



University of Sassari

Department of Architecture, Design and Urban Planning

Ph.D. in Architecture and Environment

XXXVII Cycle

SSD BIO/07 Ecology

CRUSTOSE CORALLINE ALGAE CONSERVATION AND RESTORATION IN THE CLIMATE CHANGE SCENARIO

Supervisor:

Prof. Giulia Ceccherelli

Doctoral dissertation of:

Alessandra Puccini

INDEX

1. **Chapter 1**: **GENERAL INTRODUCTION**……1

   1.1 Coralligenous reefs……..1
   1.2 Crustose Coralline Algae…….2
   1.3 Animal forest……...4
   1.4 Restoration……..6
      1.4.1 Restoration of Coralligenous reefs…….6
   1.5 Structure of the thesis and aims of the work……...8

2. **Chapter 2**: **AN *EX-SITU* APPROACH FOR CULTIVATING CORALLINE ALGAE IN A RESTORATION PERSPECTIVE**……..9

   Abstract……...10
   1 Introduction…….11
   2 Materials and methods……14
      2.1 Sampling site……14
      2.2 Experimental setup……14
      2.3 Collected data……16
      2.4 Statistical analysis…….17
   3 Results…….18
   4 Discussion……...30

3. **Chapter 3**: **INVESTIGATING TILE MATERIALS FOR ENCRUSTING CORALLINE ALGAE RECRUITMENT: A TOOL FOR FUTURE CORALLIGENOUS RESTORATION**……...34

   Abstract……....35
   1 Introduction……..36
   2 Materials and methods…….38
      2.1 Sampling site……..38
      2.2 Experimental setup…….39
      2.3 Statistical analysis……..40
      2.4 DNA extraction, PCR amplification and sequencing……41
      2.5 Sequence alignments and phylogenetic analysis……42
      2.6 Morpho-anatomical analysis……...43
   3 Results……..44
      3.1 Molecular and morphological results……46
   4 Discussion……48

4. **Chapter 4**: **ADAPTABILITY OF CRUSTOSE CORALLINE ALGAE RECRUITS TO DIFFERENT FIELD AND TEMPERATURE CONDITIONS: INSIGHTS FOR *TITANODERMA* SPECIES**……51
Abstract……52
1 Introduction……..53
2 Materials and methods…….56
2.1 Study site and experimental setup…….56
2.2 Data analysis……..61
2.3 DNA extraction, PCR amplification and sequencing……61
2.4 Sequence alignments and phylogenetic analysis……...62
2.5 Morpho-anatomical analysis……..63
3 Results……..64
3.1 Molecular and morphological results…….66
4 Discussion……68

5. **Chapter 5**: **CIVITAVECCHIA (CENTER WESTERN ITALY) PORT ENLARGEMENT AND COMPENSATION MEASURES: THE SPATIAL VARIABILITY OF CORALLIGENOUS REEFS AND LOCAL BYCATH MATERIAL TO IMPROVE HABITAT QUALITY**……..71
Abstract……72
1 Introduction……..73
2 Materials and methods…….76
2.1 Study area……...……76
2.2 Statistical analysis……..78
2.3 ESCA index calculation……78
2.4 Civitavecchia bycatch……78
3 Results……..79
3.1 Coralligenous reefs variability……...79
3.2 Civitavecchia bycatch……85
4 Discussion……87

6. **Chapter 6: GENERAL DISCUSSION AND CONCLUSION**……..96

7. **Chapter 7: REFERENCES**……..99

To my mom and my sister, for never leaving me alone.

You are my family.

Chapter 1

GENERAL INTRODUCTION

1.1 Coralligenous reefs

The coralligenous habitat is one of the most important and diverse benthic ecosystems in the Mediterranean Sea, where numerous species of calcareous encrusting algae, mainly belonging to *Corallinaceae*, build complex and heterogeneous structures on the substrate (Ballesteros 2006). These biogenic structures represent suitable niches for various marine species such as sponges, bryozoans, mollusks, and echinoderms and significantly affect the Mediterranean marine wildlife and the functioning of the ecosystem (Martin et al. 2014).

Coralligenous assemblages are in the sublittoral zone between 20 and 120m depth; light is available for the establishment of photophilous organisms, but not sufficient for the competition of fast-growing algae. The general architecture of coralligenous formations raises habitat complexity, which creates a great number of microhabitats promoting species richness (Ballesteros 2006). In addition, they are crucial to store carbon and are sources of fish supply: this gives these habitats a measure of both ecological and economic importance (Ponti et al. 2018).

Nonetheless, coralligenous habitats are exposed to numerous anthropogenic impacts such as bottom trawling, pollution, invasive species, and other climate change impacts, such as ocean acidification and warming (Cerrano et al. 2019; Silva et al. 2024). These stressors cause changes in the physical environment such as habitat degradation and loss of biodiversity, thus requiring conservation and

management measures. Recent efforts have concerned the assessment and the constant survey of coralligenous assemblages like the adoption of MPA to protect these biotopes (Gatti et al. 2015).

1.2 Crustose Coralline Algae

Crustose coralline algae (CCA), belonging to the *Corallinales*, are among the most important benthic species in the Mediterranean Sea, especially in the coralligenous communities. These algae deposit calcium carbonate inside their cell walls and thus form hard encrusting thalli attached to the rocky substrate (Ballesteros 2006). In the Mediterranean Sea, CCA are involved in the bioconstruction and consolidation of coralligenous communities; these organisms are among the primary builders of the framework of this ecosystem supporting a high level of species richness (Ballesteros 2006): in fact, due to CCA surface roughness and biochemical properties, they promote larval settlement (Piazzi et al. 2022), similarly to coral reefs where they assist reef restoration and strengthening (Harrington et al. 2004). Consequently, the role of crustose coralline algae within the context of the Mediterranean ecosystem is of fundamental importance: it provides substrate for the colonization of numerous marine species, such as invertebrates, sponges, and other algae, which increases the structural complexity and improves the habitat heterogeneity on the benthic zone (Ballesteros 2006). Further, the calcification framework in CCA is vital in the enhancement of structure and longevity of these habitats counteracting bioerosion and offering foundation to all other biogenic organisms (Valisano et al. 2019).

However, health and distribution of CCA are becoming vulnerable due to the various anthropogenic threats such as eutrophication, the development of coastal structures, and effects of climate change (e.g. floods and marine heat waves). Ocean acidification (OA), for instance, has emerged as a major threat that may compromise the CCA calcification process by reducing their growth rates and weakening the coralligenous structures (Martin et al. 2014; McCoy and Kamenos et al. 2015), thus compromising the foundation role they provide (Kamenos et al. 2013). CCA are mainly composed of calcium carbonate ($CaCO_3$), their structural material (Borowitzka 1982), which exists in two

predonimant polymorphs: calcite and aragonite. $CaCO_3$ is the key element, providing hardness and mineralized structure to the algae and is formed by Calcium (Ca^{2+}) which is incorporated into the structure of $CaCO_3$, bringing strength and rigidity to cell walls and thallus and by Carbonate ion (CO_3^{2-}) bond with the calcium ions in order to form the lattice structure of $CaCO_3$. Magnesium (Mg^{2+}) to some extent can substitute for calcium in the $CaCO_3$ lattice and its proportion varies with environmental conditions and coralline algal species. In scenarios of ocean acidification, when decreasing pH alters seawater chemistry, CCA tend to deposit more Mg^{2+} into cell walls (high Mg-Calcite form) (Nelson 2009; Kroeker et al. 2013; McCoy and Kamenos 2015). Substitution makes their structure more soluble and less resistant to climate change. Such alterations could have cascading effects on the ecosystem, evidencing the need for specific and preventive conservation and management measures to protect the organisms and their habitat (Cerrano et al. 2019).

The CCA are also highly influenced by environmental conditions such as light and temperature, both crucial for their functions and distribution in the ecosystem. Light is an essential determining factor influencing both photosynthesis and calcification of CCA (Littler and Doty 1975). The optimal light conditions are CCA species specific; nevertheless, the most common species of CCA are usually photoadapting to low light conditions as found in deep water or shaded area of the coral reef where CCA tend to form the base of the benthic community (Adey and Vassar 1975). Light quantity (intensity) and quality (wavelengths) are other factors critical in determining the depth of distribution of CCA. Thus, for example, in deeper or turbid waters, CCA have increased pigment concentration and changes in thallus form and size in order to be able to acclimatize (Foster 2001). These adaptations help CCA to support photosynthetic activity and continue calcifying even in low light environment, critical to reef building and maintenance (Dean et al. 2015).

Temperature is also a critical factor that influences CCA growth, reproduction and calcification, although each CCA species seems adapted to a specific temperature zone (and thus range). Heat-stress under global warming conditions affects growth rates, bleaching and, in some cases, mortality

of CCA (McCoy and Kamenos 2015): in fact, heat stress can injure their photosynthetic apparatus, leading to a breakdown of the association with the photosynthetic pigments, and reducing calcification (Anthony et al. 2008). On the other hand, suboptimal low temperature conditions also have negative effects on the growth and calcification of CCA especially in the temperate reefs where there are seasonal changes in temperature. This may have an impact on the enzymatic activities which are involved in calcification and thus lead to a weaker skeletal structure which cannot withstand erosion (Kuffner et al. 2008). In addition, temperature fluctuations can affect the relationships between CCA and other benthic organisms, including macroalgae and turf algae, in terms of potential shifts in community structure and function in the marine environment (Nelson 2009). In conclusion, light and temperature can be considered as key factors to control the distribution, physiological condition and ecological role of CCAs. It is therefore important to understand the relationships between these two environmental conditions and CCAs, given the future intensification of climate change effects and the prediction of their increasing impact on marine life (Guinotte et al. 2003). Future studies aiming to provide a detailed understanding of how light and temperature affect CCA performance and whether there are ways to reverse or minimize the effects of climate change for these significant organisms would be more than welcome.

1.3 Animal Forest

Animal forests create very complex and biodiverse communities in the Mediterranean Sea. Such habitats are mostly made up of suspension-feeding organisms, such as sponges, gorgonians, corals, bryozoans, and tunicates, that form dense, three-dimensional structures which are key for a wide range of marine species (Rossi et al. 2017; Orejas et al. 2022). The structural complexity of this habitat is significant in increasing the local biodiversity, as it provides shelter, breeding areas, and feeding grounds for various invertebrates and fish species (Ballesteros 2006).

Animal forests in the Mediterranean are for the most part in deep-sea environments, rocky reefs, and coralligenous habitat. For example, gorgonian forests are common in the Mediterranean and are

composed of slow-growing and long-lived species such as *Paramuricea clavata* and *Eunicella cavolini*. These gorgonians are an important part of the vertical complexity of the habitat and are especially important for the biodiversity they support (Gori et al. 2017). Likewise, the deep-sea coral reefs, which are mainly formed by scleractinian corals such as *Madrepora oculata* and *Lophelia pertusa*, are essential for the survival of many deep-sea species, some of which are endemic in the Mediterranean basin (Freiwald et al. 2004). The ecological importance of these animal forests is beyond their role as biodiversity hotspots, since they are also important in biogeochemical cycles, especially in carbon and nutrient cycling, thanks to their high biomass and productivity (Rossi et al. 2017). In addition, the thick structures that these organisms form can lead to changes in hydrodynamic processes, which might result in sedimentation patterns and water flow, and these, in turn, may have an effect on the larger marine environment (Orejas et al. 2022). However, Mediterranean animal forests are very vulnerable to anthropogenic pressures including overfishing, pollution, and climate change. Recreational fishing and trawling can cause irreparable damage to these ecosystems, due to mechanical damage and sediment resuspension respectively, which leads to decades-long processes of loss of biodiversity (Bo et al. 2014). Pollution, such as marine debris and chemical contaminants further accelerates the deterioration of these ecosystems by directly damaging the organisms that form the animal forest (Cerrano et al. 2010). Climate change and its impacts, such as the rise of temperatures and acidification of the oceans, represents a new problem that might change the distribution and health of these habitats because the physiology and calcification of the keystone species are affected (Garrabou et al. 2009). The protection and administration of animal forests in the Mediterranean are consequently of vital significance. The preservation and conservation of these habitats requires the combination of marine protected areas, methods of sustainable fishing, and efforts to mitigate the climate change impacts. Monitoring programs and further research on the ecological functions and vulnerabilities of these ecosystems are very necessary to ensure their conservation.

1.4 Restoration

Ecological restoration is one of the most important measures aimed to counteract the decline of the majority of marine habitats, especially in areas with high levels of anthropogenic pressures, such as the Mediterranean Sea. Restoration efforts are shifting more and more towards the target of habitat-forming species like the seagrass *Posidonia oceanica* (Duarte 2002; Mancini et al. 2022, 2024; Pergent-Martini C. et al. 2024) and *Cymodocea nodosa (*Zarranz et al. 2010; Boudouresque et al. 2021; Da Ros et al. 2020*)*, and fucoids such as *Cystoseira* species (Verdura et al. 2018; Falace et al. 2018) which are all very important for maintaining marine ecosystem services, such as carbon sequestration, sediment stabilization, and habitat provision.

Each of these habitats has been destroyed by factors like coastal development, anchoring, pollution, in combination with climate change effects (Boudouresque et al. 2009), and the restoration has been challenged by several difficulties: in particular, the restoration of *P. oceanica* is difficult due to the slow growth rate and sensitivity to changes in the environment, while it has been observed that *C. nodosa* exhibits lower sensitivity to environmental degradation and is capable of rapidly colonizing new substrates, thereby creating a favorable environment for the settlement of *P. oceanica* (ecological succession) (Montefalcone et al. 2024). Scientific studies also demonstrate that *C. nodosa* usually lives in relatively warmer waters (Boudouresque et al. 2021), which may support habitat restoration under the influence of future climate change.

1.4.1 Restoration of Coralligenous reefs

The restoration of coralligenous reefs is particularly difficult because of species fragile and slow-growing nature. Methods of restoration include both passive and active techniques: the former being a way of preventing further damage to existing sites, so that natural recovery can take place, the latter deployed by transplantation of the key species such as gorgonians and bryozoans (Bianchelli et al. 2023). The use of artificial substrates to promote the development and growth of coralligenous species

has been also suggested (Cerrano et al. 2010). Experimental methods have demonstrated that gorgonians transplantation from the nursery can be an essential tool for restoration, especially in combination with protection from further disturbances (Bo et al. 2014). In coralligenous habitats, most of the studies have focused on the active transplantation of sessile macroinvertebrates, especially gorgonians (Fava et al. 2010; Cerrano et al. 2018; Montseny et al. 2019; Casoli et al. 2021), following the techniques described by Linares et al. (2008). However, such interventions can only be successful when the ecological requirements of the species, as well as the environmental conditions at the restoration site, are well understood (Ponti et al. 2014). Overall, no intervention based on the transplantation of the CCA, the foundation species of the habitat, has ever been established.

The major obstacles in the restoration of coralligenous reefs are the low growth rates of target species, the complexity of these systems, and the long-term commitment and monitoring required. Moreover, restoration activities should be combined with general conservation methods, such as the reduction of local stressors and the creation of marine protected areas (MPAs), to achieve the long-term gain and sustainability of restored habitats (Boudouresque et al. 2021). Future studies are required to optimize restoration practices, to expand the knowledge of species-specific reactions to restoration interventions, and to design new ways to bring back these essential habitats on a large scale. Furthermore, future restoration projects should include adaptive management approaches, enabling the adjustment of strategies based on monitoring outcomes and changing environmental conditions (Abelson et al. 2020).

1.5 Structure of the thesis and aims of the work

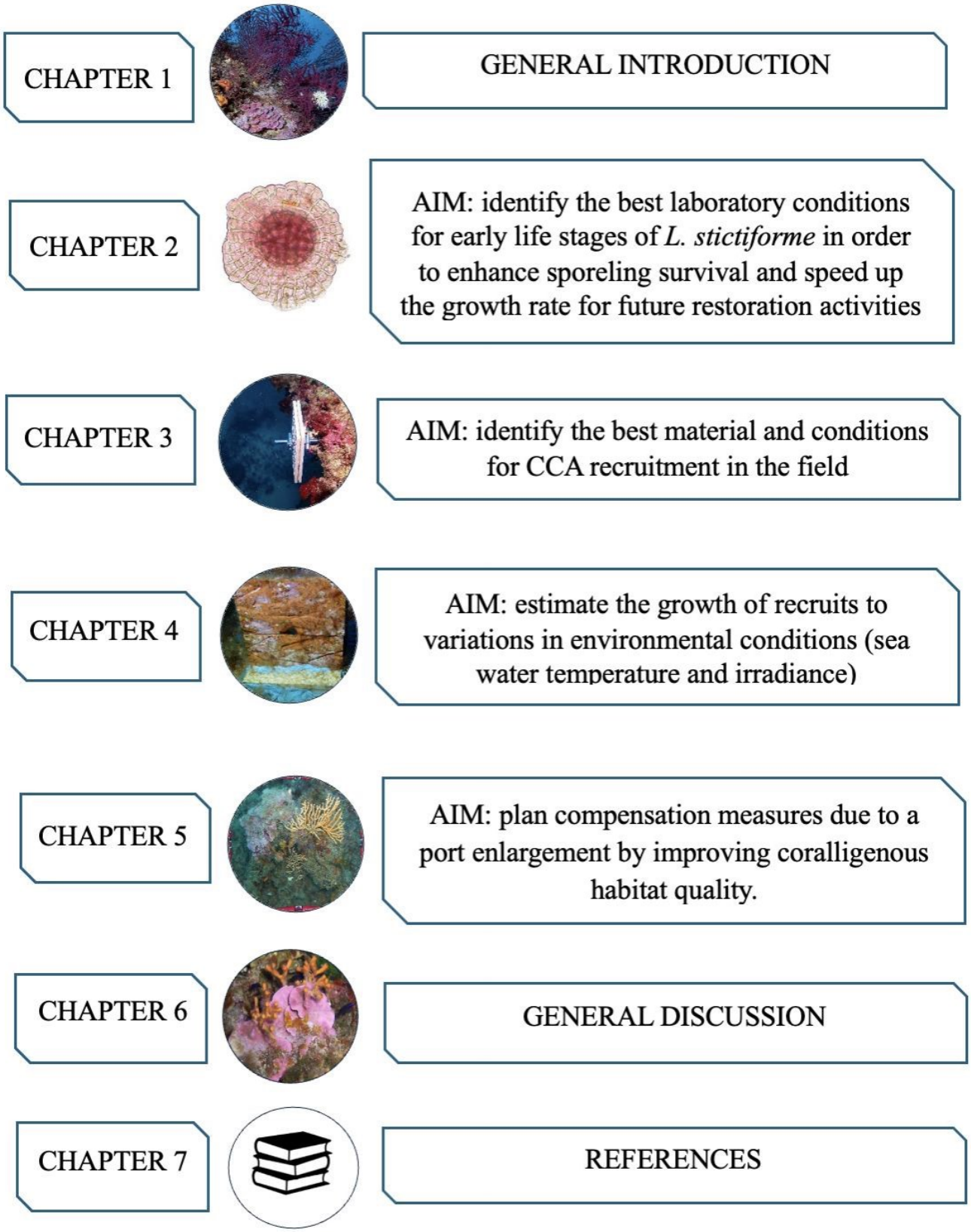


Figure 1. Structure of the thesis: the different objectives are developed in 3 experiments (1 in laboratory and 2 in the field) and in 1 study respectively.

Chapter 2

AN *EX-SITU* APPROACH FOR CULTIVATING CORALLINE ALGAE IN A RESTORATION PERSPECTIVE

Puccini A.[1*], Kaleb S.[2], Ceccherelli G.[3], Porrelli D. [4], Falace A.[2,5]

[1] *Department of Architecture, Design and Urban Planning, University of Sassari, via Piandanna 4, 07100 Sassari, Italia*

[2]*Department of Life Sciences, University of Trieste, via L. Giorgieri 10, 34127 Trieste, Italy*

[3]*Department of Chemical, Physical, Mathematical and Natural Sciences, University of Sassari, via Piandanna 4, 07100 Sassari, Italia*

[4]*Department of Life Sciences, University of Trieste, Via Alexander Fleming 31/B, 34127, Trieste, Italy*

[5] *National Institute of Oceanography and Applied Geophysics – OGS, Trieste, Italy*

*e-mail: alessandrapuccini4@gmail.com

ABSTRACT

Mediterranean coralligenous reefs are hotspots of biodiversity, but they are declining due to multiple impacts, prompting a growing number of conservation and restoration efforts. Restoration through the outplanting of the habitat foundation species, crustose coralline algae (CCA, Rhodophyta, Corallinophycidae), is still largely unexplored, although they make a major contribution to building these valuable ecosystems. In this study, we tested the feasibility of *ex-situ* culture of *Lithophyllum stictiforme*, one of the most common CCA of the coralligenous reefs, with nutrient-enriched artificial seawater medium, contributing to the knowledge of early life stage development and calcification. Thalli fragments with conceptacles were collected at a depth of 28 m (Costa Paradiso, northern Sardinia). We tested two light intensities, (L^- 40 µmol photons $m^{-2}s^{-}1$; L^+160 µmol photons $m^{-2}s^{-1}$) and two temperatures, (T^- 14 °C; T^+ 20 °C), the lower settings were chosen to simulate conditions at the sampling site, while the higher values were explored as potential growth promoters. Spore settlement, density and growth were estimated for two weeks. Calcification was analyzed at the end of the culture period using Scanning Electron Microscopy in combination with Energy Dispersive Spectroscopy (SEM-EDS).

The L^- treatments had a positive effect on germination success and density, whereby the growth of the germination disc was enhanced in the L^-T^+ treatment. SEM-EDS showed that the marginal area of the germination discs had a higher Ca content than the core area, and that the L^- treatments promoted a higher cell wall Ca percentage.

Keywords: *Lithophyllum stictiforme*, habitat restoration, biogenic reefs, calcification, Mediterranean

1. INTRODUCTION

Coralligenous reefs are among the most important subtidal habitats in the Mediterranean for important ecosystem services they provide, acting as hot spots of marine biodiversity (Ådal et al. 2006; Ballesteros 2006; Piazzi and Ceccherelli 2020), providing habitat for economically valuable species (Canals and Ballesteros, 1997; Ballesteros, 2006), and playing a role as natural carbon sinks (Thierry de Ville d'Avray et al. 2019, Cebrián et al. 2000; El Haïkali et al. 2004; Canals and Ballesteros 2007; Bracchi and Basso 2012; van der Heijden and Kamenos 2015). Their importance as marine calcifiers lies in their ability to precipitate calcium carbonate in their cell walls, predominantly as high-Mg calcite and, in the case of tropical species, aragonite (Nash and Adey 2017). Recently, CCA have garnered increased attention in the context of global environmental changes, mainly due to the susceptibility of their high-Mg calcite skeletons (McCoy and Kamenos 2015) and their capacity to adjust the geochemistry of their thallus to environmental conditions (Moberly 1968; Ulrich et al. 2021). Studies have repeatedly shown that Mg/Ca ratios in CCA can serve as indicators of seawater temperature (Kamenos et al. 2008; Diaz-Pulido et al. 2014; Ragazzola et al. 2020; Hetzinger et al. 2023) since the incorporation of Mg into calcite is an endothermic process, leading to an increase in the Mg/Ca ratio with rising temperatures (Mucci and Morse 1985; Rosenthal et al. 1997).

They are characterized by biogenic assemblages, from 20 to about 150 m depth, mainly formed by the accumulation of crustose coralline algae (CCA), mostly *Lithophyllum, Lithothamnium* and *Mesophyllum,* which constitute a secondary substrate hosting highly diverse and stratified assemblages including erect algae, sponges, bryozoans, gorgonians, and other alcyonaceans (Ballesteros 2006). Spatial patterns of colonization of *L. stictiforme* thalli by epibionts have recently been detected and differences in diversity between assemblages recruited on the CCA and on rock have provided evidence of the importance of CCA in promoting the biodiversity of coralligenous reefs (Piazzi et al. 2022). These reefs are currently facing significant threats that are leading to their decline (Ballesteros 2006; Bevilacqua et al. 2018). Overfishing, pollution, invasive species and

climate change are disrupting the demography, phenology and biogeography of numerous species, jeopardizing the integrity of the entire ecosystem (Ballesteros 2006; Bevilacqua et al. 2021; Cebrian et al. 2021; Garrabou et al. 2022). Regression of coralligenous reefs is due to both impacts on the animal (*e.g.* gorgonians, bryozoans and sponges) (Garrabou et al. 2009) and CCA (Piazzi et al. 2011; Martin et al. 2013b)

Given these challenges, both passive restoration (i.e. removal or mitigation of stressors) and active restoration strategies (i.e. transplantation of organisms), have been proposed as viable approaches to expedite the recovery of coralligenous reefs with the aim of promoting the recovery of the community structure, and thus restoring its functioning. The active restoration consists in transplanting sessile macroinvertebrates, mostly gorgonians, onto disturbed bioconstructions (Cerrano et al. 2018; Casoli et al. 2021), following Linares et al. (2008) techniques. As coralligenous restoration is still in its infancy, the effectiveness of transplants is quite uncertain due to the lack of long-term data and the scant number of case studies that have resulted in a wide range of survival outcomes (Linares et al. 2008; Fava et al. 2010; Montseny et al. 2019; Casoli et al. 2021).

In this frame, the possibility of promoting the development of CCA by producing recruits from fertile material in hatcheries for release into the sea remains to be explored. In fact, several studies indicate that these calcifying macroalgae not only serve as a selective primary substrate but are also actively involved in the recruitment of a large number of invertebrate species by inducing larval settlement and metamorphosis (Lau et al. 2005; O'Leary et al. 2017; Gómez-Lemos et al. 2018; Seabra et al. 2019; Siboni et al. 2020; Jorissen et al. 2021). In the Mediterranean, *Lithophyllum stictiforme* (Areschoug) Hauck, one of the principal bioconstructors of coralligenous (Garrabou and Ballesteros 2000; Ballesteros 2006; Piazzi et al. 2022), has been shown to positively influence the density and dynamics of settlement of some gorgonians (Zelli et al. 2020). The outplanting of *L. stictiforme* recruits could therefore provide natural substrates for the whole assemblage to develop.

However, one of the bottlenecks in planning large-scale restoration efforts is the need to outplant a large number of seedlings. Therefore, planning an efficient, effortless and cost-effective seedling production system that fits the breeding requirements of a specific species can be particularly challenging. However, the high potential of *Lithophyllum stictiforme* to generate gametes and zygotes under optimal conditions has been highlighted (Rodrìguez-Prieto 2016), supporting the hypothesis that the cultivation of seedlings from fertile conceptacles of this species could be a reliable option for the restoration of coralligenous reefs without depleting natural populations. This technique has also been explored for canopy forming species restoration such as algae of the genus *Cystoseira sensu lato* (Falace et al. 2006; Verdura et al. 2018; Falace et al. 2018) and large fucoids (e.g. Hwang et al. 2007; Pang et al. 2009; Yu et al. 2012; Yoon et al. 2014).

With the perspective of cultivating CCA seedlings, the development of an effective protocol is essential. As temperature and light play a key role in the growth of macroalgae (Hurd et al. 2014), with responses being species-specific, they should be adequately addressed in *ex-situ* culture. As well, optimal growth conditions likely vary depending on the ontogenetic stage, and early-life stages may have different requirements than adults (Yoshioka et al. 2020). Several studies have been conducted on the optimal temperature and light requirements for both adult CCA growth (e.g. Adey 1970; Leukart1994; Rodríguez-Prieto 2016; Martin et al. 2024) and early-life stages (sporelings) (e.g. Jones and Woelkerling 1983; Ichiki et al. 2000; Hwang et al. 2002; Song et al. 2013a,b; Ordoñez et al. 2017; Yoshioka et al. 2020). However, to our knowledge, there is no information on the development of germination discs of *L. stictiforme*.

Coralline algal spores follow a characteristic pattern of cell division to form a germination disc that is symmetrical and clearly visible even after few days of development (Chamberlain, 1993). The germination disc develops in the early stages of growth of the alga, originating from a spore. After being released, the spores settle on the substrate and anchor themselves to it thanks to the secretion of adhesive polysaccharides. Once adhered, the spores begin to germinate, producing flat and

compact cells that are arranged radially, thus forming the germination disc, a stable and circular structure. This disc expands thanks to cell division and, in CCA, begins to deposit calcium carbonate, which gives rigidity and resistance to the structure. From this base, the thallus of the alga will subsequently develop.

In this study, we therefore tested the feasibility of *ex-situ* culture of *L. stictiforme* using a crossed experimental design to investigate the effects of light and temperature conditions on development and calcification at the early life stage and to determine the optimal laboratory combinations.

2. MATERIALS AND METHODS

2.1 Sampling site

Thalli fragments of *L. stictiforme* (N=40), measuring approximately 15 x 15 cm and bearing developed conceptacles, were harvested by scuba divers from a depth of 28 meters at Costa Paradiso (Northern Sardinia, Tyrrhenian Sea, N 41.06546º, E 008.95039º) in March 2023. The algae were stored in seawater in the dark for 24 hours before being transported to the facilities of the University of Trieste (Italy) within 10 hours in dark and cold conditions.

2.2 Experimental setup

The culture was established in environmentally controlled rooms and the photoperiod was set to 10:14 h (L: D), which corresponds to the conditions during sampling. We tested two light intensities and two temperatures in a crossed experimental setup. Light was provided by LED lamps (AM366 Sicce USA Inc., Knoxville, USA) at 40 µmol photons m^{-2} s^{-1} (L^-) and 160 µmol photons m^{-2} s^{-1} (L^+), while the temperature was set to 14°C ± 0.5 (T^-) and 20°C ± 0.5 (T^+). These two temperature values reflected variation in the Mediterranean site where the adult thalli were collected, due to the presence of a thermocline (a transition layer between the warmer water at the surface and the cooler deep water

below). The air temperature was automatically set by the room controller and the water temperature was measured twice daily in each tank. The lower light and temperature values were chosen to reflect the environmental conditions at the sampling site, while the higher values were based on measurements at shallower depths within the sampling site where *L. stictiforme* typically thrives (Pinna et al. 2022) and were tested as growth promoters. Each treatment combination was replicated with 3 tanks of 5 liters each containing 12 glass slides (25x75 mm).

Thalli fragments were examined under the stereomicroscope to identify the sporophytes, and the presence of tetraspores was verified by removing the roof of randomly selected conceptacles for examination. The sporophyte fragments were then cleared from epiphytes with tweezers and rinsed with artificial seawater (Tropic Marin® Pro- Reef Sea salt). The thalli were then arranged in tanks suspended on rigid PVC grids (mesh size 25 mm) with the surface of the thalli facing the glass slides at the bottom of tanks. The tanks were filled with artificial seawater (37 PSU) supplemented with 10% Von Stosch's solution (Von Stosch 1963; Guiry and Cunningham 1984). Seawater pH, calcium (Ca), magnesium (Mg) and alkalinity (TAlk) were measured every three days. The pH measurements were performed using a pH meter (HI-5521-02, Hanna Instruments) calibrated on the total scale with Tris/HCl buffers (according to Dickson et al. 2007). [Ca], [Mg] and TAlk were determined using a high-precision titration test (Foundation^{TM} Pro Test Kit). To ensure consistent chemical conditions in the culture medium, it was renewed every three days, with both tanks and slides repositioned to ensure randomized culture conditions.

Each slide was seeded with a *L. stictiforme* fragment of approx. 3 x 4 cm and then removed after 24 hours. To prevent possible anoxia, the slides were lifted from the bottom during cultivation using a grid, while continuous oxygenation in the tanks was ensured by air pumps and bubblers. Seedlings were cultured for 14 days and data acquired at three time points post germination: two days (T1), seven days (T2) and fourteen days (T3). At each sampling time, three slides per tank for each treatment were collected and fixed in 4% formalin for the subsequent analyses. To guarantee independence of data, the slides sampled were never reused.

2.3 Collected data

The following data were collected during culture:

- Germination success (= *N. germination discs with at least one cell division/total number of spores x 100*) was assessed at T1 on 20 subareas, corresponding to 20 photos per slide (i.e., full field of view of the microscope at 4x magnification; area per field of view: 7.033 mm^2; total area per slide: 140.66 mm^2). Photographic sampling was done with an Axiocam 208 color camera mounted on a Zeiss Primostar 3 microscope.

- Germination disc density (*i.e., mean N. germination discs per slide*) was counted at T2 and T3 under an inverted microscope.

- Germination disc growth: at T3 the area of 20 randomly selected germination discs per slide was measured by ImageJ Software.

- Germination discs growth stages: seven growth stages were identified: I- undivided spore, II- up to four-cells, III-up to eight cells, IV- up to sixteen-cells, V- disc with meristematic peripheral cells, VI- disc with two rows of peripheral cells and VII- disc with three or more rows of peripheral cells. Development was assessed by counting live (red-colored) germination discs at different stages on 20 subareas, corresponding to 20 photos per slide (i.e., full field of view of the microscope at 4x magnification; area per field of view: 7.033 mm^2; total area per slide: 140.66 mm^2) at T1, T2 and T3.

To investigate the elemental composition and the distribution of calcification in the germination discs, Scanning Electron Microscopy in combination with Energy Dispersive Spectroscopy (SEM-EDS) analyses were performed at T3. To obtain the Ca and Mg atomic percentage, and the Mg/Ca atomic ratios, elemental maps were acquired on 3 randomly chosen germination disc per slide. The measurements were performed over the entire area of the germination disc, the central area and the marginal area (Fig. 1). For SEM-EDS analysis, the slides were mounted on aluminum stubs coated with double sided carbon tape. Samples were carbon-coated using the pulsed rod evaporation method with the Q150T Es plus sputter coater (Quorum Technologies). Samples were analyzed with

Gemini300 SEM (Zeiss) in secondary electron mode, at an acceleration voltage of 10 kV and a working distance of 8.5 mm. EDS analysis was performed at a working distance of 8.5 mm and an acceleration voltage of 10 kV, using an XFlash 610 M probe (Bruker), obtaining maps (with a scan time of 5 minutes) and semi-quantitative analysis of the regions of the germination discs (with a scan time of 1.5 minutes for each region).

