm coral gravel and lastly a layer of 10-mm oyster shell fragments, and a clay pot to serve as a hideout. These boxes were placed in a water bath (Figure 10). Two of the boxes housed 15 juveniles each and the other two boxes were left empty to receive new-borns. The sump was equipped with a Deltec SC 1455 internal protein skimmer, 0,006 m³ of 20 mm diameter bioballs, a TMC UV steriliser P1 of 55 Watts, two 300 W Eheim Jäger heaters and connected to a Hailea 500AC chiller with an Eheim universal 1200 pump. Water was returned to the tanks with an Eheim universal 2400 pump that creates a flow of 380 L/h to each tank. The boxes which housed the animals each had an individual water supply with

a flow of 12L/h. The organisms were maintained at a photoperiod of 10h Light: 14h Dark. The artificial water was produced as described in the section 2.2 and was kept at 27 °C, with salinity at 35. Water quality was ensured with biweekly 70-L water changes. The ammonia, nitrite and nitrate were kept below the 0.10 ppm level. The levels of these compounds were checked weekly with API and Salifert colorimetric tests.

During captive culture *B. stephanieae* specimens were fed *ad libitum* everyday with *E. diaphana* specimens from the monoclonal culture detailed above. Adults were fed with large anemones (5 to 8 mm of pedal disk diameter) , while juveniles were supplied with smaller sized specimens (2 to 4 mm of pedal disk diameter) (Monteiro et al., 2019).
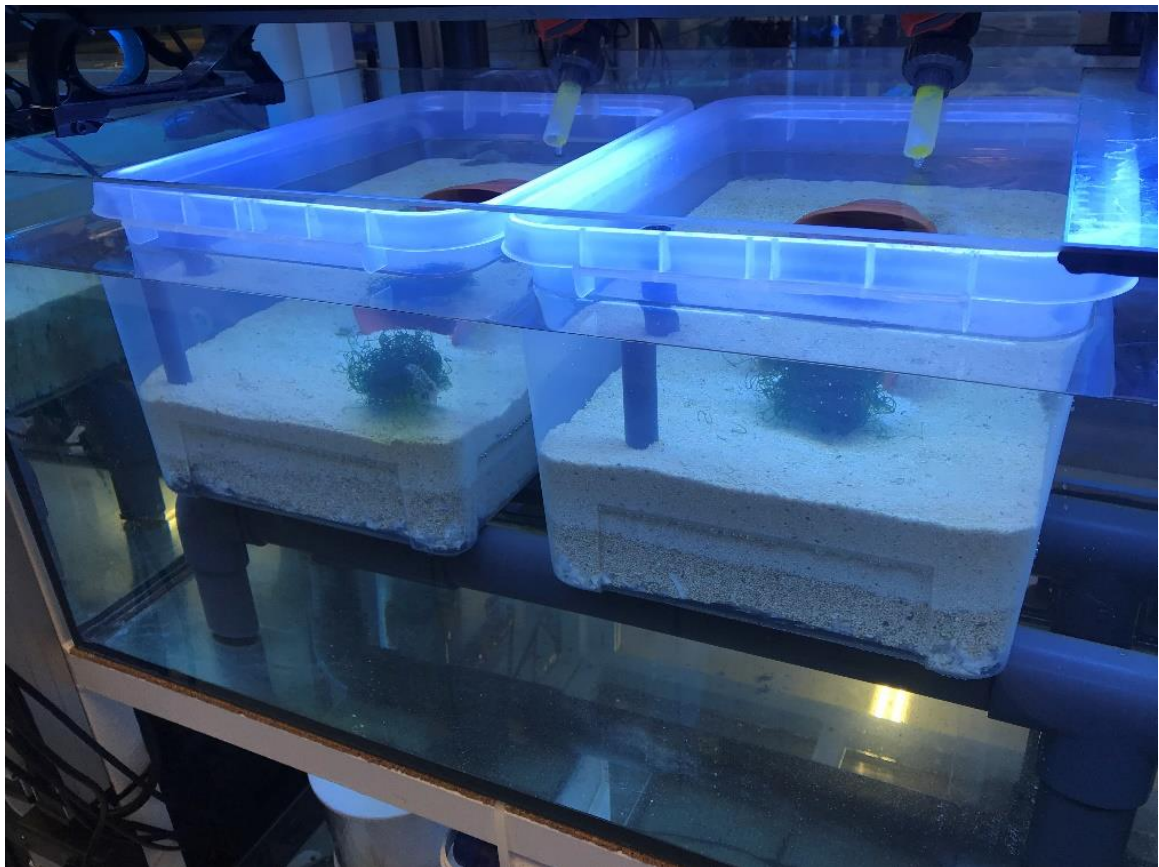

Figure 10 – *Berghia stephanieae* rearing system.

2.4 Relation between the abundance of photosynthetic endosymbionts of B. stephanieae *and* F_o *fluorescence parameter*

To establish a non-invasive methodology to access the number of photosynthetic dinoflagellate endosymbionts in *B. stephanieae*, 10 nudibranchs were paired and distributed over five 60-L (300 mm x 400 mm x 500 mm) aquariums, fitted with an Eheim thermocontrol 50 heater (50 W) and an Eheim Liberty 75 trickle filter. These aquariums were filled with synthetic sea water, made with fresh water previously purified by reverse osmosis, with a V2 Pure 360 Reverse Osmosis System (Tropical Marine Centre, United Kingdom), and Coral Pro Salt produced by Red Sea according to the manufacturer's instructions.

PAM measurements and symbiont counting were performed in cerata of fed organisms and animals starved for a period of 5 or 10 days. Before each PAM measurement, each pair of sea slugs was dark adapted for 45 min. A ceras was collected from each animal and F_o was measured. The ceras were then smeared between a slide and a cover glass and multiple pictures were taken to cover the whole area of the cover glass. All pictures were taken with a light microscope Leica DM2500 (Leica Microsystems GmbH, Germany) coupled to Leica ICC50 w Camera (Leica Microsystems GmbH, Germany). Images were then analysed with the help of ICY bioimage analysis software (as described by de Chaumont, et al., 2012) and ImageJ.

2.5 Effect of light intensity and starvation on the abundance and photosynthetic efficiency of Berghia stephanieae*'s photosynthetic endosymbionts*

To access the impact of light intensity and feeding regimes of the host on the photobiology of the photosynthetic dinoflagellate endosymbionts hosted by the nudibranch *B. stephanieae*, 20 sea slugs were randomly distributed under four different experimental treatments combining two light intensities and two feeding regimes. As for the light intensity factor, two intensities were tested: 80 µmol photon m^{-2} s^{-1} (High Light - HL), as used by Monteiro et al. (2019) and 10 µmol photon m^{-2} s^{-1} (Low Light - LL). The two different feeding regimes tested were *B. stephanieae* provided with symbiotic *E. diaphana* and *B. stephanieae* deprived of any feeding (starved individuals). Fed *B. stephanieae* under high light were considered as control organisms.

