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EXPLORING THE FACTORS INFLUENCING THE PROLIFERATION OF ALIEN *CAULERPA* SPECIES IN CYPRUS

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Abstract

Key words: Alien species, Eastern Mediterranean Sea, *Caulerpa* spp.

Alien species, also referred to as exotic or introduced species, are recognized for their potential positive and/or negative effects on the ecosystems into which they have been introduced. The Mediterranean Sea, a semi-enclosed basin, has always been the center of commercial and touristic routes, making it a region potentially threatened by the invasion and establishment of non-endemic species. This region is often described as a biodiversity hotspot, hosting >17,000 species. Among its many subregions, the Eastern Mediterranean Sea (EMED) stands out for the highest number of documented introductions, as a result of its proximity to the Suez Canal. This study focuses on key species, namely *Caulerpa taxifolia* and *Caulerpa racemosa*, whose presence and establishment has been acknowledged for years, but whose distribution and proliferation in Cyprus remain widely unknown. By performing statistical analysis on data from grab samples collected around the island at approximately 30 m depth, it was possible to gather information about the factors driving these species' distribution. In addition, the complex interplay of physiochemical factors driving the proliferation and distribution of alien *Caulerpa* species was evaluated. The statistical analysis confirmed the hypothesis that both *C. taxifolia* and *C. racemosa* thrive in areas closer to ports, even though not exhaustive conclusions can be drawn regarding organic matter content and sediment type. The present results might represent an essential starting point to further investigate into the presence and distribution of alien algal and plant species in the Mediterranean Sea, especially considering that more traditional monitoring methods often fall short in detecting these species. A deeper understanding of the drivers influencing *Caulerpa* proliferation could improve the ability to predict future colonization patterns, enabling the implementation of measures to mitigate the ecological and economic impact associated to their spread.

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1. Introduction

1.1. Alien species: definition, effects, and pathways of introduction

Alien species, also referred to as exotic, non-indigenous (NIS) or non-native, are those species that have been transported to areas where they do not typically occur as a result of human activities, allowing them to overcome geographical barriers to their natural dispersal (Blackburn et al., 2014). These species are recognized for their potential positive and/or negative effects on the ecosystems into which they have been introduced, including alterations in space and food availability, depletion of resources originally allocated to native organisms within the ecosystem. In contrast, invasive species, a subset of alien species, are responsible for overall negative impacts onto the recipient ecosystems (Russel & Blackburn, 2017), among these, direct impacts due to predation, transmission of novel diseases and an overall loss of biodiversity, due to the biotic homogenization at large spatial scales, which, to a major extent, can lead to animal extinction. Indirect impacts, instead, include all those related to associated risks and economic costs (Tarkan et al., 2021). Moreover, exotic species are likely to displace native species, cause the loss of genotypes, modify habitats, and change community structures. On the other hand, by supporting ecosystem functioning in degraded ecosystems or acting as ecosystem engineers, they may also exert positive effects on the recipient ecosystems (Katsanevakis et al., 2014). The threat posed by the presence and spread of new species is highly evident in the context of globalization, an era in which the increased pace of worldwide transports is facilitating the distribution of alien species (Meyerson & Mooney, 2007).

The effects of climate change tend to manifest themselves by altering local ecosystem dynamics and by negatively affecting their ability to react to invasions (Occhipinti-Ambrogi, 2007). Furthermore, climate change could disrupt tourism or commerce, it could alter international transport routes, and, in some instances (extreme weather events and changes in circulation patterns), it could enhance the dispersal of certain invasive species to previously isolated geographical areas (Hellmann et al., 2008).

Shipping is often regarded as the main pathway of introduction of non-indigenous species, representing the most ancient vector of organisms' spread, responsible for about 43.8% of introductions since the 1950s in the European seas (Zenetos, et al., 2020). When referring to shipping as a vector for the spread of NIS, two main phenomena need to be highlighted: the accumulation of microorganisms in ships' ballast water and their accidental release (both in ports and during their routes), as well as the accumulation of these organisms on ships' hulls, known as biofouling (Ulman et al., 2019). As for transports, invasive species can be purposefully introduced in a new environment (namely for biocontrol, agriculture or aquaculture), but accidental introductions occurring during other economic activities are common as well (Hellmann et al., 2008). Despite shipping and canals are often regarded as the most common pathways of introduction of NIS, some other vectors need to be considered. Among these, aquaculture seems to be responsible for the introduction of about 11% of the species in the Mediterranean Sea, followed by trades in ornamental species, although it mainly concerns aquatic plants (Keller et al., 2011). Overall, the major pathways of introduction, both worldwide and in the Mediterranean Sea, include corridors like the Suez Canal, aquaculture and aquarium trade, shipping and marine litter, responsible for primary and secondary introduction of species (Katsanevakis & Crocetta, 2014).

1.2. Alien species in the Mediterranean basin

Even though the Mediterranean Sea represents less than 0.8% of the world's ocean area, it comprises a high number of endemic flora and fauna, with a rate of endemism reaching 30% (Boudouresque & Verlaque, 2005), making it a biodiversity hotspot. Many of its ecoregions are nowadays severely threatened by numerous human activities, condition that it's worsened by the action of climate change in this region (Katsanevakis et al., 2014). Of the 400 recorded introduced species, about 10% are considered invasive, and therefore significantly impacting the ecosystem, by posing threats to native communities and by having adverse economic effects on public health, aquaculture, and tourism (Boudouresque & Verlaque, 2005). In this context, the Mediterranean Sea is becoming a hotspot of global change, and it is subjected to a strong "tropicalization", represented by the northward migration of thermophilic species and the invasion of tropical allochthonous species entering the basin through the Suez Canal and the Gibraltar Strait (Corrales, et al., 2018). In particular, the role of the Suez Canal should be further addressed. Opened in 1869 to shorten the commercial routes between the Mediterranean Sea and Indian Ocean, it gave rise to a concerning biological phenomenon consisting in the migration of biota from the Red Sea to the Mediterranean (Lessepsian migration or erythrean invasion) (Zakaria, 2015). It represents an entry point for species, which highly concerns the Eastern part of the Mediterranean, where, because of the proximity to the canal, the recorded number of NIS is higher (Ulman, et al., 2023). Out of the 420 species introduced through the Suez Canal, the vast majority (308 species) were introduced likely in relation to shipping activities (ballast waters and hull-fouling), and about 64 were likely introduced through aquaculture. The highest estimated values of the ratio of alien to native species richness are observed in the eastern Mediterranean (especially in the Levantine and Aegean Sea), with a decreasing trend towards the western and northern part of the basin. Overall, this unprecedented change in biodiversity patterns needs to be attributed to the opening of the canal and its continuous enlargement, but the rise in seaborne trade has also played a role, facilitating numerous introductions via shipping activities, along with the introduction of alien commodity species (Katsanevakis et al., 2014).

Although many are the stressors acting upon the Mediterranean Sea, the abrupt increase in species introduction, establishment and expansion rates must be found in the synergistic action and combination of some of them, including Lessepsian migrations, and climate change (Raitsos et al., 2010). In addition, environmental conditions, habitat fragmentation and human activities resulting in anthropogenic disturbances (namely aquaculture, oil and mining) are factors affecting the rates of invasions, explaining why this basin is facing an unprecedented acceleration in this phenomenon (Houngnandan et al., 2022). All the above-mentioned factors seem to be, in fact, responsible for the exceptional rate of biodiversity changes we must deal with today, with a rate of introductions in the Mediterranean Sea up to 1 species every 1.5 weeks (Raitsos et al., 2010). Understanding the factors promoting this species' expansion might be crucial to assess their effects in the Mediterranean Sea, where the consequences of climate change are already in place, with major impacts exerted on the Eastern Mediterranean basin, or EMED (already saltier and warmer than the rest of the basin, Winters et al., 2020).

1.3. *Caulerpa taxifolia*: evaluating this species' invasive success and its interaction with *P. oceanica*

Of the ca. 1000 alien species documented in this region (Musco et al., 2014; Katsanevakis et al., 2014), the Mediterranean Sea seems to host the largest number of introduced marine algae ever recorded, with more than 60 recorded exotic macroalgae, of which at least eight are considered invasive (Houngnandan et al., 2022). In particular, some invasive macroalgae have raised serious concerns because of their ability to alter the physical and chemical characteristics of the habitats they invade (such as sediment deposition rate, redox potential depth, etc.) and to compete with native flora, potentially disrupting biodiversity and ecosystem functioning (Musco et al., 2014). Two of the most relevant alien species in the Mediterranean Sea, *Caulerpa racemosa* and *Caulerpa taxifolia,* are here analyzed. Given their extensive distribution, their presence in Cyprus and specific factors causing their proliferation should be further addressed.

From a morphological point of view, species belonging to the *Caulerpa* genus are characterized by a structure consisting of two main parts: a creeping portion, the stolon, which attaches to substrates thanks to the rhizoids (root-like structures), and an upright portion known as fronds, which exhibit a variety of shapes depending on the species (Infantes et al., 2011). The green macroalga *C. taxifolia* (Vahl) C. Agardh, 1817 (**Figure 1**) presents pinnate, fern-like fronds (up to 25 cm long and 2 cm wide) that tend to extend upwards, stemming from horizontal stolons which are up to 3 m in length (Mateu-Vicens et al., 2010). It is a highly invasive marine species well established in the Mediterranean Sea, where it was imported to be displayed as a decorative alga in an aquarium. Its accidental release and consequent spread enabled the species to colonize approximately 4700 ha as in 2002 (Phillips & Price, 2002).

Figure 1 - *Caulerpa taxifolia* var. *distichophylla* (Sonder) Verlaque, 1817. Picture captured in Limassol Bay in March 2023.

The "*killer alga*" was named after its ability to rapidly colonize and spread on seagrass habitats (Ellul et al., 2019). Mainly found in shallow waters, *C. taxifolia* is known to have a distribution range between 0 m and 100 m in depth. Even though *C. taxifolia* is commonly found on sand, it can also grow on mud, native macroalgae, in the proximity of *P. oceanica* (**Figure 2**) meadows and on its dead matte. Furthermore, *C. taxifolia* flourishes on rocky and artificial substrates (e.g., shipwrecks) which may provide suitable habitat for marine NIS but also favors their further spread. These substrates can act as steppingstones or even corridors for the expansion of alien species. In this respect, artificial structures could serve as sentinel sites for monitoring the emergence of new NIS (Mannino et al., 2019). Its remarkable success in expansion is likely due to minimal grazing pressure, coupled with its adaptability to new environments and efficient vegetative propagation (Montefalcone et al., 2015).

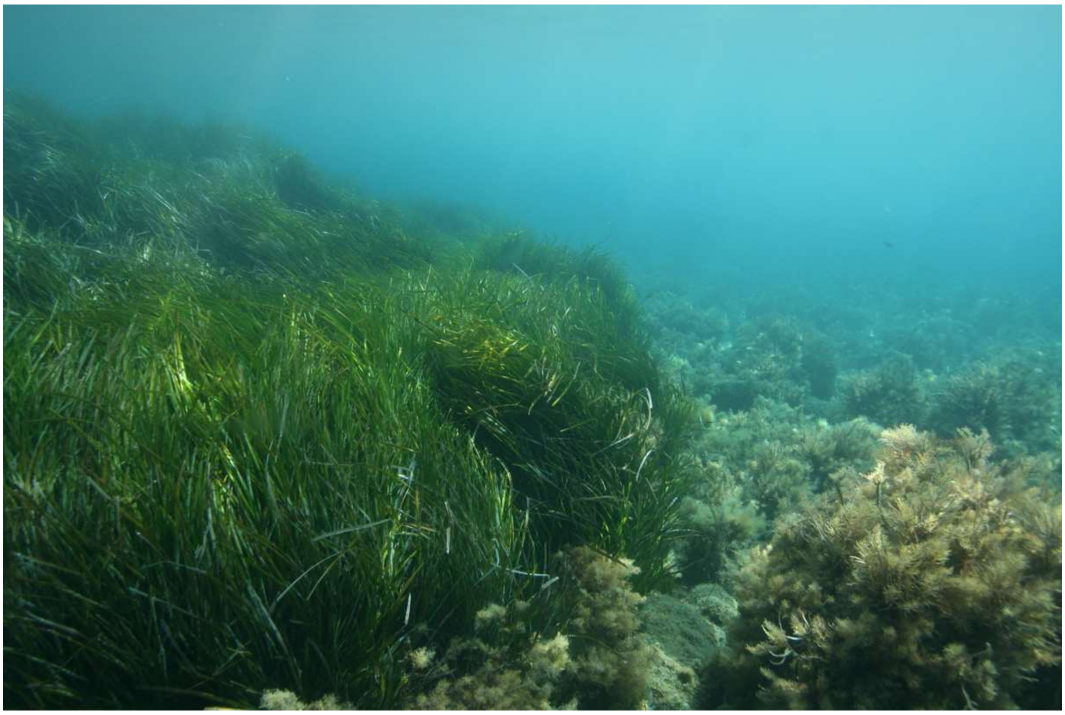


Figure 2- Posidonia oceanica (L.) Delile, 181. Picture captured in Limassol Bay in March 2023 (approximately 20 m depth).

