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Analysis of phytobenthic assemblages characterizing the upper infralittoral, in view of the "restoration" of canopy-forming species

PhD thesis

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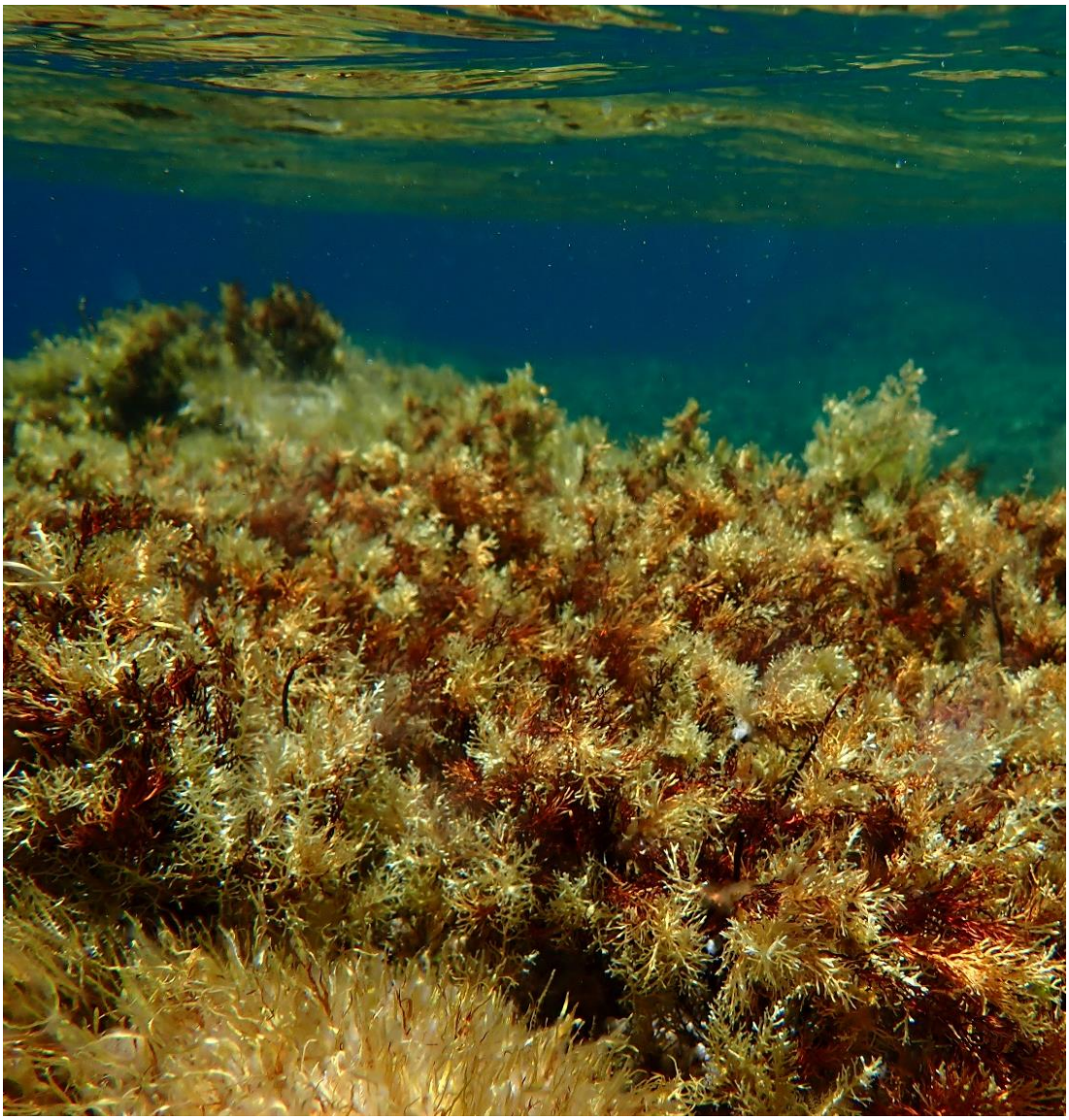
Preface

In light of the ongoing climate changes which have already affected the biodiversity of both marine and terrestrial ecosystems, the concern of scientists and researchers is increasingly focused on restoring the degraded or lost habitats. The main purposes of this PhD research project were to investigate on the upper infralittoral *Cystoseira s.l.* species which could be potentially more exposed to temperature fluctuations, identify the most threatened species and promote the natural recovery of *Cystoseira s.l.* populations to protect biodiversity and enhance CO2 drawdown. The starting point of this PhD research was to examine the past and current presence of upper infralittoral *Cystoseira s.l.* species along the eastern coast of Sicily though extensive bibliographic analysis and intensive field activity. After obtaining a complete overview of the actual distribution of these species, the attention was focused on four threatened species: *E. brachycarpa*, *E. mediterranea* and *Gongolaria montagnei* var. *tenuior* (three regressing species along the Ionian coast of Sicily) and *E. giacconei* (a cold-affinity endemic species). Their reproductive phenology and embryology were studied in detail to confirm the belonging embryological group of these species, especially of *E. giacconei*, whose embryology had never been examined. Furthermore, to understand the potential impacts of climate change on the future viability of these species, experimental trials were performed to test the effects of temperature on the reproduction and growth of embryos, which are commonly more sensitive than adults. The study on *E. giacconei* was realized in collaboration with the University of Trieste and some of the data here reported were object of a published paper. Through these trials, it was possible to gain a picture of the potential fate of these species under a scenario of further increased temperatures and thermal anomalies. Moreover, it was achieved knowledge on the thermal optimum of the early life stages of these species, which could be used for future restoration purposes. Once acquired information on embryology and thermal optimums of the first developmental stages, it was selected *E. brachycarpa* as target species to conduct an experimental restoration plot in the M.P.A. Isole Ciclopi, where this species was historically present. The juveniles of

this species were grown though *ex-situ* cultivation on volcanic rock tiles and were outplanted at the restoration site, where they were monitored and followed for one month.

To date *Cystoseira s.l.* species have not yet been included in the IUCN Red lists. Through this thesis, it could be proposed to include *E. giacconei* and *E. brachycarpa* in the IUCN Red Lists and classify them as "Critically endangered". Further restoration actions applied to M.P.A.s and adjacent unprotected areas, will represent the best future perspective for *Cystoseira s.l.* forests preservation in the Mediterranean Sea.

Chapter I
General introduction



1.1 Mediterranean marine forests: their role and importance for the marine ecosystem

The Mediterranean Sea is a semi-enclosed basin, considered a hotspot of biodiversity (Bosc et al., 2004; Coll et al., 2010). The macroalgal communities of Mediterranean rocky coasts are mainly represented by canopies consisting of *Cystoseira sensu lato* (*s.l.*) species (Fucales, Phaeophyceae) (Sales & Ballesteros, 2009; Mariani et al., 2019), which are comparable to the Atlantic laminarian forests (Grech, 2017). These communities represent key primary producers, which produce organic matter from carbon dioxide, water, and minerals (Rodríguez-Prieto et al., 2013). In the Mediterranean marine ecosystems, they are considered as engineering species, which thanks to the structural three-dimensionality of their branches modify the colonized environment and promote biological diversity by providing settlement substrate, food and shelter for a highly diverse biota (Mangialajo et al., 2008; Gianni et al., 2013). Indeed, *Cystoseira s.l.* communities host a high diversity of fish species (Orlando-Bonaca & Lipej, 2005), juvenile forms of several invertebrate species (crustaceans, molluscs and polychaetes) (Gozler et al., 2010; Çulha et al., 2010; Pitacco et al., 2014; Chiarore et al., 2019) and an extensive and diversified epiphytic flora (Ballesteros et al., 2009). In particular, *Cystoseira s.l.* are the principal erect species which contribute to construct the "elevated photophilic layer". This latter is colonized by photophilous species of small size, which use the frond of *Cystoseira s.l.* species as a substrate, forming an "epistrate" on their thallus. Finally, there is a "sub-layer", consisting of sciaphilous species both calcareous and with soft thallus, which are situated on the substrate or on the basal parts of the species of the "elevated layer" (Cormaci et al., 2003).

Cystoseira s.l. communities provide crucial ecosystem services in coastal environments, maintaining the biogeochemical cycles and the water oxygenation, protecting the coasts from physical agents (De La Fuente et al., 2019), contributing to nutrient fixing and finally constituting an important blue carbon sink (Ballesteros, 1990a; Ballesteros et al., 1998; Cheminée et al., 2013; Mineur et al., 2015) (Figure 1).

Figure 1: Main ecosystem services provided by *Cystoseira s.l.* communities (from Gianni & Mangialajo, 2016, modified)

In addition, several studies demonstrated that many *Cystoseira s.l.* species produce secondary metabolites with antioxidant, anti-inflammatory, antifungal, antiviral and antibacterial activities, which could be exploited in medicine (Orlando-Bonaca et al., 2021).

These habitat-forming species dominate several stands, from the littoral fringe down to the lower sublittoral zone (Feldmann, 1937; Ballesteros, 1988, 1990 a, b; Giaccone et al., 1994). Their zonation depends on different environmental conditions: light, salinity, temperature, hydrodynamics and grazing (Sauvageau, 1912; Ollivier, 1929; Vergés et al., 2009).

Cystoseira s.l. species present a k-strategy (long life cycle), are stenoecious (narrow ecological valence) (Robvieux, 2013) and are differently sensitive to anthropogenic disturbances (Ballesteros et al., 2009). For this reason, their presence is index of high ecological quality and were selected as biological indicators of environment quality in the frame of the Water Framework Directive (WFD, 2000/60/EC; Panayotidis et al., 1999; Buia et al. 2007; Pinedo et al., 2007). They represent useful tools to monitor the marine ecosystem health and hence the water quality (Arevalo et al., 2007; Ballesteros et al., 2007; Panayotidis et al., 2004; Asnaghi et al., 2009; Nikolic et al., 2013; Pinedo et al., 2007; Mangialajo et al., 2007).

Cystoseira s.l. species are under surveillance by international organizations such as the Council of Europe, the United Nations, the IUCN, the RAC/ASP, the WWF and MedPan. Moreover, they are protected by the Berne Convention, Barcelona Convention and Habitat Directive (Orlando-Bonaca, et al., 2021)

1.2 Main threats and current status of *Cystoseira s.l.* communities along the Mediterranean coasts

During the last decades, *Cystoseira s.l.* communities have experienced a substantial decline in several Mediterranean coasts (Cormaci & Furnari, 1999; Thibaut et al., 2005; Falace et al., 2006; Serio et al., 2006; Tsiamis et al. 2013; Capdevila et al., 2015; Thibaut et al., 2015; Catra et al., 2019). In particular, the most considerable retraction of their range was observed near the urbanized areas (Benedetti-Cecchi et al., 2001; Ballesteros et al., 2007; Mangialajo et al., 2007; Perkol-Finkel & Airoldi, 2010). Indeed, the increasing coastal urbanization caused the destruction of natural habitats and alteration in environmental conditions, provoking the regression of these species (Panayotidis et al., 2004). Moreover, the coastal development determined an increase in nutrient intake, contaminants and sediment loads (Gianni et al., 2013). Point sources of pollution such as oil spills, detergents, and vegetative paints (Airoldi et al., 2014), as well as effluents from aquaculture facilities, agricultural and industrial activities (Tamburello et al., 2022) contributed to an increase in eutrophication, damaging these communities which prefer oligotrophic and transparent waters (Furnari et al., 2003). Moreover, the increase in overgrazing of sea urchins *Paracentrotus lividus* (Lamarck, 1816) and *Arbacia lixula* (Linnaeus, 1758) (Figure 2c), which results from reduced predator populations, is one of the main causes for the global loss of macroalgal marine forests (Medrano et al., 2020). Both the two species have an important role in the maintenance of sea urchin barrens once they are established (Agnetta et al., 2015). Indeed, when settled, these barren grounds become stable alternate states maintained by several feedback mechanisms that prevent the recovery of macroalgal forests. These hysteresis loops affect both sea urchin populations, by increasing the probability of recruitment and juvenile survival, and algal establishment, by reducing the supply of propagules as neighbour algae populations become scarcer (Filbee-Dexter & Scheibling 2014; Ling et al. 2015). Loss of *Cystoseira s.l.* species has been also ascribed to the outbreak of herbivorous fishes (Verlaque, 1990; Thibaut et al., 2005). In particular, it was observed that more than

60% of the gut contents of the teleost *Sarpa salpa* (Linnaeus, 1758) (Figure 2a), the only true herbivore in the western Mediterranean (Verlaque, 1990), is composed by these species. In addition, invasive species represent another documented source of stress for these communities (Tamburello et al., 2022), since they can compete for space, become grazers or change the environmental and ecological conditions.
The two alien fishes *Siganus luridus* (Rüppell, 1829) and *Siganus rivulatus* (Forsskål and Niebuhr, 1775) (Figure 2b), introduced into the Mediterranean after the opening of the Suez Canal, have already depleted *Cystoseira s.l.* communities particularly along the eastern Mediterranean coasts (Sala et al., 2011; Gianni et al., 2017; Tsirintanis et al., 2018).

Figure 2: Some examples of herbivores threatening *Cystoseira s.l.* communities: a) *Sarpa salpa* (photo: A. Lombardo); b) *Siganus rivulatus* (from Lejeusne et al., 2010); c) *Arbacia lixula* (from Medrano, 2020)

These different forms of stress act over time and in unison, with a possible synergistic effect, on species and ecosystems (Worm et al., 2006; Halpern et al., 2008). Indeed, the conjunction of these processes are contributing everywhere to a gradual shift towards communities characterised by a lower structural complexity, leading to sea bottoms dominated by turf-forming, ephemeral and filamentous algae, mussels and sea urchins, contributing to a habitat homogenisation (Benedetti-Cecchi et al., 2001; Connell et al., 2014; Strain et al., 2014; Agnetta et al., 2015). The loss of these habitat-forming species reduces the habitat tridimensionality,

affecting the biodiversity and the ecosystem functioning (Bulleri et al., 2002; De La Fuente et al., 2019; Bianchelli & Danovaro, 2020) (Figure 3).

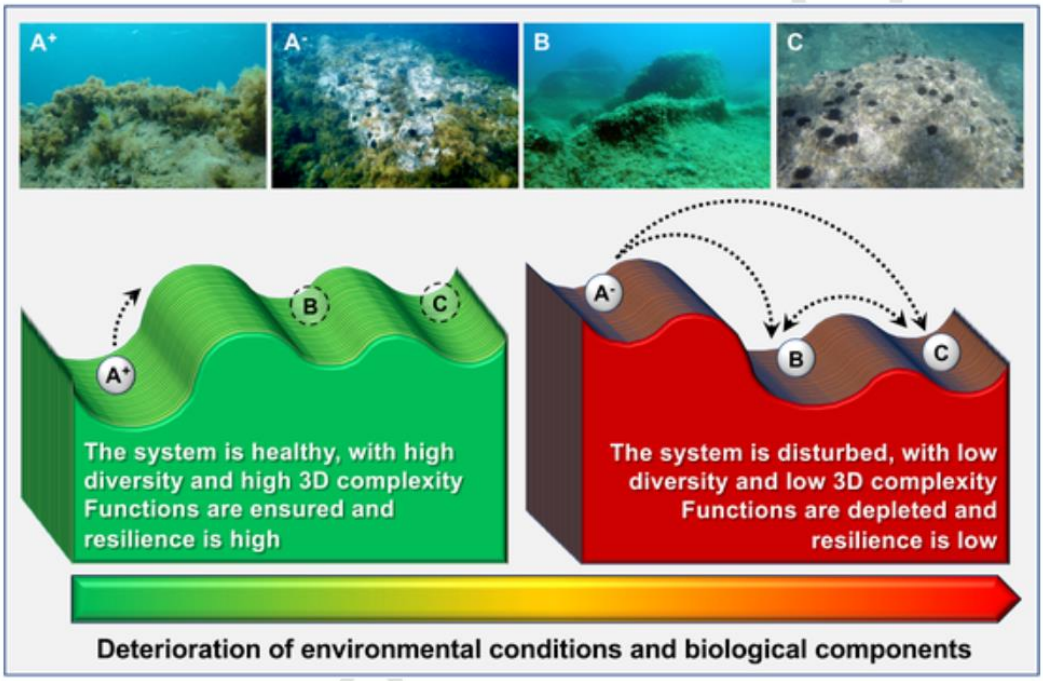


Figure 3: Schematic representation of regime shifts from Mediterranean algal forests towards barren grounds and turf-dominated assemblages (from Bevilacqua et al., 2021)

The effects of these disturbances lead to quantitative changes in the biomass of the guide species and qualitative-quantitative variations in the specific associated biodiversity. The effects on the biomass are observed not only by reduced vegetative growth of individuals, but also by a decrease in the density of the entire population. The effects on biodiversity involve a reduction in the floristic contingent, changes in the specific composition, a reduction in the number of photophilic algae compared to sciaphilic algae due to increased turbidity, increase in red algae compared to brown algae, increase in wide ecological valence species, increase in short-cycle species that can survive more extreme conditions than perennial species (Cormaci et al., 2003).

If on one hand many regions have experienced dramatic reduction of these ecosystems (Airoldi et al., 2014; Thibaut et al., 2015), in other areas losses have been limited to the most severely impacted sites and some populations have surprisingly persisted in a relatively healthy status (Thibaut et al., 2014).

Therefore, understanding what factors or combinations of factors control the distribution and condition of these ecosystems is a key priority to establish effective conservation measures (Mancuso et al., 2018).

1.3 The impacts of global change on Mediterranean marine forests

Currently, climate warming is the main driver of change in ecosystems around the world (Gruner et al., 2017). The combination of temperature increases, and a higher frequency of short-term extreme events affect the biology and ecology of most organisms in the sea. Most visible changes influence the life cycle, reproductive effort, and demography of marine organisms. Generally, these changes involve adaptive responses (such as physiological adjustments and microevolutionary processes). A direct consequence of warming is the increase in the abundance of thermotolerant species and the disappearance or rarefaction of 'cold' stenothermal species. Such changes occur as shifts in distribution ranges and/or population dynamics (Lejeusne et al., 2010). Indeed, global warming and thermal anomalies induce a redistribution of species, causing a shift to higher latitudes, higher altitudes, or deeper waters (Hsieh et al., 2009). This results in a general reorganization of local communities as species are added or deleted, and as interactions among species change in importance (Wootton et al., 2008; Harley, 2011).

The Mediterranean Sea has been considered a "hot-spot" for climate change (Giorgi, 2006) since its warming is occurring at a faster rate than in the global ocean, particularly because of its small size and semi-enclosed shape, and thermal anomalies here are increasing in intensity, frequency and duration (IPCC, 2021) (Figure 4). Marine heat waves (MHWs), defined as a discrete and prolonged anomalous warm water event in a particular location (Hobday et al., 2016), have recently caused devastating impacts on Mediterranean marine biodiversity and ecosystem services and functions (Garrabou et al., 2009; 2022). Indeed, Mediterranean MHWs have triggered unprecedented climate driven mass mortalities during the last decades and their occurrence is expected to increase in

the coming decades (Darmaraki et al., 2019; Garrabou et al., 2019). Under these rapidly changing climate conditions, this trend could affect the fate and biogeographic responses of Mediterranean species (Boero et al., 2008; Marbà et al., 2015).

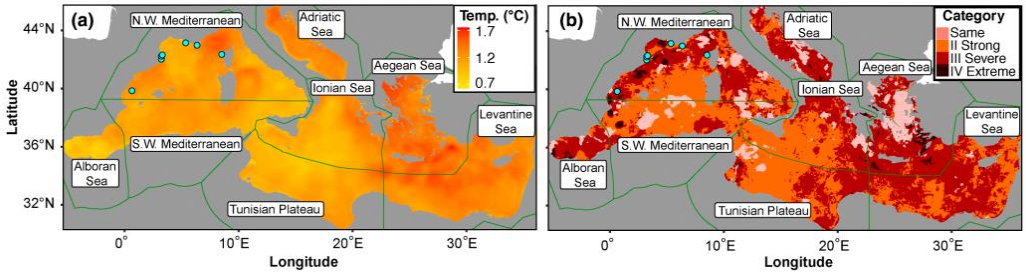


Figure 4: Patterns of warming (a) and marine heatwaves (MHWs) (b) across the Mediterranean Sea (from Garrabou et al., 2022)

Temperature is the most important factor in the distribution of marine algae, as it limits their survival, growth and reproduction (Lüning, 1990; Orfanidis, 1991). There is evidence that thermal anomalies can affect the phenology and physiology of Fucales, impairing their reproductive performance, increasing their vulnerability to other stressors and ultimately leading to population decline and local extinction events (Wernberg et al., 2010; 2016; Gouvêa et al., 2017; de Bettignies et al., 2018). In particular, regarding *Cystoseira s.l.* species, it was observed that marine heatwaves and warming can have repercussions on their reproductive phenology, germling growth and viability (Capdevila et al., 2019; Bevilacqua et al., 2019; Savonitto et al., 2021; Verdura et al., 2021; Falace et al., 2021). Indeed, the early life stages of these macroalgae are generally more susceptible to physical and biological stress than adults (Falace et al., 2018; Cáliz et al., 2019) and represent a "bottleneck" in the development and maintenance of populations (Steen & Scrosati, 2003; Schiel & Foster, 2006; Andrews et al., 2014). The negative effects of warming are exacerbated by the limited dispersal abilities of eggs and zygotes of *Cystoseira s.l.*, the low population connectivity and the cumulative effects of other disturbances (Soltan et al., 2001; Buonomo et al., 2017; Capdevila et al., 2018), thus hindering the population viability and contributing to the ongoing decline of these populations under climate change (Engelen et al., 2008; Buonomo et al., 2017). Indeed, Buonomo et al. (2018) predicted substantial retractions of suitable

habitats and limited possibilities for northward extension of *Cystoseira s.l.* species, with the consequent loss of unique evolutionary lineages in the future. Understanding the sensitivity of different life stages to temperature and their performance at thermal limits is essential to comprehend the processes causing their geographical range variations due to global warming and extreme climatic events (Walther et al., 2002; Root et al., 2003; Wernberg et al., 2011). This knowledge also allows to predict their possible future under climate change and manage their conservation and restoration more adequately (Fabbrizzi et al., 2020).

1.4 Conservation and restoration of marine Mediterranean forests: passive and active measures

The decade 2021–2030 has been defined as the UN Decade of Ecosystem Restoration, due the urgent need to reverse ecosystem damage and halt the climate changes and the biodiversity crisis (UN General Assembly, 2019). If much of this effort has been so far focused on increasing forests on land, only recently the attention has been centred on restoring marine forests (Filbee-Dexter et al., 2022), which represents a way to protect biodiversity, enhance CO_2 drawdown, and provide other benefits (Teagle et al. 2017; Ortega et al. 2019; Feehan et al. 2021) reported in Figure 5.

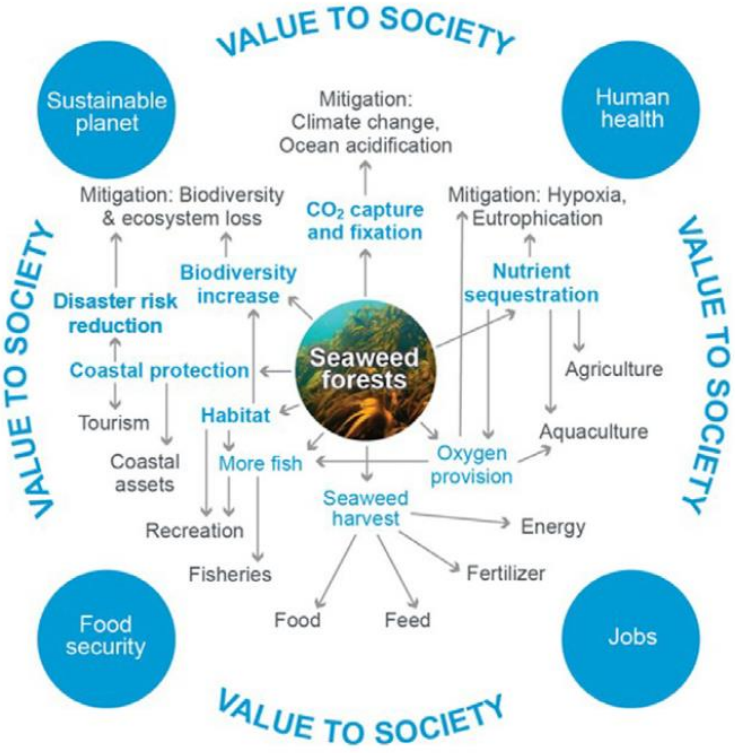


Figure 5: Key ecosystem services and functions provided by marine forests (from Filbee-Dexter et al., 2022)

Regarding *Cystoseira s.l.* communities, despite the implementation of conservations actions, only a few evidence of natural recovery of these habitats were reported (Perkol-Finkel & Airoldi, 2010; Iveša et al., 2016). For this reason, in the last years the concern for these ecosystems has increased and many restoration actions were realized, according to the Biodiversity Strategy to 2030 (Target 2), which involves the reintroduction of relevant species where they were historically present and where factors that led to their loss have been removed (Falace et al., 2018). In particular, two strategies (passive and active) were deployed to safeguard these habitats.

The marine protected areas (MPAs) are recognised as passive conservation tools to protect and restore the marine ecosystems (Medrano, 2020). In particular, the No-Take marine reserves or No-Take Zones (NTZs) are MPAs implementing the strongest protection strategies, where no extractive activity is allowed (Sala & Giakoumi, 2017). These areas prevent the overexploitation of key structural species, reducing or removing the associated impacts, maintaining and restoring

ecosystem functioning, improving the trophic regulation on populations of consumers (e.g., trophic cascades) (Medrano et al., 2020). However, in case of degraded canopies, even when the environmental stressors are mitigated or removed through the passive conservation management strategies, such as NTZs, it is difficult to recover the habitat if there are not nearby healthy populations which can produce propagules and hence restore the damaged site (Medrano, 2020). Indeed, there is little evidence that the NTZs are sufficiently effective in restoring degraded marine ecosystems (e.g., Sangil et al., 2012). Consequently, active restoration, which implies the active assistance in the recovery of a degraded, damaged, or destroyed habitat, has been recently adopted as a more suitable tool for the restoration of marine habitats in the shorter term [Society for Ecological Restoration (SER), 2004]. Active restoration allows to speed up the ecosystem recovery through improved management techniques (such as transplantation) (Perrow & Davy, 2002; Holl & Aide, 2011; Bayraktarov et al., 2016). It was demonstrated that only by combining well-designed active and passive restoration measures it is possible to reverse widespread ecosystem degradation (Lotze et al., 2006; Mitsch, 2014; Possingham et al., 2015). For example, Medrano et al. (2020) proved that the combination of active (e.g., algal transplantation and sea urchin removal) and passive restoration (e.g., establishment of marine protected areas), provides better results than isolated approaches, but also indicates that more effectively managed No-Take marine reserves can be essential for both management purposes and ecological restoration.

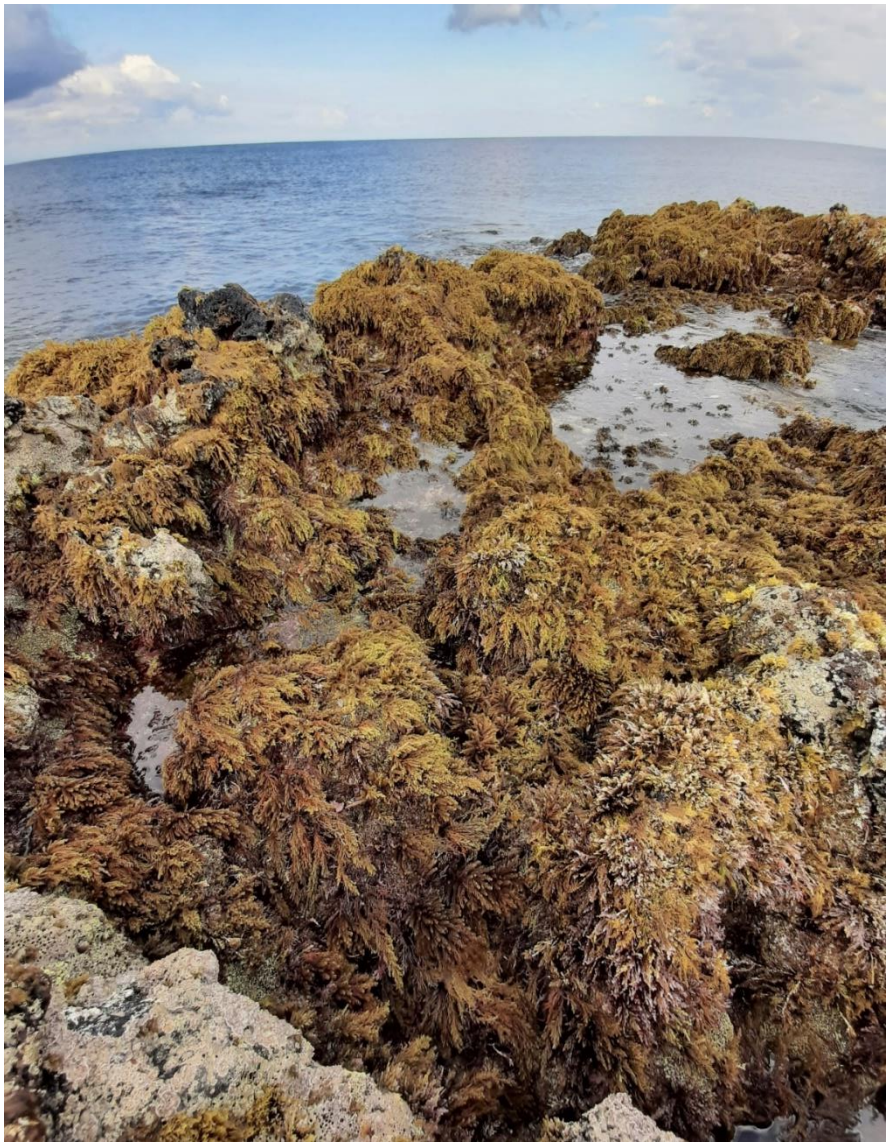
1.5 Objectives of the thesis

The main aims of this PhD thesis were: to analyse the past and current presence of upper infralittoral *Cystoseira s.l.* species, which could be more exposed to temperature fluctuations and thermal anomalies; to identify the most threatened species; to promote the natural recovery of *Cystoseira s.l.* populations through an experimental restoration plot in order to preserve the associated biodiversity and ecosystem services.

