POLITÉCNICO DE LEIRIA
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The Effect of Temperature and Substrates on Polyp Settlement in the Spotted Jellyfish, *Phyllorhiza punctata* (Von Lendenfeld, 1884)

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The Effect of Temperature and Substrates on Polyp Settlement in the Spotted Jellyfish, *Phyllorhiza punctata*

Escola Superior de Turismo e Tecnologia do Mar
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Abstract

In recent decades, human activity has been known to influence Earth's marine ecosystems in a multitude of different ways, such as through global warming, overfishing, coastal or marine construction, pollution, among others. These impacts will then produce different effects on the affected organism populations. One of these groups is the gelatinous one, which has gained a lot of scientific interest due to the increased frequency and abundance jellyfish populations worldwide. It is speculated that human activities may have an impact on the development and reproduction of these species. Therefore, the aim of this work was to evaluate the effect of temperature, and different substrates on the asexual reproduction and polyp settlement of the jellyfish species *Phyllorhiza punctata* (Von Lendenfeld, 1884).

Polyps of *P. punctata* were subjected to two different experiments. In the first experiment, the polyps were exposed to four different temperatures (22, 24, 26, and 28 ºC), and provided with five different substrate types (glass, rock, shell, plastic (PVC), and fishing net). After 60 days, the total number of polyps for each substrate and temperature was observed. In the second experiment, a different group of polyps was exposed to the same five substrates at the temperature of 24 ºC, and after 60 days, the total number of polyps, and their preferred distribution were compared. The results showed significant differences between the temperatures of 22 and 26 ºC, showing that higher temperatures have a positive effect on polyp asexual reproduction. It was also verified that a lower number of polyps was obtained at 28 ºC when compared with to 26 ºC, which indicates that this temperature may be outside the optimal range for *P. punctata* polyps, and therefore might restrict polyp reproduction instead. This leads us to believe that slight warming may benefit polyp asexual reproduction of *P. punctata*, but only within the limits supported by the species. Furthermore, polyp settlement occurred in all the provided substrates, although significant differences were observed between the plastic and the glass, rock, and shell substrates, being the later three significantly preferred over the first one. It was also possible to perceive a higher preference by the polyps to positions further away from the bottom of the goblet (negative geotaxis). In conclusion, marine-bound human activities appear to influence gelatinous organisms, and as such, further studies are required to better understand their influence on these organisms.

Keywords: Scyphozoa, warming, substrate selectivity, benthic life stage

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1 Introduction

Medusae are a part of the marine gelatinous zooplankton whose presence in the oceans seems to be increasing during the last few decades. This increase in the populations of gelatinous organisms may be derived from the numerous alterations in the marine ecosystems caused by human activities and the impact that these have on the environment (Purcell et al., 2007; Richardson et al., 2009). Some examples of these impacts are:

- Climate change is caused by the release of greenhouse gases into the atmosphere that increases Earth's natural greenhouse effect resulting in an irregular increase of the global temperature (Canadell et al., 2007).

- The overfishing of marine organisms can lead to profound changes in the food webs where gelatinous organisms are inserted. Such changes may include, a decrease in the number of organisms that directly compete with the gelatinous zooplankton for food and that might feed on the adult or earlier stages of these organisms, resulting in an increased number of gelatinous organisms on the oceans due to the lack of predators and/or competitors (Jaspers et al., 2015; Purcell & Arai, 2001).

- The construction of structures in marine environments may also contribute to the increase of the populations of gelatinous organisms by possibly providing these organisms with a larger area where the benthonic organisms or the sessile phase of classes such as the Scyphozoa can settle, grow, and reproduce (Dafforn et al., 2015; Dong et al., 2012).

- Finally, an additional impact can be associated with the transport of these organisms when attached to anthropogenic materials and vessels, such as ships or in the ballast water inside them. These may then spread into new environments with favorable characteristics to their development and absence of natural predators leading to the creation of a new invasive population. This new population can then affect the native populations either by competing with them for resources or by feeding on them, and possibly leading to their disappearance in such habitats (Graham et al., 2003; Lynam et al., 2010).

The study of gelatinous organisms is therefore crucial to understand and predict the growth of their populations and evaluate the impact of a bigger presence of these organisms in the marine environments and in the human activities within them in a more robust way.

1.1 Gelatinous organisms and climate change

Climate change is one of the biggest threats that the planet faces, and if nothing is made to reduce or attenuate its influence on Earth's ecosystems it may cause irreversible changes to them (Goodess et al., 1992). Climate change is mainly caused by the release of large quantities of greenhouse gases into the Earth's atmosphere, such as carbon dioxide (CO_2) or methane (CH_4), which in turn increase the natural greenhouse effect of Earth's atmosphere and consequentially its atmospheric and water temperature (Canadell et al., 2007; Ferreira, 2006; Stern & Kaufmann, 2014)(Fig. 1).

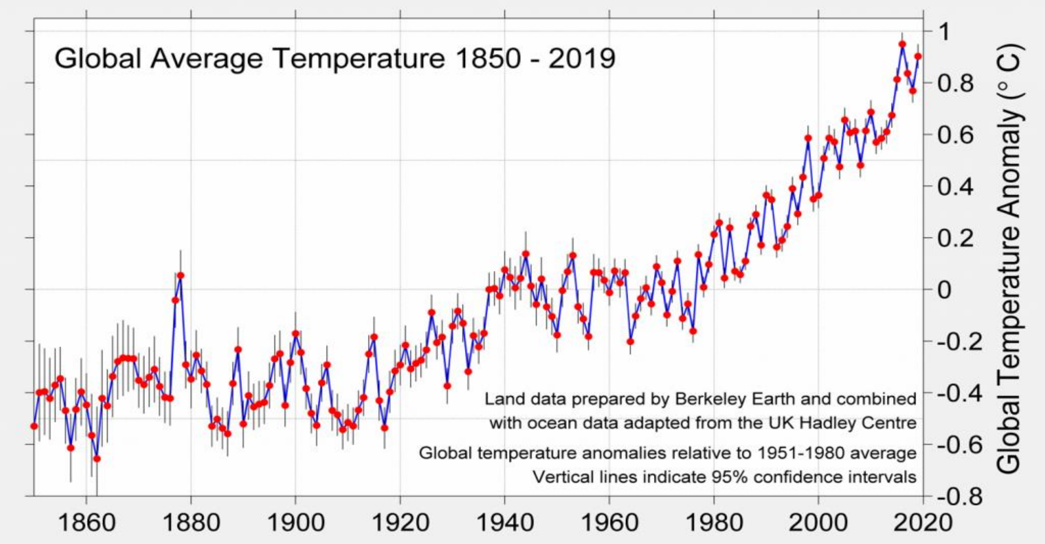

Figure 1 - Variation of global temperature from 1850 until 2020. Source: http://berkeleyearth.org/2019-temperatures/

These consequences have multiple impacts on Earth's ecosystems, with marine ecosystems and their inhabitants especially sensible to them. Some examples of these effects are the increase of the oceanic water level due to the melting of the planet's ice caps and to the thermal expansion of the ocean waters (Cazenave & Llovel, 2009; McKay et al., 2011), ocean acidification due to the high concentration of carbon dioxide in the atmosphere, and the decrease of the amount of diluted oxygen (O_2) in the ocean's water resulting from the increase in the water's temperature (Hansen et al., 2016; Levermann et al., 2013; Munday et al., 2012). In turn, these impacts can have various consequences on the organisms that inhabit marine environments, such as, organism migrations or death due to the change of environmental conditions (temperature, pH, or salinity), reduced calcification rates, and other biological processes, like enzymatic activities or photosynthesis due to the lower water pH (Anderson et al., 2013; Kroeker et al., 2013; Nagelkerken & Munday, 2016).