After its escape from the Oceanographic Museum in Monaco, *C. taxifolia* quickly established itself as a dominant species along the northwestern Mediterranean coastline, reaching Monaco, Croatia, France, Spain, and Italy, where its presence was mainly concentrated along the western coast of Liguria starting from 1992 (Montefalcone et al., 2015). Initially reported in Monaco in 1984 (Ellul et al., 2019), the macroalga was then recorded in 2007 along the southern coast of Turkey, and subsequently in Sicily, Cyprus, Malta, Rhodes, and Libya (respectively in 2009, 2013, 2016, 2016 and 2017) (Di Martino et al., 2018). Molecular studies revealed that the Mediterranean and Californian populations are of Australian origin, suggesting that its spread was human mediated through aquarium trade (hence its name "invasive aquarium strain"). An exception is represented by the Tunisian strain, which strongly suggests that its origin needs to be reconducted to a second introduction event in the Mediterranean Sea (Cevik, et al., 2007). After reaching a peak in 2007 along the French and Monaco coastlines, its expansion was followed by a phase of decreased dispersal speed. Factors contributing to this slowdown may include local eradication efforts, natural regulation, competition with other congeneric species, or changes in environmental conditions (Houngnandan, et al., 2022). Its active spread in the Mediterranean Sea is proven by records of this species along the coasts of Tunisia, Sardinia and Sicily, suggesting that *C. taxifolia* is expanding beyond its northern and western limit. Additionally, it has been suggested that the intense maritime traffic between Sicily and the other Mediterranean countries plays an important role in the distribution of allochthonous species, producing a constant spillover of new invaders in nearby areas. In this context, both Sicily and Turkey might have played (and still are) a crucial role as receivers, transit, and source for secondary dispersal of NIS in the region (Mannino et al., 2019).

The peculiar structures of *P. oceanica* known as "matte" seem to be suitable for the colonization of the invasive algae, threatening the invertebrate communities that these structures host (Musco, et al., 2014). The potential effects exerted by *C. taxifolia* on *P. oceanica* are multiple. First and foremost, species belonging to the genus *Caulerpa* are characterized by the production of secondary metabolites, namely *caulerpeyne*, whose main functions include defense against herbivores and epiphytes (Mateu-Vicens et al., 2010), and important bioactivities such as cytotoxic effects, alteration of sexual reproduction in sea urchins, toxicity against fishes and growth inhibition of marine fungi (Cevik et al., 2016). In addition, the production of *caulerpeyne* favors *Caulerpa* spp. in the competition with *P. oceanica* by reducing both growth and longevity of its leaves, resulting in a higher turnover of the seagrass. This is exacerbated by a competition for light and nutrients between the two species, with *Caulerpa* spp. being favored by higher water temperatures, which result in a higher biomass. Overall, the replacement of *P. oceanica* by *Caulerpa* spp. is causing a structural change, resulting in a shift from a complex three-dimensional system to an almost two-dimensional one (Mateu-Vicens, et al., 2010). As for its distribution in the proximity of seagrass meadows, studies have shown that the presence of *C. taxifolia* not only leads to a decrease in the number of leaves (often exhibiting signs of etiolation and chlorosis), but it also appears to be associated with a decline in the meadows themselves. Specifically, there is a correlation between sparse meadows (<50% coverage) and susceptibility to the impacts of *C. taxifolia*. However, this correlation is not observed in areas with dense seagrass coverage (>50%), where the native seagrasses negatively affect the exotic algae through resource competition (e.g., for light). In other words, *C. racemosa* and *C. taxifolia* are associated with a regression of *P. oceanica*, and this phenomenon is exacerbated by the increasing temperatures, favoring *Caulerpa* in higher water temperatures (Glasby, 2013). In conclusion, several studies suggested that a higher abundance of *Caulerpa* spp., specifically *C. prolifera* and *C. racemosa,* could be causing significant alteration to sediment conditions in areas they colonize, therefore creating adverse conditions for *P. oceanica*'s growth, resulting in its decreased abundance (Holmer et al., 2009). In other words, when *Caulerpa* colonizes habitats previously dominated by *P. oceanica*, it impairs the ability of *Posidonia* to recolonize these areas, with negative impacts for restoration efforts, whose goal is to recover these valuable ecosystems.

Light and temperature, together with substratum type and hydrodynamics conditions are the main factors determining the spatial distribution of marine macrophytes. Overall, hard substrata (e.g., rocks) offer a more stable surface compared to unconsolidated ones (e.g., sand, mud), which are mobile and usually colonized by seagrasses or macroalgae with strong root-like structures. Thanks to its rhizoids, structures able to bind sediment particles, *Caulerpa* spp. can inhabit both wave-exposed rocky bottoms and sheltered sandy-muddy sediments (Infantes, et al., 2011). Furthermore, *C. taxifolia* shows resilience to both low and high light conditions, though it is more abundant and with longer fronds in shaded conditions. Despite it being often associated with *P. oceanica, C. taxifolia* is also found in association with other NIS, namely *C. cylindracea* and *Halophila stipulacea* (Forsskål) (Mannino, et al., 2019).

1.4. *Caulerpa racemosa*: an invasive species threatening the Mediterranean Sea

Caulerpa racemosa (Forsskal) J. Agardh, 1873 (**Figure 3**) is a *Chlorophyta* of the order *Bryopsidales* that belongs to the family *Caulerpaceae*. Similar to the congeneric green algae *C. taxifolia*, this species is easily recognized thanks to its spherical, club-shaped or mushroom- to disc-shaped branchlets. Molecular analyses confirmed that the introduced algae observed in the Mediterranean Sea was a hybrid species, resulting from the green algae *C. cylindracea,* native to Australia. These findings allowed to rule out the hypothesis of a lessepsian migration, leading to believe that the species was unintentionally introduced. At last, morphological and genetic studies classified this taxon as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman and Boudouresque (hereafter *C. racemosa*). By ruling out the possibility of the species being introduced through the Suez Canal, it was possible to identify the most likely pathways of introduction, including aquaria and ship traffic (ballast water, ship hull fouling). Though there's many ongoing speculations regarding its first introduction, the species was first observed in the Mediterranean basin along the coasts of Libya (1990), and since then its presence was recorded in Italy, followed by Greece, Albania, Cyprus, France, Turkey, Malta, Spain, Tunisia. In the two decades following the species' first encounter in the Mediterranean Sea, *C. racemosa* had colonized 12 countries and all the major islands in the basin, with a further expansion in the Atlantic Ocean, where it was recorded in the Canary Islands (Klein & Verlaque, 2008).

Figure 3 - *Caulerpa racemosa* (Forsskål) J.Agardh, 1873 Cabo de Palos, Murcia, Spain. Photo by Isabel Rubio Perez. Copyright © marmenormarmayor.es. Image Reference: 25084.

Several hypotheses have been carried out for the invasive success of this species. The key has been found in several factors including efficient reproductive strategies, secondary dispersal mechanisms, the lack of predators and pathogens in this region and various favorable environmental conditions (Klein & Verlaque, 2008). In its native range (southeastern Australia), *C. racemosa* is a common and opportunistic species, growing from the intertidal down to only 6 m depth and occurring intermixed with other species, without forming monospecific meadows. In contrast, it thrives under a large array of environmental conditions in the

Mediterranean Sea, where it is found in sheltered and unsheltered shores, and on all kinds of substrata (both soft and hard) such as sand, mud, detritic and coralligenous assemblages, pebbles, rocks, and on dead *P. oceanica* matte. Moreover, it has been demonstrated that this species can survive under a wider temperature range, from 8°C in Croatia to an average of 28°C in the Eastern Mediterranean basin (Cyprus, Libya, and Turkey), along with a wide depth range (0-70m), though its abundance is higher between 0 and 30m. Given its recent occurrence in the proximity of large cities and harbors, it has been concluded that *C. racemosa* can proliferate in both relatively pristine and polluted areas. This might suggest a secondary dispersal mechanism carried out by ship traffic and fishing activities. Under stressful environmental conditions, *C. racemosa* showed higher levels of enzyme activity (SOD, CAT) compared to other macrophytes found in the Mediterranean Sea, suggesting a higher capability to cope with environmental stress. Ultimately, this suggests the species' tolerance towards high levels of pollution, but not its affinity (Klein & Verlaque, 2008).

This species' potential harmful effect probably resides in its ability to produce secondary metabolites, similarly to *C. taxifolia*. Also in this case, the production of metabolites is associated with a function of defense towards herbivores, but it also gives an advantage in the competition with other species. In contrast to what happens in its native environment, *C. racemosa* is usually capable of forming meadows dominated by different species, including *C. prolifera* (**Figure 4**) *Cymodocea nodosa, Cystoseira* spp*., H. stipulacea*, but also red algal turfs, *Zostera noltii* and sessile macrofauna (bryozoans, sponges, gorgonian corals, and anemones). Though its presence is often associated with the endemic seagrass *P. oceanica*, this species doesn't seem to be able to penetrate its meadows, rather it proliferates in the proximity or within sparse meadows. Nevertheless, there seems to be a correlation between the health of seagrass meadows and this algae's invasion success, with a higher growth rate at low density of *P. oceanica* and vice versa. Furthermore, the presence of *C. racemosa* has been proven to have effects on the vegetative cycle of the seagrass, inducing a reduced leaf length and leaf area index when the alien species is present, coupled with a higher turnover rate (Klein & Verlaque, 2008).

Figure 4- Caulerpa prolifera (Forsskål) J.V. Lamouroux, 1809. Picture captured in Limassol Bay in April 2023

Given that the focus of this study is the occurrence of alien species, specifically *C. taxifolia* and *C. racemosa*, along the coastlines of Cyprus, it is imperative to delve deeper into their presence and distribution within the region. In Cyprus, *C. taxifolia* var. *distichophylla* was found in shallow waters as well as at a depth of 42 meters. Its occurrence has been often recorded in sediment comprising fine biogenic sand in areas dominated by the non-native seagrass *H. stipulacea* (**Figure 5**). It has also been observed on muddy substrates dominated by *C. racemosa* var. *cylindracea* and on biogenic hard substrates. Dense monospecific mats of *C. taxifolia* appear to be common between 9 and 18 meters, growing on various substrates including rocky, pebbles/shells, and sand (Mystikou et al., 2015). *C. racemosa* was first recorded in 1991 at around 30 m depth and was soon after documented all around Cyprus, hinting a proliferative growth in this region. This is likely achieved by successfully colonizing a wide range of habitat (sandy or muddy bottoms) and depths (from the intertidal zone up to 60 m depth), and enhanced by several favorable environmental conditions, namely mild temperatures during winter. Overall, these invasive algae's colonization might cause ecological problems, including habitat alteration and changes in the microbenthic assemblages, with significant alteration in species diversity and abundance (Argyrou et al., 1999). For this reason, there is an urgent need to implement basic knowledge regarding these species, with a focus on their updated distribution and on the factors enhancing their proliferation in the region.

Figure 5 – *Halophila stipulacea (Forsskål) Ascherson, 1867.* Picture captured at Marine and Environmental Research Lab (MER) of a sample collected around Limassol Bay in April 2023.

1.5. Assessing the distribution of species in marine habitats: a future perspective

Understanding the distribution of marine habitats and the factors contributing to the colonization and spread of key species is essential for marine spatial planning, for the proper management of marine environment and for the protection of its ecosystems. It represents a useful approach for quantifying the spatial extent of key habitats and providing an inventory of critical or sensitive areas and hotspots of biodiversity. These insights characterize the background for environmental impacts assessment and monitoring activities, but they are also useful for developing restoration initiatives to mitigate the loss of both degraded and carbon-rich ecosystems (Montefalcone et al., 2021). While traditional efforts are usually focused on the assessment of commercially relevant species, it is equally important to focus on other ecologically relevant species which are often overlooked, as in the case of macroalgal forests. A few key species, like seagrasses and macroalgae, represent foundational elements in coastal ecosystems, providing essential habitats and foraging areas for a wide range of marine faunal species. They also function as a protection from predation and a temporary nursery area for juvenile development of some commercially relevant fish species (Macreadie et al., 2017). Nowadays, many studies are focusing on macroalgae's ability to sequester carbon and their potential role in climate change mitigation. Nevertheless, this growing attention contrasts with a limited knowledge of their global extent (Duarte et al., 2022). Therefore, it is imperative to implement the efforts aimed at assessing the distribution and extent of crucial species, by also understanding the environmental factors that influence their colonization and spread.

Study aims and objectives

The Eastern Mediterranean Sea is strongly affected by the presence of alien species, which often pose threats to native ecosystems. Among the many recorded species, a significant portion is represented by plant species, including the notorious algae of the genus *Caulerpa.* This thesis aims to provide further insights into the presence and distribution of alien algal species in Cyprus, by focusing on *C. taxifolia* and *C. racemosa*. Additional understanding of their distribution would provide a novel outlook, increasing our knowledge of the factors driving their proliferation in this region.

The main research objectives of this thesis are:

- Mapping the presence and distribution of alien *Caulerpa* species in Cyprus.
- Elucidating the complex interplay of physiochemical factors that drive the proliferation and distribution of alien *Caulerpa* species, in particular sediment composition and proximity to the main ports.
- Understanding the effect of organic matter, granulometry and distance from ports on their invasion dynamics on the population abundance of *Caulerpa* species

2. Materials and methods

2.1. Study site

The Eastern Mediterranean Sea (EMED) encompasses the Levantine, Aegean, Ionian and Adriatic Sea. It is connected to the western Mediterranean through the Strait of Sicily (Ionian Sea), the Otranto Passage (Adriatic Sea) and the Cretan Passage, located in the Levantine Sea, which opens to the Red Sea and Indian Ocean through the Suez Canal. This region is highly subjected to biological invasions; this is not only due to its placement, but also to busy maritime traffic, the presence of several shellfish farms and, most importantly, the Suez Canal (Galil & Zenetos, 2002). As for its oceanographic conditions, the Mediterranean Sea is generally oligotrophic with strong environmental gradients, making the eastern end more oligotrophic than the western part. The eastern Mediterranean basin is characterized by a gradual nutrient and chlorophyll depletion starting from the Aegean until the Ionian and Levantine Sea, and primary production rates are usually about three times lower in the eastern part compared to the rest of the basin (Galil & Zenetos, 2002). Nonetheless, coastal areas are enriched by regional features, including changing wind conditions, currents, and temporal thermoclines. The biological production, inversely related to the increase in temperature and salinity, follows a decreasing trend going from north to south and west to east (Coll, et al., 2010).