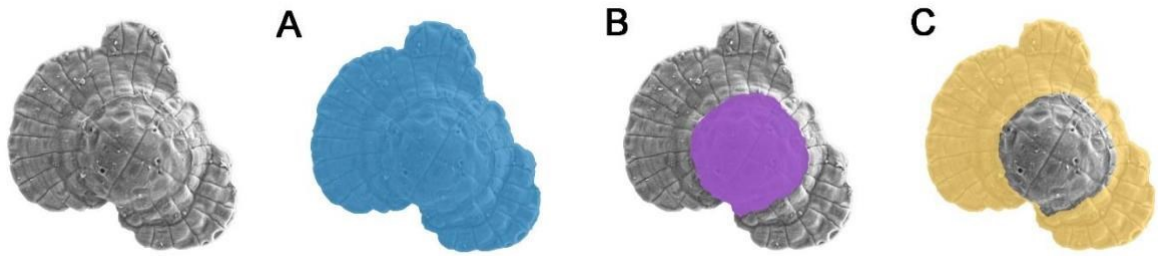


Figure 1. SEM-EDS maps acquired at T3 on *L. stictiforme* germination discs over A) the total germination disc area, B) the core area and C) the marginal area.

2.4 Statistical analyses

Permutational analysis of variance (PERMANOVA) was performed to test the effects of temperature and light on germination success at T1, germination disc density (at T2 and T3), growth at T3 and development at each sampling time. For each variable tested, the design for the analysis consisted of two factors: Temperature (Te) and Light (Li), fixed and crossed, with three tanks (n= 3, each with 10 slides) for each combination of factors: tanks (n=3) were considered replicates for the germination success and the germination disc density, while slides (n=9) were considered replicates for the growth and development at each sampling time. Significant effects were examined by performing a post hoc pairwise test. PERMANOVA and Pair-Wise tests were performed with PRIMER v7 software. Statistical analysis of the calcification data was performed using PRIMER Software. A PERMANOVA was also performed to test the effects of temperature, light and disc portion (core or

peripheral) on germination disc Ca% and Mg/Ca content. The design consisted of three factors, fixed and crossed: Temperature (Te), Light (Li) and Disc Portion (DP) with two levels each.

3. RESULTS

Germination success of *Lithophyllum stictiforme* at T1 only depended on temperature (p= 0.038, Tab. 1) with the higher percentage of germination discs with at least one cell division at T^+. Conversely, light conditions did not affect the germination success (Fig. 2).

Table 1. Germination success. Results of univariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on germination disc abundance at T1. In bold the Pseudo-F corresponding to a significant result.

Source of variation	**df**	**MS**	**Pseudo-F**	**P(perm)**	**Perms**
Temperature	1	0.011	5.599	**0.038**	980
Light	1	0.011	5.661	0.054	977
TeXLi	1	0.00009	0.047	0.832	983
Residual	8				

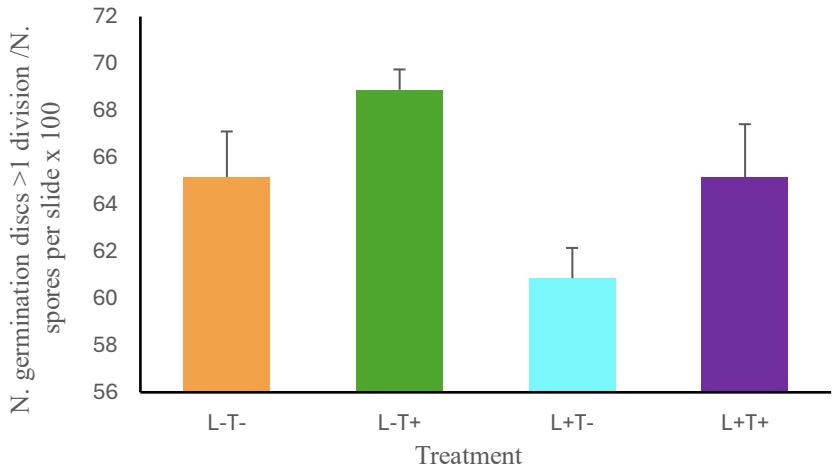


Figure 2. Germination success. Percentage (mean ± SE, n=3) of germination discs with at least one cell division out of the number of spores per slide (over 140.66 mm^2) for each treatment at T1: L^-T^-: low light-low temperature; L^-T^+: low light-high temperature; L^+T^-: high light- low temperature; L^+T^+: high light-high temperature.

However, a significant effect of light was found on germination disc density with the low light condition promoting disc abundance, regardless of the temperature (Tab. 2 and Fig. 3).

Table 2. Gemination disc density. Results of univariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on disc survival at T2 and T3. In bold the Pseudo-F corresponding to a significant result.

Source of variation	**df**	**Pseudo-F T2**	**Pseudo-F T3**
Temperature=Te	1	3.458	1.527
Light=Li	1	**5.116**	**18.391**
TeXLi	1	0.359	0.761
Residual	8		

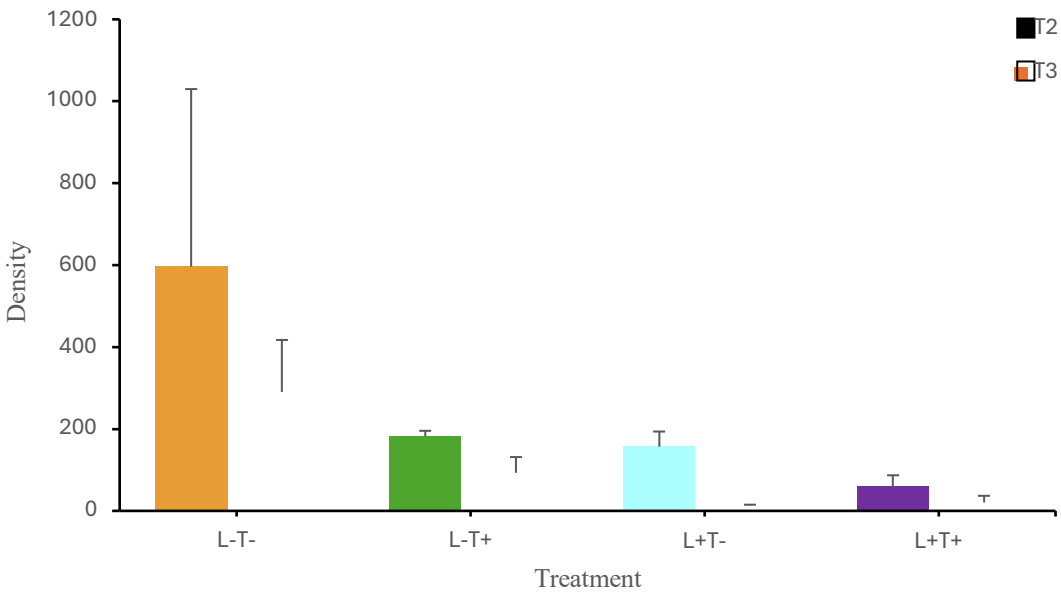


Figure 3 Germination disc density. Number of germinative discs per slide (mean +SE n=3) for each treatment at T2 and T3: L^-T^-: low light-low temperature; L^-T^+: low light-high temperature; L^+T^-: high light- low temperature; L^+T^+: high light-high temperature.

Germination of the spores followed the segmentation mode of the *Amphiroa* type, which was also described for *Lithophyllum* (Chihara, 1974) (Fig. 4). While no difference in segmentation pattern was observed among treatments, there was variability with respect to timing: multivariate PERMANOVA showed significant effects of temperature and light on seedling development through time (Tab. 3).

Table. 3. Germination disc development. Results of multivariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on disc development. Pair-Wise tests on the interaction Temperature X Light. In bold the Pseudo-F corresponding to a significant result.

Source of variation	**df**	**Pseudo-F T1**	**Pseudo-F T2**	**Pseudo-F T3**
Temperature=Te	1	**9.190**	**9.929**	**14.863**
Light=Li	1	**16.308**	**24.854**	**13.393**
TeXLi	1	**5.635**	**9.807**	**3.745**
Residual	32			

Pair-Wise tests

Temperature X Light

	T1	**T2**	**T3**
	T-L+ = T-L-	T-L+≠T-L-	T-L+ ≠ T-L-
	T+L+ ≠ T+L-	T+L+≠T+L-	T+L+ ≠ T+L-
	L-T+ ≠ L-T-	L-T+≠L-T-	L-T+ ≠ L-T-
	L+T+ = L+T-	L+T+=T-	L+T+ ≠ L+T-

Pairwise tests confirmed that a difference in development by the different treatments was detectable from the beginning of the experiment (T1). The first and second cell divisions occurred crosswise, resulting in a germination disc that was mostly four-celled (Fig. 4, stage II) two days after spore release (T1), although eight-celled (Fig. 4, stage III) and sixteen-celled stages (Fig. 4, stage IV) were also reached, especially in the L^-T^+ treatment. Univariate PERMANOVA showed significant differences between treatments for stages II and IV (Tab. 4A). In L^- treatment the development of stage IV was promoted at high temperature.

Table. 4. Germination disc development. Results of univariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on the percentage of germination disc at each stage of development at T1, T2 and T3 (Tab. 4A, 4B and 4C, respectively). Pair-Wise tests on the interaction Temperature x Light are provided at the bottom of each table. In bold the Pseudo-F corresponding to a significant result.

A

Source of variation	**df**	**Pseudo-F Stage 1**	**Pseudo-F Stage 2**	**Pseudo-F Stage 3**	**Pseudo-F Stage 4**	**Pseudo-F Stage 5**	**Pseudo-F Stage 6**	**Pseudo-F Stage 7**
Temperature=Te	1	3.713	**7.118**	**15.072**	**68.151**			
Light=Li	1	2.282	**27.529**	**9.514**	**52.478**			
TexLi	1	0.390	**7.631**	0.003	**50.857**			
Residual	32							

Pair – Wise tests

Temperature x Light

Stage 2	Stage 4
L-T- >T+	L-T+ > T-
L+T- = T+	L+T+= T-
T-L- = L+	T-L+ = L-
T+L- < L+	T+L+< L-

B

Source of variation	df	Pseudo-F Stage 1	Pseudo-F Stage 2	Pseudo-F Stage 3	Pseudo-F Stage 4	Pseudo-F Stage 5	Pseudo-F Stage 6	Pseudo-F Stage 7
Temperature	1		4.574	0.683	0.268	**6.681**	**58.903**	
Light	1		**27.044**	**12.214**	7.515	**28.272**	**58.903**	
Te x Li	1		0.977	0.079	**7.623**	**12.708**	**58.903**	
Residual	32							

Pair – Wise tests

Temperature x Light

	Stage 4	Stage 5	Stage 6
	T- L- > L+	T- L- > L+	T- L- = L+
	T+ L+ = L-	T+L- = L+	T+L- > L+
	L- T- > T+	L-T- > T+	L-T+ > T-
	L+ T- = T+	L+T- = T+	L+ T+ = T-

C

Source of variation	df	Pseudo-F Stage 1	Pseudo-F Stage 2	Pseudo-F Stage 3	Pseudo-F Stage 4	Pseudo-F Stage 5	Pseudo-F Stage 6	Pseudo-F Stage 7
Temperature=Te	1		**11.807**	0.189	0.002	**5.465**	**16.396**	**55.343**
Light=Li	1		2.107	**7.057**	1.738	**4.596**	**29.404**	**32.928**
Te x Li	1		0.627	0.296	1.051	**5.630**	**22.302**	**10.177**
Residual	32							

Pair – Wise tests

Temperature x Light

	Stage 5	Stage 6	Stage 7
	T- L- >L+	T-L- > L+	T-L- > L+
	T+L- =L+	T+L- =L+	T+L- > L+
	LT- > T+	L-T- > T+	L-T+ > T-
	L+T- =T+	L+T- =T+	L+T+ > T-

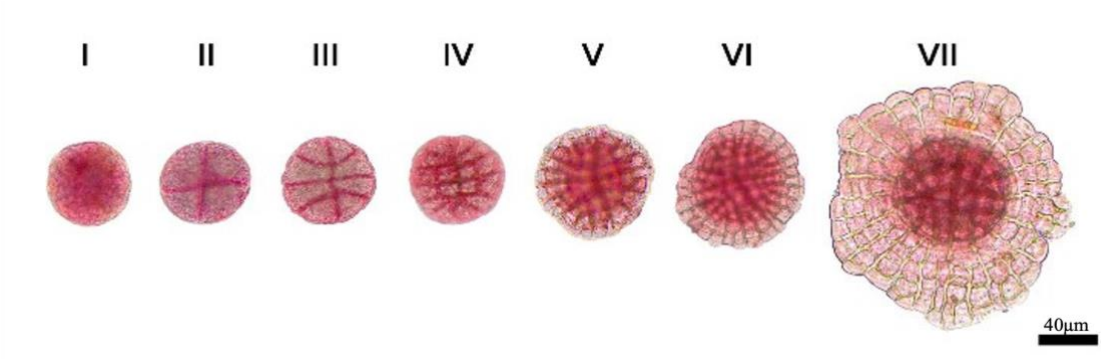


Figure 4. Developmental stages of *L. stictiforme* recruits during 14 day-culture: I- undivided spore, II- up to four-celled, III-up to eight-celled, IV- up to sixteen-celled, V- disc with meristematic peripheral cells, VI- disc with two rows of peripheral cells and VII- disc with three or more rows of peripheral cells.

The differences between treatments increased over time (Fig. 5) as highlighted by pairwise tests at T2 and T3 at all treatment conditions (Tab. 3), with significant differences observed at stages IV, V and VI at T2 (Tab. 4B). The germination disc developed one row of meristematic peripheral cells (stage V) in all treatments except at $L^{+}T^{-}$. Under L^{-}, the stage VI was induced by high temperature and under T^{-} it was promoted by low light.

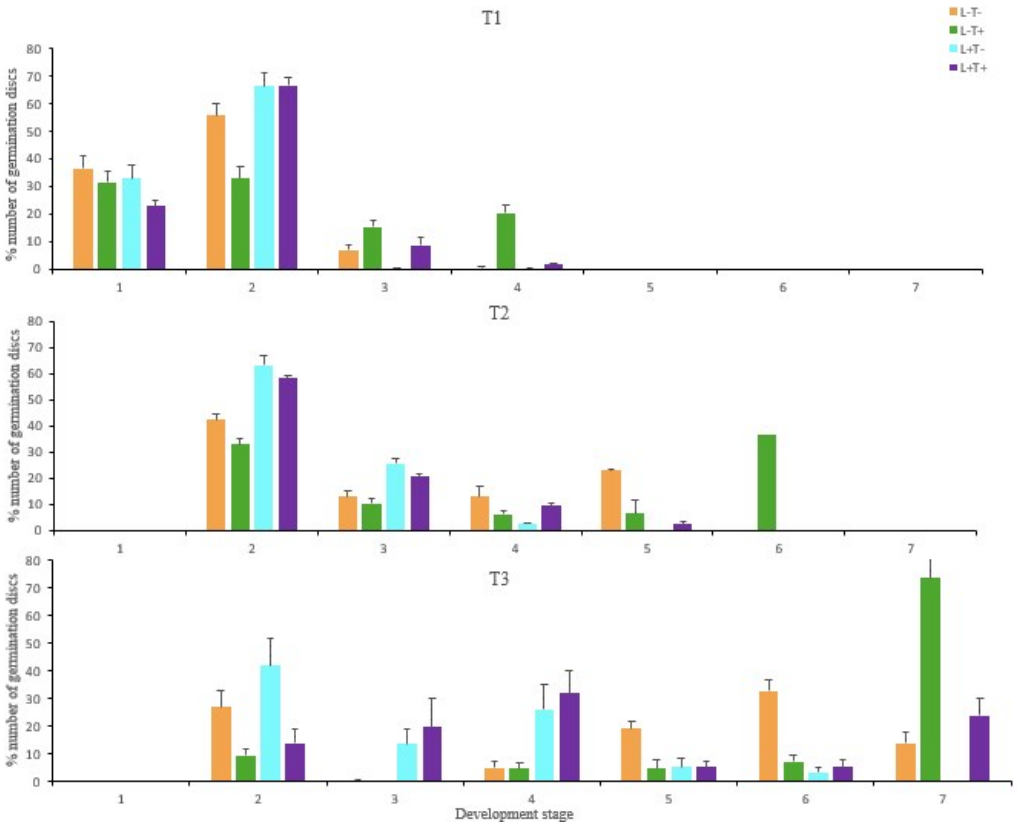


Figure 5. Developmental stages of *L. stictiforme*. Percentage of germination discs (mean +SE) at each developmental stage at T1, T2 and T3. L-T-: low light-low temperature; L-T+: low light- high temperature; L+T-: high light-low temperature; L+T+: high light-high temperature.

At T3, significant differences in the proportion of stages V, VI and VII were found (Table 4C). In L^-T^+, stage VII was predominant (73.6%), while in L^+T^+ the percentage was lower (23.8%) and the germination discs remained mainly at stage IV. In L^-T^-, stage VII accounted for 13.8% of the germination discs and in L^+T^- this stage was not reached at T3, a significant effect of light was also found on the germination disc area, indicating a promoting effect on disc size of the low light condition independently of the temperature (Fig. 6, Tab. 5).

Table 5. Germination disc growth. Results of univariate PERMANOVA testing the effect of Temperature (Te) and Light (Li) on the disc area at T3 (14 days AR). In bold the Pseudo-F corresponding to a significant result.

Source of variation	df	MS	Pseudo-F	P(perm)	perms
Temperature = Te	1	1048.6	1.124	0.331	987
Light = Li	1	6901.1	7.402	**0.005**	984
TeXLi	1	1669.1	1.793	0.213	986
Residual	8	932.26			

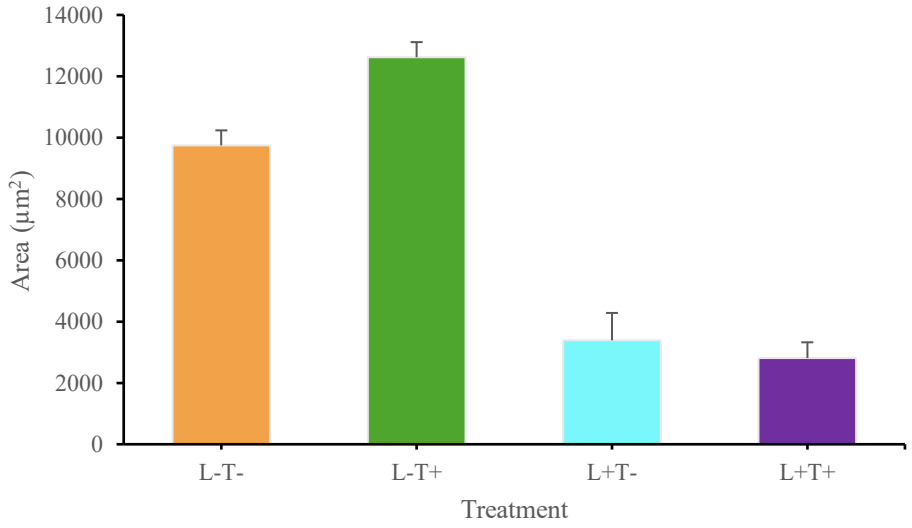


Figure 6. Germination disc area. Mean (+SE) disc area (µm^2) for each treatment at T3 (14 days after spore release): L^+T^+: high light-high temperature; L^+T^-: high light-low temperature; L^-T^+: low light- high temperature; L^-T^-: low light-low temperature.

At T3, the EDS analyses (Fig. 7) showed that treatments affected the Mg/Ca ratio of the germination discs (Fig. 8 A,B): a positive influence was found of both temperature and light intensity, but not in interaction (Tab. 6), with the core area having the highest Mg/Ca content.

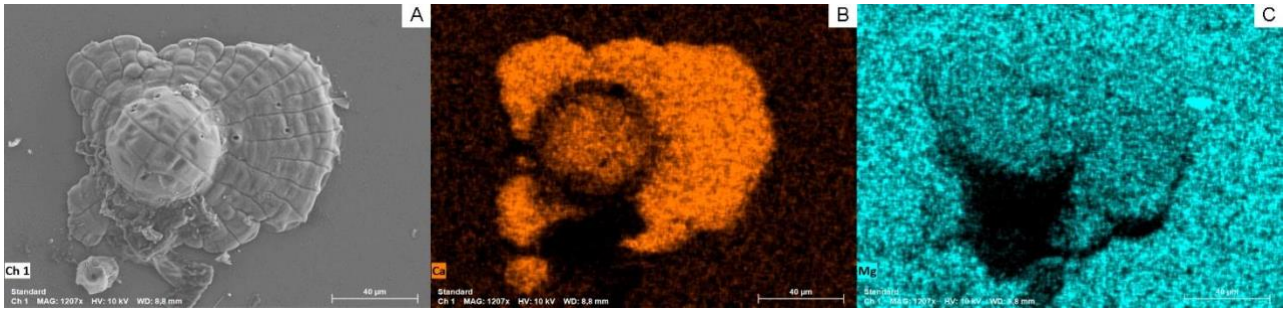


Figure 7. *L. sticitiforme* germination disc at T3. A) Scanning Electron Microscopy image. B) EDS map of calcium. C) EDS map of magnesium.

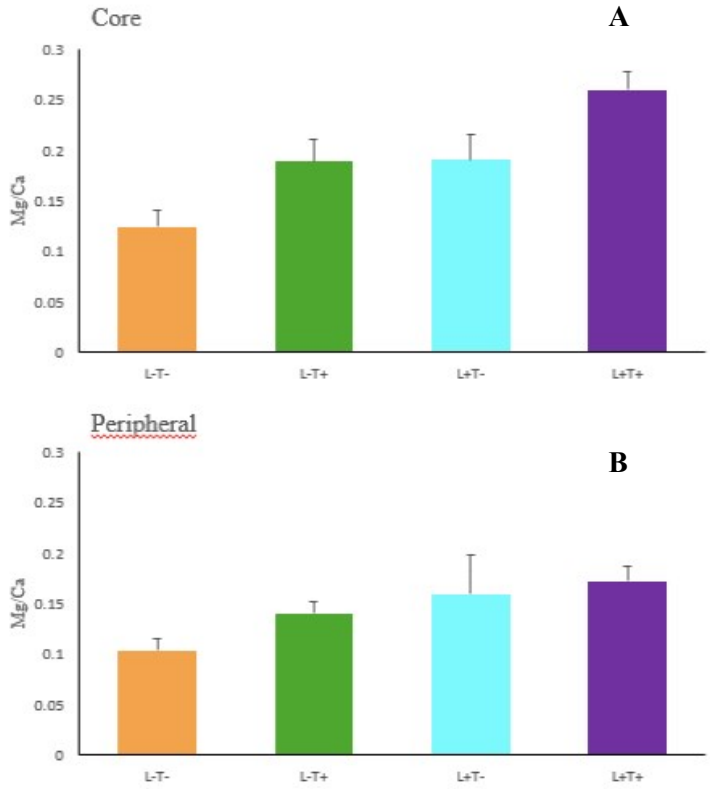


Figure 8. Calcification disc distribution. Mg/Ca content, measured over the core (A) and peripheral area (B) of the germination discs from each treatment at T3 (14 days after spore release): $L^{+}T^{+}$: high light-high temperature; $L^{+}T^{-}$: high light-low temperature; $L^{-}T^{+}$: low light- high temperature; $L^{-}T^{-}$: low light-low temperature.

Table 6. Calcification. Results of univariate PERMANOVA testing the effect of Temperature (Te), Light (Li) and Disc Portion (DP) on the Mg/Ca ratio at T3 (14 days AR). In bold the Pseudo-F corresponding to a significant result.

Source of variation	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Temperature = Te	1	0.022	5.806	**0.024**	996
Light = Li	1	0.485	12.269	**0.003**	995
Disc Portion = DP	1	0.044	11.328	**0.001**	998
TeXLi	1	0.001	0.274	0.587	999
LiXDP	1	0.002	0.753	0.391	995
TeXDP	1	0.006	1.738	0.174	996
LiXTeXDP	1	0.001	0.492	0.487	998
Residual	78				

4. DISCUSSION

This study is the first *ex-situ* experiment providing significant insights into the effects of the interaction of light and temperature on laboratory-developed recruits of *L. stictiforme*. Light and temperature conditions tested in the present study affected the germination success of *L. stictiforme*, influencing both density and growth of germination discs through time, as well as their elemental composition and the distribution of calcification after two weeks of culture.

In particular, light had a significant effect on the germination discs with density and growth promoted by lower light treatment. This is not surprising since *L. stictiforme* mainly occurs deeper than 20 m (Sartoretto et al. 1996; Ballesteros 2006; Pinna et al. 2022) and the L^- irradiance tested corresponds to the light intensity at the thalli collection depth. Furthermore, the results obtained indicate that the germination discs of *L. stictiforme* do not acclimate to higher irradiance which has reduced germination disc density and growth. This overall supports the evidence already provided for the growth of adult thalli of this species (Pinna et al. 2022). However, experiments carried out on early life stages of CCA, have produced heterogeneous results since the response to irradiance appears to be species-specific. For example, some evidence provided for *Lithophyllum yessoense* Foslie germination discs indicate their growth was favored by higher irradiance (i.e. 240µmol photons $m^{-2}s^{-1}$), although the species is a subtidal alga (Ichiki et al. 2000). Inconsistently, the fact that in intertidal CCAs, such as the *Lithophyllum okamurae* Foslie (Yoshioka et al. 2020), or in the coral reef builder *Porolithon onkodes* (Heydrich) Foslie (Ordoñez et al. 2017), germination disc growth rate was reduced at low irradiance suggests an adaptation to light condition specific of the environment where the species normally thrive.

A significant effect on the germination success of *L. stictiforme* discs was observed under high temperature treatment that promoted the germination of discs regardless of light intensities. In the location where adult thalli were collected for the experiment, the seawater temperature was

approximately 14°C (as usually at that time of year, Ceccherelli et al. 2020); the higher germination success was observed in laboratory raising the temperature to 20°C, which has been simulated the thermocline deepening that naturally occurs in that area in August and September. These results are consistent with previous studies, showing that higher temperatures can enhance the metabolic processes that promote the germination disc growth in the laboratory (Ichiki et al. 2000; Ordoñez et al. 2017). Little is known about the effects of seawater warming on Mediterranean CCA and especially on the first life stages, although it is known that the calcification of *L. cabiochae*, is negatively affected by an increase in seawater temperature (Martin and Gattuso 2009).

Our study also highlights that an increase in temperature does not have a significant effect on the growth of germinative discs that, conversely, is only influenced by light. This is consistent with observations that adult thalli of *L. stictiforme* exhibited greater marginal growth in areas where the water temperature was lower (Pinna et al. 2022). However, no evidence was ever provided for recruits.

Valuable information was also provided by the interactive effect of light and temperature on the development time of *L. stictiforme* germination discs: it was predominantly observed that the segmentation of germination discs was accelerated when temperatures were high and irradiance low. Optimal conditions do not only favor growth, but also mineralogical processes (Cornwall et al. 2017; Nash et al. 2019; McCoy et al. 2023). The EDS analysis performed in this study showed that lower Mg/Ca content was promoted at lower irradiance, conferring higher hardness and dissolution resistance, likely an advantage both in mesocosms and in the field (Martin et al. 2013a, 2013b). This result supports the assumption that *L. stictiforme* is a low-light-adapted species, as higher irradiances than in the field would trigger the calcification suppression (Martin et al. 2013b; Egilsdottir et al. 2016). Apparently, not all the CCAs are low-light adapted, since in other species the calcification of adult thalli is light-enhanced (Borowitzka 1981; Borowitzka and Larkum 1987; Martin et al. 2013a). Studies on adult thalli of CCAs have also found a close relationship between growth rate, degree of calcification and Mg content (Kamenos et al. 2008, 2009; Nash et al. 2019), with faster calcification

being associated with a lower density of calcite and increased incorporation of Mg into the cell walls (Kamenos et al. 2009; Kamenos and Law 2010; Sletten et al. 2017; Krieger et al. 2023). In the present study, germination discs of *L. stictiforme* cultured under L^+T^+ had the slowest growth, but the Mg/Ca ratio was the highest among the treatments. A possible explanation for the higher Mg substitution could lie in the effect of both light and temperature, as a correlation between higher Mg/Ca and higher temperatures in adult CCAs is widely recognized (Halfar et al. 2008, 2013; Nash et al. 2016, 2017). It is likely that the germination discs from this treatment are more vulnerable to dissolution after outplanting, as dissolution is more pronounced with increasing Mg substitution in biogenic calcite (Andersson et al. 2008).

In general, CCAs control the mineralization process during the development of germination discs (de Carvalho et al. 2022): mineralization starts only in the innermost walls in 8-cell specimens and in 16-cell germination discs the outer part is not yet calcified (de Carvalho et al. 2022). In all treatments, the Ca content was higher in the peripheral portion of germination discs, which begins to grow once the marginal meristem has formed, allowing horizontal expansion of the young thallus. The studies conducted on adult thalli of CCAs indicate that their calcification is highest in the growth regions where most chloroplasts are located with a higher photosynthetic activity) (McCoy et al. 2023). This work corroborates the finding that CCAs germination discs can control their calcification process to a certain extent; however, further investigations are needed to elucidate the mechanisms involved in their biomineralization in relation to light intensity and temperature.

Overall, several questions remain to be answered about the response to the combination of these two conditions ex situ. Further studies should investigate the spatial consistency, by collecting thalli across Mediterranean sites, and temporal variability of our findings to investigate the importance of local and seasonal adaptations: for example, research is also necessary to understand if spores released from thalli collected below the thermocline during warm periods (summer) respond differently to a major temperature increase of 5-6°C in terms of germination success, density, development and growth, exhibiting seasonal adaptations.

In conclusion, the present study expands upon the knowledge on development, growth and calcification of CCA germination discs. In particular, it assessed the feasibility and determined the optimal combination of light and temperature conditions for the *ex-situ* culture of *L. stictiforme*. Further experiments are needed to identify the best substrate (*i.e.* tile) for *L. stictiforme* recruitment to be used in mesocosm conditions, so that outplanting in the field the recruited tiles would be a technically possible and environmentally friendly action.

Chapter 3

INVESTIGATING TILE MATERIALS FOR ENCRUSTING CORALLINE ALGAE RECRUITMENT: A TOOL FOR FUTURE CORALLIGENOUS RESTORATION

Puccini A.[1]*, Peña V.[2], Ceccherelli G.[3]

[1] *Department of Architecture, Design and Urban Planning, University of Sassari, via Piandanna 4, 07100 Sassari, Italia*

[2]*BioCost Research Group, Facultad de Ciencias, and CICA – Centro Interdisciplinar de Química e Bioloxía, Universidad de A Coruña, A Coruña, Spain*

[3]*Department of Chemical, Physical, Mathematical and Natural Sciences, University of Sassari, via Piandanna 4, 07100 Sassari, Italia*

*e-mail: alessandrapuccini4@gmail.com

ABSTRACT

In recent years, the need to restore marine habitats damaged due to human impact has increasingly grown. Most of the Mediterranean studies carried out to date concern the *Posidonia oceanica* habitat and *Cystoseira/Euricaria* species. For the coralligenous habitat some gorgonian transplantations have been attempted, but no efforts have been deployed to transplant the foundations species of the habitat, the crustose coralline algae (CCA). Hence the need to develop techniques for marine transplantation of CCA has emerged. In this study, carried out in Costa Paradiso (North Sardinia, Italy), we tried to identify the most suitable artificial material (10x10 cm tiles) for the recruitment of CCA, in order to be able to use it in future restoration works. For this purpose, a manipulative field experiment was carried out (at a depth of approximately 28m depth) with an orthogonal experimental design consisting of three factors: material (two levels: ceramic and terracotta), orientation of the substrate (two levels: vertical and horizontal) and surface (two levels: surface exposed to light and surface not exposed to light). The artificial tiles were left on the seabed for a total of approximately 6 months (from February to June 2023). They were subsequently photographed in laboratory and analyzed with the Image-J Software to evaluate the percentage recruitment coverage. A posteriori, molecular and morphological analyses were carried out on random individuals of CCA grown on the substrates in order to identify the species that recruited. Results highlighted that the most suitable material for recruitment was terracotta placed on the substrate in a horizontal position under conditions of exposure to light. From genetic and morphological analyses, however, it emerged that the individuals analyzed belonged to five different species of the *Titanoderma* genus regardless of the material used, the position of the substrate or the exposure to light.

Keywords: coralline algae, restoration, artificial substrates, climate change

1. INTRODUCTION

Coralligenous reefs are deep subtidal systems of Mediterranean Sea and are considered one of the most important habitats thanks to the services they provide (Laborel 1987; Martin et al. 2014; UNEP, 2017): they act as hot-spot of marine biodiversity (Ådhal et al. 2006; Ballesteros 2006; Piazzi and Ceccherelli 2020), they are important carbon sinks (Martin et al, 2014) and habitat for economically valuable species (Canals and Ballesteros 1997; Ballesteros 2006; Rindi et al. 2019).

Coralligenous reefs are included in the Habitat Directive being part of the habitat type 1170 "reefs", and are part of the "special habitat types" under the Marine Strategy Framework Directive (E.C., 2008) which ensures the maintenance of the ecological quality of marine coastal ecosystems, protects them against threats and promotes sustainable use of marine resources (Deter et al. 2012; Piazzi et al. 2017; Sartoretto et al. 2017).

The coralligenous habitat includes several assemblages due to its great heterogeneity. Reefs are mainly produced by the acculumation of calcareous coralline algae (CCA) growing in dim light conditions and in relatively calm waters (Ballesteros 2006). CCA are ecologically important calcareous red macroalgae that create the reef substrate for a diversified assemblage of epibiota and vagile fauna. They are key component in providing an important biological habitat for many marine organisms (Cornwall et al. 2020). In Mediterranean Sea, coralligenous reefs are edified by encrusting Rhodophyta belonging to the genus *Lithophyllum, Lithothamnion, Mesophyllum, Neogoniolithon* and *Spongite* (Ballesteros 2006).

