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# Recovery and restoration of marine endangered habitats

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**ATTACHED PAPERS**

# RIASSUNTO

La biodiversità marina regola il funzionamento ecosistemico, responsabile della produzione di beni e servizi importanti per la biosfera ed il benessere umano. I cambiamenti climatici globali e le attività umane stanno alterando la biodiversità degli oceani ed il funzionamento ecosistemico. Al momento, partendo dalla consapevolezza che le sole misure di conservazione non sono sufficienti a invertire la degradazione degli ecosistemi, è riconosciuto che il restauro ambientale è una azione di cruciale importanza per far fronte a questa minaccia. Tuttavia, è necessaria una maggiore conoscenza scientifica per rendere efficaci azioni di restauro, specialmente negli ambienti profondi che sono largamente sconosciuti. Tra le maggiori attività che avranno impatti sugli habitat marini vi sono lo sfruttamento di risorse minerarie e la pesca a strascico, le quali, risospendendo particelle polimetalliche e sedimenti, avranno effetti principalmente sulle specie bentoniche. In questa tesi, il *Corallium rubrum*, specie strutturante di elevata valenza ecologica, è stato esposto a questi due tipi di particolato. Dopo la rimozione della fonte di impatto, questo organismo è in grado di recuperare parzialmente tassi di alimentazione e la sua integrità tissutale. Questo esperimento fornisce utili informazioni non solo per meglio comprendere gli effetti di tali attività su organismi bentonici, ma anche per definire possibili misure di mitigazione attraverso una modulazione appropriata della loro intensità e durata.

Allevare in acquario specie di corallo minacciate potrà essere utile per futuri progetti di restauro che prevedono il trapianto di colonie donatrici. Una dieta appropriata da somministrare durante l'allevamento può avere effetti benefici sulla loro crescita ed il loro successo riproduttivo. In questa tesi, è stata studiata la selezione di cibo da parte di alcuni coralli profondi (*Desmophyllum pertusum*, *Madrepora oculata* and *Dendrophyllia cornigera*), i quali hanno mostrato una preferenza per il crostaceo *Mysis relicta*. I risultati ottenuti dalle analisi condotte sugli isotopi stabili hanno inoltre fornito nuove informazioni sulle nicchie trofiche occupate nel Mar Mediterraneo da queste specie.

Una volta ampliate le conoscenze sugli habitat e le specie da restaurare, è necessario valutare l'efficacia delle procedure di rispristino che si vogliono applicare. In questa tesi, sono stati studiati gli effetti di due esperimenti pilota di trapianto della fanerogama *Cymodocea nodosa* e della gorgonia *Eunicella singularis* su attributi di funzionamento ecosistemico. I risultati ottenuti indicano che il trapianto di tali organismi, se opportunamente pianificato, non solo garantisce la loro sopravvivenza, ma può anche avere effetti positivi su processi ecosistemici chiave.

Tuttavia, sono necessari ulteriori studi per valutare la possibile estensione di queste azioni per affrontare l'attuale scala spaziale di perdita di specie/habitat. Questo lavoro fornisce nuovi elementi

per la comprensione dei potenziali benefici ecologici derivanti dal restauro ambientale e delle sue ricadute per la conservazione del capitale naturale.

# ABSTRACT

Marine biodiversity regulates ecosystem functions, which are responsible for the production of goods and services for the biosphere and human well-being. Global changes and human activities are altering ocean biodiversity and ecosystem functioning. At present, stemming from the awareness that conservation and management are often not enough to halt and revert the degradation of threatened ecosystems, it has been recognized that active restoration is crucial to cope with this issue. More knowledge is needed to make restoration actions effective, especially for the largely unknown deep ocean. Two of the main activities that will alter marine habitats are ore exploitation and bottom trawling that, resuspending polymetallic and sediment particles, will affect benthic species. In this thesis, the habitat-forming species *Corallium rubrum* was exposed to these types of particles. After the removal of the disturbance, its feeding rates and tissue integrity partially recovered. This experiment provides new insights on the consequences of these activities as well as on potential mitigation strategies by properly modulating their intensity and duration.

Rearing endangered corals in aquaria can be useful for future projects that aim to restore degraded reefs by transplanting healthy colonies. An appropriate diet may positively impinge on their growth or reproduction success. In this perspective, I studied the food selection of cold-water corals (*Desmophyllum pertusum*, *Madrepora oculata* and *Dendrophyllia cornigera*) was studied and these species showed a preference for the crustacean *Mysis relicta*. Stable isotope analyses provided also novel information on the trophic niches occupied by these coral species in the Mediterranean Sea.

After expanding the knowledge on the habitat or the species to be restored, it is necessary to evaluate the effectiveness of the restoration actions that it might be applied. In this thesis, the effects of two pilot transplantation experiments of the seagrass *Cymodocea nodosa* and the gorgonian *Eunicella singularis* on ecosystem functioning of surrounding sediments have been studied. The results showed that transplantation can be effective and that it can have also positive effects on key-ecological processes. However, further studies are needed to assess the potential of scaling-up these actions addressing the present scale of species/habitat loss. This work provides new elements for a better understanding of the potential ecological benefits that can contribute to the conservation of the natural capital.

# CHAPTER 1: INTRODUCTION

Marine ecosystems provide a plethora of goods and services such as food, waste treatment, protection of shorelines from storms, regulation of the climate and biogeochemical cycles of water and nutrients, recreational opportunities (Covich et al., 2004; Millennium Ecosystem Assessment, 2005). Several anthropogenic activities are altering marine biodiversity integrity, threatening the ecosystem functioning and the provisioning of goods and services (Lotze et al., 2006; Halpern et al., 2008; Lotze et al., 2018).

## 1.1 Ecological restoration

The Society of Ecological Restoration (SER) defined the "International Standards for the Practice of Ecological Restoration" and clearly distinguished between "ecological restoration" and other forms of ecosystem repair. Restoration is the process of "assisting the recovery of ecosystems that have been degraded, damaged or destroyed" (SER, 2004). Indeed, the "recovery" of an ecosystem is the achievement of a target environment that is similar to an appropriate local native model or reference ecosystem, in terms of its specific compositional, structural, and functional ecosystem attributes, (Suding et al., 2015; McDonald et al., 2016). The concept of "restoration" includes an action or multiple actions that together place a degraded ecosystem on a trajectory for complete recovery (Edwards & Gomez, 2007; Aronson et al., 2017), regardless of the period required to achieve the outcome (McDonald et al., 2016). Ecosystem restoration aims to establish a self-supporting habitat like the "original" habitat prior to the impact. Restored ecosystems can be resilient to perturbations and capable to adapt to existing and future environmental changes (Suding et al., 2015; Timpane-Padgham et al., 2017; Török & Helm, 2017). The selection of the reference ecosystem guides the restoration and should be both historically inspired and grounded in social processes that include multi-party stakeholders and restoration scientists and practitioners (Aronson et al., 2017). Remediation, reparation, and rehabilitation are instead a group of restoration actions that can help to achieve restoration purposes. Unlike restoration, rehabilitation is less ambitious in not necessarily achieving full recovery of processes and a return to ' 'pre-disturbance' ' conditions (Elliott et al., 2007; Aronson et al., 2017). This kind of action replaces structural or functional characteristics damaged by an impact, and aims to enhance the social, economic, and ecological value of the new ecosystem (Elliott et al., 2007; Suding et al., 2015; McDonald et al., 2016).

The model for all these kinds of actions, the reference ecosystem, should resemble a near undamaged site (SER, 2004). The reference ecosystem can also be derived from multiple sources of information on diverse ecological and biological variables (e.g. biodiversity, life cycles, functional variables, food webs) supported by abiotic measurements (Suding et al., 2015; Danovaro et al., 2017). In any case, ecosystem baselines should be clearly defined. Full recovery requires several steps, each encompassing different interventions (e.g. removal of the disturbance, removal of invasive species, enhancement of native species reproduction) (McDonald et al., 2016). Ecological experts in restoration have abandoned the idea of complete recovery, recognising that ecosystems are dynamic. Thus, restoration may not follow the appropriate ecological trajectory to re-establish over time, especially considering the need for ecosystems to adapt and evolve over time in response to climate change (Aronson et al., 2016).

## 1.2 Directives, legislation and projects on marine ecosystem restoration

In 1992, the biologist E.O. Wilson wrote the sentence: "The next century, will, I believe, be the era of restoration in ecology". During last decades, more and more projects of ecological restoration have been started, especially in terrestrial habitats. Ecological restoration is now recognized as a global priority and several are the directives or laws that talk about this concept, especially for terrestrial and shallow-water ecosystems (Aronson & Alexander, 2013). In contrast, restoration in the deep-sea has yet to receive much attention (Da Ros et al., 2019). Since 2010, the European Marine Strategy Framework Directive emphasized the importance of sustainably manage and protect also marine ecosystems, achieving a Good Environmental Status (GES) and avoiding significant losses of biodiversity and ecosystem functioning. In 2012, at the XI Conference of the Parties (COP11), the Convention on Biological Diversity (CBD), together with the 173 Contracting Parties, identified the 15% of the degraded habitat for each type of ecosystem (including marine ecosystems) for which the ecological restoration should be completed by 2020 (Convention on Biological Diversity, 2012). Still in 2012, the International Union for Conservation of Nature (IUCN) provided guidelines for restoration practices of coastal marine ecosystems (Keenleyside et al., 2012). The restoration of marine degraded habitats is also expected by the goal number 14 (SDG14, life below water) of the United Nations Sustainable Development Goals (SDGs) for 2030 (United Nations General Assembly, 2015). Furthermore, the United Nations General Assembly also declared 2021-2030 as the UN Decade on "Ecosystem Restoration" (United Nations General Assembly, 2019), inviting Member States to reinforce existing restoration programs, found new restoration projects and share knowledge on good experiences and practices of ecological restoration.

Among the several projects that deal with marine restoration, the European Union funded in 2016 an entire project on European marine ecosystem restoration, the MERCES project (Marine Ecosystem Restoration in Changing European Seas) (grant agreement No. 689518, Horizon 2020 research and innovation programme, www.merces-project.eu). This project focuses on the conduction of pilot restoration experiments in different degraded marine habitats: shallow soft-bottoms habitats, shallow hard-bottom habitats and deep-sea habitats.

The principal aims of this project are 1) assessing the potential of different technologies and approaches; 2) quantifying the returns in terms of ecosystems services and their socio-economic impacts; 3) defining the legal-policy and governance frameworks needed to optimize the effectiveness of the different restoration approaches. Specific aims include: a) improving existing and developing restoration actions of degraded marine habitats; b) increasing the adaptation of EU degraded marine habitats to global change; c) enhancing marine ecosystem resilience and services; d) conducting cost-benefit analyses for marine restoration measures; e) creating new industrial targets and opportunities. This PhD thesis is included in the framework of the MERCES project. I have focused my attention over pilot experiments for the restoration of deep-sea habitats, shallow soft-bottom habitats and shallow hard-bottom habitats. The principal features of the studied habitats are presented below.

## 1.3 Features of studied cases

### 1.3.1 Deep-sea habitats

The deep ocean, Earth's largest ecosystem (Danovaro et al., 2008; Ramirez-Llodra et al., 2010), remains largely unexplored (Danovaro et al., 2008; Webb et al., 2010; Costello & Chaudhary, 2017), primarily because of the technical challenges and associated high costs of investigation (Kintisch, 2013; Danovaro et al., 2017). Despite these challenges, it is widely recognised that deep-sea ecosystems host a large portion of Earth's biodiversity that plays a key role in the functioning of our planet (Danovaro et al., 2010; Rex & Etter, 2010; Thurber et al., 2014; Danovaro et al., 2017; Folkersen et al., 2018), supporting key ecological processes (including production, consumption and decomposition of organic matter and nutrient regeneration)(Danovaro et al., 2008) and providing essential goods and services for human well-being (Millennium Ecosystem Assessment, 2005; Armstrong et al., 2012; Thurber et al., 2014; Inniss et al., 2016; Sweetman et al., 2017). In the deep sea, industries are moving rapidly toward exploitation of its resources (Ramirez-Llodra et al., 2011; Gross, 2015), partially in response to the depletion of those provided by terrestrial and coastal habitats.

Plans for expansion of mineral/oil exploitation and bottom-contact fisheries in the deep sea suggest increasing potential for degradation of deep-sea ecosystems and call for regulation, protection and eventually funding of possible restoration actions (Barbier et al., 2014; Mengerink et al., 2014; Van Dover et al., 2014; Danovaro et al., 2017). Despite some pilot restoration experiments on cold-water corals provided promising results (Brooke et al., 2006; Brooke & Young, 2009; Strömberg et al., 2010; Dahl, 2013), there is still no evidence of successful ecological assisted restoration as a management tool to reverse environmental degradation caused by human activities in the deep sea (Niner et al., 2018). However, since protection alone can be insufficient to reverse habitat degradation in the deep sea, restoration actions should be properly defined to remediate and compensate human-induced damages.

Successful application of most restoration efforts will first require a deeper understanding of biodiversity and functioning of deep-sea ecosystems, and better knowledge of ecosystem resilience and recovery rates of deep-sea fauna (Da Ros et al., 2019). In addition to limited data availability, expensive technologies (with estimated costs up to millions of dollars ha$^{-1}$)(Van Dover et al., 2014) represent a major obstacle to large-scale deep-sea restoration, but international cooperation (like a stronger collaboration between industry and scientists belonging to the academia) could significantly reduce this operational cost (Danovaro et al., 2017). Future deep-sea ecosystem restoration could offer an important business opportunity for technological development and application and an investment in natural capital for a new and competitive blue-growth sector (Da Ros et al., 2019).

Main reference for this paragraph (the paper is attached at the end of this thesis):

The deep sea: the new frontier for ecological restoration
Da Ros Z, Dell'Anno A, Morato T, Sweetman AK, Carreiro-Silva M, Smith CJ, Papadopoulou N, Corinaldesi C, Bianchelli S, Gambi C, Cimino R, Snelgrove P, Van Dover CL, Danovaro R (2019) The deep sea: The new frontier for ecological restoration. *Marine Policy*, 108. https://doi.org/10.1016/j.marpol.2019.103642

### 1.3.2 Soft-bottom habitats: seagrass meadows

One of the main habitats that characterize soft bottoms are seagrass meadows, which are widely recognized to form ecosystems that host high biodiversity and provide numerous services (Nellemann et al., 2009; Barbier et al., 2011; Mtwana Nordlund et al., 2016). For example, together with mangroves and saltmarshes, seagrasses are responsible for capturing up to some 70% of the carbon permanently stored in the marine realm (Nellemann et al., 2009; Unsworth et al., 2015; Dahl et al., 2016). They provide habitat and nursery areas for a multitude of fish and invertebrate species, support complex trophic networks, filter freshwater discharges from land, stabilise sediments, and significantly reduce coastal erosion (Duarte et al., 2002). Due to their ecological role, seagrass meadows are protected under various directives (e.g. Council Directive 92/43/EEC 1992). In the Mediterranean Sea, the most common seagrasses are *Posidonia oceanica*, *Cymodocea nodosa*, *Zostera marina* and *Z. noltii*. The first one is a slow-growing species, while the other three ones are faster-growing species (Marbà & Duarte, 1997; Marbà & Duarte, 2010). The vegetative reproduction of these species ensures local growth. Seagrass meadows can be found in European waters from the intertidal zone to 40 m depth, in waters with a wide salinity range (from 5‰ in the Baltic to 37‰ in Mediterranean waters).

Due to their wide distribution along coastal areas, seagrass meadows are prone to many anthropogenic pressures such as eutrophication, dredging, anchoring, invasive species, fisheries activities, coastal development, pollution and climate change (Short & Wyllie-Echeverria, 1996; Short & Neckles, 1999; Williams, 2001; Milazzo et al., 2004; Orth et al., 2006; Williams, 2007; Boudouresque et al., 2009; Waycott et al., 2009). A study reported that the 30% of the seagrass meadows have been lost since seagrass areas were initially recorded in 1879 (Waycott et al., 2009). As an example, in the Mediterranean Sea the accidental introduction of the invasive algae *Caulerpa* spp. and the eelgrass disease caused by *Labyrinthula zosterae*, provoked a relevant decrease of the extension of seagrass meadows (Marbà & Duarte 2010, Cecherelli & Cinelli 1997, Cecherelli & Campo 2002, Muehlstein 1988).

Many seagrass restoration techniques have been experimented with different species all over the world (Paling et al., 2009; Eriander et al., 2016). However, the overall success of seagrass restoration efforts has been quite low (37-38 %) (van Katwijk et al., 2009; van Katwijk et al., 2016). Successful restoration of seagrass ecosystems likely depends on several traits and characteristics that must be carefully considered prior to attempting active actions and at all stages throughout the recovery process (Weslawski et al., 2017). Important factors for the success include those related to the characteristics of the seagrass species in question, such as the growth rate and reproduction modality (i.e., slow-growing species will require longer restoration time scales than fast-growing species) as well as the traits of the donor population (genetic diversity, plant species diversity, spatial distribution, depth, and tidal height) (Paling et al., 2009). Another important thing to consider is the receiving site (Paling et al., 2009). An ideal restoration site should have high recovery potential, including similar physical (sediment type, depth, temperature, exposure, salinity, and nutrients) and biological characteristics (presence of grazers feeding on eelgrass or preventing algal blooms, bioturbators, facilitating species) as the donor site or, at least, appropriate for the for the seagrass species being restored (Peralta et al., 2003; van Katwijk & Wijgergangs, 2004; Di Maida et al., 2013). Proximity to natural seagrass meadows may also increase restoration potential as it ensures connectivity (spread of seeds) between populations. Most importantly, given the vulnerability of seagrasses, restoration projects should ensure that anthropogenic pressures must be reduced before starting the transplantation (Burkholder et al., 2007; Park et al., 2009; García et al., 2013). Moreover, choosing the right technique for transplantation of seagrass is essential for a successful restoration.
In the framework of the MERCES Project, some trials for establish the best technique for transplanting seagrasses in the Western Central Adriatic Sea have been conducted in 2017 and 2018. The restoration case of Gabicce Mare will be presented in this thesis.

### 1.3.3 Hard-bottom habitats: coralligenous habitats

Coralligenous assemblages are hard bottoms of biogenic origin that are mainly produced by the accumulation of calcareous encrusting algae (red algae, Rhodophyta) growing in dim light conditions (Ballesteros, 2006; Ingrosso et al., 2018). This type of framework thrives in the Mediterranean Sea between 20 and 120 m of depth (Ballesteros, 2006) depending on the local environmental variables, mainly irradiation conditions (Martin et al., 2014) (Fig. 1). Coralligenous outcrops are considered as hotspots of biodiversity, hosting the 20% of the Mediterranean species. The most important are long-living algae and sessile invertebrates that belong to taxonomic groups such as sponges, corals, bryozoans and tunicates (Ballesteros, 2006), many of which exhibit low growth rates (between 0.1 and 4 cm per year) (Cocito et al., 1998; Coma et al., 1998; Garrabou & Harmelin, 2002; Linares et al., 2010; Teixido et al., 2011; Linares et al., 2012; Sartoretto & Francour, 2012; Munari et al., 2013; Priori et al., 2013) and recruitment rates (Cocito et al., 1998; Linares et al., 2007; Teixido et al., 2011; Linares et al., 2012; Montero-Serra et al., 2015).

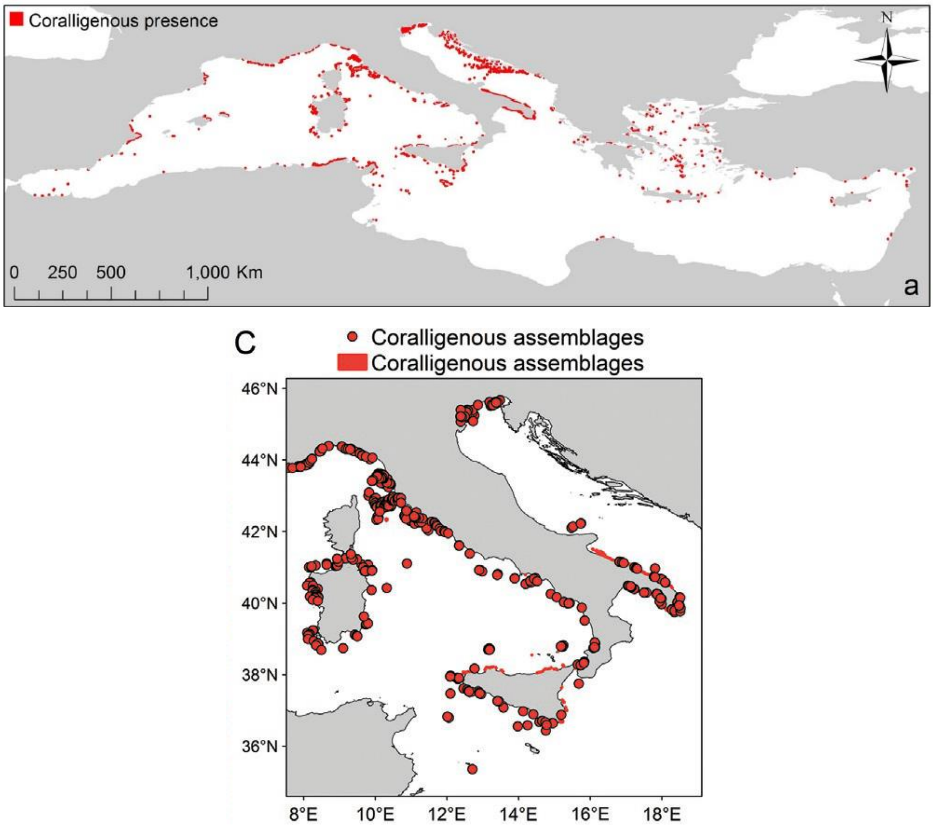

**Figure 1. Spatial distribution of coralligenous formations along the Mediterranean and Italian coasts. Images courtesy taken and modified from Martin et al., 2014 and Ingrosso et al., 2018.**

At present, coralligenous communities are threatened by several impacts that act synergistically, like nutrient enrichment, arrival of invasive species like the macroalgae *Womersleyella setacea*, *Acrothamnion preissii*, *Caulerpa cylindracea* and *C. taxifolia*, overexploitation (especially for the precious red coral *Corallium rubrum*), increase of sedimentation, mechanical impacts mainly derived from fishing activities and anchoring, as well as mucilage outbreaks, climate change and mass mortality events (Giuliani et al., 2005; Ballesteros, 2006; Balata et al., 2007; Garrabou et al., 2009; Piazzi et al., 2012; Garrabou et al., 2017). Efforts on the restoration of this kind of habitat have been focused on the transplantation of fragments of habitat-forming species like gorgonians and sponges (Linares et al., 2008; Fava et al., 2009; Montero-Serra et al., 2017). This method seems to be feasible on the local spatial scale. Expected dynamic of recovery is low and, because of this, timescales for complete restoration of coralligenous habitat are long. Moreover, the natural recovery rates of some habitat-forming species are usually not known.

In this thesis, I present the results of two experiments on the recovery capacity of *C. rubrum*, after simulated polymetallic particles exposition and sediment resuspension caused by simulated mining and trawling activities. Moreover, to evaluate the effectiveness of restoration techniques, the success of transplantation experiments of *Eunicella singularis* in the Marine Protected Area (MPA) of Portofino has been assessed.

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# CHAPTER 2: OBJECTIVES

This Ph.D. thesis deals with the issue of marine restoration. At present, stemming from the awareness that conservation and management are often not enough to halt and revert the degradation of threatened ecosystems, it has been recognized that active restoration is crucial to cope with this issue. The introduction of this work presents the principal features of the different marine habitats that will be interested by the experiments presented in the following chapters. In particular, in the attached paper, it is reviewed the state of art on ecological restoration in deep-sea ecosystems, highlighting knowledge gaps and the main scientific, technological and economic challenges. Restoration actions in the deep sea will be increasingly required in the future to face the expected progressive habitat degradation and as such they should be included in international conventions as a management tool to support conservation strategies of deep-sea ecosystems (Da Ros et al., 2019).

In the third chapter, the principal aim was to assess the recovery capacity of endangered hard-bottom species following simulated anthropogenic impacts to determine the potential of unassisted restoration. To reach this aim, two experiments were conducted to assess the impacts of simulated mining and trawling activities on the endangered species *Corallium rubrum*. Specimens of this habitat-forming species (Cerrano et al., 2013) were exposed to the resuspension of material caused by these anthropogenic activities. After the exposition, the disturbance was removed, and their natural recovery capacity was assessed. Sediment or polymetallic particle resuspension due to anthropogenic activities are expected to increase in the next future (Worm & Lenihan, 2013), highlighting the need to better understand their impacts on benthic biota and identify mitigation measures able to minimise biodiversity loss as much as possible.

In the fourth chapter, the goal was to define how to maintain hard-bottom species in aquaria in order to assess the best feeding conditions to rear them prior to transplanting operations for restoration projects. To reach this goal, an experiment was conducted to establish the feeding preferences of Cold-Water Corals collected during two oceanographic campaigns in the Ionian Sea and in the South Adriatic Sea.

In the last chapters, the principal aim was to evaluate the effects of two active restoration actions. In the framework of MERCES Project, pilot experiments on the transplantation of seagrass have been done at Gabicce Mare (North Adriatic Sea) and a transplantation of the gorgonian *Eunicella singularis* has been conducted in the Marine Protected Area of Portofino (Ligurian Sea). This two chapters report the effectiveness of these actions and their effects on ecosystem functioning through analyses on transplanted seagrasses and surrounding sediments and through analyses on transplanted *E. singularis* and surrounding sediments.

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# CHAPTER 3: RECOVERY OF THE RED CORAL *CORALLIUM RUBRUM* FOLLOWING SIMULATED PARTICLE RESUSPENSION INDUCED BY MINING AND TRAWLING ACTIVITIES

## 3.1 Introduction

Anthropogenic activities and global climate changes are modifying the biodiversity and the functioning of marine ecosystems, with cascade effects on the goods and services they provide to human wellbeing (Crain et al., 2008; Ramirez-Llodra et al., 2011; Mora et al., 2013; Levin & Le Bris, 2015). During the last decade, due to the progressive depletion of terrestrial mineral resources (Gross, 2015), industrial companies are even more interested in exploiting marine resources (Rona, 2003), including those present in the deep ocean (Van Dover, 2014; Vanreusel et al., 2016). Thus, the exploitation of marine mineral deposits is expected to become one of the major threats for marine biodiversity in the near future (Van Dover, 2011). Gold, zinc, silver, copper, lead, barium, nickel, arsenic, calcium, molybdenum, platinum and cobalt are just some of the metals whose ores are becoming economically relevant for exploitation (Baker & German, 2009). Areas included in the Exclusive Economic Zone or legal continental shelf are controlled by national jurisdiction also for what concerns mining activities (Boschen et al., 2013). Since 1994, the International Seabed Authority (ISA) has the legal responsibility for deep-sea mining activities in the "Area Beyond National Jurisdiction" (ABNJ, or the Area) (Baker & German, 2009). In the Area, all contractors have to respect the ISA's directives: establishment of environmental impact assessment, environmental monitoring programmes to conduct before, after and during the exploration and exploitation of resources, mitigation and restoration measures to reduce habitat disruption and pollution. Particular attention is given to vulnerable ecosystems like hydrothermal vent fields (Boschen et al., 2013). These ecosystems are present not only in the deep oceans (Van Dover, 2001; Tarasov et al., 2005), but also at shallower depths (Zeppilli & Danovaro, 2009). Coastal hydrothermal vents have been much less studied than deep-sea vents, despite their abundance and wide distribution (Manini et al., 2008).

Seafloor Massive Sulphides (SMS) deposits are one of the three types of ore deposits that attract the attention of the mineral companies (Boschen et al., 2013; Dunn et al., 2018). These polymetallic deposits are formed from the coalescence of collapsed hydrothermal vents' chimneys (Colman Collins et al., 2013). New chimneys build up from the precipitation of metal sulphides as acidic fluids that exit the seafloor in volcanically active regions (Colman Collins et al., 2013), along every principal ridge system on the planet (Van Dover, 2001). Nautilus Minerals Ltd. has proposed a project for SMS mining (Coffey Natural Systems 2008). This company expects to use different "Seafloor Mining Tools": an "Auxiliary Cutter" for preparing seafloor, a "Bulk Cutter" for crashing rocks and a Collecting Machine that will be able to disaggregate the ore to the size required for transfer to the surface. A “Riser and Lift System” (RALS) will pump mined ore to the “Mining Support Vessel” (MSV) (Coffey Natural Systems 2008). On the vessel, ore will be dewatered; the return water will contain particles of 8 µm diameter and it will be discharged in the sea at a height of 25-50 m above the seabed (Coffey Natural Systems 2008). This kind of operations will have both direct and indirect impacts on the ecosystem. The disruption of an exclusive habitat like hydrothermal vents and the loss of the associated biodiversity, the production of sediment and mineral plumes that will affect benthic fauna, the alteration of the flow of hydrothermal vents together with noise pollution, disposal of wastewaters, mobilization of nutrients and heavy metals will be the principal and most evident impacts of mining activities (Van Dover, 2001; International Seabed Authority 2004; Coffey Natural Systems 2008; Baker & German, 2009; Boschen et al., 2013; Van Dover, 2014). Indirect effects of mining will include the alteration of primary productivity due to the shading caused by plume resuspension during mining operations and the modification of benthic fauna behaviour (Davies et al., 2007; Baker et al., 2010; Van Dover, 2011; Boschen et al., 2013; Gena, 2013; Sharma, 2015; Levin et al., 2016). The discharge of the return water derived from dewatering ores operations could affect the environment, especially because it is expected that material will be fine-grained: benthic fauna is expected to be most affected by clogging and hyper sedimentation (Boschen et al., 2016) but tailings will probably release also metals that could be toxic for the environment (Ramirez-Llodra et al., 2015). Mining and tailings discharge will also create a potentially toxic plume that can be transported by the sea, affecting habitats far from the mining site (Ramirez-Llodra et al., 2015). Modelling studies of plume dispersal during and after mining activities at Solwara 1 (Papua Nuova Guinea) demonstrated that sedimentation of plume’s particulate consists in a layer up to 500 mm of thickness within 1 km from the discharge point (Coffey Natural Systems 2008). Some material may deposit up to 10 km from the site (Boschen et al., 2013).

Another relevant threat for marine ecosystems is represented by trawling activities (Pusceddu et al., 2014). Indeed, trawling can have a plethora of impacts on the sea bottom, including stock impoverishment, alterations to the sea-bottom morphology, sediment resuspension, increased bottom-water turbidity, epibenthos mortality, altered nutrient cycles, and alteration of the benthic biodiversity (Pauly et al., 1998; Thrush & Dayton, 2002; Clark & Rowden, 2009; Worm & Lenihan, 2013; Clark et al., 2016). Historical records of this fishing practice date back to the mid-1300s (Davis et al., 1958) and it became widely practiced with the industrialization of fisheries in the late 19th century (Myers & Worm, 2003; Puig et al., 2012). Because shallow coastal water resources have steeply declined in the last 50 years, fisheries are expanding offshore and trawling is being carried out at progressively increasing depths (Roberts, 2002; Morato et al., 2006; Pusceddu et al., 2014).

Thus, there is an urgent need to assess the effects of the expected increasing exploitation of biological and mineral resources on benthic ecosystems. In particular, while an extensive literature information exists on the direct effects induced by human exploitation on fish stocks, the effects of the increased sediment resuspension on benthic sessile organisms are still to be accurately quantified.

Octocorals are sessile invertebrates that include some of the most important ecosystem engineers in temperate coastal areas and in deep sea, contributing to the high levels of biodiversity and ecosystem functioning (Levin et al., 2001). The family Coralliidae (and in particular the genus *Corallium*) is distributed worldwide from the Pacific (where more than half of the species are reported) to the Atlantic Oceans and Mediterranean Sea (Bruckner, 2014). Among this, *Corallium rubrum* is an endemic species of the Mediterranean Sea predominantly distributed in the western basin (Ballesteros, 2006). It is an asymbiotic coral that lives more than 50 years with low growth and recruitment rates (Marschal et al., 2004; Santangelo et al., 2012), and it is one of the key engineering species of the coralligenous assemblages (Cerrano et al., 2013). This species is typical of circalittoral and semi-dark cave communities from 10 to 130 m depth (Priori et al., 2013), but it is also distributed at greater depths, down to ca. 600-800 m (Costantini et al., 2010). Colonies of *C. rubrum* were found also at 1016 m of depth (Knittweis et al., 2016). Principal deep banks are located in south Sardinia, at Portofino (Ligurian Sea), in the Channel of Sicily, in the Tuscany Archipelago (Cattaneo-Vietti et al., 2016). Some deep colonies were found also along the Calabrian Coast between 70 and 130 m (Bo et al., 2011; Bo et al., 2012) and along the coast of Naples, Ischia, Procida and Salerno (Bavestrello et al., 2014). Despite intense harvesting with the “ingegno” instrument during the last 150 years, shallow populations are widely distributed along Italian rocky coasts, but they are characterized by high densities of small colonies (Cattaneo-Vietti et al., 2016). It is known, thanks to some records reported in the ancient literature (Panceri, 1871; Canestrini & Canestrini, 1882), that the red coral was present with high colonies’ densities also at Vulcano, Lipari and Basiluzzo islands in the Aeolian Volcanic Archipelago in the Southern Tyrrhenian Sea (Mediterranean Sea) (Cattaneo-Vietti et al., 2016). Now, only small population are still observed in this area, especially along the north coast of Stromboli island (Reef Check Italia Onlus, 2019).

Marine invertebrates, like the precious *C. rubrum*, are ideal model organisms to perform mesocosm experiments and to gather useful information on a variety of environmental conditions and bathymetric ranges (Cerrano et al., 2013). This model species is usually considered to be a suspension feeder (Picciano & Ferrier-Pagés, 2007), even if its diet is based also on small zooplankton organisms captured with the help of the polyp tentacles (Tsounis et al., 2005). This model organism has been used for laboratory experiments (Cerrano et al., 2013) and offers also the opportunity to assess the extent of the impact of particle resuspension and redeposition due to anthropogenic activities, such as mining and trawling, on benthic organisms of the coralligenous habitats.

In the present study, I investigated the impact of particle resuspension and redeposition potentially induced by mining operations and trawling activities on nearby colonies of *C. rubrum* and I analysed the natural recovery capacity of the survived corals once reported in un-impacted conditions. The overall objective was to provide new information on the impact of particle resuspension on sessile organisms of the coralligenous ecosystem, such as habitat-forming hard-branching corals, and their potential recovery capacity. Indeed, sediment or polymetallic particle resuspension due to anthropogenic activities are expected to increase in the next future, highlighting the need to better understand their impacts on benthic biota and identify mitigation measures able to minimise as much as possible biodiversity loss.