2.2. Data collection

To evaluate the factors behind the proliferation of alien *Caulerpa* species in this region, a study was conducted around the entire area controlled by the Republic of Cyprus. Sampling took place in September 2022, by collecting grabs at a depth of approximately 30 m (± 10 m). Grabs were collected by deploying a Van Veen Sediment Sampler directly from the boat at predetermined locations. Once the location was reached, the sampler was lowered on the seafloor, opened and closed after sediment collection. **Table 1** provides details on the number of samples and respective sampling locations, whereas **Figure 6** portrays them on a map.

Table 1 - *Sample number and respective location and depth (m). Depth wasn't recorded for samples n. 7, 8 & 54*

Sample n.	Latitude	Longitude	Depth (m)	Sample n.	Latitude	Longitude	Depth (m)
7	34°58.180'N	33°56.659'E	-	34	34°35.331'N	32°54.391'E	36.5
8	34°56.562'N	33°54.189'E	-	35	34°37.545'N	32°52.607'E	38.6
9	34°56.341'N	33°51.367'E	31.2	36	34°38.952'N	32°49.794'E	39.5
10	34°57.874'N	33°48.669'E	35.2	37	34°38.221'N	32°46.574'E	39.8
11	34°58.219'N	33°45.435'E	35.5	38	34°37.624'N	32°43.312'E	40.1
12	34°57.754'N	33°42.188'E	34.8	39	34°38.073'N	32°40.195'E	~ 40
13	34°55.954'N	33°39.727'E	32.5	40	34°38.741'N	32°37.069'E	40.5
14	34°53.271'N	33°39.405'E	35.5	41	34°39.467'N	32°33.944'E	40.7
15	34°50.640'N	33°39.100'E	35.1	42	34°40.682'N	32°31.139'E	38.6
16	34°48.386'N	33°38.208'E	38.6	43	34°41.673'N	32°28.085'E	39.3
17	34°47.509'N	33°35.512'E	34.7	44	34°42.888'N	32°25.570'E	40.6
18	34°46.848'N	33°32.626'E	34.6	45	34°45.125'N	32°23.502'E	37.3
19	34°45.763'N	33°29.678'E	37.8	46	34°47.738'N	32°22.717'E	41.5
20	34°44.738'N	33°26.712'E	39.6	47	34°49.996'N	32°21.858'E	40.4
21	34°43.338'N	33°24.058'E	37.5	48	34°52.220'N	32°20.032'E	36.3
22	34°42.759'N	33°21.997'E	34.5	49	34°54.628'N	32°18.661'E	33.6
23	34°41.997'E	33°19.841'E	37.5	50	34°57.860'N	32°18.193'E	22.0
24	34°42.293'N	33°17.454'E	38.0	51	35°00.109'N	32°17.995'E	33.0
25	34°41.960'N	33°13.806'E	36.3	52	35°01.746'N	32°15.315'E	33.5
26	34°41.933'N	33°10.550'E	39.3	53	35°04.479'N	32°15.430'E	35.4
27	34°41.573'N	33°07.297'E	38.6	54	35°07.122'N	32°15.863'E	-
28	34°40.376'N	33°04.352'E	38.4	55	35°05.889'N	32°18.305'E	32.3
29	34°37.895'N	33°02.247'E	36.5	56	35°04.094'N	32°20.734'E	36.2
30	34°35.875'N	33°01.801'E	36.3	57	35°03.704'N	32°23.806'E	30.3
31	34°33.280'N	33°02.998'E	40.3	58	35°04.525'N	32°27.013'E	36.7
32	34°33.600'N	32°59.750'E	39.2	59	35°06.458'N	32°29.204'E	35.0
33	34°33.071'N	32°56.523'E	40.6	60	35°08.850'N	32°30.640'E	36.4

Figure 6 - *Sampling locations around Cyprus. Numbers on the map represent each sample number*

For each location, grabs were taken onboard, photographed (for a total of 54 samples and 456 pictures) and then placed in labeled buckets. Following data collection, all grabs were transported to a coastal site and placed in a sieve to collect the sediment. Using wave motion, the samples were washed to remove mud and sand. The remaining sediment was then stored in smaller, labeled buckets with lids for the subsequent granulometry and organic matter analysis.

2.3. Data processing

Data was processed using PhotoQuad v1.4 (Trygonis & Sini, 2012), a software for image processing of photographic samples, with the intent of evaluating the percentage of coverage for the species of interest. After careful selection, pictures were imported in PhotoQuad and a species library was created (**Table 2**) based on the species that occurred the most throughout the study. Given the irregular shape of the grabs, for each one of them a freehand polygon was designed, following the border of the sample. Subsequently, 100 random points were generated using Photoquad software, which allows for systematic random sampling within an image. By selecting the "100 random points" option and then "Spawn", this software automatically distributes 100 points (numbered from 1 to 100) across the image. Following this, each point was named according to the species list. A picture of this process is shown in **Figure 7.** For this part of the analysis, "Soft Substrate" was used without making a distinction of the type of sediment, which varied substantially for each grab (e.g., silt, gravel, pebbles…).

Species	Species ID	Group	Group ID
Posidonia oceanica	1	Seagrass	1
Halophila stipulacea	2	Seagrass	1
Penicillus capitatus	3	Algae	2
Caulerpa prolifera	4	Algae	2
Caulerpa racemosa	5	Algae	2
Caulerpa taxifolia	6	Algae	2
Other	7	Algae	2
Soft substrate	8	Sediment	3

Table 2 - *Species library*

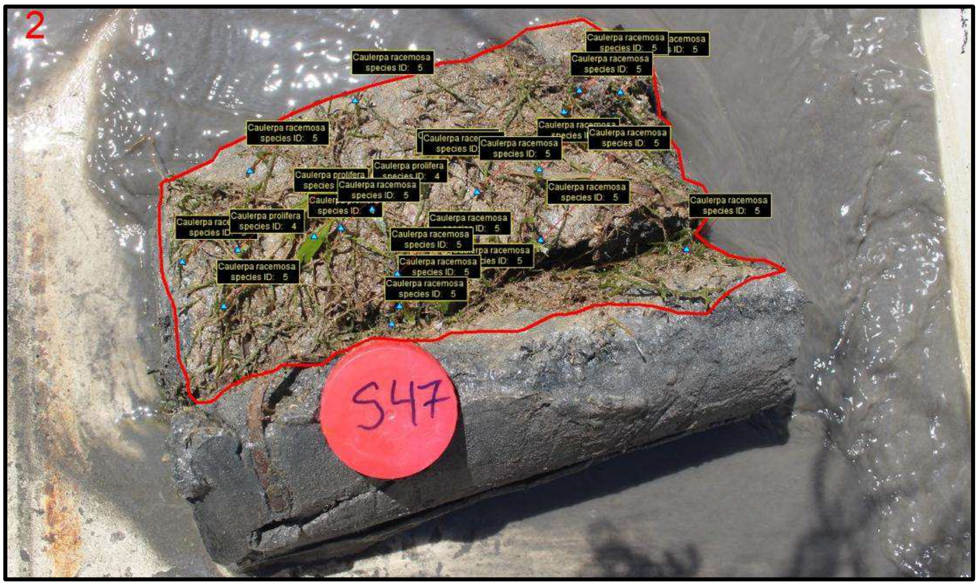


Figure 7 – *Picture showing the process of preparing the sample for the 100 random points analysis*

Given that both *C. taxifolia* and *C. racemosa* seem to thrive in relatively polluted areas (Klein & Verlaque, 2008), the distance from the major ports of Cyprus, Larnaca, here after named Port 1, (34° 55,0122'N 33° 37,9998'E) and Limassol, Port 2 (34° 39'N 33° 1,0002'E), was considered in this study. The exact coordinates for these locations were found on Google Earth, by searching, respectively, "Larnaca port" (port 1) and "Limassol port" (port 2). The distance of each sample from port 1 and port 2 was calculated on QGIS 3.36.0, and the results were included in the statistical analysis. Depth wasn't considered to be a factor in this analysis, since it didn't vary much between the samples (22-40.6 m).

2.4. Granulometry and organic matter analyses

For the second part of this study, factors such as the type of sediment and the amount of organic matter present in each sample (grab) was analyzed. To assess the particle size composition, samples were let dry for 48 hours in a dry oven at 100°C and subsequently they were weighted using a precision balance (3 decimal places). Sediment granulometry was performed using the *Endecotts Octagonal Digital* sieve-shaker, consisting of six sieves, each of different mesh size (respectively 0.075 mm, 0.15 mm, 0.212 mm, 0.3 mm, 0.6 mm and 2 mm). For each sample, the sediment of each sieve was weighted, and the respective weight was recorded. In order to determine the sediment type of each sample, mean grain size was calculated using the graphical method by Folk and Ward (1957) with the software GRADISTAT v8 (Blott & Pye, 2001). Mean grain size refers to the average grain size of the sediment distribution and provides an indication of the dominant grain character.

To assess the organic matter content, the loss of ignition (LOI) method was used, by following the guidelines of the certified Quality System ISO CYS EN 15169:2007. Following the granulometric analysis, all the sediment that passed through the 212 mm sieve (ISO3310-1) was collected and placed in plastic containers. The remaining sediment was re-homogenized into smaller grains using a mortar and a pestle, and then repassed through the 212 μm sieve. The resulting permeated sediment was used in the organic matter analysis. The homogenized sediments of the weight of 1.5 g collected from the sieve were placed in a laboratory oven at a constant temperature of 500°C for 3 hours. After removal of the sediment from the oven, each sample was weighted and compared with the initial weight. The resulting percentage difference between the initial and final weight for each sample represented the percentage of organic material in each sediment. The percentage of organic matter shown in the results represents the average of three replicates from each sample.

2.5. Statistical analysis

Data processing and analyses were carried out using Microsoft Excel for Microsoft 365 MSO (Microsoft Corporation, 2024) and R version 4.4.0 (R Core Team, 2024). Initially, the results of the 100 random points analysis were exported into Excel, where a single file comprehensive of the grab number, distance from main ports, Larnaca and Limassol, minimum distance from the port (entitled DP1 and DP2 respectively) and % of each of the species in species list was used as initial dataset for the following analysis. The choice of considering the distance from the main ports is related to the fact that alien *Caulerpa* species are known to thrive in more polluted areas. For this reason, it was hypothesized that the presence of these species was going to be higher when closer to the ports. Then, a Principal Component Analysis (PCA) was performed using R version 4.4.0, using the following packages *FactMineR (*Lê, Josse, & Husson, 2008), *ggplot2* (Wickham, 2016) and *factoextra* (Kassambara, 2021), with the intent of clearly visualizing potential clusters of species based on their location and occurrence. Following the PCA, a Spearman's correlation was also performed on R (package *ggplot2*, Wickham, 2016, method = "spearman"), with the intent of evaluating whether there was any correlation in the input data (% of *C. racemosa* for each grab, DP1, DP2 and Dmin, the minimum distance from either one of the two ports). Because of its abundance, the main of focus of the analysis turned out to be *C. racemosa*, which was assessed after the analysis carried out on Photoquad (see Results). Other species of interest, namely *C. taxifolia* and *H. stipulacea*, were not as expected, therefore were considered less relevant for this kind of analysis. The same happened with *P. oceanica*, in particular because healthy seagrass samples were discarded during data collection, with the exception of one sample, grab number 55, which consisted in one healthy shoot on a rocky substrate.