Glass anemones, *E. diaphana*, were cultured as referred above (please see section 2.2).

Seaslugs were kept individually in 2 customized PVC trays similar to the ones used by Carvalho et al. (2018) (Figure 11) , with the difference that our trays were composed only by the top portion. Sea slugs were stocked only on the centre rows to avoid differences in light intensity due to light diffusion. The pits were kept in a water bath, within the system used to rear the sea slugs, to avoid any additional stress. Light was provided by two Hailea sunshine T5 80W fluorescent lamps (80 µmol photon m^{-2} s^{-1}), under a 10h Light: 14h Dark photoperiod. Feeding was performed after every PAM measurement with the placement of two medium size anemones in the pits of the fed individuals.

PAM fluorometry measurements were made as described in the section 2.8, every other day, starting at day 0 and ending on day 14, at the same time of the day, approximately at the middle of the daytime photoperiod.
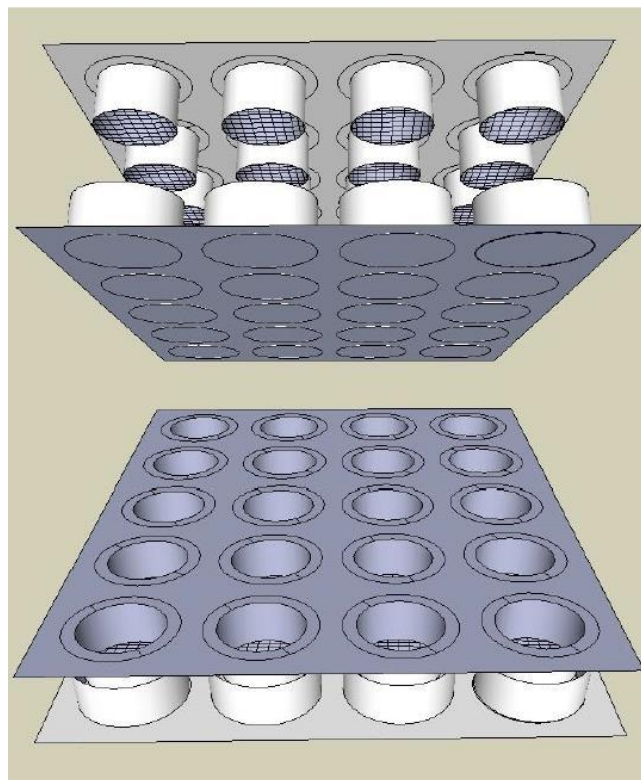

Figure 11 – Pits used to keep the sea slugs during the experiment, only the top tray was used (after Carvalho & Calado, 2018).

2.6 Effect of different trophic regimes on the abundance and photosynthetic efficiency of Berghia stephanieae's photosynthetic endosymbionts

To further evaluate the impact of different trophic regimes of *B. stephanieae* on the photobiology of its photosynthetic dinoflagellate endosymbionts, a new experiment was performed with 30 sea slugs where 10 were fed with symbiotic anemones (Fed S), 10 fed with bleached anemones (Fed B) and 10 starved (Starved).

Glass anemones were produced as referred in the sections 2.2. To produce bleached anemones, 60 *E. diaphana* were placed in two 360-L (300 mm x 1000 mm x 1200 mm) PVC boxes, Domplex 702P4N0 (Domplex SA., Portugal), with a lid, Domplex 749L (Domplex SA., Portugal), and were kept in the dark for three months. Each box was filled with natural water captured from Ria de Aveiro during high tide. The boxes were equipped with an airlift system (Figure 12) powered by an air pump to promote water circulation and an oyster shell biofilter. To ensure water quality a water change of 50 L was done weekly.

Figure 12– Airlift system used to keep *Exaiptasia diaphana* on the dark to bleach them.

This experiment was carried out in the system already described in section 2.5, with two customized PVC trays housed in a water bath within the system used to rear the sea slugs. Light was provided by two Hailea sunshine T5 80 W fluorescent lamps. Fed sea slugs were supplied

with two large anemones (5 to 8 mm of pedal disk diameter) after every measurement being performed.

F_0 and Fv/Fm were measured, following the method described in section 2.8, every other day, starting at day 0 and ending on day 14, at the same time of the day (approximately at midpoint of the daytime photoperiod).

2.7 Effect of different trophic regimes on the photosynthetic pigment profile of photosynthetic endosymbionts hosted by B. stephanieae and E. diaphana

To ensure the success of the bleaching process and study the effect of different trophic regimes on the photosynthetic pigment profile of photosynthetic dinoflagellate endosymbionts hosted by *B. stephanieae* and *E. diaphana*, ten sea slug were divided by two 60-L (300mm x 400 mm x 500mm) aquariums, fitted with an Eheim thermocontrol 50 (50 watt) heater, and an Eheim Liberty 75 trickle filter. Each aquarium was filled with synthetic sea water at 35 salinity, made with RedSea Coral Pro Salt and reverse osmosis water, produced from fresh water with the help of a V2 Pure 360 Reverse Osmosis System (Tropical Marine Centre, United Kingdom). Five of these sea slugs were only fed with bleached anemones, while the other five were solely fed with symbiotic anemones for a period of 30 days. They were collected and flash-frozen with liquid nitrogen and stored at -80 °C until further analysis. Five symbiotic and five bleached anemones were also collected and stored as previously described.

Pigment extraction and analysis by means of high-performance liquid chromatography (HPLC) was then preformed on collected samples as described below (see section 2.9).

2.8 PAM fluorometry measurements

The measurements of F_0 and Fv/Fm were performed using a chlorophyll fluorometer Imaging-PAM, M-series, Mini-version (Walz, Germany) with an IMAG-K7 camera and an IMAG-Min/B head (blue) (Figure 13).