## 3.2 Materials and methods

### 3.2.1 Sample collection

Apical branches of *Corallium rubrum* (5-7 cm of length) were collected from different colonies at the Marine Protected Area of Portofino (Ligurian Sea). In the same area, sediment samples were also collected for the experiment aiming at assessing the impact of sediment plume potentially determined by bottom trawling. Two hydrothermal chimneys were collected around the Basiluzzo island using a ROV (remotely operated vehicle) at water depths of 120 and 180 m in order to simulate the impact of tailing release due to mining activities.

### 3.2.2 Experimental set-up

Once collected, the apical branches of *Corallium rubrum* were maintained at *in situ* conditions and placed in 15 aquaria each of which containing 12 L of prefiltered seawater (0.7 µm with Whatman GF/F 47 mm). Each aquarium contained 5 nubbins (similar in shape and number of polyps) which were maintained for one month, prior to the experiments, at *in-situ* temperature (17°C ± 0.1°C) and with an oxygen concentration of 7.8 ± 0.1 mg $O_2$ $L^{-1}$ (Previati et al., 2010). The polymetallic particles were obtained by grinding the hydrothermal chimneys collected around the shallow hydrothermal field of Basiluzzo Island and only particles with a size below 125 µm were used for the experiments. The modal size of the particles was <10 µm of diameter, which is in the same range of size expected for particles discharged after dewatering processes of extracted ores (8 µm) (Coffey Natural Systems, 2008). The sediment samples were sieved and sediment particles with a size <125 µm (modal size of the sediment particles less than 10 µm) were used for the experiments. To simulate the impact of tailing discharge due to mining, three aquaria were added with polymetallic particles at concentration of 25 mg $L^{-1}$ (based on offshore drilling activities on the cold-water coral *Lophelia pertusa*, Larsson et al., 2013) and three at concentration of 100 mg $L^{-1}$, to simulate the effects of a severe resuspension event (Browne et al., 2015). Similarly, three aquaria were added with sediment particles at concentration of 25 mg $L^{-1}$ and three at concentration of 100 mg $L^{-1}$ to simulate the impact of sediment resuspension due to trawling. Three additional aquaria without particle addition were used as controls. In all experimental systems, a mechanical apparatus was present to maintain polymetallic and sediment particles in suspension (Fig. 1). In particular, a PVC-tube was used like a harm that moved continuously forward and backward over 2 parallel rows of tanks, thanks to a widescreen wiper motor controlled with a printed circuit board. The harm supported inside every tank a paddle built up with PVC-tubes that supported, in the middle of the lower part, a 4W water pump with the nozzle pointed toward the bottom of the tank. The paddles moved simultaneously along all the tanks, keeping the water in movement and keeping the polymetallic particles in suspension. The paddles minimise particle sedimentation to the bottom of the tanks.

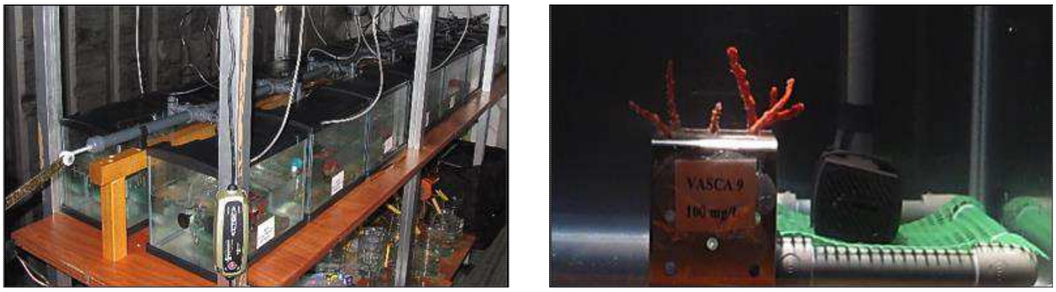


**Figure 1. Experimental apparatus used for simulating particle resuspension.**

Corals were arranged over a stainless-steel support that was positioned over the height that the paddle reached in every tank, at about 7 cm from the bottom. The corals in the aquaria were maintained in the shadow except during sample collection (Cerrano et al., 2013). Physical parameters were daily monitored using a YSI Professional Plus handheld multiparameter meter. During the experiments, pictures of the nubbins were taken twice a day at 8.30 am and at 4 pm using a Canon G12 camera. All the experiments were carried out for 2 weeks.

### 3.2.3 Polymetallic and sediment particles composition and seawater soluble metals

To assess shape and dimensions of polymetallic and sediment particles, sub-samples were observed using a scanning-electron microscope (SEM) (Philips XL20). To investigate the composition of the polymetallic and sediment particles, sub-samples were also analysed by SEM coupled with an Energy-Dispersive X-ray Analyser (EDX EDAX PHOENIX coupled with the ECON IV detector) (SEM-EDX) and by an X-Ray Diffractor (Bruker D8 ADAVANCE). Finally, about 1 gr of the grinded polymetallic material was used to determine the concentration of iron, manganese, copper, lead, aluminium, cobalt, chromium, calcium and potassium by ICP-MS (EPA 3050B 1996 + EPA 6010D 2014). The same metals were also quantified in the seawater contained in the different tanks, in order to assess their possible dissolution In particular, after 2 week of exposure to polymetallic particle resuspension (at the end of the experiment), samples of seawater from each treatment were filtered with 47 mm Whatman GF/F filters (0.7 μm nominal filtration rating) and stored at 4°C inside pre-treated HDPE bottles (washed with $HNO_3$ 1M, HCl 1 M and rinsed with MilliQ water). Metal concentrations in seawater were quantified by ICP-MS (Method APAT CNR IRSA 3010 A Man 29 2003 + APAT CNR IRSA 3020 Man 29 2003).

### 3.2.4 Feeding rates

During the experiment, the feeding rate was evaluated for each treatment. Fresh-hatched (24 hours) nauplii of *Artemia salina* were used during the acclimatization period to feed corals. *Artemia*'s dry cysts (1 g) were placed inside 2 L of pre-filtered 0.7 μm seawater (filtered using Whatman GF/F 47 mm). After 24 hours, 5 aliquots of this culture were taken and the number of nauplii was counted. It was calculated the total volume in which an amount of 12000 nauplii was contained (Baussant et al., 2018) and this quantity was given to each aquarium (1000 nauplii $L^{-1}$ final density) (Purser et al., 2010). 30 mL of water (x 3 replicates) were took from each tank (Orejas et al., 2016; Xu et al., 2016), immediately after the provisioning of food (t0) and after 3 and 4 hours (at each time by mixing the water in the aquaria). Samples were preserved with 4% aqueous formaldehyde (Sigma F8775) (Orejas et al., 2016) and nauplii of *A. salina* were counted using a Dolphus cuve (Tsounis et al., 2010) and a stereomicroscope (LEICA WILD L3B). Prey capture rate was normalized to the number of polyps present in each mesocosm per hour.

### 3.2.5 Scanning Electron Microscope analyses

Subsamples from each mesocosm (about 10-15 polyps for each fragment) were stored in 0.7 μm pre-filtered sea water with 4% formalin. After 24 hours, samples were washed with 0.7 μm pre-filtered sea water and dehydrated for 3 h in 20% ethanol. After 3 hours they were washed in the same way and dehydrated in ethanol 50%. After 3 hours, samples were stored in ethanol 70%. Samples were stored at +4°C. Samples were further dehydratated using different gradients of ethanol solutions (70-80%,80-90%, 90-95%, 95-99%) in a couple of days (Luna et al., 2007). After that, samples were prepared using HMDS (Hexamethyldisilazane, Aldrich 440191). Then, coral fragments were completely dried using the critical point drying technique. Dehydrated samples were mounted on aluminium stubs using Leit-C glue (conductive carbon cement, Neubauer Chemikalien) and sputter-coated with gold. Samples were examined with a SEM (Philips XL20). Ten pictures (90×

magnification) for each of the replicated samples were taken for each sample and these were analysed using the software PhotoQuad® (Trygonis & Sini, 2012). From each picture, the percentage of degraded tissue was established and the integrity of the portion of the entire tissue was scored with a value ranging between 0 and 100 (tissue integrity= 100-|% of altered tissue|). After the analysis, the mean value of tissue integrity from each sample was established and scored into one of the four categories of the "tissue-state index" (Table 1).

| | % | Rating scale | Colour |
|---|---|---|---|
| **TISSUE-STATE INDEX** | 0-40 | Highly damaged | 🟥 |
| | 41-60 | Moderately damaged | 🟧 |
| | 61-80 | Slightly damaged | 🟨 |
| | 80-100 | Not damaged | 🟩 |

**Table 1. Tissue-state index used for assessing the level of damage of the tissue of the exposed corals. This index vas used to evaluate the tissue state of the exposed corals and the level of damage caused by the particle resuspension.**

### 3.2.6 Prokaryotic abundance in seawater

Because stressing conditions can promote bacterial infections of corals (Vezzulli et al., 2010), at different time intervals, water samples were collected for the analysis of prokaryotic abundance. I wanted to ensure that changes in coral's behaviour and health were not related to infectious disease (Danovaro et al., 2008; Cerrano et al., 2013). Samples of 10 mL of water from each mesocosm were stored at -20°C until analysis. Replicated sub-samples (1 mL) were filtered onto 0.2 μm pore size filter (Whatman Anodisc; diameter, 25 mm) and stained with 20 μL of SYBR Green (stock solution diluted 1:500). The filters were incubated in the dark for 20 min, washed three time with 3 mL of prefiltered Milli-Q water and mounted onto glass slides with 20 μL of antifade solution (50% phosphate buffer, pH 7.8, 50% glycerol, 0.5% ascorbic acid). Prokaryotic abundances were determined by epifluorescence microscopy (Zeiss Axioskop 2 MOT). For each slide, at least 20 microscope fields were observed and at least 200 prokaryotes were counted per filter (Danovaro, 2010).

### 3.2.7 Recovery after the exposure

After 2 weeks of each treatment, the survived corals were reported into clean conditions (i.e. I removed the seawater and the particles from the treatment tanks and from the control tanks and I filled all the aquaria with clean and filtered seawater, keeping the resuspension apparatus in movement). The corals were maintained in the same conditions (no light, stable temperature and oxygen concentration). Feeding rates were assessed during the recovery period and tissue integrity was assesses after 28 days of recovery.

### 3.2.8 Statistical analyses

For each of the variables analyzed in this study, univariate distance-based permutational analyses of variance (PERMANOVA) was applied to assess differences between the different treatments and sampling times. The analyses were followed by the pair-wise test when significant differences were encountered ($p<0.05$). All the analyses were carried out on Euclidean distances using 9999 permutations with Monte Carlo simulation, considering the factors (treatment and time) as fixed and using unrestricted permutation of raw data. All the analyses were performed using PRIMER v.6.1.12 software.

## 3.3 Results

### 3.3.1 Polymetallic and sediment particle composition

X-Ray Diffraction analysis highlighted that the main minerals present in the polymetallic particles were iron oxides (Fig. 2a). SEM-EDX analysis indicated that polymetallic particles were mainly composed by iron (82%), followed by manganese (Fig. 2b). Such a composition was further comfirmed by ICP-MS analysis which revelaed high iron content (223 g $Kg^{-1}$), followed by manganes (41 g $Kg^{-1}$; Table 2). Other elements were also present such as potassium, copper, cromium and lead but with concentrations at least one order of magnitude lower compared to that of the other metals.

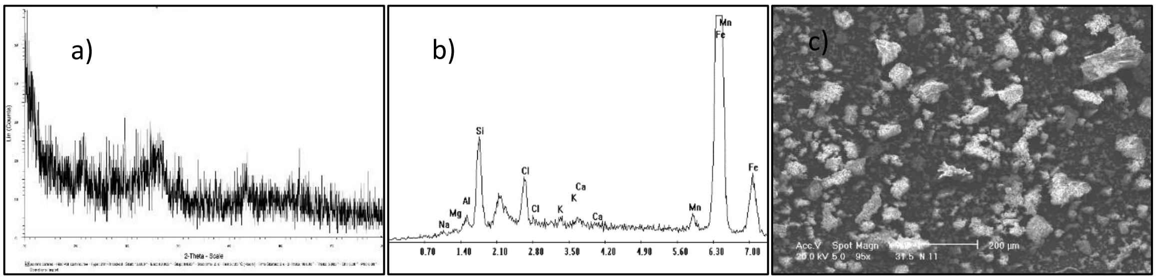


**Figure 2. a) The results of the X-Ray Diffraction analysis show that the polymetallic particles are principally made of amorphous iron oxydes. b) The results of the analysis made with SEM-EDX show that the principal elements that compound the polymetallic particles are iron, manganese and aluminium. The peak of silicium is not considered because this signal derives from the aluminium stub. c) A magnification of the polymetallic particles observed at SEM.**

| | Concentration (mg/Kg) |
|---|---|
| **Aluminium** | $1.42\times10^4$ |
| **Manganese** | $4.10\times10^4$ |
| **Potassium** | 799 |
| **Calcium** | $2.51\times10^4$ |
| **Copper** | 36.2 |
| **Iron** | $2.23\times10^5$ |
| **Cromium** | 5.2 |
| **Lead** | 19.7 |

**Table 2. Concentration of the different metals in the polymetallic particles.**

The analysis of sediment particles by X-Ray Diffraction revealed that the most important minerals were quartz, calcium carbonate and aragonite (Fig. 3a). Major elements of the sediment particles as highlighted by SEM-EDX were calcium, silicium, chloride, aluminium, magnesium and potassium (Fig. 3b).

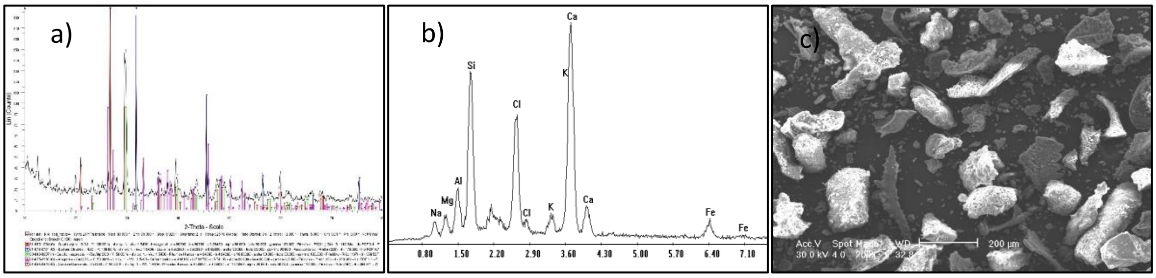


**Figure 3. a) The results of the X-Ray Diffraction analysis show that the sediment particles are principally made of quartz, calcium carbonate, aragonite. b) The results of the analysis made with SEM-EDX show that the principal elements that compound the sediment particles are calcium, silicium, chloride, aluminium, magnesium and potassium. The peak of chloride is not considered because this signal derives from the presence of some salt contained in the seawater. c) A magnification of the sediment particles observed at SEM.**

The analysis of dissolved metal concentrations in seawater at the end of the experiments with polymetallic particles revealed a significant manganese dissolution proportional to the used concentrations of polymetallyc particles. Indeed, Mn concentration in seawater was 60 (± 2.32) µg $L^{-1}$ for the 25 mg $L^{-1}$ treatment and 216 (± 48) µg $L^{-1}$ for the 100 mg $L^{-1}$ treatment (Fig. 4)

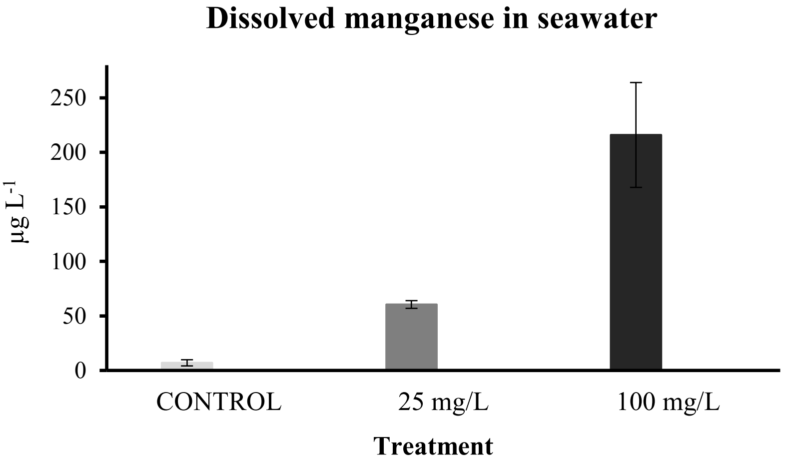


**Figure 4. Dissolved manganese concentrations in seawater at the end of the exposure to polymetallic particles. Mean and standard deviations are reported.**

### 3.3.2 Feeding rates

Feeding rates were determined at the beginning of both experiments, after 2 and 5 days for the exposure to polymetallic particle resuspension (Fig. 5) and after 2 and 10 days for the exposure to sediment particle resuspension (Fig. 6). At the beginning of both experiments (time 0) the capture rates of the corals under different treatments were not significantly different. After 2 days of exposure to polymetallic particles, the corals exposed to 25 mg $L^{-1}$ and 100 mg $L^{-1}$ of particles showed a decreased feeding rate (respectively 0.15 ± 0.06 and 0.13 ± 0.06 nauplii $L^{-1}$ $polyp^{-1}$ vs 0.39 ± 0.04 nauplii $L^{-1}$ $polyp^{-1}$ in the controls) (Fig. 5). After 5 days of exposure to polymetallic particles, the corals exposed to 25 mg $L^{-1}$ and 100 mg $L^{-1}$ were not more able to feed on nauplii of *A. salina*, whereas in the control a feeding rate of 0.37 ± 0.10 nauplii $L^{-1}$ $polyp^{-1}$ was observed.

After 2 days of exposure to sediment particles, the corals exposed to 25 mg $L^{-1}$ and 100 mg $L^{-1}$ of particles showed a decreased prey-capture rate (respectively 0.14 ± 0.06 and 0.08 ± 0.05 nauplii $L^{-1}$ $polyp^{-1}$ vs 0.36 ± 0.07 nauplii $L^{-1}$ $polyp^{-1}$ captured by the control treatment) (Fig. 6). After 10 days of exposure to sediment resuspension, the exposed corals showed prey-capture rates similar to that observed at the day 2 (0.16 ± 0.08 and 0.10 ± 0.08 nauplii $L^{-1}$ $polyp^{-1}$ respectively for the 25 mg $L^{-1}$ treatment and 100 mg $L^{-1}$ treatment vs 0.36 ± 0.06 nauplii $L^{-1}$ $polyp^{-1}$ in the controls).

In both the experiments, the univariate distance-based permutational analyses of variance showed a significant difference among the treatments ($p<0.001$). In the exposition to polymetallic particles, this statistical test showed also a strong temporal pattern; $p<0.01$ (Table 3).

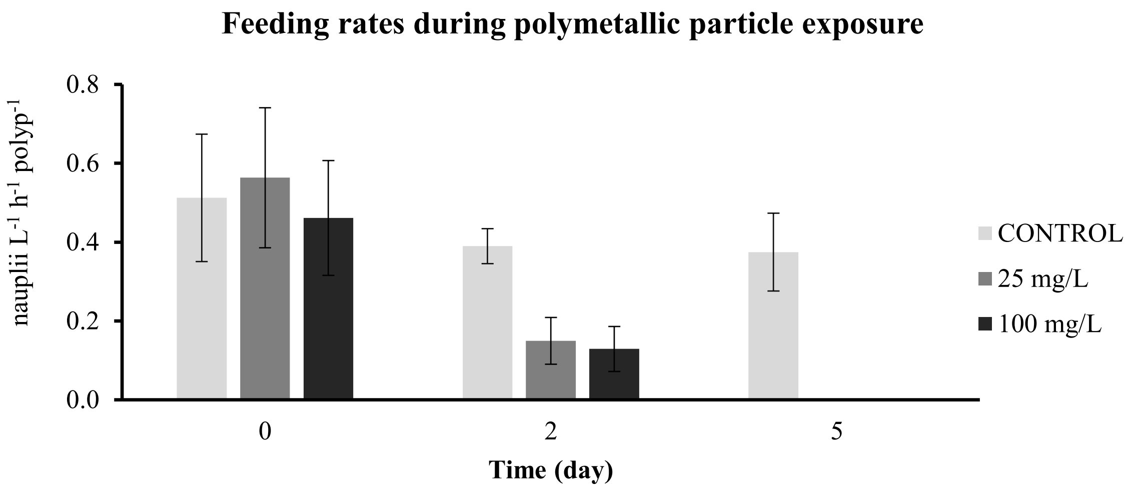


**Figure 5. Feeding rates of exposed and unexposed corals to polymetallic particles. Results are expressed as nauplii $L^{-1}$ $h^{-1}$ $polyp^{-1}$. Corals were fed with 24 h-fresh hatched nauplii of *Artemia salina*. Mean and standard deviations are reported.**

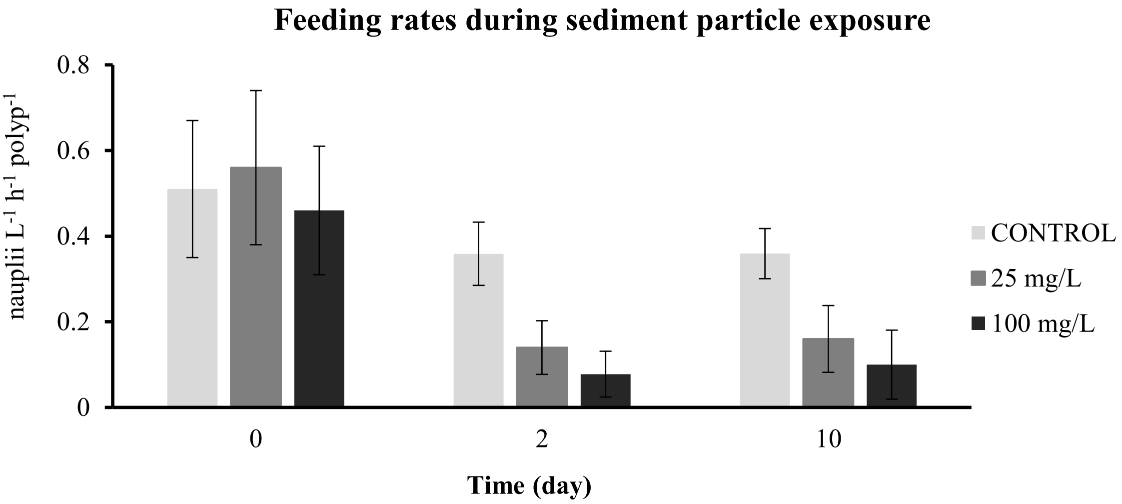
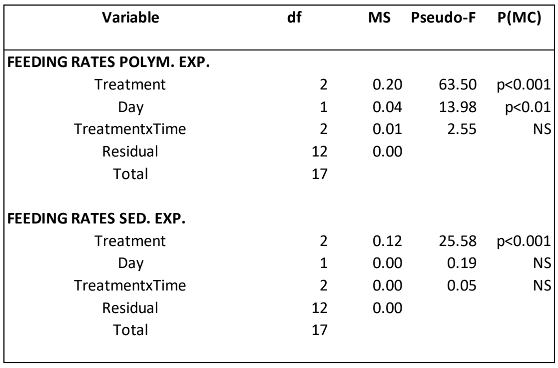


**Figure 6. Feeding rates of exposed and unexposed corals to sediment particles. Results are expressed as nauplii $L^{-1}$ $h^{-1}$ $polyp^{-1}$. Corals were fed with 24 h-fresh hatched nauplii of *Artemia salina*. Mean and standard deviations are reported.**



| Variable | df | MS | Pseudo-F | P(MC) |
|---|---|---|---|---|
| **FEEDING RATES POLYM. EXP.** | | | | |
| Treatment | 2 | 0.20 | 63.50 | p<0.001 |
| Day | 1 | 0.04 | 13.98 | p<0.01 |
| TreatmentxTime | 2 | 0.01 | 2.55 | NS |
| Residual | 12 | 0.00 | | |
| Total | 17 | | | |
| **FEEDING RATES SED. EXP.** | | | | |
| Treatment | 2 | 0.12 | 25.58 | p<0.001 |
| Day | 1 | 0.00 | 0.19 | NS |
| TreatmentxTime | 2 | 0.00 | 0.05 | NS |
| Residual | 12 | 0.00 | | |
| Total | 17 | | | |

**Table 3. PERMANOVA test for the feeding rates assessed during the two exposures. Fixed levels of contrast include control aquaria, aquaria with 25 mg $L^{-1}$ and 100 mg $L^{-1}$ of resuspended**

**particles (n = 3 replicated plots). Analyses were carried out using unrestricted permutations of the raw data and 9999 permutations. df, degrees of freedom; MS, mean square; P(MC), probability level after Monte Carlo tests; Pseudo-F, statistic F. NS= not significant.**

### 3.3.3 Tissue damage induced by particles

The tissue integrity of corals was determined after 4 and 9 days of exposure to polymetallic particles and after 9 and 12 days of exposure to sediment particle resuspension. At the beginning of both exposures (day 0), the tissue state was classified as "not damaged" for each treatment. During both treatments, the tissue state of the exposed corals was altered (Fig. 7 and Fig. 8). After 4 days of polymetallic particle resuspension, the corals exposed to a concentration of 25 mg $L^{-1}$ of particles showed a "slightly damaged" tissue while the coral exposed to the higher concentration showed a "moderately damaged" tissue. After 9 days of exposure, all the exposed corals showed a "highly damaged" tissue. After 9 days of sediment particle resuspension, all the exposed corals showed a "slightly damaged" tissue and in both treatments, after 12 days of exposure, corals showed a "highly damaged" tissue.

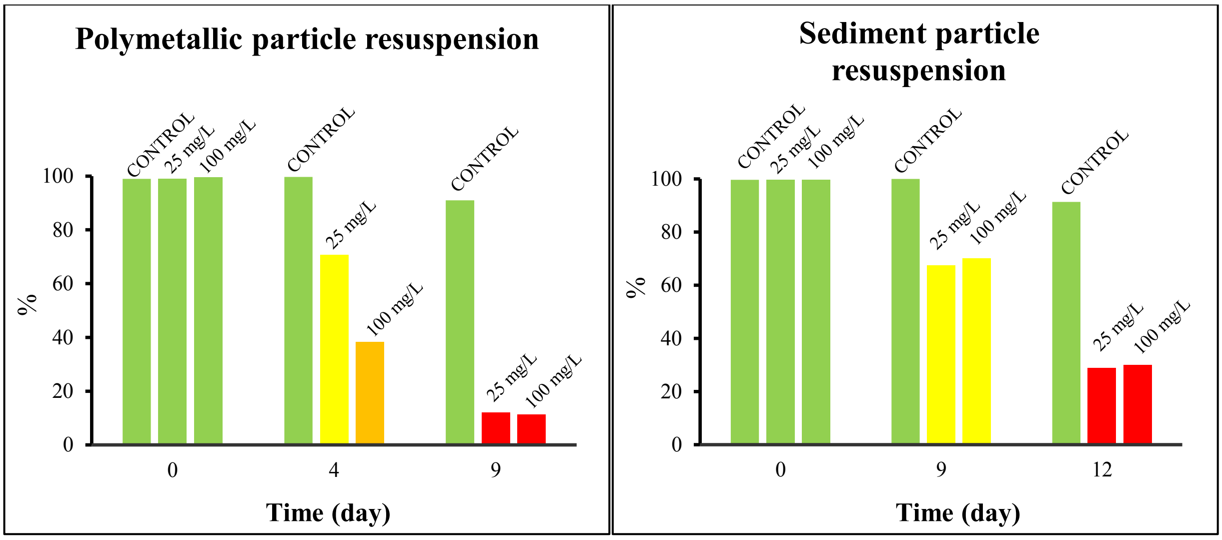


**Figure 7. Tissue-state index of the coral samples collected during time-course experiments carried out using 25 mg $L^{-1}$ and 100 mg $L^{-1}$ of polymetallic particles (Fig. 7a) and sediment particles (Fig. 7b). The colours of the columns represent the grade of damage of the tissue and correspond to one of the four levels of tissue-state index (for the correspondence see Table 1).**

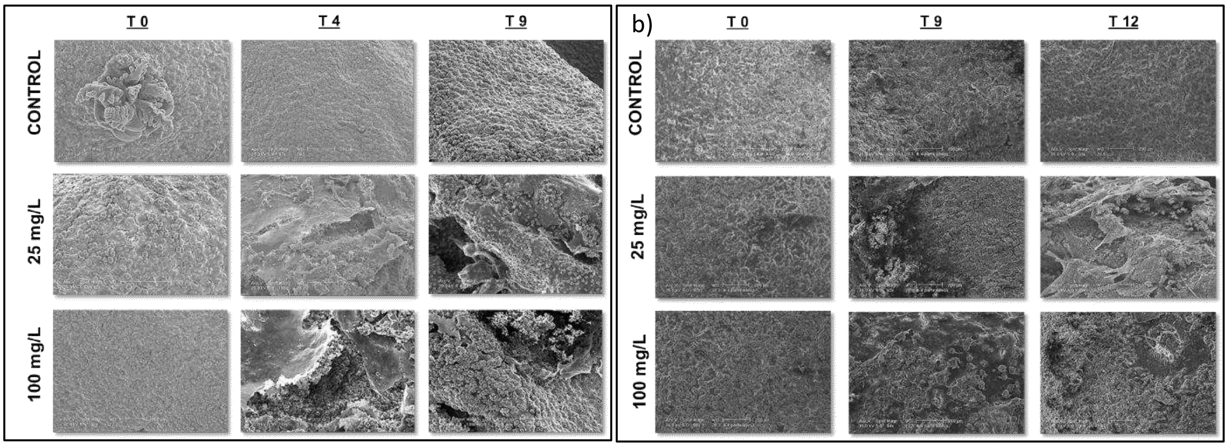


**Figure 8. Examples of corals' tissue images obtained using a scanning electron microscope. 25 mg $L^{-1}$ and 100 mg $L^{-1}$ refer to the two different concentrations of polymetallic particles used. a) Samples were analysed immediately after the addition of polymetallic particles (T0), and after 4 (T4) and 9 days (T9) of exposure to polymetallic particles. b) Samples were analysed immediately after the addition of sediment particles (T0), and after 9 (T9) and 12 days (T12) of exposure.**

### 3.3.4 Prokaryotic abundance in seawater

In both the exposures, prokaryotic abundance in the seawater increased over time (Figures 9 and 10). Such an increase was also observed in the aquaria used as controls (i.e. un-exposed corals). No significant differences were assessed among the two treatments and the control in the exposure to polymetallic particle resuspension, with some exceptions at day 10 (Table 4). In the exposure to sediment particles, the pair-wise test showed several significant differences, especially at day 1 and day 5.

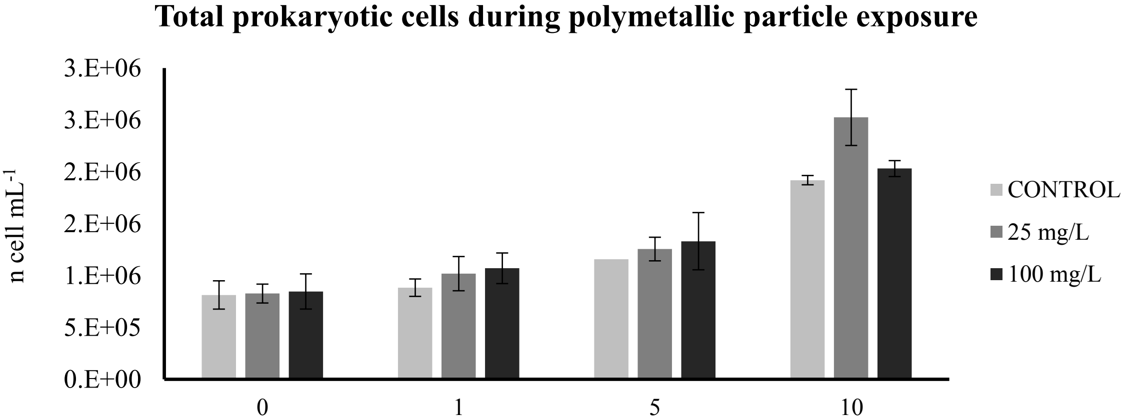

Total prokaryotic cells during polymetallic particle exposure

**Figure 9. Total prokaryotic cells in the seawater during polymetallic particle exposure. Mean and standard deviations are reported.**

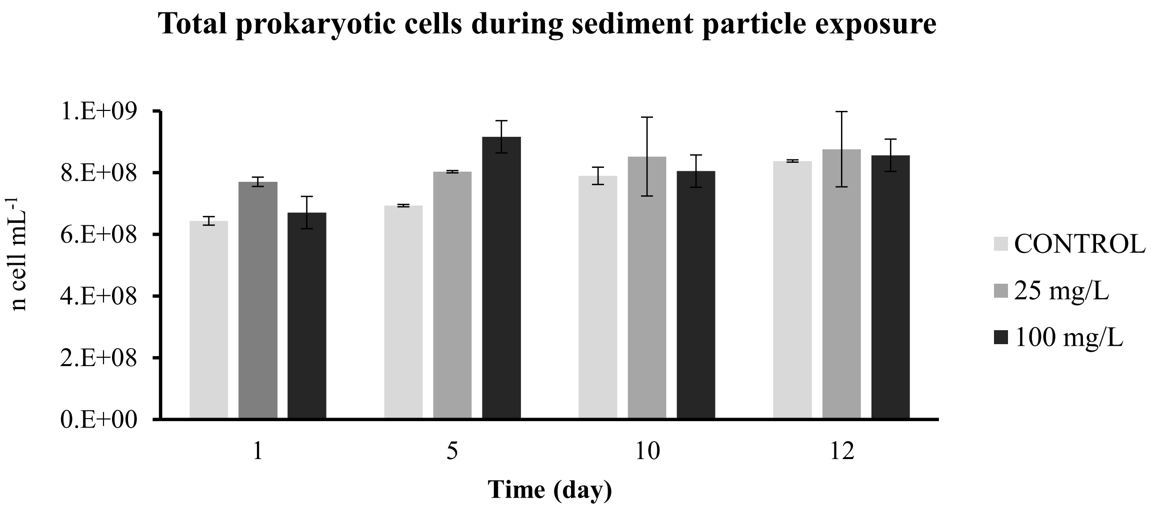


**Figure 10. Total prokaryotic cells in the seawater during sediment particles' exposure. Mean and standard deviations are reported.**

| Variable | df | MS | Pseudo-F | P(MC) | Pairwise comparisons | t | p (MC) |
|---|---|---|---|---|---|---|---|
|  |  |  |  |  | Term 'TreatmentxTime' for pairs of levels of factor 'Treatment' |  |  |
| **PROK. ABUNDANCE POLYM. EXP.** |  |  |  |  |  |  |  |
| Treatment | 2 | 1.38E+11 | 5.88 | p<0.01 | Day 0, Control, 25 mg/L | 0.143 | NS |
| Day | 3 | 3.17E+12 | 135.16 | p<0.001 | Day 0, Control, 100 mg/L | 0.269 | NS |
| TreatmentxTime | 6 | 7.49E+10 | 3.19 | p<0.05 | Day 0, 25 mg/L, 100 mg/L | 0.183 | NS |
| Residual | 24 | 2.35E+10 |  |  | Day 1, Control, 25 mg/L | 1.267 | NS |
| Total | 35 |  |  |  | Day 1, Control, 100 mg/L | 1.908 | NS |
|  |  |  |  |  | Day 1, 25 mg/L, 100 mg/L | 0.404 | NS |
|  |  |  |  |  | Day 5, Control, 25 mg/L | 1.505 | NS |
|  |  |  |  |  | Day 5, Control, 100 mg/L | 1.097 | NS |
|  |  |  |  |  | Day 5, 25 mg/L, 100 mg/L | 0.439 | NS |
|  |  |  |  |  | Day 10, Control, 25 mg/L | 3.821 | p<0.05 |
|  |  |  |  |  | Day 10, Control, 100 mg/L | 2.168 | NS |
|  |  |  |  |  | Day 10, 25 mg/L, 100 mg/L | 3.040 | p<0.05 |
| **PROK. ABUNDANCE SEDIMENT EXP** |  |  |  |  |  |  |  |
| Treatment | 2 | 2.47E+16 | 114.30 | p<0.001 | Day 1, Control, 25 mg/L | 27.881 | p<0.001 |
| Day | 3 | 4.30E+16 | 199.19 | p<0.001 | Day 1, Control, 100 mg/L | 6.742 | p<0.01 |
| TreatmentxTime | 6 | 1.01E+16 | 46.82 | p<0.001 | Day 1, 25 mg/L, 100 mg/L | 45.319 | p<0.001 |
| Residual | 24 | 2.16E+14 |  |  | Day 5, Control, 25 mg/L | 89.082 | p<0.001 |
| Total | 35 |  |  |  | Day 5, Control, 100 mg/L | 27.254 | p<0.001 |
|  |  |  |  |  | Day 5, 25 mg/L, 100 mg/L | 13.929 | p<0.001 |
|  |  |  |  |  | Day 10, Control, 25 mg/L | 3.099 | p<0.05 |
|  |  |  |  |  | Day 10, Control, 100 mg/L | 1.400 | NS |
|  |  |  |  |  | Day 10, 25 mg/L, 100 mg/L | 2.374 | NS |
|  |  |  |  |  | Day 12, Control, 25 mg/L | 2.169 | NS |
|  |  |  |  |  | Day 12, Control, 100 mg/L | 8.394 | p<0.01 |
|  |  |  |  |  | Day 12, 25 mg/L, 100 mg/L | 1.112 | NS |

**Table 4. PERMANOVA and pair-wise tests for the prokaryotic abundances assessed in the seawater during both exposures. Fixed levels of contrast include control aquaria, aquaria with 25 mg $L^{-1}$ and 100 mg $L^{-1}$ of resuspended particles (n = 3 replicated plots). Analyses were carried out using unrestricted permutations of the raw data and 9999 permutations. df, degrees of freedom; MS, mean square; P(MC), probability level after Monte Carlo tests; Pseudo-F, statistic F; t, statistic t for pairwise comparisons. NS= not significant.**

### 3.3.5 Recovery of corals after the exposure

No specimen of *C. rubrum* survived at the end of the exposure to 25 mg $L^{-1}$ of polymetallic particles, so it was possible to assess the recovery in terms of prey-capture rates and tissue state only for the controls and the corals exposed to a concentration of 100 mg $L^{-1}$ of polymetallic particles. Prey-capture rates were determined after 12 and 20 days from the polymetallic particle exposure (Fig. 11) and after 9 days for the sediment particle exposure (Fig. 12). Corals exposed to 100 mg $L^{-1}$ of polymetallic particles showed an increased prey-capture rate after 20 days of the recovery period.