The first step of this research was the evaluation of the past and current presence of upper infralittoral *Cystoseira s.l.* species along the eastern coast of Sicily. After achieving a complete overview, the four species *Ericaria brachycarpa*, *E. mediterranea*, *Gongolaria montagnei* var. *tenuior* (three regressing species along the eastern coast of Sicily) and *E. giacconei* (a cold affinity dotted endemism), were selected to conduct an in deep study on their reproductive phenology and embryology in order to examine the zygote segmentation, embryo development and confirm the belonging embryological group. Subsequently, to understand the potential impacts of climate change on the future viability of these species, experimental trials were performed to test the effects of temperature on the reproduction and embryo growth, in order to identify the thermal optimums of their early stages and recognise how they could respond to the foreseen increases in temperature and thermal anomalies. Afterward, among these examined species, it was selected *E. brachycarpa* to realise an experimental restoration plot in the M.P.A. Isole Ciclopi, where the historical presence of this species was reported by Pizzuto (1999). For this purpose, it was performed *ex-situ* cultivation of *E. brachycapa* juveniles and outplanting in the M.P.A. Isole Ciclopi, where they were monitored for a month, in order to follow juveniles' growth in the field.

Finally, during the study period at the laboratory "Biogeographical Ecology and Evolution" of the "Centro de Ciencias do Mar" (CCMAR, University of Algarve, Portugal), it was performed a genetic pre-screening of the target species through DNA barcoding to characterise its genotype for future restoration purposes and contextualise it in the Mediterranean biogeographical scenario.

Chapter II
The species *Cystoseira sensu lato*



2.1 Historical information

The genus *Cystoseira* belongs to the family Sargassaceae Kützing, order Fucales Kylin. This genus was described in 1820 by the Swedish phycologist Carl Adolph Agardh. The name means 'chain of vesicles' (τυσΤ8 = vesicle and σεΤḍα= chain), which refers to the presence of air vesicles in the thallus. These species are mainly distributed in the Mediterranean and along the North Atlantic coasts (Roberts, 1978), with about 40 endemic species listed in AlgaeBase (Guiry & Guiry, 2021). These species were defined by Feldmann (1937) as an example of neoendemism, which includes "the species and varieties of the Mediterranean, having close affinities with other forms existing in neighbouring regions, in particular in the North Atlantic". Indeed, these species originated from Atlantic species that were present in the area of the current Mediterranean Sea before the "Messinian salinity crisis" (ca. 6-7 million of years ago), characterized by the closure of the Strait of Gibraltar and the subsequent desiccation of the basin resulting in the disappearance of almost all the flora and fauna.

The high plasticity of this genus and the occurrence of a great number of ecotypes in different geographical zones and environmental conditions (Giaccone & Bruni, 1973) led to the assumption that the speciation process of these species is still active today (Giaccone & Geraci, 1989). This phenomenon is indicative of a possible hybridization process among species, which suggests a recent speciation event that may lead to vicariance in different areas of the Mediterranean basin (Amico, 1995). Over time the genus was characterized by a high number of varieties and formae, which made difficult the identification of taxa (Robvieux, 2013). Indeed, some diacritical characters were found to be uncertain and variable, leading to misidentifications over the years (Serio & Furnari, 2021). This is the reason why over time different taxonomic approaches were proposed: laboratory cultures for the study of the ways of reproduction and the first environmental stages (Guern, 1962; Colombo et al., 1982); anatomy of vegetative and reproductive characters through morphological groups (Roberts, 1978); chemotaxonomy of secondary metabolites (Amico, 1995); biogeography (Giaccone, 1980); numerical taxonomy (Giaccone & Bruni, 1971; 1973). Nevertheless, only in recent years thanks to

improvements in the field of molecular and genetic analyses, the taxonomy and phylogeny of *Cystoseira* was clarified, splitting it in three different genera: *Cystoseira sensu stricto*, *Gongolaria* Boehmer and *Ericaria* Stackhouse (Draisma, 2010; Bruno de Sousa et al., 2019; Orellana et al., 2019; Neiva et al., 2022). Overall, these species are referred as *Cystoseira sensu lato* (Orellana et al., 2019).

2.2 Morphology

The species *Cystoseira s.l.* are characterized by an arborescent habit and are fixed to the substrate through a holdfast that can be a discoid base or by digitiform aptera. The only pleustophyte species is *Cystoseira aurantia*, which lives floating in the lagoons (Cormaci et al., 2012; Battelli & Catra, 2021). The thallus can be non-caespitose (with a single axis) or caespitose (with several axes emerging from the base) (Figure 6). The apex is defined prominent, if it overcomes the level of insertion of primary branches, and not prominent if it is enclosed in the basal portion of primary branches and is barely visible. Moreover, the apex can be spinose or smooth depending on whether thorny processes are present or absent on it. Primary branches are cylindrical or flattened over all or part of their length and, normally during the unfavourable season, they are partially eliminated. With the growth of new primary branches, the older ones, which have already reached their full development, are progressively discarded and on the cauloid it is possible to observe scars of fallen branches or stumps that in some species originate adventitious ramifications. For this reason, the *Cystoseira s.l.* species were identified by Feldmann (1937) as hemiphanerophytes (i.e., perennant algae for part of their thallus). Indeed, during the vegetative resting period, generally during autumn-winter, it occurs the disappearance of higher order branches, while the axis and primary branches are maintained. Therefore, it is possible to distinguish a winter habit, characterised by the absence of the fronds, and a spring-summer habit, during which there is the maximum development of them.

In some species, there are swellings at the base of primary branches called tophules, in which reserve substances accumulate (Figure 6). After the vegetative resting period, from the tophules new primary branches emerge. Beyond the primary

branches, there are secondary and tertiary branches that can be cylindrical or flattened. In many species, both primary branches and higher-order ones show spinose appendages with variable shape and size. Almost all *Cystoseira s.l.* species possess cryptostomata, small cavities penetrating the cortical zone that communicate with the external environment through an opening called ostiole from which hyaline hairs come out. In sheltered environments, some species show gas vesicles called aerocysts, which promote buoyancy (Gómez-Garreta et al. 2001; Cormaci et al., 2012).

At the end of each secondary or tertiary branching, during the reproductive period, it occurs the development of receptacles (Figure 6), specialised fertile areas of the thallus (Hamel, 1931). The position of receptacles generally is terminal, but in some species, as in *Ericaria zosteroides* (C. Agardh) Molinari & Guiry, they can be at the base of the branches or in intercalary position. Within the receptacles, there are several conceptacles, spherical cavities which contain the female and male reproductive cells. The conceptacles can be considered as fertile cryptostomata (Robvieux, 2013).

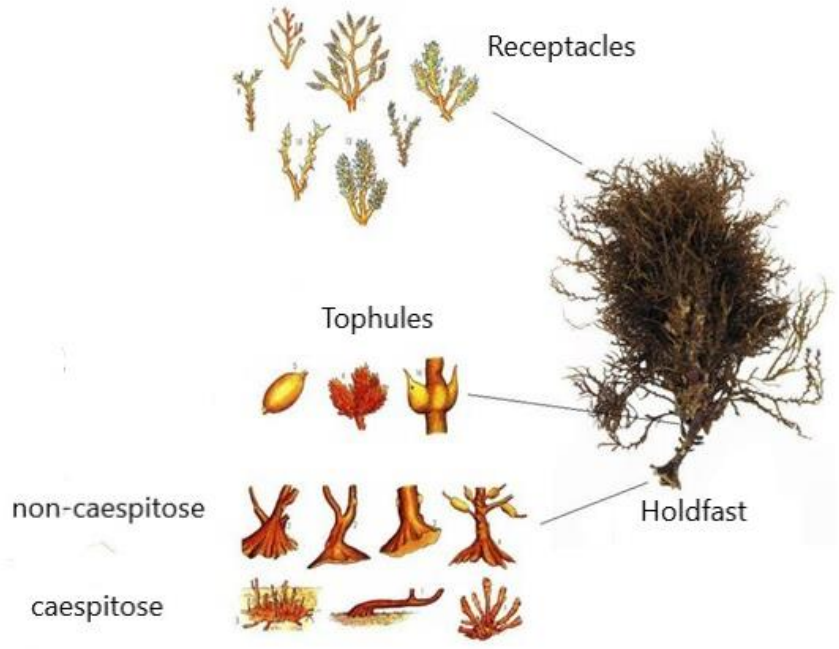


Figure 6: Diacritical characters of *Cystoseira s.l.* species (from Mannino & Mancuso, 2009, modified)

2.3 The fertilisation in *Cystoseira s.l.* species

In *Cystoseira s.l.* species the fertilisation occurs through oogamy. These species are monoecious with hermaphrodite conceptacles. The interior of the latter is taped by paraphyses, and fertile hairs which will give male and female gametocysts: antheridia and oogonia, respectively (Figure 7). The oogonia generally occupy the bottom of the conceptacle, while the antheridia are located near the ostiole where they are arranged in a crown (Sauvageau, 1912; Guern, 1962).

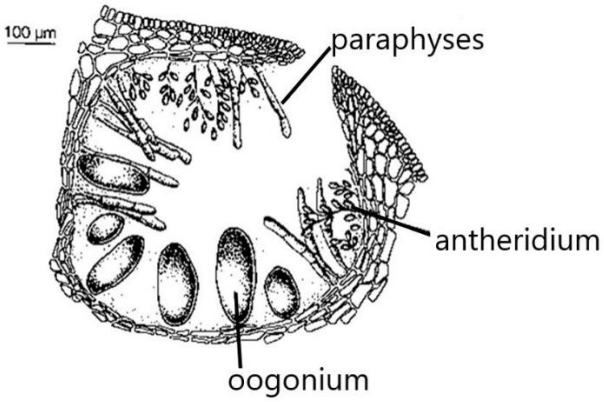


Figure 7: Section of a conceptacle (from Gómez-Garreta et al., 2001, modified)

The oogonia are sessile and are surrounded by three layers (exochiton, mesochiton and endochiton) within which first a meiotic division takes place, followed by a mitotic division (Guern, 1962). The number of oospheres contained in each oogonium changes according to the family or genus (Clayton, 1984) (Figure 8). In *Cystoseira s.l.* species only one oosphere remains, due to the extrusion of the remaining seven nuclei (Gómez-Garreta et al., 2001).

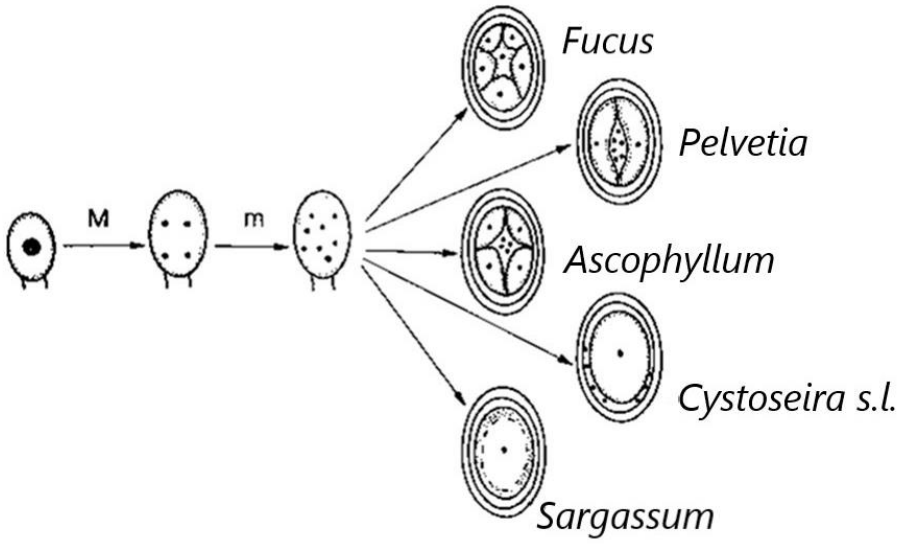


Figure 8: Different types of oogonium development in Fucales (from Jensen, 1974, modified)

The antheridia can be sessile or carried by branching filaments, surrounded by two layers in which a meiotic division takes place, followed by several mitotic divisions, forming 64 antherozoids. Depending on the species, within the chloroplasts, the antherozoids may have or not a stigma that is pigmented for the presence of carotenoids. Antherozoids are biflagellated with the anterior flagellum having mastigonemes and the posterior one having a smooth, swollen portion (Gómez-Garreta et al., 2001).

Cystoseira s.l. species possess a diplobiontic monogenetic cycle, with the dominance of a diploid sporophyte (2n) and a haploid phase represented only by the gametes (Gómez-Garreta et al., 2001; Rodríguez-Prieto et al., 2013; Robvieux, 2013) (Figure 9). According to Jensen (1974), the adults are considered sporophytes, and the oogonia and antheridia represent megasporocysts and microsporocysts respectively, containing extremely reduced female and male gametophytes. Even in other brown algae such as Laminariales the gametophyte subsists at the stage of a few cells within the conceptacle contained in the sporophyte (Sauvageau, 1915; Dayton, 1985).

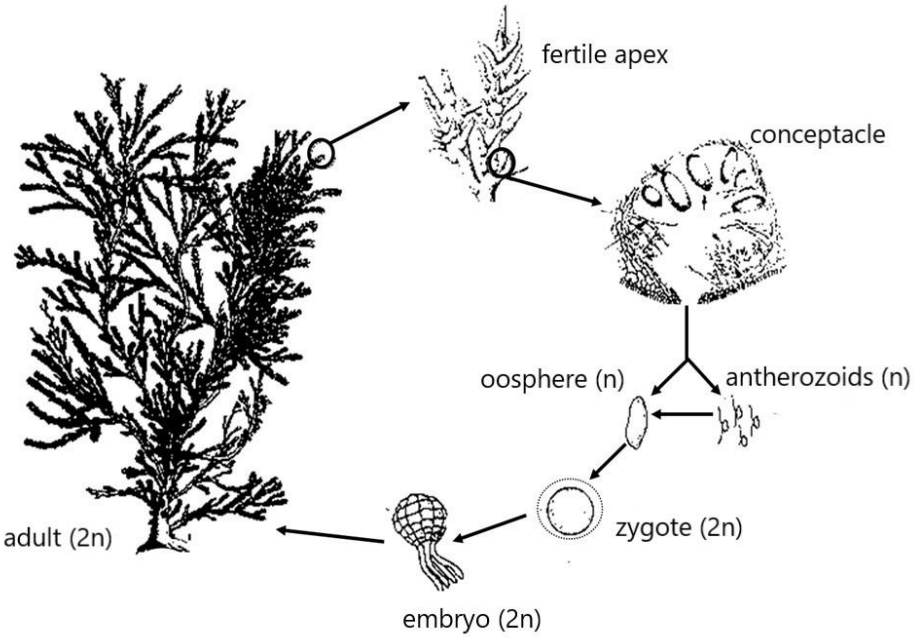


Figure 9: Life cycle of *Cystoseira s.l.* species (from Gómez-Garreta et al., 2001, modified)

During the fertilisation, the male gametes are the first to be released. According to Guern (1962), the inner membrane of the male gametocyst is expelled externally and jellifies before releasing a cloud of male gametes. The male gametes are active for several hours (Thuret, 1850) and remain near the ostiole (Guern, 1962). Subsequently, also the oospheres are expelled externally. Guern (1962) described two types of emission of female gametes:

- The first type, described in *Ericaria mediterranea* (Sauvageau) Molinari & Guiry happens in one step. Once the female gametes (of spherical shape) are ejected from the conceptacle, they encounter the male gametes, the fertilisation takes place, and the zygote falls directly on the substrate;
- The second type, described in *C. compressa* (Esper) Gerloff & Nizamuddin, occurs in two steps. Once the female gametes (of ovoid shape) are expelled from the conceptacle, they remain attached to it through a thick mucilaginous layer. This latter protects from bacteria and diatoms but does not prevent the fertilisation. Only in a second time the embryos will detach and fall to the substrate, attaching themselves on the substrate through their rhizoids.

2.4 The four embryological groups

By analysing the development of zygotes and embryos in several *Cystoseira s.l.* species, Guern (1962) identified three main embryological groups. Subsequently, Gil-Rodríguez et al. (1988) proposed a fourth embryological group. These groups are distinguished by the following characters:

- Shape of the oosphere once released from the conceptacle;
- Number and branching of the antheridia;
- Presence or absence of a coloured stigma in the antherozoids;
- Presence or absence of basal growth hairs;
- Pattern of zygote segmentation;
- Number of primary rhizoids.

2.4.1 First group

The first group, which includes the majority of *Cystoseira s.l.* species, is characterised by the following features:

- oosphere with a spherical shape, which falls immediately on the substrate;
- branched and numerous antheridia;
- antherozoids with a pigmented stigma;
- segmentation pattern of the zygote which occurs with a first equatorial division, a second division parallel to the first forming a rhizoidal cell and a third division perpendicular to the other two at the opposite pole to the rhizoidal one (Figure 10);
- absence of basal growth hairs;
- four primary rhizoids dividing from the rhizoidal cell.

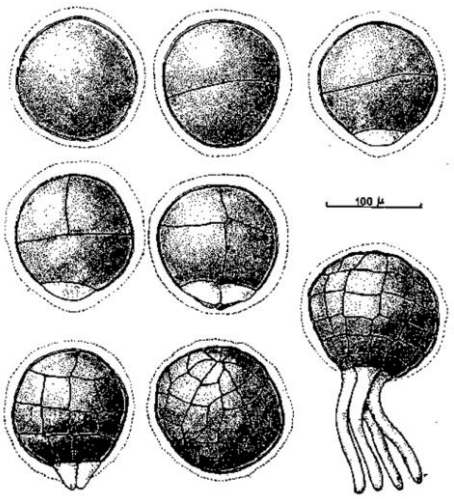


Figure 10: First developmental stages in the first embryological group (from Guern, 1962)

2.4.2 Second group

The second group comprehends the species belonging to *Cystoseira s.s.*, which possess the following features:

- ovoid oosphere that remains close to the conceptacle through a mucilaginous membrane and a pedicel penetrating into the conceptacle;
- poorly branched and few antheridia;
- antherozoids lacking a stigma;
- segmentation pattern of the zygote that occurs with a first equatorial division, a second division parallel to the first forming a rhizoidal cell and a third division perpendicular to the other two at the opposite pole to the rhizoidal one (Figure 11);
- presence of basal growth hairs;
- eight primary rhizoids dividing from the rhizoidal cell.

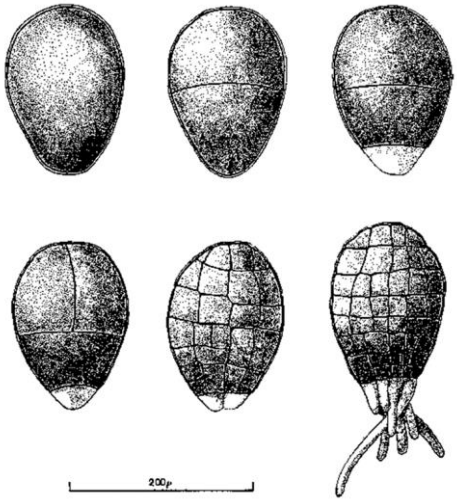


Figure 11: First developmental stages in the second embryological group (from Guern, 1962)

2.4.3 Third group

In this group only two species were included: *Gongolaria baccata* (S. G. Gmelin) Molinari & Guiry and *Ericaria sedoides* Neiva & Serrão. Colombo et al. (1982) considered them as ancient species, from which the speciation of the genus *Cystoseira* originated in the Atlantic and Mediterranean, respectively. The features of this group are:

- spherical oosphere, falling immediately onto the substrate;
- branched and numerous antheridia;
- antherozoids with a pigmented stigma;
- segmentation pattern that occurs in the following way: initial formation of a protuberance at one of the poles of the oosphere, where the first division takes place forming a rhizoidal cell, second division perpendicular to the first and subsequent divisions (third and fourth) oblique to the second (Figure 12);
- absence of basal growth hairs;
- four primary rhizoids dividing from the rhizoidal cell.

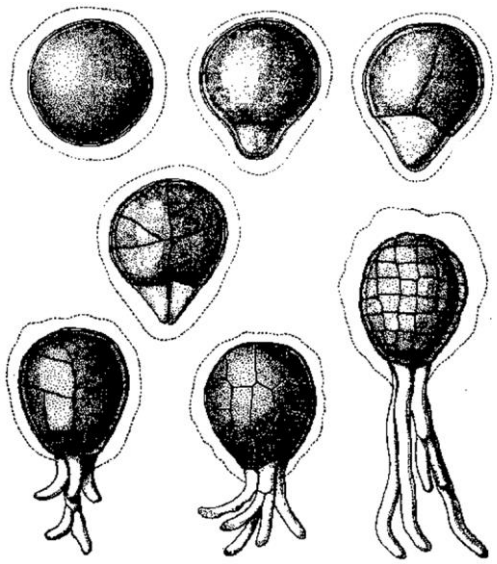


Figure 12: First developmental stages in the third embryological group (from Guern, 1962)

2.4.4 Fourth group

Gil-Rodríguez et al. (1988) described this group based on the culture observations of the first developmental stages in *Gongolaria abies-marina* (S. G. Gmelin) Kuntze, which did not correspond to any of the previous groups. More recently, Savonitto et al. (2019) studied the reproductive traits and embryology of *Ericaria barbatula* (Kützing) Molinari & Guiry and found the same characteristics highlighted by Gil-Rodríguez et al. (1988), listed below:

- spherical oosphere, sinking immediately onto the substrate;
- branched and numerous antheridia;
- antherozoids with a pigmented stigma;
- segmentation pattern with a first division at equatorial level from which two identical daughter cells are formed; second division perpendicular to the first one occurring in one of the two daughter cells and third division perpendicular to the first one occurring in the other daughter cell (Figure 13);
- absence of basal growth hairs;
- four primary rhizoids arising from a rhizoidal cell.

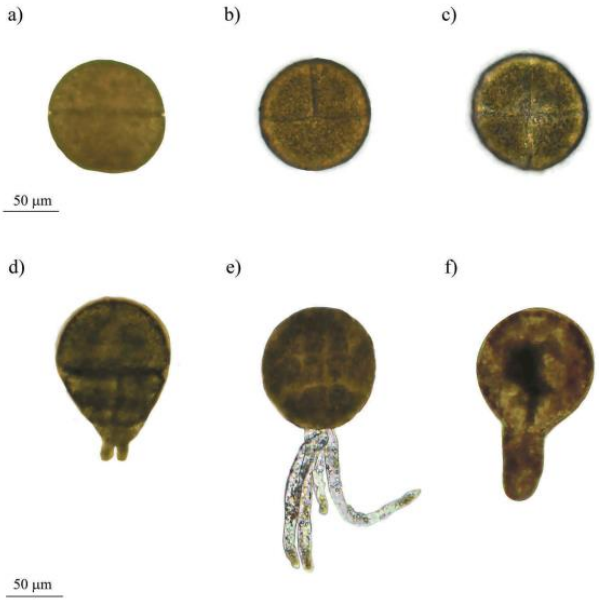
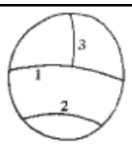
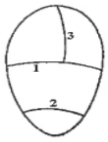
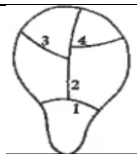


Figure 13: First developmental stages in the fourth embryological group: a) the first division zygote segmentation; b) the second; c) the third one; d) emission of the rhizoids; e) rhizoids with different lengths; f) no division of the rhizoidal cell (from Savonitto et al., 2019)

In Table 1 the species and the main features of the four embryological groups are summarised.

Table 1: Summary table of the main embryological groups

Species	Shape of the oosphere	Pattern of segmentation	Number of primary rhizoids	Place of embryo development	Antheridia	Basal growth hairs	Antherozoids
E. mediterranea *E. selaginoides* *E. brachycarpa* *G. elegans* *G. usneoides* *G. montagnei* *E. zosteroides* *E. crinita* *G. barbata* *G. sauvageauana* *C. jabukae* *E. amentacea* *G. susanensis* *E. giacconei*	spherical		4	on the substrate	numerous and branched	absent	with stigma
C. compressa *C. foeniculacea* *C. humilis*	ovoid		8	near the conceptacle	not numerous and not branched	present	without stigma
G. baccata *E. sedoides*	spherical		4	on the substrate	numerous and branched	absent	with stigma
G. abies-marina *E. barbatula*	spherical		4	on the substrate	numerous and branched	absent	with stigma

2.5 Taxonomic and phylogenetic revision

The first phylogenetic study on the genus *Cystoseira* was realized by Susini (2006), who proved that this genus was polyphyletic, suggesting splitting it into three new genera. Then, Draisma et al. (2010), who gave a larger contribution on the family Sargassaceae, confirmed the polyphyly of this genus and divided the Mediterranean and Atlantic species into three separate clades: *Cystoseira-4*, *Cystoseira-5* and *Cystoseira-6*. According to Draisma et al. (2010) the clade *Cystoseira-4* corresponded to the group ‘‘*C. discors-abrotanifolioides*’’ in Amico et al. (1985), which included *Cystoseira compressa*, *Cystoseira humilis* Schousboe ex Kützing and *C. foeniculacea* (Linnaeus) Greville. Therefore, only these three species remained related to this genus, keeping their names. Moreover, the clade *Cystoseira-4* differed from the clades *Cystoseira-5* and *-6* in having antherozoids lacking eyespots (stigmata), few antheridial branches with few ramifications, conceptacles with trichothallic hairs, ovoid oospheres that were larger than in the other two clades, and eggs that after discharge were retained at the surface of the receptacle until after fertilisation by means of a mucilaginous substance (Draisma et al., 2010). The species *Cystoseira*-4 were separated from the species in clade *Cystoseira-6* on the basis of the number of erect axes, being the clade *Cystoseira-4* characterized by caespitose species and the clade *Cystoseira-6* by species with one erect axis. The clade *Cystoseira-5* was separated from the *Cystoseira-4* for the absence of distichous branches and iridescence present in the species of the former group. For the species of clade *Cystoseira-5* the names *Carpodesmia* Greville and *Ericaria* Stackhouse were proposed, while for the species in the group *Cystoseira-6* the names *Baccifer* and *Gongolaria* Boehmer were suggested. However, Draisma et al. (2010) did not proposed the reinstatement of these genera because of the incomplete taxon sampling (Table 2).

Table 2: Classification proposed by Draisma et al. (2010)

Clade	Proposed genus name	Species included	Distribution
Bifurcaria-1	*Brassicophycus* gen. nov.	*B. brassicaeformis*	South Africa
Bifurcaria-2	*Bifurcaria* Stackhouse 1809	*B. bifurcata*[a]	Atlantic Europe
Cystoseira-1	*Sirophysalis* Kützing 1843	*S. trinodis*	Tropical Indo-West-Pacific
Cystoseira-2	*Polycladia* J. F. C. Montagne in Orbigny 1847	*P. heinii, P. indica, P. myrica*[b]	Eastern Indian Ocean
Cystoseira-3	*Stephanocystis* Trevisan 1843	*S. crassipes, S. dioica, S. geminata, S. hakodatensis, S. neglecta, S. osmundacea, S. setchellii*	Temperate-cold North Pacific
Cystoseira-4	*Cystoseira* C. Agardh 1820	*C. compressa, C. foeniculacea, C. humilis*	Mediterranean, northeast Atlantic, and Bermuda[c]
Cystoseira-5	No name proposed yet	*C. amentacea, C. barbatula, C. brachycarpa, C. crinita, C. funkii, C. mediterranea, C. tamariscifolia, C. zosteroides*	Mediterranean and northeast Atlantic
Cystoseira-6	No name proposed yet	*C. abies-marina, C. baccata, C. barbata, C. elegans, C. jabukae, C. nodicaulis, C. sauvageauana, C. sonderi, C. spinosa, C. squarrosa, C. susanensis, C. usneoides*	Mediterranean and northeast Atlantic
Halidrys	*Halidrys* Lyngbye 1819	*H. siliquosa, H. murmanica*	Atlantic and Arctic Europe
Sargassum-1	*Sargassum* C. Agardh 1820	All currently recognized species except *S. decurrens*	Circumtropical-temperate waters
Sargassum-2	*Sargassopsis* Trevisan 1843	*S. decurrens*	Tropical Indo-West-Pacific

Clades refer to the names used in the main text and in Figures 1 and 2.
[a]The generic status of *Bifurcaria galapagensis* is considered incertae sedis.
[b]The generic status of *Cystoseira myrica* var. *occidentalis* is considered incertae sedis.
[c]Schneider and Lane (2007).

Subsequently, Bruno de Sousa et al. (2019) improved the phylogeny of the Mediterranean and Atlantic *Cystoseira* species, through the implementation of different mitochondrial markers. The results of this study validated the polyphyly of the genus and the existence of three well supported clades: Cystoseira-I, II and III (Figure 14). The group Cystoseira-III included *C. compressa, C. foeniculacea* and *C. humilis*; the group Cystoseira-I included *C. amentacea* (C. Agardh) Bory, *C. barbatula* Kützing, *C. brachycarpa* J. Agardh, *C. crinita* Duby, *C. funkii* Schiffner ex Gerloff & Nizamuddin, *C. mediterranea* Sauvageau, *C. tamariscifolia* (Hudson) Papenfuss and *C. zosteroides* C. Agardh; and the group Cystoseira-II contained *C. abies-marina* (S. G. Gmelin) C. Agardh, *C. baccata* (S. G. Gmelin) P. C. Silva, *C. barbata* (Stackhouse) C. Agardh, *C. elegans* Sauvageau, *C. mauritanica* Sauvageau, *C. nodicaulis* (Withering) M. Roberts, *C. sonderi* (Kützing) Piccone, *C. montagnei* J. Agardh, *C. squarrosa* De Notaris and *C. usneoides* (Linnaeus) M. Roberts. According to Bruno de Sousa et al. (2019), the last two groups were more closely related than Cystoseira-III. Moreover, they found a connection between this phylogeny and the chemotaxonomic classification, as also observed by Susini (2006): linear diterpenoids and rearranged meroterpenoids were exclusive to Cystoseira-I taxa (the most "chemically evolved" group), while all Cystoseira-III taxa lacked diterpenoids and lipophilic secondary metabolites (Valls & Piovetti, 1995).