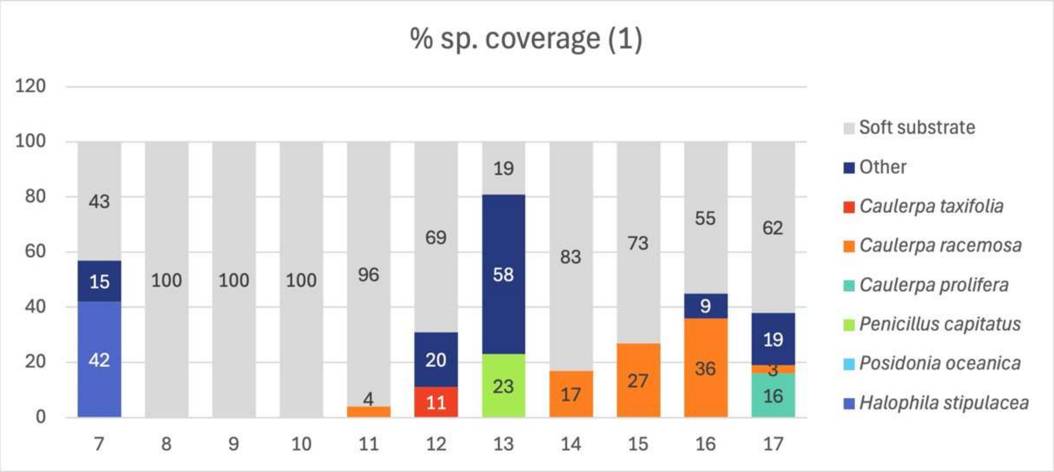
2.6. Generalized Linear Mixed Model (GLMM)

Data collected from the Photoquad analysis (% species coverage), OM and Granulometry analyses were grouped together with Minimum Distance from the ports for each sample to perform a GLMM. Given that *C. racemosa* and *C. taxifolia* counts showed many zeros in the analysis, a zero-inflation model was considered to be more appropriate. The aim of the analysis was to assess whether factors like type of sediment (hereafter "Sediment", with 3 levels: Sand, Clay and Silt), distance (Dmin) and organic matter (OM) significantly influenced the distribution of alien species like *C. racemosa* and *C. taxifolia*. Because of the low number of species that were obtained, % of *C. racemosa* and *C. taxifolia* for each sample were added up together for this analysis, and generally named "*Caulerpa*". In addition, because of multicollinearity problems, the number of levels for the factor "Sediment" had to be reduced, while it initially counted 9 different levels, as resulting from the granulometry analysis: Coarse Sand, Coarse Silt, Fine Sand, Fine Silt, Medium Sand, Medium Silt, Very, Coarse Sand, Very Fine Sand and Very Fine Silt. A total of 6 different models were fitted using the '*glmmTMB*' package on R (version 4.4.1): a base model without interaction terms (*family* = *nbinom2*()), a model with interaction terms, and four zero-inflated models (one with all predictors in the zero-inflation component ~Dmin + OM + Sediment, a second one with Dmin and Sediment in the zero-inflated component, a third one with only Sediment in the zero-inflation component, a fourth one, a zero-inflated model with the interaction between OM and Sediment). Akaike's Information Criterion (AIC) (Akaike, 1973) was used to rank the GLMM models. The zero-inflated model with interaction between OM and Sediment and Dmin was evaluated as the most suitable given that it resulted in the lowest AIC score. Finally, Model diagnostics were checked with '*DHARMa'* packages.

3. Results

3.1. Species coverage assessment

In the present study, a total of 54 grab samples were considered. By analyzing 100 random points in Photoquad for each grab, it was possible to assess the coverage of the species of interest, here represented by a percentage. The results are here portrayed in the following graphs (**Figure 8**).



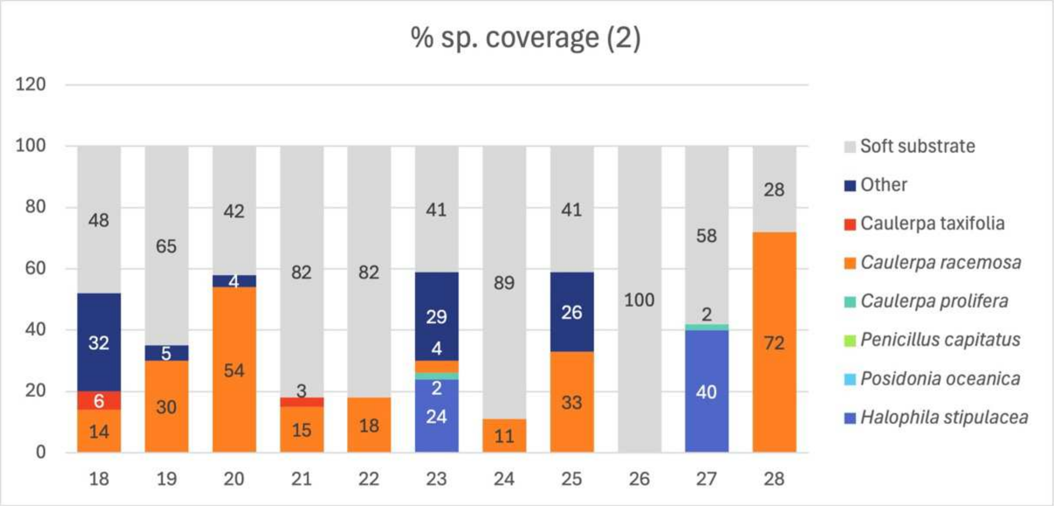


Figure 8- *Graphs representing the % of species found in samples 7-60 according to the estimate made on Photoquad. To make the graphs more readable, samples were divided in 5 charts.*

From a first analysis it can be concluded that 14 samples (25% of total samples) did not have any species but were only made of substrate. As for the rest, most samples (32 samples, 58% of total samples) had at least 3% of *C. racemosa.* The rest of the species evaluated in this project were not as abundant, though a vast part of the samples were characterized by roots, rhizomes or fragmented parts of species (here named "*other*"), which made the species recognition challenging, as they could have covered *C. racemosa*, *C. taxifolia* or *H. stipulacea*, making it difficult to spot them through the evaluated pictures. By using QGIS 3.36.0, it was possible to portray these results on a map, with the aim of better visualizing the species distribution around the study site. **Figure 9** shows how *C. racemosa*, *C. taxifolia* and *C. prolifera* were located in the southern part of the island, characterized by the richest samples (in terms of species composition) out of all the ones collected. It needs to be noted that although sample 61 was recorded (both its depth and location), no grab was collected and/or photographed, and it was therefore excluded from this analysis, as it wasn't possible to assess its species coverage assessment (empty graph in this picture).

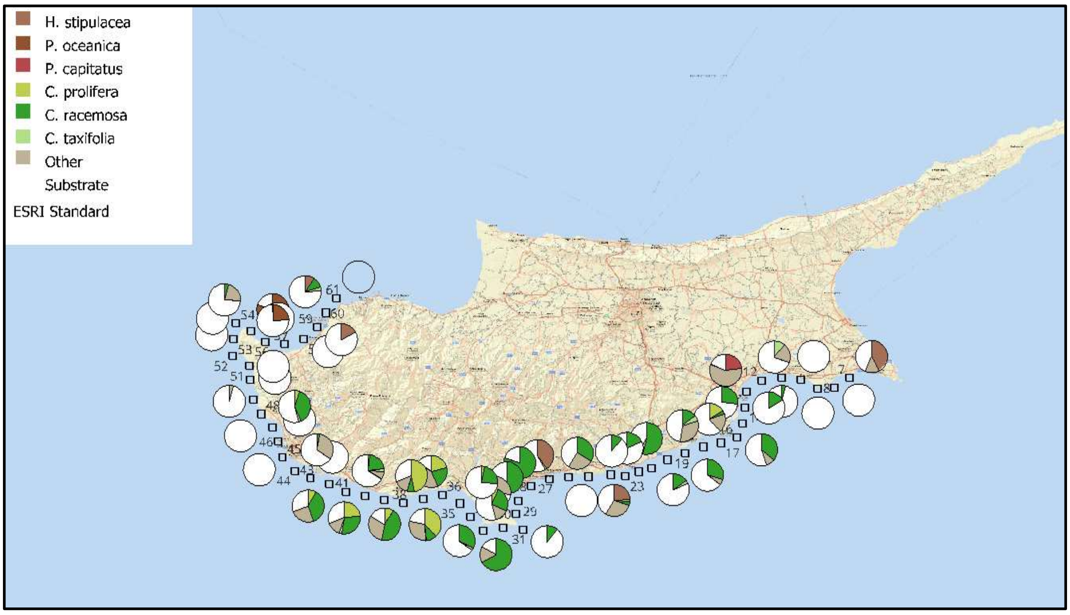


Figure 9- *Map of the study site showing sample locations and respective results from the analysis on PhotoQuad. The empty circle is referred to sample 61, which was recorded but released after collection and therefore no data are available for this analysis*

3.2. PCA and Spearman's correlation

For this part of the analysis, a dataset comprehensive of sample number and species % was used to run the Principal Component Analysis on R. As the goal was to assess whether there is a structure in the species distribution among the samples, "Soft Substrate" was not included in this analysis. From a first analysis (**Figure 10**) it can be concluded that there's not much structure in the evaluated samples, except for a small cluster of samples in the left part of the graph, between the vectors of *C. racemosa* and *P. oceanica*. In addition, it is possible to highlight some structuring in samples 37-42, which showed a higher % of *C. prolifera* than the other samples. This suggests that these stations have a distinct composition compared to others. Interestingly, the species that showed a strong correlation with the most stations is *P. oceanica*, highlighting its widespread presence in the study area. It can also be stated that there's only a few outliers in these results, as represented by samples n. 55, 27, 7 and 13. This is not peculiar, rather coherent with the results of the species coverage assessment: samples n. 7 and 27, for instance, showed a higher amount of *H. stipulacea* (respectively 42% and 40%), which was uncommon in all the other evaluated samples. Similarly, sample n. 13 was the only one characterized by soft substrate and *P. capitatus* exclusively, while sample n. 55 was characterized by a single shoot of *P. oceanica*, as the others were discarded and, therefore, not used in this thesis. As for the remaining samples, it cannot be concluded that they all had a similar composition in species assemblage, rather most of them showed a significant % of *C. racemosa*, indubitably the most abundant species found in the collected grabs.

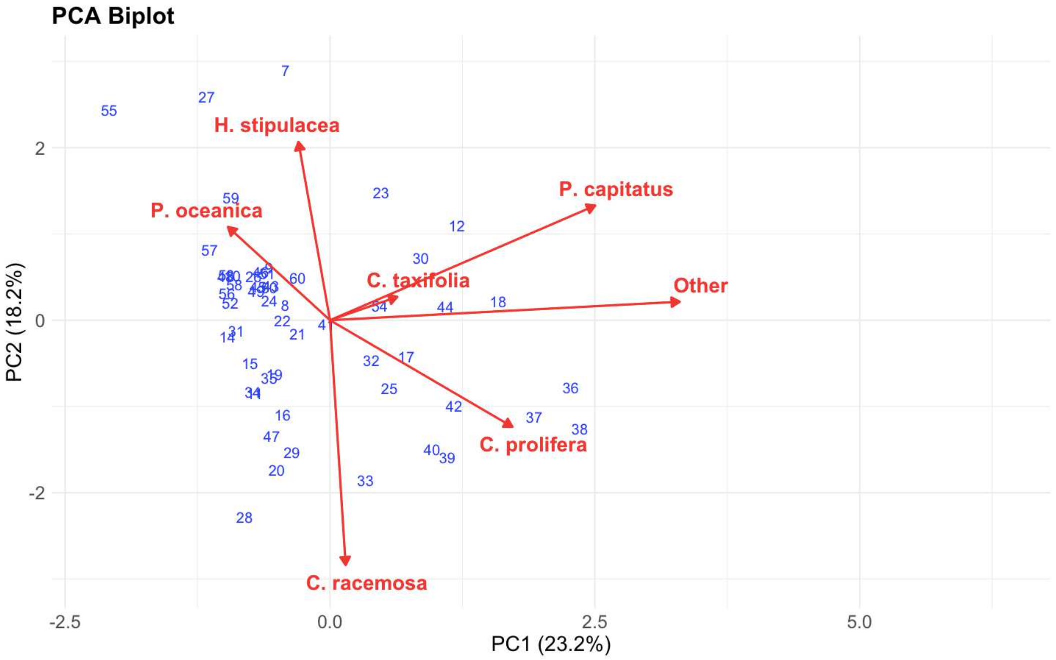


Figure 10- *Principal Component Analysis of samples 7-60. Biplot showing species vectors with labels representing species names (e.g., C. prolifera, P. oceanica). Sample numbers are plotted based on their species association*

In addition, in order to investigate if the presence of one species correlates with the presence of another, a correlation plot was created on R using the package *corrplot* (Wei & Simko, 2017, **Figure 11**). The results show a slight negative correlation for all the evaluated species, with the exception of *C. prolifera* and *C. racemosa*, suggesting that the presence of *C. prolifera* might be associated with this invasive species, potentially enabling and/or facilitating its establishment. Notably, a slight stronger negative correlation is observable between *C. racemosa* and the two seagrasses' species evaluated in this project, *H. stipulacea* (-0.20) and *P. oceanica* (-0.13). This result might be interesting when considering that invasive algal species are associated with a regression of endemic species like *P. oceanica*, especially with higher temperatures that tend to be favoring *Caulerpa*.

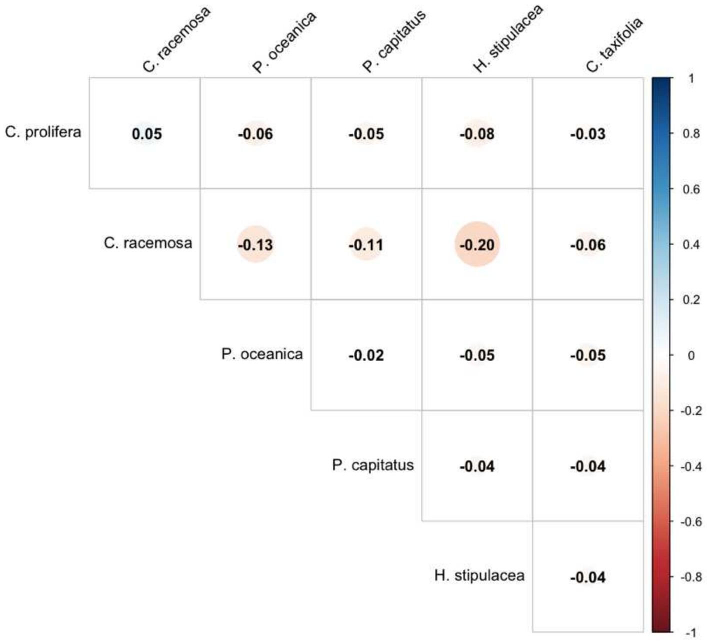


Figure 11- *Correlation plot of species abundance. Negative correlations are represented in red, positive correlations are represented in blue.*

Based on these results, a Spearman's correlation was performed on R (version 4.4.0), since it is a method that doesn't assume the normal distribution of the data. To do so, *Caulerpa*'s presence or absence, distance from the two ports (DP1 and DP2) and Minimum Distance (Dmin) were evaluated. Samples number and respective minimum distance are shown in **Table 3**. The results of the Minimum Distance are shown in km, and they were computed on QGIS. The results of the Spearman's correlation (**Table 4**) show a negative correlation of all the comparison evaluated in this analysis, suggesting that the presence of *C. racemosa* decreases as the distance increases. Out of the 3 comparisons, *C. racemosa* and DP2 showed the stronger negative correlation (-0,534634), suggesting that the port of Limassol might have a stronger influence on the presence of this species.