However, the feeding rates of exposed corals were still significantly lower ($p<0.01$; Table 5). Such corals, in fact, captured 0.35 ± 0.10 nauplii $L^{-1}$ $polyp^{-1}$ despite the unexposed corals captured 0.68±0.07 nauplii $L^{-1}$ $polyp^{-1}$. After 9 days after the exposure to sediment particle, the corals exposed to 25 mg $L^{-1}$ and 100 mg $L^{-1}$ of sediment showed a prey-capture rate of 0.64 ± 0.09 and 0.69 ± 0.05 nauplii $L^{-1}$ $polyp^{-1}$, respectively, vs the unexposed corals that captured 1.32 ± 0.06 nauplii $L^{-1}$ $polyp^{-1}$. The PERMANOVA test showed that the differences among the corals maintained under control treatment and recovered after the expositions to the two different concentrations were still significant ($p<0.001$; Table 5).

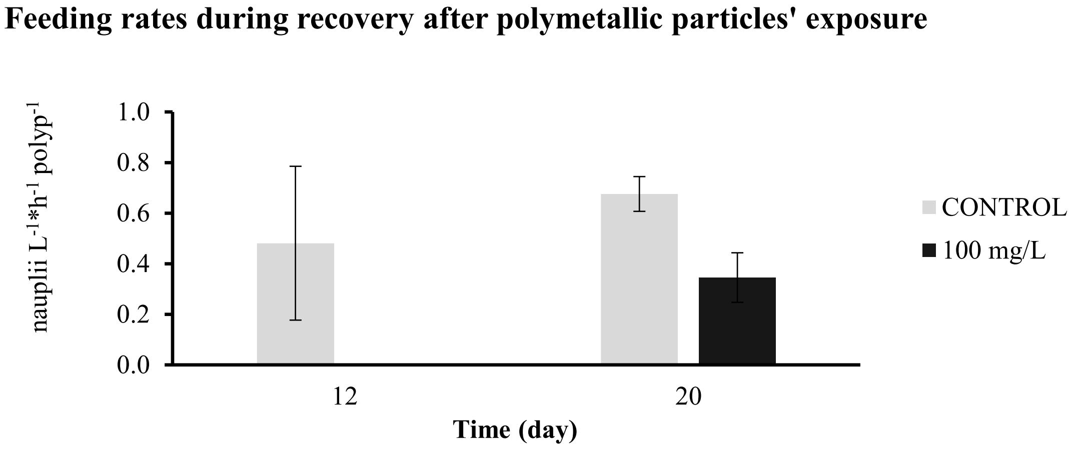


**Figure 11. Feeding rates of exposed and unexposed corals to polymetallic mineral particles once reported in clean seawater (i.e. seawater without polymetallic particles). Results are expressed as nauplii $L^{-1}$ $h^{-1}$ $polyp^{-1}$. Corals were fed with 24 h-fresh hatched nauplii of *Artemia salina.* Mean and standard deviations are reported.**

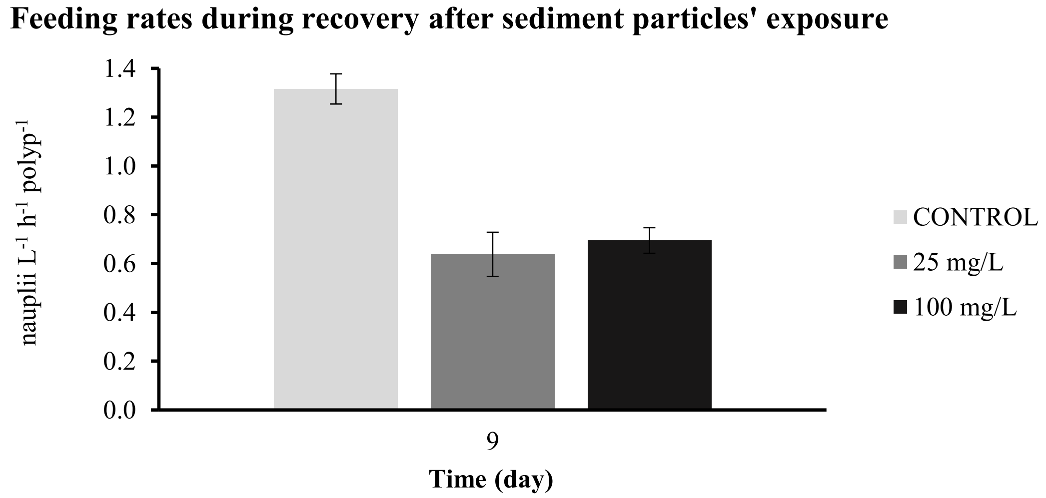


**Figure 12. Feeding rates of exposed and unexposed corals to sediment particles once reported in clean seawater (i.e. seawater without sediment particles). Results are expressed as nauplii $L^{-1}$ $h^{-1}$ $polyp^{-1}$. Corals were fed with 24 h-fresh hatched nauplii of *Artemia salina*. Mean and standard deviations are reported.**

| Variable | df | MS | Pseudo-F | P(MC) |
|---|---|---|---|---|
| **FEEDING RATES POLYM. RECOVERY** | | | | |
| Treatment | 1 | 0.49 | 18.79 | p<0.01 |
| Day | 1 | 0.22 | 8.54 | p<0.05 |
| TreatmentxTime | 1 | 0.02 | 0.64 | NS |
| Residual | 8 | 0.03 | | |
| Total | 11 | | | |
| **FEEDING RATES SED. RECOVERY** | | | | |
| Treatment | 2 | 0.42451 | 86.272 | p<0.001 |
| Residual | 6 | 4.92E-03 | | |
| Total | 8 | | | |

**Table 5. PERMANOVA and pair-wise tests for the feeding rates assessed during recovery after both exposures. Fixed levels of contrast include control aquaria, aquaria with 25 mg L$^{-1}$ and 100 mg L$^{-1}$ of resuspended particles (n = 3 replicated plots). Analyses were carried out using unrestricted permutations of the raw data and 9999 permutations. df, degrees of freedom; MS, mean square; P(MC), probability level after Monte Carlo tests; Pseudo-F, statistic F; t, statistic t for pairwise comparisons. NS= not significant.**

At the end of the recovery period (after 28 days), the tissue state was “not damaged” for all the treatments with the exception of the corals that were exposed to a concentration of sediment particles of 100 mg L$^{-1}$ that did not recover completely, showing a “slightly damaged” tissue (Fig. 13).

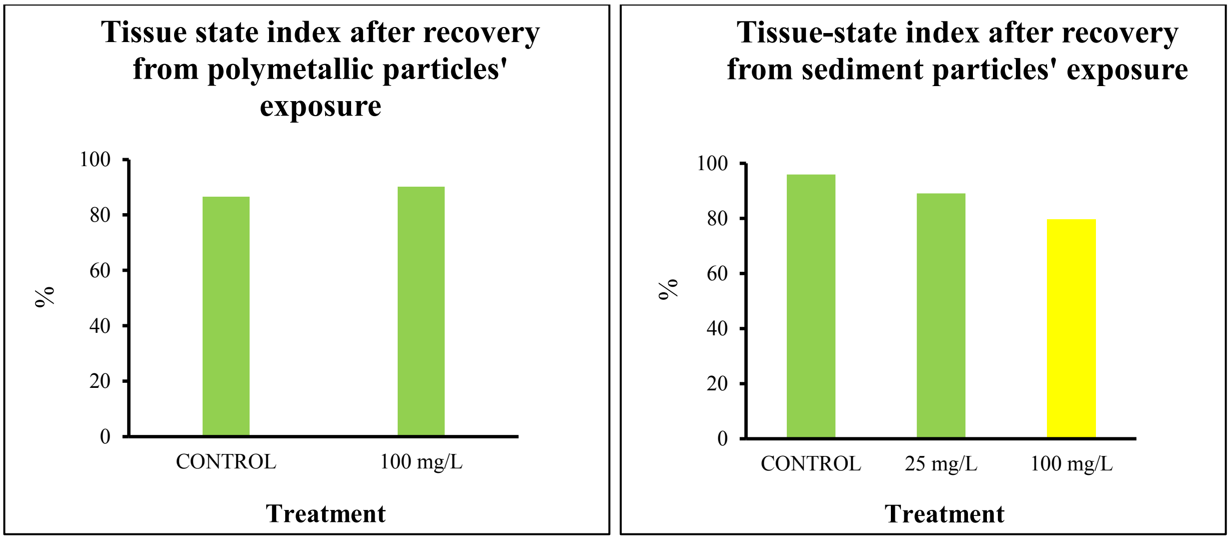


**Figure 13. Tissue-state index of unexposed and exposed corals once reported in clean seawater (i.e. seawater without polymetallic or sediment particles).**

### 3.4 Discussion

The results of the present study provide evidences that polymetallic and sediment particles resuspension and redeposition can severely impact benthic organisms, such as the endangered species *Corallium rubrum.* In only 2 days from the beginning of the experiment, a significant reduction of the polyps’ expansion and feeding activity (capture of preys) was observed for the corals exposed both to polymetallic and sediment particles compared to the control corals. Such an impact was even more evident after 5 days of treatment with polymetallic particles. In fact, corals were no more able to feed on their preys, independently from the particles’ concentration. The prolonged effect of the exposure to polymetallic particles resuspension, with the retraction and the burial of the polyps, could have severely affected the nutritional uptake rates (Larsson et al., 2013). Conversely, no major changes in feeding rates of corals were observed after 2 and 10 days in the exposure with sediment particles, indicating that, at least in the short-term, sediment particles resuspension has a lower impact compared to polymetallic particles resuspension.

Polyps' clogging and tentacular retraction together with mucus production represent a natural mechanism of many scleractinian corals to reduce the impact of high sedimentation conditions (Erftemeijer et al., 2012; Duckworth et al., 2017). Studies on tropical corals revealed that soft alcyonacean corals are usually passive sediment shredders and are more sensitive compared to scleractinian corals (Riegl & Bloomer, 1995; Riegl & Branch, 1995). The last ones are able to actively remove sand particles inflating polyps and creating a smothering surface that favours the run-off of the deposited material (Riegl, 1995), in order to avoid damages or loss of tissue (Stafford-Smith, 1993). This may explain the differences between the effects of the exposure of the scleractinian cold-water coral *Lophelia pertusa* (Larsson et al., 2013) and that of the alcyonaceans *C. rubrum* (present study) and *Dentomuricea meteor* (Carreiro-Silva, personal communication) to the same concentration (25 mg $L^{-1}$) of similar materials maintained in suspension in simulated-laboratory experiments. In the first case, authors demonstrated that *L. pertusa* actively removed both natural sediments and drill-cuttings, even after repeated exposures, without significant effects on coral mucus production, respiration and growth rates (Larsson et al., 2013). Conversely, the gorgonian *D. meteor* showed a reduction of the metabolic rates and an alteration of its antioxidant defence system. Moreover, tissue necrosis till death of all coral nubbins was observed at the end of the experiment (Carreiro-Silva, personal communication). The lack of visible signs of particles removal from *D. meteor* suggests the existence of a not efficient cleaning system in this species (Carreiro-Silva personal communication). Similarly, in our experiment, *C. rubrum* did not show signs of active removal of the deposited polymetallic particles or sediment particles.

Previous studies highlighted that the deposition of particulate material over corals can affect their metabolism by increasing respiration rates and carbon-loss through greater mucus production (Riegl & Branch, 1995), and can induce tissue damage and promote necrosis (Jones et al., 2016). In the present study, particle resuspension determined an evident effect also on the tissue integrity of *C. rubrum*. Indeed, signatures of a certain tissue damage were observed just after 4 days of exposure to polymetallic particles and such damage became severe after 9 days. During the sediment exposure, after 9 days, corals exposed both to 25 and 100 mg/L of concentration of the resuspended material showed only a slightly damaged tissue.

Moreover, I found that polymetallic particle resuspension can also determine a relevant dissolution of metals (in this case manganese) in the surrounding seawater and that such an effect was proportional to the concentration of polymetallic particles present, thus potentially enhancing their impact on corals. Manganese (Mn) is an essential element for marine life, but it can exert a toxic effect at high concentrations (Summer et al., 2019). For instance, the Norway lobster *Nephrops norvegicus* exposed for 10 days to 20 mg $L^{-1}$ of concentration of Mn displayed impairment of the immune system (Hernroth et al., 2004). The same species showed also a reduced capacity of detecting food when exposed for 12 days to lower Mn concentrations (0.1 and 0.2 mM) (Krang & Rosenqvist, 2006). The exposure to Mn of the echinoderm *Asterias rubens* can cause an increase in the number of coelomocytes involved in the immunologic response of this organism (Oweson et al., 2010). A five-days exposure of *N. norvegicus*, *A. rubens* and *Mytilus edulis* to 15 mg $L^{-1}$ of Mn affected the response to infections of the lobster and the bivalve but did not cause changes in the bactericidal response of the echinoderm (Oweson & Hernroth, 2009). A concentration of 12 mg $L^{-1}$ of Mn induced neuromuscular disturbance in the echinoderms *A. rubens* and *Ophiocomina nigra* (Sköld et al., 2015). Corals can uptake metals from the seawater through a direct uptake of soluble elements and through feeding (Ramos et al., 2004). Summer et alii (2019) reported that concentrations of Mn from 1 mg $L^{-1}$ to 50 mg $L^{-1}$ are able to determine a relevant impact on the scleractinian coral *Acropora spathulate.* In particular, the coral tissue rapidly sloughed away from the hard skeleton of the fragments, a deleterious effect that is usually preceded by increased mucus production. Altogether these findings suggest that, besides the direct effect exerted by polymetallic particles on corals, also the high concentrations of manganese in the dissolved phase can have a role in exacerbating their impact.

Potential additional impacts of particles' exposure can be exerted by bacteria growing on damaged tissue and potentially accelerating tissue degradation (Allers et al., 2013). In this study, microscopic observations did not highlight the presence of bacterial aggregates on coral tissue or major changes in microbial contamination in surrounding seawater, suggesting a minor role of bacteria in tissue degradation processes.

Results presented in this study indicate a higher impact of polymetallic particles on corals when compared to sediment particles, possibly due to a synergistic effect exerted by metal dissolution from mineral ores and physical abrasion by the polymetallic particles. Indeed, SEM observations provide evidence that polymetallic particles had more jagged edges than the sediment particles and this could increase the action of particles abrasion, till coral death in the treatment at the lowest particles' concentration (25 mg $L^{-1}$) after only 2 weeks of exposure.

This laboratory experiment provides evidence of the potential effects of polymetallic and sediment particles resuspension that future mining operations and trawling activities could cause. However, further studies based on *in-situ* experiments are needed to assess the actual impact of mining and trawling plumes on benthic sessile organisms. At the same time, also the grain size of plume's particles should be considered as the ability of corals to remove them from their tissue generally decreases with the decrease of particle size (Weber et al., 2006).

Despite the negative effects I observed, the survived coral nubbins showed a certain degree of resilience as indicated by their recovery in terms of tissue state and feeding capacity once brought back in clean conditions (i.e. filtered seawater). However, feeding rates of exposed corals remained lower compared to those of un-exposed ones, suggesting that the negative consequences of particle exposures could last over than 10-20 days.

Overall, results from this study provide new information on the impact of the resuspension of polymetallic and sediment particles on sessile organisms such as hard-branching corals. These results can be used, in the future, to analyse the potential interaction among different stressors, to combine these assessments under laboratory conditions with the consideration about habitat loss in the field. Moreover, these results pave the way for the definition of mitigation measures to minimise as much as possible the effects of such anthropogenic activities on benthic biota.

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# CHAPTER 4: FOOD SELECTION OF MEDITERRANEAN COLD-WATER CORALS

## 4.1 Introduction

Cold water corals (CWCs) are important habitat-forming species belonging to azooxanthellate stony corals (Scleractinia), and include alcyonaceans and gorgonians (Octocorallia), black corals (Antipatharia) and hydrocorals (Stylasteridae) (Roberts et al., 2006). CWCs and their habitats are receiving great interest from the scientific community due to their high ecological value (Roberts et al., 2009). In particular, deep-water reefs formed by stony corals are complex three-dimensional structures and are biodiversity hotspots (Henry & Roberts, 2007; Hovland, 2008; Bongiorni et al., 2010; Mastrototaro et al., 2010; Rueda et al., 2019) acting as nurseries, refugia or feeding grounds for several species of fishes and invertebrates (Baillon et al., 2012; D'Onghia, 2019).

CWCs are threatened by several anthropogenic activities (D'Onghia et al., 2016; Ragnarsson et al., 2016). Among them, deep-sea fishery directly impacts CWC reefs (D'Onghia, 2019), up to affecting at places an estimated 95-98% of the total coral cover (Gianni, 2004). Oil and gas exploitation continue to extend deeper, increasing the risks of oil-spills accidents or dispersal near CWC reefs (Cordes et al., 2016). Also, deep-sea mining will affect CWC reefs that are often located near exploiting sites (Roberts & Cairns, 2014; Ragnarsson et al., 2016). In addition, global climate change threats CWCs due to decrease of pH and the consequent decrease of the depth of aragonite saturation horizon (ASH) (Ragnarsson et al., 2016). Projections indicate that 70 % of the actual locations of CWC reefs could become undersaturated by 2099 (Guinotte et al., 2006).

In the Mediterranean Sea, the most common reef-building CWCs are the 'white corals' *Desmophyllum pertusum* (Linnaues 1758)*,* formerly known as *Lophelia pertusa* (Addamo et al., 2016), and *Madrepora oculata* (Linnaeus, 1758). These two taxa often co-occur and engineer the CWC-grounds with maximum areal occupancy in the coral provinces identified in the central and western Mediterranean Sea between ca. 200-800 m (Fanelli et al., 2017; Taviani et al., 2017; Chimienti et al., 2019; Taviani et al., 2019). The "yellow coral" *Dendrophyllia cornigera* (Lamarck, 1816), forms colonies from the mesophotic zone down to bathyal depths where it often mingles with white corals (Castellan et al., 2019).

The restoration of degraded coral grounds through the transplantation of nubbins taken from healthy colonies or reared in aquaria has been proposed in literature as a possible restoration action (Da Ros et al., 2019 and references therein). Pilot experiments on transplantation of nubbins of *D. pertusum* were carried out in the Gulf of Mexico and in the North Sea with encouraging results of high survival rates (Brooke & Young, 2009; Dahl, 2013). These studies, together with the hope that mineral accretion technology effective for tropical corals could also enhance the survival and growth of CWCs (Strömberg et al., 2010), are opening new possibilities for restoring damaged CWC habitats.

One of the difficulties in rearing CWCs in captivity conditions is their maintenance under laboratory conditions: variations of physical conditions (especially environmental pressure) and manipulations are the principal causes of stress affecting CWCs in laboratory experiments (Orejas et al., 2019). Technological improvements facilitating the manipulation of deep-sea organisms include instruments preventing damages from pressure variations during transportation and manipulation, but their use is still limited by their cost (Danovaro et al., 2014). Experiments could be easily conducted with high-pressure tanks, but such structures occur only in a few laboratories in the world (Garel et al., 2019). Moreover, also variations in the feeding behaviour can affect the success of breeding CWCs. In captivity, nauplii of *A. salina* and algae are routinely used to feed *D. pertusum* and *M. oculata* (Orejas et al., 2019) while *Mysis* sp. and *A. salin*a are used to feed *D. cornigera* (Gori et al., 2015; Orejas et al., 2019).

Here I investigated the feeding preferences of *D. pertusum*, *M. oculata* and *D. cornigera* in order to establish the best conditions for their breeding and for maintaining them on-board during the oceanographic campaigns. Four food sources were chosen to feed the corals and establish their food preferences: 1) nauplii of *A. salina*, 2) the green algae *Tetraselmis subcordiformis,* (Wille) Butcher 1959, 3) two species of rotifers (*Brachionus plicatilis*, Müller, 1786, and *B. rotundiformis*, Tschugunoff, 1921) and 4) the eumalacostracan *Mysis relicta*.

The aims of this study are: 1) to assess prey-capture rates of the different CWCs and their capture preference; 2) to determine the isotopic signatures of the exposed specimens in order to discriminate between ingested *vs*. assimilated food items (i.e. which of the captured preys were preferentially assimilated by the three species) in order to assess the better feeding conditions to rear them in aquaria prior to transplanting operations for restoration projects 3) to increase our understanding on the feeding ecology of CWCs occurring in the Mediterranean Sea, by also determining their trophic niche. To achieve these two last goals, I used stable isotope analysis (here after SIA), generally used in food-web studies to study the community structure (Post, 2002). Here I have determined the isotopic ratios of carbon ($C^{13}/C^{12}$) (here after $\delta^{13}C$) and nitrogen ($N^{15}/N^{14}$) (here after $\delta^{15}N$). $\delta^{13}C$ is used to identify food sources at the lower level of the trophic web (Fry, 2006) and $\delta^{15}N$ is considered a proxy of a species' trophic position (Post, 2002). SIA also enables to determine the isotopic niche of a species that includes its ecological niche (Layman et al., 2012) and allows the comparison between different isotopic niches across the same community (Jackson et al., 2011).

### 4.2 Materials and methods

Three species of cold-water corals were sampled using an ROV (Remotely Operated Vehicle) during two oceanographic campaigns conducted in the Mediterranean Sea on-board of RV *Minerva Uno*. Live colonies of *D. pertusum* and *M. oculata* were collected during the SIRIAD16 oceanographic campaign at depth of 244 and 400 m, respectively, in the south-western Adriatic Sea (Fig. 1). *D. cornigera* was collected at 139 m depth, during the RISD_16 campaign, in the northern Ionian Sea (Fig. 1). Corals were kept alive inside 20 L PVC darkened aquaria filled with bottom seawater, without feeding them, both due to the logistical limitations on board, and because as reported in literature is better not to feed them during the first days after capture (Orejas et al., 2019).

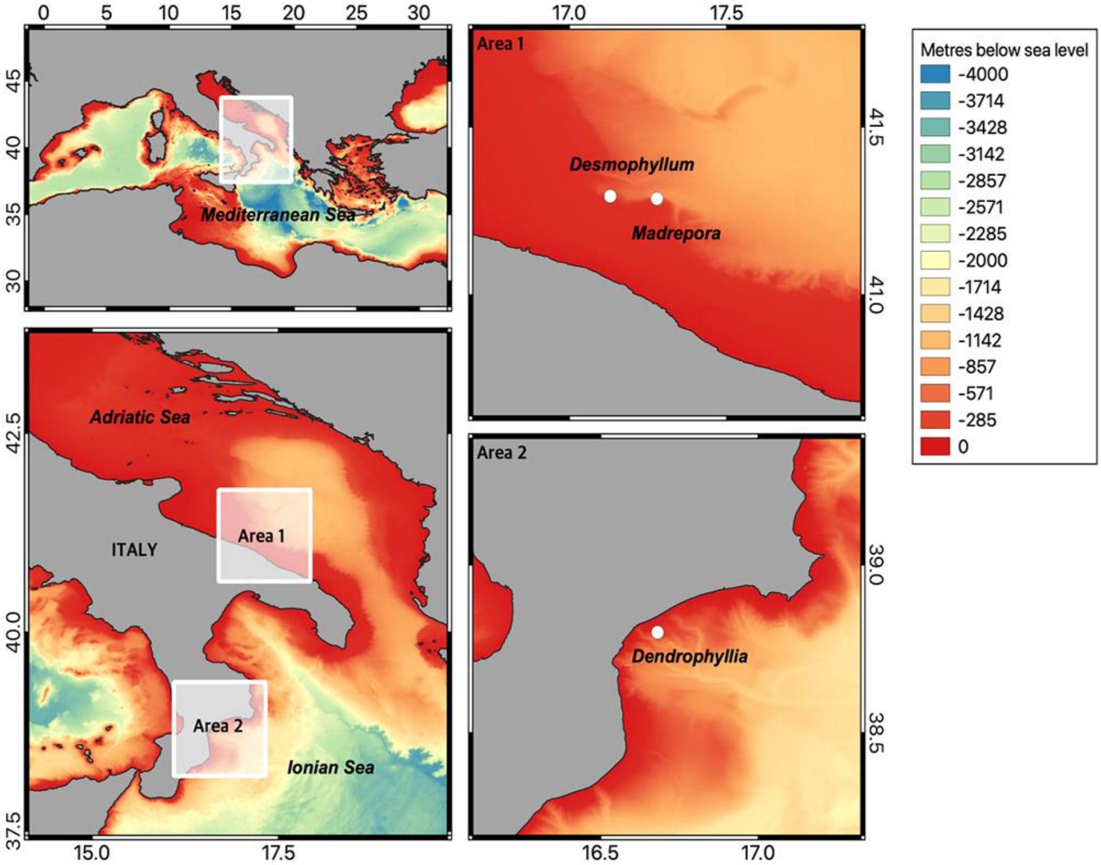

**Figure 1. The map shows the points in which corals were collected. The location of sites is discussed in this study. “Area 1” highlights the zone of Bari canyon in which *Desmophyllum pertusum* and *Madrepora oculata* were collected. “Area 2” highlights the position of the collecting site of *Dendrophyllia cornigera*. Bathymetry from EMODnet (European Marine Observation and Data Network) Bathymetry portal (http://www. emodnet-bathymetry.eu).**

Once in the laboratory, the colonies were kept in different aquaria in the dark and at *in-situ* temperature (13°C ± 0.1°C). Temperature was maintained constant through a common water bath and a refrigerator (TECO SeaChill Chiller TR5). Seawater was sampled in the central Adriatic Sea (salinity 37 ± 0.2) and was filtered with a 20 µm-mesh prior to use it for water change every 2 weeks.

A mechanical pump continuously mixed up seawater, and an air stone, placed at the top of the aquaria preventing any influence on polyps' activity, oxygenated the seawater. The oxygen concentration was maintained at *in situ* conditions (7.1 ± 0.2 mg $L^{-1}$) (Davies et al., 2008). The acclimatization period lasted around one month for each species. During this period, I provided only small quantities of *A. salina* to the corals in order to acclimatize them (total biomass of ca. 3 µgC $L^{-1}$).

### 4.2.1 Behavioural observations

Observations on coral behaviour and response to presence of food or light, as well as mucus production, during collection, transport and acclimatization, were filed to gather information essential at best maintenance in aquaria.

### 4.2.2 Set up of the feeding experiments

*Artemia*'s dry cysts (1 g) were placed inside a conical *Artemia* hatchery filled with 2 L of pre-filtered 0.7 µm seawater (filtered using Whatman GF/F filters) with intensive light. After 24 hours, nauplii were hatched and used for the experiment. The green algae *T. subcordiformis* was cultured in 500 mL Erlenmeyer flasks filled with sterile F/2 medium (Guillard, 1975). The cultures were maintained at 21°C, lightened by a continuous light with a photon flux density of 100 µmol $m^{-2}$ $s^{-1}$ (400-700 nm). *B. plicatilis* and *B. rotundiformis* were cultured at 21°C in a tank with mechanical aeration filled with pre-filtered 0.7 µm seawater at a salinity of 33 (Yufera et al., 1997). Rotifers were fed with baker's yeast (Orejas et al., 2019). These food sources were selected for the highly standardised rearing protocols and the variety of the composition, which can simulate a wide range of potential coral preys (Orejas et al., 2019). On the contrary, mysids are not easy to rear due to the common cannibalism of the adults towards juveniles (Mauchline, 1980). For this reason, *M. relicta* was supplied from frozen stocks and kept in suspension using an intense water recirculation.

Each food source was provided in the same total amount (total biomass expressed as µg of C), twice a week to *D. pertusum* and *M. oculata* (around 75 µgC $L^{-1}$ from each food source) and three times a week to *D. cornigera* (around 250 µgC $L^{-1}$ for each food source). Food sources' biomass was determined using literature data (*T. subcordiformis*), and by using bio-volumetric measurements (zooplankton). Body volumes of *M. relicta*, *A. salina*, *Brachionus* spp. were determined measuring with a stereomicroscope (LEICA WILD L3B) with micrometric grids. The biovolume and the biomass of rotifers was calculated accordingly to Gradinger et al., 1999. For the crustaceans, the biovolume was calculated from the body width (W) and length (L) (ten specimens for each food source) using the formula $V=L\times W^2\times C$, where C is an a-dimensional factor (Gambi et al., 2019). I assumed an average density of 1.13 g $cm^{-3}$ to calculate the wet biomass and then the dry weight (µg dry weight: µg wet weight = 0.25) (Wieser, 2007). The carbon content was considered as the 40% of the dry weight (Higgins & Thiel, 1988).

### 4.2.3 Prey-capture rate experiments

The prey-capture rate was evaluated for each of the three species. For this experiment, I followed the protocol reported in Tsounis et al. (2010). Fresh living zooplankton and phytoplankton were mixed and added in the aquaria, twice a week (for 30 days) for *D. pertusum* and *M. oculata*, and three times a week for *D. cornigera. F*rozen individuals of *M. relicta* were added at the same time. Seawater in the aquaria was gently mixed by a continuous slightly aeration (Piccinetti et al., 2016) and by recirculation pumps. Seawater was vigorously mixed, and samples (100 mL for 3 replicates) were took from each tank after a couple of seconds and after 5 hours (Orejas et al., 2016). Samples were preserved with 4% formaldehyde (Sigma-Aldrich, CAS Number 50-00-0) (Orejas et al., 2016) and, after 24 hours, individuals of *A. salina* and *Brachionus* spp. were counted using a Dolphus cuve (Tsounis et al., 2010) and a stereomicroscope (LEICA WILD L3B). For counting *T. subcordiformis* cells, replicates of 60 mL of seawater were collected at the same time from each tank and preserved for 24 hours with 2% formaldehyde. Replicated subsamples of 1 mL of seawater were observed using a Sedgewick-rafter counting chamber and algae cells were counting under a microscope (Orejas et al., 2016). Averaged prey capture rate was normalized to the number of polyps present in each mesocosm to determine the number of nauplii captured by each polyp each hour. Each time after feeding, the uneaten food was removed (Orejas et al., 2019).

### 4.2.4 Stable Isotope analysis (SIA)

Isotopic values of all the food items were also determined in this study. Once arrived at the laboratory, one third of the sampled colonies of the coral samples was immediately frozen at –20°C ($T_0$). SIA were conducted only on the soft bodies of the animals to avoid the interference of the C signal provided by the analysis of the entire calyx. Samples from the different cultures of the food sources were collected and frozen. At the end of the experiment ($T_f$), samples of each of the coral colonies were collected and immediately frozen. The samples were then dried for 24 h at 60°C. Samples were ground to a fine powder with a mortar and a pestle (Fanelli et al., 2011). Except for algae and rotifers, subsamples were acidified adding drop by drop HCl 1M (Sigma-Aldrich, CAS Number 7647-01-0) to remove inorganic carbonates. Cessation of bubbling was used as signal of completion of the reaction. These subsamples were dried again at 60 °C for 24 h (Jacob et al., 2005). Some samples were acidified (and dried) once again until complete removal of inorganic carbonates. All the samples were weighed (ca. 1 mg of dry weight) in tin capsules (Elemental Microanalysis Tin Capsules

Pressed, Standard Weight 5 x 3.5 mm). Stable isotope measurements were carried out by a Thermo Fisher FlashEA 1112 elemental analyser coupled to a Thermo Electron Delta Plus XP isotope ratio mass spectrometer (IRMS), according to standard protocols (Fanelli et al., 2009; Fanelli et al., 2011; Rumolo et al., 2016). Briefly, the samples were run against blank cups and known urea standards. Three capsules of urea were analysed at the beginning of each sequence and one every six samples as a quality control measure and to compensate for potential machine drift. Experimental precision (based on the standard deviation of replicates of the internal standard) was <0.1 ‰ for $\delta^{15}N$ and <0.2 ‰ for $\delta^{13}C$. The $\delta^{15}N$ and $\delta^{13}C$ values were obtained in parts per thousand (‰) relative to Vienna Pee Dee Belemnite (VPDB) and atmospheric $N_2$ standards, respectively, according to the following formula:

$\delta^{13}C$ or $\delta^{15}N$ =[(Rsample/Rstandard)−1]×103, where R=$^{13}C/^{12}C$ or $^{15}N/^{14}N$.