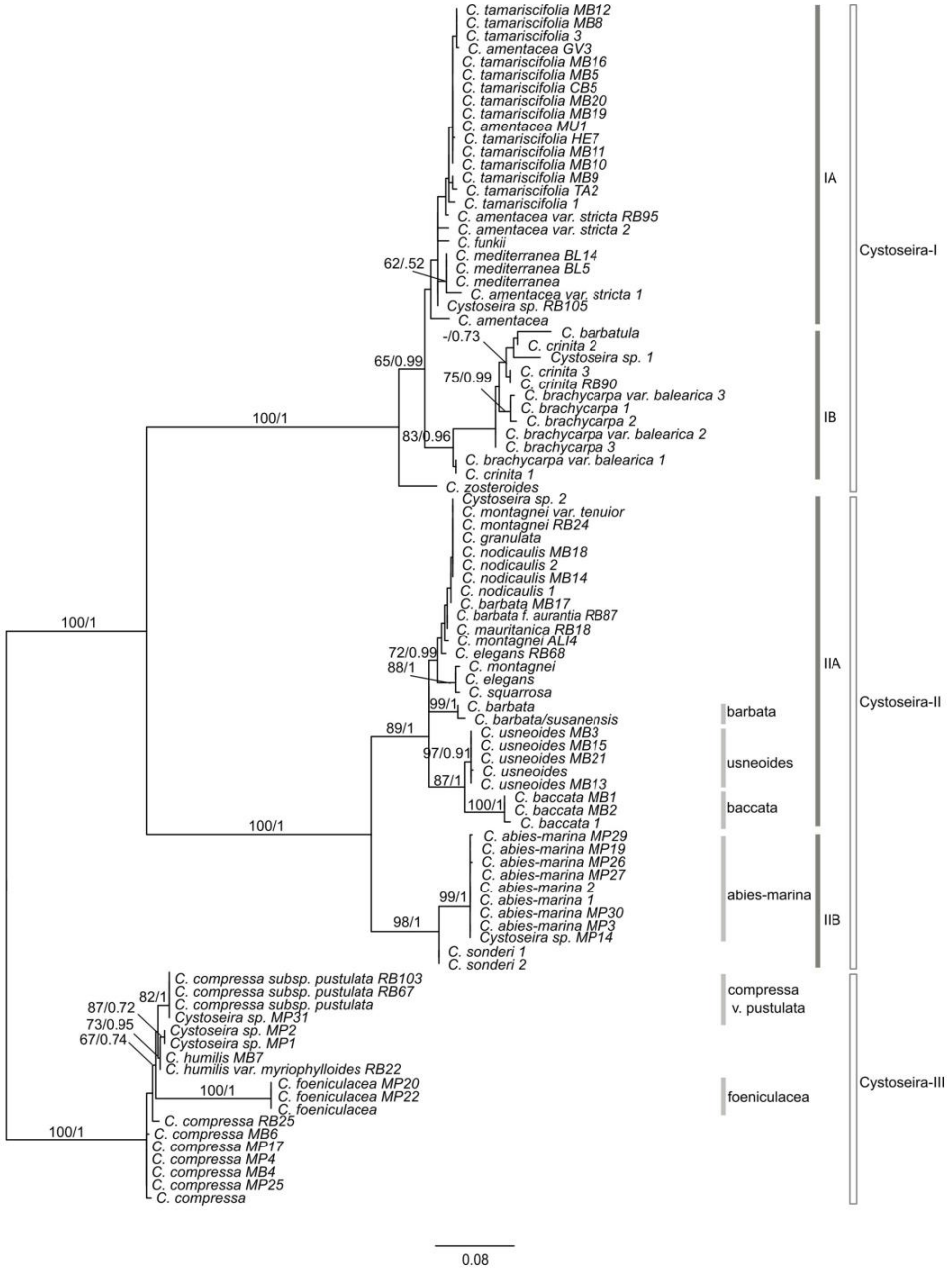


Figure 14: Phylogenetic tree reported by Bruno de Sousa et al. (2019)

Orellana et al. (2019) studied the diversity and phylogenetic relationships of eastern Atlantic and Mediterranean species on the basis of DNA sequences and morphological observations. They found three clades Cystoseira I, Cystoseira II and Cystoseira III, which corresponded respectively to three different genera: *Cystoseira sensu stricto* (*s.s.*) (*C. foeniculacea*, *C. humilis*, *C. compressa* and *C. aurantia*), *Treptacantha* Kützing (*T. abies-marina*, *T. algeriensis*, *T. baccata*, *T.*

barbata, *T. elegans*, *T. mauritanica*, *T. montagnei*, *T. nodicaulis*, *T. sauvageauana*, *T. squarrosa*, *T. susanensis* and *T. usneoides*) and *Carpodesmia* Greville (*C. zosteroides*, *C. amentacea*, *C. barbatula*, *C. crinita*, *C. funkii*, *C. mediterranea* and *C. tamariscifolia*).

The clade Cystoseira I was characterized by species with a caespitose habit, warty non spinose receptacles with grouped conceptacles, cylindrical or flattened primary branches, cortex cells with thickened walls and square meristoderm cells. The clade Cystoseira II included species with non-caespitose habit, smooth primary branches (at least in the basal region) with widely spaced spiny appendages upwards, spiny receptacles with few conceptacles at the base of each fertile spine, cortex cells with thickened walls and square meristoderm cells. Finally, the clade Cystoseira III contained species with a caespitose or non-caespitose habit, branches bearing spinose appendages, receptacles with spiny or filiform appendages and grouped conceptacles, cortex cells with thin walls and rectangular meristoderm cells (Table 3).

Novoa and Guiry (2019) claimed that the names *Treptacantha* and *Carpodesmia* had to be preceded by *Gongolaria* and *Ericaria* respectively, which had the priority on the former names. Therefore, they proposed *Gongolaria* and *Ericaria* as the correct names for the clades “Cystoseira 2” and “Cystoseira 3” defined by Orellana & al. (2019).

Table 3: Morphological characters of the three clades: Cystoseira 1, Cystoseira 2 and Cystoseira 3 (from Orellana et al., 2019)

Species	Group	Caespitose habit	Holdfast	Apex of axis	Spines in primary branches at basal region	Spines in secondary branches	Aerocysts	Receptacles
C. abies-marina (S.G. Gmelin) C. Agardh	*Cystoseira* 2	-	Haptera	-	-	+	-	With spines, single conceptacles at base of spine
C. algeriensis Feldmann	*Cystoseira* 2	-	Disc	Smooth, not prominent	-	+	-	With spines, single conceptacles at base of spine
C. amentacea (C. Agardh) Bory	*Cystoseira* 3	+	Haptera	Spinose, not prominent	+	+	-	With spines, few conceptacles at base of spine
C. baccata (S.G. Gmelin) P. C. Silva	*Cystoseira* 2	-	Disc	Smooth, prominent	-	-	+	Without spines, few conceptacles
C. barbata (Stackhouse) C. Agardh	*Cystoseira* 2	-	Disc	Smooth, prominent	-	-	+	Without spines, few conceptacles
C. barbatula Kützing	*Cystoseira* 3	+	Haptera/ Disc	Smooth, prominent	+	+	-	With spines, numerous conceptacles
C. brachycarpa J. Agardh	*Cystoseira* 3	+	Haptera	Smooth, not prominent	+	+	-	With spines, few conceptacles
C. compressa (Esper) Gerloff & Nizamuddin	*Cystoseira* 1	+	Disc	Smooth, not prominent	-	-	-	Without spines, numerous conceptacles
C. crinita Duby	*Cystoseira* 3	+	Disc	Spinose, prominent	+	-	-	With spines, few conceptacles
C. elegans Sauvageau	*Cystoseira* 2	-	Disc	Spinose, not prominent	-	+	-	With spines, numerous conceptacles at base of spines
C. foeniculacea (Linnaeus) Greville	*Cystoseira* 1	+	Disc	Spinose, not prominent	+	-	+	Without spines, numerous conceptacles
C. funkii Schiffner ex Gerloff & Nizamuddin	*Cystoseira* 3	-	Disc/ Haptera	Smooth, not prominent	+	+	-	With spines, few conceptacles at the base of spines
C. humilis Schousboe ex Kützing	*Cystoseira* 1	+	Disc	Smooth, not prominent	-	-	+	Without spines, numerous conceptacles
C. mauritanica Sauvageau	*Cystoseira* 2	-	Disc	Spinose, not prominent	-	+	-	With spines, few conceptacles at base of spines
C. mediterranea Sauvageau	*Cystoseira* 3	-	Haptera/ Disc	Spinose, not prominent	+	+	+	With spines, few conceptacles at base of spines
C. montagnei J.Agardh	*Cystoseira* 2	-	Disc	Spinose, not prominent	-	+	-	With spines, few conceptacles at base of spines
C. nodicaulis (Withering) M. Roberts	*Cystoseira* 2	-	Disc	Smooth, prominent	-	+	+	With spines, few conceptacles
C. sauvageauana Hamel	*Cystoseira* 2	-	Disc	Spinose, prominent	-	+	-	With spines, single conceptacles at base of spine
Cystoseira sp. (as *C. aurantia* Kützing)	*Cystoseira* 1	+	Disc	Smooth, not prominent	-	-	+	Without spines, numerous conceptacles
C. squarrosa De Notaris	*Cystoseira* 2	-	Disc	Spinose, not prominent	-	+	+	With spines, few conceptacles
C. susanensis Nizamuddin	*Cystoseira* 2	-	Disc	Smooth, prominent	-	-	+	Without spines, numerous conceptacles
C. tamariscifolia (Hudson) Papenfuss	*Cystoseira* 3	-	Haptera/ Disc	Spinose, not prominent	+	+	+	With spines, numerous conceptacles
C. usneoides (Linnaeus) M. Roberts	*Cystoseira* 2	-	Haptera	Smooth, not prominent	-	+	+	With spines, few conceptacles
C. zosteroides (Turner) C. Agardh	*Cystoseira* 3	-	Haptera	Smooth, prominent	+	+	-	With spines, numerous conceptacles

Recently, Neiva et al. (2022) used DNA barcoding to update the systematics and biogeography of *Cystoseira s.l.* species. Within the group *Cystoseira s.s.*, they found two main lineages. One lineage was characterized by *C. foeniculacea* with a distribution in the Atlantic and Mediterranean Sea. The other lineage grouped three related entities: *C. compressa* represented by samples coming from all the Mediterranean and from Azores and Canary Islands; *C. humilis* found exclusively in Atlantic samples; and *C. pustulata* (Ercegovic) Neiva & Serrão including samples from both Atlantic and Mediterranean (Figure 15).

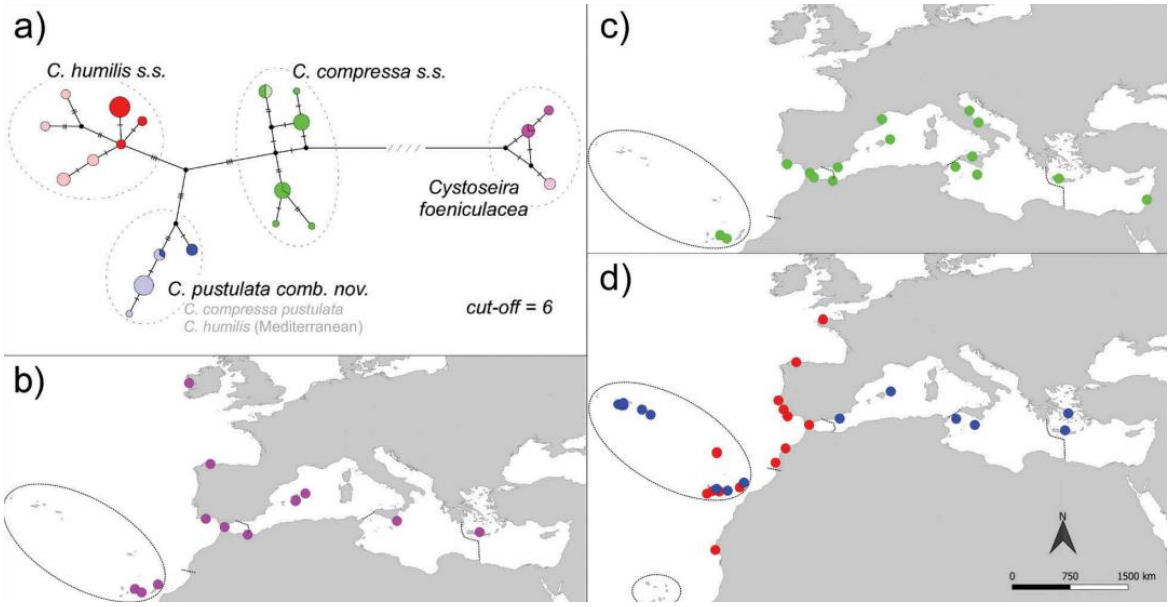


Figure 15: Genetic entities and distribution of *Cystoseira s.s.*: a) haplotype network; b) geographic sampling of *C. foeniculacea*; c) *C. compressa*; d) *C. humilis* and *C. pustulata* (from Neiva et al., 2022)

The genus *Ericaria* was composed by five lineages. Three of them were represented by *E. zosteroides* (C.Agardh) Molinari & Guiry, *E. sedoides* and *E. dubia* (Valiante) Neiva & Serrão, respectively. A more diversified Mediterranean lineage included *E. brachycarpa s.s.* (with samples coming from the northern coast of Sicily and southern Crete), *E. balearica* (Sauvageau) Neiva, Ballesteros & Serrão, (represented by samples from the Balearic Sea and the Sicilian Island of Pantelleria), *E. corniculata* (Turner) Neiva & Serrão (from Crete) and finally *E. crinita s.l.* (comprising samples identified as *E. crinita*, *E. barbatula* and *E. giacconei* D. Serio & G. Furnari coming from Sicily, the Island of Pantelleria, Adriatic Sea, Crete, Black Sea, and Menorca). Finally, they found three lineages corresponding to three separated haplogroups (A, B and C) represented by the *E. selaginoides* complex. The haplogroup A included Atlantic single-cauloid samples identified as *E. selgainoides* (Linnaeus) Molinari & Guiry, as well as Mediterranean caespitose algae, identified as *E. amentacea*, from the south-eastern Iberian Peninsula, Balearic Islands and Pantelleria. The haplogroup B included non-caespitose samples (identified as *E. mediterranea*) from the Spanish Catalonia and caespitose samples from Sicily (identified as *E. amentacea*). Finally, the haplogroup C grouped caespitose algae (identified as *E. amentacea*) from Malta,

Adriatic and Crete. Therefore, haplogroup A was mainly distributed in western Mediterranean, haplogroup B in Sicily and haplogroup C in the eastern Mediterranean (Figure 16).

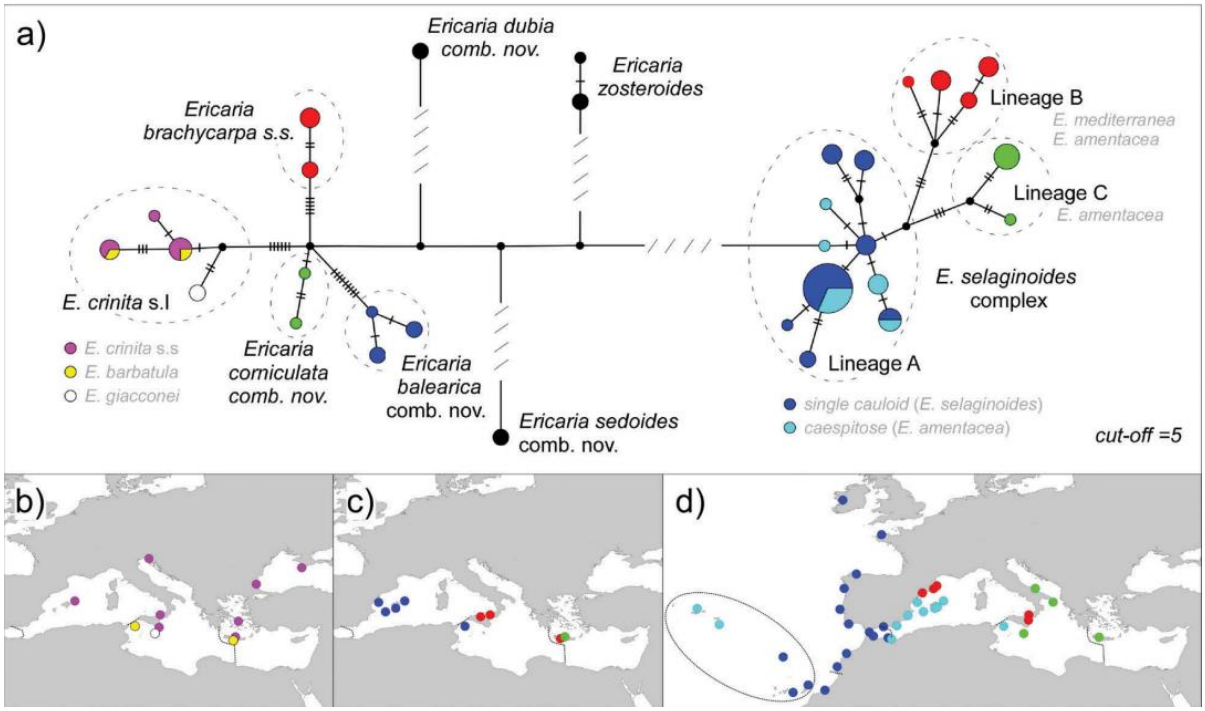


Figure 16*:* Genetic entities and distribution of *Ericaria*: a) haplotype network*;* b) Geographic sampling of *E. crinita s.l.*; *c) E. corniculata, E. balearica* and *E. brachycarpa s.s.; d) E. selaginoides* complex (from Neiva et al., 2022)

The genus *Gongolaria* comprised two main clades (A and B). The first one was represented by the two caespitose species, *G. abies-marina* (from the Macaronesia) and *G. sonderi* (Kützing) Neiva, João Soares & Serrão (from Cape Verde archipelagos). The clade *Gongolaria* B included three main lineages widely distributed in the Atlantic and the Mediterranean. One lineage comprised the two large Atlantic species *G. baccata* and *G. usneoides* (Linnaeus) Molinari & Guiry. Another lineage was composed by *G. barbata* (Stackhouse) Kuntze from the Mediterranean and Black Sea and the Sicilian samples previously identified as *G. susanensis* (Nizamuddin) Molinari & Guiry. Finally, the last lineage comprised multiple species complexes with very low genetic differentiation: *G. nodicaulis* (Withering) Molinari & Guiry (sampled from Ireland to Mauritania), *G.*

gibraltarica Sauvageau) Neiva, Bermejo & Serrão (represented by samples from Tarifa, Nador, Ria Formosa, Cádiz Bay and Nador lagoons), *G. montagnei s.l.* (including samples identified as *C. spinosa* Sauvageau and C. *algeriensis* from both shallow to deeper waters of the Spanish coasts), *G. elegans s.l.* [including Spanish samples identified as *G. elegans* (Sauvageau) Molinari & Guiry and *G. sauvageauana* (Hamel) Molinari & Guiry from both shallow and deep environments], *Gongolaria* sp. 2 [with samples identified as *G. montagnei* (J. Agardh) Kuntze and *G. elegans* from Crete and Sicily]. Finally, the most divergent complex included samples of the Levantine-endemic *G. rayssiae* (Ramon) Molinari & Guiry and a closely related entity from Macaronesia, referred to as *Gongolaria* sp. 1 [comprising algae from Tenerife originally identified as *G. mauritanica* (Sauvageau) Molinari & Guiry, and *Gongolaria* sp. from Madeira] (Figure 17).

Neiva et al. (2022) concluded that, despite the sampling effort, many areas of the Mediterranean remained still unexplored, thus it would have been needed to clarify the validity and affinities of poorly sampled taxa, to clarify patterns of diversity and species assembly.

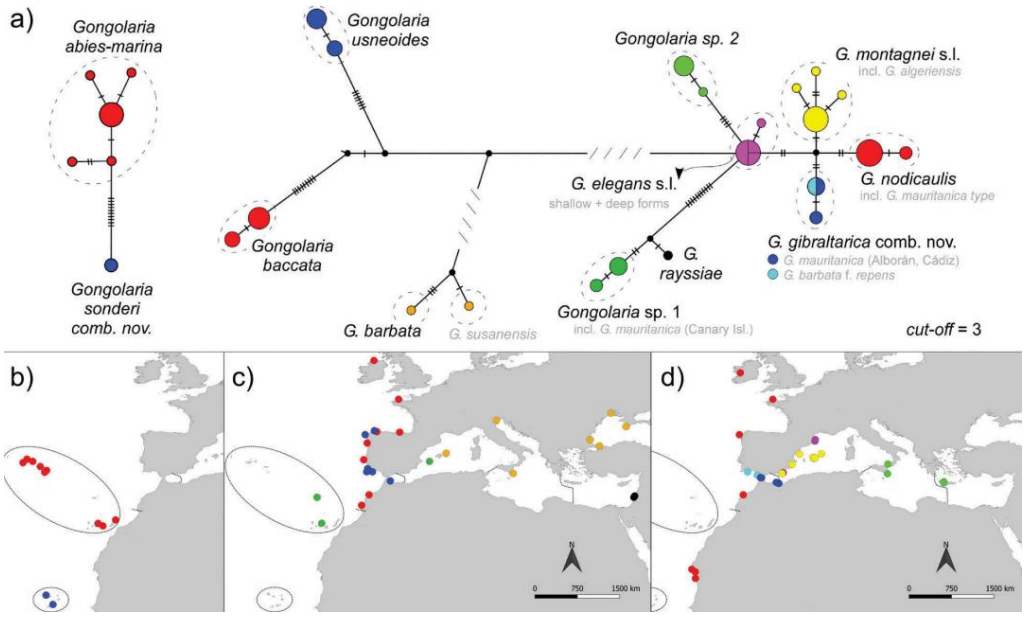


Figure 17: Genetic entities and distribution of *Gongolaria: a)* haplotype networks of clade A (left) and clade B (right); b) Geographical sampling depicting *G. abies-marina* and *G. sonderi;* c) *G. baccata*, *G. usneoides, G. barbata* (incl. Marzamemi's *'G. susanensis'*), *G. rayssiae* and *Gongolaria* sp. 1; *d) G. nodicaulis, G. montagnei s.l., G. elegans s.l.* (incl. Columbrete's *G. sauvageauana), G. gibraltarica* and *Gongolaria* sp. 2 (from Neiva et al., 2022)

2.6 The infralittoral *Cystoseira s.l.* species

The *Cystoseira s.l.* species are distributed in the benthic domain determining a true "zonation" of the photophilic vegetation of hard substrates, and the factors that mainly regulate their bathymetric distribution are light intensity and the type of hydrodynamics (Giaccone et al., 1994). The highest diversity of *Cystoseira s.l.* species is observed in the infralittoral plan, which extends from the minimum tidal level to the maximum depth where photophilic algae and seagrasses grow. This lower limit varies from a few metres to 40 m of depth, according to the water quality and topography (Robvieux, 2013).

The infralittoral plan is subdivided in three horizons:

- upper horizon (from 0 to -50 cm), where light intensity and hydrodynamics are strong;
- middle horizon (from -50 cm to -15 m), where light intensity and hydrodynamics are attenuated. This horizon is subdivided in high (from -50 cm to -2 m) and low (from -2 m to -15 m);
- deep horizon (from -15 to -40 m), where light intensity and hydrodynamics are weak.

This thesis focused on *Cystoseira s.l.* species distributed in the upper and middle horizons of the infralittoral plan, which could be potentially more exposed to the effects of climate change. Therefore, this section will provide information concerning only these two horizons, without deepening deep communities.

The upper horizon is denoted by the presence of *E. amentacea* (that mainly occupies the infralittoral fringe) and *E. mediterranea* (Figure 18). These communities develop in well-lit environments, exposed to disruptive hydrodynamics. In areas characterised by intense upwelling phenomena (as in the Strait of Messina or in the Alboran Sea) *E. amentacea* is substituted by *E. selaginoides* (Cormaci et al., 2003).

Figure 18: The species of the upper horizon: on the left *E. amentacea* and on the right *E. mediterranea* (photos: G. Marletta)

The high middle horizon is distinguished by biotopes with light intensity not less than 60% of the incident light intensity at the surface, and with a multidirectional hydrodynamics, which tends to become bidirectional more in depth. The typical vegetational association of this horizon is *Cystoseiretum crinitae*, described in 1958 by Molinier (1960) for the coastline of Cape Corse (Corsica, France). According to Giaccone et al. (1994), the dominant species of this association, *E. crinita*, is rich in geographical and ecological vicariants:

- in more exposed environments, with intense oscillating hydrodynamics, the dominant species is replaced by *E. brachycarpa* (J. Agardh) Molinari & Guiry (Figure 19a);
- in the northern and colder biotopes (e.g., in the Adriatic Sea) there is instead *Cystoseira crinitophylla* Ercegovic, which can also extend to the lower horizon;
- on sub-horizontal platforms with reduced hydrodynamics (such as in the Ionian Sea or in the Strait of Sicily) it is often substituted by *E. barbatula* (Figure 19b);
- in infralittoral pools and sheltered bays, it is usually replaced by *G. elegans*, *G. barbata* and *C. compressa* (Figure 20).

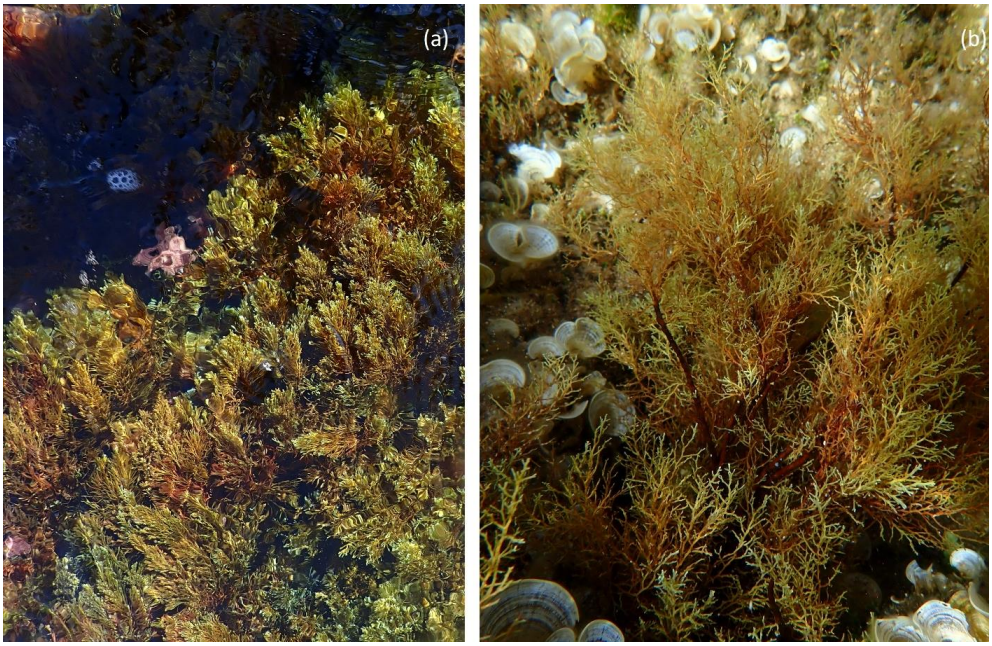


Figure 19: The species of the high middle horizon: a) *E. brachycarpa*; b) *E. barbatula* (photos: G. Marletta)

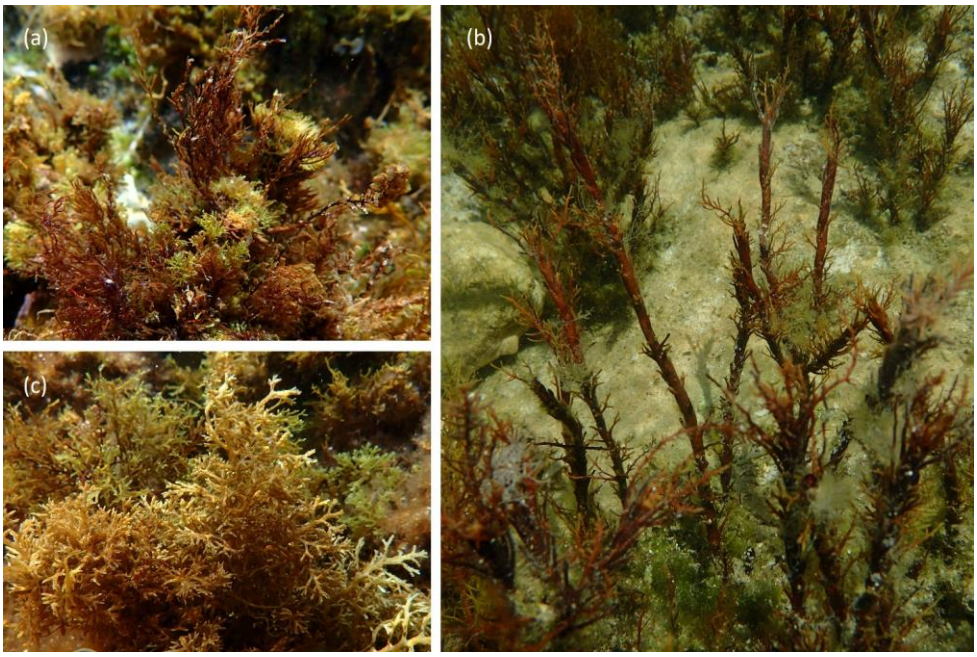


Figure 20: The species of the high middle horizon: a) *G. elegans*; b) *G. barbata*; c) *C. compressa* (photos: G. Marletta)

The low middle horizon is characterised by biotopes with light intensity not less than 15% of that incident at the surface and an oscillating unidirectional hydrodynamics. According to Giaccone et al. (1994) the dominant species of these

biotopes is *G. sauvageauana*, which in the eastern Mediterranean can be replaced by *G. montagnei* var. *tenuoir* (Ercegović) Molinari & Guiry (Figure 21) and in the Adriatic Sea by *E. corniculata*.

