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XXXIV CICLO DEL DOTTORATO DI RICERCA IN AMBIENTE E VITA

BASE KNOWLEDGE AND IMPLEMENTATIONS IN THE RESTORATION OF MEDITERRANEAN MARINE FORESTS

Settore scientifico-disciplinare: BIO/03

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ἀνᾰτεθηκός τῷ εὐδαίμονι ἐμοῦ

Opus est enim ad notitiam sui experimento;

quid quisque posset nisi temptando non didicit.

Seneca – De Providentia, IV

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ABSTRACT

Brown macroalgae of the genus *Cystoseira sensu lato* (Fucales, Phaeophyceae, henceforth *Cystoseira*) form dense canopy forests along the coasts of the Mediterranean Sea, sustaining biodiversity and biogeochemical cycles. In the last four decades, these habitats have undergone substantial and widespread regression, because of local natural and anthropogenic pressures and, more recently, global climatic stressors.

Despite various conservation policies have been adopted to passively counteract the deterioration of these habitats, the natural recovery is infrequent. For this reason, active restoration efforts are required and strongly recommended. In particular, restoration by *ex situ* recruitment enhancement is to be favoured. This is a sustainable and non-destructive methodology, which foresees the collection of fertile apices in a donor site, the reproduction and cultivation of propagules in mesocosm and the further outplanting of the cultivated germlings in a receiving site to be restored.

In this doctoral dissertation, the theme of restoration of *Cystoseira* forests by *ex situ* recruitment enhancement is addressed. The first part of the thesis provides base-knowledge on three *Cystoseira* species, useful to underpin further restoration efforts. In the first chapter, the early development of *Ericaria barbatula* (= *Cystoseira barbatula*) is described. This threatened species, particularly requiring in terms of water quality, could be a possible candidate for restoration efforts, thus the acquired knowledge could be exploited for the development of culturing protocols for *ex situ* recruitment enhancement. The second chapter represents the first description of the detrimental effects of a marine heatwave on the reproductive phenology of a macroalga, providing some considerations on the possible role of thermal anomalies and climate change in explaining the decline of *Gongolaria barbata* (= *Cystoseira barbata*) observed in the Gulf of Trieste (Northern Adriatic Sea) in the last thirty years, and of *Cystoseira* species in general. The third chapter focuses on the thermotolerance of early-life stages and adults of *Ericaria giacconei* (= *Cystoseira hyblaea*), a dotted endemism of the south-central Mediterranean Sea, discussing its conservation under ocean warming. Also the first description of the species' embryology is provided.

The second part of this work focuses on some of the first restoration efforts of *Cystoseira* species by *ex situ* recruitment enhancement in the Mediterranean Sea and the application of

novel methods towards its upscaling. The first chapter reports the first restoration of *G. barbata* in the Adriatic Sea by *ex situ* recruitment enhancement, addressing the effects of the abovementioned marine heatwave and fish grazing on the restoration performance. Also in this case, the description of the species' embryology is published for the first time. The second chapter investigates the effect of the donor population and the receiving site on the restoration performance and the feasibility of decreasing the restoration costs by reducing the cultivation time in mesocosm.

The present PhD thesis contributes to the science behind the restoration of *Cystoseira* forests in the Mediterranean Sea, which is still at the outset. The results obtained in the first part provide new knowledge on the biology of *Cystoseira* species from different parts of the Mediterranean Sea, useful to underpin future restoration efforts. In the second part of the thesis, the implementation of restoration efforts shows how, despite biological and logistic challenges can be overcome, complex environmental dynamics and unpredictable climatic events represent the major threat to the success of restoration of *Cystoseira s.l.* forests, strongly limiting the possibilities of upscaling and the predictability of outcomes. In a context of ever increasing anomalous climatic events and environmental pressures on coastal habitats, the implementation of mitigation actions thus results as a crucial strategy to manage the stochasticity of restoration results.

INTRODUCTION

The genus *Cystoseira sensu lato*: ecological relevance and status

In the Mediterranean Sea, brown macroalgae of the genus *Cystoseira sensu lato* (Fucales, Phaeophyceae) are pivotal habitat-forming species, which dominate the biomass in several assemblages along rocky shores from the intertidal to the deep subtidal zone (e.g., Feldmann, 1937; Pignatti, 1962; Giaccone, 1973)(Figure 1). This genus has recently undergone different taxonomical revisions: the latest by Molinari-Novoa & Guiry (2020) divides the genus *Cystoseira sensu lato* into the three genera *Cystoseira sensu stricto* (24 species), *Ericaria* (10 species) and *Gongolaria* (13 species).

Cystoseira sensu lato species (henceforth *Cystoseira*) are key primary producers and deliver crucial ecosystem services in coastal environments, by providing settlement substrate, food and shelter for a highly diverse biota, contributing to nutrient fixing and constituting a blue carbon sink (e.g., Ballesteros, 1990; Ballesteros et al., 1998; Cheminée et al., 2013; Pitacco et al., 2014; Mineur et al., 2015; Chiarore et al., 2019). Because of their foundation role, the decline of *Cystoseira* species and their replacement by opportunistic or alien algae or by invertebrates (e.g., molluscs) can have negative cascading impacts on the biodiversity of associated communities (e.g., Thibaut et al., 2015b; Fabbrizzi et al., 2020; Bianchelli & Danovaro, 2020). For these reasons, *Cystoseira* species are used as bio-indicators of the "good ecological status" in European coastal waters according to the Water Framework Directive (2000/60/EC), being implemented in various ecological indexes (i.e., CARLIT – Ballesteros et al., 2007; Blanfuné et al., 2017; EEI – Orfanidis et al., 2003).

Figure 1 – *Cystoseira* assemblages photographed in different parts of the Mediterranean: a) Gozo Island, Malta (August 2019); b) Strunjan Natural Park, Slovenia (March 2021).

Since the second half of the 20th century, numerous studies have documented regressions or local extinctions of assemblages of *Cystoseira* species, due to the interplay of natural and anthropogenic stressors, like coastal urbanization, eutrophication, pollution, overgrazing, outcompetition by mussels, human trampling and species' introductions (e.g., Bellan-Santini, 1966; Munda, 1974; 1980; 1982; 1993a; 1993b; Gros, 1978; Verlaque, 1987; Hoffman et al., 1988; Chryssovergis & Panayotidis, 1995; Cormaci & Furnari, 1999; Benedetti-Cecchi et al., 2001; Soltan et al., 2001; Hereu, 2004; Thibaut et al., 2005; 2015a; 2015b; Mangialajo et al., 2008; Sales & Ballesteros, 2009; Falace et al., 2010; Mačić et al., 2010; Sales et al., 2011; Gianni et al., 2013; Pinedo et al., 2013; Strain et al., 2014; Blanfuné et al., 2016; Valdazo et al., 2017; Mancuso et al., 2018). More recently, growing evidence is pointing out to the decisive role of climate change as well (e.g., Bevilacqua et al., 2019; Rilov et al., 2018; Campos Cáliz et al., 2019; Capdevila et al., 2019; Falace et al., 2021; Verdura et al., 2021). Indeed, the Mediterranean Sea is considered a "hot-spot" for climate change (Giorgi, 2006): its warming is occurring at a faster rate than in the global ocean, due to its small size and semi-enclosed shape, and thermal anomalies are occurring with increasing intensity, frequency and duration (IPCC, 2021). Consequently, these rapidly changing climatic conditions raise concerns about the fate and the biogeographic responses of Mediterranean species, and in particular of habitat-forming species like *Cystoseira* (Boero et al., 2008; Marbá et al., 2015).

The conservation of *Cystoseira* forests

Given the crucial ecological role played by *Cystoseira* species, different conservation policies have been adopted internationally to halt and possibly reverse the observed trends of widespread degradation. These include:

i. The Bern Convention, which defines various *Cystoseira* species (i.e., *C. amentacea*, *C. mediterranea*, *C. sedoides* and *C. spinosa*) as "strictly protected" (Appendix I; Council of Europe, 1979);
ii. The Habitats Directive, where *Cystoseira* assemblages are considered "habitats of critical importance" in the EU (Directive 92/43/EEC; Annex I, included in "Rocky reefs" – Council of Europe, 1992);
iii. The European Red List of Habitats, which classifies as "endangered" the photophilic communities with canopy-forming algae in Mediterranean infralittoral and upper circalittoral rock (Part 1, Annex A; Gubbay et al., 2016);

iv. The Barcelona Convention, which includes all Mediterranean *Cystoseira* species (apart from *C. compressa*) on the "List of endangered or threatened species" (amended Annex II of the "Protocol concerning Specially Protected Areas and biological diversity"; UNEP, 2019);

However, these conservation programs have resulted to fall short of being effective (Katsanevakis et al., 2020). Indeed, to date, the records of natural recovery of these populations have been infrequent (but see Munda, 2000; Iveša et al., 2016; Orlando-Bonaca & Rotter, 2018; Medrano et al., 2020b), owing to the scarce population connectivity and the low propagule dispersal capability characterizing *Cystoseira* species (e.g., Soltan et al., 2001; Buonomo et al., 2017; Capdevila et al., 2018; Riquet et al., 2021).

Restoring *Cystoseira* forests

Since unassisted recovery of *Cystoseira* is hardly ever successful despite conservation measures and management of the stressors that caused regression, active restoration is now largely advocated. Procedures of "actively assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed" (SER, 2004) are in fact largely recognized as an effective way to enhance actual efforts of conservation, to the point that the United Nations General Assembly has proclaimed the period 2021-2030 as the "UN Decade on Ecosystem Restoration" (United Nations General Assembly, 2019). Ecosystem restoration is also encouraged at the European level, for example in the framework of the "Biodiversity Strategy to 2030" (European Commission, 2020). Nonetheless, in the marine realm the restoration of ecosystems is still in the "innovation phase" (Waltham et al., 2020), and, contrarily to other coastal ecosystems (e.g., coral reefs, seagrass meadows, mangroves, saltmarshes), marine forests have gotten less consideration worldwide (Bekkby et al., 2020). In particular, the restoration of marine forests is a new, rather unexplored, discipline in the Mediterranean Sea. In fact, there have been few studies focused on the restoration of *Cystoseira*, especially before the beginning of the present thesis work (i.e., November 2018). Moreover, most of these efforts have been performed on a small scale within single sites (never exceeding a few meters) and with a limited number of specimens, aiming to assess the effectiveness of the tested method or to apply specific hypotheses within experimental studies (Tamburello et al., 2019).

As for the tested methodologies, transplantation of adult thalli has been used as a prime procedure for restoring *Cystoseira* forests (Falace et al., 2006; Susini et al., 2007; Perkol-Finkel

& Airoldi, 2010; Perkol-Finkel et al., 2012; Robvieux, 2013). This technique consists in collecting adult thalli from a donor population and transplanting them in the selected receiving site. However, considering the critical status of *Cystoseira* assemblages and that large-scale restoration actions could require multiple transplantation efforts for being consistent, reiterated collections may compromise the long-lasting persistence of the donor populations.

Therefore, recruitment enhancement is now widely considered as the most suitable technique, by virtue of its sustainability. It consists in the non-destructive sampling of fertile apices of *Cystoseira* during the reproductive period in a selected donor site (Figure 2). Fertile portions can then be directly placed in the receiving site to allow propagule release (*in situ* recruitment enhancement – Verdura et al., 2018; Medrano et al., 2020a), or can be transferred to nursery facilities, where reproduction occurs in controlled conditions and the cultivated germlings are then outplanted in the receiving site (*ex situ* recruitment enhancement – Falace et al., 2018; Verdura et al., 2018, De La Fuente et al., 2019; Medrano et al., 2020a; Orlando-Bonaca et al., 2021; Savonitto et al., 2021). The cultivation of germlings in controlled mesocosms allows to maximize the production of recruits by setting the best culture conditions, i.e., temperature, light, photoperiod, and nutrients (Falace et al., 2018; Verdura et al., 2018), "capitalising" the short reproductive period (Gianni et al., 2013; Savonitto et al., 2021). This aspect appears crucial considering the increasing frequency of short-term extreme events (heatwaves), which have been shown to alter the reproductive timing and efficiency of *Cystoseira* species (Bevilacqua et al., 2019).

Figure 2 – Collection of fertile apices of *Gongolaria barbata* in April 2021 in the Strunjan Natural Park (Slovenia), as a first step of the subsequent restoration effort.

Many knowledge gaps and biological, ecological and operative bottlenecks actually challenge large-scale *Cystoseira* restoration, hence research is essential to develop effective and sustainable solutions. As for *ex situ* recruitment enhancement, three are the main challenges that restoration practitioners have to face (Figure 3). In mesocosm, the main goal is to optimize the cultivation. This is possible by thoroughly knowing the target species, their reproduction and early-life stages (which have been poorly explored so far), their optimal physiological requirements and the factors affecting their viability. In the field, the main goal is the reduction of mortality of outplanted germlings, which is already naturally elevated but can be exacerbated by human-induced overgrazing or extreme meteorologic and climatic events. Finally, methodologies have to be cost-effective, since there would be a lower likelihood of getting funding as costs rise.

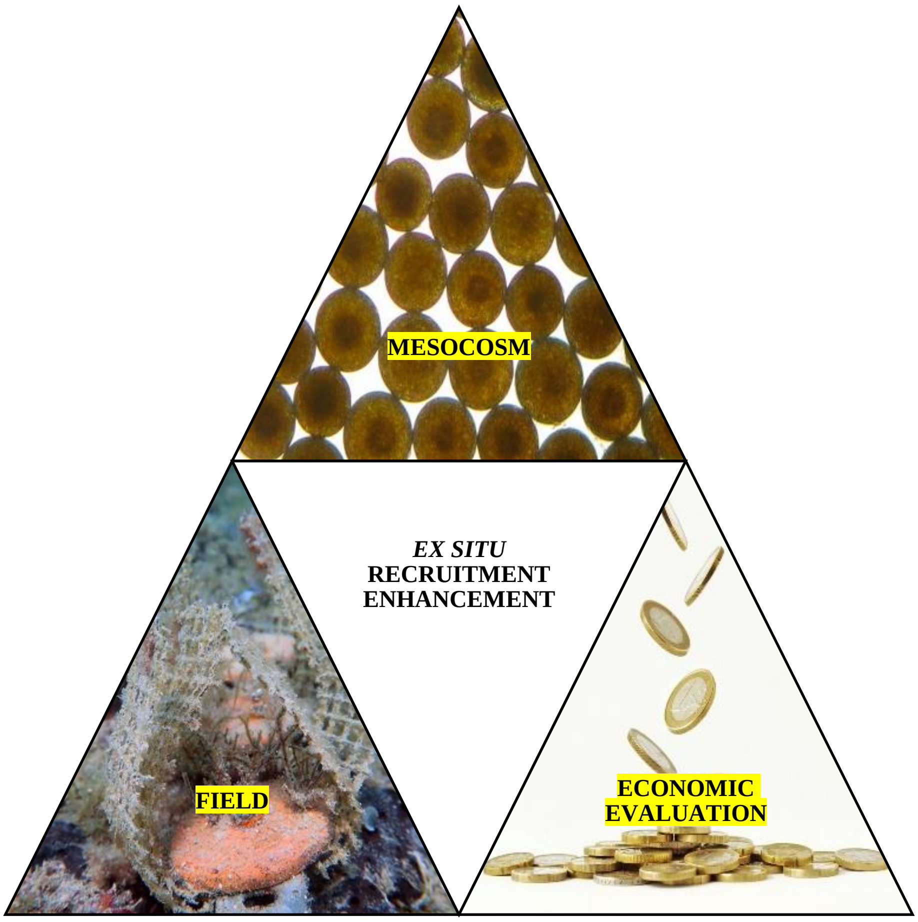

Figure 3 – Scheme summarising the main areas challenging the *ex situ* recruitment enhancement of *Cystoseira* forests and actually limiting its up-scaling. These are the optimization of cultivation in mesocosm, the reduction of mortality of outplanted germlings in the field, and the reduction of overall costs.

Thesis outline

Considering the impellent necessity to restore threatened Mediterranean *Cystoseira* habitats on an adequate scale, and the abovementioned paucity of studies addressing this topic, the present doctoral dissertation aims at:

i. Enlarging the biological base knowledge on *Cystoseira* species, which is necessary to underpin restoration efforts (Part 1);
ii. Applying novel restoration methods for the restoration of *Cystoseira* by *ex situ* recruitment enhancement towards its upscaling (Part 2).

In Part I, the first chapter (Savonitto et al., 2019) describes the reproductive phenology, embryology and early development of *Ericaria barbatula* (formerly *C. barbatula*). This species is highly requiring in terms of water quality and has undergone declines and local extinction, elements that make it a possible candidate for restoration. Knowing its reproductive strategy and its early development is a pivotal step in implementing mesocosm cultivation protocols for *ex situ* recruitment enhancement. Studying the species' response to environmental cues is also crucial to unravel the possible causes of regression and understand their vulnerability in a climate change scenario. In the second chapter (Bevilacqua et al., 2019), the detrimental effects of a marine heatwave (= MHW) on the reproductive phenology are for the first time reported for a macroalga, providing some considerations on the possible role of thermal anomalies and climate change in explaining the decline of *Gongolaria barbata* (= *C. barbata*) observed in the Gulf of Trieste (Northern Adriatic Sea) in the last thirty years, and of *Cystoseira* species in general. The third chapter (Falace et al., 2021) investigates the thermal sensitivity of adults and early developmental stages of *E. giacconei* (formerly *C. hyblaea*), a dotted endemism of the south-central Mediterranean Sea, discussing its conservation under ocean warming and providing the first description of its embryology.

In Part II, the first chapter (Savonitto et al., 2021) deals with the first restoration of *G. barbata* by *ex situ* recruitment enhancement in the Adriatic Sea, whose main challenges were represented by the species reproductive stochasticity due to the MHW and grazing by herbivorous fish. As a mitigation strategy against the low cultivation efficiency induced by the MHW, a suspended outdoor cultivation was proposed. Notably, the species' embryology is published as well. The last chapter (Orlando-Bonaca et al., in preparation) explores the feasibility of reducing the cultivation time in mesocosm through the previously introduced suspended culture, and considers the influence of the donor population and the receiving site on the success of *G. barbata* restoration.

The results presented in this thesis mostly derive from research activities performed in the framework of the EU-funded project ROC-POP Life (LIFE16 NAT/IT/000816).

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PART I - BASE KNOWLEDGE UNDERPINNING THE RESTORATION OF MARINE FORESTS

Reproductive phenology, zygote embryology and germling development of the threatened *Carpodesmia barbatula*[1] (= *Cystoseira barbatula*) (Fucales, Phaeophyta) towards its possible restoration

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[1] Currently, the species is referred to as *Ericaria barbatula* (Kützing) Molinari & Guiry.

Abstract

Carpodesmia barbatula (= *Cystoseira barbatula*) (Fucales, Phaeophyta) is a species endemic to the Southern Mediterranean Sea, which grows in clean waters on exposed sublittoral rocky shores. Together with other species of the genus *Cystoseira sensu lato*, it is an ecosystem engineer that forms dense canopy forests, sustaining biodiversity, productivity, and nutrient cycling in temperate rocky reefs. Human impacts and climate stressors are seriously hampering these ecosystems and actual conservation efforts appear insufficient for their preservation. Restoration actions could be a solution for the loss of these habitats, but they require the deep knowledge of the target species. In the present study, we describe egg release, zygote development, and germling growth of *C. barbatula*, poorly known, despite its importance in the upper sublittoral seaweed flora. The aim is to improve the knowledge on the early life stages of this rare and threatened species, towards its possible future restoration.

Keywords: zygote segmentation; polarization; germination; macroalgae reproduction; Mediterranean Sea

Introduction

*Carpodesmia barbatula (*Kützing) S. Orellana & M. Sansón (= *Cystoseira barbatula*) (Fucales, Phaeophyta) is a species endemic to the Southern Mediterranean Sea (Berov et al., 2015). Kützing firstly described it in the Gulf of Naples (Italy) in 1860. The species has been recorded in Sicily (i.e. Catania, Lampedusa Island – Italy), Maltese Islands, Cyclades Islands (Greece), the Eastern coast of Libya (Cormaci et al., 1990, 1992, 1997; Furnari et al., 2003, 2010), Tunisia and the North-Western coast of Africa (Bouafif et al., 2014), and Cyprus (Gerloff & Nizamuddin 1975, as *C. graeca*).

The diversity and phylogenetic relationships of the genus *Cystoseira sensu lato* have been recently revised using DNA sequences and morphological analyses (Orellana et al., 2019). This study distinguished three genera and identified characters that differentiate species. One clade corresponds to *Cystoseira sensu stricto,* the second one corresponds to *Treptacantha* gen. emend. and the third one corresponds to *Carpodesmia* gen. emend., to which *C. barbatula* belongs[2].

C. barbatula is a caespitose species that inhabits the upper sublittoral zone, on exposed/moderately exposed unpolluted rocky shores at 1-1.5 m depth and in littoral rockpools (Cormaci et al., 1992; Berov et al., 2015). It lives in association with other species of the genus *Cystoseira sensu lato* (like *Carpodesmia crinita, Treptacantha barbata*, *T. elegans*[3]*, Cystoseira crinitophylla, C. compressa*; e.g. Cormaci et al., 1992; Alongi et al., 1999; Montesanto & Panayotidis 2001), which act as ecosystem engineers, forming dense canopy forests. These assemblages increase the spatial heterogeneity of rocky shores, thus sustaining rich communities of organisms: they provide food, shelter and nursery to rocky reef fishes (Cheminée et al., 2013) and invertebrates (Chiarore et al., 2019), and control the structure of the associated benthic community (Mineur et al., 2015). They also foster coastal biogeochemical cycles and productivity (Blanfuné et al., 2019).

Both human impacts and climate stressors are severely hampering these ecosystems, causing regression or, in some cases, even local extinction of *Cystoseira* species in the Mediterranean Sea (Mangialajo et al., 2008; Irving et al., 2009; Sales & Ballesteros, 2009; Sales et al., 2011; Thibaut et al., 2015; Mancuso et al., 2018; Bevilacqua et al., 2019). Owing to their great ecological value and their endangered status, all the *Cystoseira s.l.* (apart from *C. compressa*)

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[2] Please refer to the Thesis' introduction for the current taxonomy.

[3] Currently these species are referred to as *Ericaria crinita*, *Gongolaria barbata*, *G. elegans*

are considered threatened and are object of conservation (e.g. amended Annex II of the Protocol of the Barcelona Convention UNEP; Habitat Directive – Habitat 1170). *C. barbatula*, in particular, is vulnerable because it has a restricted areal distribution and is highly requiring in terms of water quality (Cormaci et al., 1992; Montesanto & Panayotidis 2001). Besides, it lives only in shallow waters at the land-sea interface, a kind of habitat that is particularly sensitive to anthropogenic changes in upstream terrestrial systems and to direct coastal impacts (e.g. Ruttenberg & Granek 2011; Falace et al., 2018). For example, in the oligotrophic waters of Cyprus, the canopy of *C. barbatula* diminished near industrialised areas and got replaced by simpler communities, dominated by stress-resistant and ephemeral species such as *Ulva* spp. and *Halopteris scoparia* (Kletou et al., 2018). Moreover, although it has been described in the Gulf of Naples (Italy), *C. barbatula* has not been recorded anymore in that area, indicating that it could be definitely disappeared (Grech, 2017).

In a context of local extinctions, with conservation efforts resulting necessary but not sufficient for an enduring survival of these habitats, the restoration of this rare species could be a solution. However, restoration techniques, mostly based on recruitment enhancement (e.g. Falace et al., 2018; Verdura et al., 2018; De La Fuente et al., 2019), cannot be applied disregarding the knowledge of the early life stages of the target species. Although *C. barbatula* is an important member in sublittoral seaweed flora along the coasts of the Southern Mediterranean Sea, to the best of our knowledge only its morphology has been described. Its life cycle, zygote embryology, and development have not been described so far, neither in the most extensive study on *Cystoseira* embryology (Guern, 1962).

In the present study, we describe its egg release, zygote development, and germling growth, in order to improve the knowledge on the early life stages of this rare and threatened species. Knowing its reproductive strategy and its first life stages is a pivotal step to develop protocols for cultivation in lab towards its possible future restoration.

Materials and methods

On February 27th, 2019, during the reproductive period of *C. barbatula*, healthy fertile apices were collected in Porto Palo di Capo Passero (Siracusa, Sicily, Southern Italy – 36.651194, 15.076121), in exposed littoral rockpools of 80 cm depth.

Samples were gently rinsed with seawater to remove epibionts, wrapped with aluminium foil and delivered refrigerated within 12 h to the laboratory at the University of Trieste (NE Italy).

There, samples were stored in the dark at 4 °C for 24 hours. The fertile apices were then placed both on glass slides in Petri dishes and on clay disks in 4-L aquaria filled with filtered seawater, in an environmentally controlled room. The temperature and light regimes were set at 18 °C under 125 μmol photons m^{-2} s^{-1}, with a photoperiod of 11L:13D, resembling the ones at the sampling site. The thermal "shock" (4 to 18 °C) was aimed at stimulating the release of fertilized oogones from the mature receptacles. Apices were removed from the slides after zygotes had been released (24 h). This time, Petri dishes and aquaria were filled with a culture medium enriched in nutrients (von Stosch's enriched filtered seawater; Von Stosch, 1963), renewed every 4 days to minimize any possible effect of nutrient limitation. Aquaria were continuously aerated by air pumps. Specimens were maintained at these conditions and monitored for 4 weeks.

Zygote development was followed by means of an inverted microscope (Leica, DM IL LED), and photographs were taken with a Canon Powershot G9 camera twice daily. Juveniles were studied at the stereomicroscope (Leica, MZ 6) and photographed with a Nikon Coolpix 4500 camera once daily. Measurement were obtained by processing photographic data with the software ImageJ (Schneider et al., 2012).

Results

Reproductive phenology

C. barbatula thalli, during the period of reproductive development, measure 30-40 cm in height. The caespitose structure is made up of 3 up to 11 primary axes (cauloids), 10 to 20 cm long and with a diameter of 2.5 – 3.5 mm (Figure 1a). The apex of cauloid is smooth and prominent (Figure 1b). Adventitious branches lay at the base of dropped primary branches, with ultimate branchlets showing a cylindrico-filiform shape. Ultimate branchlets are 5 cm long and bring receptacles (Figure 1c). The receptacles appear compact, humpy, subulate, mucronate, and sometimes they show ephemeral lateral spine-like appendages (Figure 1d).

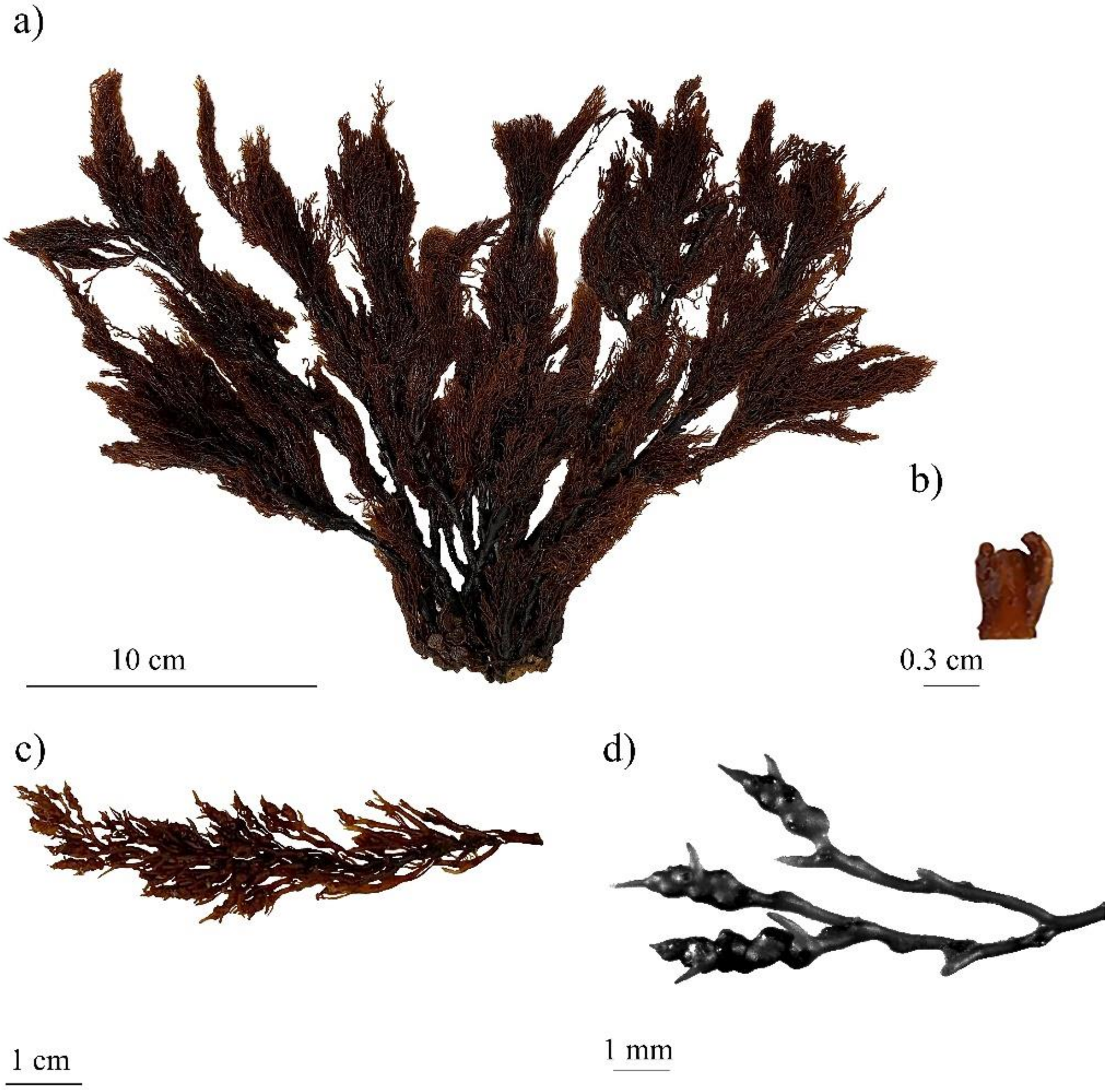

Figure 1 – a) A *C. barbatula* thallus during the reproductive period, b) the apex of the cauloid, c) an ultimate branchlet bringing receptacles, and d) mucronate receptacles with ephemeral lateral spine-like appendages.

Each receptacle hosts several conceptacles of circular section (diameter around 450-500 μm) and communicating with the external environment through an apical ostiole. Conceptacles are hermaphrodite, as they contain antheridia and oogones, and paraphyses (Figure 2a). There are no basal hairs developing at the bottom of the conceptacles.

Antheridia contain 30 – 45 μm long antherozoids that, when mature, look orange owing to carotenoids colouring their stigma (Figure 2b). Oospheres in the conceptacle measure 115-170 μm and have an ovoid shape (Figure 2c), while after their emission they become round (Figure 2d). Eggs are fecundated when they exit from the operculum and show the external membrane typical of other *Cystoseira* species (Guern, 1962).

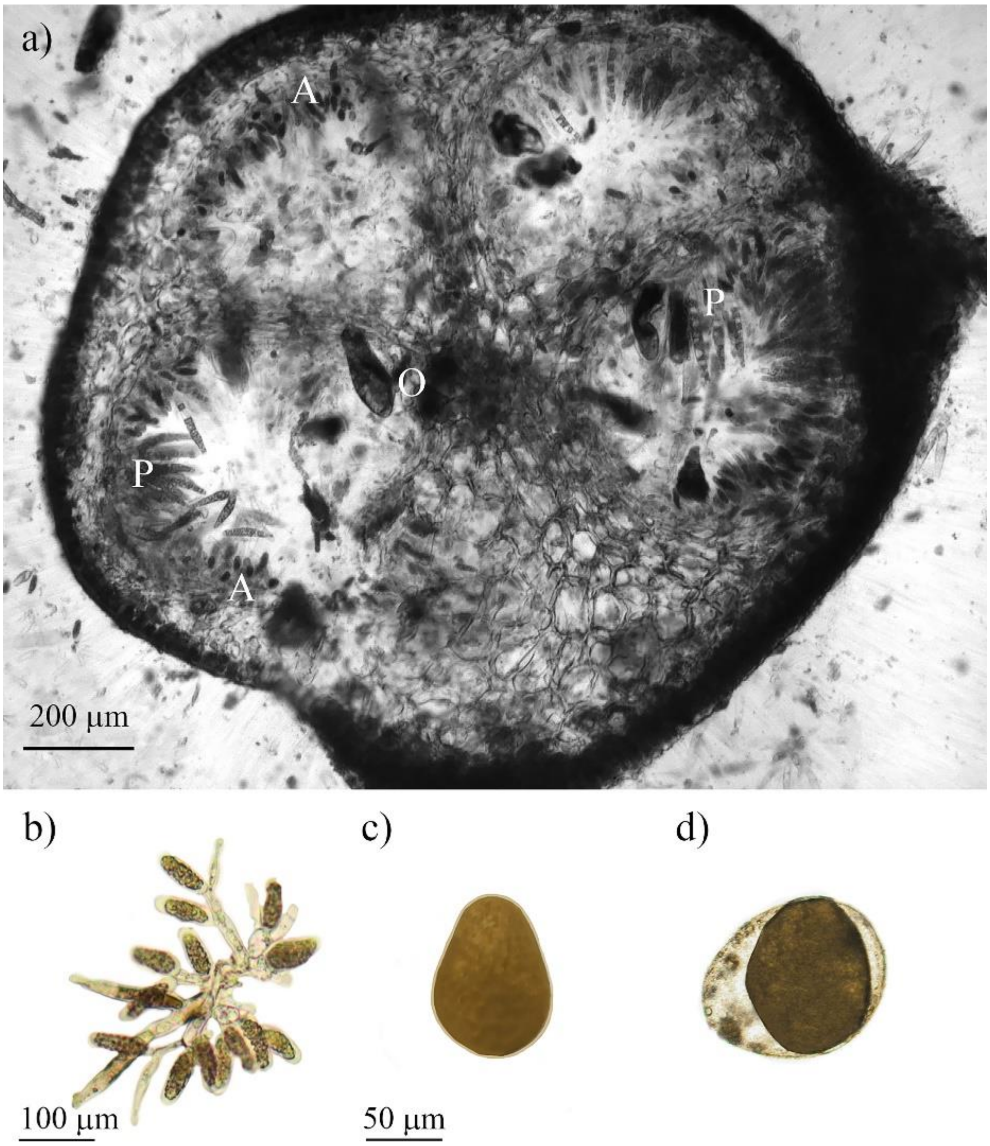

Figure 2 – a) Horizontal section of a conceptacle (A = antheridia, O = oogones, P = paraphyses). b) detail of the antheridia. c) Oogone before and d) after the emission from the conceptacle, surrounded by the fecundation membrane.

Zygote and first stages of development

Shortly after fertilization (AF), zygotes of *C. barbatula* adhere to the substratum by means of their fecundation membrane. Once this is formed, the first segmentation appears equatorially within 12 h AF (Figure 3a).

A second division perpendicular to the first one takes place at one of the poles (Figure 3b). A third division, equal to the previous one, but occurring in the opposite pole, leads to the formation of the fourth cell within 12-24 h AF (Figure 3c).