Gelatinous zooplankton is also affected by these impacts which can have various consequences depending on the affected population. It has been shown that the higher temperatures resulting from global warming can lead to higher populations of certain species. However, it can also hinder the development of species that prefer lower water temperatures, typically having a positive effect on temperate species, but a negative effect on species that live near their thermal maximum (Lynam et al., 2010; Purcell, 2005; Schiariti et al., 2008). Purcell et al.'s (2007) results show that 11 out of the 15 populations of temperate jellyfish reviewed in their work increased in size as the water warmed. On the other hand, Lynam et al. (2010) describes that cooler water temperatures led to an increase in the populations of *Aurelia aurita* and *Cyanea* sp. in the northern part of the North Sea. There is also evidence that climate change may cause shifts in bloom formation and trophic mismatches in the food web. An example of these changes is the phenomenon found by Costello et al. (2006), where an earlier appearance of *Mnemiopsis leidyi* in Narragansett Bay (Rhode Island, USA) in recent years was influenced by the amplification of pulsed Spring warming events that permitted early reproduction of the species during winter. Another example of the trophic mismatches that can happen was also identified for the same species by Sullivan et al. (2001), where the peak occurrence of *M. leidyi* occurred two months earlier than expected, which was not the case for one of its preys, *Acartia tonsa*. These phenomena then lead to a discrepancy between the seasonal timing between predator and prey, which almost eradicated *A. tonsa* from the plankton in Narragansett Bay, as a result of the fomented predation pressure from *M. leidyi*.

1.2 Overfishing impacts on the marine ecosystems

Overfishing of marine resources has become a problem in recent decades. The ever-increasing appetite for fish of the growing world population has led to the overexploitation of many fish stocks and even to the depletion of some (Fig. 2). Even though significant efforts are being made to regulate marine organism capture and increase aquaculture production to decrease the strain in the marine ecosystems, some problems have already been identified a result from the overexploitation of these resources. Problems such as the overtake of the ecological role of the overfished species by underfished species of the same trophic level possibly leading to significant changes in the ecological communities and their behavior (Jackson et al., 2001; Möllmann et al., 2008; Scheffer et al., 2005). Another identified issue is the decrease or even disappearance of certain species due to the overfishing of other species which would keep competitors and/or predators’ numbers in check and their overfishing leads to the uncontrolled growth of these competitors/predators. An example of this interaction is the one verified by Zaneveld et al. (2016) in coral reefs, where the overfishing of herbivorous paired with nutrient pollution led to an increase in algal cover and consequently to a decrease in coral recruitment.

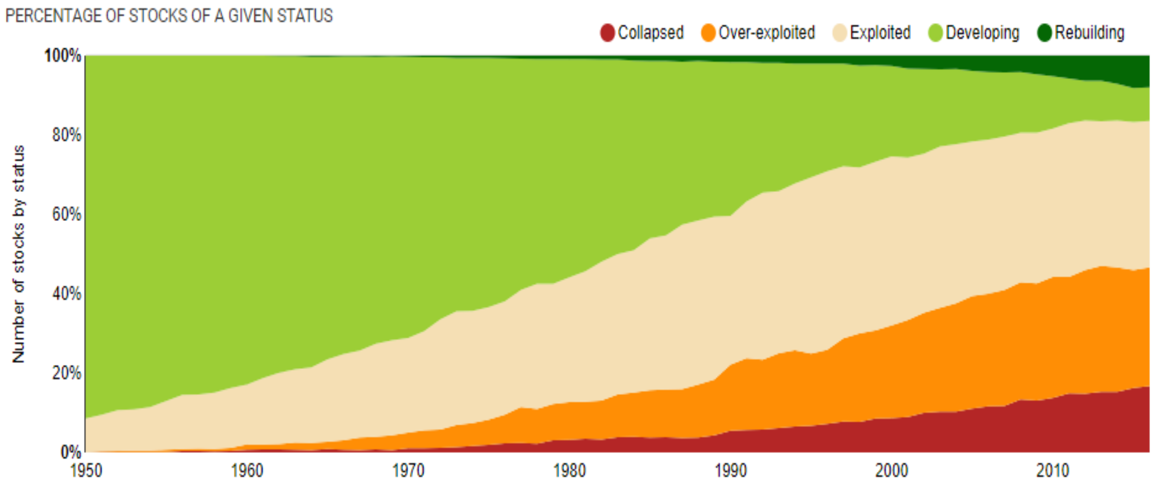

Figure 2 - Status of the global fishing stocks. Source: http://www.seaaroundus.org/data/#/global/stock-status

Nonetheless, the gelatinous zooplankton may also benefit from these impacts, as the removal of predators and zooplanktivorous fish competitors can cause changes in the ecosystem that improve conditions for gelatinous zooplankton. The removal of species that feed on gelatinous zooplankton such as the bluefin tuna *Thunnus thynnus*, spearfish *Tetrapturus belone*, and swordfish *Xiphias gladius*, among others, can lead to an increase in the population of gelatinous organisms (Cardona et al., 2012; Jaspers et al., 2015; Purcell & Arai, 2001; Roux et al., 2013). Besides, the removal of zooplanktivorous forage fish species lowers the amount of competition with gelatinous predators for food, consequently leading to an increase in the population of the gelatinous organisms (Purcell et al., 2007; Robinson et al., 2014).

This combination of multiple factors can lead to even greater consequences than the ones verified for when they affect ecosystems separately (Arai, 2001).

1.3 Impacts of the construction of ocean-bound infrastructures

The construction of ocean-bound structures can affect marine life in various ways, such as through the destruction of the habitat of local communities or the addition of new substrates. This can promote the settlement of some species over others, these gaining then an advantage over possible competitors, which can lead to a shift in the population dynamics within the ecosystem (Fig. 3). Many of these structures are also known to release pollutants and/or nutrients to the environment, possibly leading to local eutrophication which in turn causes various impacts on the local environment (Bulleri & Chapman, 2010; Jiang et al., 2001; Nabe-Nielsen et al., 2018; Stelzenmüller et al., 2010). Finally, these structures can also emit noise and light pollution that may affect the local fauna and flora (Dafforn et al. 2015).

