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INTERVENTO DI RESTAURO DI *GONGOLARIA BARBATA* IN UN SITO CARATTERIZZATO DA FORTE PRESSIONE DI GRAZING, LUNGO LA COSTA DEL MAR ADRIATICO CENTRALE (SIN, FALCONARA MARITTIMA)

GONGOLARIA BARBATA RESTORATION IN A SITE CHARACTERIZED BY STRONG GRAZING PRESSURE, ALONG THE COAST OF THE CENTRAL ADRIATIC SEA (SNI, FALCONARA MARITTIMA)

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RIASSUNTO

L'incessante declino su scala globale delle foreste di alghe brune provoca profondi cambiamenti agli ecosistemi marini di cui fanno parte, portando alla perdita di biodiversità e al conseguente abbassamento della qualità ambientale. Sono state sviluppate differenti tecniche per intraprendere interventi di restauro su larga scala, soprattutto nel Mar Mediterraneo. In questo lavoro di tesi è stato testato, per un periodo di sei mesi, il restauro di *Gongolaria barbata* sulle barriere frangiflutti del SIN (Sito di Interesse Nazionale) di Falconara Marittima (Ancona), con l'utilizzo di gabbie per l'esclusione dei *grazers*. Sono state trapiantate al SIN, dopo il reclutamento *in situ* avvenuto alla Piscinetta del Passetto, tre strutture su cui erano cresciuti giovanili di *G. barbata*, e sono state posizionate all'interno di apposite gabbie con maglia da 12 mm. Parallelamente al monitoraggio della crescita delle alghe, è stata condotta l'analisi della biodiversità associata (meio- e macrofauna) e un esperimento in acquario per testare la pressione dovuta all'erbivoria su *G. barbata* da parte del granchio comune (*Pachigrapsus marmoratus*). I risultati di questo studio hanno dimostrato l'efficacia delle gabbie per la sopravvivenza dei giovanili delle alghe, nonostante l'influenza delle mareggiate che hanno caratterizzato il periodo invernale. In particolare, fatta eccezione per la struttura 3 che è stata colpita da una forte mareggiata, gli altri individui di *G. barbata* sono cresciuti

nel tempo rispondendo positivamente anche all’apertura e alla completa rimozione delle gabbie avvenuta alla fine dell’esperimento, a causa di una mareggiata. Per quanto riguarda la biodiversità associata, invece, è stata riscontrata una risposta in termini di ricchezza di taxa sia nella meio- che nella macrofauna, e nella presenza/assenza di taxa rari nella meiofauna. Questo suggerisce che il restauro di *G. barbata* può avere un effetto positivo sulla biodiversità, ma è necessario prolungare i monitoraggi nel tempo per corroborare i dati ottenuti. I risultati dell’esperimento in acquario, invece, hanno dimostrato che *P. marmoratus* può essere considerato uno dei principali *grazers* di *G. barbata* sulle barriere frangiflutti, specialmente in aree caratterizzate dall’assenza di pesci erbivori.

In conclusione, un elevato numero di individui di macroalghe e una maggiore densità algale dovrebbero essere punti chiave di un intervento di restauro su substrati artificiali, in modo da controbilanciare la pressione di *grazing* e promuovere anche l'aumento dell'abbondanza, biomassa e diversità della fauna associata.

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1. INTRODUCTION

1.1 Ecological importance of *Cystoseria s.l.* species

The taxonomic group *Cystoseira sensu latu* refers to the genera *Cystoseira*, *Ericaria,* and *Gongolaria* (Molinari and Guiry, 2020), which are brown algae (Phaeophyceae) belonging to the order Fucales. Together with the genus Sargassum, these macroalgae are important habitat-forming species. They live from the surface to the upper circalittoral zone and down to 100 meters depth (Jódar-Pérez et al., 2020), along the rocky coasts of the Mediterranean Sea (Gianni et al., 2013), and possess large canopies that create a three-dimensional habitat, a source of enrichment of environmental complexity.

Cystoseira s.l. species are photosynthetic organisms, producing oxygen and increasing coastal primary productivity (Gianni & Mangialajo, 2013), providing food and shelter to many associated organisms. Epiphytic algae, sessile and vagile invertebrates find shelter among the macroalgae forest and the juveniles of different species of fish, also of commercial interest. Brown algae, therefore, play an important role in providing reproductive and nursery habitats, helping to increase the density of coastal fish assembly (Orlando-Bonaca et al., 2021). In addition, they are involved in the biogeochemical cycles, export organic matter to other systems, and regulate water quality, also bringing benefits to ecotourism (Bianchelli & Danovaro, 2020).

Macroalgae forests can also affect the surrounding habitat by modifying the physical factors related to it, such as the intensity of light, the movement of water, and the amount of substrate that can be swept away by the movement of their fronds (Bulleri et al., 2001). Accordingly, the *Cystoseira s.l.* species can also influence the recruitment rates and post-settlement mortality of the larvae of different organisms, such as other algae or barnacle crustaceans (Bulleri et al., 2001). In addition, several studies confirmed that many species of *Cystoseira s.l.* can produce secondary metabolites with important antioxidant, anti-inflammatory, antifungal, antiviral and antibacterial activities, which could be exploited in medicine (Orlando-Bonaca et al., 2021).

These macrophytes play a fundamental role in the supply of ecosystem goods and services, and therefore in maintaining high biodiversity that is strongly linked to the proper functioning of shallow rocky habitats (Tamburello et al., 2021). Indeed, it has been shown that habitat-forming species, such as those belonging to the *Cystoseira s.l.* group, can determine the vertical distribution pattern of seaweed assemblages in coastal environments (Bulleri et al., 2001). A study carried out in the North-West Mediterranean has shown that, following the removal of *Cystoseira* from coastal areas, a homogenization of coastal habitats occurred at lower and greater depths, with a drastic loss of biodiversity (Bulleri et al., 2001). The absence of *Cystoseira* is also related to the disappearance of red encrusting algae, sponges, *Vermetus sp*., anemones, and bryozoans, which are replaced by algal turfs typical of shallow rocky environments (Bulleri et al., 2001).

The ecological importance of these macroalgae is impaired by their decline, which has increased considerably in recent years due to the numerous anthropogenic threats. Therefore, since 2009, all species of the group *Cystoseira s.l.* (except for *C. compressa*) are included in the list of endangered or threatened Mediterranean marine species of the Barcelona Protocol (Annex II) and Annex I of the Bern Convention (https://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2009:0585:FIN:IT:PDF). In addition, all *Cystoseira* species are considered "Habitats of Community Interest by the EU (Habitat reef-code 1170 Annex I)" under the Habitats Directive; the IUCN (International Union for the Conservation of Nature), the RAC/SPA (Regional Activity Centre for Specially Protected Areas established under the Barcelona Convention) and the Medpan (Mediterranean network of Marine Protected Areas) keep these species under close surveillance (Orlando-Bonaca et al., 2021).

The *Cystoseira s.l.* species, because of their restricted habitat and low tolerance to pollution, are excellent bioindicators; for this reason, in the context of the

Water Framework Directive (2000/60/EU) are used to determine the ecological quality of the coast (CARLIT Index) (Orlando-Bonaca et al., 2021).

1.2 Main threats and degradation state of *Cystoseria s.l.* and *Gongolaria barbata* forests in the Mediterranean Sea

The human-induced threats to macroalgae forests in the Mediterranean Sea are numerous and act from a local to a global scale.

Locally, one of the main stress sources is coastal urbanization, which modifies environmental characteristics and is responsible for the destruction of many marine habitats. In addition, coastal development determines nutrient intake, contaminants, and sediment loads at sea (Gianni et al, 2013). As a consequence, an increase in eutrophication, point sources of pollution such as oil spills, detergents, and vegetative paints (Airoldi et al., 2014), as well as effluents from aquaculture facilities, agricultural and industrial activities (Tamburello et al., 2021) can be detected in most of the coastal ecosystems. The increase in turbidity and pollution, which results from the increase of all these substances at sea, endangers coastal ecosystems and, among them, macroalgae forests, which prefer oligotrophic environments and transparent waters.

Another important threat for the *Cystoseira s.l.* species is the excessive and illegal fishing, in particular targeting the species belonging to the highest trophic levels, because it determines trophic cascades, leading to an increase of the grazers (Airoldi et al., 2014). Invasive animal and plant species represent another source of stress for macroalgae forests (Tamburello et al., 2021), with which they can compete for space, may become grazers/predators, or change the environmental and ecological conditions of the habitats in which the macroalgae live.

The abovementioned threats overlap with the wider stress induced by climate change, which is causing the rise of the sea temperature and increasing the acidification of waters (Bianchelli & Danovaro, 2020).

The *Cystoseira s.l.* species present their greatest diversity in the Mediterranean Sea, which hosts one-third of all the species of the group described so far (Jódar-Pérez et al., 2020); however, this and the associated great biodiversity is threatened and in the last years a dramatic loss of 50%, and up to >80%, of *Cystoseira* forests has been observed (Danovaro, unpublished data). Along the coasts of many states bordering the Mediterranean, such as Spain, France, Italy, Croatia, Albania, Greece, and Turkey, huge losses of Fucales have been documented, and in some cases also the local extinction of some species (Airoldi et al., 2014). In the coast of Albères, in France, of the 14 species of

Fucales present in 1912, only 5 were recorded in 2003 (Thibaut et al., 2005); in Catalonia, species like *Carpodesmia crinita, Gongolaria elegans, Treptacanta ballesterosii var. tenuior, Gongolaria sauvageauana,* and *Gongolaria barbata*, have restricted their habitat to a few selected sites, mainly due to the destruction of the coast caused by urbanization (Mariani et al., 2019). Along the coasts of eight Italian regions, 371 populations of 19 different species of *Cystoseira s.l.,* for many of which the causes are unknown, are locally extinct since the 2000s (Tamburello et al., 2021). In some areas of the Gulf of Naples, 7 out of 15 species of *Cystoseira s.l.* disappeared and a clear decline of the remaining ones has been recorded (Grech, 2017). Species such as *Cystoseira compressa, Cystoseira dubia, Cystoseira foeniculacea, Cystoseira platyclada, Cystoseira amentacea, Cystoseira brachycarpa, Cystoseira crinita, Cystoseira mediterranea, Cystoseira zosteroides, G. barbata, G. elegans, G. sauvageauana* have become extinct in some Italian islands, also where marine protected areas are in force (Punta Campanella, Regno di Nettuno, Porto Cesareo, Egadi Islands, Pelagie, Cyclops and Tremiti) (Tamburello et al., 2021).

In particular, between 1879 and 1999, 187 populations of the species *G. barbata* were surveyed in 10 Italian regions and all the seas of the peninsula; in the last years, the populations have been reduced to 107 in 6 regions, exclusively of the central and southern Tyrrhenian, Sardinia, the Adriatic and the North Ionian Sea (Tamburello et al., 2021). The extinctions were detected between 1979 and 2016 for 40 populations in Campania, Puglia, Sicily, Marche, and Veneto (Tamburello et al., 2021). Along the Conero Riviera, in the Marche region, despite the species is still well diffused in some sites (Rindi et al., 2020), regression has been recorded probably due to several climatic and anthropogenic pressures, such as extreme storm events, extraction of rocks (Perkol-Finkel and Airoldi, 2010), trampling and swimming due to summer tourism (Rindi, personal observation).

Due to the increased state of forests' degradation of *Cystoseira s.l.*, in recent years the percentage of protected populations has increased by 77.8%, and most of these are included in national parks, marine protected areas, or sites of Natura 2000 or SPAMIs (Special Protected Areas of Mediterranean Importance, *sensu* Protocol concerning Specially Protected Areas and Biological Diversity in the Mediterranean - SPA/BD Protocol) (Tamburello et al., 2021). However, this does not ensure their safety, as in these areas the regulations are very often limited and there are no management and monitoring plans, making poor protection of *Cystoseira s.l.* forests (Airoldi et al., 2014).

1.3 Restoration as a solution to forests' loss

The decline of the macroalgae forests leads to biodiversity and ecosystem functioning loss of the affected area (Bianchelli & Danovaro, 2020; Medrano et al., 2020). Their disappearance induces the formation of less structural complexity environments, consisting of algal turfs, filamentous or ephemeral algae, mussels' belts, or barren grounds (Airoldi et al., 2014). These ecosystem's alternative states are persistent and make difficult the macroalgae forests recovery, even after the cessation of the stress sources that triggered the decline (Airoldi et al., 2014). Therefore, the conservation and restoration of these species are the best solutions to counteract the loss of *Cystoseira s.l.* species (Fraschetti et al., 2021). However, in many degraded areas, the natural recovery of forests is made difficult by the low dispersion potential of the Fucales zygotes (Orlando-Bonaca et al., 2021).

The restoration effort of *Cystoseira s.l.* in the Mediterranean Sea can be carried out through different techniques, which must be chosen according to the species and the habitat to be restored (Gianni et al., 2013, Cebrian et al., 2021). The first method is the transplantation of adult thalli from healthy macroalgae forests, fixed at the desired site. In this case, different artificial supports can be used on which the thalli are attached by epoxy glue, polyurethane foam, or hooks (Gianni et al., 2013; Orlando-Bonaca et al., 2021). The same method can be performed using rock fragments covered by an adult or juvenile thalli, which are transferred and attached to the substrate of the site to be restored by epoxy glue (S. Perkol-Finkel and L. Airoldi, 2012, Gianni et al., 2013). These techniques have been tested in different parts of the Mediterranean Sea and with different species of macroalgae, such as *G. barbata, C. crinita, C. amentacea, C. compressa, and C. foeniculacea f. tenuiramosa*, always obtaining a transplant success of more than 30% (Gianni et al., 2013). However, the approach based on adult individuals is nowadays strongly disputed, since healthy populations and their habitat (in this case the rocky substrate) should not be exploited or destroyed (Gianni et al., 2013, Cebrian et al., 2021).

Another restoration method is based on the use of gametes and/or algal zygotes, which are collected on artificial substrates of different materials and grown *in situ*, or *ex-situ* (recruits grown in the laboratory and then transplanted to the sea) (Verdura et al., 2018). The recruitment can be done through the positioning of artificial support in areas with high settlement potential (*in situ* recruitment), or in the laboratory through the use of mature receptacles placed on appropriate structures to collect the zygotes (*ex situ* recruitment) (Gianni et al., 2013). An alternative method is to place mature receptacles directly on rocky substrate at sea to encourage recruitment (Orlando-Bonaca et al., 2021).

Even in this case, the success has been demonstrated by numerous restoration experiments of different *Cystoseira s.l.* species. For example, in 2018, a *G. barbata* forest was successfully restored in Menorca (Spain) through *in situ* and *ex situ* techniques, which proved to be comparable in terms of intervention success (Verdura et al., 2018). In Slovenia, in 2019, was tried by Orlando-Bonaca et al. to restore *G. barbata* through *ex situ* laboratory cultivation, with the use of clay tails for the collection of zygotes. The structures with the tails were then transferred to the sea after 20 days of growth in the laboratory and protected inside cages to exclude the grazing fish; the survival overtime of the *G. barbata* juveniles on the clay tails was very high (89%) (Orlando-Bonaca et al., 2021).