After 24 h AF, the development of the rhizoidal pole takes place. There, one cell divides into four new cells, which develop into rhizoids by elongation (Figure 3d). It can happen that one or some of the rhizoids, which appear like colourless simple tubes containing physodes, do not grow at the same rate and have different lengths (Figure 3e). In some rare cases, there is no division of the rhizoidal cell at all, and consequently only one big rhizoid develops (Figure 3f).

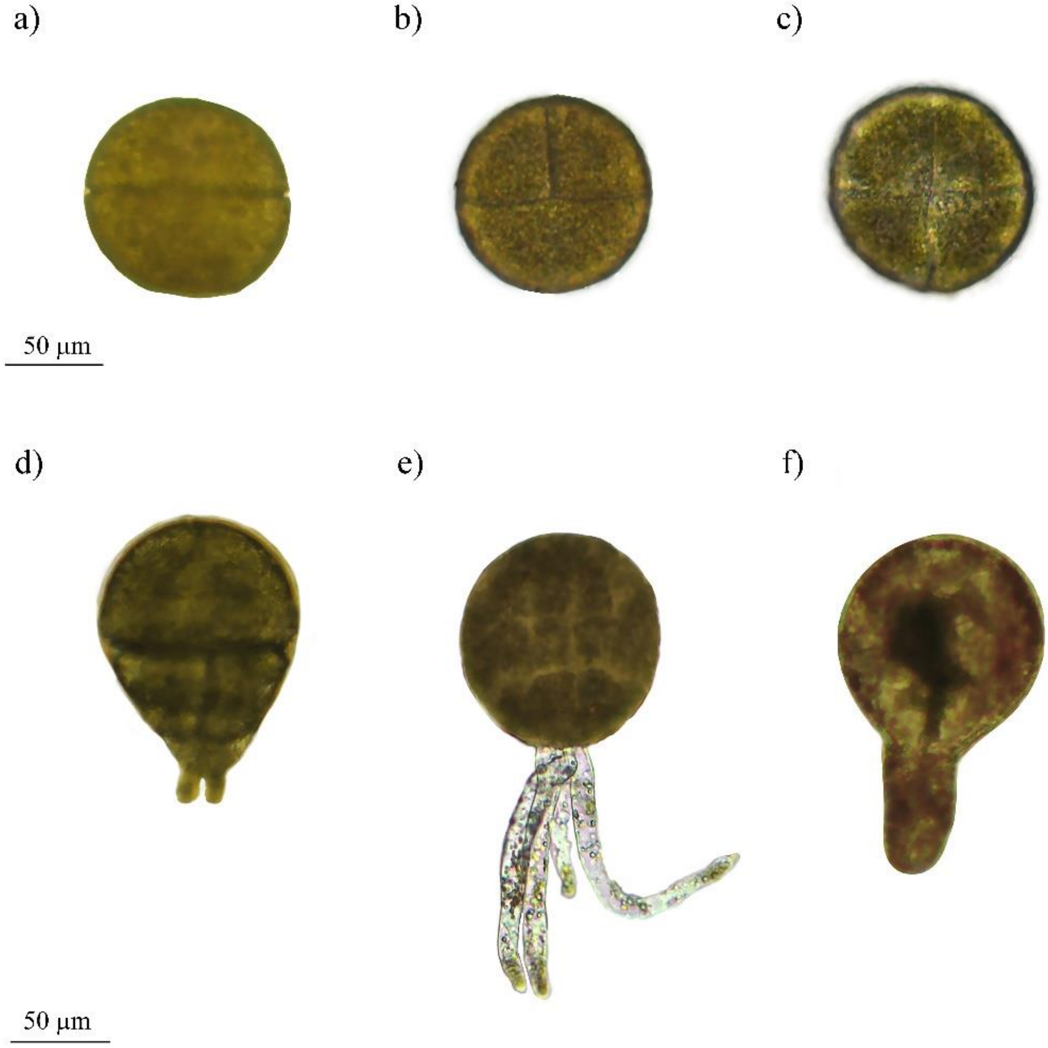

Figure 3 – a) The first zygote division followed by b) the second and c) the third one. d) Emission of the rhizoids. e) rhizoids can show different lengths and, in rare cases, f) there is no division of the rhizoidal cell.

During division, the embryo acquires a square patterned aspect, while its volume remains approximately the same. The embryo keeps its spherical shape during germination, while being enclosed in the fecundation membrane.

Three to four days AF, embryos gradually acquire an elongated shape, a characteristic morphology of embryos of Fucales. From day 5 AF, in some embryos, a little invagination is present in the apical pole (Figure 4a), where after 7 days AF, one hyaline hair (or trichothallic filament) appears (Figure 4b). There can be also more trichothallic filaments in a bush, sometimes growing into lateral depressions (Figure 4c). Two weeks after its appearance, this hair falls, representing the end of the embryo development stage.

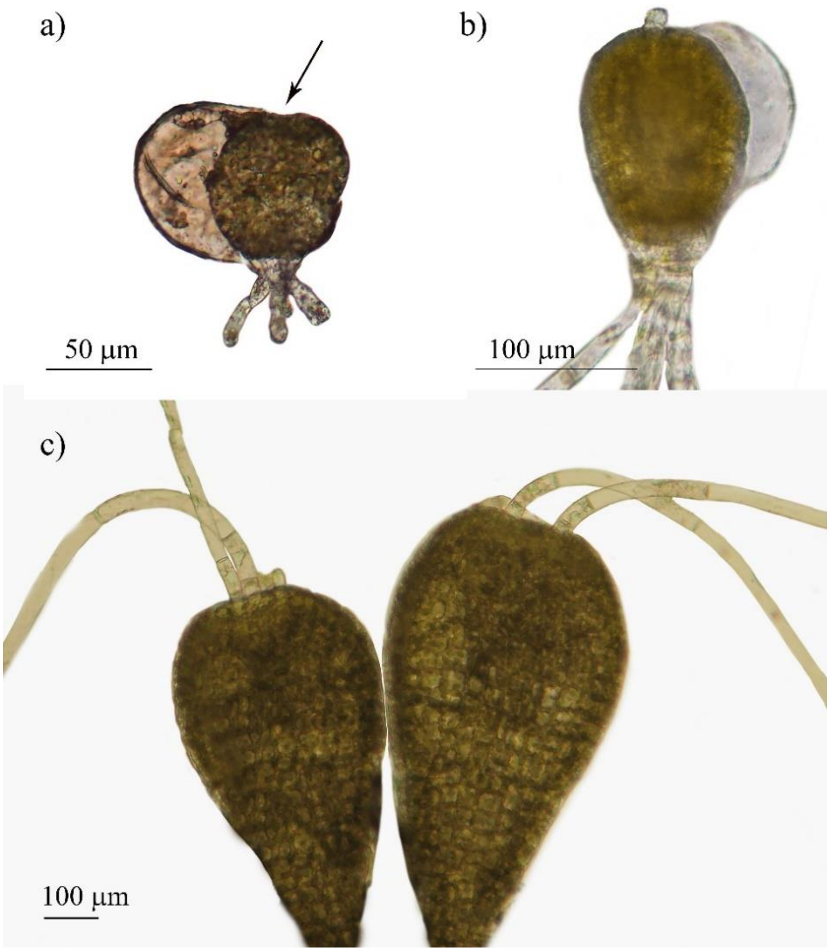

Figure 4 – a) Some embryos show a little invagination in the apical pole (pointed out by the arrow), where b) after 7 days AF one hyaline hair appears. c) There can be also more trichothallic filaments in a bush.

After two weeks, individuals start to develop the first lateral branches and cryptostomata (Figure 5), with a length ranging between 1.1 and 2.6 mm after 4 weeks of culture. This species lacks aerocysts and has got spaced and prominent cryptostomata.

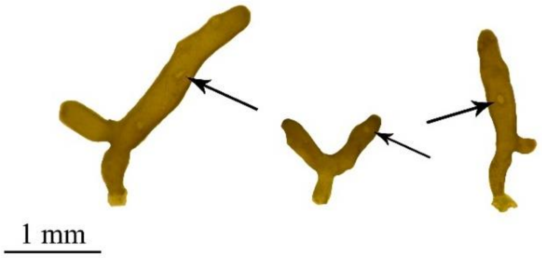

Figure 5 – Germlings after four weeks of culture. Cryptostomata are pointed out by the arrows.

Discussion

The obtained results show that *C. barbatula* shares many traits with the species of the groups I and III identified by Guern (1962): the spherical shape of the oogones, the number of primary rhizoids (i.e. four), the embryo development on the substratum after jellification of the fecundation membrane, the structure of conceptacles (numerous and branched antheridia and absence of basal hair), and coloured antherozoids (stigma with carotenoids).

Considering the first stages of the zygote segmentation, *C. barbatula* cannot however be ascribed to any of the groups identified by Guern (1962). On the contrary, the sequence of segmentations is similar to another species of the genus *Cystoseira s.l.*, namely *Treptacantha abies-marina*[4], for which the creation of a fourth group for embryogenic classification had been proposed (Gil-Rodriguez et al., 1988).

Another peculiarity observed in the embryo development of *C. barbatula* is that immediate cytoplasm polarization and asymmetry of the cell wall do not take place, differently from the majority of fucoids (Kropf, 1997; Bogaert et al., 2013). Due to the lack of a polar axis development affected by environmental signals, the first division thus does not separate the rhizoid and the thallus cells (Goodner & Quatrano 1993, Bogaert et al., 2016). The absence of an immediate polarization of the zygote shows that this species could be not sensitive to the direction of light, at least in the first phases of development, contrarily to *Fucus* for example (Bogaert et al., 2015).

C. barbatula maintains a spherical shape during germination, and this is a feature common for other Fucales, like *Himanthalia elongata* (Ramon, 1973), *Pelvetia canaliculata* (Moss, 1974; Hardy & Moss, 1979) and *Halidrys siliquosa* (Hardy & Moss, 1978), and for osmotically stressed zygotes of *Fucus vesiculosus* (Torrey & Galun, 1970).

Conclusions

Considering the early life stages of *C. barbatula*, it is evident that it shares many traits with the other species of the genus *Cystoseira s.l.* and, generally, with Fucoids. However, the sequence of the first zygote segmentations and the lack of early cell polarization are striking traits that distinguish this species and preclude its inclusion in any of the groups identified by Guern (1962).

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[4] Currently, *Gongolaria abies-marina*.

In a scenario of global changes, the currently observed abundance diminution, areal reduction or disappearance of *C. barbatula* (Grech, 2017; Kletou et al., 2018) make it urgent to actively intervene to restore these endangered habitats. Thus, this study provides basic knowledge on the early life stages of *C. barbatula*, pivotal for the further development of culture protocols for restoration purposes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Climatic anomalies may create a long-lasting ecological phase shift by altering the reproduction of a foundation species

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The resilience of ecological communities is often defined by one or a few species that have disproportionately important roles influencing many other species in the community. This is true for some areas of the Mediterranean Sea that are characterized by large brown fucoid algae of the genus *Cystoseira*[5] that form dense underwater forests structurally similar to the giant kelps of the Pacific. While shorter than the giant kelp, *Cystoseira* species form dense underwater stands, contributing to the three-dimensional complexity of the seascape (Figure 1).

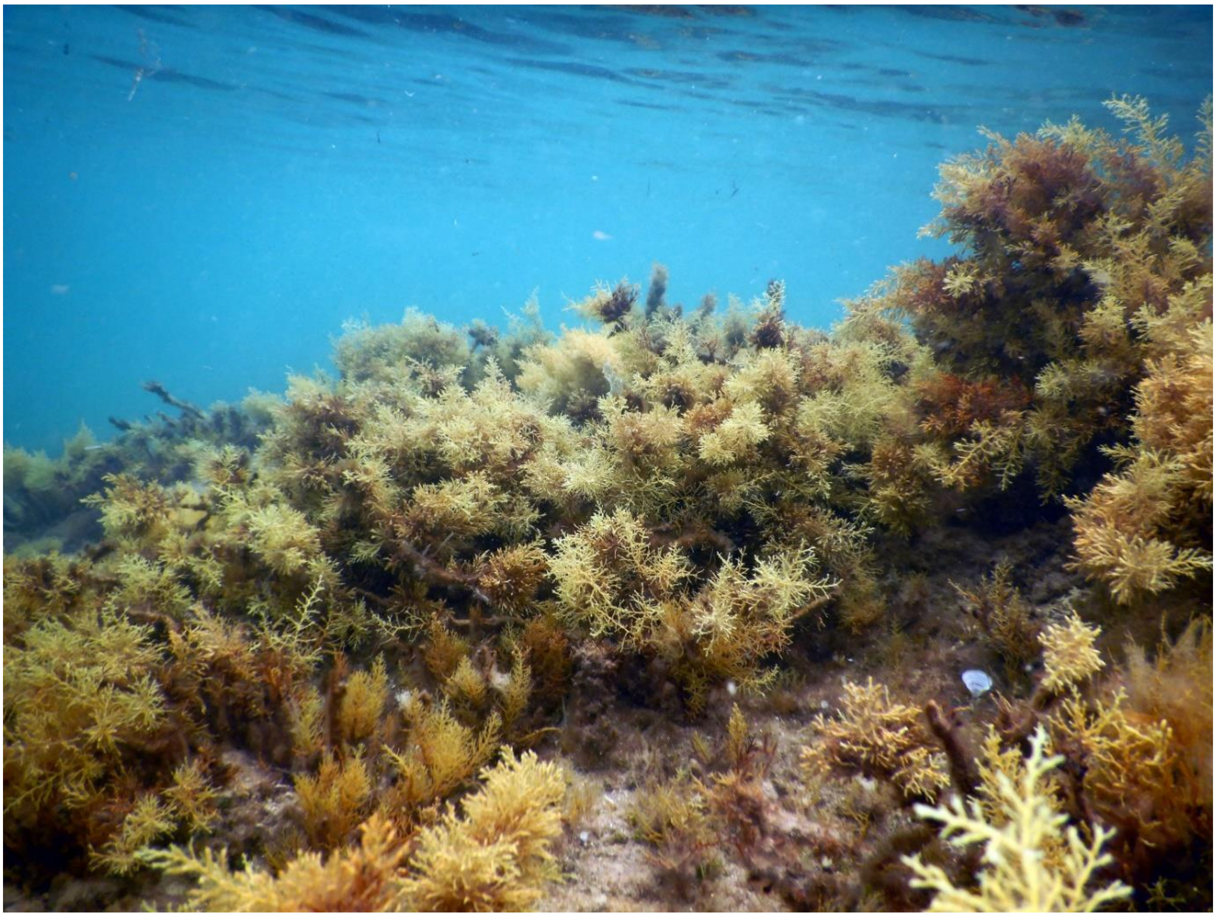

Figure 1 – Mediterranean underwater *Cystoseira* spp. forest.

These canopy-forming seaweeds play a crucial role in primary production and nutrient cycling of temperate coastal ecosystems from the Mediterranean Sea to the Atlantic Ocean (Mineur et al., 2015) and act as "ecosystem engineers," providing food, nursery, and shelter for a rich

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[5] In the whole chapter, *Cystoseira sensu lato* is referred to as *Cystoseira*.

associated biota. Interacting human pressures are causing the widespread disappearance of these complex and productive species (Strain et al., 2014). As they are being replaced by simpler assemblages, biodiversity declines and ecosystem functions are altered (Falace et al., 2010; Sales et al., 2011). The natural recovery of these populations is hampered by their very limited dispersal ability (Capdevila et al., 2018).

In February 2019, during a routine survey within the Marine Protected Area of Strunjan (Slovenia) to monitor the vegetative and reproductive cycle of the fucoid *Cystoseira barbata* (Stackhouse) C. Agardh[6], we made a singular discovery. Even though they were in their winter resting phase, dozens of *C. barbata* thalli had reproductive structures (receptacles; Figure 2a,b).

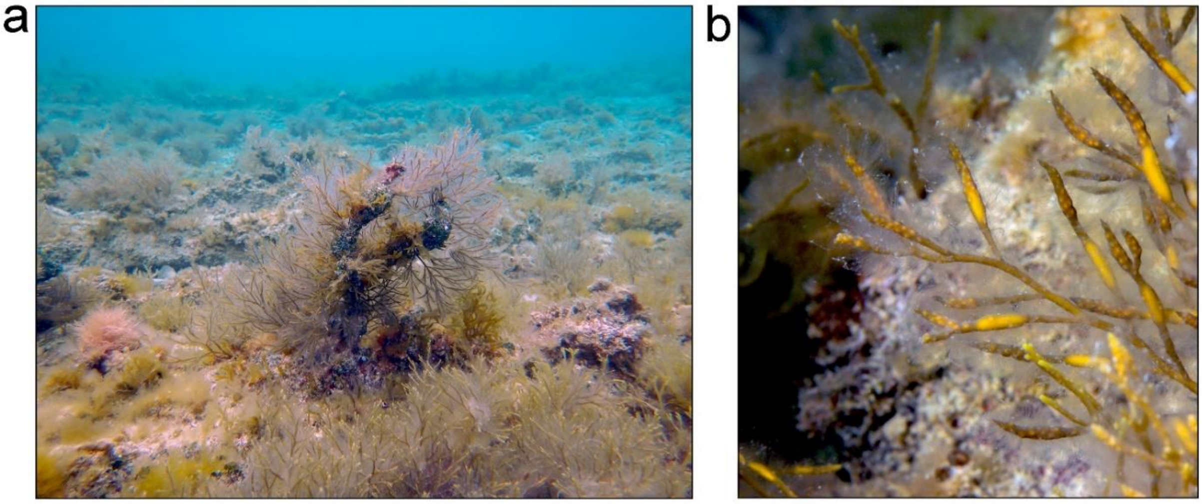

Figure 2 – (a) *Cystoseira barbata* winter habitus, with short primary and adventitious branches. (b) Branches with reproductive structures (receptacles).

In addition and even more amazing, the receptacles were also developing on adventitious branches, which are sterile by definition. Subsequent surveys revealed this unusual phenology in other areas as well, from Strunjan to the Marine Protected Area of Miramare (Italy), suggesting that this phenomenon spanned at least 50 km of rocky coast in the region. Like other congenerics, *C. barbata* has marked seasonal variations of vegetative growth. In the Northern Adriatic Sea, as temperature and photoperiod increase in early spring, branches usually start to develop from a perennial cauloid (Falace & Bressan, 2006). Later, during late spring–early summer, the branches become fertile as they reach their maximum development. At the end of summer, upright branches are shed and the cauloid persists in a quiescent state during the fall–winter cold season.

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[6] Currently, this name is regarded as a synonym of *Gongolaria barbata* (Stackhouse) Kuntze.

During our study, instead of this normal life cycle, *C. barbata* was fertile almost 3 months earlier than usual, yielding mature receptacles (Figure 3a) able to release zygotes and produce viable embryos (Figure 3b).

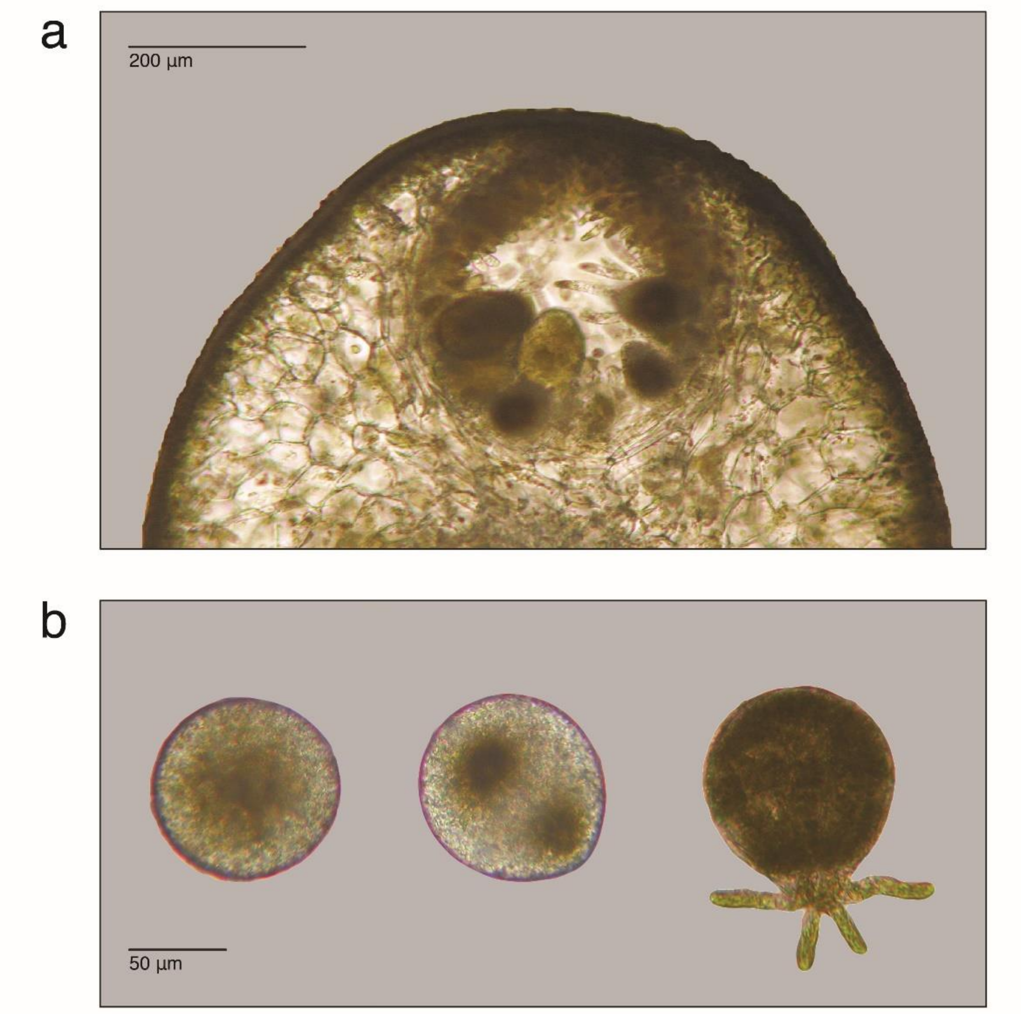

Figure 3 – (a) Transverse section of a receptacle with a fully mature conceptacle (gametes are fully developed and close to release). (b) Zygote and early life stages of *C. barbata* embryos.

Interestingly, this early unexpected reproductive event followed a period of 2 yr where *C. barbata* was infertile in the Northern Adriatic Sea (personal observation of the authors). Although drawing conclusions on the reasons behind the prolonged infertility of *C. barbata* and this mistimed reproductive event is still premature, there are some clues. After a period of relatively normal temperature from 2003 to 2013, there have been recurring episodes of exceptional warming in the cold season in the Northern Adriatic Sea during the last 6 yr (Appendix S1: Figure S1). Immediately before the extraordinary reproductive event, in February 2019, a thermal anomaly of 2.65 °C higher than the average during the last four decades has struck the whole basin. Near the Marine Protected Area of Strunjan, the coastal oceanographic buoy MAMBO OGS (National Institute of Oceanography and Applied Geophysics) recorded a warming in the intermediate waters of about 1.5 °C occurring in the middle of February and persisting for more than 1 month. This warming is 1 month prior to the average timing of warming during the first 10 yr (1999–2009) of measurements (Appendix S1: Figure S2).

As temperature is one of the main variables regulating reproductive and vegetative phenology in seaweeds (Eggert, 2012), early and extreme episodes of warming could play a decisive role in triggering the survival of subsequent life stages. For example, the release of zygotes during early warm spells in winter could cause later mass mortality of embryos because of the unfavorable environmental conditions. To make matters worse, if the algae allocated much of their resources for unusual reproductive events, then they may not be able to become fertile during the normal season or to produce healthy propagules. Receptacles from *C. barbata* sampled in May 2019 released 10 times fewer zygotes than during the previous exceptional event in February 2019, and the subsequent growth of germlings from them differed consistently (Figure 4).

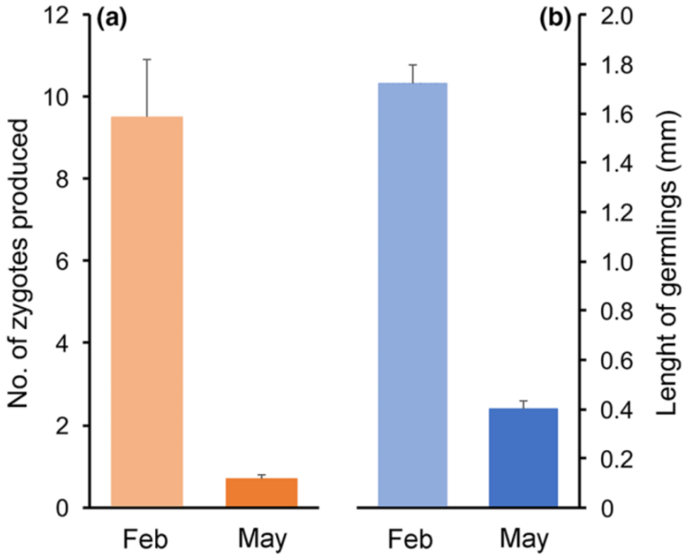

Figure 4 – (a) Mean (SE) number of zygotes produced by receptacles (n = 33) collected in February and May 2019. (b) Mean (SE) length of germlings after 4 weeks (n = 60) from cultured zygotes produced in the two periods.

Intense reservoir depletion under reiterated reproductive efforts could also affect the vegetative phase of adults. Available data corroborated this hypothesis, showing that the average length of *C. barbata* thalli in the study area greatly contracted during the last 15 yr, which coincided with an increased frequency of hot spells (Figure 5).

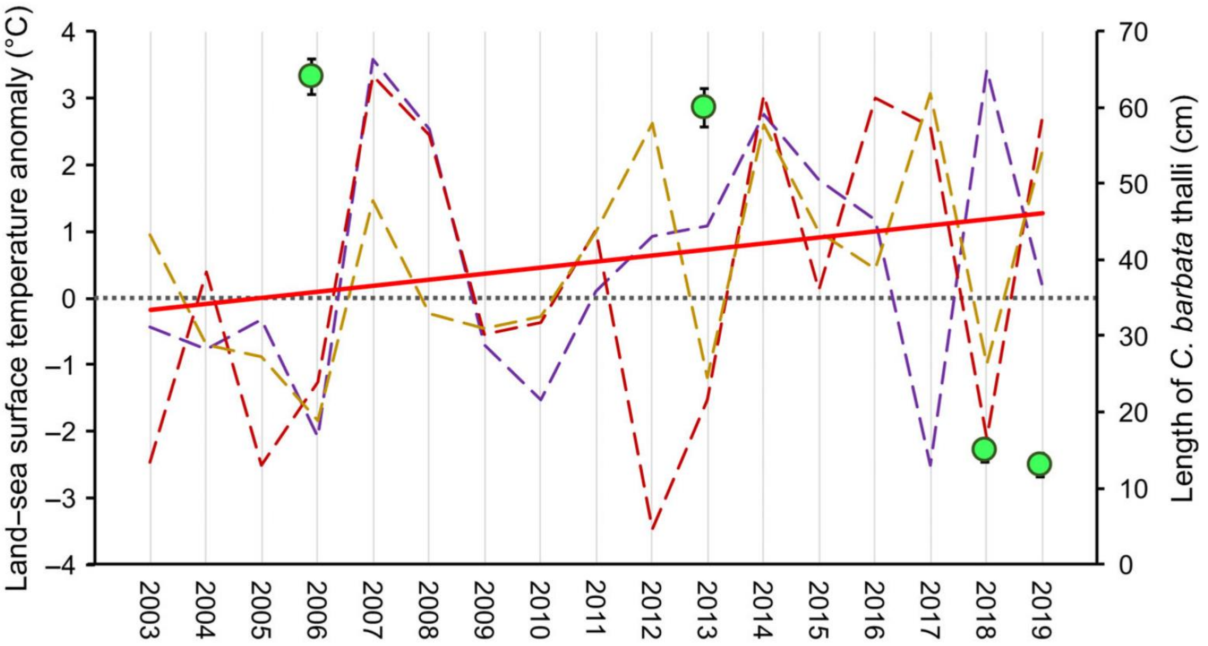

Figure 5 – (Left axis) Land–sea surface temperature anomalies (dashed lines) in the northern Adriatic Sea recorded in January (violet), February (red), and March (gold) in different years from 2003 to 2019. The solid red line indicates the overall trend in temperature increase. Anomalies were based on the average temperature since 1981 (black dotted line); data retrieved from https://www.ncdc.noaa.gov/cag; see supporting information for further details. (Right axis) Mean (SE) length of *C. barbata* thalli (green circles) from Strunjan in 2006, 2013, 2018, and 2019 late spring (n = 35).

In the Mediterranean Sea, direct habitat destruction, pollution, and overgrazing by sea urchins and herbivorous fish are acknowledged as the main drivers of disappearing algal forests, with climate change representing an emerging threat to these ecosystems (Blanfuné et al., 2019). Our observations and preliminary assessments highlighted potential disruptive effects of winter hot spells on reproductive timing, recruitment, and adult survival that could severely affect the persistence of *Cystoseira* populations. However, most reproductive anomalies, like the one we had the chance to observe and report here, are likely to remain unnoticed because of the widespread preference of surveying macroalgae in late spring or summer. Thus, the contribution of climate change and related extreme events, such as heat waves, to the ongoing decline of *Cystoseira* canopies could have been largely overlooked.

Because extreme climatic episodes are increasing in intensity and frequency, implementing coordinated initiatives connecting centers for climate alerts and algologists may shed light on how these phenomena impact population dynamics of *Cystoseira* species, and help current attempts to restore algal forests. The *ex situ* restoration of *Cystoseira* requires three main steps, including the collection of fertile apices in healthy populations, the setup of mesocosms for controlled reproduction and generation of juveniles, and the implant of juveniles in the restoration sites (Falace et al., 2018). Understanding how climate unpredictability can affect the physiology and phenology of *Cystoseira* species is, therefore, crucial. Networks of early warning against extreme events might allow restoration ecologists to be in right place at the right time, in order to monitor phenological responses of *Cystoseira*, collect premature zygotes that can be cultured under controlled conditions and used for implantation, or select lineages with higher plasticity to temperature extremes, increasing the chance for successful restoration actions of these valuable marine habitat formers.

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Supporting Information

Appendix S1

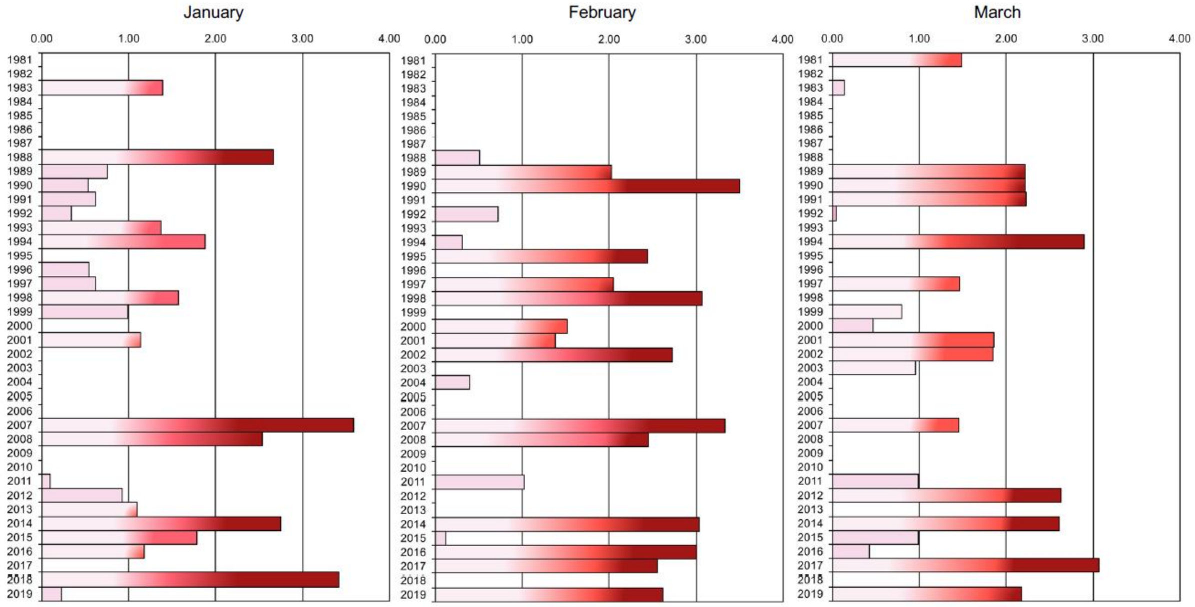

Figure S1 – Land-sea surface temperature anomalies in the Northern Adriatic Sea recorded in January, February, and March in different years from 1981 to 2019. Anomalies were based on the average temperature since 1981. Negative anomalies were not reported. Data retrieved from https://www.ncdc.noaa.gov/cag, accessed in May 2019. Data for the studied region was mined from the Global Historical Climate Network- Monthly (GHCN-M), Europe, and selecting the 5°×5°-degree geographic cell including the Northern Adriatic Sea. Sea surface temperatures are determined using the extended reconstructed sea surface temperature (ERSST) analysis.

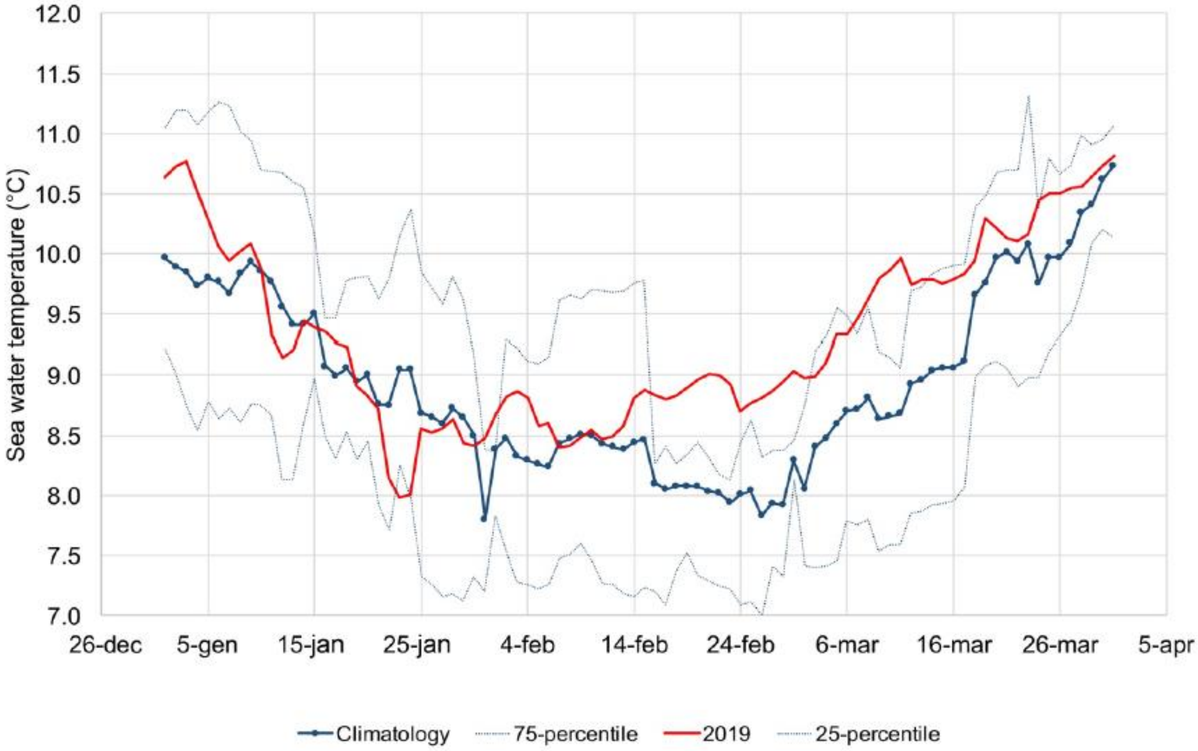

Figure S2 – Sea water temperature (solid red line) recorded from December to April 2019 at 10 m depth by the coastal oceanographic buoy MAMBO-OGS (National Institute of Oceanography and Applied Geophysics) (Italy) positioned in the Northern Adriatic Sea. The solid blue line (Climatology) indicates the average temperature for the same period calculated during the first ten years (1999-2009) of measurements, whereas dotted thin lines are the 25 and 75 percentiles.