At least three replicates for samples were analysed.

### 4.2.5 Data treatment

In order to provide an estimate of the relative contributions of the different sources to the isotopic content of the samples, the Bayesian mixing model SIAR (Stable Isotopes Analysis in R) was used (Parnell et al., 2010). SIAR uses a Markov-chain Monte Carlo modelling; it fits a Bayesian model of the diet habits of the animals taking data of stable isotopes. This model assumes that each stable isotope value for each animal comes from a Gaussian distribution with an unknown mean and standard deviation. The mean results as a weighted combination of the different isotopic values of food sources (Conese et al., 2019). The standard deviation depends on the intraspecific variability among the individuals and on the uncertainty of fractionation corrections. In this study, I used for $^{13}C$ the Trophic Enrichment Factor (TEF) of 1.0 ± 0.1 ‰ (Ferrier-Pagès et al., 2011) and for $^{15}N$ the TEF of several consumers' diets that is 2.5 ± 0.1 ‰ (Carlier et al., 2009).

Prior to run the model, the isotopic values of the sources and of the corals were plotted, applying the correct TEFs, to determine the mixing polygon (Phillips et al., 2014). I excluded *T. subcordiformis* as food source due to its very high $\delta^{15}N$ isotope value which remains far outside the mixing polygon (Jackson et al., 2011). The corals' isotopic values did not fall completely within the range of the food source isotopic values, so I evaluated to use some literature data to better construct the mixing polygon and to define the sources with which to run the model (Phillips et al., 2014). One of the potential food sources for CWCs is the particulate organic matter (POM) contained in the seawater (Mueller et al., 2014). In this study, I used two different POM values as inputs in the SIAR model, based on the hypothesis that CWCs can be influenced by both the Bari Canyon POM during the collection, transportation and the beginning of the experiment and by the Northern Adriatic Sea POM for the other part of the experiment, as I used Adriatic seawater for maintaining corals in our laboratory. The values of isotopic content of the POM of the Bari Canyon ($\delta^{15}$N = –0.1 ‰ and $\delta^{13}$C = –20.7 ‰) and POM of the Adriatic Sea (summer period, $\delta^{15}$N = 7 ‰ and $\delta^{13}$C = –22 ‰) were taken from literature (respectively from Carlier et al., 2009 and Faganeli et al., 2009).

To examine the trophic niche of each species, SIBER package (Stable Isotope Bayesian Ellipses in R) was used (Jackson et al., 2011). This R package was used to determine the structure of the trophic niches of the three species. Layman metrics were calculated in SIBER: $\delta^{13}$C range (CR), $\delta^{15}$N range (NR), total area of the convex hull (TA), mean distance to centroid (CD) (Layman et al., 2007). CR provides information on the diversity of the resources at the base of the trophic web with higher values that indicate multiple basal carbon sources; NR gives information on the trophic length of the community and CD estimates trophic diversity within a food web and is a function of the degree of species spacing: lower numbers indicate that distinct taxa are exhibiting similar ecological functions (Jackson et al., 2011). TA gives an indication of the niche width but is highly sensitive to sample size (Layman et al., 2007). SIAR and SIBER were used also to calculate the corrected Standard Ellipse Areas ($SEA_C$) that is the sample-size corrected population isotopic niche (Jackson et al., 2011) allowing the comparison between the niches of the three species (classified as groups of the same community). It contains approximately 40% of the data within a set of bivariate data and thus represents the core niche area for a population or community (Layman et al., 2007; Jackson et al., 2011). Overlap of isotopic niches suggests, at least in part, an overlap of resource usage by the groups (Layman et al., 2007; Jackson et al., 2011). The percent overlap is calculated by assessing the percent of the overlapping area over the total area covered by the two ellipses (Krumsick & Fisher, 2019). All the analyses were carried out using R version 3.5.1 (R Development Core Team, 2018).

## 4.3 Results

### 4.3.1 Behavioural observations

Once in the laboratory, corals were acclimatized in stable *in-situ* temperature conditions and in the dark. *D. cornigera* was the most reactive species. It reacted to each food provisioning by moving tentacles in order to capture preys. *D. pertusum* seems to be the most light-sensible species. It avoided exposure to the weak light used during samplings, withdrawing polyps and producing mucus. *M. oculata* expanded the polyps' tentacles when it detected the presence of food items.

### 4.3.2 Prey-capture rates

Each polyp of *D. pertusum* captured an average of 3.6 ± 1.8 (SE) nauplii of *A. salina* per hour, 3.7 ± 2 (SE) individuals of *Brachionus* spp. and about 3.2 ± 0.7 × $10^3$ (SE) cells of *T. subcordiformis*. Each polyp of *M. oculata* captured an average of 1 ± 0.3 (SE) nauplii of *A. salina* per hour, 2.1 ± 0.7 (SE) rotifers and about 9.8 ± 2.6 × $10^3$ (SE) cells of the algae. Each polyp of *D. cornigera* preyed, on average, 2 × $10^3$ ± 53 (SE) nauplii per hour, 1.6 × $10^3$ ± 5.6 × $10^2$ (SE) individuals of *Brachionus* spp. and about 4.8 ± 1.9 ×$10^4$ (SE) cells of algae (Table 1). Specimens of *M. relicta* were completely removed each time by all the three species of corals.

| **Species** | **Food source** | **Prey-capture rate (ind polyp$^{-1}$ h$^{-1}$) ± SE** | **Total C (µg C polyp$^{-1}$ h$^{-1}$)** |
|---|---|---|---|
| *D. pertusum* | *Mysis relicta* | 0.004 | 14.2 |
| *D. pertusum* | *A. salina* | 3.6 ± 1.8 | 0.8 |
| *D. pertusum* | *Brachionus* spp. | 3.7 ± 2 | 0.3 |
| *D. pertusum* | *T. subcordiformis* | 3.2 ± 0.7 × $10^3$ | 0.7 |
| *M. oculata* | *Mysis relicta* | 0.001 | 3.6 |
| *M. oculata* | *A. salina* | 1 ± 0.3 | 0.2 |
| *M. oculata* | *Brachionus* spp. | 2.1 ± 0.7 | 0.2 |
| *M. oculata* | *T. subcordiformis* | 9.8 ± 2.6 × $10^3$ | 0.2 |
| *D. cornigera* | *Mysis relicta* | 0.2 | 783.2 |
| *D. cornigera* | *A. salina* | 2 × $10^3$ ± 53 | 47.2 |
| *D. cornigera* | *Brachionus* spp. | 1.6 × $10^3$ ± 5.6 × $10^2$ | 13.9 |
| *D. cornigera* | *T. subcordiformis* | 4.8 ± 1.9 × $10^4$ | 10 |

**Table 1. Different prey-capture rates of the three species of corals and total quantity of carbon captured per polyp. SE = standard error**

In terms of biomass, most of the organic carbon (µg C polyp$^{-1}$ h$^{-1}$) was obtained from the largest preys (*M. relicta*; Table 1). In fact, both *D. pertusum* and *D. cornigera* preys a similar number of nauplii of *A. salina* and specimens of *Brachionus* spp., but the mass of carbon supplied by the crustaceans is higher than that of the rotifers. All coral species fed also upon *T. subcordiformis*, which provided a food supply higher than that of the rotifers for the species *D. pertusum* and *M. oculata*.

### 4.3.3 Results of SIA

The isotopic content of the food items provided to the three coral species, varied from 8.9 ‰ (in *M. relicta*) to 17.1 ‰ (in *T. subcordiformis*) for $\delta^{15}$N and from –19.5 ‰ (in *M. relicta*) to –13.5 ‰ (in *Brachionus* spp.) for $\delta^{13}$ C (Table 2).

| **Food sources** | **$\delta^{15}$N** | **SD** | **$\delta^{13}$C** | **SD** |
|---|---|---|---|---|
| *Mysis relicta* | 8.9 | <0.05 | –19.5 | 0.1 |
| *Artemia salina* | 14.5 | 0.1 | –18.6 | <0.05 |
| *Brachionus* spp. | 11.5 | 0.1 | –13.5 | 0.2 |

| | | | | |
|---|---|---|---|---|
| *Tetraselmis subcordiformis* | 17.1 | <0.05 | –13.8 | <0.05 |

**Table 2. Mean $\delta^{15}N$ and $\delta^{13}C$ of the food sources provided to the corals. SD = standard deviation.**

The isotopic content was determined analysing samples taken from the soft body of the corals. At the end of the experiment ($T_f$, after 30 days from the beginning of the feeding experiment) $^{15}N$ values were more enriched in all the species. *D. pertusum* shows an increase in the $\delta^{15}N$ value of 2.9 ‰ (from 3.4 ± 0.4 ‰ to 6.3 ± 0.5 ‰). In *M. oculata* the increase is of 0.8 ‰ (from 3.3 ± 0.9 ‰ to 4.1 ± 1.8 ‰) and in *D. cornigera* the increment is of 1.2 ‰ (from 4.8 ± 0.6 ‰ to 6 ± 0.6 ‰). The isotopic signals in *D. pertusum* and *M. oculata* were $^{13}C$-depleted (respectively from –19.9 ± 0.3 ‰ to –20.7 ± 0.8 ‰ and from –19.5 ± 1.9 ‰ to –20 ± 1.7 ‰) but for *D. cornigera* the values of $\delta^{13}C$ showed an increase (from –21 ± 0.5 ‰ to –20.4 ± 1.7 ‰) (Table 3).

| **Time** | **Species** | **$\delta^{15}N$** | **SD** | **$\delta^{13}C$** | **SD** |
|---|---|---|---|---|---|
| $T_0$ | *D. pertusum* | 3.8 | 0.4 | –19.9 | 0.3 |
| | *M. oculata* | 3.3 | 0.9 | –19.5 | 1.9 |
| | *D. cornigera* | 4.8 | 0.6 | –21 | 0.5 |
| $T_f$ | *D. pertusum* | 6.3 | 0.5 | –20.7 | 0.8 |
| | *M. oculata* | 4.1 | 1.8 | –20 | 1.7 |
| | *D. cornigera* | 6 | 0.6 | –20.4 | 1.7 |

**Table 3. Mean $\delta^{15}N$ and $\delta^{13}C$ of the corals at the beginning ($T_0$) and at the end ($T_f$) of the experiment. SD= standard deviation.**

Concerning potential food sources for the three species selected, based on the output of SIAR mixing model, the isotopic values of corals' samples at $T_0$ (empty circles) are closer to the signal of the POM recorded in the Bari Canyon (POM_BariC; Fig. 2). At $T_f$, isotopic values (empty triangles) are closer to the value of POM recorded in the Northern Adriatic Sea (POM_NAdr) and *M. relicta* (Fig. 2).

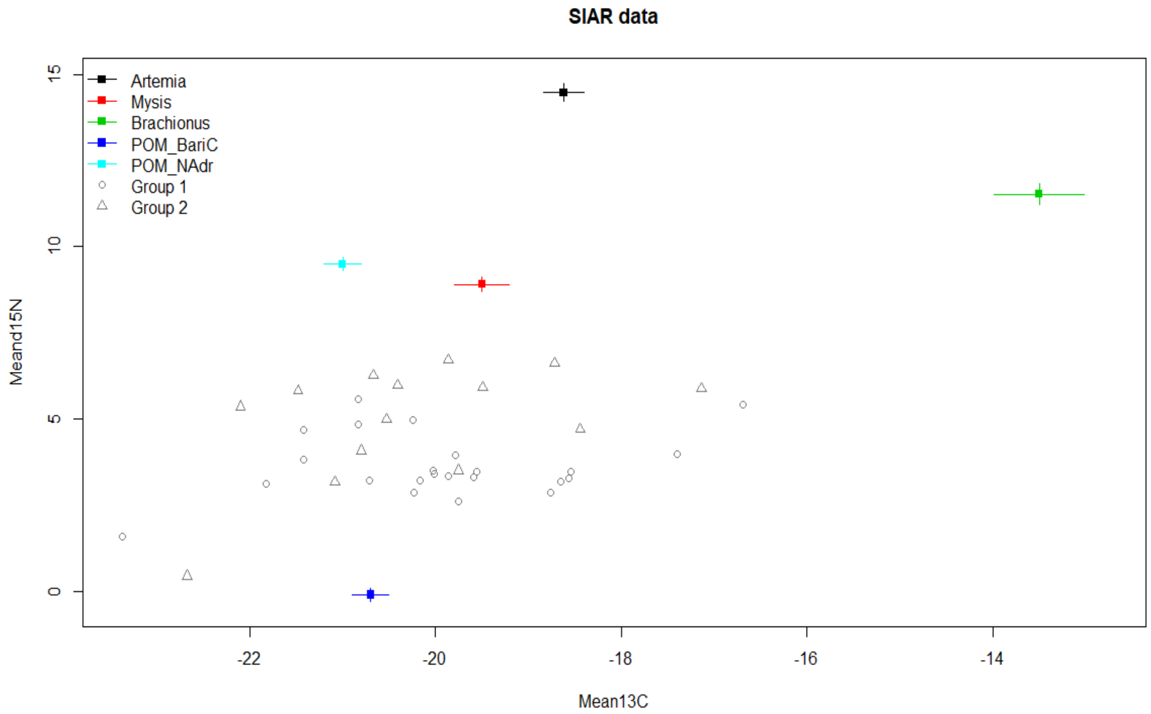

**Figure 2. Results of isotopic content analysis. Filled squares represent the sources (POM_BariC indicates the POM of Bari canyon and POM_NAdr indicates the POM of North Adriatic Sea; Artemia indicates *Artemia salina* nauplii), circles represent isotopic content of the consumer at time $T_0$ (Group 1) and triangles represent the isotopic content of consumers at time $T_f$ (Group 2).**

SIAR mixing model also provided the proportions of each food source to the diet of the three CWC species (Fig. 3). At $T_0$, the principal contribution to the isotopic composition of the three species was given by the POM of the South Adriatic Sea (64.8 %) (Fig. 3a). After 30 days, the proportion of the contribution of POM of the North Adriatic Sea to CWC's diet increased (19.3 %) together with the contribution of mysids (17.1%) (Fig. 3b).

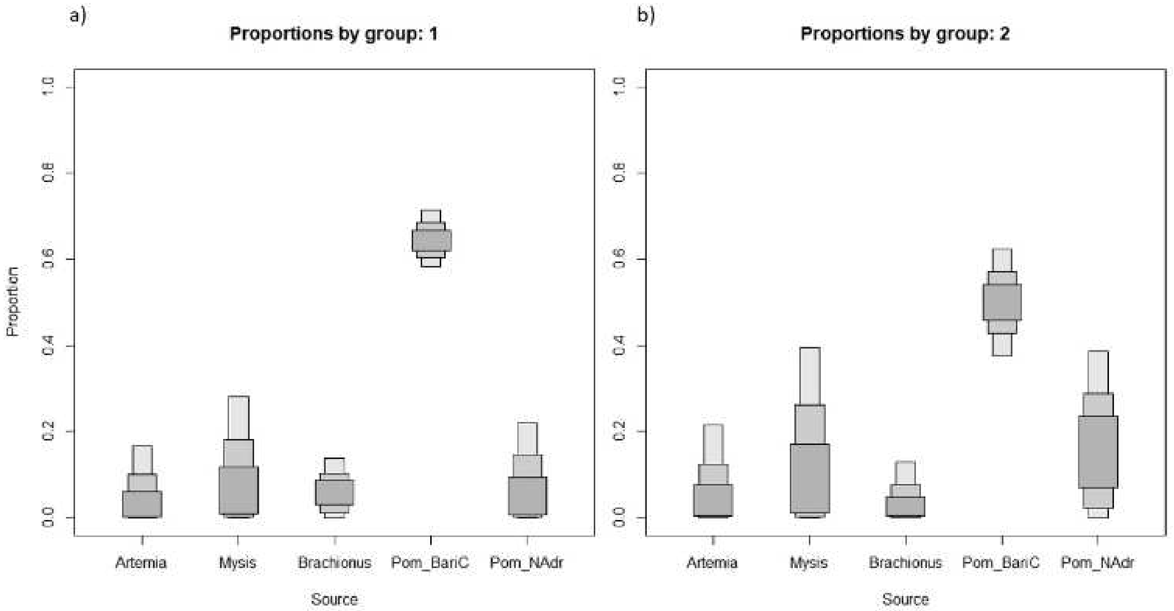

**Figure 3. Proportions of each food source in the corals diet obtained with stable isotope analysis mixing models. Each plot shows proportions for each food source at the beginning ($T_0$) and at the end ($T_f$) of the experiment. Boxes indicate 50%, 75% and 95% Bayesian confidence intervals.**

Standard ellipses showed that *M. oculata* has the widest and *D. cornigera* the smallest isotopic niche (Fig. 4). Additionally, Layman metrics (Table 4) indicated that *D. cornigera* had the smallest total area (TA) (3.12), followed by *D. pertusum* (4.12), while *M. oculata* showed a TA of 16.2. The corresponding values of the different $SEA_C$ are 2.75, 2.38 and 5.83, for *D. pertusum, D. cornigera* and *M. oculata*, respectively. While the $SEA_C$ of *M. oculata* and *D. cornigera* are expanded along the x-axis (pointing out to a wider $\delta^{13}$C range), the SEAc of *D. pertusum* is stretched along the y-axis (corresponding to a greater $\delta^{15}$ N range) (Fig. 4 and Table 4). *M. oculata* has the greatest $\delta^{13}$C and $\delta^{15}$N ranges and mean CD, which is a proxy of trophic diversity (Table 4). Overall, there is a partial overlap of the SEAc of the three species. The SEAc of *D. pertusum* and *M. oculata* overlapped for 16.1%, while those of *D. pertusum* and that of *D. cornigera* for 15.4%. The overlap of the SEAc of *M. oculata* and *D. cornigera* is largely <0.1%.

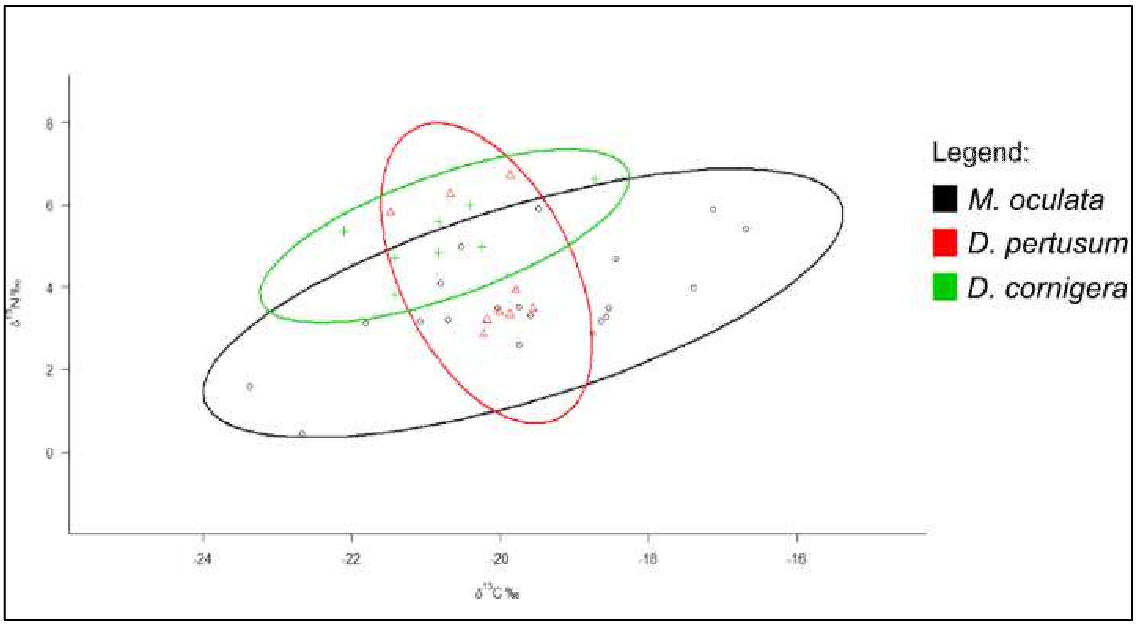

**Figure 4. $\delta^{13}$C - $\delta^{15}$N scatterplot with standard ellipses corrected for small sample size population (SEAc) overlaid for the three CWC species.**

| | *D. pertusum* | *M. oculata* | *D. cornigera* |
|---|---|---|---|
| **TA** | 4.12 | 16.20 | 3.12 |
| **SEAc** | 2.75 | 5.83 | 2.38 |
| **$\delta^{15}$ N range** | 3.85 | 5.46 | 2.80 |
| **$\delta^{13}$ C range** | 1.92 | 6.68 | 3.38 |
| **CD** | 1.39 | 1.79 | 1.05 |

**Table 4. Total area (TA), Standard ellipse area (SEAc) and Layman metrics calculated for each species.**

### 4.4 Discussion

This study elucidates the food preferences of the CWCs reared in aquaria, adding useful information for the maintenance of these species in laboratory, a mandatory step for restoration experiments. In this context, this study provides important clues for understanding trophic preferences of CWCs in captivity. CWCs need stable conditions for their successful breeding in aquaria (in terms of temperature, absence of light, gradual water change; Orejas et al., 2019).

During the whole experiment, the three CWC species captured all the different types of preys including algae, rotifers and crustaceans (both branchiopods and mysids). Available literature on coral feeding experiments (Table 5) shows that prey-capture rates differ among the three CWC species, depending on the type of prey and on the different life stage of the specimens offered as food (e.g., naupliar stages vs adults).

| **Species** | **Prey** | **Capture rate Ind. polyp$^{-1}$ h$^{-1}$** | **Capture rate µg C polyp$^{-1}$ h$^{-1}$** | **Temp. (°C)** | **Reference** |
|---|---|---|---|---|---|
| *D. pertusum* | *M. relicta* | 0.004 | 14.2 | 13 | present study |
| *D. pertusum* | adults of *A. salina* | 7.8 ± 2.5 (SD) | 3269 ± 1042 (SD) | 12 | Tsounis et al., 2010 |
| *D. pertusum* | Copepods | n.d. | 126.3 ± 32.6 (SD)/ 250.4 ± 79.5 (SD) | 8 | Orejas et al., 2016 |
| *D. pertusum* | nauplii of *A. salina* | 3.6 ± 1.8 (SE) | 0.8 | 13 | present study |
| *D. pertusum* | nauplii of *A. salina* | 283.7 ± 130.1 (SD) | 462 ± 212 (SD) | 12 | Tsounis et al., 2010 |
| *D. pertusum* | nauplii of *A. salina* | 2.5 ± 1 / 16.2 ± 2.1 (SD) | 2.2 ± 0.9 (sd)/ 14.6 ± 1.9 (SD) | 8 | Purser et al., 2010 |
| *D. pertusum* | *Brachionus* spp. | 3.7 ± 2 (SE) | 0.3 | 13 | present study |
| *D. pertusum* | *T. subcordiformis* | $3.2 \pm 0.7 \times 10^{3}$ | 0.7 | 13 | present study |
| *D. pertusum* | *Skeletonema marinnoii* | n.d. | −264.3 ± 158.4 (SD) / 798.4 ± 343 (SD) | 8 | Orejas et al., 2016 |
| *M. oculata* | *M. relicta* | 0.001 | 3.6 | 13 | present study |
| *M. oculata* | adults of *A. salina* | 2.4 ± 2.3 (SD) | 994 ± 996 (SD) | 12 | Tsounis et al., 2010 |
| *M. oculata* | nauplii of *A. salina* | 1 ± 0.3 (SE) | 0.2 | 13 | present study |
| *M. oculata* | nauplii of *A. salina* | 47.9 ± 33.3 (SD) | 78.1 ± 54.3 (SD) | 12 | Tsounis et al., 2010 |
| *M. oculata* | *Brachionus* spp. | 2.1 ± 0.7 (SE) | 0.2 | 13 | present study |
| *M. oculata* | *T. subcordiformis* | $9.8 \pm 2.6 \times 10^{3}$ (SE) | 0.2 | 13 | present study |
| *D. cornigera* | *M. relicta* | 0.2 | 783.2 | 13 | present study |
| *D. cornigera* | adults of *A. salina* | 5.3 ± 1.5 (SD) | 2225 ± 632 (SD) | 12 | Tsounis et al., 2010 |
| *D. cornigera* | adults of *A. salina* | 160 ± 70 (SD) | nd | 16 | Gori et al., 2015 |
| *D. cornigera* | nauplii of *A. salina* | $2 \times 10^{3} \pm 53$ (SE) | 47.2 | 13 | present study |
| *D. cornigera* | nauplii of *A. salina* | 33.3 ± 25.1 (SD) | 189 ± 41 (SD) | 12 | Tsounis et al., 2010 |
| *D. cornigera* | nauplii of *A. salina* | 889 ± 250 (SD) | nd | 16 | Gori et al., 2015 |
| *D. cornigera* | *Brachionus* spp. | $1.6 \times 10^{3} \pm 5.6 \times 10^{2}$ (SE) | 13.9 | 13 | present study |
| *D. cornigera* | *T. subcordiformis* | $4.8 \pm 1.9 \times 10^{4}$ (SE) | 10 | 13 | present study |

**Table 5. Feeding rates of the three CWC species considered in this experiment, reported in literature. nd = not determined. SD= standard deviation; SE= standard error**

In this study, for the first time, four different types of food sources have been provided in the same amounts (in terms of total μg of C) in order to allow corals to select the preferred food items. Prey-capture rates expressed in terms of μg of C captured each hour by each polyp of the three species of CWCs show a preference for the mysid *M. relicta*, and subordinately by the branchiopod *Artemia salina*. Several studies reported that the diet of CWCs in the field is based on zooplankton, such as copepods (Kiriakoulakis et al., 2005; Naumann et al., 2015). For this reason, *A. salina* of the same size of target copepods is often used in CWC rearing in aquaria (Orejas et al., 2019 and references therein) and in feeding experiments. The capture rates of *A. salina* nauplii observed for *D. pertusum* and *D. cornigera* in the present study are consistent with literature values (Purser et al., 2010; Gori et al., 2015), while *M. oculata* showed lower capture rates than previously observed (Tsounis et al., 2010). Moreover, in terms of μg of C captured each hour by each polyp, the total amount of μg of C assumed by this last species feeding on *A. salina* and on *Brachionus* spp. is similar (respectively 0.24 and 0.18 μg C polyp$^{-1}$ h$^{-1}$). Usually, rotifers in aquaria are provided as feeding supplementary to corals with very small polyp size, like black corals (e.g. *Bathypathes* sp. and *Leiopathes glaberrima*) or gorgonians (es *Dentomuricea meteor*) (Orejas et al., 2019). Among the three different species, *M. oculata* is the one with smallest polyps (5-10 mm in diameter) (Orejas et al., 2019) so the greater capture rates for rotifers (around 350 μm in length) could be related to the suitable size of this prey compared to the size of the corals' polyps.

Measurements of prey-capture rates are difficult to compare, because capture rates may overestimate the real ingestion rate if the prey is not efficiently transferred to the gut (Purser et al., 2010). Trapped food may be lost due to the phenomenon of sloppy feeding (Moller, 2004) or could not be assimilated so prey-capture rates cannot directly be translated in ingestion rates or even in assimilation rates (Orejas et al., 2019). Because of this, I integrated capture-rates results with SIA results that allowed us to evaluate the degree of assimilation of the provided food. SIA were used to determine the assimilated food and results were compared with the evaluation of feeding rates.

The values of $\delta^{13}$C measured at $T_0$ in this study for the three species of CWCs (from –19.5 to –21 ‰) fit well with that of North Atlantic CWCs (from –22.2 to –19.3 ‰) (Duineveld et al., 2004; Sherwood et al., 2008). These values are slightly more negative than that for CWCs from the Strait of Sicily, South of Malta (from –18.9 to –18.2 ‰, CNR cruise CORAL of the RV 'Urania', 450–600 m) (unpublished M. Taviani). Our results are consistent also with data from the CWCs coral province of Santa Maria di Leuca for *D. pertusum* and *M. oculata* ($\delta^{13}$C from –19 to –21 ‰) (Carlier et al., 2009). In our experiment, a decrease in $\delta^{13}$C in *D. pertusum* and *M. oculata* is observed at $T_f$ (respectively –20.7 and –20 ‰), although $\delta^{13}$C values remained similar to those reported for the Ionian Sea (Carlier et al., 2009). These results likely support the hypothesis of a high similarity, in terms of food sources, between the South Adriatic (Bari Canyon) and S. Maria di Leuca CWC provinces. On the contrary, an increase in $\delta^{13}$C for *D. cornigera* was observed probably due to the greater ingestion and also assimilation of *M. relicta.* This species has a greater size (the polyps measure 20-40 mm in diameter) (Orejas et al., 2019) and mysids are likely more adapted to the dimension of the polyps of this species (Gori et al., 2015; Orejas et al., 2019).

The values of $\delta^{15}$N at $T_0$ in all the species were more negative (about halved) (from 3.3 to 4.8 ‰) compared to the results obtained for specimens of *D. pertusum* and *M. oculata* collected and immediately frozen in the Santa Maria di Leuca CWC province (values of $\delta^{15}$N from 6.9 to 10.1 ‰) (Carlier et al., 2009) or that observed for CWCs from the Atlantic (Duineveld et al., 2004; Kiriakoulakis et al., 2005). The very high $^{15}$N-depletion could be caused by both stressors (thermal shock during the journey from the seabed to the surface, light exposition immediately after the collection, variations in pressure, retraction of polyps that are unavoidable stressors induced by the collection) (Orejas et al., 2019) and starvation. During the first five days after the collection of all the colonies, corals were not fed because of logistical limitations during the oceanographic campaigns. In addition, feeding is not recommended in this phase as polyps are generally closed, as also observed in our experiment, and thus not ingested food could decompose and remain in the aquaria, which are difficult to manage when on-board (Orejas et al., 2019). Thus, corals started eating after the period of acclimatization, at the beginning of the experiment that was around one month for all the collected corals. I assumed that during this starving period, they fed principally through filtration of POM from the seawater. This is consistent with the results observed from SIAR mixing models, as the isotopic values observed in $T_0$ for the three species is closed to that of the POM of the Bari Canyon ($\delta^{15}$N = –0.1 ‰ and $\delta^{13}$C = –20.7 ‰; Carlier et al., 2009). Indeed, I used bottom seawater collected in this area to fill the aquaria during the oceanographic cruise. POM is easier to assume and less energy-expensive to capture compared to whole animal preys (living or frozen), so our results confirm that CWCs are able to use also fine organic particles as food source (Mortensen, 2001; Mueller et al., 2014; Orejas et al., 2016). Environmental stress generally increases the energy that aquatic organisms require to recover and, accordingly, their food demand increases (Sokolova et al., 2012). In order to maintain their basal functioning and to recover after the collection, corals filtered POM from seawater. It is probable that CWCs can sustain their basal metabolism feeding only on POM during periods in which other food sources are not available. As an example, the values of $\delta^{15}$N of *D. pertusum* and *M. oculata* in the Rockall Bank (colonies samples between 750-845 m of depth, North East Atlantic) are very closed to that of obligate filter-feeding taxa like tunicates and bivalves that are known to feed only on organic particles (Duineveld et al., 1997). After 30 days from the beginning of the experiment, the isotopic values of corals were closer to those of POM of the northern Adriatic Sea. I used seawater collected from the Central Adriatic Sea to fill the aquaria during the experiment, so these results support the idea that corals still used POM as main food sources also during this second phase.

Among the other food sources, according to SIAR results, the mysids *M. relicta*, contributed to the corals' diet for 17.14%. This food item seemed to be the preferred one, also in terms of feeding rates (expressed in µg of C assumed per polyp each hour). This crustacean is member of the family Mysidae, the same family of *Boreomysis arctica* (Krøyer, 1861) and *B. megalops* (G.O. Sars, 1872). These two mysids are among the most abundant suprabenthic species on the upper and middle slope of the Ionian Sea (Madurrel & Cartes, 2003) and of the Catalan Sea (Cartes & Sorbe, 1995) and thus they can be the principal natural preys of CWCs living in these areas, also considering they are common prey for Dendrophylliidae corals (Orejas et al., 2019). *In-situ* observations on *D. pertusum* reported that this coral preferentially captures copepods (*Calanus*) and cumaceans (Henrich & Freiwald, 1997). These results suggest that, for the three species of CWCs, maintenance in aquaria could be better supported by a diet based on the provisioning of frozen *Mysis* sp. instead of *A. salina*, being the first one more similar to the natural preys of CWCs. Supply the corals with *Mysis* sp. can solve also the problem of the depletion of components like fatty acids in the tissue of corals fed only with *A. salina* (Larsson et al., 2013).

Our findings provide important results also for assessing the trophic niches of the three CWC species and their relationship. *M. oculata* seems to be a more generalist species, with the greatest trophic diversity, as evidenced by the wide $\delta^{13}$C range and the greatest CD values among the three species. This means that *M. oculata* is the CWC species with the highest feeding plasticity, able to feed on different preys and to adapt its diet to a large number of food sources compared to the other two CWC species. This is confirmed also by the prey-capture rates observed during this experiment, since *M. oculata* captured primarily *M. relicta* and then, in the same quantity (in terms of biomass) *A. salina*, rotifers and algae. In the Mediterranean basin, *M. oculata* seems to be the most abundant CWC species: it is up to 50 times more abundant over *D. pertusum* in Cape de Creus and Lacaze-Duthiers canyons in the Gulf of Lion (Gori et al., 2013) and also dominates the CWC communities in the Santa Maria di Leuca coral province (Vertino et al., 2010), in Bari Canyon (Freiwald et al., 2009) and in the Alboran Sea (Corbera et al., 2019). The higher abundance of this species in the basin may be related also to its greater trophic generalism and thus to a higher adaptation to different conditions and food availability. Opportunistic feeding behaviour was previously reported for *D. pertusum* (Mortensen, 2001; Mueller et al., 2014; Orejas et al., 2016) and *D. cornigera* (Gori et al., 2018). A generalist behaviour in deep-sea ecosystems represents an advantage as the species can rely on available food source (POM, zooplankton, phytodetritus) in an environment where food availability may be heterogeneous in time and space (Gori et al., 2018).