Over the past two decades, particular attention has been paid to increasing marine climate change effects, particularly to the threats of ocean acidification and warming (Gattuso et al. 2015). Corallingenous reefs are among the most threatened ecosystems by environmental changes caused by the increase in atmospheric CO_2 correlated to increase in ocean temperature, also called ocean warming (OW), and in ocean acidification (OA) (Page and Pulido 2020). Because of the crucial

ecological role of CCA, it is therefore of interest to understand how their future ecological role will be altered in the future scenario.

Ocean warming is a great threat to key foundation species such as corals and kelps (Hughes et al. 2017), but little is known about its effect on coralline algae, while they are ecologically fundamental in most hard-bottom habitats. Average sea surface temperature will rise by 2.7-4°C by 2100 under Shared Socioeconomic Pathways 8.5 (SSP5-8.5) (IPCC, AR6 2023), while atmospheric carbon dioxide (CO_2) concentration has risen from 280 ppm before the industrial revolution to a current value of 410 ppm due to human activities and is expected to reach more than 950 ppm by the end of the century considering the IPCC (Intergovernmental Panel on Climate Change) emission scenario SSP5-8.5 (IPCC, AR6 2023). CCA are among the calcifying organisms most sensitive to ocean acidification: a review by Cornwall et al. (2019) identified 78 articles on the impacts of OA on CCA, while only 14 of them provide evidence of impact of OW or the interaction of the two factors. Most of these studies were conducted on adult thalli of coralline algae (Martin and Gattuso, 2009; Diaz-Pulido et al. 2012; Martin et al. 2013; Page and Pulido 2020) but little is known about the effects of climate change on recruits (Cornwall et al. 2020). All of these experiments were conducted in mesocosm or laboratory, and evidence gained in the field are particularly needed (but see Pinna 2022). To restore the structure and functionality of coralligenous reefs that have been degraded, damaged or destroyed, active restoration by means of removing the source of disturbance, implements strategies to accelerate recovery and address challenges to remove barriers to this recovery (Holl and Aide 2011) are badly needed. Restoration efforts for coralligenous reefs have primarily concentrated on transplanting fragments of habitat-forming species such as gorgonians and sponges (Linares et al., 2009; Fava et al. 2009; Montero-Serra et al. 2017). Given that the recruitment, growth and recovery time of the species that lived in this habitat are very long, there are currently no studies that demonstrate the effects of restoration on large temporal and spatial scales. Consequently, restoration efforts on coralligenous habitats are still in a very early stage. However, to my knowledge, no attempts

have still be made to restore this habitat by transplanting CCA recruits. At this aim, this study was designed to identify an artificial structure that, depending on the material and orientation, would favor the natural recruitment of the CCA. This was done by means of a field experiment using structures of the same size but different material (Kennedy et al. 2017). By positioning such structures with different orientations respect to the reef, and by evaluating differences between upper and lower surface of the structures, we tested the hypothesis that CCA recruit abundance and growth would change depending on the combination of such conditions. A posteriori, the identification of CCA recruits was done in order to identify the species whose recruitment was promoted by the materials. The results are useful to produce structures covered by CCA to be transplanted in sites to be restored and/or use them in further experiments testing the effects of climate conditions linked to OW and OA.

2. MATERIALS AND METHODS

2.1 Sampling site

This study was conducted on coralligenous reefs of Costa Paradiso (N 41.06546º, E 008.95039º), North Sardinia (Fig.1), from February 2022 and will last until February 2023. This site is far from any anthropogenic sources of disturbance (e.g., urbanization, industries, aquaculture and harbors) and is characterized by oligothropic conditions (Barisiello et al. 2002; Regione Autonoma della Sardegna, 2016). In this area the coralligenous reef is formed by massive bioconstructions from 15 m of depth (Piazzi et al. 2021) where CCA are large in thallus size and very abundant.

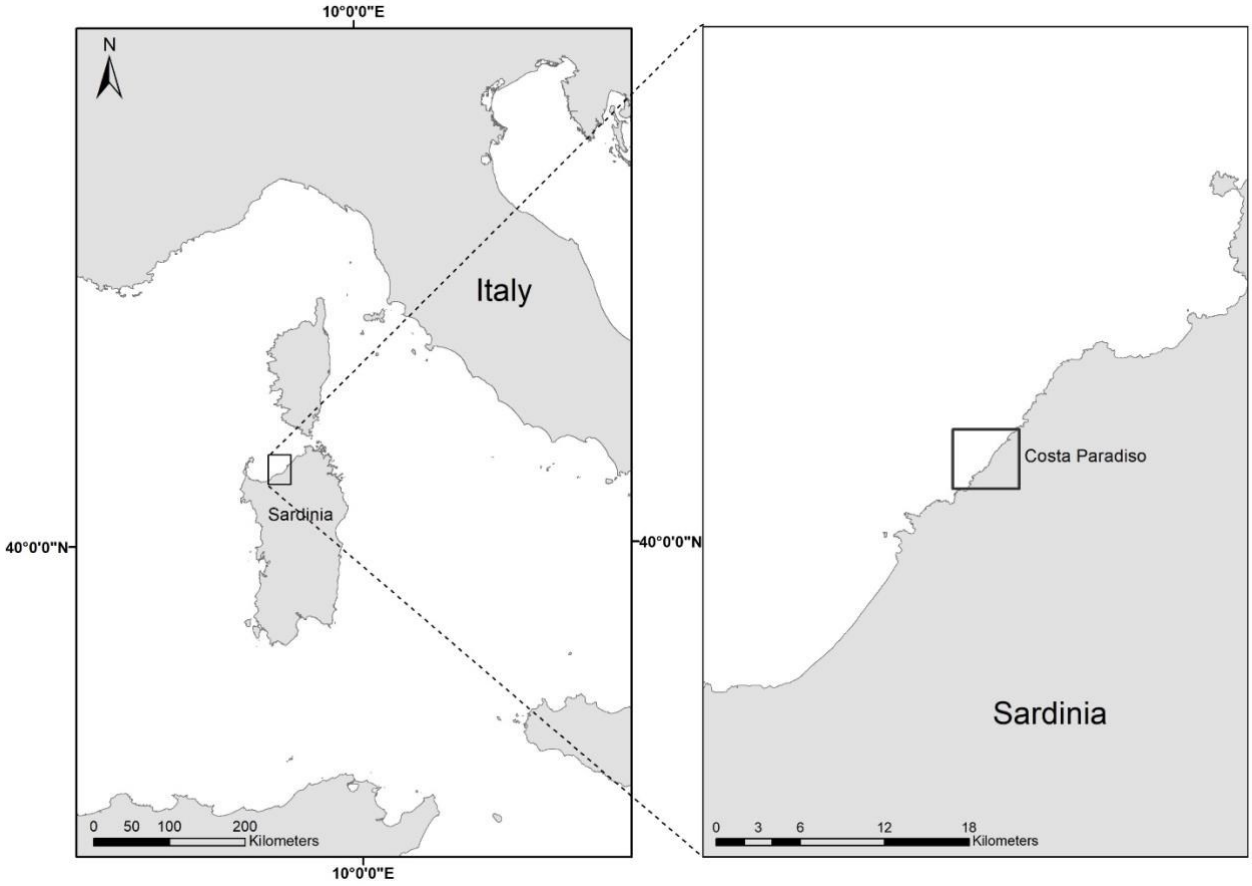


Figure. 1. Map of the study area.

2.2 Experimental setup

In the selected site, an area was chosen at 28 m of depth where an array of 16 settlement structures (9.5 x 9.5 x 1.5 mm in size), 8 made of terracotta and 8 of ceramic, was hammered directly to the rocky substrate thanks to threaded rod, so that for each material type 4 were oriented horizontally and 4 oriented vertically (n=4) (Fig.2). Each structure was made of two tiles attached to each other so that the two surfaces (exposed and not exposed) were equal and available for CCA settlement: this allowed testing the differences in recruitment between the two sides. At the end of the experimental period (5 months, from February to June 2023), all the tiles were recollected from the field and transported to the laboratory to be photographed. All the images taken were analyzed using ImageJ Software to calculate the total percentage coverage.

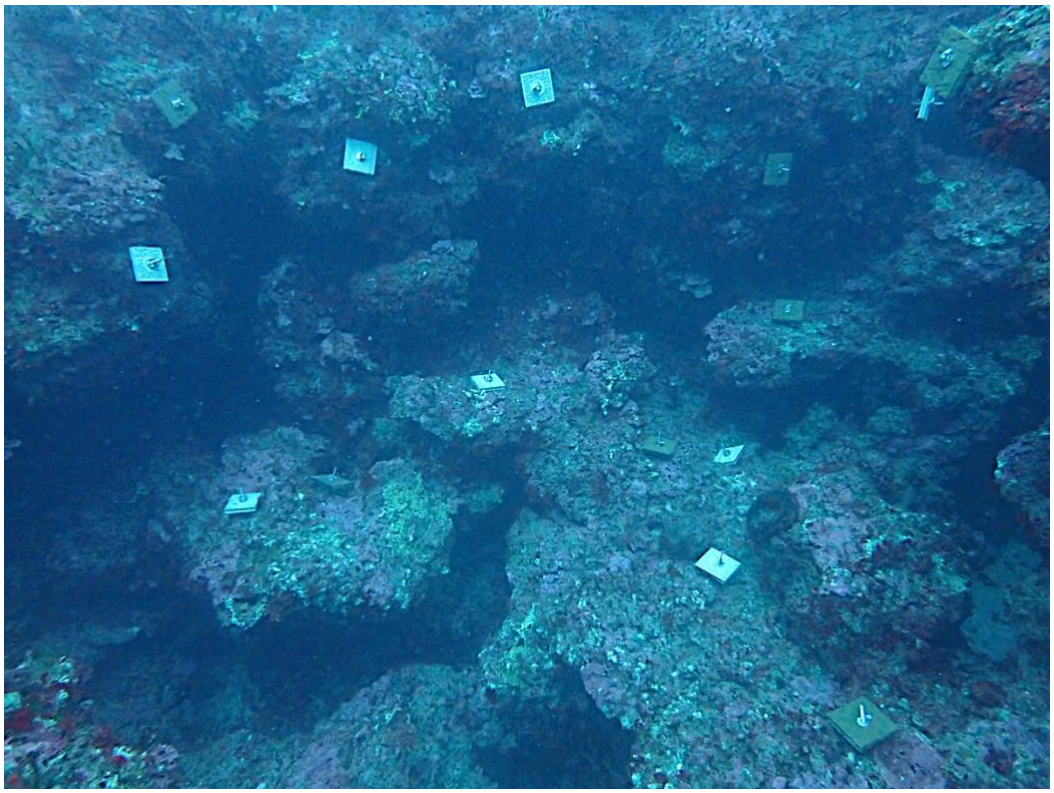


Figure 2. Tiles of terracotta and ceramic hammered to the substrate both in vertical and horizontal position.

2.3 Statistical Analysis

The total percentage coverage of the CCA recruited was evaluated through a three-way permutational analysis of variance (PERMANOVA, Anderson, 2001) based on Bray-Curtis distance using an orthogonal design where material (Terracotta and Ceramic, two levels), orientation (Vertical and Horizontal, two levels), and surface (Exposed and Not Exposed, two levels) will be treated as fixed crossed factors. A posteriori comparison of means was done using Pair-Wise comparisons after PERMANOVAs. PERMANOVA was performed with PRIMER v7 software.

2.4 DNA extraction, PCR amplification and sequencing

The tiles with coralline algae used for DNA extraction were air-dried for 48 h at 40ºC (oven) and subsequently placed in bags with silica gel. DNA extractions were performed using material that appeared visually clean and devoid of contaminations. When the presence of epiphytes or other possible contaminants was observed, the material was carefully cleaned with a brush and tweezers. Fragments selected were ground to a fine powder using sterilized microdrill (Dremel 4000, Breda, Netherlands), tweezers and razor blade. DNA was extracted using a NucleoSpin Tissue XS Kit (MACHEREY-NAGEL, GmbH & Co, Düren, Germany) (Fig.3).



Figure 3. Example of some steps of the DNA extraction process.

PCR amplification was performed on the plastid *psbA* gene using the primers psbA-F1 and psbA-R1 (Yoon et al. 2002). We performed all amplifications in a final volume of 10 µL, using the Supreme NZYTaq II Colorless Master Mix (2X), from NZYTech, and the concentrations detailed as follows: DNA 1.0 µL, H_2Od 3.6 µL, Primer Forward (10 µM) and Reverse (10 µM) 0.2 µL each and 1 Supreme NZYTaq II Colourless M. Mix (2X). The PCR protocol for the amplification of *psbA* gene consisted of an initial denaturation at 95ºC for 4 min, and then 45 cycles at 95ºC for 30s, 52 for 40s, 72 for 50s and a final extension step at 72ºC for 5 minutes and storage at 10ºC. PCR products were visualized and quantified in 2% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA) and

visualized under UV light. PCR product with expected lengths and yields were purified and custom-sequenced by Macrogen Europe (Amsterdam, The Netherlands).

2.5 Sequence alignments and phylogenetic analysis

Sequences were assembled ad aligned with the use of CodonCode Aligner (CodonCode Corporation, USA) and adjusted by eye using SeaView version 5 (Gouy et al. 2010). Alignments were assembled for *psb*A using ClustalW in SeaView with default settings and refined by eye. For the molecular analyses, sequences of species belonging to the genera *Phymatolithon* and *Mesophyllum* (*Hapalidiales* outgroup) and sequences of the genus *Titanoderma*, downloaded from the Genbank database, were included. The quality of the sequences was assessed by visual examination of the electopherograms in CodonCode Aligner and only high-quality sequences (i.e. lacking double peaks or other ambiguities that could make the readings questionable) were included in the phylogenetic analyses.

Phylogenetic analyses were performed on all data sets using p-distances in MEGA version 6 (Tamura et al. 2013), with nodal support assessed by 1000 bootstrap (BP) resampling. Phylogenetic inference by Maximum Likelihood (ML) was performed using MEGA Software. The GTR+G+I model was used for all reconstructions.

2.6. Morpho-anatomical analysis

For scanning electron microscopy (SEM), the air-dried material was fractured using tweezers and razor blade. The pieces were placed both perpendicularly and horizontally, to analyze their internal anatomy and surface respectively, on stubs, using Silver Conductive Paint 503 (Electron Microscopy Sciences), coated with gold for 30 minutes in an Edwards S150B sputter coater and examined with a JEOL JSM 6400 scanning electron microscope with an accelerating voltage of 25 kV (Fig.4).

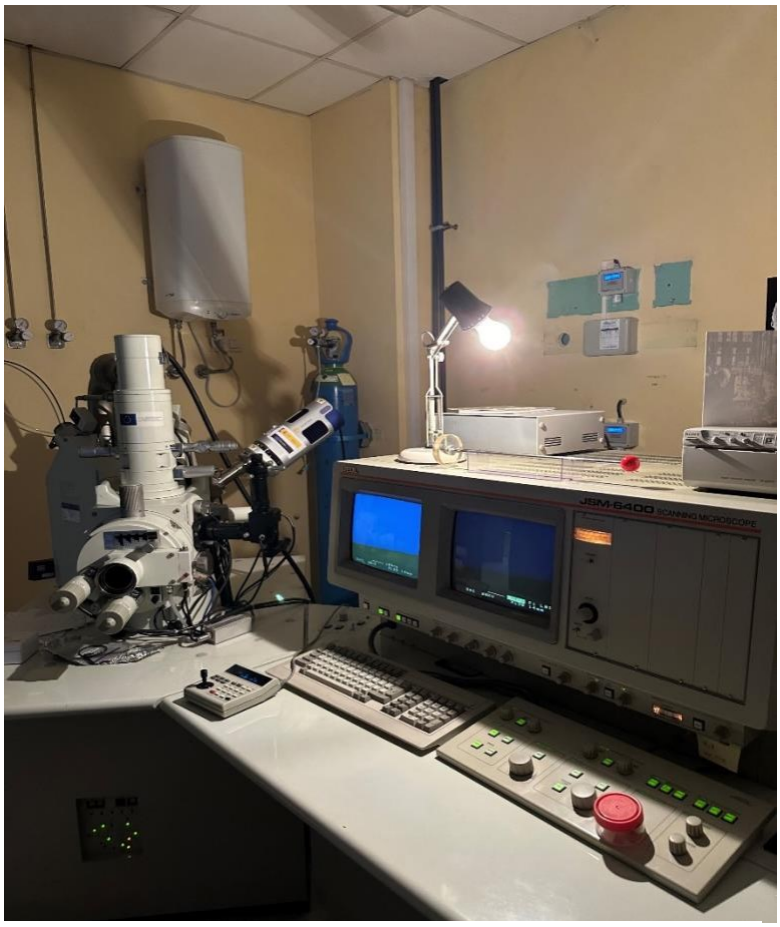


Figure 4. Scanning Electron Microscope (SEM) used for the morpho-anatomical analysis.

3. RESULTS

After one year of exposure, CCA recruits were found on all tiles at all conditions, although with a wide range of total cover. Significant differences in the percent cover of all encrusting corallinales were found depending on the orientation and exposition combination, highlighting that horizontal tiles had an higher cover than the vertical ones (Figure 5, Table 1). Furthermore, for both horizontal and vertical orientation, CCA coverage was larger on the exposed side (Figure 5, Table 1). Another significant difference in encrusting corallinales cover was found between materials depending on the orientation, where horizontal terracotta tiles were more encrusted than the horizontal ceramic and more than the vertical terracotta ones (Figure 5, Table 1).

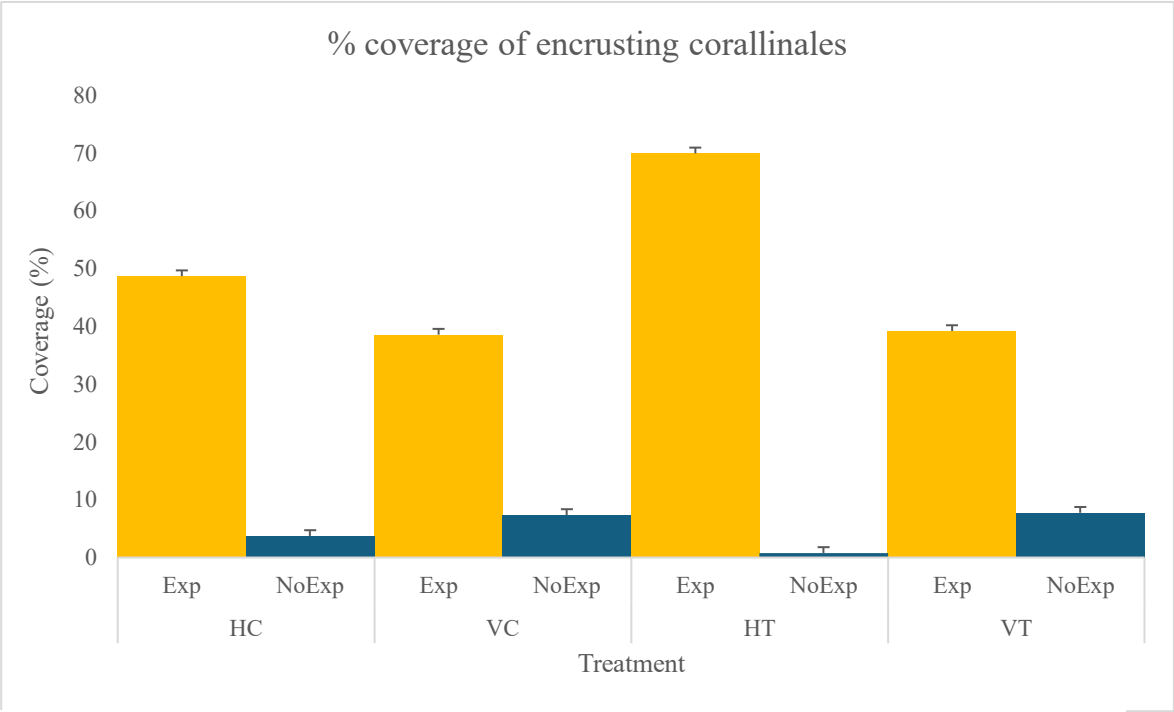


Figure 5. Average (+SE) percent coverage of encrusting corallinales between treatments. Material (T= terracotta and C=ceramic), Orientation (H=horizontal and V=vertical), Exposition (Exp=exposed or NoExp=not exposed surfaces).

Table 1. Results of PERMANOVA testing the effect of Material, Orientation and Exposition on the CCA recruitment. In bold the Pseudo-F corresponding to a significant result.

Source of variation	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Material = Ma	1	3632.4	3.14	**0.0257**	9946
Orientation = Or	1	2171.8	1.87	0.1130	9945
Exposition = Ex	1	72814	63.00	**0.0001**	9947
MaXOr	1	3329.3	2.88	**0.0347**	9943
MaXEx	1	2722.6	2.35	0.6860	9948
OrXEx	1	3197.4	2.76	**0.0331**	9948
MaXOrXEx	1	2213.3	1.91	0.1128	9952
Residual	52	60095			

Pair-Wise test			
Material X Orientation		Orientation X Exposition	
Horizontal	T>C	Exposed	H>V
Vertical	T=C	Not Exposed	H=V
Terracotta	H>V	Horizontal	E>NE
Ceramic	H=V	Vertical	E>NE

3.1 Molecular and morphological results

Molecular analyses revealed the presence of individuals belonging exclusively to the genus *Titanoderma*, and specifically to five species, in all experimental conditions, as shown in Fig. 6. This genus is poorly studied in the Mediterranean Sea, and the absence of *psbA* gene sequences in GenBank prevents the identification of the full species names.

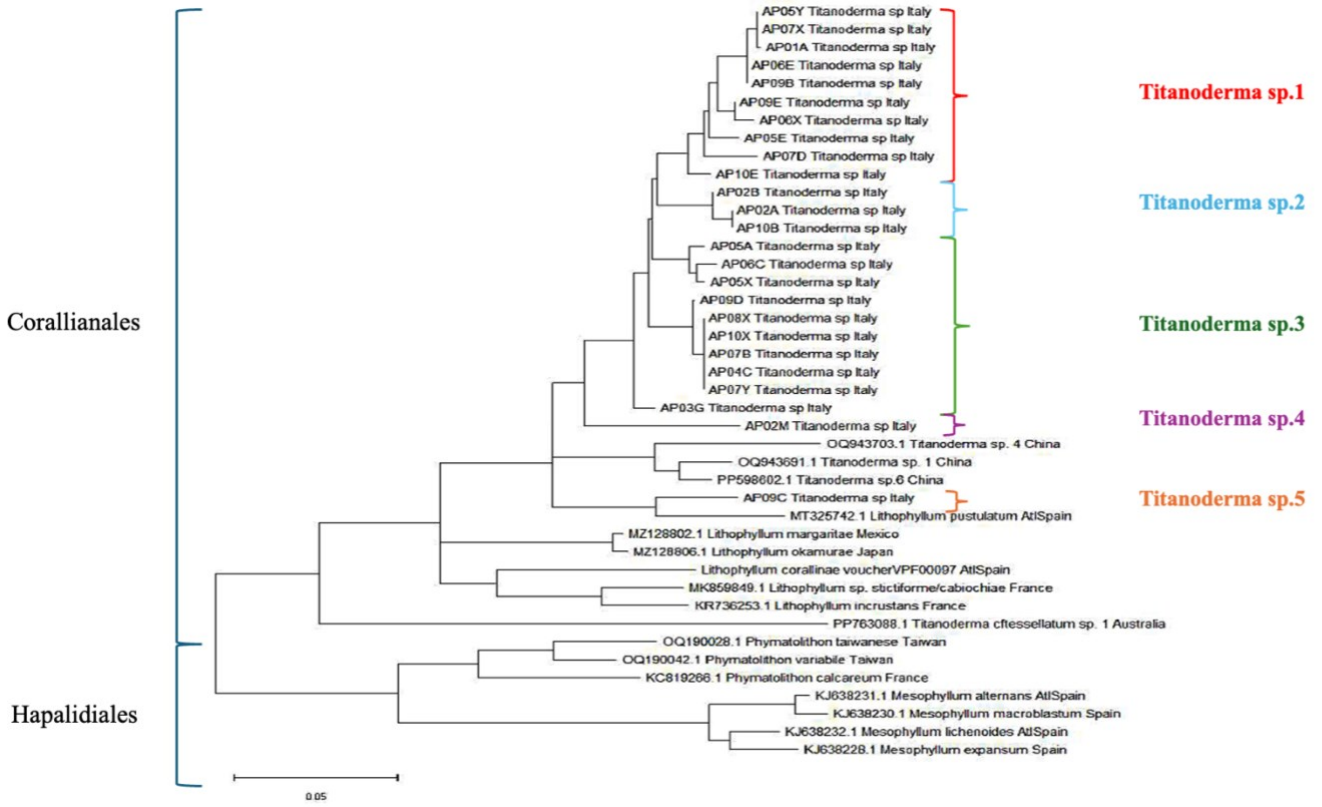


Figure 6. Phylogram inferred from Maxim Likelihood (ML) analysis of *psbA* data set of *Titanoderma*.

These results were subsequently confirmed by SEM morphological analyses (Fig. 7), where characteristic structures of the order Corallinales can be identified, such as the presence of secondary pit connections (Fig. 7c), and in particularly of the genus *Titanoderma*, with the presence of palisade cells (Fig. 7d).

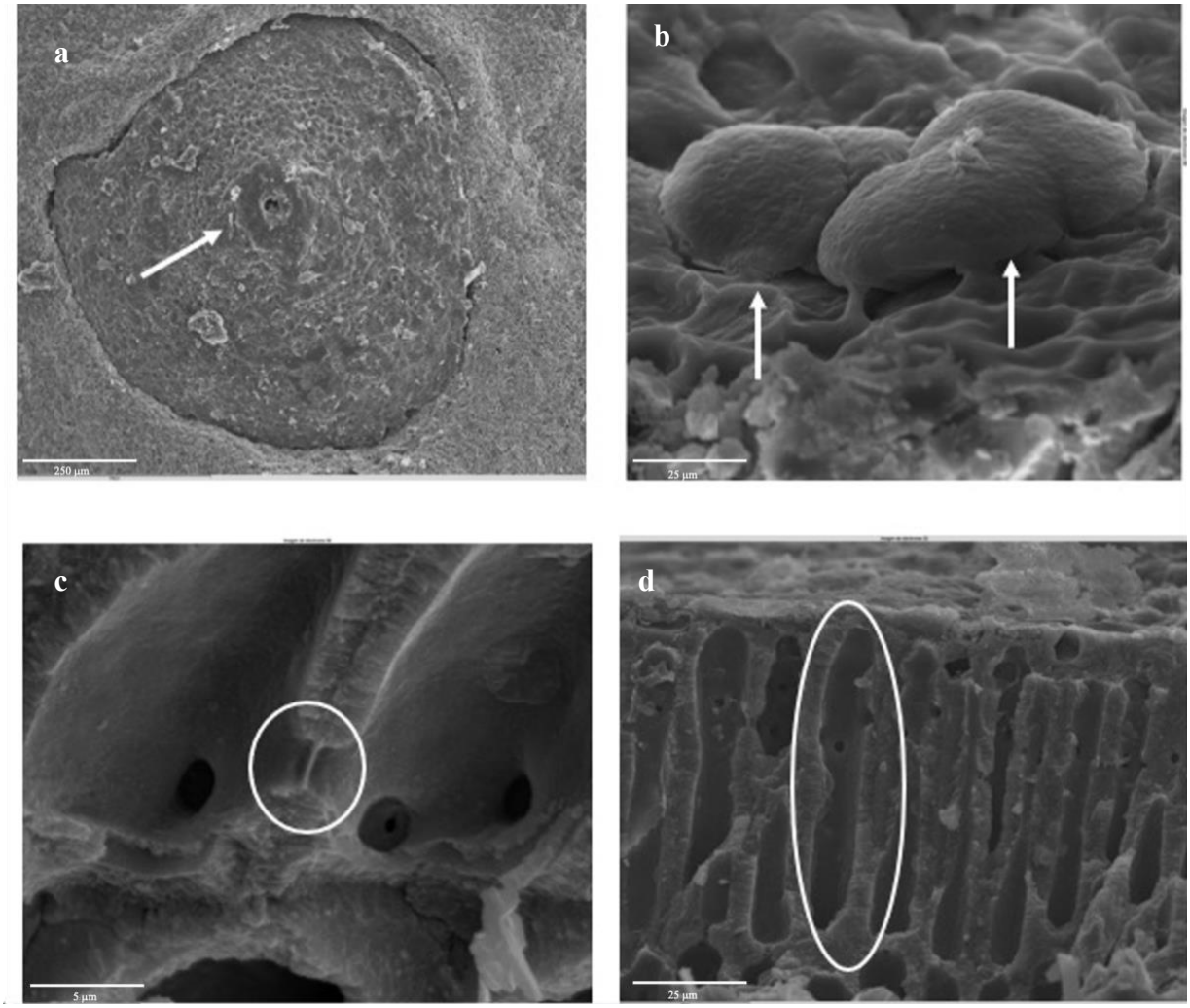


Figure 7. Scanning Electron Microscope (SEM) images. Reproductive (a-b) and vegetative (b-c) anatomy of *Titanoderma* sp. (a) Uniporate conceptacle. (b) Carpospores. (c) Secondary pit connections. (d) Palisade like-cells.

4. DISCUSSION

This study represents one of the first field experiments conducted on the recruitment of CCAs on artificial substrates. The material used, exposure and substrate orientation tested here revealed significant differences in the recruitment of the CCA.

Artificial tiles have been yet utilized in *in situ* experiments of CCA to investigate a wide range of topics. These include investigating community responses to elevated CO_2 levels (Fabricius et al. 2011; Fabricius et al. 2015), evaluating natural variability in recruitment and life history traits on coral reefs (Adey and Vassar 1975; Villas-Bôas et al. 2005) and conducting focused research on calcification rates and the role of CCA in reef accretion (Mallela 2013).

As indicated by the analysis, the surface not exposed and thus much closer to the seabed shows a significantly lower percentage of algal coverage compared to the exposed surface. This could be due to the negative effect of limited light, which is insufficient for photosynthesis and algal growth (Ballesteros 2006). When light is scarce, photosynthesis is inhibited, leading to reduced algal growth and, consequently, difficulty in forming calcareous structures, which are essential for their ecological role. A decrease in the ability to deposit calcium carbonate may weaken the algal structure, making it more vulnerable to erosion and competition with other species. This would be in agreement with Guntur et al. (2023) where the upward surface presented a higher coverage than the downward one probably due to higher levels of light and nutrients (Littler and Littler 2013).

Another possible explanation for the reduced algal cover on the less light-exposed surface could be that recruits stabilize near adult individuals, if not directly on the underside of the adult thallus, as observed during bottom explorations. This hypothesis should be tested with future research to determine whether there is some interaction between the adult individuals, the released spores and subsequent recruits.

Our study also highlighted that the recruitment of CCA was greater on horizontal surfaces compared to vertical ones. Substrate inclination is a factor that can influence the presence and development of crustose coralline algae by altering the angle of sunlight incidence, thus affecting the amount of light available for photosynthesis (Ballesteros 2006). This agrees with Kennedy et al. (2017) and Mallela (2013) where a greater total cover on horizontally oriented tiles (upward facing surfaces) compared with vertically oriented tiles was reported. In 2016, Fava et al., with a three-year recruitment experiment, evaluated the role of colonization processes in the formation and maintenance of the heterogeneity of coralligenous assemblages on the northern Adriatic shelf. They used travertine tiles anchored horizontally to the substrate and they observed that at the end of the experiment encrusting calcareous Rhodophyta covered approximately 46% of the surface. Although a vertical substrate is generally more exposed to currents and favors the removal of sediment and nutrients, factors that could promote algal growth, in the artificial substrates used in this experiment a lower algal percent coverage was found, regardless of the material used.

Another significant difference was observed in the material used, where terracotta was found to promote algal growth more than ceramic. Terracotta is generally rougher and more porous than ceramic, and this natural texture favors colonization by CCA, probably retaining more nutrients and humidity. This difference could also be attributed to the distinct chemical composition of the two materials, making terracotta more favorable for algal growth and calcification. This is in contrast with Kennedy et al. (2017) and Guntur et al. (2023) where total cover of the CCA on the tiles did not depend on the material. Our result is partially in agreement with what Adey and Vassar (1975) had found on the existence of a material that favors the settlement of CCA even if the two materials are different (terracotta vs PVC). Other studies found that physical (e.g. hydrodynamics, irradiance) and/or biological processes (e.g. spore supply, competition, predation and grazing) may promote establishment more than the material used (Littler 1975). For example, Field et al. (2007) observed no significant difference in CCA cover on unglazed vs galzed terracotta, while Burt et al. (2009)

found strong variations in benthic communities between experimental sites but not between the material of the substrata.

The fact that only *Titanoderma* sp. recruited on tiles was totally unexpected, as well as the finding that no individuals belonging to the keystone species of the coralligenous habitat (e.g. such as *Lithophyllum* sp. or *Mesophyllum* sp.) were found on the artificial substrates placed on the bottom. Therefore, further studies are necessary to identify the ecological role of *Titanoderma* sp. in the Mediterranean coralligenous habitat especially on other algal species present at the site and to understand if this species represents the pioneer species in the CCA succession and whether there are facilitative or competitive interactions with the other common CCA species. Conversely, the fact that in a whole year no other CCA species have recruited would be worrying.