Is the south-Mediterranean canopy-forming *Ericaria giacconei* (= *Cystoseira hyblaea*) a loser from ocean warming?

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Abstract

Canopy-forming brown algae support highly productive ecosystems whose decline has been attributed to the interplay of several anthropogenic disturbances. Climate change could have disruptive effects on the biology of these species, but the role of temperature in the development of early life stages is poorly understood. The aim of this study was to assess the response of *Ericaria giacconei*, a winter-reproducing Southern–Mediterranean endemic species, to thermal stress by testing five temperatures (12, 15, 18, 24, and 28 °C) on adults and early stages. Chlorophyll a fluorescence of adult plants was measured at 0, 24, 72, and 120 h on nine fronds in each of the three aquaria per treatment. To assess egg release, zygote settlement, and embryo growth rate, approximately 1.200 receptacles were cultured on six Petri dishes per temperature treatment, and 10 random subsections of 2×2 mm were examined in three Petri dishes at 0, 20, 44, and 92 h after fertilization. Adult plants showed a plastic physiological response, and thermal stress had no significant effect on PSII efficiency. Embryos fully developed only at 12 and 15 °C. Mortality increased at 18 and 24 °C, and no zygotes survived at 28 °C. In a scenario of further increasing temperatures, the effects of warming could affect the recruitment of *E. giacconei* and increase its vulnerability to further stresses. These effects on the survival of early stages, which are the bottleneck for the long-term survival of the species, should be taken into account in conservation and restoration measures to maintain canopy-forming macroalgal populations and associated biodiversity and ecosystem services.

Keywords: thermal stress, early life stages, photosynthetic efficiency, marine forest, climate change, conservation

Introduction

Canopy-forming algae of the order Fucales and Laminariales (Phaeophyceae) are among the most ecologically and socio-economically valuable marine species in temperate waters (Steneck et al., 2002; Smale et al., 2013; Bennett et al., 2015). They provide a structural and trophic framework that supports rich biodiversity by providing food, shelter, and habitat for other associated species (Bustamante et al., 2017; Teagle et al., 2017), and are responsible for nutrient cycling and CO_2 storage (Krause-Jensen & Duarte, 2016; Filbee-Dexter & Wernberg, 2020).

Macroalgal forests are undergoing major regressions worldwide due to a combination of multiple natural and anthropogenic sources of disturbance (Steneck et al., 2002; Strain et al., 2014; Mineur et al., 2015; Krumhansl et al., 2016). In recent years, an increasing number of studies have reported changes in the distribution and abundance of these macroalgal populations as a result of ocean warming and thermal anomalies (especially marine heat waves, MHWs) (e.g., Smale, 2020 and the references therein; Bevilacqua et al., 2019; Savonitto et al., 2021; Verdura et al., 2021). Populations at the edge of their range appear to be particularly affected by this trend (e.g., Viejo et al., 2011; Nicastro et al., 2013; Araújo et al., 2014; Álvarez-Losada et al., 2020; Gurgel et al., 2020).

Thermal anomalies may affect the phenology and physiology of these species, impairing their performance, increasing their vulnerability to other stressors, and eventually leading to population declines and local extinction events (Wernberg et al., 2010, 2016; Gouvêa et al., 2017; de Bettignies et al., 2018). These events could also lead to changes in associated species and their interactions (Vergés et al., 2016; Wernberg et al., 2016; Provost et al., 2017), which may ultimately result in detrimental cascading effects on ecosystem functions and the resulting provision of goods and services (Smale et al., 2013; Vergés et al., 2014; Straub et al., 2019).

To date, most works addressing the effects of heat stress on the physiology and biology of brown algae have been manipulative and laboratory-based, focusing on kelps, with the 'sporophyte phase' being the most studied (e.g., Wilson et al., 2015; Burdett et al., 2019; Nepper-Davidsen et al., 2019; Fernández et al., 2020; Hereward et al., 2020; Diehl et al., 2021; Umanzor et al., 2021). As for the Fucales, the genus *Fucus* is the most extensively studied, and works on adults predominate over those on early life stages (e.g., Strömgren, 1977; Pearson et al., 2009; Jueterbock et al., 2014; Nielsen et al., 2014; Graiff et al., 2015; Mota et al., 2015;

Smolina et al., 2016; Rothäusler et al., 2018; Figueroa et al., 2019). Overall, the trend that emerges is a high sensitivity in the early life stages and a relative ability of adults to grow and survive over broader temperature ranges and to physiologically compensate for thermal stress.

In the Mediterranean Sea, macroalgal forests are dominated by *Cystoseira sensu lato* (*s.l.*) species (Fucales, Phaeophyceae). In recent decades, they have declined or become locally extinct due to anthropogenic pressure (e.g., Thibaut et al., 2005, 2015; Falace et al., 2010; Perkol-Finkel & Airoldi, 2010; Blanfuné et al., 2016). To date, there is little evidence of natural recovery of damaged *Cystoseira* populations (e.g., Munda, 2000; Iveša et al., 2016; Orlando-Bonaca & Rotter, 2018; Medrano et al., 2020), because once losses have occurred, recovery from nearby populations tends to be difficult due to the short dispersal of eggs/zygotes and low connectivity of populations (e.g., Soltan et al., 2001; Buonomo et al., 2017; Capdevila et al., 2018).

There is evidence that thermal anomalies and warming can alter the reproductive phenology, germling growth, and viability of *Cystoseira s.l.* species (Celis-Plá et al., 2017; Capdevila et al., 2018; Savva et al., 2018; Bevilacqua et al., 2019; Campos Cáliz et al., 2019; Mancuso et al., 2019; Savonitto et al., 2021; Verdura et al., 2021). As the Mediterranean Sea is warming faster than the oceans and thermal anomalies occur with increasing intensity, frequency, and duration (Diffenbaugh et al., 2007; Vargas-Yáñez et al., 2008; IPCC, 2019; Pastor et al., 2020; Pisano et al., 2020), examining the response of *Cystoseira s.l.* species to temperature may provide useful insights into their potential future fate under global warming.

The present study focuses on *Ericaria giacconei* Serio et G. Furnari (= *Cystoseira hyblaea* Giaccone), a species endemic to the Sicily Channel (Central Mediterranean Sea) that lives in the intertidal and upper sublittoral at depths of 0.2–1.5 m on semi-exposed and exposed rocky shores. Maximum vegetative and reproductive development occurs in winter, from January to March, when mean seawater temperature at 1 m depth ranges from 14.7 to 16.1 °C. This species was described at Punta D’Aliga (southern coast of Sicily, Italy) (Giaccone, 1985), where it is locally extinct (Cormaci et al., 2012). Its current range is fragmented and restricted to two localities: Cap Bon (Kelibia) along the Northern Tunisian coast (Bouafif et al., 2016) and Portopalo di Capo Passero (Isola delle Correnti) in Southern Italy (present study). Its disappearance from the type locality, its limited range, and the fact that it lives in shallow waters raise concerns about the possible fate of *E. giacconei* in the current warming regime. This species, like other *Cystoseira s.l.* species, is listed in some international agreements (e.g., Barcelona Convention, Directive 92/43/EEC), but these are not legally binding.

The objective of this study is to determine the thermal tolerance of both early developmental stages and adults of *E. giacconei*. Adult photosynthetic efficiency and egg release, zygote settlement, and embryo development were examined at five temperature treatments from 12 to 28 °C. Evidence for the likely response of this species to projected climate change is provided, together with a thorough discussion on its conservation status. Another outcome of this work is the embryology of *E. giacconei*, which has never been described before.

Materials & Methods

Sampling Site

Samples were collected from a semi-exposed rocky shoreline on the southern coast of Sicily (Sicily Channel, Mediterranean Sea: 36° 38’ 49’’ N; 15° 04’ 45’’ E). On the seabed, sandy substrates covered by *Posidonia oceanica* (L.) Delile alternate with shallow rocky reefs dominated by dense and well-structured stands of *E. giacconei* in the upper subtidal. This species also occurs in the intertidal, replacing the typical fringe of *Ericaria amentacea* (C. Agardh) Molinari et Guiry as previously described by Giaccone (1985) at the type locality.

The mean seawater temperature on the Ionian coast of Sicily at 1 m depth is 15.6 °C in winter, with values ranging from 15.1 to 16.7 °C, and 25.5 °C in summer, with values ranging from 22.0 to 27.5 °C (Clementi et al., 2019).

Experimental Set-up

Approximately 6000 receptacles and 135 primary branches (approximately 10 cm long) of *E. giacconei* were collected in March 2020. Samples were wrapped in aluminium foil, stored at 4 °C in the dark and transported to the Phycological Laboratory, University of Trieste, within 24 h after collection. At the laboratory, the receptacles were stored at 4 °C for 36 h, while the adult fronds were acclimatized at 18 °C for 48 h.

Five temperature treatments were replicated in environmentally controlled rooms: 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 and 28 °C, i.e., temperatures the species is normally exposed to in summer. Light intensity was set to 125 mmol photons m^{-2} s^{-1} supplied by LED lamps (AM366 Sicce USA Inc., Knoxville, TN, United States) and measured with a LI-COR LI-190/R Photometer (LICOR-Biosciences, Lincoln, NE, United States); photoperiod was set to 12:12 h light:dark. For each heat treatment, three aquaria were filled with 10 l of filtered seawater (0.22 µm filter membrane), and each aquarium contained nine adult primary branches. Pumps (Sicce Syncra Nano, Sicce S.r.l., Pozzoleone, IT) were placed at the bottom of each aquarium, to ensure that the medium was properly oxygenated. The experiment on adult fronds lasted 120 h (Figure 1).

Early life stages, up to the end of the embryonic stage (i.e., the fall of apical hair; Nienburg, 1931; Galun & Torrey, 1969; Savonitto et al., 2019), were studied for 92 h (Figure 1). Six replicate Petri dishes per treatment were filled with 10 ml of filtered seawater (0.22 µm filter membrane) and incubated at the five temperatures listed above. Each Petri dish was seeded with approximately 200 receptacles. To counteract evaporation, additional aquaria filled with filtered seawater were kept at the same temperatures to refill the experimental aquaria and Petri dishes.

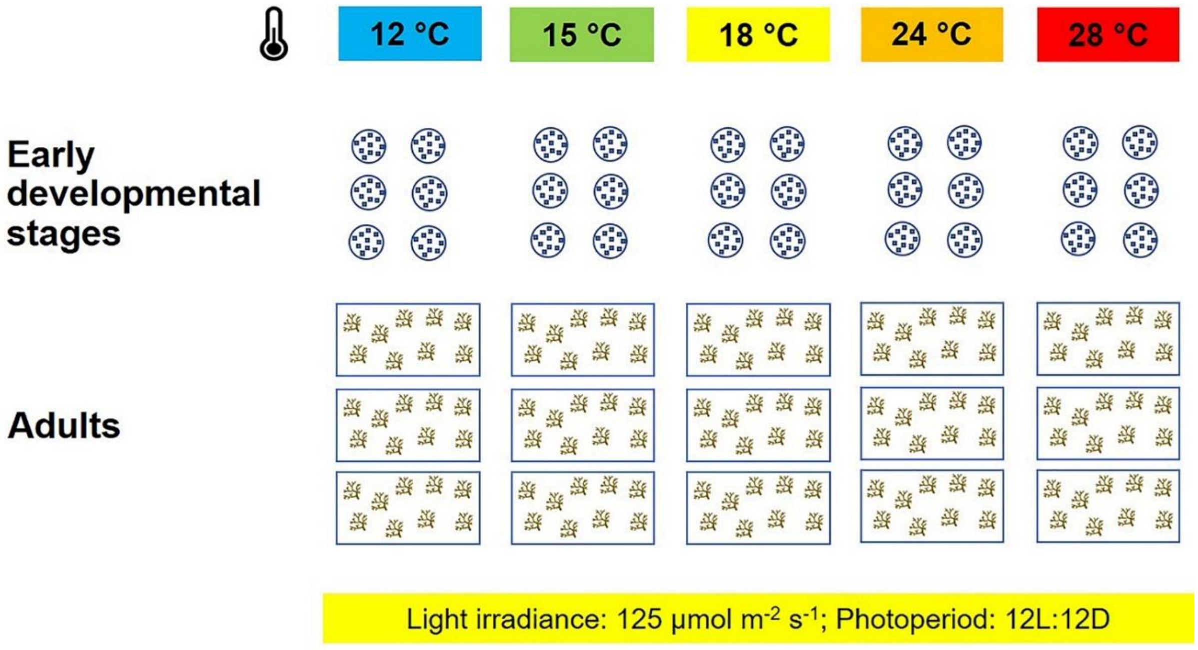

Figure 1 – Experimental setup: for each temperature treatment (°C), six Petri dishes and three aquaria were used to test the thermal tolerance of early life stages and adults of *Ericaria giacconei*.

Response Variables

Adult Plants

Chlorophyll a fluorescence (Chl_aF) of each adult specimen was measured at the end of acclimation (t0) and after 24 h (t1), 72 h (t2) and 120 h (t3) using a Photosynthetic Efficiency Analyzer Fluorimeter Handy-PEA (Hansatech, King’s Lynn, United Kingdom). Measurements were taken after a 30 min dark adaptation using the standard Handy-PEA clip. A saturating redlight pulse of 3500 mmol photons m^{-2} s^{-1} for 0.8 s was emitted to obtain the 0JIP fluorescence transient, i.e., the time resolved Kautsky induction, and hence F_m (transient maximum Chl_aF level). F_0 (minimum Chl_aF level), needed to calculate F_v (variable Chl_aF level, i.e., F_m–F_0) and thus F_v/F_m (maximum quantum yield of PSII photochemistry), was measured 50 ms after the onset of illumination. The performance index (PI_{abs}) was also calculated from the analysis of the Chl_aF transient from F_0 to F_m, the so-called JIP test (Strasser et al., 2000; Bussotti et al., 2010). PI_{abs} is calculated from three independent expressions related to (a) the density of reaction centers, (b) the maximum quantum yield of primary photochemistry, and (c) the efficiency of the electron transport chain between PSII and PSI (Strasser et al., 2000). PI_{abs} is commonly used to test the effects of environmental factors such as temperature, salinity and high intensities of visible and UV-light on the viability and efficiency of the photosynthetic apparatus (Misra et al., 2001).

Early Developmental Stages

Receptacles were removed from Petri dishes after fertilization (AF; 30 h after seeding). To avoid experimental bias and to ensure that the receptacles of all thermal treatments had the same reproductive potential (RP), it was estimated as follows:

$$\boldsymbol{RP\ (conceptacles\ mg^{-1}) = \frac{mean\ no.of\ conceptacles\ receptacle^{-1}}{mean\ receptacle\ dry\ weight}}$$

The number of conceptacles per receptacle was counted under a stereomicroscope (Leica MZ 6, Leica Microsystems, Wetzlar, Germany). Receptacles were then dried at 70 °C for 48 h. To quantify egg release and zygote settlement at different temperatures, 10 subareas of 0.2×0.2 cm^2 in three Petri dishes were randomly selected per treatment and photographed under a stereomicroscope with a Nikon Coolpix 4500 camera (Nikon Corporation, Tokyo, Japan) at each sampling time. To reduce stress on the algae, photographs were taken within a few minutes. Three Petri dishes were randomly selected to assess egg release and the remaining three were used to assess zygote settlement. Photographic sampling was carried out at the time of fertilization (i.e., 30 h after seeding) and 20 h AF. The digital images were analyzed to count the number of specimens in each subarea. The counts were then extrapolated to the entire culture area (i.e., 23.76 cm^2). Release (RE) and settlement (SE) efficiencies were calculated as follows:

$$\boldsymbol{RE\ (eggs\ mg^{-1}) = \frac{no.of\ eggs\ cm^{-2}}{mean\ receptacle\ dry\ weight\ cm^{-2}}}$$

$$\boldsymbol{SE\ (zygotes\ mg^{-1}) = \frac{no.of\ zygotes\ cm^{-2}}{mean\ receptacle\ dry\ weight\ cm^{-2}}}$$

Embryo growth was assessed by taking digital images of 10 randomly selected subareas (0.2×0.2 cm^2) under an inverted microscope (Leica DM IL LED, Leica Microsystems, Wetzlar, Germany) using a Canon Powershot G9 camera (Canon Inc., Tokyo, Japan) at 20, 44, and 92 h AF. 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 (Supplementary Figure 1). To describe the embryo development, additional dedicated slides were seeded with receptacles at 15 °C (i.e., the average seawater temperature during the reproductive period) and observed over time under an inverted microscope.

Statistical Analysis

Repeated-measures ANOVA was used to test the effects of temperature (five levels: 12, 15, 18, 24, and 28 °C) and time (three levels: t1, t2, and t3) on F_0, F_m, F_v/F_m, and PI_{abs} (n = 27). The assumption of normality of response variables was tested with the Shapiro–Wilk test. In all cases, the assumption of normal distribution was fulfilled. Tuckey's HSD post hoc test was used to examine pairwise significant differences between treatment combinations.

One-way ANOVA was performed to test for differences between temperature treatments on RP, RE, and SE. The assumption of normality of response variables was tested with the Shapiro–Wilk test. In all cases, the assumption of normal distribution was fulfilled.

Significant terms were examined by performing a *post hoc* pairwise t-test to compare the different treatments. Cochran's C-test (Underwood, 1997) was used to test the assumption of homogeneity of variances prior to analysis. For RP, data were square root-transformed to remove heterogeneous variances. To explain the observed bell-shaped patterns, a quadratic regression model was fitted to RE and SE against temperature.

Distance-based permutational multivariable analysis of variance (PERMANOVA, Anderson, 2001) was used to test for differences in temporal patterns of embryonic development between treatments. Data from treatments at 28 °C were not included in the analysis since the number of settled zygotes at 20 h AF was extremely low (mean 0.7 zygotes/subarea ± 0.1 SE), and zygote mortality at later sampling times was 100%. The analysis was based on Bray–Curtis dissimilarities (Bray & Curtis, 1957) on untransformed data, and each term in the analysis was tested by 5,000 random permutations. The design for the analysis included two crossed factors: Treatment (Tr, four levels, and fixed) and Time (Ti, three levels, and fixed), with n = 3. Non-metric multidimensional scaling ordination (nMDS) of the Tr×Ti centroids was used to represent the multivariate patterns.

Results

After acclimation, the adult primary branches of *E. giacconei* had F_v/F_m values ranging from 0.606 to 0.768, attesting the viability and good physiological status of the photosynthetic apparatus of the samples. F_v/F_m values were stable throughout the experiment, although slight but significant changes were observed as a function of temperature and time (Figure 2A, Table 1, and Supplementary Table 1). Specifically, at 12 °C F_v/F_m statistically increased over time by 5.3% (Supplementary Table 1). The interaction between temperature and time had a significant effect on F_0 and F_m (Table 1 and Supplementary Table 1); from t1 to t3, both parameters were stable at 18 and 24 °C, whereas F_0 significantly decreased in samples at 12 and 15 °C and F_m at 15 and 28 °C (Figures 2B,C and Supplementary Table 1). PI_{abs} was only affected by temperature (Table 1 and Supplementary Table 1): it was highest at 28 °C and gradually decreased from 24 to 15 °C, with the lowest values at 12 °C at t3 (Table 1, Figure 2D, and Supplementary Table 1).

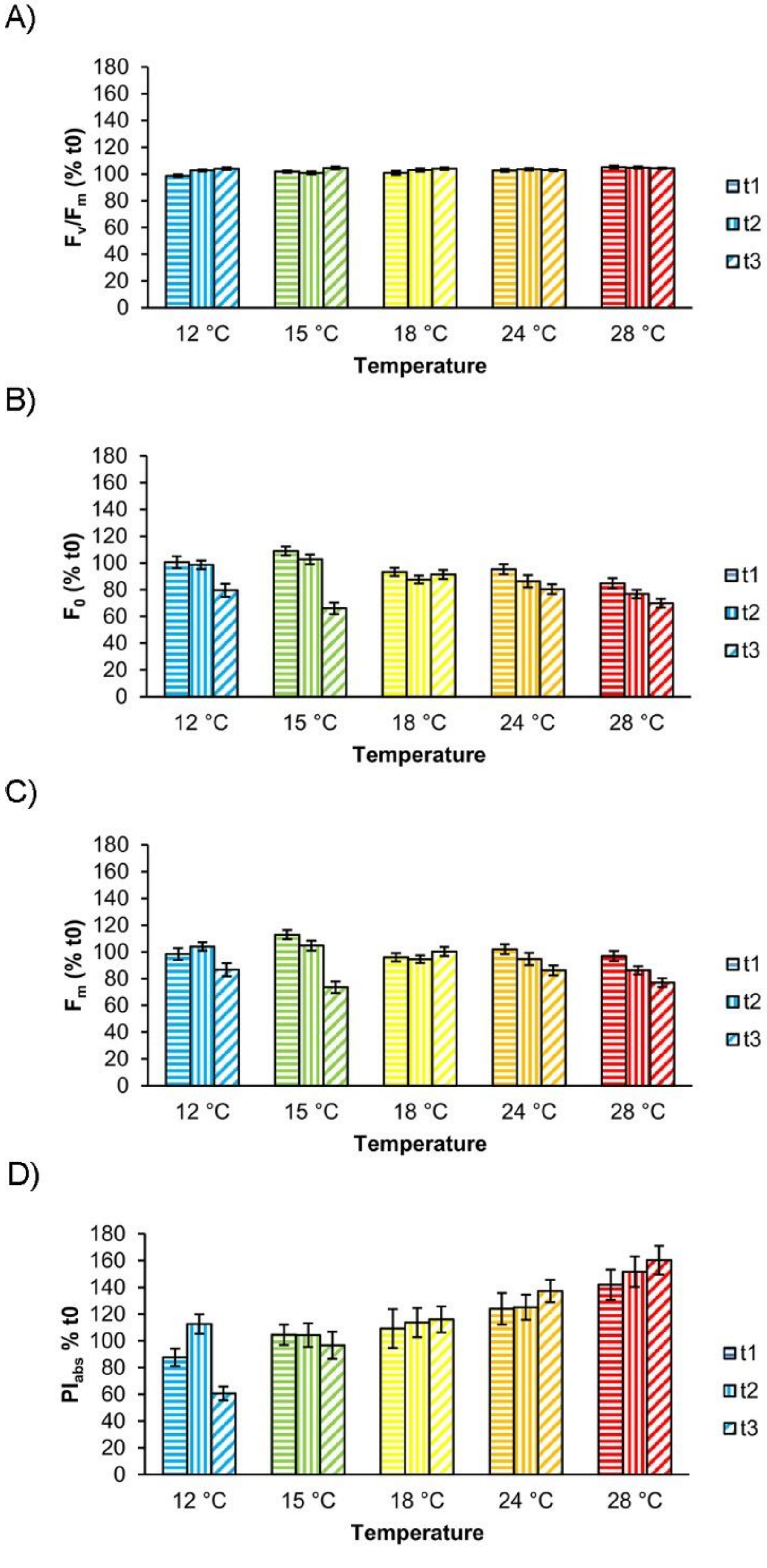

Figure 2 – Chlorophyll a fluorescence parameters of *Ericaria giacconei* adults as a function of temperature: F_v/F_m (A), F_0 (B), F_m (C), and PI_{abs} (D) (color-coded as in Figure 1). Fronds were exposed for 24 (t1), 72 (t2), and 120 h (t3) to the tested temperatures. Values (mean±SE; n = 27) are expressed as percentage (%) of the mean value at t0.

Table 1 – Summary of ANOVAs on F_v/F_m, F_0, F_m, PI_{abs}. ^{NS} = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

			F_v/F_m			F_0			F_m			PI_{abs}		
	Num df	Den df	SS	Error SS	*F*	SS	Error SS	*F*	SS	Error SS	*F*	SS	Error SS	*F*
Time	2	256	232	6787.1	4.3684^{*}	25644	67433	48.6773^{***}	20596	96958	27.1894^{***}	4452	651958	0.8740^{NS}
Treatment	4	128	349	3708.9	3.0138^{*}	13972	43935	10.1769^{***}	6561	49513	4.2402^{**}	197157	362166	17.4203^{***}
Time × Treatment	8	256	368	6787.1	1.7343^{NS}	17155	67433	8.1408^{***}	16436	96958	5.4246^{***}	37618	651958	1.8464^{NS}

Ericaria giacconei has branched pigmented antheridia and ovoid oospheres retained in the conceptacle (Supplementary Figure 2). The following embryological traits were observed: the first and second division of the zygote are parallel to each other, and the third division is perpendicular to the previous ones. The embryo development takes place directly on the substratum: four primary rhizoids are formed fixing it (Supplementary Figure 3).

The RP did not differ significantly among thermal treatments, making them comparable at the beginning of the experiment (Table 2 and Figure 3A). No significant effects of temperature were detected on RE (Figure 3B and Table 2). In contrast, temperature significantly affected SE (Figure 3C and Table 2).

Table 2 – 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	2.148^{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		$W = 0.919^{NS}$			$W = 0.907^{NS}$			0.944^{NS}		
Cochran's *C* test		$C = 0.705^{*}$			$C = 0.533^{NS}$			$C = 0.528^{NS}$		
Transformation		Square root			None			None		

Specifically, SE at 28 °C was lower than all other treatments. RE (Figure 3B) and SE (Figure 3C) showed a bell-shaped response to temperature, and the quadratic model fitted to the data explained 51 and 61% of the variability for RE and SE, respectively (Table 3).

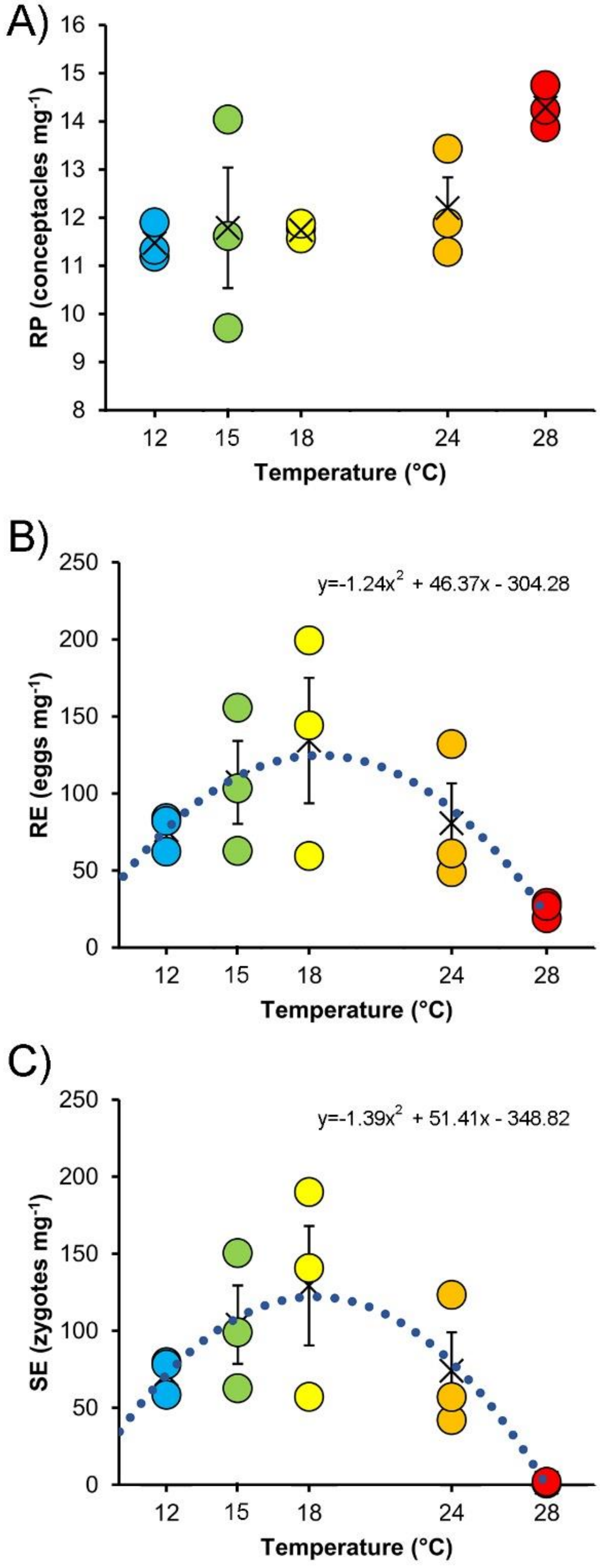

Figure 3 – Mean values (±SE) of reproductive potential (A), release efficiency (B), and settlement efficiency (C) at the different temperatures. The values of each replicate are also indicated (color-coded as in Figure 1). The dotted curves show the quadratic model fitted to the data (see Table 3).

Table 3 – Summary of quadratic regression fitted to data of release and settlement efficiency against temperature.

	Multiple R-squared	*F*	*P*
Release efficiency	0.51	6.272	0.014
Settlement efficiency	0.61	9.577	0.003

PERMANOVA on embryo status revealed a significant Tr×Ti interaction (Table 4), indicating that temporal patterns of embryonic development differed significantly between temperature treatments.

Table 4 – PERMANOVA testing for differences in the proportion of different developmental stages of embryos at varying times and temperature treatments after fertilization. Analysis was based on Bray-Curtis dissimilarities and untransformed data, with 5000 permutations.

Source	df	SS	MS	Pseudo-*F*	*P* (perm)
Time	2	19282.0	9641.0	23.973	0.000
Treatment	3	4104.0	1368.0	3.402	0.000
Time × Treatment	6	18611.0	3102.0	7.713	0.000
Residual	347	139550.0	402.2		

These differences were evident in the nMDS ordination of Tr×Ti centroids (Figure 4). The centroids of 12 and 15 °C clustered alongside those of 18 and 24 °C, the latter also showing marked separation between 20 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 4A). In contrast, multicellular embryos or rhizoids were found in the treatments at 12 and 15 °C in each time interval (Figure 4B), 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 4C).

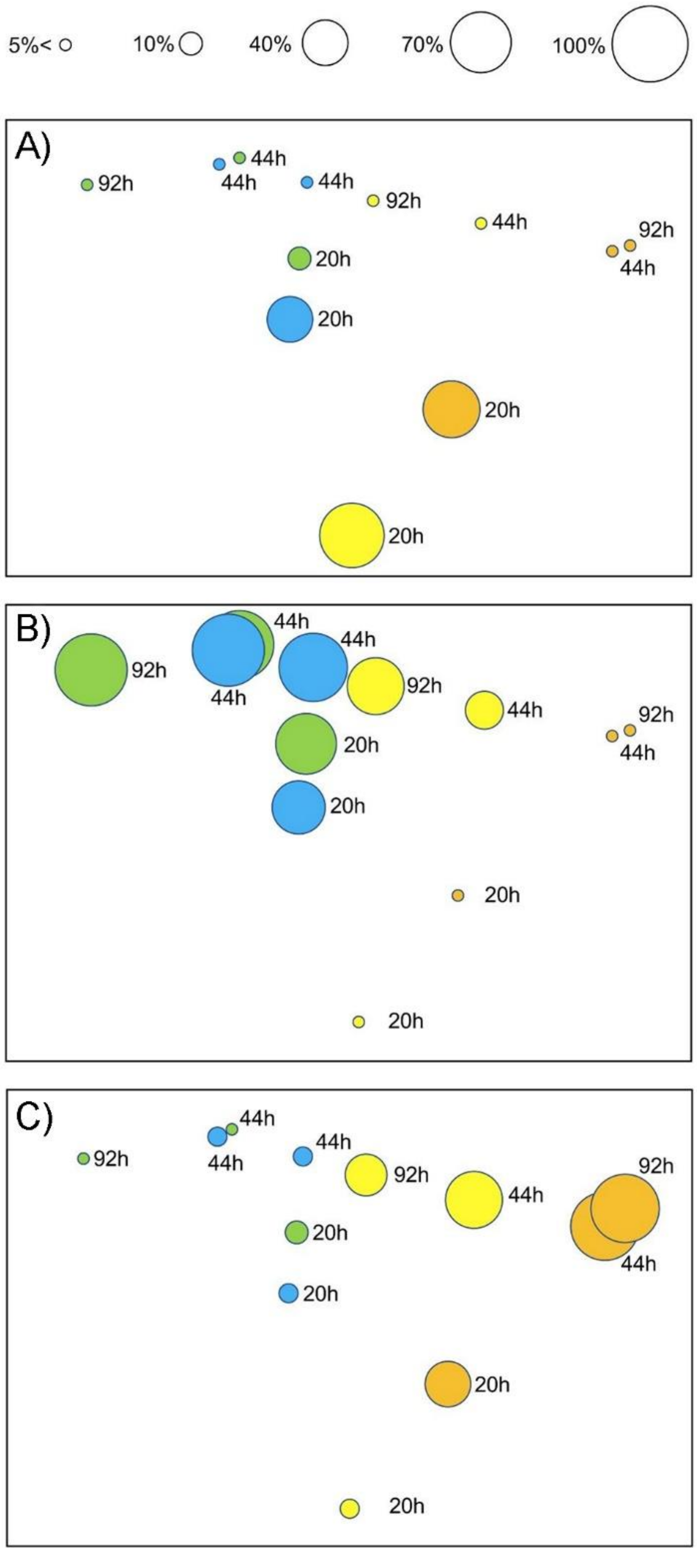

Figure 4 – nMDS ordination of Tr×Ti centroids (stress: 0.04) based on Bray–Curtis dissimilarities (untransformed embryo development data). The ordination plot is presented in three versions highlighting three developmental stages, with superimposed bubbles, indicating the corresponding percentage of 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 1 for 12, 15, 18, and 24 °C).

Discussion

Climate change, coupled with multiple anthropogenic and natural stressors occurring in coastal ecosystems, poses a major threat to the long-term survival of marine forests. From this perspective, studying species vulnerability to temperature stress can provide relevant insights that can be used to make more robust and integrated predictions for marine forest conservation and management.

In our experiment, adults of *E. giacconei* were not negatively affected by temperatures, indicating an expected ability to acclimatize to a wide range of temperatures typical of the Mediterranean Sea and especially the intertidal zone. All temperatures to which thalli were exposed had a statistically significant, but not physiologically relevant effect on F_v/F_m (max increase +5% at 12 °C, from 0.659 to 0.694; max decrease -1% at 28 °C, from 0.731 to 0.725), which remained generally steady and within the range of values indicative of a healthy PSII, i.e., >0.6 (e.g., Celis-Plá et al., 2014; Smolina et al., 2016; Falace et al., 2018b; Savva et al., 2018; Campos Cáliz et al., 2019; Verdura et al., 2021). In contrast, temperatures above 18 °C caused an almost equal decrease in F_0 and F_m (Figures 2B,C).