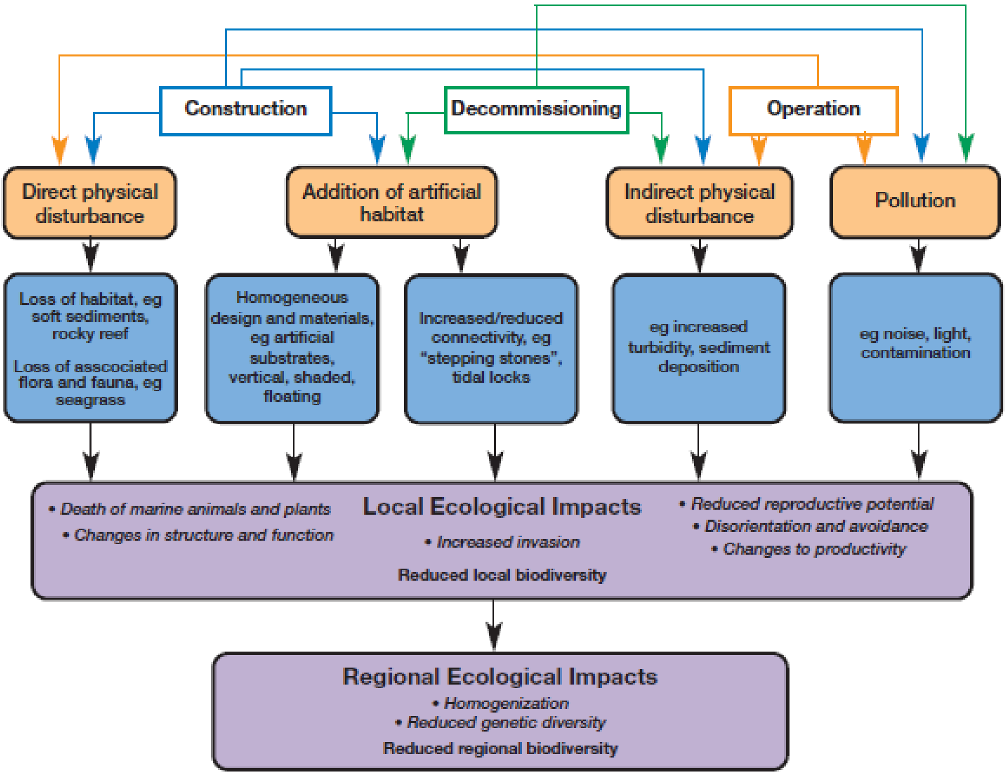

Figure 3 - Ecological Impacts caused by different human activities on coastal environments. Image adapted from Dafforn et al. (2015).

The construction in marine environments has increased over the last few years, it was estimated that the physical footprint of built structures was at least 32000 km^2 worldwide as of 2018 and that this value is expected to reach 39400 km^2 by 2028 (Bugnot et al., 2021). This construction can then destroy or alter local ecosystems and despite the construction of artificial structures in marine or coastal ecosystems, the measures to mitigate the impact of these structures are still insufficient or need to be updated, which has led to a lack of control of the damage caused by construction on the surrounding ecosystem. A possible mitigating measure identified by some authors was to make the built structures multifunctional for these to fulfill their goal but also to, for example, function as pollution mitigators or biodiversity enhancers (Dafforn et al., 2015; Gaston et al., 2013).

Most of the gelatinous organisms are either benthic or possess a sessile stage in their life cycle, for example, most of the organisms of the Anthozoa class are benthic and most of the Scyphozoa class possess a sessile stage, the polyp, and are therefore also subjected to these types of changes to their environment. Although the destruction of habitat can harm the gelatinous populations, the addition of new types of substrates to the environment may also provide them with an advantage over other species, as this provides them with more surface area for the benthic organisms to settle. These can also provide favorable conditions for jellyfish development, such as nutrient-rich or warmer waters (Dong et al., 2012; Purcell, 2011; Purcell et al., 2007). The introduction of new substrates in the marine environment is also speculated to be one of the ways invasive gelatinous species are using to invade new environments. It is considered that the sessile phase of different gelatinous organisms can go attached anthropogenic materials and vessels, such as ships, as part of its biofouling community, and through this way invade new locations where they can then proliferate as new invasive species (Graham et al., 2003).

1.4 Impacts of gelatinous organisms on human activity

The increase of gelatinous populations promoted by the aforementioned factors causes great impacts in both the marine ecosystems and on the human activities conducted and/or depending on them. These organisms are known for interfering in various human activities, such as net-based fisheries, aquaculture, electric generation, and tourism (Graham et al., 2014; Lucas et al., 2014; Ocaña-Luna et al., 2011) (Fig. 4A and B). The latter may be due to many species of the phylum Cnidaria possessing nematocysts with enzymes, neurotoxins, or potent pore-forming toxins, that can affect human health (Jouiaei et al., 2015). The increase of these species was also attributed to an indirect impact on fishing activities since gelatinous organisms compete and/or feed on fish larvae possibly leading to a decrease of the fishing stock (Graham et al., 2003).

Even though such interferences give gelatinous organisms a negative connotation, as they are usually perceived as harmful and "pests" (Graham et al., 2014), these organisms can also be useful as resources for multiple industries. For example, some species are being introduced or used for culinary purposes in some cultures (Leone et al., 2019; Omori & Nakano, 2001) (Fig. 4C), while others produce important compounds for multiple high end applications, such as fluorescent compounds that can be used in medical and scientific research (Leone et al., 2013; Markova et al., 2010). It has also been investigated a possible use of liquified jellyfish as a seeding growth enhancer and as insecticides (Hussein, Sayed, & Saleh, 2015). Beyond functional uses, this type of organism is also highly valued by its looks, and numerous species are currently reared in aquaculture and traded in the marine ornamental industry (Fig. 4D) (Schaadt et al., 2017; Ukaonu et al., 2011).

Figure 4 - Examples of jellyfish impacts and uses on human activities. A - Jellyfish being removed from a power plant in Israel. B - Jellyfish clogging the fishing nets of fishing boats, C – Jellyfish exposed in a public aquarium in Japan, D – A prepared plate of jellyfish salad. Sources: https://www.smh.com.au/environment/jellyfish-force-shutdown-of-power-plants-20110711-1haa6.html; https://www.abc.net.au/news/2014-04-01/jellyfish/5359924?pfm=ms&nw=0; https://www.laprensalatina.com/tokyo-aquarium-launches-mesmerizing-panoramic-jellyfish-tank/; https://www.thehongkongcookery.com/2012/06/jellyfish-salad.html respectively.

1.5 *Phyllorhiza punctata*

The species studied in the present work, *Phyllorhiza punctata* (Von Lendenfeld, 1884), belongs to the Scyphozoa class inside the Cnidaria phylum. It is characterized by its brownish blue color with white crystalline inclusions evenly spread across its umbrella, giving the appearance of spots (Fig. 5A). Their stomach is connected to four radial canals that are contained inside four oral arms that present nematocysts and are used for prey capture. They also normally possess eight sensory structures evenly spread across the margin of their umbrella, the rophalia. Additionally, these organisms can also present symbiotic associations with zooxanthellae (i.e., Symbiodiniaceae dinoflagellates) that confer them a brownish coloration. *P. punctata* can reach up to 62 cm in diameter (CABI, 2021; Graham et al., 2001). The polyp phase (Fig. 5B) is usually small, milky white, and typically sessile. When fully matured they normally have sixteen tentacles which they can utilize for capturing prey and as a defense (Calder, 1971; Peach & Pitt, 2005). The polyp phase also typically presents a longer life spam than its pelagic counterpart, and is also more resistant to variations in environmental conditions, such as temperature, salinity, and lower levels of dissolved oxygen (Prieto et al., 2010).