This experiment was not specifically set to study the behaviour of corals, but, nevertheless, some general considerations can be made, *D. cornigera* was the most reactive species during the entire experiment, especially after the food administration that, as reported in literature, stimulates its polyps' reaction (Orejas et al., 2019). *D. pertusum* was the most sensitive species, secreting mucus during the brief weak-light exposition at sampling times (feeding time and collection of the water samples for the evaluation of prey-capture rates). This species reacted slowly at the presence of food, confirming previous observations (Mortensen, 2001). Behaviour differences among species could possibly reflect also their reaction to pressure, in the order of ca. 20 bars for *D. pertusum* and ca. 40 for *M. oculata* (Orejas et al., 2019). The eurybathic *D. cornigera* was instead collected at ca. 14 bars, thus facing lower pressure variations, in principle less stressing for the animal. Also *M. oculata* shows much higher tolerance to environmental fluctuations (Wienberg et al., 2009). On the contrary, *D. pertusum* is a species more commonly found in habitats with temperatures between 4-12 °C (Freiwald et al., 2004) and it was observed that its abundance increases compared to that of *M. oculata* with higher latitudes in boreal areas (Arnaud-Haond et al., 2017). In the central Mediterranean Sea temperatures measured in live coral habitats during oceanographic campaigns range between 13.4 and 13.9°C (Freiwald et al., 2009). These temperatures are close to the ecological limit of *D. pertusum* (Brooke et al., 2013) while the optimal temperatures for this species were estimated to be around 6.2-6.7°C (Davies et al., 2008). This could support behavioural observations which indicated slower reactions to the presence of food of this species compared to that of *D. cornigera* and *M. oculata*, at the experimental temperature (13°C), which is likely more suitable for the latter two species (Naumann et al., 2014; Gori et al., 2015) than for *D. pertusum*.

### 4.5 Conclusions

There are evidences that measures of protection such as the creation of offshore MPAs can be very successful against the impacts of trawling activities (Huvenne et al., 2016), but these actions are no longer sufficient to conserve cold-water coral reefs. These habitats will face numerous and increasing impacts acting synergistically (trawling, mining/oil exploiting activities, climate changes) (Ragnarsson et al., 2016). Moreover, it is known that CWC colonies take hundreds of years to reach a diameter of 1.5-2 m and thousands of years to build a reef patch 10-30 m thick (Fosså et al., 2002). In the Mediterranean Sea, the observed *in-situ* growth rates of *D. pertusum*, are between 1.3 ± 1.5 mm year$^{-1}$ (old polyps) (Lartaud et al., 2013) while, for *M. oculata*, the observed *in-situ* growth rates are between 1.2 ± 1.2 mm year$^{-1}$ (old polyps) (Lartaud et al., 2013). For this reason, the expected natural recovery rates are very slow. Active restoration actions should be properly defined to remediate and compensate human-induced damages. Future restoration actions for CWC reefs will include rearing in aquaria of nubbins taken from healthy donor colonies and transplantation of these fragments in degraded mounds (Van Dover et al., 2014). Promising results of pilot transplantation experiments have been reported in literature for *D. pertusum* (Da Ros et al., 2019 and references therein).

The outcomes of this experiment bear on the successful maintenance of CWC in aquaria, an important prerequisite for laboratory experiments aimed at evaluating the recovery potential of CWCs and to define the best restoration techniques. In fact, rearing corals in aquaria and improving their wellness in captivity may positively impinge on their growth or reproduction success, which may turn into the generation of nubbins successfully transplantable in degraded reefs. Finally, this study provides novel information on the trophic niches occupied by *D. pertusum*, *M. oculata* and *D. cornigera* in the Mediterranean Sea.

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# CHAPTER 5: TRANSPLANTATION EXPERIMENT OF THE SEAGRASS *CYMODOCEA NODOSA*: ASSESSMENT OF THE EFFICIENCY AND EFFECTS ON ECOLOGICAL PROCESSES

## 5.1 Introduction

Seagrass meadows cover about 0.1-0.2% of the global ocean (Duarte, 2002), and ca. 30% of their known areal extent has disappeared in the last century (Waycott et al., 2009). Seagrass habitats are highly productive (Duarte, 2002) and play a key role in the provisioning of ecosystem's goods and services (Nellemann et al., 2009; Barbier et al., 2011; Mtwana Nordlund et al., 2016). Seagrass meadows provide habitats and nurseries for diverse assemblages of fish and invertebrate species, support complex trophic networks, stabilise sediments, reduce coastal erosion, increase water quality, contribute to pollution buffering and nutrient and carbon sequestration (Hemminga & Duarte, 2000; Nellemann et al., 2009; Unsworth et al., 2015; Dahl et al., 2016). Due to their ecological role, seagrass meadows are protected under different directives (e.g. Council Directive 92/43/EEC 1992).

Despite the large number of ecosystem services that they provide, seagrass meadows are affected by several anthropogenic activities that threat their conservation and the maintainment of their ecological role. Direct human impacts on seagrass meadows include mechanical damage due to dredging, fishing, anchoring, eutrophication, aquaculture, and coastal constructions (Duarte, 2002; Milazzo et al., 2004; Orth et al., 2006). Climate changes and global warming can also affect seagrass meadows causing alteration of their distribution, reproduction, germination, growth and productivity (Short & Neckles, 1999; García et al., 2013). As an example, the Mediterranean seagrass *Posidonia oceanica* is highly vulnerable to water warming, which can induce declines in shoot abundance (Marbà & Duarte, 2010). The loss rates of these ecosystems are comparable to those reported for mangroves and coral reefs, placing seagrass meadows among the most threatened ecosystems in the world (Waycott et al., 2009). In the Mediterranean Sea, major losses have occurred due to coastal development, pollution, trawling, fish farming, moorings, dredging, dumping and presence of alien species (Boudouresque et al., 2009). Considering the important ecological role of seagrass ecosystems, the restoration of degraded meadows is one of the main topics in the field of ecological restoration (Paling et al., 2009).

The success of seagrass restoration actions conducted in coastal marine ecosystem is generally relatively low (Bayraktarov et al., 2016). For example, the outcomes for the success are as low as 40% for most of the seagrass restoration projects described in literature (Bayraktarov et al., 2016). However, in some cases a much higher success of seagrass transplantation has been reported, in which planted areas resembled natural meadows in terms of plant density (Bastyan & Cambridge, 2008).

Considering the increasing amount of impacts that threat seagrass meadows, there is an urgent need to develop new techniques and approaches to improve the success of the restoration of degraded seagrass meadows. In the frame of the MERCES project (Marine Ecosystem restoration in Changing Europeans Seas; http://www.merces-project.eu/), I carried out transplantation experiments of the seagrass *Cymodocea nodosa* in a shallow-coastal ecosystem of the North Western Adriatic Sea. *Cymodocea nodosa* is one of the most common seagrass species of shallow-soft bottoms of the warm waters of the Mediterranean Sea. This species lives on sandy and muddy bottoms, from the sea level to 50 m of depth (Olesen et al., 2002). This species is fast growing (up to 70 mm day$^{-1}$) and the leaves reach a maximum length of 40 cm during summer (Cancemi et al., 2002). These seagrasses can spread vegetatively through the production of new shoots from the horizontal rhizome; with sexual reproduction that could eventually occur during spring (Buia & Mazzella, 1991).

The aims of this study were 1) to evaluate the efficiency of a new transplantation technique by monitoring the shoot density and leaf biomass of *Cymodocea nodosa* over time and 2) to assess the effects of transplantation of the seagrass on key-ecological processes (i.e. organic matter cycling).

## 5.2 Materials and methods

### 5.2.1 Study area

The investigated area is located in Gabicce Mare (North-western coastline, 43°57.967′N and 12°45.859′E) inside a Site of Community Importance (Fig. 1).

**Figure 1. Location of the study area in the North-western Adriatic Sea (Central Mediterranean).**

The northern part of this area is limited by a touristic harbour and the Tavollo River, and the southern portion is confined by the Monte San Bartolo, which gives the name to the Regional Natural Park created in 1997 to protect an area of great naturalistic importance (Colantoni et al., 2004). Gabicce Mare presents eroded Messinian deposits, mainly composed of sand and pelites (Tramontana et al., 2005). This area is characterized by the presence of seagrass meadows (Fig. 2) in the coastal area and is limited by breakwater structures that are located ca. 100 m from the shore and parallel to the coast.

**Figure 2. Spatial distribution of seagrass meadows in Gabicce Mare. Seagrass meadows were mapped during summer 2017.**

The meadows are constituted by *Zostera marina*, *Zostera noltei* and *C. nodosa* (Coccioni, 2003; Balsamo et al., 2011). Shoot density was measured during preliminary surveys using 40 × 40 cm PVC-quadrats. Spring's shoot density was 925-1925 shoots m$^{-2}$ for *C. nodosa*, 269-1246 shoots m$^{-2}$ for *Z. noltii* and 216-1093 shoots m$^{-2}$ for *Z. marina*. These values were comparable to densities reported in literature for different Mediterranean seagrass meadows (Guidetti et al., 2002; Buia et al., 2003).

### 5.2.2 Transplantation technique

The experimental design included 9 delimited squared plots (1m × 1m). 6 of these plots, located at the same depth of the 3 replicated plots with transplanted seagrasses, were used as control plots (3 in the bare sediments and 3 in the existing seagrass meadow) (Fig. 3b). Due to logistic difficulties encountered in the study area (in particular, the presence of a high number of tourists during the summer season), it was not possible to include control plots for the artifacts. The seagrass transplantation was carried out using biodegradable bags inserted in biodegradable jars anchored with U-shaped stainless-steel rods (Fig. 3a). A manual stainless-steel corer was used to dig a clod from the donor seagrass meadow. This clod was immediately inserted in a biodegradable bag to maintain the clod integrity. The biodegradable bag was included in a biodegradable jar to maintain the consistency of the clod. The jar was then easily anchored with a U-shaped stainless-steel rod in the sediments. Several jars were planted in each 1m × 1m plot in order to cover their entire experimental surface. Each jar contained 11-13 shoots of seagrass.

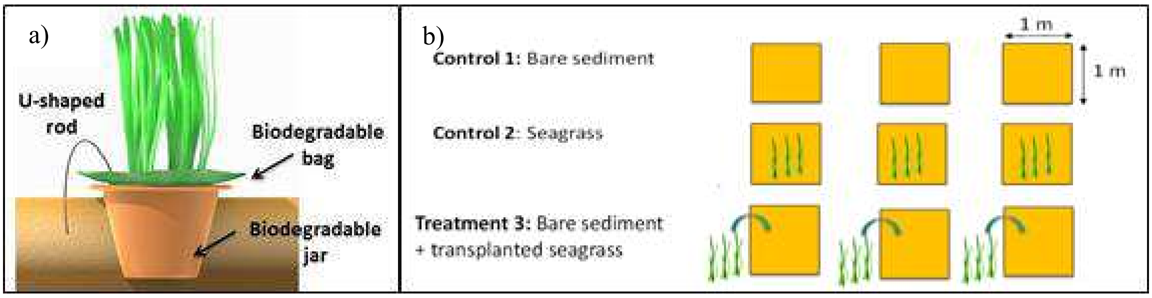


**Figure 3. a) The seagrass transplantation was carried out using biodegradable bags inserted in biodegradable jars anchored with U-shaped stainless-steel rods. b) Experimental design with the replicated plots.**

### 5.2.3 Monitoring and samplings

Seagrass density and biomass (both from the donor meadows and from the transplanted plots) were assessed from May 2018 to February 2019 in order to cover the seasonal variability. During the same time interval, sediment samples were collected using plexiglass corers from each plot. The superficial slide of the corer was immediately frozen at -20°C and used, once at the laboratory, for the determination of the quantity and biochemical composition of organic matter. Moreover, during each sampling, sterile plastic test-tubes were filled with the superficial sediment collected from each experimental plot to assess the values of the extracellular enzymatic activity to investigate the effects of seagrass transplantation on the trophic status and organic matter cycling. Samples were collected in May 2018 (May'18), July 2018 (Jul'18), September 2018 (Sep'18) and February 2019 (Feb'19).

### 5.2.4 Shoot density and leaf biomass

Shoot density was assessed at each sampling by counting the number of the shoots present inside each experimental plot (Li et al., 2014). Shoots were also collected from each vegetated (seagrass meadow and transplanted seagrass) plot for laboratory determination of the leaf biomass. Once at laboratory, the shoots were washed with freshwater and cleaned of epiphytes and sediments. The biomass was determined by drying the leaves for 24-48 hours at 60°C, following the protocols by Buia et al. (2003).

Biomass was multiplied with the shoot number of the same experimental plot to estimate the "leaf biomass" (Buia et al., 2003).

### 5.2.5 Biochemical composition of organic matter in sediments

Proteins, carbohydrates and lipids were determined spectrophotometrically, following the protocols detailed in Danovaro (2010), and their sedimentary contents (mg/g dry sediment) were expressed as bovine serum albumin, glucose and tripalmitine equivalents, respectively. Carbohydrate, protein and lipid sedimentary contents were converted into carbon equivalents using the conversion factors of 0.40, 0.49 and 0.75 μgC $\mu g^{-1}$, respectively, and their sum defined as biopolymeric carbon (Dell'Anno et al., 2002).

### 5.2.6 Extracellular enzymatic activities in sediment samples and turnover of organic matter

Extracellular aminopeptidase and β-glucosidase activities were determined in sediment samples by the analysis of the cleavage rates of their artificial fluorogenic substrates L-leucine-4-methylcoumarinyl-7-amide and 4-methylumbelliferyl MUF-b-D-glucopyranoside, respectively (Danovaro, 2010). Briefly, undiluted water samples and sediment sub-samples diluted with 0.02-μm pre-filtered seawater collected at the water-sediment interface from each station, were incubated in the dark at the *in-situ* temperature for 1 hour. The fluorescence of the samples was measured immediately after the addition of the substrate and after the incubation, and all the assays were carried out in triplicate. At the end of the incubation, the water samples and the supernatant of sediment samples (obtained following centrifugation at 3000 × g, 10 min) were analyzed fluorometrically at 380 nm excitation and 440 nm emission (for Leu-MCA) or at 365 nm excitation and 455 nm emission (for Glu-MUF). The fluorescence was converted into C degradation rates using standard curves of 7-amino-4-methylcoumarin for Leu-MCA and of 4-methylumbelliferone for Glu-MUF (Danovaro, 2010). The aminopeptidase and β-glucosidase activity was converted into C degradation rates, using 72 μg of C per μmole of degraded substrate as conversion factor and the turnover rates of the whole protein and carbohydrate pools were calculated as the ratios of the daily C degradation rates and the whole protein and carbohydrate C contents in the sediment (Pusceddu et al., 2014).

### 5.2.7 Statistical analyses

In order to assess differences in the investigated variables among experimental plots in the different sampling periods (May/July/September 2018 and February 2019), distance-based permutational analysis of variance (PERMANOVA) was used. When significant differences were encountered ($p<0.05$), a pair-wise test was also carried out. The analyses were carried out using 9999 permutations with Monte Carlo simulation and unrestricted permutation of raw data. Each statistical analysis was carried out by using the PRIMER v.6.1.12 software.

## 5.3 Results

### 5.3.1 Shoot density and leaf biomass

Transplanted seagrasses were still present in February 2019 (50.3 ± 20 shoots $m^{-2}$), after the severe storm events occurred during winter. In all periods, shoot density of transplanted seagrasses was significantly lower compared to the density of the donor meadow, except for September 2018 (Fig. 4a and Table 1). In September 2018, transplanted seagrasses showed the highest density (619.7 ± 271.4 shoots $m^{-2}$), comparable to the values observed in the donor meadow (874.1 ± 134.4 shoots $m^{-2}$). A similar pattern was observed also for leaf biomass which was significantly lower in the transplanted seagrasses compared to that observed in the donor seagrass meadows, with the exception of the sampling at the end of summer (100.5 ± 47.7 µgC $m^{-2}$ of transplanted seagrass vs. 282.9 ± 131.5 µgC $m^{-2}$ in the donor meadow) (Fig. 4b and Table 1).

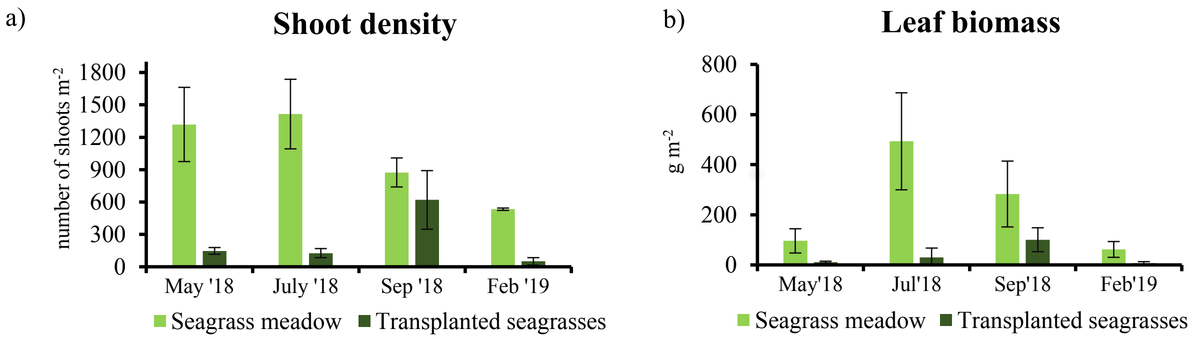
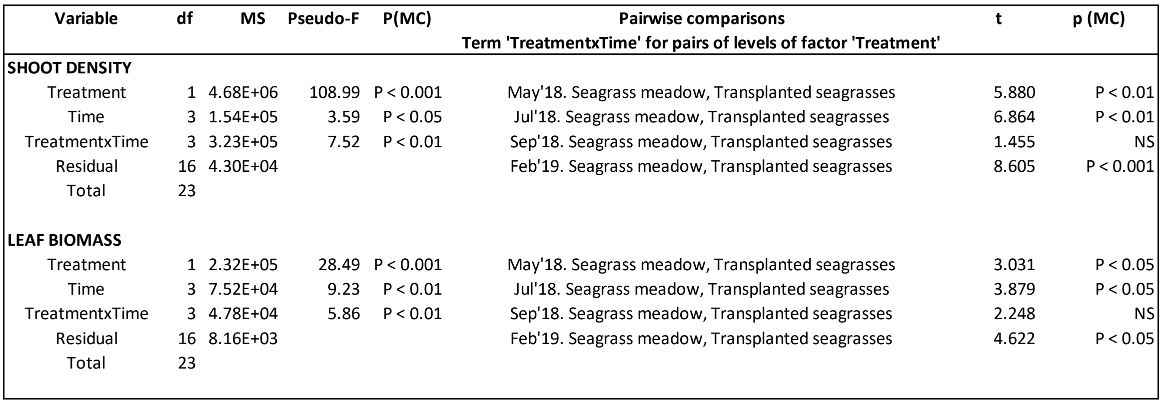


**Figure 4. a) Shoot density in the experimental plots. b) Leaf biomass of the shoots collected in the different experimental plots. In both graphs, error bars indicate the standard deviation from the mean value.**



| Variable | df | MS | Pseudo-F | P(MC) | Pairwise comparisons<br>Term 'TreatmentxTime' for pairs of levels of factor 'Treatment' | t | p (MC) |
|---|---|---|---|---|---|---|---|
| **SHOOT DENSITY** | | | | | | | |
| Treatment | 1 | 4.68E+06 | 108.99 | P < 0.001 | May'18. Seagrass meadow, Transplanted seagrasses | 5.880 | P < 0.01 |
| Time | 3 | 1.54E+05 | 3.59 | P < 0.05 | Jul'18. Seagrass meadow, Transplanted seagrasses | 6.864 | P < 0.01 |
| TreatmentxTime | 3 | 3.23E+05 | 7.52 | P < 0.01 | Sep'18. Seagrass meadow, Transplanted seagrasses | 1.455 | NS |
| Residual | 16 | 4.30E+04 | | | Feb'19. Seagrass meadow, Transplanted seagrasses | 8.605 | P < 0.001 |
| Total | 23 | | | | | | |
| **LEAF BIOMASS** | | | | | | | |
| Treatment | 1 | 2.32E+05 | 28.49 | P < 0.001 | May'18. Seagrass meadow, Transplanted seagrasses | 3.031 | P < 0.05 |
| Time | 3 | 7.52E+04 | 9.23 | P < 0.01 | Jul'18. Seagrass meadow, Transplanted seagrasses | 3.879 | P < 0.05 |
| TreatmentxTime | 3 | 4.78E+04 | 5.86 | P < 0.01 | Sep'18. Seagrass meadow, Transplanted seagrasses | 2.248 | NS |
| Residual | 16 | 8.16E+03 | | | Feb'19. Seagrass meadow, Transplanted seagrasses | 4.622 | P < 0.05 |
| Total | 23 | | | | | | |

**Table 1. PERMANOVA and pair-wise tests for testing differences in the shoot density and leaf biomass between transplanted seagrasses and the donor meadow. Fixed levels of contrast include seagrass meadow and transplanted seagrasses (n = 3 replicated plots). df, degrees of freedom; MS, mean square; P(MC), probability level after Monte Carlo tests; Pseudo-F, statistic F; t, statistic t for pairwise comparisons. NS= not significant.**

### 5.3.2 Biochemical composition of organic matter in the sediments

Biopolymeric C concentrations (range: 0.9 ± 0.1 - 4.3 ± 0.4 mgC $g^{-1}$ Fig. 5a) displayed significant temporal variability both in unvegetated (bare) and vegetated sediments. No significant differences were found between BPC concentrations in unvegetated sediments and in the sediments surrounding both transplanted and donor seagrass, with some exceptions (Table 2). In May 2018, BPC concentrations were significantly lower ($p<0.01$, Table 2) in sediments surrounding transplanted seagrasses than in sediments surrounding donor seagrasses. In September 2018, a significant difference ($p<0.01$, Table 2) was observed between BPC concentrations in unvegetated sediments and in sediments surrounding donor seagrasses.

The values of the protein to carbohydrate ratio (used as a proxy of organic matter quality) ranged from 0.2 ± 0.1 to 1.8 ± 0.01 (Fig. 5b). The sediments surrounding transplanted seagrasses displayed, except for July 2018, a significantly higher quality of organic matter compared to that of the unvegetated sediments (Fig. 5b and Table 2). In May and September 2018, the quality of organic matter was significantly higher in sediments surrounding transplanted seagrasses compared to that determined in sediments surrounding the donor seagrass ($p<0.001$, Table 2) and vice versa in July 2018.

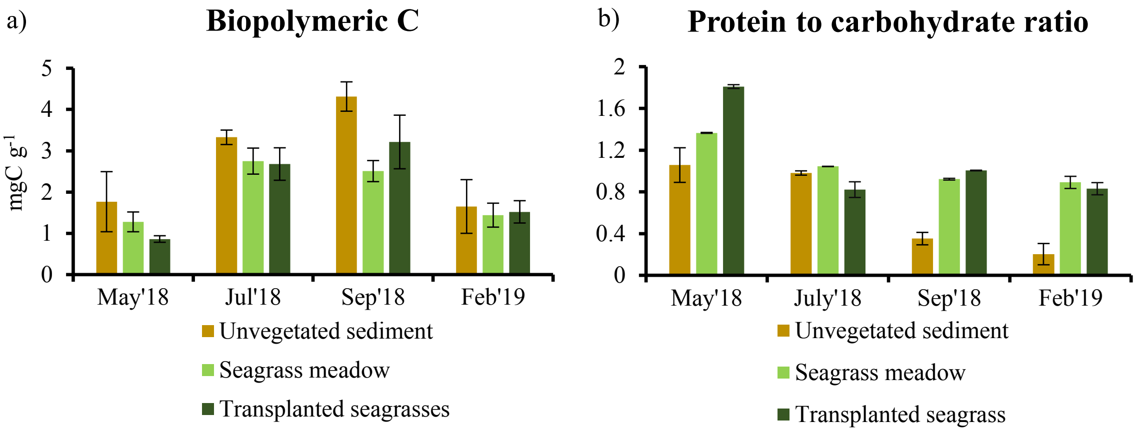


**Figure 5. a) Temporal variability of the biopolymeric C concentrations in the sediments of the different experimental plots. b) Temporal variability of the protein to carbohydrate ratio in sediments of the different experimental plots. Mean values and standard deviations are reported.**

### 5.3.3 Extracellular enzymatic activities and turnover of organic matter

Aminopeptidase activity varied between showed 12 ± 0.8 and 74.4 ± 16.5 nmol g$^{-1}$ h$^{-1}$, whereas β-glucosidase activity ranged from 0.2 ± 0.1 to 8.1 ± 3.1 nmol g$^{-1}$ h$^{-1}$ (Fig. 6). In July 2018, aminopeptidase activity was significantly lower in the donor meadow compared to transplanted seagrass, whereas in all the other periods no significant differences were observed among the different plots investigated (including bare sediments; Table 2). β-glucosidase activity displayed significant temporal changes in all the different plots, but not significant changes were observed among plots with different treatments (Table 2).

In May and September 2018, the turnover rate of carbohydrate pools was significantly faster in the sediments of both transplanted seagrass and donor meadow compared to that observed in unvegetated sediments (Fig. 7; Table 2). In February 2019, the turnover rate of carbohydrate pools was faster in sediments surrounding transplanted seagrasses compared to the values observed both in unvegetated sediments and in sediment surrounding the donor meadow (Table 2).

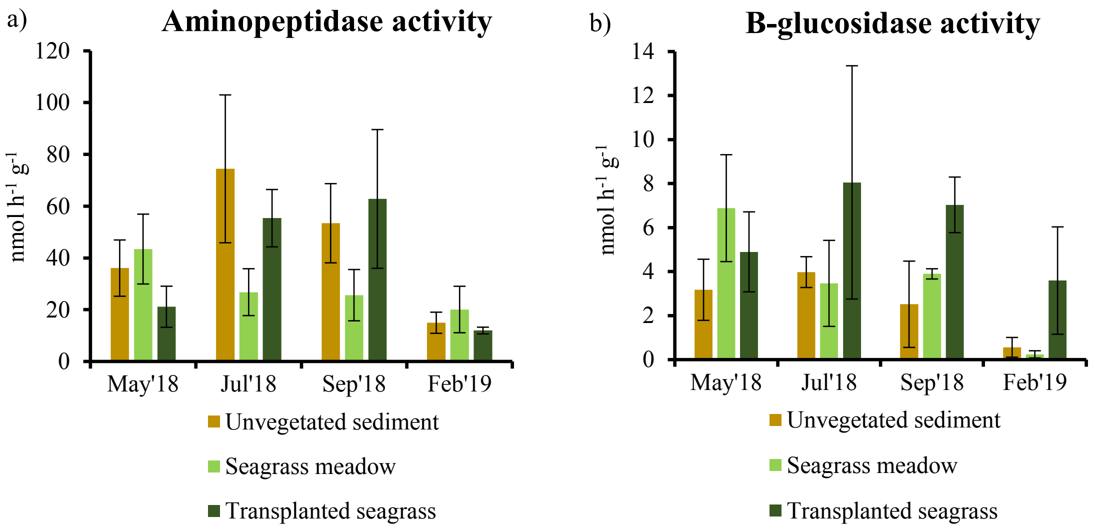


**Figure 6. a) Temporal variability of extracellular aminopeptidase activity. b) Temporal variability of β-glucosidase activity. Mean values and standard deviations are reported.**

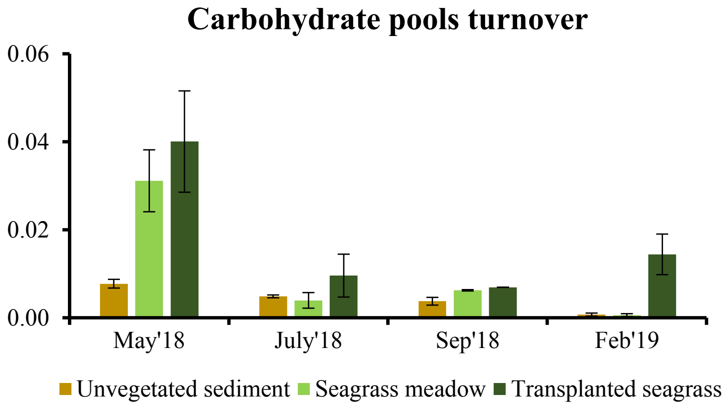


**Figure 7. Temporal variability of the turnover rates (expressed as $d^{-1}$) of carbohydrate pools. Mean values and standard deviations are reported.**

| Variable | df | MS | Pseudo-F | P(MC) | Pairwise comparisons | t | p (MC) |
|---|---|---|---|---|---|---|---|
| | | | | | Term 'TreatmentxTime' for pairs of levels of factor 'Treatment' | | |
| **BIOPOLYMERIC CARBON** | | | | | | | |
| Treatment | 2 | 1.68 | 9.26 | P < 0.001 | May'18. Unvegetated sediment, Seagrass meadow | 0.832 | NS |
| Time | 3 | 9.44 | 51.97 | P < 0.001 | May'18. Unvegetated sediment, Transplanted seagrasses | 2.139 | NS |
| TreatmentxTime | 6 | 0.83 | 4.56 | P < 0.01 | May'18. Seagrass meadow, Transplanted seagrasses | 4.822 | P < 0.01 |
| Residual | 24 | 0.18 | | | Jul'18. Unvegetated sediment, Seagrass meadow | 0.462 | NS |
| Total | 35 | | | | Jul'18. Unvegetated sediment, Transplanted seagrasses | 2.607 | NS |
| | | | | | Jul'18. Seagrass meadow, Transplanted seagrasses | 2.148 | NS |
| | | | | | Sep'18. Unvegetated sediment, Seagrass meadow | 8.151 | P < 0.01 |
| | | | | | Sep'18. Unvegetated sediment, Transplanted seagrasses | 2.576 | NS |
| | | | | | Sep'18. Seagrass meadow, Transplanted seagrasses | 2.245 | NS |
| | | | | | Feb'19. Unvegetated sediment, Seagrass meadow | 0.321 | NS |
| | | | | | Feb'19. Unvegetated sediment, Transplanted seagrasses | 0.321 | NS |
| | | | | | Feb'19. Seagrass meadow, Transplanted seagrasses | Negative | NS |
| **PROTEIN TO CARBOHYDRATES RATIO** | | | | | | | |
| Treatment | 2 | 0.81 | 177.83 | P < 0.001 | May'18. Unvegetated sediment, Seagrass meadow | 3.219 | P < 0.05 |
| Time | 3 | 0.99 | 218.16 | P < 0.001 | May'18. Unvegetated sediment, Transplanted seagrasses | 7.809 | P < 0.01 |
| TreatmentxTime | 6 | 0.17 | 37.25 | P < 0.001 | May'18. Seagrass meadow, Transplanted seagrasses | 41.380 | P < 0.001 |
| Residual | 24 | 0.00 | | | Jul'18. Unvegetated sediment, Seagrass meadow | 5.333 | P < 0.01 |
| Total | 35 | | | | Jul'18. Unvegetated sediment, Transplanted seagrasses | 3.540 | P < 0.05 |
| | | | | | Jul'18. Seagrass meadow, Transplanted seagrasses | 5.132 | P < 0.01 |
| | | | | | Sep'18. Unvegetated sediment, Seagrass meadow | 16.394 | P < 0.001 |
| | | | | | Sep'18. Unvegetated sediment, Transplanted seagrasses | 18.945 | P < 0.001 |
| | | | | | Sep'18. Seagrass meadow, Transplanted seagrasses | 16.080 | P < 0.001 |
| | | | | | Feb'19. Unvegetated sediment, Seagrass meadow | 10.153 | P < 0.001 |
| | | | | | Feb'19. Unvegetated sediment, Transplanted seagrasses | 10.153 | P < 0.001 |
| | | | | | Feb'19. Seagrass meadow, Transplanted seagrasses | Negative | NS |
| **AMINOPEPTIDASE ACTIVITY** | | | | | | | |
| Treatment | 2 | 746.49 | 3.54 | P < 0.05 | May'18. Unvegetated sediment, Seagrass meadow | 0.735 | NS |
| Time | 3 | 2409.30 | 11.41 | P < 0.001 | May'18. Unvegetated sediment, Transplanted seagrasses | 1.921 | NS |
| TreatmentxTime | 6 | 845.38 | 4.00 | P < 0.01 | May'18. Seagrass meadow, Transplanted seagrasses | 2.465 | NS |
| Residual | 24 | 211.10 | | | Jul'18. Unvegetated sediment, Seagrass meadow | 2.754 | NS |
| Total | 35 | | | | Jul'18. Unvegetated sediment, Transplanted seagrasses | 1.078 | NS |
| | | | | | Jul'18. Seagrass meadow, Transplanted seagrasses | 3.458 | P < 0.05 |
| | | | | | Sep'18. Unvegetated sediment, Seagrass meadow | 2.640 | NS |
| | | | | | Sep'18. Unvegetated sediment, Transplanted seagrasses | 0.527 | NS |
| | | | | | Sep'18. Seagrass meadow, Transplanted seagrasses | 2.253 | NS |
| | | | | | Feb'19. Unvegetated sediment, Seagrass meadow | 0.899 | NS |
| | | | | | Feb'19. Unvegetated sediment, Transplanted seagrasses | 1.217 | NS |
| | | | | | Feb'19. Seagrass meadow, Transplanted seagrasses | 1.553 | NS |
| **B-GLUCOSIDASE ACTIVITY** | | | | | | | |
| Treatment | 2 | 41.53 | 9.03 | P < 0.01 | | | |
| Time | 3 | 17.80 | 3.87 | NS | | | |
| TreatmentxTime | 6 | 9.37 | 2.04 | NS | | | |
| Residual | 24 | 4.60 | | | | | |
| Total | 35 | | | | | | |
| **CARBOHYDRATE POOLS TURNOVER** | | | | | | | |
| Treatment | 2 | 5.92E-04 | 28.777 | P < 0.001 | May'18. Unvegetated sediment, Seagrass meadow | 5.699 | P < 0.01 |
| Time | 3 | 9.36E-04 | 45.484 | P < 0.001 | May'18. Unvegetated sediment, Transplanted seagrasses | 4.841 | P < 0.01 |
| TreatmentxTime | 6 | 1.78E-04 | 8.6334 | P < 0.001 | May'18. Seagrass meadow, Transplanted seagrasses | 1.145 | NS |
| Residual | 24 | 2.06E-05 | | | Jul'18. Unvegetated sediment, Seagrass meadow | 0.878 | NS |
| Total | 35 | | | | Jul'18. Unvegetated sediment, Transplanted seagrasses | 1.671 | NS |
| | | | | | Jul'18. Seagrass meadow, Transplanted seagrasses | 1.879 | NS |
| | | | | | Sep'18. Unvegetated sediment, Seagrass meadow | 4.829 | P < 0.01 |
| | | | | | Sep'18. Unvegetated sediment, Transplanted seagrasses | 6.242 | P < 0.01 |
| | | | | | Sep'18. Seagrass meadow, Transplanted seagrasses | 7.650 | P < 0.01 |
| | | | | | Feb'19. Unvegetated sediment, Seagrass meadow | 0.396 | NS |
| | | | | | Feb'19. Unvegetated sediment, Transplanted seagrasses | 4.596 | P < 0.01 |
| | | | | | Feb'19. Seagrass meadow, Transplanted seagrasses | 4.629 | P < 0.01 |

**Table 2. Output of the PERMANOVA and pair-wise analysis for testing differences in the quantity (as BPC concentrations), quality (as protein to carbohydrate ratio), extracellular enzymatic activities and turnover rates of carbohydrate pools in the sediments surrounding transplanted seagrass, donor meadow and bare sediments at the different time intervals. Fixed levels of contrast include unvegetated sediments, sediments surrounding existing seagrasses, and sediments surrounding transplanted seagrasses (for all the treatments, n = 3 replicated plots). Analyses were carried out using unrestricted permutations of the raw data and 9999 permutations. df, degrees of freedom; MS, mean square; P(MC), probability level after Monte Carlo tests; Pseudo-F, statistic F; t, statistic t for pairwise comparisons. NS= not significant.**

## 5.4 Discussion

The success of seagrass restoration depends on different factors including the suitability of the transplantation site, the sampling period and the methodology used (Calumpong & Fonseca, 2001; Short et al., 2002; Campbell & Paling, 2003; Bos & van Katwijk, 2007; Park & Lee, 2007; Bastyan & Cambridge, 2008; Hovey et al., 2012; Suykerbuyk et al., 2012). Seagrass transplantation should be conducted by considering also that the seasonal biological cycle varies among the species (Calumpong & Fonseca, 2001; Park & Lee, 2007). In the present study, the transplantation experiment of *C. nodosa* was conducted in an area as far as possible from the impact of thousands of bathers present in the Gabicce Mare during summer season (e.g. 604070 touristic presences between May and September in 2015 in the whole municipality of Gabicce Mare, http://statistica.turismo.marche.it/DatiTurismo) which could potentially damage experimental plots and transplanted seagrasses. In this study, I conducted the transplantation experiment during the spring season, after the winter period that is characterised by heavy storms that can compromise the maintenance of the transplanted seagrasses. This is expected to favour the settlement, maintenance and the vegetative growth of the underground rhizome of the transplanted seagrasses. In this way, transplanted plants will have a longer period for recovery from physical stress (Park & Lee, 2007). Moreover, in temperate ecosystems, the spring period has been identified to be more suitable for the success of seagrass transplantation (Paling et al., 2009).