Fucales forests restoration can be carried out in degraded habitats where historically macroalgae were present, but also in areas where they have never been recorded, to increase the area's ecological value (Gianni et al., 2013). Coastal infrastructures, such as breakwater barriers, ports, and offshore installations, are an ideal harsh substrate for the conservation of endangered marine species (Perkol-Finkel et al., 2012). Restoration work by *G. barbata* on the breakwater was carried out in the northern Adriatic Sea (locality Marotta, Ancona), with encouraging results (Perkol-Finkel et al., 2012).

All the restoration techniques mentioned above are viable alternatives when the conservation or natural restoration of macroalgae forests is not sufficient. In general, it’s better to prefer non-destructive restoration techniques for the natural populations, especially for those species with a low dispersion capacity of the zygotes (Gianni et al., 2013). It should also be considered that *ex situ* experiments require more resources and consequently a higher cost than *in situ* experiments (Verdura et al., 2018). To improve the probability of the success of the intervention and the costs/benefits ratio, it’s suggested the integration of different restoration techniques (Gianni et al., 2013) and the implementation of these within Marine Protected Areas, to facilitate the survival of forests in the long term (Medrano et al., 2020).

Restoration methodologies are highly heterogeneous, and this makes results scarcely comparable. It may be useful to develop standardized approaches to allow comparisons between different restoration interventions and provide important information to researchers for the restoration of macroalgae in the Mediterranean Sea (Cebrian et al., 2021).

1.4 The effect of grazing pressure on restoration success

The grazing can be a stressing factor for the macroalgae forests of the *Cystoseira s.l.* species, leading also to regime shift and the formation of alternative states, as barren grounds. Overfishing and illegal fishing, and the introduction of new alien species in the Mediterranean Sea, increase this phenomenon, often creating irreversible scenarios without direct human intervention (Airoldi et al., 2014; Tamburello et al., 2021;). One of the main predators of the *Cystoseira s.l.* is the sea urchin (*Paracentrotus lividus* and *Arbacia lixula*), which populations outbreaks come from the lack of its predators, mainly overfished fishes. These echinoderms can predate entire macroalgae forests forming the so-called barren grounds, less productive environments with low biodiversity levels (Bianchelli et al., 2016). A second important predator of the hard substrata in the Mediterranean is the goldline (*Salpa salpa*), a bony fish recognized as herbivorous selective towards some species of *Cystoseira s.l.*; also, in this case, it's assumed that the increase in goldline is due to imbalances in its predators caused by overfishing (Gianni et al., 2013). The grazing is also one of the main difficulties encountered in restoration and is often responsible for their failure, especially on artificial substrates (Gianni et al., 2013). This was demonstrated by an experiment carried out by Perkol-Finkel et al. (2012), which led to an attempt to restore *G.*

barbata on the breakwaters of the locality Marotta (Ancona), transferring four stones with numerous seaweed recruits taken from the beach of the Due Sorelle (Riviera del Conero). After 3 days, the stones no longer contained any individual of *G. barbata*; the experiment was then repeated surrounding the stones with plastic cages to test the grazers exclusion. The survival of the recruits occurred with the use of cages with a net mesh of 1x1 cm in area, which allowed to exclude macro- and mesograzers (Perkol-Finkel et al., 2012). The use of nets of different mesh sizes allowed researchers to detect among small organisms, such as crabs and small fish, those responsible for the grazing.

This thesis work is the consequence of the results obtained in summer 2020 by a transplant experiment on the breakwater barriers of *G. barbata*, conducted within the project AFRIMED. Stones containing macroalgal recruits were taken from the Piscinetta del Passetto (Ancona), where there is a healthy natural population, and transferred to the artificial reefs at the Site of National Interest (SNI) of Falconara Marittima and Marina Dorica (Ancona tourist harbor). The attempt was unsuccessful, as after only two days the stones at both sites were completely cleaned, with no more *G. barbata* recruits (Figure 1). The environmental conditions of the experiment have led to the assumption that grazers were responsible, but they have not been identified.

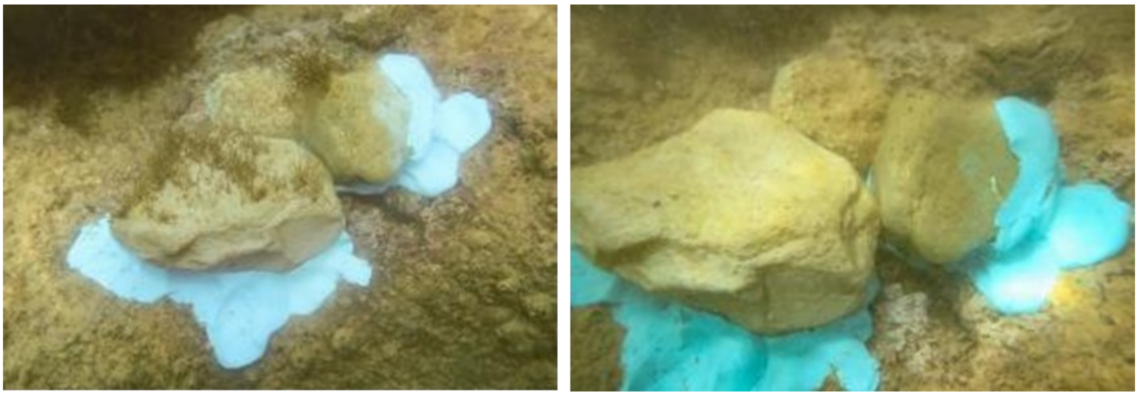

Figure 1: Stones with *G. barbata* recruits at the SNI at time 0 and after 48h from the transfer

Both of these experiments showed how grazing in restoration on artificial substrates can be more decisive than abiotic factors, such as sedimentation and substrate inclination (Gianni et al., 2013). These environments, in fact, often represent "oases" of hard substratum inside sandy habitats, therefore able to attract a great number of predators compared to the nearby natural habitats (Perkol-Finkel et al., 2012). The use of cages could be a solution to this problem, which would make it possible to exploit the ecological potential of artificial structures at sea.

2. OBJECTIVES

The first aim of this thesis work is to test, using cages for the exclusion of grazers, the survival and consequently the *G. barbata* restoration on the breakwater in a previously impacted habitat, that is the Site of National Interest (SNI) of Falconara Marittima. The SNI is a historically polluted and degraded environment subject to strong grazing pressure, and there is no information about the presence/absence of *G. barbata* in this area in the past. However, the SNI putatively represents an optimal site for restoration, since the anthropogenic pressure has finished ten years ago and it's still interdicted to any human use (Corinaldesi et al, 2022).

Another goal is to verify the biodiversity associated with *G. barbata* in the restoration site and to identify the main grazers of this algae on the breakwater, to measure their grazing rate.

The creation of real self-sustaining *G. barbata* forests in breakwater environments is the ambitious long-term goal of this experiment, with the aim of ecological rehabilitation of this habitat, followed by an increase in biodiversity and an increase in the coverage of this protected and seriously declining species in the Mediterranean Sea.

This work is part of the EU AFRIMED (Algal Forest Restoration in Mediterranean Sea) project (funded by the Executive Agency for Small and

Medium Enterprise - EASME and the European Maritime and Fisheries fund – EMFF, between 2019 and 2022), dealing with the restoration of degraded macroalgal forests dominated by *Cystoseira spp*, and involving eight countries bordering the Mediterranean Sea.

3. MATERIALS AND METHODS

3.1 Species and study area

G. barbata is a Fucales living in shallow and sheltered rocky environments and considered a threatened species by the Barcelona Convention. This species is rare along the coasts of the Mediterranean Sea, due to the few habitats that reflect the environmental conditions required by the species, except for the northern Adriatic Sea, where there are several populations (Verdura et al., 2018). In particular, *G. barbata* is sporadically present along the Ancona coast and the Conero Riviera where, however, it's suffering a rapid decline. For this reason, it's of fundamental importance to act with conservation and restoration interventions.

The study area is the Site of National Interest (SNI) of Falconara Marittima (coordinate 43°38'54''N 13°21°35''E), established under Law No. 179 of July 31, 2002, and surrounded by Decree of the Minister of Environment and Territorial Protection of February 26, 2003 (Corinaldesi et al., 2022; Figure 2). The SNI includes a marine-coastal area of about 1200 ha and territory of about 108 ha; it includes industrial settlements with high environmental impact, such as the API Raffineria S.p.A. and the disused area of the industrial plant "ex Montedison", which produced phosphatic fertilizers from pyrite and phosphorite.

Figure 2: Geographical framework of the study area

The area is also affected by the presence of the Esino River, which flows upstream of the API, and influences the site with emissions of freshwater, sediment, and soil contaminants into the sea.

Although the SNI is included in the Area with High Risk of Environmental Crisis Falconara and Lower Esino Valley (AERCA) (ARPAM website source), the anthropogenic impact is no longer detectable in the marine area: the marine sediment could be classified as "meso-eutrophic" (Pusceddu et al., 2009), a common condition in many areas of the Adriatic Sea (Bianchelli et al., 2016, 2018). However, in the terrestrial environment, soil and groundwater show contaminants levels above the threshold (Legambiente website source, 2021).

The marine environmental context of the SNI is characterized by an accentuated hydrodynamism, shallow and sandy seabed, and big inter-seasonal water temperature range, due to the shallowness and the contribution of the Esino river freshwater (ARPAM website source).
In the coastal area facing the SNI, there are 11 breakwater barriers, extending from the "ex Montedison" to the mouth of the river. These structures represent a hard substrate different from the surrounding sandy habitat, so they could be exploited by different organisms. Indeed, the fauna associated with the barriers is composed of fishes, such as the golden mullet (*Liza aurata*), the look (*Oblada melanura*), different species of bream (*Diplodus spp*.), small blennids and gobids that find refuge among the crevices of the rocks. Also, taxa such as crustaceans (especially crabs as *Pachigrapsus marmoratus*), some species of cnidarians (anemones), and cephalopods (*Sepia spp.*) can be observed, whereas attached to the rocks there are present large quantities of oysters (*Ostrea spp*.), encrusting crustaceans (balanidi) and mussels (*Mytilus galloprovincialis*) (Figure 3).

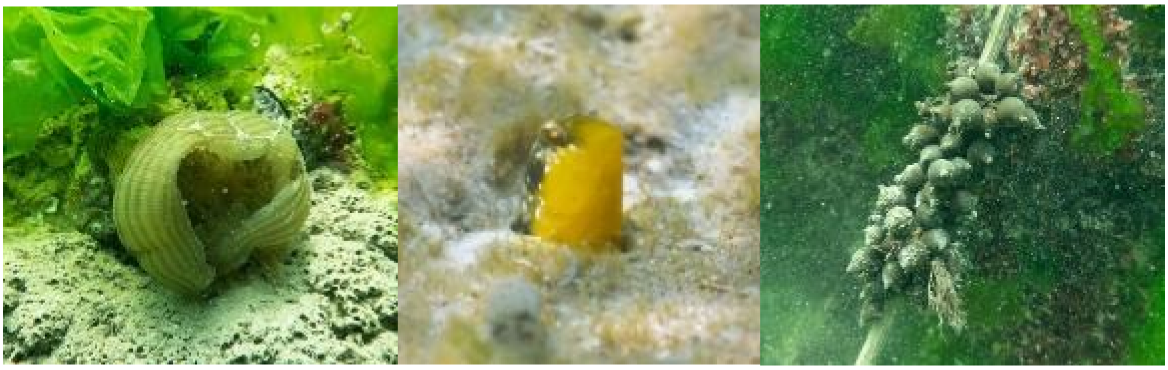

Figure 3: Examples of fauna species present on the breakwater barriers of Falconara's SNI

The flora is dominated by green and red algae (Figure 4), as *Ulva cf. lacinulata* and *Hypnea spinella*, which dominate most of the substrate. Also, species as *Codium fragile*, *Gracilaria gracilis*, *Gracilaria turuturu*, *Schottera micaeensis,* and *Ellisolandia elongata* can be observed.

However, brown algae are not present and the presence of *G. barbata* could represent a remarkable enrichment to the ecosystem and a source of food for the organisms.

Figure 4: Examples of flora species present on the breakwater barriers of Falconara's SNI

The SNI of Falconara Marittima, and in particular the breakwater barrier chosen for this thesis work, aren't the most suitable habitat for the settlement of *G. barbata*. This is due to the numerous critical issues present in the area, such as the high rate of sedimentation, the influence of fresh water and nutrients from the Esino river, and grazing. However, the donor population living at Piscinetta del Passetto (Ancona) supports similar environmental conditions.

3.2 Site selection and experiment description

During a preliminary survey, the third breakwater barrier starting from the area "ex Montedison" was selected as a restoration site, because it's distant from the Esino river, corresponding to low levels of transport of sediment, freshwater, and nutrients. In particular, the cages have been positioned at about 1 meter depth in the landward and lateral right part of the barrier, where hydrodynamism and sedimentation are low. The accumulation of sediment is, however, greater in the innermost areas of the barrier, due to the transport by lateral currents.

In situ recruitment

The *G. barbata* specimens used for transplantation in the SNI were obtained from an *in situ* recruitment experiment carried out at Piscinetta del Passetto (Ancona); three structures have been built to recruits the zygotes derived from mature specimens of *G. barbata*, forming the donor population.

The structures are composed of a 50 cm long aluminum bar, bent at the end by levering with a vice, to form two support points and raise the structure by 3,5 cm from the bottom (Figure 5).