Considering the use of tiles in restoration efforts for coralligenous reefs, these findings suggest that the use of these artificial structures can be encouraged in the management of degraded coralligenous reefs. Based on the evidence collected, the terracotta tiles can be a more suitable substrate for the colonization of coralline algae which can better settle, grow, and potentially perform their ecological function as a substrate for other coralligenous species. The highest CCA recruitment would be expected in the exposed surface of terracotta tiles fixed horizontally. These artificial tiles could also be useful for stabilizing a substrate mechanically damaged (e.g. by anchoring or trawling) or they could promote biodiversity by increasing the heterogeneity of the surrounding substrate by attracting different marine species.

Chapter 4

ADAPTABILITY OF CRUSTOSE CORALLINE ALGAE RECRUITS TO DIFFERENT FIELD TEMPERATURE AND LIGHT CONDITIONS: INSIGHTS FOR *TITANODERMA* SPECIES

Puccini A.[1]*, Peña V.[2], Ceccherelli G.[3]

[1] *Department of Architecture, Design and Urban Planning, University of Sassari, via Piandanna 4, 07100 Sassari, Italia*

[2]*BioCost Research Group, Facultad de Ciencias, and CICA – Centro Interdisciplinar de Química e Bioloxía, Universidad de A Coruña, A Coruña, Spain*

[3]*Department of Chemical, Physical, Mathematical and Natural Sciences, University of Sassari, via Piandanna 4, 07100 Sassari, Italia*

*e-mail: alessandrapuccini4@gmail.com

ABSTRACT

Crustose coralline algae (CCA) are among the major calcifying organisms in the Mediterranean Sea and are important foundation taxa in the photic zone. They enhance the structural complexity of marine ecosystems, promoting settlement and metamorphosis of various invertebrates. Due to multiple local factors and climate change effects, coralligenous reefs are in decline. Investigations on CCA, the foundation species, are therefore necessary to know their adaptability to different conditions and thus to evaluate the possibility of restoration actions based on CCA transplantation. To this end, a manipulative field experiment was conducted in Costa Paradiso (North Sardinia, Italy) where CCA recruited at 35 m of depth on artificial substrates (terracotta and ceramics 10x10 cm tiles) with varying initial coverage (high and low): to disentangle the effects of irradiance from those of the temperature, three treatments were used by fixing the tiles at 1) 15 m of depth where they were placed in a natural cavity so that the algae could experience the 35 m light irradiance but water temperature above the thermocline (LLHT), at 2) 15 m of depth outside the cavity (HLHT), and at 3) the same origin depth, 35 m, where irradiance was low and temperature below the thermocline (LLLT). The recruits exhibited greater growth when exposed to higher temperature compared to those in combined low light and low temperature conditions. A posteriori, molecular and morphological analyses were conducted to identify the CCA species which allowed estimating the species-specific growth rate at the studied conditions. These analyses revealed that the individuals analyzed belonged to five different species of the genus *Titanoderma*, regardless of the light and temperature conditions.

Keywords: coralline algae, recruits, coralligenous reefs, environmental conditions, climate change

1. INTRODUCTION

Coralline algae (CA) are among the major calcifying organisms in the Mediterranean Sea and are one of the most important foundation taxa in the photic zone. They belong to the division Rhodophyta and the subclass Corallinophycidae that encompass several orders (*Corallinales*, *Hapalidiales*, *Corallinapetrales*, *Sporolithales* and *Rhodogorgonales*) (Le Gall and Saunders 2007; Nelson et al. 2015; Jeong et al. 2021). They are found in a wide range of marine ecosystems, from polar to tropical regions, both in intertidal and subtidal habitats growing in dim light conditions (Laborel 1961: Pérès and Picard 1964; Ballesteros 2006) and they are important in the formation of reef framework through carbonate deposition and in providing habitats or chemical cues for larval settlement of important marine invertebrates increasing benthic biodiversity (McCoy and Kamenos 2015; Huggett et al. 2018). Some of them act as important ecosystem engineers (Steneck 1986; Nelson et al. 2009; Fragkopoulou et al. 2021). Their morphological patterns include encrusting forms (crustose coralline algae, CCA), geniculate articulated thalli and free-living thalli (rhodolith/maerl). The term "crustose coralline" refers to all forms that grow roughly radially on hard bottoms with a thallus thickness lower than 1 cm (McCoy and Kamenos 2015). Bioconstructor CA are slow-growing and long-lived organisms and are considered highly vulnerable to long term climate change (Cornwall et al. 2019; Rindi et al. 2019; Peña et al. 2021).

In the Mediterranean Sea, the main builders of coralligenous assemblages are the non-geniculate species of the *Lithophyllum stictiforme* (Areschoug) Hauck complex (Pezzolesi et al. 2019) together with *Mesophyllum alternans* (Foslie) Cabioch & M.L. Mendoza, *M. expansum* ((Philippi) Cabioch & M.L.Mendoza and *Neogoniolithon mamillosum* (Hauck) Setchell & L.R. Mason, together with *Peyssonnelia rosa-marina* Boudouresque & Denizot (Hong 1980; Ballesteros 2006; Peña et al. 2015). Coralligenous reefs are Mediterranean subtidal systems which protected for the important services they provide (Laborel 1987; Martin et al. 2014; UNEP 2017): in fact, they are hot-spots of marine

biodiversity (Ådhal et al. 2006; Ballesteros 2006; Piazzi and Ceccherelli 2020), providing habitat for economically valuable species (Canals and Ballesteros 1997; Ballesteros 2006; Rindi et al. 2019), and they are important carbon sinks (Martin et al. 2014).

Although temperature are considered to be the main factors determining the development of CCA (Adey 1970), little is known about the influence of temperature on their presence and distribution in the Mediterranean Sea, even if several diseases due to thermal effects have been evidenced, like bleaching events (Hereu and Kresting 2016; Hereu et al. 2018). Elevated temperature can cause coral bleach (Hughes et al. 2017, 2018) and damage kelp and gorgonians forests (Garrabou et al 2009; Krumhansl et al. 2016), leading to mass mortalities (Garrabou et al. 2023). Because the average sea surface temperature is predicted to rise by 2.73˚C by 2100 under the RCP (Representative Concentration Pathway) 8.5, understanding the responses of some habitat-froming species such as CCA to the the increasing temperature has become crucial.

Response of CCA to climate change is limited compared with the abundant information from canopy-forming seaweeds as evidenced by Capdevila et al. (2018) who evidenced that high temperature on early life stages of the habitat-forming *Cystoseira zosteroides* C. Agardh (currently regarded as *Ericaria zosteroides* (C. Agardh) Molinari & Guiry) increased the vulnerability of the species and delayed the population recovery or compared with other foundation species such as corals and kelps (Wernberg et al. 2016; Hughes et al. 2017). In a review by Cornwall et al. (2019), is evidenced a net negative impact of increasing temperatures on some species of coralline algal calcification: the decline in net calcification and net photosynthesis with the consequent reduction in growth rate and increase in percent bleaching has been found in temperate CCA under temperature conditions resembling all emissions scenarios by IPCC (2014) (Britton et al. 2021), while in adult thalli of *Lithophyllum stictiforme* was evidenced a higher marginal growth, higher growth in thickness and conceptacles production at higher temperature but low light conditions, highlighting the interactive role of temperature and light irradiance. Although the recruit development under ocean acidification has been investigated in many CCA species (*Lithophyllum incrustans* Philippi, Cumani et al. 2010;

Phymatolithon lenormandii Adey, Bradassi et al. 2013), recruit growth under different temperature and light conditions has not been adequately investigated, especially in CCA recruits. A mesocosm study where on CCA recruits has evidenced an effective interaction between low light, high temperature and fast flow rate as the optimal conditions for CCA recruit photosynthesis (Villas-Boas et al. 2023).

Within the order Corallinales, the subfamily Lithophylloideae comprises relevant and highly diverse genera such as *Lithophyllum* and the articulated *Amphiroa.* This subfamily is characterized by having uniporate tetrasporangial conceptacles and cells of contiguous filaments joined by secondary pit-connections (Irvine and Chamberlain 1994). The genus *Titanoderma* also belongs to this subfamily. It forms crustose and flat thallus or composed of regenerating imbricating layers, or mainly of branches. The thallus is dimerous and is composed of palisade basal cells. This internal structure is quite different from that of *Lithophyllum* which do not have palisade basal cells. This morphological feature is a key characteristic for distinguishing between species of the two genera. However, in the scientific literature, there has been an ongoing discussion for many years regarding the distinction between the genus *Lithophyllum* and the genus *Titanoderma* based on this character. Furthermore, most *Lithophyllum* species exhibit an epilithic habit, whereas many species of *Titanoderma* prefer an epiphytic existence (Richards et al. 2014) such as *T. cystoseirae* (Hauck) Woelkerling, Y.M.Chamberlain & P. Silva and *T. laminariae* (P. & H. Crouan) Y.M.Chamberlain recorded in the European coasts (Irvine and Chamberlain 1994; Bressan and Babbini 2003). In this regard, Yan et al. (2024) conducted a study to document the cryptic diversity, biogeography, and potential evolutionary patterns of taxa within the Lithophylloideae by reconstructing the phylogenetic tree of this subfamily species on a global scale, integrating both biogeographical and evolutionary analyses. Their research revealed a highly robust phylogenetic framework for the Lithophylloideae, supporting the hypothesis of polyphyly in the *Lithophyllum* group and a separation of *Titanoderma*. Additionally, they demonstrated that *Lithophyllum*, in contrast to *Titanoderma*, exhibits an isolated geographical pattern.

This could result from the Tethyan Transequatorial Exchange (TTE) that has led to two distinct distribution regions: one along the European/Mediterranean coasts, potentially including the genus *Lithophyllum*, and the other, more widely distributed, comprising at least five distinct groups in the subfamily of Lithophylloideae. In these groups was not included the cryptic genus *Titanoderma* who exhibited different morphological features, differences in distribution and differences in the evolutionary pattern resulting in a separation of this genus from the others. This study has clarified the longstanding debate between the genera *Lithophyllum* and *Titanoderma*, demonstrating that they can be considered two distinct and independent groups.

At this aim an experiment was conducted in Sardinia (Italy, Western Mediterranean) where the response of CCA recruits to different light and temperature combinations was tested in a manipulative field experiment, so that algae adaptability to changes in thermal conditions was evaluated by separating the effects of light irradiance and temperature on the recruit growth. This study gained information on the biology of CCA algal recruits, predicting if future changes in temperature, and the deepening of thermocline, will affect their growth. Identification of CCA recruits, which was only possible a posteriori of the growth analysis, provide valuable insights on the specific species biology.

2. MATERIALS AND METHODS

2.1 Study site and experimental setup

The manipulative experiment was conducted in Costa Paradiso, North Sardinia (41°04'N, 08°57'E) (Fig. 1) from June 2023 to December 2023. This site is far from any anthropogenic sources of disturbance and is characterized by high oligotrophy (Barisiello et al. 2002; Regione Autonoma della Sardegna 2016). A total of thirty terracotta tiles 10x10 cm in size were positioned on the sea bottom from February to June 2023 so that CCA could recruit. Once recollected, all the tiles were photographed, and images were analyzed to estimate the cover of CCA. Based on the position they

were fixed on the substratum they had either a High (from 27.015 to 79.448 cm^2) or Low (from 0.483 to 25.595 cm^2) cover of CCA recruits (15 for each type).

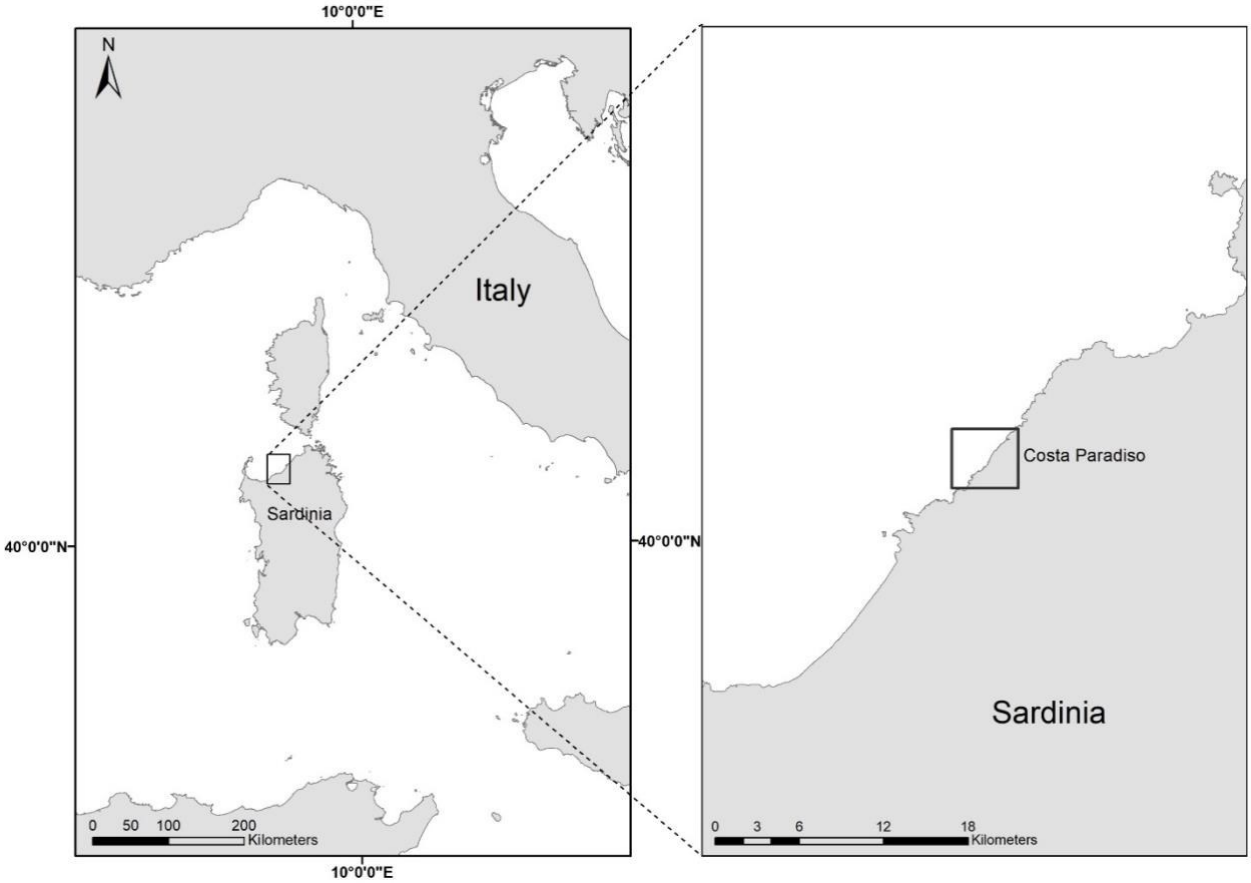


Figure. 1. Map of the study area.

After being photographed, tiles of each group (high and low CCA cover) were attributed in groups of five to three treatments (Fig. 2): a natural cavity within the bioconstruction at 15m of depth (LLHT: LowLight HighTemperature), outside the cavity at 15 m (HLHT: HighLight HighTemperature), and at 35m of depth (LLLT: LowLight LowTemperature), where the 15 m of depth were involved in the water stratification for most of the experimental time and light irradiance at 35 m was similar to the 15m inside the cavity.

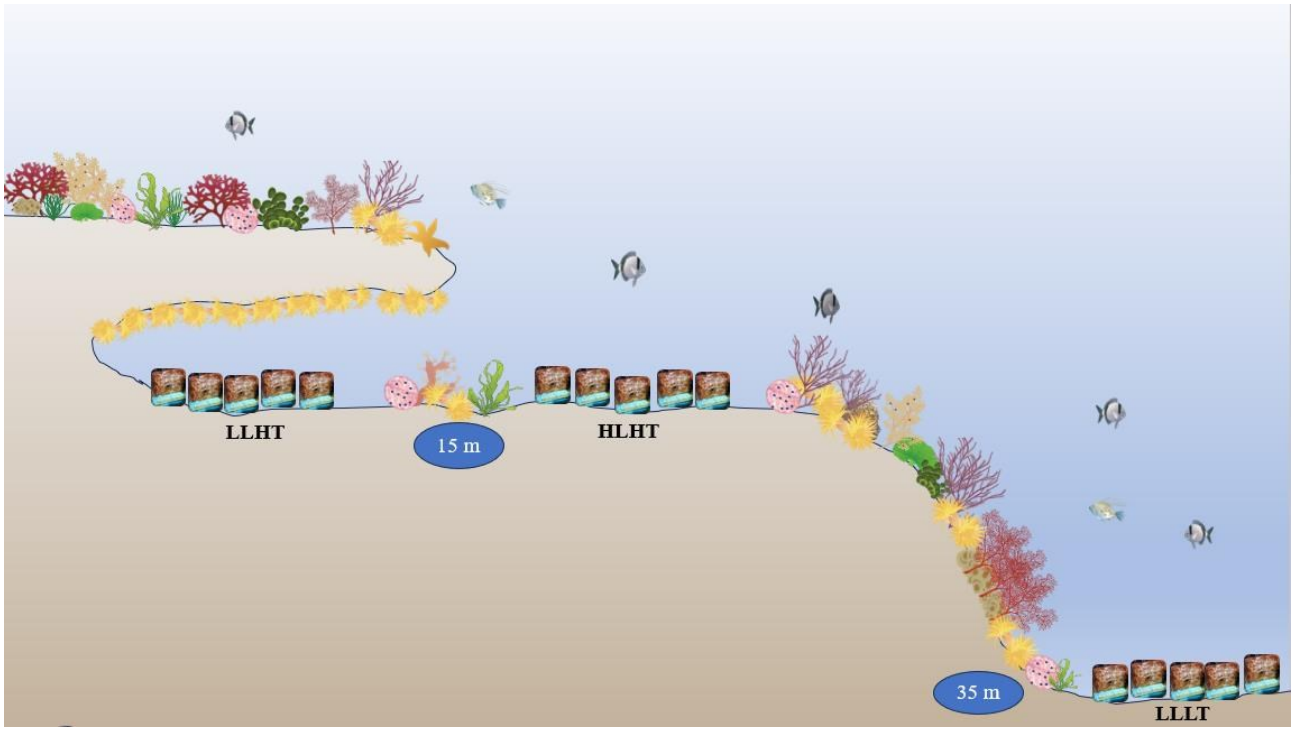


Figure. 2. Sampling design: two treatments at 15 m depth LLHT (LowLight HighTemperature) and HLHT (HighLight HighTemperature); one treatment at 35 m depth LLLT (LowLight LowTemperature).

Therefore, the comparison of the CCA recruit performance between LLHT and HLHT conditions will measure the effect of light at high temperature, while the comparison between LLHT and LLLT will measure the effect of temperature at low light condition, giving insights on the effects of thermocline deepening. In the field, tiles were fixed vertically using two-component epoxy underwater filler (Sub Coat Veneziani).

In all the three experimental conditions, two data loggers (HOBO Pendant Temp/Light, 64K UA-002-64, Onset Computer Corporation, USA) were fixed to record temperature (Fig. 3A) and irradiance (Fig. 3B) data during the whole experimental time.

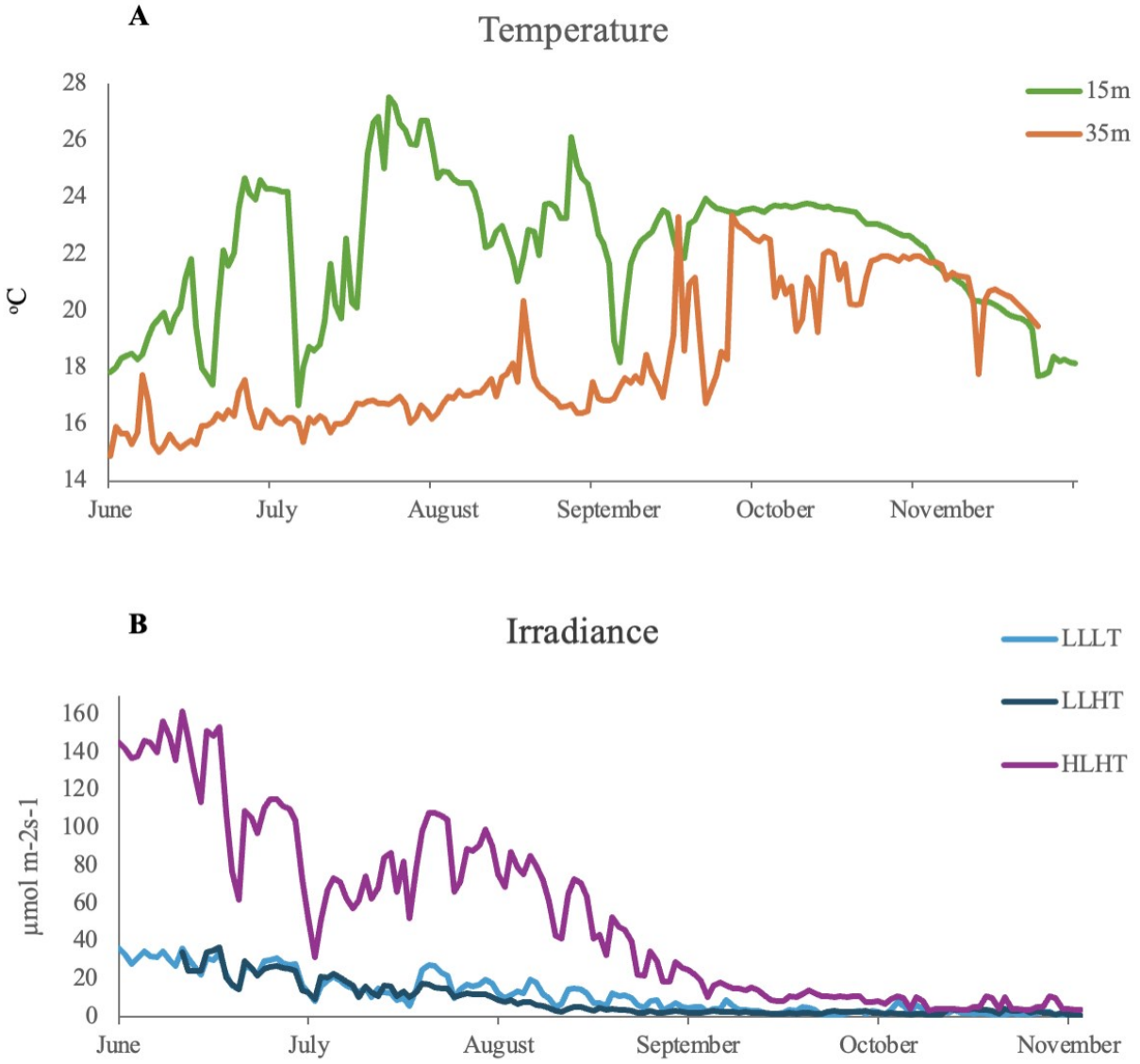


Figure 3. (A) Daily seawater temperature from June to November in the study area. 15 m depth in green and 35 m depth in orange; (B) Daily irradiance from June to November in the study area at each depth (15 and 35 m from loggers). LLLT in light blue, LLHT in blue and HLHT in purple.

At the end of the experimental period (6 months, from June to December 2023), all the tiles were recollected from the field and transported to the laboratory to be rephotographed. All the images taken before and at the end of the experimental conditions were analyzed using ImageJ Software to evaluate differences in CCA size due to treatments (Fig. 4). The CCA coverage between times was compared and the growth was evaluated (difference in the CCA coverage between June and December). Then, molecular and morphological analyses have been performed to identify CCA species.



Figure. 4. Comparison in High Initial Cover (Red) and Low Initial Cover (Blu) from June (Green) and December (Purple).

2.2 Data analysis

The growth of CCAs was evaluated through a two-way permutational analysis of variance (PERMANOVA, Anderson 2001) based on Euclidean distance using an orthogonal design where Initial coverage (High and Low) and Treatment (LLHT, HLHT, and LLLT) were considered as fixed crossed factors. A posteriori comparison of means was done using a Pair-Wise test. The analysis was performed with PRIMER v7 software.

2.3 DNA extraction, PCR amplification, and sequencing

The tiles with coralline algae used for DNA extraction were air-dried for 48 h at 40ºC (oven) and subsequently placed in bags with silica gel. DNA extractions were performed using material that appeared visually clean and devoid of contaminations. When the presence of epiphytes or other possible contaminants was observed, the material was carefully cleaned with a brush and tweezers. Fragments selected were ground to a fine powder using sterilized microdrill (Dremel 4000, Breda, Netherlands), tweezers and razor blade. DNA was extracted using a NucleoSpin Tissue XS Kit (MACHEREY-NAGEL, GmbH & Co, Düren, Germany) (Fig. 5).



Figure 5. Example of some steps of the DNA extraction process.

PCR amplification was performed on the plastid *psbA* gene using the primers psbA-F1 and psbA-R1 (Yoon et al. 2002). We performed all amplifications in a final volume of 10 µL, using the Supreme

NZYTaq II Colorless Master Mix (2X), from NZYTech, and the concentrations detailed as follows: DNA 1.0 µL, H_2Od 3.6 µL, Primer Forward (10 µM) and Reverse (10 µM) 0.2 µL each and 1 Supreme NZYTaq II Colourless M. Mix (2X). The PCR protocol for the amplification of *psbA* gene consisted of an initial denaturation at 95ºC for 4 min, and then 45 cycles at 95ºC for 30s, 52 for 40s, 72 for 50s and a final extension step at 72ºC for 5 minutes and storage at 10ºC. PCR products were visualized and quantified in 2% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA) and visualized under UV light. PCR product with expected lengths and yields were purified and custom-sequenced by Macrogen Europe (Amsterdam, The Netherlands).

2.4 Sequence alignments and phylogenetic analyses

Sequences were assembled ad aligned with the use of CodonCode Aligner (CodonCode Corporation, USA) and adjusted by eye using SeaView version 5 (Gouy et al. 2010). Alignments were assembled for *psb*A using ClustalW in SeaView with default settings and refined by eye. For the molecular analyses, sequences of species belonging to the genera *Phymatolithon* and *Mesophyllum* (*Hapalidiales* outgroup) and sequences of the genus *Titanoderma*, downloaded from the Genbank database, were included. The quality of the sequences was assessed by visual examination of the electopherograms in CodonCode Aligner and only high-quality sequences (i.e. lacking double peaks or other ambiguities that could make the readings questionable) were included in the phylogenetic analyses.

Phylogenetic analyses were performed on all data sets using p-distances in MEGA version 6 (Tamura et al. 2013), with nodal support assessed by 1000 bootstrap (BP) resampling. Phylogenetic inference by Maximum Likelihood (ML) was performed using MEGA Software. The GTR+G+I model was used for all reconstructions.

2.5 Morpho-anatomical analyses

For scanning electron microscopy (SEM), the air-dried material was fractured using tweezers and razor blade. The pieces were placed both perpendicularly and horizontally, to analyze their internal anatomy and surface respectively, on stubs, using Silver Conductive Paint 503 (Electron Microscopy Sciences), coated with gold for 30 minutes in an Edwards S150B sputter coater and examined with a JEOL JSM 6400 scanning electron microscope with an accelerating voltage of 25 kV (Fig. 6).

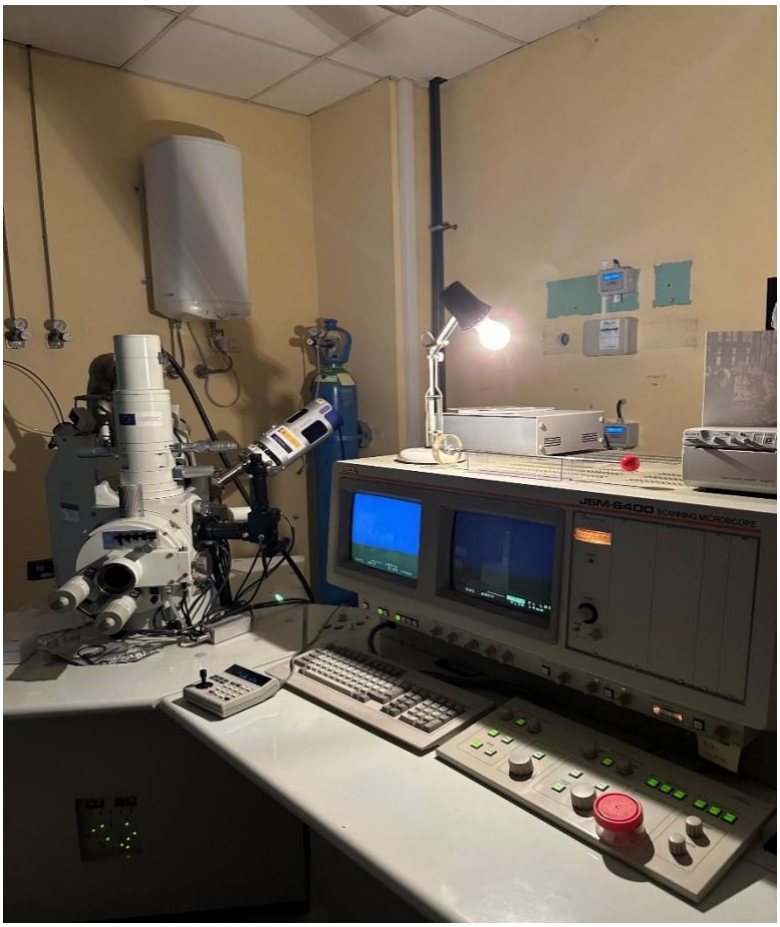


Figure 6. Scanning Electron Microscope (SEM) used for the morpho-anatomical analysis.

3. RESULTS

Overall, the growth of CCA recruits varied from 4.941 to 35.476 cm^2/six months per tile. Significant differences in the change between June and December in CCA recruit coverage was observed depending on the interaction between the two factors, Initial coverage and Treatment (Fig. 7, Table 1). Specifically, in the LLHT (LowLight HighTemperature) and HLHT (HighLight HighTemperature) conditions where the initial CCA coverage was higher, a higher increased was observed, while in the LLLT (LowLight LowTemperature) treatment the change in CCA coverage did not depend on the initial coverage. Another significant difference was found between treatments since in LLHT and HLHT a greater growth of CCA recruits was found compared to LLLT, but only for the high initial coverage tiles. In fact, for the low initial coverage tiles, no difference was observed between treatments (Figure 7, Table 1).

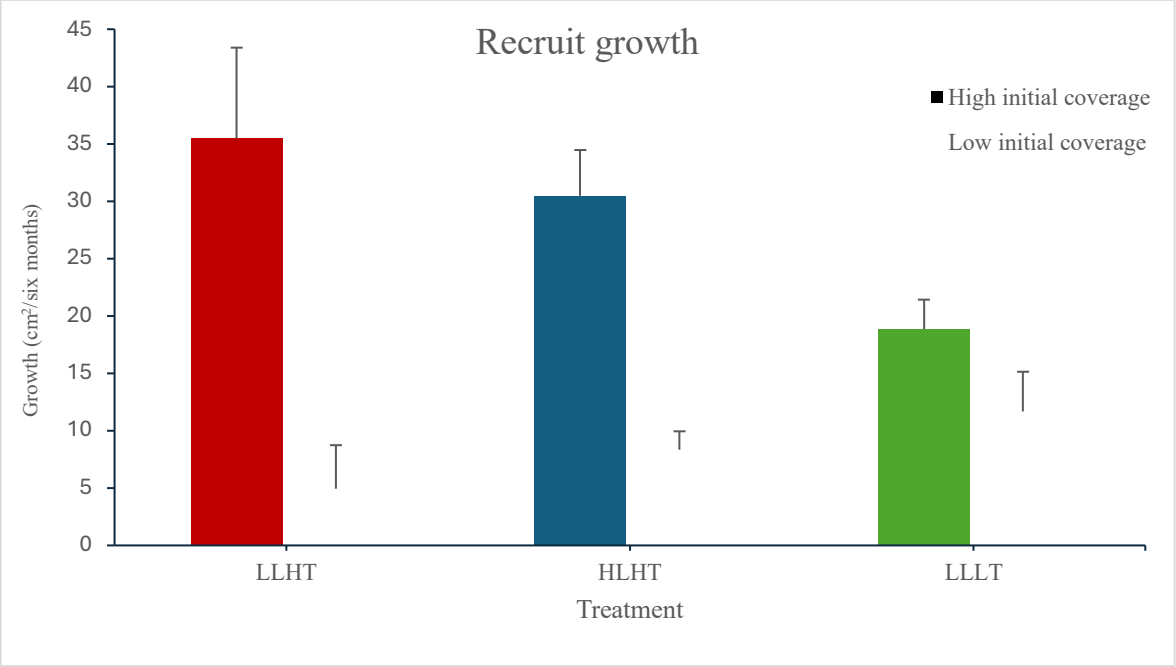


Figure 7. Average (+SE) growth of CCA recruits among the treatments LLHT (LowLight HighTemperature), HLHT (HighLight HighTemperature), LLLT (LowLight LowTemperature), depending on the initial coverage (High or Low).

Table 1. Encrusting coralline algae growth: results of PERMANOVA testing the effect of Initial coverage (High vs Low) and Treatments (LLHT, HLHT, LLLT). In bold the significant results.

Source of variation	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Initial Coverage =IC	1	46.886	45.782	**0.001**	9998
Treatment =Tr	2	0.396	0.386	0.677	9998
ICxTr	2	5.292	5.167	**0.011**	9998
Residual	22	1.024			

Pair-Wise test

Treatment	*Initial Coverage*
LLHT	H > L
HLHT	H > L
LLLT	H = L

Initial Coverage	*Treatment*
High	LLHT = HLHT
	LLHT > LLLT
	HLHT > LLLT
Low	LLHT = HLHT
	LLHT = LLLT
	HLHT = LLLT

3.1 Molecular and morphological results

Molecular analyses revealed the presence of individuals belonging exclusively to the genus *Titanoderma*, and specifically to five species, in all experimental conditions, as shown in Fig. 8. This genus is poorly studied in the Mediterranean Sea, and the absence of *psbA* gene sequences in GenBank prevents the identification of the full species names.