<u>Table 3</u> - Minimum distance (Dmin) from each sample to either one of the two reference points, Larnaca and Limassol ports. These results were computed using QGIS.

Sample	Dmin (km)	Sample	Dmin (km)
7	28.172	34	12.051
8	24.048	35	12.827
9	19.731	36	16.777
10	16.184	37	21.762
11	11.794	38	26.83
12	7.101	39	31.505
13	2.148	40	36.223
14	4.29	41	40.99
15	8.957	42	45.354
16	13.074	43	50.143
17	15.36	44	54.202
18	18.225	45	57.966
19	22.362	46	60.217
20	26.702	47	62.677
21	31.363	48	66.686
22	33.095	49	70.316
23	29.597	50	73.562
24	26.131	51	75.852
25	20.572	52	80.903
26	15.792	53	83.462
27	10.921	54	85.756
28	5.915	55	81.421
29	3.251	56	76.475
30	6.276	57	72.341
31	11.393	58	69.613
32	10.4	59	69.719
33	12.998	60	71.553

<u>Table 4</u> – *Spearman's correlation values. Dmin refers to the minmum distance of the samples from either one of the two reference ports. DP1 refers to "Distance Port 1" (Larnaca) and DP2 refers to "Distance Port 2" (Limassol). C. racemosa here does not refer to the specie's abundance, rather its presence or absence. Statistically significant values are shown in bold.*

Comparison	Correlation	p-value
C.racemosa-DP1	-0,2943491	**0,03073263**
C. racemosa-DP2	-0,534634	**0,00003121357**
C. racemosa-Dmin	-0,3327947	**0,01393863**

3.3. Organic Matter & Granulometry analyses

The organic matter and granulometry analyses comprised of several different results. The most important findings are reported below, including particle size distribution % (**Figure 12**), sediment type (**Figure 13**) and % of OM per sample (**Figure 14**). As for % particle composition, the major contribution to the evaluated samples corresponds to <0.075 mm and 0.075-0.15 mm sieves. The particle size distribution was quite abundant in the 0.6-2 mm sieve only for a few of the samples (n. 7, 8, 9, 45, 48, 49, 50, 52 & 53) (**Figure 12**).

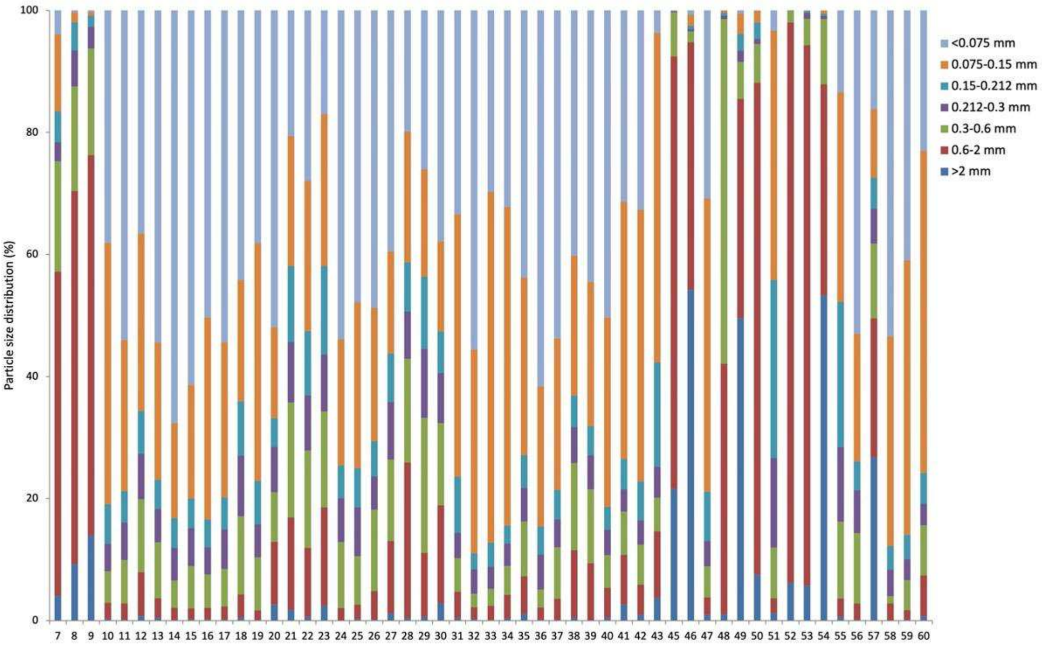


Figure 12- *Particle size distribution. The results are shown as a % per each type of sieve: <0.075 mm, 0.075-0.15 mm, 0.15-0.212 mm, 0.3-0.6 mm, 0.6- mm2, >2 mm*

Figure 13 aims at better visualizing all the sediment types found in the samples of this study. Out of 10 types, the most recurrent ones were Fine Silt (10%), followed by Clay (13%) and Very Coarse Sand (13%).

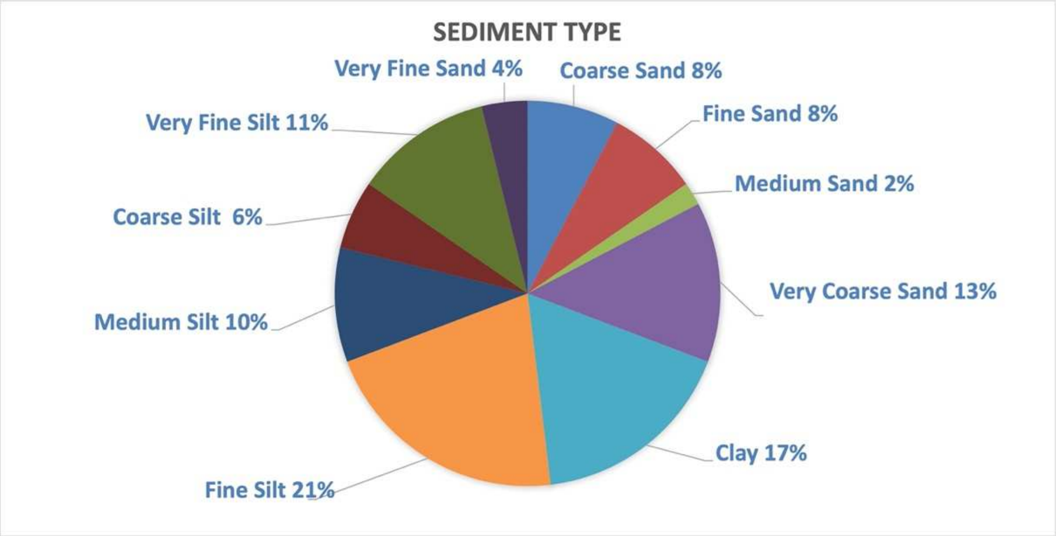


Figure 13 - *Sediment types found in the evaluated samples and respective %*

As shown in **Figure 14**, the highest content of organic matter was found in samples number 27, 28, 29 & 30, which were located closer to the city of Limassol, together with sample n. 57, unlike the samples collected in the same area, which showed lower OM contents. On the contrary, the lowest OM content was found in 43, 50, 8 & 9, and their content was overall similar to the one of nearby samples, with the exception of sample n. 43. On average, the amount of organic matter in the collected samples was 6.90%, though no similar value has been found for the Mediterranean Sea, making a direct comparison challenging.

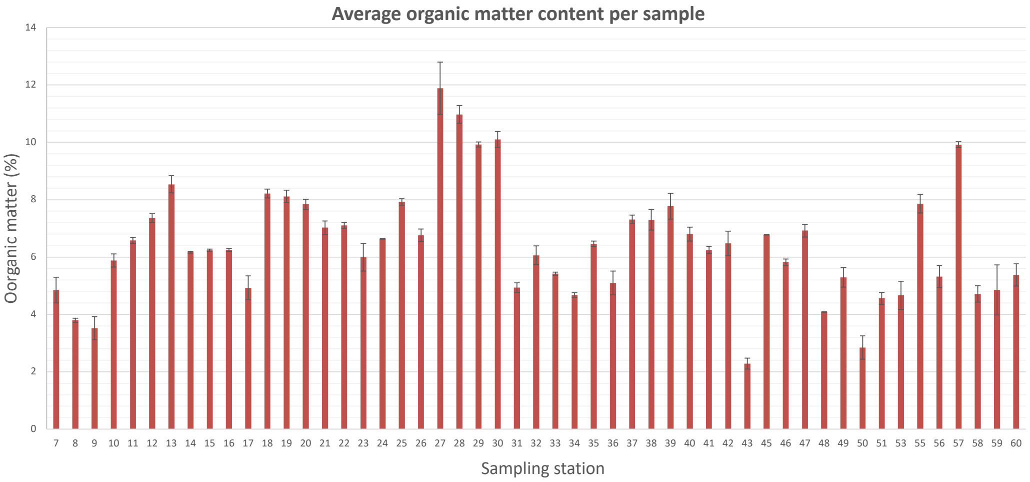


Figure 14 - *Average % of OM (± SD) in the samples 7-60*

3.4. Generalized Linear Mixed Model

The results of the GLMM, as shown in **Table 5**, provide comprehensive insight into how organic matter (OM), minimum distance from ports (Dmin), and sediment type (sand, clay, and silt) influence the presence and distribution of Caulerpa (including *C. taxifolia* and *C. racemosa*). The conditional part of the model gives insights on the extent of *Caulerpa*'s distribution in the study area. Because none of the evaluated factors are significant (p-value <0.05) it can be concluded that the relationship between the predictors and *Caulerpa* coverage may be weak, or there might be other factors influencing the species' distribution, which are not captured by this model. Nevertheless, because of the zero-inflated part of the model, some interesting conclusions can be drawn in this situation. For instance, increases in OM and the presence of Silt are associated with an increase in the probability of observing zeros (absence of *Caulerpa*), though none of these findings is statistically significant. On the contrary, Sand significantly increases the likelihood of zeros, indicating that in sandy sediments, zeros are more common ($p = 0.0416$). A small increase in distance from ports (Dmin) slightly but significantly increases the likelihood of observing zeros ($p = 0.0135$). The interaction between organic matter and sandy sediment decreases the likelihood of zeros, hinting that higher organic matter in sandy sediments makes zeros less likely, with a result close to significance ($p = 0.0641$). At last, the interaction effect (OM*Silt) similarly decreases the likelihood of zeros, though less dramatically, however this result is not statistically significant ($p = 0.5829$). In other words, if both organic matter and sand, and organic matter and silt are present, this is likely associated with the presence of *C. taxifolia* or *C. racemosa,* but their extent is not well captured by this analysis.

Table 5- GLMM results for the model "Zero Inflation with Interaction between OM and Sediment". The first part of the table (above) represents the conditional results of the model, while the second (below) includes the results of the zero-inflated part. Values in bold indicate statistical significance at $p < 0.05$

Factor	Odds Ratio	95% CI	z-Value	p-Value
Conditional fixed effect				
(Intercept)	8.45	0.20–357.43	1.12	0.264
OM	1.15	0.63–2.11	0.46	0.647
Sediment Sand	0.06	0.00–6.57	-1.17	0.243
Sediment Silt	2.72	0.05–144.24	0.49	0.622
Dmin	0.99	0.98–1.01	-0.72	0.471
OM: Sand	1.38	0.69–2.73	0.91	0.363
OM: Silt	0.92	0.49–1.74	-0.25	0.802
Conditional Zero Inflation				
(Intercept)	0.00005	8.48e-10–2.43	-1.80	0.072
OM	3.06	0.66–14.15	1.43	0.151
Sediment Sand	**3.32e+05**	**1.62e+00–6.79e+10**	**2.04**	**0.042**
Sediment Silt	5.01	0.00–1.15e+05	0.31	0.753
Dmin	**1.07**	**1.01–1.12**	**2.47**	**0.013**
OM: Sand	0.17	0.02–1.11	-1.85	0.064
OM: Silt	0.66	0.15–2.89	-0.55	0.583

Out of all the models, the "interaction model" proved to be the most promising: both interactions have negative coefficients, suggesting they mitigate the effects of their individual components on the probability of zeros, which might hint complex relationships between Organic Matter and all Sediment types regarding the presence and absence of *Caulerpa*. In conclusion, the significant predictors identified in the zero-inflation part of the model, particularly sediment type and distance from ports, indicate that Caulerpa is less likely to be present in sandy sediments and areas farther from ports, possibly due to unfavorable environmental factors or pollution levels. However, when Caulerpa is present, these factors, along with their interactions (OM*Silt, OM*Sand), do not significantly influence its coverage, highlighting the need for further research to uncover other factors that may drive the distribution of *Caulerpa*.