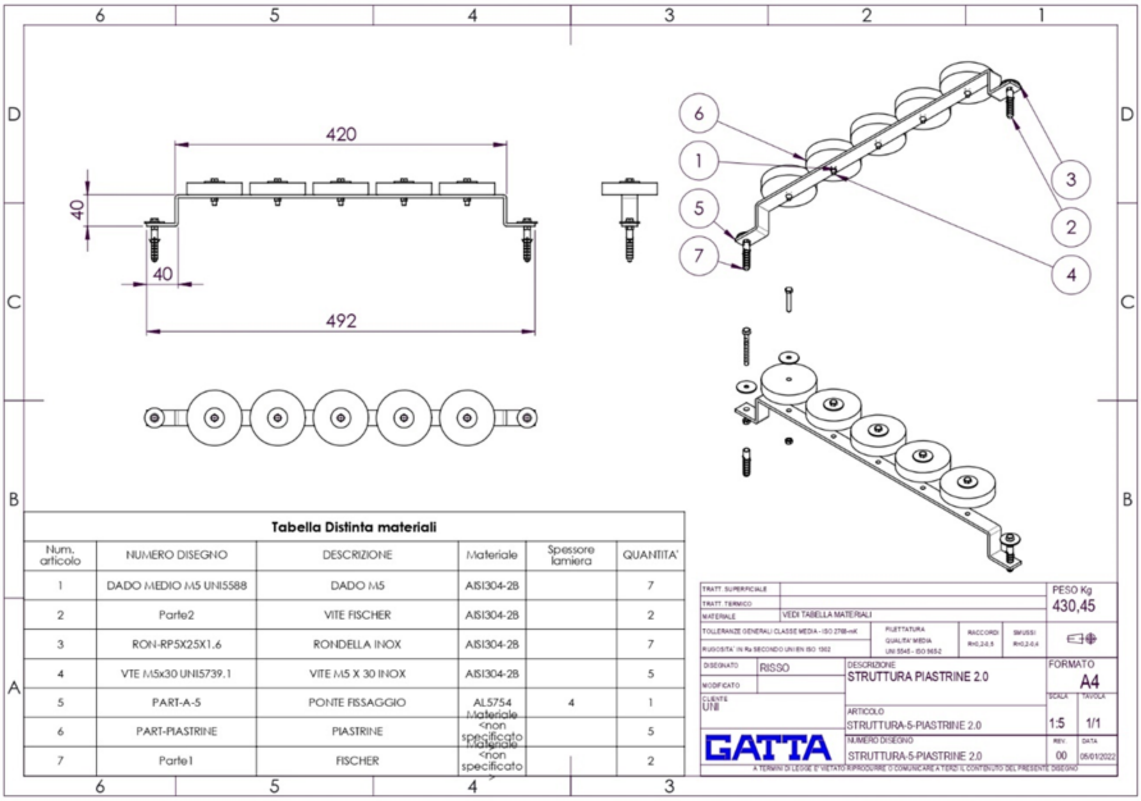

Figure 5: Structure used for the in situ recruitment

On the bar, were made five holes 7,5 cm spaced one from each other, where were fixed, through stainless steel screws, five clay tiles previously pierced with a drill. The latter has a diameter of 7 cm and a height of 1,5 cm, and their rough surface represents an ideal hard substrate for the settlement of the *G. barbata* zygotes.

On 5th February 2021, the structures were transferred to Piscinetta del Passetto (Ancona, coordinate: 43° 37’ 05.20’’ N; 13° 32’ 01.73’’ E), a sheltered bay at a shallow depth where there are numerous adult specimens of *G. barbata*, which resulted mature at that time (according to the phenological data, collected in the framework of AFRIMED project). One structure was placed in the middle of a mature adult’s forest, and the other two less than 1 m away from other adults. The structures were fixed to the substrate using stainless steel screws, with the use of a drill.

The cages, used to protect *G. barbata* from grazing, were built with a galvanized iron mesh with 12 mm mesh and assembled with plastic ties. These structures measure 40 cm in length, 25 cm in width, and 20 cm in height and have a removable lid.

Restoration intervention

On 21st July 2021, the 3 structures with the *G. barbata* recruited juveniles were taken from the Passetto and transferred, inside 3 cages, to the SNI of Falconara Marittima. Three nearby rocks were identified to fix the experimental units (structure + cage): the first and second cages were placed on two adjacent rocks, well sheltered and with a flat surface. The third, however, was placed in a nearby rock less sheltered and with a slight slope (Figure 6).

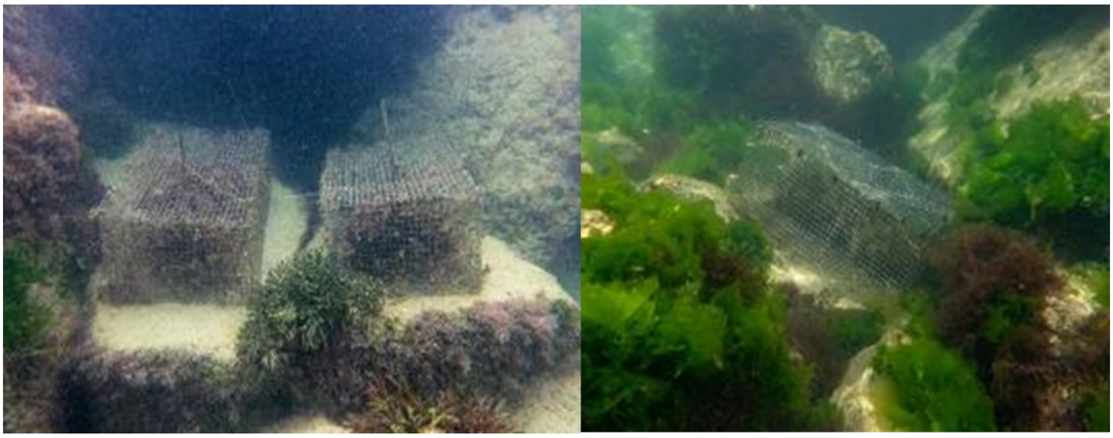

Figure 6: Experimental units (structure + cage) in the SNI of Falconara Marittima

Once the most suitable area for the transplant operation was identified, the cages were placed on the rocks with the structures inside, and the entire assemblage was fixed with the drill and stainless-steel screws.

On 21st October 2021, following a strong storm, the cage of the third structure was uprooted and one of the five clay tails was lost (Figure 7). The same day, the lid of the first cage was removed to test the resistance of *G. barbata* grown against grazers.

On 13 st December 2021 was made an attempt to sampling but, due to another strong sea storm in the days before, there was insufficient visibility. However, algae and cages were still present.

Figure 7: Third structure without the cage after the sea storm

During the sample on 19 st January 2022, it was possible to see that another sea storm in the previous days broke the cage of structure 1, while that of structure 2 was torn away from the sea (Figure 8).

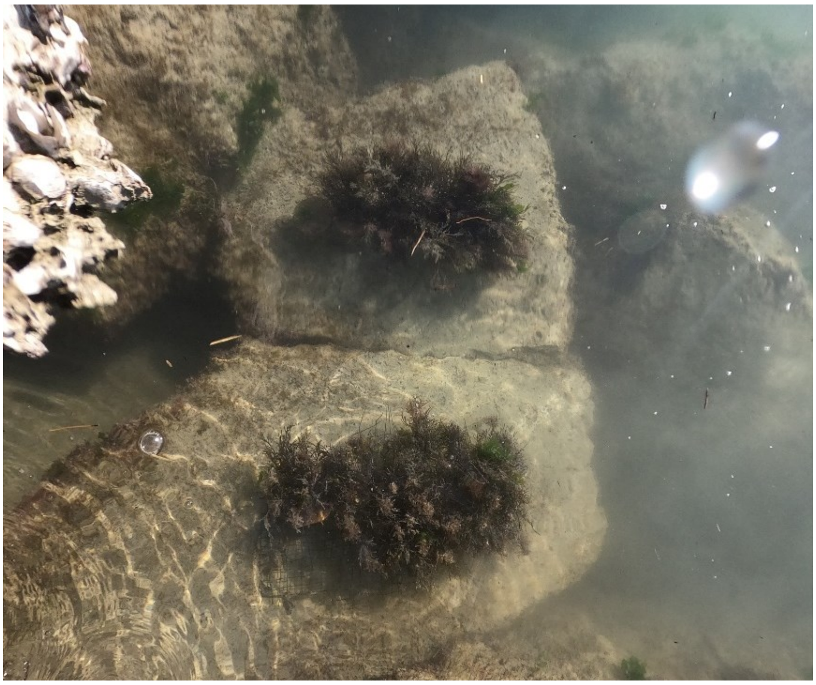

Figure 8: Structures 1 and 2 without cages after the January sea storm

During the restoration intervention, nine monitoring were carried out to measure the height of the algae and for the measurement of the canopy, and four monitoring times for the analysis of the associated biodiversity.

Grazing experiment

During the sampling survey of 21st October, a crab belonging to the species *P. marmoratus* was photographed while was eating a specimen of *G. barbata* settled on the third structure, deprived of the cage after the sea storm (Figure 9).

Figure 9: *P. marmoratus* eating *G. barbata* on the third structure

After 18 days, on 8th November, the third structure was completely removed by grazers and didn't present any *G. barbata* specimen. Since no herbivorous fish have been observed along the breakwaters during the entire period, an experiment was conducted in the aquarium to test whether crabs of the species *P. marmoratus* could be one of the main grazers of the alga. To do this, on 2nd December, were taken 4 specimens of crab from the Piscinetta del Passetto, 3 females and 1 male, together with a stone with numerous individuals of *G. barbata* less than 15 cm in height, grown on a small rock (Figure 10).

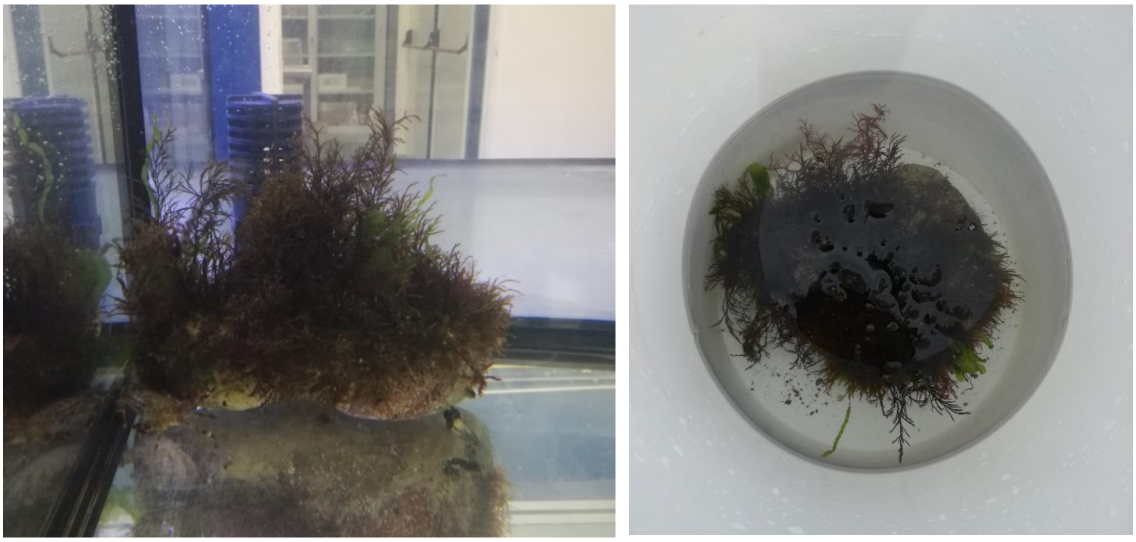

Figure 10: Stone with numerous individuals of *G. barbata* taken from Piscinetta del Passetto

The choice of a donor site different from the restoration area in Falconara depended on the greater ease of detection and capture of crabs at the Passetto, where natural algae populations are also present. Moreover, here are present detached small rocks with *G. barbata* recruits, which have no possibility to survive due to the unstable substrate.

The crabs and the rock with the algae were transferred inside a tank of 200 L, inside which was placed a rubber mat as a substrate to facilitate the movement of animals, which was not given any type of additional food. The photoperiod of the tank was set at seven hours of light and 17 h of dark, and the water temperature was 13°C. On 6th December, after 4 days from the beginning of the experiment, one of the 4 crabs was found dead, probably attacked and eaten by the other specimens. The tank was decommissioned in January, six sampling times have been carried out for the variable "grazing rate" (Figure 11).

3.3 Response variables and sampling methodologies

The response variables chosen allowed us to monitor the growth of *G. barbata* over time and changes in associated biodiversity.

In particular, the height of the algae in centimeters, the amplitude and variation of the canopy, and the biodiversity associated with the structures have been analyzed through the analysis of meiofauna and macrofauna.

The grazing rate was considered for the grazing experiment in the aquarium.

3.3.1 Algae height

The height of the *G. barbata* in centimeters was used to monitor its growth rate and was recorded with two different methodologies: as for the *in situ* experiment, through the ImageJ application were measured from 10 to 15 thalli every two weeks, from March to April 2021, then every month until transplantation to SNI in July (Figure 11).

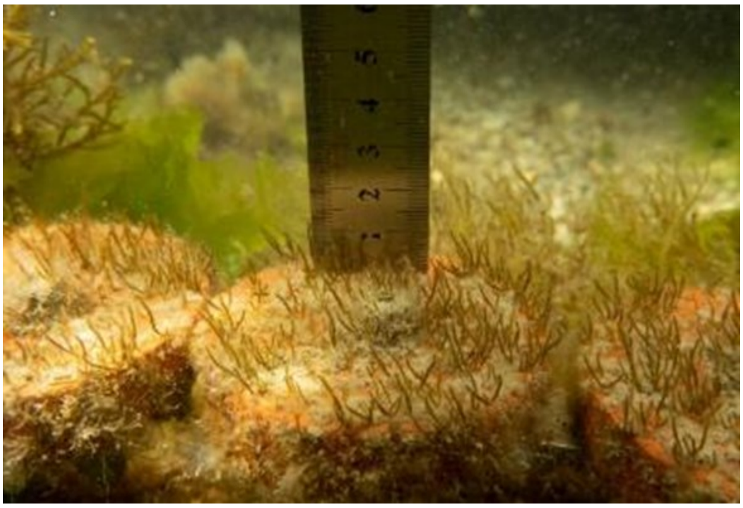

Figure 11: Example of a photo used to measure the recruits with ImageJ

The photographs used in ImageJ were taken during periodic snorkeling. During the transplant experiment at Falconara Marittima, the height of the algae was measured from July 2021 to January 2022 every about three weeks (Figure 12), except the penultimate sampling that was done after 7 weeks due to bad weather conditions, and the last sampling that was made 11 days after.


Figure 12: Height measurement of the thalli of structure three at the restoration site

At each sampling date, the height of 2 thalli was taken for each clay tile. An exception is the structure one that on tile number 5 had a single specimen of *G. barbata*; to compensate, were measured, at each sampling time, 3 thalli in the clay tile number four. In total, for each structure, 10 algae were measured using a steel ruler, starting from the basal disk up to the highest tip of the frond of each individual of *G. barbata*. At the T5, T6, T7, and T8 have been measured only 9 algae in structure 1, due to the disappearance of the single specimen on tile number 5.

3.3.2 *G. barbata* canopy extention

The canopy was measured by calculating the percentage of fronds' coverage of *G. barbata*, taking the area of the cages as a reference. Again, the ImageJ application and the pictures taken at each sampling date were used, approximately once every 3 weeks. On 1st December 2021 was taken a photo only at cage number 1, due to problems during the sampling. The photos were taken with the lid of the cages open and from above, to frame the surface of the algae (Figure 13).