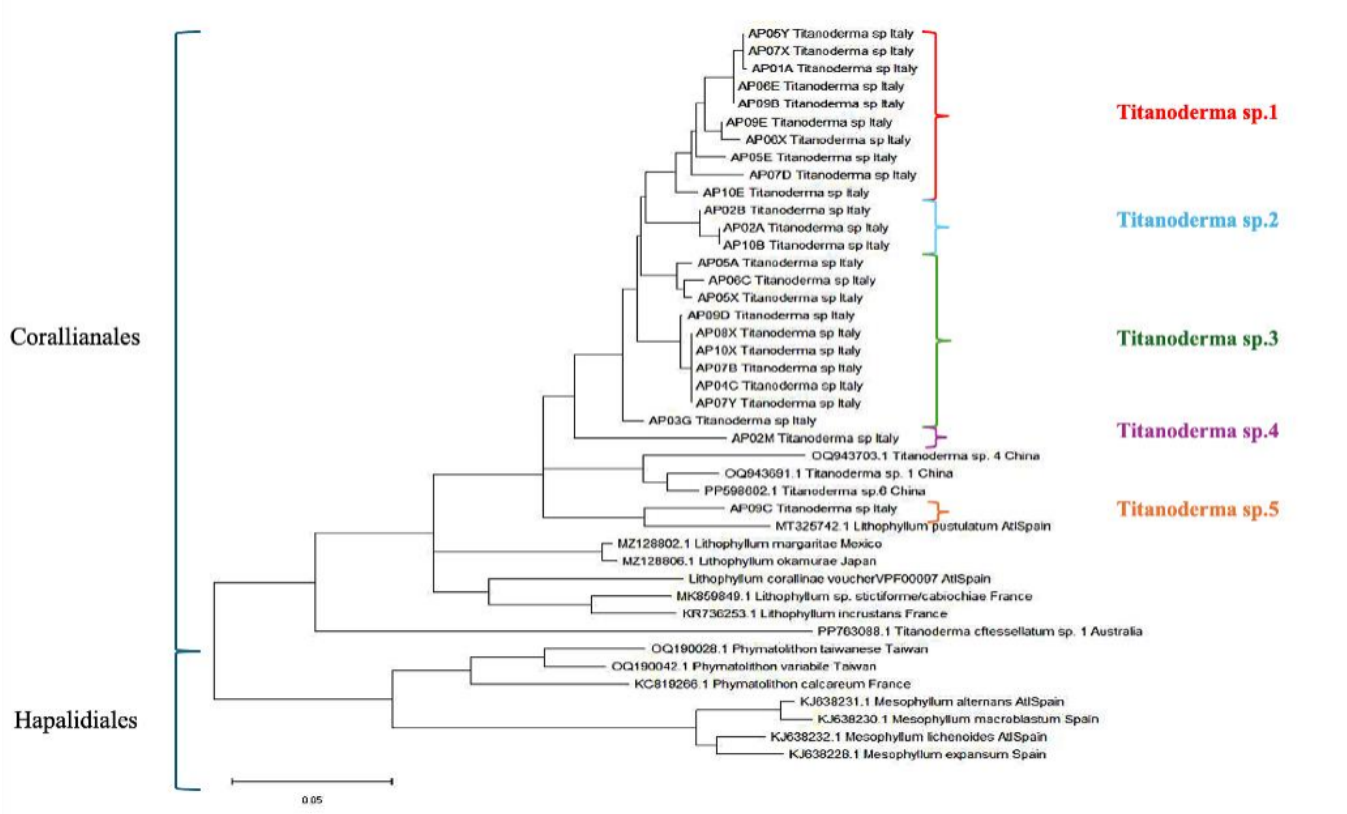


Figure 8. Phylogram inferred from Maxim Likelihood (ML) analysis of *psbA* data set of *Titanoderma*.

These results were subsequently confirmed by SEM morphological analyses (Fig. 9), where characteristic structures of the order Corallinales can be identified, such as the presence of secondary pit connections (Fig. 9c), and in particularly of the genus *Titanoderma*, with the presence of palisade cells (Fig. 9d).

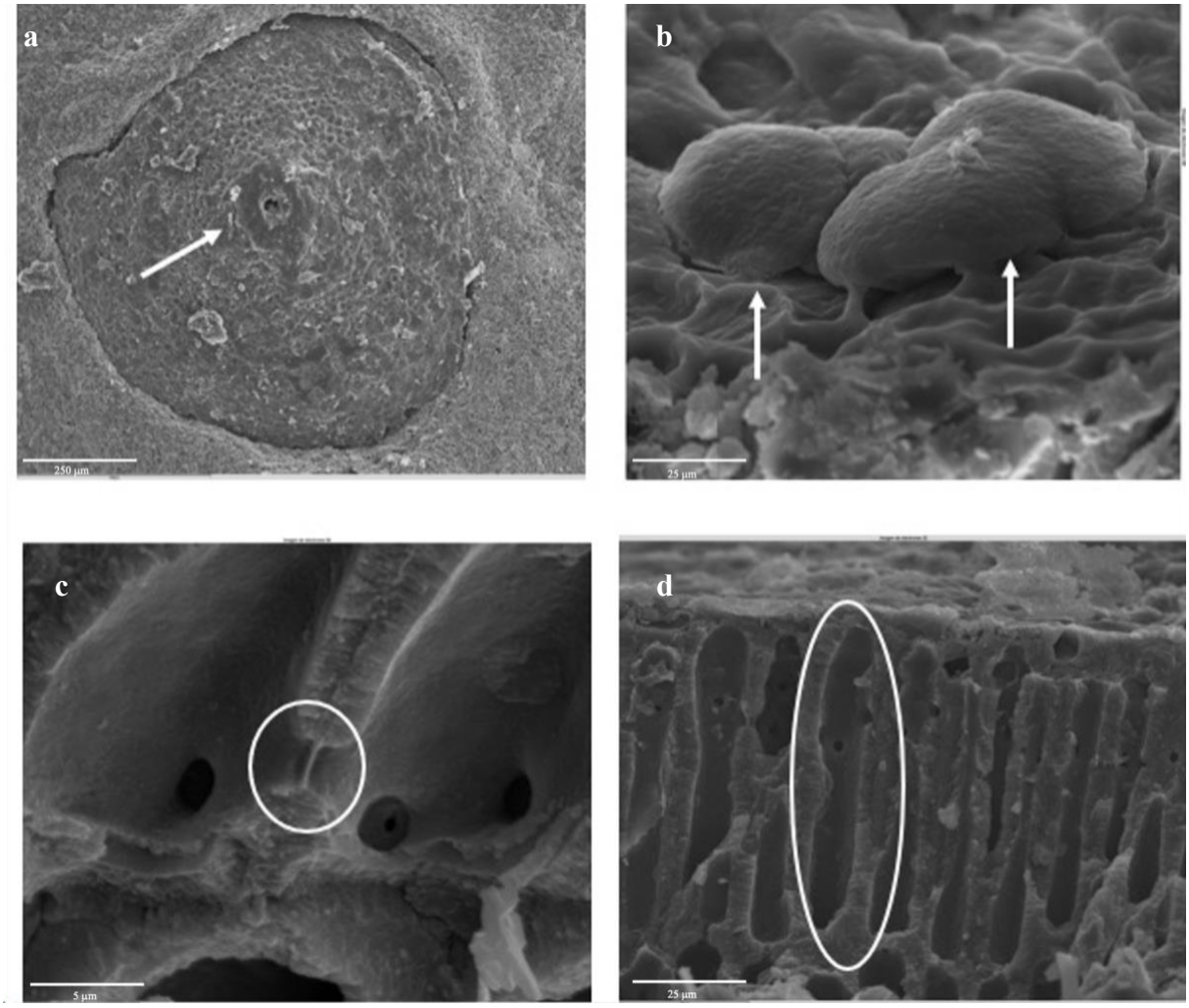


Figure 9. Scanning Electron Microscope (SEM) images. Reproductive (a-b) and vegetative (b-c) anatomy of *Titanoderma* sp. (A) Uniporate conceptacle. (B) Carpospores. (C) Secondary pit connections. (D) Palisade like-cells.

4. DISCUSSION

This study, for the first time, provides important information about the growth of CCA recruits under different environmental condition: particularly, at the light of the species identification that was done a posteriori, in this study we tested the adaptability of *Titanoderma* sp. recruits to different field conditions of temperature and light. The effects of conditions were only appreciable in tile where the initial recruit cover was high. For this treatment, the results showed that an increase in temperature can promote growth at low light intensity, with *Titanoderma* recruits under the LLHT treatment exhibiting greater growth compared to those under the LLLT treatment. This contrasts with the study by Rodriguez-Prieto (2016) gained for *Lithophyllum stictiforme* which found that, in adult individuals, light was the limiting factor for growth compared to temperature. It also contradicts an additional observation in our study, where no significant differences were observed between LLHT and HLHT recruits, thereby excluding a light effect at high temperature condition. However, in such literature comparison, it is important to consider first, that laboratory studies (as the Rodriguez-Prieto 2016) do not account for the natural variability that instead is observed in field studies (such as this), and second, that the CCA species considered in these studies are different. So, if the comparison with Rodriguez-Prieto (2016) results may seem inappropriate, it is important to consider that to date, no field studies exist on *Titanoderma* recruits except for Fong et al. (2024) who studied the interactive effects of temperature and light on recruits of a tropical species of *Titanoderma* growth and found that best condition was under low levels of temperature and high levels of light. Therefore, the inconsistency between the two studies may be interpreted at the light of the intensity treatment and species identification: in Fong et al. (2024) the low-temperature condition was 27.5°C (and the high temperature of 30°C), which is instead comparable to our average high-temperature condition and the two studies both deal with *Titanoderma* but likely different species, one being tropical and the other temperate with distinct adaptations to temperature ranges.

Then, some evaluations may be done considering other CCA species of the study site, even if at an adult stage. Pinna et al. (2022) reported that adult CCA thalli of *Lithophyllum stictiforme* exhibited greater growth in areas where water temperature was lower. This finding contrasts with the results observed in recruits, as our study highlighted significant higher growth under LLHT conditions compared with those under LLLT. This is an important observation for all coralline algae species living in deep subtidal environments, particularly those living below the thermocline. It can be hypothesized that, in the event of a thermocline deepening (Hoegh-Guldberg and Bruno 2010) due to future climate change effects, *Titanoderma* recruits may exhibit a positive response with increased size and faster growth.

Finally, to complete ecological and physiological studies on CCA, it is crucial to validate the results with genetic analyses that allow for the correct identification of the species involved in the study. In our study, the molecular and morphological analyses revealed that the only one species that grew on our artificial substrates was a CCA belonging to the genus *Titanoderma* and no other CCAs were found. From the literature emerges an ongoing debate regarding the characterization and description of this genus in comparison to other CCA: for example, the different evolutionary patterns observed for the genera *Titanoderma* and *Lithophyllum* (Yan et al. 2024) may reflect their ecological differences, and while *Lithophyllum* tends to be epilithic, *Titanoderma* exhibits an epiphytic tendency. Such divergence from these two genera could by their evolutionary history. The oldest fossil record of *Lithophyllum* species dates to the early Miocene (Aguirre et al. 2010; Caragnano et al. 2020), when the Mid-Miocene Tethyan Transequatorial Exchange (TTE) occurred, leading to a separation into two distinct groups due to geographical isolation. Based on the oldest *Titanoderma* fossil record found in the central Pacific Ocean, *Titanoderma* origin dates back from the Oligocene; other fossil records from the late Miocene were discovered in all oceans across low to mid latitudes, suggesting that *Titanoderma* was capable of rapidly growing on various substrates, allowing the genus to expand globally (Basso et al. 2009). This could explain the presence of individuals belonging to the genus

Titanoderma on the artificial substrates used in this study rather than those of *Lithophyllum,* although this latter species seems much more abundant at the study site.

At the biological species level, future research should develop our findings by testing the two conditions (light and temperature) effects on algal performance across environmental gradients, possibly measuring also metabolic responses by using respiration chambers equipped with sensors for dissolved oxygen and temperature. However, the overall most important ecological issue to address is the role of *Titanoderma* species in the coralligenous reefs that should be investigated with several manipulative field experiments with the aim of identifying the interactions with the other CCA species and the role with the invertebrate community. The information collected here represents an important information base to be developed.

Chapter 5

CIVITAVECCHIA (CENTER WESTERN ITALY) PORT ENLARGEMENT AND COMPENSATION MEASURES: THE SPATIAL VARIABILITY OF CORALLIGENOUS REEFS AND LOCAL BYCATCH MATERIAL TO IMPROVE HABITAT QUALITY

Puccini A.[1]*, Scagnoli E. [2], Stipcich P. [3,4], Pinna F. [5], Piazzi L. [6], Ceccherelli G.[7]

[1] *Department of Architecture, Design and Urban Planning, University of Sassari, via Piandanna 4, 07100 Sassari, Italia*

[2] *Laboratory of Experimental Oceanology and Marine Ecology, Department of Ecological and Biological sciences DEB, University of Tuscia, Port of Civitavecchia, Civitavecchia (RM), Italy*

[3] *Department of Biology, University of Naples Federico II, Naples, Italy*

[4] *National Biodiversity Future Centre, Palermo, Italy*

[5] *Departement of Integrative Marine Ecology, Genoa Marine Centre, Stazione Zoologica Anton Dohrn, Genoa, Italy*

[6] *Consorzio per il Centro Interuniversitario di Biologia Marina "G. Bacci". V.le Nazario Sauro4, Livorno, Italia*

[7] *Department of Chemical, Physical, Mathematical and Natural Sciences, University of Sassari, via Piandanna 4, 07100 Sassari, Italia*

*e-mail: alessandrapuccini4@gmail.com

ABSTRACT

The coralligenous habitat is considered a biodiversity hotspot of fundamental importance in the Mediterranean Sea. Like much of the marine ecosystem, this habitat is also subject to the impact of man and his activities. Developing infrastructures usually leads to a loss of biodiversity and it is of fundamental importance to identify compensation measures to mitigate the unavoidable damage. This work presents the preliminary results of a study carried out to mitigate the impact of the enlargement of the port of Civitavecchia (Lazio, Italy) throughout the coastal zone of the area. At this aim, six study sites were selected and based on their location, they were divided respectively into control sites and sites impacted by the works. Photographic sampling was carried out at about 25m of depth on these sites (20 photos per site) in order to evaluate the structure of the coralligenous community present there and to calculate the ecological state of the environment through the use of the ESCA index. In addition, organisms from the bycatch of small-scale local commercial fishing activities were collected to estimate the quantity and species present with the aim of using these organisms to restore or increase the habitat quality in the degraded sites. The results showed that in three of the sites analyzed there was a "high" level of quality, in two there was a "good" level of quality and only in one was the level "moderate". Finally, it emerged that the most collected organisms from bycatch belonged to the species *E. cavolini* and *Cladocora caespitosa*. This approach of study can be useful for habitat restoration ecologists to plan compensation strategies and plan habitat requalification operas.

Keywords: coralligeenous reefs, bycatch, restoration, spatial variability

1. INTRODUCTION

Coralligenous habitat (1170, ISPRA 2020) is one of the most important benthic ecosystems in the Mediterranean Sea. Its conservation represents an important challenge for the management of coastal marine systems (Ballesteros 2006). Coralligenous cliffs are a biodiversity hot-spot, due to carbonate deposition by encrusting coralline algae (for example *Lithophyllum spp., Lithothamnium spp.* and *Mesophyllum spp.*) and several invertebrates, mainly Cnidaria, Polychaeta, Porifera and Bryozoa (Ballesteros 2006; Martin et al. 2014). Coralligenous reefs (Fig. 1) have been included in "special habitats" to be assessed and protected under the European Marine Strategy Framework Directive (EC, 2008) for the ecological services they provide for fisheries and the CO_2 balance (Thierry de Ville d'Avray et al. 2019).

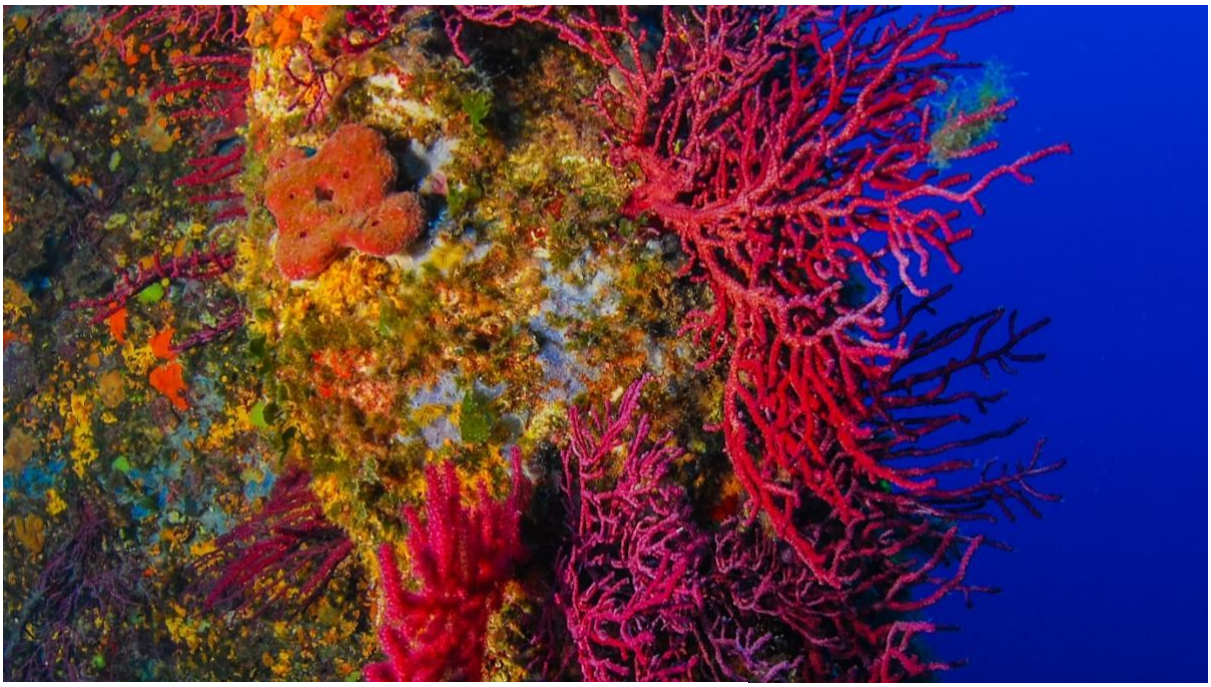


Figure 1. Example of coralligenous cliff with gorgonians.

Due to vulnerability of this habitat to local anthropogenic stressors, as sediment deposition (Balata et al. 2005), eutrophication due to wastewater discharge or aquaculture (Piazzi et al. 2011, 2019a), direct habitat destruction due to fishing, anchoring and coastal construction activities (Ferrigno et al. 2018;

Betti et al. 2020), assessing the ecological status of coralligenous through visual census techniques is commonly needed (Piazzi et al. 2021a). The scenario on the conservation status of coralligenous assemblages and on the assessment methods is complicated by factors acting on larger scale such as those related to climate change. For example, it is known that heatwaves affect the coralligenous reducing habitat structure and eroding biodiversity (Garrabou et al. 2009; Verdura et al. 2019) as evidenced by widespread mortality events involving numerous species during heatwaves (Garrabou et al. 2019). In general, coralligenous is described as degraded without gorgonians (Fig. 2) and with a low cover of erect briozoa and massive sponges, but with a high algal cover (Ponti at al. 2014; Verdura et al. 2019).

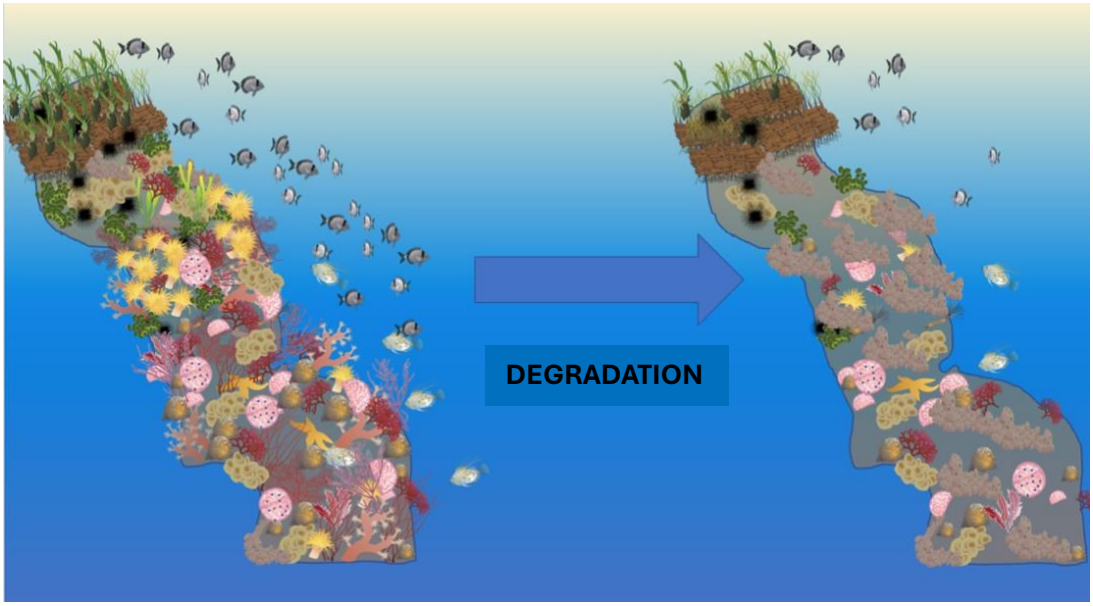


Figure 2. Representation of coralligenous population with gorgonians (left) and after the degradation without gorgonians and with lower taxa richness (right).

Spatial variability of coralligenous assemblages have been widely studied (Piazzi et al. 2004, 2016; Virgilio et al. 2006; Ponti et al. 2011; Casas-Guell et al. 2015, 2016) and recently the influence of temperature on community changes have been evaluated (Ceccherelli et al. 2020). In addition,

differences in the structure of this habitat, have been assessed between distant biogeographical regions (Cinar et al. 2020) whereby it's known that coralligenous cliffs with no gorgonians are not necessarily the result of habitat degradation (i.e., Sardinian west coasts, Piazzi et al. 2021b).

Bycatch refers to the incidental capture of non-target species as fish, invertebrates, seabirds, marine mammals, turtles, but also algae during commercial fishing operations (Davies et al. 2009). This incidental capture is a significant problem in global fisheries because it can lead to unsustainable harvest of non-target species, damaging marine ecosystems and leading to the decline of vulnerable species. Bycatch can also be distinguished in "unused" and "unmanaged" (Davies et al. 2009) where "unused" catch is the one which is not used for consumption, sold or reuse as a bait and it includes discards and wasted catch, while "unmanaged" catch represents individual species or groups of different species that don't have a specific management to ensure that harvesting is sustainable, in accordance with the FAO Code of Conduct for Responsible Fishing (Boopendranath 2012). Bycatch has direct and indirect effects as biodiversity loss, decline in populations of non-target species, ecosystem disruption due to the removal of organisms that can alter the structure and function of the ecosystem with potential economic costs.

Recent studies have illustrated the complexity of the bycatch and discard problem in fishing. Lively and McKenzie (2023), within their review, consider the global problem of discards and bycatch, focusing on how increasing the use of more selective gear and regulations has reduced discards, but also on the challenge of collecting consistent data on discard rates and the species involved. Also, Squires et al. (2021), introduce new approaches for solving the problem of bycatch through multidisciplinary approaches. This study analyses solutions to merge new technologies, management policies and international cooperation, aiming to reduce the ecological impacts and promote more sustainable fishing. The authors highlight that the solution of this complex problem requires the integration of scientific, economic and social knowledge in order to intensify the sustainable management of fishery resources.

The aim of this study is to identify useful sites around Civitavecchia (western central Italy) where to transplant bycatch material (algae and invertebrates) and thus partially compensating the damage that will be provoked by the port enlargement. To reach this goal it is first crucial to gain estimates of the spatial variability of coralligenous reefs in the coastline near Civitavecchia and of the ecological quality of the habitat, allowing the identification of receiving sites of the available bycatch organisms. The second aim is to estimate the type (species and status) and the quantity of bycatch collected by Civitavecchia fishermen to be able to plan the transplants to the receiving sites.

2. MATERIALS AND METHODS

2.1 Study area



Figure 3. The six sites selected near Civitavecchia port.

This study was conducted along the coasts of Civitavecchia where six different sites have been selected: Murata, Frasca, Scoglio del Corallo, Pecoraro, Marangone and Mano Aperta (Fig. 3). In

each site two areas (A1 and A2) tens of meters apart have been selected. These sites were selected after surveys of the area using ROV and SSS (Side Scan Sonar) instruments to evaluate their conservation status.

In October 2022 in each area, at a depth between 18 and 27 m, 20 images of coralligenous assemblages on vertical walls were collected randomly using a camera connected with a 40x50 cm frame (Fig. 4, Piazzi et al. 2019b).

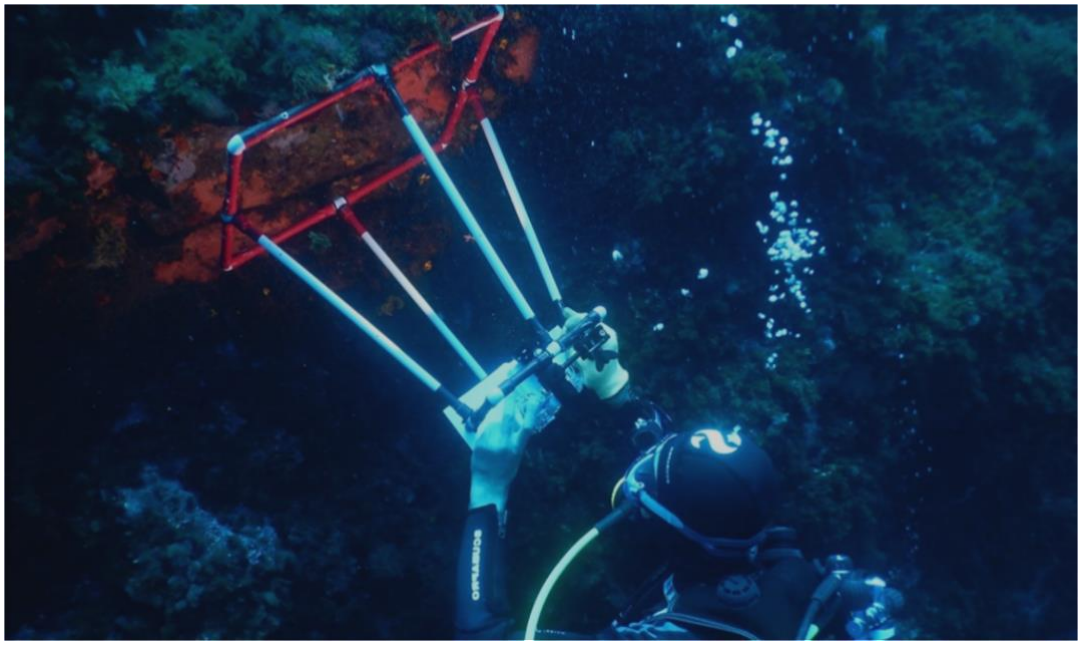


Figure 4. Photographic sampling on coralligenous cliff.

In laboratory, the 240 images collected were analyzed. The organisms easily identifiable in the photographic samples were considered as taxa (species identification was not always possible), while those who showed similar morphological features were clustered in morphological groups (Piazzi et al. 2017). Percent cover of taxa/morphological groups have been evaluated by manual contouring technique using ImageJ software (Cecchi et al. 2014). Percent cover of sediment was also calculated for each image.

2.2 Statistical Analysis

Assemblage diversity was evaluated as number of taxa/morphological groups through a permutational analysis of variance (PRIMER 7+ PERMANOVA, Anderson 2001) based of Euclidean distance. A two-way model was used with Site (six levels) as fixed factor and Area (two levels) as random factor nested in Site. Spatial differences in the assemblage structure (presence and abundance of taxa/groups) were analyzed by PERMANOVA based on similarity matrix (Bray-Curtis index) of fourth-root transformed data. Similarities were shown through an nMDS and the Pair-Wise test was used to identify similarity between sites. SIMPER was used to find which taxa/groups contribute to differences between sites.

2.3 ESCA Index Calculation

Coralligenous habitat quality was assessed using ESCA index (Ecological Status of Coralligenous Assemblages, Piazzi et al. 2021a). According to Water Frame Directive (EC, 2000), for ESCA index five ecological classes were defined based on different Ecological Quality Ratio (EQR') value ranges: 1.0-0.81 High; 0.80-0.66 Good; 0.65-0.51 Moderate; 0.50-0.36 Poor; 0.35-0 Bad. EQR' value comes from the average of EQR_{SL}, EQR_{α}, EQR_{β}. EQR_{SL} is obtained adding all SL values (Sensitivity Level) for each taxa/group; EQR_{α} represents the number of taxa/morphological groups per sample, and EQR_{β} represents the heterogeneity of population calculated with PERMIDISP. At the aim of calculating the ESCA index, for each site 3 plots, with 10 photos each, were considered. Data resulting from the image analysis of photos have been included in the calculation of the index.

2.2 Civitavecchia bycatch

To estimate the species and quantity of the bycatch a total of twenty-two recovery campaigns were carried out from February to August, fifteen in the Port of Civitavecchia and seven in the port of Santa Marinella. The operations mainly focused on small-scale local fishing, particularly lobster fishing. In these operations, gillnets were deployed at depths ranging from 15 to 50 meters, depending on the

fishing area. The recovered species will then be transplanted into two different sites where restoration will be carried out (Frasca and Mano Aperta).

3. RESULTS

3.1 Coralligenous reefs variability

Overall were found 30 taxa/morphological groups (Tab. 1)

Table 1. Mean percentage coverage ± SD of taxa/morphological groups found at the six sites investigated.

Taxa/morphological groups	Murata (%)	Mano Aperta (%)	Frasca (%)	Pecoraro (%)	Marangone (%)	Scoglio del corallo (%)
Algal turf	19.05±0.28	2.53±0.03	3.83±0.06	4.38±0.20	3.23±0.22	2.43±0.03
Codium spp.	0	0.16±0.01	0	0	0	0
Corallium rubrum	0	0	0	0	0	3.15±0.10
Dyctiotales	0	0	0.01±0.00	0	0	0
Encrusting briozoans	0.21±0.01	0	0.001±0.00	0	0	0
Encrusting corallinales	32.18±0.27	14.95±0.43	3.43±0.07	18.94±0.72	12.44±0.76	11.29±0.16
Encrusting Ochrophyta	0.06±0.00	3.78±0.09	4.63±0.09	0.71±0.10	2.12±0.24	5.37±0.11
Encrusting sponges	4.31±0.11	0.46±0.03	0.81±0.04	0.02±0.006	0	0.53±0.01
Erect ascidians	0.02±0.00	0.01±0.00	0.07±0.00	0.02±0.006	0	0.07±0.004
Erect briozoans	0.22±0.01	0	0	0	0	0
Erect Ochrophyta	0.42±0.03	0	0	0	0	0
Erect Rhodophyta	0.0008±0.00	0.45±0.05	0.05±0.01	0	0	0
Eunicella cavolini	4.22±0.14	0.84±0.05	0.14±0.01	0.5±0.09	0	0.25±0.02
Flabellia petiolata	0.02±0.00	0.02±0.00	0.009±0.00	0	0	0
Hydrozoa	1.77±0.06	0	0	0	0	0
Large serpulids	0.01±0.00	0	0	0	0	0

Leptogorgia sarmentosa	0.74±0.07	0	0	0	0	0
Massive sponges	0.15±0.02	0.06±0.01	0	0	0	0.12±0.01
Myriapora truncata	0.008±0.00	0.02±0.00	0.002±0.00	0	0	0.002±0.0004
Palmophyllum crassum	0	0	0.04±0.00	0	0	0
Paramuricea clavata	4.46±0.35	0	0	0	0	0
Parazoanthus axinellae	0.75±0.04	0.43±0.02	1.06±0.03	0.01±0.003	0.28±0.06	2.88±0.07
Peyssonnelia sp.	11.56±0.21	10.67±0.18	21.1±0.27	23.35±1.53	8.88±0.62	11.02±0.17
Prostrate sponges	0.46±0.02	0.31±0.01	0.22±0.01	0.14±0.02	0.21±0.03	0.96±0.02
Pseudoclorodesmis furcellata	0.32±0.01	0	0	0	0	0
Reteporella grimaldii	0.03±0.00	0.029±0.00	0.16±0.01	0.005±0.001	0.01±0.003	0.003±0.0005
Salmacina-Filograna complex	0.13±0.02	0	0	0	0	0
Scleractinie Azooxantellate individuali	0.006±0.00	0	0	0	0	0
Scleractinie Zooxantellate	0.27±0.01	0.47±0.05	0.16±0.02	0.54±0.07	1.70±0.24	0.85±0.05
Valonia spp.	0.002±0.00	0	0	0	0	0

The PERMANOVA analysis on the number of taxa/morphological groups per sample has identified a significative difference between sites (Tab. 2 and Fig. 5).