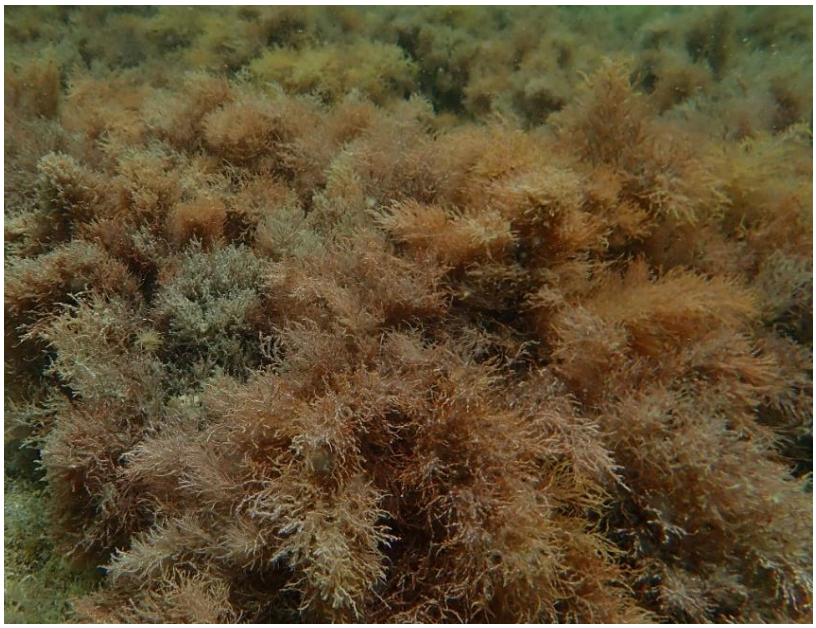


Figure 21: The species of the low middle horizon: *G. montagnei* var. *tenuior* (photo: G. Marletta)

Chapter III
Upper infralittoral *Cystoseira s.l.* species along eastern Sicily

3.1 Past and current presence of the upper infralittoral *Cystoseira s.l.* species along the Ionian coast of Sicily

The first phase of this research project investigated on the presence of the upper infralittoral *Cystoseira s.l.* species along the eastern coast of Sicily, comparing the historical data with the current distribution. To conduct this analysis, it was consulted the past literature (from the 1970s to 2000) to identify the past distribution of *Cystoseira s.l.* species along the Ionian coast of Sicily. Subsequently, the sampling was carried out either in areas where the presence of these species was previously documented and in sites which could host *Cystoseira s.l.* communities. For each found species, only two thalli, presenting all diacritical features, were collected to avoid impacting the populations. These thalli were collected with a pick to include the base, in order to gather all necessary information for the species identification. Subsequently the thalli were stored with seawater and alcohol and identified in the laboratory. Then, the identified thalli were stored *in exsiccata* for future consultation.

From all obtained information it was produced a table containing the past and current presence of these species (Table 5). For the past presence the following terms were used "common" (presence in several sites), "frequent" (presence in 3-4 sites), "rare" (presence only in 1-2 sites), while the actual presence was indicated as "stable" (species loss only in one site), "in regression" (species loss in several sites), "locally extinct" (species never found during the monitoring).

3.2 The study area

The eastern Sicily is the area that borders the Ionian Sea and embraces the coastline between Punta Faro (Messina) and Capo Passero (Portopalo) (Figure 22). The morphology of this area is extremely variable: the coasts of the north-eastern Sicily are generally volcanic, while predominantly calcarenitic elements prevail along the south-eastern coasts (Giaccone & Sortino, 1974). Subvolcanic as well as subaerial

and submarine volcanic rocks constitute the coastal area of the northern side of the Gulf of Catania (Corsaro & Cristofolini, 1997). These rocks, dissected by faults and dismantled by wave action, produce a belt of blocks, up to a few meters in size, all along the coast and at the base of local shallow cliffs (Rosso et al., 2019).

The south-eastern Sicily, mostly constituted by the Hyblean Plateau, is characterised by the occurrence of anomalous calcareous boulders, which are scattered along large terraces located at 2–5 m above the sea level, gently sloping towards the sea. Several boulders show biogenic encrustations (serpulids, balanids, lithophaga) all over their surface which suggest that they were dragged from the mid-sublittoral zone (Scicchitano et al., 2007).

To conduct this survey, the following sites were chosen to assess the current presence of *Cystoseira s.l.* communities distributed in the upper infralittoral (from 0 cm to – 2m): Santa Tecla (37.64001, 15.18443), Santa Maria La Scala (37.61338, 15.17442), Capo Mulini (37.57444, 15.17345), M.P.A. Isole Ciclopi (37.56118, 15.16731), Catania (37.53513, 15.12878), Bay of Brucoli (37.29247, 15.19733), Augusta (37.24277, 15.25708), Magnisi Peninsula (37.14518, 15.24246), Maddalena Peninsula (37.04119, 15.30854), M.P.A. Plemmirio (37.00382, 15.33639), Vendicari Wildlife Oasis (36.80459, 15.10392), Marzamemi (36.74438, 15.11905) and Portopalo di Capo Passero (36.65129, 15.07604) (Figure 22).

Figure 22: On the left, investigated area located along the eastern coast of Sicily; on the right, sites monitored during this study

3.3 Historical data

The consulted literature begins approximately from the '70s when numerous phycologists started to carry out several studies on the flora and marine benthic vegetation of eastern Sicily. One of the first papers on this area was "Ricerche floristiche sulle alghe marine della Sicilia orientale" (Furnari & Scammacca, 1970a), in which the authors reported some information on the benthic flora of the Acireale. Further research on the biodiversity of macrophytobenthos in the eastern sector of Sicily were produced in the following years (e.g., Furnari & Scammacca, 1970b; 1971, 1973, 1975; Cormaci et al, 1976, 1979, 1985; Cormaci & Furnari, 1979a,b; Battiato & Ponte, 1975, 1978; Battiato et al., 1978, 1980). Later, Giaccone et al. (1986), published a review on the marine flora of Sicily and the minor Islands based on both bibliographic and original data. During the '90s, numerous studies more focused on the dynamics and on the morphological and reproductive phenology of *Cystoseira s.l.* populations were performed (e.g., Pizzuto & Serio, 1994; Serio, 1995; Pizzuto et al., 1996; Pizzuto, 1999; Alongi et al., 1999a,b).

3.4 Results and Discussion

The following taxa, distributed in the upper infralittoral, were considered: *E. amentacea*, *G. barbata*, *E. barbatula*, *E. mediterranea*, *E. brachycarpa*, *C. compressa*, *E. crinita*, *G. elegans*, *C. foeniculacea*, *E. giacconei*, *G. sauvageauana* and *G. spinosa* var. *tenuior*. Table 4 reports the list of the species with the sites (including those reported in historical data) where they were found.

Regarding the past presence of the considered species, before the 2000s the most common taxa along the eastern coast of Sicily were *E. amentacea*, *E. bracycarpa*, *C. compressa*, *C. foeniculacea* and G. *sauvageauana*. Meanwhile, *G. barbata*, *E. barbatula*, *E. mediterranea*, *E. crinita*, *G. elegans* and *G. spinosa* var. *tenuior* were frequent species. Instead, *E. giacconei* was rare since it was only reported in its type locality (Punta D'Aliga), in the southern coast of Sicily (Giaccone, 1985).

As concerns the actual presence of these species, it can be noted that *E. amentacea*, *G. barbata* and *C. compressa* have disappeared only in one site where they were historically present, while in other areas (both already documented and investigated here for the first time) they remained stable. The populations of *E. barbatula*, *E. mediterranea*, *E. brachycarpa*, *G. elegans*, *C. foeniculacea* and *G. montagnei* var. *tenuior* were subjected to a decline in some sites of historical presence. However, during the samplings, these species (except *E. barbatula*) were found in other areas where their presence were not previously documented. Two species, *E. crinita* and *G. sauvageauana*, were never found during this study and were considered as locally extinct. Finally, *E. giacconei*, which was not previously recorded along the eastern coast of Sicily, during our samplings and the research of Serio & Furnari (2021) was found in two sites (Portopalo di Capo Passero and Vendicari).

Table 4: Sites investigated during this study: ST = Santa Tecla, SM= Santa Maria La Scala, CM= Capo Mulini, IC= M.P.A. Isole Ciclopi, CT= Catania, BRU=Brucoli, AUG=Augusta, MP= Magnisi Peninsula, MAD= Maddalena Peninsula, PLE= Plemmirio, VEN= Venicari, MAR=Marzamemi, PCP= Portopalo di Capo Passero. The symbol + indicates the presence of the species in the site, the symbol - indicates the absence of the species in the site and the symbol * denotes the historical presence of the species in that site.

Sites													
Species	ST	SM	CM	IC	CT	BRU	AUG	MP	MAD	PLE	VEN	MAR	PCP
E. amentacea	+	+	+	+*	+*	+	+	+	+*	+	+*	-	-*
G. barbata	-	-	-	-	-*	-	+	-	+*	-	+	-	+*
E. barbatula	-	-	-	-	-	-	-	-	-*	-	+*	-*	+*
E. mediterranea	-	-	-	-*	-*	+	+	+	+	+	+*	-	-
E. brachycarpa	-	-*	-	-*	-*	+	+	-	-*	-	-*	-	+*
C. compressa	-	-	+	+	-*	+*	+	+	+*	+	+*	+	+*
E. crinita	-	-	-	-	-	-	-	-	-*	-*	-*	-	-*
G. elegans	-	-	-	-*	-	+	-	-	-	-	-*	-	+*
C. foeniculacea	-	-	-	-*	-*	+	+	+	-*	-	+	+*	+*
E. giacconei	-	-	-	-	-	-	-	-	-	-	+	-	+
G. sauvageauana	-	-	-	-*	-*	-	-*	-	-*	-*	-*	-	-*
G. montagnei var. *tenuior*	-	-	-	-	-	+*	-	-	-*	-	+	-*	-

Table 5 reports the information obtained through the consultation of the previous data on past presence of *Cystoseira s.l.* species and the current data acquired through the samplings in both the sites of historical presence and the additional ones.

Table 5: Past (before the 2000s) and actual presence of *Cystoseira s.l.* species along eastern Sicily

Species	Past presence	Present presence
E. amentacea	common	stable
G. barbata	frequent	stable
G. barbatula	frequent	in regression
E. mediterranea	frequent	in regression
E. brachycarpa	common	in regression
C. compressa	common	stable
E. crinita	frequent	locally extinct
G. elegans	frequent	in regression
C. foeniculacea	common	in regression
E. giacconei	rare	stable
G. sauvageauana	common	locally extinct
G. montagnei var. *tenuior*	frequent	in regression

Future monitoring activities in the areas here investigated will be necessary to evaluate the status of *Cystoseira s.l.* populations and understand the impacts that could threaten them. In addition, conservation measures (i.e., No-Take Zones) should be implemented to improve the protection of these species.

Chapter IV
Reproductive phenology and embryology of four *Cystoseira s.l.* species

4.1 The species under study

The previous chapter reported the results on the past and current presence of the upper infralittoral *Cystoseira s.l.* species along the Ionian coast of Sicily. In this phase of the research project, we focused on four of these species: *E. brachycarpa*, *G. montagnei* var. *tenuior*, *E. mediterranea* (three regressing species along the Ionian coast of Sicily) and *E. giacconei* (a cold-affinity endemic species). Of these taxa, it was performed an in-deep study of the reproductive phenology and embryology. In particular, the principal aims were: 1) to examine the reproductive traits, a preparatory phase to the subsequent embryological study; 2) to analyse the zygote segmentation and embryo development, to confirm the belonging embryological group (see Chapter II) of these species, especially of *E. giacconei*, whose embryology had never been studied in detail. The study of the embryology of *E. giacconei* was realised in cooperation with the University of Trieste and the relative data on the first developmental stages here reported were published in the paper by Falace et al. (2021).

4.2 Materials and Methods

During 2021, the reproductive phenology of *E. brachycarpa*, *E. mediterranea*, *E. giacconei* and *G. montagnei* var. *tenuior* was surveyed by collecting two thalli every two months at Brucoli (*E. brachycarpa*, *E. mediterranea*, *G. montagnei* var. *tenuior*) and at Portopalo di Capo Passero (*E. giacconei*). The collected thalli were used to perform the morphological observations at the stereomicroscope, necessary for the correct identification of the species. Moreover, fertile apices were used to measure the morphometric variables of the receptacles, conceptacles and gametes (length of the receptacle, diameter of the conceptacles, length and width of antheridia and oogonia). To execute these measures, longitudinal sections of the receptacles and transverse sections of the conceptacles were made through a razor blade on glass slides. Then, the sections were observed and measured at optical microscope, and photographed through a camera Nikon D40.

To examine the embryology of these species, some fertile apices were wrapped in aluminium foil, transported to the laboratory, and stored in dark and refrigerate conditions (6°C) for 12 h. Subsequently, for each species, about 30 fertile apices were placed on 9 glass slides located inside 3 petri dishes filled with filtered seawater, in controlled mesocosm at 20°C, saturating light and photoperiod 12L:12D (Figure 23). This thermal shock (6–20°C) stimulated the release of fertilised oospheres from the mature receptacles. After the release of zygotes, the apices were removed from the slides, and the filtered seawater inside the petri dishes was changed with a pipette. For all the species, the pattern of zygote segmentation and embryo development were followed for four weeks, during which the filtered seawater was changed every two days to avoid the proliferation of diatoms and bacteria. The first phases of zygote segmentation and the embryo development were checked at optical microscope and photographed through Nikon D40.

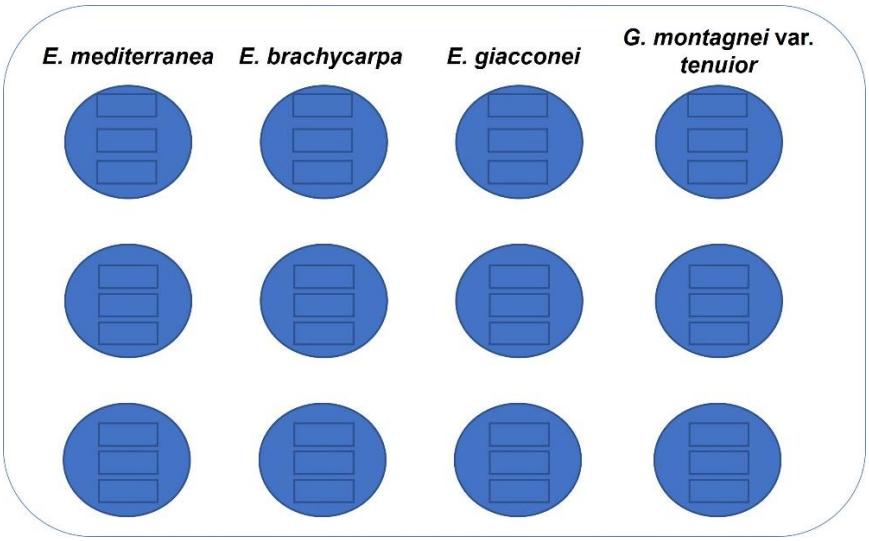


Figure 23: Experimental setup for the embryological study of *E. mediterranea*, *E. brachycarpa*, *E. giacconei* and *G. montagnei* var. *tenuior*

4.3 Results and discussion

4.3.1 Reproductive traits and embryology of Ericaria mediterranea

E. mediterranea is non-caespitose species, attached to the substrate by a robust basal disc, more often by radiating haptera. The apex of the cauloid is not very prominent and covered with spines. The primary branches are cylindrical, with a pyramidal shape. Secondary and tertiary branches are covered with long and pointed spines. Branches are mostly deciduous and those that persist are gracile and sterile. The receptacles are terminal and surrounded by short spinose appendages. During spring, the receptacles are very conspicuous, compact and cylindrical, while in autumn they start to be smaller. *E. mediterranea* grows in well or moderately exposed rocky coasts, in the upper infralittoral (Figure 24). The maximum vegetative and reproductive development of this species is in spring and summer, while from the beginning of autumn it tends to lose the frond.

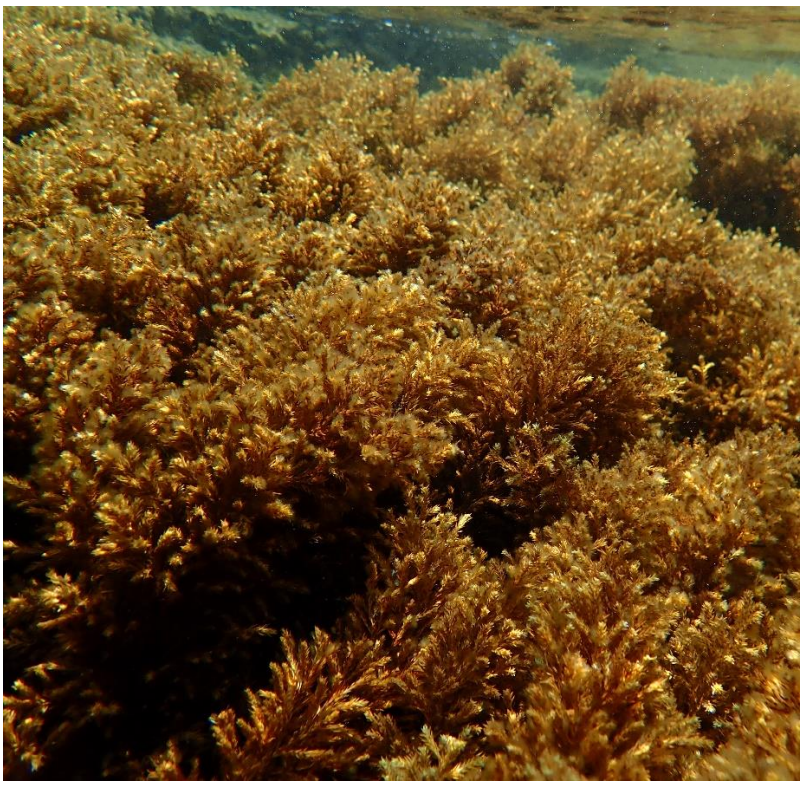


Figure 24: *E. mediterranea* community at Brucoli (photo: G. Marletta)

The receptacles (length = 1.4 ± 1.4 cm) are compact and covered with spines (Figure 25a). The receptacle contains 3–6 conceptacles. In cross section, the conceptacle (diameter = 546.7 ± 23.7 µm) has an oval shape and contains oogonia, antheridia and paraphyses (Figure 25b). The oogonia (length = 116.7 ± 26.8 µm; width = 57.4 ± 5.6 µm) are ovoid (Figure 25c) and located at the bottom of the conceptacle, far from the ostiole (Figure 25b). The antheridia (length = 26.6 ± 12.4 µm; width = 9.4 ± 1.6 µm) are branched and coloured for the presence of a pigmented stigma (Figure 25d). They are sited on the roof of the conceptacle together with the paraphyses, which cover even the walls (Figure 25b).

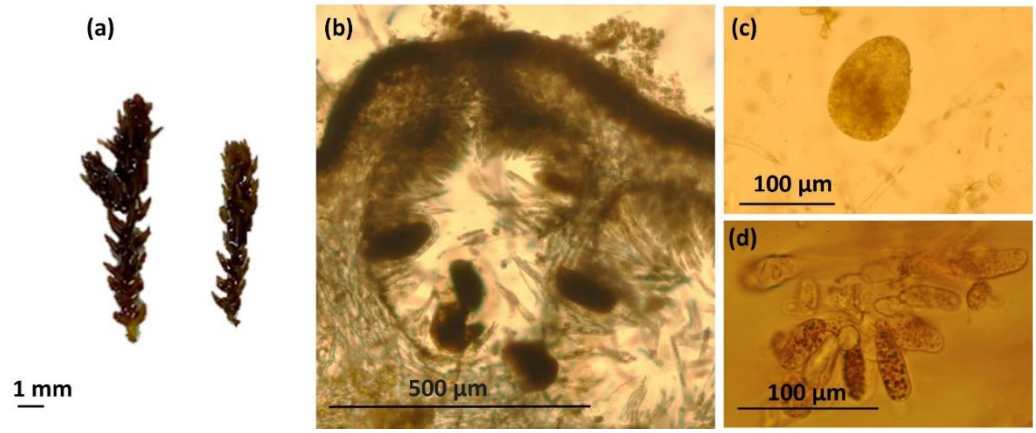


Figure 25: Reproductive traits of *E. mediterranea*: a) receptacles; b) transverse section through a conceptacle; c) ovoid oogonium; d) branched and coloured antheridia

Once the oosphere is fertilized, it assumes a spherical shape and adheres on the substrate through the fertilization membrane (Figure 26a). After about 24 hours, the first division appears at the equatorial level (Figure 26b). In one of the two daughter cells, it occurs a second division which is parallel to the first one and creates the rhizoidal pole. At the opposite pole, the third segmentation is formed perpendicularly to the previous divisions (Figure 26c). After about two days, the embryo assumes greater dimensions and the fertilization membrane starts to detach. At the same time, the rhizoidal cell divide forming four cells which will develop four primary rhizoids (Figure 26d). After about five days, in some embryos a small invagination is present at the apical pole, from which a hyaline hair grows. The

detachment of this hyaline hair after about a week represents the end of embryogenesis.

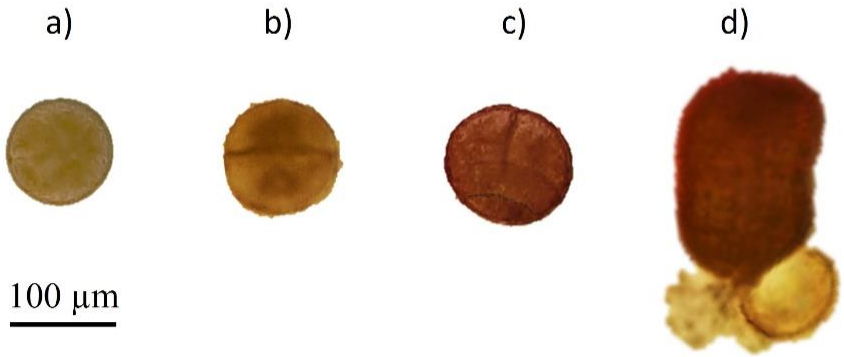


Figure 26: Embryogenesis of *E. mediterranea*: a) zygote; b) first division; c) third division; d) embryo showing the detachment of the fertilization membrane

4.3.2 Reproductive traits and embryology of Ericaria brachycarpa

This species shows a caespitose habit, with cylindrical erect cauloids, rough for the presence of basal stumps of the fallen branches. It is attached to the substrate by a discoid base formed by aptera. The apices are not prominent and are spinose during the vegetative activity, whereas they tend to get smoother during the resting period. Primary branches are cylindrical and can have smooth or slightly spinose bases, depending on the season. Secondary and tertiary branches are cylindrical and covered with spinose appendages. The receptacles are at the apices of terminal branchlets, and are spinose initially diffuse, and at maturity become closer and more compact. Prominent conceptacles are at the base of the spinose appendages.

E. brachycarpa lives in the upper infralittoral, from the surface to several meters' depth, along exposed rocky coasts (Figure 27). The maximum vegetative and reproductive development of this species is between spring and summer, while at the beginning of the autumn it starts to lose the frond, which is covered by other

algae such as *Ellisolandia elongata* (J. Ellis & Solander) K. R. Hind & G. W. Saunders.

Figure 27: *E. brachycarpa* community at Brucoli (photo: I. Pagana)

The receptacles (length = 0.6 ± 0.08 cm) often present a terminal spine (Figure 28a). Within each receptacle, there are overall 2-4 conceptacles (Figure 28b). In cross section, the conceptacles (diameter = 619.1 ± 80.3 µm) have an oval shape and contain oogonia, antheridia and paraphyses (Figure 28c). The separation layers between two conceptacles are normally 2-3. The oogonia (length = 159.6 ± 13.4 µm; width = 85.3 ± 9.1 µm) are oval, sessile and located on the bottom of the conceptacle. The antheridia (length = 30 ± 2.2 µm; width = 12.5 ± 1 µm), which contains a pigmented stigma, are branched and arranged on the walls of the conceptacle with the paraphyses.

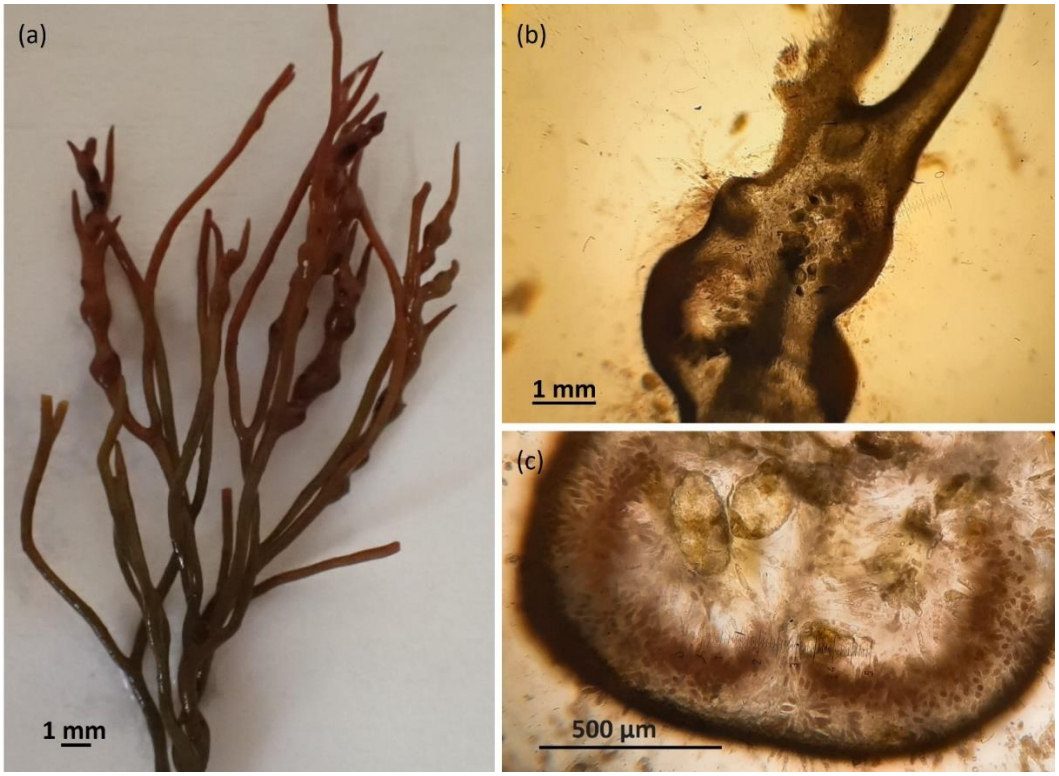


Figure 28: Reproductive traits of *E. brachycarpa*. a) receptacles; b) longitudinal section through a receptacle; c) transverse section through a conceptacle.

Once the fertilization occurred, the zygote (diameter = 44 ± 1.64 µm) adheres to the substrate through the fertilization membrane (Figure 29a). Soon, it begins to divide, and the first segmentation occurs at the equatorial level (Figure 29b). The second division, which is parallel to the first one, forms a rhizoidal pole (Figure 29c). The third segmentation appears at the opposite pole to the rhizoidal one and is perpendicular (Figure 29d). Subsequently, the rhizoidal pole splits into 4 primary rhizoids (Figure 29e). After few days, the embryo acquires greater size, develops in length and the number of its rhizoids grows. At the same time, the fertilization membrane breaks away and from the apical pole, a hyaline hair grows (Figure 29f). After a week this hair detaches, thus representing the end of embryo stage (Figure 29g). During the following weeks, the juveniles continue to grow and develop upwards (Figure 29h).