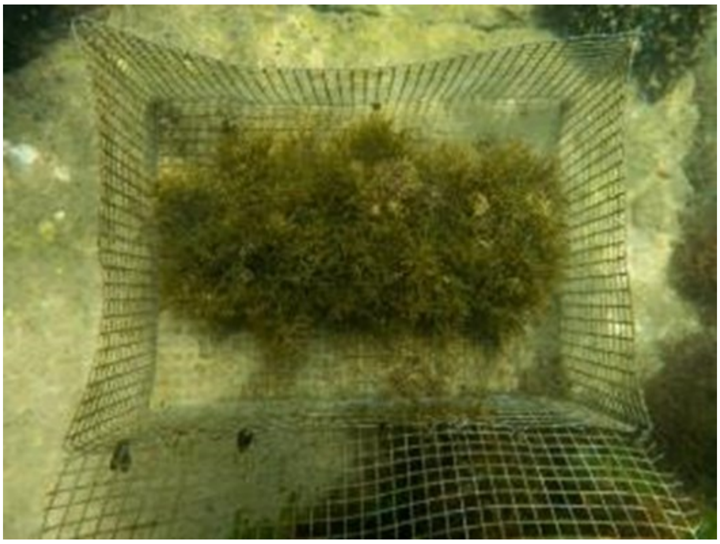

Figure 13: Photo used to calculate the surface percentage with ImageJ

After the October and January sea storms that tore away the cage of the third structure first and then the cage of the other two structures, a ruler was used as a reference in ImageJ to measure the surface of *G. barbata* specimens (Figure 14).

Figure 14: Photo used to calculate the canopy with ImageJ after the sea storm

In December 2021 was made a sampling but wasn't possible to take photos to analyze the *G. barbata* canopy due to the turbidity of the water.

3.3.3 Associated biodiversity

For the study of the associated biodiversity, meiofauna and macrofauna were analyzed; in particular, their abundance, richness of taxa, and taxonomic composition were investigated. Meiofauna are increasingly used as an indicator of environmental quality and anthropogenic impact (Bianchelli et al., 2016) due to its sensitivity to environmental disturbances, ubiquity, high abundances, biomass and diversity, a short life cycle with rapid turnover times, and the absence of larvae with pelagic dispersal (Danovaro et al., 2004). Sampling for meio- and macrofauna analyses was done approximately every 2 months, from transplantation to SNI in July until January 2022. A modified manual corer with a diameter of 9 cm was used to take samples from the hard substrate, which were then collected in plastic bags and closed with rubber bands (Danovaro and Fraschetti, 2002). The samples were then frozen at -15°C and then defrosted to be fixed and analyzed.

In total, for each sampling time were collected 6 samples, 3 near the cages and 3 at the control site (Figure 15).

Figure 15: Breakwater barriers at SNI with control and restoration site highlighted

The latter is located along the breakwater barrier in a mirror position concerning the structures and was chosen to replicate the same environmental conditions of the restoration site.

3.3.3.1 Laboratory analysis

Macrofauna

For the extraction of macrofauna, the samples were subjected to ultrasonic treatment (3 x 1 minutes) and filtered on a 500 µm mesh. All the material passed through the filter was collected in a becker. Subsequently, from the filter, the samples were placed in 50 ml falcon with 70% ethanol and Rose Bengal and kept refrigerated for 2-3 days to allow the staining of the sample.

For microscopic analysis, the sample was again filtered with a 500 µm mesh to remove ethanol and placed in a Delfuss cuvette. The organisms' count was carried out at the stereomicroscope with magnifications from 16 to 40X (Figure 16).

Figure 16: Different macrofauna taxa found in the SNI during the microscope analysis (hydrozoa, pycnogonida, isopods)

Meiofauna

Due to a large amount of sediment present in the samples, was applied an extraction protocol by centrifugation in a density gradient. The extraction was carried out with Ludox HS40 gel solution (Heip et al.,1985). The samples, after filtered on a 500 µm mesh for the extraction of macrofauna and collected in a beaker, were filtered with a 20 µm mesh and collected in a 50 ml falcon. Ludox 100% was added with a sediment ratio: Ludox = 1:3. The samples were re-suspended and centrifuged for 10 minutes at 3000 rpm, 3 times, to obtain an extraction efficiency of more than 90%. After each centrifugation, the supernatant was collected on a 20 µm filter, thoroughly rinsed to remove Ludox residues. The samples on the filters were then placed in a falcon with 70% ethanol and Rose Bengal. The residual sediment after centrifugation has been checked at the stereomicroscope to verify the absence of organisms and therefore the success of the extraction protocol. As with macrofauna, organisms' counting was done through stereomicroscope analysis, after filtering the sample and placing it in a Delfuss cuvette (Figure 17).

Figure 17: Different meiofauna taxa found in the SNI during the microscope analysis (gastropods and amphipods)

3.3.4 Grazing rate

The grazing rate allows us to understand how much the grazers consume *G. barbata* individuals. To persist, the growth of algae must prevail over the amount of algal biomass eaten by grazers. For this experiment were measured, with a steel ruler, the heights of *G. barbata* 11 individuals present on the stone taken from the Piscinetta of Passetto, and the total number of individuals was counted. These measurements were taken from the 2nd of December 2021 (T0) until 21st December (T5, 19 days later), with an interval of 4 days, except between T2 and T3 (5 days) and between T3 and T4 (2 days). Although the tank was decommissioned in January, were made only six samplings, because the aquarium wasn't accessible for pandemic reasons. However, it was possible to take photos in January which provided information on the progress of the experiment (Figure 18).

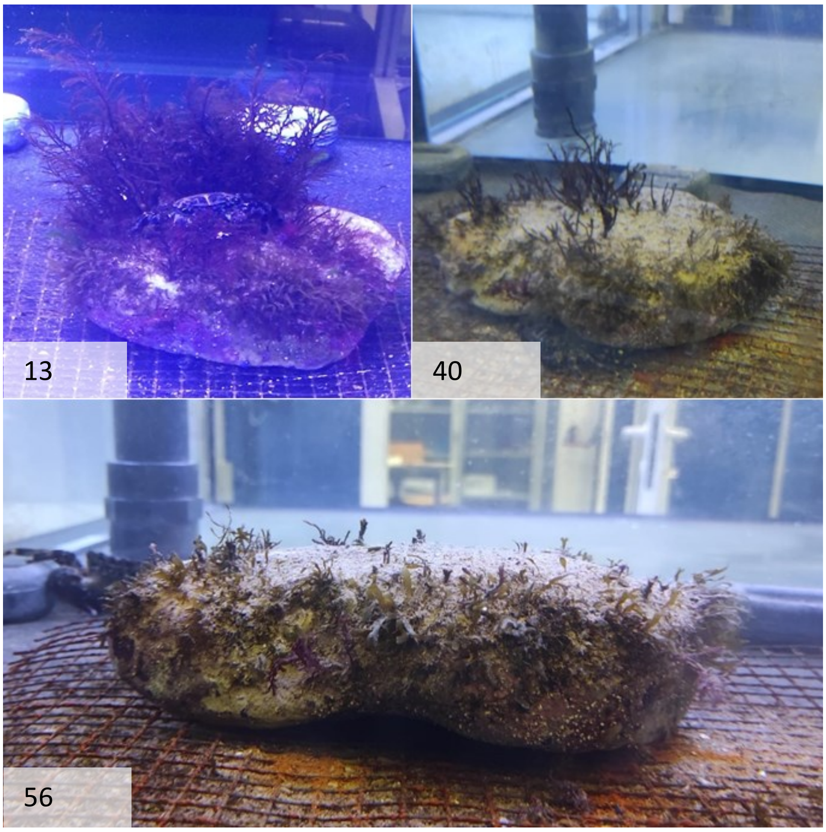

Figure 18: Stone with *G. barbata* specimens at 13 days, 40 days, and 56 days from the beginning of the experiment

Through the use of an underwater camera, mini-videos were also made taking photos at an interval of 30 seconds one from each other, to shoot crabs while eating *G. barbata*.

3.4 Experimental design

The aim of the experiment is to assess the feasibility of *G. barbata* restoration on breakwater barriers in a Site of National Interest, using cages for the grazer's exclusion. For algal growth during the recruitment phase, the experimental design considered 2 factors as source of variability: Time, fixed, with 8 levels, and Structure, fixed with 3 levels.

For algal growth during the restoration experiment, the experimental design considered 2 factors as source of variability: Time, fixed with 9 levels, and Structure, fixed with 3 levels. In both cases, the analysis considered 9-10 replicated measures for each level of both factors.

For the analysis of the associated biodiversity, the experimental design considered 2 factors as source of variability: Site (fixed, 2 levels: cage and control) and Time (fixed, 4 levels), with 3 replicates.

3.5 Data processing and statistical analysis

The data, for all variables, has been processed with the Excel package office 365. Algae height has been expressed in cm as average ± standard deviation, and the *G. barbata* abundance in the grazing experiment has been reported as the number of individuals. The canopy was expressed as a percentage of coverage on a standardized surface.

Regarding the associated biodiversity, the values of the abundance of meio-macrofauna have been reported as the average of individuals/10 cm^2 for meio- and individuals/50 cm^2 for macrofauna ± standard error on three replicates. Taxa richness was expressed as the number of cumulative taxa of the three replicates, while the taxonomic composition was reported as the percentage of the cumulative taxa abundance on three replicates.

The experimental designs were applied to permutational analyses of variance (PERMANOVA), in uni- (for algal growth, total meio- and macrofaunal abundance) and multivariate context (for taxonomic composition of the meio- and macrofaunal assemblages) and based on Euclidean distance and Bray-Curtis similarity matrices, respectively. Analyses on meio- and macrofaunal taxonomic composition were repeated considering the whole assemblages, excluding the 2 dominant taxa (for meiofaunal assemblages) and considering only the rare taxa. When significant differences were observed, pair wise tests were performed to establish between which levels significant differences were present. To visualize differences in the taxonomic composition, bi-plot were also produced following CAP (Canonical Analysis of Principal coordinates) analysis.

All statistical analyzes were done with the PRIMER7 software package.

4. RESULTS

4.1 Gongolaria barbata growth

Height

During the recruitment phase (from 05/03/21 to 20/07/21) at Piscinetta del Passetto, *G. barbata* average growth was from 0,063 ± 0,016 cm to 5,72 ± 1,7 cm (Figure 19).

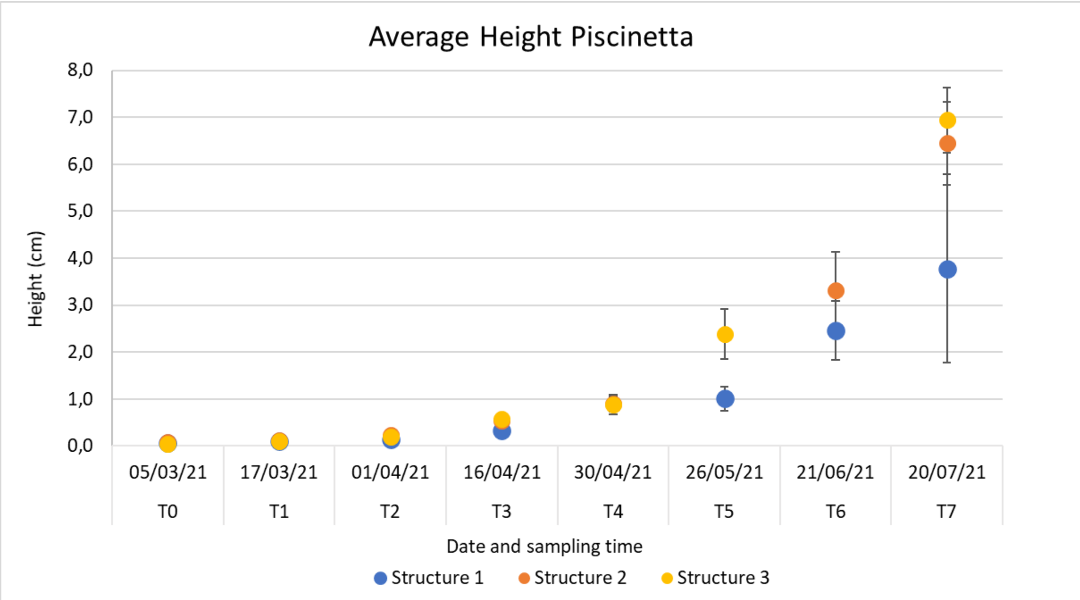

Figure 19: Temporal variation of the height of *G. barbata* in the Piscinetta site. The bar represents the standard deviation

G. barbata on the three structures showed an exponential increase in height, with the highest values recorded in July 2021 (T7). The maximum average height has been reached on structure 3 at T7 with 6,9 ± 0,7 cm. In the last 2 sampling times, structure 1 showed lower values than the other two structures, which have a similar growth pattern. The average height of the three structures at T7, before transplantation, is 5,7 ± 1,7 cm.

The PERMANOVA reveals a significant effect of the factors Time, Structures, and Structures X Time (Table 1) on the average height during the recruitment.

Table 1: PERMANOVA analysis conducted on the average algae height in the in situ experiment. Source = source of variability, df = degree of freedom, MS = means of squares, Pseudo-F = pseudocasual function, P = P level (same legend for the following tables)

Source	df	MS	F	P
Structure	2	3,2	43,7	0,001
Time	6	30,4	418,7	0,001
Structure X Time	9	1,1	15,1	0,001
Residuals	231	0,1		

The *pair wise* test conducted on the term Structure x Time showed statistical differences between structures all the time, except in T0 between structure 1 and 3, T1 between structure 1 and 2, and 1 and 3, T2 between structure 2 and 3, T3 between structures 2 and 3, and in T4. The *pair wise* test conducted on the term Structure x Time also reveals a difference in all the structures all the time, this indicates that the algae have grown steadily over time.

After the transplant at SNI (Figure 20), the average growth of the algae continued to increase on the 3 structures, until the end of September (respectively 14,1 ± 3,6 cm structure 1, 13,7 ± 0,9 cm structure 2 and 15 ± 2 cm structure 3). In the following 2 sampling times, the *G. barbata* average height resulted stable on structure 1 and 2, whereas on structure 3 decreased and completely disappeared in December (T6), after a strong sea storm that provoked the destruction of cage 3.