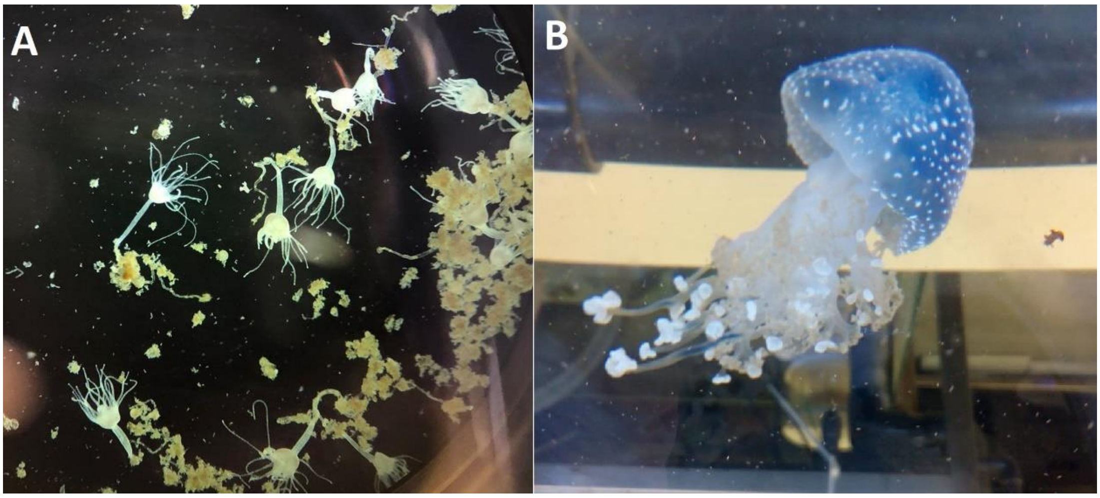

Figure 5 - A- Small group of *Phyllorhiza punctata* polyps. B - A *Phyllorhiza punctata* adult medusa

Phyllorhiza punctata is a native species of the West Pacific Ocean, even though it has already spread across various other regions worldwide, such as the North of Golf of Mexico, the Californian coast, the Atlantic Ocean, and the Mediterranean Sea (CABI, 2021; Ocaña-Luna et al., 2011). Due to the fact that this species has a relatively high euryhaline tolerance it can inhabit coastal and estuarine zones (Gunter, 1961). It also has the capacity of taking refuge in deeper zones when the conditions are not favorable to its survival and to adapt to new conditions, granting it with a high potential to become an invasive species, as shown in the figure below (Graham et al., 2001; Rippingale & Kelly, 1995)(Fig. 6).

Figure 6 - Global map representing the points where report of *Phyllorhiza punctata* appearances have been registered (red dots). Image adapted from https://www.haus-des-meeres.at/en/Our-Animals/Research/Reaserach-in-HdM.htm

Most scyphozoan species have a life cycle divided between two different phases, the pelagic medusa phase with restricted motile capability, and the sessile polyp phase (Fig. 7) (Lotan et al., 1994). Its reproduction is also divided between these two phases, where sexual reproduction occurs during the adult medusa phase, and asexual reproduction occurs during the polyp phase. Sexual reproduction starts with the release of the male gametes into the surrounding water which then find their way into the female ovaries, present near its gastric cavity, and where these fecund the oocytes inside them.

After the fecundation, the eggs will grow into planula larvae which are then released by the female in the surrounding waters. These new planulae will then search for a substrate to settle on, and transform into a new polyp, which will grow until fully mature (possessing 16 tentacles) (Holst & Jarms, 2006; A. Schiariti et al., 2008). During this life stage, the organism can asexually reproduce through methods such as budding or podocyst formation, leading to a bigger colony of organisms, or, it can also suffer a metamorphic process called strobilation (Helm & Dunn, 2017). During this process, the tentacle and mouth area of the polyp will transform itself into a small medusa-like structure, the ephyra, that will later be released by the polyp. Since each *P. punctata* polyp can only produce one ephyra at a time, this strobilation is called monodisc strobilation, in contrast with polydisc strobilation, where one polyp releases more than one ephyra during the strobilation process (Helm & Dunn, 2017).

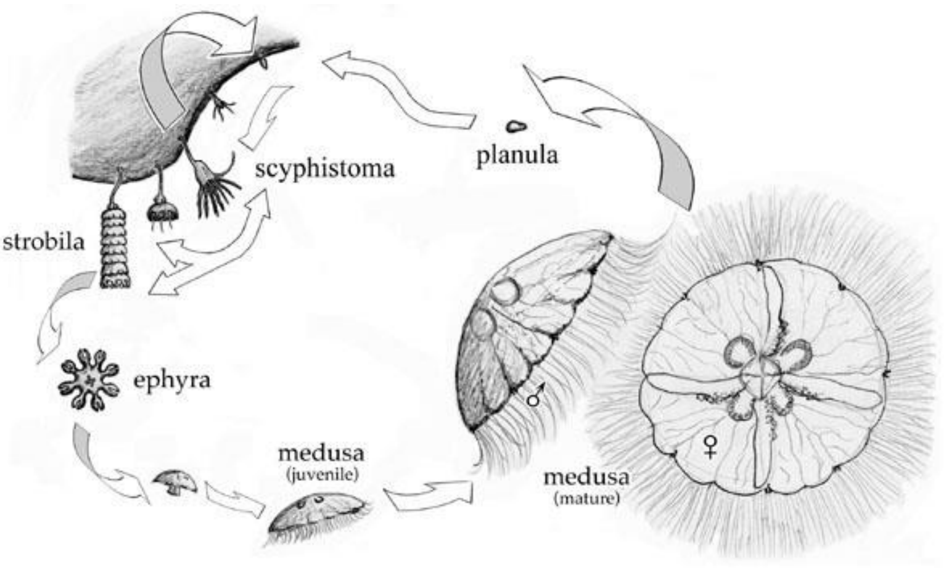

Figure 7 - Typical Scyphozoan life cycle. Source: http://thescyphozoan.ucmerced.edu/Biol/Ecol/LifeHistory/ScyphozoaLH.html

The ephyrae normally possess eight small rhopalar arms, split into two rhopalar lappets at their tip, that in their middle harbor a small sensory structure called a rhopalium, four small undeveloped oral arms and a manubrium, through which it will feed, and a brownish coloration due to the zooxanthellae lodged in the mesoglea. It has also been denoted that stress-induced mutations can occur during this process, which can create ephyrae that have a lower or higher number of oral arms than usual (3-4 in the lower end and up to 16 on the higher end) (Berking & Herrmann, 2002). These ephyrae will then go on to grow into adult medusae starting the cycle anew (Holst & Jarms, 2006).

1.6 Objectives

Due to the attention that gelatinous organisms have gained in recent years, consequent of the increasing populations of these beings in Earth’s oceans, and an increased search for these organisms in the marine ornamental trade, the lack of knowledge that exists surrounding these organisms and their biological processes has become more apparent. The possibility that they can also affect multiple human activities and their potential use as a sustainable marine resource for biotechnological application and bioprospecting calls for an increased amount of global monitoring of their ecology and biochemical composition. It is then imperative that more information about gelatinous organisms is found and analyzed in order to better predict how they can influence and be influenced by the changing oceanic ecosystems, to better prevent the impacts that gelatinous organisms can cause in human activities. The analysis of these variables is also very important for the optimization of polyp and medusae rearing in aquaculture, in order to better understand the optimal conditions for the welfare and reproduction of these organisms.

This study investigated the effect of different types of substrata on the settlement rate of *Phyllorhiza punctata* polyps and determined the impact of temperature variability on their asexual reproductive rates. It was also analyzed if there was a substrate among the provided ones that would be favored over the others possibly demonstrating selective fixation by the species.

2 Materials and methods

The experiments were conducted in CETEMARES, Marine Sciences R&D, Education Centre MARE – Polytechnic of Leiria (Peniche, Portugal).

The polyps of *Phyllorhiza punctata* were obtained via the asexual reproduction of polyp colonies that were maintained in the laboratory, which were provided by the Oceanário de Lisboa, and the brine shrimp nauplii utilized for feeding were obtained through the decapsulation and hatching of Commercial artemia cysts (Ocean Nutrition®, USA).