To better visualize the relationship between the amount of organic matter and sediment type, and how these affect the distribution of *Caulerpa* in the evaluated samples, a scatterplot was created (**Figure 15**). By separating each sediment type, it can be easily concluded that Sand is indeed associated with the higher 0 counts of *Caulerpa* coverage % (species absence). In contrast, both Clay and Silt did not show a strong positive correlation between organic matter and *Caulerpa* coverage. Notably, none of the Clay or Silt samples had organic matter levels below 5.0, whereas Sand samples did. Out of the three, Silt had both the higher amount of organic matter content and the higher amount of *Caulerpa* species detected, though there is not strong evidence that *Caulerpa* presence increases significantly with either low or high organic matter levels. This relationship seems to suggest that Silt might provide a better environment for *Caulerpa*'s growth and proliferation, though further research is needed to confirm this hypothesis.

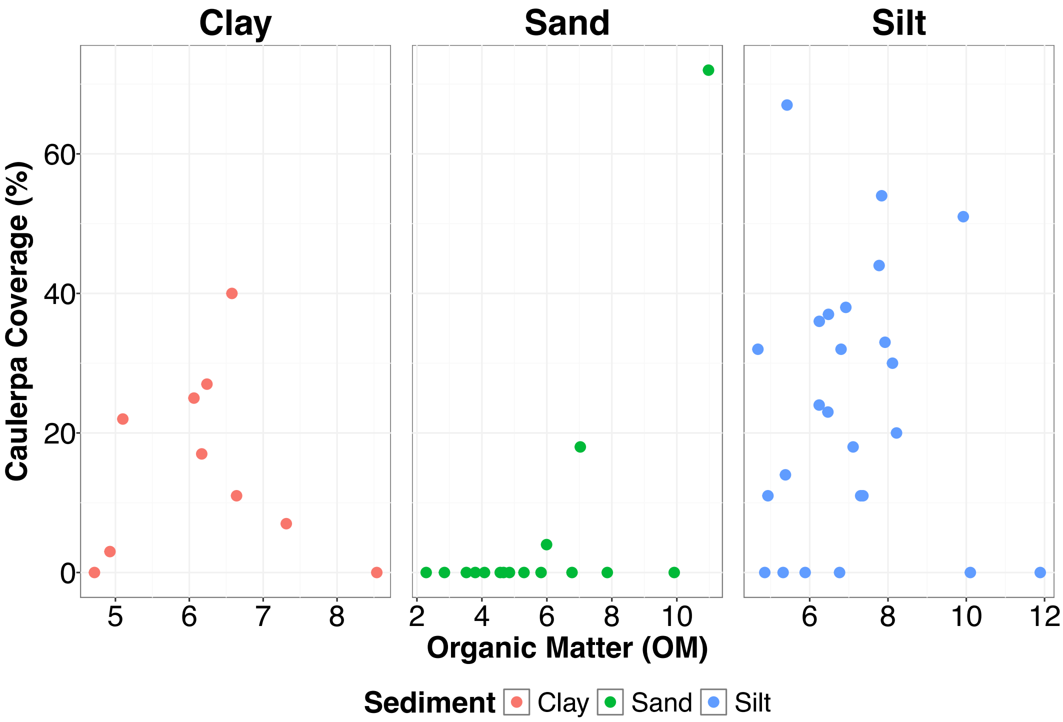
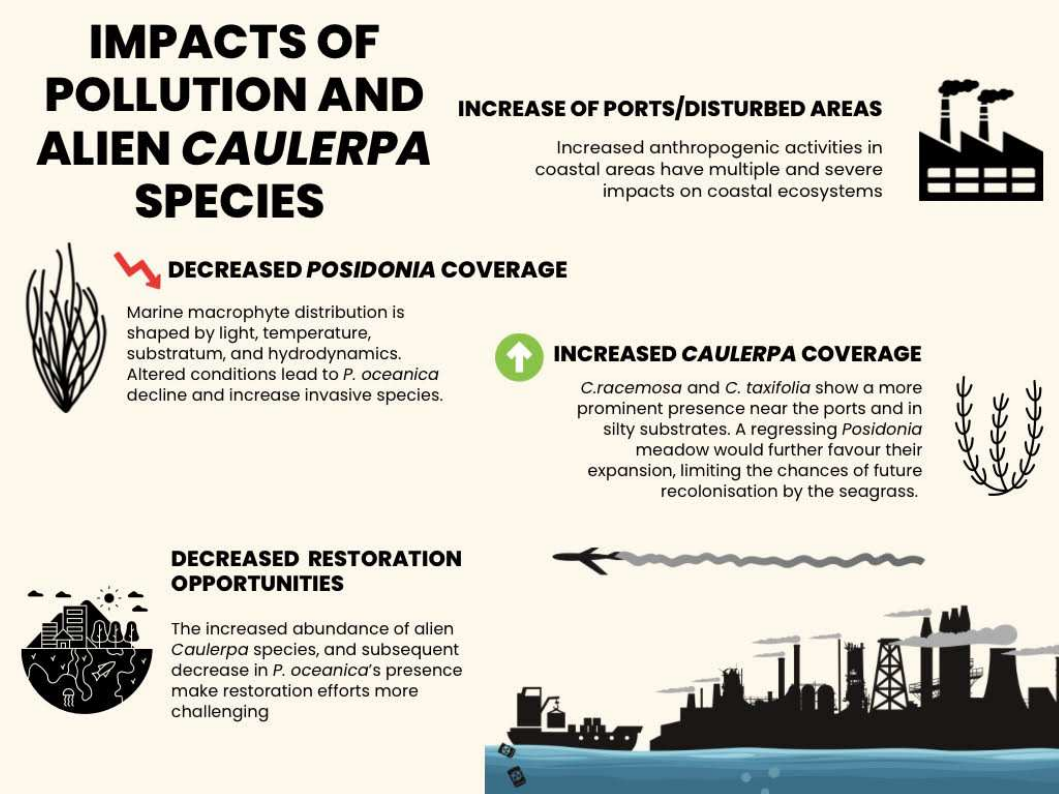


Figure 15- Scatterplot showing the interaction between organic matter content and *Caulerpa* coverage for each of the three different types of sediment.

4. Discussion

The factors driving the widespread expansion of *Caulerpa* remain unclear across large spatial extents. In this context, this thesis project is both novel and impactful as it identifies the key factors responsible for the colonization and proliferation of *Caulerpa*. This study reveals that *C. racemosa* and *C. taxifolia* show a tendency to colonize habitats with a higher organic matter content and with a preference for silt and clay substrate, whilst sandy areas are generally associated with the absence of these species. However, the invasive success of *Caulerpa* species might reside in the complex interaction between multiple environmental factors, which was not fully captured by this analysis. In addition, the role of distance from ports, as an indicator for secondary introductions, proved to be consistent with previous findings. These insights could facilitate targeted management actions, enable the identification of risk areas, and support proactive and early response strategies. In the following section, the significance of each factor will be examined in detail, with a focus on their broader implications for future research.

Figure 16 visually represents the key ecological interactions and impacts observed in the study. It provides a summary of the most important findings of this research project, highlighting the tight relationship between *P. oceanica* and *Caulerpa*.



IMPACTS OF POLLUTION AND ALIEN *CAULERPA* SPECIES

INCREASE OF PORTS/DISTURBED AREAS

Increased anthropogenic activities in coastal areas have multiple and severe impacts on coastal ecosystems

DECREASED *POSIDONIA* COVERAGE

Marine macrophyte distribution is shaped by light, temperature, substratum, and hydrodynamics. Altered conditions lead to *P. oceanica* decline and increase invasive species.

INCREASED *CAULERPA* COVERAGE

C.racemosa and *C. taxifolia* show a more prominent presence near the ports and in silty substrates. A regressing *Posidonia* meadow would further favour their expansion, limiting the chances of future recolonisation by the seagrass.

DECREASED RESTORATION OPPORTUNITIES

The increased abundance of alien *Caulerpa* species, and subsequent decrease in *P. oceanica*'s presence make restoration efforts more challenging

Figure 16 – *Graphical abstract showing the most important findings of this study*

4.1. Results interpretation

Macroalgal *Caulerpa* species are able to colonize habitats that are characterized by different biotic and abiotic factors, owing their invasive success in the Mediterranean Sea to the lack of natural predators, their ability to synthesize secondary metabolites that act as a deterrent for herbivory, and to their ability to thrive in unfavorably areas, like ports or marinas, characterized by higher pollution levels (Klein & Verlaque, 2008). Given the threat they pose into the areas they colonize, numerous studies have been carried out throughout the years with the purpose of monitoring their ever-growing spread in the basin (Mannino et al., 2019; Ellul et al., 2019; Musco et al., 2014), and, since their physical factors may be considered the main regulators of their spread, also their ecology (Klein & Verlaque, 2008). Though a few factors including the effects of light and temperature (Meinesz et al., 1995; Komatsu et al., 1997) have been widely investigated (Piazzi et al., 2005), many others, namely the organic matter content and type of sediment, were often overlooked.

4.1.1. Sediment type

It has been hypothesized that *C. taxifolia* and *C. racemosa* can thrive in different conditions and habitat, showing a preference for dead matte of *P. oceanica* and rocky bottoms (which favor the "rooting" of rhizoids and therefore the growth of stolons, Piazzi et al., 2016) over sand and *P. oceanica* meadows, which are less favorable for their colonization (Infantes et al., 2011). All the sediment collected in this study were "Soft Sediment", later characterized as three general categories: "Sand", "Clay" and "Silt". The prevalence of *Caulerpa* in silt sediment types does not seem conclusive per se, but the Generalized Linear Mixed Model results suggest that there could be an interesting interaction between higher amount of organic matter detected in the substrate and the sediment type, especially silt and clay. However, the lack of statistically significant findings prevents us from drawing definitive conclusions on the sediment type. Nevertheless, it can be stated that most samples characterized by sandy substrate were associated to 0% in *Caulerpa* coverage, thought this might be due to an insufficient sample size. Furthermore, given the limited cohesive properties of sand, it could be hypothesized that sediment sampling was not an adequate method for this type of sediment, potentially leading to a loss of species during collection, unlike clay or silt.

4.1.2. Organic matter content

The content of OM found in the evaluated samples did not show a clear pattern, with values averaging 6.90%. The highest amount of organic matter content was found in samples 27, 28, 29 & 30 (10-12%), which were collected closer to the city of Limassol, hinting that a higher OM content might be correlated to human activities and/or pollution. Previous studies concluded that sediments colonized by *C. racemosa* showed increased organic matter (OM), total protein and carbohydrate contents and organic C, N, and P contents, exerting positive and/or negative effects on native assemblages (Rizzo et al., 2020). Similarly, it has been hypothesized that conditions such anoxia, high content of organic matter and hydrogen sulfide could promote the growth of *C. taxifolia,* especially in areas affected with urban pollution, which, in turn, cause significant mortality of ecologically relevant species like *P. oceanica.* Nonetheless, previous experiment results do not provide significant proof to support the hypothesis that *C. taxifolia'*s higher invasive capability is due to sediments with high loads of organic matter (Terrados & Marba, 2006). The zero-inflated interaction model hints that no definitive conclusion can be drawn regarding organic matter alone. Nonetheless, the relationship between sediment type and organic matter suggests that *Caulerpa*'s presence may be influenced by other factors, or by a complex interaction between these predictors that the model could not fully capture. For instance, the interaction between OM and sand suggests that higher levels of organic matter might promote the presence of *Caulerpa* in otherwise less favorable sandy environments. Similarly, the interaction with silt also points to a potential mitigating effect on the absence of *Caulerpa*. However, how OM exactly impacts the extent of *C. racemosa* and *C. taxifolia* remains widely unknown, needing further investigation. In other words, the zero-inflated interaction model hints that monitoring *Caulerpa* spp. might be challenging, but it is essential to account for factors leading to its absence (i.e. sand) and factors affecting its extent when present. In particular, sand and minimum distance proved to be the most significant predictors in the zero-inflation model, and they could be essential in understanding why certain areas might have no *Caulerpa* coverage.

4.1.3. Distance from ports

These findings are in accordance with the hypothesis that *C. racemosa* and *C. taxifolia* might have used ship traffic and fishing activities as a pathway for secondary introductions (Klein & Verlaque, 2008), which is proved by their greater abundance in proximity of ports. In addition, these findings are conclusive with the hypothesis that these species tend to take advantage of habitat degradation for their spread, given their greater occurrence in marine areas exposed to high levels of human pressure rather than areas far from urban areas (Gennaro & Piazzi, 2014). This is represented by a decrease in *Caulerpa*'s presence as the minimum distance (Dmin) from the reference points (Limassol and Larnaca ports) increases, making this predictor one of the few significant ones in this analysis. Since the most interesting observations are the ones resulting from an interaction, it could be suggested that this might be case for distance too, but this wasn't evaluated in the present thesis because of multicollinearity problems. Further studies might concentrate on this aspect, possibly by coupling the interaction of minimum distance with either depth or type of sediment, specifically soft sediments, which are often overlooked. The results of the GLMM do not enable us to conclude that *C. taxifolia* shows the same behavior, as the response variable used in the analysis was generally "*Caulerpa*", a result obtained by adding the values of *C. racemosa* and *C. taxifolia*. Similarly, it can be concluded that the correlation between *C. racemosa* and DP2 (Limassol harbor) is stronger, but this couldn't be highlighted in the model because the "minimum distance" from one of the two ports was considered. These results might hint that a possible stronger influence of Limassol port is exerted on the invasive *C. racemosa*, possibly because of higher habitat degradation or pollution which, in turn, cause the regression of endemic seagrasses.