In this experiment, seagrasses were present also after the winter season, confirming that the used technique is suitable for transplanting seagrasses in the study area, despite seagrass density was lower compared to that observed in the donor meadow. Plants in well-established substrates show a higher resistance to hydraulic forces compared to transplants that are exposed to higher erosive forces (Christensen & Tackney, 1983). In general, flowing water, buoyant forces, and gravity act synergistically with hydrodynamic forces upon plants and their anchoring system (Li et al., 2014). Immediately after the transplantation, waves and currents could dislodge poorly anchored plants that did not have the enough time to establish and expand their roots (van Katwijk & Hermus, 2000; Bos & van Katwijk, 2007). In this study, the use of the descripted technique and the choice to carry out the transplant during spring favoured the settlement of the plants and the growth of the roots.

In September 2018, after five months from the beginning of the experiments, shoot density and biomass of transplanted *C. nodosa* were not significantly different to values of the donor meadow. Moreover, in the same month, the shoot biomass reached about the 70% of the density observed in the control sites and a biomass equal to about the 35% of that assessed for existing seagrasses. The lack of significant differences between transplanted *C. nodosa* and the original meadow has been also reported in the lagoon of Venice after 7 and 17 months by using a different technique (i.e. the sod technique (Curiel et al., 2003; Curiel et al., 2005). Considering the short duration of this monitoring, these findings suggest that transplanted *C. nodosa* can reach density and biomass similar to those reported in original meadows only after a certain time interval that is needed to anchor its roots to the new substrate and to a vegetative expansion of the underground rhizome.

The “Society for Ecological Restoration” has established that successful ecological restoration actions should also allow the recovery of ecosystem function and services (SER, 2004). Many of the restoration studies reported in literature do not consider the recovery of ecosystem function and services. Rather, they consider the expansion of propagules, the survival of transplanted plants and the cover percentage as success criteria of restoration (Bayraktarov et al., 2016).

In this study, I investigated the effects of the presence of transplanted seagrasses on the trophic state and organic matter degradation rates of the surrounding sediments. In the different experimental plots, I found high BPC concentrations typically encountered in highly productive coastal ecosystems (Dell’Anno et al., 2002; Pusceddu et al., 2009; Bianchelli et al., 2016). BPC concentrations were characterized by a strong temporal pattern, and the concentrations assessed in unvegetated sediments were, generally, not significantly different than those encountered in vegetated sediments. Such an effect could have been caused by the limited distance between vegetated and unvegetated sediments along with the presence of breakwaters which act as a physical trap for the particles, thus causing a high availability of organic matter for benthic heterotrophic consumers in the whole area investigated.

Conversely, the quality of organic matter, in terms of the protein to carbohydrate ratio was significantly higher in sediments surrounding transplanted *C. nodosa* and in sediments collected in the donor meadow than in unvegetated ones. Such trophic characteristics allow us to hypothesize, according to the optimal foraging theory (MacArthur and Pianka, 1966), that benthic detritus feeders inhabiting the sediments surrounding transplanted seagrass and the donor meadow may have relevant energetic/metabolic advantages compared to those inhabiting bare sediments.

Aminopeptidase and β-glucosidase activities were generally not statistically different between unvegetated and vegetated sediments, suggesting a minor effect of the presence of seagrasses on organic matter degradation processes. However, turnover rates of the carbohydrate pools were significantly higher in vegetated sediments (including transplanted ones) than in unvegetated sediments. Since carbohydrates represent the less readily fraction of organic matter for benthic heterotrophic consumers and are rich-carbon organic compounds, their faster degradation in vegetated sediments can have important consequences for benthic trophodynamic and biogeochemical processes (i.e. C cycle). Thus, *C. nodosa* transplantation, in addition to the enhancement of the quality of organic matter in surrounding sediments, can potentially improve benthic ecosystem performance. Ecological benefits have been also highlighted by monitoring activities carried out to follow the natural recovery of *Zostera marina* meadow in the Baltic Sea (Jankowska et al., 2018). In particular, an increase of food items utilized by meiofauna copepods and macrofaunal grazers have been observed along with an increase of feeding efficiency of omnivores.

In conclusion, despite longer time series (at least 5 years, Calumpong & Fonseca, 2001) are required before drawing definitive conclusions, the transplantation experiment of *C. nodosa* at Gabicce Mare was rather successful, not only because transplanted seagrasses persisted also after harsh winter conditions, but also because they can improve ecosystem functioning, thus overall contributing to the success of ecological restoration efforts.

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# CHAPTER 6: THE RESTORATION OF SHALLOW CORALLIGENOUS ECOSYSTEMS: ASSESSMENT OF THE HEALTH STATUS OF TRANSPLANTED GORGONIANS IN THE PORTOFINO MARINE PROTECTED AREA (NW MEDITERRANEAN SEA)

## 6.1 Introduction

The coralligenous habitat is defined by the Regional Activity Center for Specially Protected Areas as a biogenic hard-substrate formed mainly by the accumulation of calcareous encrusting algae growing under dim light conditions (UNEP-RAC/SPA, 2003). The coralligenous framework thrives exclusively in Mediterranean waters at water depths from 20 to120 m (Ballesteros, 2006).

This habitat harbours approximately 20% of the marine Mediterranean biodiversity and is protected under the Habitat Directive as “1170 Reefs” (Council Directive 92/43/EEC, 1992) (Ballesteros, 2006). In general, the species inhabiting coralligenous assemblages (e.g. *Corallium rubrum*, *Scalarispongia scalaris*, *Chondrosia reniformis*) show low growth rates (between 0.1 and 4 cm per year) (Cocito et al., 1998; Coma et al., 1998; Garrabou & Harmelin, 2002; Linares et al., 2010; Teixido et al., 2011; Sartoretto & Francour, 2012; Munari et al., 2013), low recruitment events and long lifespan that can vary from decades to centuries (Teixido et al., 2011). These characteristics made the coralligenous particularly vulnerable to anthropogenic impacts and global climate changes. In particular, a plethora of threats have be identified including fishing activities and anchoring, scuba diving activities (Coma et al., 2004; Betti et al., 2019), mucilage phenomena (Giuliani et al., 2005), ocean acidification (especially for calcifying organisms) (Cerrano et al., 2013), algal blooms, natural events like strong storms, temperature anomalies related to global warming (Ballesteros, 2006; Garrabou et al., 2009) and invasive species like the macroalgae *Caulerpa cylindracea* and *Womersleyella setacea* (Ballesteros, 2006; Piazzi & Balata, 2009).

Among the anthozoans, several species of gorgonians inhabit this habitat. In the last two decades, these animals experienced several mass-mortality events caused by sea temperature rises (Cerrano et al., 2000; Perez et al., 2000; Garrabou et al., 2009). These phenomena are often linked to climate anomalies and alteration of water circulation (Cerrano & Bavestrello, 2008). For example, during the mass mortality of late summer 2003, up to 80% of sea fans in the Gulf of Genoa were affected by the event, showing at least 10% of colony surface with necrosis signs (Garrabou et al., 2009). These animals are considered ecosystem engineers (sensu Jones et al., 1994) being able to i) build a greater variety of micro-habitats and refuges for invertebrates, ii) decrease current flow but increase turbulent flow, enhancing the probability that suspension feeders can catch planktonic preys (Shashar et al., 1995), iii) increase the delivery of pelagic larvae to benthic substrates (Walters et al., 1997), iv) indirectly, control the coralligenous dynamics affecting the community structure in terms of sessile and vagile fauna and meiofauna (Cerrano et al., 2010; Ponti et al., 2016; Valisano et al., 2016), v) increase the flux rate of metabolically important substances as result of a thinner diffusive boundary-layer (Hearn et al., 2001). Gorgonians are among the most vulnerable organisms of the coralligenous assemblages. The main direct anthropogenic impact is exerted by fishing, followed by anchorage and diving (Bavestrello et al., 1997). The removal and/or damage of these organisms following anthropogenic impacts severely modify community structure with cascade effects on ecosystem functioning (Cebrian et al., 2012). Since recovery of gorgonians following damages are slow, several efforts have been made to find suitable transplantation techniques (Linares et al., 2008a; Fava et al., 2009) for gorgonian restoration. For example, to study usefulness methods, Linares et al. (2008a), testing different transplanting techniques for restoring a population of *Paramuricea clavata* in Medes Islands, reported a survival rate up to 80% (Linares et al., 2008b). Another transplantation experiment was conducted using *P. clavata*, *Eunicella cavolini*, *Eunicella singularis*, and *Eunicella verrucosa*: the fragments survived in the short term (mean survival rate of 98%), but after three months from transplantation a climate anomaly determined the death of the organisms (Portofino, Ligurian Sea) (Fava et al., 2009).

**Figure 1. A specimen f *E. singularis* inside the MPA of Portofino.**

In this work, I focused the attention on the gorgonian *E. singularis* (Fig. 1). This is an endemic species of the Mediterranean Sea and is the only gorgonian of this basin that can host endosymbiotic zooxanthellae (Carpine & Grasshoff, 1975). They belong to the *Symbiodinium* clade (Forcioli et al., 2011). *E. singularis* thrives principally on horizontal and sub-horizontal substrates (Weinberg & Weinberg, 1979) at water depth from 5 to 50 m with the highest population densities between 15 and 30 m (Linares et al., 2008b). It has an elongation rate that ranges from 2.2 to 4.52 cm $yr^{-1}$ (Weinberg & Weinberg, 1979; Munari et al., 2013). The maximum density that the colonies of *E. singularis* can reach is of 84 colonies $m^{-2}$ (Weinberg & Weinberg, 1979). More recent assessments of the mean density of this species range from 13 ± 6.8 colonies $m^{-2}$ for Tyrrhenian populations (Munari et al., 2013) and 37 colonies $m^{-2}$ for the populations of Cape de Creus (Northwestern Mediterranean Sea) (Gori et al., 2011). *E. singularis* is classified as a “near threatened species” because of the decline comprised between

20% and 90% observed in shallow populations (Kipson et al., 2015). Once degraded, populations of this gorgonian are not able to support ecosystem functioning.

In this study I carried out an experiment to assess the efficiency of transplantation actions for the restoration of gorgonians by using fragments of *E. singularis* collected from donor colonies of the Marine Protected Area of Portofino and transplanting them within the same MPA. In particular, the aims of this study, which has been carried out in the frame of the European project MERCES (Marine Ecosystem Restoration in Changing Europeans Seas, www.merces-project.eu), were: 1) to assess the survival capacity of transplanted *E. singularis* and its health status based on the analyses of the associated microbiome and of the symbiotic zooxanthellae and 2) to investigate the effects of transplanted gorgonians on the trophic status and key-ecological processes (i.e. organic matter degradation rates) of the surrounding environment.

## 6.2 Materials and methods

### 6.2.1 Study area and transplantation experiment

The study area is located inside the MPA of Portofino (Ligurian Sea, Northwestern Mediterranean Sea). This area is subject to the typical cyclonic circulation of the Ligurian Sea (Astraldi & Manzella, 1983). The chosen source population was that inhabiting the cliff at 28-30 m depth in the diving site called “Punta dell’Indiano” located in the Western part of the AMP (Fig. 2). Instead, the experimental area was located at “Punta del Faro”. Inside this area, two experimental plots were done, one in the “West site” and one in the “East site”, both on a horizontal substrate at depth of 28 m.

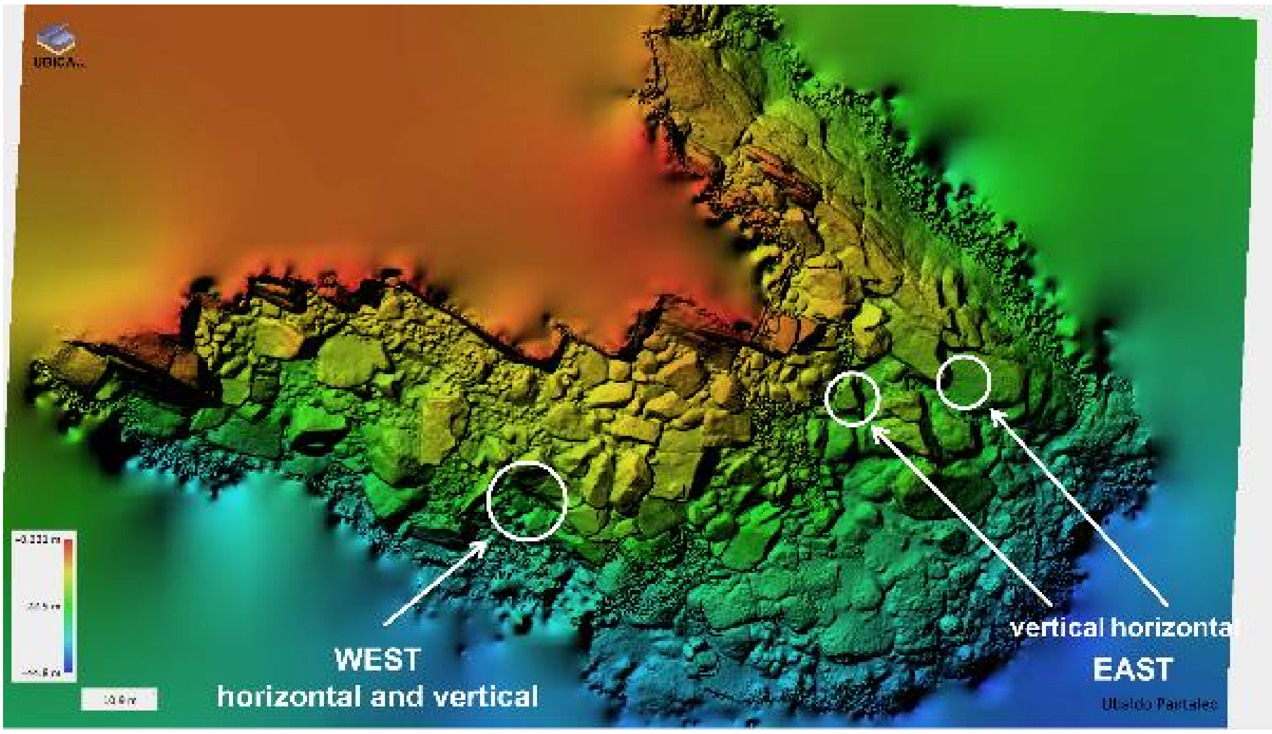


**Figure 2. A 3-D reconstruction of the site chosen for carry out the transplantation experiments (Punta del Faro). On the western side, there is a diving site highly frequented by divers, while the eastern side is less frequented. Both the transplantation areas are located on a horizontal plate at 27 m of depth.**

For each site, 15 fragments were transplanted with a density of about 18.8 colonies m$^{-2}$. Due to logistic difficulties, no more replications (in terms of experimental plots) have been performed. After 4, 10 and 16 months from the transplantation (hereafter indicated respectively as t0, t6, t12), fragments of the transplanted gorgonians and samples of the donor colonies were collected. Gorgonian samples were then utilised for the analyses of zooxanthellae and prokaryote abundances and prokaryote diversity and assemblage composition.

At the same sampling intervals, sedimentary particles (hereafter defined sediment samples) were collected at the water-bottom interface (using a syringe with a silicone tube) of three different 10 cm x 10 cm squares positioned near the transplanted gorgonians. Additional sediment samples were collected at ca. 100 m far from the transplanted gorgonians (using the same device and approach) an were used as controls. Once in the laboratory, sediment particles were separated from the seawater by centrifugation and analysed for the determination of organic matter quantity and biochemical composition, extracellular enzymatic activities (as a proxy of organic matter degradation rates) and prokaryotic abundance. Due to harsh weather conditions and poor visibility, in October 2017 (t0) it was not possible to collect sediment and gorgonians samples from the East site.

### 6.2.2 Zooxanthellae abundance and classification

Before the analysis, each fragment of gorgonian was gently defrosted, weighted and measured for length, width and height in sterile conditions. Then, each fragment was crushed in a sterile mortar and pestle (Luna et al., 2007) adding 5 mL of pre-filtered and autoclaved seawater. The samples were then centrifuged (4000×g, for 10 minutes) to separate the algae from the host tissue. The extracted liquid was discarded, and the pellet was re-suspended with pre-filtered and autoclaved seawater (Cervino et al., 2001). Aliquots of the samples were filtered through 2.0 µm polycarbonate filters and, then, filters were mounted on glass slides. Then, filters were mounted on glass slides. Zooxanthellae were counted under a Zeiss Axioplan epifluorescence microscope (Carl Zeiss Inc., Jena, Germany; ×400 and ×1,000) under blue-light excitation (Nguyen-Kim et al., 2015) and the number of cells were normalized to the gorgonians' tissue area (Danovaro et al., 2008). Based on the auto fluorescence and the structure of the cells, the zooxanthellae were classified as a) healthy (H, brown/bright yellow colour, intact zooxanthellae); b) pale (P, pale yellow colour, vacuolated, partially degraded zooxanthellae); transparent (T, lacking pigmentations, mostly empty zooxanthellae) (Mise & Hidaka, 2003; Danovaro et al., 2008).

### 6.2.3 Prokaryotic abundance in gorgonian samples

Prokaryotic abundances were determined at each time interval to ensure that changes in polyps' behaviour and eventual mortality were not ascribed to the outbreak of pathogenic infections (Danovaro et al., 2008; Cerrano et al., 2013). Before the analysis, each fragment of gorgonian was gently defrosted, weighted and measured for length, width and height in sterile conditions. Then, each fragment was rinsed 3 times with pre-filtered and autoclaved seawater and the gorgonian was crushed in a sterile mortar and pestle (Luna et al., 2007) adding 5 mL of pre-filtered and autoclaved seawater. Aliquots of the suspension were collected for assessing the total prokaryotic abundance. This was determined filtering the samples through a 0.2 µm pore-size filter (Whatman® 110656 Nuclepore™ Track-Etched Membranes) and staining the filters for 20 minutes in the dark with SYBR® Green I nucleic acid gel stain (Sigma-Aldrich, CAS Number 163795-75-3) diluted 1:40 (Luna et al., 2007). Filters were then washed three times with 3 mL of prefiltered and sterile Milli-Q water and mounted on a microscopic glass-slide with 20µL of antifade solution (50% phosphate buffer, pH 7.8, 50% glycerol, 0.5% ascorbic acid). Prokaryotic abundances were determined by epifluorescence microscopy (Zeiss Axioskop 2 MOT, Carl Zeiss Inc., Jena, Germany). For each slide, at least 20 microscope fields were observed and at least 200 prokaryotes and viruses were counted per filter (Danovaro, 2010).

### 6.2.4 Analyses of gorgonians' associated microbiome

Fragments of gorgonians were cut from the donor and transplanted colonies using clean stainless-steel scissors. The fragments were maintained in sterile test-tubes and immediately frozen at -20°C once arrived on boat. Fragments were maintained at -27°C till molecular analyses. Total genomic DNA content was extracted from the replicated gorgonians' samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturers' protocols. DNA extracts were sent to LGC Genomics GmbH (Berlin, Germany) for amplification, library construction, and multiplexed sequencing on an Illumina MiSeq V3 platform (Illumina, San Diego, CA, USA) using Klindworth primers 341-F (CCTACGGGNGGCWGCAG) and 785-R (GACTACHVGGGTATCTAAKCC).

Raw sequences were analyzed through the QIIME2 pipeline (version 2017.12; https://qiime2.org/). Paired-end sequence files were loaded, and sequence pairs merged by means of the VSEARCH plugin (Rognes et al., 2016) using the standard values set by the pipeline. Merged reads were then trimmed to 250 bp and analysed by means of the DADA2 plugin (Callahan et al., 2016), which infers community composition in each sample by partitioning sequences according to the respective error models, thus filtering for erroneous reads and chimeras and resolving minimal variations between prokaryotic taxa. From the OUT (operational taxonomic unit) table obtained from the analysis of each sample we subsampled 1200 sequences, thus obtaining a normalized OTU table. The community composition was analysed at the level of families (D4).

### 6.2.5 Prokaryotic abundance in sediment samples

Total prokaryotic cell counts were performed using the SYBR Green I direct count procedure (Danovaro, 2010). Sediment samples were treated 3 times for 1 minute with a sonicator (Branson Sonifier 2200, 60 W) with the addition of 0.2 µm pre-filtered tetrasodium pyrophosphate solution (final concentration 5mM). After these procedures, samples were treated in the same way of that used for the assessment of the prokaryotic abundance in the gorgonians' tissue. Data of prokaryotic abundance were expressed as number of cells per gram of dry weight sediment (Danovaro, 2010).

### 6.2.6 Biochemical composition of organic matter in the sediments

Protein, carbohydrate and lipid concentrations in the sediments were determined spectrophotometrically, following the protocols detailed in Danovaro (2010), and their sedimentary contents (mg × g dry sediment$^{-1}$) expressed as bovine serum albumin, glucose and tripalmitine equivalents, respectively. Carbohydrate, protein and lipid sedimentary contents were converted into carbon equivalents using the conversion factors of 0.40, 0.49 and 0.75 μgC $\mu g^{-1}$, respectively, and their sum defined as biopolymeric carbon (BPC) (Dell'Anno et al., 2002).

### 6.2.7 Extracellular enzymatic activities in sediments

Extracellular aminopeptidase and β-glucosidase activities were determined in the collected sediments by the analysis of the cleavage rates of their artificial fluorogenic substrates L-leucine-4-methylcoumarinyl-7-amide (Leu-MCA) and 4-methylumbelliferyl MUF-b-D-glucopyranoside (MUF-Glu), respectively (Danovaro, 2010). Briefly, sediment sub-samples were diluted with 0.02-μm pre-filtered seawater. The fluorescence of the samples was measured immediately after the addition of the substrate and after the incubation in the dark at the in-situ temperature for 1 hour. All the assays were carried out in triplicate. At the end of the incubation, the supernatant of sediment samples (obtained following centrifugation at 3000×g, 10 min) were analyzed fluorometrically at 380 nm excitation and 440 nm emission (for Leu-MCA) or at 365 nm excitation and 455 nm emission (for MUF-Glu). The fluorescence was converted into C degradation rates using standard curves of 7-amino-4-methylcoumarin for Leu-MCA and of 4-methylumbelliferone for MUF-Glu. The aminopeptidase and β-glucosidase activity was converted into C degradation rates, using 72 μg of C per μmole of degraded substrate as conversion factor and the turnover rates of the whole protein and carbohydrate pools were calculated as the ratios of the daily C degradation rates and the whole protein and carbohydrate C contents in the sediment (Pusceddu et al., 2014).

### 6.2.8 Statistical analyses

To identify differences in the investigated variables between wild and transplanted colonies at the different sampling interval, distance-based permutational analysis of variance (PERMANOVA) was used. When significant differences were encountered ($p<0.05$) a pair-wise test was carried out. All the analyses were carried out using 9999 permutations with Monte Carlo simulation, considering all the factors as fixed and unrestricted permutation of raw data. To visualize differences in the zooxanthellae assemblage composition among the treatment, the site and the times, bi-plots after a Canonical Analysis of Principal Coordinates (CAP) were also prepared (Anderson and Willis, 2003). For the analysis of the composition of the prokaryotic community, beside PERMANOVA, a Similarity Percentage (SIMPER) analysis were used to identify the taxa contributing to the greatest extent to the observed patterns. Such analysis was performed both at family and OTU level. All analyses were performed using PRIMER v.6.1.12 software.

## 6.3 Results

The density of zooxanthellae in gorgonians' tissue ranged from $1.2\times10^5 \pm 1.2\times10^4$ to $4.5\times10^6 \pm 1.4\times10^6$ algal cells $cm^{-2}$ (Fig. 3a). The density of zooxanthellae displayed significant temporal changes both in the donor and transplanted gorgonians ($p<0.001$). However, no significant differences were found between the density of zooxanthellae in wild colonies and transplanted ones (Table 1). The composition of zooxanthellae assemblage (Fig. 3b) showed temporal changes (Fig. 3c), with a higher number of pale and transparent cells in both the donor and transplanted gorgonians at t12. Significant differences ($p<0.05$) were observed only at t6 between wild and transplanted colonies in the East site.

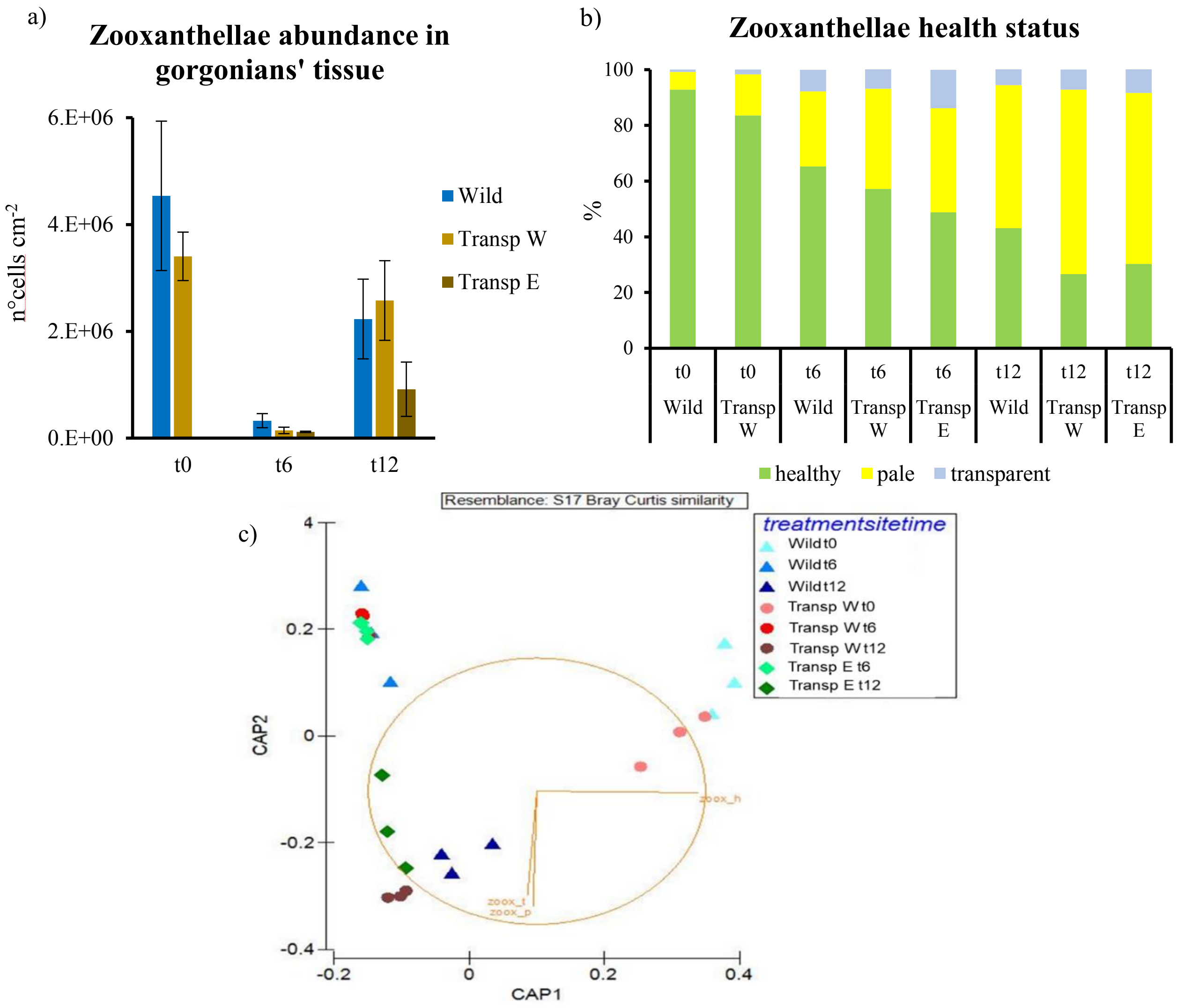


**Figure 3. a) Total abundance of zooxanthellae in the gorgonians' tissue. In this graph, error bars indicate the standard deviation from the mean value. b) Health status of the zooxanthellae hosted in the gorgonians' tissue. c) Variations in the composition of the zooxanthellae communities (biplot after canonical analysis of the principal coordinates, mis-classification error: 29.2%). Wild=wild colonies, Transp W=colonies transplanted in the West site, Transp E=colonies transplanted in the East site. zoox_h= healthy zooxanthellae; zoox_t= transparent zooxanthellae; zoox_p= pale zooxanthellae.**

Prokaryotic abundances in gorgonians' tissue ranged from 1×$10^7$ ± 5.7×$10^6$ to 5.3×$10^8$ ± 2.1×$10^8$ cells $cm^{-2}$ and displayed significant temporal changes (Fig. 4 and Table 1). At t6, the abundance of prokaryotes was significantly higher in transplanted gorgonians compared to the value observed in wild colonies ($p<0.05$, Table 1).

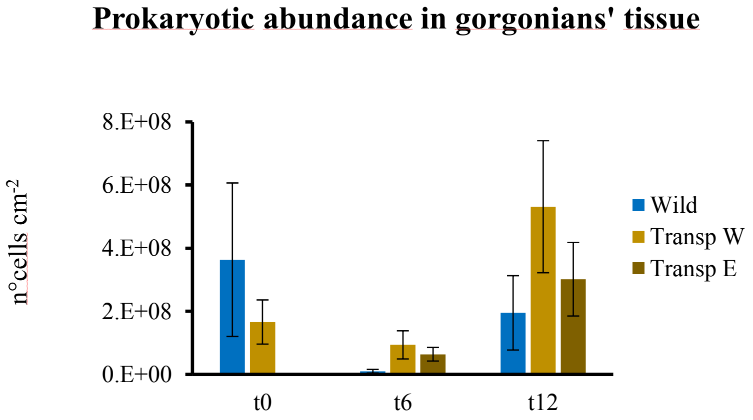


**Figure 4. Total abundance of prokaryotic cells in the gorgonians' tissue. Wild=wild colonies, Transp W=colonies transplanted in the West site, Transp E=colonies transplanted in the East site. Mean and standard deviations are reported.**

| Variable | df | MS | Pseudo-F | P(MC) | Pairwise comparisons<br>Term 'TreatmentxTime' for pairs of levels of factor 'Treatment' | t | p (MC) |
|---|---|---|---|---|---|---|---|
| **Zooxanthellae/$cm^2$ of tissue** | | | | | | | |
| Sitetreatment | 2 | 1.52E+12 | 3.43 | NS | | | |
| Time | 2 | 2.06E+13 | 46.46 | p<0.001 | | | |
| SitetreatmentxTime | 3 | 1.19E+12 | 2.67 | NS | | | |
| Residual | 16 | 4.44E+11 | | | | | |
| Total | 23 | | | | | | |
| **Prokaryotes/$cm^2$ of tissue** | | | | | | | |
| Sitetreatment | 2 | 1.36E+16 | 0.79 | NS | t6. Donor, Transplanted W | 3.216 | p<0.05 |
| Time | 2 | 1.92E+17 | 11.13 | p<0.01 | t6. Donor, Transplanted E | 4.179 | p<0.05 |
| SitetreatmentxTime | 3 | 7.31E+16 | 4.24 | p<0.05 | | | |
| Residual | 16 | 1.72E+16 | | | | | |
| Total | 23 | | | | | | |

**Table 1. PERMANOVA and pair-wise tests (showed only where significant results were obtained) for the zooxanthellae abundance in gorgonians' tissue and prokaryotic abundance in gorgonians' tissue. Fixed levels of contrast include donor colonies and transplanted colonies in the West site (Transplanted W) and in the East site (Transplanted E) (n = 3 replicates). Analyses were carried out using unrestricted permutations of the raw data and 9999 permutations. df, degrees of freedom; MS, mean square; P(MC), probability level after Monte Carlo tests; Pseudo-F, statistic F; t, statistic t for pairwise comparisons. NS= not significant.**

Bacterial OTU richness ranged from 17 ± 4.2 in the wild colonies at t12 to 117.5 ± 3.5 in transplants of the East site at t6 (Fig. 5a). With the exception of t0, wild colonies displayed significantly lower bacterial OTU richness when compared to transplanted colonies ($p<0.01$). Pielou index varied between 0.43 and 0.79 (Fig. 5b) with higher values observed in transplanted colonies at all sampling intervals when compared to the wild colonies.