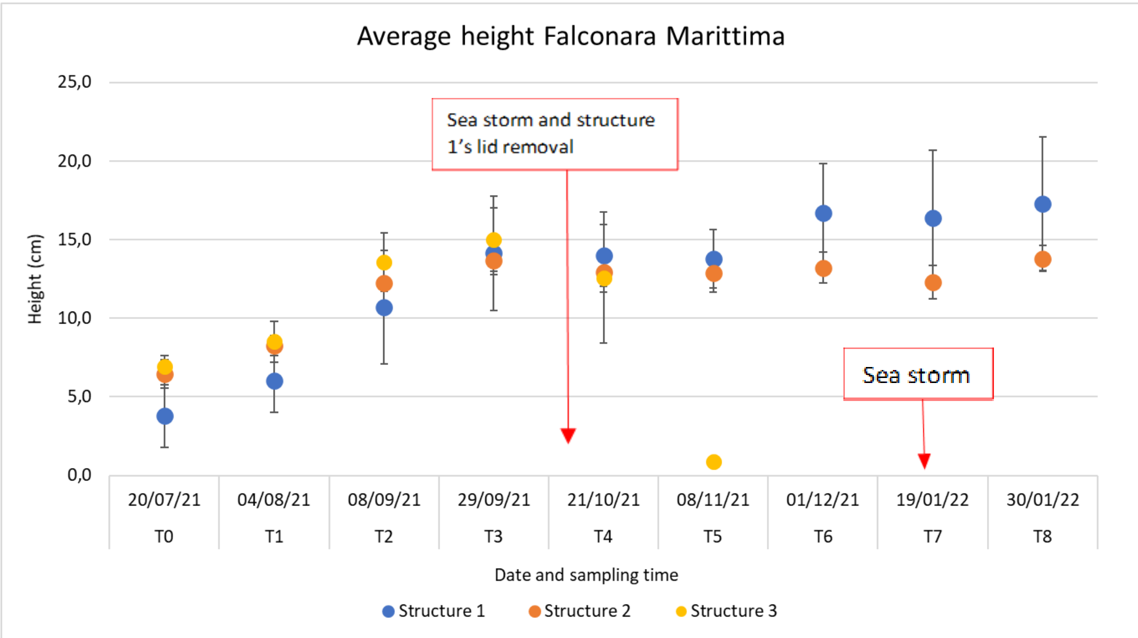

Figure 20: Average algae height after the transplantation at the SNI. The bar represents the standard deviation

In December, the *G. barbata* heights were on average 16,7 ± 3,1 cm and 13,2 ± 1 cm on structures 1 and 2, respectively. In January 2022 (T7), after another strong sea storm that destroyed cage 1 and pulled out cage 2, the algae heights were on average 16,4 ± 4,3 cm on structure 1 and 12,3 ± 1,1 cm on structure 2. In the last sampling time (T8), the data reported are 17,3 ± 4,2 cm on structure 1, and 13,8 ± 0,8 cm on structure 2.

The PERMANOVA analysis conducted on the average algae height in Falconara reveals a significant effect of the factors in Time, Structure, and Time X Structures (Table 2).

Table 2: PERMANOVA analysis conducted on the average algae height in Falconara Marittima

Source	df	MS	F	P
Structure	2	20,6	3,6	0,034
Time	8	299,2	51,9	0,001
Structure X Time	13	34,5	6,0	0,001
Residuals	202	5,8		

Based on the results obtained with the *pair wise* test conducted between the Structures at any time, the difference is already noticed by the T0. From the time T6 to T8, the test shows significant differences between structures 1 and 2, while structure 3 has disappeared.

The *pair wise* test, based on the term Structures x Time for pairs of factor Time, shows mainly differences between the first and the last times, without any significant growth in the last observations.

Canopy

Figure 21 shows the variation of the canopy of the *G. barbata* specimens in the three structures at the restoration site.

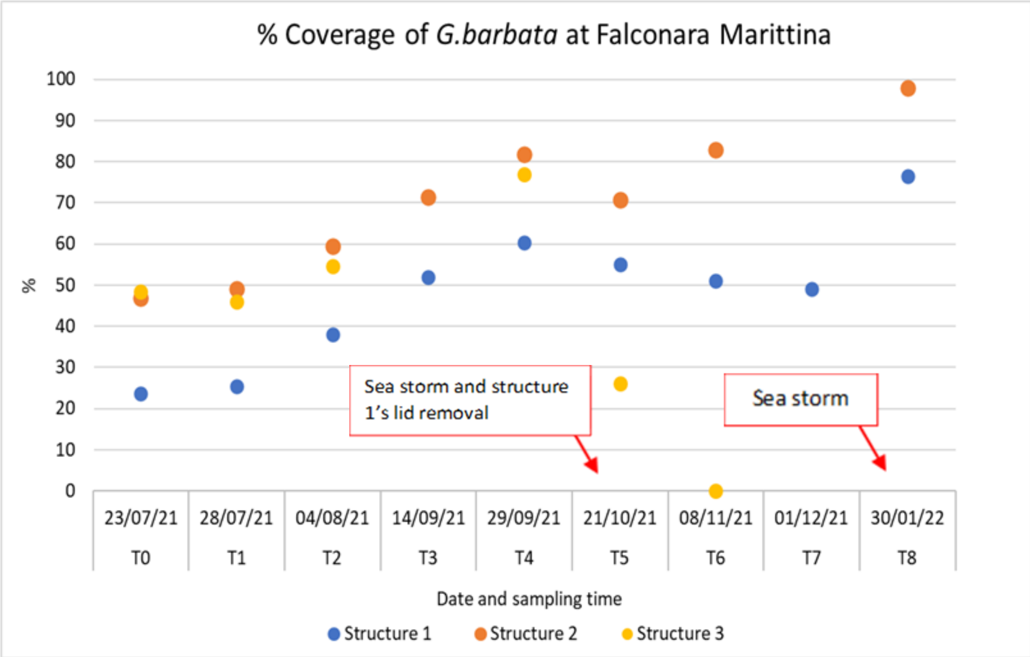

Figure 21: Percentage of coverage of *G. barbata* at the restoration site

The percentage of coverage in the three structures increases from July (T0) until September (T4), with a maximum value of 60-82%, reached by algae on structures 1 and 2, respectively. In October (T5), immediately after the storm, was observed a decrease in the canopy coverage in all the cages (55% structure 1, 71% structure 2, and 26% structure 3). In the following sampling time (T6), the percentage of coverage increased in structure 2 (83%), was constant in structure 1 (51%), while structure 3, destroyed by the storm, has no more coverage. At T7, only the coverage of structure 1 was reported, with a value of 49%. In the last sampling time, the percentage of coverage increased in the two structures: 77% for structure 1 and 98% for structure 2.

4.2 Associated fauna

4.2.1 Meiofauna

Total abundance

In Figure 22 the total meiofauna abundance in control and restored points is reported.

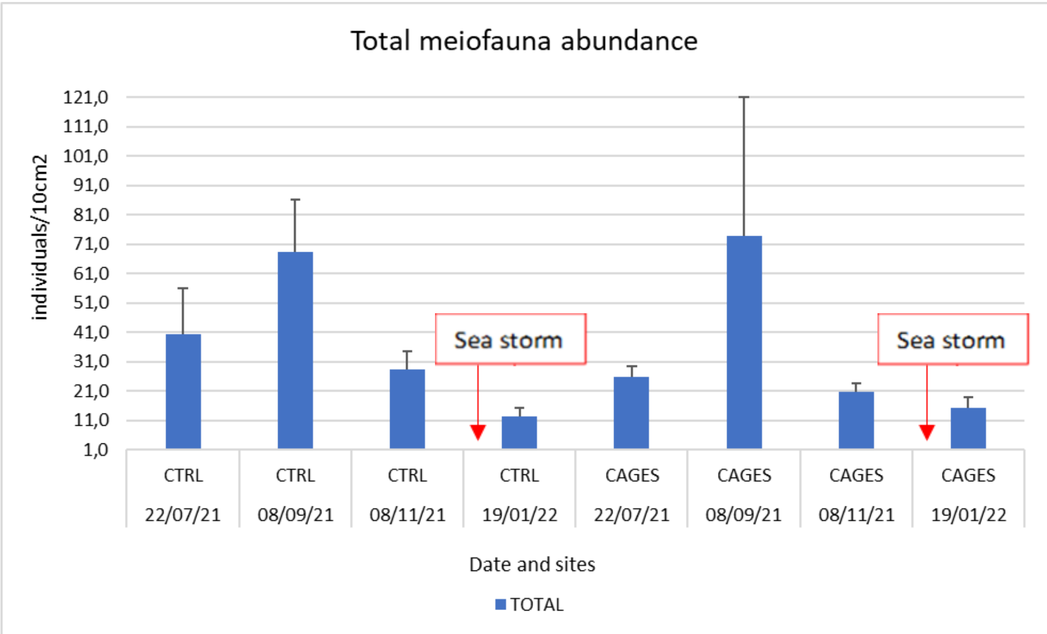

Figure 22: Total meiofauna abundance (n. ind/10 cm^2) in the control stations and near the restoration site in the SNI. The data are reported as average, and the bar represents the standard error over 3 replicates

The values in the control site vary from 40,24 ± 15,66 ind/10 cm^2 in T0, to 68,22 ± 17,86 ind/cm^2 in T1, 28,29 ± 6,14 ind/10 cm^2 in T2 and to 12,47 ± 2,78 ind/10 cm^2 in T3.

At the restoration site, the values are 25,83 ± 3,65 ind/10 cm^2 at T0, 73,98 ± 46,92 ind/10 cm^2 at T1, 20,70 ± 2,84 ind/10 cm^2 at T2 and 15,46 ± 3,45 ind/10 cm^2 in T3.

The PERMANOVA analysis detected significant effect only for the variables Time, and no difference was found between the two Sites and between the two Sites and Time (Table 2).

Table 2: PERMANOVA analysis conducted on the total meiofauna abundance in the SNI

Source	df	MS	F	P
Sites	1	65,9	0,1	0,807
Time	3	3716,7	3,4	0,023
Site X Time	3	131,8	0,1	0,959
Residuals	16	1066,7		

This test shows that the total abundance in the control site and under the cages has the same fluctuation over time.

The richness of meiofaunal taxa

The richness of meiofaunal taxa is represented in Figure 23 and reported cumulatively for each sampling point.

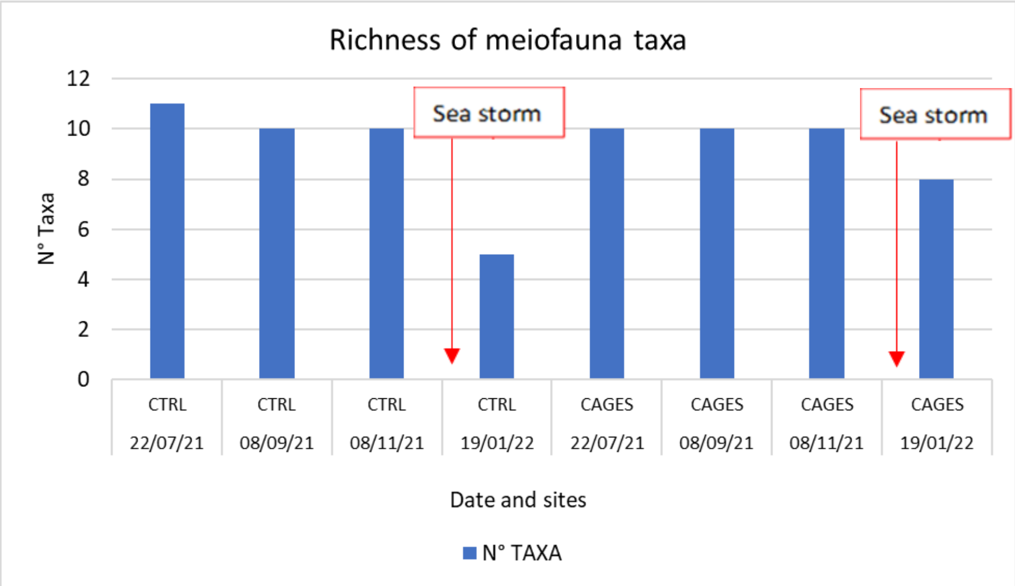

Figure 23: Total richness of meiofauna taxa (number of taxa) in the control stations and in the SNI restoration site, the data are reported cumulatively in the analyzed replicates

At the control site were observed 11 meiofauna taxa at T0 and 10 taxa at T1 and T2; 5 taxa are observed at T3 after a sea storm in the previous days. In the restoration site, instead, 10 taxa were counted in the first three sampling times (T0, T1, and T2), and 8 taxa are reported at T3, the last sampling time.

Meiofaunal taxonomic composition

Figure 24 reported data on the community structure of the meiofaunal assemblages. A total of 15 taxa have been identified: nematodes, copepods and nauplii, ostracods, isopods, polychaetes, oligochaetes, kinorhynchs, tanaidacea, cumacea, cladocera, bivalves, mites, amphipods, gastropods, and hydrozoa.

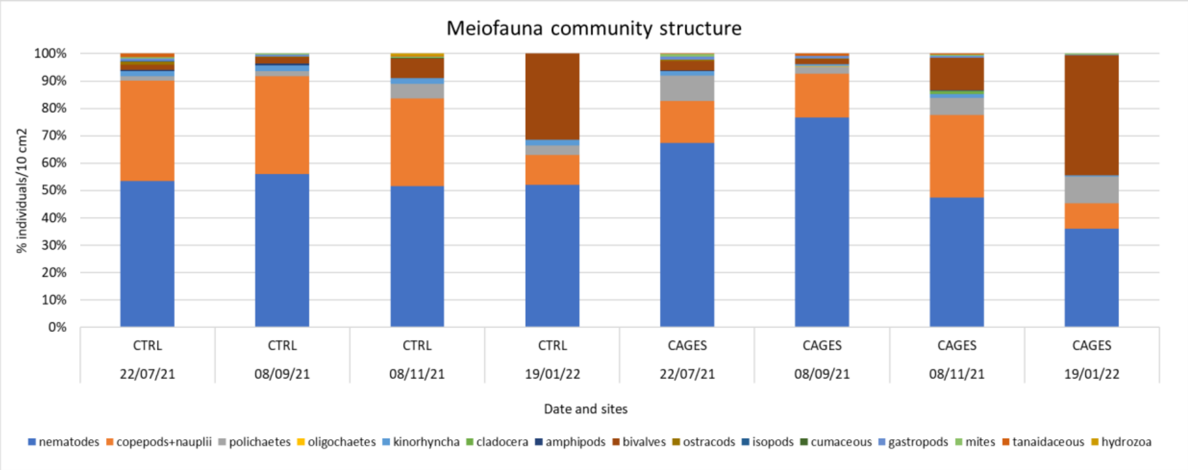

Figure 24: Percentages of the abundance of meiofauna taxa in the control and restoration site in the SNI, the data are reported cumulatively in the analyzed replicates

The structure of the meiofaunal assemblages, from T0 to T2, is dominated by nematodes both at the control (53,5 % T0, 56% T1 and 51,7% T2) and at the restoration site (63,3% T0, 47,3% T1 and 76,6% T2), and by copepods (36,6% T0, 35,8% T1 and 31,9% T2 at the control and 15,4% T0, 15,9% T1, 30,1% T2 at the restoration site). Also, at T3 in the control site nematodes are the prevalent taxa (52,1%) followed by bivalves (31,5%), while in the restoration site the bivalves are the first taxa (43,7%) and nematodes are the second one (35,9%).