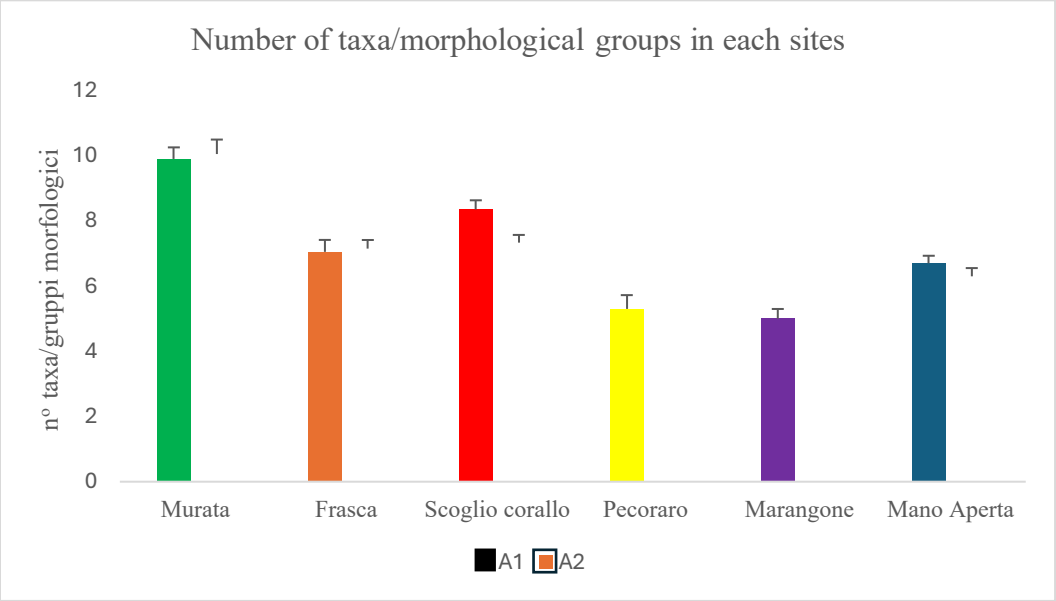


Figure 5. Mean (+SE) of taxa/morphological groups per sample in the areas (A1 and A2) investigated in each site.

Table 2. PERMANOVA results on the number of taxa/morphological groups per sample. In bold the significant values.

Source of variation	df	MS	Pseudo F	P
Site	5	3250.3	27.403	**0.011**
Area (S)	4	126.9	1.552	0.171
Residual	168	82.0		

A special focus should be made on the presence of sediment on the substrate: in all sites, except for Murata, on average more than 40% of the substrate is covered with sediment, although there is a considerable difference between areas of the same site (Fig. 7 and Tab. 3).

Table 3. PERMANOVA results on the amount of sediment. In bold the significant values.

Source of variation	df	MS	Pseudo F	P
Site	5	24226	41.041	**0.034**
Area (S)	4	651	2.021	**0.025**
Residual	168	321		

Overall, the assemblage resulted homogeneous at the site and area scale (Tab. 4): a significative difference was found between sites, where Murata was the site that deviated the most from the others due to the lower amount of sediment and the higher cover of encrusting coralline algae (Tab. 5 and Fig. 6). However, differences were found also in the higher abundance of *Eunicella* sp., almost absent in the other sites, and in the presence of *Leptogorgia sarmentosa* and *Paramuricea clavata* totally absent in the other sites.

Table 4. PERMANOVA results on the coralligenous assemblage structure. In bold the significant values.

Source of variation	df	MS	Pseudo F	P
Site	5	21922	8.742	**0.015**
Area (S)	4	2988	7.461	**0.001**
Residual	168	400		

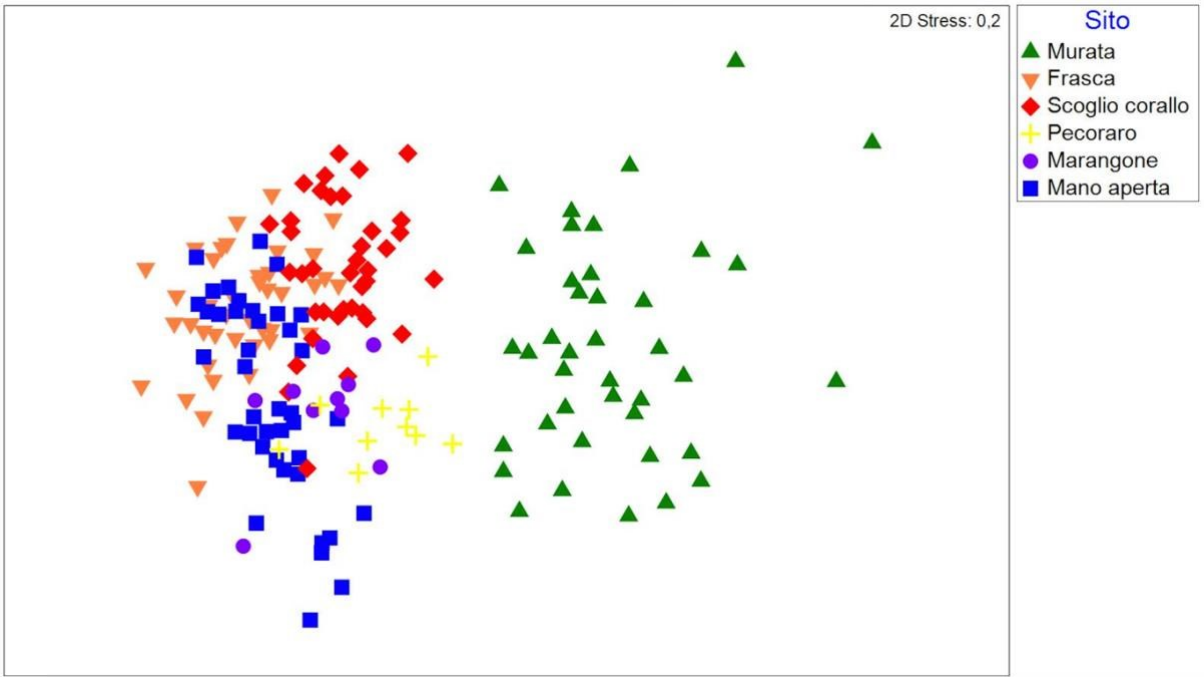


Figure 6. nMDS of the coralligenous assemblage where each centroid represents a sample.

Table 5. SIMPER analysis results on the coralligenous assemblage structure between sites.

Species	Contribution %	SD	Cumulative contribution %
Murata vs. Frasca			
Sediment	46.891	2.991	46.891
Encrusting corallinales	29.814	2.841	76.692
Peyssonnelia sp.	13.145	1.286	89.836
Paramuricea clavate	4.342	0.324	94.174
Murata vs. Mano Aperta			
Sediment	52.715	2.766	52.715
Encrusting corallinales	26.853	2.0686	79.563
Peyssonnelia sp.	9.309	1.208	88.863
Paramuricea clavata	4.760	0.329	93.637

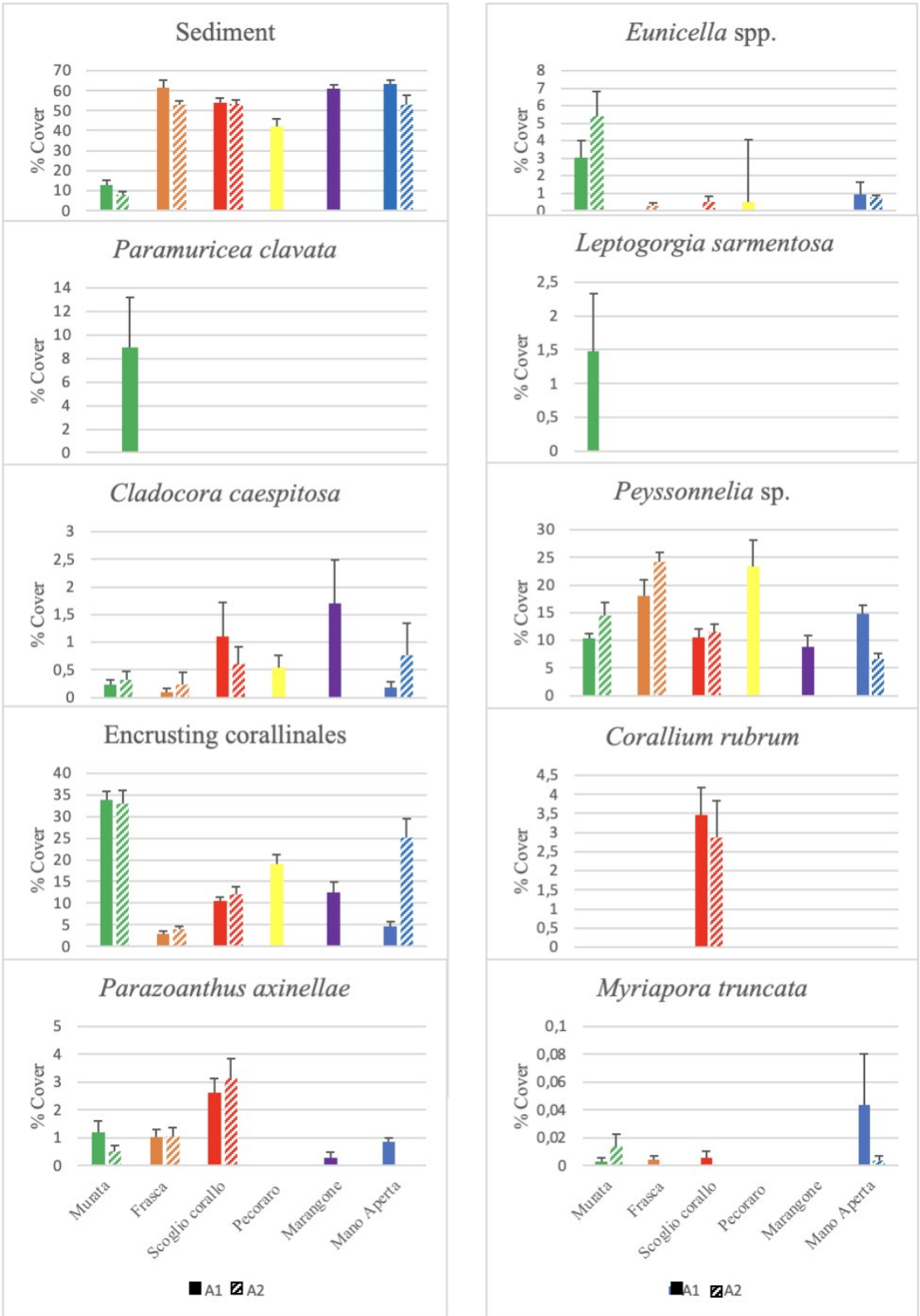


Figure 7. Percent cover (+SE) of taxa/morphological groups more relevant in the six sites investigated and in the areas for each site (A1 and A2). Top left the sediment cover.

The site named Scoglio del Corallo is the only one where specimens of *Corallium rubrum* were found, with a higher abundance of *Cladocora ceaspitosa* and *Parazoanthus axinellae* compared to the other sites (Fig. 7).

The analysis of habitat quality, done by using ESCA index, has assessed that differences in the community structure between sites are also in the quality level (Tab. 6): three sites (Murata, Mano Aperta and Scoglio del Corallo) were of high quality with an EQR' > 0.8; two sites (Frasca and Marangone) were of good quality with and EQR' between 0.6 and 0.8; only one site, Pecoraro, was of moderate quality with an EQR' between 0.6 and 0.4.

Table 6. Results of ESCA index calculation in the six sites investigated.

Sito	EQR_{SL}	EQR_{α}	EQR_{β}	EQR'		
Murata	0,78	0,67	1,30	0,92	EQR ≥ 0.8	High
Frasca	0,54	0,49	1,01	0,68	0.6 ≤ EQR< 0.8	Good
Mano Aperta	0,57	0,43	1,68	0,90	0.4 ≤ EQR< 0.6	Moderate
Scoglio del corallo	0,65	0,54	1,37	0,85	0.2≤ EQR< 0.4	Poor
Marangone	0,35	0,33	1,25	0,64	EQR < 0.2	Bad
Pecoraro	0,37	0,34	0,83	0,52		

3.2 Civitavecchia Bycatch

A variety of species were recovered live, including erect coralligenous organisms such as *Eunicella cavolini*, *Paramuricea clavata,* sponges and bryozoans (Fig. 8). These species are particularly important for their role in increasing spatial complexity and supporting biodiversity within the coralligenous habitats. The organisms detected in greater quantities were *Eunicella cavolinii* Koch and *Cladocora caespitosa* (Linnaeus 1758). *E. cavolinii* (Fig.9) is a Mediterranean yellow gorgonian that grows on rocky bottoms usually between 10 and 150 metres of depth, is an endemic species and often lives associated with *Paramuricea clavata*. It forms arborescent colonies, with the coenchyma ranging from light yellow to light orange and the polyps are generally transparent yellow, which

usually reach 40–50 cm in height and are arranged perpendicular to the current, with soft and flexible branches. *Cladocora caespitosa* (Fig.10), is the most important bioconstructor coral endemic of the Mediterranean Sea. It is a species of madrepore which, although often forming isolated colonies, in some conditions can create aggregations. This species lives in a symbiotic relationship with the zooxanthellae of the *Symbiodinium* genus. These unicellular algae live inside madrepore polyps, providing energy through photosynthesis and contributing to colony growth. The coral, in turn, deposits calcium carbonate, forming the limestone cases that make up the structure of the colony. With a diameter that can reach 50 cm, *C. caespitosa* is the largest madrepore in the Mediterranean Sea. It is common on rocky seabeds, between a few meters and 60 meters deep, often in environments characterized by sand, rock and *Posidonia oceanica* meadows. With an average generation length of around 30 years, this species is fundamental to the Mediterranean ecosystem, but is also vulnerable to climate change, in particular rising sea temperatures and ocean acidification. Its conservation is therefore essential to maintain the biodiversity and stability of Mediterranean marine ecosystems.

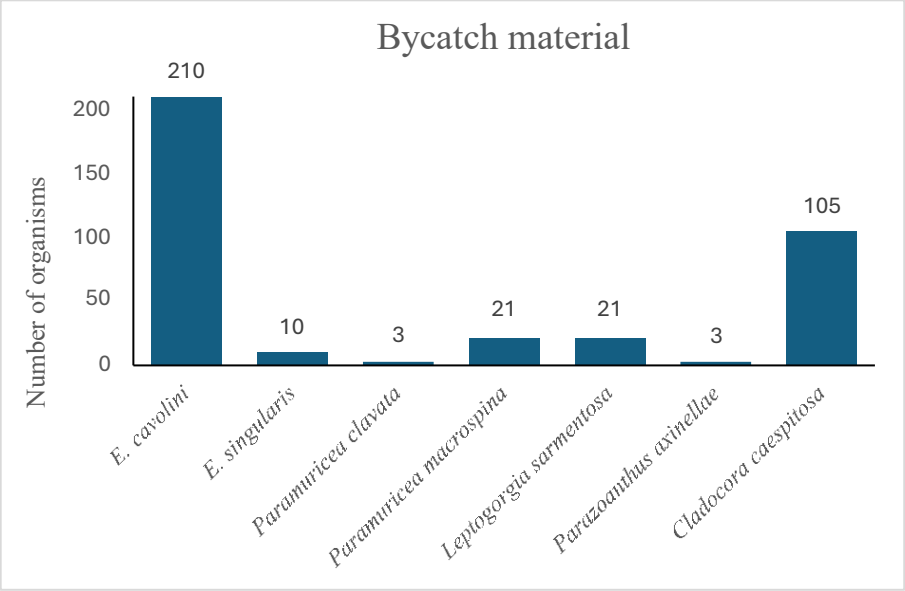


Figure 8. Total number of live organisms collected with bycatch.



Figure 9. *Eunicella singularis*



Figure 10. *Cladocora caespitosa*

4. DISCUSSION

The analysis of coralligenous assemblages and habitat quality revealed high heterogeneity between sites, and it was possible to identify Murata as the one with the best conservation status (especially in one of the two areas where is still present *Paramuricea clavata*). Overall, given the quality found in the other sites, experimental activities are planned to recover coralligenous assemblages in Mano Aperta and Frasca, where the bycatch material could be allocated.

The effectiveness of ESCA index in assessing the ecological status of coralligenous reefs across various environmental conditions and for impact evaluation studies is well known (Cecchi et al. 2014). This is the case of Penna et al. 2017 who used ESCA index to assess ecological status of habitats in the area affected by the Costa Concordia shipwreck (Giglio Island, Italy). Also, Piazzi et al. (2018) were able to evaluate the impact of benthic mucilaginous aggregates on coral reefs thanks to the use of this index. In 2019 (Piazzi et al.) ESCA was also used to evaluate the impact of fishing cages on coralligenous reefs in the Gorgona Island in the Tuscan Archipelago National Park (North-

Western Mediterranean Sea, Italy). In this context, the usefulness will rely in the temporal change of the quality status of the coralligenous reefs depending on the proximity to the port works in the Civitavecchia area: the calculation of the ESCA index should thus be repeated over the years similarly to Piazzi et al. in 2021 after a 10-year monitoring of 42 sites in the Mediterranean taking into consideration the anthropic impacts present in the different areas.

The performance (survival, growth and necrosis) of transplanted organisms will be evaluated in the two receiver sites compared with the performance in Murata, which will represent the control site for transplanting activities (Fig. 11).

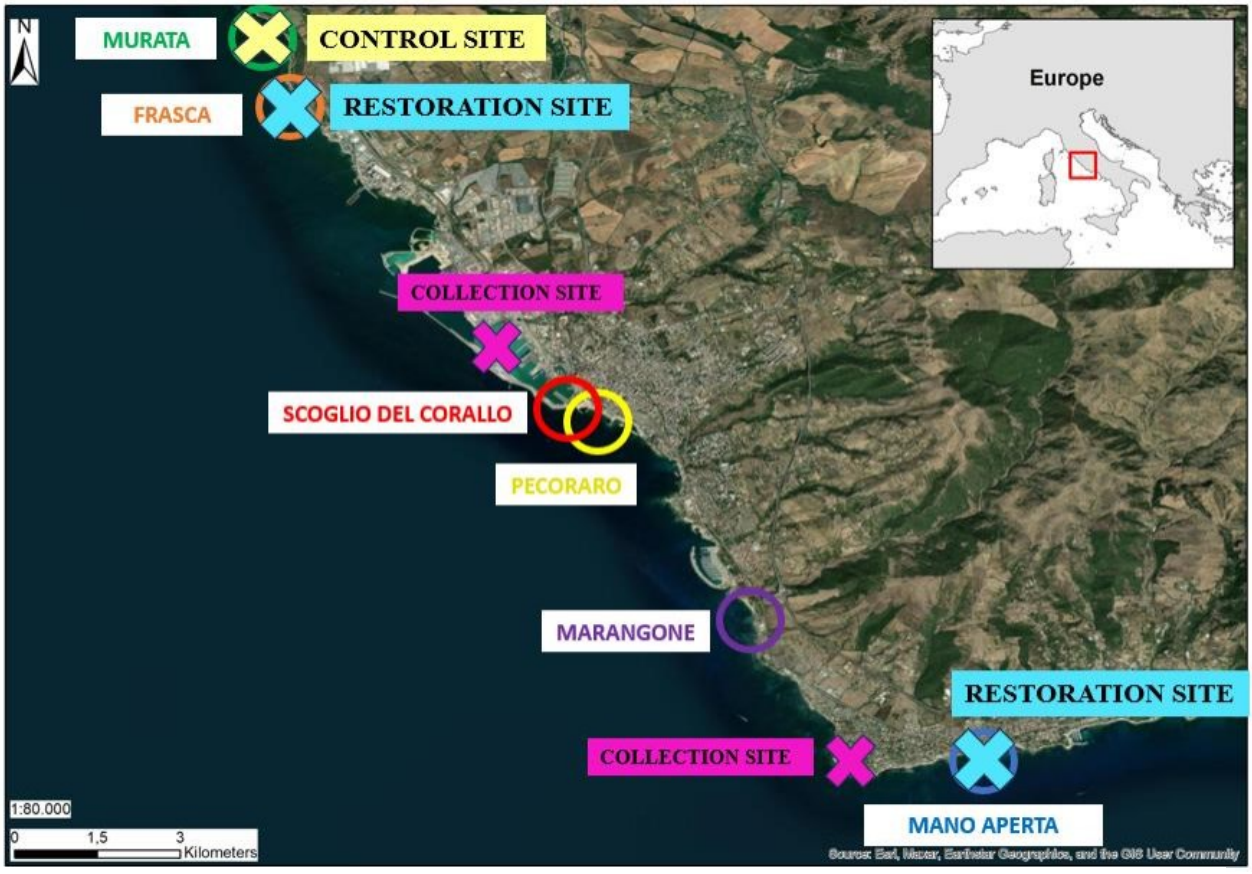


Figure 11. In light blue (Frasca and Mano Aperta) the receiver sites where experimental transplants will be made. In yellow (Murata) the control site. In pink (Civitavecchia port and Santa Marinella port).

The use of bycatch material in restoration efforts is a relatively new, and thus an evolving technique. The first pilot effort was conducted in France by Monteseny et al. in 2019, who transplanted 120 gorgonians obtained from bycatch on stainless steel structures in the Cap de Creus area (north-western Mediterranean Sea) between 70 and 100 meters of depth: one year after the transplantation, the survival rate of the transplanted organisms was about 87.5%. Similarly, Casoli et al. (2021) reported results from approximately three years of monitoring after the transplantation of 135 gorgonians (*E. cavolini, Eunicella singularis*, and *Paramuricea clavata*) from local bycatch in the area where the Costa Concordia sank in 2012, area where restoration efforts have been underway since 2019 to repair the damage from the shipwreck: in this study, the survival rate of the transplanted organisms was around 82.1%, with the most significant decline occurring within the first two months after transplantation. It was also noted that about 1.5 years after transplantation, the epoxy putty used to attach the organisms to the seabed had been fully colonized by encrusting benthic organisms, becoming part of the new ecosystem. These new colonies were compared with control colonies present in the area (untouched organisms), and it was found that they exhibited comparable rates of epibiosis and necrosis, which depended only by seasonal fluctuations in both cases.

Overall, these results have suggested that gorgonians obtained from bycatch of local artisanal fishers can indeed be effectively used as transplant material, and the employment of this technique can be an excellent solution for restoring damaged habitats. Overall, the restoration of coralligenous reefs using bycatch material has never included other taxa, besides gorgonians, such as encrusting coralline algae, briozoa or sponges.

MURATA

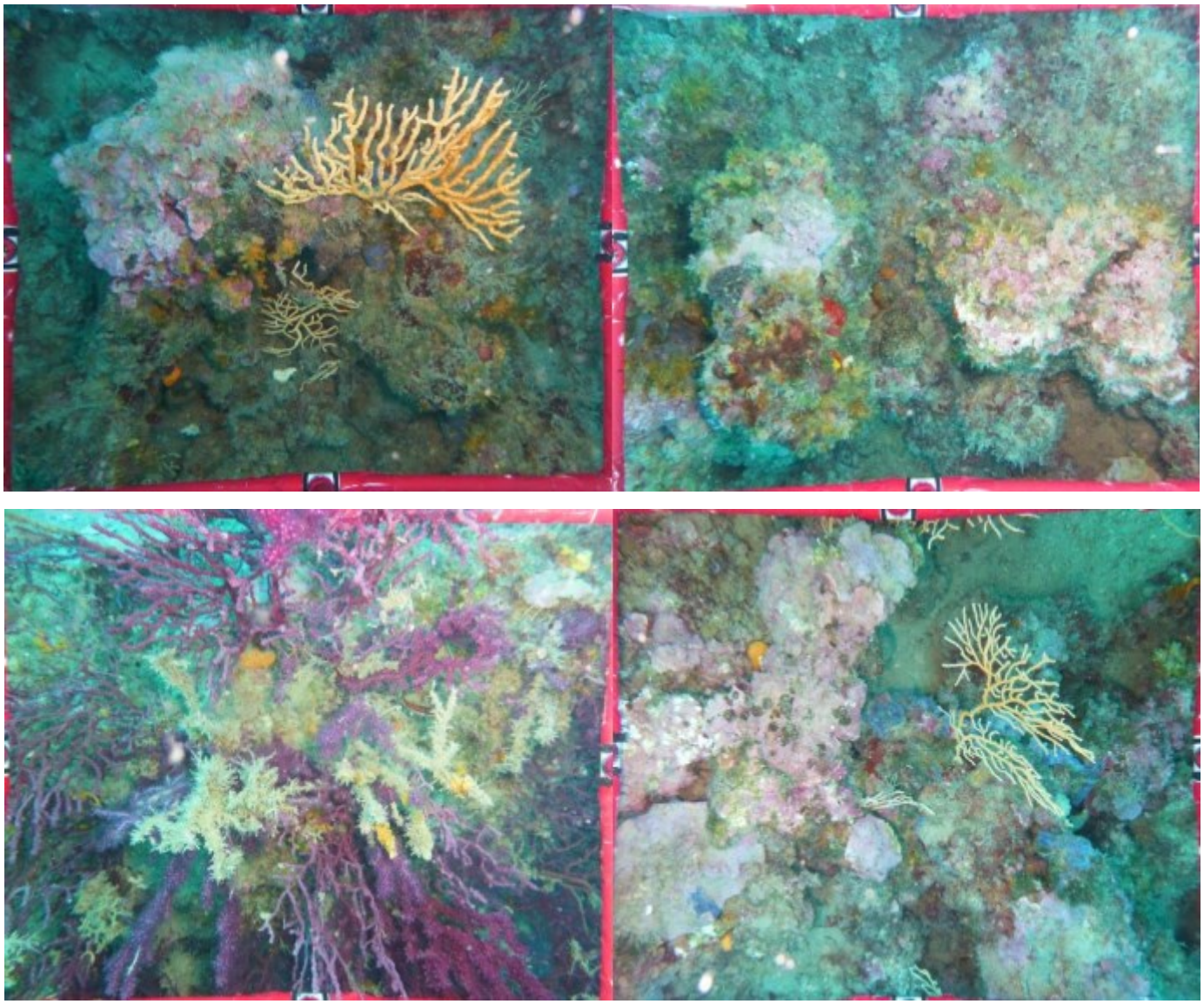


Figure 13. Photographic images of sampling done at Murata site. Frame size: 40x50 cm.

FRASCA

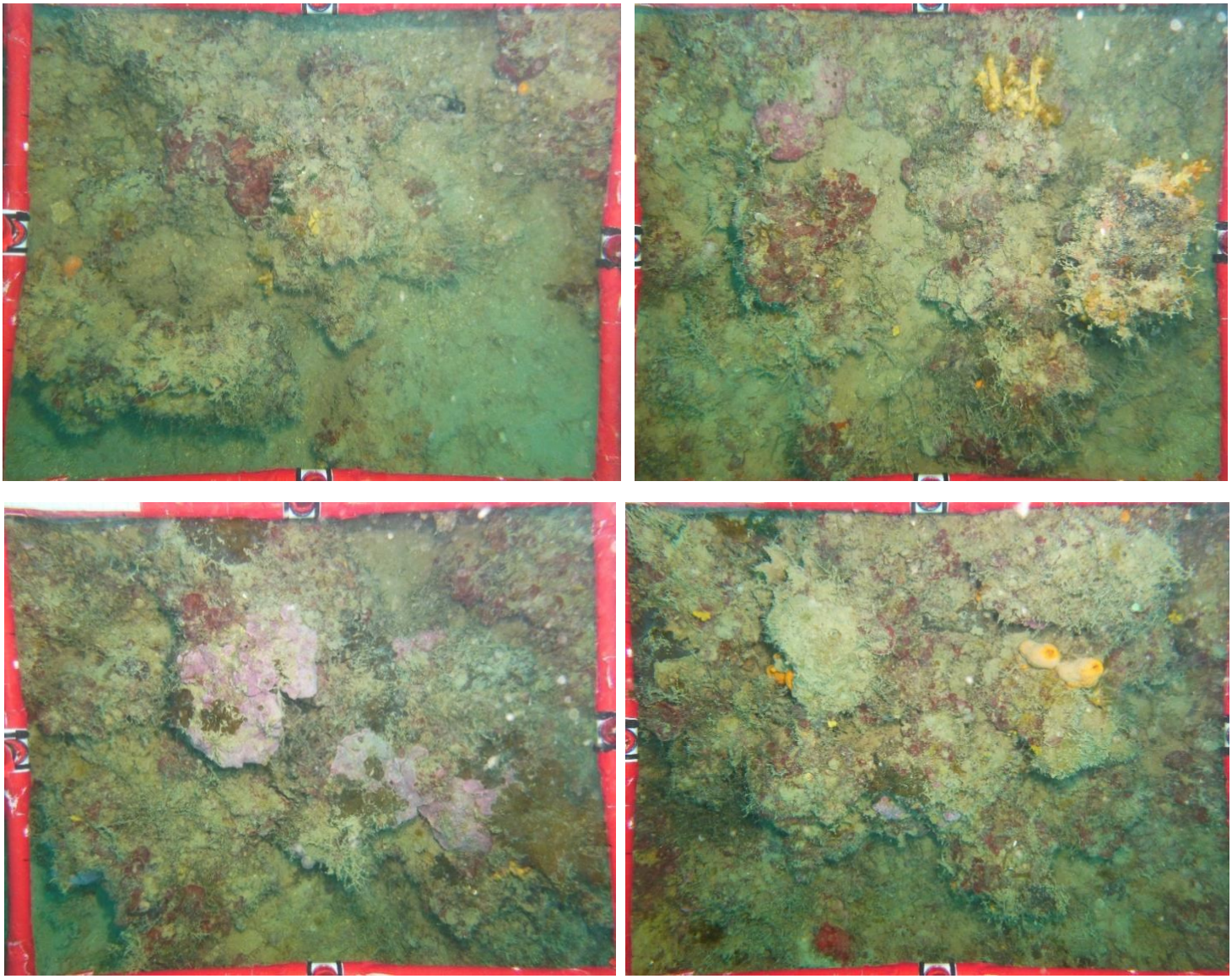


Figure 14. Photographic images of sampling done at Frasca site. Frame size: 40x50 cm.

SCOGLIO DEL CORALLO

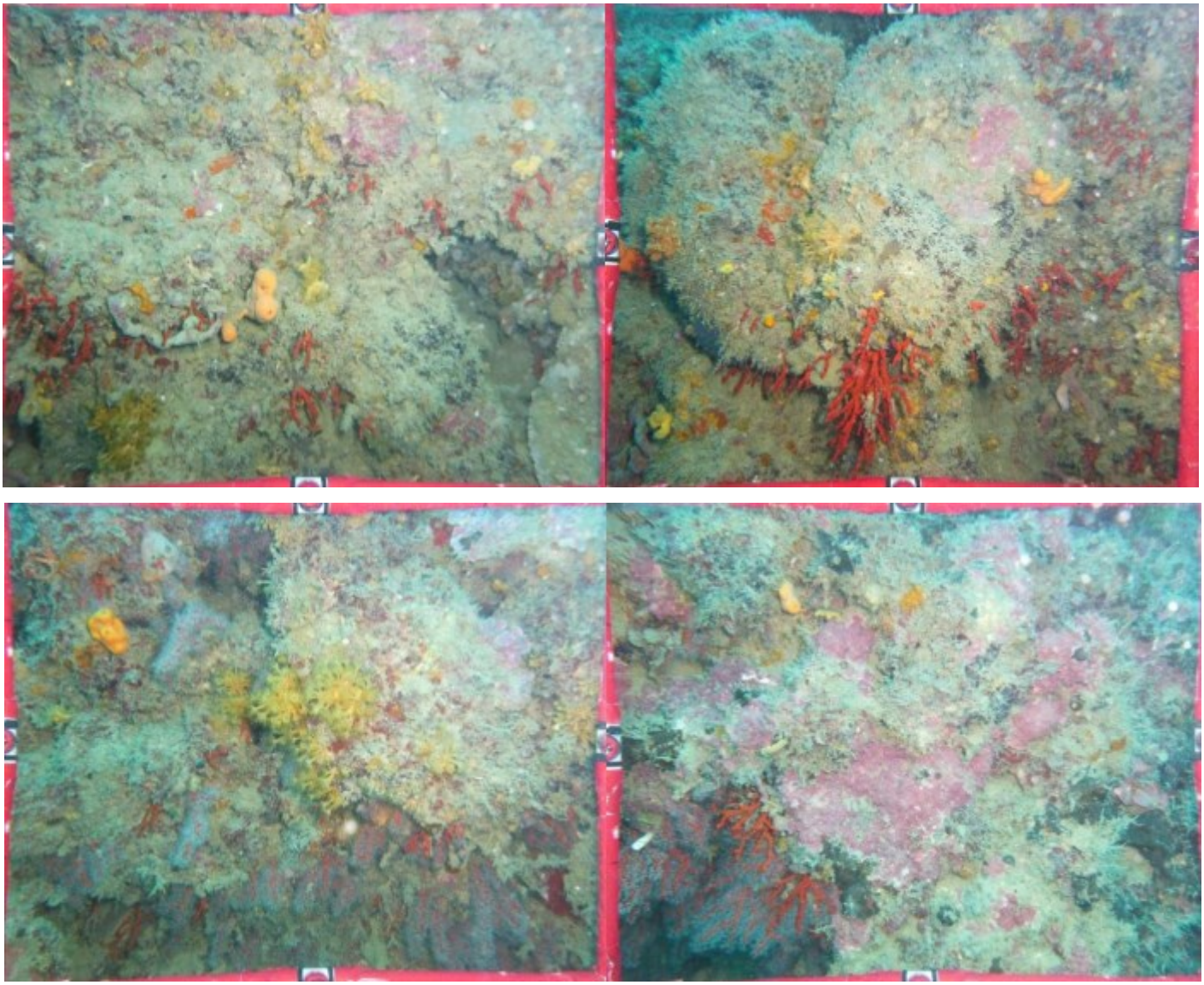


Figure 15. Photographic images of sampling done at Scoglio del Corallo site. Frame size: 40x50 cm.