Photosystem II is considered the most heat-sensitive component of the photosynthetic apparatus, especially at the level of the oxygen-evolving complex (Oukarroum et al., 2016). Impairment of this component leads to a progressive decrease in electrons entering the electron transport chain from PSII until its complete inactivation (Allakhverdiev et al., 2008). Several parameters of the fast Chl_aF transients, such as the maximal and basal fluorescence (F_m and F_0) and the derived maximum quantum yield (F_v/F_m), are the most appropriate tools for detecting early effects of heat stress, as they have been shown to correlate with heat sensitivity/tolerance (Allakhverdiev et al., 2008). In particular, the increase in F_0 is closely related to the temperature at which PSII is inactivated (Yamane et al., 2000). For the aforementioned reasons, this could be interpreted as a transient adaptation of the photosynthetic apparatus to the temperature change rather than heat stress.

Several works reporting the effects of temperature on photosynthetic efficiency of brown algae have shown that adults are generally tolerant of temperature fluctuations. For instance, Chl_aF of *E. selaginoides* adults was not affected after exposure to temperatures up to 28 °C for 15 days (Campos Cáliz et al., 2019). Savva et al. (2018) reported that F_v/F_m of *Cystoseira compressa* exposed from 12 to 34 °C maintained values close to the optimum in the range of

19.2–30.9 °C. Similarly, Mancuso et al. (2019) observed an increase in F_v/F_m in the field up to 28 °C when the algae were submerged, and a marked decrease during tidal emersion only when air temperature exceeded 28 °C. Accordingly, populations of *Fucus serratus* from southern areas of North Atlantic showed a decrease in PI_{abs} only when temperatures ranged from 28 to 36 °C (Jueterbock et al., 2014), although *F. serratus* is a coldaffine species. In our case, adults of *E. giacconei* showed higher PI_{abs} at the upper extreme of the tested temperature range (Figure 2D), suggesting that they have better PSII efficiency in warm seasons. Negative effects on Chl_aF parameters were observed in *Fucus distichus* only when thalli were exposed to temperatures 10–15 °C above their optimum (Smolina et al., 2016), and in *E. selaginoides* when dissolved CO_2 and nutrients were also altered (Celis-Plá et al., 2017).

The tolerance of adult thalli of *E. giacconei* and the other intertidal *Cystoseira s.l.* species to temperatures up to 28 °C might be related to an adaptation to the highly dynamic habitat they colonize. Indeed, the intertidal is characterized by large temperature fluctuations due to tidal cycles, especially during the warmer months. Notably, during summer tidal cycles, at the site where *E. giacconei* was sampled, these algae can experience temperatures ranging from 28 °C (seawater temperature) at 1 m depth at high tide to 41 °C (air temperature) at low tide within a few hours (Servizio Informativo Agrometereologico Siciliano, 1995; Clementi et al., 2019). In contrast, species that are not adapted to such extreme environmental changes might be more sensitive to temperature increases. For example, Verdura et al. (2021) reported that adults of the subtidal species Ericaria crinita showed a marked decrease in biomass, F_v/F_m, and C:N ratio during a 30-day period at 28 °C. Similarly, Sato et al. (2020) observed a decrease in PSII efficiency in the subtidal kelp *Saccharina sculpera* maintained at temperatures ≥28 °C, while the optimal range for the tested population was 22– 24 °C.

Despite the high tolerance of *Cystoseira s.l.* adults, especially of intertidal species, to temperature fluctuations, little is known about the possible effects on early developmental stages and developmental processes. Apart from the oldest embryological studies (e.g., Guern, 1962; Colombo et al., 1982; Gil-Rodríguez et al., 1988; Motta et al., 1988; Alongi et al., 1999), the embryogenesis of many *Cystoseira s.l.* species is still poorly known (Falace et al., 2018a; Savonitto et al., 2019). Based on reproductive traits and zygote division sequence, *E. giacconei* fits into the first embryological group described by Guern (1962), which includes most *Cystoseira s.l.* species (e.g., *Ericaria mediterranea*, *Gongolaria elegans*, and *E. selaginoides*).

Regarding the effect of seawater temperature on early developmental stages, we found that the eggs' release efficiency did not vary significantly among the tested temperatures. However, greater exudate production was observed at higher temperatures (Supplementary Figure 4). Exudates, typically phlorotannins, are released by macroalgae under stress conditions (Sieburth & Jensen, 1969; Kroes, 1970; Abdala-Díaz et al., 2006). The settlement efficiency of the zygotes of *E. giacconei* increased from 12 to 18 °C, but no statistically significant difference was found, then it started to decrease (24 °C) and dropped significantly at 28 °C. Remarkably, the extremely low settlement efficiency at 28 °C was due to the fact that eggs and zygotes had undergone cell lysis and clustered together (Supplementary Figure 5).

The detrimental effect of heat was even more pronounced during germling development. 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 observed at 15 °C (highest percentage of embryos with rhizoids already after 20 h AF) suggests that this temperature represents the thermal optimum for reproduction and development of the early life stages. Actually, it corresponds to the mean seawater temperature during the winter months when the species reproduces.

To date, very few studies have investigated the potential effects of warming on the early life stages and in adults of *Cystoseira s.l.* species (e.g., Campos Cáliz et al., 2019; Capdevila 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. In particular, a tolerance threshold of 24 °C was found in *Ericaria zosteroides* (as *C. zosteroides*), a deep-sea species (Capdevila et al., 2019), and 28 °C in *Ericaria selaginoides* (as *C. tamariscifolia*) (Campos Cáliz et al., 2019) and *Ericaria crinita* (Verdura et al., 2021), two species from shallower waters. These results are only partially consistent with ours, as almost all germlings in this study failed to settle or survive at 28 °C. However, in contrast to previous studies, we tested a broader temperature range and found that although *E. giacconei* is an intertidal to upper sublittoral species endemic to the southern Mediterranean, and thus hypothetically adapted to high temperatures, its thermal optimum is at much lower temperatures (12–15 °C) than the other *Cystoseira s.l.* species examined.

Our findings suggest that *E. giacconei* is a stenothermic, cold adapted macroalga that requires an extremely narrow range of low temperatures for embryonic development and survival. These results support the findings of Bouafif & Langar (2019) who, by modeling the potential spatial distribution of *Cystoseira s.l.* species in Tunisia, reported that *E. giacconei* occurs only in the colder waters of northern Tunisia. Sites where *E. giacconei* thrives could represent climatic refugia where the species still survives (e.g., Lourenço et al., 2016; Abelson et al., 2020; Verdura et al., 2021). The Sicilian Channel is characterized by a surface current called "Modified Atlantic Water" (MAW), forming two flows: one along the Sicilian shelf and the other off the Tunisian coast (Robinson et al., 1999; Béranger et al., 2004; Jouini et al., 2016). The complex bathymetry, as well as the water circulation, favor a semi-permanent upwelling regime, which is enhanced by local winds (e.g., Mistral) along the southern coast of Sicily. Therefore, the interplay of surface currents and upwelling provides lower sea surface temperatures along the coast (Raffa et al., 2017), but cannot prevent the occurrence of adverse climatic conditions.

Exceptionally high temperatures for several consecutive days during the reproductive season (e.g., Supplementary Figure 6) may actually lead to massive mortality of zygotes/embryos, thus defeating the reproductive efforts of the species. Furthermore, the negative effects of warming on recruitment could be exacerbated by other stressors that have been shown to negatively affect the early developmental stages of *Cystoseira s.l.*, such as herbicides and pollutants (de Caralt et al., 2020).

From this point of view, the recruitment of new individuals seems to be the real bottleneck for the population dynamics of *E. giacconei*, as recruitment failures, if they occur over several years, can lead to lower population densities, ultimately affecting their long-term survival.

The stenothermic nature of the early life stages and the warmer sea areas that evenly surround the few localities with favorable conditions make this species a dotted endemism (Giaccone & Di Martino, 1996). Consequently, *E. giacconei* may become extinct if climate change continues with the current pattern. In the Sicilian Channel, several studies have already reported the disappearance of infralittoral stenoecious species of *Cystoseira s.l.* due to the increase in sea surface temperature and changes in deep circulation (Alongi et al., 2004; Catra et al., 2006; Serio et al., 2006), further evidence of the tropicalization process caused by climate change affecting the Mediterranean Sea (Boero et al., 2008; Furnari & Cormaci, 2009; Marbà et al., 2015).

Together with all Mediterranean species of *Cystoseira s.l.* (except *C. compressa*), *E. giacconei* is included in the "List of Threatened or Endangered Species" of Barcelona Convention (modified Annex II of the "Protocol on Specially Protected Areas and Biological Diversity"; United Nations Environment Agency, 2019; Verlaque et al., 2019), but its conservation status has not yet been defined by the IUCN (like the fucoid *Sargassum*, see Thibaut et al., 2016). In our opinion, *E. giacconei* should be included in the IUCN Red List of Species (International Union for Conservation of Nature, 2021) and classified as Critically Endangered due to its limited distribution and high vulnerability. As a conservation strategy, the climate refugia that ensure the persistence of *E. giacconei* should receive the highest level of protection.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author Contributions

AF, MT, and GA conceived the ideas and designed the methodology. GM and GA collected samples in the field. AF, GS, and MS performed the experiments in aquaria. FC and SB performed the statistical analysis. AF led the writing of the manuscript. AF, GM, GS, MS, FC, SB, and MT contributed significantly to the draft of the manuscript and approved the submitted version. All authors contributed to the article and approved the submitted version.

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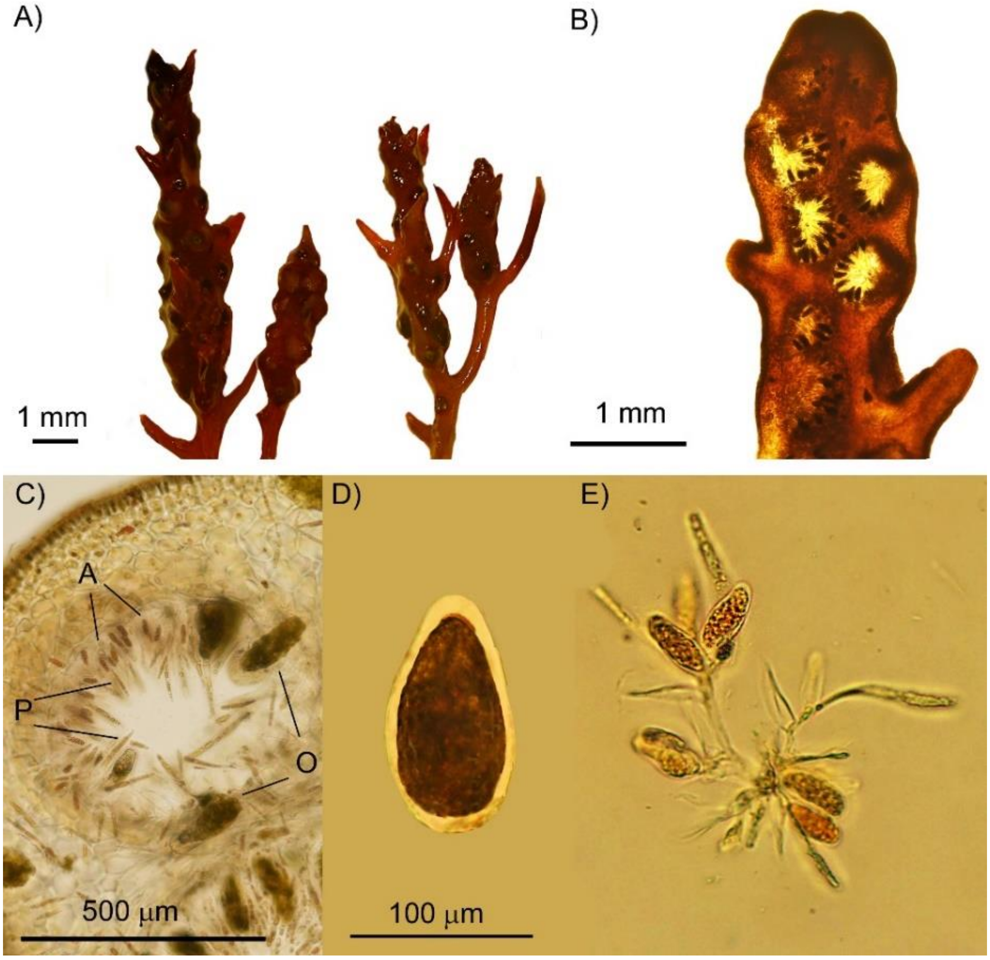
Supporting information

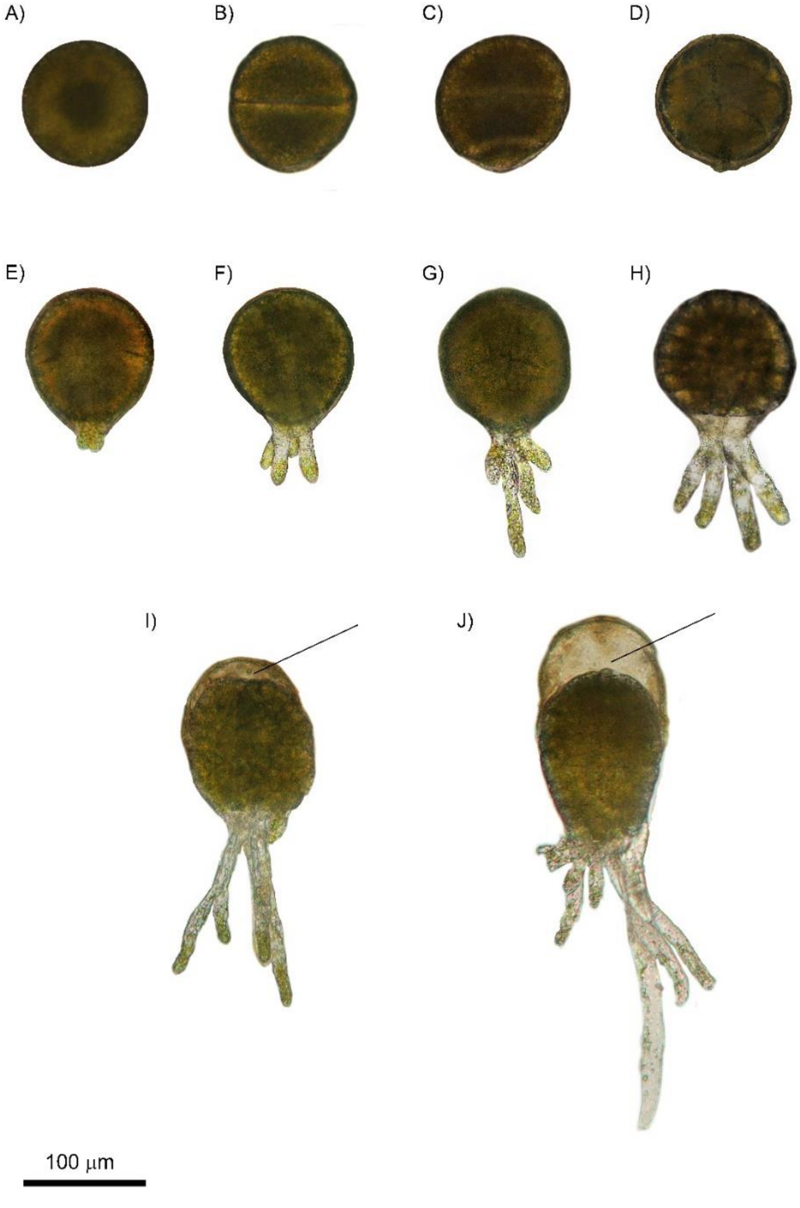
Supplementary Figures and Tables

Supplementary Figures

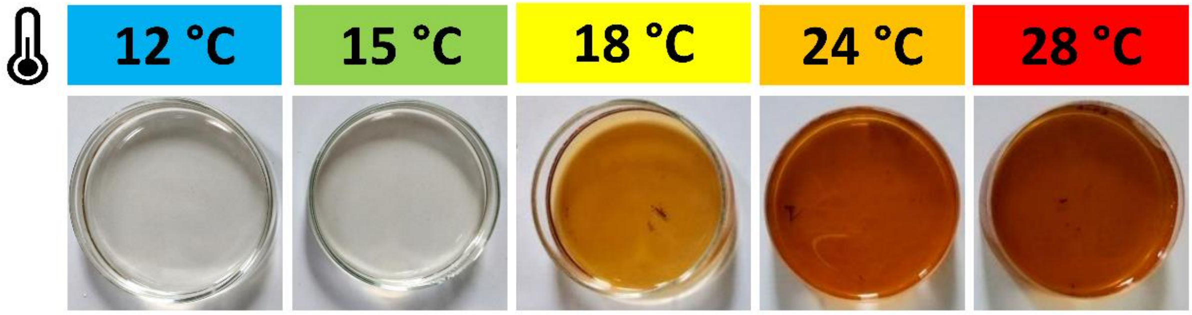
Supplementary Figure 1 – Developmental stages considered in the study of the effects of temperature on the embryo development of *Ericaria giacconei*.



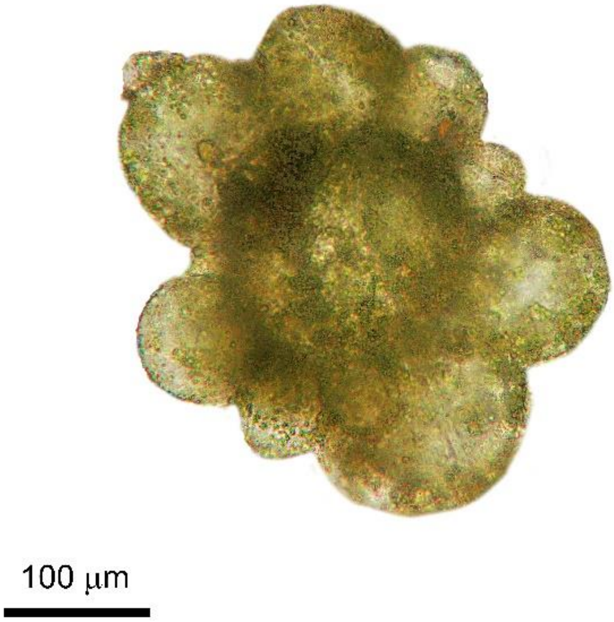
Supplementary Figure 2 – *Ericaria giacconei*: A) receptacles (length = 3-10 mm; width = 1-1.7 mm) with spiny appendages; B) longitudinal section through a receptacle; C) transverse section through a conceptacle (length = 554.8 ± 64.0 µm; width = 514.9 ± 49.5 µm). Black lines show the oval sessile oogonia on the underside of the conceptacle (O), antheridia located on both lateral walls and underside of conceptacle (A) and paraphyses (P). Basal hairs were never observed; D) oogonia (length = 157.9 ± 11.8 µm; width = 67.1 ± 3.8 µm); E) branched antheridia (length = 53.2 ± 4.2 µm; width = 16.7 ± 2.7 µm). The antheridia contain antherozoids with orange stigma due to carotenoids.



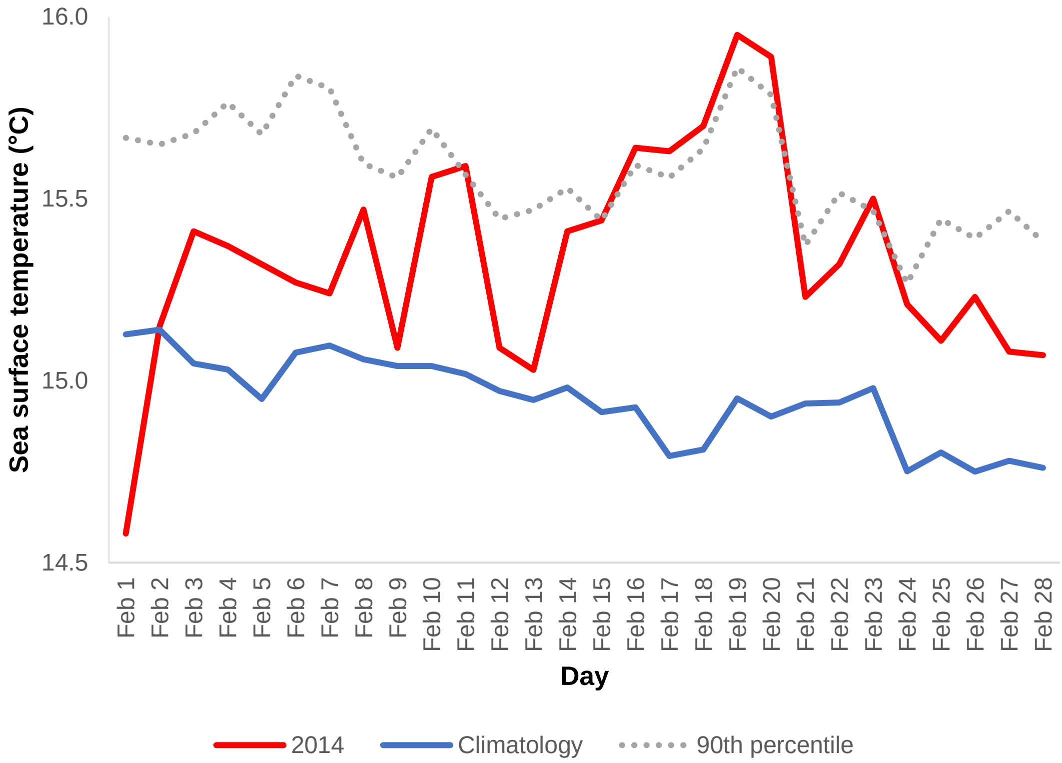
Supplementary Figure 3 – Embryogenesis of *Ericaria giacconei*. After fertilization, A) the zygote, which has a spherical shape and a diameter of ca. 95 ± 5.4 µm, adheres to the substrate through the fertilization membrane. Subsequently, the zygote begins to divide, and B) the first division occurs in an equatorial position, forming two cells of equal size. C) the second division occurs parallel to the first and isolates the rhizoidal pole, giving rise to the rhizoidal cell. The third division occurs at the opposite pole and is perpendicular to the previous divisions. Successively (D-H), the embryo begins to actively divide, and 4 primary rhizoids are formed from the rhizoid cell. After a few days (I-J), the fertilization membrane begins to detach, and the embryo increases its volume and assumes an elongated shape. Finally, a small hyaline hair appears from an invagination at the apical pole (indicated by the black line), and the fertilization membrane is released.



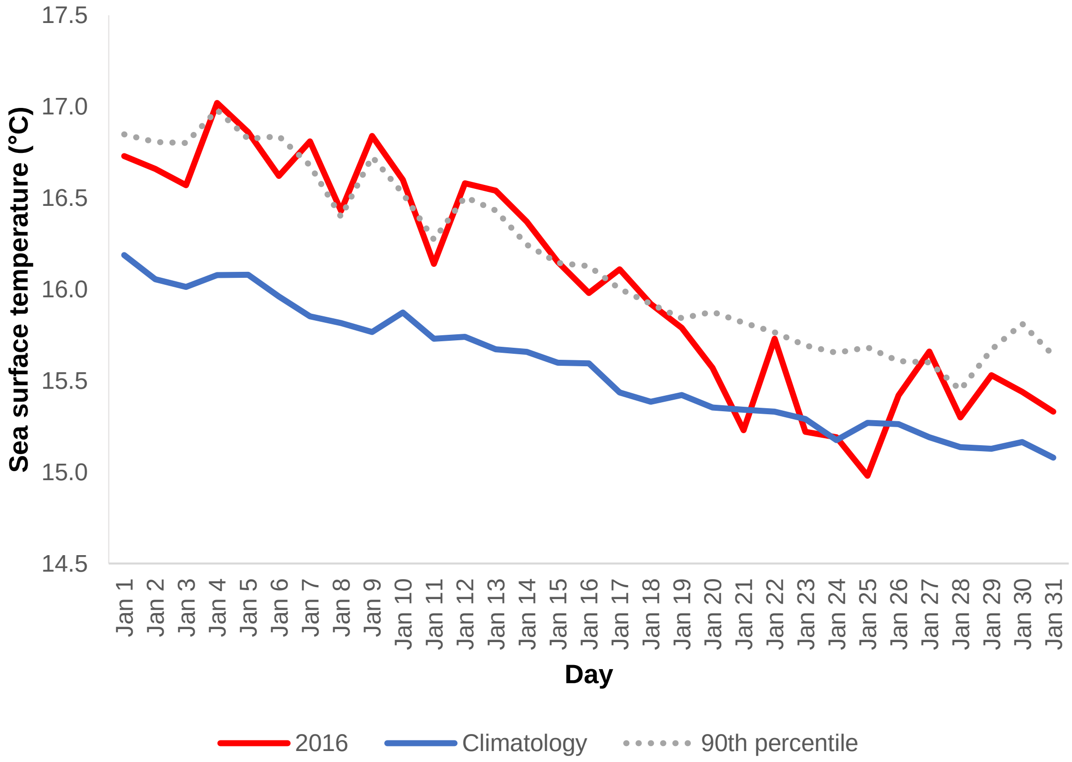
Supplementary Figure 4 – Exudates, collected from experimental Petri dishes under various temperature treatments.



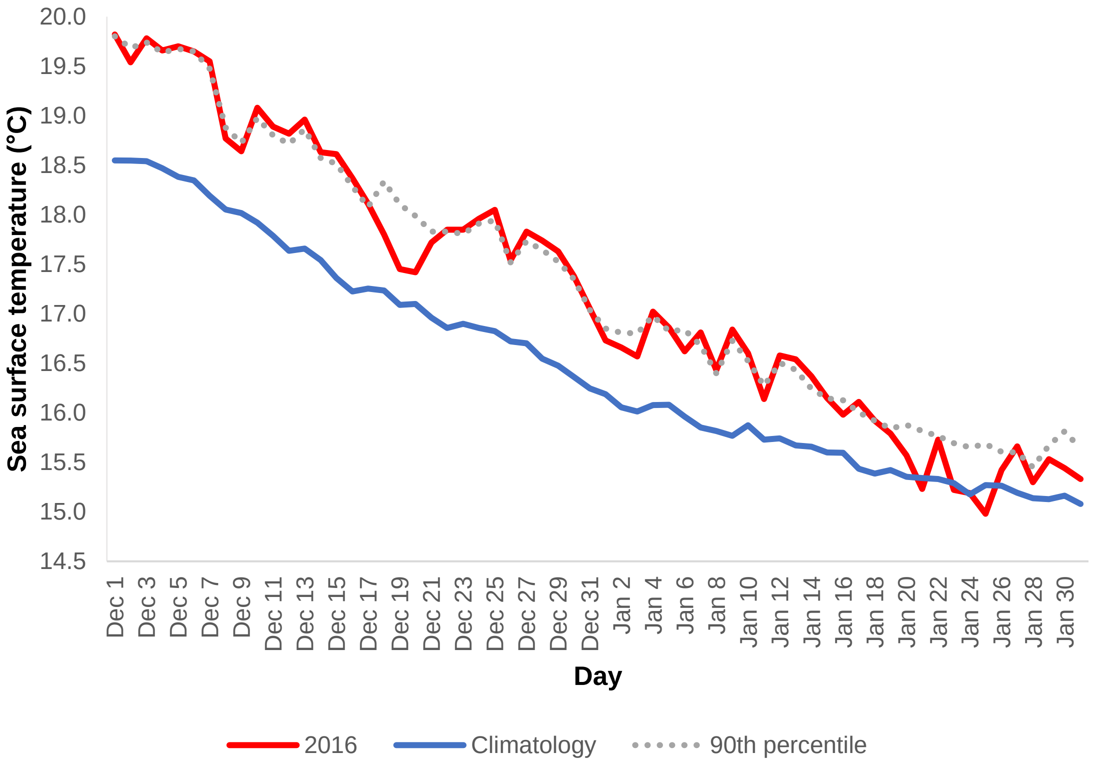
Supplementary Figure 5 – Clustered dead zygotes of *E. giacconei* at 28 °C.


16.0
15.5
15.0
14.5
Sea surface temperature (°C)
Feb 1
Feb 2
Feb 3
Feb 4
Feb 5
Feb 6
Feb 7
Feb 8
Feb 9
Feb 10
Feb 11
Feb 12
Feb 13
Feb 14
Feb 15
Feb 16
Feb 17
Feb 18
Feb 19
Feb 20
Feb 21
Feb 22
Feb 23
Feb 24
Feb 25
Feb 26
Feb 27
Feb 28
Day
2014
Climatology
90th percentile

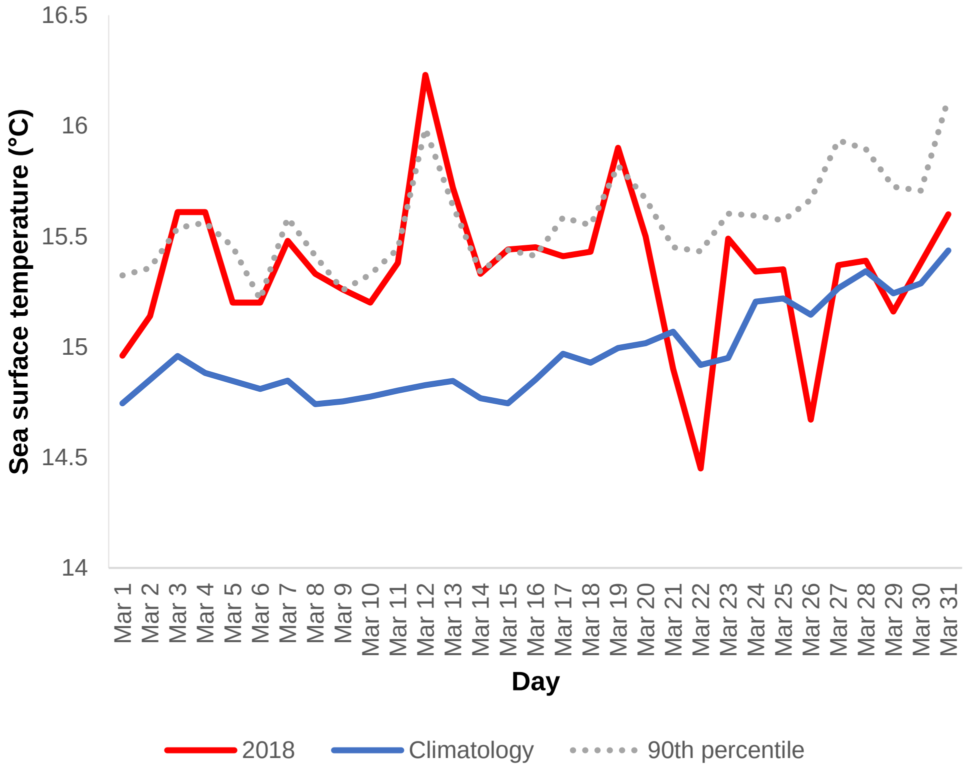
a)


17.5
17.0
16.5
16.0
15.5
15.0
14.5
Sea surface temperature (°C)
Jan 1
Jan 2
Jan 3
Jan 4
Jan 5
Jan 6
Jan 7
Jan 8
Jan 9
Jan 10
Jan 11
Jan 12
Jan 13
Jan 14
Jan 15
Jan 16
Jan 17
Jan 18
Jan 19
Jan 20
Jan 21
Jan 22
Jan 23
Jan 24
Jan 25
Jan 26
Jan 27
Jan 28
Jan 29
Jan 30
Jan 31
Day
2016
Climatology
90th percentile

b)


Sea surface temperature (°C)
20.0
19.5
19.0
18.5
18.0
17.5
17.0
16.5
16.0
15.5
15.0
14.5
Dec 1
Dec 3
Dec 5
Dec 7
Dec 9
Dec 11
Dec 13
Dec 15
Dec 17
Dec 19
Dec 21
Dec 23
Dec 25
Dec 27
Dec 29
Dec 31
Jan 2
Jan 4
Jan 6
Jan 8
Jan 10
Jan 12
Jan 14
Jan 16
Jan 18
Jan 20
Jan 22
Jan 24
Jan 26
Jan 28
Jan 30
Day
2016
Climatology
90th percentile

c)


Sea surface temperature (°C)
16.5
16
15.5
15
14.5
14
Mar 1
Mar 2
Mar 3
Mar 4
Mar 5
Mar 6
Mar 7
Mar 8
Mar 9
Mar 10
Mar 11
Mar 12
Mar 13
Mar 14
Mar 15
Mar 16
Mar 17
Mar 18
Mar 19
Mar 20
Mar 21
Mar 22
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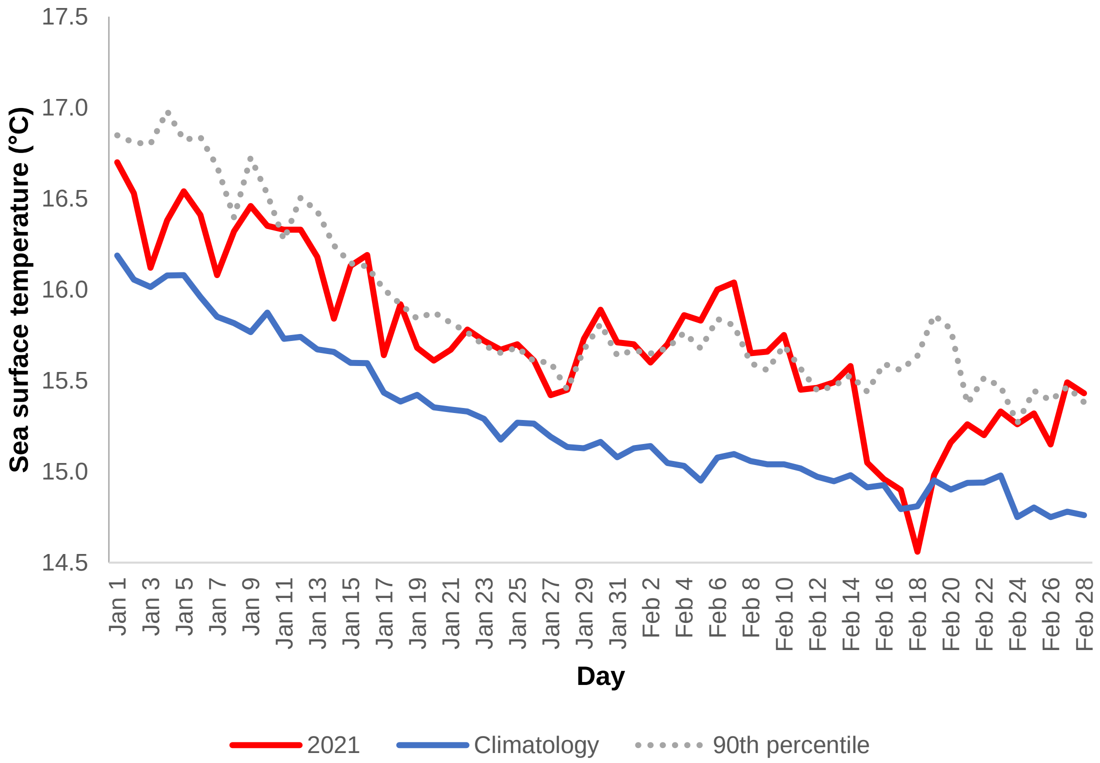
Mar 24
Mar 25
Mar 26
Mar 27
Mar 28
Mar 29
Mar 30
Mar 31
Day
2018
Climatology
90th percentile

d)



e)

Supplementary Figure 6 – Most relevant anomalies in mean daily sea surface temperature observed in the winter months (December, January, February, March) during 2010-2021 in the study site (southern coast of Sicily, Sicily Channel, Mediterranean Sea): a) February 2014; b) January 2016; c) December 2016 - January 2017; d) March 2018; e) January-February 2021. The red line indicates the mean daily temperature; the blue line (Climatology) indicates the mean daily temperature during 2010-2021, the grey dotted line indicates the 90th percentile of daily temperature during 2010-2021. Credits: E.U. Copernicus Marine Service Information.