The third most abundant taxon in the T0, T1 and T2 in both sites is represented by bivalves (2% T0, 2,2% T1, 7% T2 at control and 1,9% T0, 3,4% T1, 11,9% T2 at restoration site), followed by polychaetes (1,7% T0, 1,8% T1, 5,4% T2 at control and 2,5% T0, 6,3% T1 and 9,3% T2 at restoration site) and kinorhynchs (1,7% T0, 2,1% T1, 2,2% T2 at control and 1,6% T0, 0,7% T1 and 1 ,5% T2 at restoration site).

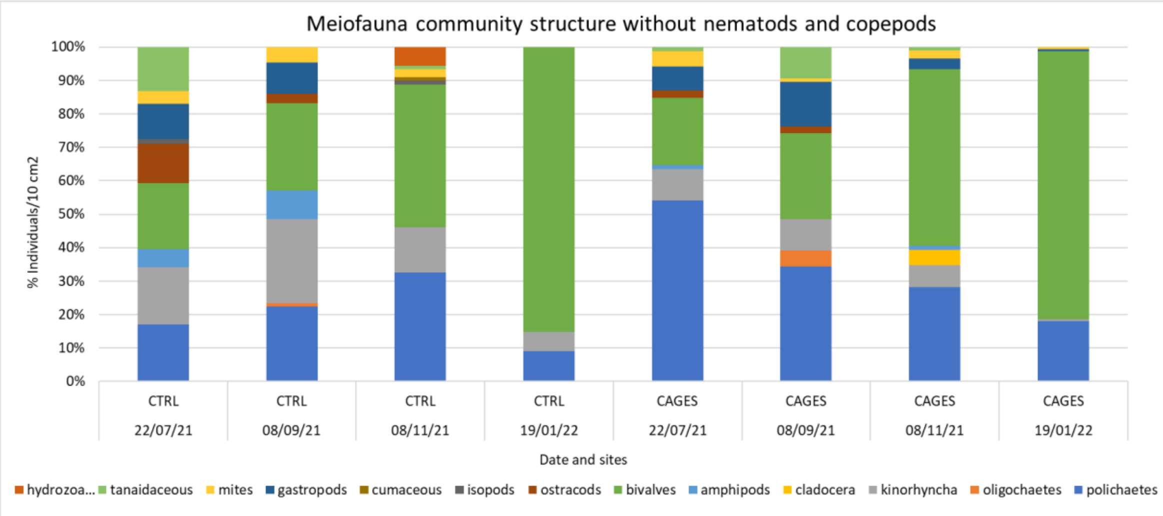

Figure 25: Taxonomic meiofauna composition without nematodes and copepods at the control and restoration site

At the T3, the third most abundant taxon at the control site is represented by copepods, followed by polychaetes (3,4%) and kinorhynchs (2,1%). In the restoration site, at the third position, there are the polychaetes (9,8%), followed by copepods (9,2%).

In the control site, a small percentage of taxa is occupied by ostracods (1,2% T0, 0,2% T1, 0% T2), tanaidaceous (1,3% T0, 0% T1, 0,2% T2) and gastropods (1% T0, 0,8% T1, 0% T2); in the restoration site there are instead cladocerans

(0% T0, 0% T1, 1% T2), gastropods (1.2% T0, 0% T1, 1% T2, 0,3% T3) and kinorhynchs only at T3 with a percentage of 0,3%.

The analysis of the variation of taxonomic composition as a function of time in the two sites, conducted through PERMANOVA, determined no significant differences for the factors Sites and Sites X Time. The only statistically significant difference is for the factor Time (Table 3).

Table 3: PERMANOVA analysis conducted on the meiofauna community structure in the SNI

Source	df	MS	F	P
Sites	1	1291,3	1,3	0,246
Time	3	5252,8	5,3	0,001
Sites X Time	3	692,3	0,7	0,682
Residuals	16	989,0		

The same results were obtained analyzing the community structure with all taxa and without the two principal ones (nematodes and copepods) (Table 4).

Table 4: PERMANOVA analysis conducted on the meiofauna community structure without nematodes and copepods

Source	df	MS	F	P
Sites	1	1749,6	1,2	0,272
Time	3	5212,3	3,5	0,001
Sites X Time	3	1757,1	1,2	0,27
Residuals	16	1468,8		
Total	23			

The contribution of other taxa is 0.1-0.9% in the control site and 0.1-0.8% in the restoration site. The latter can be considered rare because, according to the definition of "rare taxa", they are present in a percentage of less than 1% of total abundance (Bianchelli et al., 2010).

The rare taxa are dominated by oligochaetes, amphipods, isopods, cumaceans, mites, and hydrozoa at the control site, and by oligochaetes, amphipods, ostracods, mites, pycnogonida, and tanaidaceous at the restoration site. In the T3, in the control site weren't detected any rare taxa (Figure 26, N.D. = Not Detectable).

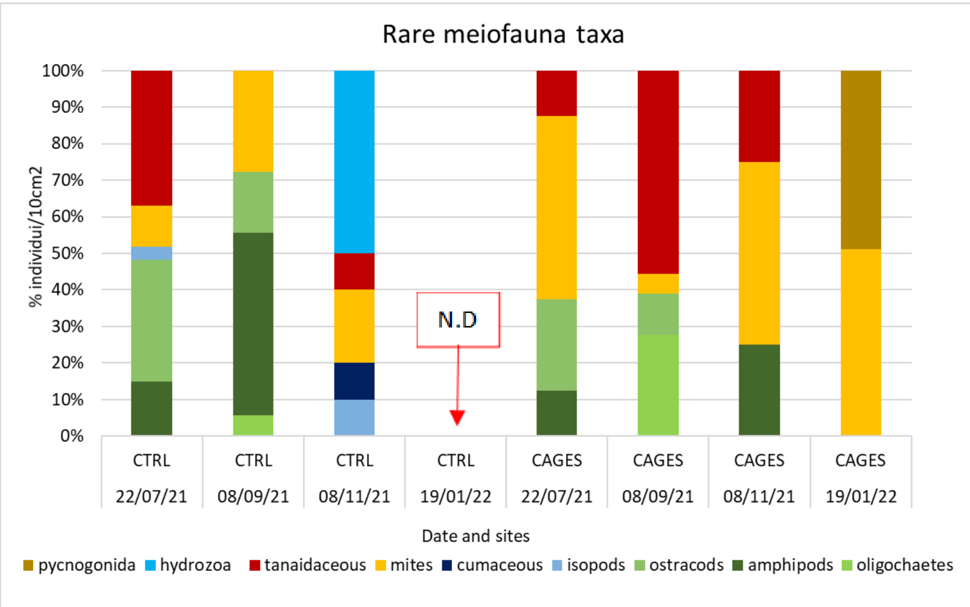

Figure 26: Rare meiofauna taxa in control and restoration site

The PERMANOVA analysis conducted on the rare meiofauna taxa revealed significant effects of the factors Time and Sites X Time (Table 5).

Table 5: PERMANOVA analysis conducted on the rare meiofauna taxa in the SNI

Source	df	MS	F	P
Sites	1	4381,8	1,6	0,143
Time	3	4866,6	1,8	0,032
Sites X Time	2	5430,5	2,0	0,02
Residuals	12	2767,3		

The pair wise tests revealed significant differences between control and cages, and it is worthy of notice that at T4 the rare taxa were present only near the cages.

Was also conducted a bi-plot after CAP analysis on rare meiofaunal taxa composition (Figure 27). This analysis shows a clear separation between the control and the cages. Moreover, the rare taxa under the cages are less dispersed than the controls and are similar to control at the T0. The graph also shows how, over time, the data relating to the two sites (control and cages) are dispersed and differentiated.

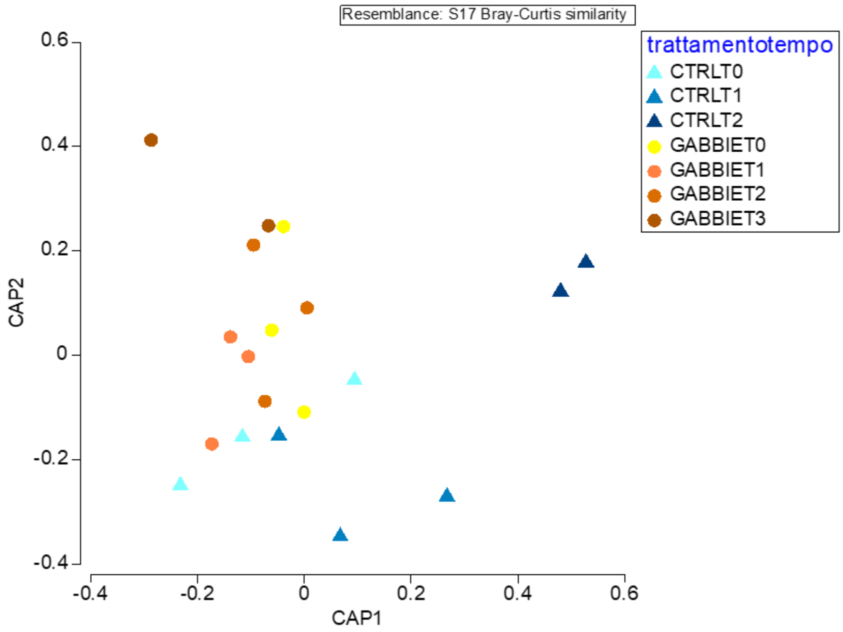

Figure 27: CAP analysis of rare meiofauna taxa data. The dots represent the rare taxa under the cages at various times, while the triangles represent the rare taxa in the control site at various times

4.2.2 Macrofauna

Total abundance

In Figure 28 the data on the total macrofauna abundance are reported.

The values in the control site vary from 10,22 ± 2,76 ind/50 cm^2 in T0, at 6,81 ± 1,64 ind/50 cm^2 in T1, at 9,17 ± 2,36 ind/50 cm^2 in T2, and at 3,14 ± 2,24 ind/50 cm^2 in T3.

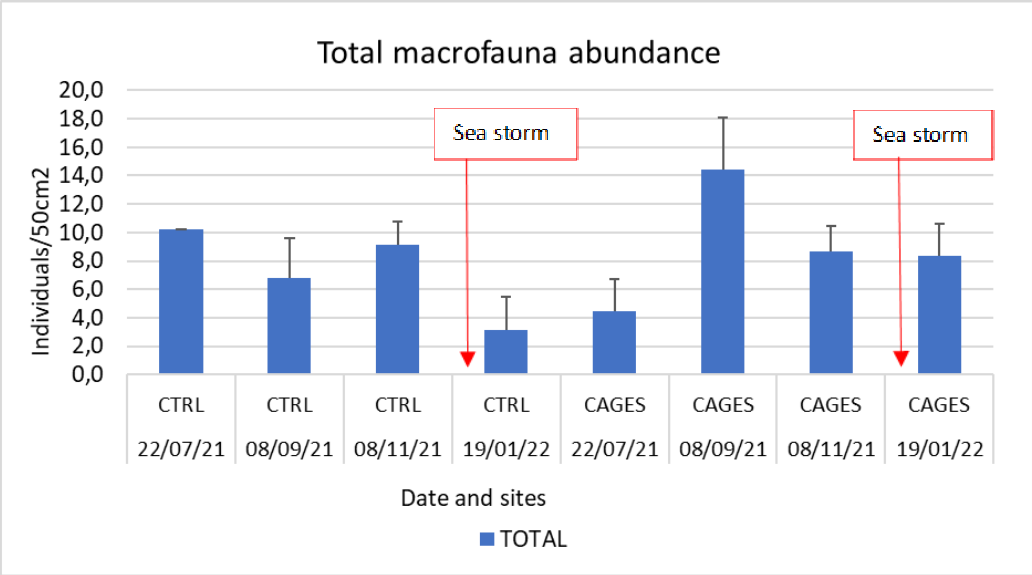

Figure 28: Total macrofauna abundance (n. ind/50 cm^2) in the control stations and near the restoration site in the SNI, the data are reported as average standard error over 3 replicates

At the restoration site, the values are 4,45 ± 3,71 ind/50 cm^2 at T0, 14,41 ± 1,83 ind/50 cm^2 at T1, 8,65 ± 2,24 ind/50 cm^2 at T2 and, at the end, 8,38 ± 3,86 ind/50 cm^2 at T3.

The PERMANOVA analysis conducted on total macrofauna abundance doesn't reveal any statistically significant differences (Table 6).

Table 6: PERMANOVA analysis conducted on the total macrofauna abundance in the SNI

Source	df	MS	F	P
Sites	1	1,5	0,7	0,444
Time	3	0,5	0,2	0,902
Site X Time	3	2,8	1,3	0,323
Residuals	16	2,2		

Taxonomic richness

Figure 29 reported the richness of macrofaunal taxa cumulatively for each sampling point.

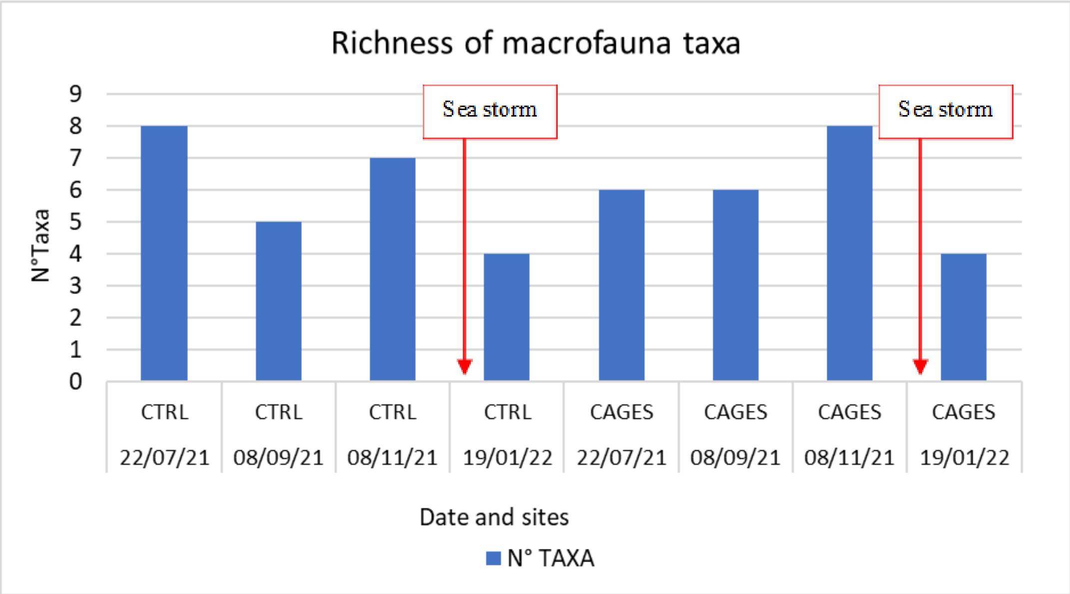

Figure 29: Total richness of macrofauna taxa (number of taxa) in the control stations and in the SNI restoration site, the data are reported cumulatively in the analyzed replicates

In the control site were detected 8 taxa at T0, 5 at T1, 7 taxa at T2, and 4 taxa at T3. At the restoration site, 6 taxa were counted in the first two sampling times (T0, T1), 8 at T2 and, as in the control, 4 in T3.

