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Settore Scientifico Disciplinare: BIO/07 – Ecologia

Brown macroalgae transplantation as habitat restoration technique: methods, effectiveness, and concerns

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CICLO XXXII
ANNO CONSEGUIMENTO TITOLO 2020

Content

General introduction

Regime shifts and alternative stable states in intertidal rocky habitats: focus on intertidal canopy forests

Human activities can affect a multitude of ecological process acting at different spatial scale, from local to global (Folke *et al*., 2004). The synergistic and long-lasting effect of both anthropogenic pressure (such as overexploitation of resources, pollution, urbanization, and introduction of invasive species) and climatic-driven stresses (e.g., temperature increase, ocean acidification, and increased frequency and strength of extreme events), can deeply modify the structure and function of ecosystems (Airoldi & Beck, 2007; Claudet & Fraschetti, 2010; Micheli *et al.*, 2013; Hawkins *et al.*, 2015). In extreme cases, ecosystems can respond to perturbations with an abrupt switch from a state to another (i.e., "regime shift"), often dramatically different from the initial state (Sheffer, 2009; Conversi *et al.*, 2014). Regime shifts are observed and verified for a great number of habitat, ranging from terrestrial (Dublin *et al.,* 1990; Wang & Eltahir, 2000; Higgins *et al.,* 2002; Peterson, 2002; Anderies *et al.*, 2003; Danell *et al.*, 2003) to marine environments (Knowlton, 1992; Norström *et al.,* 2009; Steneck *et al.,* 2013; Bozec & Mumby, 2014; Filbee-Dexter & Scheibling, 2014; Jouffray *et al.*, 2015; Bulleri *et al.*, 2016; Piazzi *et al.*, 2016).

Regime shifts are commonly observed in many marine systems: in particular, coral reefs (Done, 1992; Knowlton, 1992; Hughes, 1994; Norström *et al*., 2009; Bozec & Mumby, 2014; Jouffray *et al*., 2015), kelp forests (Simenstad *et al*., 1978; Steneck *et al*., 2013; Filbee-Dexter & Scheibling, 2014), and sea urchin-coralline barrens (Sala et al., 1998; Agnetta *et al*., 2015; Bulleri *et al*., 2016; Piazzi *et al*., 2016) are those studied most in-depth.

Rocky intertidal habitats are complex systems with unique characteristics, since these open ecosystems are subject to steep environmental gradients and characterized by a high associated biodiversity (Thompson *et al*., 2002), which gives them considerable ecological, socio-economic, and conservation value (Raffaelli & Hawkins, 1996). Furthermore, the fact

that rocky environments are constantly exposed to natural and anthropogenic pressures, makes the occurrence of regime shifts in these systems more likely (Hawkins *et al.*, 2015).

Particular attention has been paid to shifts involving habitat-forming organisms, since they may have severe repercussion on entire ecosystems and, consequently, on the services that they provide to human society (Huges *et al.*, 2003; Folke *et al.*, 2004). In fact, often the loss of a habitat-forming species can lead to shifts from complex and heterogeneous habitats to degraded and simpler habitats, which are considered less desirable as they are characterized by a lower productivity and biodiversity (Folke *et al.*, 2004).

Canopy-forming brown macroalgae (mostly kelps and fucoids, respectively belonging to Laminariales and Fucales) are the dominant habitat-forming organisms of both intertidal and subtidal environments of temperate shores (Steneck *et al.*, 2002). These species, which are able to form extensive canopies, are often compared to terrestrial forests (Jones *et al.*, 1994; Gianni *et al.*, 2013), and play a fundamental role as primary producers in coastal areas, regulating nutrient cycling (Verdura *et al.*, 2018). Furthermore, structurally complex macroalgae are able to host more heterogeneous and abundant assemblages with respect to other algae with a simpler structure (Chemello & Milazzo, 2002; Hooper & Davenport, 2006). In fact, they provide a complex three-dimensional habitat that enhance the available living space and supply a higher variety of food resources (MacArthur, 1965; Orav-Kotta & Kotta, 2004; Willis *et al.*, 2005). Moreover, they can reduce the negative interspecific interactions, such as competition (Marx & Hermkind, 1985) and predation (Warfe & Barmuta, 2004), and the effect of the wave stress (Gregg & Rose, 1982). Providing shelter, food, and disposable habitats for a great variety of organisms, these macroalgae are, thus, able to host and sustain highly heterogeneous communities, enhancing the biodiversity and trophic complexity of rocky ecosystems (Angel & Ojeda, 2001; Graham, 2004; Schiel & Foster, 2006).

Compared to other marine and terrestrial complex systems which are showing a substantial global decrease, marine forests are displaying a different trend with an average small global decline and a massive local-scale decline (Krumhansl *et al.*, 2016). This means that local stressors, in particular those that involve habitat destruction or modification of environmental conditions (mainly of anthropic origin, such as pollution, overfishing, harvesting, etc.), have a crucial role in determining the disappearance of these species in many coastal areas, especially the urbanized ones (Airoldi & Beck, 2007; Strain *et al.,* 2014). Combined impacts of both

anthropogenic pressures and environmental/climate stresses have as most noticeable consequence the loss of canopy-forming seaweeds, which are replaced by smaller and opportunistic algae (e.g. turf and ephemeral algae), invertebrate beds (e.g. mussels/barnacle), or barren environments (e.g. urchin/coralline barrens) (Petraitis & Latham, 1999; Benedetti-Cecchi *et al.*, 2001; Mangialajo *et al.*, 2008; Falace *et al.*, 2010; Templado, 2014). Once established, these communities are able to inhibit the recolonization of space from macroalgae, forming persistent, simpler, and less productive habitats (Strain *et al*., 2014).

Several are the examples of macroalgae recovery from disturbances at different spatial scales, ranging from local- (e.g. outbreaks of herbivores; Estes *et al.*, 1989; Guidetti, 2006) to large-scale (e.g. El Nino-Southern Oscillation Dayton *et al.*, 1992; Edwards & Estes, 2006), but they are mostly referred to short-lived and/or high dispersal capacity species (Blanfuné *et* al., 2019). In fact, most of the degraded canopy habitats have not recovered, becoming locally extinct in many temperate areas around the globe, even though the implementation of substantial conservation measures (e.g. establishment of Marine Protected Areas, inclusion in international protection agreements, improvement of water quality, etc.). This lack of recovery is likely due to a combination of different drivers, such as modification of habitat and biotic conditions, demographic factors (e.g. reduction of fertility), and/or low dispersal potential (Airoldi *et al.,* 2009; Reed *et al.,* 2004; Schiel & Foster, 2006; Perkol-Finkel & Airoldi. 2010). In particular, low dispersal capacity of these species is a factor that may impair their natural recovery of canopy habitats, especially when natural populations disappear and adult individuals are missing (Falace *et al.*, 2018). In these cases, passive conservation measures are not enough to restore degraded habitats**,** highlighting the necessity for developing active interventions to recover missing canopy forests (Falace *et al.*, 2006; Susini *et al.*, 2007; Campbell *et al.*, 2014; Marzinelli *et al.*, 2016).

Macroalgae transplantation as habitat restoration method

Because of the relevant effect that habitat-former species have on ecosystems, canopy macroalgae are often the primary objective of restoration efforts, with the double aim of resettling both the biogenic structure of the habitat (structural recovery), and its associated

biodiversity and services (functional recovery) (Goodsell & Chapman, 2009). Despite the fact that the practicability of active restoration remains a discussed and controversial topic, especially for what regards habitat of remarkable ecological importance (such as coral reefs and seagrasses meadows; Jaap, 2000; Fonseca *et al*., 1996; Bell *et al.,* 2008), some life traits of macroalgae (e.g., fast growth-rates and short life spans) seem to play a relevant role in making the restoration of canopy forests an interesting conservation option (Carney *et al.*, 2005; Hernàndez-Carmona *et al.*, 2005; Yu *et al*., 2012).

To date, transplantation of individuals collected from natural and healthy populations has been proposed as a primary method for restoring brown macroalgae forests. Several attempts with different brown macroalgal species, both kelp (Vàsquez & Tala, 1995; Hernandez-Carmona *et al*, 2005; Dean & Jung, 2001; Carney *et al.,* 2005; Correa *et al.*, 2006) and fucoids (Falace *et al.*, 2006; Susini *et al.*, 2007; Sales *et al.*, 2011; Perkol-Finkel *et al.*, 2012), have been made using diverse techniques and obtaining variable outcomes. All of these techniques were simple and low-cost, so they represented attractive restoration options; nevertheless, logistic difficulties (such as the time and efforts needed to appropriately fixing the individuals to the substratum, or for the installation of transplanting devices in the chosen area) and ethical reasons, given the impact they have on natural populations, allowed only small-scale attempts.

Given the huge ecological importance and the critical conservation status of most of these species, the search for less disruptive transplanting solutions is increasingly a topic of primary importance (Gianni *et al.*, 2013). The most frequently used techniques are the release of gametes and zygotes, or the installation of fertile material (such as mature fronds and receptacles) in the area of interest in order to improve the recruitment potential. Outplanting is also a feasible option, involving the production of recruits under controlled laboratory conditions with the purpose of being subsequently transplanted into the field. Outplanting is also an ecologically sustainable method, which ensures the production of large numbers of germlings, allowing to plan large-scale restoration measures (Falace *et al.,* 2018).

Objective of the study

The aim of this project is to evaluate different transplantation methodologies, using as model organisms brown macroalgae of ecological interest present in the Mediterranean Basin and the Atlantic temperate region.

Chapter 1 is focused on the evaluation of the main proposed method for the transplantation of the Mediterranean fucoid *Cystoseira amentacea* var. *stricta*, assessing the efficiency and possible secondary effects of the transplantation method itself. The used technique involved the transplantation of adult thalli, giving us the possibility to examine also eventual changes in the structure of the associated epifaunal community.

In the **Chapter 2**, the efficiency of the transplantation method for the kelp species *Laminaria ochroleuca* was assessed. The experiment involved the transplantation of three different life stages of the macroalga, examining eventual differences in terms of mortality and growth rates as proxy of the success of the transplantation. Furthermore, it was evaluated the influence of macroalgal canopy on the survival of the individuals after the transplantation, placing the replicates at different distances from the kelp patch.

Chapter 1

Adult thalli transplantation: a manipulative experiment with *Cystoseira amentacea* var. *stricta* on the Northern Sicily coast

Abstract

Brown macroalgae of genus *Cystoseira* play a key role in structuring intertidal communities. In the Western Mediterranean basin, *Cystoseira amentacea* var. *stricta* is the dominant organism in upper infralittoral rocky shores, forming monospecific belts that can sustain complex and heterogeneous communities. Due to its fundamental ecological role and to its endemic status in the Mediterranean, *Cystoseira amentacea* has been protected by several international conventions. Despite significant conservation efforts, however, in the last few years *Cystoseira* populations have experienced a dramatic decline in many Mediterranean coastal areas, highlighting the urgent need for active intervention. For this reason, transplantation of individual adult thalli of *Cystoseira* from healthy populations has been proposed in order to restore degraded forests. While previous experiments focused on the development of an adequate transplantation method, with the survival rate of the transplanted thalli as the main outcome, none of these studies ever considered possible side effects that could lead to early death of the transplanted individuals. Here, we conducted a manipulative experiment using *Cystoseira amentacea* to evaluate, in addition to the success of the transplant, possible secondary effects due to the transplantation technique itself, and examining variables concerning morphology, structural complexity and stress response of the transplanted algae, as well as the structure of the associated community.

Results showed that the transplantation technique may modify the physical structure and the complexity of the transplanted thalli, potentially influencing also their fatty acid composition and phenolic content. Furthermore, it can affect species composition and abundance of individuals of the macrofaunal community.

1.1. Introduction

The Mediterranean is an enclosed sea characterized by extremely reduced tide cycles, oligotrophic waters, high salinity, and high superficial water temperature (Ros *et al.*, 1985). All of these features prevent the development of kelp beds, but are relatively favorable for Fucales, in particular for what regards the genus *Cystoseira* (Roberts, 1978).

Cystoseira species C. Agardh (Fucales, Phaeophyceae) are caespitose algae characterized by a single thallus attached to the substratum by a conical disc or by haptera (Gòmez-Garreta *et al.*, 2002). These algae are perennial, but they undergo seasonal morphological changes; they have a monogenetic diplontic life cycle and a high reproductive potential, since they produce numerous, large, and easily sinking zygotes. Whilst these reproductive features are an advantage for what regards the formation of dense mono-specific forests, they allow for lower the dispersal capability of the species (Johnson *et al*., 1998; Gaylord *et al.*, 2002).

Cystoseira species are well distributed along all the Mediterranean Sea and in the Atlantic Ocean (Draisma *et al*., 2010), ranging from superficial waters up to the upper circalittoral zone (Falace *et al.*, 2018). Their distribution range is controlled by several environmental factors, such as water temperature, depth, wave exposure and nutrient concentration, geomorphology and substratum features (Falace & Bressan, 2005; Sales & Ballesteros, 2009; Lasinio *et al.*, 2017; Mancuso *et al*., 2018).

The genus *Cystoseira* is represented by a total of 42 species in all of its distribution area, of which about 30 species are present and endemic in the Mediterranean Basin, which is thus considered a hot-spot for *Cystoseira* (Cormaci *et al*., 2012). In particular, in the Western Mediterranean basin *Cystoseira* populations represent the dominant organisms of the infralittoral fringe (e.g., the shallowest level of the infralittoral zone), forming dense forests that occupy large areas (Menge & Branch, 2000; Mangialajo *et al.*, 2012). Due to its morphological characteristics, the genus *Cystoseira* has a crucial functional role in structuring intertidal and subtidal rocky systems, providing also a lot of ecosystem services (Jones *et al*., 1994): in fact, *Cystoseira* forests are some of the most important “habitat engineers”, increasing three-dimensional structural complexity and spatial heterogeneity of rocky

systems, and providing refuge and food to their associated communities; furthermore, they enhance the productivity, sustain complex food webs, and maintain high levels of biodiversity in coastal systems (Gianni *et al.,* 2013; Mineur *et al.*, 2015; Piazzi, 2018).

In the last few years, populations of *Cystoseira* have been experiencing a dramatic decline in many Mediterranean locations, in particular in urbanized coastal areas (Benedetti-Cecchi *et al.*, 2001; Thibaut *et al*., 2005; Perkol-Finkel & Airoldi, 2010), due to the synergistic and cumulative effects of anthropogenic local disturbances and climate stresses. The human pressures that contribute more to the disappearance of *Cystoseira* populations are those which cause habitat destruction and modification of environmental factors: in particular, the recent increase in coastal urbanization can alter substrate, hydrodynamic conditions, sediments, nutrients, and chemical pollutants (Airoldi, 2003; Airoldi & Beck, 2007; Arévalo *et al*., 2007; Ludwig *et al.*, 2009; Sales & Ballesteros, 2009; Sales *et al.*, 2011). Loss of *Cystoseira* patches can also be attributed to the outbreaks of herbivores, in particular, sea urchins (Verlaque, 1984; Gianguzza *et al.*, 2011) and the fish *Sarpa salpa* (Vergés *et al.,* 2009), usually in relation with over-fishing of their main predators. Furthermore, other potential impacts may be agricultural practice, aquaculture, and sampling for scientific research (Gianni *et al.*, 2013).

With the loss of canopy, *Cystoseira* forests are often replaced by other algal species with lower structural complexity, such as turf, encrusting, and filamentous algae. Once established, these organisms are able to inhibit recolonization of space from canopy macroalgae, often forming undesirable and irreversible alternative stable states (Strain *et al.*, 2014; Chemello *et al.*, 2018).

Because of their sensitivity to anthropogenic pressures, all of the *Cystoseira* species (with the exception of *Cystoseira compressa*) are included in the Annex II of the Barcelona Convention, and protected by the Bern Convention; furthermore, *Cystoseira* species are listed as "of community interest" according to the Habitat Directive (92/43/EEC; EEC, 1992), are indicators of good environmental quality according to the Water Framework Directive (2000/60/EC; EC, 2000) and CARLIT method (Ballesteros *et al.*, 2007; Blanfuné *et al.*, 2017), and considered vulnerable species by several international organization (such as IUCN and MedPan).

Despite substantial conservation efforts, many of the degraded systems are not able to naturally recover (probably because of the small dispersal range of the macroalga; Clayton,

1990; Dìez *et al.,* 1999; Soltan *et al.*, 2001), highlighting the need of active intervention measures. For this reason, *Cystoseira* transplantation has recently been attempted, using different techniques: the most common method is the transplantation of juveniles or adult thalli (Falace *et al.*, 2018). Previous studies focused mostly on the development of an adequate methodology of transplantation, examining as main result the survival rate of the transplanted thalli over time, without considering possible causes of early death or eventual changes, both morphological and physiological, in the transplanted individual with respect to natural populations, which might be due to the transplantation technique itself.

1.1.1 Macroalgae morphology, complexity, and relation with epifaunal community structure

Complexity is defined by the small-scale features of a habitat such as the "size, shape, surface texture and degree of angularity of a substrate, and their relationship to inter-substrate spaces" (Gee & Warwick, 1994). Previous studies generally showed a positive trend in the relationship between habitat complexity and structure of the associated community: in fact, increasing complexity may lead to an increase in diversity and abundance of organisms, in relation to modification of environmental features such as living space (Morse *et al.*, 1985), variety of food resources or feeding surfaces (Fretter & Manley, 1977), changes in microenvironmental condition, and reduction of predation (Coull & Wells, 1983; Gibbons, 1988, Warfe & Barmuta, 2004) and competition (Edgar, 1983; Marx & Hermkind, 1985).