Supplementary Tables

Supplementary Table 1 – Mean ± SE values of F_v/F_m, F_0, F_m, PI_{abs} of adults of *E. giacconei*. All values are reported as percentage (%) of the mean value at t0. Different letters indicate significant differences as determined using ANOVA followed by Tukey's HSD post hoc test ($P<0.05$).

	F_v/F_m		F_0		F_m		PI_{abs}	
t1								
12 °C	98.7	± 1.1 ^{a}	100.6	± 4.2 ^{fgh}	98.3	± 4.4 ^{cde}	87.6	± 6.5 ^{cde}
15 °C	101.8	± 0.9 ^{ab}	108.9	± 3.0 ^{h}	112.9	± 3.4 ^{e}	104.5	± 7.7 ^{bcde}
18 °C	100.9	± 1.5 ^{ab}	93.2	± 2.8 ^{defgh}	96.0	± 3.1 ^{cde}	109.2	± 14.5 ^{bcd}
24 °C	102.7	± 1.1 ^{ab}	95.3	± 3.5 ^{defgh}	102.0	± 3.7 ^{cde}	123.9	± 11.8 ^{abcd}
28 °C	105.1	± 1.1 ^{b}	84.8	± 3.1 ^{bcde}	96.8	± 3.8 ^{cde}	141.9	± 11.4 ^{ab}
t2								
12 °C	102.7	± 0.7 $_{ab}$	98.6	± 2.7 $_{efgh}$	104.0	± 3.2 ^{cde}	112.5	± 7.3 ^{de}
15 °C	100.8	± 1.0 $_{ab}$	102.7	± 3.1 $_{gh}$	104.7	± 3.7 ^{de}	104.2	± 8.8 ^{bcde}
18 °C	103.0	± 1.2 $_{ab}$	87.6	± 2.1 $_{cdefg}$	94.5	± 2.9 ^{bcd}	113.6	± 10.9 ^{bcde}
24 °C	103.5	± 0.9 $_{b}$	86.3	± 3.7 $_{cdef}$	94.7	± 4.6 ^{bcd}	125.1	± 9.4 ^{abc}
28 °C	104.8	± 0.9 $_{b}$	76.8	± 2.7 $_{abc}$	86.2	± 3.1 ^{abc}	151.7	± 11.3 ^{bcd}
t3								
12 °C	104.0	± 1.0 ^{b}	79.6	± 4.6 ^{abcd}	86.6	± 4.9 ^{abcd}	60.6	± 5.1 ^{e}
15 °C	104.5	± 1.1 ^{b}	66.0	± 3.4 ^{a}	73.6	± 4.3 ^{a}	96.6	± 10.1 ^{cde}
18 °C	104.0	± 1.0 ^{b}	91.3	± 3.3 ^{cdefg}	100.3	± 3.4 ^{cde}	116.0	± 9.8 ^{bcde}
24 °C	103.0	± 0.7 ^{ab}	80.4	± 3.1 ^{abcd}	86.2	± 3.6 ^{abc}	137.2	± 8.4 ^{abc}
28 °C	104.3	± 0.6 ^{b}	69.9	± 3.1 ^{ab}	77.0	± 3.3 ^{ab}	160.3	± 10.8 ^{a}

PART II – APPLYING METHODS FOR THE RESTORATION OF MARINE FORESTS

Addressing reproductive stochasticity and grazing impacts in the restoration of a canopy-forming brown alga by implementing mitigation solutions

Aquatic Conservation: Marine and Freshwater Ecosystems (2021), 1-13

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Abstract

1. The worldwide decline of marine forests, due to human impacts and climate change, emphasizes the need to develop and implement effective and sustainable solutions to restore these endangered habitats and to re-establish the services they provide.

2. In this study, the *ex situ* restoration of *Gongolaria barbata* (= *Treptacantha barbata*), a Mediterranean subtidal habitat-forming species of brown seaweed, was for the first time implemented in a marine protected area in the Adriatic Sea. Two restoration efforts were performed in 2019. The first was started in winter, after a marine heatwave that triggered early fertility, and the second in spring, when the species usually reproduces.

3. This study aimed to evaluate: (i) the disruptive effects of a thermal anomaly on the reproductive biology and performance in culture of *G. barbata*; and (ii) the impact of the grazing pressure on juveniles after the outplanting.

4. The first cultivation was more productive than the second, in terms of zygote release and germling growth. To mitigate the low efficiency of the second culture and to avoid prolonged highly demanding maintenance in the mesocosms, the cultivation period was extended outdoors using a structure suspended in the water column.

5. The modular frames conceived for outplanting *G. barbata* proved to be effective because of their easy operability and low cost. Controlling for herbivorous fish had significant positive effects on both juvenile survival and growth.

6. The outcomes highlighted that an unpredictable climatic event and fish grazing were major threats that impaired the restoration process of *G. barbata*. These stressors should be considered when developing plans to implement effective large-scale restoration of canopy-forming macroalgae.

Keywords: climate change, early life stages, *ex situ* restoration, fucoid, heatwave, macroalgae, marine forests, phenology, subtidal

Introduction

In the last 4 decades, the decline of *Cystoseira sensu lato* (henceforth referred to as *Cystoseira*) (Fucales, Phaeophyceae) forests in the Mediterranean Sea has been widely documented (e.g. Munda, 1980; 1982; 1993a; Hoffman et al., 1988; Cormaci & Furnari, 1999; Thibaut et al., 2005; 2015a; 2015b; Mangialajo et al., 2008; Blanfuné et al., 2016; Valdazo et al., 2017; Mancuso et al., 2018) and has been mainly attributed to the interplay of several human impacts on the coastal environment, such as habitat destruction, pollution and overgrazing by sea urchins and herbivorous fish (e.g. Bellan-Santini, 1966; Arnoux & Bellan-Santini, 1972; Munda, 1974; 1982; 1993b; Verlaque, 1987; Hoffman et al., 1988; Chryssovergis & Panayotidis, 1995; Cormaci & Furnari, 1999; Benedetti-Cecchi et al., 2001; Soltan et al., 2001; Hereu, 2004; Sales & Ballesteros, 2009; Sales et al., 2011; Gianni et al., 2013; Pinedo et al., 2013; Strain et al., 2014). Recent studies have also suggested or reported the disruptive effect of climate change on the distribution, abundance and biology of these species (e.g., Buonomo et al., 2018; Bevilacqua et al., 2019; Campos Cáliz et al., 2019; Capdevila et al., 2019).

Cystoseira forests provide critical ecosystem services in littoral habitats by increasing the complexity of rocky bottoms and thus supporting rich biodiversity and complex food webs (e.g. Sauvageau, 1912; Funk, 1927; Boudouresque & Lück, 1972; Giaccone, 1973; Ballesteros, 1988; Rull & Gómez Garreta, 1989; Otero-Schmitt & Pérez-Cirera, 1996; Ballesteros et al., 1998; Pizzuto, 1999; Ballesteros et al., 2009; Cheminée et al., 2013; Pitacco et al., 2014; Chiarore et al., 2019; Cuadros et al., 2019).

Due to their compromised status and key ecological role, all Mediterranean *Cystoseira* species (except *Cystoseira compressa*) are included on the “List of endangered or threatened species” of the Barcelona Convention (amended Annex II of the “Protocol concerning Specially Protected Areas and biological diversity”; UNEP, 2019), and some species are ‘strictly protected’ by the Bern Convention (Appendix I; Council of Europe, 1979). In addition, *Cystoseira* assemblages are considered habitats of critical importance in the EU (Directive 92/43/EEC; Annex I, included in “Rocky reefs” – Council of Europe, 1992) and are indicators of the good ecological status of coastal waters in the context of the Water Framework Directive (2000/60/EC; i.e. CARLIT – Ballesteros et al., 2007; Blanfuné et al., 2017; EEI – Orfanidis et al., 2003).

Although establishing marine protected areas (MPAs) can reduce some of the human impacts on these forests, it is insufficient to ensure their resilience (Gianni et al., 2013; Medrano et al., 2020b). To date, there has been little evidence of the natural recovery of degraded *Cystoseira* stands (e.g. Munda, 2000; Iveša et al., 2016; Orlando-Bonaca & Rotter, 2018; Medrano et al., 2020b) because, when large-scale losses have occurred, replenishment from nearby populations is rather difficult due to the shortdistance dissemination of the eggs/zygotes and the low population connectivity (e.g. Soltan et al., 2001; Buonomo et al., 2017; Capdevila et al., 2018).

For these reasons, active restoration of *Cystoseira* forests has been recommended, and different methods have been tested, such as the transplantation of adult thalli (e.g., Falace et al., 2006; Susini et al., 2007; Perkol-Finkel et al., 2012) and recruitment enhancement, both *ex situ* (Falace et al., 2018; Verdura et al., 2018; De La Fuente et al., 2019; Medrano et al., 2020a) and *in situ* (Verdura et al., 2018; Medrano et al., 2020a). The implementation of the recruitment enhancement showed positive outcomes and is preferable because it is more sustainable and has a lower impact on donor populations than adult transplantation.

Nevertheless, some knowledge gaps and bottlenecks hinder large-scale *Cystoseira* restoration, and more research is needed to develop effective, sustainable, and cost-effective solutions. Improving species-specific cultivation protocols could maximize germling survival, given the multiple stressors that can cause mortality in the early developmental stages (e.g., Dudgeon & Petraitis, 2005; Schiel & Foster, 2006; Irving et al., 2009; Araujo et al., 2012). Optimal culture conditions can also maximize germling growth (De La Fuente et al., 2019) and support the attainment of a ‘refuge’ size (Vadas et al., 1992), which reduces the consumption and bulldozing impact of macro- and mesograzers (e.g., crustaceans, molluscs) in the outplants. However, grazing by fish and sea urchins frequently represents a major threat to the survival and growth of outplanted organisms (e.g., Mangialajo et al., 2012; Perkol-Finkel et al., 2012; Gianni et al., 2013; Ferrario et al., 2016; Gianni et al., 2018; Gianni et al., 2020; Medrano et al., 2020a). Verlaque (1990) reported that up to 60% of the gut contents of *Sarpa salpa* can be made up of *Cystoseira*. This herbivore can remove up to 90% of the surface area of transplanted adults in a few days (Gianni et al., 2018) and sometimes even in a few hours (Gianni et al., 2017), completely nullifying reforestation efforts. Finally, extreme climatic events (i.e., thermal anomalies, storm surges) can alter the reproductive timing of species (Bevilacqua et al., 2019) or hamper the survival/growth of outplanted juveniles (De La Fuente et al., 2019).

The present study, developed within the framework of the European project ROCPOPLife (LIFE16 NAT/IT/000816), focuses on the restoration of *Gongolaria barbata* (=*Treptacantha barbata*) by recruitment enhancement in the Miramare MPA (Gulf of Trieste, Northern Adriatic Sea). This species thrives in shallow coastal waters with low hydrodynamism and is fairly tolerant of eutrophic environments (e.g., Feldmann, 1937; Montesanto & Panayotidis, 2001; Sfriso et al., 2009; Falace et al., 2010).

In large areas of the Gulf of Trieste, the distribution of *G. barbata* has declined in the last 30 years (Falace & Bressan, 2003; Falace et al., 2005; Falace et al., 2010) to the point that *G. barbata* is now only present along the Slovenian coast (Orlando-Bonaca & Rotter, 2018). It is worth noting that, despite extensive monitoring, the species was not found to be fertile in 2017 and 2018, while it was found to be fertile twice in 2019: first in winter, prematurely, after a marine heatwave (MHW) (Bevilacqua et al., 2019), and then in spring, when it usually reproduces (Falace & Bressan, 2006). Both times, *ex situ* restoration techniques were used, which represents the first implementation of *G. barbata* restoration in this geographical area. Overall, this study aimed to evaluate the following:

1. the effects of a thermal anomaly on the reproductive biology and performance in culture of *G. barbata* (the first phase of *ex situ* restoration), and
2. the impact of fish grazing pressure on juveniles in the first, most critical, months following outplanting (the second phase of the *ex situ* restoration).

The outcomes of these restoration efforts led to the implementation of mitigation solutions to overcome some of the bottlenecks in subtidal canopy-forming macroalgae restoration (i.e., reproductive stochasticity, fish grazing) from the perspective of upscaling these restoration techniques.

Methods

Study sites

The donor and the receiving sites are both located in the Gulf of Trieste, the northernmost part of the Adriatic Sea (Eastern Mediterranean), approximately 30 km apart (Figure S1). The source of fertile material was Strunjan Natural Park in Slovenia (45.535105, 13.628437), which is characterized by healthy *Cystoseira* forests in which *G. barbata* occurs in association with *C.*

compressa and *Ericaria crinita* (=*Carpodesmia crinita*). The restoration site was the Miramare MPA in Italy (45.701835, 13.714113), where *G. barbata* was once present (Bussani & Vukovic, 1992) but has not been observed in the last 20 years and only isolated thalli of *C. compressa* can be found (Falace et al., 2005). The cultured germlings were outplanted in the ‘no-take’ area of the Miramare MPA (30 ha out of the total 120 ha), where human activities are prohibited.

Laboratory **ex situ** *cultivation*

The *ex situ* cultivation protocol of Falace et al. (2018), as improved by De La Fuente et al. (2019), was applied for the first time to *G. barbata*. Fertile apices were harvested in Strunjan Natural Park and transported under dark and cold (4 °C) conditions to the University of Trieste facilities within 1 h (Figure S2). Then, the apices were rinsed with filtered sea water and stored in dark conditions at 4 °C for 36 h. Under controlled culture conditions, clay tiles (4.5 cm in diameter, with a 0.6-cm hole at the centre to screw the tile onto the outplanting modular supports, total area of 12.65 cm^2) were seeded with five apices each in 8-L aquaria. The light intensity was set at 125 μmol photons m^{-2} s^{-1} with a photoperiod of 15:9 h light:dark, and the temperature was set at 18 °C. These conditions were set to resemble those in which the species typically reproduces in the Gulf of Trieste (considering the reference period April–May, as reported in Falace & Bressan, 2006). The medium (Von Stosch's enriched filtered sea water) was renewed every 3 days to minimize any possible effect of nutrient limitation and was continuously aerated through bubbling and pumping (approximately 300 L h^{-1} flow) to ensure that the medium was oxygenated. The culture period lasted 4 weeks.

This protocol was applied in two separate cultures in 2019 as described below.

First **ex situ** *cultivation and outplanting*

In February 2019, a MHW (+2.65 °C than the average monthly sea-surface temperature during the last 40 years) occurred in the northern Adriatic Sea, causing a phenological shift that abruptly triggered fertility in *G. barbata* at the donor site (Bevilacqua et al., 2019). At the end of February, the thalli still showed a winter habitus without well-developed primary branches (Figure 1a, b) such that the receptacles grew on the adventitious branches (Figure 1c). The receptacles (Figure 1c, d) were smaller than those that typically develop in spring on the primary branches (Figure 1e, f, g, h); thus, some of the receptacles were sampled to test whether they were able to release viable zygotes.

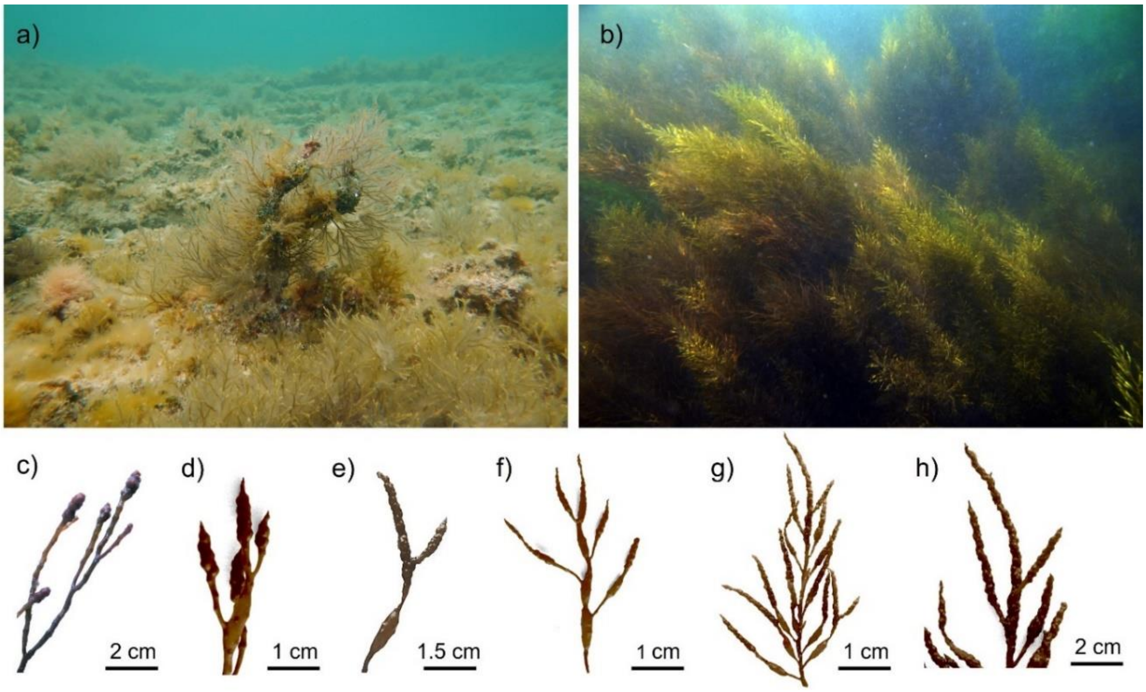

Figure 1 – *Gongolaria barbata* (a) in February 2019, in the donor site with the winter *habitus*, and (b) as it usually appears during the springtime maximum development in the Gulf of Trieste (never observed during this study; credits: D. Curiel). (c) Receptacles developing on adventitious branches (i.e. first *ex situ* cultivation). Receptacles developing on primary branches: (d) small (as collected for the first and second *ex situ* cultivation) and (e, f, g, h) fully developed (never observed during this study).

Since the sampled receptacles released zygotes that developed into viable embryos, in March, approximately 2500 fertile apices of *G. barbata* were harvested, and 420 clay tiles were seeded. Twenty-four hours after seeding, the apices were removed, and this time was considered the time of fertilization (T_0).

During the laboratory culture, data were collected and processed as follows (Table S1):

- Thirty tiles were randomly selected and tagged at T_0. These tiles were used for: (i) calculating the release efficiency at T_0 as the no. zygotes released per tile/no. receptacles per tile; and (ii) calculating the germling survival rate between 2 (T_1) and 4 (T_2) weeks after fertilization as the % survival = no. germlings per tile at T_2 × 100/no. germlings per tile at T_1 through photographic sampling at T_1 and T_2 with a Nikon D300 camera (Nikon Corporation, Tokyo, Japan).
- In total, 120 tiles were randomly selected at both T_1 (Figure S3a) and T_2 (Figure S3b) and photographed with a Nikon D300 camera (Nikon Corporation, Tokyo, Japan) to assess germling density (i.e., the number of germlings per tile).
- Twenty tiles were randomly selected for morphometric measurements at T_2. All the germlings were carefully removed from the tiles, and 1200 of them were selected at

random and observed under a stereomicroscope (Leica MZ 6, Leica Microsystems, Wetzlar, Germany), where they were photographed with a Nikon Coolpix 4500 camera (Nikon Corporation, Tokyo, Japan; Figure S4).

The number of zygotes released per tile (n = 30), the number of germlings per tile at T_1 and T_2 (n = 120), and the germling length at T_2 (n = 1200) were obtained by processing photographs with ImageJ software (Schneider et al., 2012). Specifically, counts were performed visually by superimposing a grid on each photograph and counting the number of items in each grid cell.

Additionally, to describe the embryology of *G. barbata*, several glass slides were seeded. Zygote and embryo development were studied with an inverted microscope (Leica DM IL LED, Leica Microsystems, Wetzlar, Germany), and photographs were taken with a Canon Powershot G9 camera (Canon Inc., Tokyo, Japan; Figure S5).

In the last 4 days of culture before outplanting (in April 2019), the temperature was gradually lowered to 13 °C to acclimate the germlings to the measured field temperature. Thereafter, 400 tiles were transported to the Miramare MPA (20 min by car) in refrigerated boxes filled with filtered sea water and attached with screws to 14 previously assembled metal modular frames in approximately 4 h. Each modular frame consisted of a 100 × 2.5 cm flat perforated bar made of galvanized iron with four identical 50 × 2.5 cm bars attached perpendicular to the first bar to form a rake-like shape (Figure 2).

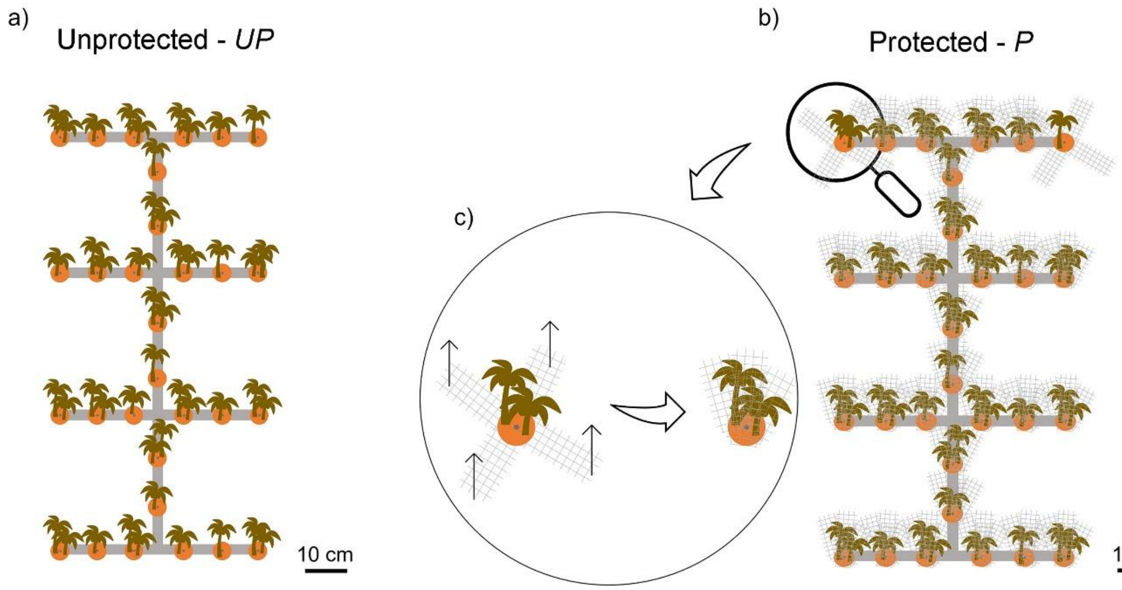

Figure 2 – The outplanting modular structure: (a) 'Unprotected' (UP) and (b) 'Protected' (P). The grazing deterrents were formed by (c) raising the flexible metallic mesh strips around the tile, as petals of a flower. Each modular structure had a surface of 0.5 m2 (0.5 m × 1 m).

Each tile was fastened to the bar with a bolt at a fixed distance of approximately 5 cm from the other tiles, for a total of approximately 28 tiles per modular structure. The modular frames were specifically designed to mimic the spatial distribution pattern of *G. barbata* adult individuals at the donor site. Therefore, by using photoquadrats, we gathered information about the density (i.e. 43 thalli m^{-2} ± 5, average ± SE) and mean adult thallus diameter (i.e. 15 cm ± 0.12) in the patches at the donor site. The final structure of the modular frames had a capacity of 30 tiles per frame, assuming the survival of one thallus per tile to the adult stage.

Two types of modular frame were conceived for testing and mitigating the effects of fish grazing (grazing trial): a basic frame and a frame with deterrents against herbivores (Figure 2a, b). Each deterrent was made of two flexible metallic mesh strips (3.5 × 20 cm, 0.5 cm mesh size) crossed at their midpoints to form an 'X' shape (Figure 2c). The ends of the strips had a spike shape to deter fish. The overlapping strips were placed under the tiles before attaching them to the substrate with screws. The protective structure was formed by bringing the ends of the strips around the tile like the petals of a flower, creating a cage-like structure around the tile.

Of the 400 tiles, half were affixed to the metallic modular frames without protection ('Unprotected', UP; Figure 2a), and the remaining 200 tiles were protected with grazing deterrents ('Protected', P; Figure 2b). The 14 modular frames (7 UP, 7 P) were randomly affixed with screws to the rocky bottom at 3 m depth, the same depth at which *G. barbata* thrives in the donor population.

In each treatment (UP, P), thallus length was measured on 30 randomly selected tiles at 15 days (Time G1, where G refers to the grazing trial), 30 days (Time G2), 60 days (Time G3), 90 days (Time G4), 120 days (Time G5) and 180 days (Time G6) after tile deployment. At Time G6, the number of tiles on which juveniles were present and the respective number of juveniles per tile were counted.

Bimonthly, the grazing deterrents were gently brushed with a wire brush to remove sediment and epiphytes that could decrease the light availability on the tiles and thus limit juvenile growth.

In June, July, and August 2019, the density of *S. salpa*, an herbivorous fish that typically grazes on *Cystoseira* (Verlaque, 1990), was assessed with a strip-transect visual census (Brock, 1954) inside the ‘no-take’ zone of the MPA. Because of the limited dimensions of this zone, the method was modified, and the counts were performed simultaneously by two scuba divers along two parallel transects of approximately 1.2 km at different depths. In addition, to document the activity of grazers on the germlings, an underwater camera (GOPRO Hero8 Black Edition, GoPro, San Mateo, CA, USA) was affixed between some of the modular frames. Video sampling was carried out every 3 weeks during the experiment.

Second **ex situ** *cultivation and outplanting*

The second cultivation effort began in May 2019. During receptacle collection in Strunjan Natural Park, the donor population appeared to be depleted of its reproductive potential, with small receptacles (Figure 1d), probably because the algae had allocated much of their resources to the earlier anomalous reproductive event. Therefore, only ca. 800 fertile apexes were harvested, and 146 clay discs were seeded. The receptacle release efficiency (n = 30) and germling length after 1 month of culture (n = 60) were obtained as described above for the first cultivation.

In this case, despite the culture settings being the same as in the previous cultivation, the germlings showed slower growth. Therefore, to avoid a prolonged culture, which would have been highly demanding, the culture period was extended outdoors. In June 2019, after 4 weeks of culture, all the tiles were transported to the Miramare MPA, where they were affixed to a one-level plastic lantern net (diameter 55 cm) at 3 m depth and in close proximity to the previous outplanting (lantern net trial; Figure 3a). This kind of structure suspended in the water column is usually employed for the fattening and confinement of bivalves and sea cucumbers. In the present study, this device was used as an intermediate step between culturing and outplanting to mitigate the low culture efficiency.

After 3 months in the lantern net, the tiles were moved to the rocky bottom using the previously assembled modular frames. In the previous outplanting, the unprotected juveniles were heavily grazed, so all the tiles were protected by grazing deterrents (P).

To observe germling growth in the lantern net, germling length was measured on 30 random tiles at 21 days (Time L1, where L refers to the lantern net trial), 30 days (Time L2) and 60 days (Time L3) after placement in the lantern net (Figure 3b). The juveniles' length was then measured on the outplanting date (Time L4) and at 30 days (Time L5) and 90 days (Time L6) after the outplanting (90, 120 and 180 days after deployment in the lantern net, respectively).

Figure 3 – Suspended in situ cultivation in the second restoration trial. (a) The lantern net and (b) a detail of the tiles fixed to the bottom of the floating structure.

Statistical analyses

In the first cultivation, the relationship between the percentage of germling survival at T_2 (response variable) and the germling density at T_1 on the tagged tiles was tested by means of a simple linear regression. To make the relationship linear, the values of the response variable were log-transformed. Simple linear regression was also used to test the relationship between the germling density at T_2 and the germling density at T_1 on the same tiles.

For the first outplanting, a linear mixed model was created using the R package 'lme4' (Bates et al., 2015) to evaluate the effect of grazing deterrence on thallus length over time. The model included the treatment (two levels: UP, P) as a fixed effect and the sampling time as a random effect. R^2 values were calculated using the 'r2glmm' package (Jaeger, 2017). Pairwise Wilcoxon rank sum tests were used to compare the two treatments independently at each sampling time. All statistical analyses were performed using R 3.6.3 (R Core Team, 2020).

Results

First ex situ *cultivation and outplanting*

In culture, the release efficiency averaged 9.5 ± 1.4 SE zygotes receptacle^{-1}.

After 2 weeks (T_1), the mean density was 687 ± 52 SE germlings tile^{-1} (corresponding to 54 germlings cm^{-2} ± 4), ranging from 36 to 2277 germlings tile^{-1}. After 4 weeks (T_2), the mean density was 93 ± 4 SE germlings tile^{-1} (corresponding to 7.35 germlings cm^{-2} ± 0.32), ranging from 13 to 239 germlings tile^{-1}.

The mean percentage of germling survival between T_1 and T_2 was 15.7% ± 1.4 SE. A negative relationship was found between the survival percentage at T_2 and the germling density at T_1 (linear regressor $\beta = -0.001$; $p < 0.001$, $R^2 = 0.43$), suggesting a density-dependent effect (Figure 4a). Nonetheless, the tiles with higher initial densities were also those with the highest final densities (Figure 4b): there was a positive relationship between germling density at T_2 and germling density at T_1 (linear regressor $\beta = 0.094$; $p < 0.001$, $R^2 = 0.63$).

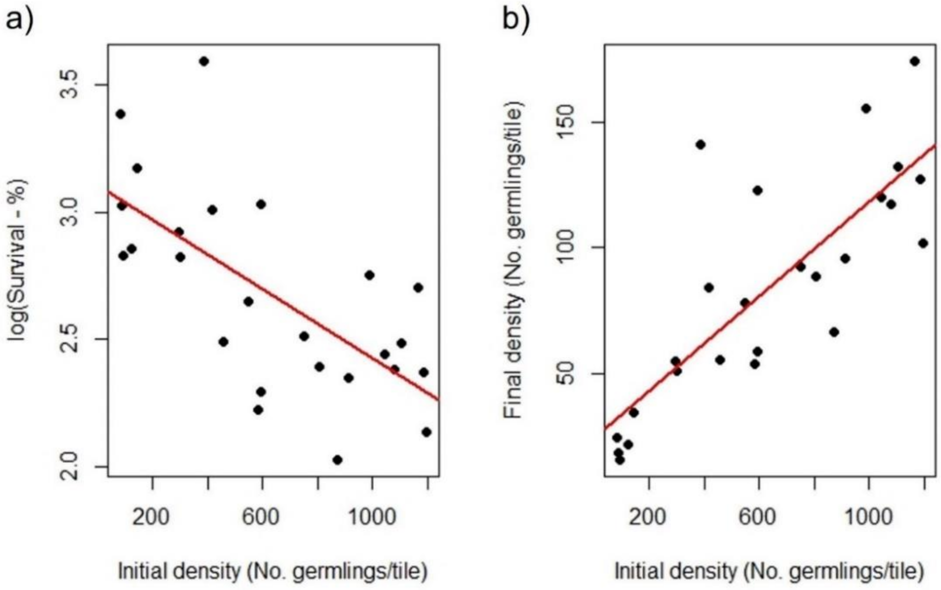

Figure 4 – Germling survival and density in the first culture. (a) Relationship between germling % survival at T_2 and the germling density at T_1 (No. of germlings/tile). (b) Relationship between germling density at T_2 and the

germling density at T_1, expressed in terms of No. of germlings per tile. The red lines represent the calculated regression trend.

At T_2, the germlings were 1.72 mm ± 0.01 SE long, with a maximum of 3.45 mm length (Figure 5).

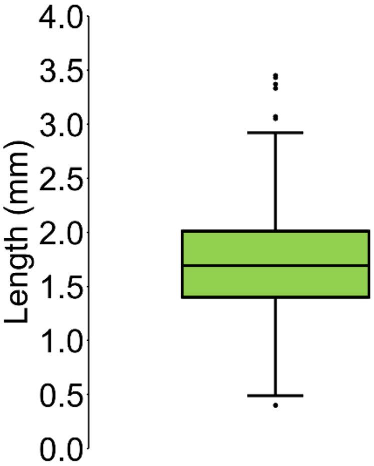

Figure 5 – Variability of germling length (mm) after 4 weeks of culture in the first cultivation (T_2). The solid line within the box represents the median.

Two weeks after outplanting (Time G1), the mean germling length was 0.8 cm ± 0.1 SE in the UP treatment, while it was 0.7 cm ± 0.1 SE in the P treatment. After 6 months (Time G6), the juveniles were 3.3 cm ± 0.3 SE long in the UP treatment and 6.2 cm ± 0.4 SE long in the P treatment. The P treatment significantly affected juvenile length ($P < 0.001$, $R^2 = 0.25$; Table S2): starting at Time G3, the juvenile length in the P treatment was always greater than that in the UP treatment (Figures 6a, S3c, d, e, f, and S6). The treatment also affected the density distribution of juvenile lengths; juvenile length was less evenly distributed on the UP tiles (Figure 6b).

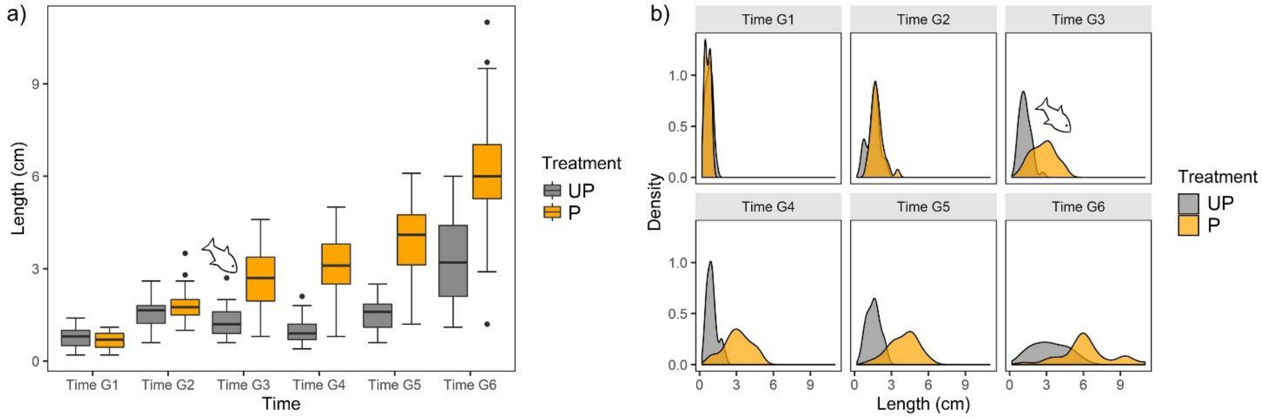

Figure 6 – Growth of the juveniles in the first outplanting. (a) Boxplots of the thallus lengths (cm) in the two treatments, ‘Unprotected’ (UP) and ‘Protected’ (P). (b) Size-class distribution of the restored population over time. The fish icon represents the sampling time at which fish grazing on the juveniles started to be detected.

After 6 months (Time G6), juveniles were present on 29% of the tiles in the UP treatment (1.2 individuals per tile ± 0.07 SE) and on 46% of the tiles in the P treatment (1.5 ± 0.09 SE; Figure 7).