Prior to these measurements, sea slugs were dark adapted for 45 min to ensure that there was no non-photoquemical quenching and, as such, ensure an accurate measurement of F_0 (Murchie & Lawson, 2013). After this procedure, specimens were placed, one at a time, on a watch glass

with a minimal amount of water to restrain their movement and an actinic saturating pulse was emitted to record F_0. This procedure was done by always keeping the same settings on the chlorophyll fluorometer (Measuring light intensity – 2, Frequency – 1, Gain – 1, Damping – 1), so that the different values of F_0 being recorded could be compared. The settings employed were previously optimized in a preliminary experiment. The animals were then returned to the dark for half an hour. The animals were subsequently placed in the same watch glass and an actinic saturating pulse was emitted to record Fv/Fm. This procedure was performed separately from the F_0 to enable the usage of different PAM settings.
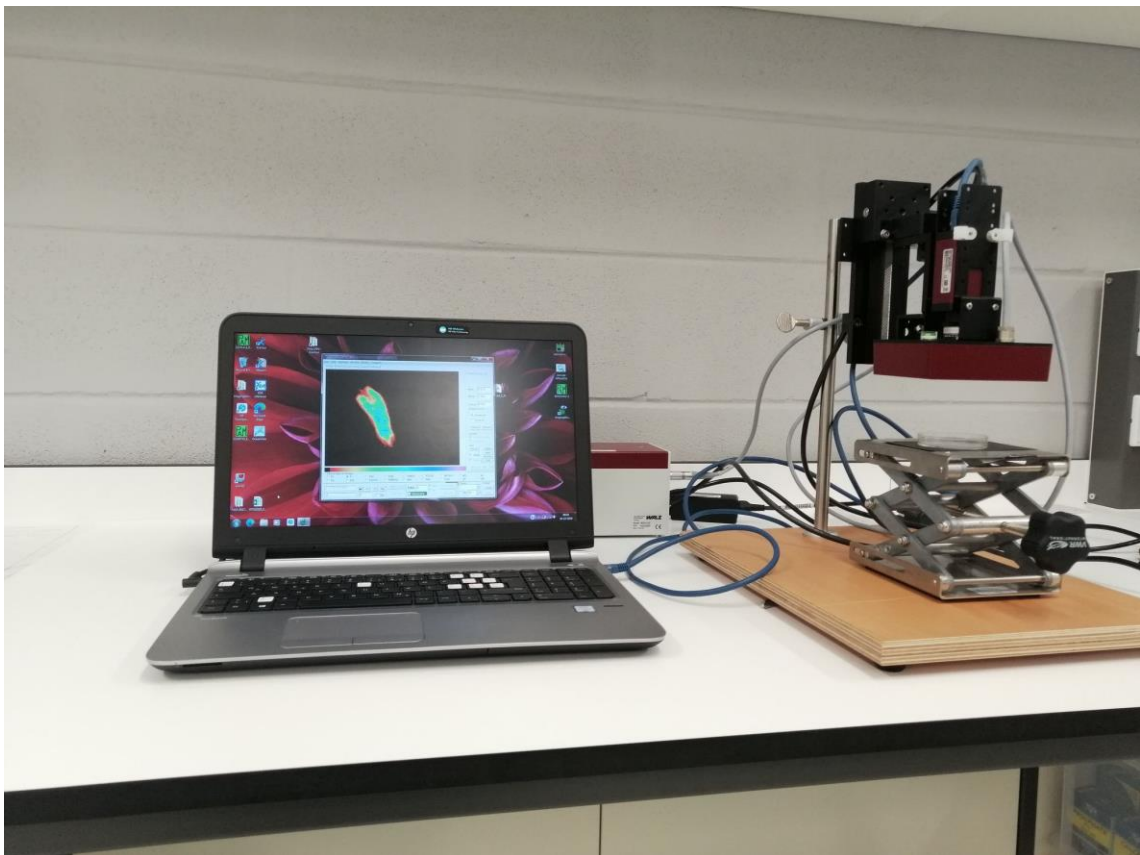

Figure 13 – Chlorophyll fluorometer used in the experiment.

2.9 Pigment extraction and analysis

Pigment extraction was done following a modified procedure from Mendes et al. (2007). Briefly: the freeze-dried samples were macerated with a cold extraction solution (95% methanol buffered with 2% ammonium acetate) and then sonicated in a VWR Ultrasonic cleaner USC-T for 30 s.

Samples were then stored in the dark at -20 °C for 20 min. Extracts were filtered, with a PTFE 0.22 µm filter and immediately injected in the HPLC.

A Shimadzu HPLC, Prominence-I LC 2030C 3D Plus (Shimadzu Corp., Japan) was used to analyse extracted pigments (Figure 14). To achieve the chromatographic separation of pigments a monomeric ODS C_{18} column was used. This method was described by Mendes et al. (2007) and consists in the use of a solvent gradient first described by Kraay et al. (1992), with a flow rate established at 0.6 mL min^{-1}, an injection volume of 100 µL and a run duration of 35 min.

The identification of photosynthetic pigments was performed by comparing absorbance peaks and retention times with available literature (Roy et al., 2011). Photosynthetic pigments concentration was calculated from the signals recorded in the photodiode array detector.

Figure 14– Identification of photosynthetic pigments performed using a Shimadzu HPLC.

2.10 Statistical analysis

To predict the concentration of photosynthetic dinoflagellate endosymbionts while using F_0 as a proxy, a linear regression model was used (Dytham, 2011).

The homogeneity of variances of data referring to the effects of light intensity and starvation on the abundance and photosynthetic efficiency of *Berghia stephanieae* photosynthetic dinoflagellate endosymbionts was tested using Levene's test. The distribution of data was tested with the Shapiro-Wilks test. Mauchly's test was used to test the sphericity of the data. As

ANOVA assumptions were violated data was analysed using non-parametric Wilcoxon's test (Dytham, 2011).

Data on the effect of different trophic regimes on the abundance and photosynthetic efficiency of *Berghia stephanieae* photosynthetic dinoflagellate endosymbionts was tested for homogeneity of variances and normality of distribution as described above. As data violated ANOVA assumptions a Kruskal-Wallis test and a post-hoc Mann-Whitney test for pairwise comparisons were used (Dytham 2011).

As the variances of the concentration of photosynthetic pigments of photosynthetic dinoflagellate endosymbionts hosted by *B. stephanieae* and *E. diaphana* was not equal, the differences on the profile of photosynthetic pigments of photosynthetic dinoflagellate endosymbionts hosted by *B. stephanieae* and *E. diaphana* were evaluated using a non-parametric Mann-Whitney U test (Dytham, 2011).

These statistical tests were performed using the software IBM SPSS Statistics 27 (IBM Corp., Armonk, N.Y., USA). All graphics presented were created using the software Microsoft Excel Office 365.