2.1 Polyp colony rearing

The polyp colony was maintained on a 4 L aquarium filled with 2 L of seawater at salinity 34, pH 8, and temperature of 21°C ± 0.5°C, previously filtered through a 40 µm sieve. To the aquarium was also added a small airflow pipe to provide weak aeration to promote its oxygenation, equal distribution of food, and to prevent the appearance of dead zones in the aquarium. The polyps were fed twice a week with an *ad libitum* amount of live brine shrimp nauplii (*Artemia franciscana*). Finally, a water exchange of 50% of the aquarium’s volume was done weekly utilizing previously filtered seawater at salinity 34, in order to control the salinity, and levels of ammonia, nitrites, and nitrates in the aquarium's water.

2.2 Experimental tests

2.2.1 Influence of temperature and substrata on polyp settlement

Initially, twelve goblets were divided into four different groups of three, and to each group was assigned one of four temperatures 22, 24, 26, and 28 ºC that were tested during this experiment. These temperatures present the range reported for the condition observed for late spring and early summer (22 and 24 ºC) in their natural conditions (Graham et al., 2003; Rippingale & Kelly, 1995), and two additional temperatures (26 and 28 ºC) representing the predictions for 2100 by the IPCC (Shukla et al., 2019). To each goblet was attached a strip and a lid of black plastic adhesive to prevent the penetration of light into the inside of the goblets, in order the better simulate the natural environment of the polyps. To them were also added 200 mL of seawater at salinity 34, which had been previously sieved through a 40 μm sieve, and lastly, against the wall of each goblet were equidistantly laid five substrates, three of them of anthropogenic origin (glass, plastic and fishing net), and two of them of a natural origin (rock and shell) (Fig. 8 and 9A). Each temperature treatment was then moved to the inside of a larger aquarium, where distilled water had been previously added and acclimatized to the desired temperature for each group (Fig. 9B). This water bath had the finality of reducing the fluctuation of the temperature inside the goblets to avoid any stimulus caused by the variation of temperature. To each goblet was then added a small tube for aeration and a small black plastic cover.

Figure 8 - Examples of the five substrates utilized during the experiment (glass, fishing net, shell, rock, and plastic.

Finally, 240 polyps were counted with a ZEISS Stemi 508 stereomicroscope and distributed among all the goblets, with 20 polyps being added to the center of each goblet to slowly acclimate to their respective temperature. The polyps were then left on the goblets for 60 days, after which the number of polyps settled onto each substrate, the goblet bottom and wall, and free-swimming polyps was observed and registered using the ZEISS Stemi 508 stereomicroscope.

The temperature and salinity of each goblet were measured daily and adjustments to both parameters were done whenever necessary. Once a week the polyps were fed with 5 mL of a live feed mixture composed of 50% rotifers (*B. plicatilis*) at a concentration of around 108 rotifers mL^{-1}, and 50% brine shrimp nauplii (*A. franciscana*) at a concentration of around 100 nauplii mL^{-1} to provide feeding for adult polyps and polyps of smaller sizes. The following day, a water exchange of 50% of the water's volume was performed to maintain the water quality inside each goblet, and any polyp removed through this exchange was then returned to the goblet.

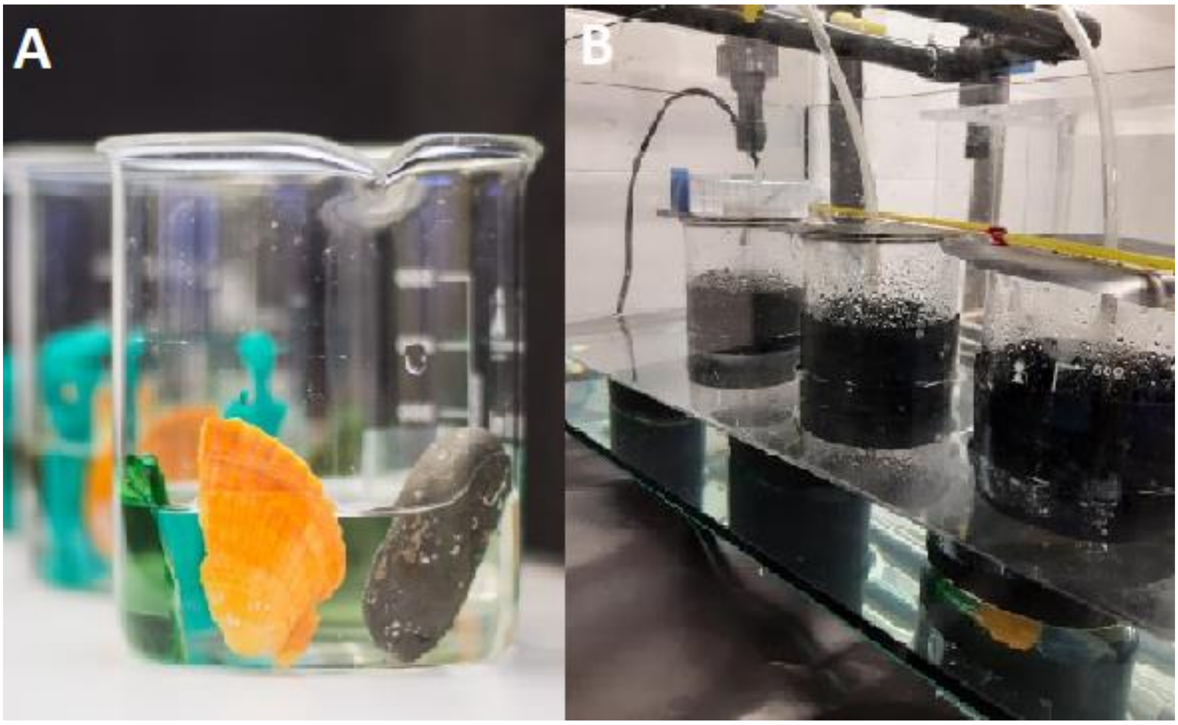

Figure 9 - A- Example of the placement of the substrates inside the goblet during the experiment. B - Goblets inside the bigger tank where they were kept during the duration of the experiment.

2.2.2 The impact of substrata and location on polyp settlement

For this experiment, three similar pieces of five different substrates (rock, shell, glass, plastic, and fishing net) were distributed by fifteen randomly assorted 200 mL goblets, filled with 100 mL of 40 µm-filtered seawater at salinity 34. After which all the goblets were placed inside a water bath at the temperature of 24º C to prevent large temperature fluctuations inside the goblets and covered with a black plastic cover to limit the exposure of the goblets to direct light and better simulate their natural environmental conditions (Fig. 10). Finally, 150 polyps were then counted under a dissecting stereomicroscope and equally distributed between all the goblets in groups of 10 per goblet. The polyps were then left for 60 days, after which the number of polyps attached to each substrate, goblet bottom, goblet wall, and free-swimming polyps were counted.

Figure 10 – Two instances of the fifteen goblets utilized on experiment 2, where it's possible to see them before the experiment (A) and during the experiment, with the plastic cover lightly pushed back (B).

The temperature and salinity of each goblet were measured daily and adjustments to both parameters were done when necessary. Once a week the polyps were fed with 2.5 mL of a mixture composed of 50% rotifers (*Brachionus plicatilis*) at a concentration of around 108 rotifers mL^{-1}, and 50% brine shrimp nauplii (*Artemia franciscana*) at a concentration of around 100 nauplii mL^{-1} to provide feeding for adult polyps and polyps of smaller sizes. The following day, a water exchange of 50% of the water volume was done to maintain the water quality inside each goblet, and any polyp removed through this exchange was then returned to the goblet.