Taxonomic composition

Figure 30 shows the macrofauna taxonomic composition. Twelve taxa have been identified: nematodes, polychaetes, copepods, picnogonidae, hydrozoa, anemones, oligochaetes, bivalves, gastropods, tanaidaceous, isopods, and amphipods.

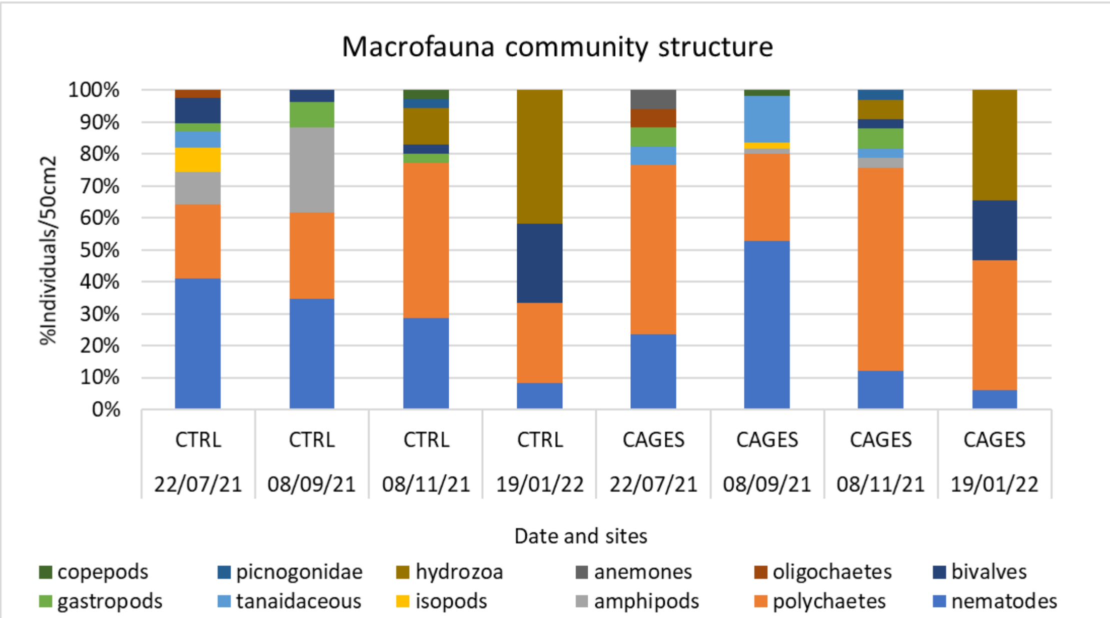

Figure 30: Percentages of the abundance of macrofauna taxa in the control and restoration site

Both at the control and at the restoration site, in the T0, T1 and T2, the prevalent taxa are nematodes (41% T0, 34,6% T1, 28,6% T2, at control and 23,5% T0, 52,7% T1, 12,1% T2 at the restoration site) and polychaetes (23,1% T0, 26,9% T1, 48,6% T2 at the control site and 52,9% T0, 27,3% T1, 63,6% T2 at the restoration). At T3, in the control site, the main taxa in terms of abundance are hydrozoa (41,7%), bivalves, polychaetes (both 25%), and nematodes (8,3%);

in the restoration site, polychaetes are the principal taxon (40,6%), followed by hydrozoa (34,4%), bivalves (18,8%) and nematodes (6,3%). In the control site, at the T0 and T1, the third most common taxon is represented by the amphipods (10,3% T0 and 26,9% T1), followed by isopods and bivalves at T0 (both 7,7%) and by gastropods and bivalves at T1 (7,7% and 3,8%). Then, at T0 there are tanaidaceous (5,1%), gastropods, and oligochaetes (both 2,6%), while at T2 there are hydrozoa with 11,4%, and then gastropods, bivalves, picnogonidae, and copepods with 2,9%.

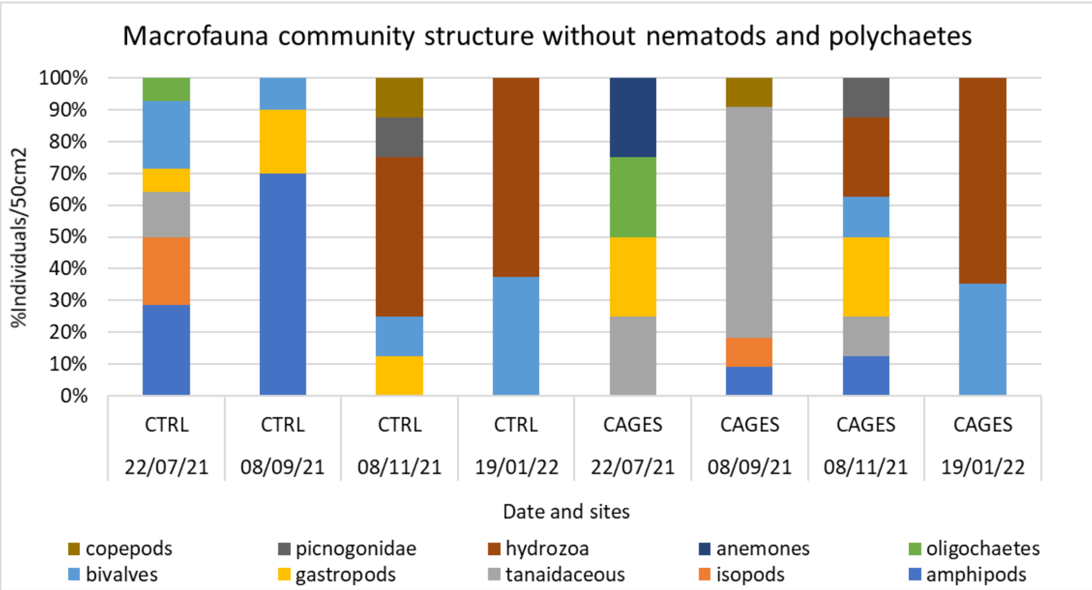

Figure 31: Taxonomic macrofauna composition without nematodes and polychaetes at the control and restoration site

At the restoration site, at the T0 the other taxa present are tanaidaceous, gastropods, oligochaetes, and anemones with a percentage of 5,9%. Then, at the T1 there are the tanaidaceous (14,5%), followed by amphipods, isopods, and copepods (1,8%). Finally, at the T2 there are gastropods and hydrozoa with the percentage of 6,1%, and amphipods, tanaidaceous, bivalves, and pycnogonids with 3%.

The PERMANOVA analysis was conducted on the macrofauna community structure with and without polychaetes and nematodes, the dominant taxa. In both cases, a statistically significant effect was detected only for the factor Time (Table 7 and 8).

Table 7: PERMANOVA analysis conducted on the macrofauna community structure

Source	df	MS	F	P
Sites	1	2137,9	0,9	0,516
Time	3	6437,2	2,7	0,001
Site X Time	3	2154,2	0,9	0,585
Residuals	16	2391,2		

Table 8: PERMANOVA analysis conducted on the macrofauna community structure without polychaetes and nematodes

Source	df	MS	F	P
Sites	1	2238,2	0,8	0,574
Time	3	8734,7	3,2	0,001
Site X Time	3	2974,8	1,1	0,348
Residuals	14	2697,0		

The bi-plot after CAP analysis on macrofauna community structure data is reported in Figure 23.

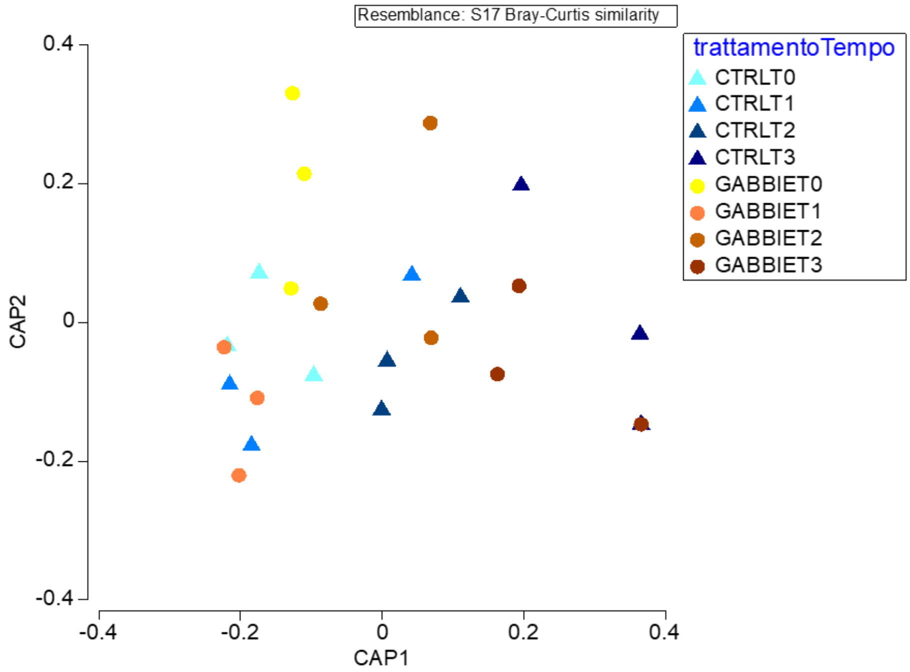

Figure 32: CAP analysis of macrofauna community structure data. The dots represent the taxa under the cages at various times, while the triangles represent the taxa in the control site at various times

This analysis doesn't show a clear disposition between the control and the cages; the graph also shows how, over time, the data relating to the two sites (control and cages) are dispersed and differentiated.

4.3 Grazing

G. barbata growth

Figure 33 reported the average variation of *G. barbata* heights in the aquarium.

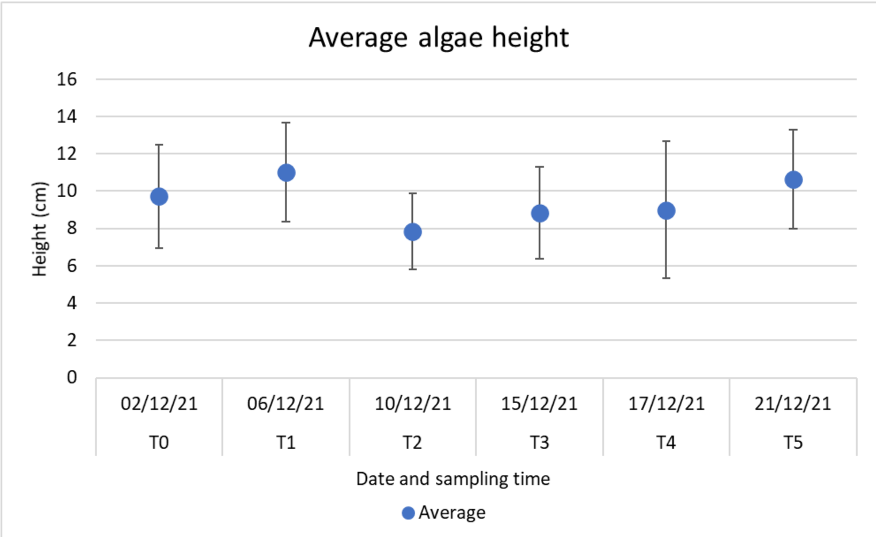

Figure 33: temporal variation of the height of *G. barbata* in the grazing experiment in the aquarium. The bar represents the standard deviation

The algae height is highest at T1, with an average of 10,8 cm, and is lower at T2 with a value of 9,8 cm. At the T3 and T4, the average height is 9,9 cm and it increases at T5 with 10,7 cm.

The PERMANOVA analysis conducted on the average algae height did not reveal a significant effect of the factor Time (Table 9).

Table 9: PERMANOVA analysis conducted on average algae height of grazing experiment. The variable Time was considered

Source	df	MS	F	P
Time	5	2,6818	0,42815	0,817
Residuals	60	6,2637		
Total	65			

Abundance of G. barbata individuals

Figure 34 reports the variation of the number of algae individuals on the rock in the aquarium.

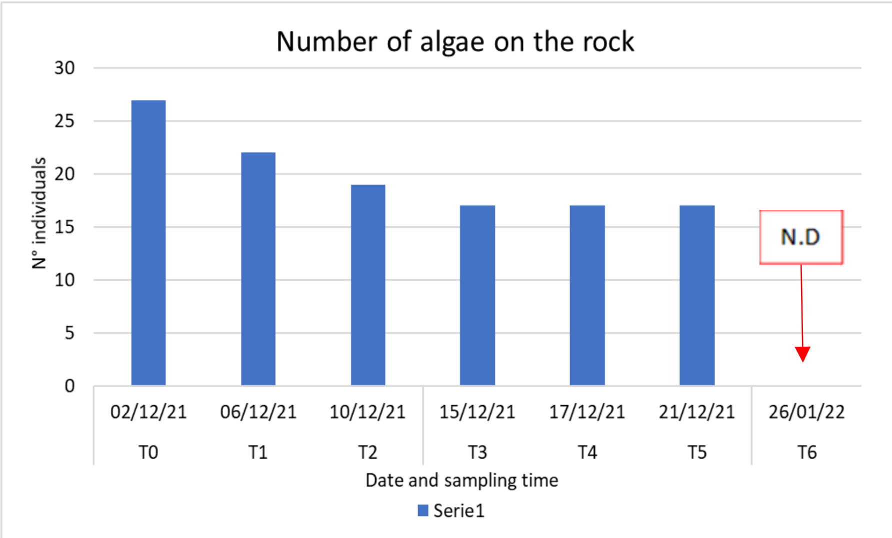

Figure 34: Algae number variation of the rock in the aquarium experiment

The total number of the algae was 27 at T0, 22 at T1, and 19 at T2; in the following sampling times (T3, T4, and T5) the value is constantly 17. 40 days after the start of the experiment, through the photos, it was possible to estimate the number of algae, equal to 12. When the tank in the aquarium was decommissioned, after 56 days, no more *G. barbata* individuals were present on the stone.

5. DISCUSSION

5.1 Evaluation of the *G. barbata* restoration success in the SNI

It's well known that the restoration of endangered species is one of the best weapons available to us in recent decades. By definition, ecological restoration is "the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed" (SER Primer, 2002).

A restoration intervention's success can provide a lot of benefits, which can lead to improvements related to biodiversity and ecosystem services, as climate, economic growth, and mental and physical well-being (Aronson & Alexander, 2013). While the restoration of terrestrial environments it's well understood, knowledge regarding coastal-marine ecosystems is still scarce or missing (Blignaut et al., 2013). In this regard, the initiative "Decade on Ecosystem restoration" by the United Nations, from 2021 to 2030, plays a fundamental role since its aim is to restore ecosystems in all continents and in all oceans of the Earth (Decade on restoration website source).