MARANGONE

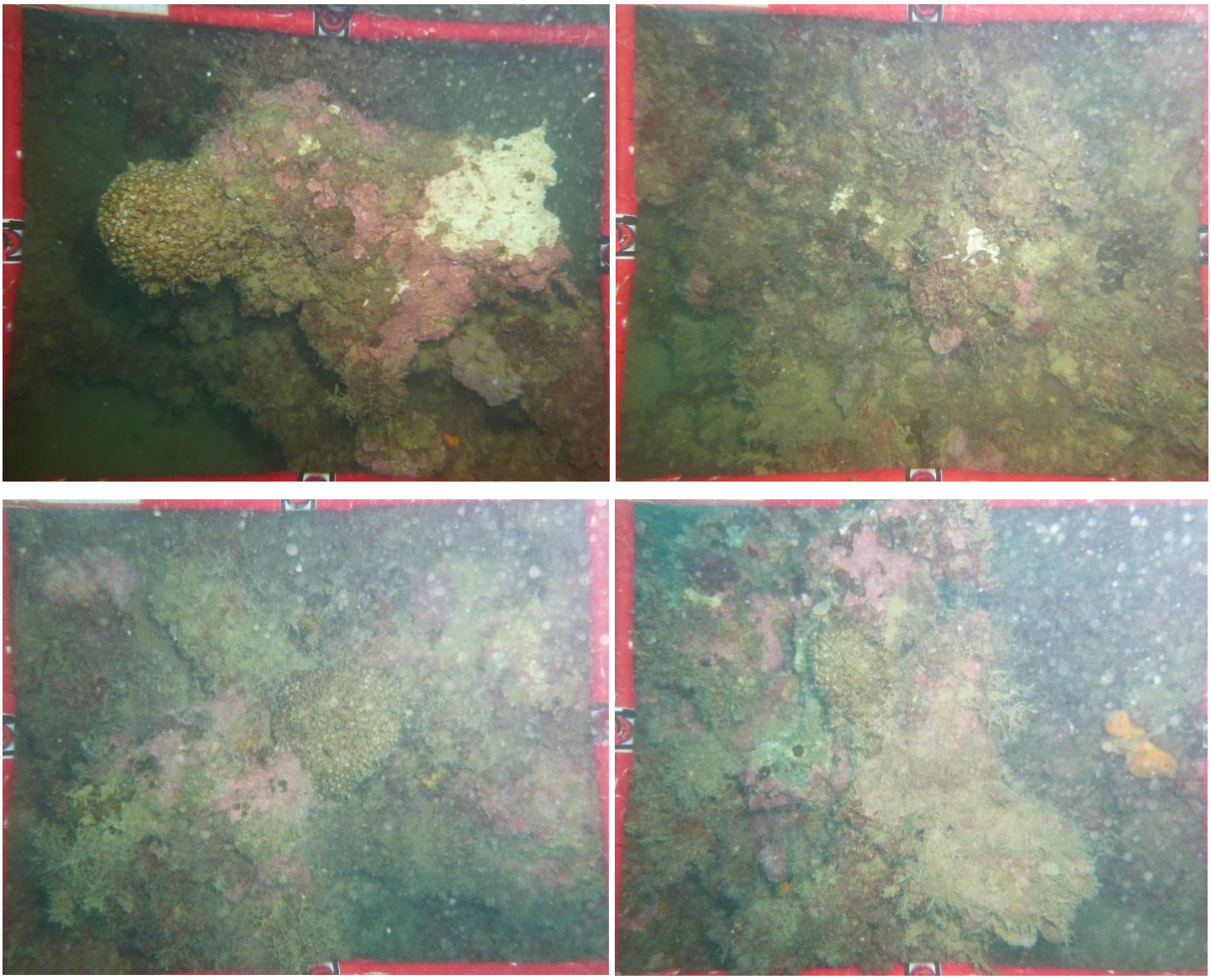


Figure 16. Photographic images of sampling done at Marangone site. Frame size: 40x50 cm.

PECORARO

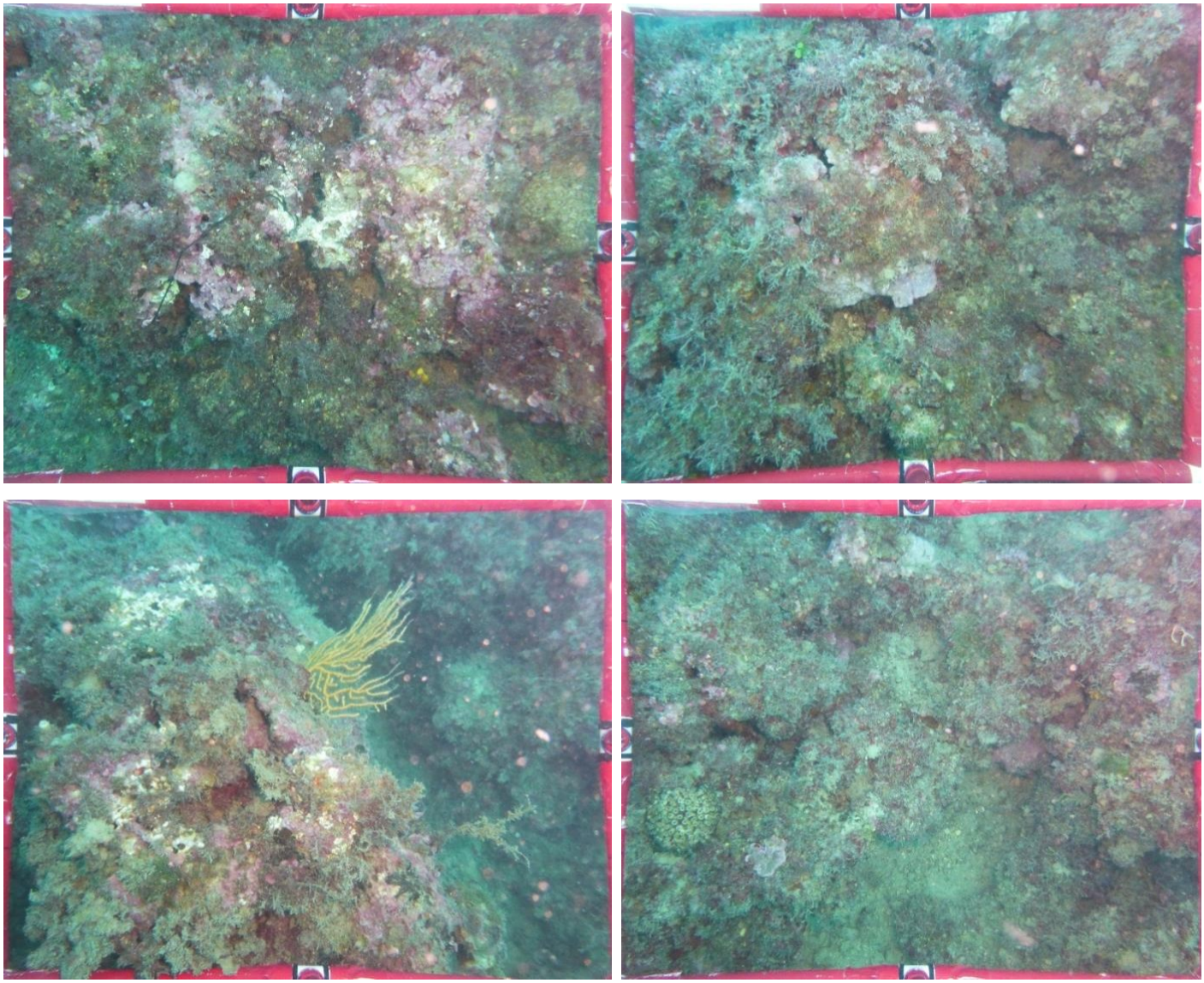


Figure 17. Photographic images of sampling done at Pecoraro site. Frame size: 40x50 cm.

MANO APERTA

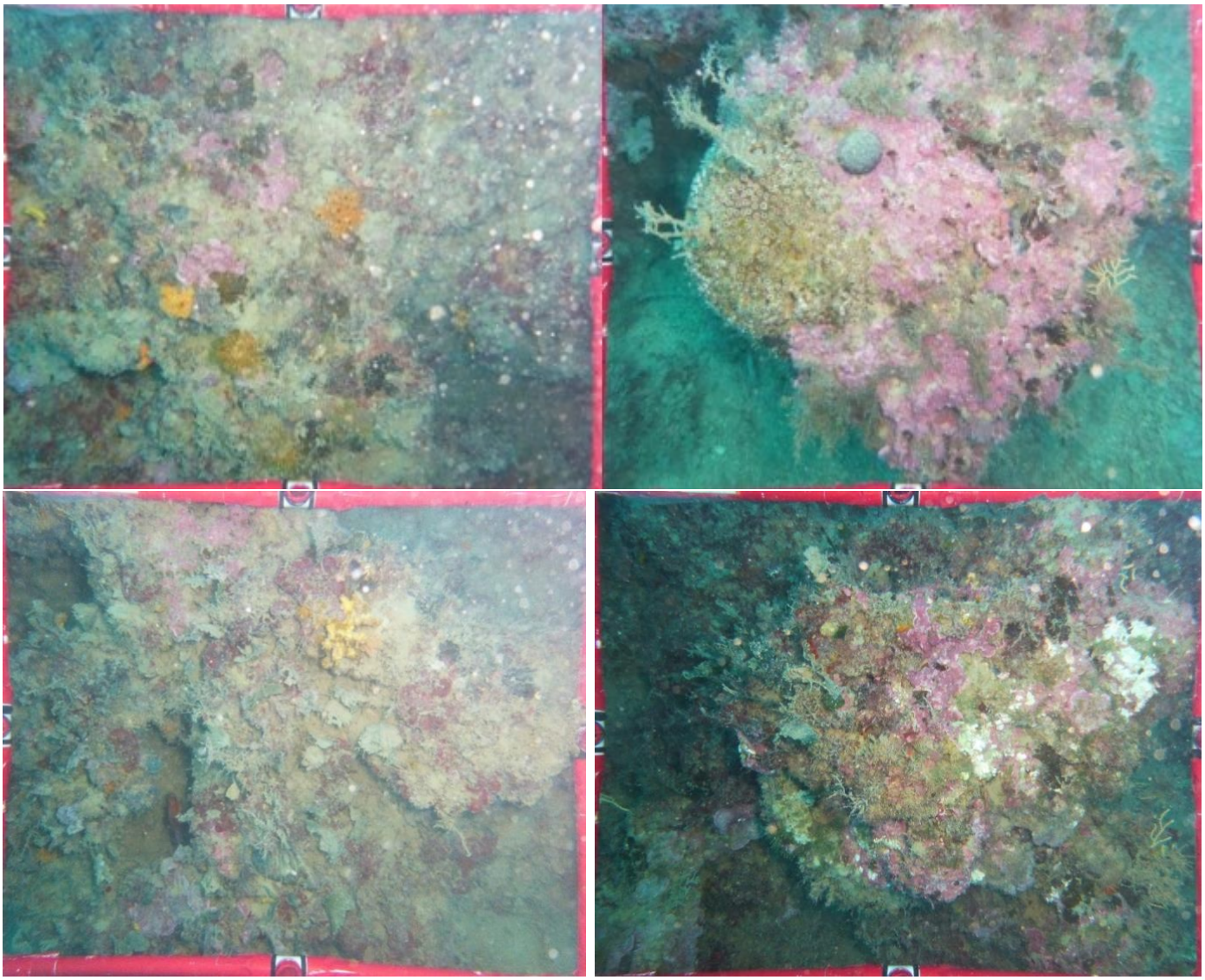


Figure 18. Photographic images of sampling done at Mano Aperta site. Frame size: 40x50 cm.

Chapter 6

GENERAL DISCUSSION AND CONCLUSION

Climate change and human impact on the marine environment are two factors that are increasing in frequency, leading to increasingly important negative effects. This leads to damage the ecosystem with a consequent loss of plant and animal biodiversity. Hence the need to restore degraded habitats to bring the ecosystem back to its initial functions and services. In recent years there has been a greater talk about this need, seeing the negative effects of multiple factors on ecosystems and a big important step was achieved in June 2024 when the first Nature Restoration Law was approved, a European-level legislation to regulate works of restoration and set objectives for EU member states to achieve to recover damaged marine and terrestrial habitats.

This thesis was focused on investigating possible restoration techniques for crustose coralline algae (CCA). These organisms are key species in the Mediterranean Sea, as they create bioconstructions that act as a substrate for many other living organisms and therefore promote a very high marine biodiversity (Ballesteros 2006).

The cultivation of CCA with the aim of restoring damaged coralligenous habitats is a very recent approach and not yet considered as the restoration studies on Mediterranean coralligenous carried out so far have focused only on gorgonians (Fava et al. 2010; Cerrano et al. 2018; Montseny et al. 2019; Casoli et al. 2021).

In addition to this, this thesis work was also aimed at studying the recruitment, growth and response of CCA recruits to different environmental conditions (e.g. light and temperature), both in the laboratory and in the field. In the literature there are many studies on the behavior and performance

of adult CCA individuals in response to environmental factors, but what is currently missing are studies regarding the recruits of these organisms and the early life stages.

When thinking about restoring a damaged habitat with a view to climate change, it is of fundamental importance to understand how the foundation habitat species develop and respond to environmental stressors starting from the first stages of life, as well as identifying what the best techniques and materials to be used to make damaged habitats recover and therefore restore the ecosystem to a good state of health and conservation status.

Lastly, the importance of carrying out in-depth studies on the areas that require recovery interventions was addressed, trying to understand what the main source of disturbance was and, where possible, trying to eliminate it. In the end, the value of reusing material from bycatch for restoration works was highlighted, material which would therefore usually be discarded after commercial fishing activities. This is useful both for recovering organisms that would otherwise die and for carrying out restoration works without damaging other intact habitats where species can be taken to transplant into the damaged areas.

The results obtained in this thesis represent a significant first step and can provide interesting ideas for future marine restoration projects. Although these findings have shown significant potential, it is important to highlight that they need to be further explored and implemented through additional studies and more specific research. It is necessary to invest further in research to refine restoration techniques and ensure greater resilience of marine ecosystems. The field of marine restoration is complex and constantly evolving, and for this very reason, the integration of new discoveries and multidisciplinary approaches will be fundamental to maximize the effectiveness of future initiatives. The results presented in this work may help stimulate further investigations and offer a useful starting point for developing more complete and targeted interventions in the context of the protection of marine ecosystems.

Considering the rapid degradation of marine ecosystems due to climate change and human impacts, the results of these studies take on even greater relevance. The proposed methodologies can in fact contribute to addressing the loss of biodiversity and restoring fundamental habitats, such as coralligenous reefs in the Mediterranean Sea.

Chapter 7

REFERENCES

(2017) United Nations Environment Programme UNEP. International Organizations.

Abelson A., Reed D.C., Edgar G.J., Smith C.S., Kendrick G.A., Orth R.J., Airoldi L., Silliman B., Beck M.W., Krause G., Shashar N., Stambler N., Nelson P. (2020). Challenges for Restoration of Coastal Marine Ecosystems in the Anthropocene. Frontiers in Marine Science. DOI:10.3389/fmars.2020.544105.

Ådahl, E., Lundberg, P. E. R., & Jonzen, N. (2006). From climate change to population change: the need to consider annual life cycles. *Global Change Biology*, *12*(9), 1627-1633.

Adey, w. H. (1970). The effects of light and temperature on growth rates in boreal-subarctic crustose corallines 1. *Journal of phycology*, *6*(3), 269-276.

Adey, W. H., & Vassar, J. M. (1975). Colonization, succession, and growth rates of tropical crustose coralline algae (Rhodophyta, Cryptonemiales). *Phycologia*, 14(2), 55-69.

Aguirre, J., Perfectti, F., Braga, J.C., (2010). Integrating phylogeny, molecular clocks, and the fossil record in the evolution of coralline algae (Corallinales and Sporolithales, Rhodophyta). Paleobiology 36, 519–533. https://doi.org/10.1666/09041.1.

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. Austral Ecol. (Devon, UK: PRIMER -E Ltd)26, 32–46. doi: 10.1046/ j.1442-9993.2001.01070.x.

Andersson AJ, Mackenzie FT, Bates NR (2008) Life on the margin: Implications of ocean acidification on Mg-calcite, high latitude and cold-water marine calcifiers. Mar Ecol Prog Ser 373: 265–273.

Anthony, K. R. N., Kline, D. I., Diaz-Pulido, G., Dove, S., & Hoegh-Guldberg, O. (2008). Ocean acidification causes bleaching and productivity loss in coral reef builders. *Proceedings of the National Academy of Sciences*, 105(45), 17442-17446.

Aronson J., Blignaut J.N., Aronson T.B. Conceptual frameworks and references for landscape-scale restoration: reflecting back and looking forward, Ann. Missouri Botan. Garden 102 (2) (2017) 188200 https://doi.org/10.3417/2017003.

Balata, D., Piazzi, L., Cecchi, E., Cinelli, F., (2005). Variability of Mediterranean coralligenous assemblages subject to local variation in sediment deposition. Marine Environmental Research 60, 403-421.

Ballesteros E (2006) Mediterranean coralligenous assemblages: a synthesis of present knowledge. Oceanogr Mar Biol 44:123–195.

Barisiello, O., Bernabei, S., Bucci, M., Casazza, G., Cavalieri, S., Comperini, W., ... & Volpi, E. (2002). Qualità delle acque marino costiere. Annuario dei dati ambientali.

Betti, F., Bavestrello, G., Bo, M., Ravanetti, G., Enrichetti, F., Coppari, M., Cappanera, V, Venturini, S., Cattaneo-Vietti, R., (2020). Evidences of fishing impact on the coastal gorgonian forests inside the Portofino MPA (NW Mediterranean Sea). Ocean and Coastal Management 187, 105105.

Bevilacqua S, Guarnieri G, Farella G, Terlizzi A, Fraschetti S (2018) A regional assessment of cumulative impact mapping on Mediterranean coralligenous outcrops. Sci Rep 8(1):1757.

Bevilacqua S, Airoldi L, Ballesteros E, Benedetti-Cecchi L, Boero F, Bulleri F, Terlizzi A et al (2021). Mediterranean rocky reefs in the Anthropocene: Present status and future concerns. Adv Mar Biol 89:1-51.

Bianchelli, S., Martini, F., Lo Martire, M., Danovaro, R., & Corinaldesi, C. (2023). Combining passive and active restoration to rehabilitate a historically polluted marine site. *Frontiers in Marine Science*, *10*, 1213118.

Bo, M., Bava, S., Canese, S., Angiolillo, M., Cattaneo-Vietti, R., & Bavestrello, G. (2014). Fishing impact on deep Mediterranean rocky habitats as revealed by ROV investigation. *Biological Conservation*, 171, 167-176.

Boopendranath, M R. (2012). FAO Code of conduct for responsible fishing operations.

Borowitzka MA (1981) Photosynthesis and calcification in the articulated coralline red algae *Amphiroa anceps* and *A. foliacea*. Mar Biol 62:17–23.

Borowitzka, M. A. (1982). Morphological and cytological aspects of algal calcification. In *International review of cytology* (Vol. 74, pp. 127-162). Academic Press.

Borowitzka MA, Larkum AWD (1987) Calcification in algae: Mechanisms and the role of metabolism. Crit Rev Plant Sci 6:1–45.

Boudouresque, C. F., Bernard, G., Pergent, G., Shili, A., & Verlaque, M. (2009). Regression of Mediterranean seagrasses caused by natural processes and anthropogenic disturbances and stress: A critical review. *Botanica Marina*, 52(5), 395-418.

Boudouresque C-F, Blanfuné A, Pergent G, Thibaut T. Restoration of Seagrass Meadows in the Mediterranean Sea: A Critical Review of Effectiveness and Ethical Issues. *Water*. (2021); 13(8):1034. https://doi.org/10.3390/w13081034.

Boudouresque, C. F., Meinesz, A., Ribera, M. A., & Ballesteros, E. (2012). Spread of the green alga *Caulerpa taxifolia* (Caulerpales, Chlorophyta) in the Mediterranean: Possible consequences of the introduction. *Invasive Aquatic Species of Europe: Distribution, Impacts and Management*, 39-46.

Bracchi VA, Basso D (2012) The contribution of calcareous algae to the biogenic carbonates of the continental shelf: Pontian Islands, Tyrrhenian Sea, Italy. Geodiversitas 34:61–76.

Bradassi, F., Cumani, F., Bressan, G., & Dupont, S. J. M. B. (2013). Early reproductive stages in the crustose coralline alga Phymatolithon lenormandii are strongly affected by mild ocean acidification. *Marine Biology*, *160*, 2261-2269.

Britton, D., Mundy, C. N., Noisette, F., McGraw, C. M., & Hurd, C. L. (2021). Crustose coralline algae display sensitivity to near future global ocean change scenarios. *ICES Journal of Marine Science*, *78*(10), 3748-3756.

Burt J, Bartholomew A, Bauman A, Saif A, Sale PF (2009) Coral recruitment and early benthic community development on several materials used in the construction of artificial reefs and breakwaters. J Exp Mar Biol Ecol 373: 72−78.

Campbell, S. J. & Woelkerling, W. J. (1990). Are *Titanoderma* and *Lithophyllum* (Corallinaceae, Rhodophyta) distinct genera? *Phycologia* 29:114-25.

Canals M., Ballesteros E. (1997). Production of carbonate particles by phytobenthic communities on the Mallorca-Menorca shelf, northwestern Mediterranean Sea. Deep Sea Research Part II: Topical Studies in Oceanography. Volume 44, Issues 3–4. Pages 611-629. ISSN 0967-0645. https://doi.org/10.1016/S0967-0645(96)00095-1.

Capdevila, P., Linares, C., Aspillaga, E., Riera, J. L., & Hereu, B. (2018). Effective dispersal and density-dependence in mesophotic macroalgal forests: Insights from the Mediterranean species Cystoseira zosteroides. *PLoS One*, *13*(1), e0191346.

Caragnano, A., Rodondi, G., Basso, D., Pena, V., Le Gall, L., Rindi, F., (2020). Circumscription of Lithophyllum racemus (Corallinales, Rhodophyta) from the western Mediterranean Sea reveals the species Lithophyllum pseudoracemus sp. nov. Phycologia 59, 584–597. https://doi.org/10.1080/00318884.2020.1829348.

Casas-Güell, E., Teixidó, N., Garrabou, J., Cebrian, E., (2015). Structure and biodiversity of coralligenous assemblages over broad spatial and temporal scales. Marine Biology 162, 901-912.

Casas-Guëll, E., Cebrian, E., Garrabou, J., Ledoux, J.B., Linares, C., Teixidó, N., (2016). Structure and biodiversity of coralligenous assemblages dominated by the precious red coral *Corallium rubrum* over broad spatial scales. Scientiphic Reports 6, 36535.

Casoli, E., Ventura, D., Mancini, G., Cardone, S., Farina, F., Donnini, L., ... & Ardizzone, G. (2022). Rehabilitation of Mediterranean animal forests using gorgonians from fisheries by-catch. Restoration Ecology, 30(1), e13465.

Cebrián E, Ballesteros E, Canals M (2000) Shallow rocky bottom benthic assemblages as calcium carbonate producers in the Alboran Sea (southwestern Mediterranean). Oceanol Acta 23: 311–322.

Cebrián E, Linares C, Garrabou J (2021) Warming may increase the vulnerability of calcareous algae to bioinvasions. Mar Pollut Bull 173:113099.

Ceccherelli G, Pinna F, Pansini A, Piazzi L, La Manna G (2020) The constraint of ignoring the subtidal water climatology in evaluating the changes of coralligenous reefs due to heating events. Sci Rep 10(1):17332.

Cecchi, E., Gennaro, P., Piazzi, L., Ricevuto, E., Serena, F., (2014). Development of a new biotic index for ecological status assessment of Italian coastal waters based on coralligenous macroalgal assemblages. European Journal of Phycology 49, 298-312.

Cerrano C, Danovaro R, Gambi C, Pusceddu A, Riva A, Schiaparelli S. (2010). Gold coral (Savalia savaglia) and gorgonian forests enhance benthic biodiversity and ecosystem functioning in the mesophotic zone. Biodiversity and Conservation 19:153–167. DOI: 10.1007/s10531-009-9712-5.

Cerrano C, Bakran-Petricioli T, Boavida J, Ferretti E, Gari A, Gomez-Gras D, Gori A, Hereu B, Kipson S, Ledoux J, Linares C, López-Sanz À, López-Sendino P, Milanese M, Montero-Serra I, Pagès M, Sarà A, Pica D, Serrao E, Torsani F, Viladrich N, Garrabou J. (2018) Restoring biodiversity in the Mediterranean coralligenous - the MERCES project. PeerJ Preprints 6:e26813v1.

Cerrano, C., Ponti, M., Brugnone, F., Coppari, M., & Danovaro, R. (2019). The impact of marine litter in Mediterranean coralligenous assemblages. *Marine Pollution Bulletin*, 138, 314-320.

Cinar, M.E., Féral, J.P., Arvanitidis, C., Davis, R., Sini, M., Doğan, A., Gerovasileiou, V., Evcen, A., Chenuil, A., Dağli, E., Aysel, V., Issaris, Y., Bakir, K., Nalmpantı, M., Sartoretto, S., Salomidi, M., Sapouna, A., Açik, S., Dimitriadis, C., Koutsoubas, D., Katağan, T., Öztürk, B., Koçak, F., Erdogan-Dereli, D., Önen, S., Özgen, O., Türkçü, N., Kirkim, F., Önen, M., (2020). Coralligenous assemblages along their geographical distribution: Testing of concepts and implications for management. Aquatic Conservation: Marine and Freshwater Ecosystems 30, 1578-1594.

Cornwall CE, Comeau S, McCulloch MT (2017) Coralline algae elevate pH at the site of calcification under ocean acidification. Glob Chang Biol 23(10):4245-4256.

Cornwall, C. E., Diaz-Pulido, G., & Comeau, S. (2019). Impacts of ocean warming on coralline algal calcification: Meta-analysis, knowledge gaps, and key recommendations for future research. *Frontiers in Marine Science*, *6*, 186.

Cornwall C.E., Diaz-Pulido G , Comeau S. (2020). Impacts of Ocean Warming on Coralline Algal Calcification: Meta-Analysis, Knowledge Gaps, and Key Recommendations for Future Research. Frontiers in Marine Science. DOI:10.3389/fmars.2019.00186.

Cornwall, Christopher Edward; Comeau, Steeve; DeCarlo, Thomas M; Larcombe, E; Moore, B; Giltrow, K; Puerzer, F; D'Alexis, Q; McCulloch, Malcolm T (2020): Seawater carbonate chemistry and calcification physiology of coralline algae [dataset]. PANGAEA, https://doi.org/10.1594/PANGAEA.925187.

Cumani, F., Bradassi, F., Di Pascoli, A., & Bressan, G. (2010). Marine acidification effects on reproduction and growth rates of Corallinaceae spores (Rhodophyta). In *CIESM Congress Proceedings* (Vol. 39, No. 735, pp. 603-610).

Da Ros, Z., Corinaldesi, C., Dell'Anno, A., Gambi, C., Torsani, F., & Danovaro, R. (2020). Restoration of Cymodocea nodosa seagrass meadows: efficiency and ecological implications. *Restoration Ecology, 29*.

Davies R.W.D., Cripps S.J., Nickson A., Porter G. (2009). Defining and estimating global marine fisheries bycatch. Marine Policy. Volume 33, Issue 4. Pages 661-672. ISSN 0308-597X. https://doi.org/10.1016/j.marpol.2009.01.003.

de Carvalho RT, Wendt CHC, Willemes MJ, Bahia RG, Farina M, Salgado LT (2022) Ontogeny and Early Steps of the Calcification Process in Coralline Algae *Lithophyllum corallinae* (Florideophyceae, Rhodophyta). Front Mar Sci 9:900607.

Dean, A. J., Steneck, R. S., Tager, D., & Pandolfi, J. M. (2015). Distribution, abundance and diversity of crustose coralline algae in the shallow tropical Pacific Ocean. *PLOS ONE*, 10(12), e0144906.

Deter J., Descamp, P., Ballesta L., Boissery P., Holon F. (2012). A preliminary study toward an index based on coralligenous assemblages for the ecological status assessment of Mediterranean French coastal waters. Ecological Indicators. 20. 345–352. 10.1016/j.ecolind.2012.03.001.

Diaz-Pulido, G., Anthony, K. R., Kline, D. I., Dove, S., & Hoegh-Guldberg, O. (2012). Interactions between ocean acidification and warming on the mortality and dissolution of coralline algae 1. Journal of Phycology, 48(1), 32-39.

Diaz-Pulido G, Nash MC, Anthony KRN, Bender D, Opdyke BN, Reyes-Nivia C, et al (2014). Greenhouse conditions induce mineralogical changes and dolomite accumulation in coralline algae on tropical reefs. Nat Commun 5:3310.

Dickson AG, Sabine CL, Christian J R (2007) Guide to best practices for ocean CO2 measurements. PICES Special Publication 3, 191 pp.

Duarte, C. M. (2002). The future of seagrass meadows. *Environmental Conservation*, 29(2), 192-206.

E.C., 2000. Directive 2000/60/EC of the European Parliament and of the Council, of 23 October 2000, establishing a framework for Community action in the field of water policy. Official Journal of the European Communities, G.U.C.E. 22/12/2000, L 327.

E.C., 2008. DIRECTIVE 2008/56/EC of the European Parliament and of the Council, of 17 June 2008, establishing a framework for Community action in the field of marine environmental policy (Marine Strategy Framework Directive). Official Journal of the European Commission, G.U.C.E. 25/6/2008, L 164/19.

Edwards A.J., Gomez E.D., Reef Restoration Concepts and Guidelines: Making Sensible Management Choices in the Face of Uncertainty, (2007), pp. i 38.

Egilsdottir H, Jon Olafsson J, Martin S (2016) Photosynthesis and calcification in the articulated coralline alga *Ellisolandia elongata* (Corallinales, Rhodophyta) from intertidal rock pools. European J Phycol 51:1, 59-70.

El Haïkali B, Bensoussan N, Romano JC, Bousquet V (2004) Estimation of photosynthesis and calcification rates of *Corallina elongata* Ellis and Solander, 1786, by measurements of dissolved oxygen, pH and total alkalinity. Sci Mar 68:45-56.

Fabricius KE, Langdon C, Uthicke S, Humphrey C, and others (2011). Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations. Nat Clim Chang 1: 165−169.

Fabricius KE, Kluibenschedl A, Harrington L, Noonan S, De'ath G (2015). In situ changes of tropical crustose coralline algae along carbon dioxide gradients. Sci Rep 5: 9537.

Falace A, Zanelli E, Bressan G (2006) Algal transplantation as a potential tool for artificial reef management and environmental mitigation. Bull Mar Sci 78(1):161-166.

Falace A, Kaleb S, de la Fuente G, Asnaghi V, Chiantore M (2018) Ex situ cultivation protocol for *Cystoseira amentacea* var. *stricta* (Fucales, phaeophyceae) from a restoration perspective. PloS One 13(2):e0193011.

Fava F, Bavestrello G, Valisano L, and Cerrano C (2009) Survival, growth and regeneration in explants of four temperate gorgonian species in the Mediterranean Sea. Italian Journal of Zoology 77:44-52. https://doi.org/10.1080/11250000902769680.

Fava F, Bavestrello G, Valisano L, Cerrano C (2010) Survival, growth and regeneration in explants of four temperate gorgonian species in the Mediterranean Sea. Ital J Zool 77(1):44-52.

Ferrigno, F., Appolloni, L., Russo, G.F., Sandulli, R., (2018). Impact of fishing activities on different coralligenous assemblages of Gulf of Naples (Italy). Journal of Marine Biological Association of the United Kingdom 98, 41-50.

Fava, F., Ponti, M., & Abbiati, M. (2016). Role of recruitment processes in structuring coralligenous benthic assemblages in the northern Adriatic continental shelf. *PLoS One*, *11*(10), e0163494.

Field SN, Glassom D, Bythell J (2007) Effects of artificial settlement plate materials and methods of deployment on the sessile epibenthic community development in a tropical environment. Coral Reefs 26: 279−289.

Fong, J., Jackson, T. L., Flores, F., Antunes, E., Abdul Wahab, M. A., Negri, A. P., & Diaz-Pulido, G. (2024). The interplay of temperature, light, and substrate type in driving growth and reproduction of an important tropical crustose coralline alga. Journal of Applied Phycology, 36(7), 3133-3145. DOI: 10.1007/s10811-024-03312-z.

Foster, M. S. (2001). Rhodoliths: Between rocks and soft places. *Journal of Phycology*, 37(5), 659-667.

Fragkopoulou, E., Serrão, E. A., Horta, P. A., Koerich, G., & Assis, J. (2021). Bottom trawling threatens future climate refugia of rhodoliths globally. *Frontiers in Marine Science*, *7*, 594537.

Freiwald, A., Fossa, J. H., Grehan, A., Koslow, T., & Roberts, J. M. (2004). *Cold-water Coral Reefs: Out of Sight – No Longer out of Mind*. UNEP-WCMC.

Garrabou J, Ballesteros E (2000) Growth of *Mesophyllum alternans* and *Lithophyllum frondosum* (Corallinales, Rhodophyta) in the northwestern Mediterranean. Eur J Phycol 35:1–10.

Garrabou, J., Coma, R., Bensoussan, N., Bally, M., Chevaldonné, P., Cigliano, M., ... & Cerrano, C. (2009). Mass mortality in Northwestern Mediterranean rocky benthic communities: effects of the 2003 heat wave. *Global change biology*, *15*(5), 1090-1103.

Garrabou, J., Gómez-Gras, D., Ledoux, J.-B., Linares, C., Bensoussan, N., López-Sendino, P., Bazairi, H., Espinosa, F., Ramdani, M., Grimes, S., Benabdi, M., Souissi, J.B., Soufi, E., Khamassi, F., Ghanem, R., Ocaña, O., Ramos-Esplà, A., Izquierdo, A., Anton, I., Rubio-Portillo, E., Barbera, C., Cebrian, E., Marbà, N., Hendriks, I.E., Duarte, C.M., Deudero, S., Díaz, D., Vázquez-Luis, M., Alvarez, E., Hereu, B., Kersting, D.K., Gori, A., Viladrich, N., Sartoretto, S., Pairaud, I., Ruitton, S., Pergent, G., Pergent-Martini, C., Rouanet, E., Teixidó, N., Gattuso, J.-P., Fraschetti, S., Rivetti, I., Azzurro, E., Cerrano, C., Ponti, M., Turicchia, E., Bavestrello, G., Cattaneo-Vietti, R., Bo, M., Bertolino, M., Montefalcone, M., Chimienti, G., Grech, D., Rilov, G., Tuney Kizilkaya, I., Kizilkaya, Z., Eda Topçu, N., Gerovasileiou, V., Sini, M., Bakran-Petricioli, T., Kipson, S., Harmelin, J.G., (2019). Collaborative Database to Track Mass Mortality Events in the Mediterranean Sea. Frontiers in Marine Science 6, 707.