2.3 Statistical analysis

For the first experiment, a two-way analysis of variance (two-way ANOVA) was performed to evaluate temperature, substrate type, and the interaction of these factors' effect on the polyp attachment rate (measured as the number of polyps settled to the substrate) (Zar, 2010). Where statistically significant effects were identified, the ANOVA was followed by a Tukey's *a posteriori* HSD test to identify the significance of differences between pairs of conditions. The normality and homogeneity of the data were evaluated and verified by the Shapiro-Wilk test complemented with Q-Q plots and histograms, and the Levene test complemented with fitted residual plots, respectively.

As for the second experiment, a similar statistical analysis was performed, however, the analyzed variables on the two-way ANOVA were the substrate type, the settlement location, and the interaction between these two.

Where applicable, results are displayed in the form of average ± standard deviation. The denoted differences were considered statistically significant at the 5% significance level (that is, $\alpha = 0.05$). All statistical analyses were conducted using R version 4.0.2 (R Development Core Team, 2020).

3 Results

3.1 The influence of temperature and substrata on Polyp Settlement

The results of the two-way ANOVA indicated differences between the tested substrates and between the different temperatures (p-value < 0.05, Table 1). No significant differences were found in the interaction between the substrate type and the temperature.

Table 1 – Results of the two-way ANOVA depicting the differences between different substrates, and temperatures, and the interaction between both parameters on *Phyllorhiza punctata* polyp settlement.

	Df	F value	P value
Substrate	7	5.415	< 0.001
Temperature	3	3.316	0.025
Substrate:Temperature	21	0.805	0.704

3.1.1 The effect of temperature on polyp asexual reproduction

Polyps in all treatments reproduced asexually during the experiment. However, the results showed that the temperature did appear to influence the asexual reproduction of the *P. punctata* polyps (p-value = 0.0253, Table 1). In general, an increase in temperature promoted a higher number of new polyps (312 ± 11.61, 391 ± 58.45, and 462 ± 13.20 for the temperatures of 22, 24, and 26 ºC respectively), although a decrease in polyp reproduction, was noted at the highest temperature (379 ± 51.19 at the temperature of 28º C). Significative differences in the number of new polyps were observed between the temperatures of 22 and 26 ºC (p-value = 0.013) thus confirming a positive influence of the temperature in polyp asexual reproduction (Fig. 11).

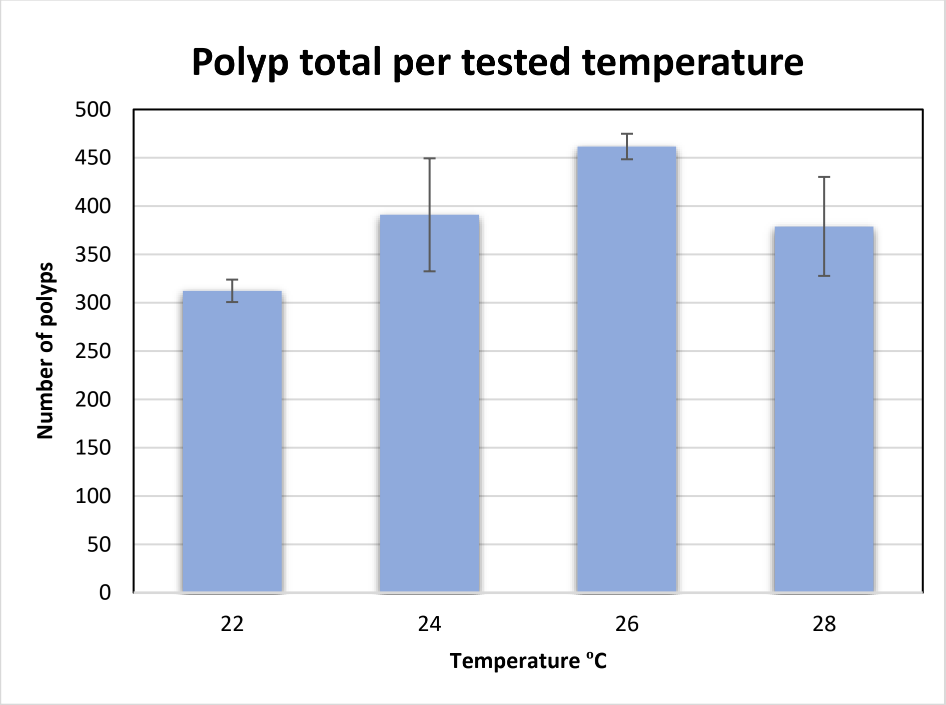

Figure 11 - Total *Phyllorhiza punctata* polyps for each temperature tested (22, 24, 26, and 28 ºC). The values are presented in the form of average ± standard deviation.

3.1.2 Effect of different substrates on polyp settlement preferences

There were no significant differences between substrates, except from the plastic, which showed significantly lower polyp fixation (22.00 ± 11.48) than the shell (66.08 ± 7.86, p-value < 0.001), glass (50.25 ± 16.49, p-value = 0.026), and rock (55.50 ± 3.13, p-value = 0.004) but not from the fishing net (48.25 ± 16.05, p-value = 0.051). Even so, polyp settlement occurred in all of the provided substrates indicating that all the tested substrates might be used by *P. punctata* polyps (Fig. 12).

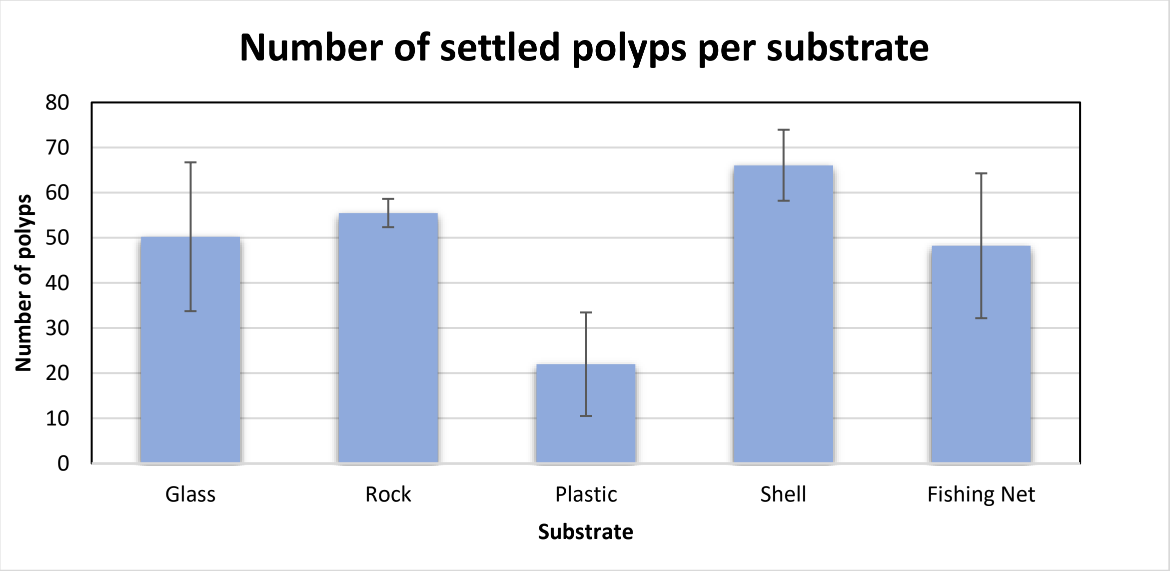

Figure 12 - Total settled *Phyllorhiza punctata* polyps for each of the experimented substrates (glass, rock, plastic, shell, and fishing net. The values presented are the average ± standard deviation.

3.2 Effect of substrata and location on polyp settlement

The results from the two-way ANOVA showed significant differences between the substrates and the locations evaluated (Table 2). Furthermore, it was also possible to verify that no significant differences were found in the interaction between substrates and settlement locations.