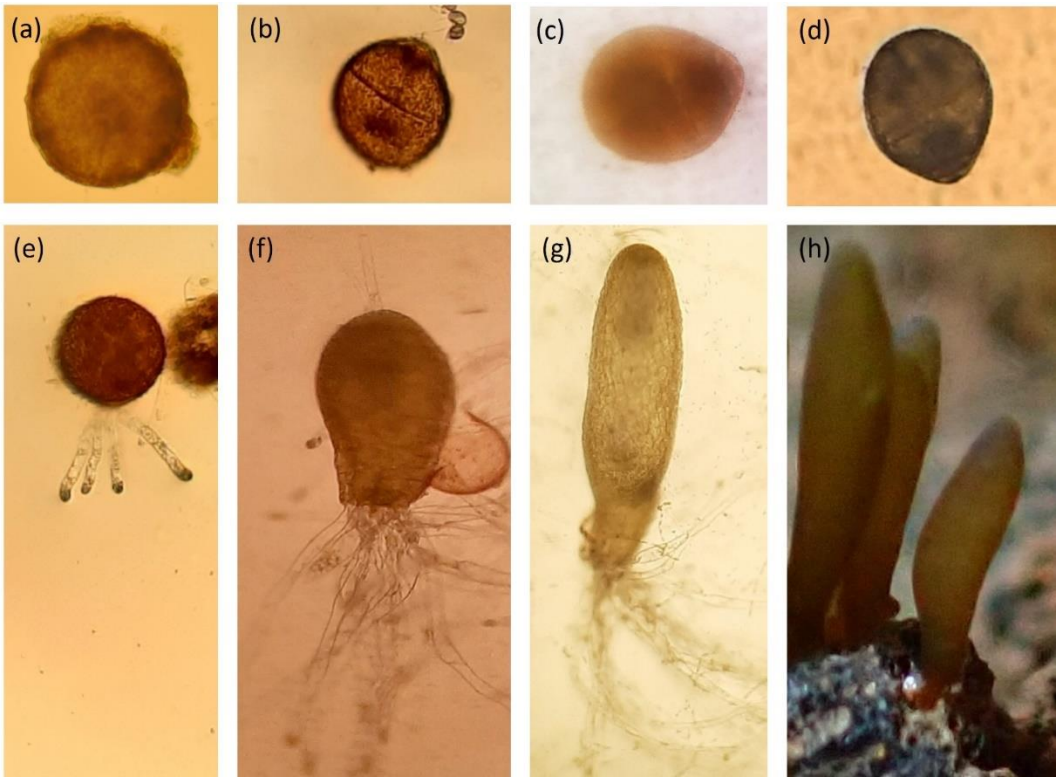


Figure 29: Embryogenesis of *E. brachycarpa*. a) the zygote (length= 44 ± 1.6 µm); b) first embryological stage (length= 57.6 ± 16.4 µm); c) second embryological stage (length= 99.6 ± 2.9 µm); d) third embryological stage (length= 99.6 ± 2.9 µm); e) embryo with 4 primary rhizoids (length= 368.8 ± 37.5 µm); f) embryo with the hyaline hair and the detachment of the fertilization membrane (length= 1017.3 ± 66.3 µm); g) stage after the fall of the hyaline hair, which represents the end of embryogenesis (length= 1417.3 ± 65.3 µm); h) juveniles developing upwards (length= 2158 ± 3.3 µm)

4.3.3 Reproductive traits and embryology of Ericaria giacconei

E. giacconei has a caespitose habit, with erect slightly rough cauloids and not prominent and smooth apices, which at the beginning of spring can be surrounded by the buds of primary branches. The latter are cylindrical and when bring the receptacles assume a cupressoid habit. The higher-order branches are cylindrical-conical. Receptacles are terminal and not very compact, with conceptacles at the base of spinose appendages, which tend to merge into apical spikes.

This species represents a dotted endemism (Giaccone & Di Martino, 1996) because it is currently distributed in two localities of the eastern coast of Sicily (see Chapter III) and at Kelibia bay, in Tunisia (Bouafif et al., 2016).

This species lives in the upper infralittoral along semi-exposed rocky coasts, where it is vicariant of *E. amentacea* (Figure 30). It does not show a true quiescence period

and its maximum vegetative and reproductive development occurs between winter and beginning of spring.

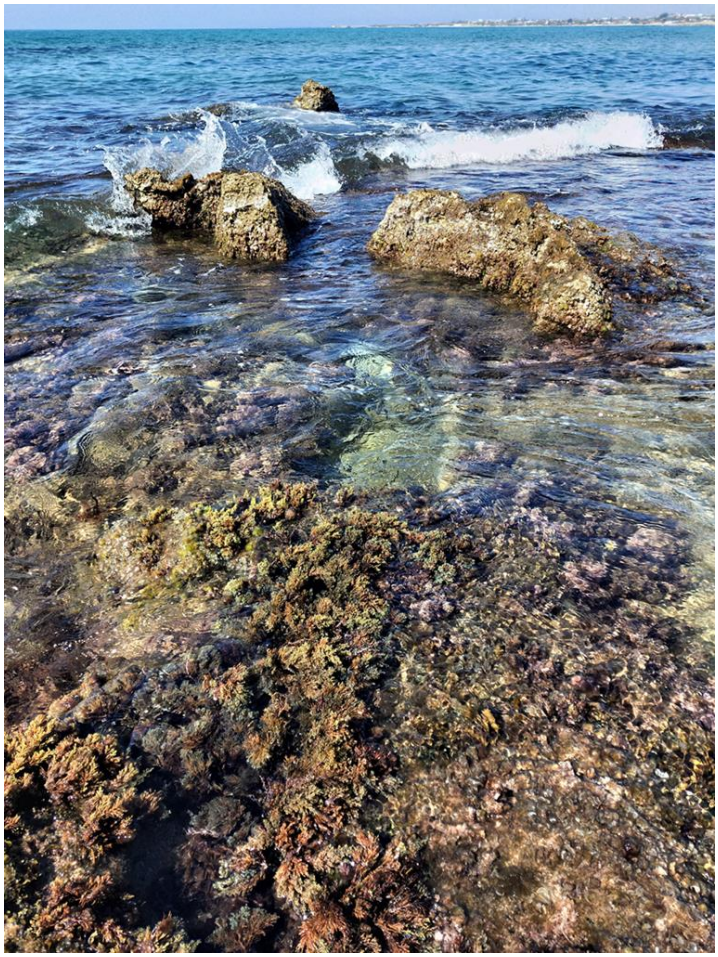


Figure 30: *E. giacconei* community at Portopalo di Capo Passero (photo: G. Savonitto)

During the reproductive period, the receptacles (length = 0.3 ± 0.03 cm) appear to be arranged in panicles and present few deciduous spinose appendages (2-3 for each receptacle) (Figure 31a). Each receptacle hosts from 5 to 9 conceptacles (Figure 31b). In cross section, the conceptacles (diameter = 554.8 ± 64.0 µm) have an oval shape and contain oogonia, antheridia and paraphyses (Figure 31c). The oogonia (length = 157.9 ±11.8 µm; width = 67.1 ± 3.8 µm) are oval (Figure 31d), sessile and located on the bottom of the conceptacle opposite to the ostiole. The antheridia (length =53.2 ± 4.2 µm; width = 16.7 ±2.7 µm) are branched and arranged on the walls of the conceptacle with the paraphyses. Antheridia contain antherozoids,

which are orange for the presence of carotenoids that colour their stigma (Figure 31e).

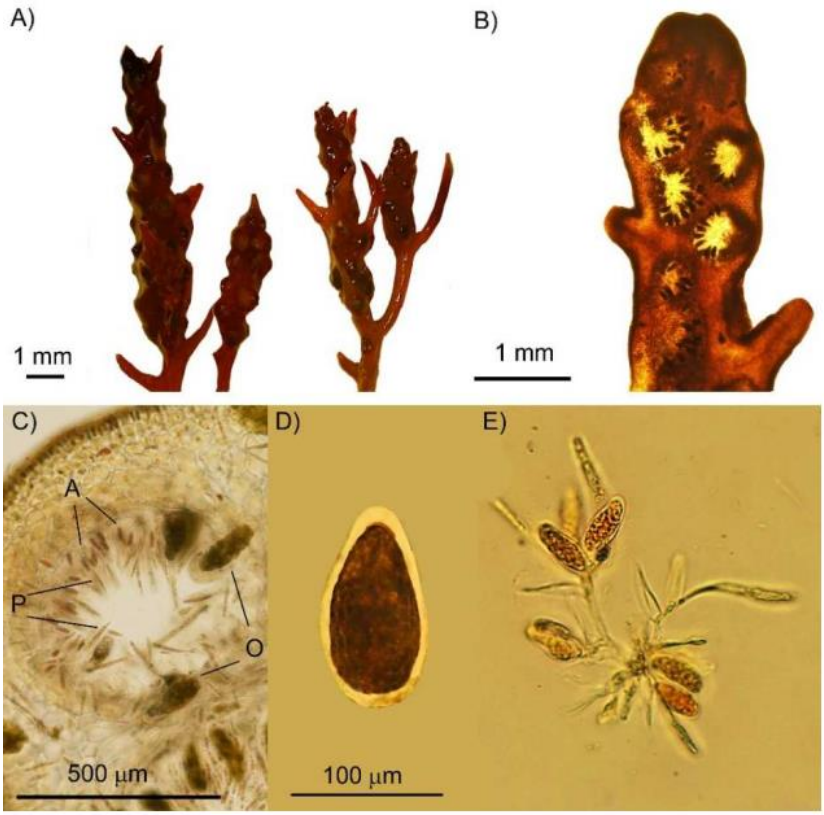


Figure 31: Reproductive traits of *E. giacconei*. a) receptacles; b) longitudinal section through a receptacle; c) transverse section through a conceptacle: black lines show the oval sessile oogonia (O) on the bottom of the conceptacle, antheridia (A) and paraphyses (P) on the walls of conceptacle; d) oogonium; e) branched antheridium with pigmented stigma

After the fertilisation, the zygote, which has a spherical shape and a diameter of ca. 95 ± 5.4 µm (Figure 32a), adheres to the substrate through the fertilization membrane. Subsequently, the zygote begins to divide, and the first segmentation phase appears at the equatorial level, forming two cells of equal size (Figure 32b). The second segmentation is parallel to the first one and isolates the rhizoidal pole, creating the rhizoidal cell (Figure 32c). The third division occurs at the opposite pole and is perpendicular to the previous segmentations. Successively, the embryo begins to actively divide, assuming a square shape, although its volume remains approximately constant (Figure 32d). Then, the rhizoidal cell divides, forming 4 new cells which through elongation, develop into 4 primary rhizoids (Figure 32e-h). After few days, the fertilization membrane begins to detach, the embryo acquires

an elongated shape, and the number of rhizoids grows (Figure 32i-j). From an invagination on the apical pole, a small hyaline hair appears, and the fertilization membrane is released (Figure 32j).

Figure 32: Embryogenesis of *E. giacconei*. a) the zygote; b) first division; c) second division; d) subsequent divisions; e-h) development of 4 primary rhizoids; i-j) gradual detachment of the fertilization membrane. The black lines show the invagination on the apical pole from which a hyaline hair will grow

4.3.4 Reproductive traits and embryology of Gongolaria montagnei *var.* tenuior

G. montagnei var. *tenuior* shows a non-caespitose habit, with a single erect cauloid originating from a discoid base, through which it is fixed to the substrate. The cauloid can be simple or branched, bringing branches up to the third-fourth order. The apex is spinose and not prominent. The primary branches are initially cylindrical and then flattened with spinose appendages. Secondary and higher order branches are cylindrical and spinose. This species presents oblong and spinose tophules, which are distributed especially near the apex. According to the ageing, they can be rough or smooth. The conceptacles are at the base of a few spinose appendages and are initially scattered but become grouped in receptacles, which are terminal and not very compact.

G. montagnei var. *tenuior* grows in sheltered and shallow waters, as lagoons or bays, between 2–5 m (Figure 33). Its maximum vegetative and reproductive development occurs between spring and summer. At the end of summer and autumn, it starts to lose the frond and only the main cauloid remains, then from the beginning of spring primary branches are produced from the tophules.

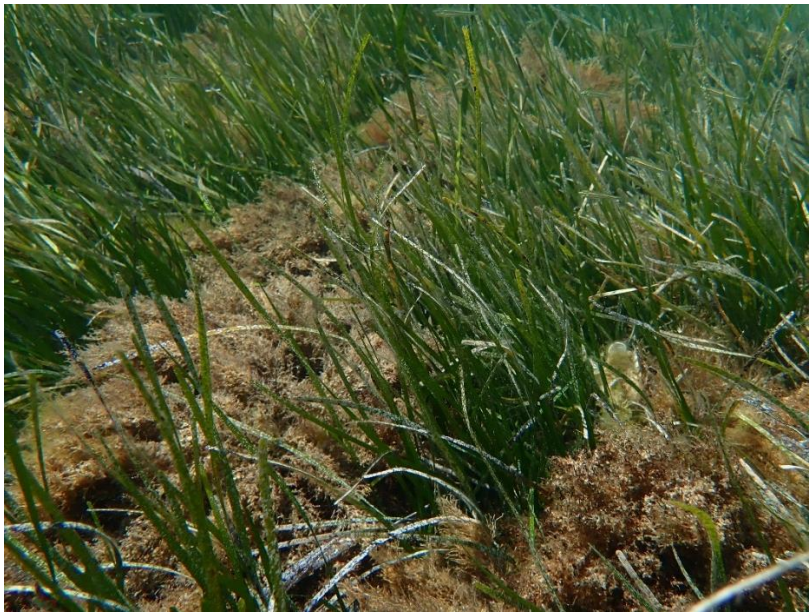


Figure 33: *G. montagnei* var. *tenuior* population among *Posidonia oceanica* (L.) Delile meadows at Brucoli (photo: G Marletta)

Within each receptacle (length = 0.7 ± 0.04 cm) (Figure 34a), there are overall 4-8 conceptacles (Figure 34b). In cross section, the conceptacles (diameter = 424 ± 7.3 µm) have an oval shape and contain oogonia, antheridia and paraphyses (Figure 34c). The separation layers between two conceptacles are normally 3. The oogonia (length = 129.2 ± 30 µm; width = 53.3 ± 6.1 µm) are oval and slightly elongated, sessile and located on the bottom of the conceptacle, together with the paraphyses (Figure 34d). The antheridia (length = 30.5 ± 10.2 µm; width = 13.4 ± 1.2 µm), which contains a pigmented stigma, are branched and arranged in a ring on the walls of the conceptacle (Figure 34d).

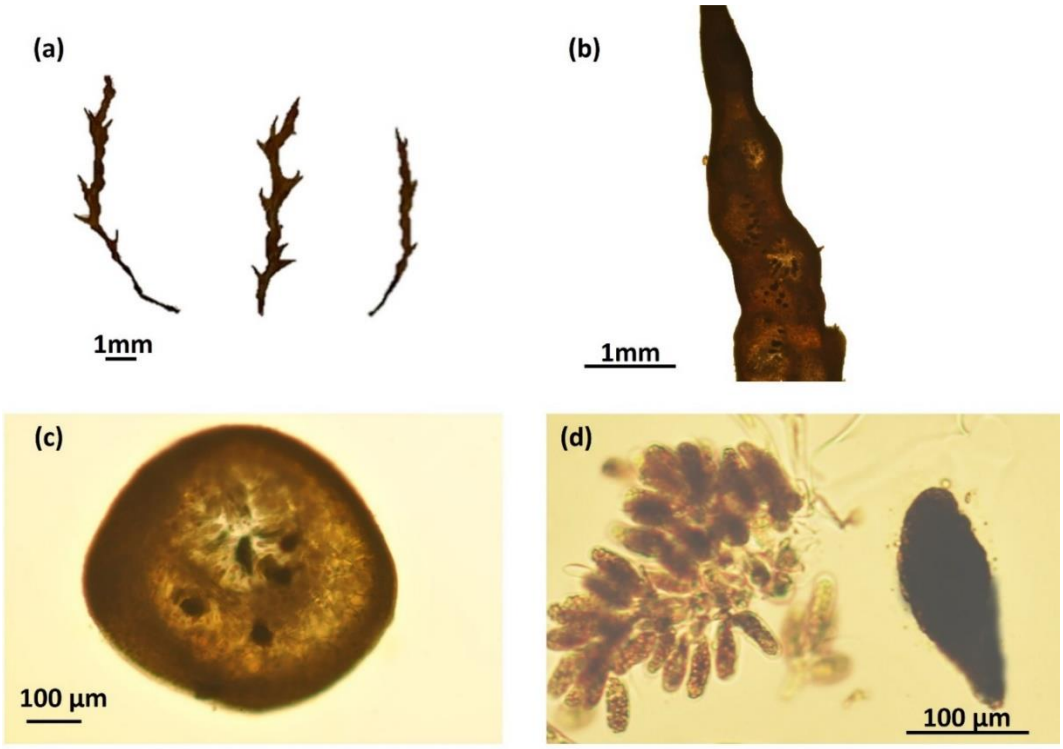


Figure 34: Reproductive traits of *G. montagnei* var. *tenuior*: a) receptacles; b) longitudinal section through a receptacle; c) transverse section through a conceptacle; d) numeros and branched antheridia and an ovoid oogonium.

After the fertilization, the zygote has a spherical shape and adheres to the substrate through the fertilization membrane (Figure 35a). The first segmentation appears at the equatorial level (Figure 35b). A second division occurs parallel to the first forming a rhizoidal cell, and a third division perpendicular to the other two appears

at the opposite pole (Figure 35c). Subsequently, the rhizoidal cell divides, forming four cells that develop four primary rhizoids by elongation (Figure 35d). From an invagination of the apical pole, a hyaline hair starts to appear, and the fertilization membrane is released. At the end of the first week, this hair is eliminated, and the embryo start to growth in height.

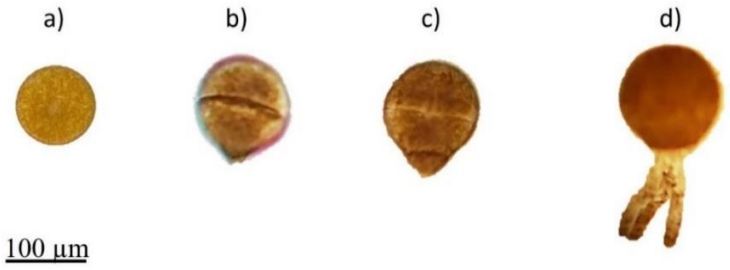


Figure 35: Embryogenesis of *G. montagnei* var. *tenuior*: a) the zygote; b) first division; c) second division with initial development of the third one; d) embryo with four primary rhizoids

4.4 Belonging embryological group of *E. mediterranea*, *E. brachycarpa*, *E. giacconei* and *G. montagnei* var. *tenuior*

The analysis of the first phases of zygote segmentation was essential to confirm the belonging of these species to the four embryological groups, and it was particularly interesting as concerns *E. giacconei*, whose embryology had not yet been studied in detail.

Through the observations of the reproductive traits and embryology of these four species, it was noted that they belong to the first embryological group described by Guern (1962), which currently includes most of *Cystoseira s.l.* species. Indeed, these species present the following features which characterise this group: oospheres which after fertilization become spherical and attach on the substrate, numerous and branched antheridia, containing antherozoids with a pigmented stigma, absence of basal hairs in the conceptacle, first and second segmentations

parallel and third division perpendicular to the previous ones, embryo development on the substrate and presence of 4 primary rhizoids.

The knowledge of the reproductive and embryological features of *Cystoseira s.l.* species could be an interesting tool to assist and improve their identification. Moreover, understanding the reproductive phenology, embryology and growth of *Cystoseira s.l.* species represents a crucial step to develop effective restoration protocols. Finally, it is an essential prerequisite to investigate the potential effects of climate change and warming impacts on the first developmental stages of these species, which could ultimately affect the long-term viability of *Cystoseira s.l.* populations.

Chapter V
Impacts of temperature on *Cystoseira s.l.* first embryological stages

5.1 The effects of climate change on the early stages of *E. mediterranea, E. brachycarpa, E. giacconei* and *G. montagnei* var. *tenuior*

In the Chapter IV, it was deepened the knowledge on the reproductive phenology and embryology of the four species, *E. mediterranea*, *E. brachycarpa*, *E. giacconei* and *G. montagnei* var. *tenuior*.

By understanding the growth and development of these species, at this stage of the research work we focused on the effects of climate change to understand how these can interact on the first developmental stages and so affect the future viability of *Cystoseira s.l.* populations. Indeed, it was demonstrated that the early life stages are generally more sensitive to warming than adults, which show a higher ability to grow and survive over broader temperature ranges and to physiologically compensate for thermal stress (Capdevila et al., 2018; Cáliz et al., 2019; Verdura et al., 2021). Therefore, the impacts on early developmental stages may compromise the ability of macroalgal forests to cope with further disturbances, with effects scaling up to the communities that they support (Capdevila et al., 2018). Anticipating the direction of change, with studies capable of determining the thermal response of *Cystoserira s.l.* first developmental stages, is crucial for the preservation of these habitat-forming organisms. In this context, the aim of this stage of the PhD thesis was to test the response to different temperatures on the early life stages of *E. mediterranea, E. brachycarpa, E. giacconei* and *G. montagnei* var. *tenuior*, to predict the potential future fate of these species under ocean warming and be able to identify their thermal optimums to improve the effectiveness of restoration protocols. The experimental trial on *E. giacconei* was conducted in collaboration with the Phycological Laboratory of the University of Trieste and some data here reported were object of the publication by Falace et al. (2021).

5.2 Materials and Methods

To conduct this research, fertile apices of *E. giacconei*, *E. brachycarpa*, *E. mediterranea* and *Gongolaria montagnei* var. *tenuior* were collected at Portopalo di Capo Passero and Brucoli, respectively. The first study species was *E. giacconei*, the only one among the investigated species which reproduces during winter (Giaccone, 1985). This trial was realised between February and March 2020, in partnership with the University of Trieste. Subsequently, due to the COVID-19 pandemic, the second trial was carried out in March 2021 on *E. brachycarpa*. Finally, the third trial on *G. montagnei* var. *tenuior* and *E. mediterranea* was performed in April 2022 through new climate-controlled rooms, in which it was possible to change the temperature between day and night, reproducing the daily temperature excursions.

Receptacles of *E. giacconei* were wrapped in aluminium foil, stored at 4 °C in the dark and transported to the Phycological Laboratory of the University of Trieste, within 24 h after collection. At the laboratory, the receptacles were stored at 4 °C for 36 h. For this species the following temperature treatments were selected: 12 °C, i.e. the lowest temperature the species can be exposed to in winter; 15 °C, i.e. the average daily seawater temperature during the reproductive period; 18 °C, i.e. the average daily temperature in early winter (December); 24 °C and 28 °C, i.e. temperatures the species is normally exposed to in summer. For each temperature, six petri dishes with about 200 receptacles and filtered seawater were prepared and incubated in controlled rooms with light intensity set to 125 μmol photons m−2 s−1 and photoperiod 12:12 h (light:dark) (Figure 36).

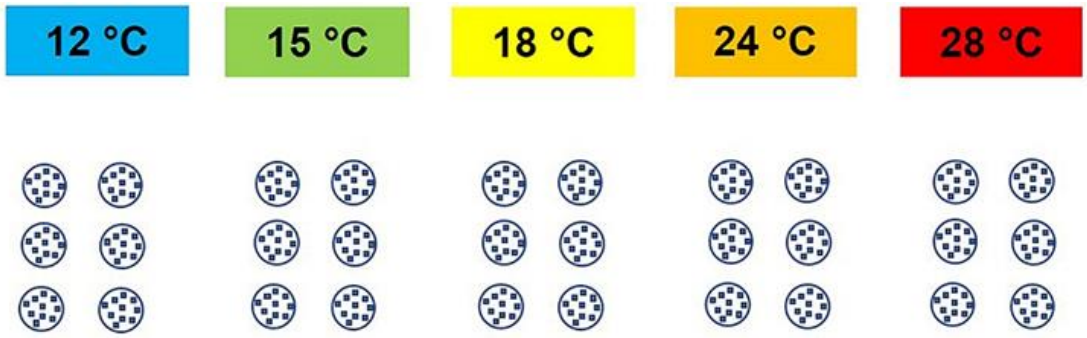


Figure 36: Temperature treatments on the first developmental stages of *E. giacconei*

Receptacles of *E. brachycarpa*, *G. montagnei* var. *tenuior* and *E. mediterranea* were wrapped in aluminium foil and stored for 24 h in dark conditions at a temperature of 6°C. The following day, the receptacles were washed with filtered sea water and the epiphytes were cleaned off with a brush.

The trial on the juveniles of *E. brachycarpa* consisted of three different temperature treatments (15°C, 17°C and 20°C). For each temperature, 3 petri dishes filled with 60 receptacles and filtered seawater were incubated in controlled rooms with light intensity set to 100 µmol photons m−2 s−1 and photoperiod of 12:12h (Figure 37).

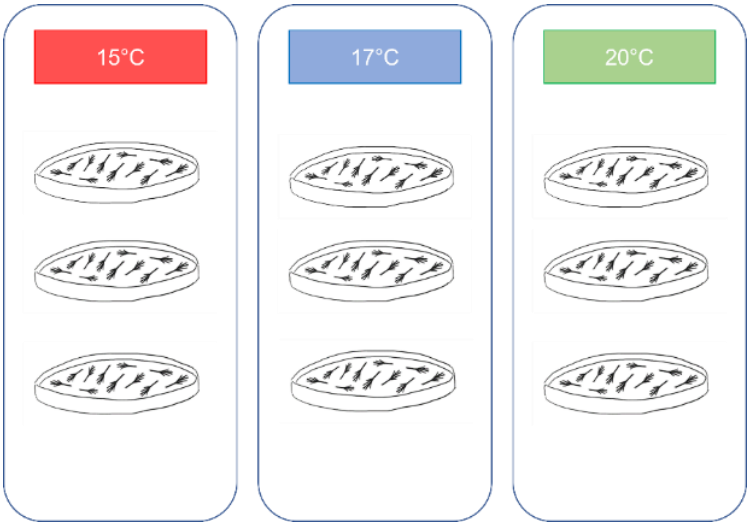


Figure 37: Temperature treatments on the first developmental stages of *E. brachycarpa*

For the trial on *G. montagnei* var. *tenuior* and *E. mediterranea*, in some of the treatments the temperature was maintained stable, while in others it was alternated between light and dark: 15°/10°C, 15°/15°C, 20°/15°C and 25°/25°C. The

experimental set up consisted of 2 petri dishes for each temperature treatment with about 100 receptacles placed on slides, incubated in the abovementioned rooms provided by three neon tubes with light intensity set to 120 µmol photons m−2 s−1 and photoperiod of 12:12h (Figure 38).

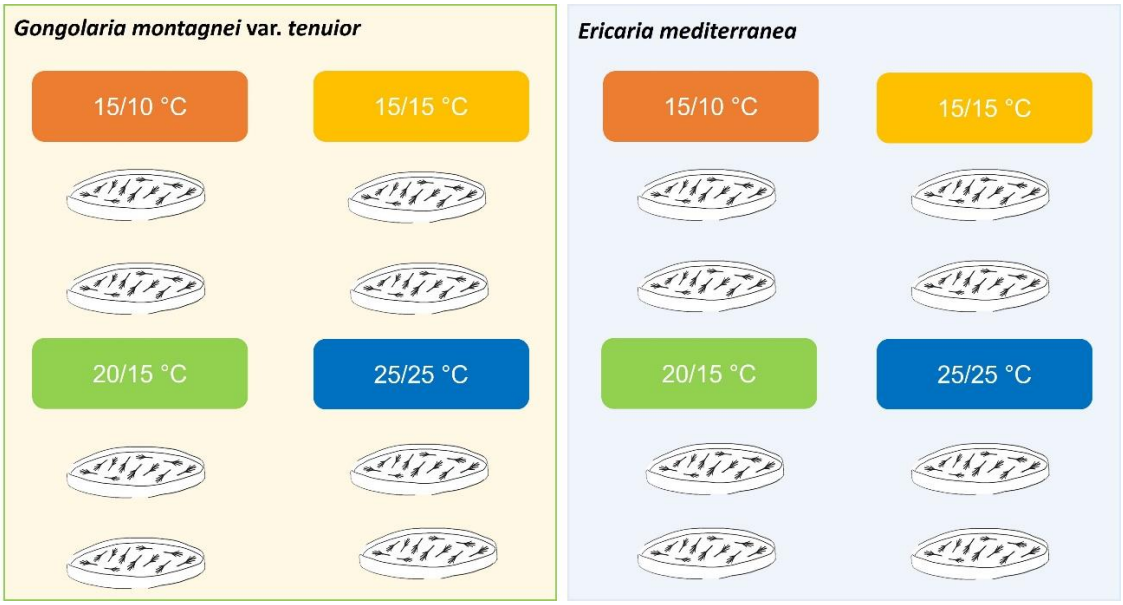


Figure 38: Temperature treatments on the first developmental stages of *G. montagnei* var. *tenuior* and *E. mediterranea*

The receptacles inside the petri dishes were removed 2 days after setting up, allowing the settlement of zygotes. The development of the early life stages up to the end of the embryonic stage (i.e., the fall of apical hair) was followed for 92 h (*E. giacconei*) and for 96 h (*E. brachycarpa*, *G. montagnei* var. *tenuior* and *E. mediterranea*).

For *E. giacconei*, we evaluated:

- reproductive potential (RP) = mean no. of conceptacle receptacle^{-1}/mean receptacle dry weight;
- release efficiency (RE) = no. of eggs cm^{-2}/mean receptacle dry weight cm^{-2};
- settlement efficiency (SE) = no of zygotes cm^{-2}/mean receptacle dry weight cm^{-2};

To calculate receptacle dry weight, receptacles were dried at 70 °C for 48 h. To quantify egg release and zygote settlement at different temperatures, 10 subareas of 0.2×0.2 cm2 in three Petri dishes were randomly selected per treatment and photographed under a stereomicroscope with a Nikon Coolpix 4500 camera. Embryo growth was assessed at 20, 44, and 92 h AF (after fertilization). In each subarea, the percentage of unfertilized eggs (= stage 0), zygotes (= stage 1), two-celled embryos (= stage 2), multicellular embryos (= stage several), multicellular embryos with rhizoids (= stage rhizoids), dead embryos (= stage dead), deformed dead embryos (= stage deformed dead), and deformed living embryos (= stage deformed living) were counted.

For the other species, we assessed:

- reproductive success = no. zygotes released per slide/no. receptacles per slide at T0 and T1;
- % survival rate = (no. juveniles per slide at T2 / no. juveniles per slide at T1) ×100;
  (no. juveniles per slide at T3 / no. juveniles per slide at T2) ×100;
- % mortality rate = no. dead juveniles /no. live juveniles at T1, T2, T3 ×100

To carry out the observations, a grid with squares of area 0.5×0.5 cm2 was placed on the microscope mechanical stage. The number of juveniles (zygotes and embryos) was monitored by randomly selecting one of the slides in each petri dish and counting them on each 0.5×0.5 cm2 surface area on the aforementioned grid. The various stages of zygote segmentation and embryo development were evaluated and photographed through a Nikon D40 at 24 (T0), 48 (T1), 72 (T2) and 96 (T3) h AF. In each subarea, the number of zygotes, first, second and third embryological stages, embryos with 4 primary rhizoids, elongated embryos with hyaline hair and dead embryos were counted.