Quantifying complexity in macroalgae can be a challenging issue, since the concept of complexity includes both qualitative and quantitative features: the term "habitat architecture" refers to relative abundance of different structural elements (qualitative aspect), whilst the "habitat size" refers to the absolute abundance of these elements (quantitative aspect) (Veiga *et al*., 2014).

Until recently, the most commonly used indexes to describe complexity have taken into consideration only its quantitative characteristic, reducing to the evaluation of biomass, volume, or surface area of algae, which are generally inappropriate as they fail to adequately represent the subtle structure of substrates, and do not allow any comparison among

different substrates (Gee & Warwick, 1994); moreover, these indexes do not consider the habitat scale, and how it can influence organisms of different sizes (Hicks, 1985; Taniguchi *et al*., 2003).

With the introduction of fractal geometry, the possibility of using an alternative method to defining structural complexity of habitat through the use of fractals has been opened up. Comparing to the other measures, fractal dimension provides a numerical expression of complexity which is entirely independent of the nature of the habitat, and is also related to the scale at which the habitat is perceived from organisms of different size which inhabit it (Gee & Warwick, 1994).

Presence and abundance of complex macroalgae can contribute in increasing the three-dimensional structure of the substratum, influencing also the characteristics of associated communities such as diversity and dimension (Chemello & Milazzo, 2002; Hauser *et* al., 2006; Hooper & Davenport, 2006; Warfe & Barmuta, 2006, Veiga *et al.*, 2014). In fact, taking into consideration small spatial scale of observation (10^{-2} - 10^{-3}m), complex macroalgae can provide a considerable amount of available space for a wide range of organisms (Jones & Andrew, 1992). However, different macroalgal species do not support benthic fauna in the same way (Williams & Seed, 1992): differences in life cycles and, in particular, their presence and persistence in a given habitat (e.g. perennial algae are available for colonization for longer periods comparing to algae with annual cycles, resulting in a greater abundance of associated fauna; Marzinelli *et al*., 2015), the production of chemical compounds used for defense and protection, or differences in algal architecture (Duffy & Hay, 1994), are characteristics that can strongly affect abundances and relative composition of associated community.

Therefore, differences in structure and complexity of macroalgae can significantly affect the associated community that use them as habitats. This assumption might be valid not only for what regards different algal species, but also in case of structural alteration of individuals belonging to the same species, since previous studies highlighted the possibility that transplantation may influence the morphology of transplanted thalli in comparison to natural populations. Falace *et al.* (2006) showed that the transplanting techniques used on a manipulative experiment on *Cystoseira compressa* caused a stress on the transplanted individuals, which resulted in changes of the algae structures: in fact, transplanted thalli showed significant differences in morphology features with respect to unmanipulated thalli,

with the transplanted ones showing loss of primary branches, and a less flourishing and more prostrate aspect.

Thus, if transplantation process may alter morphology and complexity of the algae, it would be plausible that it could also likely influence the community associated to the transplanted plants, which might significantly differ, in terms of biodiversity and abundance, from those that characterize natural macroalgal populations. This negative effect could represent a potential issue in the management of reforestation plans: indeed, although all of the restoration projects that involve habitat-forming organisms (such as coral reefs and seagrass meadows) have as primary objective the structural recovery of the habitat, it must also be remembered that the real success of a restoration process is achieved when also the functional recovery of the system is obtained, i.e. the recovery of the associated community in terms of biodiversity and trophic structure (Marzinelli *et al*., 2016; Nordström *et al.*, 2015; McSkimming *et al.*, 2016). Recolonization of epifauna back onto its host macroalga after an experimental disturbance can take from 1 to 10 days, but this concerns only small spatial scales (e.g. tens of meters; Poore, 2005). Nevertheless, little is known for what regard experiments on larger spatial scales, but previous studies suggest that restoration of associated organisms can be a complex process that potentially requires a long period of time (>18 months; Marzinelli *et al*., 2015).

1.1.2 Stress response

Changes in environmental variables (e.g. desiccation, changes in temperature, pH, salinity, etc.) and biotic factors (such as herbivory) can act as stress drivers, influencing life cycle, distribution, and also survival of seaweeds (Flores-Molina *et al.*, 2013; Diaz-Pulido *et al.*, 2012; Vergés *et al.,* 2009). Seaweeds, especially those which inhabit the intertidal zone and thus are exposed to stressful conditions, have developed survival strategies that involve biochemical and physiological adaptations to the stress (Eggert, 2012), such as accumulation of lipidic antioxidants, the activation of antioxidant enzymes (Cockell & Knowland, 1999), and the production and accumulation of secondary metabolites that can be used for

protection (in particular in the role of photoprotectors), and defense against grazers and epiphytes (Amsler & Fairhead, 2006; Amsler, 2008; Sudatti *et al*., 2011).

Fatty acids - With the term "fatty acids" (FA) are defined carboxylic acids with long aliphatic chains that can be classified on the number of present double bonds as monounsaturated FAs (MUFAs, 1 double bond), and polyunsaturated FAs (PUFAs, ≥ 2 double bonds). Furthermore, PUFAs are classified as *n-3* or *n-6* FAs depending on the position of the first double bond from the methyl end (Kumari *et al.*, 2013).

Fatty acids are fundamental components of the cell membrane and of storage lipids, and their composition can undergo to seasonal variations in response to changes in environmental variables, depending on the physiological state of the algae (Gerasimenko *et al.*, 2011; Kim *et al.*, 1996; Nelson *et al.*, 2002). Environmental stressors, such as variation in salinity, temperature, light, nutrients, as well as chemical pollutants and desiccation are known to be associated to the formation in the seaweed cells of reactive oxygen species (ROS), causing "oxidative stress" (Dring, 2006): this kind of stresses are able to inducing fluctuation in the fluidity of the cell membranes (Mikami & Murata, 2003), which is one of the first adaptive response to achieve the acclimation to these stresses.

Fatty acids (especially PUFAs and sterols) are considered functional healthy compounds of human diet, since they are known to have antioxidant, antidiabetic, anti-inflammatory, and antibacterial properties (Mendis & Kim, 2011; Mišurcová *et al.*, 2011; Nomura *et al.* 2013) and, for this reason, are being increasingly exploited for nutritional purposes (Pereira *et al.*, 2012). At the present date, the main source of these elements are marine fishes like tuna and mackerel, but the serious depletion of fish stocks has highlighted the need to find new sustainable alternative sources (Vizetto-Duarte *et al,* 2015).

Although algae contain significantly smaller amounts than marine fishes in lipid content, they can still be considered valid alternatives due to their natural large stocks. In particular, brown macroalgae are one of the most promising algal class, in relation to the considerable amount of PUFAs contained in several species with respect to other algal phyla (Colombo *et al.*, 2006; Silva *et al.*, 2013).

Depending on this, the majority of the existent studies on macroalgae focuses on a nutraceutical perspective, encompassing lipid and fatty acid composition, their metabolic

pathways, and genes and enzymes involved in their synthesis and regulation, while the response mechanisms of macroalgae to environmental changes, in terms of variation of fatty acid composition, are still poorly understood.

Phenolic compounds - Phenolic compounds (or polyphenols) are products of the secondary metabolism of algae, and consist of a hydroxyl group (–OH) directly bonded to an aromatic hydrocarbon group (Waterman & Mole, 1994). Polyphenols assume different roles during the entire life cycle of the algae, both for early stages and adult plants, such as cell-wall formation, adhesion, as well as contribution to growth and reproduction phases (Schoenwaelder, 2002). Differences in the content of these compounds among various algal species have multiple reasons, both taxonomic, ecological, and environmental. Previous studies showed that concentration of polyphenols is subjected to changes in response to different seasons, habitats, and variation in environmental variables such as salinity, UV irradiation, light conditions, and nutrient availability (Hemmi & Jormalainen, 2002; Fairhead *et al*., 2006; Connan *et al.*, 2006; Svensson *et al*., 2007; Jormalainen & Honkanen, 2008). Phenol concentration may vary depending on the algal species (Van Alstyne *et al.*, 2001), but also intra-specific differences are known, with variation in the distribution of these compounds within the same individual, and among different plants of the same species (Freile-Pelegrìn *et al.*, 2013).

Normally in brown macroalgae, polyphenols are more abundant in young and actively growing individuals. Species characterized by apical growth, such as Fucales, have the highest amount of these compounds in the upper part of the branches (Hay & Fenical, 1988), whilst those with meristems responsible for the algal growth (e.g. *Laminaria* species, *Ascophyllum nodosum* or *Sargassum muticum*) have the highest concentration in the meristematic area (Connan *et al.*, 2006).

Fucales are relatively rich in phenolic compounds (Targett & Arnold, 2001; Lee, 2008), which are known to be mainly used as photoprotectors and deterrent against herbivores. In particular, *Cystoseira* species which are highly palatable macrolgae subjected to be grazed by both fishes and sea urchins, contain a high diversity of phenols, a characteristic that could concern the deterrent properties of these compounds, making these macrolgae a less-attractive food in comparison with other algal species (Amico, 1995).

The chemical processes involved in the defense mechanism against biotic factors (deterring properties for predators, pathogens, and other competitors) are the most and best studied, but, despite the ecological importance of these macroalgae, still few are the studies dealing with variation in phenolic content in relation to changes in environmental variables (Mannino *et al*, 2016).

All the adult transplantation procedures involve the displacement of individuals from a donor site to a transplantation site, in which the habitat conditions could be significantly different from the initial location, so the transplanted thalli could be easily subject to a distress due to the changes in environmental variables. Furthermore, physical damage of the thalli due to the mechanical removal of individuals from the substratum and their manipulation is likely to happen.

Since all of these factors could trigger a stress response in the macroalgae, the examination of both the two metabolic pathways could be a potential useful tool in order to identify eventual subtle effects of the proposed transplantation technique that could lead to an early death of the transplanted thalli.

1.1.3 Objective of the study

The aim of this study is to assess the effectiveness of the adult transplantation method (proposed by Susini et al., 2007, and Mangialajo et al., 2012) for the intertidal species *Cystoseira amentacea* var*. stricta* (Montagne, 1846), also exploring possible side effects that have not been considered previously. For this reason, a manipulative experiment has been conducted in order to evaluate, in addition to the success of the transplant, also possible secondary effects due to the manipulation of the thalli, and to the transplantation technique itself. In detail, the success of the transplantation will be assessed by counting the remaining transplanted thalli over time, whilst eventual secondary effects will be evaluated by comparing the transplanted individuals with unmanipulated thalli, and examining variables concerning morphology, structural complexity and stress response of the algae, as well as the structure of the associated community, in terms of biodiversity and trophic structure.

Cystoseira amentacea var. *stricta* is diffused in all the Italian seas with the exception of northern Adriatic. In the Mediterranean basin, the species is also found in Spain and Balearic Islands, France and Corsica, Turkey, Malta, Greece, Algeria, Libya, Morocco, and Tunisia. *C. amentacea* is a caespitose plant up to 20–35 cm height, which forms extensive belts on shallow and wave-exposed rocky shore, and is able to tolerate regular emersion caused by wave-movements and tides. Thalli are brown-green colored, due to the accumulation in the tissue of pigments such as fucoxanthin and other xanthophylls, and sometimes can present iridescent fronds. The basis of the thallus is made by creeping axis which adhere to the substratum through rhizoids; from the basal structure cylindrical axes develop, ranging from 5 to 10-15 cm long. Primary branches are cylindrical, the secondary ones are inserted on the primaries quite high, showing decreasing length from the basis to the apex, and giving to the alga a conical and elongated appearance. *C. amentacea* is a perennial species: the axis is present year-round, while the branches are deciduous. Sexual reproduction is guaranteed by production of gametes, which are produced in fertile areas called receptacles located at the apex of terminal branches. Reproduction and production of embryos take place in the spring season, whit fertile thalli found from spring until late autumn.

We chose *Cystoseira amentacea* var. *stricta* because of its peculiar characteristics: it is a Mediterranean endemic species that colonizes the upper infralittoral zone, where it forms dense monospecific belts; also, this species is known to be able to sustain complex associated communities, and it is easy to sample. All of these features make this brown macroalga an ideal species for these kind of manipulative field experiments.

1.2. Materials and methods

Sampling sites

The field experiment was carried out near Palermo (Sicily, Tyrrhenian Sea), in a coastal area approximately 10 km away from the city (38° 12’ 37,4’’N, 13° 17’ 11,8’’E).

A pre-survey has been conducted in order to properly choose the experimental area. We selected two different sampling sites a few hundred meters apart (Barcarello and Capo Gallo; fig.1) on natural rocky shore characterized by carbonate substrate, which are similar in terms of orientation, light, hydrodynamic conditions, and presence of natural and homogeneous *C. amentacea* populations. In both the two sites, *Cystoseira amentacea* var*. stricta* forms dense and continuous belts in the infralittoral fringe, in strict association with calcareous algae (*Lithophyllum lichenoides* Philippi, 1837), and biogenic constructions formed by vermetid molluscs of the species *Dendropoma cristatum* Biondi, 1859, which can host complex communities, increasing considerably the local biodiversity (Chemello *et al.*, 1988; Donnarumma *et al*., 2018). The biocenosis of photophilic algae of the infralittoral zone are also characterized by the presence of diverse species of *Cystoseira* (Mannino & Mancuso, 2009), which alternate with sandy areas occupied by tufts of *Posidonia oceanica* Delile, 1813.

Figure 1 - Study area and sampling sites (Barcarello and Capo Gallo)

Experimental setup

The experiment started at the end of May 2018, since previous experiments found that late spring seems to be the best period to attempt a *Cystoseira* transplant, as the alga is in its reproductive phase (Falace *et al.*, 2006), and ended in September 2018, when thalli begin the regression after the reproductive period. We chose to create interspersed removal plots inside the natural *Cystoseira* belt, to have a greater certainty of an adequate site selection.

The transplantation technique was made in three phase: 1) clearing of all the removal plots (20x20 cm each) by removing all the macroalgal canopy, and scraping the area to bare rock; 2) drilling the substratum creating 18 mm diameter holes; 3) individual thalli were collected using a chisel with their holdfast still attached to the substrate, and transplanted to a single hole using epoxy putty (Stucchi Veneziani). In this phase, particular attention was paid to hydrodynamic conditions, since the epoxy putty requests a long amount of time to catalyze (about 24 hours), and the adequate fixation of the thalli to the substratum is a fundamental step for the success of transplantation.

For each removal plot 8 thalli were transplanted (all of them with similar size), since due to the procedure of the transplantation technique (i.e. the time and the considerable physical effort requested to drilling the substratum, and also the long catalyzation time of the epoxy putty), it results impossible to simulate the natural density of *Cystoseira* individuals (*C. amentacea* holdfasts are able to cover more than 30% of the substratum; Mangialajo *et al*., 2008). Thalli were taken from nearby donor *Cystoseira amentacea* populations, removing the epifauna before the transplantation by quickly washing each thallus in freshwater before transplanting it. For each site, 20 random plots were cleared, with a total number of 320 transplanted thalli.