3 Results

3.1 Species identification through DNA Barcoding

DNA amplification was confirmed for both 16S (Figure 15) and COI (Figure 16) genes with an electrophoresis in agarose gel visualized under UV light.
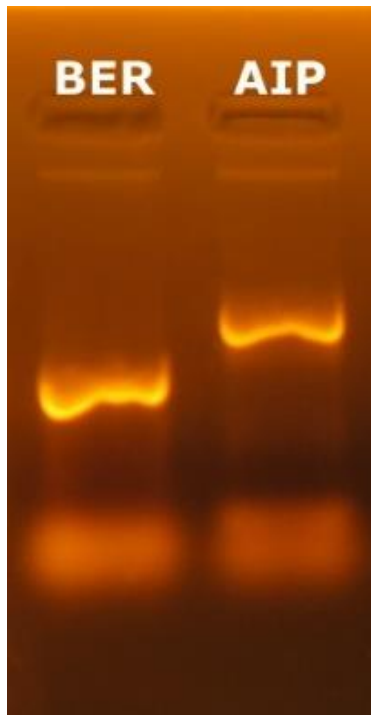
Figure 15 – Agarose gel from an electrophoresis of the 16S amplification results. BER – *Berghia stephanieae*, AIP – *Exaiptasia diaphana*.
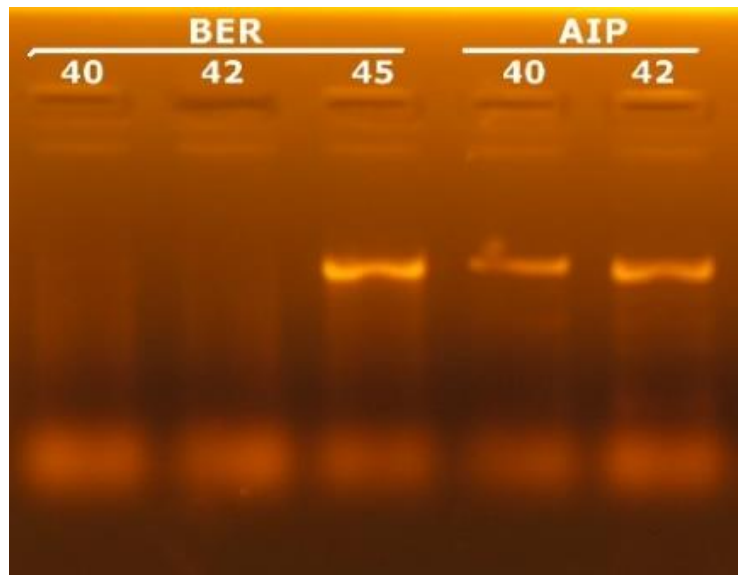

Figure 16 – Agarose gel from an electrophoresis of the COI amplification results, with the different annealing temperatures tested (40 °C, 42 °C and 45 °C). BER – *Berghia stephanieae*, AIP – *Exaiptasia diaphana*.

The BLAST analysis of the sea slug's 16S (Figure 17) and COI (Figure 18) consensus sequence against NCBI GenBank database resulted in the identification of this sea slug as a *B. stephanieae.* Both consensus sequence queries returned a result with high query cover (16S – 98%, COI – 96%), a low E value (both – 0.0) and a high percentage of identity (16S – 99.75%, COI – 99.70%).

Figure 17 – Output of the NCBI BLAST of the consensus sequence of the 16S gene of *Berghia stephanieae*.

Figure 18 – Output of the NCBI BLAST of the consensus sequence of the COI gene of *Berghia stephanieae*.

The anemone's COI consensus sequence BLAST against NCBI GenBank database (Figure 19) resulted in the identification of this anemone as *Aiptasia pulchella*, a taxonomic identification currently unaccepted for *E. diaphana.* This query returned a result with high query cover (99%), a low E value (0.0) and a high percentage of identity (99.56%).

Figure 19 – Output of the NCBI BLAST of the consensus sequence of the COI gene of *Exaiptasia diaphana*.

3.2 Relation between the abundance of photosynthetic endosymbionts of B. stephanieae and F_o fluorescence parameter

F_o was noticed to decrease when *B. stephanieae* endured starvation, while conspecifics fed with symbiotic anemones displayed a stable F_o (Figure 20).
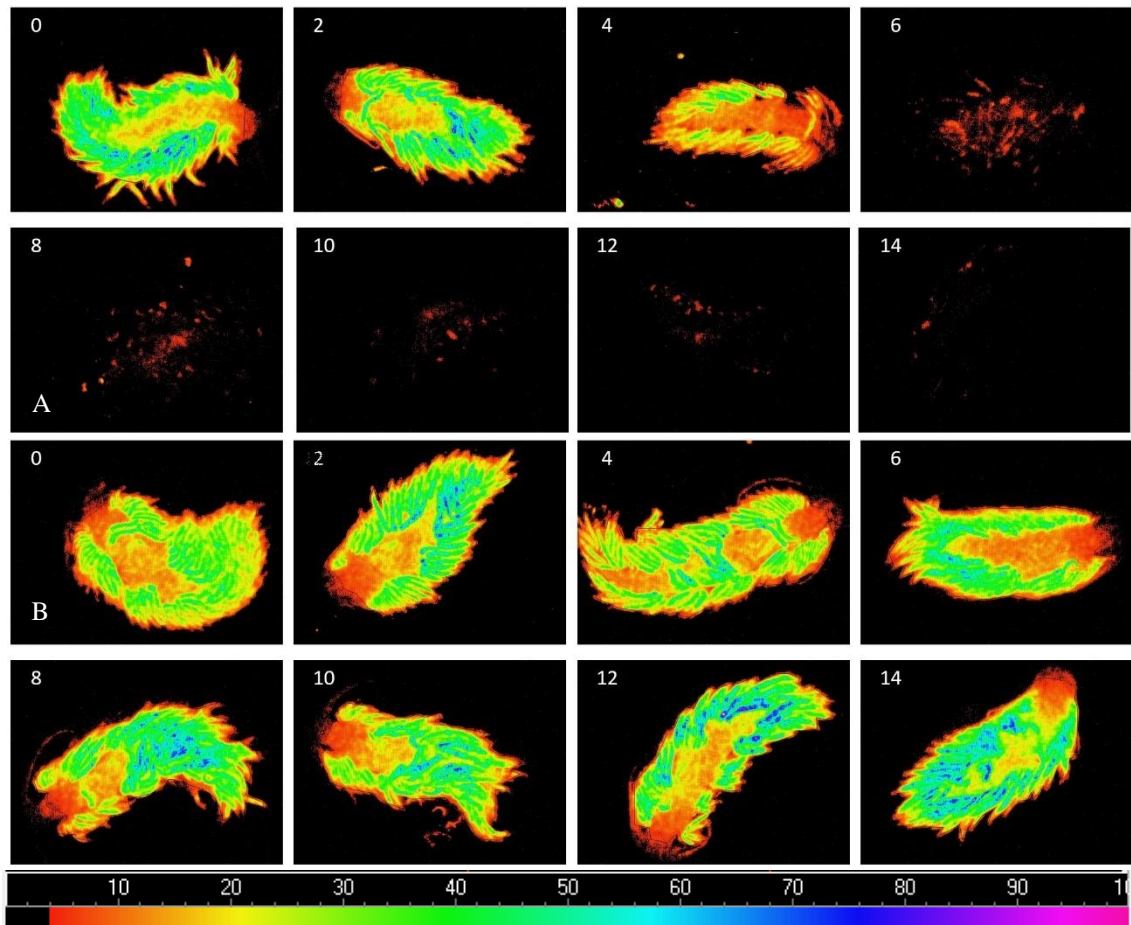
Figure 20 – Evolution of the fluorescence parameter F_0 measured with an Imaging-PAM fluorometer in *Berghia stephanieae* enduring starvation (A) or being fed with symbiotic anemones (*Exaiptasia diaphana*) for 14 days (B). Top left corner digit in each image represents the number days the animal has been exposed to each of the feeding treatments.