For all species, the Analysis of Variance (ANOVA) was performed to test for differences between temperature treatments on the calculated parameters. The assumption of normality of response variables was tested with the Shapiro-Wilk test. Significant terms ($p < 0.05$) were examined by performing a post hoc test to

compare the different treatments. Moreover, to test for differences in temporal patterns of *E. giacconei* embryo developmental stages between treatments, it was used the distance-based permutational multivariable analysis of variance (PERMANOVA, Anderson, 2001). Data from treatments at 28 °C were not included in this analysis since the number of settled zygotes at 20 h AF was extremely low (mean 0.7 ± 0.1 zygotes/subarea), and zygote mortality at later sampling times was 100%.

5.3 Experimental trials

5.3.1 Ericaria giacconei

The reproductive potential did not differ significantly among thermal treatments, making them comparable at the beginning of the experiment (Figure 39A and Table 6). Even the release efficiency did not vary significantly among the tested temperatures (Figure 39B and Table 6). In contrast, the settlement efficiency of *E. giacconei* zygotes increased from 12 to 18 °C, but no statistically significant difference was found, then it started to decrease at 24 °C and dropped significantly at 28 °C (Figure 39C and Table 6). The extremely low settlement efficiency at 28 °C was due to the fact that eggs and zygotes had undergone cell lysis and clustered together (Figure 39C).

Table 6: Summary of ANOVAs on reproductive effort, release efficiency and settlement efficiency. The assumption of normality was checked through the Shapiro-Wilk test. Pairwise tests were also reported. NS = not significant; * = P < 0.05

		Reproductive effort			Release efficiency			Settlement efficiency		
	df	SS	MS	*F*	SS	MS	*F*	SS	MS	*F*
Treatment	4	0.3	0.1	2.15^{NS}	19893.0	4973.0	2.664^{NS}	27108.0	6777.0	3.997^{*}
Residual	10	12.5	1.2		18665.0	1867.0		17041.0	1704.0	
Pairwise *t*-test			-			-		28 °C≠12 °C=15 °C=18 °C=24 °C		

Shapiro-Wilk test	$W = 0.92^{NS}$	$W = 0.91^{NS}$	0.94^{NS}
Cochran's *C* test	$C = 0.71^{*}$	$C = 0.53^{NS}$	$C = 0.53^{NS}$
Transformation	Square root	None	None

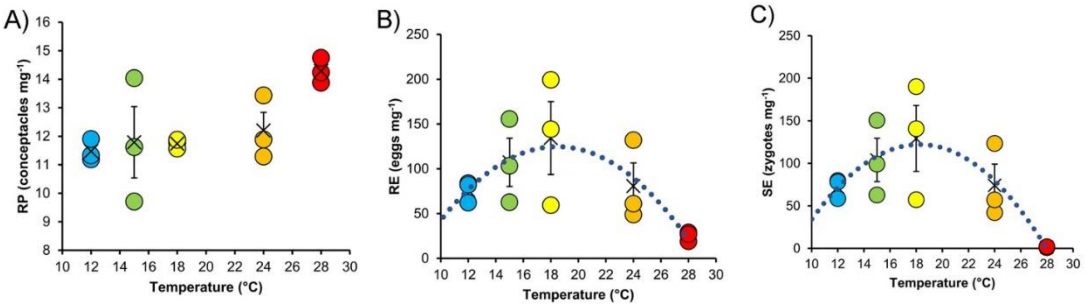


Figure 39: Mean values (±SE) of reproductive potential (A), release efficiency (B), and settlement efficiency (C) at the different temperatures

PERMANOVA on embryo status revealed a significant Tr × Ti interaction (Table 7), indicating that temporal patterns of embryonic development differed significantly between temperature treatments.

Table 7: PERMANOVA testing for differences in the proportion of different developmental stages of embryos at varying times and temperature treatments after fertilization.

Source	df	SS	MS	Pseudo-*F*	*P* (perm)
Time	2	19282.0	9641.0	23.97	0.00
Treatment	3	4104.0	1368.0	3.40	0.00
Time × Treatment	6	18611.0	3102.0	7.71	0.00
Residual	347	139550.0	402.2		

These differences were evident in the nMDS ordination of Tr × Ti centroids (Figure 40). The centroids of 12 and 15 °C clustered alongside those of 18 and 24 °C, the latter also showing marked separation between 20 h and 44-92 h AF. These differences were mainly due to the fact that at 20 h AF a higher percentage of eggs, zygotes or two-celled embryos were found in the treatments at 18 and 24 °C than

in those at 12 and 15 °C (Figure 40A). In contrast, multicellular embryos or rhizoids were found in the treatments at 12 and 15 °C in each time interval (Figure 40B), suggesting that the development rate was faster at lower temperatures. In addition, embryo mortality was consistently higher at 18 and 24 °C than at 12 and 15 °C, with the highest percentage of dead embryos recorded at 24 °C (Figure 40C).

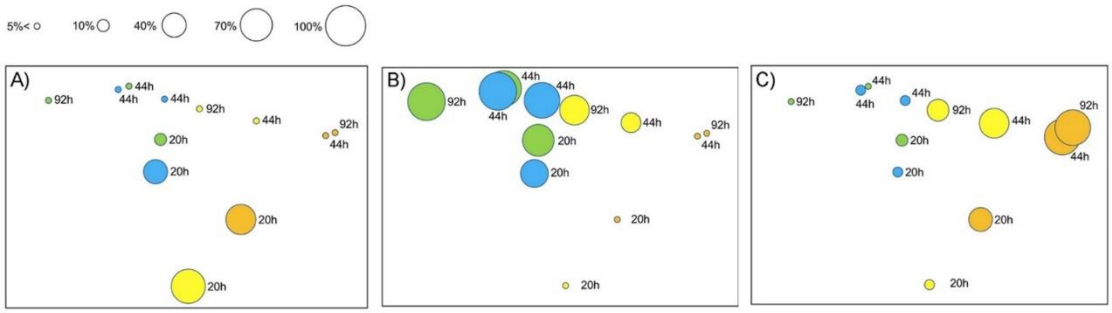


Figure 40: The ordination plot is presented in three versions highlighting three developmental stages, with superimposed bubbles, indicating the corresponding percentage of embryos in earlier (cumulative for stages 0, 1, and 2) (A) and later (cumulative for stages "several" and "rhizoid") (B) developmental stages, and dead (cumulative for stages "dead" and "deformed dead") embryos (C) for each time point (20, 44, and 92 h AF) and treatment (color-coded as in Figure 44 for 12, 15, 18, and 24 °C).

5.3.2 Ericaria brachycarpa

The reproductive success of *E. brachycarpa* was significantly different according to the temperature and the two crossed factors (temperature × time) (Table 8).

Table 8: ANOVA on reproductive success of *E. brachycarpa*. The assumption of normality was checked through Shapiro-Wilk test

	Sum of Squares	df	Mean Square	F	p
temperature	4.21	2	2.10	4.57	0.03
time	0.56	1	0.56	1.22	0.29
temperature ✻ time	6.78	2	3.39	7.37	0.008
Residuals	5.51	12	0.46		

Table 8: ANOVA on reproductive success of *E. brachycarpa*. The assumption of normality was checked through Shapiro-Wilk test

	Sum of Squares	df	Mean Square	F	p
Normality Test (Shapiro-Wilk)					
Statistic	**p**				
0.97	0.83				

In particular, Tukey's post hoc test highlighted that the most significant differences in the reproductive success were found between the temperature treatment at 17°C and at 20°C (Table 9).

Table 9: Tukey's Post Hoc test highlighting the most significant terms in the reproductive success of *E. brachycarpa* among temperature treatments

Comparison							
temperature		**temperature**	**Mean Difference**	**SE**	**df**	**t**	**p_{tukey}**
15	-	17	0.37	0.39	12.0	0.94	0.63
	-	20	-0.79	0.39	12.0	-2.02	0.15
17	-	20	-1.16	0.39	12.0	-2.96	0.03

At T0 the reproductive success was higher at 15°C than the other temperature treatments, while at T1 it was more elevated at 20°C (Figure 41).

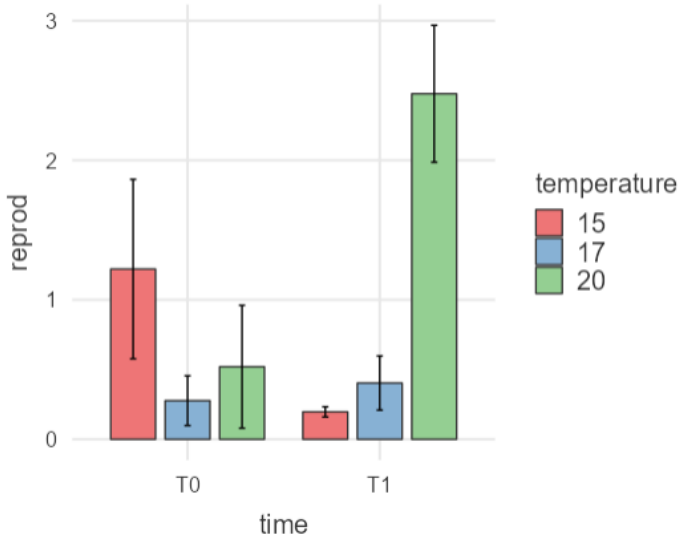


Figure 41: Reproductive success of *E. brachycarpa* at T0 (24h) and T1 (48h) for all temperature treatments

The survival rate of *E. brachycarpa* was significantly different according to the temperature treatments, but it did not show significant differences according to the time of the experiment and the two crossed factors (temperature × time) (Table 10).

Table 10: ANOVA on the survival rate of *E. brachycarpa*. The assumption of normality was checked through Shapiro-Wilk test.

	Sum of Squares	df	Mean Square	F	p
temperature	21.35	2	10.67	4.23	0.04
time	1.69	1	1.69	0.67	0.43
temperature ✻ time	17.49	2	8.75	3.47	0.07
Residuals	30.28	12	2.52		

Normality Test (Shapiro-Wilk)

Statistic	p
0.91	0.099

The post hoc test underlined that the most significant differences in the survival rate of *E. brachycarpa* were between the treatments at 17°C and 20°C (Table 11).

Table 11: Tukey's Post Hoc test highlighting the most significant terms in the survival rate of *E. brachycarpa* among temperature treatments

Comparison							
temperature		temperature	Mean Difference	SE	df	t	p_{tukey}
15	-	17	0.55	0.92	12.0	0.60	0.82
	-	20	-1.98	0.92	12.0	-2.16	0.12
17	-	20	-2.54	0.92	12.0	-2.77	0.04

At 20°C the survival rate of *E. brachycarpa* was higher at T2/T1 than at T3/T2. Conversely, at T2/T1 the survival rate at 15°C was lower and then increased at

T3/T2. At 17°C, the survival of *E. brachycarpa* juveniles did not remarkably change between T2/T1 and T3/T1. In general, the highest survival rate was observed at 20°C (Figure 42).

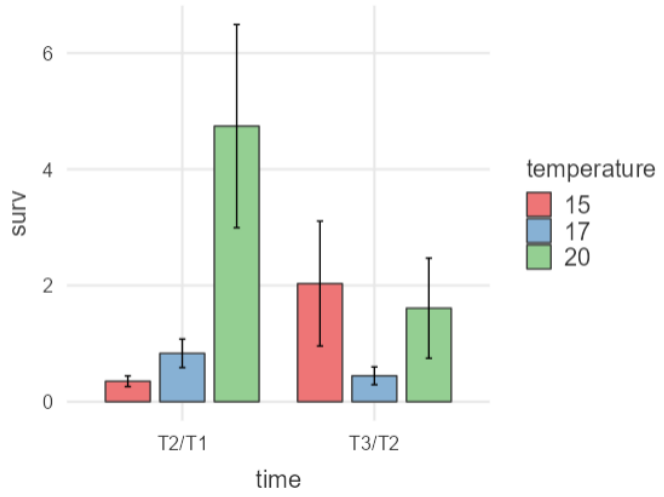


Figure 42: Survival rate of *E. brachycarpa* at T2/T1 (72h/48h) and T3/T2 (96h/72h) for all temperature treatments

The mortality rate of *E. brachycarpa* showed significant differences according to the temperature treatment, while no significant variations were observed according to the time and the two crossed factors (temperature × time) (Table 12).

Table 12: ANOVA on the mortality rate of E. *brachycarpa*. The assumption of normality was checked through Shapiro-Wilk test.

	Sum of Squares	df	Mean Square	F	p
temperature	4.89	2	2.44	4.19	0.03
time	0.03	2	0.02	0.03	0.97
temperature ✻ time	1.73	4	0.43	0.74	0.58
Residuals	10.49	18	0.58		

Normality Test (Shapiro-Wilk)

Statistic	p
0.93	0.08

Tukey's post hoc showed that the most significant differences were observed between the temperature treatments at 15°C and 17°C (Table 13).

Table 13: Tukey's Post Hoc test highlighting the most significant terms in the mortality rate of *E. brachycarpa* among temperature treatments

Comparison							
temperature		**temperature**	**Mean Difference**	**SE**	**df**	**t**	**p_{tukey}**
15	-	17	-0.95	0.36	18.0	-2.63	0.04
	-	20	-0.10	0.36	18.0	-0.28	0.96
17	-	20	0.85	0.36	18.0	2.36	0.07

During all the experiment, the highest mortality rate was observed at 17°C, without changing remarkably from T1 to T3. Instead for the other temperature treatments it was observed a variation in the mortality rate according to the time of the experiment. Indeed, at 15°C we observed a higher mortality at T1 than T2 and T3, while at 20°C this rate progressively increased from T1 to T3 (Figure 43).

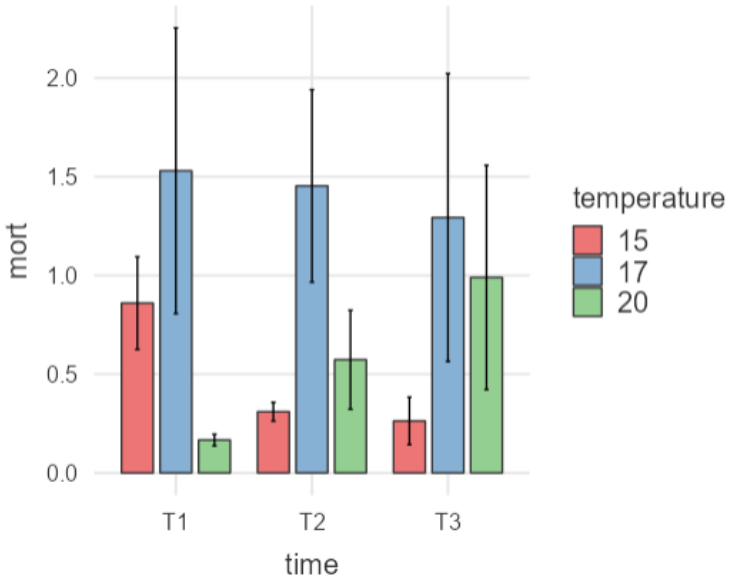


Figure 43: Mortality rate of *E. brachycarpa* at T1(48h), T2 (72h) to T3 (96h) for all temperature treatments

5.3.3 Gongolaria montagnei var. *tenuior* and *Ericaria mediterranea*

The reproductive success was similar among the two species. It was significantly different among the temperature treatments, but not significant as regards time (T0 and T1) and the two crossed factors (temperature × time) (Table 14).

Table 14: ANOVA on reproductive success of *G. montagnei* var. tenuior and *E. brachycarpa*. The assumption of normality was checked through Shapiro-Wilk test

	Sum of Squares	df	Mean Square	F	p
temperature	728.2	3	242.7	5.27	0.03
time	76.6	1	76.6	1.66	0.23
temperature ✻ time	69.7	3	23.2	0.50	0.69
Residuals	368.5	8	46.1		

Normality Test (Shapiro-Wilk)

Statistic	p
0.93	0.26

Tukey's post hoc test revealed that the most evident difference was between the temperature treatments at 15/15 °C and 20/15 °C (Table 15).

Table 15: Tukey's Post Hoc test highlighting the most significant terms in the reproductive success of *G. montagnei* var. *tenuior* and *E. brachycarpa* among temperature treatments

Comparison							
temperature		temperature	Mean Difference	SE	df	t	p_{tukey}
15/10	-	15/15	5.50	4.80	8.00	1.15	0.67
	-	20/15	-12.25	4.80	8.00	-2.55	0.13
	-	25/25	2.50	4.80	8.00	0.52	0.95
15/15	-	20/15	-17.75	4.80	8.00	-3.70	0.03
	-	25/25	-3.00	4.80	8.00	-0.63	0.92
20/15	-	25/25	14.75	4.80	8.00	3.07	0.06

The reproductive success was higher at 20/15°C than the other temperature treatments for both times (T0 and T1). At T0 the number of released zygotes was similar between 15/10 °C and 25/25 °C. The lowest value was observed at 15/15°C. At T1 a higher number of zygotes were released for all the temperature treatments. However, the reproductive success remained almost similar between the two temperature treatments of 15/10 °C and 25/25 °C, with the lowest value at 15/15 °C (Figure 44).

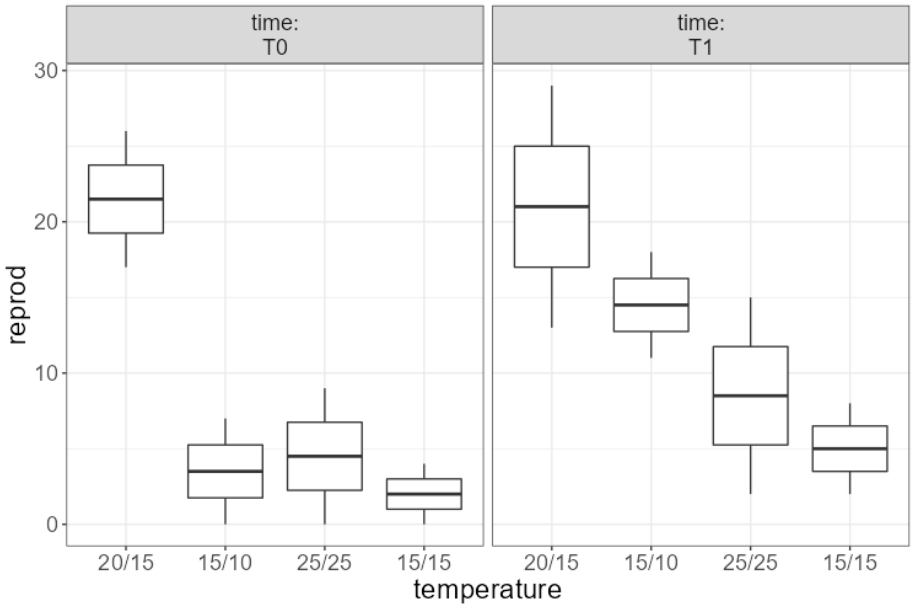


Figure 44: Reproductive success of the two species for all temperature treatments at time T0 (24h) and T1 (48h)

As regards the survival rate, it did not vary significantly among temperature treatments, but it was significantly different in relation to the two crossed factors time × species (Table 16).

Table 16: ANOVA on the survival rate of *G. montagnei* var. *tenuior* and *E. mediterranea*. The assumption of normality was checked through Shapiro-Wilk test

	Sum of Squares	df	Mean Square	F	p
time	0.31	1	0.31	0.27	0.61
species	1.17	1	1.17	1.01	0.33

Table 16: ANOVA on the survival rate of *G. montagnei* var. *tenuior* and *E. mediterranea*. The assumption of normality was checked through Shapiro-Wilk test

	Sum of Squares	df	Mean Square	F	p
time ✻ species	15.02	1	15.02	13.03	0.004
Residuals	13.83	12	1.15		

Normality Test (Shapiro-Wilk)

Statistic	p
0.89	0.06

Tukey's post hoc test showed that the most significative difference was found between the survival rate of *G. montagnei* var. *tenuior* and *E. mediterranea* at T3/T2 (Table 17).

Table 17: Tukey's post hoc test highlighting the most significant terms in the survival rate relative to time x species

Comparison					Mean Difference	SE	df	t	p_{tukey}
time	species		time	species					
T2/T1	*G. montagnei*	-	T2/T1	*E. mediterranea*	1.40	0.76	12.0	1.84	0.3
		-	T3/T2	*G. montagnei*	1.66	0.76	12.0	2.13	0.18
		-	T3/T2	*E. mediterranea*	-0.82	0.76	12.0	-1.08	0.71
	E. mediterranea	-	T3/T2	*G. montagnei*	0.26	0.76	12.0	0.34	0.97
		-	T3/T2	*E. mediterranea*	-2.22	0.76	12.0	-2.92	0.05
T3/T2	*G. montagnei*	-	T3/T2	*E. mediterranea*	-2.48	0.76	12.0	-3.26	0.03

In particular, it was observed an opposite trend in the survival rate between the two species. At T2/T1, *G. montagnei* var. *tenuior* showed a high survival rate at all temperature treatments, particularly at 25/25 °C. Instead at T2/T1 the survival rate

of *E. mediterranea* juvenile was lower. Conversely, at T3/T2 the survival rate of *G montagnei* var. *tenuior* recruits decreased in all temperature treatments, while the survival rate of *E. mediterranea* juveniles rose, with higher values at 15/15°C and 25/25°C (Figure 45).

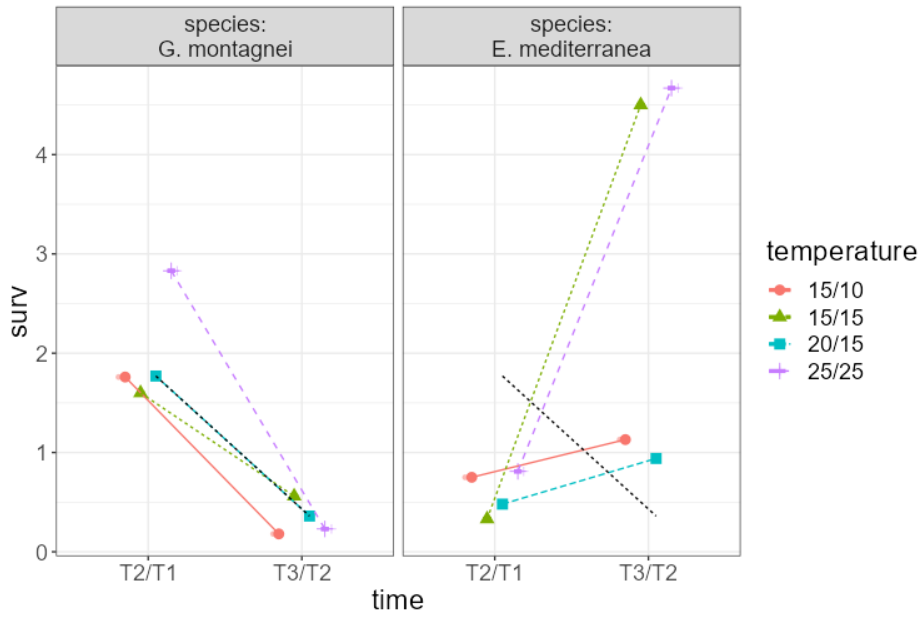


Figure 45: Survival rate of *G. montagnei* var. *tenuior* and *E. mediterranea* at T2/T1 (72h/48h) and T3/T2 (96h/72h) for each temperature treatment

The mortality rate changed significantly according to the species and the two crossed factors temperature × species (Table 18). No significant variations in the survival rate were observed according to the time of the experiment.

Table 18: ANOVA on the mortality rate of *G. montagnei* var. *tenuior* and *E. mediterranea*. The assumption of normality was checked through Shapiro-Wilk test.

	Sum of Squares	df	Mean Square	F	p
species	0.19	1	0.19	5.95	0.03
temperature	0.31	3	0.10	3.17	0.05

Table 18: ANOVA on the mortality rate of *G. montagnei* var. *tenuior* and *E. mediterranea*. The assumption of normality was checked through Shapiro-Wilk test.

	Sum of Squares	df	Mean Square	F	p
species ✻ temperature	0.68	3	0.23	6.89	0.003
Residuals	0.52	16	0.03		

Normality Test (Shapiro-Wilk)	
Statistic	**p**
0.96	0.42

The results of Tukey's post hoc test were listed below, and the most significant terms are marked with an asterisk (Table 19).

Table 19: Tukey's post hoc test highlighting the most significant terms in the mortality rate relative to temperature x species

species	temperature	species	temperature	Mean Difference	SE	df	t	ptukey
G. montagnei	15-10	*G. montagnei*	15/15	0.08	0.15	16.0	0.52	0.999
		G. montagnei	20/15	-0.08	0.15	16.0	-0.54	0.999
		G. montagnei	25/25	-0.64	0.15	16.0	-43.16	0.01*
		E. mediterranea	15-ott	-0.03	0.15	16.0	-0.18	1.00
		E. mediterranea	15/15	-0.04	0.15	16.0	-0.25	1.00
		E. mediterranea	20/15	0.04	0.15	16.0	0.25	1.00
		E. mediterranea	25/25	0.11	0.15	16.0	0.72	0.99
	15/15	*G. montagnei*	20/15	-0.16	0.15	16.0	-10.62	0.96
		G. montagnei	25/25	-0.71	0.15	16.0	-48.35	0.004*
		E. mediterranea	15-ott	-0.10	0.15	16.0	-0.7	0.996
		E. mediterranea	15/15	-0.11	0.15	16.0	-0.77	0.99
		E. mediterranea	20/15	-0.04	0.15	16.0	-0.27	1.00
		E. mediterranea	25/25	0.03	0.15	16.0	0.2	1.00
	20/15	*G. montagnei*	25/25	-0.55	0.15	16.0	-37.73	0.03*
		E. mediterranea	15-ott	0.05	0.15	16.0	0.36	1.00

		E. mediterranea	15/15	0.04	0.15	16.0	0.29	1.00
		E. mediterranea	20/15	0.12	0.15	16.0	0.79	0.99
		E. mediterranea	25/25	0.19	0.15	16.0	12.65	0.99
	25/25	*E. mediterranea*	15-10	0.61	0.15	16.0	41.35	0.01*
		E. mediterranea	15/15	0.6	0.15	16.0	40.67	0.02*
		E. mediterranea	20/15	0.67	0.15	16.0	45.64	0.006*
		E. mediterranea	25/25	0.74	0.15	16.0	50.39	0.002*
E. mediterranea	15/10	*E. mediterranea*	15/15	-0.01	0.15	16.0	-0.07	1.00
		E. mediterranea	20/15	0.06	0.15	16.0	0.43	1.00
		E. mediterranea	25/25	0.13	0.15	16.0	0.91	0.98
	15/15	*E. mediterranea*	20/15	0.07	0.15	16.0	0.5	1.00
		E. mediterranea	25/25	0.14	0.15	16.0	0.97	0.97
	20/15	*E. mediterranea*	25/25	0.07	0.15	16.0	0.48	1.00

Juveniles of *G. montagnei* var. *tenuior* showed the highest mortality rate at 25/25°C particularly at T3, while for the other temperature treatments this rate maintained almost low throughout the experiment. Instead, the mortality rate of *E. mediterranea* juveniles at T1 was higher at 15/15 °C than the other temperature treatments. Subsequently, this rate decreased from T2 to T3 for all temperature treatments (Figure 46).

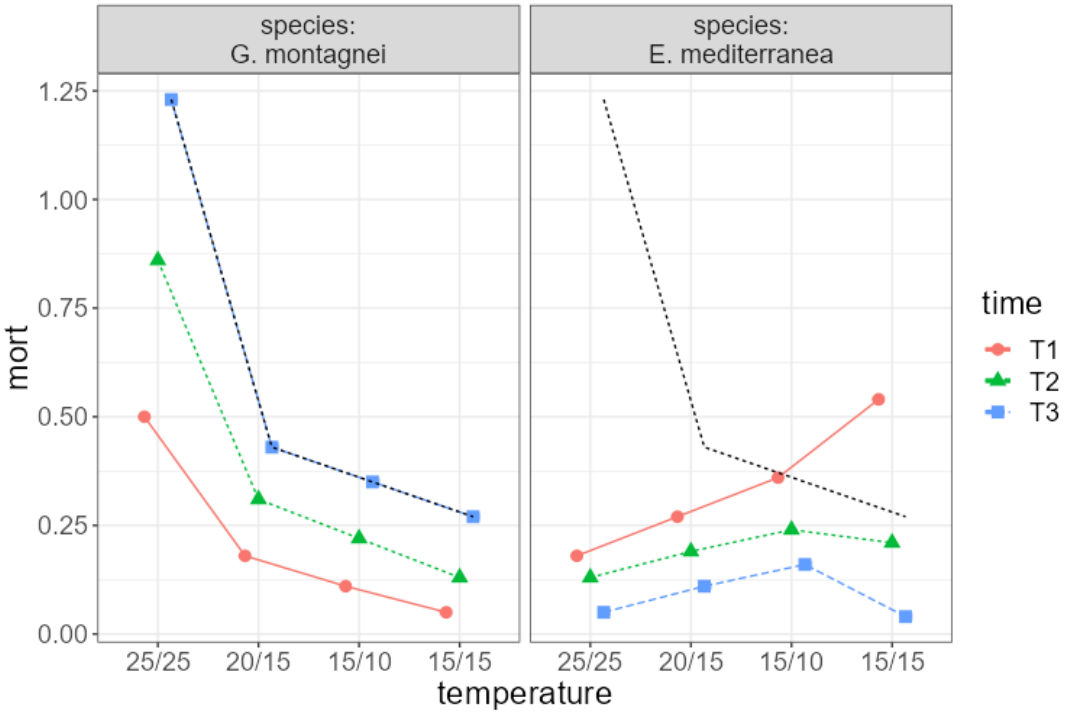


Figure 46: Mortality rate of the two species at T1 (48h), T2 (72h) and T3 (96h), according to the different temperature treatment

5.4 Discussion and conclusions

To date, very few studies have investigated the potential effects of warming on *Cystoseira s.l.* first embryological stages and juveniles' growth (e.g., Capdevila et al., 2019; Cáliz et al., 2019; Verdura et al., 2021). These studies focused specifically on the effects of high temperatures on the settlement and survival of recruits, showing that higher temperatures lead to embryo death. A tolerance threshold of 24

°C was found in *Ericaria zosteroides*, a deep-sea species (Capdevila et al., 2019), and 28 °C in *E. selaginoides* (Cáliz et al., 2019) and *E. crinita* (Verdura et al., 2021), two species from shallower waters.