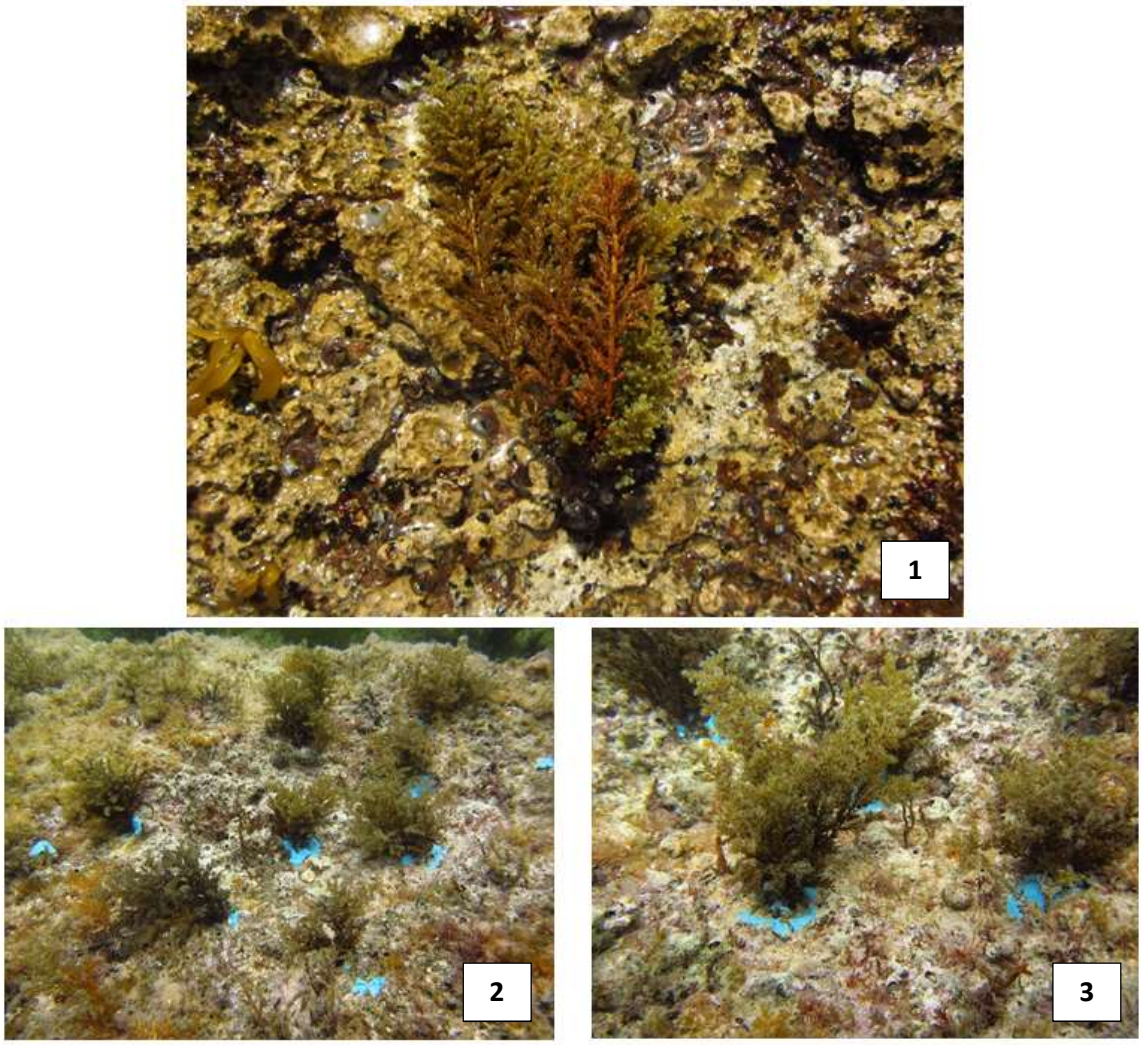

Figure 2 – 1) Individual thallus of Cystoseira amentacea var. stricta before the transplantation; 2) removal plots, and 3) close-up on transplanted individuals

Sample collection

We selected four sampling dates after the transplantation (T0): T1, after two weeks; T2, after a month; T3, after two months, and T4, after three months. In each date, ten thalli were randomly harvested (five for each type of analysis, e.g. morphology, stress) from the sampling plots, and the same amount of thalli were harvested from the unmanipulated natural population, in order to use them as control. Each individual was carefully placed in a separated plastic bag, preventing the escape of the vagile organisms associated to the algae.

At T0 and at each subsequent sampling date, data on physicochemical variables (e.g. water temperature, salinity, and conductibility) were obtained for each sites, using a multi parametric probe.

All the collected samples were kept under cold conditions until arrival at the laboratory, and stored at -20°C (samples dedicated to morphology/complexity and community structure), or rinsed in deionized water, and then stored at -80°C (stress analysis) before the processing phase and subsequent analysis.

Sample processing and laboratory analysis

Macroalgal samples, both transplanted and unmanipulated thalli, went throug different procedures depending on the type of performed analysis.

Macroalgal morphology/structural complexity and epifaunal assemblage biodiversity

Individual thalli were placed on a sieve (0.5 mm mesh size) and washed under running freshwater to remove the associated macrofauna. Obtained specimens were later identified with the aid of a binocular microscope to the lowest possible taxon (usually species level) and counted. Furthermore, eventual presence of algal epiphytes on the thalli was noted.

To obtain information on structure of the macroalgae (sensu Hacker & Steneck, 1990), three types of volume were measured:

- Canopy Volume (CV): volume (expressed in milliliters) defined by length, width, and height of the alga immersed in the water;
- Thallus Volume (TV): volume (expressed in milliliters) of the thallus given by the water displacement that occurs when the alga is totally immersed in a graduate cylinder;
- Interstitial Volume (IV): volume (expressed in milliliters) of water that occupies the space within the alga frond, obtained by subtracting TV from CV.

Canopy Volume was obtained as volume of a cylinder $CV = \pi \times r^2 \times h$ and h is the length of the alga from the base to the apical part of the frond, including eventual epiphytes; the radius

r was calculated by measuring the width in three portions of the thallus: apical (L1), median (L2), and basal (L3), and using the following formula: $r = \left(\frac{L1+L2+L3}{3}\right)/2$.

Thallus Volume was measured using a graduated glass cylinder (250 ml ± 2 ml) filled up to volume $v1$; after the immersion of the thallus, the new volume $v2$ is noted, and the thallus volume is calculated by the formula: $TV = v2 - v1$.

Interstitial Volume was simply obtained subtracting the Thallus Volume to the Canopy Volume: $IV = TV - CV$.

After the calculation of the volumes, a photographic sampling was performed to measure area, perimeter, and bi-dimensional fractal dimension of each thallus. Every individual was placed on a plasticized sheet of graph paper (28x38 cm), and then photographed with the aid of a copy-stand on which the camera is positioned, keeping the focal plane parallel to the subject. Subsequently, the photos were processed using the graphic software Photoshop CS5, as follows: 1:1 Image resizing, Background elimination, and application of the Stamp filter (light/dark balance 30, gradient 1). Each image is saved in .JPG format, and then imported into the fractal3 software (Sasak *et al.*, 1994), calculating both area and perimeter of individuals, and their fractal dimension (function: "Fractal dimension → Black"). Thalli with presence of epiphytes were photographed twice (before and after the removal of the epiphytes), always following the same procedure, in order to evaluate possible changes in structural and complexity parameters due to the presence of the epiphytes.

Stress analysis

Fatty acid analysis was carried out both on transplanted and unmanipulated thalli (n=5 for each condition). Only the apical portion of the frond of each thallus, corresponding to newly grown tips, was selected for the analysis. Samples were freeze-dried and ground. Lipid extraction was performed with an aliquot of 100 mg for each sample, following a modified version of the Bligh and Dyer (1959) method, and using a MilliQ distilled water:methanol:chloroform mixture (1:2:1 v:v:v) with 0.01% BHT (butylated hydroxyltoluene) to prevent lipid oxidation. Samples were then sonicated to improve lipid extraction, and then centrifuged twice to separate the lipid from the aqueous phase. The lipid extracts were evaporated to dryness under gentle nitrogen stream, weighed, and expressed as percentage

of total lipids. Then, lipid extracts were subjected to acid-catalysed transesterification using methanolic hydrogen chloride to obtain fatty acid methyl esters (FAMEs), which were then analyzed by a gas chromatograph (GC-2010, Shimadzu) equipped with a BPX-70 capillary column (30 m length; 0.25 mm ID; 0.25 µm film thickness, SGE Analytical Science), and detected by a flame ionization detector (FID). Peaks were identified by retention times from mixed commercial standards (37FAME from Supelco; QUALFISH from Larodan). Tridecanoic and tricosanoic acids (C13:0 and C23:0) were used as surrogate standards, while pentacosanoic acid methyl ester (ME C25:0) was used as internal standard for FAME quantification.

Analysis on **total phenolic compounds** was carried out both on transplanted and unmanipulated thalli (n=5), following a modified protocol from Harrison & Durance (1989) and Bolser *et al*. (1998). Samples were freeze-dried and ground, adding 2 ml of MetOH 80% to 4 mg of sample. Phenol extraction took place in 24 hours of incubation in dark conditions at 4°C. After centrifugation, the supernatant fraction was collected, and a calorimetric determination of total phenols was carried out with 20% Na_2CO_3 and Folin Ciocalteu reagents. Reaction time was about 2 hours, with constant stirring at room temperature. Finally, samples were read in a spectrophotometer at 765 nm. The results are expressed in mg/g of dry weight.

Data elaboration and statistical analysis

Transplantation success

Number of surviving transplanted individuals was considered as response measure of the transplantation success, and was obtained by counting the individuals left in all the clearing plots in each sampling date. Survival rate relative to each sampling time was also calculated by assessing the percentage of surviving thalli with the respect of the previous sampling time.

Macroalgae morphology and complexity

In order to test eventual differences in morphology and complexity between transplanted and unmanipulated thalli over the course of the experiment, data on volumes and fractal dimension were analyzed through the permutational analysis of variance (PERMANOVA, 9999 permutations) using PRIMER v6.1.10 & PERMANOVA software (Plymouth, UK). The model included three fixed othogonal factors, Site (2 levels, Barcarello and Capo Gallo), Treatment (2 levels, Unmanipulated and Transplanted) and Time (5 levels, T0, T1, T2, T3 and T4). Unfortunately, Capo Gallo site lost all the transplanted thalli before the end of the experiment: the last collected individuals (sampling date T3) were too small to allow a morphology examination and were therefore excluded from the analysis.

Community biodiversity

To gather information about the structure of the epifaunal community present on *Cystoseira* thalli, and to evaluate eventual differences between the communities associated to the two different treatments (Transplanted thalli vs Unmanipulated thalli), we assessed the main generic descriptors (i.e., abundance in terms of total number of individuals, taxa richness as total number of taxa, and the number of individuals per taxa).

PERMANOVA analysis was carried out for abundance and taxa richness data, in order to test if there were significant differences between treatments (Factor Treatment: 2 levels, Transplanted and Unmanipulated) in the two sites (Factor Site: 2 levels, Barcarello and Capo Gallo) over the time (Factor Time: 5 levels, T0, T1, T2, T3, and T4), with all the factors considered fixed and orthogonal. When PERMANOVA showed significant differences ($p < 0.05$), a pair-wise comparison was performed to explore differences among all pairs of levels of the factors.

In order to assess differences in the epifaunal community among sites, sampling times, and treatments, data on community diversity were were square-root transformed to downweight the influence of dominant taxa and consider all the species occurences, and resembled using Euclidean similarity distance matrix. Data were analyzed performing a Principal Coordinates Analysis (PCO) considering the interaction of all the factors “TimexSitexTreatment”, superimposing the main macrofaunal taxa to the graph.

Additionally, a SIMPER test was performed on the untransformed data to determine which taxa mostly contribute to differences between the different communities.

To explore the relationship between the architecture of the substratum and the structure of the associated community, correlation analyses among data of interstitial volumes and fractal dimensions of the thalli (both proxy of the complexity of the macrolgal substratum and the availability of living space for the epifaunal organisms), and data on abundance and taxa richness of the community were performed.

Epifaunal taxa richness hosted by *Cystoseira* thalli was evaluated using species accumulation curves, comparing the accumulation curve of the number of observed taxa to the accumulation curves calculated by two estimators: Chao1 estimator is based on abundance data, and is derived as a lower bound of species richness in terms of numbers of singletons and doubletons (species represented in a single abundance sample by, respectively, only one or two individuals); Chao2 is based on incidence data (species presence/absence), relying on the assumption that non-observed taxa are rare species, considering "rare" a taxon when it is found in less than 3 samples (Veiga *et al*., 2014; Chao & Chiu, 2016).

Stress response

Differences in both fatty acid (FA) profiles and content of phenols between treatments in the two different sites over time were tested using PERMANOVA with 9999 permutations through PRIMER v6.1.10 & PERMANOVA software (Plymouth, UK).

PERMANOVA was performed to test differences in total phenolic content (expressed as mg/g of dry weight of extract) between treatments (Factor Treatment: 2 levels, Transplanted and Unmanipulated) in the two sites (Factor Site: 2 levels, Barcarello and Capo Gallo) over the time (Factor Time: 5 levels, T0, T1, T2, T3, and T4), with all the factors considered fixed and orthogonal. Pair-wise tests were used to check significant post-hoc differences.

Individual FA data were expressed as percentage of total FAs, and resembled using Euclidean similarity distance matrix, after being transformed using the arcsine function. PERMANOVA and pair-wise tests were carried out on the transformed data with the same design used for the analysis of the phenolic content.

Moreover, Principal Coordinates Analysis (PCO) was performed on the FA profiles considering the interaction of all the factors “TreatmentxSitexTime”, superimposing the main classes of FAs to the graph.

Furthermore, analysis of similarity percentage (SIMPER) was carried out on untransformed data to identify which FA contributed more to the similarity within, and the dissimilarity between the two treatments and between the transplanted and unmanipulated individuals over the time.

Since the Capo Gallo site lost all the transplanted individuals before the end of the experiment, the last sampling date (T4) was not included in the statistical analysis.

1.3 Results

1.3.1 Environmental variables

Throughout the course of the experiment, mean temperature of surface water ranged between 17,45°C in May and 25,22°C in September, with a maximum of 27,92°C in August. Salinity varied between 37,31‰ in May and in 37,99 ‰ in September, with a peak of 38,02‰ in August (Fig.3).

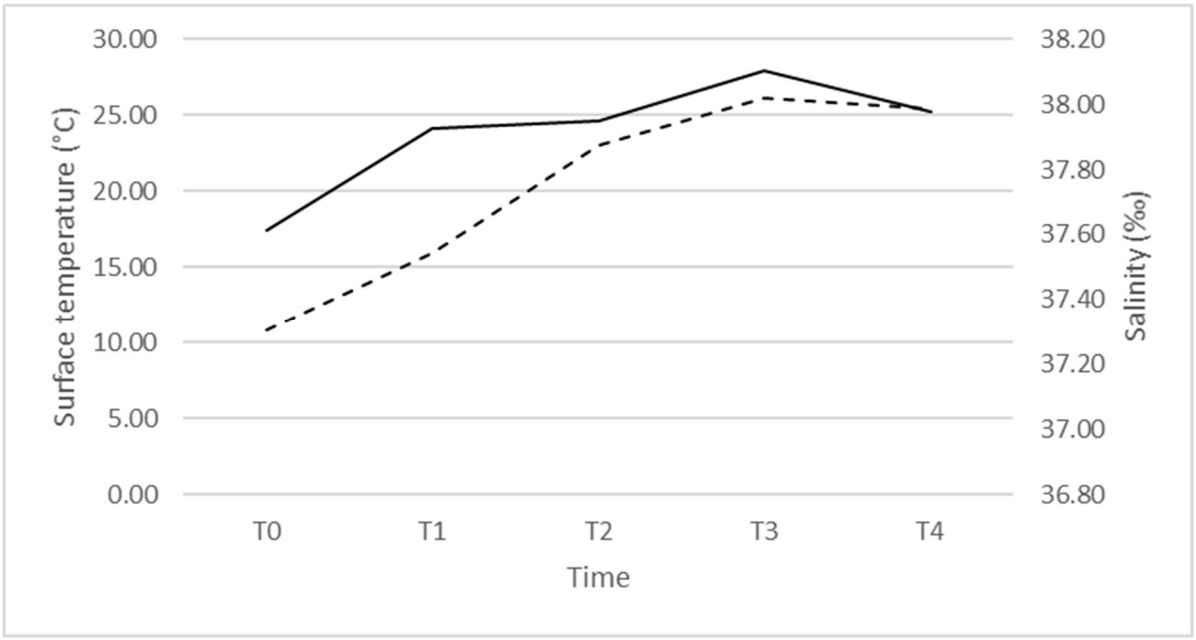

Figure 3 – Surface water temperature (°C) and salinity (‰) measured in seawater during the course of the transplanting experiment

1.3.2 Transplantation success

Total number of surviving thalli (fig. 4) and relative survival rate (fig. 5) varied over the time and between the two different sites. For what regards the survival rate (considered as proxy of the transplantation success) of the transplanted thalli, the results showed dramatic differences between the two sites: in fact, whilst survival rate at the Barcarello site was about 50% in the last three sampling dates, survival rate of Capo Gallo suddenly collapsed since the

first two sampling date, and reached zero before the end of the experiment, without any living individual left in the site. However, observing directly the total number of surviving thalli over the course of the experiment, a strong mortality of the transplanted individuals results evident in both the two sites.