A linear regression was calculated to predict the concentration of symbionts in the cerata with the use of F_0. A significant regression equation was found (F $(1, 28) = 33.899$, p = 0.000), with an R of 0.740. The predicted concentration of symbionts equal to -758.73 + 15662 (F_0) symbionts mm of cerata^{-1} (Figure 21).
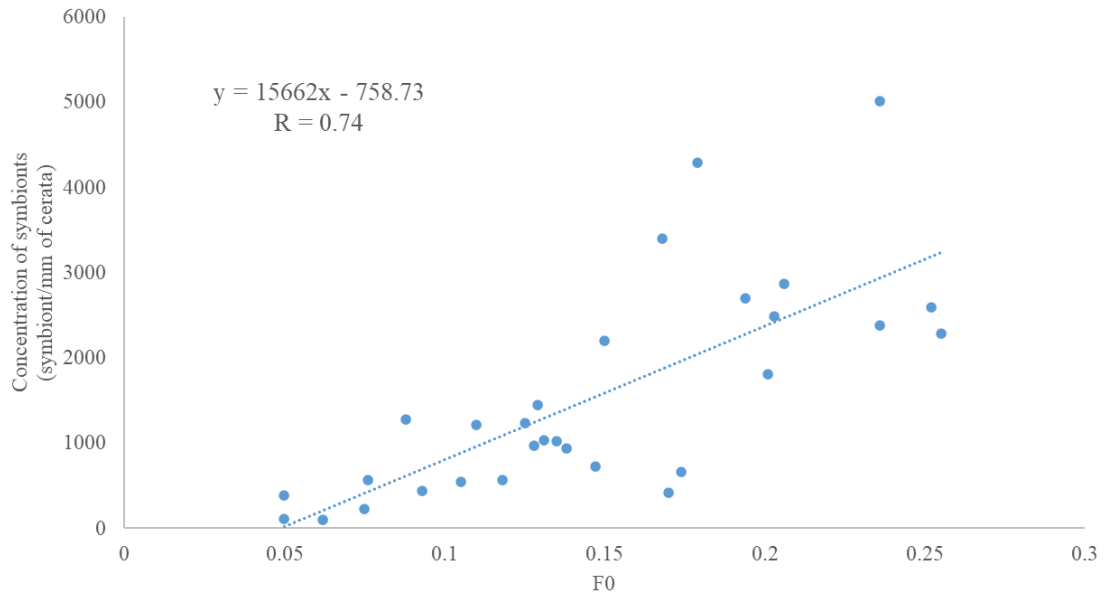
Figure 21 – Correlation between the number of photosynthetic dinoflagellate endosymbionts within cerata of *Berghia stephanieae* and the F_0 fluorescence parameter (n = 30).

3.3 Effect of light intensity and starvation on the abundance and photosynthetic efficiency of Berghia stephanieae*'s photosynthetic endosymbionts*

When testing for differences in the effect of light intensity and starvation on the abundance and photosynthetic efficiency of *B. stephanieae*'s photosynthetic dinoflagellate endosymbionts mortality was recorded across all treatments, with starved specimens displaying 30% mortality and fed ones only 10% mortality.

Starvation had a statistically significant effect in minimum chlorophyll fluorescence of sea slug's photosynthetic dinoflagellate endosymbionts (Z = -2.366, p = 0.018), with fed specimens displaying an average F_0 of 0.285 at the end of the experimental period, while starved conspecifics displayed an average F_0 of 0.022 (Figure 22).
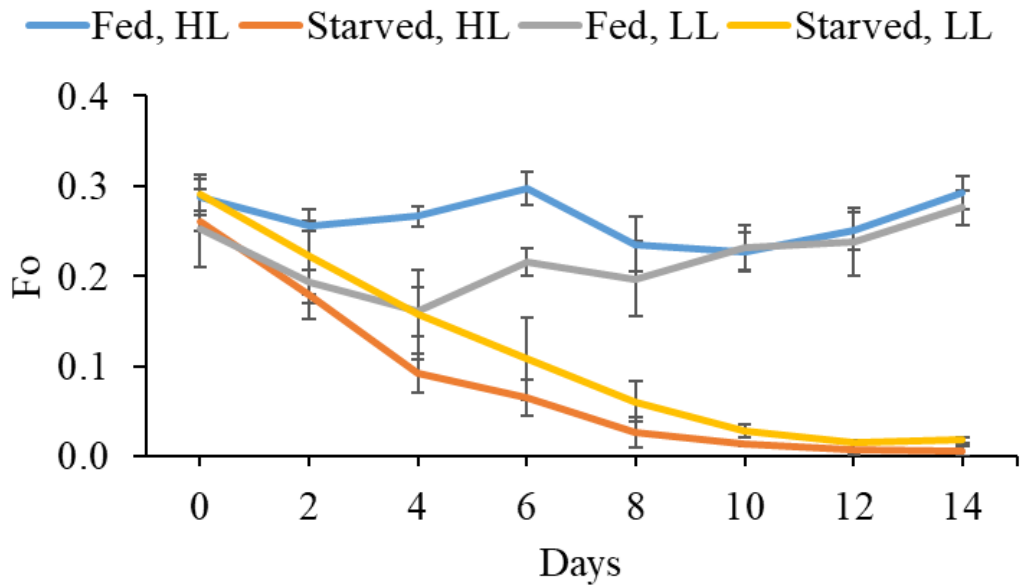
Figure 22 – Evolution of F_0 of *Berghia stephanieae* reared under different light intensities and feeding regimes (mean ± std.error, n = 5).