In our study, we found that the release efficiency of *Ericaria giacconei* did not vary significantly among the tested temperatures, but the settlement efficiency of the zygotes increased from 12 to 18 °C, starting to decrease at 24°C and furtherly at 28°C. Conversely, the reproductive success of the other species varied significantly according to the temperature treatment, being higher at 20°C for *E. brachycarpa* and at 20/15 °C for both *G. montagnei* var. *tenuior* and *E. mediterranea*, probably because these species reproduce during spring (Gómez-Garreta et al., 2001, Cormaci et al., 2012) when the seawater temperature is around 20°C. Indeed, for *G. montagnei* var. *tenuior* and *E. mediterranea*, higher temperatures (as 25/25 °C) resulted in low reproductive success.

The detrimental effect of heat was more pronounced during *E. giacconei* germlings' development. Indeed, embryos were able to fully develop only at 12 and 15 °C, while mortality increased sharply at 18 °C and all germlings died at 28 °C. The highest development rate was observed at 15 °C, which corresponds to the mean seawater temperature during the winter months when the species reproduces. This suggests that this temperature represents the thermal optimum for reproduction and development of *E. giacconei* early life stages.

The survival rate of *E. brachycarpa* and *G. montagnei* v. *tenuior* juveniles was higher in the initial steps of the experiment and then decreased at the end of it. On the contrary, the survival rate of *E. mediterranea* juveniles was lower in the initial phases of the experiment and then subsequently increased. As regards the mortality rate, for *E. brachycarpa* higher values were observed at the end of the experiment at 17°C and 20°C. Also, for *G. montagnei* var. *tenuior,* this rate increased considerably at the end of the experiment and particularly at 25/25°C. Instead, the mortality rate of *E. mediterranea* was higher in the initial steps of the experiment, even though this rate was always lower than that observed in the other two species during the entire experiment. This suggests that high temperatures might trigger greater survival in the early developmental stages of *E. brachycarpa* and *G. montagnei* var. *tenuior*, but over time, these temperatures can have a detrimental

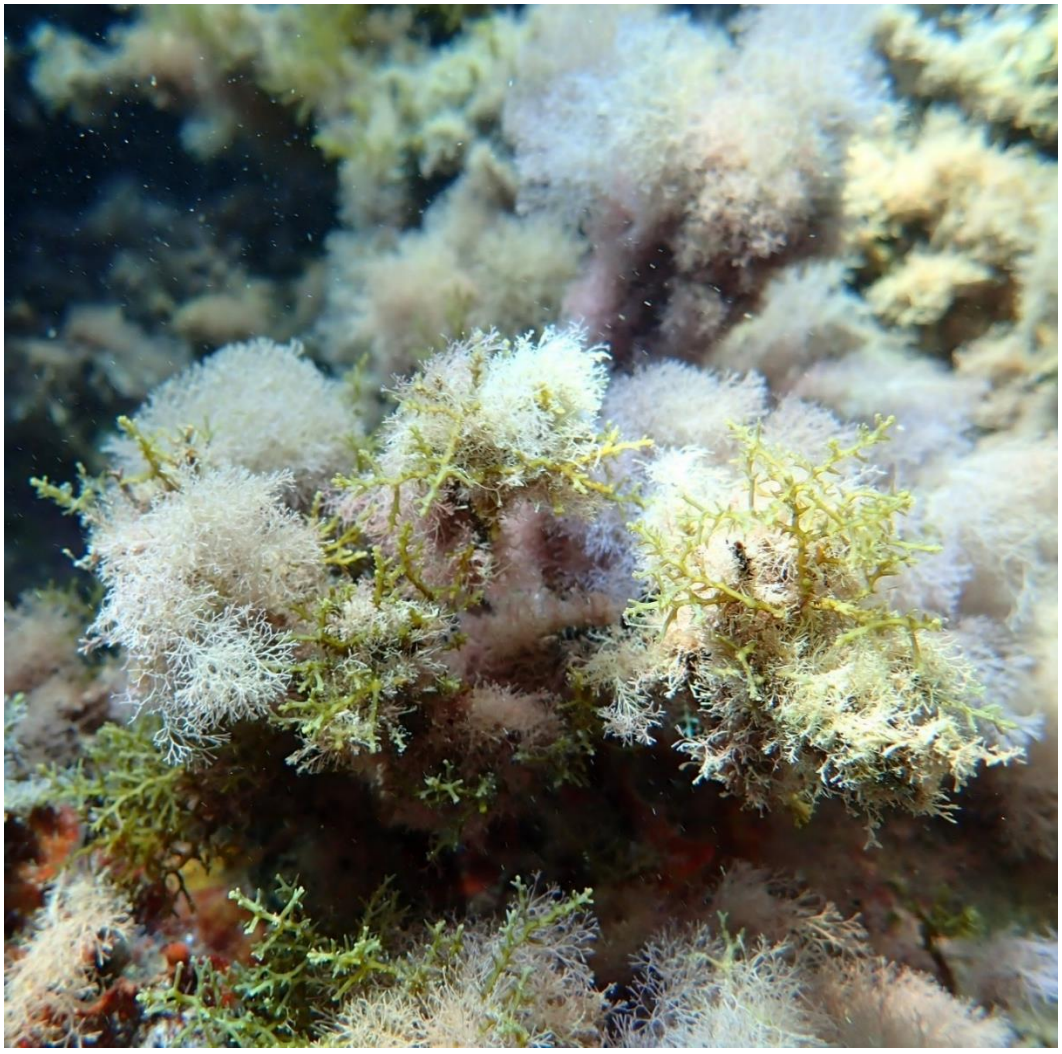
effect on the juveniles of these species. On the other hand, *E. mediterranea* recruits would appear to be more able to deal and withstand high temperatures for long periods than *E. brachycarpa* and *G. montagnei* var. *tenuior* juveniles. This could depend on the fact that *E. mediterranea* is a species of the upper horizon of infralittoral, which differently to the species of the middle horizon, is usually subjected to variations in salinity, hydrodynamics and temperature (Cormaci et al., 2003). Thus, the juveniles of this species may also be better adapted to cope with different environmental stresses than juveniles of other infralittoral species.

In conclusion, it was found that the thermal optimum of *E. giacconei* juveniles is lower (12-15°C) and narrower compared to that found in the juveniles of the other three species. This is because this species reproduces in winter and has a cold affinity, occurring only in the colder waters of northern Tunisia (Bouafif et al., 2016) and in the Sicilian Channel, where there is semi-permanent upwelling regime which provides lower sea surface temperatures (Falace et al, 2021).

The juveniles *E. brachycarpa* showed high reproductive success and survival at both 15°C and 20°C, while the highest mortality was observed at 17°C. The recruits of *E. mediterranea* and *G. montagnei* var. *tenuior* had an elevated reproductive success at the temperature treatment of 20/15°C. Moreover, the juveniles of *G. montagnei* var. *tenuior* revealed a high survival rate and low mortality at the temperature treatment of 15/15°C. Instead, for *E. mediterranea* juveniles the lowest mortality and the highest survival was registered at 25/25°C.

The knowledge of the thermal optimums of these species could be useful to develop species-specific protocols, which might ultimately favour the effectiveness of restoration actions. Furthermore, in light of the undergoing climate changes, these types of studies represent useful tools to predict and understand the future fate of *Cystoseira s.l.* species.

Chapter VI
Experimental restoration plot of
Ericaria brachycarpa



6.1 Experimental restoration plot of *E. brachycarpa* in the M.P.A. Isole Ciclopi

Among the species analysed in the previous chapters, it was selected *E. brachycarpa* to realize an experimental *ex-situ* restoration plot in the M.P.A. Isole Ciclopi, area where a population of this species extended from 2 to 5 m of depth until the late 1990s (Pizzuto, 1999). Today, as seen in the Chapter III, many populations of this species have disappeared from some areas where they were previously documented. Catra et al. (2019) reported a severe loss of *E. brachycarpa* populations in the M.P.A. Isole Ciclopi and in Santa Maria La Scala, both sites where this species was present in 1994 locally reaching a 100% coverage at a depth of 3 m (Pizzuto, 1999). The main factors that could have contributed to this decline are the high increase of water turbidity (and sediment deposition) (Costanzo et al., 2021) and the overgrazing by the sea-urchins *Paracentrotus lividus* and *Arbacia lixula* (Catra et al., 2019). Indeed, both these two species have high density population in these areas since 2000 (Cantone & Beninato, 2004) and have increased in the last decade (Catra et al., 2019). Consequently, to halt this decline and enhance the natural recovery of *E. brachycarpa* populations and their associated biodiversity, there is an urgent need of upscaling the restoration efforts. Therefore, the aims of this stage were: to identify a species-specific protocol for the *ex-situ* cultivation and outplanting of *E. brachycarpa*, in order to implement an effective experimental restoration plot; to understand the strengths and weaknesses of this intervention with the intent to realize future sustainable and large-scale restoration actions.

6.2 *Ex-situ* cultivation and outplanting

Fertile apices of *E. brachycarpa* were collected in April 2022 from the donor site of Brucoli, where a healthy population of this species is present in high hydrodynamic and shallow waters (about -50 cm), just below the infralittoral fringe.

The receptacles were wrapped in aluminium foil and were stored for 24h in dark conditions and at a temperature of 6°C. The following day, the receptacles were washed with filtered seawater and the epiphytes were cleaned off with a brush. Then, ca. 240 fertile apices were placed on 24 Etna's volcanic rock tiles placed into two aquaria filled with filtered seawater (Figure 47). This type of substrate was chosen to reproduce the basaltic substrate present at the restoration site of the M.P.A. Isole Ciclopi, where the species was historically present.

The remaining 40 receptacles were positioned on glass slides within two petri dishes containing filtered seawater (Figure 47). These latter were used to observe and photograph zygote and embryo development with an optical microscope, without stressing the juveniles in the aquaria. Both aquaria and petri dishes were held inside a climate-controlled room at 20/15°C, with light intensity set to 120 µmol photons m−2 s−1 and photoperiod of 12:12 hours light:dark, following the protocol by Falace et al. (2018) modified according to the species requirements. Indeed, these conditions were selected because in the experiment of the effects of temperatures on the early developmental stages (see Chapter V), promoted high reproductive success and survival rates in the juveniles of *E. brachycarpa*.

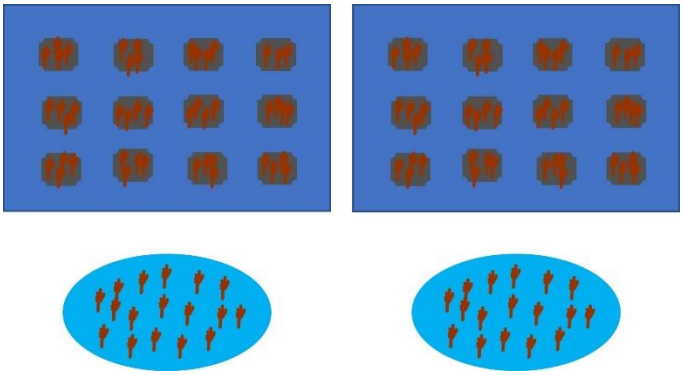


Figure 47: Experimental set up for *ex-situ* cultivation of *E. brachycarpa*

After, 2 days the receptacles were removed from both aquaria and petri dishes. During the laboratory cultivation, the selected culture medium was Von Stosch (1964), with addition of germanium dioxide and potassium tellurium solutions, to accelerate the growth of *E. brachycarpa* juveniles and prevent diatom and bacteria proliferation.

The aquaria were filled with 2 l of filtered seawater and 2 l of this culture medium, renewed every 2 days to minimize any possible effect of nutrient limitation, and were aerated by air pumps.

To monitor juveniles' development, a grid with squares of area 0.5×0.5 cm2 was placed on the microscope mechanical stage. The number of juveniles (zygotes and embryos) was monitored by randomly selecting one of the slides in each petri dish and counting them on each 0.5×0.5 cm2 surface area on the aforementioned grid. The various stages of zygote segmentation and embryo development were evaluated and photographed through a Nikon D40 at 24 h (T0), 48 h (T1), 1 week (T2), 2 weeks (T3), 3 weeks (T4) and 4 weeks (T5) after fertilisation.

During the laboratory culture, the following data were collected and processed:

- reproductive success = no. zygotes released per slide/no. receptacles per slide at T0 and T1;
- % survival rate= (no. juveniles per slide at T2 / no. juveniles per slide at T1) × 100;
  (no. juveniles per slide at T3 / no. juveniles per slide at T2) × 100;
  (no. juveniles per slide at T4 / no. juveniles per slide at T3) × 100;
  (no. juveniles per slide at T5 / no. juveniles per slide at T4) × 100;
- % mortality rate = no. dead juveniles /no. live juveniles from T1 to T5 × 100;
- average length of juveniles from T1 to T5, obtained by processing photographs with ImageJ software;
- juveniles' density from T1 to T5, calculated as the number of zygotes and embryos/on a surface 0.5 cm×0.5 cm2.

One month after the laboratory cultivation, the 24 rock tiles were brought to the restoration site. The tiles, previously protected by purpose-built plastic cages, were fixed to the sea bottom at ca -50 cm of depth through the epoxy putty BCR400V-Plus. Photographic monitoring of the tiles in the field was performed for one month.

The average length (mm) of juveniles at the first, second, third and fourth week (week 1, 2, 3 and 4) after outplanting was measured by processing the photographs with ImageJ. The cages were cleaned from epiphytes monthly in order to prevent shading.

One-way ANOVA was performed to check for differences in the abovementioned parameters during the times of the *ex-situ* cultivation (from T0 to T5) and outplanting (from week 1 to week 4). The assumption of normality was verified through Shapiro-Wilk test. Significant terms ($p < 0.05$) were subsequently examined by performing Tukey's pairwise post hoc test. Moreover, Pearson's linear correlation coefficient was used to the test the relation between juveniles' density and their average length during time. Statistical analyses were performed using jamovi 2.3 software (jamovi project, 2022).

6.3 Results of laboratory culture and outplanting

The One-ANOVA analysis did not show a significant difference in the reproductive success between the two times (T0 and T1) (Table 20 and Figure 48), being 38.33 ± 2.45 at T0, and 34.75 ± 6.23 at T1.

Table 20: One-way ANOVA on the reproductive success

Shapiro-Wilk test			**W**	**p**
			0.98	0.97
One-way ANOVA	**F**	**df1**	**df2**	**p**
	1.32	1	3.98	0.32

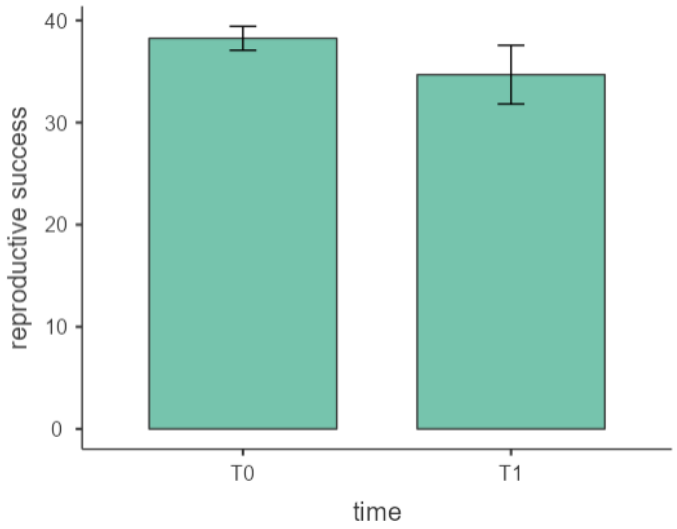


Figure 48: Bar plots of the reproductive success at time T0 (24h) and T1 (48h)

On the contrary, the survival rate varied significantly among times (T2/T1; T3/T2; T4/T3; T5/T4) (Tables 21 and 22). The highest value was observed at T3/T2 with 1.61% of survived juveniles, while the lowest value was found at T2/T1 with only 0.72% of survived juveniles (Figure 49).

Table 21: One-way ANOVA on the survival rate

Shapiro-Wilk test			**W**	**p**
			0.94	0.52
One-way ANOVA	**F**	**df1**	**df2**	**p**
	8.42	3	4.15	0.03

Table 22: Tukey's post hoc test showing differences in the survival rate among times

		T2/T1	T3/T2	T4/T3	T5/T4
T2/T1	Mean difference	—	-0.89	-0.29	0.003
	p-value	—	0.004	0.38	1.00
T3/T2	Mean difference		—	0.6	0.9
	p-value		—	0.03	0.004
T4/T3	Mean difference			—	0.3
	p-value			—	0.37
T5/T4	Mean difference				—
	p-value				—

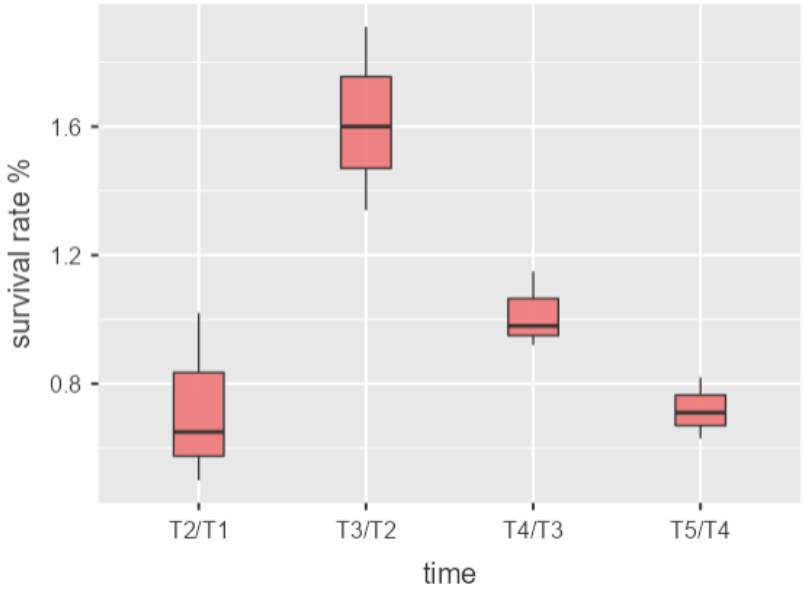


Figure 49: Box plot depicting the survival rate at T2/T1 (1 week/48 h), T3/T2 (2 week/1 week), T4/T3 (3 week/2 week) and T5/T4 (4 week/3 week)

The mortality rate changed significantly according to the time after fertilisation (T1, T2, T3, T4 and T5) (Table 23 and 24). The highest mortality was observed at T1, with the 11.13% of dead juveniles. Then, this rate decreased with 5.86% of dead juveniles at T3 and a new rise was observed at the final stages of the experiment with 7.04% and 9.64% of dead juveniles at T4 and T5, respectively (Figure 50).

Table 23: One-way ANOVA on the mortality rate

Shapiro-Wilk test			**W**	**p**
			0.96	0.67
One-way ANOVA	**F**	**df1**	**df2**	**p**
	8.11	4	4.93	0.021

Table 24: Tukey's post hoc test showing differences in the mortality rate among times

		T1	T2	T3	T4	T5
T1	Mean difference	—	2.65	5.27	4.09	1.50
	p-value	—	0.08	0.001	0.007	0.49
T2	Mean difference		—	2.62	1.44	-1.16
	p-value		—	0.09	0.52	0.7
T3	Mean difference			—	-1.18	-3.77

Table 24: Tukey's post hoc test showing differences in the mortality rate among times

		T1	T2	T3	T4	T5
	p-value			—	0.68	0.01
T4	Mean difference				—	-2.59
	p-value				—	0.09
T5	Mean difference					—
	p-value					—

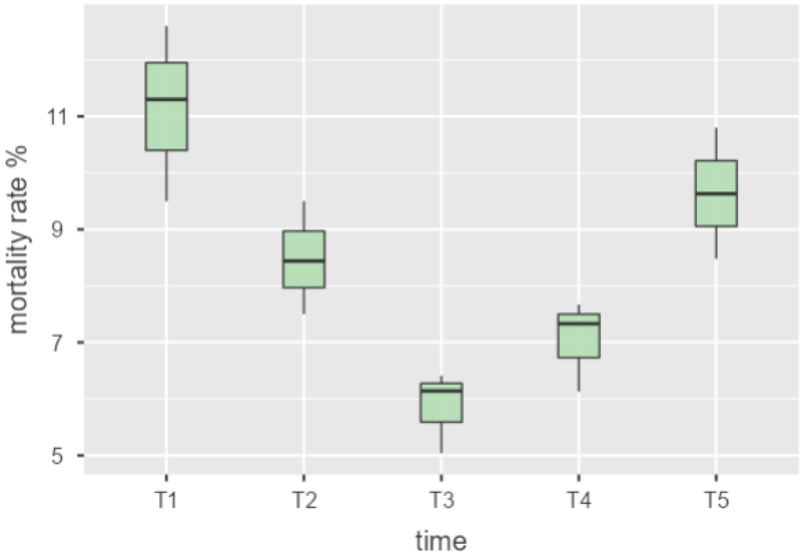


Figure 50: Box plot depicting the mortality rate at T1 (48h), T2 (1 week), T3 (2 week), T4 (3 week), T5 (4 week)

The average length (µm) of juveniles increases significantly throughout the *ex-situ* cultivation (Table 25 and 26). Indeed, at T1 the juveniles were 379 ± 60.2 µm long and they progressively grew reaching 1550 ± 23.5 µm in length (Figure 51).

Table 25: One-way ANOVA on the average length (µm) of juveniles during the cultivation

Shapiro-Wilk test			**W**	**p**
			0.99	1.00
One-way ANOVA	**F**	**df1**	**df2**	**p**
	709	4	9.69	< 0.001

Table 26: Tukey's post hoc test showing differences of the average length of juveniles among times of cultivation

		T1	T2	T3	T4	T5
T1	Mean difference	—	-478	-641	-1041	-1172
	p-value	—	< .001	< .001	< .001	< .001
T2	Mean difference		—	-163	-563	-694
	p-value		—	< .001	< .001	< .001
T3	Mean difference			—	-400	-531
	p-value			—	< .001	< .001
T4	Mean difference				—	-131
	p-value				—	< .001
T5	Mean difference					—
	p-value					—

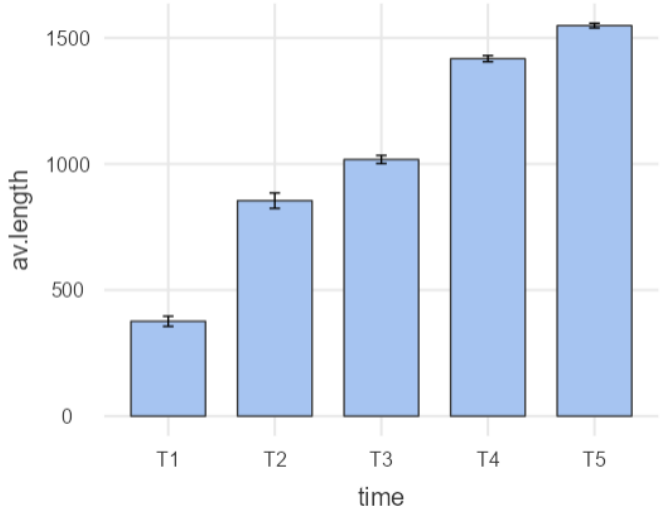


Figure 51: Bar plot depicting the average length (µm) of juveniles at T1 (48h), T2 (1 week), T3 (2 week), T4 (3 week), T5 (4 week)

On the contrary, juveniles' density showed an opposite trend than the previous one. It was significantly different according to the time of the experiment (Table 27 and 28), with the highest value observed at T1 (53.87 ± 1.53 juveniles cm^{-2}), and a decline at T5 (34.7 ± 1.02 juveniles cm^{-2}) (Figure 52).

Table 27: One-way ANOVA on the juveniles' density

Shapiro-Wilk test			**W**	**p**
			0.85	0.004
One-way ANOVA	**F**	**df1**	**df2**	**p**
	125	4	7.29	< 0.001

Table 28: Tukey's post hoc test showing differences of juveniles' density among times

		T1	T2	T3	T4	T5
T1	Mean difference	—	3.67	8.85	10.8	18.93
	p-value	—	0.432	0.006	< .001	< .001
T2	Mean difference		—	5.18	7.12	15.26
	p-value		—	0.149	0.027	< .001
T3	Mean difference			—	1.94	10.08
	p-value			—	0.881	0.002
T4	Mean difference				—	8.13
	p-value				—	0.01
T5	Mean difference					—
	p-value					—

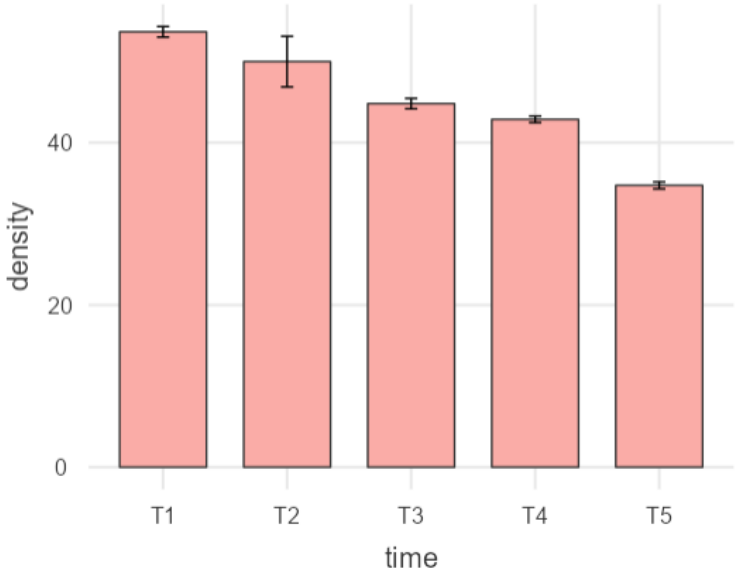


Figure 52: Bar plot depicting juveniles' density at T1 (48h), T2 (1 week), T3 (2 week), T4 (3 week), T5 (4 week)

In particular, during the cultivation it was observed a negative correlation between the average length and density of juveniles (Table 29). Indeed, at T1 in correspondence of the lowest value in the average length, it was observed the highest density of juveniles. Conversely, at T5 juveniles reached their maximum length, but their density decreased sharply (Figure 53).

Table 29: Correlation Matrix of the average length and density of juveniles

		average length	density
average length	Pearson's r	—	
	p-value	—	
density	Pearson's r	-0.88 ***	—
	p-value	< .001	—

Note. * p < .05, ** p < .01, *** p < .001

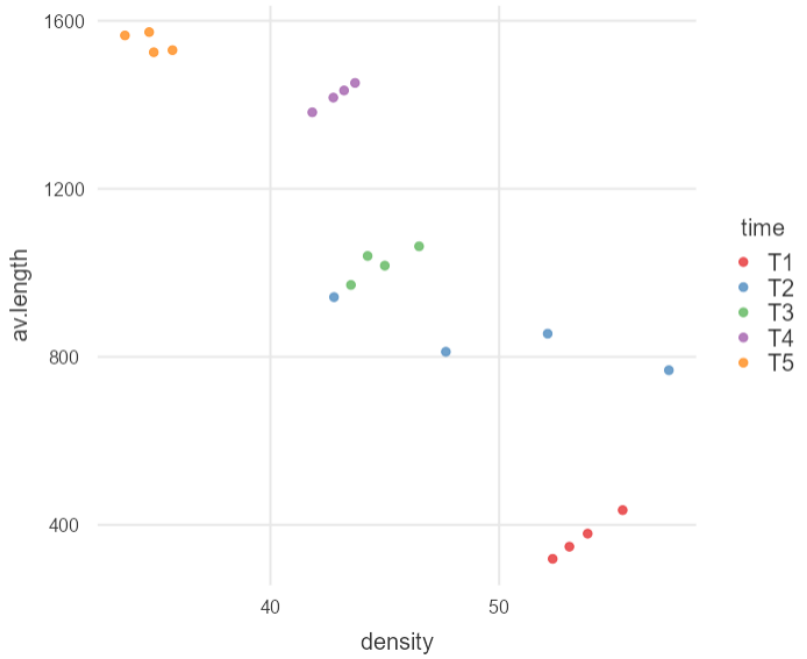


Figure 53: Scatterplot displaying the relation between the average length and density of juveniles at time T1 (48h), T2 (1 week), T3 (2 week), T4 (3 week) and T5 (4 week)

The average length (mm) of juveniles after the outplanting was significantly different according to the week (1,2, 3 and 4) (Table 30 and 31). At the first week the juveniles were 1.55 ± 0.32 mm long, while they gradually increased reaching 5.18 ± 0.22 mm at the fourth week (Figure 54).