4.1.4. Species assemblage

Not many conclusions can be drawn from the PCA analysis, which failed to show a clear clustering of species in the samples. Nevertheless, the results seem to be in accordance with what previously known about *Caulerpa*'s ecology. *C. racemosa*, can form dense meadows dominated by several different species, including *C. prolifera* and *H. stipulacea* (Klein, & Verlaque, 2008). While the species assemblage in these habitats is likely more complex, grab sampling only captures a few species, possibly sessile species anchored to the seafloor by rhizomes or roots, primarily macroalgae and macrophytes. On the other hand, thanks to the species coverage analysis it was possible to conclude that the southern part of Cyprus is the richest in terms of species composition, whilst many of the samples collected in the Western and Eastern part of the island often showed no significant presence of species, as they were often made entirely of substrate. When present, the species that is most abundant is indeed *C. racemosa*, confirming its invasive properties. *C. taxifolia* proved to be less abundant than initially thought, though it can be argued that this is due to the reduced number of samples, to the high number of samples characterized by "sand", less favorable for this species' colonization, and possibly less cohesive than other sediment types.

4.2. Significance of the study and future prospects

The results of this study provide new insights towards a more comprehensive understanding of the factors that drive the distribution of alien *Caulerpa* species. Understanding how these species colonize new habitats and what are the circumstances in which they thrive the most is crucial for present and future monitoring efforts, especially considering that eradication efforts are too extensive and costly for being put in place (Rizzo & Vega Fernández, 2023). In addition, these results represent essential data that could be integrated into species distribution models (SDMs) whose goal is to predict where *Caulerpa* spp. are going to proliferate next. By using environmental and geographical variables, SDMs are in fact able to project potential species distribution under current and future scenarios (Guisan & Thuiller, 2005). In this context, the present findings on sediment types, organic matter, and distance from ports could represent a first step towards building models that better identify areas at risk of invasion, with the goal of prioritizing monitoring and management efforts in certain areas. Analogously, these findings might help pinpoint areas that are (or will be) susceptible to *Caulerpa* colonization, preventing its further spread. In conclusion, prioritizing this research might also benefit the productivity of local fisheries. *C. taxifolia* and *C. racemosa* significantly alter marine habitats therefore affecting the composition of native species assemblages: for instance, they tend to outcompete native seagrasses, which often represent crucial habitats for many different species, including commercially relevant ones (Klein, & Verlaque, 2008). Consequently, identifying "hotspots" of invasion might be important to make predictions on the stock productivity, therefore avoiding extensive economic losses.

4.3. Climate change & study challenges

Climate change and biological invasions are key processes affecting global biodiversity, with a deeply interconnected relationship. Evidence shows that global warming has facilitated - and continues to facilitate - the expansion of alien species into regions previously inaccessible to them (Walther et al., 2009). The era in which we are living is strongly characterized by global changes and accelerated trades, making seaweeds organisms of major concerns. Representing about 40% of the non-native species introduced into the world's oceans, seaweeds pose severe social and economic threats to the worldwide coastal economy, and their outstanding invasive success is to be attributed to a complex interplay of environmental variables such as temperature, light, salinity and nutrients. Because of climate change, all these variables are expected to change, gradually or rapidly, on a local or global scale, ultimately leading to substantial changes in species distribution (Zanolla, & Andreakis, 2016). Similarly, climate change, particularly rising temperature, might further accelerate the ongoing spread of alien *C. taxifolia* and *C. racemosa,* and this phenomenon could exacerbate the decline of endemic species, ultimately leading to an overall loss of biodiversity in the Mediterranean Sea. Understating how climate change impacts alien species might be essential to identify regions where eradication and containment efforts should be focused (Roger et al, 2015).

Managing NIS in marine environments is rather challenging and requires the involvement of conservation managers, policy makers and citizens to gather comprehensive information on their distribution (Mannino et al, 2021). Though many important management actions and policy measures have been carried out in Mediterranean EU and non-EU countries, the European Commission tends to discourage the application of measures with excessive costs and uncertain outcomes. In this context, adaptive management becomes crucial, along with monitoring plans and effort to limit further spread of well-established NIS (Rizzo & Vega Fernández, 2023). However, traditional monitoring and mapping methods (field surveys and sampling campaigns) not always represent the best solution for monitoring these species' distribution and spread overtime (Mannino et al., 2021). The present study addresses the challenges of mapping the invasive *Caulerpa* species, which require management actions given the size of their impact and the pace at which they are spreading in the Mediterranean Sea. In this context, each surveying method shows pros and cons for habitat mapping. Traditional methods like field surveying are indeed more accurate, but the complexity of natural habitats limits them, while making it time-consuming and costly. More recent methods like multispectral sensors might not be suitable for discriminating between macroalgae at a species level, though they can be adapted to achieve accurate results in a context of broader taxonomic groups or low spatial heterogeneity. Given that macroalgal communities can be spatially and spectrally diverse, mapping them might require newly developed solutions. For instance, remote sensing technologies allow to acquire information on both spatial coverage and biomass estimates for much larger sites in a much shorter time (Rossiter, 2020). Amongst the most effective tools, side scan sonars (SSS) provide high resolution imagery of the seafloor, offering details on sediment texture, topography and seagrass meadows, though it does not provide bathymetric data (Pandian et al., 2009). The results of this project suggest that grab sampling could be an efficient method in evaluating macroalgal and seagrasses coverage, which are often missed in more traditional monitoring efforts. *Caulerpa* species are difficult to detect with conventional methods like side-scan sonars or video techniques, especially in deeper areas and/or because of *Caulerpa*'s patchy distribution. Video techniques, while effective in surveying both shallower and deeper areas, may struggle with visibility in greater depths and in turbid waters (Komatsu et al., 2003). In contrast, sediment grabs offer a more reliable method for assessing *Caulerpa*'s presence and its density, allowing for more precise quantification of coverage assessment and providing insights into the interactions between invasive algae and their surrounding environment. In addition, sampling collection directly from the seafloor enables further analysis of biotic and abiotic factors that might highlight key drivers responsible of these species' expansion. In conclusion, future studies perspectives might consider the idea of coupling sediment grabs with other techniques, in order to obtain a holistic approach, essential for developing effective management strategies to mitigate the spread of invasive species.

5. Conclusion

The present study aims at deepening the existing knowledge on alien *Caulerpa* species in the Eastern Mediterranean Sea, by focusing on the presence and distribution of *C. racemosa* and *C. taxifolia* in Cyprus. Though their presence has been widely known to the public for more than two decades, a detailed conclusion on their extent along the coasts of Cyprus was lacking from the existing literature. By combining video techniques and grab sampling, it was possible to conduct a more thorough study on the factors that might influence the presence and spread of these species in the Mediterranean Sea. Among the evaluated factors, organic matter, type of sediment (sand, clay or silt) and distance from reference polluted areas (Limassol and Larnaca harbors) stood out, hinting that a complex interplay of these predictors affects these species' presence, though results were not too conclusive on the extent of this phenomenon. A Generalized Linear Mixed Model (GLMM) was used to assess which predictors might have had a more significant influence on *C. racemosa* and *C. taxifolia*'s distribution. The result show *Caulerpa* is less likely to be found in sandy substrates. In addition, a slight increase in distance from ports is strongly correlated with *Caulerpa*'s decrease in abundance, hinting that these species might in fact thrive from habitat degradation, also colonizing more polluted areas. Results from the Principal Component Analysis (PCA) were less conclusive but showed that the Southern part of the island was the richest in terms of species composition, with *C. racemosa* overall dominating the evaluated samples. Similarly, findings in species assemblage were not different from the existing literature, with *C. taxifolia* and *C. racemosa* often found together with *H. stipulacea, C. prolifera* and *P. oceanica*. In addition, it was highlighted how sampling grab could represent a valid and easier alternative for species mapping, specifically regarding *Caulerpa* spp., which are of challenging detection with traditional methods due to their patchy nature or ability to colonize deeper and more turbid waters. In conclusion, the presented evidence might be valuable for developing species distribution models (SDMs) aimed at identifying potential invasion "hotspots", thereby prioritizing monitoring and management efforts. This is particularly relevant in the context of global changes, which are expected to further favor the expansion of NIS. Additionally, species distribution models might be crucial in identifying areas with less productive stocks, helping to prevent significant economic losses and allowing fishing efforts to be directed more effectively.

Appendix I

Considering the need to expand existing knowledge on the presence and distribution of alien *Caulerpa* species in Cyprus, an initial project was carried out a few years prior to this thesis project. The initial setup was similar to the one described in this thesis, though the coverage assessment of the species of interest was based on video footage operated by a SpotX tow camera which was also equipped with a GoPro, for a better video quality. In this instance, data (video) was collected by deploying the tow camera from a boat, aiming to maintain a steady frame for ground-truthing. As a result, two videos were obtained for each of the 52 transects: one from the tow camera, which provided coordinates and occasionally depth, and one from the GoPro. The transects were portrayed on a map using QGIS 3.36.0, resulting in a display like the one shown in the following picture (**Figure 17**).

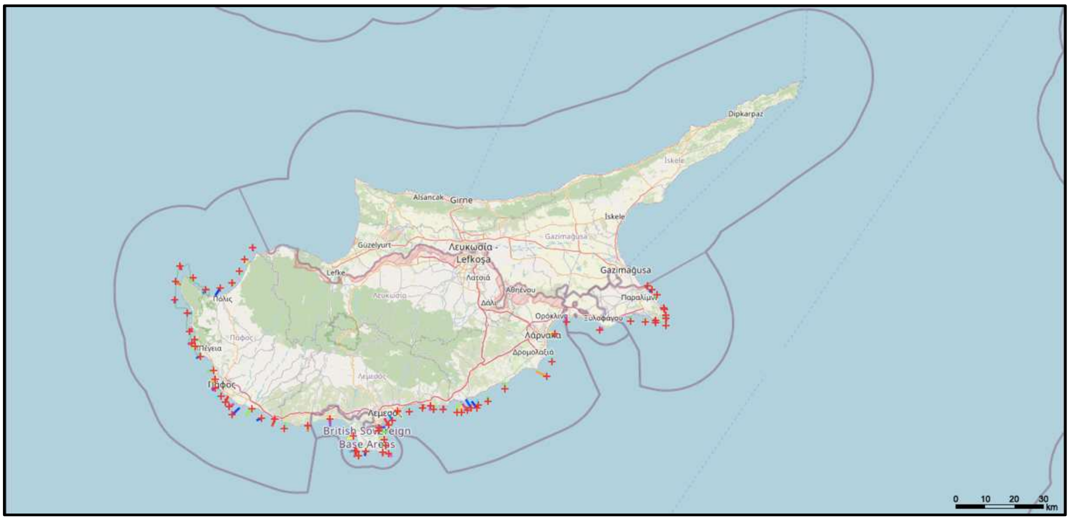


Figure 17 - *Transects location*

Data collection encompassed most of the island's coast, with transect lengths varying significantly (from less than 1 km to a maximum of 3.7 km, averaging 1.3 km) and recorded depths. Unfortunately, depth data was not recorded for many transects due to the tow camera's limitations. For the next step, a point was selected every 100 meters along each transect using QGIS 3.36.0, and the coordinates of each point were used to extract the corresponding frame from the GoPro footage. A total of 703 pictures (see **Figure 18** for an example) were obtained and subsequently imported into Photoquad for the 100 random points analysis. The chosen species library was the following (**Table 6**).

Table 6 - *Species list for footage analysis*

Species	Species ID	Group	Group ID
Caulerpa prolifera	1	Algae	1
Other algae	2	Algae	1
Vegetated matte	3	Seagrass	2
Non vegetated matte	4	Seagrass	2
Posidonia oceanica	5	Seagrass	2
Halophila stipulacea	6	Seagrass	2
Bare reef	7	Habitat	3
Dead *Posidonia oceanica*	8	Seagrass	2
Caulerpa racemosa	9	Algae	1
Caulerpa taxifolia	10	Algae	1
Reef with algae	11	Habitat	3
Penicillus capitatus	12	Algae	1

Also in this case, the species library was created based on the species (or substrate features) that were more important for the analysis, but also the most present in the analyzed footages. In this case, a big part of the recorded videos consisted in healthy *P. oceanica* meadows and/or dead matte. Evaluating the distribution of seagrass meadows is fundamental when considering the presence and expanding establishment of alien species, such the ones considered in this project. As the extensive literature shows, both *C. taxifolia* and *C. racemosa* have an intertwined relationship with the endemic seagrass, often causing its decline. Though this initial project was continued due to its interruption, the recorded footages could be of essential importance in setting a database of the distribution of these extensive meadows, for potential future comparison. In conclusion, though the methodology was almost mirrored in the project carried out during my thesis, the data resulting from this initial part of the experiment was not considered. The reason behind this is that the main goal was to assess the presence of *C. taxifolia* and *C. racemosa*, which are of difficult detection due to their smaller size. Furthermore, even when considering the GoPro footages, which had a higher resolution, the turbidity of the water was a bigger limit than expected, making it difficult to clearly distinguish the single species. At last, depth had to be excluded from the evaluated factors; although important, it wasn't often recorded from the ROV, due to mechanical errors.