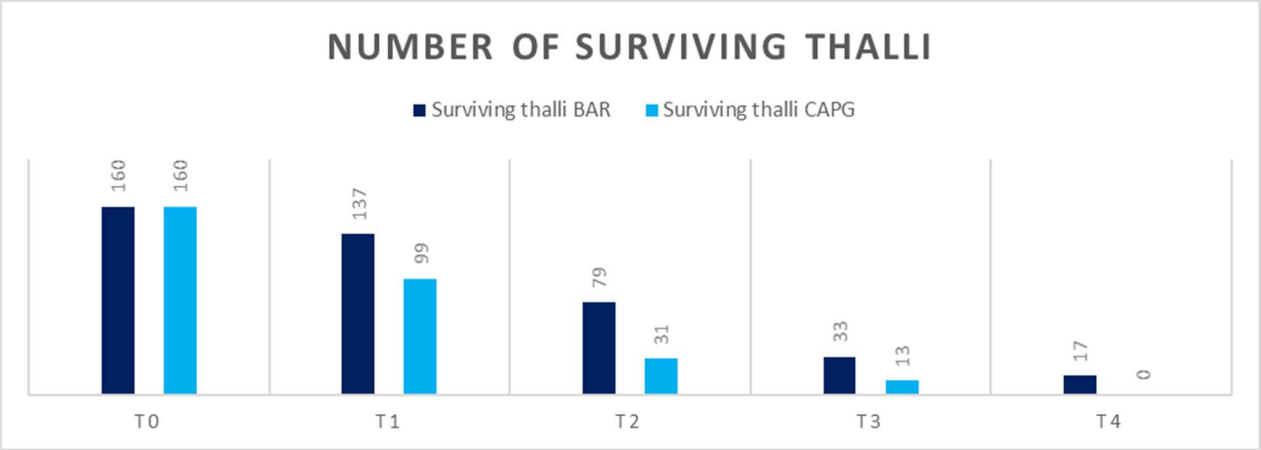

Figure 4 – Total number of surviving transplanted thalli in the two sites (BAR, Barcarello and CAPG, Capo Gallo) throughout the experiment

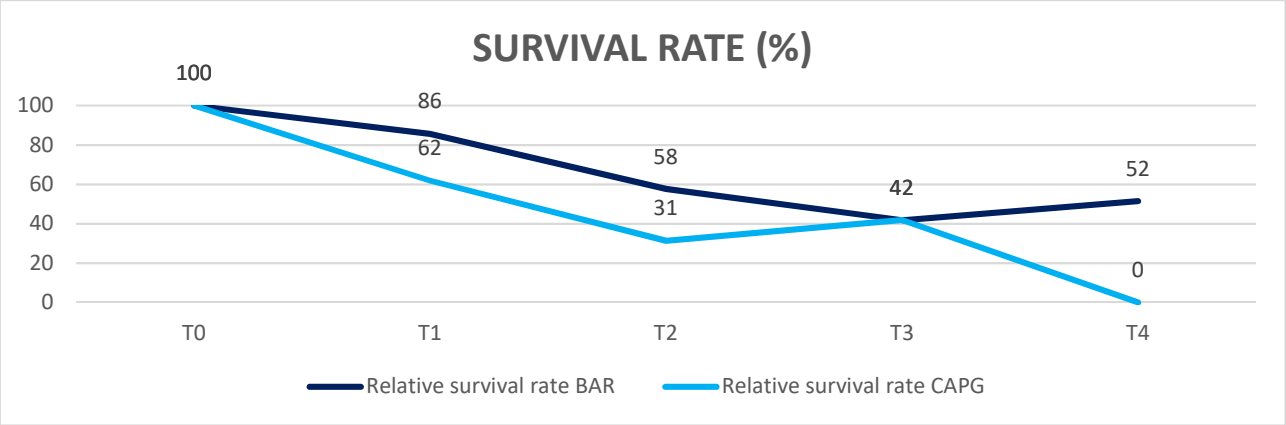

Figure 5 - Survival rate (%) of transplanted thalli in the two sites (BAR, Barcarello and CAPG, Capo Gallo) during the course of the experiment

1.3.3 Macroalgae morphology and complexity

Volumes

Results obtained from the analysis of volumes revealed that, basically, volumes of the transplanted individuals were smaller than those occupied by unmanipulated thalli, except for T1 in the Barcarello site (fig. S1). This tendency is displayed for all the types of volume that have been measured (Canopy, Interstitial, and Thallus volumes). The highest values were found at the beginning of the experiment in both the sites for the natural populations, but all the volumes showed a dramatic decrease from the first sampling date.

PERMANOVA results carried out on CV and IV evidenced that these volumes are significantly different for the interaction SitexTime (CV: PERMANOVA, SitexTime, p=0,001, F(2)= 72,49; IV: PERMANOVA, SitexTime, p=0,034, F(2)=72,4331), and for the treatment (CV: PERMANOVA, treatment, p=0,01, F(1)=89,143; IV: PERMANOVA, treatment, p=0,004, F(1)=88,311). Pair-wise tests performed to examine the differences in the two sites during the course of the experiment showed no differences in the two sites for the first three sampling dates, for bot CV and IV (tab.S1-S2). For what regards CV, significant differences are found between T0 and T1, and T1 and T3 for the Barcarello site, while Capo Gallo site showed differences only between T0 and the two subsequent sampling dates (tab.S1). Regarding IV, in Barcarello the differences are between T0 and T1, and T0 and T4, whilst volumes in Capo Gallo showed the same differences found for CV (T0 different from both T1 and T2).

PERMANOVA performed on TV data highlighted significant differences for the factors Treatment (PERMANOVA, treatment, p=0,034, F(1)=50,234) and Time (PERMANOVA, time, p=0,001, F(4)=42,628), but not for the Site (Tab.S3).

Fractal dimension

Fractal dimensions of *Cystoseira* thalli did not exhibit marked trends, assuming very similar values in both the two sites. Furthermore, it seems that fractal dimension slightly decreased

throughout the course of the experiment, ranging from 1,81±0,04 at T0 to 1,70±0,04 at T3, and generally with higher values in the unmanipulated individuals (fig.S2).

PERMANOVA results showed that fractal dimension significantly differs for the interaction between treatment and time (PERMANOVA, TreatmentxTime, p=0,009, F(3)=38,052), but not between the different sites. Pair-wise tests revealed that fractal dimension for the two treatments did not differ across the sampling times, excepted on T3. Moreover, fractal dimension of the unmanipulated thalli on T0 was significantly different from all the other sampling times. Transplanted individuals collected in T3 differed from sampling times T1 and T2, but not from T4 (Tab.S4; fig.6)

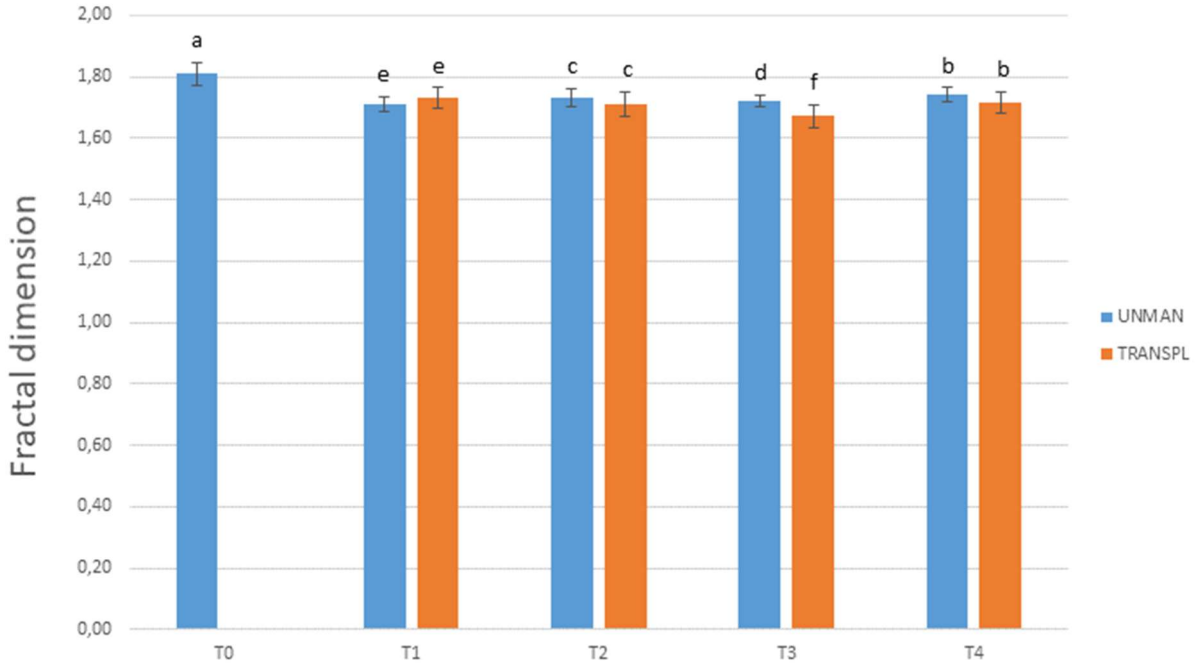

Figure 6 – Fractal dimension of Cystoseira amentacea *thalli varied with the interaction between treatments and sampling times (PERMANOVA, TreatmentxTime, p=0,009, F(3)=38,052). Unman, unmanipulated; Transpl, transplanted. Based on pair-wise test results, different letters were attributed to significantly different points*

1.3.4 Community biodiversity

The analysis of the macrofaunal community showed 91 different taxa (22 Polychaeta 37 Mollusca, and 32 Crustacea; tab.S5), with a total number of 12606 individuals counted and identified.

Crustaceans were the most abundant organisms, representing in terms of number of number of individuals about the 91% of the whole macrofaunal community with 11387 individuals. The most common species were *Parhyale aquilina* (Costa, 1857), *Caprella acanthifera* Leach, 1814, and *Ischyrocerus inexpectatus* Ruffo, 1959.

Polychaeta accounted for 592 individuals, with *Platynereis dumerilii* (Audouin & Milne Edwards, 1833), *Syllis prolifera* Krohn, 1852, and *Salvatoria limbata* (Claparède, 1868) as the most frequent species.

Mollusca was the least abundant phylum with 572 organisms, with Rissoa sp., Gibbula sp., and *Sinezona cingulata* Costa, 1861 as the most numerous taxa.

Data on epifaunal abundance revealed that, overall, for Barcarello site the average number of individuals was higher for the transplanted than the unmanipulated thalli (fig.S3). Additionally, their abundance in unmanipulated thalli increased from T0 to T2 (from 144,40±22,57 to 174,40±36,51 individuals), and then decreased in T3, reaching the minumum value in T4 (65,80±21,38 individuals). The transplanted thalli showed similar trend, with a dramatic decrease in T3 (from 279,40±33,51 in T1 to 73,60±27,32 individuals in T3).

Capo Gallo showed an opposite trend, with higher values of macrofaunal individuals in unmanipulated thalli compared to the transplanted ones (fig.S3). Unmanipulated thalli showed the maximum number of epifaunal individuals on T0 (469,40±53,17 individuals), then dropped in T1 and T2 (respectevely 140,40±44,75 and 175,60±43,04 individuals), whilst for the transplanted thalli an increase from T1 to T2 (98,80±30,66 individuals in T1 and 144,20±32,80 individuals in T2) was observed.

PERMANOVA carried out on abundance data highlited significative differences for the interactions between sites and treatments (PERMANOVA, SitexTreatment, p=0,029, F(1)=50,588)and between sites and sampling times (PERMANOVA, SitexTime, p=0,024,

F(2)=3,542) (tab.S6; fig.7). Pair-wise tests performed on the interactions revealed the two sites differed from each other in the transplantation treatment, but not in the natural population. Additionally, significative differences bewteen the two sites were found just for the first sampling date (T0).

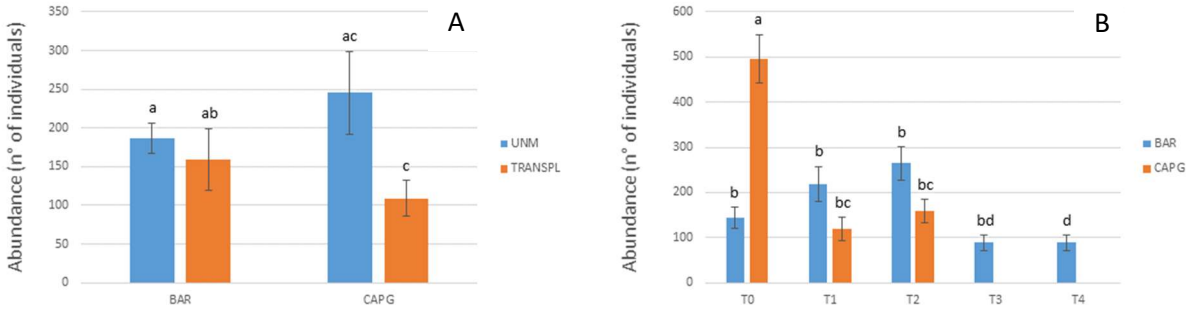

Figure 7 – A) Macrofaunal abundance varied with the interaction between sites and treatment (PERMANOVA, SitexTreatment, p=0,029, F(1)=50,588). Unman, unmanipulated; Transpl, transplanted B) and between sites and sampling times (PERMANOVA, SitexTime, p=0,024, F(2)=3,542). Bar, Barcarello; CapG, Capo Gallo. Based on pair-wise test results, different letters were attributed to significantly different points

Taxa richness followed the same trend of organism abundance, with, generally, higher values in transplanted thalli for Barcarello, and higher values for the unmanipulated thalli in Capo Gallo site (fig.S4). Number of taxa in Barcarello site was more or less stable during the first three sampling time for the unmanipulated thalli (about 17 taxa per date), increased in T3 (22,20±2,15 taxa) to then decline again in the last sampling time (14,40±1,57 taxa); in the transplanted thalli, taxa richness remained almost the same in T1 and T2 (22 taxa per sampling time on average), then decreased to in T3 and T4 (about 18 taxa per time).

Capo Gallo site showed a decrement in taxa richness for the unmanipulated thalli from T0 (17,40±1,86 taxa) to T1 (13±1,55 taxa), and then a slight increase in T2 (14,40±1,94 taxa). The transplanted thalli showed no significant trend over the two sampling dates. Furthermore, number of taxa resulted higher in Barcarello site compared to Capo Gallo site, with an average number of 18 taxa in Barcarello and 14 taxa in Capo Gallo.

PERMANOVA performed on taxa richness showed significant differences for the interaction between sites and treatments (PERMANOVA, SitexTreatment, p=0,028, F(1)=48,184) (tab.S7; fig.8). While there are no relevant differences for the unmanipulated population, the two sites resulted different from each other for the transplantation treatment.

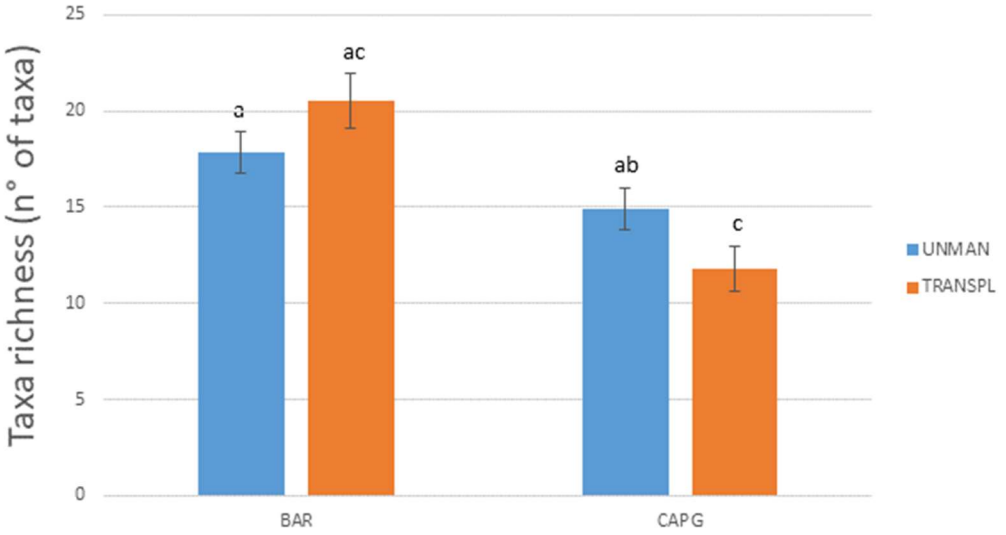

Figure 8 – Macrofaunal taxa richness varied with the interaction between sites and sampling times only (PERMANOVA, SitexTreatment, p=0,028, F(1)=48,184). Unman, unmanipulated; Transpl, transplanted. Based on pair-wise test results, different letters were attributed to significantly different points

Analysis of principal coordinates did not show a clear division between the different treatments, but a slight separation of the two last sampling dates in the Barcarello site, driven mainly by the two taxa *Syllis prolifera* Krohn,1852 and *Ampithoe ramondi* Audoin, 1826, can be observed (fig.9).

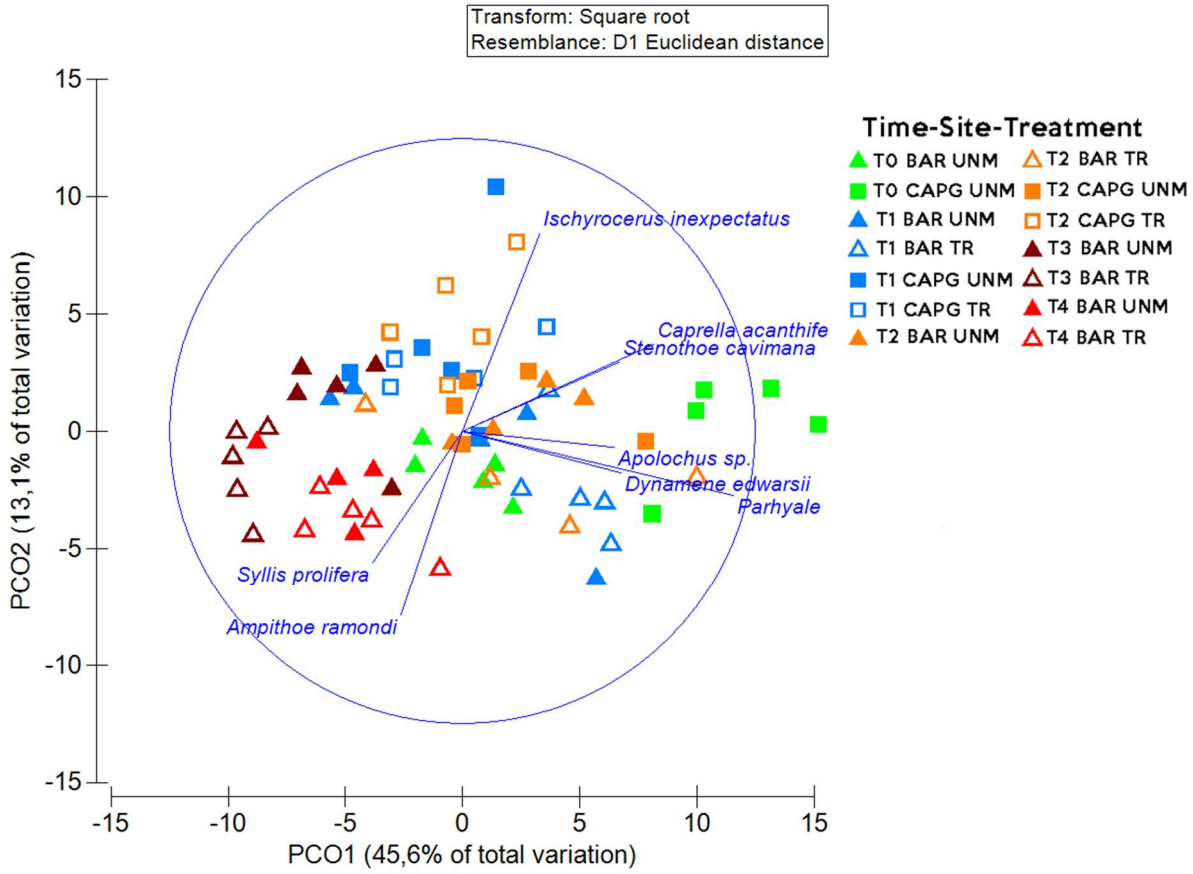

Figure 9 – Analysis of principal coordinates (PCO) of epifaunal community strucure of both unmanipulated and transplanted Cystoseira amentacea *in the two sites across time. The main taxa are superimposed to the graph.*

SIMPER analysis performed on the structure of the community revealed that *Parhyale aquilina*, *Ischyrocerus inexpectatus*, and *Caprella acanthifera* contributed the most to the differences observed between the different treatments.