As for maximum quantum yield of PSII, starvation also had a statistically significant effect in Fv/Fm of sea slug's photosynthetic dinoflagellate endosymbionts (Z = -2.201, p = 0.028), with fed specimens maintaining an average Fv/Fm of 0.600, while starved conspecifics displayed an unmeasurable Fv/Fm (Figure 23).
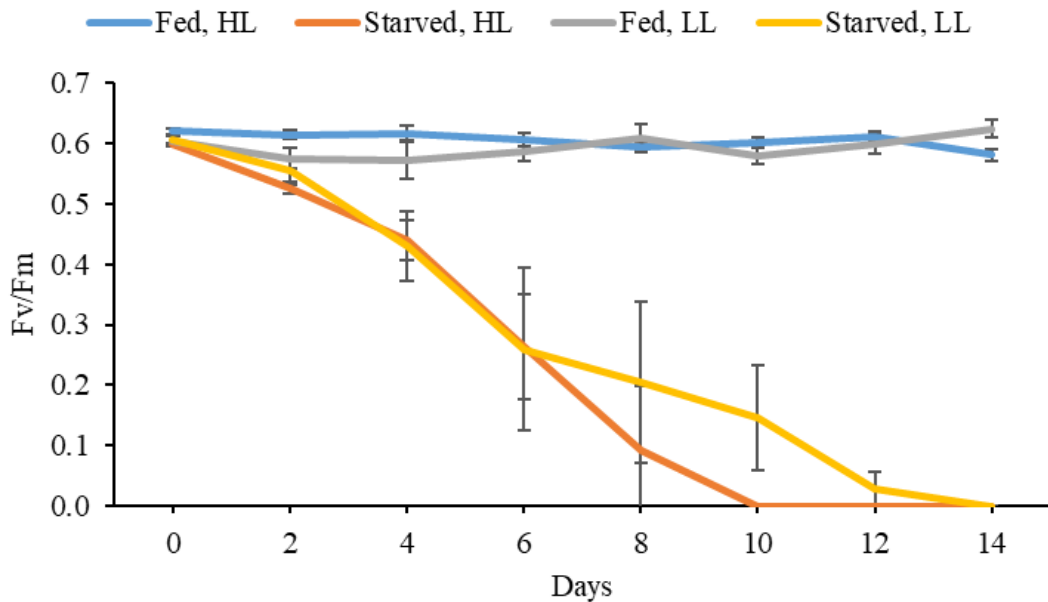
Figure 23 – Evolution of Fv/Fm of *Berghia stephanieae* reared under different light intensities and feeding regimes (mean ± std.error, n = 5).

Light was shown to have no significant impact in the abundance (Z = -1.355, p = 0.176) and photosynthetic efficiency (Z = -1.826, p = 0.068) of the sea slug's photosynthetic dinoflagellate endosymbionts.

3.4 Effect of different trophic regimes on the abundance and photosynthetic efficiency of Berghia stephanieae's photosynthetic endosymbionts

During the evaluation of the effects of different trophic regimes on the photobiology of photosynthetic dinoflagellate endosymbionts hosted by *B. stephanieae* mortality was recorded across all treatments, with starved specimens showing the highest mortality (at 60%) and fed specimens showing the lowest one (at 10%). Individuals fed with bleached anemones showed a mortality of 20%.

Statistically significant differences were found for the effect of different feeding regimes on minimum chlorophyll fluorescence (H (2) = 15.012, p = 0.001). The post-hoc Mann-Whitney test revealed that the individuals fed with symbiotic anemones presented a significantly higher F_o at 0.218 than its conspecifics fed with bleached anemones at 0.018, $U(N_{Fed\ S} = 9, N_{Fed\ B} = 8) = 0,000$, $Z = -3.475$, $p = 0.001$ and starved ones at 0.022, $U(N_{Fed\ S} = 9, N_{Starved} = 4) = 0.000$, $Z = -2.777$, $p = 0.005$ (Figure 24).
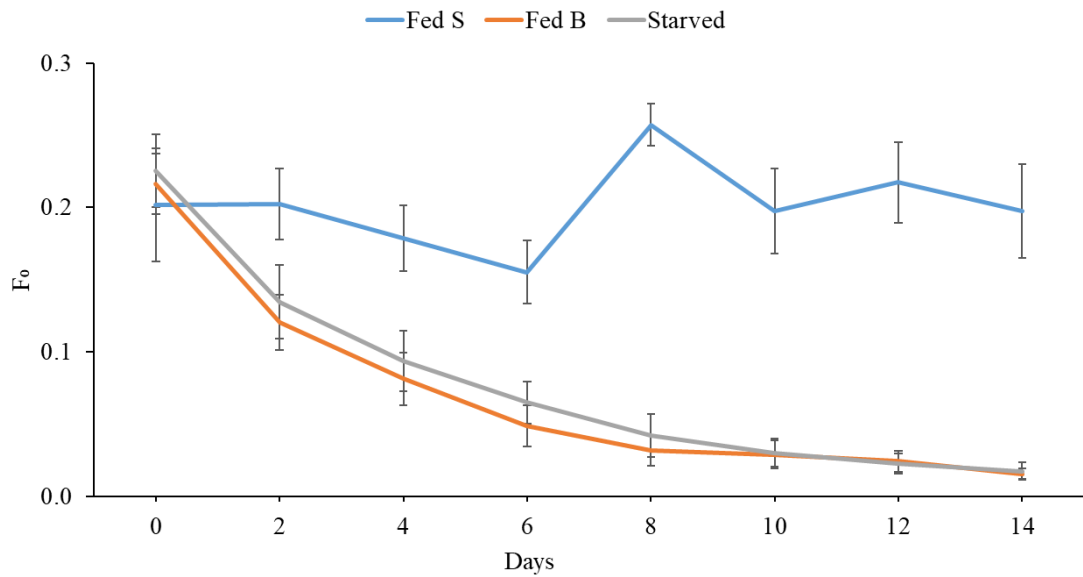
Figure 24 – Evolution of F₀ of *Berghia stephanieae* reared under different trophic regimes (mean ± std.error, n = 10).

The different feeding regimes also promoted a significant impact in the maximum quantum yield of PSII of *B. stephanieae*'s photosynthetic dinoflagellate endosymbionts (H (2) = 15.202, p < 0.000). A post-hoc Mann-Whitney test showed that sea slugs that were fed with symbiotic anemones had a significantly higher Fv/Fm at 0.473 than those fed with bleached anemones at 0.043, $U(N_{Fed\ S} = 9, N_{Fed\ B} = 8) = 0,000,\ Z = -3.486, p < 0.001$ and starved ones at 0.090, $U(N_{Fed\ S} = 9, N_{Starved} = 4) = 0.000, Z = -2.781, p = 0.005$ (Figure 25).
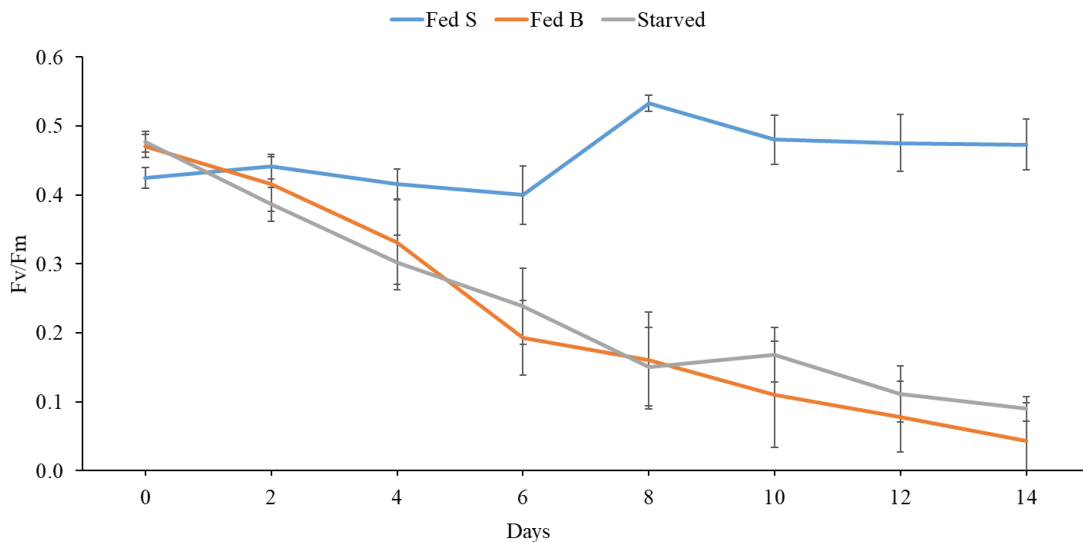
Figure 25 – Evolution of the Fv/Fm of *Berghia stephanieae* when exposed to different trophic regimes (mean ± std.error, n = 10).