Garrabou, J., Gómez-Gras, D., Medrano, A., Cerrano, C., Ponti, M., Schlegel, R., ... & Harmelin, J. G. (2022). Marine heatwaves drive recurrent mass mortalities in the Mediterranean Sea. *Global Change Biology*, *28*(19), 5708-5725.

Gatti, G., Bianchi, C. N., Morri, C., Montefalcone, M., Sartoretto, S., & Parravicini, V. (2015). Coralligenous reefs state along anthropized coasts: Application and validation of the COARSE index, a synthetic approach based on a rapid visual assessment. *Ecological Indicators*, 52, 567-576.

Gattuso JP, Magnan A, Billé R, Cheung WW, Howes EL, Joos F, Allemand D, Bopp L, Cooley SR, Eakin CM, Hoegh-Guldberg O, Kelly RP, Pörtner HO, Rogers AD, Baxter JM, Laffoley D, Osborn D, Rankovic A, Rochette J, Sumaila UR, Treyer S, Turley C. OCEANOGRAPHY. Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. Science. (2015) Jul 3;349(6243):aac4722. doi: 10.1126/science.aac4722. PMID: 26138982.

Gómez-Lemos LA, Doropoulos C, Bayraktarov E, Diaz-Pulido G, (2018) Coralline algal metabolites induce settlement and mediate the inductive effect of epiphytic microbes on coral larvae. Sci Rep 8(1):17557.

Gori, A., Bavestrello, G., Grinyó, J., Dominguez-Carrió, C., Ambroso, S., Bo, M. (2017). Animal Forests in Deep Coastal Bottoms and Continental Shelf of the Mediterranean Sea. In: Rossi, S., Bramanti, L., Gori, A., Orejas , C. (eds) Marine Animal Forests. Springer, Cham. https://doi.org/10.1007/978-3-319-17001-5_5-2.

Guinotte, J. M., Buddemeier, R. W., & Kleypas, J. A. (2003). Future coral reef habitat marginality: Temporal and spatial effects of climate change in the Pacific basin. *Coral Reefs*, 22(4), 551-558.

Guiry MD, Cunningham EM (1984) Photoperiodic and temperature responses in the reproduction of north-eastern Atlantic *Gigartina acicularis* (Rhodophyta: Gigartinales). Phycologia 23(3):357–367.

Guntur, Luthfi O. M., & Asadi M. A. (2023). Recruitment of crustose coralline algae on tiles material for monitoring coral larvae settlement's consolidators at Nature Reserve Pulau Sempu, East Java, Indonesia. Brazilian Journal of Biology, 83, e245922. https://doi.org/10.1590/1519-6984.245922.

Halfar J et al (2013) Artic sea-ice decline archived by multicentury annual-resolution record from crustose coralline algal proxy. Proc Natl Acad Sci 110(49):19737–19741.

Halfar J, Steneck M, Joachimski A, Kronz A, Wanamaker AD Jr. (2008) Coralline algae as high-resolution climate recorders. Geology 36(6):463–466.

Harrington, L., Fabricius, K., De'Ath, G., & Negri, A. (2004). Recognition and selection of settlement substrata determine post-settlement survival in corals. *Ecology*, 85(12), 3428-3437.

Hereu, B., & Kersting, D. K. (2016). Diseases of coralline algae in the Mediterranean Sea. *Coral reefs*, *35*(2), 713-713.

Hereu, B., Matamalas, N., Aspillaga, E., Capdevila, P., & Rovira, G. (2018). Bleaching of crustose coralline alage in the Mediterranean Sea. In *3rd Mediterranean Symposium on the Conservation of Coralligenous and Other Calcareous Bio-Concretions* (pp. 63-68).

Hetzinger S, Grohganz M, Halfar J, Hathorne E, Ballesteros E, Kersting DK (2023). Elemental cycles in the coralline alga Neogoniolithon hauckii as a recorder of temperature variability in the Mediterranean Sea. Front Mar Sci 10:1151592.

Hoegh-Guldberg O, Bruno JF. The impact of climate change on the world's marine ecosystems. Science. (2010) Jun 18;328(5985):1523-8. doi: 10.1126/science.1189930. PMID: 20558709.

Holl, Karen & Aide, T. Mitchell. (2011). When and where to actively restore ecosystems? For Ecol Manag. Forest Ecology and Management - FOREST ECOL MANAGE. 261. 10.1016/j.foreco.2010.07.004.

Hong, J. S. (1980). Etude faunistique d'un fond de concrétionnement de typre coralligène soumis à un gradient de pollution en Méditerranée nord-occidentale (Golfe de Fos) (Doctoral dissertation).

Huggett, M. J., McMahon, K., & Bernasconi, R. (2018). Future warming and acidification result in multiple ecological impacts to a temperate coralline alga. *Environmental microbiology*, *20*(8), 2769-2782.

Hughes, T. P., Kerry, J. T., Álvarez-Noriega, M., Álvarez-Romero, J. G., Anderson, K. D., Baird, A. H., ... & Wilson, S. K. (2017). Global warming and recurrent mass bleaching of corals. *Nature*, *543*(7645), 373-377.

Hughes, T. P., Kerry, J. T., Baird, A. H., Connolly, S. R., Dietzel, A., Eakin, C. M., ... & Torda, G. (2018). Global warming transforms coral reef assemblages. *Nature*, *556*(7702), 492-496.

Hurd CL, Harrison PJ, Bischof K, Lobban CS (2014) Seaweed ecology and physiology. Cambridge University Press, USA.

Hwang EK, Kim EJ, Kim HG, Sohn CH (2002) Tetraspore release and growth of a crustose coralline alga, *Lithophyllum yessoense* (Rhodophyta, Corallinaceae). Kor J Fish Aquat Sci 35:242–246.

Hwang EK, Park CS, Baek JM (2007) Artificial seed production and cultivation of the edible brown alga, *Sargassum fulvellum* (Turner) C. Agardh: developing a new species for seaweed cultivation in Korea. In Eighteenth International Seaweed Symposium: Proceedings of the Eighteenth International Seaweed Symposium, held in Bergen, Norway, 20–25 June 2004 (pp. 25-31). Springer Netherlands.

Ichiki S, Mizuta H, Yamamoto H (2000) Effects of irradiance, water temperature and nutrients on the growth of sporelings of the crustose coralline alga *Lithophyllum yessoense* Foslie (Corallinales, Rhodophyceae). Phycol Res 48:115–120.

IPCC, 2023 (Intergovernmental Panel on Climate Change): Climate Change 2023: Synthesis Report. Sixth Assessment Report. IPCC, Geneva, Switzerland.

Irvine, L.M. & Chamberlain, Y.M. (1994). *Seaweeds of the British Isles, 1 (2B). Corallinales, Hildenbrandiales*. pp. vii + 276. London: HMSO.

ISPRA 2020. Monitoraggio e valutazione dello stato ecologico dell'habitat coralligeno. Il coralligeno di parete. Manuale e linea guida. https://www.isprambiente.gov.it/files2020/pubblicazioni/manuali-e-linee-guida/mlg_191_2020.pdf.

Jeong, S.Y., Nelson, W.A., Sutherland, J.E., Peña, V., Le Gall, L., Diaz-Pulido, Won, B.Y. & Cho, T.O. (2021). Corallinapetrales and Corallinapetraceae: a new order and family of coralline red algae including *Corallinapetra gabrielii* comb. nov. *Journal of Phycology* 57(3): 849-862.

Jones PL, Woelkerling WJ (1983) Some effects of light and temperature on growth and conceptacle production in *Fosliella cruciata* Bressan (Corallinaceae, Rhodophyta). Phycologia 22(4):449-452.

Jorissen, H., Galand, P. E., Bonnard, I., Meiling, S., Raviglione, D., Meistertzheim, A. L., ... & Nugues, M. M. (2021). Coral larval settlement preferences linked to crustose coralline algae with distinct chemical and microbial signatures. Scientific Reports, 11(1), 14610.

Kamenos NA, Cusack M, Moore PG (2008) Coralline algae are global palaeothermometers with bi–weekly resolution. Geochim Cosmochim Acta 72:771–779.

Kamenos NA, Cusack M, Huthwelker T, Lagarde P, Scheibling RE (2009) Mg-lattice associations in red coralline algae. Geochim Cosmochim Acta 73:1901–1907.

Kamenos NA, Law A (2010) Temperature controls on coralline algal skeletal growth. J Phycol 46:331–335.

Kamenos, NA, Perna, G., Gambi, M. C., Micheli, F., & Kroeker, K. J. (2013). Coralline algae in a naturally acidified ecosystem persist by maintaining control of skeletal mineralogy and size. *Proceedings of the Royal Society B: Biological Sciences*, 280(1773), 20131171.

Kennedy, E. V., Ordoñez, A., Lewis, B. E., & Diaz-Pulido, G. (2017). Comparison of recruitment tile materials for monitoring coralline algae responses to a changing climate. Marine Ecology Progress Series, 569, 129-144.

Krieger, E. C., Taise, A., Nelson, W. A., Grand, J., Le Ru, E., Davy, S. K., & Cornwall, C. E. (2023). Tolerance of coralline algae to ocean warming and marine heatwaves. PloS Climate, 2(1), e0000092.

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., ... & Gattuso, J. P. (2013). Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. *Global change biology*, *19*(6), 1884-1896.

Krumhansl, K. A., Okamoto, D. K., Rassweiler, A., Novak, M., Bolton, J. J., Cavanaugh, K. C., ... & Byrnes, J. E. (2016). Global patterns of kelp forest change over the past half-century. *Proceedings of the National Academy of Sciences*, *113*(48), 13785-13790.

Kuffner, I. B., Andersson, A. J., Jokiel, P. L., Rodgers, K. S., & Mackenzie, F. T. (2008). Decreased abundance of crustose coralline algae due to ocean acidification. *Nature Geoscience*, 1(2), 114-117.

Laborel, J. (1961). Le concrétionnement algal "coralligène" et son importance géomorphologique en Méditerranée. *Rec. Trav. Stat. Mar. Endoume*, *23*(37), 37-60.

Laborel, J. (1987). Marine biogenic constructions in the Mediterranean, a review. Sci. Rep. Port-Cros Nat. Park 13, 97–126.

Lau SC, Thiyagarajan V, Cheung SC, Qian PY (2005) Roles of bacterial community composition in biofilms as a mediator for larval settlement of three marine invertebrates. Aquat Microb Ecol 38(1):41-51.

Le Gall, L. & Saunders, G.W. (2007). A nuclear phylogeny of the Florideophyceae (Rhodophyta) inferred from combined EF2, small subunit and large subunit ribosomal DNA: establishing the new red algal subclass Corallinophycidae. *Molecular Phylogenetics and Evolution* 43: 1118-1130.

Leukart P (1994) Field and laboratory studies on depth dependence, seasonality and light requirement of growth in three species of crustose coralline algae (Rhodophyta, Corallinales). Phycologia 33:281–290.

Linares C, Coma R, Zabala M (2008) Restoration of threatened red gorgonian populations: an experimental and modelling approach. Biol Conserv 141(2):427-437.

Linares C., Coma R., Garrabou J., Bianchimani O., Drap, P., Serrano, E., & Zabala, M. (2009). Contribution to the conservation of coralligenous communities through studies on population ecology

of Mediterranean gorgonians. In Proceedings of the 1st Mediterranean symposium on the conservation of the coralligenous and other calcareous bio-concretions (pp. 107-112).

Littler M.M. and Littler D.S., (2013). The Nature of crustose Coralline Algae and their interactions on reefs. Smithsonian Contributions to the Marine, no. 39, pp. 199-212.

Littler, M. M., & Doty, M. S. (1975). Ecological components structuring the seaward edges of tropical Pacific reefs: The distribution, communities and productivity of Porolithon. *Journal of Ecology*, 63(4), 117-129.

Lively, J. A., & McKenzie, J. (2023). Discards and bycatch: A review of wasted fishing. *Advances in Marine Biology*, *95*, 1-26.

Mallela J (2013) Calcification by reef-building sclerobionts. PLOS ONE 8: e60010.

Mancini G., Ventura D., Casoli E., Belluscio A., Ardizzone G.D., Transplantation on a Posidonia oceanica meadow to facilitate its recovery after the Concordia shipwrecking, Marine Pollution Bulletin, Volume 179, (2022), 113683, ISSN 0025-326X, https://doi.org/10.1016/j.marpolbul.2022.113683.

Mancini, G., Ventura, D., Casoli, E., Michelangeli, F., Panarello, G., Belluscio, A., & Ardizzone, G. (2024). Posidonia oceanica (L.) Delile, 1813 seedlings as a potential integrative tool for restoring damaged meadows. *Restoration Ecology*, *32*(5), e14156.

Martin S, Gattuso JP (2009) Response of Mediterranean coralline algae to ocean acidification and elevated temperature. Glob Change Biol 15(8):2089-2100.

Martin S, Charnoz A, Gattuso JP (2013a) Photosynthesis, respiration and calcification in the Mediterranean crustose coralline alga *Lithophyllum cabiochae* (Corallinales, Rhodophyta). European J Phycol 48:163–172.

Martin S, Cohu S,Vignot C, Zimmerman G, Gattuso JP (2013b) One-year experiment on the physiological response of the Mediterranean crustose coralline alga, *Lithophyllum cabiochae*, to elevated pCO2 and temperature. Ecol Evol 3:676–693.

Martin, C. S., Giannoulaki, M., De Leo, F., Scardi, M., Salomidi, M., Knitweiss, L., ... & Fraschetti, S. (2014). Coralligenous and maërl habitats: Predictive modelling to identify their spatial distributions across the Mediterranean Sea. *Scientific Reports*, 4, 5073.

Martin S, Calvert V, Chenuil A (2024) Physiological differences among cryptic species of the Mediterranean crustose coralline alga *Lithophyllum stictiforme* (Corallinales, Rhodophyta). Eur J Phycol 59(2):184–195.

McCoy, S. J., & Kamenos, N. A. (2015). Coralline algae (Rhodophyta) in a changing world: Integrating ecological, physiological, and geochemical responses to global change. *Journal of Phycology*, 51(1), 6-24.

McCoy SJ, Pueschel CM, Cornwall CE, Comeau S, Kranz SA, Spindel NB, Borowitzka MA (2023) Calcification in the coralline red algae: a synthesis. Phycologia 62:648-666.

Moberly R (1968). Composition of magnesian calcites of algae and pelecypods by electron microprobe analysis. Sedimentology 11 (1–2): 61–82

Montefalcone M. Challenges in Restoring Mediterranean Seagrass Ecosystems in the Anthropocene. *Environments*. (2024); 11(5):86. https://doi.org/10.3390/environments11050086.

Montero-Serra I, Garrabou J, Doak DF, Figuerola L, Hereu B, Ledoux J-B, and Linares C (2017) Accounting for life-history strategies and timescales in marine restoration. Conservation Letters 11. https://doi.org/10.1111/conl.12341.

Montseny, M., Linares, C., Viladrich, N., Olariaga, A., Carreras, M., Palomeras, N., ... & Gori, A. (2019). First attempts towards the restoration of gorgonian populations on the Mediterranean continental shelf. Aquatic Conservation: Marine and Freshwater Ecosystems, 29(8), 1278-1284.

Mucci A, Morse JW (1985). Auger-spectroscopy determination of the surface-most adsorbed layer composition on aragonite, calcite, dolomite, and magnesite in synthetic seawater. Am J Sci 285: 306–317.

Nash MC, Martin **S**, Gattuso JP (2016) Mineralogical response of the Mediterranean crustose coralline algae *Lithophyllum cabiochae* to near-future ocean acidification and warming. BG 13:5937–5945.

Nash MC, Adey W (2017) Multiple phases of mg-calcite in crustose coralline algae suggest caution for temperature proxy and ocean acidification assessment: Lessons from the ultrastructure and biomineralization in *Phymatolithon* (Rhodophyta, Corallinales). J Phycol 53:970–984.

Nash MC, Diaz-Pulido G, Harvey AS, Adey W (2019) Coralline algal calcification: A morphological and process-based understanding. PloS One 14(9):e0221396.

Nelson W. A. (2009). Calcified macroalgae–critical to coastal ecosystems and vulnerable to change: a review. *Marine and Freshwater Research*, *60*(8), 787-801.

Nelson, W. A., Sutherland, J. E., Farr, T. J., Hart, D. R., Neill, K. F., Kim, H. J., & Yoon, H. S. (2015). Multi-gene phylogenetic analyses of New Zealand coralline algae: Corallinapetra Novaezelandiae gen. et sp. nov. and recognition of the Hapalidiales ord. nov. *Journal of Phycology*, *51*(3), 454-468.

o'Leary, J. K., Barry, J. P., Gabrielson, P. W., Rogers-Bennett, L., Potts, D. C., Palumbi, S. R., & Micheli, F. (2017). Calcifying algae maintain settlement cues to larval abalone following algal exposure to extreme ocean acidification. Scientific reports, 7(1), 5774.

Ordoñez A, Kennedy EV, Diaz-Pulido G (2017) Reduced spore germination explains sensitivity of reef-building algae to climate change stressors. PloS One 12:e0189122.

Orejas, C., Carreiro-Silva, M., Mohn, C., Reimer, J., Samaai, T., Allcock, A. L., & Rossi, S. (2022). Marine animal forests of the world: definition and characteristics. *Research Ideas and Outcomes*, *8*, e96274.

Padilla-Gamiño, J. L., Gaitán-Espitia, J. D., Kelly, M. W., & Hofmann, G. E. (2016). Physiological plasticity and local adaptation to elevated pCO 2 in calcareous algae: an ontogenetic and geographic approach. *Evolutionary applications*, *9*(9), 1043-1053.

Page, T. M., & Diaz- Pulido, G. (2020). Plasticity of adult coralline algae to prolonged increased temperature and pCO2 exposure but reduced survival in their first generation. PLoS One, 15(6), e0235125. https://doi.org/10.1371/journal.pone.0235125.

Pang, S. J., Liu, F., Shan, T. F., Gao, S. Q., & Zhang, Z. H. (2009). Cultivation of the brown alga Sargassum horneri: sexual reproduction and seedling production in tank culture under reduced solar irradiance in ambient temperature. Journal of Applied Phycology, 21, 413-422.

Peña, V., De Clerck, O., Afonso-Carrillo, J., Ballesteros, E., Bárbara, I., Barreiro, R. & Le Gall, L. (2015). An integrative systematic approach to species diversity and distribution in the genus *Mesophyllum* (Corallinales, Rhodophyta) in Atlantic and Mediterranean Europe. *European Journal of Phycology* 50(1): 20-36.

Peña, V., Harvey, B. P., Agostini, S., Porzio, L., Milazzo, M., Horta, P., ... & Hall-Spencer, J. M. (2021). Major loss of coralline algal diversity in response to ocean acidification. *Global Change Biology*, *27*(19), 4785-4798.

Pérès, J. M., & Picard, J. (1964). *Nouveau manuel de bionomie benthique de la mer Méditerranée*. Station Marine d'Endoume.

Pergent-Martini C., André S., Castejon I., Deter J., Frau F., Gerakaris V., Mancini G., Molenaar H., Montefalcone M., Oprandi A., Pergent G., Poursanidis D., Royo L., Terrados J., Tomasello A., Ventura D., Villers F., (2024). Guidelines for *Posidonia oceanica* restoration. Report Cooperation agreement Mediterranean Posidonia Network (MPN), French Biodiversity Agency (OFB) & University of Corsica Pasquale Paoli (UCPP) N°OFB-22-1310: 29 p. + Appendices.

Pezzolesi, L., Peña, V., Le Gall, L., Gabrielson, P.W., Kaleb, S., Hughey, J.R., Rodondi, G., Hernandez-Kantun, J.J., Felace, A., Basso, D., Cerrano, C. & Rindi, F. (2019). Mediterranean *Lithophyllum stictiforme* (Corallinales, Rhodophyta) ia a genetically diverse species complex: implications for species circumscription, biogeography and conservation of coralligenous habitats. *Journal of Phycology* 55(2): 473-492.

Piazzi, L., Balata, D., Pertusati, M., Cinelli, F., (2004). Spatial and temporal variability of Mediterranean macroalgal coralligenous assemblages in relation to habitat and substratum inclination. Botanica Marina 47, 105-115.

Piazzi, L., Gennaro, P., Balata, D., (2011). Effects of nutrient enrichment on macroalgal coralligenous assemblages. Marine Pollution Bulletin 62, 1830-1835.

Piazzi, L., La Manna, G., Cecchi, E., Serena, F., Ceccherelli, G., (2016). Protection changes the relevancy of scales of variability in coralligenous assemblages. Estuarine, Coastal and Shelf Science 175, 62-69.

Piazzi, L., Gennaro, P., Cecchi, E., Serena, F., Bianchi, C.N., Morri, C., Montefalcone, M., (2017). Integration of ESCA index through the use of sessile invertebrates. Scientia Marina 81, 283-290.

Piazzi, L., Cecchi, E., Cinti, M.F., Stipcich, P., Ceccherelli, G., (2019a). Impact assessment of fish cages on coralligenous reefs: an opportunity to use the STAR sampling procedure. Mediterranean Marine Science 20, 627-635.

Piazzi, L., Gennaro, P., Montefalcone, M., Bianchi, C.N., Cecchi, E., Morri, C., Serena, F., (2019b). STAR: an integrated and standardized procedure to evaluate the ecological status of coralligenous reefs. Aquatic Conservation: Marine and Freshwater Ecosysistems 29, 189-201.

Piazzi L, Ceccherelli G (2020) Alpha and beta diversity in Mediterranean macroalgal assemblages: relevancy and type of effect of anthropogenic stressors vs natural variability. Mar Biol 167(3): 32.

Piazzi, L., Cinti, M. F., Guala, I., Grech, D., La Manna, G., Pansini, A., et al. (2021). Variations in coralligenous assemblages from local to biogeographic spatial scale. Mar. Environ. Res. 169, 105375. doi: 10.1016/j.marenvres.2021.105375.

Piazzi, L., Gennaro, P., Cecchi, E., Bianchi, C.N., Cinti, F., Gatti, G., Guala, I., Morri, C., Sartoretto, F., Serena, F., Montefalcone, M., (2021a). Ecological Status of Coralligenous Assemblages: ten years of application of the ESCA index from local to wide scale validation. Ecological Indicators 121, 107077.

Piazzi, L., M.F. Cinti, I. Guala, D. Grech, G. La Manna, A. Pansini, F. Pinna, P. Stipcich, G. Ceccherelli (2021b). Variations in coralligenous assemblages from local to biogeographic spatial scale. Marine Environmental research 169, 105375.

Piazzi L, Pinna F, Ceccherelli G (2022) Crustose coralline algae and biodiversity enhancement: The role of *Lithophyllum stictiforme* in structuring Mediterranean coralligenous reefs. Estuar, Coast Shelf Sci 278:108121.

Pinna F, Caragnano A, Piazzi L, Ragazzola F, Stipcich P, Rindi F, Ceccherelli G (2022) The Mediterranean bioconstructor *Lithophyllum stictiforme* shows adaptability to future warming. Front Mar Sci 9:930750.

Ponti, M., Fava, F., Abbiati, M., (2011). Spatial-temporal variability of epibenthic assemblages on subtidal biogenic reefs in the northern Adriatic Sea. Marine Biology 158, 1447-1459.

Ponti, M., Perlini, R.A., Ventra, V., Grech, D., Abbiati, M., Cerrano, C., (2018). Ecological shifts in Mediterranean coralligenous assemblages related to gorgonian forest loss. PLoS One 9. 9, e102782.

Ponti, M., Turicchia, E., Ferro, F., Cerrano, C., & Abbiati, M., (2014). The influence of the spatial scale of observation on the detection of human impacts on coralligenous assemblages. *Marine Environmental Research*, 101, 29-36.

Ragazzola F, Caragnano A, Basso D, Schmidt Daniela N, Fietzke J (2020). Establishing temperate crustose early Holocene coralline algae as archives for paleoenvironmental reconstructions of the shallow water habitats of the Mediterranean Sea. Palaeontology 63:155–170.

Regione Autonoma della Sardegna (2016). Riesame e aggiornamento del piano di gestione del distretto idrografico della Sardegna. Monitoraggio e classificazione delle acque marino-costiere. Monitoraggio e classificazione delle acque superficiali, 121– 1148.

Richards, J.L., Gabrielson, P.W., Fredericq, S., (2014). New insights into the genus Lithophyllum (Lithophylloideae, Corallinaceae, Corallinales) from deepwater rhodolith beds offshore the NW Gulf of Mexico. Phytotaxa 190, 162–175. https:// doi.org/10.11646/phytotaxa.190.1.11.

Rindi, F., Braga, J. C., Martin, S., Peña, V., Le Gall, L., Caragnano, A., & Aguirre, J. (2019). Coralline algae in a changing Mediterranean Sea: how can we predict their future, if we do not know their present? Frontiers in Marine Science, 6, 723.

Rindi, F., Falace, A., Moro, I., Ivesa, L., Caragnano, A., & Sfriso, A. (2019). Diversity patterns and long-term changes in the benthic macroalgal vegetation of the Northern Adriatic Sea. EUROPEAN JOURNAL OF PHYCOLOGY, 54, 98-98.

Rodríguez-Prieto C (2016) Light and temperature requirements for survival, growth and reproduction of the crustose coralline *Lithophyllum stictaeforme* from the Mediterranean Sea. Bot Mar 59(2-3):95-104.

Rosenthal Y, Boyle EA, Slowey N (1997). Temperature control on the incorporation of magnesium, strontium, fluorine, and cadmium into benthic foraminiferal shells from Little Bahama Bank: prospects for thermocline paleoceanography. Geochim Cosmochim Acta 61 (17):3633–3643.

Rossi, S., Rizzo, L., Di Camillo, C., Gambi, C., Gili, J. M., & Bavestrello, G. (2017). Animal forests of the world: An overview. *Marine Animal Forests*, 1-28.

Sartoretto S, Verlaque M, Laborel J (1996) Age of settlement and accumulation rate of submarine "coralligène"(− 10 to− 60 m) of the northwestern Mediterranean Sea; relation to Holocene rise in sea level. Mar Geol 130(3-4):317-331.

Sartoretto, S., Schohn, T., Bianchi, C. N., Morri, C., Garrabou, J., Ballesteros, E., ... & Gatti, G. (2017). An integrated method to evaluate and monitor the conservation state of coralligenous habitats: The INDEX-COR approach. Marine pollution bulletin, 120(1-2), 222-231.

Seabra M, Cruz T, Fernandes J, Silva T, Hawkins S, (2019) Recruitment of the limpet *Patella ulyssiponensis* and its relationship with crustose coralline algae: Patterns of juvenile distribution and larval settlement. J Mar Biol Assoc UK 99(8):1787-1796.

Siboni, N., Abrego, D., Puill-Stephan, E., King, W. L., Bourne, D. G., Raina, J. B., ... & Harder, T. (2020). Crustose coralline algae that promote coral larval settlement harbor distinct surface bacterial communities. Coral Reefs, 39, 1703-1713.

Silva, S., Grande, U., Rendina, F., Buonocore, E., Guidato, M., Contegiacomo, M., & Franzese, P. P. (2024). The scientific literature on coralligenous habitat and fishing impacts . *Ecological Questions*, *35*(1), 1–18. https://doi.org/10.12775/EQ.2024.010.

Sletten HR, Gillikin DP, Halfar J, Andrus CFT, Guzmán HM (2017) Skeletal growth controls on Mg/Ca and P/Ca ratios in tropical Eastern Pacific rhodoliths (coralline red algae). Chem Geol 465:1–10.

Song JN, Park SK, Heo JS et al (2013a) Effects of temperature on the spore release and growth of *Lithophyllum yessoense* and *Hildenbrandia rubra*. Kor J Fish Aquat Sci 46:296–302.

Song JN, Park SK, Oh JC et al (2013b) The effects of environmental factors on the growth of *Lithophyllum yessoense* and *Hildenbrandia rubra* sporelings in laboratory culture. Kor J Fish Aquat Sci 46:827–834.

Squires, D., Ballance, L. T., Dagorn, L., Dutton, P. H., & Lent, R. (2021). Mitigating bycatch: novel insights to multidisciplinary approaches. Frontiers in Marine Science, 8, 613285.

Steneck, R. S. (1986). The ecology of coralline algal crusts: convergent patterns and adaptative strategies. *Annual review of ecology and systematics*, *17*(1), 273-303.

Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution:30 2725-2729.

Thierry de Ville d'Avray, L., Ami, D., Chenuil, A., David, R., Feral, J.P., (2019). Application of the ecosystem service concept at a small-scale: the cases of coralligenous habitats in the North-western Mediterranean Sea. Marine Pollution Bulletin 138, 160-170.

UNEP, 2017. Action Plan for the Conservation of the Coralligenous and Other Calcareous Bio-Concretions in the Mediterranean Sea. UNEP/MAP Athens, GR.

Ulrich RN, Guillermic M, Campbell J, Hakim A, Han R, Singh S, Eagle RA (2021). Patterns of element incorporation in calcium carbonate biominerals recapitulate phylogeny for a diverse range of marine calcifiers. Front Earth Sci 9: 641760.

Valisano L, Palma M, Pantaleo U, Calcinai B, Cerrano C. Characterization of North-Western Mediterranean coralligenous assemblages by video surveys and evaluation of their structural complexity. Mar Pollut Bull. (2019) Nov;148:134-148. doi: 10.1016/j.marpolbul.2019.07.012. Epub 2019 Aug 20. PMID: 31442852.

van der Heijden L, Kamenos N (2015) Reviews and syntheses: Calculating the global contribution of coralline algae to total carbon burial. BG 12:6429-6441.

Verdura J, Sales M, Ballesteros E, Cefalì ME, Cebrian E (2018) Restoration of a canopy-forming alga based on recruitment enhancement: methods and long-term success assessment. Front Plant Sci 9:1832.

Verdura, J., Linares, C., Ballesteros, E., Coma, R., Uriz, M.J., Bensoussan, N., Cebrian, E., (2019). Biodiversity loss in a Mediterranean ecosystem due to an extreme warming event unveils the role of an engineering gorgonian species. Scientific Reports. 9, 5911.

Villas-Bôas AB, Figueiredo MAdO, Villaça RC (2005). Colonisation and growth of crustose coralline algae (Corallinales, Rhodophyta) on the Rocas Atoll. Braz J Oceanogr 53: 147−156.

Villas-Boas, A. B., Tâmega, F. T. S., Figueiredo, M. A. O., & Coutinho, R. (2023). Photosynthetic responses of crustose coralline algae recruit from an upwelling area to light intensity, temperature and current flow rate in a mesocosm. *Marine Environmental Research*, *190*, 106118.

Virgilio, M., Airoldi, L., Abbiati, M., (2006). Spatial and temporal variations of assemblages in a Mediterranean coralligenous reef and relationships with surface orientation. Coral Reefs 25, 265-272.

Von Stosch HA (1963) Wirkungen von Jod und Arsenit auf Meeresalgen in Kultur. Proc Int Seaweed Symp 4:142–50.

Wernberg, T., Bennett, S., Babcock, R. C., De Bettignies, T., Cure, K., Depczynski, M., ... & Wilson, S. (2016). Climate-driven regime shift of a temperate marine ecosystem. *Science*, *353*(6295), 169-172.

Woelkerling, W. J., Chamberlain, Y. M. & Silva, P. C. (1985). A taxonomic and nomenclatural reassessment of *Tenarea*, *Titanoderma* and *Dermatolithon* (Corallinaceae, Rhodophyta) based on studies of type and other critical specimens. *Phycologia* 24:317-37.

Yan S-H, Wang X. L., Xia B.M., Wang G.C., A multiscale analysis of coralline algae Lithophylloideae (Corallinophycidae, Rhodophyta) shedding new light on understanding cryptic diversity. Molecular Phylogenetics and Evolution.Volume 199, (2024), 108140. ISSN 1055-7903. https://doi.org/10.1016/j.ympev.2024.108140.

Yoon, H. S., Hackett, J. D. & Bhattacharya, D. A single origin of the peridinin- and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis. *Proc. Nat. Acad. Sci. USA* **99,** 11724–11729 (2002).

Yoon JT, Sun SM, Chung G (2014) Sargassum bed restoration by transplantation of germlings grown under protective mesh cage. J Appl Phycol 26:505-509.

Yoshioka S, Kato A, Koike K et al (2020) Effects of water temperature, light and nitrate on the growth of sporelings of the non-geniculate coralline alga *Lithophyllum okamurae* (Corallinales, Rhodophyta). J Appl Phycol 32:1923–1931.

Yu Y.Q., Zhang Q.S., Tang Y.Z., Zhang S.B., Lu Z.C., Chu S.C., Tang X.X. (2012) Establishment of intertidal seaweed beds of *Sargassum thunbergii* through habitat creation and germling seeding. Ecol Eng 44:10-17.

Zarranz, Maite E., González-Henríquez, Nieves, García-Jiménez, Pilar and Robaina, Rafael R.. "Restoration of *Cymodocea nodosa* (Uchria) Ascherson seagrass meadows through seed propagation: seed storage and influences of plant hormones and mineral nutrients on seedling growth *in vitro*" *Botanica Marina*, vol. 53, no. 5, (2010), pp. 439-448. https://doi.org/10.1515/bot.2010.051.

Zelli, E, Quéré, G, Lago, N, Di Franco, G, Costantini, F, Rossi, S, Bramanti L (2020). Settlement dynamics and recruitment responses of Mediterranean gorgonians larvae to different crustose coralline algae species. J Exp Mar Biol Ecol 530:151427.