Correlation analyses among epifaunal abundance and taxa richness revealed that, considering the whole community, abundance data showed a stronger positive correlation with both interstitial volume and fractal dimension (Person's correlation index ρ= 0,50 and ρ= 0,46, respectively). Taxa richness showed a weak positive correlation with both the two morphology variables (ρ= 0,10 for interstitial volume and ρ= 0,23 for fractal dimension) (fig.10; tab.8).

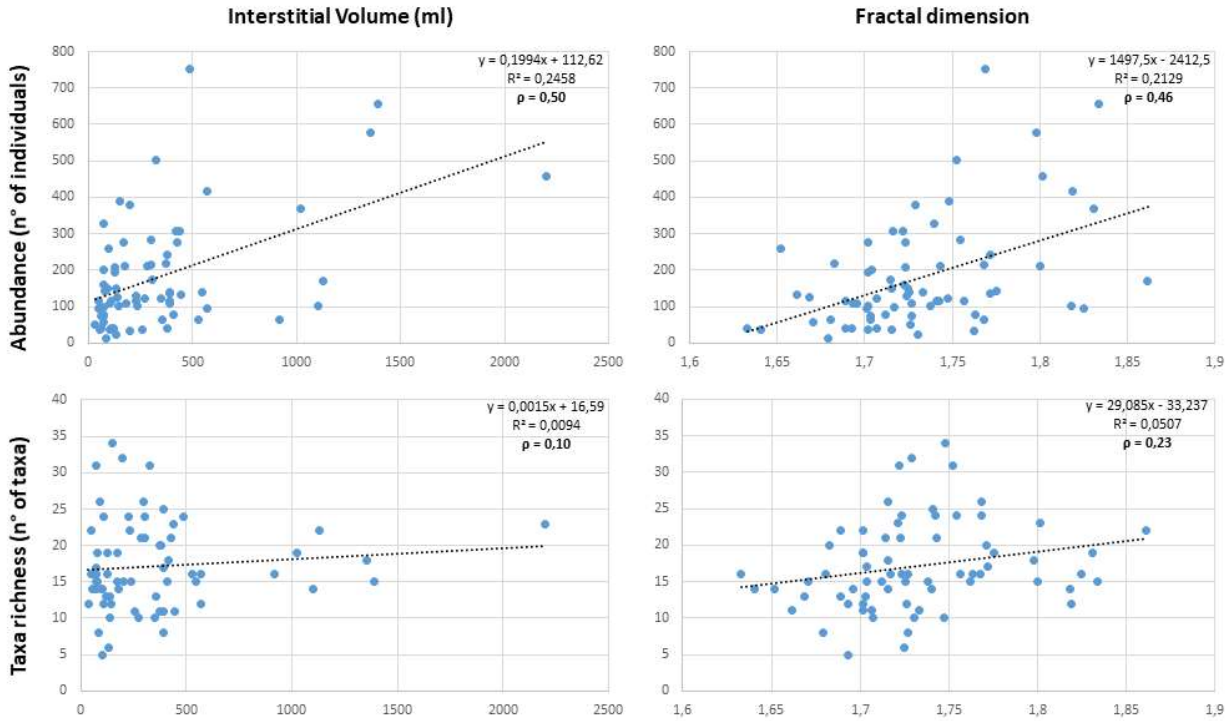

Figure 10 – Correlation analyses between epifaunal abundance and taxa richness data, and interstitial volume and fractal dimension of Cystoseira amentacea *thalli. ρ: Pearson's correlation index values*

Correlation analyses performed separately on the different classes of organisms evidenced that abundance values of Crustacean had a moderate positive correlation with both interstitial volume and fractal dimension of the thalli. Taxa richness showed a weak correlation with the two morphological variables (tab.8a).

Mollusca showed a weak negative relation between interstitial volume and both abundance and taxa richness, whilst there was a poor positive correlation between fractal dimension and the two community variables (tab.8b).

Polychaeta class displayed a weak negative interaction among abundance and the two macroalgal measures, and between taxa richness and interstitial volume, while there was a poor positive interaction between fractal dimension and taxa richness (tab.8c)

Table 1 – Correlation analyses between epifaunal community variables and measures of macroalgal morphology for both the entire community and the different classes of organisms

Class	Variables	Pearson's correlation index (ρ)
Entire community		
	Interstitial vol. - Abundance	0,50
	Fractal dimen. - Abundance	0,46
	Interstitial vol. - Taxa richness	0,10
	Fractal dimen. - Taxa richness	0,23
a) Crustacea		
	Interstitial vol. - Abundance	0,37
	Fractal dimen. - Abundance	0,45
	Interstitial vol. - Taxa richness	0,18
	Fractal dimen. - Taxa richness	0,26
b) Mollusca		
	Interstitial vol. - Abundance	-0,02
	Fractal dimen. - Abundance	0,16
	Interstitial vol. - Taxa richness	-0,04
	Fractal dimen. - Taxa richness	0,09
c) Polychaeta		
	Interstitial vol. - Abundance	-0,26
	Fractal dimen. - Abundance	-0,03
	Interstitial vol. - Taxa richness	-0,17
	Fractal dimen. - Taxa richness	0,01

Values of taxa richness estimated by the Chao1 (Unmanipulated, 117 taxa; Transplanted, 79,90 taxa) and Chao2 estimators (Unmanipulated, 134,6 taxa; Transplanted, 84,5 taxa) were higher than the observed values for both the unmanipulated and transplanted samples. However, both observed and estimated values of taxa richness showed similar patterns (fig.11).

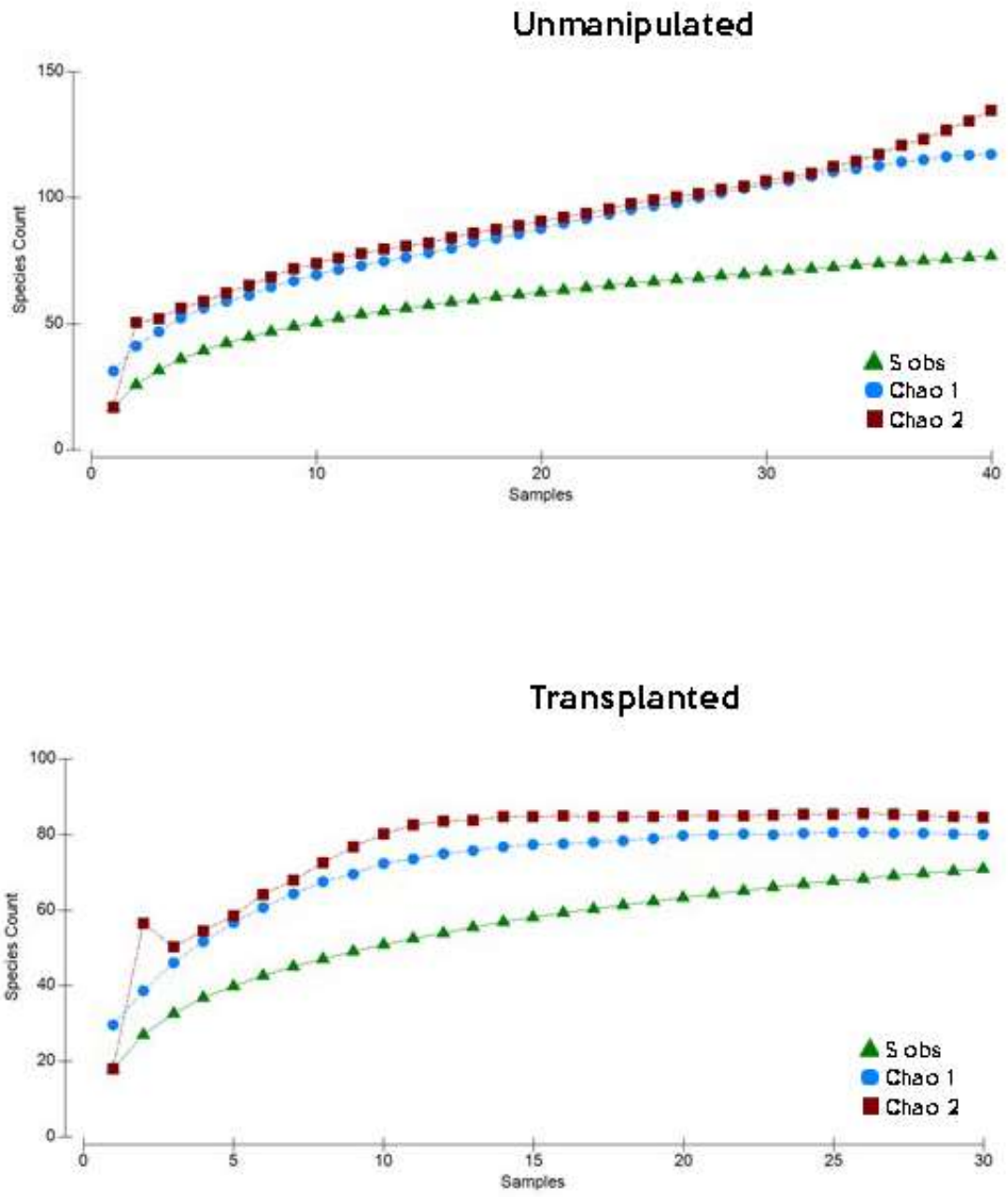

Figure 11 – Species accumulation curves obtained from the Chao1 and Chao2 estimators and for the observed taxonomic richness for both the two different treatments (Unmanipulated and Transplanted thalli)

1.3.5 Stress response

Total phenolic content

Total phenolic content showed a similar trend for both of the sites, with higher average values found in the transplanted individuals. Furthermore, TPC increased from T0 to T1 (from 5,23±1,25 mg/g at T0 to 6,32±2,59 mg/g at T1), and then progressively decreased in all the other sampling dates, reaching the minimum value at the end of the experiment (T4, 2,07±0,70 mg/g). This general trend of decline during the course of the experiment is observable in both the transplanted and unmanipulated individuals (fig.S5).

PERMANOVA results highlighted significant differences in TPC between treatments (PERMANOVA, Treatment, p=0,007, F(1)=94,513) and for the interaction between sites and sampling times (PERMANOVA, SitexTime, p=0,002, F(3)=67,073) (tab. S5; fig.12). Pair-wise tests, carried out to examine the differences of the TPC in the sampling dates in the two different sites, showed that, while in the Barcarello site only T4 results significantly different from all the other sampling dates, in Capo Gallo site all the sampling date are significantly different from each other.

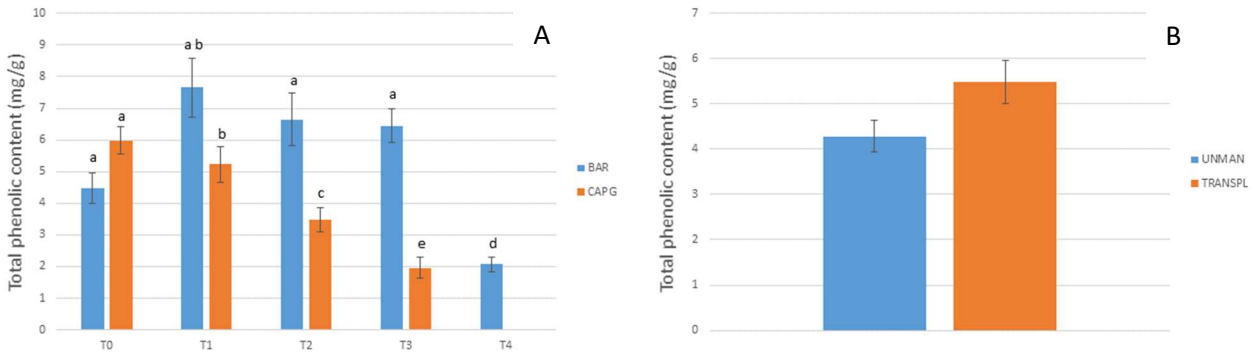

Figure 12 – A) Total phenolic content varied with the interaction between sites and sampling times (PERMANOVA, SitexTime, p=0,002, F(3)=67,073) B) and between different treatments (PERMANOVA, Treatment, p=0,007, F(1)=94,513). Unman, unmanipulated; Transpl, transplanted. Based on pair-wise test results, different letters were attributed to significantly different points

Fatty acids

Analysis performed on fatty acids identified 28 individual FAs for Barcarello site and 26 for Capo Gallo. The dominant FAs were 16:0 (palmitic acid), 18:1 n9-cis (oleic acid), 18:3 n3 (acido α-linolenico, ALA), and 20:4 n6 (arachidonic acid, AA) in both the two sites (tab.10-11).

Natural population of *Cystoseira* in Barcarello site showed similar trends over the time for both SFAs and MUFAs, which displayed an increase from T0 to T4 (SFAs, T0: 29,36±2,23%; T4: 31,26±1,76%; MUFAs, T0: 18,23±2,95%; T4: 25,10±3,11%). On the contrary PUFAs showed a different trend, peaking at T1 and accounting for the 59,53±5,63% of the all FAs, and then decreasing to 41,38±4,43% at the end of the experiment. Bacterial markers showed just a slight increase, going from 1,11±0,34% at T0 to 2,56± at the last sampling date. Transplanted thalli displayed a different pattern: although MUFAs followed the same trend of unmanipulated individuals (incrementing from 17,82±2,21% at T0 to 29,31±3,12% at T4), SFAs showed a relevant increase from 24,42±1,27% to 33,73±1,55% at T3, to then decrease to 29,42±2,50% at the last sampling date. PUFAs content dropped just after the first sampling date, from 57,84±5% at T0 to 41,05±6,17% at the end of experiment. Bacterial markers as in the natural population, showed just a little increment from 0,95±0,07% at T0 to 1,96±0,46% at T4 (fig.13).

Data for Capo Gallo site were available for just T0 and the two subsequent sampling dates: unmanipulated thalli showed a small increase for SFAs (from 27,82±2,66% at T0 to 29,21±1,53% at T2), whilst MUFAs and bacterial markers remained almost stable. PUFAs decremented from 52,14±9,98% at T0 to 50,74±6,43% at T2; for what regards transplanted thalli, both MUFAs and bacterial markers showed an increase from T1 to T2, SFAs increased from 28,02±1,45% to 32,97±2,89%, while PUFAs dropped from 51,91±4,83% to 45,26±4,84% (fig.13).