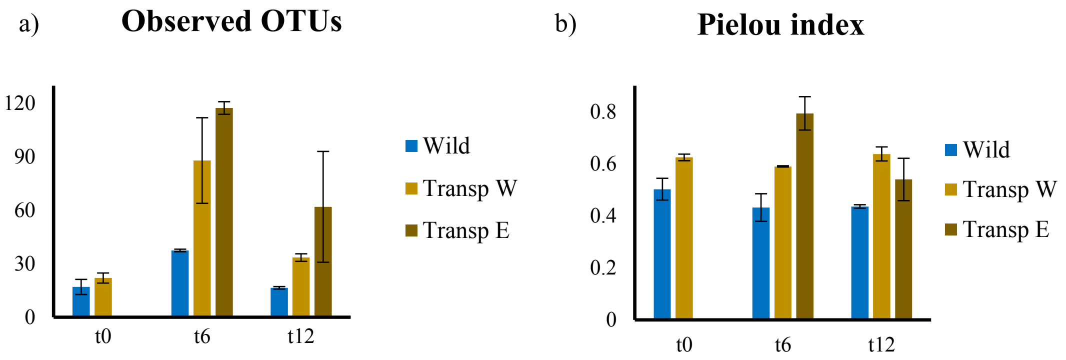


**Figure 5. a) Bacterial OTU number and b) Pielou index in the investigated samples. Wild=wild colony, Transp W=colonies transplanted in the West site, Transp E=colonies transplanted in the East site.**

In all gorgonian samples *Endozoicomonas* was the main bacterial genus. The quantitative importance of this genus (in terms of number of reads) to the bacterial assemblages did not show significant temporal changes in wild colonies, whereas it displayed significant temporal variation in transplanted ones. Transplanted fragments were characterized by a higher number of reads affiliated to the genera *Meiothermus* and *Anoxybacillus* when compared to the wild colonies (Fig. 6).

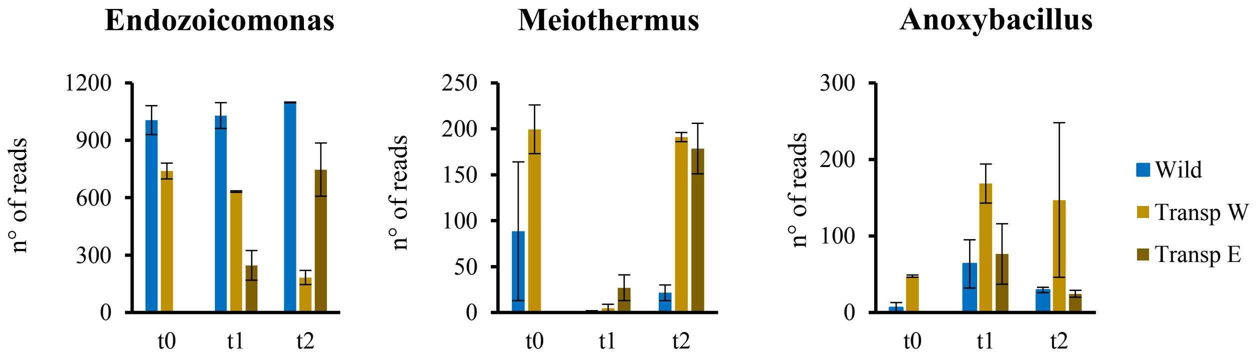


**Figure 6. Main genera of bacteria (in terms of number of reads) associated with gorgonians' tissue of wild and transplanted colonies.**

The microbiomes of gorgonians were mainly represented by the family *Hahellaceae* with values ranging from 12.2 % in transplanted colonies of the West side (r2) at t12 to 91.7 % in wild colonies (r2) at t12 (F. 7). Within this family it was present only one genus, *Endozoicomonas.* In wild colonies, this genus represented an average of 87% of bacterial community, and its presence explained about 69% of the similarities of the microbial assemblages among the wild colonies at all the sampling times. The families *Hahellaceae* and that of *Propionibacteriaceae* were the only shared by all the specimens, even if with a wide variability, from 0.4% in wild colonies (r1) at t0 to 27.8% in in transplanted gorgonians (r1) at t12. The families *Alteromonadaceae* and *Sphingobacteriaceae* and the marine-group BD7-8 (level of order) were found only in in transplanted gorgonians (r1) of the East side at t6 with a contribution to the assemblages, respectively, of 2.4, 4.2 and 6.2%. Bacteria belonging to the family *Bifidobacteriaceae* were found only in transplants of the West side (r1) at t0 (contributing for 2.5%). Bacteria of the family *Mycoplasmataceae* were associated with all the transplanted colonies at t6, with values from 1.3% in transplanted gorgonians of the West side (r2) at t6 to 6.3% in transplanted gorgonians of the East side (r1) at t6. The family *Thermaceae*, that was present with bacteria of the genus *Meiothermus*, was found in all the samples with the exception of wild colonies (r1) at t6 and in transplanted gorgonians of the West side (r1) at t6, with values up to ca. 19% in transplants of the West site (r1) at t0. *Anoxybacillus* of the family *Bacillaceae* represented another relevant genus of the microbiomes contributing for up to 16% of the assemblages in in transplanted gorgonians of the West site (r2) at t6.

SIMPER analysis highlighted an average dissimilarity of the bacterial assemblage composition in wild and transplanted colonies of the West site of ca. 50%, half of which explained by the contribution of *Hahellaceae*, *Thermaceae*, *Rhodospirillaceae*, *Bacillaceae*, *Propionibacteriaceae* and *Rhodobacteraceae*. Similar results were also observed comparing the bacterial assemblage composition of the wild and transplanted colonies of the East side.

PERMANOVA analysis revealed significant temporal changes in the bacterial assemblage composition both in wild and transplanted colonies ($p<0.01$). Conversely, no significant differences were observed between the bacterial assemblage composition between wild and transplanted colonies or between transplanted colonies of the two sites, except for transplanted gorgonians of the East site vs wild colonies at t6 ($p<0.05$).

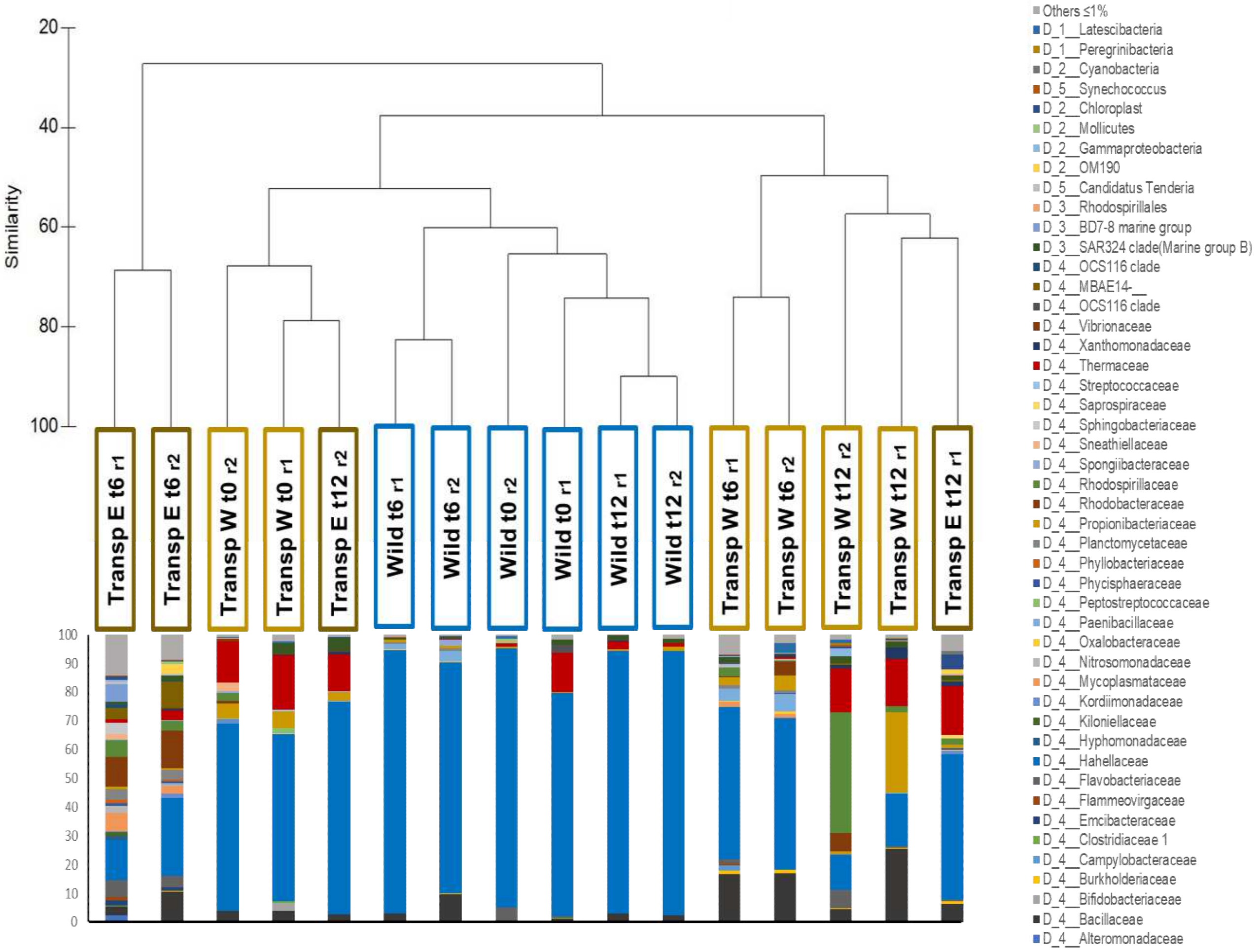


**Figure 7. Taxonomic composition (at the family level on OUT data normalized to 1200 sequences) of the bacterial assemblages in the different gorgonians' samples. the output of cluster analysis is also reported. Wild=wild colony, Transp W=colonies transplanted in the West site, Transp E=colonies transplanted in the East site.**

Biopolymeric C (BPC) concentrations in the sediments were highly variable, ranging from 3.9 ± 1.5 to 11.4 ± 0.6 mgC $g^{-1}$ (Fig. 8a). BPC concentrations in bare sediments and sediments surrounding transplanted gorgonians did not differ significantly with only few exceptions (Table 2). At t0, BPC concentrations in bare sediments of the West site (Bare W) was significant higher ($p<0.05$, Table 2) than those of sediments surrounding transplanted colonies in the West site (Transp W). At t6, significant differences ($p<0.01$, Table 2) were observed between sediments around transplanted gorgonians of the West site and those around transplanted colonies in the East site (Transp E). The values of protein to carbohydrate ratio ranged from 0.2 ± 0.02 to 1.6 ± 0.3 (Fig 8b) and did not display significant changes between sediments surrounding transplanted or bare sites (Table 2).

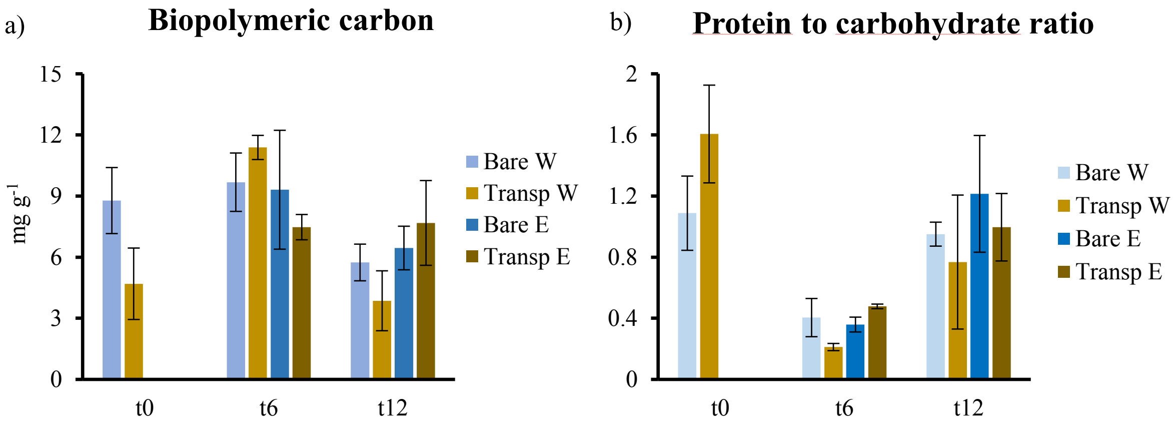


**Figure 8. a) Biopolymeric carbon content and b) protein to carbohydrate ratio in the sediment surrounding transplanted or bare sites. Bare W=bare sediments in the West site, Transp W=sediments surrounding transplanted colonies in the West site, Bare E=bare sediments in the East site, Transp E=sediments surrounding transplanted colonies in the East site. Mean and standard deviations are reported.**

Also, organic matter composition (in terms of the relative importance of protein, carbohydrate and lipid to biopolymeric C pools) did not show significant differences between sediments surrounding transplanted or bare sites (Fig. 9, Table 2).

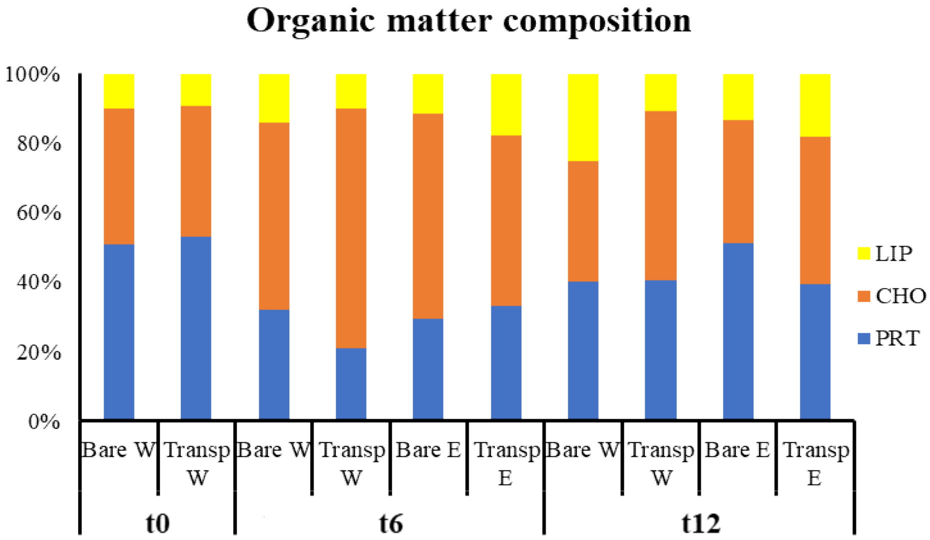


**Figure 9. Organic matter composition. LIP= lipids, CHO= carbohydrates, PRT=proteins. Bare W=bare sediments in the West site, Transp W=sediments surrounding transplanted colonies in the West site, Bare E=bare sediments in the East site, Transp E=sediments surrounding transplanted colonies in the East site.**

Prokaryotic abundances in sediment samples ranged from $3.4\times10^7 \pm 1.4\times10^7$ prokaryotes $g^{-1}$ in sprig (t6) to $1.5\times10^9 \pm 1.8\times10^8$ prokaryotes $g^{-1}$ after 12 months (Fig. 10). No significant differences were found between prokaryotic abundances in the sediments surrounding the transplanted gorgonians and the wild colonies. Prokaryotic abundances both in the sediments surrounding the transplanted gorgonians and the wild colonies showed significant temporal changes with the highest values at t0 and the lowest at t12 (Table 2).

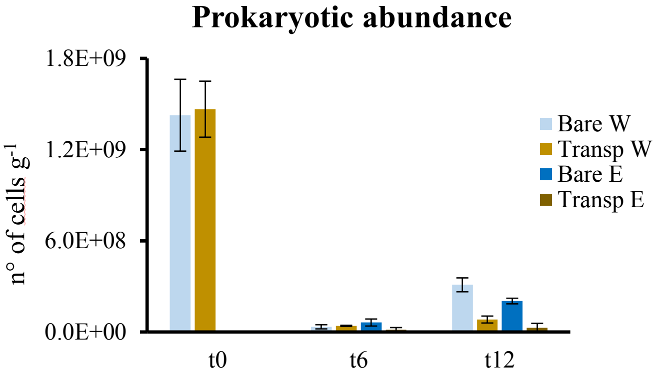


**Figure 10. Total abundance of prokaryotes in the sediment samples. Bare W=bare sediments in the West site, Transp W=sediments surrounding transplanted colonies in the West site, Bare E=bare sediments in the East site, Transp E=sediments surrounding transplanted colonies in the East site. Mean and standard deviations are reported.**

Aminopeptidase activity ranged from 18.3 ± 5.1 to 735.8 ± 141.7 nmol g$^{-1}$ h$^{-1}$, whereas β-glucosidase activity varied from .3 ± 0.1 to 27.4 ± 6.2 nmol g$^{-1}$ h$^{-1}$. Aminopeptidase and β-glucosidase activities displayed significant temporal changes both in the sediments surrounding the transplanted gorgonians and in bare sediments (Fig. 11, Table 2). β-glucosidase activity at t0 and t12 was significantly higher in the bare sediments of the West site compared to sediments surrounding transplanted gorgonians in the same site (Table 2). Significant differences in both extracellular enzymatic activities were also found between sediments surrounding transplanted gorgonians of the West and East sites (Table 2).

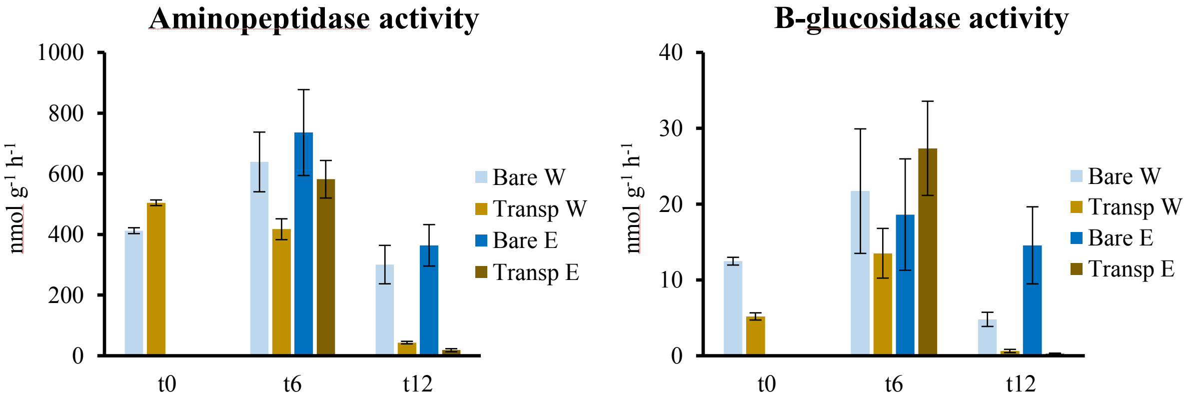
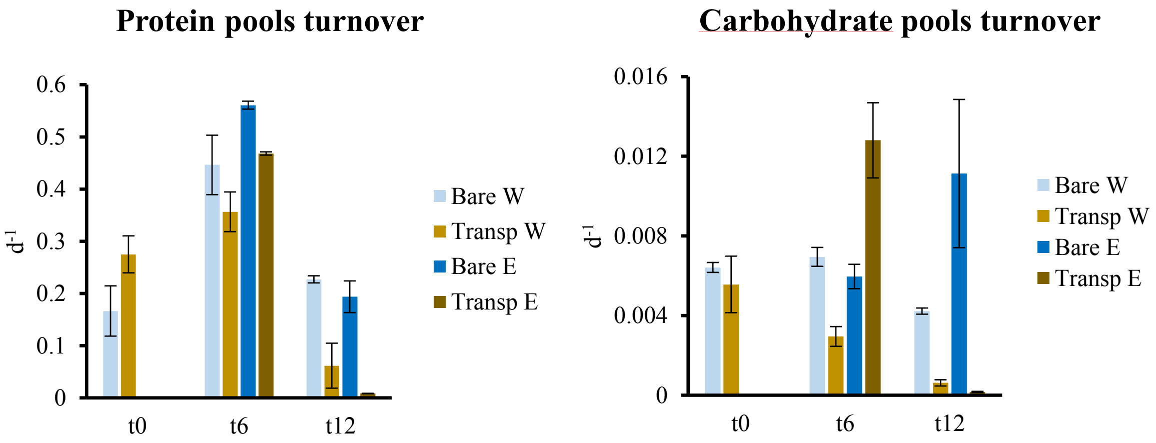


**Figure 11. Extracellular enzymatic activities in sediment samples. Bare W=bare sediments in the West site, Transp W=sediments surrounding transplanted colonies in the West site, Bare E=bare sediments in the East site, Transp E=sediments surrounding transplanted colonies in the East site. Mean values and standard deviations are reported.**

The turnover rates of both protein and carbohydrate pools showed significant temporal changes (Fig. 12; Table 2). In general, the turnover rates of protein and carbohydrate pools were significantly slower in the sediment surrounding the transplanted gorgonians of the West and East site when compared to those of their respective bare sites.


Protein pools turnover

Carbohydrate pools turnover

**Figure 12. Turnover rates of protein and carbohydrate pools in sediment samples. Bare W=bare sediments in the West site, Transp W=sediments surrounding transplanted colonies in the West site, Bare E=bare sediments in the East site, Transp E=sediments surrounding transplanted colonies in the East site. Mean and standard deviations are reported.**

| Variable | df | MS | Pseudo-F | P(MC) | Pairwise comparisons<br>Term 'TreatmentxTime' for pairs of levels of factor 'Treatment' | t | p (MC) |
|---|---|---|---|---|---|---|---|
| **PROKARYOTIC ABUNDANCE IN SEDIMENTS** | | | | | | | |
| Sitetreatment | 3 | 6.51E+15 | 0.31 | NS | | | |
| Time | 2 | 3.59E+18 | 173.53 | P<0.001 | | | |
| SitetreatmentxTime | 4 | 1.65E+16 | 0.79 | NS | | | |
| Residual | 20 | 2.07E+16 | | | | | |
| Total | 29 | | | | | | |
| **BIOPOLYMERIC CARBON** | | | | | | | |
| Sitetreatment | 3 | 3.12 | 1.23 | NS | t0. Bare W, Transplanted W | 2.962 | p<0.05 |
| Time | 2 | 39.09 | 15.35 | p<0.001 | t6. Transplanted W, Transplanted E | 7.893 | p<0.01 |
| SitetreatmentxTime | 4 | 15.44 | 6.06 | p<0.01 | | | |
| Residual | 20 | 2.55 | | | | | |
| Total | 29 | | | | | | |
| **ORGANIC MATTER COMPOSITION** | | | | | | | |
| Sitetreatment | 3 | 945.12 | 1.9006 | NS | | | |
| Time | 2 | 13943 | 28.039 | p<0.001 | | | |
| SitetreatmentxTime | 4 | 933.92 | 1.8781 | NS | | | |
| Residual | 20 | 497.27 | | | | | |
| Total | 29 | | | | | | |
| **PROTEIN TO CARBOHYDRATES RATIO** | | | | | | | |
| Sitetreatment | 3 | 6.9E-02 | 1.21 | NS | | | |
| Time | 2 | 2.3E+00 | 40.38 | p<0.001 | | | |
| SitetreatmentxTime | 4 | 1.5E-01 | 2.67 | NS | | | |
| Residual | 20 | 5.7E-02 | | | | | |
| Total | 29 | | | | | | |
| **AMINOPEPTIDASE ACTIVITY** | | | | | | | |
| Sitetreatment | 3 | 9.9E+04 | 22.59 | p<0.001 | t0. Bare W, Transplanted W | 11.905 | p<0.001 |
| Time | 2 | 5.3E+05 | 121.90 | p<0.001 | t6. Bare W, Transplanted W | 3.685 | p<0.05 |
| SitetreatmentxTime | 4 | 3.9E+04 | 8.99 | p<0.001 | t6. Transplanted W, Transplanted E | 4.029 | p<0.05 |
| Residual | 20 | 4.4E+03 | | | t12. Bare W, Transplanted W | 7.016 | p<0.01 |
| Total | 29 | | | | t12. Transplanted W, Transplanted E | 6.608 | p<0.01 |
| | | | | | t12. Bare E, Transplanted E | 8.747 | p<0.01 |
| **B-GLUCOSIDASE ACTIVITY** | | | | | | | |
| Sitetreatment | 3 | 99.27 | 6.31 | p<0.01 | t0. Bare W, Transplanted W | 18.101 | p<0.001 |
| Time | 2 | 618.14 | 39.27 | p<0.001 | t6. Transplanted W, Transplanted E | 3.414 | p<0.05 |
| SitetreatmentxTime | 4 | 137.17 | 8.71 | p<0.001 | t6. Bare E, Transplanted E | 2.978 | p<0.05 |
| Residual | 20 | 15.74 | | | t12. Bare W, Transplanted W | 7.525 | p<0.01 |
| Total | 29 | | | | t12. Bare E, Transplanted E | 4.861 | p<0.01 |
| **PROTEIN POOLS TURNOVER** | | | | | | | |
| Sitetreatment | 3 | 2.53E-02 | 22.73 | p<0.001 | t0. Bare W, Transplanted W | 3.144 | p<0.05 |
| Time | 2 | 0.34251 | 307.30 | p<0.001 | t6. Transplanted W, Transplanted E | 5.069 | p<0.01 |
| SitetreatmentxTime | 4 | 2.58E-02 | 23.14 | p<0.001 | t6. Bare E, Transplanted E | 19.499 | p<0.001 |
| Residual | 20 | 1.11E-03 | | | t12. Bare W, Transplanted W | 6.572 | p<0.01 |
| Total | 29 | | | | t12. Bare E, Transplanted E | 10.584 | p<0.001 |
| **CARBOHYDRATE POOLS TURNOVER** | | | | | | | |
| Sitetreatment | 3 | 4.55E-05 | 22.31 | p<0.001 | t6. Bare W, Transplanted W | 10.087 | p<0.001 |
| Time | 2 | 4.00E-05 | 19.63 | p<0.001 | t6. Transplanted W, Transplanted E | 8.741 | p<0.01 |
| SitetreatmentxTime | 4 | 6.22E-05 | 30.50 | p<0.001 | t6. Bare E, Transplanted E | 5.968 | p<0.05 |
| Residual | 20 | 2.04E-06 | | | t12. Bare W, Transplanted W | 28.304 | p<0.001 |
| Total | 29 | | | | t12. Transplanted W, Transplanted E | 4.937 | p<0.01 |
| | | | | | t12. Bare E, Transplanted E | 5.103 | p<0.01 |

**Table 2. PERMANOVA and pair-wise tests (showed only where significant results were obtained) for different variables measured in sediments collected in all the experimental plots. Fixed levels of contrast include bare sediments both in the West site and in the East site (Bare W and Bare E) and sediments surrounding transplanted colonies both in the West site and in the East site (Transplanted W and Transplanted E) (for all the treatments, n = 3 replicates). Analyses were carried out using unrestricted permutations of the raw data and 9999 permutations. df, degrees of freedom; MS, mean square; P(MC), probability level after Monte Carlo tests; Pseudo-F, statistic F; t, statistic t for pairwise comparisons. NS= not significant.**

## 6.4 Discussion

At the end of the monitoring period carried out in the present study, 50% of the gorgonians transplanted in the West site were present vs. 75% in the East site. The lower persistence of gorgonians transplanted in the West site may be due either to harsher hydrodynamic conditions able to detach the anchored fragments from the sea bottom and/or detachment caused by diver pressure which is more relevant in the West site of the Portofino MPA. These findings reinforce previous findings (Linares et al., 2008a; Fava et al., 2009) on the importance of selecting suitable sites for improving the success of restoration actions of hard bottom species. Independently from the sites were experiments were carried out, the remaining transplanted gorgonians were visually healthy and microscopic observations carried out on collected fragments did not reveal any major sign of tissue stress and/or damage.

The species *E. singularis* is the only Mediterranean gorgonian hosting symbiotic algae (Munari et al., 2013). Changes of the zooxanthellae community along with of the whole microbiome can have important consequences on the health of the holobiont. In this study, I analyzed and compared for the first-time temporal changes of zooxanthellae and prokaryotes associated to donor and transplanted colonies of *E. singularis.* Zooxanthellae abundances in gorgonians' tissue displayed similar temporal patterns in the donor and transplanted gorgonians with values significantly lower in April 2018 (i.e. t6) than in October 2017 (i.e. t0) and October 2018 (i.e. t12). Zooxanthellae abundances in donor and transplanted colonies of *E. singularis* were not significantly different and fall within the range of values previously reported for this species (Forcioli et al., 2011). The lack of significant changes between zooxanthellae abundances in donor and transplanted colonies of *E. singularis* suggests that transplantation does not affect the symbiotic algae of the holobiont, if this is properly done in a suitable receiving site. This is also confirmed by the analyses of the assemblage composition of the algal symbiont which with only one exception, did not differ significantly between donor and transplanted colonies of *E. singularis*. A significant temporal change of the assemblage composition of zooxanthellae was observed with a higher quantitative relevance of pale algal cells present in all of the colonies in October 2018 and was likely driven by thermohaline conditions (Mise & Hidaka, 2003).

Prokaryotic abundances associated to gorgonians displayed similar temporal patterns than those observed for zooxanthellae, suggesting a coupling between symbiotic algae and prokaryotes and their holobiont. Previous studies reported a major increase of total prokaryotic abundance in diseased colonies of different tropical corals compared to healthy colonies (Luna et al., 2007; Luna et al., 2010). In this study, prokaryotic abundances did not differ significantly between donor and transplanted colonies of *E. singularis,* providing further support of the good health status of transplanted gorgonians.

Increasing evidence indicate that associations between marine invertebrates and microbes play fundamental roles in driving host functions, nutrition and health (McFall-Ngai, 2008; McFall-Ngai et al., 2013), but studies on such associations in Mediterranean ecosystems are still limited. The bacterial assemblages associated to donor gorgonian colonies were much less diverse (higher OTU richness) and less evenly distributed among taxa than those associated to transplanted colonies, with values in donor colonies displaying a limited temporal variability. Such limited temporal variability has been already documented in gorgonians (van de Water et al., 2017), suggesting that temporal stability of the microbiome can be relevant for the maintenance of healthy gorgonians.

The microbiomes of donor colonies were always largely dominated by bacteria belonging to the genus *Endozoicomonas* (on average 87% of the assemblage), which is typically associated with gorgonians (Vezzulli et al., 2013). These bacteria are important for the functioning of the holobiont, since they may also protect corals from diseases: they produce antimicrobial chemical defences and they prevent colonization by bacterial pathogens, occupying all the available ecological niches (Kelman et al., 2006; Ritchie, 2006; Rypien et al., 2010). To these regards, a strong decrease of the bacteria belonging to the genus *Endozoicomonas* has been observed in diseased colonies of the Mediterranean gorgonian *P. clavata* (Vezzulli et al., 2013). In this study, the relative quantitative importance of the genus *Endozoicomonas* was less pronounced and more variable over time in transplanted gorgonians than in donor colonies. The decrease of the quantitative importance of the genus *Endozoicomonas* in transplanted gorgonians was coupled with an increase of the genera of *Meiothermus* and *Anoxibacillus*, also found in donor colonies but with a lower contribution to the bacterial assemblages. Bacteria of the genera *Meiothermus* and *Anoxybacillus* (facultative anaerobic) are typical of hot spring systems (Chan et al., 2015), although the latter has been reported also in association with the gills of freshwater fishes (Wang et al., 2010) and with the tropical coral of the genus *Sarcophyton* (Lee et al., 2012).

In the present study, SIMPER analysis indicates that an important percentage of the dissimilarity between the microbiomes of transplanted and donor colonies was due to the presence of bacterial taxa affiliated with *Thermaceae* and *Bacillaceae* families to which *Meiothermus* and *Anoxybacillus* genera belong, respectively. This suggests that transplantation can lead to a certain variation of the composition of the microbiome associated to gorgonians by inducing stressful conditions on the holobiont. However, such a microbiome composition of the transplanted gorgonians in October 2018 (at t12) was not statistically different compared to that of the donor colonies. These results suggest that the transplanted gorgonians were healthy as much as the donor colonies inhabiting the MPA. To support this consideration, the absence in the bacterial assemblages of opportunistic pathogens (e.g. belonging to the genus *Vibrio*), which have been previously documented to be responsible of various diseases both in tropical and Mediterranean corals (Luna et al., 2010; Vezzulli et al., 2010), is remarkable.

Gorgonians can promote particle sedimentation by decreasing current flow, thus enhancing trophic availability for other benthic heterotrophic consumers (Cerrano et al., 2010). Despite the general oligotrophic conditions of the Ligurian Sea (Fabiano et al., 1995), in the present study I have found that biopolymeric C concentrations were high both in bare sediments and in sediments surrounding transplanted gorgonians. Such values, indeed, are typically encountered in eutrophic coastal systems (Dell'Anno et al., 2002; Pusceddu et al., 2009) and are much higher than those observed in bare sediments and sediments surrounding *Savalia savaglia* at 50-60 m depth in the same study area (Cerrano et al., 2010). No effects on the trophic status of sediments due to gorgonian transplantation were observed, since sediments surrounding transplanted colonies were generally characterised by BPC concentrations not significantly different to those determined in bare sediments. Also, the quality and the overall biochemical composition of organic matter did not display significant changes between sediments surrounding transplanted gorgonians and bare sediments. The lack of significant differences in the quantity and quality of organic matter was also associated to the lack of significant differences of benthic prokaryotic abundances between bare and transplanted sites.

Benthic prokaryotes play an important role in C cycling and nutrient regeneration through degradation processes mediated by extracellular enzymatic activities (Danovaro et al., 2010). In the present study, aminopeptidase and β-glucosidase activities in the sediments were similar to values reported in other shallow Mediterranean ecosystems (Pusceddu et al., 2005) and displayed significant temporal changes both in the sediments surrounding the transplanted gorgonians and in bare sediments. Aminopeptidase and β-glucosidase activities were generally significantly lower in the sediments surrounding the transplanted gorgonians than in bare sediments. Although further studies are needed to better understand such differences, these findings suggest that, at least on the time scale investigated, transplanted gorgonians in the Portofino MPA are not able to enhance key ecological processes like gorgonian forests formed by the gold coral *Savalia savaglia* do (Cerrano et al., 2010), probably due to the small dimension and reduced density of the colonies used.

Overall results presented here indicate that the analysis of the diversity of the microbiome can be a useful complementary tool for evaluating the health status of hard-bottom transplanted invertebrates. Besides, I stress the need of long-term monitoring to evaluate the effectiveness of restoration actions basing it not only on the assessment of the survival rate of transplanted organisms but also on the determination of benefits on ecosystem performance through a before-after control-impact paired (BACIP) approach.

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# CHAPTER 7: CONCLUSIONS

The results obtained in this work will be useful to better develop restoration actions. This thesis provides advances on the knowledge of the effects of the potential anthropogenic impacts and the way in which threatened species are able to recover. Moreover, this work provides useful information on the effectiveness and the environmental effects of two ecological restoration practices addressed to the species (seagrass) and to the habitats (coralligenous).