Table 2 – Two-way ANOVA results depicting the main effect and interactions between substrata and local of settlement.

	Df	F value	P value
Material	4	5.245	0.002
Local	3	38.192	< 0.001
Material:Local	12	1.925	0.060

3.2.1 Effect of different locations on polyp settlement

It was possible to note a significant difference in polyp fixation between the settlement locations (p-value < 0.001, Table 2). No significant differences were observed in polyp settlement between the substrate and the goblet bottom. Although, it was possible to verify a significant preference for the goblet wall by the polyps (64.47 ± 23.94) when compared to the provided substrate (26.60 ± 5.17) and the goblet bottom (17.33 ± 3.44) (p-value < 0.001 in both instances) (Fig. 13).

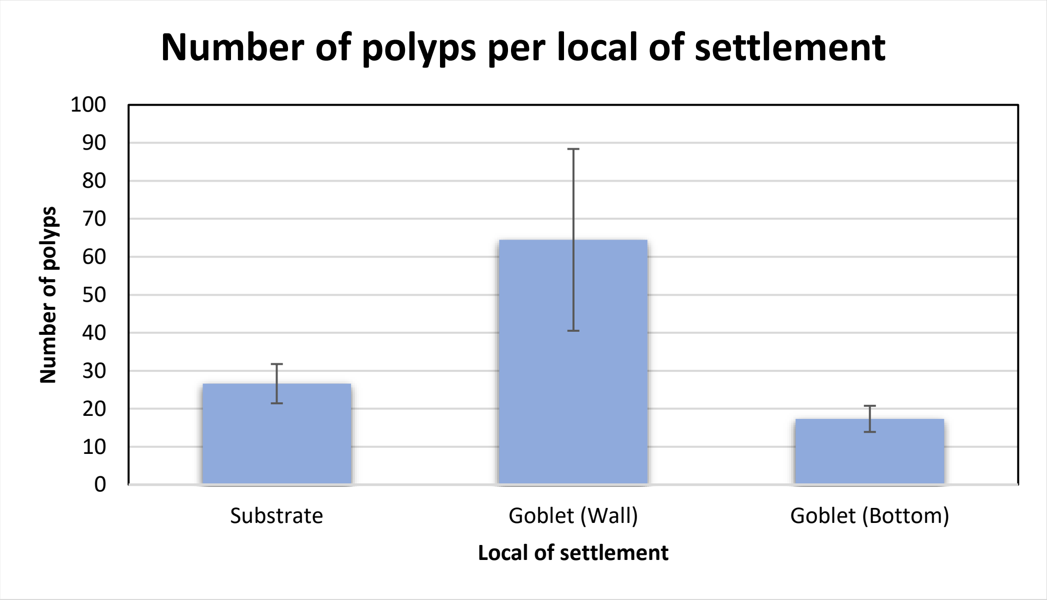

Figure 13 - Number of total *Phyllorhiza punctata* polyps settled onto all of the analyzed positions (substrate, goblet (wall), and goblet (bottom). The values presented are the average ± standard deviation.

3.2.2 The influence of substrate on polyp settlement

In general, there were no significant differences between most of the provided substrates. Still, it was possible to note significant differences between the fishing net (18.33 ± 7.41) and the rock substrates (27.67 ± 5.31), as well as between the fishing net and the glass substrates (34.00 ± 9.63) (p-value = 0.008 and p-value = 0.001, respectively). It was also observed that the number of non-settled polyps in this experiment was higher when compared to the first experiment (83 ± 22.36 compared to 52 ± 9.07 non-settled polyps) (Fig. 14).

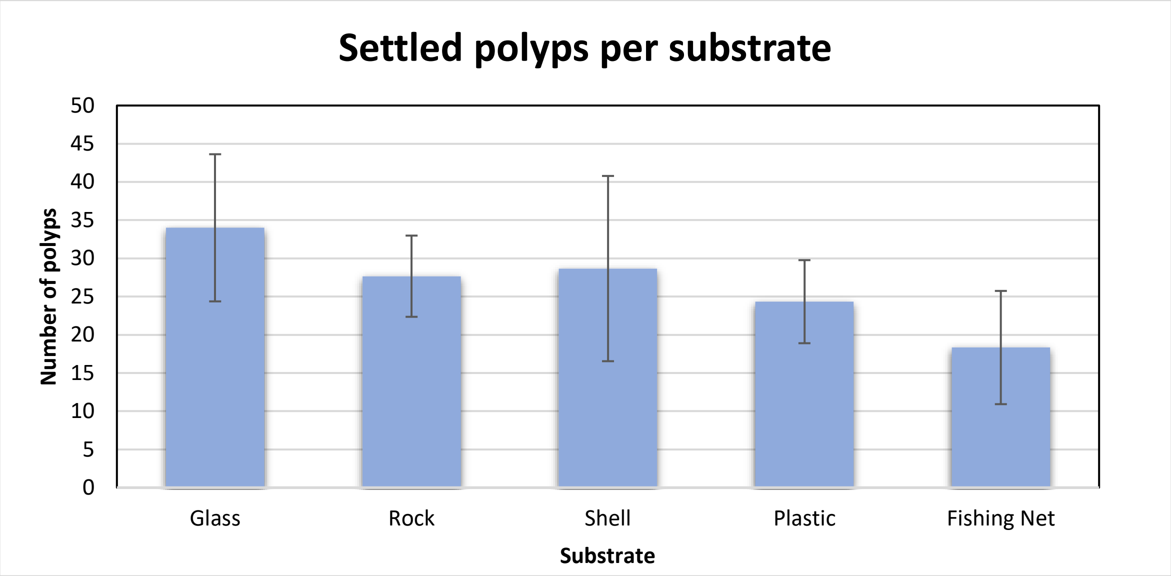

Figure 14 - Total of settled *Phyllorhiza punctata* polyps per experimented substrate (glass, rock, shell, plastic, and fishing net. The values presented are the average ± standard deviation.

4 Discussion

The study of the impact of global warming in gelatinous organisms, as well as in other types of organisms, is paramount for a better comprehension of the changes it can cause on marine environments. However, the sessile phase of *Phyllorhiza punctata* and many other jellyfish is still poorly understood and so are its mechanisms. As such, it is also important to discover as much information as possible about this life stage in order to have a better comprehension of the life cycle of these organisms, and how different environmental factors can affect their development and reproduction.

Accordingly, the effects of different temperatures on polyp asexual reproduction and the effect of different substrates on polyp fixation, plus a possible connection between these two variables, were evaluated throughout this experimental work. The results make apparent a positive effect of increasing temperature on polyp asexual reproduction but only up to a critical temperature value (26 ºC), after which the effect becomes negative (Fig. 11). It was also possible to note that there were significant results among the provided substrates, which might indicate a preference of some substrates over others and possibly a limited capability of substrate selection by the polyp buds (Table 1).

4.1 Temperature's effect on polyp asexual reproduction

The analysis of the tested population showed that the asexual reproduction of the polyps was positively influenced by the increase in temperature. Nonetheless, this positive effect on polyp reproduction was only shown up to a certain limit (26 ºC), after which higher temperatures (28 ºC) became detrimental for the asexual reproduction of the species (Fig. 11). Various authors have obtained similar results for polyps of the same species, where the increase of the water temperature led to an increase in the reproductive activity of the polyps (Miranda, 2016; Rippingale & Kelly, 1995). This was also the case for many different *Scyphozoa* species, Treible & Condon (2019), for example, found this to be true for the *Aurelia aurita*, *Chrysaora quinquecirrha*, and the *Chrysaroa fuscescens* species. A few other examples are the results of Feng et al. (2015), which obtained similar results for the *Nemopilema nomurai* species, and the work of (Schiariti et al., 2014) that found this to also be true for 11 different Scyphozoa species, including *P. punctata*, further supporting the results obtained in this experiment.