Table 2 – Fatty acid profiles (mean±SD) of C.amentacea *thalli for both two treatments, Unmanipulated and Transplanted, during the course of the experiment (Barcarello site). FAs <0.1% in all samples are omitted*

Site	BAR UNM										BAR TRAN							
Time	T0		T1		T2		T3		T4		T1		T2		T3		T4	
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
FAs																		
14:0	4,0	0,3	3,0	0,3	3,5	0,7	3,6	0,2	4,2	0,4	3,0	0,2	3,4	0,4	3,9	0,3	4,1	0,2
15:0	0,2	0,0	0,2	0,0	0,2	0,1	0,2	0,1	0,3	0,0	0,1	0,0	0,2	0,0	0,2	0,0	0,3	0,0
16:0	23,3	1,6	19,6	1,1	22,9	0,9	24,0	1,4	25,4	1,0	20,3	0,9	23,0	0,9	27,7	0,6	23,9	2,1
17:0	0,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
18:0	0,5	0,1	0,3	0,1	0,4	0,1	0,5	0,1	0,4	0,0	0,3	0,1	0,5	0,0	0,8	0,1	0,4	0,0
19:0	0,0	0,0	0,0	0,0	0,1	0,0	0,0	0,0	0,1	0,0	0,0	0,0	0,1	0,1	0,0	0,0	0,1	0,0
20:0	0,1	0,0	0,1	0,0	0,0	0,0	0,1	0,1	0,1	0,1	0,0	0,0	0,1	0,0	0,1	0,1	0,1	0,0
21:0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,0	0,0	0,1	0,0	0,0
22:0	0,1	0,1	0,0	0,0	0,0	0,0	0,1	0,1	0,1	0,0	0,0	0,0	0,1	0,0	0,1	0,1	0,0	0,0
LCFAs (>22:0)	0,5	0,1	0,4	0,1	0,6	0,2	0,6	0,1	0,6	0,1	0,4	0,1	0,5	0,1	0,5	0,1	0,5	0,1
ΣSFA	**29,1**	**2,2**	**23,6**	**1,6**	**27,7**	**2,1**	**29,0**	**1,9**	**31,2**	**1,7**	**24,2**	**1,2**	**28,0**	**1,5**	**33,5**	**1,4**	**29,4**	**2,5**
16:1 n7	5,0	1,0	4,2	0,5	5,6	1,4	4,4	0,5	6,3	0,5	4,0	0,5	4,5	0,3	4,4	0,5	6,2	0,6
18:1 n7	0,9	0,3	0,9	0,1	1,1	0,1	1,6	0,1	1,8	0,2	0,9	0,1	1,2	0,1	1,4	0,2	1,6	0,3
18:1 n9c	13,2	1,9	11,5	0,6	15,5	0,4	16,5	1,1	18,5	2,5	12,8	1,6	14,5	0,7	16,6	1,5	21,4	2,2
22:1 n9	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,1	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
ΣMUFA	**19,1**	**3,2**	**16,7**	**1,3**	**22,2**	**1,9**	**22,6**	**1,8**	**26,8**	**3,3**	**17,7**	**2,2**	**20,2**	**1,1**	**22,5**	**2,2**	**29,2**	**3,1**
18:2 n6c	3,9	0,8	2,7	0,3	3,0	0,5	3,7	0,5	3,6	0,6	2,7	0,5	3,3	0,4	4,6	0,3	2,5	0,4
18:3 n3	9,9	0,7	13,2	1,3	12,1	1,4	12,6	1,0	10,8	0,6	12,8	1,8	12,8	0,8	11,4	0,5	10,3	0,5
18:3 n6	0,4	0,1	0,4	0,1	0,3	0,1	0,2	0,0	0,3	0,0	0,3	0,1	0,3	0,0	0,3	0,2	0,2	0,0
18:4 n3	4,5	1,7	10,8	1,7	5,1	0,7	3,0	1,1	2,1	0,6	9,3	0,5	5,0	0,6	2,4	0,2	1,6	0,7
20:2 n6	0,5	0,1	0,6	0,0	0,8	0,3	0,8	0,2	1,2	0,2	0,7	0,2	0,6	0,2	0,4	0,1	1,3	0,2
20:3 n3	0,2	0,0	0,3	0,1	0,3	0,1	0,3	0,1	0,3	0,1	0,4	0,1	0,4	0,1	0,1	0,1	0,3	0,1
20:3 n6	1,4	0,2	1,0	0,2	0,9	0,2	0,6	0,2	1,8	0,7	1,0	0,2	1,1	0,2	1,2	0,2	2,0	0,5
20:4 n3	1,1	0,1	2,0	0,2	1,7	0,1	1,7	0,1	1,3	0,2	2,0	0,2	1,8	0,2	1,5	0,2	1,4	0,1
20:4 n6	22,2	1,3	16,1	0,7	17,6	0,9	15,8	0,8	15,3	0,5	17,5	0,5	17,4	0,6	15,8	0,8	17,2	2,5
20:5 n3	7,0	1,4	12,1	0,9	7,5	0,8	7,9	0,6	4,3	0,7	11,0	0,7	8,0	0,8	4,6	0,9	3,8	0,9
22:2 n6	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,1	0,0	0,0	0,1	0,1	0,0	0,0	0,1	0,1
22:4 n6	0,1	0,0	0,2	0,0	0,2	0,1	0,2	0,1	0,3	0,1	0,1	0,0	0,1	0,0	0,1	0,1	0,2	0,0
22:6 n3	0,0	0,0	0,1	0,0	0,0	0,0	0,9	0,3	0,0	0,0	0,0	0,0	0,1	0,1	0,8	0,9	0,0	0,0
ΣPUFA	**51,4**	**6,7**	**59,5**	**5,5**	**49,4**	**5,1**	**47,5**	**4,9**	**41,3**	**4,3**	**57,8**	**4,9**	**51,1**	**4,1**	**43,3**	**4,5**	**41,0**	**6,1**
Σn3	**22,7**	**4,0**	**38,5**	**4,2**	**26,6**	**3,1**	**26,3**	**3,1**	**18,8**	**2,2**	**35,5**	**3,3**	**28,0**	**2,6**	**20,8**	**2,8**	**17,4**	**2,3**
Σn6	**28,7**	**2,7**	**20,9**	**1,3**	**22,7**	**2,0**	**21,2**	**1,8**	**22,5**	**2,1**	**22,3**	**1,6**	**23,1**	**1,5**	**22,5**	**1,7**	**23,6**	**3,8**
Iso	0,0	0,0	0,0	0,0	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,0	0,0	0,0
Anteiso	0,1	0,0	0,0	0,0	0,3	0,2	0,2	0,0	0,3	0,1	0,0	0,0	0,1	0,0	0,1	0,1	0,2	0,0
ΣBR	**0,1**	**0,0**	**0,0**	**0,0**	**0,4**	**0,3**	**0,2**	**0,1**	**0,3**	**0,2**	**0,0**	**0,0**	**0,2**	**0,0**	**0,2**	**0,1**	**0,2**	**0,1**

ΣOH	**0,0**	**0,0**	**0,0**	**0,0**	**0,2**	**0,2**	**0,2**	**0,1**	**0,1**	**0,1**	**0,0**	**0,0**	**0,2**	**0,1**	**0,1**	**0,1**	**0,0**	**0,1**
ΣCYS	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,1**	**0,1**	**0,1**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**

Table 3 - Fatty acid profiles (mean±SD) of C.amentacea *thalli for both two treatments, Unmanipulated and Transplanted, during the course of the experiment (Capo Gallo site). FAs <0.1% in all samples are omitted*

Site	**CAPG UNM**						**CAPG TRAN**			
Time	**T0**		**T1**		**T2**		**T1**		**T2**	
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
FAs										
10:0	0,07	0,02	0,03	0,02	0,01	0,01	0,16	0,12	0,02	0,02
12:0	0,08	0,01	0,03	0,01	0,04	0,03	0,12	0,04	0,04	0,01
14:0	3,56	0,38	2,10	0,09	3,38	0,18	3,07	0,29	3,66	0,68
15:0	0,19	0,02	0,09	0,01	0,17	0,02	0,17	0,04	0,20	0,05
16:0	22,62	1,98	23,50	0,57	24,41	0,95	23,45	0,79	27,61	1,63
18:0	0,45	0,09	0,13	0,03	0,51	0,10	0,34	0,03	0,65	0,12
20:0	0,15	0,04	0,05	0,03	0,11	0,05	0,09	0,04	0,12	0,07
LCFAs (>22:0)	0,43	0,05	0,35	0,05	0,48	0,11	0,51	0,06	0,53	0,16
ΣSFA	**27,55**	**2,59**	**26,27**	**0,81**	**29,10**	**1,45**	**27,89**	**1,40**	**32,83**	**2,74**
16:1 n7	5,94	0,63	5,44	0,60	4,96	0,51	5,11	0,50	4,89	0,15
18:1 n7	0,86	0,04	0,89	0,06	1,11	0,20	0,96	0,13	1,32	0,19
18:1 n9c	13,06	1,15	13,58	0,60	13,56	1,41	13,54	1,19	15,22	0,96
20:1 n11	0,00	0,00	0,00	0,00	0,00	0,00	0,32	0,72	0,00	0,00
ΣMUFA	**19,86**	**1,82**	**19,91**	**1,26**	**19,62**	**2,12**	**19,94**	**2,53**	**21,43**	**1,31**
18:2 n6c	4,08	0,49	2,50	0,16	3,76	0,47	3,06	0,39	4,41	0,94
18:3 n3	9,30	0,62	10,74	0,64	11,82	1,23	10,99	1,00	10,12	0,69
18:3 n6	0,44	0,08	0,12	0,05	0,29	0,06	0,25	0,06	0,31	0,08
18:4 n3	5,83	3,28	9,14	1,19	4,23	1,07	6,51	0,96	2,92	0,49
20:2 n6	0,52	0,15	0,56	0,11	0,43	0,16	0,61	0,16	0,35	0,22
20:3 n3	0,27	0,08	0,20	0,05	0,19	0,03	0,30	0,07	0,12	0,12
20:3 n6	1,15	0,32	0,88	0,08	1,05	0,17	0,98	0,28	1,14	0,07
20:4 n3	1,36	0,09	2,06	0,15	1,69	0,10	1,92	0,23	1,46	0,13
20:4 n6	20,58	2,95	16,29	0,49	19,29	1,98	17,81	0,67	18,11	0,87
20:5 n3	8,41	1,86	10,97	0,88	7,80	1,03	9,24	0,83	5,87	0,92
22:2 n6	0,15	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
22:4 n6	0,04	0,02	0,09	0,03	0,11	0,06	0,17	0,06	0,15	0,04
22:5 n3	0,00	0,00	0,02	0,05	0,02	0,05	0,04	0,09	0,14	0,09
22:6 n3	0,02	0,02	0,00	0,00	0,05	0,05	0,05	0,02	0,17	0,17
ΣPUFA	**52,14**	**9,98**	**53,59**	**3,89**	**50,74**	**6,43**	**51,91**	**4,83**	**45,26**	**4,84**
Σn3	**25,19**	**5,95**	**33,15**	**2,96**	**25,81**	**3,55**	**29,04**	**3,21**	**20,80**	**2,61**
Σn6	**26,95**	**4,03**	**20,44**	**0,93**	**24,93**	**2,88**	**22,87**	**1,62**	**24,47**	**2,23**

Anteiso	0,07	0,01	0,00	0,00	0,14	0,02	0,00	0,00	0,18	0,05
ΣBR	**0,07**	**0,01**	**0,00**	**0,00**	**0,14**	**0,02**	**0,00**	**0,00**	**0,18**	**0,05**
ΣOH	**0,00**	**0,00**	**0,00**	**0,00**	**0,07**	**0,11**	**0,00**	**0,00**	**0,00**	**0,00**

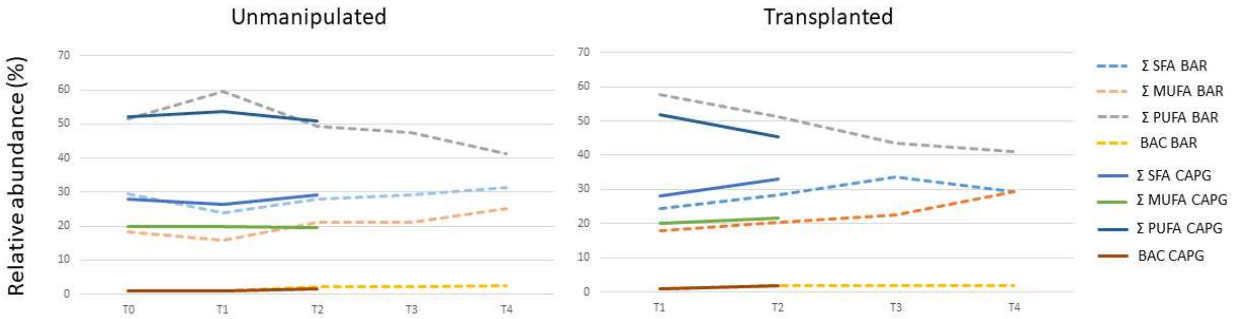

Figure 13 – Trends over the time for both the two treatments (Unmanipulated and Transplanted thalli) of the main classes of fatty acids in the two sites

FAs profiles were significantly different for the interactions between all the factors (PERMANOVA, SitexTreatmentxTime, p=0,009, F(1)=36,658). Pair-wise tests carried out on the different interactions showed that the two sites had different FA compositions, for both natural population and transplanted thalli. Moreover, FA composition in unmanipulated thalli differed from the composition of transplanted thalli in all the sampling times from T1 toT4. Additionally, at the beginning of the experiment (T0) FAs profiles did not show any difference between the two sites, but they start to differ since T1, showing relevant differences in all the other sampling dates (tab.S10).

Analysis of principal coordinates (PCO) of fatty acid profiles did not show a clear distinction of the samples according to the treatment, although it is possible to notice a separation of the first sampling dates (T0 and T1), characterized by a high PUFA content along the axis: T1 samples clustered on the right side of the graph in function of the high abundance of (n-3)PUFA, while the high presence of (n-6)PUFA drove the separation of T0 samples along the second axis. Moreover, it is possible to observe a slight separation along the first axis of T3 and T4 samples, driven by the sum of SFA, MUFA, as well as bacterial markers (fig.14)

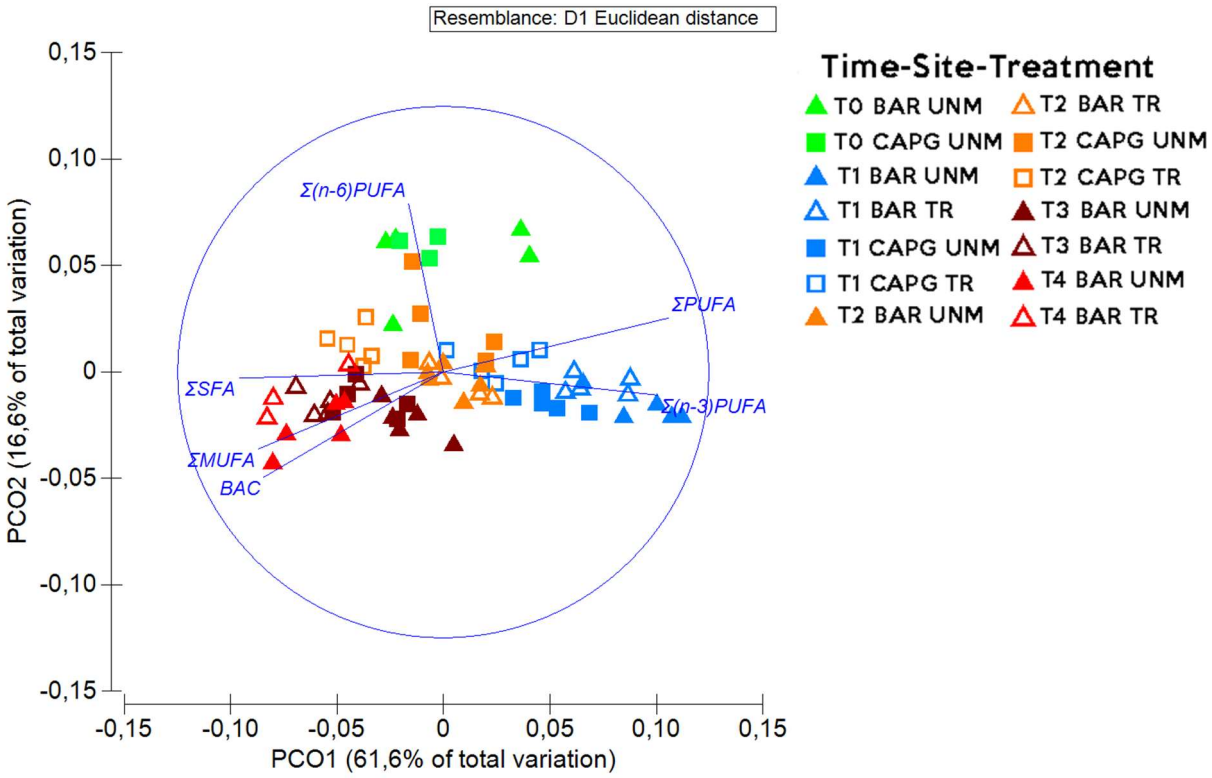

Figure 14 – Analysis of principal coordinates (PCO) of fatty acid profiles for both unmanipulated and transplanted C. amentacea thalli *in the two sites across time. The main classes of FAs are superimposed to the graph*

SIMPER analysis revealed that, within MUFAs and PUFAs, ALA, oleic acid, and arachidonic acid, were responsible of the main difference; among the other FAs, palmitic acid, 20:5 n3 (eicosapentaenoic acid, EPA), and 18:4 n3 (stearidonic acid, SDA) contributed to the differences observed between the two different treatments over the time.

1.4 Discussion

From a perspective of habitat restoration and conservation management, macroalgae transplantation assumed a relevant role in recent years, with special focus on species that play important ecological functions, such as forming species (De La Fuente *et al*., 2019).

Still, while studies have been focusing mostly on testing the efficacy of different techniques, little attention has been devoted to drivers that could negatively affect the survival of

transplanted individuals (Falace & Bressan, 2004; Falace *et al.*, 2006; Susini *et* al., 2007; Perkol-Finkel *et al.*, 2012).

This study represents a first attempt to understand the possible side effects of a commonly used transplantation procedure on the macroalga *Cystoseira amentacea* var. *stricta*. The main objectives have been the examination of multiple ecological aspects related to the transplantation, such as morphological modifications of transplanted individuals and their response to the manipulation stress, as well as variations in the structure of the macrofaunal associated to the macroalgae.

Results showed that an effect of the transplantation technique could be likely to happen, modifying the architecture and the complexity of the thalli, and potentially affecting their biochemical response.