The brown algae belonging to the order Fucales, in particular to the group *Cystoseira s.l.*, play an important ecological role that is endangered by their constant decline, especially in the Mediterranean Sea. For this reason, targeted restoration interventions of these species are necessary, especially for those populations that are not able to recover naturally (Gianni et al., 2013, Fraschetti et al., 2021). This thesis work aims to test the restoration of *G. barbata*, a brown algae in rapid decline, on breakwaters barriers in a formerly polluted site (SNI of Falconara Marittima), with high grazing pressure. The SNI's breakwaters were chosen as a restoration site in order to increase their ecological value in habitats that are often uniform and degraded.

The *in situ* recruitment of *G. barbata* at the Passetto was successful, with the exponential growth of the recruits along time. The algae on structures 2 and 3 have had a comparable growth rate, while structure 1 has reached lower heights. This is probably due to the movement of the adult's fronds of *G. barbata* that surrounded structure 1, which prevented a correct growth especially in the last part of the structure.

The use of cages to exclude grazers has allowed the algae to grow and survive even six months from the start of the transplantation in the SNI (except for structure 3 damaged by the sea storm). It has been demonstrated that, especially in the early stages of the experiment, the presence of cages is essential to avoid grazing, and this has also been proven by other studies conducted on artificial reefs. In this regard, Perkol-Finkel et al. (2012) demonstrated that the use of cages with 1x1 cm mesh for the *G. barbata* restoration in artificial barriers allows the algae to survive and to have a growth comparable to natural populations.

In our case, during the experiment at the SNI, however, some critical issues related to the presence of the cages were evident: first, it's essential to choose the most suitable site where to place them, possibly on a flat surface and sheltered from the wave motion (Perkol-Finkel et al., 2012, Gianni et al., 2013, Cebrian et al., 2021). In fact, in October, a sea storm has torn away the cage of the third structure, which was less sheltered than the other two, leading to the complete disappearance of *G. barbata* individuals in about 2 weeks, due to grazing. It should be noted that the algae of structure 3 have been affected by the sea storm with a decrease of the canopy from 77 to 26%, probably also for this reason they have not survived the grazing pressure. Despite their more sheltered position, even the cages of the first two structures were torn away by the January sea storm, but probably the highest coverage of *G. barbata* individuals allowed them to survive at least until the last sampling, which happened 9 days later (77% of coverage for structure 1 and 98% for structure 2 at this time). This suggests that, in addition to the height reached by the algae, the canopy and the number of individuals also play a key role in survival against grazers.

Another problem related to the use of cages is the presence of epiphytes, in particular green and red algae (like *Ulva cf. lacinulata* and *Hypnea spinella*) abundantly present on the breakwaters at Falconara. These organisms, in fact, epiphyte the walls of the cages and are in competition for the resources with *G. barbata*; moreover, they could modify the irradiance, and the water exchange conditions (Cebrian et al., 2021). Removing only the cover of the cages could be a solution, in order to create an open environment around the *G. barbata* individuals; in this way, the water movement could contribute to cleaning the epiphytic organisms. This operation should be done once the algae have reached a height allowing them to withstand the grazers. In this experiment, after the October storm that swept away one of the cages, the cover of cage 1 was removed with excellent results: the algae survived and grew over time, and the presence of epiphytes reduced, probably also due to their seasonal fluctuation. The survival of *G. barbata* on structure 1, after uncovering, can also be attributed to the presence of the cage's walls, which could prevent predators from reaching the algae. However, the total removal of cages 1 and 2 due to the January sea storm testifies, at least until the last sampling time, that the algae are able to survive at the grazers once reached a certain height and canopy.

The results of this experiment also suggest that cages, and in particular lids, after a certain period of time, can prevent algae from expanding and growing properly. This can be noted from the comparison of the growth trend of algae of the structures 1 and 2: since the lid was removed from cage 1, in fact, the individuals of *G. barbata* have reached heights that, over time, have significantly exceeded those of structure 2, which has not been uncovered (until the January storm). At the T8 (8 weeks after the lid removal) indeed, the maximum height reached by the algae of structure 1 was 26,1 cm, while that of structure 2 was 15,4 cm. Instead, the trend of the canopy is inverse: during all the restoration experiments, the percentage of coverage of structure 1 is lower than that of structure 2 (already at the beginning of the transplant structure 1 had less density of individuals than the other two). This could suggest that the absence of the lid favors the growth in height of the algae.

Despite the difficulties encountered mainly due to stochastic events (bad weather and sea storms) *G. barbata* survived and grew over time; this shows that the cages play a fundamental role in the success of restoration interventions in artificial reefs, at least for the survival of juvenile algae and until they become pressure resistant to grazing.

5.2 Response of the fauna community associated with the *G. barbata* restoration

The macroalgae forests play an important role in increasing the ecosystem biodiversity, due to their role as primary producers and the higher organic matter availability which they provide (Bianchelli et al., 2016). Some recent studies have been proved that the total or partial disappearance of macroalgae forests leads to an impoverishment of biodiversity, with the diminution of both important components of benthic ecosystems and ecosystem functions (Danovaro and Fraschetti, 2002, Bianchelli et al., 2016, Bianchelli and Danovaro, 2020).

The meio- and macrofauna are important ecological indicators that can provide information on the ecosystem's ecological quality, so their abundance, biomass, and biodiversity can be investigated in restoration interventions of different macroalgae species and used as indicators of success (Bianchelli et al., 2016, Danovaro and Fraschetti, 2002, Pearson and Rosenberg, 1978 and Pusceddu et al., 2011). Meiofauna have an ecological role in linking the detrital and prokaryotic components with the higher trophic levels; is, therefore, a fundamental trophic resource for macro- and megafauna, helping to maintain proper ecosystem efficiency and functionality (Bianchelli et al., 2016, Pusceddu et al., 2011). The macrofauna component also represents a fundamental step in the trophic chain, eating meiofauna and representing the temporary meiofauna which in turn is fundamental for macrofaunal recruitment. In addition, macrofauna is characterized by high trophic-functional heterogeneity and complex and diversified life cycles, for all these reasons it's suitable for environmental quality assessment (Pearson and Rosenberg, 1978).

In this restoration experiment has been investigated the total abundance, the number of taxa, and the community structure of meio- and macrofauna under the cages and in the control site, to test the biodiversity changes led by *G. barbata*. The results of meio- and macrofauna abundance show no significant differences between the control and the restoration site, as the two temporal trends of the values are comparable. This is probably due to seasonal fluctuations and the sea storms that characterized the period of the experiment, and that influence especially the meiofauna abundance (Danovaro and Fraschetti, 2002).

The meiofauna abundance values recorded in this restoration intervention are comparable to those found in some barren grounds ecosystem (Bianchelli et al., 2016); the SNI breakwater barriers, in fact, represent a hard and bare substrate comparable to barrens systems, which are very stable environment. This suggests that a longer period of time is probably required to obtain a response from the benthic community after a restoration intervention. The variation of the richness of taxa in meio- and macrofauna in the two sites do not present strong differences; however, at the T3, the meiofauna presents a higher number of taxa under the cages compared to the control site. The macrofaunal richness of taxa was similar or even higher near the cages than in the control site, at almost all sampling time.

Regarding the meio- and macrofauna total community structure, significant differences were found for the variable "time"; also, in this case, the change of taxonomic structure is probably due to seasonal fluctuations of benthic fauna and there isn't yet influenced by the presence of *G. barbata*. However, previous studies highlighted that the most dominant taxa, as nematodes and copepods, may mask differences in the taxonomic composition (Bianchelli et al., 2010). Indeed, in this study, when we considered only the rare taxa, we observed significant differences in the taxonomic composition of the meiofaunal assemblages between control site and cages with *G. barbata*. Rare meiofauna taxa are different in time and between time and the two sites, and at the T3 they were not found at the control site but only under the cages. At each sampling time, the total richness of taxa observed, considering control and cages' sites, is higher than the richness observed at each site. This let us hypothesize that the presence of *G. barbata* promotes the overall richness of the area in which it is transplanted (Figure 35), particularly due to the rara taxa. In particular, in the cages site, there is a prevalent abundance of tanaidaceous and mites, taxa that are probably able to respond quickly to the enrichment of habitats brought by the transplantation of *G. barbata*.

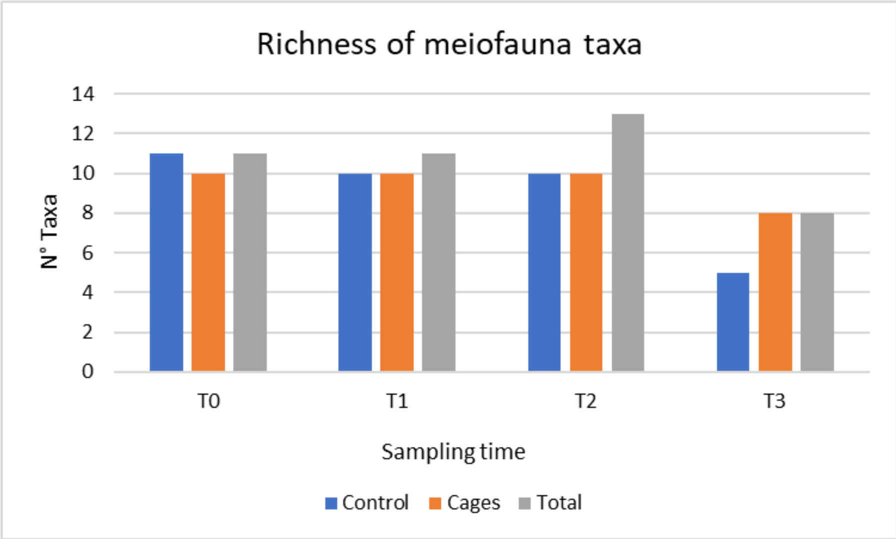

Figure 35: Overall richness of meiofauna taxa observed at each sampling time

In the control site, however, there is a greater abundance of amphipods and hydrozoa in respect to the restoration site, even if the latter taxon appears with high values of abundance also in the macrofauna under the cages. This lets us hypothesize that the *G. barbata* outlplants could have an important role in the macrofaunal recruitment, hosting temporary meiofauna.

The macroalgae forests create an attractive response towards the benthic community and this involves an increase in the taxa richness (Danovaro and Fraschetti, 2002). The greater is the complexity of the macroalgae habitat and the greater is the biodiversity associated with it, due to the high number of ecological niches available (Veiga et al., 2014). Longer monitoring times are therefore required in order to obtain a response from the meio- and macrobenthic community in a restoration intervention, allowing *G. barbata* specimens to grow and thus create a more complex three-dimensional habitat.

5.3 Response and evaluation of grazing pressure on *G. barbata*

The artificial marine substrates, like breakwaters barriers, are more and more numerous along the coasts, contributing to the alteration of the habitats and influencing the ecosystem functioning (Perkol-Finkel, 2021, Gianni et al., 2013). Several studies have shown that artificial structures are environments with lower levels of biodiversity than natural ones, often dominated by opportunistic and invasive species (Bulleri and Chapman, 2010, Ferrario et al., 2016). For these reasons, they can be exploited as a substrate for macroalgae restoration interventions, in order to increase their ecological value and, at the same time, protect species in severe decline (Gianni et al., 2013). However, these structures are characterized by a high rate of grazing on the algae, probably because they represent an "oasis" of hard substratum in sandy habitats and, moreover, the absence of other more attractive species leads the predators to eat restored macroalgae, which represent a new trophic resource of high quality in an extremely oligotrophic system (Ferrario et al., 2015).

The grazing experiment in the aquarium was conducted after the observation that *P. marmoratus* was apparently the only species eating outplanted *G.*

barbata on the breakwaters at SNI. The experiment gave positive results, in fact, it was possible to film crabs eating the algae.

The macroalgae individuals, in the 19 days in which they were measured, didn't have a significant growth/decrease over the entire period, while their number decreased from 27 to 22 after 4 days from the beginning of the experiment. On the following sampling time, the algae number dropped to 19. In the last three times, however, the number of algae has remained stable at 17. On 11th January, the presence of 12 algae was estimated on the rock and, 16 days after, on 27th January, no more *G. barbata* individuals were detected, while other red and green algae species were still present.

Considering the lack of algal growth and the concurrent reduction of their number, it can be assumed that the crabs have the grazing modalities by detaching the algae from the base. This hypothesis could help to explain the progressive and rapid disappearance of all the *G. barbata* specimens present on structure 3, after the elimination of the cage due to the sea storm.

These findings suggest that, in future restoration interventions, it would be necessary to transplant a very large number of algae individuals. In addition, it is confirmed that algae of reduced height (probably less than 10 cm) need cages to survive in environments with high grazing pressure. The resistance to the grazing pressure of the algae of greater dimensions is certainly greater, but we still don’t know how much percentage of cover and what height is necessary for the algae to survive, in the time, to the grazers without human intervention.

6. CONCLUSIONS AND PERSPECTIVES

This experiment has demonstrated that the use of cages for the grazer's exclusion, in habitats with high grazing pressure, it's efficient and allows the survival of macroalgae over time. We can also affirm that the *in situ* recruitment in a different area from the restoration one represents a good strategy and allow to obtain juvenile algae without damaging the natural donor populations. In addition, field observations and the aquarium experiment confirmed that crabs can be considered among the main herbivores of *G. barbata* in the environments of the breakwaters, especially when they represent an oasis of rocky substratum.

As far as biodiversity is concerned, we observed different responses by meio- and macrofauna. The main response for both components was observed in the richness of taxa and for meiofauna in the presence/composition of rare taxa. Overall, our results let us suggest that restoration interventions may have a positive role for enhancing the biodiversity level, however in order to corroborate our data, it would be necessary to lengthen the monitoring times at least until the spring/summer, when the environmental conditions are more favorable, and the benthic community has greater seasonal abundances and diversity. Restoration on artificial substrates is a great possibility (Ferrario et al, 2015), but to ensure lasting success over time it's necessary to expand the temporal and spatial scale of the interventions (Fraschetti et al., 2021). A high number of individuals of macroalgae and increased algal density should be key points of a restoration intervention on artificial substrates, because they help to counterbalance the grazing pressure and also promote the increase of faunal abundance, biomass, and diversity. The success of a restoration is such when the self-maintaining of the transplanted population is reached (Verdura et al., 2018). The breakwater barriers, however, are characterized by a high rate of sedimentation, which could reduce the recruitment of macroalgae thus becoming an obstacle to self-sufficiency (Irving et al., 2009).). Further monitoring is thus necessary also to control the reproductive success of the transplanted individuals. Finding tipping points (i.e., the proper number of transplanted individuals, canopy %) could be a challenge for the future, with the aim of creating real macroalgae forests that succumb to the impoverishment of habitats caused by artificial substrates.

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