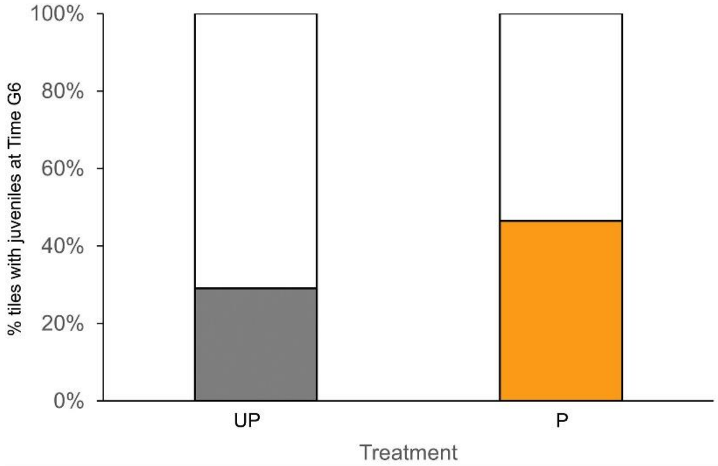

Figure 7 – Percentage of tiles with juveniles after 6 months in the field (Time G6) in the two treatments (‘Unprotected’ – UP, ‘Protected’ – P) from the first outplanting.

The visual census of *S. salpa* detected the presence of approximately 1600 specimens inside the 30-ha ‘no-take’ area of the MPA.

Second **ex situ** *cultivation and outplanting*

In culture, the release efficiency averaged 0.7 ± 0.1 SE zygotes receptacle^{-1}, and the mean germling length after 4 weeks of culture was 0.4 mm ± 0.03 SE.

In the field, the germlings in the lantern net were 1.4 cm ± 0.1 SE long after 1 month (Time L2) and 1.6 cm ± 0.11 SE long after 2 months (Time L3); in the third month (Time L4), a decrease in length due to fish grazing was detected (1.2 cm ± 0.08 SE). In fact, signs of fish bites were evident on many juveniles (Figure 8).

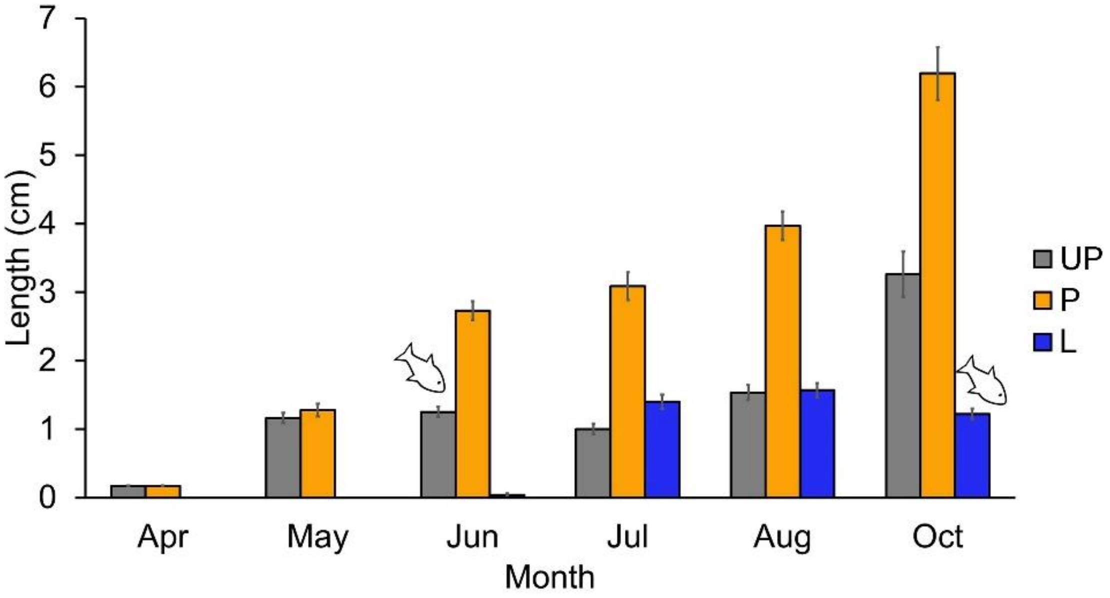

Figure 8 – Length of juveniles from the first ('Unprotected' – UP, 'Protected' – P) and the second ('Lantern net' – L) outplanting. The fish icon represents the sampling time at which signs of fish grazing on the juveniles started to be detected.

Once the juveniles moved to the rocky bottom and obtained protection (the P condition), they grew to 2.9 cm ± 0.3 SE long at Time L6 (Figure 9). The juveniles in the second experiment, unlike those in the first experiment, did not become fertile in the following spring (Figure S7).

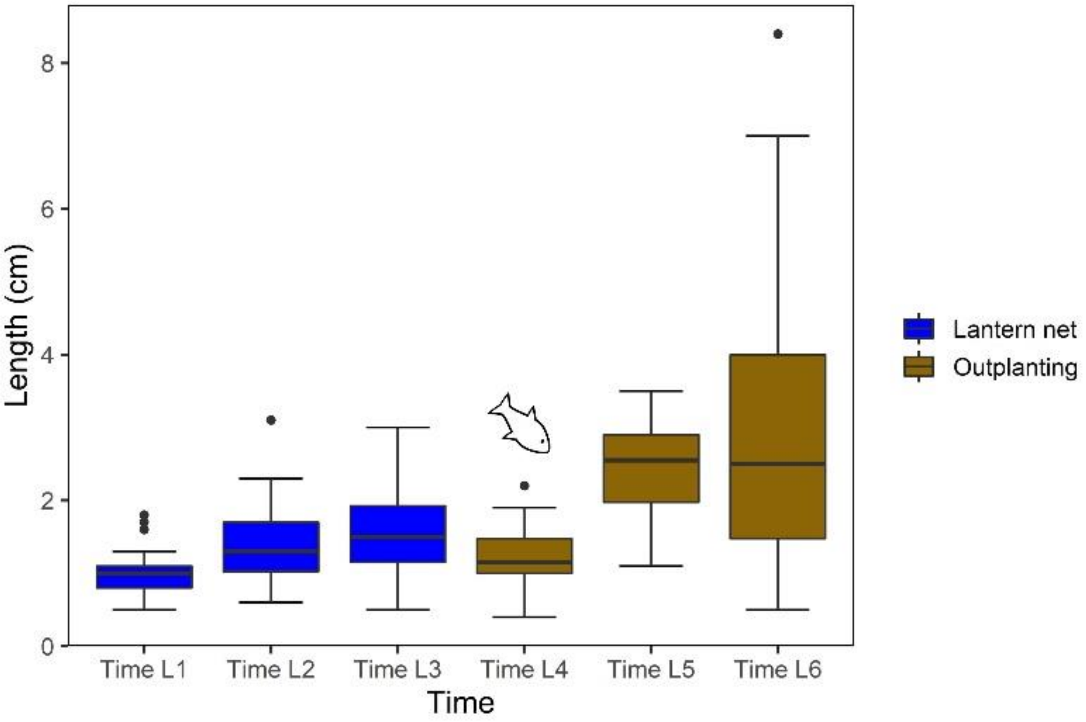

Figure 9 – Juvenile length (cm) when the tiles were in the lantern net (blue) and on the sea bottom (brown). Juveniles were moved to the sea bottom at Time L4. The fish icon represents the sampling time at which signs of fish grazing on the juveniles started to be detected.

Discussion

The restoration of key species and habitats is promoted internationally to reverse the shift from highly diverse ecosystems to less diverse ones and thus to re-establish the services that ecosystems provide (Biodiversity Strategy to 2020 – European Commission, 2011; Biodiversity Strategy to 2030 – European Commission, 2020; UN Decade on Ecosystem Restoration – General Assembly of the United Nations, 2019).

Among the factors that can affect the restoration process, climate change, whose effects on target species are poorly known, can impair restoration success in unpredictable ways. Although various studies support the hypothesis that temperature plays a key role in the gametogenesis and gamete release of Fucales (e.g., Norton, 1981; Bacon & Vadas, 1991; Pang et al., 2009; Kraufvelin et al., 2012; Falace et al., 2018), information on its role in these processes is insufficient (de Bettignies et al., 2018). In addition, although the anticipation of spring phenology due to global warming is on average stronger for marine species than for terrestrial species (as reviewed in Poloczanska et al., 2013 and in de Bettignies et al., 2018), this is the first study in which the effects of a reproductive shift in response to a sea thermal anomaly are assessed for seaweed in a restoration context.

In this study, the second culture was less efficient than the first culture in terms of zygote release (10 times lower) and germling growth (four times shorter), despite both cultures being carried out under the same laboratory conditions. The natural variability in propagule release among fucoid species is well known (Gunnill, 1980; Reed, 1990; Vadas et al., 1992); therefore, differences in the release efficiency could be expected. In this case, however, the MHW probably exacerbated this natural variability and affected germling growth as well. As it was already fertile in February, *G. barbata* was most likely to be at the end of its reproductive cycle in May, as also evidenced by the poor development of branches and receptacles compared to the usual spring phenology in the Gulf of Trieste (Falace & Zanelli, 2006).

This result highlights how reproductive stochasticity may represent a major threat to the implementation of large-scale restoration actions in the future, as thermal anomalies are being recorded with increasing frequency throughout the ocean and are likely to shape future marine ecosystems (Frölicher & Laufkötter, 2018; Pastor et al., 2018; Wernberg et al., 2019). Early-warning networks regarding extreme climatic events might allow restoration practitioners to monitor the phenological responses of *Cystoseira*, collect prematurely fertile receptacles in order to cultivate *Cystoseira* under controlled mesocosm conditions, or select lineages with higher plasticity to thermal extremes, increasing the chance for successful restoration actions (Bevilacqua et al., 2019).

After 1 year (March 2020), the taller thalli (approximately 14–17 cm) grown in the first outplanting were starting to develop receptacles. This development did not occur in plants in the second outplanting, probably because after 6 months, the juveniles in the first outplanting (the P treatment) were twice as long as those in the second outplanting (6.2 cm ± 0.4 SE versus 2.9 cm ± 0.3 SE). The difference in length probably occurred because the juveniles in the first outplanting benefited from favourable seasonal conditions (e.g., the extended photoperiod in the summertime) for a longer period. This outcome suggests that, in a restoration context, altered life cycles and delayed growth could also hinder spill-over dynamics in the restored plantlets as adults and slow the re-establishment of self-sustaining populations.

Despite the anomalous receptacle development, in the first culture, the cultivation protocol resulted in a higher final germling length than in a previous study on the same species. After 1 month, the germling length was 1.72 mm ± 0.01, which is six times longer than that reported by Verdura et al. (2018) (i.e., 0.2–0.4 mm), and germlings in that study reached 5 mm after 6 months. Our results are comparable to those obtained with *E. amentacea* in Falace et al. (2018) (i.e., 1.38 mm ± 0.13 SE after a 3-week culture).

The germling density averaged 54 germlings cm^{-2} ± 4 SE after 2 weeks of culture, a lower value than in Falace et al. (2018) for *E. amentacea* (160 germlings cm^{-2} ± 47). However, the mean density value obtained after a month (7.35 germlings cm^{-2} ± 0.32) was similar to the value reported for *G. elegans* by Medrano et al. (2020a) after 2 months of culture (i.e., 10.14 germlings cm^{-2} ± 5.30).

As expected, the germling survival rate in culture (average 16% after 4 weeks) was higher than that reported in natural stands (e.g., Gunnill, 1980; Schiel, 1988; Lamote & Johnson, 2008; Capdevila et al., 2018). For example, Wright et al. (2004) reported that in *Fucus gardneri* less than 0.0004% of embryos survived to become visible recruits, while Chapman (1995) observed that in *Fucus distichus*, only 0.4–12% of settlers became visible recruits.

The negative relationship between survival rate and germling density can be explained by the progressive decline in the density of a population of growing plants (i.e., the ‘self-thinning’ process; e.g., Black, 1974; Santelices et al., 1980; Dean et al., 1989; Ang & De Wreede, 1992; Creed et al., 1997; Steen & Scrosati, 2004).

Controlling for grazers improved the performance of the outplantings. After 6 months, the number of juveniles per tile ranged between one and two in both treatments, but, in the P treatment, the percentage of tiles with at least one juvenile was almost 2-fold that in the UP treatment. In addition, the juveniles were on average 2 times larger in the P treatment, and their length distribution resembled that of a natural population. According to Verdura et al. (2018), a population with a well-represented size distribution is indicative of a successful restoration effort.

Sarpa salpa, which is highly abundant inside the MPA, was most likely to be the main grazer on the outplanted juveniles; schools were often observed around the experimental plots during monitoring by scuba divers and on camera, and the plantlets showed evident signs of fish bites. Conversely, grazing by sea urchins (one of the major drivers of the loss of *Cystoseira* stands; e.g., Verlaque, 1984; Hereu, 2004; Nikolić et al., 2013) does not need to be considered in the present study because sea urchins were never observed on the tiles and are scarce in the MPA. The impact of mesograzers (e.g., molluscs, crustaceans) as observed in the videos was also likely to be negligible, probably because the germlings were outplanted having attained 'refuge' size; this finding highlights the importance of maximizing germling growth in nurseries.

The grazing deterrents, in addition to successfully protecting the outplanted juveniles, reduced the risks of detachment and loss related to hydrodynamism compared to large cages affixed with epoxy. However, they require the same periodical maintenance; thus, their deployment in large-scale interventions would be unsustainable. A possible, though controversial, option that has been suggested for achieving long-term restoration success in sites with high grazing pressure is the restoration of trophic interactions or herbivore culling (e.g., Filbee-Dexter & Scheibling, 2014; Gianni et al., 2018; Carlsson & Christie, 2019; Eger et al., 2020; Medrano et al., 2020a).

The modular frames proved to be a suitable approach for outplanting subtidal *Cystoseira* species. Screwing the tiles onto the preassembled modular structures decreases the required time, costs and personnel compared with other methods, thus showing great upscaling potential. In fact, (i) tile screwing can be rapidly performed on land (3 h and four people for 400 tiles); (ii) scuba divers can easily and rapidly deploy a large number of tiles, reducing the stress on germlings (2 h and two people, considering both the drilling activities before deployment and fastening the module to the rock); (iii) the modular frames can be arranged to mimic the different patterns of *Cystoseira* distribution in natural stands; and (iv) not using epoxy putty to fix each tile to the rocky substrate minimizes the aesthetic and environmental impacts of the outplanting and guarantees the stability of the outplants in the first hours after deployment, before putty hardening (De La Fuente et al., 2019).

The lantern net allowed us to reduce the prolonged cultivation duration that would have been required for the germlings to reach the 'refuge' size in the second experiment because of the slow germling growth. The suspended in situ cultivation also seemed to restrict the presence of grazers, which can typically be found on vegetated rocky bottoms. However, fish bites started to be detected when the juveniles were approximately 1.5–2 cm long (Time L4) as in the UP treatment on the sea bottom (Time G3); this result suggests that juveniles start to become attractive to *S. salpa* when they reach approximately 1.5–2 cm in length.

Large-scale restoration must be cost-effective and requires the production of large quantities of germlings; however, production is hampered by the availability of facilities (e.g., the dimension and number of environmentally controlled rooms, the number of aquaria, labour), which requires many consecutive cultivation cycles to be performed. However, the number of possible cultivation cycles is limited by the narrow reproductive period of the majority of fucoids (e.g., 2 months in the Northern Adriatic Sea for *G. barbata* – Falace & Bressan, 2006). Coupling the culture in the mesocosms to a transitional step in the field (e.g., a floating lantern net) before outplanting on rocky bottoms in a three-step process allows the advantages of both ex situ and in situ techniques to be combined. The recruitment enhancement achieved through optimized culture conditions in mesocosms would: (i) reduce the impact of unpredictable natural events; (ii) increase settlement success; (iii) maximize the survival of the most critical early developmental stages; and (iv) allow the production of more and larger germlings in a shorter time. Suspended in situ cultivation, which reduces the amount of time that the germlings stay in the mesocosms, is beneficial because: (i) it prevents the risk of culture contamination by bacteria and epiphytes, which often occurs in prolonged laboratory cultures; and (ii) it is cost-effective; with a low investment, it avoids time-consuming and costly maintenance in the laboratory.

Currently, comparisons of the restoration costs between the proposed methodology and those reported in other studies (i.e., Verdura et al., 2018; Medrano et al., 2020a) are almost impossible, considering that the restoration parameters (such as the hourly cost per person, depreciation of the costs incurred in building the nurseries, culture yield) have not been standardized. Meaningful comparative cost analyses would be feasible only in an aquaculture context, with optimized protocols and with specialized semi-industrial infrastructure, and are not feasible while this methodology is still in an experimental phase.

In conclusion, it is evident that the restoration of fucoids is still in its infancy. It is necessary to proceed with experimental trials to test culturing and outplanting methods tailored to the target species and habitats before planning large-scale interventions. In addition, a better understanding of the relationships of the early fucoid life stages to disturbance/variation in the physical environment as well as to climate change should be achieved, and these relationships should be considered within the context of effective large-scale restoration efforts.

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Supporting Information

Figure S2 – Map of the study sites. The green circle indicates the Miramare Marine Protected Area in Italy (Receiving site) and the orange circle indicates the Strunjan Natural Park in Slovenia (Donor site).

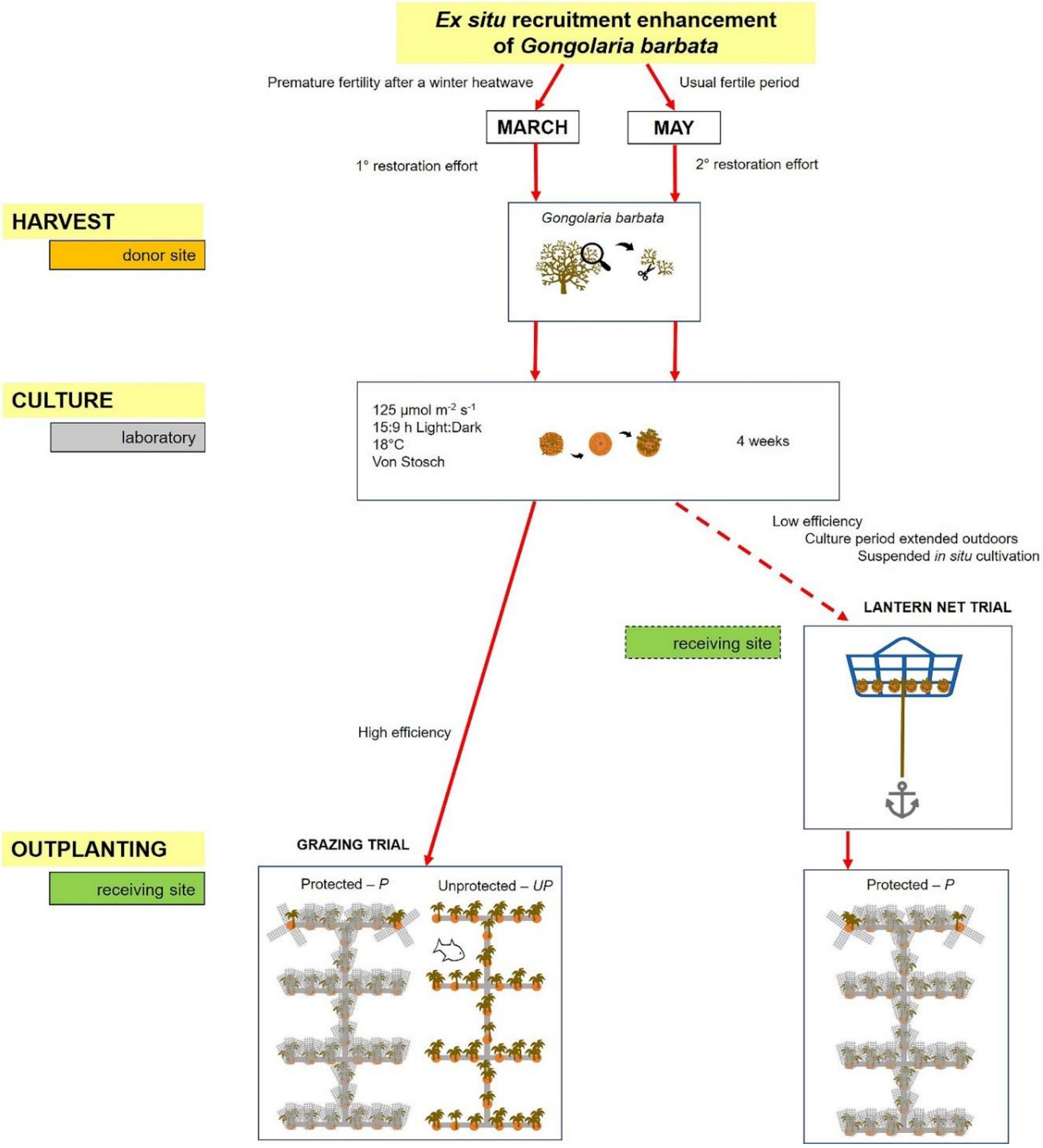

Figure S3 – Outline of the two restoration efforts implemented in the present study.

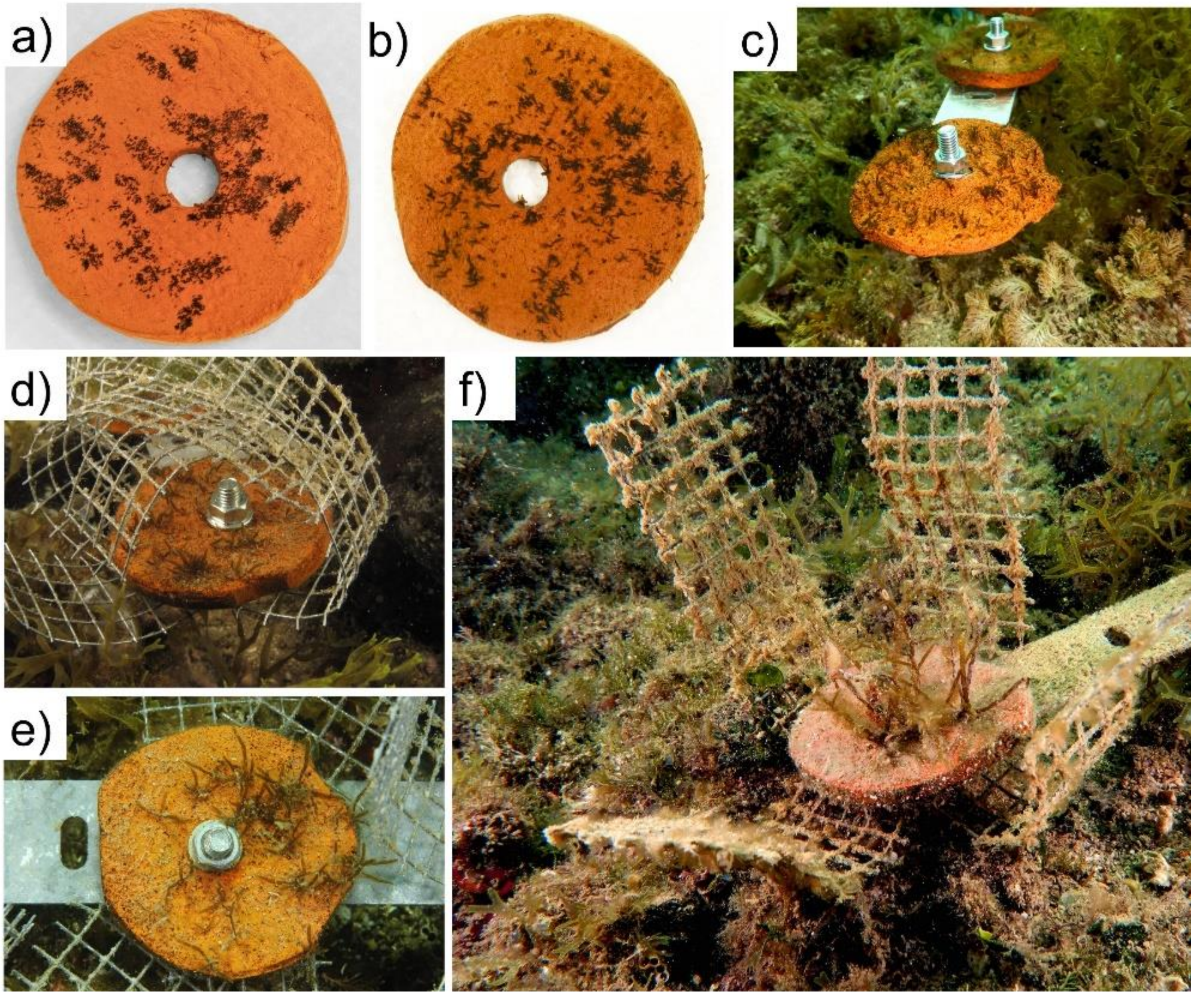

Figure S3 – Germling/juvenile development over time in the first experiment. Germlings after (a) two and (b) four weeks of culture (respectively at T_1 and T_2). Juveniles c) at the deployment time, d) after two weeks (Time G1), e) after one month (Time G2) and f) after five months in the field (the devices were opened to allow photos).

Figure S4 – Germling morphologies after four weeks in culture (T_2).

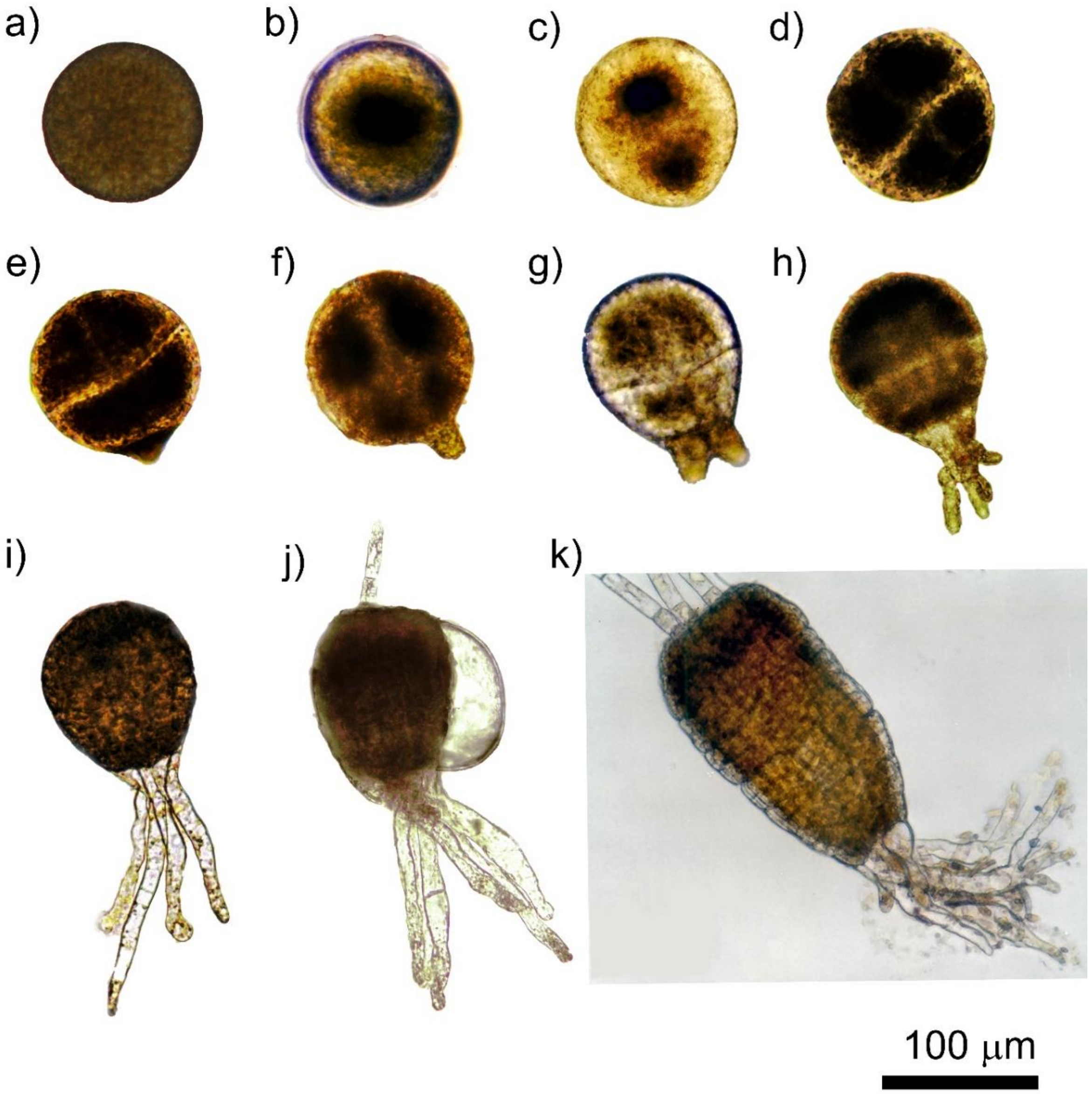

Figure S5 – Early morphogenesis of *Gongolaria barbata* (= *Treptacantha barbata*). a) The oogone before fecundation. The zygote cytoplasm, b) at first homogeneous, becomes metabolically differentiated (polarization) with the establishment of a growth axis expressed by the cell wall protrusion. Twenty-four hours after fertilization (AF) it is possible to observe c) the first division of the zygote, which is an equatorial asymmetrical division (perpendicular to the growth axis), leading to the formation of d) the first daughter cells. The lower one gives rise to the rhizoidal portion and the upper one to the thallus. e) Thirty-six hours AF a longitudinal division (perpendicular to the previous one) takes place in the thallus cell, and 48 h AF many rapid divisions occur in the apical cell, without increase of the cell volume (constant diameter 100-105µm). The process of rhizoid development is as follows: e, f) the mother cell of rhizoids forms g) several sprouts, which further elongate and develop into rhizoid buds. The rhizoid buds continue growing, forming h, i) long filaments. The rhizoidal cell, by means of perpendicular divisions, gives rise to four cells that differentiated into the four primary rhizoids. The rhizoids elongate, while repeated divisions of the thallus cell 4-5 days AF result in j) the formation of an embryo with the apical hair (on the right part of the embryo the fecundation membrane is visible). k) The embryo then starts to elongate and to develop many apical hairs and secondary rhizoids.

Figure S6 – Evident signs of grazing from fishes on the juveniles in the "Unprotected" treatment, as indicated by the black arrows with the fish icon.

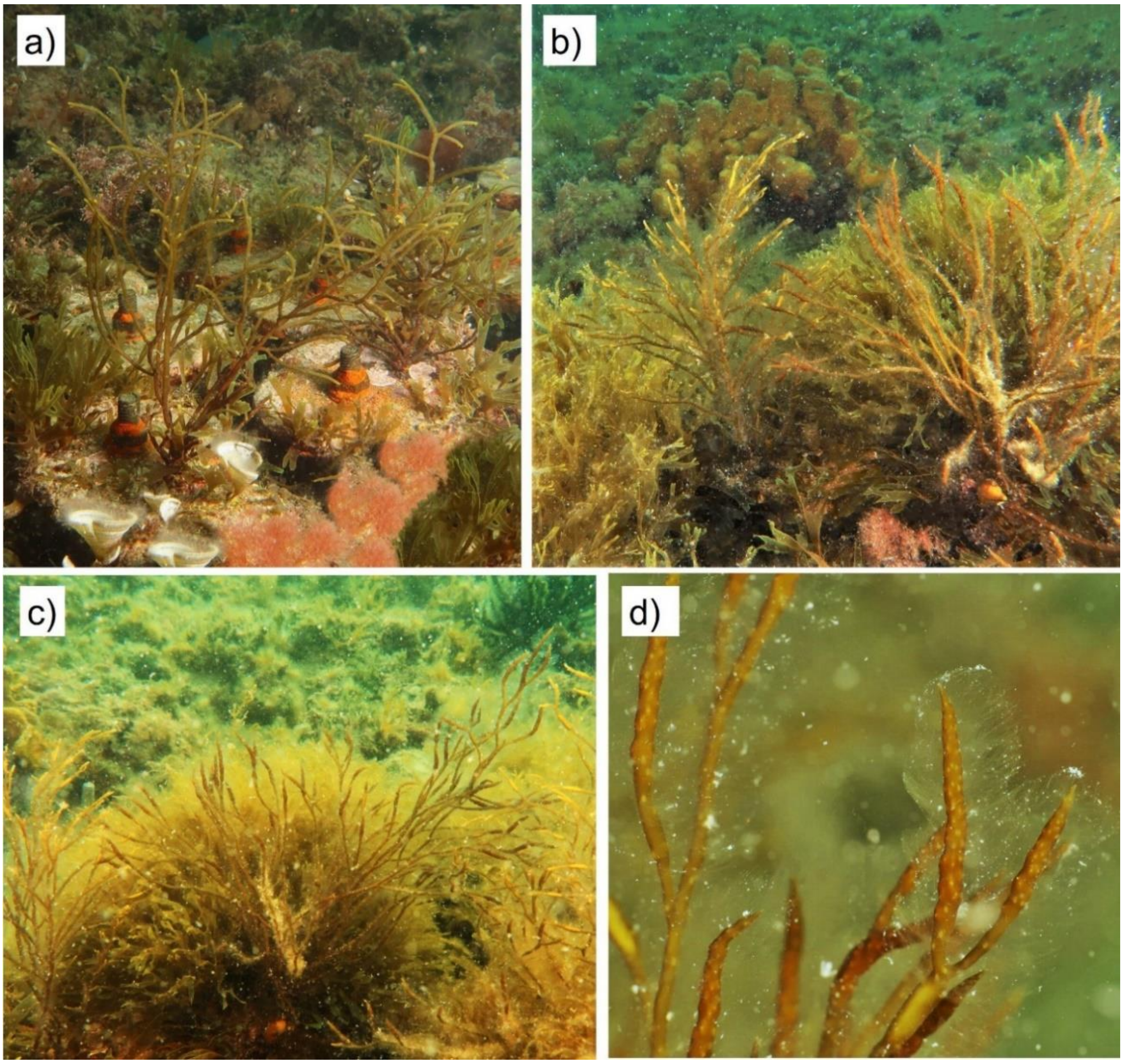

Figure S7 – Fertile young thalli in the year following outplanting: a) in February 2020, and b,c) in April 2020; d) detail of the receptacles.

Table S1 – Description of the data obtained in the first cultivation experiment. All data were obtained after processing the photographs with ImageJ software.

Sampling time	**Sample size**	**Variable**	**Calculation/Unit**
T_0	30 tiles tagged	Release efficiency	No. zygotes released per tile/No. receptacles per tile
T_1 = 2 weeks	30 tiles, tagged at T_0	Germling survival rate	No. germlings per tile for % survival
	120 tiles, randomly selected at T_1	Germling density at T_1	No. germlings per tile
T_2 = 4 weeks	30 tiles, tagged at T_0	Germling survival rate	No. germlings per tile for % survival; % survival = No. germlings per tile at T_2 x100/No. germlings per tile at T_1
	120 tiles, randomly selected at T_2	Germling density at T_2	No. germlings per tile
	1,200 random germlings from 20 random tiles	Germling length	Length measurements in mm

Table S2 – Summary of the Linear Mixed Model computed using germling length as a function of the treatment.