In particular, the following conclusions can be drawn:

1. the resuspension and redeposition of particles due to mining and trawling activities can severely damage habitat-forming species, like the octocoral *Corallium rubrum*; the consequences of these activities might be minimized to a certain extent by properly modulating the intensity and the duration of these activities. In our case, the exposed benthic species showed a certain recovery capacity in terms of reduction of the tissue damage and partial recovery of its feeding capacity;
2. if properly fed, deep-sea habitat-forming species could be maintained in aquaria to be used for future restoration actions. Crustaceans of the genus *Mysis* are the best food item for breeding *Desmophyllum pertusum*, *Madrepora oculata* and *Dendrophyllia cornigera* in aquaria;
3. seagrasses, if properly transplanted, can survive and improve ecosystem functioning. The transplantation technique used for the experiment conducted at Gabicce Mare allowed the transplanted seagrasses to survive even after the strong hydrodynamic events occurred during the winter. Moreover, transplanted seagrass can improve ecosystem functioning;
4. gorgonians can survive after transplantation, but further studies are needed to better understand the effects of these restoration actions on key-ecological processes and to assess the timing for the recovery of gorgonians forests.

However, further studies are needed to assess the potential of scaling-up these actions addressing the present scale of species/habitat loss. Overall results presented here expand our knowledge on actions needed for the recovery and restoration of marine habitats and open new perspectives for a better understanding of the potential ecological benefits that can support the conservation of the natural capital.

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# The deep sea: The new frontier for ecological restoration

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ABSTRACT

Deep-sea ecosystems are the most extensive on Earth and provide key goods and services for human well-being, such as genetic resources and climate regulation. Maintaining the sustainable functioning of the global biosphere therefore requires protection of deep-sea ecosystems, particularly because these ecosystems face major changes related to human and climate-induced impacts. Although we lack data to evaluate the spatial scale of degraded deep-sea habitats, numerous studies document human impacts on the whole ocean. However, protection alone can be insufficient to reverse habitat degradation in the deep sea. Scientifically, deep-sea restoration actions may be feasible, but whether such actions will achieve sustainability goals when applied at broad spatial scales of impact remain questionable. Successful application of most restoration efforts will first require a deeper understanding of biodiversity and functioning of deep-sea ecosystems, and better knowledge of ecosystem resilience and recovery rates of deep-sea fauna. In addition to limited data availability, expensive technologies (with estimated costs up to millions of dollars $ha^{-1}$) represent a major obstacle to large-scale deep-sea restoration, but international cooperation (like a stronger collaboration between industry and scientists belonging to the academia) could significantly reduce this operational cost. Future deep-sea ecosystem restoration could offer an important business opportunity for technological development and application and an investment in natural capital for a new and competitive blue-growth sector.

## 1. Introduction

The deep ocean, Earth's largest ecosystem [1,2], remains largely unexplored [1,3,4], primarily because of the technical challenges and associated high costs of investigation [5,6]. Despite these challenges, it is widely recognized that deep-sea ecosystems host a large portion of Earth's biodiversity that plays a key role in the functioning of our planet [6–10], supporting key ecological processes (including production, consumption and decomposition of organic matter and nutrient regeneration; [1]) and providing essential goods and services for human well-being [9,11–14]. Marine biodiversity loss can fundamentally alter ocean stability, food and habitat provisioning, water quality, recovery from perturbations and biogeochemical cycles [1,15–20]. Anthropogenic activities and global climate changes threaten life in the global ocean [21–24]. Humans are modifying the environment and ecology of the oceans through top-down (e.g. fisheries overexploitation) and bottom-up impacts (e.g. eutrophication processes induced by excess nutrient release into coastal waters) [18]. Marine ecosystems worldwide are thus subjected to multiple stressors [13,25,26].

The deep sea is not an exception since industries are moving rapidly

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toward exploitation of its resources [22,27], partially in response to the depletion of those provided by terrestrial and coastal habitats. Rising demand for deep-ocean resource exploitation has generated the need for international laws to protect marine Areas Beyond National Jurisdiction (ABNJs, [6]). The United Nations Convention on the Law of the Sea (UNCLOS) works towards Conservation and Sustainable Use of Marine Biological Diversity beyond Areas of National Jurisdiction (BBNJ) and will likely apply in a multi-sectoral and integrated approach towards management, cooperation and coordination [28]. However, the protection of ABNJs is fragmented under different institutions [29] that concern fisheries (Regional Fisheries Management Organizations - RFMOs), mineral resources and mining activities (International Seabed Authority - ISA), and the BBNJ (UNCLOS). Despite this, at time, there is insufficient coordination between these organizations.

Plans for expansion of mineral exploitation and bottom-contact fisheries in the deep sea suggest increasing the potential for degradation of deep-sea ecosystems and call for regulation, protection and eventually funding of possible restoration actions [6,30–32]. Regulation of these activities for sustainable environmental management should follow a mitigation hierarchy [33], which firstly should avoid the impacts that can cause significant loss of biodiversity. The second step should include minimization measures that could reduce biodiversity loss, investing in engineering solutions and technical innovations that can be able to reduce the impacts. As third step, when biodiversity loss occurs, remediation/restoration actions are needed.

There is evidence that protection measures for the deep sea can be effective. As an example, the closure of the zone of the Darwin Mounds from trawling activities was successful, with coral recolonization and re-growth of damaged colonies [34]. The creation of deep-water Marine Protected Areas (MPAs) like that near Okinawa Island provided evidence of the potential success of a right management program. With protection, deep-water snappers can achieve sexual maturity and their reproduction rate could be improved [35]. For what concerns mining activities, ISA has identified some Areas of Particular Environmental Interest (APEIs) in the Clarion-Clipperton Zone located in the Pacific Ocean in which mining is forbidden [36] and the General Fisheries Commission for the Mediterranean and Black Sea established six Fisheries Restricted Areas (FRAs) in the high seas to protect Essential Fish Habitats and Vulnerable Marine Ecosystems (VME). To identify the last ones, new multi-criteria assessment methods are also available [37].

Requirements for remediation are often included in terrestrial projects, but similar approaches in the deep sea will face numerous challenges [38]. Several international conventions and directives, such as the European Marine Strategy Framework Directive or the UN 2030 Agenda for Sustainable Development, emphasize the importance of sustainably managing and protecting marine ecosystems, achieving good environmental status, avoiding significant adverse impacts such as biodiversity loss and loss of ecosystem functions [16], and taking actions for restoration of marine ecosystems. Despite some pilot restoration experiments on cold-water corals provided promising results [39–42], there is still no evidence of successful ecological assisted restoration as a management tool to reverse environmental degradation caused by human activities in the deep sea [38]. However, since protection alone can be insufficient to reverse habitat degradation in the deep sea, restoration actions should be properly defined to remediate and compensate human-induced damages.

The aim of this paper is to review the state of art on ecological restoration in deep-sea ecosystems, highlighting knowledge gaps and the main scientific, technological and economic challenges. We stress that restoration actions in the deep sea will be increasingly required in the future to face the expected progressive habitat degradation and as such they should be included in international conventions as a management tool to support conservation strategies of deep-sea ecosystems.

### *1.1. Which are the main impacts in the deep sea?*

Industrialised states and private companies are expanding exploration of the oceans into deeper waters [43,44] to exploit biotic and abiotic resources [22,43,45–47]. Three major activities (trawling, mining and oil and gas exploitation) can affect deep-sea ecosystems over wide spatial scales (several square kilometres for oil and gas exploitation, thousands of square kilometres for mining and trawling activities) and at different depths. Depending on the type of catch, deep-sea trawling now operates from ca. 200 m down to 1500–2000 m depth [48,49], whereas mining activities are projected to extend to depths from ca 1000–1500 m down to more than 4000 m.

Steep declines in shallow fisheries resources in recent years [50,51] coincide with expansion of fisheries offshore to progressively greater depths [27,46,52–54], threatening ecologically important and sensitive habitats such as canyons and seamounts [52,55,56]. Among all human activities, bottom trawling represents the most serious threat worldwide to cold-water coral reefs and deep-sea sponge grounds, which are both biodiversity hotspots [52,57,58]. These deep-sea habitats are also characterized by species with slow growth rates (e.g., growth rates of the cold-water coral species *Lophelia pertusa* are around 4–25 mm per year and the observed growth rate of the deep-sea sponge *Rossella racovitzae* is around 2.9 mm per year) [59,60]. The use of fishing devices such as otter trawls impacts deep-sea ecosystems in several ways, including stock depletion, alteration of sea-bottom morphology, sediment resuspension, increased bottom-water turbidity, faunal mortality, altered nutrient cycles, and reduced benthic biodiversity [49,55,61–65]. These impacts are increasing worldwide. For example, 90% of the North Sea seabed has been trawled at least once and about 15 million $km^2$ of seabed—an area nearly the size of Russia—are trawled every year [66]. Bottom fishing is so far the major threat for seamount ecosystems that cover an estimated area of 21% of the ocean floor (about 76.5 million $km^2$) [56].

Mineral exploitation to support increasing metal demands [67] represents the main future threat for the integrity of large swaths of deep seabed [57,68,69]. Nowadays, there is a strong commercial interest in manganese nodules in abyssal plains [70], cobalt-rich crust on seamounts [45], and seafloor massive sulphides at hydrothermal vents [67,71]. It is known that future mining activities could cause multiple impacts on deep-sea ecosystems, including habitat destruction, smothering of benthic communities by sediments, and increased toxicity associated with the release of heavy metals contained in the mineral ores [43,68,71–76], as well as alteration of a wide range of ecosystem services [77]. Exploration contracts have already been awarded for more than 1,800,000 $km^2$ of seabed [78]. Around 1.3 million $km^2$ of international seabed have been set aside for mineral exploration in the Pacific and Indian Ocean and along the Mid-Atlantic Ridge. Moreover, about 1,000,000 $km^2$ of deep seabed have been licensed and occurred in areas beyond national jurisdiction (ABNJs) [78,79].

Exploitation of oil and gas continues to extend deeper, with increasing risks for drilling-muds dispersal and oil-spills accidents [22,80,81]. More than 500 oil platforms are currently present in waters below 200 m depth in European seas linked to several thousand kilometres of pipelines [82]. These structures often occur near continental slopes, seeps, vents, and coral mounds [22]. Hydrocarbon extraction from the deep seafloor also releases heavy metals, discharges hazardous compounds such as barium in synthetic fluids contained in drilling muds, and leads to loss of habitat and benthic communities [83]. For example, the oil spill from the Deepwater Horizon in 2010 contaminated surrounding ecosystems with polycyclic aromatic hydrocarbons and barium and caused oxygen anomalies together with an alteration of food input to deep-sea benthic communities [84,85]. Laboratory-simulated experiments demonstrated that resuspension of sediments that could be provoked by both oil/gas exploitation and trawling activities causes several damages to deep-sea benthic organisms like corals and sponges [86–88].

Another threat for the deep sea is bioprospecting activity that can lead to genetic exploitation of deep-sea biological resources [89]. Several marine species, including microorganisms, provide a significant source of compounds of interest for biotechnological development [90,91]. For example, pharmaceutical companies are exploring deep-sea microbes for their potential to produce antioxidant, anti-microbial and other bioactive substances [90,92,93]. Enzymes including esterase, lipase, protease, peptidase, polymerase (e.g. Taq-polymerase) from deep-sea microorganisms offer multiple applications because of their capacity to expand the conditions in which biocatalysis can occur [94]. Deep-sea animals, such as the hard coral *Dendrophyllia cornigera,* produce steroids with potential anti-cancer properties [95].

Beyond impacts from resources' exploitation, numerous studies report contamination by multiple chemical compounds (introduced from sewage waters and coastal runoff, marine litter, plastic debris, discharge of industrial or military refuse; [22,96–104]). Scientific activities can alter deep-sea ecosystems using dredges, moorings, and other technical instruments that may affect benthic fauna [22,105].

Due to fossil-fuels use, land deforestation and degradation of habitats, the content of carbon dioxide in the atmosphere has increased by ca. 50% during the last two centuries, one-quarter of which is absorbed by the oceans [96,106]. This oceanic uptake of $CO_2$ has resulted in a reduction in pH and changes in the carbonate chemistry of the oceans from the surface to the deep ocean [14,23], with expected significant impacts on cold-water coral reefs [107,108]. At the same time, the rise of sea-surface temperature, due to the increase of greenhouse gases in the atmosphere, by enhancing water stratification and reducing mixing, is expected to reduce primary productivity of the ocean surface and the export of carbon to the ocean floor with severe consequences on the structure, function and biodiversity of deep-sea ecosystems [32,109–112]. Structural and functional alterations of deep-sea biodiversity can also occur due to temperature shifts of the deep-water masses [113]. Direct and indirect global climate change impacts will likely exacerbate all the other impacts on deep-sea ecosystems [14,23,32,96,106–113]. Moreover, at present, the actual extension of marine ecosystems impacted by human activities is still largely unknown [114], especially that in the deep sea [22]. Due to our scarce knowledge of the deep-sea functioning, cumulative consequences of all the impacts are not easy to assess [115].

## 2. Planning the “restoration” of deep-sea ecosystems

The Society of Ecological Restoration (SER) defines the International Standards for the Practice of Ecological Restoration and clearly distinguishes between “ecological restoration” and other forms of ecosystem repair. Although restoration is the process of “assisting the recovery of ecosystems that have been degraded, damaged or destroyed” [116], the “recovery” of an ecosystem refers to the achievement of a target environment similar to an appropriate local native model or reference ecosystem, in terms of its specific compositional, structural, and functional ecosystem attributes [117,118]. “Restoration” includes an action or multiple actions that jumpstart recovery and place a degraded ecosystem on a trajectory for recovery [119,120], regardless of the period required to achieve the recovery outcome [118]. Ecosystem restoration principally aims to establish a self-supporting habitat similar to the ‘original’ habitat prior to the impact. Restored ecosystems can be resilient to perturbations, including the capacity for the ecosystem to adapt to existing and anticipated environmental change [117,121,122]. The selection of the desired reference system guides ecological restoration and should be both historically inspired and grounded in social processes that include multiparty stakeholders and restoration scientists and practitioners [120]. Application of a group of restoration actions at a site can help achieving restoration goals, including remediation, reparation, and rehabilitation. As a restoration goal, rehabilitation is less ambitious in not necessarily achieving full recovery of processes and a return to “pre-disturbance” conditions [120,123]. It simply replaces structural or functional characteristics damaged by an impact, and aiming to enhance the social, economic, and ecological value of the new ecosystem [117,118,123].

SER states that the reference ecosystem, as a model for all of these kinds of actions, should resemble a near undamaged site. In alternative, the reference ecosystem can be derived from multiple sources of information on diverse ecological and biological variables (e.g. biodiversity, life cycles, functional variables, food webs) supported by abiotic measurements [6,117]. In any case, ecosystem baselines should be clearly defined. Full recovery requires several steps, each encompassing several interventions (e.g. removal of the disturbance, removal of invasive species, enhancement of native species reproduction; [118]). Ecological experts in restoration have abandoned the idea of total recovery, recognising the dynamic nature of ecosystems, and restoration may not follow the appropriate ecological trajectory to re-establish over time, especially considering the need for ecosystems to adapt and evolve over time in response to climate change [124].

Regarding the deep sea, some new industrial projects, such as the pipeline deployment in the deep Mediterranean Sea and the exploitation of mineral ores in Papua New Guinea, include mitigation actions for reducing the environmental impact. For example, the mitigation plan for the exploitation of deep-sea mineral ores in Papua New Guinea includes the selection of an area similar in size and characteristics to the mining one, as a possible source for natural repopulation of the impacted site (i.e., natural unassisted recovery; [68]). Despite this, if deep-sea mining will be realized in the next future, restoration actions are needed to remediate and compensate the habitat degradation induced by such activities [120,123]. Managers may sometimes consider replacement, re-creation or creation of new undamaged habitats that can compensate for the loss of another [118,119].

Furthermore, for most deep-sea ecosystems, we lack information on ecosystem baselines to establish an appropriate reference for effective restoration, an attribute that most terrestrial restoration efforts can provide through experimental ecology. We require robust information on biodiversity, trophic interactions, distribution ranges, dispersal distance and connectivity [112]. The scientific community recognizes an urgent need to increase knowledge on deep-sea ecosystem functioning prior to deep-sea resource exploitation [100,125,126]. The limited information on deep-sea ecosystems (less than 0.0001% of the deep ocean's surface has been investigated [112]) precludes the assessment of restoration effectiveness in case of degradation due to future possible activities such as deep-sea mining [32]. Even when baselines are better known, like for some deep-sea habitats (e.g. cold-water coral reefs and seamounts) or for certain areas of the oceans (e.g. some areas of the north-east Atlantic Ocean), restoration actions will be a major challenge, especially due to the need of sophisticated technologies and infrastructures (e.g. autonomous underwater vehicles, remotely operated vehicles, oceanographic vessels) and high costs [5,112]. In addition, the prolonged timeframe for natural recovery of threatened deep-sea ecosystems by slow rates of recruitment and growth of resident fauna will further constrain deep-sea restoration efforts [22,127,128]. Thus, in parallel with monitoring ecosystem recovery following anthropogenic disturbances, we need to develop and apply restoration strategies to degraded/damaged deep-sea ecosystems.

### 2.1. *Passive restoration: the natural unassisted recovery of deep-sea ecosystems*

Multiple studies have investigated anthropogenic impacts on deep-sea ecosystems and the natural recovery capacity of benthic fauna (Table 1) with passive restoration, i.e. unassisted natural recovery of ecosystems following cessation of an activity. Although the unassisted restoration can be considered the less expensive practice, economic resources should be allocated by governments, research institutions, academia, industries, and non-governmental organizations for monitoring activities aiming to assess the natural recovery of the

**Table 1**
Benthic fauna recovery capacity in different deep-sea habitats. n.a. = not available.

| Type of action | Habitat | Depth | Biotic component | Duration | Spatial scale | Result/Efficacy | References |
|---|---|---|---|---|---|---|---|
| Natural recovery | *Soft bottom* | 1760 m | Macrofauna | 2 years | $< 10\ m^2$ | no significant recovery | [129] |
| | | 1525 m | Macrofauna, meiofauna | 3 months | $\simeq 200\ m^2$ | no recovery | [84] |
| Fertilisation with kelp powder | | 1300 m | Macrofauna | 5 months | $< 10\ m^2$ | low colonisation rates, no effects of enrichment | [130] |
| Natural recovery | *Seamount and coral mounds* | ca. 1200 m | Stony corals, associated megafauna | 5 years | $\simeq 0.002\ km^2$ | no significant recovery | [131] |
| | | from 750 to 1600 m | Macrofauna | about 20 years | $\simeq 0.006\ km^2$ | no recovery of community composition | [132] |
| | | from 750 to 1600 m | Megafauna | 5–10 years | n.a. | no complete recovery | [133] |
| Natural recovery | *Manganese-nodule fields* | ca. 4000 m | Prokaryotes | 6 months | $< 10\ m^2$ | abundances similar to undisturbed sites | [138] |
| | | | Megafauna, macrofauna, meiofauna | 3 years | $< 10\ m^2$ | high abundances but no completely recovery of community structure | |
| | | | Polychaeta | | | differences in taxa and functional group composition | |
| | | 4140 and 4160 m | Soft-bottom megafauna | 7 years | $\simeq 0.05\ km^2$ | differences in abundance and taxa composition | [136] |
| | | | Hard-bottom megafauna | | | no recovery due to hard-bottom removal | |
| | | from 4122 to 4201 m | Macrofauna | 7 years | $< 10\ m^2$ | recovery of the overall abundances, no recovery of community structure | [137] |
| | | ca. 5000 m | Meiofauna (Nematoda) | 26 years | $< 10\ m^2$ | abundance, biomass and biodiversity significantly lower within dredging tracks | [141] |
| | | ca. 5000 m | Macrofauna | 44 months | $< 10\ m^2$ | recolonization in progress but at slow rates | [143] |
| | | 200–800 m | Epifauna | 37 years | $\simeq 0.02\ km^2$ | no significant recovery | [142] |

investigated ecosystems, including funding for ship time, personnel involved and sampling activities [6]. For restoration/rehabilitation/conservation practices, a global strategy could be the principle of "polluter pays": stakeholders who are responsible for the damage should fund deep-sea ecosystem recovery actions [31]. Despite ISA could allocate a part of the money derived from the payment of licences for funding future protection projects on mined areas, activities like high seas fishing and oil extraction have free access to deep-sea resources and are not obliged to fund such programs. In these cases, a tax corresponding to the 1% of their revenues will generate a big fund to support actions for deep-sea conservation in any form [31]. In addition to the very high costs, the long-time scale needed for the recovery of degraded deep-sea ecosystems will further constrain restoration efforts [22,127,128].

The first assessments of the natural unassisted recovery of deep-sea assemblages in the early 1970s compared rates of macrofaunal re-colonisation of defaunated sediments placed in boxes at 1760 m depth (using the submersible Alvin) to those at shallow depths (10 m depth; [129]). This experiment provided the first evidence of much slower recovery rates of macrofauna in benthic deep-sea ecosystems compared to shallow environments (years *vs.* few months). A similar study in the Santa Catalina Basin (California) at 1300 m depth used defaunated sediments to explore the potential of organic enrichment using kelp algae (*Macrocystis pyrifera*) powder to enhance deep-sea macrofaunal recovery [130]. Colonisation rates in the enriched sediments were i) lower than those in the un-enriched deep-sea sediments and ii) an order of magnitude lower than rates at shallow depths. Moreover, several time-series observations of seamount benthic communities impacted by bottom trawling [49,131–134] revealed no consistent signs of recovery in megafauna or macrofauna 5–10 years after fishing cessation, again suggesting very slow recovery for these benthic organisms.

Recovery of deep-sea benthic assemblages was also investigated in a manganese nodule area of the deep South Pacific (Peru Basin) after a simulated disturbance in 1989 [135–137]. Researchers simulated mining impacts by intensively ploughing manganese nodules near the sediment surface with a plough-harrow disturber designed to mimic manganese extraction. Half a year later, only prokaryote abundances resembled those of the undisturbed sites [138]. After 7 years, neither macrofaunal nor megafaunal abundances had completely recovered [136,137] and after 26 years there are still no evidence of complete recovery of the ecosystem functioning [139,140]. In the Clarion Clipperton Zone (North-eastern Pacific), the most commercially attractive deep-sea deposit of manganese nodules, several experiments simulated mining operations using benthic disturbers: meiofauna had not recovered 26 years after the experiment [141], and epifauna had not recovered after 37 years [142]. In the Central Indian Basin, 44 months after researchers' simulated disturbance by manganese nodule collection, the sedimentary macrofauna still had not recovered [143]. A recent meta-analysis suggested impacts and recoveries from deep-sea mining comparable to those associated with bottom fisheries or volcanic eruptions [144]. This study reinforces expectations of slow recovery rates in benthic deep-sea assemblages [22,76] and that deep-sea mining, by removing the hard substrates on which associated fauna depends, can significantly impact assemblage composition and result in long-term community changes [142,144]. Recovery rates of disturbed taxa differ widely, with some taxa increasing above pre-disturbance abundance and others that do not recover even in the long term [144]. Other studies of recovery of benthic deep-sea fauna examined impacts of oil and gas exploitation. For example, oil dispersal from the Gulf of Mexico Deepwater Horizon blowout resulted in slow recovery of deep-sea invertebrate assemblages [84], supporting previous findings obtained after the Amoco Cadiz oil spill off France in 1978 [145].

Therefore, multiple lines of evidence indicate (very) delayed recovery of altered deep-sea ecosystems, principally because of slow rates of faunal recruitment and growth [22,112]. Thus, the establishment of Marine Protected Areas and Marine Reserves in high seas is needed not

**Table 2**
Proposed methods for the restoration of damaged deep-sea habitats. Evaluation of the costs for the first year has been made using data from literature [30,161,162]. The time required for the recovery is based on the data/opinion of the authors reported in the reference list. n.e. = not estimated.

| Habitat | Impact | Type of recovery | Proposed method | Costs for the 1st year (in millions USD x ha$^{-1}$) | Time for recovery | References |
|---|---|---|---|---|---|---|
| *Soft bottoms* | trawling, dredging, bottom longlines | Passive restoration | Protected Areas to enhance natural recovery | 1.2 | > 40 years | [142] |
| *Seamounts* | trawling, dredging, bottom longlines | Passive restoration | Protected Areas to enhance natural recovery | 1.2 | > 10 years | [49,131–133,144] |
| | mining of Fe–Mg crust | Active restoration | Increasing of the rugosity of mined substrata to promote larval settlement | n.e. | n.e. | [163] |
| *Coral mounds* | trawling, dredging, bottom longlines | Passive restoration | Protected Areas to enhance natural recovery | 1.2 | at least one decade to reach the same proportion of dead/alive corals of control areas | [34,43,164] |
| | trawling, dredging, bottom longlines | Active restoration | Rearing and transplant of nubbins of deep corals | ≥15 | several decades | [30] |
| | | | Electrified artificial reefs to enhance survival/growth/recruitment rate of Cold-Water Corals | 4.4 | n.e. | [41,152] |
| | | | Recruitment of larvae in shallow depths and translocation in deeper areas | 3.3 | n.e. | [30] |
| | | | Transplanting fragments from donor colonies | 3 | n.e. | [30,39,40] |
| *Manganese nodules fields* | mining | Passive restoration | Protected Areas to enhance natural recovery | 1.2 | > 40 years | [142] |
| | | | Networks of Areas of Particular Environmental Interest where mining is not allowed | 5 | n.e. | [36] |
| | | Active restoration | Deployment of hard artificial substrata | n.e. | n.e. | [163] |
| | | | Addition of artificial sponges to enhance recruitment of associated fauna | n.e. | n.e. | |
| | | | Recreating nodule moulding mined sediments | n.e. | n.e. | |
| *Hydrothermal vents* | mining | Passive restoration | Protected Areas to enhance natural recovery | 1.2 | n.e. | [36,71,147] |
| | | Active restoration | 3D structures for the recruitment and/or transplant of vent fauna/enhancement of sulphides precipitation and eventually drilling of the seafloor to recreate the vent | ≥15 | several decades | [30,163] |

only to protect deep-sea habitats from human exploitation activities, but also to favour the natural recovery of already degraded habitats. More areas with restrictions for resource exploitations, like APEIs and Mediterranean FRAs, should be established for preserving deep-sea habitats. If specific exploitation activities will be allowed, they should be strongly regulated asking to the industries to provide specific environmental management plans that includes baseline assessment, monitoring and mitigation measures. This kind of management will be crucial to assess and reduce the impacts [146]. Finally, the application of restoration tools will be necessary to remediate and compensate unavoidable human impacts on deep-sea ecosystems [31].

### 2.2. Active restoration of deep-sea ecosystems

Active restoration of deep-sea ecosystems has received little attention, and until now lack any discussion on how to enhance natural recovery [30]. One major scientific research challenge is to develop new approaches and methodologies to support and accelerate natural recovery of deep-sea habitats affected by trawling and oil exploitation, and in the future by mining. Researchers have proposed several methods for deep-sea habitat restoration (Table 2), but all of these approaches require testing and evaluation of costs with applications in the field [147]. In some cases, however, active restoration (e.g., transplantation of artificial nodules in polymetallic nodule fields) can be unfeasible. For example, assuming mining within a contract area (75,000 $km^2$) removes nodules over an area of 15,000 $km^2$ and the average nodule density prior to removal is 15 nodules $m^{-2}$, it would require $2.25 \times 10^{11}$ nodules to be produced and carefully deposited at the seafloor. Assuming each nodule costs 0.1 USD to produce, it would equate to 22.5 billion USD to produce the nodules required to restore the hard habitat in 1 claim area.

The maintenance of deep-sea species in aquaria has significantly advanced in the last decade through technological improvements that allow researchers to manipulate deep-sea organisms, avoiding damage caused by pressure variations during transportation and manipulation [148,149]. High-pressure tanks can maintain deep-sea organisms at *in situ* pressures, facilitating laboratory experiments to test recovery potential (e.g. in terms of growth rates, budding rates, larval settling and growth) from damage/*ex-situ* transplantation. Laboratory experiments on the cold-water coral *L. pertusa*, reveal high recovery potential because of its capacity to regenerate coenosarc tissue after skeletal damage caused by physical disturbance actions, for example, from mining or trawling [150]. One study advocates that young polyps of fragments of Mediterranean *L. pertusa* maintained in aquaria can have higher growth rates than *in-situ* [151]. Moreover, mineral accretion technology (based on the seawater electrolysis induced by low voltage and a low intensity electric field; [152]) that has proven to be effective for tropical coral restoration [153] can significantly enhance the survival and growth of *L. pertusa*, opening new possibilities for rehabilitating and restoring cold-water coral habitats [41]. Pilot transplantation experiments of nubbins of *L. pertusa* were carried out in the Gulf of Mexico and in the North Sea. Other transplantation experiments off the south-eastern coast of Florida were carried out with *Oculina varicosa*. These experiments provided evidence of high survival rates of both species [39,40,42], suggesting that transplantation of nubbins from healthy colonies to degraded habitats can be an effective method for cold-water coral reef restoration [42].

The MERCES project (Marine Ecosystem Restoration in Changing European Seas; www.merces-project.eu) aims to provide additional evidence that deep-sea restoration is feasible, either using coral nubbins transplantation techniques or using artificial habitats enabling a better recruitment of the larvae of some deep-sea species. In this way, it will be possible to assist regeneration and aid the recovery of areas impacted by human activities, although the long-term efficacy of these transplants has yet to be assessed. MERCES is an EU-funded project started in 2016 which focuses on developing new restoration actions of some specific kinds of degraded deep-sea habitats (hard bottoms). New technologies and approaches are now available to quantify the returns of restoration either in terms of ecosystem services and socio-economic impacts and defining the legal-policy and governance frameworks needed to optimize the effectiveness of the different restoration actions. In the framework of this project, active restoration experiments are focused on deep-water corals (in the Condor Seamount located in the north-eastern Atlantic Ocean) and compared with coral garden restoration at shallower depths. In the framework of the Work Package 4 ("Restoration of deep-sea habitats"), fragments of *Dentomuricea meteor* were collected, maintained in aquaria and transplanted with the use of benthic landers. Analyses of survival rate, growth rate and success of the experiment are still ongoing.

Further studies on active restoration protocols are needed, especially because the majority of available studies is focused only on cold-water corals that are also the most well mapped European deep-sea habitats [154]. Funding of scientific projects (as MERCES) could improve the comprehension of how restoration can be applied in the deep sea, how much time restoration actions could require to be effective and successful, how pilot experiments could be transposed at a broader spatial scale, which can be the criteria to establish the level of success of a restoration action.

Although the development of some promising tools to restore deep-sea degraded areas without compromising pristine habitats are in progress, the costs for scaling-up these pilot experiments are still uncertain. In addition, the long-term success of these experiments is unknown yet and we need to monitor the restored habitats to assess the efficiency and efficacy of the restoration action.

### 2.3. Deep-sea restoration: costs and benefits

Restoration of deep-sea ecosystems will surely challenge future decision makers evaluating the costs and benefits of maintaining ecosystem functioning and provisioning of goods against the economic benefits of exploitation activities. The costs required for restoration and major uncertainties of the outcomes raise major concern, in that restoration of deep-sea ecosystems might be 3–4 orders of magnitudes more expensive than for shallow water ecosystems [31]. As an example, the costs for the restoration of coastal habitats range from 0.8 to 1.6 million USD per hectare [155]. Despite these high costs, it appears that restoration in coastal areas can be successful and can provide tangible and social-economic benefits [156]. Both costs and benefits are more difficult to evaluate for deep-sea ecosystems [12].

Previous studies have considered hypothetical restoration actions in two impacted deep-sea ecosystems: the Darwin Mounds (UK) and Solwara I (Papua New Guinea) [30]. Economic estimates highlighted that restoring "Darwin Mounds" would cost about 75 million USD per hectare, whereas restoring "Solwara 1" would cost about 740 million USD per hectare [30]; these estimates for the deep sea exceed by orders of magnitude the average cost of restoring one hectare of marine coastal habitats [30]. In particular, for restoration of deep-sea ecosystems, the use of ships and equipment such as remotely operated vehicles would account for approximately 80% of these costs [30].

As far as the duration of the restoration action is concerned, restoration projects in coastal areas often last less than five years (due to the duration of grants, academic thesis, experimental trials; [155]). However, the expected lower recovery rates in the deep sea let to hypothesise decades for reaching comparable results (Table 2). Another problem is how to determine the success of restoration efforts. For example, restoration programs in saltmarshes and coral reefs demonstrate high survival rates of transplanted organisms that promote habitat recovery [155]. However, it is difficult to evaluate the real success of these operations because most of these restoration actions have been carried out on a spatial scale of few hectares or less, whereas the spatial scale of human impacts on coastal habitats is in the order of several thousands of hectares [119]. Clearer criteria for assessing the success of restoration actions should be established either for coastal (e.g., survival rate of transplanted seagrasses and corals; [119]) and deep-sea ecosystems, where in addition we need to expand our knowledge on the baseline conditions needed to establish the success of the recovery [30,157].

## 3. Conclusions and future perspectives

Despite high costs and uncertainties of the success, restoration of degraded habitats is considered as a priority for the next decades. On March 2019, the UN General Assembly has declared 2021–2030 as UN Decade on "Ecosystem restoration" [158]. In 2015, the United Nations defined a list of 17 Sustainable Development Goals (SDGs) for 2030. One of these goals (SDG14, life below water) is specifically dedicated to the conservation and sustainable management of marine life, including restoration of marine degraded habitats [159].

The IUCN (International Union for Conservation of Nature) has provided guidelines for restoration practices of coastal marine ecosystems [160]. To date, no guidelines have been established for the restoration of the deep sea. As a step toward such guidelines, scientists have proposed that governments and the UN should commit to the development of a global Deep-Sea Ecosystem Monitoring Network (DEMN) that can provide new scientific knowledge to inform sustainable management of the deep ocean [6]. DEMNs will require a high level of funding to sustain the research that will support development of effective conservation and restoration actions.

Policy makers, before making decisions on exploitation of deep-sea resources, should take into consideration the following: i) avoid actions of irreversible degradation, ii) plan in advance the mitigation of the impacts caused on deep-sea ecosystems and iii) assess the costs for restoring the degraded deep-sea habitats [30,38]. It is also evident that restoration (both passive and active) actions for the deep sea should be identified before exploitation, and when restoration actions are deemed unrealistic or inconvenient after a cost-benefit analysis, the society should be properly informed. Policy makers should also consider that a loss of ecosystem functions could cause long-term loss of goods and services and have negative economic consequences. If the costs of restoration overtake the benefits obtained by exploiting activities, policy makers should consider carefully the convenience of such activities. Protection alone can be insufficient to reverse the degradation of deep-sea habitats so that restoration will be increasingly required in the future. Finally, policy makers should require the adoption of common internationally standardised guidelines for the restoration of the deep sea. We also need to improve and share our knowledge through international cooperative research and understand that ecosystem restoration will be the priority action for the compensation of the environmental impacts caused by the exploitation of natural resources in the deep sea. With global imperatives to restore marine ecosystems, accumulating knowledge from scientific research, environmental baseline studies, and other sources, an optimistic view is that the science of deep-sea restoration will mature rapidly and that application of restoration actions in the deep sea may be an opportunity for a new and competitive blue-growth sector.

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## Author contributions

All authors equally contributed to this work.

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