Table 30: One-way ANOVA on the average length (mm) of juveniles after outplanting

Shapiro-Wilk test			**W**	**p**
			0.90	0.09
One-way ANOVA	**F**	**df1**	**df2**	**p**
	32.3	3	5.48	< 0.001

Table 31: Tukey's post hoc test showing differences among the weeks after outplanting

		1 week	2 week	3 week	4 week
1 week	Mean difference	—	-1.67	-3.3	-3.62
	p-value	—	0.003	< .001	< .001
2 week	Mean difference		—	-1.63	-1.96
	p-value		—	0.004	< .001

Table 31: Tukey's post hoc test showing differences among the weeks after outplanting

		1 week	2 week	3 week	4 week
3 week	Mean difference			—	-0.33
	p-value			—	0.81
4 week	Mean difference				—
	p-value				—

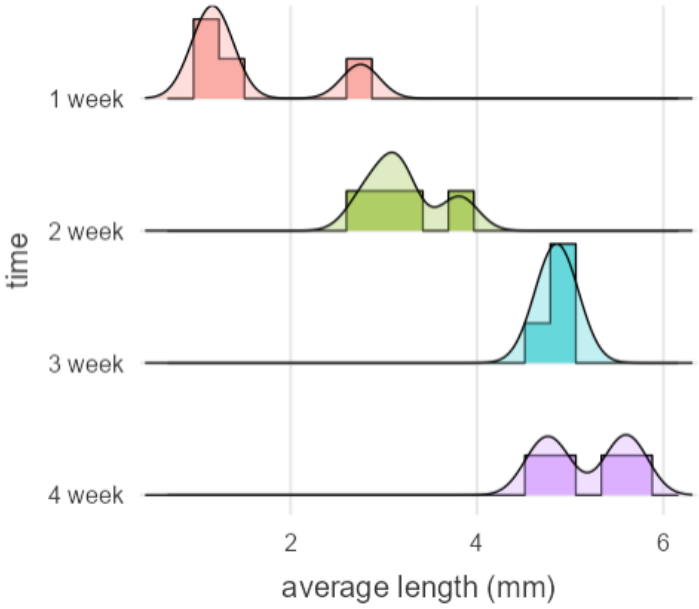


Figure 54: Histogram displaying the variations in the average length (mm) of juveniles at the different weeks after outplanting

6.4 Expectations and learned lessons

Due to the ongoing decline of habitat-forming species of the order Fucales, there is an urgent need to develop efficient restoration protocols and studies aimed on acquiring the best practices to undertake to manage coastal ecosystems (Falace et al., 2018).

In this step of the PhD work, we focused on identifying a species-specific protocol to implement an experimental restoration plot for the recovery of a population of *E. brachycarpa* in the M.P.A. Isole Ciclopi, where this species was historically

present. The first prerequisite, before realising the *ex-situ* cultivation of this species, was to follow its reproductive phenology (Chapter IV) and collect fertile receptacles during the period of the maximum maturity of this species. This indeed resulted in a high reproductive success (36.5 ± 2.45), compared to the values found in test of temperatures (Chapter V) on *E. brachycarpa* at 20°C (1.5 ± 0.39), *E. mediterranea* (19.4 ± 1.98) and *G. montagnei* var. *tenuior* (22.8 ± 4.1) at 20/15°C. Moreover, the selected temperature treatment for this cultivation (20/15°C), extrapolated from the previous experiment on temperatures (Chapter V), led to a maximum survival rate of juveniles of 1.6% at T3.

During the *ex-situ* cultivation of *E. brachycarpa*, it was observed that the mortality rate was higher (11%) during the first week, as also highlighted by Falace et al. (2018), who found a high embryonic mortality after one week of cultivation and explained that this is due to the very high stochastic gamete and zygote mortality of *Cystoseira s.l.* in the natural environment.

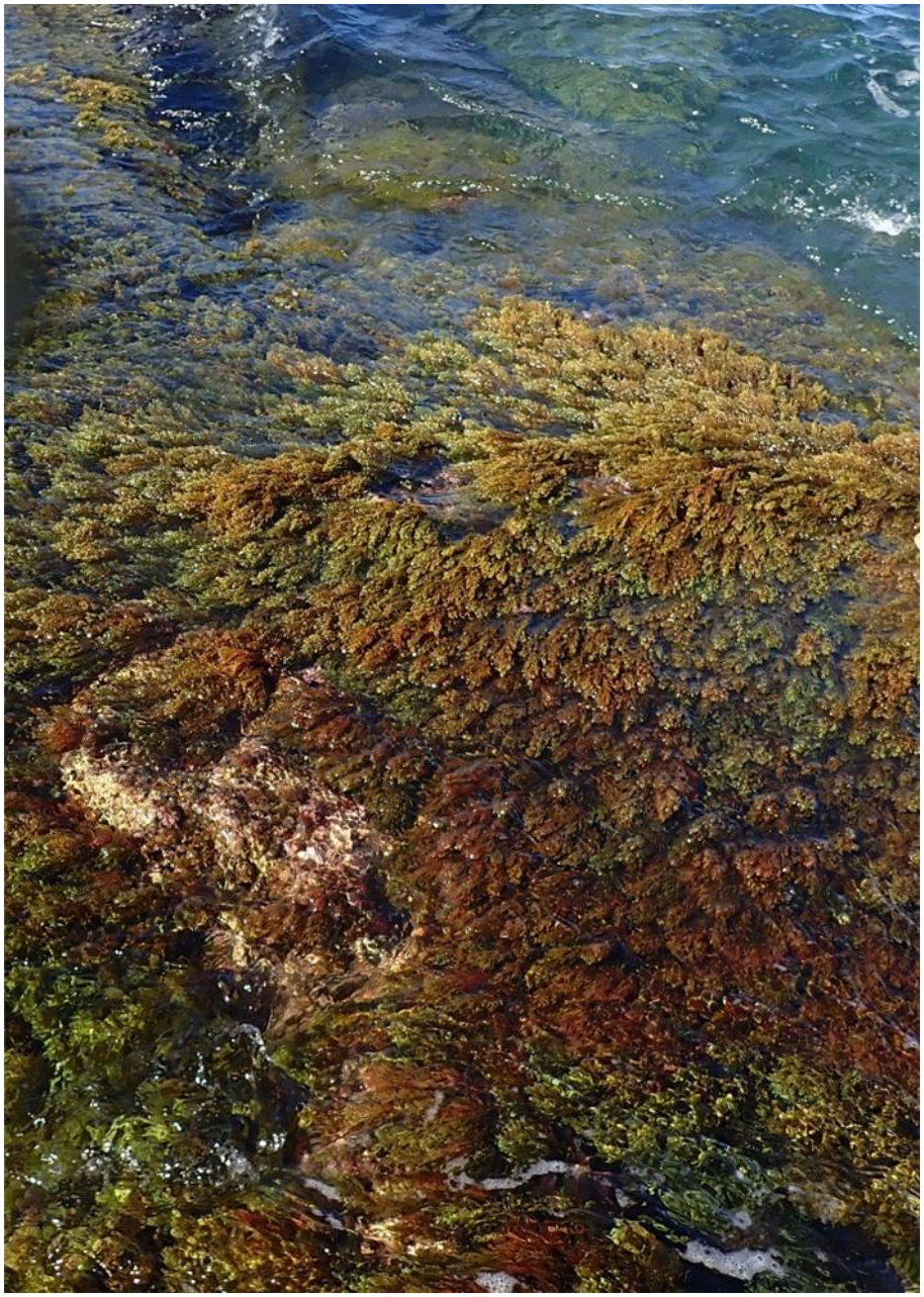
Moreover, it was also detected a negative correlation between the density and average length of juveniles. Indeed, with the progressive increase of length, the density of juveniles dropped at the final stages of the cultivation, with a rise in the mortality rate. This relationship was observed also by Savonitto et al. (2021), which attributed this trend to the process of "self-thinning" (Ang & De Wreede, 1992; Steen & Scrosati, 2003). Indeed, with the increment in sizes of germlings, the competition for space takes place, causing an increase in mortality and thus resulting in a decrease in of individuals density. This was noted at T5, when the juveniles reached a length 1550 ± 23.5 μm, but the density was below 40 juveniles cm^{-2}. It was reported that sharp drops in density are common also in natural populations due to the high natural sensitivity of the first fucoid life stages (Vadas et al., 1992; Irving et al., 2009). After two weeks of culture, juveniles' density (45 ± 1.48 juveniles cm^{-2}) was slightly lower than that reported by Savonitto et al. (2021) (54 ± 4 juveniles cm^{-2}). After one month of cultivation, the average length of juveniles was 1.5 ± 23,5 mm, a value similar to that reported by Savonitto et al. (2021) (1.72 ± 0.01 mm) and Falace et al. (2018) (1.38 ± 0.13 mm).

The outplanting technique revealed to be efficient indeed the tiles with cages were not removed by the wave motion. After one month in the field, the average length

of juveniles was 5.18 ± 0,22 mm, slightly lower than that reported by De La Fuente et al. (2019) (6.02 ± 0.18 mm), but higher than that observed by Falace et al. (2018) (4.73 ± 0.05 mm). Nevertheless, at the end of the month, it was noted that if on one hand the cages prevented grazing by fish and urchins, they failed to protect the young seedlings from the impact of small crustaceans and molluscs, which were the only ones able to enter inside the cages. Unfortunately to date the grazing pressure by mesograzers (e.g., amphipods, small crustaceans and gastropods, polychaetes) on small-sized recruits is still hardly prevented (Tamburello et al., 2019). Some studies highlighted that small-size species can be crucial in regulating the success of recruitment of Laminariales or Fucales (Sjøtun et al., 2007; Korpinen & Jormalainen, 2008; Henríquez et al., 2011). Indeed, some juveniles of macroalgae, having lower content of polyphenolic compounds, may be more palatable for mesograzers than adult conspecifics (Chenelot & Konar, 2007). Furthermore, it was demonstrated that mesograzing increases with higher temperatures (Weinberger et al., 2011; IPCC, 2021). Therefore, under a warming climate change scenario the impacts of mesograzing are expected to increase in the future.

In conclusion, it was demonstrated that to increase the potential for restoration success it is important: to collect fertile material in the proper time, following the reproductive phenology of the target species; to identify species-specific protocols adapted to the target species requirements (e.g., thermal optimum); to use an appropriate culture medium and a suitable substrate to guarantee good culture performance and obtain high densities of healthy embryos; to use anti-grazing devices strictly adapted to the grazer populations present at the restoration site. Unfortunately, this last point represented a limit during our outplanting, and highlighted the necessity to deepen knowledge of the invertebrates and fish communities present at the restoration site, before conducting a recovery intervention. Moreover, ad hoc experimental studies in the laboratory would help elucidating the overlooked impact of mesograzers on *Cystoseira s.l.* juveniles, to allow to implement new strategies to reduce their abundance for restoration purposes.

Chapter VII
Genetic characterisation of *Ericaria brachycarpa* from Brucoli



7.1 Biogeography of *Ericaria brachycarpa* and *E. balearica*

During my study period of three months at the laboratory "Biogeographical Ecology and Evolution" of the CCMAR (Centro de Ciências do Mar) of University of Algarve (Faro, Portugal), we analysed genetically some samples from Sicily in order to contextualise them in the Mediterranean biogeographical scenario. The biogeography of *Cystoseira s.l.* species has been recently studied by Neiva et al. (2022), who defined species delimitation and their geographic distribution (see Chapter II). Whitin the genus *Ericaria*, they recognised a Mediterranean lineage, which included four Molecular Operational Taxonomic Units (MOTUs): one with samples of *E. crinita*, E. *crinita* f. *bosphorica* (Sauvageau) Sadogurska, Neiva & Israel, *E. barbatula* and *E. giacconei*; one comprising only *E. corniculata*; one containing *E. balearica*; and finally, one including only *E. brachycarpa s.s*. Previously samples belonging to these last two taxa were all considered and identified as *E. brachycarpa*. However, the molecular analyses revealed that these two entities represent cryptic species, which are well genetically differentiated and geographically separated. In particular, *E. balearica* is distributed in the Balearic Sea and the Sicilian Island of Pantelleria, while *E. brachycarpa* is present in the northern coast of Sicily and Greece. Therefore, Neiva et al. (2022) pointed out that these species are both distributed around Sicily, but due to the limited sampling, their eastward and westward range limits could only be guessed. Consequently, they encouraged future studies to better identify their distribution and potential areas of overlapping. Moreover, they suggested to integrate molecular pre-screening of species before taking reforestation actions. Indeed, by understanding species boundaries, ranges and affinities, it is possible to recognise, anticipate and manage any diversity losses. Moreover, combining the knowledge on species thermal tolerance with its genetic characterisation, allow to identify which individuals or populations are "preadapted" to future climates, in order to assist restoration efforts and improve the success of reforestation (Carvalho et al., 2021).

Considering the above, samples morphologically identified as *E. brachycarpa* collected at Brucoli (the donor population selected for the experimental restoration

plot) were analysed through DNA barcoding. The aims of this step were: to understand if this entity belongs to *E. brachycarpa* or to *E. balearica*; to contribute improving the knowledge on the distribution of these species; to perform a molecular pre-screening on the target species selected for the *ex-situ* cultivation, in order to identify genetically this entity for future restoration projects.

7.2 DNA extraction, amplification and sequencing

To perform the genetic analyses, 5 branchlets of *E. brachycarpa* (of about 4 cm in length) were collected from different individuals in the site of Brucoli. The samples were cleansed by epiphytes and preserved inside small zip-lock bags with dehydrated silica gel. Species identification was performed according to Gómez-Garreta et al. (2001) and Cormaci et al. (2012).

These samples were analysed through DNA barcoding at the laboratory of "Biogeographical Ecology and Evolution" of the CCMAR, with the supervision of the Dr João Neiva.

For DNA extraction and amplification, it was followed the same method used by Neiva et al. (2022). DNA extraction was carried out using the 250 Nucleospin® Plant II kit (Macherey-Nagel Duren, Germany), following the manufacturer protocol. DNA was diluted 1:100 for the Polymerase chain reactions (PCRs). The samples were amplified and sequenced through the primers *cox1* (590 pb) (Saunders & McDevit, 2012) and *gaz2* (600 bp) (Saunders, 2005). PCR reactions were performed in 20 µl total volume, which included 5µl of diluted DNA, 6.95 µl of H_2O, 4 µl of GoTaq Flexi buffer, 1.6 µl of $MgCl_2$ (25mM),1.25 µl of each dNTP (2mM), 0.5 of Forward primer (10µM) (*cox1*-789F or *gaz2*F) and 0.5 of Reverse primer (10µM) (*cox1*-1378R or *gaz2*R), 0.2 µl of Go Taq Flexi DNA Polymerase (5U). Both PCRs started with an initial denaturation step (94°C, 5 minutes), followed by 35 cycles of 94°C for 30 seconds, 49°C (the annealing temperature for the used primers) for 45 seconds, 72°C for 60 seconds, finishing with an extension step of 72°C for 10 min (Table 32).

Amplified fragments were sequenced in an ABI PRISM 3130xl automated capillary sequencer (Applied Biosystems) at the CCMAR. Sequences were aligned, proofread and concatenated in Geneious Prime 2020 (http://www.geneious.com).

Table 32: Loci, primers' sequences, and annealing temperatures. At the bottom, PCR reagents and conditions.

Locus [primers]	**Primers 5' → 3'**	**T_a (ºC)**	**Reference**
*cox*1 [Gaz2]	F: 5'-CCAACCAYAAAGATATWGGTAC -3' R: 5'-GGATGACCAAARAACCAAAA-3'	49	Lane et al., 2007
*cox*1 [cox1-789F /cox1-1378R]	F: 5'-TNTAYCARCATTTATTTTGGTT-3' R: 5'-TCYGGNATACGNCGNGGCATACC-3'	49	Silberfeld et al., 2010
PCR Reagents | Final Conc.	**PCR Step**	**T(ºC)**	**t(m's)**
H_2O | to 10uL Buffer | 1x $MgCl_2$ | 2mM dNTP's | 0.125 µM Primer F | 0.5 µM Primer R | 0.5 µM GoTaq | (5U/µL) | 1 U DNA (1:100) (5 µL)	initial denaturation	94	5'00
	denaturation annealing extension	94 ***T_a*** 72	0'30 0'45 1'00
	final extension	72	10'00

7.3 Results and Discussion

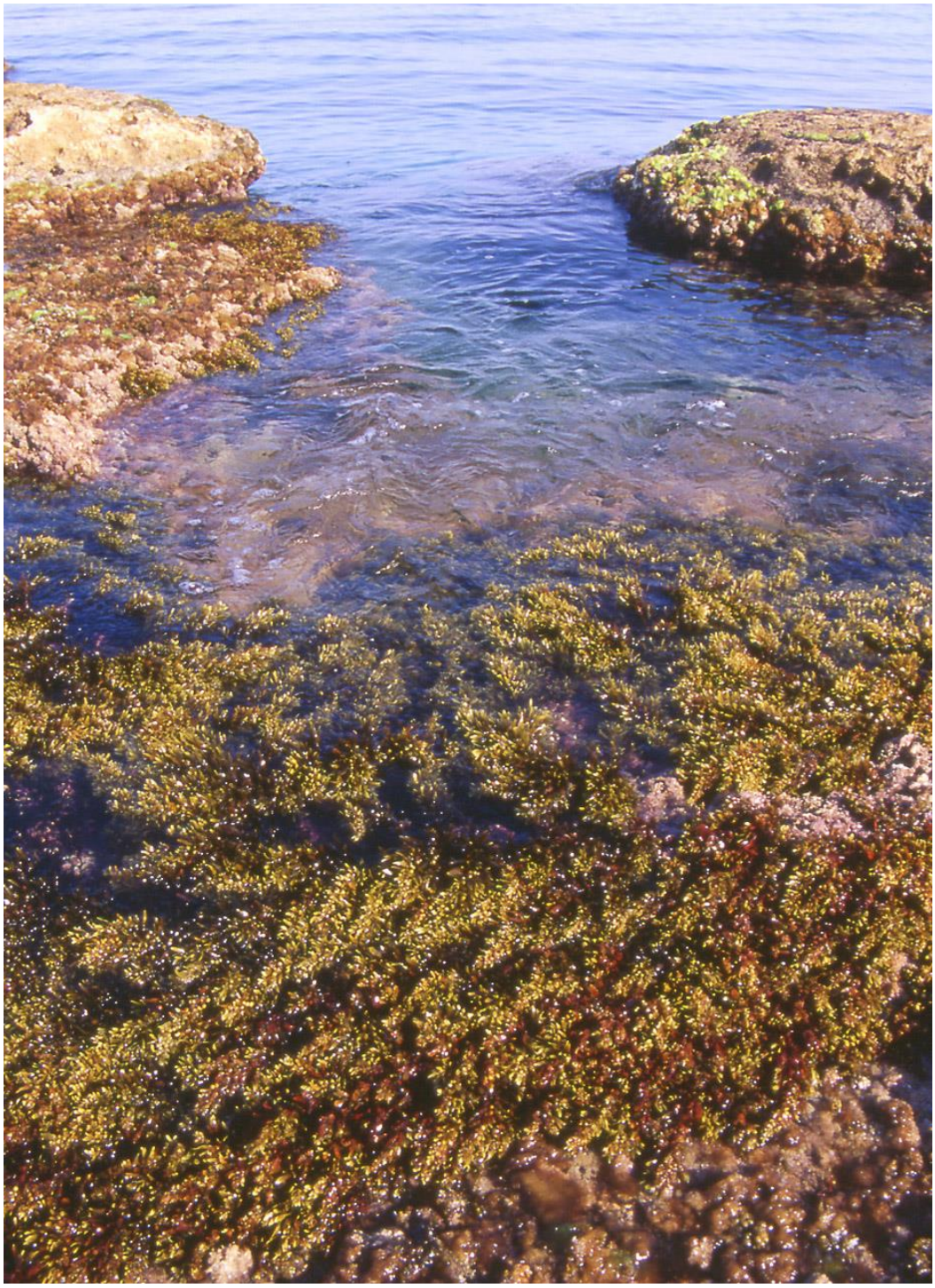
Through the barcoding analysis, it was observed that the samples morphologically identified as *E. brachycarpa* fall into the genus *Ericaria* (Orellana et al., 2019; Novoa & Guiry, 2019) and belong to the MOTU of *E. brachycarpa s.s,* which includes samples from the northern coast of Sicily and Crete (Neiva et al., 2022). However, as already observed by Neiva et al. (2022), the samples from Sicily (including our samples) are morphologically distinct from those from the southern Crete. Indeed, Cretacean samples showed more robust cauloids and primary branches, with strongly spinose apices, to the extent that it was initially hypothesised that they corresponded to *Cystoseira crinitophylla*. Therefore, Neiva et al. (2022) suggested the presence of two different entities within the group *E. brachycarpa s.s.*. Future samplings could confirm this theory or otherwise reveal the existence of a single polymorphic taxon, with different morphological formae connected with different types of environments. Indeed, the samples from the northern Sicily, but also our samples were collected in shallow and exposed waters,

while those coming from Crete were collected in deeper waters (Neiva et al., 2022). A similar case was observed by Sadogurska et al. (2021) for *G. barbata* from the Black Sea. Indeed, they found that specimens from sheltered locations had long sickle-shaped receptacles with numerous chained aerocysts, while specimens from exposed shores had few aerocysts and were characterized by smaller, spindle-shaped or oval receptacles. Nevertheless, COI sequences of all *G. barbata* samples were almost identical, also compared to Mediterranean samples. Therefore, they concluded that there was a single entity which showed high morphological variability, depending on the season and wave exposure.

Between the two primers, *cox1* provided better results, while the PCRs with *gaz2* did not amplify, even after several changings in the PCR conditions (e.g., annealing temperature, quantity of $MgCl_2$). According to Neiva et al. (2022) *cox1* alone can provide a good first proxy to reconstruct broad phylogenetic patterns and species affinities. Moreover, contrary to the mtDNA-based approaches, it is capable to discriminate past and ongoing species hybridization (Neiva et al., 2022).

Through the barcoding of our samples, we performed the genetic characterisation of *E. brachycarpa* from Brucoli and locate it genetically within the groups described by Neiva et al. (2022). This population had never been molecularly investigated, thus along the eastern coast of Sicily the distribution of *E. brachycarpa* rather than *E. balearica* could only be guessed.

Chapter VIII
General discussion



8.1 Overview of the main results

Cystoseira s.l. species are one of the most productive ecosystems in the Mediterranean Sea, supporting high biodiversity and valuable ecosystem services. However, in the last decades, these species experienced a several decline in many Mediterranean coastal areas, due to the synergistic effect of various anthropogenic impacts. Therefore, in light of the UN Decade of Ecosystem Restoration, increasing attention have been focused on restoring these threatened marine habitats as a measure to mitigate climate change over large scale.

The aims of this thesis were to investigate the past and current presence of upper infralittoral *Cystoseira s.l.* species which are potentially more prone to the effects of climate changes, identify the most threatened species and promote the natural recovery of *Cystoseira s.l.* populations through an experimental restoration plot.

The first step was the evaluation of the past and actual presence of upper infralittoral *Cystoseira s.l.* species along the Ionian cost of Sicily, based on in-depth bibliographic research and intense activity in the field. After this evaluation, among these upper infralittoral species, four threatened species were identified: *E. brachycarpa, E. giacconei*, *G. montagnei* var. *tenuior* and *E. mediterranea*. Their reproductive phenology and embryology were studied in detail, in order to examine the zygote and embryo development and check the belonging embryological group. Through these observations, it was observed that all these species belong to the first embryological group described by Guern (1962). These observations were interesting particularly as regards *E. giacconei*, whose embryology had never been studied in detail.

Being the early developmental stages of *Cystoseira s.l.* species extremely sensitive to anthropogenic impacts, are generally considered as a bottleneck for their future persistence and viability. Consequently, considering the foreseen scenario of increasing temperatures and thermal anomalies, it was tested the effect of different temperatures on the reproduction and embryos' growth of *E. giacconei, E. brachycarpa, G. montagnei* var. *tenuior* and *E. mediterranea*. Through these trials, we obtained information on the thermal optimum of the juveniles of these species, which could be used for future restoration purposes. Moreover, it was found that

the most sensitive species to warming is *E. giacconei*, whose juveniles have a very narrow thermal optimum compared to that of the other species, thus this species could suffer more the increase of temperatures. On the contrary, the recruits of *E. mediterranea* could be more able to withstand high temperatures for long periods, being the highest temperature-tolerant species among those here studied.

Among the investigated species, it was selected *E. brachycarpa* as target species to conduct an experimental restoration plot in the M.P.A. Isole Ciclopi, where this species was historically present, and Brucoli was chosen as donor site since there is still a healthy and well-preserved population of *E. brachycarpa*.

The juveniles of *E. brachycarpa* were obtained through *ex-situ* cultivation in the laboratory and were grown directly on volcanic rock tiles for the outplanting in the M.P.A. Isole Ciclopi, where there is a basaltic seabed. After one month of cultivation, we obtained juveniles of about 1.5 mm in length, a value comparable to those reported in other studies, and the tiles were transported to the restoration site. Here, the tiles were located on the seabed with the appropriately selected outplanting technique and were monitored for one month, during which it was observed a further growth in length of juveniles, reaching similar sizes (5.20 mm) to those reported in literature. These results underlined the importance: to follow the reproductive phenology of the target species, collecting the fertile apices in the period of maximum maturity of the species; identify species-specific protocols adapted to the target species requirements (e.g., thermal optimum); to use an appropriate culture medium and a suitable substrate to guarantee good culture performance and obtain high densities of healthy embryos; to use anti-grazing devices strictly adapted to the grazer populations present at the restoration site.

Since most of *Cystoseira s.l.* species present high morphological plasticity, it is often difficult to be sure of their taxonomic identification and understand their biogeographic boundaries. Furthermore, for some species as *E. brachycarpa*, some range limits are still unexplored and the presence of cryptic species (as *E. balearica*) that can only be discerned trough molecular tools, makes the identification even more challenging. Therefore, considering the above, during my study period at the "Biogeographical Ecology and Evolution" of the CCMAR (University of Algarve, Portugal), some samples of the donor population of Brucoli were analysed through

DNA barcoding, to perform a molecular pre-screening of the target species and identify it genetically for future restoration projects. Through the genetic analyses, it was found that this entity falls in the group identified by Neiva et al. (2022) as *E. brachycarpa s.s.*, whose known distribution hitherto includes the northern coast of Sicily and Crete.

In conclusion, it was demonstrated that the conjunction of active (realization of the experimental restoration plot) and passive (safeguard of the restoration plot in the M.P.A.) conservation measures is the best strategy for the recovery of *Cystoseira s.l.* populations and their associated biodiversity and ecosystem services. Further restoration actions applied to M.P.A.s and adjacent unprotected areas, will represent be the best future perspective for *Cystoseira s.l.* forest preservation in the Mediterranean Sea.

8.2 Conservation measures

Although *Cystoseira s.l.* species are listed in the annexes of some important European Conventions (i.e., Barcelona Convention, Bern Convention) to date they are not still enough safeguarded. As regards the priority species of the Habitat Directive, *Posidonia oceanica* (L.) Delile is well documented in the Natura 2000 sites and overall, there is good knowledge on its health status and distribution. On the contrary, *Cystoseira s.l.* communities are not considered as priority habitats by the Habitats Directive and the cartography of the Natura 2000 sites simply refers to "photophilic algae of rocky substrate", without a specific distinction between encrusting Corallinales deserts, filamentous algae forming dense turfs, bushes of photophilous algae or forests of large brown algae (Gianni & Mangialajo, 2016). Moreover, up to now these species have not yet been assessed in the IUCN Red List, although they urgently need a strong and true protection in the Mediterranean Sea (Grech, 2017). Therefore, assessing their status and the magnitude of regression at local scale according to the defined criteria could be crucial to improve the conservation of these species (Blanfuné et al., 2016). Through this thesis, it could be proposed to include *E. giacconei* and *E. brachycarpa* in the IUCN Red List and classify them as "Critically endangered" in accordance with criterions B and A,

respectively. Indeed, the distribution area of *E. giacconei* is very limited probably due to the restricted thermal tolerance of its early life stages (Chapter V). Regarding *E. brachycarpa*, it was observed an almost total disappearance of most populations previously present along the eastern coast of Sicily (Santa Maria La Scala, M.P.A. Isole Ciclopi, Capo S. Alessio, Catania, Maddalena Peninsula, Syracuse and Vendicari) (Chapter III).

8.3 Future perspectives

New advancements in restoration tools and approaches could optimize the success and cost-effectiveness of the recovery actions. Since marine forests are one of the major carbon sinks, the restoration of these ecosystems can help to mitigate climate change over large scales (Gattuso et al., 2018). When combined with other local management actions, the restoration interventions could also help to buffer the climatic impacts and compensate for critical ecosystem services that are impaired (Duarte et al., 2013; Possingham et al., 2015; Darling & Côté, 2018). However, this would require at least decades to affect the Earth's climate (Solomon et al., 2009). Therefore, there is a growing recognition of the need to promote climate adaptation, so the coastal marine ecosystems manage to function and provide ecosystem services under future environmental conditions (Webster et al., 2017; Darling & Côté, 2018; Abelson, 2020). In this context, restoration tools could be used to promote adaptation management to cope with future climate-change conditions (Abelson et al., 2020). One of the most innovative approaches makes use of the "intrinsic resistance" of some ecosystem engineer species, which are able to resist climate change and other stressors. This approach involves the identification of resistant genotypes, their cultivation, and finally their reintroduction in areas most influenced by changing conditions (Darling & Côté, 2018; Coleman & Goold, 2019). Another intriguing strategy exploits the "extrinsic resistance" of the environment, through the identification of spatial refuge sites (Verdura et al., 2021). Examples of suitable refugia can include locations that are less vulnerable to climate disturbances (i.e., cool currents and deeper sites; Darling & Côté, 2018), or stressful and disturbed habitats (i.e., high sedimentation, elevated temperature, acidified

waters) whose constituent species are locally adapted to tolerate exposure to chronic stressors (Webster et al., 2017). These local refugia could be promoted as priority sites to conduct relevant restoration interventions.

One of the major questions of the ecosystem restoration in the Anthropocene is if the implementation of restoration practices could be at the service of the needs of society and promote ecological functions and values (Abelson et al., 2020). The adoption of a socio-ecological approach to restoration could help to delineate clearer goals and contribute to the "blue economy" (World Bank United Nations Department of Economic and Social Affairs, 2017). Indeed, connecting seaweed cultivation and the restoration industry, may transform marine forest restoration into a commercial-scale enterprise capable of significant contribution to the blue economy and to global restoration efforts (Filbee-Dexter et al., 2022). This would provide new possibilities of work, enhancing the economic development, but it could also raise awareness and connectedness to the marine environment. Therefore, societal involvement in the planning, implementation, and monitoring of restoration projects will play a key role in the future of restoration success.

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