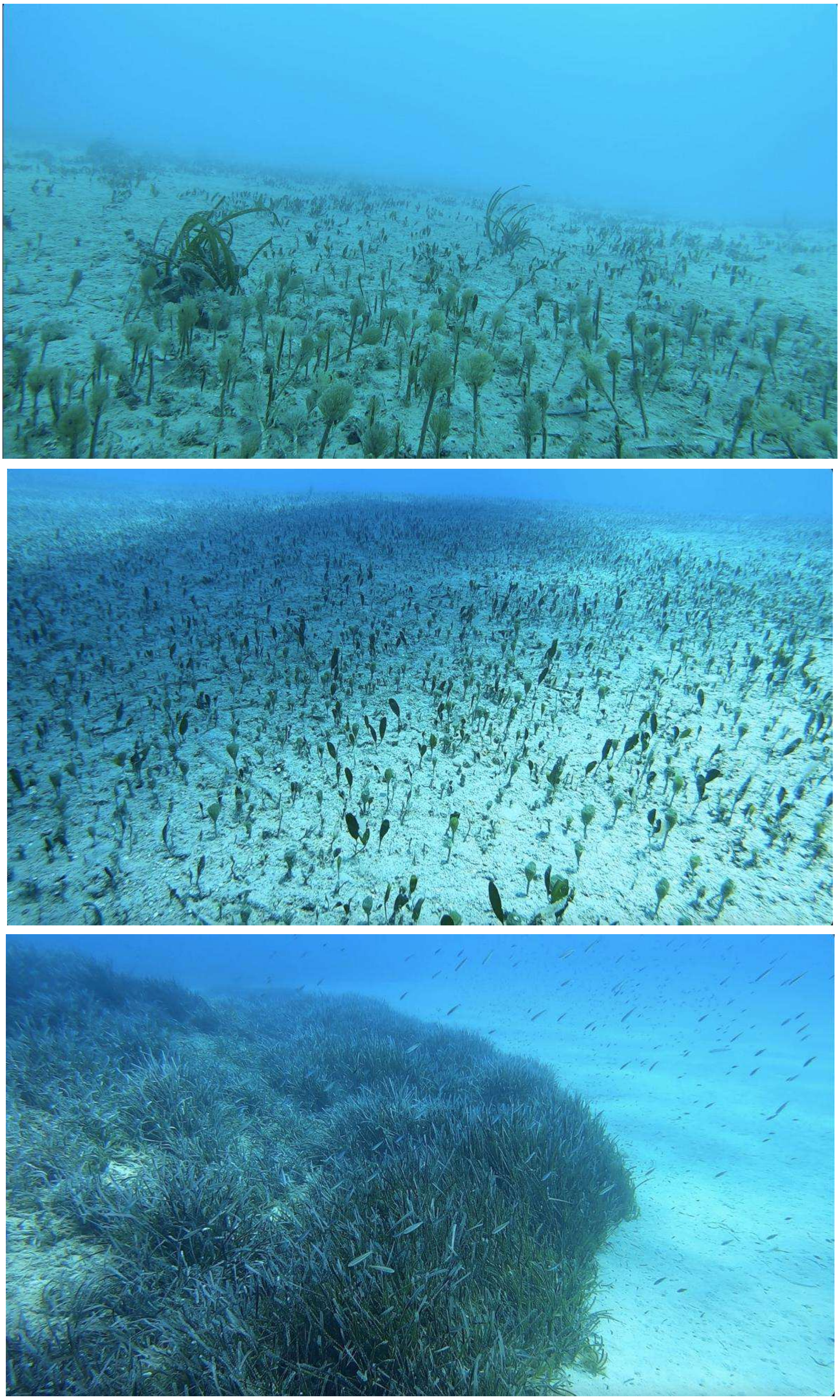


Figure 18 - *Example of pictures initially used for the analysis. The pictures in question respectively belong to transects 14, 16 and 68*

The following graphs (**Figure 19, Figure 20**, **Figure 21** and **Figure 22** represent some preliminary results of the species coverage assessment analysis conducted using PhotoQuad. **Figure 19** shows the results of Transect 1, which was surveyed starting from 34.715895°, N 33.359903° E and spanning depths from 20 to 46 m. **Figure 20** shows the results of Transect 2, collected at 34.72914° N 33.41741° E with depth ranging from 35 to 47 m. **Figure 21** presents the results of Transect 4, recorded from a starting point of 34.77253° N, 33.48064° E, covering a depth range from 5 to 30 m. **Figure 22** illustrates the results of Transect 8, which was surveyed starting from 34.70176° N, 33.25122° E but for which the depth was not recorded.

From a first analysis it can be concluded that the species percentages vary greatly from the ones reported in the grab sampling experiment, largely attributable to the greater presence of *P. oceanica* and its matte. In addition, most of the observations report the presence of "Other" species, underscoring the challenges of species identification through footage alone, which strongly depended on weather and sea conditions. Consequently, these four transects provide limited insights into the abundance of alien *Caulerpa* species, as their smaller size often made them difficult to identify with certainty.

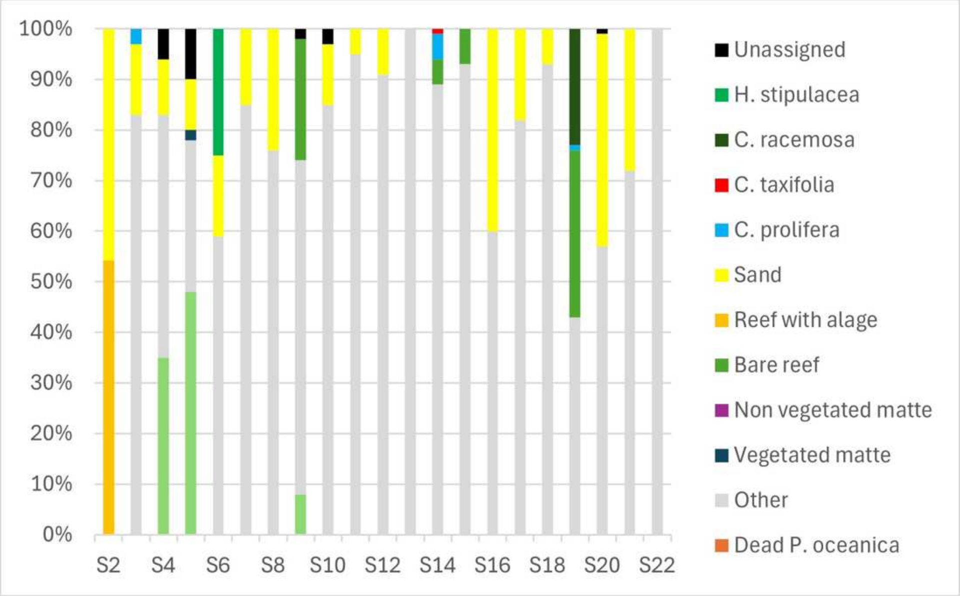


Figure 19- *Graph representing species coverage (%) of Transect 1 performed on PhotoQuad. Samples showed in the graph (S2-S22) were collected by selecting a point every 100 m on the transect line. Sample 1 was not included as the tow camera footage started recording later than the chosen sample. Unassigned points represent those for which the species recognition was challenging.*

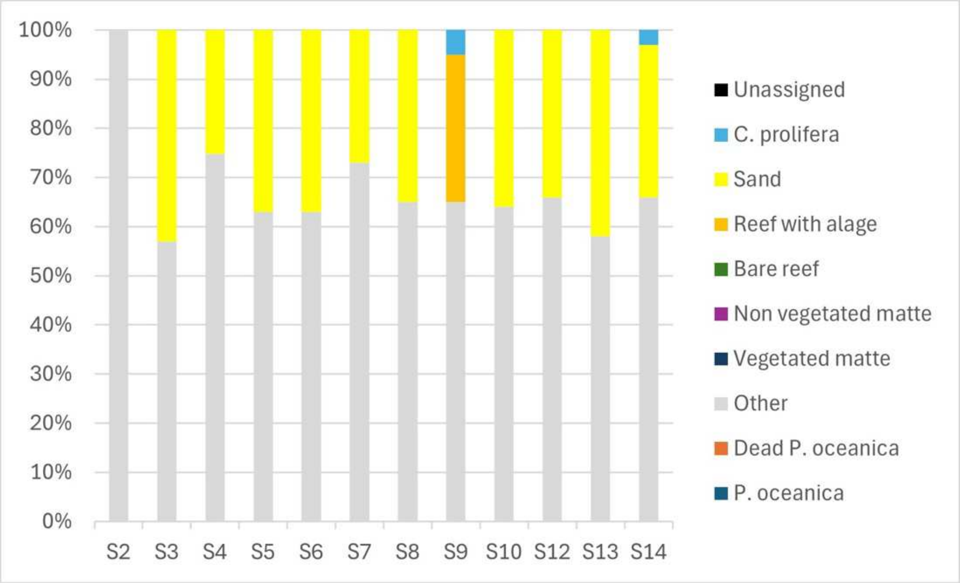


Figure 20 - *Graph representing species coverage (%) of Transect 2 performed on PhotoQuad. Samples showed in the graph (S2-S14) were collected by selecting a point every 100 m on the transect line. Sample 1 and Sample 11 were not included as the tow camera footage started recording later than the chosen samples. Unassigned points represent those for which the species recognition was challenging.*

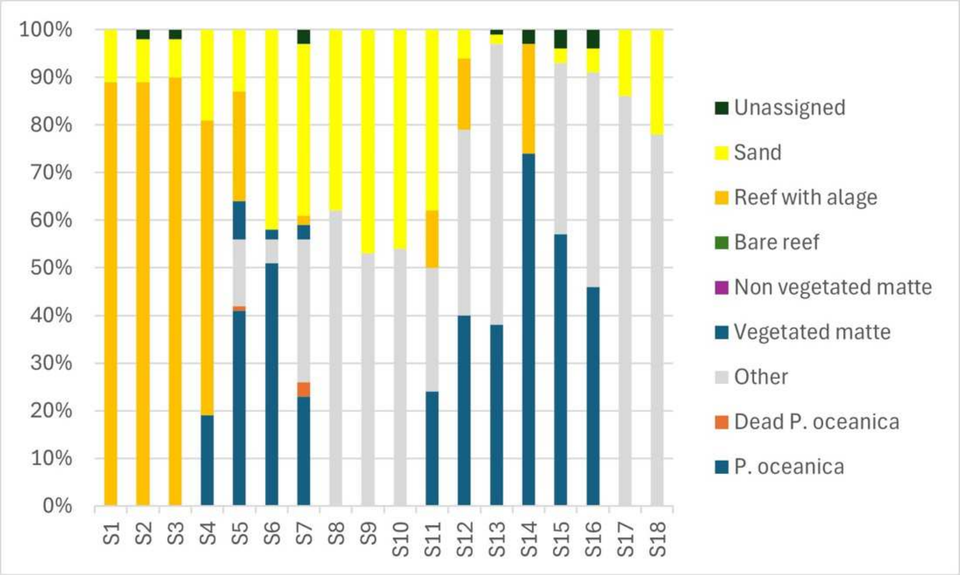


Figure 21- *Graph representing species coverage (%) of Transect 4 performed on PhotoQuad. Samples showed in the graph (S1-S18) were collected by selecting a point every 100 m on the transect line. Unassigned points represent those for which the species recognition was challenging.*

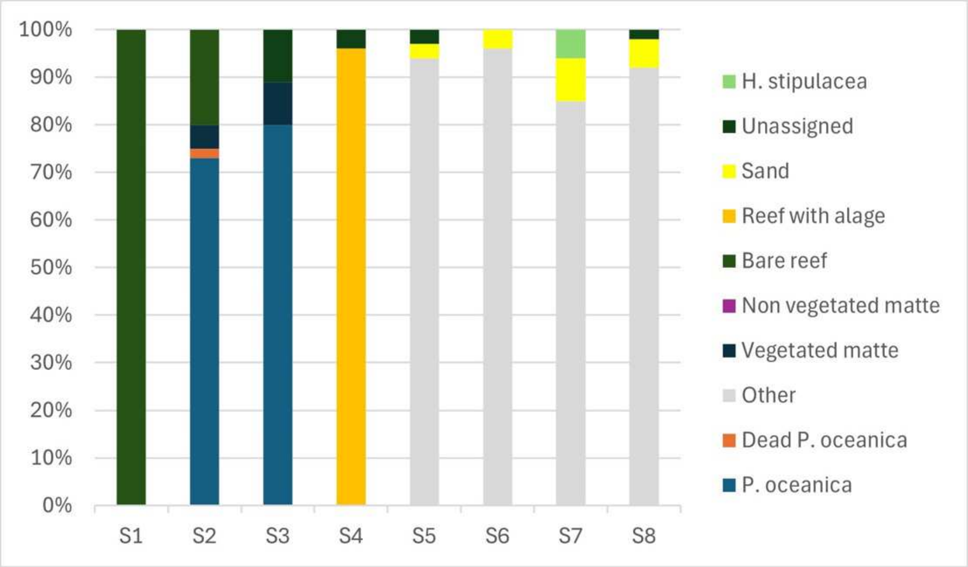


Figure 22- *Graph representing species coverage (%) of Transect 8 performed on PhotoQuad. Samples showed in the graph (S1-S8) were collected by selecting a point every 100 m on the transect line. Unassigned points represent those for which the species recognition was challenging.*

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