Variable	**Estimate**	**Std. Error**
Intercept	1.62	0.70
Treatment	1.30***	0.12

***p < 0.001

“Where” and “how” implications in brown algal forest restoration in the Northern Adriatic Sea

Under review in Frontiers in Marine Science

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Preliminary abstract

Multiple anthropogenic factors are responsible for the decline of *Cystoseira sensu lato* (thereafter, *Cystoseira*) forests along Mediterranean coasts. Some *Cystoseira* species are already regionally extinct, and their decline has been widely recorded. Sustainable and efficient techniques for the restoration of *Cystoseira* are needed. In this context, the objectives of this study were to analyse the reproductive traits of three populations of *Gongolaria barbata* from three nearby donor sites in the northern Adriatic Sea, assessing the differences in their reproductive potential and reproductive success, and to evaluate the outplanting success in terms of the effectiveness of *G. barbata* restoration in relation to the different donor and receiving sites (Miramare MPA and in front of the Marine Biology Station Piran (MBSP)) and implemented methods (*ex situ* and hybrid method combining mesocosm cultivation and a suspended culture in the field).

Fertile apexes of *G. barbata* were cultivated on tiles in two laboratories. Half of the tiles were transported in the receiving sites after 2 weeks of cultivation and placed on suspended lantern nets (hybrid method), which were later (after 3 months) transferred to the seabed on concrete plates with protective cages. The remaining tiles were placed directly on concrete plates with protective cages on the seabed (*ex situ* method) after a 4-week culture. At both sites, the lantern nets and plates were randomly placed at 3 m depth. In each treatment, thallus length was measured monthly.

The seedlings in suspended culture showed lower performance at Miramare receiving site, most likely due to the unfavourable environmental conditions. The satisfactory results obtained at MBSP demonstrate the efficiency of the hybrid method and confirm its potential to reduce costs and time required for cultivation. Given that unpredictable climate events pose the greatest threat to restoration performance, those challenges have to be taken into account in setting new restoration practices. Moreover, herbivore regulation is extremely urgent and should be planned and implemented on a larger regional scale.

Key words: *Gongolaria barbata*, canopy-forming algae, restoration, *ex situ*, cultivation, Mediterranean

Preliminary introduction

Canopy-forming fucoids are dominant foundation species found on almost all Mediterranean coasts (Bulleri et al., 2012; Gianni et al., 2013; Assis et al., 2020; Bringloe et al., 2020). In particular, *Cystoseira sensu lato* (Fucales, Phaeophyceae) species, recently subdivided into the three genera *Cystoseira*, *Ericaria* and *Gongolaria* (Molinari-Novoa and Guiry, 2020), can thrive from intertidal to circalittoral rocky bottoms, forming dense forests that are among the most productive assemblages in the Mediterranean coastal zone, with different species replacing each other along a bathymetric gradient (Boudouresque & Lück, 1972; Giaccone, 1973; Ballesteros, 1988; Ballesteros, 1989; Rull & Gómez Garreta, 1989; Otero-Schmitt & Pérez-Cirera, 1996; Ballesteros et al., 1998; Pizzuto, 1999; Ballesteros et al., 2009).

Their complex spatial heterogeneity favours high primary production (Ballesteros et al., 2009; Mačić & Svirčev, 2014), rich understory communities of algae and invertebrates (Pitacco et al., 2014; Bianchelli et al., 2016; Mancuso et al., 2021), and outstanding fish densities and diversity (Orlando Bonaca & Lipej, 2005; Cheminée et al., 2013). Other ecosystem services provided by kelp and fucoid canopy algae, including *Cystoseira sensu lato* (thereafter, *Cystoseira*) forests (De La Fuente et al., 2019a), include their role as carbon sinks (Queirós et al., 2019; Filbee-Dexter et al., 2022), as well as wave attenuation and mitigation of coastal erosion, services that are becoming increasingly important in the face of climate-driven sea level rise and increases in extreme storm surges (Smale et al., 2013).

Further benefits of *Cystoseira* spp. to humans are underscored by their production of many potentially bioactive metabolites (Bruno de Sousa et al., 2017a), which have been shown to have antioxidant, anti-inflammatory, antifungal, antiviral and antibacterial effects (Mhadhebi et al., 2011; Vizetto-Duarte et al., 2016; Bruno de Sousa et al., 2017b; De La Fuente et al., 2020).

As a result of multiple anthropogenic impacts (e.g., coastal urbanisation, eutrophication, sediment loading and overgrazing), *Cystoseira* stands have gradually disappeared in many Mediterranean coastal areas in recent decades, often being replaced by filamentous or ephemeral taxa (e.g., Mangialajo et al., 2008; Falace et al., 2010; Vergés et al., 2014; Thibaut et al., 2015; Rindi et al., 2017, 2018; Catra et al., 2019; Mariani et al., 2019; Bernal-Ibáñez et al., 2021; Orlando-Bonaca et al., 2021a; Orfanidis et al., 2021). In addition, human modifications in the marine environment also affect population connectivity, leading to habitat fragmentation and loss, and genetic disjunction even at small spatial scales (Alberto et al., 2010).The first strategy to address the decline of *Cystoseira* spp. and their habitat was their protection through international agreements (e.g., Bern Convention, Barcelona Convention, Directive 92/43/EEC, European Red List of Habitats). Nevertheless, there is little evidence of natural recovery of degraded *Cystoseira* forests (but see Munda, 2000; Perkol-Finkel & Airoldi, 2010; Iveša et al., 2016; Medrano et al., 2020a).

An important feature of the reproduction and settlement of *Cystoseira* spp. is that their zygotes sink rapidly (Clayton, 1990), so that they fall and stick to the substrate near the parent algae rather than being blown away by the current. Thus, their low dispersal capacity makes the colonisation of new or damaged areas extremely difficult and therefore habitat fragmentation has a major impact on this species. Restoring affected areas, but only those where previous disturbances are no longer present or have been reduced (e.g., after human intervention in the coastal zone), is so important as it can greatly accelerate habitat recovery.

Restoration is increasingly recognized as an appropriate strategy to actively trigger or accelerate the recovery of degraded coastal habitats (Abelson et al., 2020), as proposed in the recently proclaimed UN Decade of Ecosystem Restoration (2021−2030; https://www.decadeonrestoration.org/). However, detailed knowledge of the current and past distribution of lost habitats, the pressures that have led to their decline, and accurate characterization of donor populations are prerequisites for implementing successful restoration efforts (Gann et al., 2019). In recent years, several restoration attempts have been made in the Mediterranean region under various projects to counteract the losses of fucoids (Falace et al., 2018; Verdura et al., 2018; De La Fuente et al., 2019b; Medrano et al., 2020b; Orlando-Bonaca et al., 2021b; Savonitto et al., 2021). Restoration of brown algal forests can be achieved through: transplanting adult thalli (Falace et al., 2006; Susini et al., 2007), deploying *in situ* bags with fertile receptacles, attached to a hoe and fixed to the seafloor at selected restoration sites (Verdura et al., 2018; Medrano et al., 2020b), and outplanting of juveniles grown *ex situ* under laboratory conditions (Falace et al., 2006; Sales et al., 2011; Falace et al., 2018; Verdura et al., 2018; De La Fuente et al., 2019b; Savonitto et al., 2021; Orlando-Bonaca et al., 2021b; Lardi et al., 2022). The latter two techniques are recommended for the recovery of endangered species to avoid depletion of donor populations (De La Fuente et al., 2019b).

A sharp decline of Fucales has also been reported in the northern Adriatic basin (Falace et al., 2010; Orlando-Bonaca & Rotter, 2018; Orlando-Bonaca et al., 2021a), leading to displacement by turf-forming taxa in shallow waters. The occurrence of these low-lying algae is more likely to be associated with human-induced hydromorphological shoreline changes and high sediment resuspension rates (Falace et al., 2010; Orlando-Bonaca & Rotter, 2018) rather than nutrient enrichment, as the northern Adriatic is considered oligotrophic (Mozetič et al., 2012). Moreover, negative impacts from native herbivorous fish have also been documented for *Sargassum vulgare* C. Agardh (Orlando-Bonaca & Mavrič, 2014) and recently for the transplanted *Gongolaria barbata* (Stackhouse) Kuntze (Orlando-Bonaca et al., 2021b; Savonitto et al., 2021). Currently, in the Gulf of Trieste *G. barbata* and *Cystoseira compressa* (Esper) Gerloff & Nizamuddin are quite common only along the Slovenian coastline, while they have almost disappeared along the Italian coastline. Other species from this group are already rare in Slovenian waters and extinct in Italian waters of the Gulf of Trieste.

Based on previous results of *G. barbata* restoration in the northern Adriatic Sea (Orlando-Bonaca et al., 2021b; Savonitto et al., 2021), the present study aimed to evaluate the performance of different donor populations in close proximity to each other in the restoration of two receiving sites. In order to successfully reduce cultivation time (to avoid lengthy maintenance and minimise costs), we compared the performance of the *ex situ* method (already consolidated at the same sites in the ROC-POP LIFE and J1-1702 projects) with a hybrid method combining cultivation in the mesocosm and suspended culture in the field.

In this context, the objectives of this study were: (1) to analyse the reproductive traits of three populations of *G. barbata* from three nearby donor sites (i.e., Izola, Strunjan, Piran), to assess the differences in their reproductive potential and reproductive success, and (2) to evaluate the restoration success in relation to the different donor and receiving sites (Miramare MPA vs. Piran Marine Biological Station) and the methods used.

Materials and Methods

Study area, donor and receiving sites

The study area is located in the Gulf of Trieste (Figure 1), a shallow, semi-enclosed bay in the northernmost part of the Adriatic and Mediterranean Seas. The gulf stretches from Cape Savudrija (Croatia) to Grado (Italy) and encompasses the entire Slovenian coast, with an average depth of about 21 m. The area is known for the lowest winter temperatures (mostly below 10 °C) in the Mediterranean, and the prevailing winds, which blow mainly from the northeast in an offshore direction (Boicourt et al., 2021). Maximum summer seawater temperatures reach 28 °C (data from the oceanographic buoy VIDA, https://www.nib.si/mbp/en/). The average salinity is about 37 PSU and is mainly influenced by the freshwater inflow from the Soča (Isonzo) River, while water circulation is mainly counter clockwise in the lower layer and clockwise in the surface layer (Stravisi, 1983).

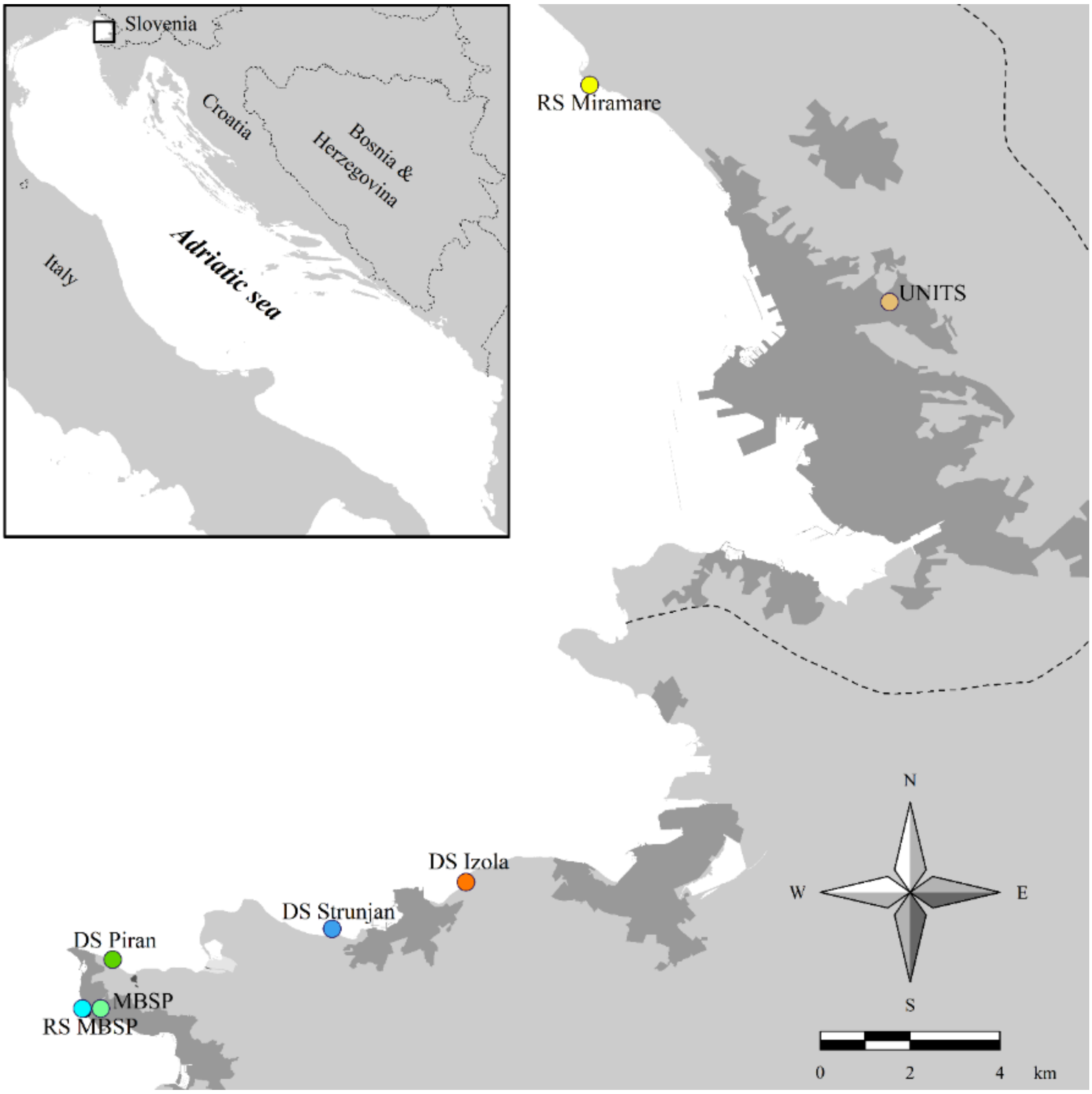

Figure 1 – Map of the study area with donor (=DS) and receiving (=RS) sites for *Gongolaria barbata*.

The rationale of the experiment, which aimed to test the effects of different (three) donor and receiving (two) sites, and cultivation methods (hybrid vs. *ex situ*) on the efficiency of *G. barbata* restoration, is shown in Figure 2.

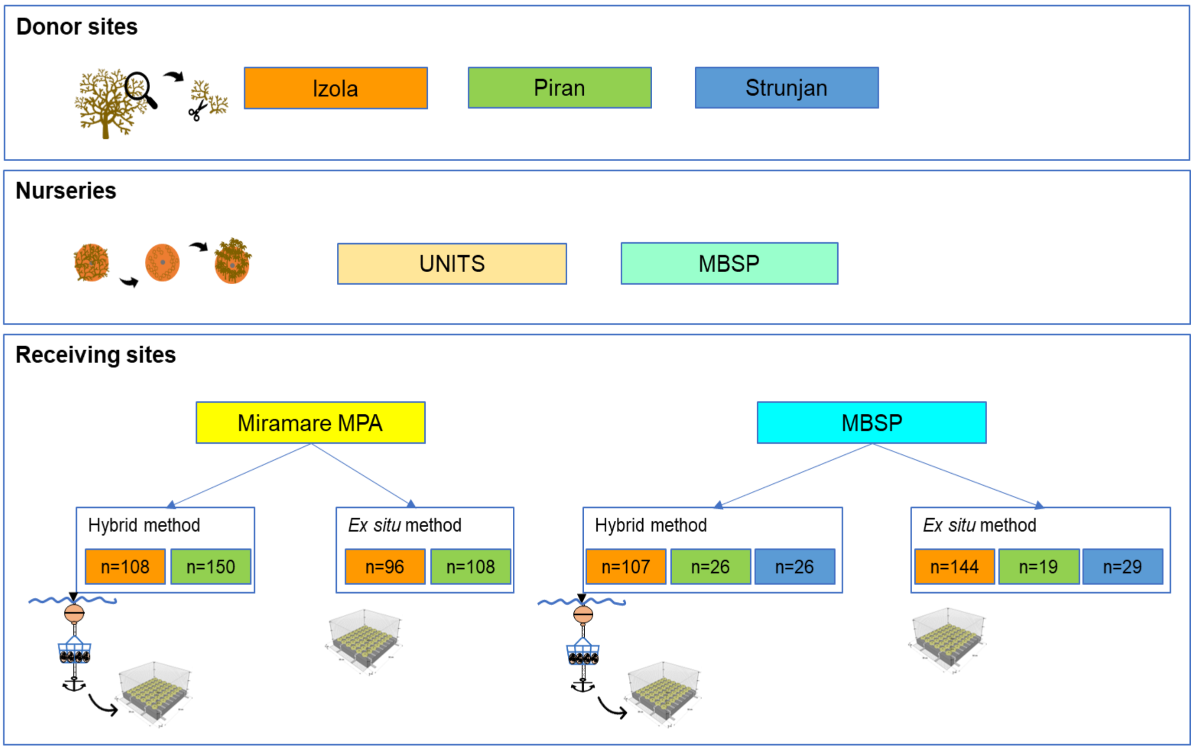

Figure 2 – Experiment on *G. barbata* restoration in the northern Adriatic Sea. UNITS = University of Trieste (Italy), MBSP = Marine Biology Station Piran (Slovenia). It shows: i) the three donor sites where fertile receptacles were collected; ii) the two nursery facilities where germlings were cultivated on clay tiles starting from the fertile receptacles; iii) the two receiving sites where the tiles coming from different donor populations were outplanted using either the hybrid or *ex situ* method.

Fertile apices of *G. barbata* were collected in Slovenian coastal waters from three donor populations located in:

- Izola (45.543567, 13.676371), which is characterised by a healthy and dense population of *G. barbata* and *C. compressa*;
- Strunjan Natural Park (45.53379, 13.638281), where *G. barbata* forms healthy stands in association with *C. compressa* and *Ericaria crinita* (Duby) Molinari & Guiry;
- Piran (45.5284, 13.5754), where belts of *G. barbata* are present in association with *C. compressa* and *E. crinita*.

The distances along the coastline between the donor sites (at an isobath depth of 2 m) are: Izola-Strunjan = 4.6 km, and Strunjan-Piran = 6.9 km. All donor sites have a shallow, rocky seabed and are moderately exposed to wave action. Due to overgrazing by native herbivorous species, such as *Sarpa salpa* (L., 1758) at the Piran sampling site in March 2021, the number of apices collected in this area was very low compared to the other two donor sites (pers. obs.).

The germlings were grown in the two nursery facilities established at the University of Trieste (UNITS) and at the Marine Biology Station Piran (MBSP).

The cultivated germlings were outplanted in:

- the ‘no-take’ zone of the Miramare MPA in Italy (45.701802, 13.714223), where *G. barbata* was once present (Bussani & Vuković, 1992) and has been recently reintroduced as part of the ROC-POP LIFE project (Savonitto et al., 2021). The MPA is located in a highly urbanized coastal system and is affected by grazing from herbivorous fish (Savonitto et al., 2021; Bevilacqua et al., 2022);
- the coastal area off the Marine Biology Station Piran (hereafter MBSP) in Slovenia (45.5177, 13.5680), where less than a decade ago, there was a dense brown algal forest, consisting mainly of *G. barbata* and *C. compressa*. This habitat was completely destroyed in 2016 due to invasive encroachment for beach construction, when no precautions were taken to limit environmental damage. This site is also affected by fish grazing pressure (Orlando-Bonaca et al., 2021b).

Culture in mesocosms

Cultures of *G. barbata* were carried out in the two nursery facilities following the protocol of Falace et al. (2018).

Apices with mature receptacles were collected at 1-2 m depth simultaneously at the three donor sites in early April 2021. They were then transported to the nursery facilities within a few hours under cool (4 °C) and dark conditions. Once in the facilities, receptacles were cleaned with filtered seawater to remove epibionts, and stored at 4 °C for 24 h, followed by thermal shock to promote gamete release.

In environmentally controlled rooms, ca. 50 mg (fresh weight) of fertile apices were placed on each rough clay tile (4.5 cm diameter with a central hole of 0.6 cm diameter), in aquaria filled with filtered seawater enriched with Von Stosch solution and germanium dioxide (for details see Falace et al., 2018; De La Fuente et al., 2019b), and oxygenated with air pumps and bubblers. At UNITS, 204 tiles were seeded with apices from Izola and 303 with apices from Strunjan; at MBSP, 251 tiles were seeded with apices from Izola and 55 with apices from Piran. Using the room controller, temperature was set at 15 °C, light intensity at 125 μmol m^{-2} s^{-1}, and photoperiod at 15:9 h light:dark to simulate conditions during the reproductive period of *G. barbata* at the donor sites. Air temperature was automatically adjusted by the room computer, and to ensure that the water temperature was constant, it was measured with a thermometer in each aquarium every day.

Twenty-four hours later, the apices were removed (T_0) and those from 20 randomly selected tiles were stored at -4 °C for their further analysis (morphometric measurements, count of conceptacles). To avoid nutrient limitation and diatom contamination, the filtered seawater was renewed every 3-5 days, since the water circuit was closed. Treatment aquaria as well as tiles within aquaria were randomly repositioned during culture medium changes to control for positional variation in light intensity.

To test whether it is possible to shorten the cultivation time in the mesocosm, the *ex situ* method (4-week cultivation) was compared with a hybrid method, combining 2-week cultivation followed by in situ suspended culture.

Hybrid method

After 2 weeks of culture in mesocosms (T_2), approximately half of the tiles were randomly selected and transported to the receiving sites, where they were fixed to plastic lantern nets (55 cm in diameter) suspended at 2 m depth, as described in Savonitto et al. (2021).

108 tiles with germlings from Izola and 150 from Strunjan were placed in the Miramare MPA, while 107 from Izola, 26 from Strunjan and 26 from Piran were placed in front of the MBSP.

After 3 months in the lantern nets (July), the tiles were transferred to the rocky bottom using outplanting modules. The modules consisted of 50x50 cm concrete plates (each with up to 48-54 tiles) covered with iron anti-grazing cages (mesh size 1x1 cm^2) and fixed with metal wedges as described in Orlando-Bonaca et al. (2021b). In Miramare MPA, the tiles without juveniles were excluded, so that finally 69 tiles from Strunjan and 7 tiles from Izola were moved to the sea bottom on 2 plates. Contrarily, all the tiles were transferred from the lantern nets to the sea bottom in front of the MBSP on three plates.

Ex situ *method*

After 4 weeks of cultivation in the mesocosms (T_4), the remaining tiles were transported to the receiving sites and fixed with screws to pre-assembled outplanting modules. Tiles from different cultures were marked and placed randomly on the plates (5 plates in the Miramare MPA and 4 in front of the MBSP). Specifically, 96 tiles with germlings from Izola and 108 from Strunjan were located in the Miramare MPA, while 144 from Izola, 19 from Strunjan and 29 from Piran were placed in front of the MBSP. Each tile was considered a replicate.

The tiles were monitored at Miramare MPA and MBSP by SCUBA-diving, and the iron cages were periodically cleaned with a wire brush, to remove epibionts and sediment.

The tiles of the hybrid method were fixed on different plates than those of the *ex situ* method, but the plates of the two methods were interspersed on the sea bottom, very close to each other.

Collected data

During mesocosm culture, data were collected and processed as follows (Table 1):

- At T_0,
    - after the removal of the apices from the tiles, 100 receptacles from each donor site (Izola, Strunjan, Piran) were randomly selected and photographed under a stereomicroscope (Leica MZ 6, Leica Microsystems, Wetzlar, Germany) with a Nikon Coolpix 4500 camera (Nikon Corporation, Tokyo, Japan) for morphometric measurements (i.e., length and width in mm);
    - 20 tiles per donor site (Izola and Strunjan) were randomly selected at UNITS to determine the:
    i. reproductive potential as *RP= N. of conceptacles per receptacle* of all the receptacles on each tile (ca. 70 receptacles per tile). The number of conceptacles on each receptacle was assessed by counting the total number of ostioles protruding on the external surface of the receptacle using a stereomicroscope;
    ii. reproductive success (*RS= N. of zygotes per tile x number of conceptacles per tile*$^{-1}$) by photographic sampling with a Nikon D300 camera (Nikon Corporation, Tokyo, Japan).
- At week 1 (T_1), 2 (T_2), 3 (T_3, only at MBSP), and 4 (T_4) after fertilisation, germling density (i.e., number of germlings per tile) was estimated on pictures randomly selecting 42 tiles from Izola (henceforth as juvIZ) and 76 from Strunjan (juvST) at UNITS, while 30 from Izola (juvIZ) and 10 from Piran (juvPI) at MBSP.
- At T_4, 40 germlings per donor site (only Izola and Strunjan donor populations were considered: juvIZ and juvST at UNITS, juvIZ at MBSP) were randomly selected and photographed directly on the tiles under a stereomicroscope to measure their length and width. No data are available at T_4 for juvPI cultured at MBSP, since. the photos were accidentally deleted during processing.

The length and width of receptacles, the number of zygotes per tile at T_0, the number of germlings per tile at T_1, T_2, T_3 and T_4, and the length and width of germlings at T_4 were determined by analysing the photos with ImageJ software (Schneider et al., 2012).

Table 1 – Summary of the data obtained during the mesocosm culture at UNITS and MBSP.

Sampling time	**Sample size**	**Variable**	**Calculation/Unit**	**Notes**
T_0	100 receptacles per donor site	Receptacle length and width	Length and width measurements in mm	
	20 randomly selected tiles	Receptacle reproductive potential	N. of conceptacles per receptacle of all the receptacles on these tiles	Only at UNITS, tiles seeded with apices from Izola and Strunjan
		Reproductive success	N. zygotes released per tile*N. receptacles per tile	
T_1 = 1 week	42 tiles from Izola and 76 from Strunjan at UNITS, 30 from Izola and 10 from Piran at MBSP, randomly selected at T_1	Germling density at T_1	N. germlings per tile	
T_2 = 2 weeks	42 tiles from Izola and 76 from Strunjan at UNITS, 30 from Izola and 10 from Piran at MBSP, randomly selected at T_2	Germling density at T_2	N. germlings per tile	
T_3 = 3 weeks	30 from Izola and 10 from Piran at MBSP	Germling density at T_3	N. germlings per tile	Only at MBSP, tiles seeded with apices from Izola and Piran
T_4 = 4 weeks	42 tiles from Izola and 76 from Strunjan at UNITS, 30 from Izola and 10 from Piran at MBSP	Germling density at T_4	N. germlings per tile	At MBSP, missing data for Piran
	40 germlings per donor site	Germling length and width	Length and width measurements in mm	

In the field, thallus' lengths were measured by SCUBA divers at Miramare MPA and MBSP on 20 randomly selected tiles per method and donor site from May to November (ca. on 40 thalli per donor site) (see Table 2). In November, % survival was counted visually by SCUBA divers as the total number of tiles on which juveniles were present multiplied per 100. Notably, no length data are available from August onwards for the hybrid method's juveniles outplanted in the Miramare MPA due to 100% mortality.

Table 2 – Summary of the data obtained in the field at both the Miramare MPA and MBSP receiving sites. *Ex situ* method refers to the direct outplanting of cultured juveniles, while hybrid method refers to juveniles that earlier left the lab for being placed sheltered in the field in lantern nets to finalise growth: therefore, juveniles cultured in the hybrid condition stayed in the field 0.5 months longer than the directly outplanted of the *ex situ*.

Time	**Event**	**Data**	**Time in the field**	
			Hybrid method	***Ex situ* method**
Mid-May	Outplanting of tiles		0.5 months	0
May	Monitoring	Thallus length (20 tiles x method x donor site)	1 month	0.5 months
June	Monitoring	Thallus length (20 tiles x method x donor site)	2 months	1.5 months
July	Monitoring, outplanting of tiles of the hybrid method	Thallus length (20 tiles x method x donor site)	3 months	2.5 months
August	Monitoring	Thallus length (20 tiles x method x donor site)	4 months	3.5 months
September	Monitoring	Thallus length (20 tiles x method x donor site)	5 months	4.5 months
October	Monitoring	Thallus length (20 tiles x method x donor site)	6 months	5.5 months
November	Monitoring	Thallus length (20 tiles x method x donor site) and % survival	7 months	6.5 months

Preliminary statistical analysis

One-way ANOVA design was applied to test for possible differences between donor sites (Izola, Piran and Strunjan) in receptacle morphometry (length and width, n=100), reproductive potential (RP, n=20), reproductive success (RS, n=20) and germling morphometry (length and width, n=40) after 4 weeks of culture. Verification of the assumptions (normality with the Kolmogorov-Smirnov test and homoscedasticity with the Bartlett's test) was performed prior to conducting the analyses. If the assumptions were not fulfilled, square root transformation of the response variable was applied and assumptions re-tested. If still not compliant with ANOVA assumptions, the non-parametric equivalent of ANOVA, the Kruskal-Wallis test, was performed.

Differences in germling density on the tiles according to donor populations (juvIZ, juvPI and juvST) were assessed applying a linear mixed model, after checking for normality of the response variables, using the variable “Donor population” as fixed, while “Time” as random.

As for the field data, analyses on the response variable thallus length measured at the last monitoring time (November 2021) were conducted separately for the two receiving sites, Miramare and MBSP, comparing the donor populations and the two different outplanting methods (n=20). A two-way ANOVA design, where “Donor population” and “Outplanting method” were used as fixed orthogonal factors, was performed. Similarly, separate analyses were performed for the two outplanting methods, ex situ and hybrid, comparing donor populations and receiving sites. A two-way ANOVA design, where “Donor population” and “Receiving sites” were used as fixed orthogonal factors, was performed. Normality and homoscedasticity assumptions were tested by Kolmogorov-Smirnov test and Bartlett’s test respectively, applying a square root transformation when necessary.

For all analyses, post-hoc comparisons on significant terms were performed by Tukey test and significant differences are shown on the corresponding plots. All statistical analyses and plots were performed with the free software RStudio (RStudio Team, 2021).

Preliminary results

- ***Mesocosm***

Morphometry of the receptacles

Significant differences in the length and width of the receptacles were found between the three donor sites, with the Izola receptacles being longer and larger (length: 6.02 ± 0.26 SE mm; width: 0.96 ± 0.02 SE mm) than those from Piran (length: 4.66 ± 0.16 SE mm; width: 0.78 ± 0.02 SE mm; p-value < 0.001) and Strunjan (length: 4.70 ± 0.18 SE mm; width: 0.83 ± 0.02 SE mm; p-value < 0.001). No significant differences were found between Piran and Strunjan (Figure 3, Table 3).

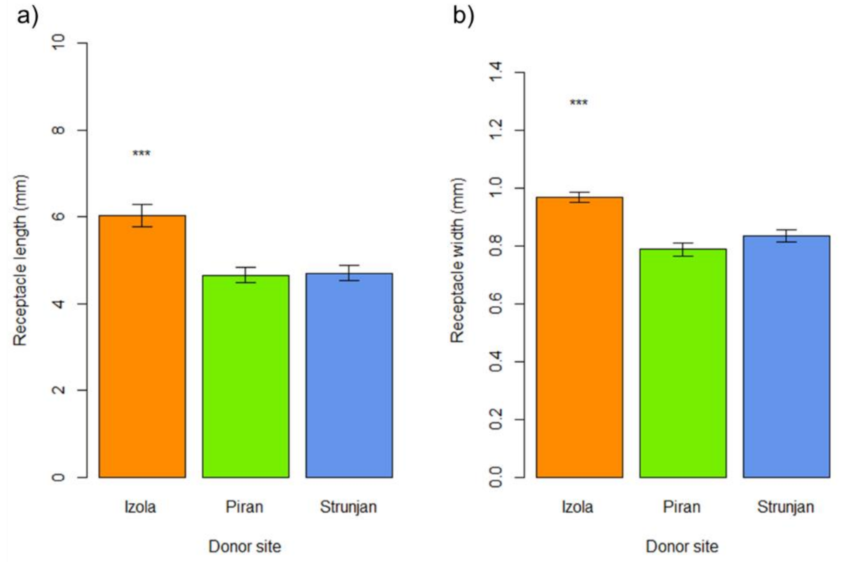

Figure 3 – *G. barbata* receptacles' length (a) and width (b) in the three donor sites.

Fertile apical fronds from Piran and Strunjan generally had short, simple and cylindrical receptacles that were sparsely mucronated and had few or no aerocysts (Figure 4). In contrast, fronds from Izola had long fusiform or mucronate receptacles, predominantly prominent conceptacles, and numerous single or concatenated aerocysts (Figure 4; Table 4).

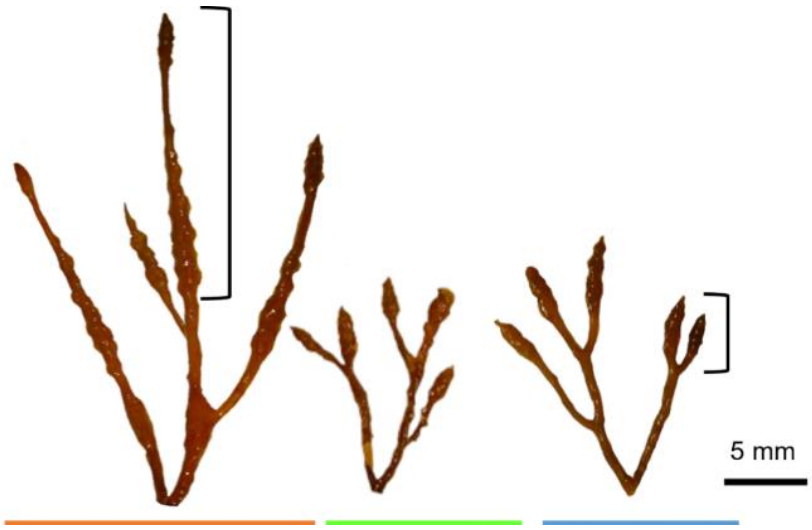

Figure 4 – Fertile apices of *G. barbata* populations from Izola (orange), Piran (green) and Strunjan (blue) sampling sites.

Table 4 – Comparison of *Gongolaria barbata* populations sampled at Izola, Piran and Strunjan.

Feature	**Donor population**		
	Izola	**Piran**	**Strunjan**
Cryptostomata	numerous	numerous	numerous
Aerocysts	abundant, oval to spindle shaped, isolated or in chains (up to 5 aerocysts)	absent or few isolated	absent or few isolated
Receptacle shape	simple, fusiform mucronate or pedicellate	simple or bifid, cylindrical, mucronate	simple or bifid, cylindrical, mucronate
Receptacle length	6.33 ± 0.33 mm, up to 19.79 mm	4.55 ± 0.17, up to 10.89 mm	4.71 ± 0.18 mm, up to 9.97
Receptacle width	0.96 ± 0.02 mm	0.84 ± 0.02 mm	0.77 ± 0.02 mm
Conceptacle	prominent	smooth or slightly prominent	smooth or slightly prominent
Conceptacles receptacle^{-1}	30.45 ± 0.45 conceptacles receptacle^{-1} (up to 93 conceptacles receptacle^{-1})	-	24.96 ± 0.25 conceptacles receptacle^{-1} (up to 65 conceptacles receptacle^{-1})

Reproductive potential and reproductive success

The receptacles from Izola had a significantly higher reproductive potential (30.5 ± 0.4 SE conceptacles receptacle^{-1}) compared to Strunjan (25.0 ± 0.3 SE conceptacles receptacle^{-1}; p-value < 0.001; Figure 5a, Table 3). Conversely, reproductive success of the Izola receptacles (0.4 ± 0.1 SE zygotes conceptacle^{-1}; p-value < 0.01) was significantly lower than in those from Strunjan (0.8 ± 0.1 SE zygotes conceptacle^{-1}; Figure 5b, Table 3).