3.5 Effect of different trophic regimes on the photosynthetic pigment profile of photosynthetic endosymbionts hosted by B. stephanieae and E. diaphana

Bleaching was successful as no photosynthetic pigments were found in *B. stephanieae* that were fed with bleached anemones. Furthermore, only two out of the five tested bleached anemones presented measurable photosynthetic pigments, at very low concentrations (peridinin – 0.03 mg g^{-1}; chlorophyll *a* – 0.01 mg g^{-1}).

The photosynthetic pigment profile recorded from both *B. stephanieae* and *E. diaphana* differed only in one of the recorded pigments, diadinochrome (Table 2).

There were statistically significant differences between chlorophyll c_2 (p = 0.027), peridinin (p = 0.016), diadinoxanthin (p = 0.028) and chlorophyll *a* (p = 0.016) concentrations within *B. stephanieae* and *E. diaphana* (Figure 26). The concentration of Chlorophyll c_2 in symbiotic anemones (0.805 mg of pigments/g of sample) was higher than that of sea slugs fed with them (0.049 mg of pigments/g of sample), $U(N_{Anemones} = 5, N_{Sea\ slugs} = 4) = 1.000, Z = -2.205, p = 0.027$. Peridinin concentration was also significantly higher in symbiotic anemones (2.436 mg of pigments/g of sample) than that of sea slugs fed with them (0.235 mg of pigments/g of sample), $U(N_{Anemones} = 5, N_{Sea\ slugs} = 5) = 1.000, Z = -2.402, p = 0.016$. Diadinoxanthin concentrations in symbiotic anemones (0.333 mg of pigments/g of sample) that was higher than that of sea slugs fed with them (0.029 mg of pigments/g of sample), $U(N_{Anemones} = 5, N_{Sea\ slugs} = 5) = 2.000, Z = -2.193, p = 0.028$. Lastly, statistically significant differences were also found between chlorophyll *a* concentrations in symbiotic anemones (1.631 mg of pigments/g of sample) that was higher than that of sea slugs fed with them (0.087 mg of pigments/g of sample), $U(N_{Anemones} = 5, N_{Sea\ slugs} = 5) = 1.000, Z = -2.402, p = 0.016$.

Table 2 – List of pigments recorded in both *Berghia stephanieae* and *Exaiptasia diaphana* samples, with average retention times and absorption maxima (λ_{max}).

Photosynthetic pigment (recommended abbreviation)	*B. stephanieae*	*E. diaphana*	Retention time (min)	λ_{max} (nm)
Peridininol	✓	✓	6,783	472
Chlorophyll c_2 (Chl c_2)	✓	✓	8,301	443, 580, 629
Peridinin (Peri)	✓	✓	10,521	476
Dinoxanthin (Dino)	✓	✓	14,264	418, 442, 472
Diadinoxanthin (Diadino)	✓	✓	14,764	420, 448, 478
Diadinochrome (Diadchr)	✗	✓	15,417	408, 430, 458
Chlorophyll a (Chl a)	✓	✓	24,258	430, 616, 663
Pheophytin a (Phe a)	✓	✓	26,888	505, 607, 665
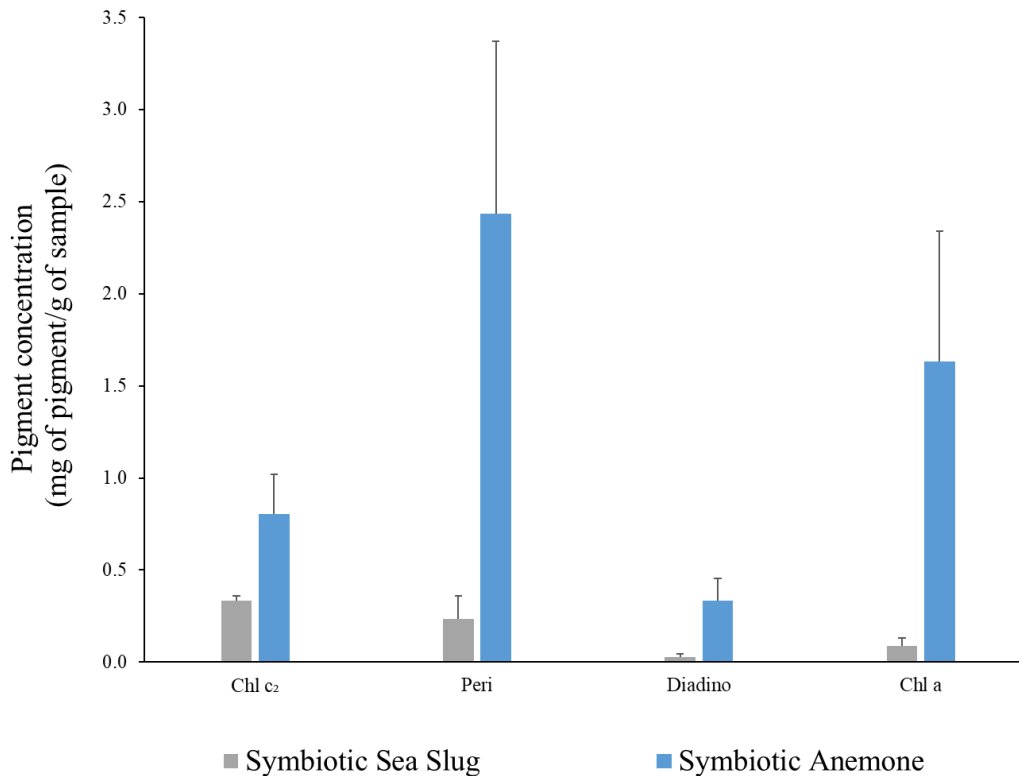
Figure 26 – Concentration of photosynthetic pigments within symbiotic anemones *Exaiptasia diaphana* and *Berghia stephanieae* fed with symbiotic anemones (mean ± std.error, n = 5).