The strong mortality rate observed in both the sites could indicate that local factors, such as different algae morphology, exposure to wave action, or different grazing pressure, can heavily affect the effectiveness of the transplantation method. Macroalgae that inhabit intertidal ecosystems are more subjected to multiple environmental stressors, especially during low tides when the individuals are exposed to elevated temperatures and undergo to significant water loss and overheating (Martinez *et al.*, 2011). Dense macroalgal canopies have the potential to ameliorate stressful environmental conditions typical of intertidal systems, modulating and mitigating local hydrodynamic regimes and reducing heat stress (Bertness & Callaway, 1994; Umanzor *et al.*, 2017; Scrosati & Ellrich, 2018). Thus, not being able to replicate natural density of *Cystoseira amentacea* populations in the experiment could make the transplanted thalli more exposed to wave action and to heat effects, as well as make them more sensitive to pressure of epiphytes and grazers, and dramatically affecting their survival.

Macroalgal complexity measures (sensu Hacker & Steneck, 1990), i.e. volumes and fractal dimension, showed differences between the natural population and the transplanted thalli. In particular, both volumes and fractal dimension, tend to assume lower values in transplanted individuals, indicating a less structured and complex substratum, as well as a lower availability of living space for the epifaunal organisms. Preceding transplanting experiments involving different *Cystoseira* species already signal the possibility that transplanted individuals may undergo to a stress that can affect their morphological features, altering the architecture and the complexity of the cauloid and reducing their size, and having as major consequence a

dramatic decline of survival rate of the transplanted thalli (Falace *et al.*, 2006). Additionally, these morphological differences between transplanted and natural thalli can potentially influence associated epifaunal assemblages, shaping the abundance of organisms and their diversity (Veiga *et al*., 2014). Previous findings, in fact, suggested that faunal composition depends mainly on habitat architecture, while the abundance of individuals is related to habitat size (Christie *et al*., 2009). Our outcomes partially supported this hypothesis, since fractal dimension showed lower correlation coefficient values for taxa richness than for abundance but, on the other hand, abundance data showed the highest value of correlation coefficient with the interstitial volumes. Moreover, variations in abundance and taxa richness data did not seem to be strictly dependent from the transplantation treatment, showing instead differences in relation to the site and sampling time. Therefore, probably, there could be other local biotic or abiotic drivers that shape the structure of macrofaunal assemblage (Veiga *et al.*, 2014).

Regarding the species composition of macrofaunal community, there are strong evidences of a mono-taxon assemblage, in which Crustacean represent the majority of the associated community, constituting approximately the 91% of the entire assemblage in terms of abundance of individuals, but not for what concern the number of detected taxa which, on the other hand, result to be similar to the other classes of epifaunal organisms (i.e., Polychaeta and Mollusca). The numerical prevalence of crustacean over the other macrofaunal classes was observable especially from May to August, a result that seems to be in line with seasonal variations already reported in previous studies, with maximum densities of crustacean signaled for spring-summer (Guerra-Garcìa *et al*., 2011; Chavanich & Wilson, 2000). This abundance pattern seems to be related to seasonal changes in environmental variables as temperature, day length and influence of wave action (Neto, 2000).

For what regards the response to an eventual manipulation stress, data on phenolic content showed that, in general, higher values of TPC were found in transplanted individuals. Moreover, phenolic content seemed to increase just right after the transplantation to, then, decrease during the course of the experiment, reaching minimum values at the end of the experiment. Previous studies assessing seasonal trends of TPC in *C. amentacea*, showed a negative correlation of TPC with seawater temperature, with the highest values measured in winter and spring, and a reduction in phenolic content in summer (Mannino *et al.*, 2016).

Fatty acid analysis revealed that the first sampling dates were mostly characterized by high PUFA content (both (n-3) and (n-6)PUFAs), while at the end of the experiment the thalli showed higher content of MUFAs and SFAs. These outcomes are coherent with previous studies, which evaluated seasonal changes and the effect of temperature on fatty acid composition in brown macroalgae species: the general trend, in fact, is a negative correlation between unsaturated fatty acid and temperature, with a decrease in total unsaturated fatty acids and an increase in total SFAs following an increase of ambient temperature (Nomura *et al*., 2013; da Costa *et al.*, 2019).

Thus, both phenolic and fatty acid content seems to respond primarily to variations in seawater temperature. However, despite a more likely prevalence of the effect of seasonal changes of local environmental factors, the manipulation treatment could still affect the ability of the macroalgae to cope with variations in environmental variables.

Under a conservation management perspective, the examination of all these ecological aspects and of the role of the main drivers that can affect transplantation process can provide useful insights, resulting in a potential improvement of the success of the procedure, especially if the main purpose is reforestation of *Cystoseira* at relevant spatial scale.

Nevertheless, longer experimental and monitoring times are needed, in order to separate and disentangle effects of seasonal fluctuations of environmental factors from the effect of a transplanting procedure.

Chapter 2

Developing reforestation methods for the kelp *Laminaria ochroleuca* using different life stages

Abstract

Kelps have a fundamental ecological role in intertidal and subtidal systems, and provide a lot of ecosystem services to humankind, but recently the combined effects of climate change and human activities are affecting their populations' resilience, shifting their distribution poleward. Since degraded kelp forests are often not able to naturally recover after disturbance, the search and implementation of new transplanting procedures in order to re-establish depleted populations is an objective of primary importance. For this reason, the transplantation of the intertidal kelp species *Laminaria ochroleuca* was attempted, involving three different life stages: adults, juveniles, and sporophytes cultured in laboratory conditions. Using different development stage allows assessing eventual differences in terms of mortality and growth rates, which can be used as proxies for transplantation success. Furthermore, the influence of macroalgal canopy on the survival of the transplanted individuals was evaluated, placing replicates of each stage at three different distance to the canopy patch.

Results evidenced the inadequacy of the chosen transplantation method for adult and juvenile individuals. In fact, few plants survived, independently from the distance to macroalgal canopy. These outcomes highlighted the need for choosing carefully the transplanting procedures in function of the characteristic of the single macroalgal species and local environmental conditions, possibly giving priority to new and more sustainable techniques capable of producing large numbers of germlings, which could also allow planning large-scale restoration measures.

2.1 Introduction

Kelp forests are among the most diverse and productive complex systems in temperate and cold coastal areas in both hemispheres (Mann, 1973). Kelps are globally widespread, inhabiting 43% of the world's marine ecoregions of all continents with the exception of Antarctica (Spalding *et al*., 2007; Krumhansl *et al.*, 2016).

Kelp forests are able to deeply modify local habitats, influencing the structure and function of ecosystems (Steneck, 2002). Because of their morphological features, their impressive biomass, and their persistence in the environment, kelp canopies have a fundamental role in attenuating the wave action, altering all the associated coastal processes, such as erosion, sedimentation, and influencing recruitment of benthic invertebrates (Duggings *et al.*, 1990).

Kelp species are important primary producers and, by supplying significant amounts of macroalgal detritus, they are also used as food source from detritivores and suspension feeders, magnifying the secondary productivity, and thus supporting complex food webs (Duggins *et al*., 1989; Mann, 2000; Krumhansl & Scheibling, 2012).

Furthermore, kelp forests support the formation of complex biogenic habitat, increasing the associated biodiversity (Dayton, 1985; Duggins *et al*., 1989). Indeed, kelps are directly used as substratum by sessile organisms (Dunton & Shell, 1987), and serve as habitat and nursery ground for a wide variety of species, providing shelter and food for many pelagic and benthic organisms, including economically relevant species (Bologna & Steneck, 1993; Anderson *et al*., 1997; Steneck *et al.*, 2002; Graham, 2004). Moreover, their canopy reduces the available light, creating favorable conditions for shade-adapted species (Santelices & Ojeda, 1984). By and large, from a human perspective, kelp beds provide a multitude of ecosystem services with an estimated value in the order of billions of dollars per year (Smale *et al.*, 2013; Vasquez *et al*., 2014; Bennet *et al.*, 2016).

Climate change and the increasing frequency of extreme events (e.g., storms, floods, heat waves, etc.) are the main threat for kelp forest, since these dramatic variations in

environmental conditions may have devastating consequences (Vasquez *et al*., 2006). In fact, catastrophic weather events, such as intense storms, can reduce the abundance of kelp by tearing out the plants and removing the spores and gametophytes from the substrate (Pereira *et al*., 2017). Furthermore, these species are highly sensitive to the warm and nutrient-poor water conditions associated to the climate change (Graham *et al*., 2007; Pereira *et al.,* 2015; Schiel & Foster, 2015), threatening the stability of populations, and influencing their distribution (Wernberg *et al.*, 2010; Filbee-Dexter *et al*., 2016; Franco *et al*., 2017), often resulting in massive mortality events (Dayton *et al.,* 1989; Johnson *et al.,* 2011).

Besides climate change and extreme weather events, kelp stands are particularly sensitive to a variety of disturbances, such as high sedimentation rates (Cheney *et al*., 1994; Shaffer & Parks, 1994), contamination of metals and pollutants (Hopkins & Kain, 1978, Chung & Brinkhuis, 1986), UV radiation (Hoffman *et al*., 2003), and invasions by competitive alien species (Britton-Simmons, 2004; Casas *et al.,* 2004). Additionally, several of these systems have shifted to barren grounds as consequence of overgrazing by sea urchins (Steneck *et al.*, 2002).

Since kelps are foundation species, it means that a multitude of species depends on their presence in the environment, and thus their decline might influence the structure and function of entire ecosystems, bringing them to collapse (Ellison *et al.*, 2005; Byrnes *et al.*, 2011). For this reason, several techniques have been used to try to enhance or restore kelp beds, such as sea urchin and competitive seaweeds control but, since often these methods seem to not have significant results, the attention shifted to n the culturing and transplanting of kelp individuals, both adults and juveniles (Hernàndez-Carmona *et al.*, 2000).

Several previous studies already assessed the feasibility of kelp transplantation, including the genera *Macrocystis, Laminaria* and *Sacchoriza.* These had the main purpose of assessing the role of habitat influence on morphological characteristics of the algae (Norton, 1969; Chapman, 1974; Druehl & Kemp, 1982; Brostoff, 1988), and restoring kelp stands in areas where those species disappeared due to biotic or abiotic factors (Mc Peak, 1977; Rice *et al*., 1989; Hernàndez-Carmona *et al.*, 2000). Since large-scale restoration plans require not only a considerable economic investment but also significant monitoring and maintenance efforts, projects mostly include small-scale experiments, developing both outplanting and transplanting techniques that use different development stages (Carney *et al.*, 2005).

The basic morphology of kelps consists in a holdfast (a root-like structure that anchors the plant to the substratum), a flexible stipe, and a frond called blade or lamina. Between the stipe and blade is located an area of meristematic tissue, which is responsible of the growth of the individuals (Kelly, 2005). The typical kelp biological cycle involves two life stages, a macroscopic diploid sporophyte that produces haploid spores, which germinate into microscopic haploid gametophytes. Gametophytes, in turn, produce haploid male and female gametes which, after fertilization originate new sporophytes (Dayton, 1985; Kelly, 2005).

Attempting transplantation with different life stages may present different advantages and disadvantages. Adults in reproductive phase are able to release spores locally, working as a first step for recolonization of areas where there is no canopy and providing protection for the juveniles. However, due to their morphological characteristics, they might not be able to adapt to the transplanting conditions, and bigger individuals are more subjected to the dragging force exerted by incoming waves and currents (Correa *et al.*, 2006). Moreover, removing adult individuals from healthy populations for transplanting may negatively affect their persistence (Carney *et al*., 2005). Transplantation of small juveniles could be more sustainable since, given the high rate of recruiting, these individuals compete for space and resources, and have a very low probability of surviving and reaching maturity. Therefore, considering the high mortality rates, their collection is less likely negatively affect natural populations. On the other hand, outplanting gametophytes and microscopic sporophytes cultured under laboratory conditions, allows a faster production of a large amount of individuals, but often with extremely high mortality rates (about 1 out of 100000 embryonic sporophyte develop into a mature individual; North, 1976). Furthermore, outplanting in areas with lack of natural canopy can result in a failure of the procedure: indeed, several previous studies documented a positive effect of the presence of adult conspecifics, since dense canopies can ameliorate abiotic environmental conditions, and protect recruits from grazers and competitors (Vadas *et al.*, 1992; Schiel & Foster, 2006).

2.1.1 Objective of the study

The main objective of the present work is to develop a valid technique to transplant the kelp *L. ochroleuca* Bachelot de la Pylaie, 1824, assessing the eventual influence of different stages of the life cycle of the species on the success of transplantation. Furthermore, a possible effect of the presence of macroalgal canopy on the survival of transplanted and outplanted individuals has been evaluated.

Laminaria ochroleuca is an Iberian species that lives in temperate waters, ranging from Portuguese to Northwestern coasts of Spain, and from Brittany to the English and Bristol channels, but it can also be found in Morocco and some Mediterranean locations, inhabiting rocky substrata from surface waters to 30 m deep (Birkett *et al.*, 1998; Flores-Moya, 2012**;** Ramos *et al*., 2016).

L. ochroleuca is the main perennial kelp species that inhabit Portuguese rocky shores, forming extensive and dense monospecific canopies. Recent studies highlighted that populations of this species seem to have shifted their distribution northward, mainly as a result of the effects of climate change (Smale *et al*., 2015; Pereira *et al*., 2019). Since the presence and persistence of the species in the habitat may influence a multitude of ecological process and ecosystem services, the development of valid transplantation techniques to restore depleted populations is particularly important.

2.2 Materials and methods

Sampling site

In Northern Portugal, *L. ochroleuca* forms dense stands in deep intertidal pools and has a patchy distribution in the upper subtidal, while the lower subtidal is dominated by the species *Saccorhiza polyschides* (Pereira *et al.*, 2017). We identified a large and dense intertidal patch (>30 individuals/m^2) of *L. ochroleuca* canopy in Viana do Castelo, northern Portugal (41°41'57.2N'', 8°51'19.84''W; fig.16).

Figure 15 - Study site and intertidal canopy patch of Laminaria ochroleuca

We transplanted both adult and juvenile individuals, and outplanted sporophytes produced in laboratory, assessing survival rates and growth of all the different life stages. Moreover, we placed replicates of the three life stage at three different distances from the *Laminaria* patch (away, in the middle, or at the edge of the patch), in order to evaluate the effect of the macroalgal canopy on the survival and growth of transplanted individuals.

Experimental setup and sampling

Adult and juvenile transplantation

30 adult and 45 juvenile individuals (<20 cm of total length) of *L. ochroleuca* were collected from the transplantation site inn July 2019. This time of the year was chosen to match with the peak of recruitment period of *Laminaria* individuals in the area. Samples were transported in cold seawater inside a refrigerated box, and reached the laboratory within 2 hours of collection in field

In the laboratory, the total length of the lamina of each adult was measured, and then the lamina was cut at the same length (30 cm) for all the individuals. Furthermore, maximum length of juvenile individuals has been recorded.

The linear growth of *Laminaria* individuals was assessed using the hole-punch method (Parke, 1948): in laboratory, two holes were punched at the center of the lamina, 3 cm above the meristem (at the junction between stipe and blade). Linear growth was calculated by measuring the distance between the holes and the meristem, and subtracting the initial 3 cm distance.

After measuring, two adults were fastened by the holdfast to a 16x4 cm PVC tile, using cotton ties to permit the natural movement of the individuals and try to prevent breakage of the stipe (fig.17). The same procedure was used for the juveniles, fixating three individuals to each PVC plate. Five tiles per life stage were numbered and, the day after the collection of individuals, the plates were screwed to the substratum in the transplantation site.

Both linear growth and individuals' survival were recorded monthly.

Figure 16 – Adult individuals of Laminaria ochroleuca *tied to PVC plate before transplantation*

Outplanting of sporophytes

In June 2019, a month before the transplantation, reproductive tissue of *L. ochroleuca* was collected from the transplantation site, and transported to the laboratory within 2 hours. The tissue was, then, cleaned from epiphytes and releasing of the spores was induced.

Spores were then maintainined in culture in 0,2 µM Provasoli Enriched Seawater (PES) (Andersen, 2005), changed weekly, with a 12:12 light:dark photoperiod and photon fluency rate of 30 µmol m^{-2} s^{-1}. After germination, the gametophytes were grown for three weeks.

The same procedure was used to produce a second set of culture, but in this case the culture was maintained under a 10 µmol m^{-2} s^{-1} red light to induce vegetative growth of gametophytes.

The two culture solutions were sprayed onto a different cotton rope and maintained in two separate tanks filled with 0,2 µM PES, which was changed once a week.

On July 2019, a day before the transplantation, the cotton ropes with the two sporophyte cultures were cut in segments of 15 cm each, and then fixed to five PVC tiles per culture. The day after, all the tiles were transported to the transplantation site, and fixated to the substrate.

2.3 Results

Adult and recruit transplantation

PVC plates with adult individuals were checked one month after the transplantation, on August 2019. More than half of the transplanted plants were lost, with a survival rate of about 41%. Nevertheless, all the remaining individuals showed an increase in length (average linear growth: 18,6±2,64 mm/month).

Two months after the transplantation (September 2019), 95% of the transplanted plants were lost, but the surviving thalli were still growing (average linear growth: 50±45 mm/month).

98% of transplanted juveniles were lost the first month after the transplantation.