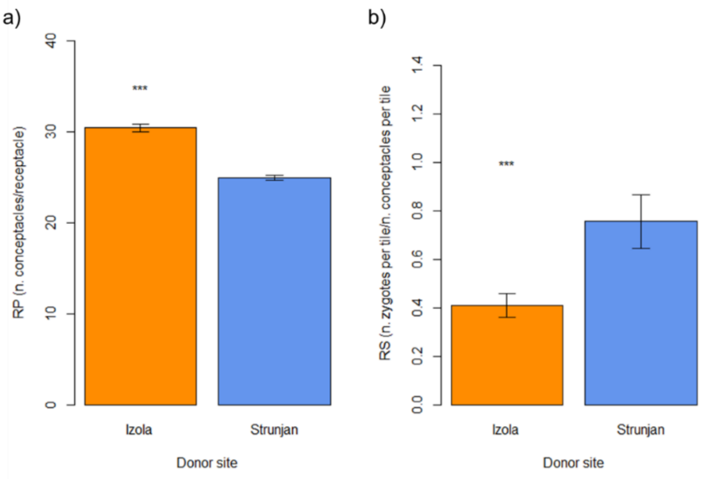

Figure 5 – Reproductive potential (a) and reproductive success (b) of receptacles from Izola and Strunjan donor sites.

Germling density

Germling density gradually decreased from the first to the fourth week in culture (Figure 6). After 4 weeks, it ranged from 132 ± 15 SE germlings per tile (juvIZ) to 73 ± 8 SE germlings per tile (juvST) (Figure 6). At earlier sampling times (T_1-T_3), juvST had significantly higher germling density values (p-value < 0.001, Table 3) than juvIZ and juvPI, which in turn differed significantly from each other (p-value = 0.02, Table 3).

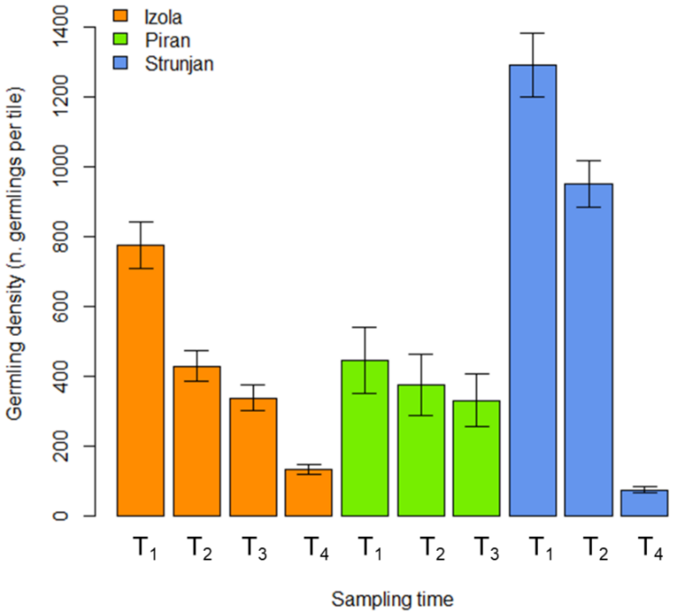

Figure 6 – Germling density on tiles from apices collected in Izola, Piran and Strunjan (data for Izola are pooled for UNITS and MBSP). No data are available for T_3 for germlings of Strunjan cultivated at UNITS, and for T_4 for germlings from Piran cultivated at MBSP.

Germling morphometry after 4 weeks

After 4 weeks in culture, significant differences in length and width were recorded between juvIZ (length: 1.15 ± 0.07 SE mm; width: 0.29 ± 0.01 SE mm) and juvST (length: 0.65 ± 0.03 SE mm; width: 0.17 ± 0.01 SE mm), with juvIZ having higher values in both morphometric parameters (p-value < 0.001) (Figure 7a, b; Table 3).

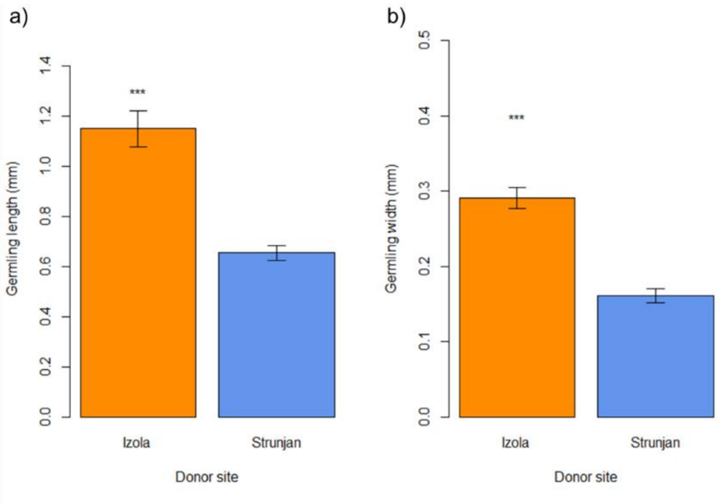

Figure 7 – Germling size (mean±SE) at T_4 (data for Izola are pooled for UNITS and MBSP). No data are available for those from Piran cultivated at MBSP.

Table 3 – Results of statistical analyses on laboratory data.

Response variable	**Statistics**	**Tested factor**			
Receptacle length	Anova		SS	F	p-value
		Donor site	3.465	11.3	<0.0001
		Residuals	47.531		
				chi-sq	p-value
Receptacle width	KS test	Donor site		36.904	<0.0001
				chi-sq	p-value
Reproductive potential	KS test	Donor site		101.09	<0.0001
			SS	F	p-value
Reproductive success	Anova	Donor site	0.455	7.7237	0.008
		Residuals	2.237		
			Std.Error	t-value	p-value
Germling density	Linear mixed model	Intercept	0.507	8.194	<0.0001
		Donor pop: juvPI	0.166	-2.224	0.027
		Donor pop: juvST	0.084	7.079	<0.0001
		Random effect: Time	1.008		
				chi-sq	p-value
Germling length	KS test	Donor population		13.433	0.0002
				chi-sq	p-value
Germling width	KS test	Donor population		31.015	<0.0001

- ***Field data***

Juvenile length

As for the hybrid method, thalli in Miramare MPA showed lower performance compared to MBSP: indeed, reduced length (0.3 ± 0.1 SE - juvIZ; 0.5 ± 0.1 SE cm - juvST) was observed after only 3 months in the field (July 2021). These tiles were heavily colonised by molluscs from June and especially in July (i.e., at the time of outplanting on the sea bottom), and in August (i.e., after 1 month on the sea bottom) the mortality of the juveniles was 100%. For this reason, monitoring of the tiles of the hybrid method was interrupted at this site. Off the MBSP, mean thallus length after 7 months (November 2021) did not differ significantly between juveniles from the three donor populations (Figure 8a; Table 5), ranging from 2.3 ± 0.4 SE cm (juvST) to 3.2 ± 0.3 SE cm (juvPI) and 3.3 ± 0.3 SE cm (juvIZ), with a maximum of 7.3 cm.

At 6.5 months (November 2021), the mean length of thalli implanted using the *ex situ* method ranged from 8.4 ± 0.7 SE (juvST) to 9.1 ± 0.6 SE cm (juvIZ), with a maximum of 15.5 cm in the Miramare MPA, with no significant differences between juvIZ and juvST. Off the MBSP, thalli rangend in length from 2.2 ± 0.3 SE (juvPI) to 4.9 ± 0.3 SE cm (juvIZ), with a maximum of 12.9 cm (Figure 8b). JuvIZ differed significantly in thalli length from juvPI and juvST (which did not differ from each other) and were significantly shorter than those implanted in Miramare (Figure 8b; Table 5). Similarly, juvST implanted in MBSP were significantly shorter than those implanted in Miramare (Figure 8b; Table 5).

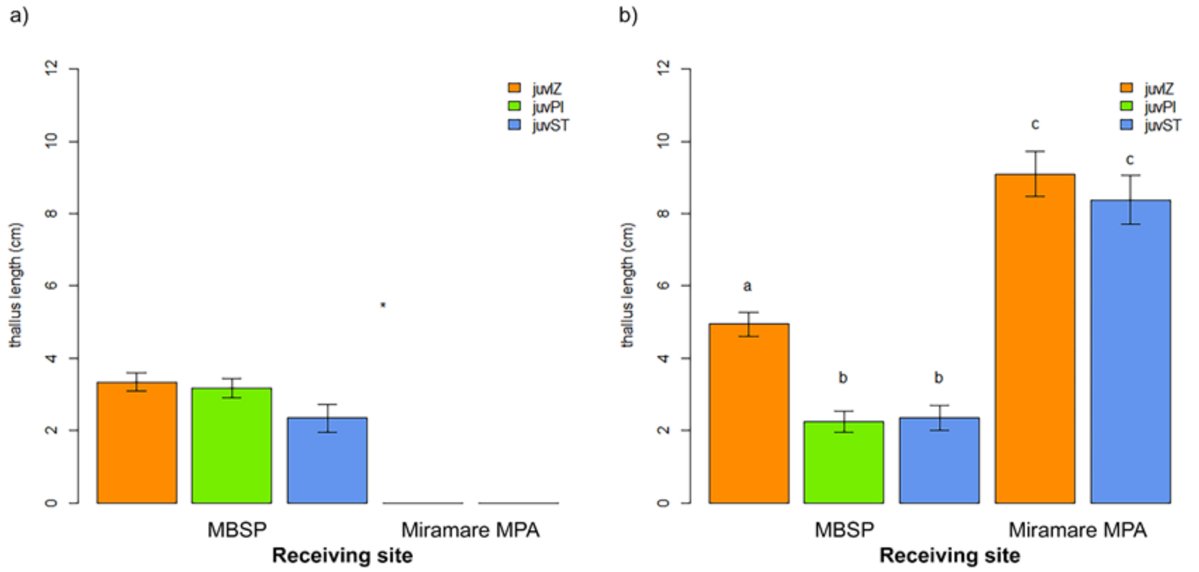

Figure 8 – Thallus length (mean±SE) after 6.5 months in the field comparing juvIZ, juvST and juvPI in the receiving sites in front of the MBSP and in Miramare MPA for a) hybrid and b) *ex situ* methods.

In front of the MBSP, no significant differences were found between the two methods, except for juvIZ implanted with the *ex situ* method, which performed better than all others (p-value < 0.0001; Figure 9a). In Miramare MPA, no differences were found between juvIZ and juvST using the *ex situ* method, while significant differences were found between the two methods (p-value < 0.001).

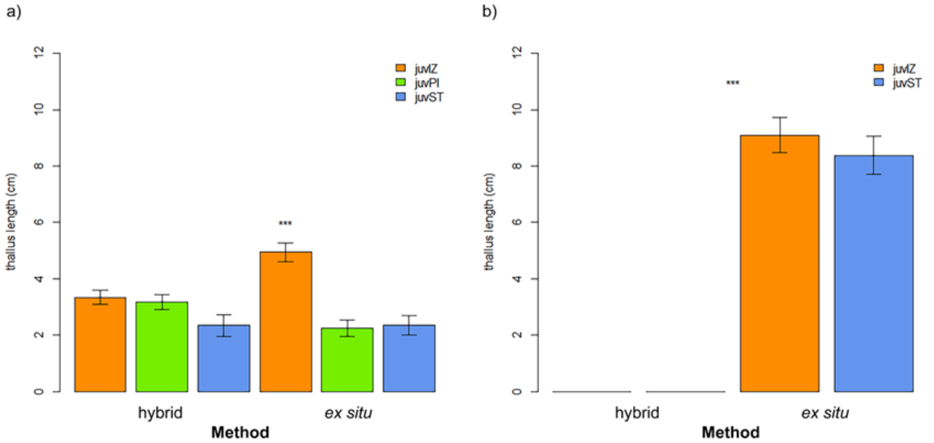

Figure 9 – Thallus length (mean±SE) after 6.5 months in the field comparing juvIZ, juvST and juvPI implanted with the *ex situ* and hybrid methods in the receiving sites a) in front of the MBSP and b) in Miramare MPA.

Percent survival

In front of the MBSP, the percentage of tiles with germlings (% survival) ranged from 58% (juvST) to 70% (juvIZ) and 90% (juvPI) for the *ex situ* method, while for the hybrid method it was 41% for juvST, 83% for juvIZ and 100% for juvPI (Figure 10a). In the Miramare MPA, the percentage of tiles with germlings in November 2021 ranged from 44% (juvST) to 78% (juvIZ) for the *ex situ*, while it was 0 % for the hybrid method for both juvIZ and juvST (Figure 10b).

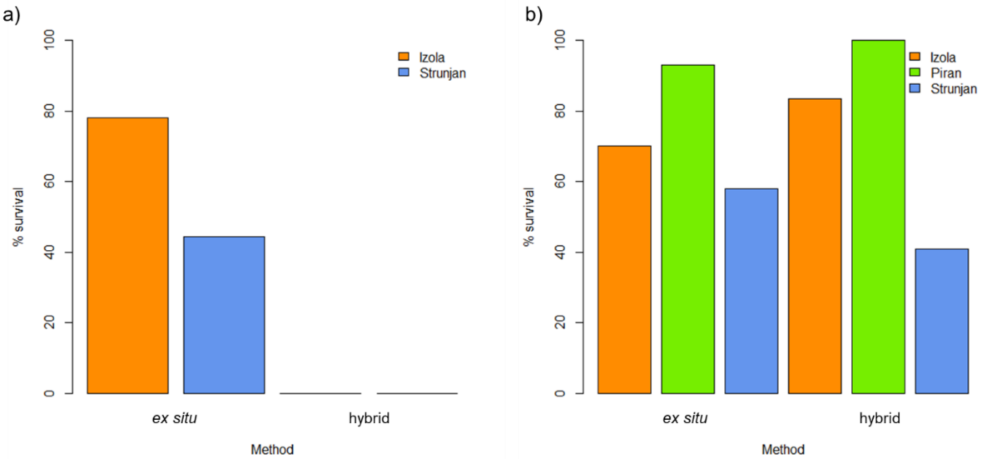

Figure 10 – Percent survival data in the *ex situ* and hybrid method in November 2021 from fertile *G. barbata* material collected in Izola, Piran and Strunjan donor sites, in the receiving sites of a) Miramare MPA and b) MBSP.

Table 5 – Statistic results for field data.

Response variable	Statistics	Tested factors			
			SS	F	p-value
Juveniles' length in MBSP	Anova	Donor pop	0.861	14.197	<0.0001
		Method	0.114	3.743	0.055
		Donor pop X Method	0.580	9.5547	0.0001
		Residuals	4.308		
			SS	F	p-value
Juveniles' length in Miramare MPA	Anova	Donor pop	4.665	0.563	0.457
		Method	145.129	17.542	0.0002
		Donor pop X Method	0.239	0.029	0.866
		Residuals	306.110		
			SS	F	p-value
Juveniles' length hybrid method	Anova	Donor pop	6.381	1.350	0.266
		Receiving site	15.314	6.480	0.013
		Donor pop X Rec site	0.471	0.199	0.657
		Residuals	160.708		
			SS	F	p-value
Juveniles' length in MBSP *ex situ* method	Anova	Donor pop	1.201	21.780	<0.0001
		Receiving site	2.142	77.696	<0.0001
		Donor pop X Rec site	0.188	6.815	0.010
		Residuals	3.060		

Preliminary discussion

For restoration purposes, it is important to gain a deeper knowledge of the phenology of the species being restored. Over the last forty years, many studies have investigated the phenology of *Cystoseira* species, highlighting that they usually undergo both morphological and reproductive changes throughout the year (e.g., Gómez-Garreta et al., 1982; Hoffmann et al., 1992; Alongi et al., 1999; Marzocchi et al., 2003; Falace et al., 2005; Falace & Bressan, 2006; Medrano et al., 2020a). Nevertheless, few studies focused on phenotypic variation among populations of fucoid species and also reported significant differences in reproductive traits (De Paula and De Oliveira 1982; Silva et al., 2004; Sadogurska et al., 2021).

Within its geographical range, *G. barbata* shows considerable ecological plasticity and outstanding morphological variability due to environmental factors (Ercegović, 1952; Falace & Bressan, 2006). In the present study, we found relevant differences in the shape and size of apical fronds and the number of aerocysts between populations of *G. barbata* from Izola and those from Strunjan and Piran (Tables 3 and 4): long spindle-shaped receptacles, bearing aerocysts and always ending with a mucron for the population from Izola (Falace et al., 2005; Falace & Bressan 2006), while the populations from Strunjan and Piran had short cylindrical receptacles that bore almost no aerocysts and were not always mucronated (Orlando-Bonaca et al., 2021b).

In the Black Sea, Sadogurska et al. (2021) reported that this variability might depend on the hydrodynamic regime and the season and observed two main morphologies. At sheltered sites, G. barbata had long, sickle-shaped receptacles, sometimes with protruding conceptacles, and numerous concatenated aerocysts. In contrast, on exposed shores G. barbata had small, oval or spindle-shaped receptacles with smooth surfaces and few aerocysts. Nevertheless, molecular analyses revealed minor differences between Black Sea samples of G. barbata, representing smaller infraspecific variations. Sadogurska et al. (2021) therefore concluded that the morphological variability of G. barbata in the Black Sea is not due to infraspecific taxa, but to considerable ecological plasticity and seasonal variation. Consequently, the populations sampled for the present study could represent two different morphotypes, likely due to local environmental differences.

Although the study sampling sites are close to each other on the Slovenian coast (see Material and methods), they have different environmental characteristics. The Izola donor site is located at the edge of Koper Bay, which is characterised by high sedimentation and suspension rates reflected in increased water turbidity (Ogorelec et al., 1991). The area is exposed to large freshwater inputs from the Rižana and Badaševica rivers (Cozzi et al., 2012) and is located close to the port of Koper. In addition, some unidentified sources of pollution (probably municipal sewage) affect the ecological status of macroalgae in this area (Orlando-Bonaca & Rotter, 2018; Orlando-Bonaca et al., 2021c). In contrast, Strunjan and Piran are exposed to lower anthropogenic pressures (Orlando-Bonaca et al., 2015), as they are located on a coastline that is still in its pristine state. Moreover, these two sites are located outside the bays of Koper and Piran, which are known to have the highest sedimentation and suspension rates in Slovenian marine waters (Ogorelec et al., 1991). Consequently, the three donor populations might be adapted to different sedimentation rates, salinity, and turbidity, affecting the length of receptacles and the presence of aerocysts. In environments with high water turbidity, the development of aerocysts stimulates algae to stretch upwards and in this way improve their access to light (Lüning, 1991). The elongated shape of the receptacles could be another adaptation to increase surface area and better capture light to enhance photosynthesis. Therefore, the Izola population could be a morphotype adapted to higher sedimentation.

The fertile apices of the Izola population not only had longer and larger receptacles (Figures 3 and 4), but also a higher number of conceptacles per receptacle (RP, Figure 5a; Table 3). However, the number of zygotes per tile relative to the number of conceptacles per tile (RS) was higher in the *G. barbata* population from Strunjan (Figure 5b; Table 3). Furthermore, the density of juvIZ and juvPI was lower than that of juvST after one (T_1) and two weeks of culture (T_2) (Figure 6). Nevertheless, the progressive decrease in seedling density over time (T_4), which can be explained at least in part by the process of "self-thinning" of a growing plant population (Ang & Wreede, 1992; Steen & Scrosati, 2004), was more pronounced in juvST. Not only the density but also the dimensions of juvST were smaller than those of juvIZ (Figure 7) at T_4. This result suggests that the population from Strunjan, although more successful in the first two weeks of culture, was less successful than the others after three to four weeks.

The success of outplanting juveniles in the field was very variable and depended on both the location of the receiving/donor sites and the method. As with the *ex situ* method, the greatest differences in Slovenia were due to the different donor sites. This is because although all experimental tiles were exposed to the same environmental conditions, juvIZ thalli grew significantly better than juvST and juvPI (Figure 8). This was probably due to preadaptation to the high sediment resuspension rate characteristic of this receiving site. The MBSP receiving site is located at the edge of Piran Bay, which has a similar water turbidity, suspension and sedimentation rate as the Izola donor site at the edge of Koper Bay (Ogorelec et al., 1991). In addition, the tiles were placed near an underwater pipe, to prevent the protective cages from being damaged by fishermen's trawls, as the site is not a protected area. However, during monitoring it was found that the proximity of the pipe reduces hydrodynamics and exposes the juvenile thalli to higher sediment resuspension during growth. This may have favoured the growth of juvIZ, which are adapted to similar environmental conditions, and disadvantaged juvST and juvPI. In fact, sedimentation may be one of the most pervasive factors affecting the survival and development of early life stages of macroalgae (Vadas et al., 1992; Schiel & Foster, 2006; Irving et al., 2009). In addition, the protective cages in Miramare were cleaned of fouling at least twice a month, whereas in MBSP they were cleaned only once a month. The lower light conditions may have contributed to the lower algal growth in front of the MBSP.

For the hybrid method, the main differences were due to the receiving site: the method performed much better at MBSP than at Miramare (Figure 10). As this method performed better at Miramare in 2019 (Savonitto et al., 2021), these poorer results could be related to a series of unfavourable environmental conditions that occurred in the Italian part of the Gulf of Trieste in spring and early summer 2021. An impressive bloom of the jellyfish *Rhizostoma pulmo*, followed by a massive bloom of *Noctiluca scintillans*, which was much more pronounced in the Italian waters than in the Slovenian ones (ARPA FVG, 2021), characterised the area during the laying of the tiles on the lantern nets and in the following crucial weeks (Figure 11). In addition, the tiles on the lantern nets were colonised by mussel and oyster recruits from June onwards, and this trend intensified in July, favoured by a mussel farm near the MPA that probably served as a larval source. The molluscs have thus probably displaced the seedlings of *G. barbata*, a phenomenon frequently observed in *Cystoseira* populations (Gros, 1978; Benedetti-Cecchi et al., 1996; Thibaut et al., 2005; Mačić et al., 2010; Perkol-Finkel & Airoldi, 2010).

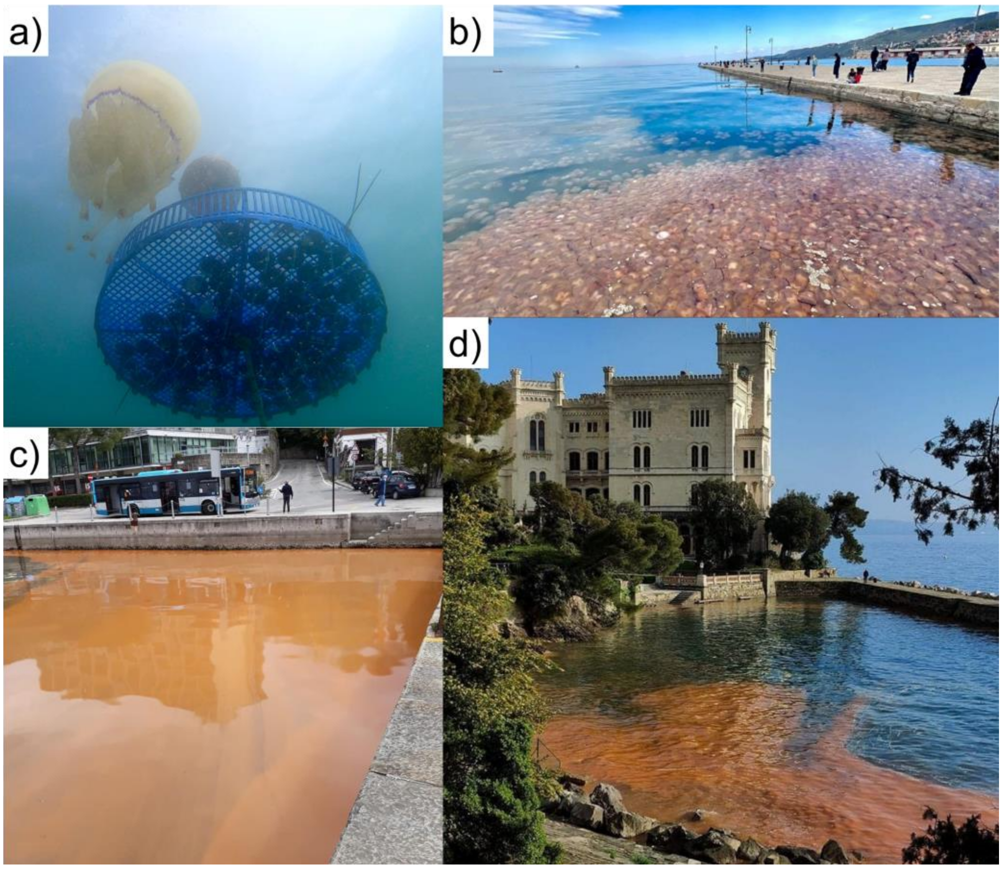

Figure 11 – Unfavourable environmental dynamics that characterized the Italian portion of the Gulf of Trieste (Northern Adriatic Sea) in spring 2021. a) *Rhizostoma pulmo* over a lantern net in the Miramare MPA (credits: Marco Segarich); b) Jellyfish bloom in Trieste, Italy (credits: Repubblica; https://www.repubblica.it/green-and-blue/2022/04/26/news/bloom_meduse_trieste_riscaldamento_mare_pesca_intensiva-346958254/); *Noctiluca scintillans* bloom c) next to Miramare MPA (credits: Area Marina Protetta Miramare) and d) inside the MPA, on the experimental plots (credits: Legambiente FVG; https://www.legambientefvg.it/component/content/article/2-uncategorised/2411-i-complessi-equilibri-degli-ecosistemi-marini-che-succede-nel-nostro-golfo?Itemid=337).

Nevertheless, the satisfactory results of the hybrid method at MBSP support the pilot application reported by Savonitto et al. (2021). At MBSP, the lantern net allowed us to successfully reduce the culture time required for the seedlings to reach a "refuge size," and it also seemed to limit the presence of mesograzers, which typically occur on vegetated rocky bottoms. In addition, this intermediate step of keeping seedlings in suspended culture in the sea prior to final outplanting on the seafloor reduced the risk of bacterial or microalgal outbreaks, which are common with prolonged culture in mesocosms (Orlando-Bonaca et al., 2021b; Lardi et al., 2022). Nevertheless, it should be further tested in Slovenian coastal waters to improve the outcomes in terms of thalli growth. Shortened times in mesocosms correspond to reduced maintenance and overall costs, which is necessary for scaling up efforts beyond a purely experimental scale, as is now required by international policies and standards (Gann et al., 2019).

Controlling grazers with protective cages improved the overall performance of outplanting , as it has already been shown that the loss of a protective cage for only one week resulted in significant overgrazing of juveniles by herbivorous fish (Orlando-Bonaca et al., 2021b). *S. salpa*, which is very abundant in the Miramare MPA and also along the Slovenian coast, is most likely the main predator of *G. barbata* recruits in the Gulf of Trieste, as schools were regularly observed around the experimental plots during monitoring by divers and with the camera also in 2021 (pers. obs.). Conversely, the impact of grazing by sea urchins and meso-grazers (e.g., molluscs, crustaceans) was likely negligible, as indicated by our monitoring videos and underwater observations. In any case, the thalli cannot be kept in a cage indefinitely. In spring 2022, the cage that had protected thalli outplanted in spring 2020 (Orlando-Bonaca et al., 2021b) was removed, and after several months without protection, thalli were biting at the tips but were at least 30 cm long overall (unpublished data). In contrast, the sharp decline of macroalgae in the Miramare MPA has not only affected *Cystoseira*, as the system has "tipped" into an alternative state dominated by turf algae and other grazing-resistant species (e.g., *Dictyota* spp., encrusting calcareous Rhodophytes; Falace et al., 2010; Bevilacqua et al., 2022). Reversing this state would require enormous efforts and the ongoing maintenance of grazing protection, with great uncertainty about the long-term viability of transplanted stands.

Our results suggest that the characteristics of the donor site may confer higher fitness to the cultured seedlings when they are in the field, as shown by the higher growth of juvIZ under sediment resuspension in front of the MBSP.

Despite its late start compared to restoration on land, marine restoration holds enormous potential as an important tool for developing strategies to ensure that the oceans continue to provide the goods and services on which the well-being of life on Earth depends (Danovaro et al., 2021). The results of this study suggest that the outplanting of infralittoral canopy-forming species such as *G. barbata* in the northern Adriatic should be further tested and improved, at least on the Slovenian coasts of the Gulf of Trieste. Although the laboratory cultivation is already consolidated albeit further improvable, positive results of the laboratory phase do not guarantee the success of outplanting at sea. Successful reestablishment of brown algal forests, even in MPAs, is influenced by factors other than nutrient enrichment and other pollutants, some of which cannot be controlled (i.e., climate change-related trends and events, complex and unpredictable environmental dynamics like jellyfish or *N. scintillans* blooms) and others that are more measurable/manageable, such as overgrazing by herbivorous fish. While it is often easy to detect changes in a community, it can be difficult to interpret the observed shifts in community structure to determine whether restoration is warranted and whether it will be successful. As protection is easier and cheaper than restoration, herbivore regulation (to reduce herbivore density) is extremely urgent and should be planned and implemented on a larger regional scale to save not only brown algal forests, but also other endangered infralittoral vegetation.

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CONCLUSIONS

The present thesis has focused on the restoration by *ex situ* recruitment enhancement of *Cystoseira* forests in the Mediterranean Sea under two main aspects. On one hand, it has contributed to expand the base knowledge on the genus *Cystoseira*, which is essential to successfully conserve, manage and restore these pivotal habitat-forming species, in particular in a climate change scenario. On the other hand, it has provided some of the first concrete implementations of the *ex situ* recruitment enhancement technique in the Mediterranean Sea, investigating how crucial factors – like climate change, fish grazing, selection of donor populations and receiving sites, mesocosm cultivation times – can determine the outcomes of restoration efforts.

Particular attention has been devoted to the early developmental stages of *Cystoseira* species, which had been poorly investigated before, in spite of their crucial importance in driving population recruitment and, consequently, persistence. For the first time, the embryology of *E. barbatula*, *G. barbata* and *E. giacconei* has been published. For the two latter species, also the effect of temperature has been investigated, thus providing evidence on the detrimental effect of climate change on their biology and their possible fate.

From an operative point of view, the fine-tuning of cultivation protocols to grant optimal culture conditions has emerged as an essential requisite for obtaining higher yields and thus larger and more numerous germlings to be outplanted. In the field, natural mortality has resulted to be exacerbated by multiple stressors, as grazing by herbivorous fish, complex environmental dynamics and climate change. Throughout this study it has clearly emerged that, despite biological and logistic challenges can be overcome, biotic interactions and unpredictable events represent the major threat to the success of restoration of *Cystoseira* forests, strongly limiting the possibilities of upscaling. Implementation of mitigation actions thus results as a crucial and – in some cases – the sole possible strategy to cope with the stochasticity of restoration results driven by these stressors.

APPENDIX – DISSEMINATION OF RESULTS & DIVULGATION ACTIVITIES

The data collected during this doctoral work have been the subject of scientific dissemination and to divulgation to the public.

Research papers in international journals[7]

Savonitto, G., Alongi, G., & Falace, A. (2019). Reproductive phenology, zygote embryology and germling development of the threatened *Carpodesmia barbatula* (= *Cystoseira barbatula*)(Fucales, Phaeophyta) towards its possible restoration. Webbia, 74(2), 317–323. https://doi.org/10.1080/00837792.2019.1692594

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[7] The corresponding author is underlined.

Presentations at Congresses

Savonitto, G., De La Fuente, G., Ciriaco, S., Srijemsi, M., Chiantore, M., & Falace, A. Thermal anomalies trigger phenological shifts in Mediterranean threatened marine forests. Youmares 11 Conference – online, 15-16/10/2020.

Marletta, G., Srijemsi, M., **Savonitto, G.**, Bevilacqua, S., Costanzo, L. G., Carniel, F. C., Tretiach, M., & Falace, A. Does ocean warming pose a significant threat to the survival of marine forests? The possible fate of *Cystoseira hyblaea* (Fucales). 12th International Phycological Congress – online, 22-26/03/2021.

Savonitto, G., De La Fuente, G., Chiantore, M., Clausing, R., Asnaghi, V., Srijemsi, M., Ciriaco, S., Caragnano, A., Falace, A. Lessons learned from the restoration of two *Cystoseira sensu lato* (Fucales, Ochrophyta) species in the Ligurian and Northern Adriatic Sea. 115° Congresso della Società Botanica Italiana – online, 09-11/09/2020.

Posters at Congresses

Falace, A., **Savonitto, G.**, De La Fuente, G., Chiantore, M. Framework underpinning procedures for canopy-forming brown algae restoration in Mediterranean ecosystems in a global change scenario. 8th World Conference on Ecological Restoration – Cape Town, South Africa, 22-27/09/2019.

Abstracts for oral communications at Congresses

Falace, A., De La Fuente, G., **Savonitto, G.**, Peplis, M., & Chiantore, M. (2019). Will the restoration of canopy-forming brown algae keep up the pace with climate changes?. Riunione scientifica del Gruppo di Lavoro per l'Algologia – Bari, 15-16/11/2019.

Falace, A., **Savonitto, G.**, De La Fuente, G., Srijemsi, M., Ciriaco, S., & Chiantore, M. (2021). Mitigation solutions in the restoration of a canopy-forming brown alga to face reproductive stochasticity and grazing impacts. 12th International Phycological Congress – online, 22-26/03/2021.

De La Fuente, G., Clausing, R., Asnaghi, V., **Savonitto, G.**, Falace, A., & Chiantore, M. (2021). Towards the restoration of canopy-forming brown algae in the Mediterranean sea: big challenges and some wins. 12th International Phycological Congress – online, 22-26/03/2021.

Marletta, G., Marina Srijemsi, **Gilda Savonitto**, Stanislao Bevilacqua, Luca Giuseppe Costanzo, Fabio Candotto Carniel, Mauro Tretiach, Giuseppina Alongi, Annalisa Falace. Impacts of climate change on the developmental stages of *Cystoseira hyblaea* (Fucales) and its possible fate under a foreseen warming scenario. 116th Congress of the Italian Botanical Society – online, 08-10/09/2021

Lessons

“Restoration of marine forests in the Mediterranean and the ROC-POP Life project” to the students of the International MSc in Marine Biological Resources (IMBRSea) – Università Politecnica delle Marche. Trieste, 04/04/2019.

Radio

"Ecosistemi marini e coste" Pianeta Azzurro, Radio Spazio 103, 13/07/2021.

"Le foreste marine" RADAR SEGNALI DALLA SCIENZA, DALLA CULTURA, DALLA SOCIETÀ, RAI FVG, 08/10/2019.

"Trieste città europea della scienza Il mare", podcast Magazzino 26, Radio3 RAI, 02/09/2020.

TV

"Contadini del mare contro i cambiamenti climatici", BuongiornoRegione, TgR RAI Fvg, 22/03/2019; TgLeonardo, 25/03/2019.

"Il restauro delle foreste marine nel Golfo di Trieste " Trieste in Diretta, Telequattro, 17/05/2019.

Newspaper

"Alto Adriatico a rischio, ma lo Stato dorme" Vita Nuova, 29/03/2019.

Instagram

1 min about marine forests on Ig stories of Davines for the project "Tuteliamo il mare: Foreste di mare e di terra", 12/06/2020.

Public events

Partecipation to Barcolana 51 with a stand dedicated to the ROC-POP Life project (2019).

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