4 Discussion

Climate change is an ever-growing problem affecting reef building organisms all over the world, with the Caribbean being the most affected region (Carpenter et al., 2008). Sea slugs, as reef inhabiting organisms, are also sensitive to climate change, and can be used together with chlorophyll fluorescence methods to study climate change impacts in the ecosystem (Goddard et al., 2011).

Berghia stephanieae's symbiotic relationship with *Symbiodinium* dinoflagellates was first studied in 1991 by Kempf, and was classified as a primitive mutualistic symbiosis. It has since then been the focal point of some ecological studies. These studies, however, use highly intrusive methodology, that sometimes require the sacrifice of the study subject (Leal et al., 2012a; Mies et al., 2018; Monteiro et al., 2019). Our data support that a non-invasive approach can successfully be used to study the concentration of photosynthetic dinoflagellate endosymbionts within *B. stephanieae*. A linear regression was established between the photosynthetic parameter F_o and the photosynthetic dinoflagellate endosymbiont abundance within *B. stephanieae*. The aforementioned linear regression allows the use of the fluorescence parameter F_o as a proxy measurement for the abundance of photosynthetic dinoflagellate endosymbionts of *B. stephanieae* and as such it opens new ways to the study of the mollusc–photosynthetic endosymbiont symbiosis without the need to sacrifice animals.

In every treatment where sea slugs were deprived of acquiring new photosynthetic dinoflagellate endosymbionts, either by starvation or by being fed bleached anemones, the values of F_o recorded were very low within four to eight days. This finding framed within the aforementioned correlation, evidenced that during the experimental trials performed sea slugs lost their photosynthetic dinoflagellate endosymbionts over time. This finding was somehow expected, as Monteiro et al. (2019) referred that the association between *B. stephanieae* and Symbiodiniaceae did not meet the criteria established for a mutualistic relationship. Indeed, the retention time of Symbiodiniaceae by *B. stephanieae* is short (3-5 days), with this sea slug being unable to sequester free-living Symbiodiniaceae and its growth being unaffected by the presence or absence of dinoflagellates (Kempf, 1991; Monteiro et al., 2019).

Some sea slugs are known to digest their photosynthetic dinoflagellate endosymbionts (Kempf, 1991). A study of the faecal matter of four sea slugs, *Melibe pilosa, Melibe* sp., *Pteraeolidia ianthina* and *Baeolidia moebii*, detected the presence of deteriorated photosynthetic

endosymbiont cells (Kempf, 1984). This finding indicates that these species may obtain some nutrients by digesting some of their photosynthetic endosymbionts when starved (Kempf, 1984). Our data showed no statistical difference when comparing the evolution of F_o of *B. stephanieae* individuals fed with bleached anemones or starved ones. This alongside the fact that Kempf (1991) found no evidence of active digestion of the photosynthetic dinoflagellate endosymbionts of *B. stephanieae* indicates that the loss of these symbionts is not due to a lack of supply of dietary nutrients, but rather due to the failure of getting new symbionts from its diet.

Regular feeding is needed to replace the photosynthetic dinoflagellate endosymbionts that *B. stephanieae* loses regularly through defecation (Kempf, 1991). In our experiment every sea slug that was kept in an environment with food available *ad libitum* (glass anemones with photosynthetic dinoflagellate endosymbionts) kept its F_o and Fv/Fm relatively stable throughout the experimental period. This finding is in line with the fact of *B. stephanieae* having to regularly replenish its photosynthetic dinoflagellate endosymbionts to keep their abundance and photosynthetic efficiency constant.

The use of non-invasive chlorophyll fluorescence methods, although promising, should be approached carefully, when employed on motile organisms, as their movement can hinder specific photobiology studies. Without the immobilization of the sea slug, every slight movement will cause non-physiological changes in the fluorescence signal emitted by the animal, which will ultimately compromise fluorescence studies such as steady-state light-response curves, rapid-response curves and Fv/Fm (Cruz et al., 2012; 2013).

The photosynthetic pigment profile of *E. diaphana* differed only from the profile recorded from *B. stephanieae* in the presence of Diadchr. This may be due to *B. stephanieae* presenting a lower concentration of photosynthetic pigments, as it was revealed by our data on the photosynthetic pigments Chl c_2, Peri and Diadino, and as such Diadchr may have been present in levels below the detection limit of the equipment.

The sea slugs fed with bleached anemones did not present any detectable concentration of photosynthetic pigment, so they may be called aposymbiotic, as if any symbiont was indeed present, there was no evidence of photosynthetic activity. Bleached anemones, on the other hand, were found to display residual amounts of Chl c_2, Peri, Diadino and Chl a. As such, it may not be fully accurate to call *B. stephanieae* fed on bleached anemones, aposymbiotic. Although bleached anemones were kept in the dark for long periods of time, they seem to still harbour a

small number of photosynthetic dinoflagellate endosymbionts. This was already suggested to be possible by Leal et al. (2012a), and later confirmed by Monteiro et al. (2019), with the latest authors counting photosynthetic dinoflagellate endosymbionts within bleached anemones to up 0.5% of its initial number after a four month exposure to complete darkness.

Overall, the present study supports the findings by Monteiro et al. (2019) that no symbiosis exists within *B. stephanieae* and the photosynthetic dinoflagellate endosymbionts acquired through the ingestion of *E. diaphana*. The use of chlorophyll fluorescence methodologies was validated for the model organisms addressed in the present work allowing to make non-destructive measurements, in real time and rapidly, with this being an advantage when investigating the impacts of climate change in marine photosynthetic animals. To our knowledge, even though this methodology is commonly employed in sacoglossan sea slugs (Cartaxana et al., 2018; 2019; Cruz et al., 2012, 2013) and some nudibranch species (Burghardt et al., 2008; Wägele, 2001), this methodology had never been used in *B. stephanieae* and it is certainly a powerful tool for the study of the evolution of the symbiosis between molluscs and their photosynthetic dinoflagellate endosymbionts.

5 Conclusion

The present study shows experimental evidence that *B. stephanieae* does not engage in a mutualistic relationship with the endosymbionts captured from its prey glass anemone *E. diaphana*. *Berghia stephanieae* requires a constant supply of new photosynthetic endosymbionts in order to maintain their abundance and photosynthetic efficiency. The present work is a first step towards futures studies using chlorophyll fluorescence parameter F_o to infer the concentration of photosynthetic endosymbionts in nudibranch species and the prevalence of their association. Additionally, this approach can also be a valuable tool to address the potential consequences of bleaching affecting marine organisms that are predated by stenophagous species. To date, the short and long-term consequences of such trophic disruption remains largely unaddressed and is certainly worth investigating.

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