Hereupon, it was also noted that the number of produced polyps diminished for the highest tested temperature (28 ºC) which might indicate a negative influence on polyp reproduction by temperature, past a certain point. This effect was also encountered in some Scyphozoa species, such as reported by Lynam et al. (2010), which denoted that cooler oceanic waters enhanced *A. aurita* and *Cyanea spp* populations in the northern part of the North Sea, and that warmer temperatures resulting from global warming might suppress these populations in the future. It has been speculated that jellyfish populations that live near their thermal maximum may suffer negative impacts of global warming (Purcell, 2005). This might be because temperatures above the range supported by the species can lead to the employment of several mechanisms to mitigate the stress caused by unfavorable conditions. Some species are known to answer this stress via rapid metabolic responses that consume energetic reserves, and even activate anaerobic metabolism pathways (Aljbour et al., 2019; Nevarez-Lopez et al., 2020). This being the case it is possible that the diversion of energy to these adaptative mechanisms can lead to a decrease in the energy available for reproduction efforts, leading to a decrease in the number of new polyps produced by the polyp population. *Phyllhoriza punctata*, being a tropical species native to the western Pacific Ocean, and a species that is usually found in estuarine environments is known to support a wide range of salinities and temperatures (CABI, 2021; Rodríguez, 2020). Nonetheless, according to the obtained results and available literature, if the effect of global warming is not mitigated in the future, the positive influence that warmer water temperatures have on the asexual reproduction of *P. punctata* might instead become a deterrent to their reproduction, especially in populations already in their maximum thermal range.

4.2 Substrate influence on polyp settlement

In both experiments, it was possible to note that plastic and fishing net substrates did not appear to be as favorable for polyp settlement as the other provided substrates, being that both the plastic and the fishing net were the ones where the lowest number of polyps settled (Fig. 12 and 14). Besides plastic-based substrates, no other differences were found between the provided substrates which might indicate that the provided anthropogenic substrates (glass, plastic and fishing net) are not substantially better or worse for polyp fixation than the natural ones (rock and shell). These results are somewhat similar to the ones obtained on the only other study with *P. punctata* polyps by Miranda (2016), which also provided various substrates, although all of them anthropogenic, for polyp settlement and found no significant differences between them. This can also be supported by the work of Marques et al.(2015), which demonstrated that *Aurelia* sp polyps from the Thau lagoon settled on the anthropogenic substrates inserted into the lagoon through the human activities that occur there, but also onto the biofouling communities that grow attached to these substrates.

Studies such as the ones made by Dong et al. (2018), Feng et al. (2017), and Hoover & Purcell (2008) with *Aurelia* spp, demonstrate the capacity that polyps have to settle in either natural or man-made materials, which then leads to the conclusion that the introduction of new substrates into the marine environment might be beneficial for gelatinous organisms. With the introduction of these materials, the area available for colonization by these organisms and others is increased, leading to bigger populations as they can take advantage of the space provided by new substrates and the space created by the biofouling communities that grow attached to them (Marques et al., 2015). Still, it is worth noting that different species do seem to prefer different materials, as is shown in this work and the works described above, and that the limitation of jellyfish populations in coastal areas is only possible through the removal of the artificial substrates (Jin et al., 2017).

4.3 Impact of substrate location on polyp settlement

As it was demonstrated that *P. punctata* polyps, did tend to prefer to settle on more vertical surfaces, instead of resting on the bottom (Fig. 13). This was seen in both experiments performed in this work where the polyps preferred the goblet walls and the substrates positioned against them, over the bottom of the goblet in experiment 1 and the sides of the goblet over the substrates and the bottom of the goblet in experiment 2.

Even though the number of studies that focus on the positioning of settled polyps in the provided substrates (i.e., underside, lateral, topside) is low a few aspects relating to the matter have been denoted. In the case of *Phyllorhiza punctata* polyps, it has been observed by Rippingale et al. (1995), that, for both planulae and ciliary buds, the underside of provided substrates is preferred by the polyps for settlement. This behavior was similar to the one observed by Holst & Jarms (2007) for five different Scyphozoan species (*Aurelia aurita, Cyanea capillata*, *Cyanea lamarckii*, *Chrysaora hysoscella,* and *Rhizostoma octopus*), in which all of the analyzed species preferred the underside of the provided substrates in their experiments. It has also been described in the literature that planulae larvae and polyp buds do appear to possess negative geotaxis, meaning that they tend to settle away from the bottom of the container (Holst & Jarms, 2006; Svane & Dolmer, 1995). This would lead us to believe that the substrate that would then be preferred by the polyps would be the water-air interface, as it is the most distant from the bottom of the container, but this hypothesis was dismissed by Holst & Jarms (2007), and Korn (1966), as the natural turbulence of oceanic water would not allow for settlement on this interface. This is further supported by the results found here in experiment 1, in which no polyps were found in this interface possibly due to the fact that to each goblet was added an aeration tube that increased water turbulence inside the goblet, and even in experiment 2, in which no aeration was added very few polyps were noted in the water-air interface. These results might also be further influenced by the water exchange that was done weekly to every goblet that would also cause turbulence in the water inside the goblet.

Finally, the high number of polyps found in the substrates in the goblet walls from experiment 2, might be explained by the negative geotaxis that polyp buds appear to show. Since settling in the water-air interface was impossible, due to water turbulence, the substrates and goblet wall might have been selected as the best substrates to settle on by the polyps amongst the provided ones. Still, further studies are necessary to corroborate these results.

5 Conclusions

During these experiments, it was possible to conclude that *Phyllorhiza punctata* polyps' asexual reproduction seems to be positively influenced by a temperature increase. However, it was also noticed that this is only true up to a point and that if the temperature is further increased, its positive influence will then turn to a negative impact and diminish the number of new polyps produced.

It was also observed that plastic and fishing net were not preferential for polyp settlement, it was seen that the rock, shell and glass substrates were preferably used by the studied population. Even so, polyp settlement was verified in all the provided substrates, indicating that *P. punctata*'s polyps have the ability to use the new materials introduced by human activities in the marine environments, to further spread and increase their population and to possibly get transported to new environments, where the species can then establish a new population. A possible way to control this behavior may be the limitation or removal of the anthropogenic materials that are used in marine environments.

Then, it was also found that the positioning of the substrate itself might be an important factor for polyp settlement and that the analyzed population preferred substrates that were perpendicular to the goblet's bottom for its settlement. However, few studies have been done on this research area, and more studies are essential to understand the true impact of substrate positioning for polyp settlement.

Through this work it was possible to better understand the effect of temperature and of the different substrates on the settlement of *P. punctata* polyps. The influence of higher temperatures then those previously experimented with this species was analyzed, and in this way a more robust idea of the temperature's impact on the asexual reproduction of this species was obtained. Various substrates were also utilized, and through the differences verified between them a better notion of the substrates that are preferred, or not by this species was also achieved.

Finally, it was noticed that even though the work done here helps us understand a little bit more of these organisms, a great lack of knowledge is still present in this research area. Therefore, more research with different conditions, species, substrates and substrate positions is required to further understand the polyp settlement mechanisms for *P. punctata* polyps and of other species. This is important to better understand the impact that human activities will have on the gelatinous organism populations around the world and how they will influence them.

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