Outplanting of sporophytes

The fixation of the plates with microscopic sporophyte was 100% successful, with no loss during the first month after transplantation (August 2019). However, sown ropes were entirely overgrown by turf, including both green and red algae species (fig.18).

Figure 17 – Turf algae growing on microscopic sporophytes experimental units

The plates were, thus, removed from the transplantation site and taken to the laboratory, to check under the stereoscope if there were kelp recruits still growing on the ropes. After being sure that there were no kelps left, all the red and green turf in the ropes was separately quantified as fresh weight. After that, turf algae were dried at 60°C for 48h until constant dry weight was reached. Furthermore, total length of each single rope was measured, in order to assess the quantity of dry weight of turf algae per linear centimeter.

Green algae, mainly *Ulva* spp., represented the largest portion (about the 62%) of the turf, with an average density of 158,16±7,92 mg DW/cm, while red algae had an average density of 96,89±10,31 mg DW/cm.

A second outplanting attempt, using macroscopic sporophytes growing on ropes, (>1mm) has been performed on August 2019. We used the same sporophyte culture obtained by the vegetative growth of gametophytes, which was left to grow for another month**.** We chose this culture because it showed a higher number of sporophytes per cm compared to the other one: higher densities of sporophytes could probably benefit kelps in a competition for space with turf algae.

2.4 Discussion

Transplanting and outplanting methods for restoration purpose have been increasingly developed in recent years (Carney *et al.*, 2005). Previous studies attempted transplantation using several kelp species, including large-sized kelps such as *Macrocystis* and *Laminaria* with very variable outcomes in terms of survival of individuals and growing rates, and often with discouraging results (Hernàndez-Carmona *et al.*, 2000; Dean & Jung, 2001; Carney *et al*., 2005). In extreme cases, all the plants were lost shortly after the transplantation (Vasquez & Tala, 1995).

Regarding adult and juvenile transplantation, our results evidenced the inadequacy of the chosen method. Even if the same technique has been previously used with *Laminaria ochroleuca* (e.g. Franco *et al*., 2017), particularly harsh local environmental conditions (e.g. strong hydrodynamism or storm events) may have determined the failure of the transplanting technique.

In detail, stipe breakage was the main cause of mortality of the transplanted individuals. For what regards adults, tying the plants from the basis of the stipe probably did not allow their free movement. Indeed, transplanted individuals were often found with only the holdfast remaining tied to the plate: most likely, since the plants were not able to follow waves and currents, the resistance offered by their voluminous fronds may have determined the break of the stipe just above the tying point. Juveniles, on the other hand, have really soft and delicate stipes, which are particularly prone to physical injuries. Additionally, some of the transplanted individuals had cuts that coincide with fish bite marks, which resulted in severe damages of both the stipes and blades.

A previous study assessed that local pressure due to herbivory may affect both distribution and abundance of *Laminaria* species in Portugal (Franco *et al*., 2015). Moreover, outcomes of a transplantation experiment conducted on *L. ochroleuca* showed that exclusion of grazers resulted in an increase of survival rate of transplanted juveniles, while transplanted adults showed an increase in the lamina extension (Franco *et al*., 2017). However, devices that protect transplanted individuals from grazers, such as exclusion cages, are a valid option in small-scale experiments, due to the consistent efforts and relevant costs needed to install them in the field.

The influence of herbivory on survival of sporophyte was not evaluated, even if precedent studies revealed that small-size grazers (e.g., amphipods and gastropods) are fundamental in determine the success of recruitment of Laminariales (Henrìquez *et al.*, 2011).

Regarding outplanting of microscopic sporophytes, the major issue seems to be the competition with algal turf. Previous studies already documented that survival of juvenile individuals can be seriously affected by the presence of algal competitors for light and substratum (Mc Peak, 1981; Kennelly, 1987). In particular, turf algae are mostly composed by fast-growing opportunistic species, which seem to be able to replace kelps on both small and large spatial scale (Filbee-Dexter & Wernberg, 2018). Outplanting should be timed in order to avoid periods when competitive algae or grazers are at their highest densities. In our case, to limit the colonization of turf on the sporophyte ropes, a second outplanting with already visible sporophytes has been attempted, since bigger sporophytes may be able to better competite for space.

Presence of canopies of conspecific individuals is reported in literature to have contrasting effects: it can either mitigate environmental condition promoting growth and survival of individuals (Schiel & Foster, 2006) or, on the contrary, inhibit their growth by competitive shading (Vadas *et al.*, 1992).

However, it seems that in our case there are no differences in the survival of both adult and juvenile individuals according to their position in the *L. ochroleuca* patch, since all of the transplanted plants were subjected to dislodgment due to the intense hydrodynamism. Moreover, the replicates of microscopic sporophytes seem to be equally affected by turf overgrowth, without any difference based on their different position in the *Laminaria* patch.

These results highlight some of the difficulties already encountered in previous studies (e.g. Hernàndez-Carmona *et al*., 2000; Carney, 2003; Carney *et al*., 2005), such us the choice of a valid planting method and a suitable restoration site. Despite these precautions, a certain degree of uncertainty has to be expected, as well as a potential failure of the transplantation method. However, combining different planting techniques can more likely increase the restoration success, especially in unpredictable environments characterized by large fluctuations of abiotic and biotic variables, such as intertidal habitats.

General conclusion

Many brown macroalgae species have a fundamental ecological role as ecosystem engineers, especially Laminariales and Fucales, which are able to build complex habitat such as kelp forests and fucoid canopies (De La Fuente *et al*., 2019; Layton *et al*., 2019). These habitats are subjected to multiple stressors, both natural and anthropogenic, which can act synergistically determining the decline and loss of macroalgal forests over extensive areas (Thibaut *et al.*, 2005; Sales *et al*., 2011). Since passive conservation strategies are not always successful in recovering degraded habitats, active restoration plans are more often required (Marzinelli *et al*., 2014).

As a consequence, macroalgal transplantation techniques are being increasingly developed but, despite their importance, restoration of macroalgal forests still remain poorly explored compared to other relevant marine habitats (Gianni *et al.*, 2013).

In the last few years, several transplantation methods have been tested, with the main objective of assessing their efficacy: the most frequently used technique was transplantation of adult and juvenile individuals (Correa *et al*., 2006; Falace *et al*., 2006; Susini *et al*., 2007). Despite the reported success of some of these transplantation attempts involving both kelp and fucoid species, most of the studies seem to have variable outcomes, often resulting in low survival rates or no remaining individuals (Mangialajo *et al.*, 2012; Perkol-Finkel *et al.*, 2012; Campbell *et al*., 2014).

The majority of transplantation experiments have been performed at small spatial scale and with a limited number of transplanted individuals collected from healthy donor populations (Hernàndez-Carmona *et* al., 2000; Correa *et al*., 2006; Falace & Bressan, 2006; Falace *et al*., 2006; Susini *et al*., 2007). However, since several kelp species have fast growth and high recruitment rates, naturals donor populations are able to naturally recover after these interventions. Fucoid species like *Cystoseira*, on the other hand, are characterized by a low dispersal capacity that compromise natural recovery of degraded canopies, an effect that is magnified in case of poor densities or absence of adults. In those cases, collecting individuals cannot be viewed as an option as it could result in a permanent damage for donor forests (Falace *et al*., 2018; Rindi *et al.*, 2018). So, from a perspective of conservation management, it

seems incoherent to remove individuals from healthy populations to recover impacted areas, especially for species characterized by a scarce resilience, since this practice can result in an unrecoverable disturbance for donor populations. Furthermore, stress due to transplantation can induce modifications in the transplanted individuals, at both morphological and physiological level, potentially affecting the transplantation success (Correa *et al*., 2006; Falace *et al.*, 2006). Given these premises, it seems obvious that adult transplantation is not a viable option, even more in case of extensive reforestation plans.

Thus, there is a growing necessity to switch to more efficient restoration methods, such as outplanting of recruits cultured in laboratory conditions (Carney *et al.*, 2005; Verdura *et al.*, 2015; Falace *et al*., 2018). This kind of approach offer several benefits compared to adult transplantation, such as fast production and the availability of large quantities of individuals with an insignificant impact on natural population, potentially allowing large-scale restoration plans (Falace *et al.*, 2018; De La Fuente *et al*., 2019).

However, several factor may affect both the cultivation and outplanting procedures. First, the availability of fertile material, which may influence the timing of the restoration procedure, depending on species demographic features, climatic conditions, and location of the donor population (Tamburello *et al*., 2019). The cultivation phase should follow species-specific protocols, in terms of conditions and duration of cultivation phase, and choice of the proper substrate (Falace *et* al., 2018). Moreover, particular attention must be paid to the use of culture medium, and to the adding of antibacterial solution and a careful cleaning of the fertile material, which can prevent blooms of epiphytes and bacteria (Verdura *et al*., 2018).

Once transported to the field, it would be advisable to implement protection devices (e.g. exclusion cages and nets) to mitigate the effect of grazers: previous studies, in fact, highlighted a positive effect of protection cages on the survival of ouplanted germlings (Dudgeon & Petraitis, 2005; Franco *et al*., 2017), although these devices can't prevent the effect of mesograzers (Korpinen *et al*., 2008; Henrìquez *et al*., 2011).

Lastly, attention has to be paid also to the technique used for the attachment to the substrate, as it can dramatically affect the success of the outplanting (e.g. Carney *et al*. (2005) lost about the 60% of all the outplanted substrates during the study due to an epoxy failure).

Recovering degraded macroalgal forest is an important topic in habitat restoration. However, there is still a lack of information about species response to transplanting and outplanting procedures, as well as about the implementation of species-specific protocols, and identification of suitable restoration sites. Pilot studies having these main objectives should be considered a priority, since they can provide useful information on best practices to follow in order to maximize the success of restoration procedures, providing also useful tools under a perspective of conservation management.

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Supplementary material

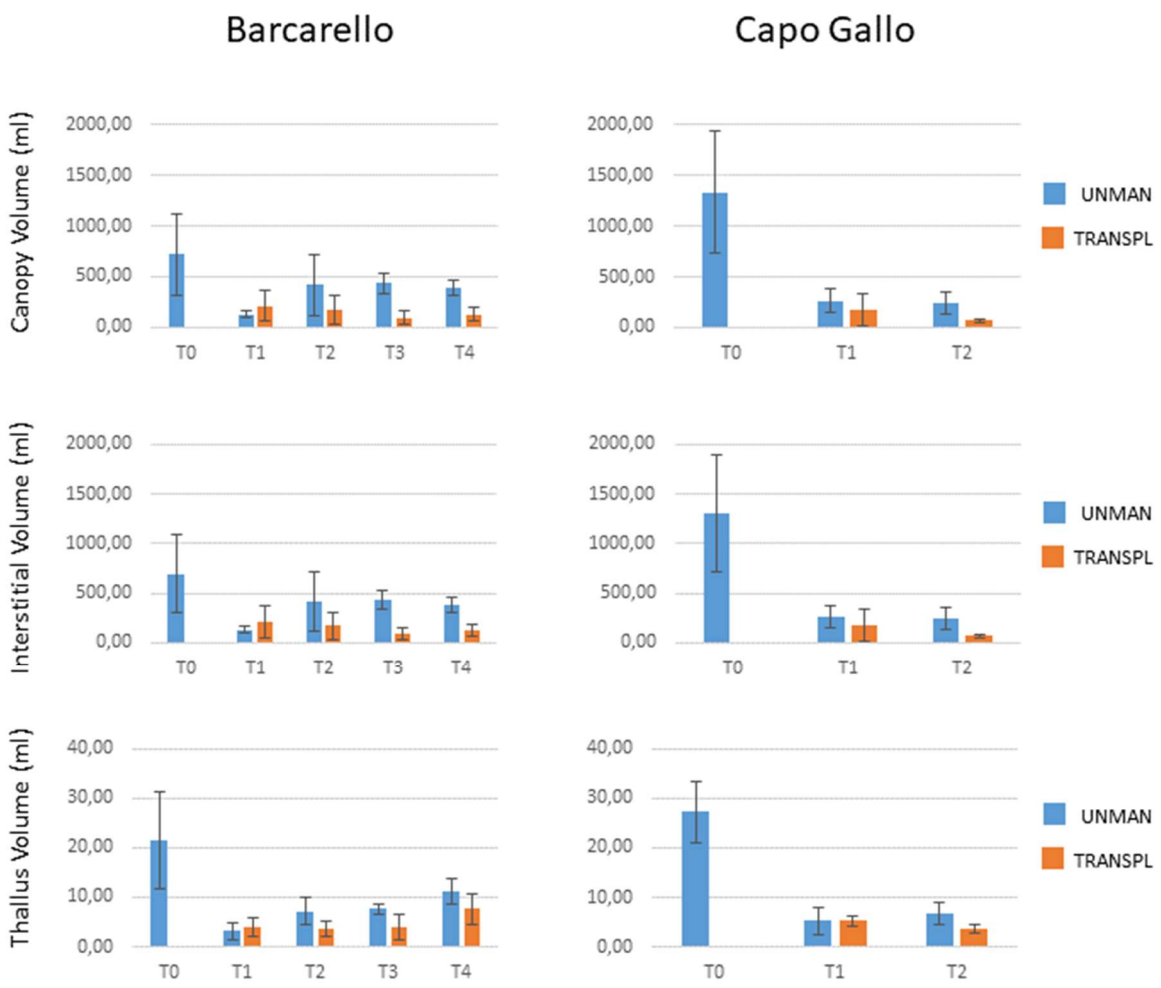

Figura S1 – Volumes (mean±SD) of C.amentacea transplanted and unmanipualated thalli, in both the two sites during the course of the experiment. Values are expressed in milliliters

Table S1 - PERMANOVA results testing the effects of the fixed and orthogonal factors Site, Treatment, and Time on the Canopy Volume of the thalli. Notes: a) Main test, b) Pair-wise tests. Significant differences are highlighted in bold font

PERMANOVA					
a) Main test					
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Site	1	2.78E+09	53,056	**0,02**	997
Treatment	1	4.67E+09	89,143	**0,01**	997
Time	4	8.26E+09	15,763	**0,001**	999
SitexTreatment	1	5830,6	0,11129	0,718	996
SitexTime	2	3.80E+09	72,491	**0,001**	999
TreatmentxTime	3	82417	15,731	0,225	999
SitexTreatmentxTime	1	34674	0,66183	0,438	997

b) Pair-wise tests				
Differences between sites within times				
Time	**Site**	**t**	**P(perm)**	**perms**
T0	BAR vs CAPG	19,068	0,073	126
T1	BAR vs CAPG	0,85823	0,409	999
T2	BAR vs CAPG	17,518	0,072	998

Differences between times within sites				
Site	**Time**	**t**	**P(perm)**	**perms**
Barcarello	T0, T1	37,123	**0,003**	996
	T0, T2	15,489	0,145	999
	T0, T3	18,127	0,098	994
	T0, T4	21,504	0,052	995
	T1, T2	1,519	0,163	998
	T1, T3	20,413	**0,044**	995
	T1, T4	21,364	0,057	998
	T2, T3	0,42959	0,694	996
	T2, T4	0,46013	0,685	992
	T3, T4	4.91E+02	0,956	997
Capo Gallo	T0, T1	46,508	**0,001**	993
	T0, T2	48,778	**0,001**	998
	T1, T2	12,017	0,244	995

Table S2 - PERMANOVA results testing the effects of the fixed and orthogonal factors Site, Treatment, and Time on the Interstitial Volume of the thalli. Notes: a) Main test, b) Pair-wise tests. Significant differences are highlighted in bold font

PERMANOVA					
a) Main test					
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Site	1	2.70E+09	52,373	**0,028**	997
Treatment	1	4.56E+09	88,311	**0,004**	996
Time	4	7.84E+09	15,179	**0,001**	999

SitexTreatment	1	5782,4	0,11202	0,755	998
SitexTime	2	3.74E+09	72,431	**0,001**	997
TreatmentxTime	3	80274	15,551	0,218	999
SitexTreatmentxTime	1	34321	0,66489	0,45	995

b) Pair-wise tests				
Differences between sites within times				
Time	**Site**	**t**	**P(perm)**	**perms**
T0	BAR vs CAPG	19,022	0,071	126
T1	BAR vs CAPG	0,83875	0,411	995
T2	BAR vs CAPG	17,622	0,09	995

Differences between times within sites				
Site	**Time**	**t**	**P(perm)**	**perms**
Barcarello	T0, T1	36,732	**0,003**	996
	T0, T2	14,976	0,16	995
	T0, T3	17,597	0,108	996
	T0, T4	21,293	**0,05**	997
	T1, T2	15,085	0,151	998
	T1, T3	20,145	0,068	997
	T1, T4	20,248	0,072	993
	T2, T3	0,43774	0,697	998
	T2, T4	0,51515	0,667	998
	T3, T4	0,1541	0,886	997
Capo Gallo	T0, T1	45,579	**0,002**	995
	T0, T2	47,864	**0,001**	997
	T1, T2	12,149	0,251	999

Table S4 - PERMANOVA results testing the effects of the fixed and orthogonal factors Site, Treatment, and Time on the Thallus Volume of the thalli. Notes: significant differences are highlighted in bold font

PERMANOVA					
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Site	1	52,9	3,931	0,059	996
Treatment	1	67,6	50,234	**0,034**	996
Time	4	573,66	42,628	**0,001**	999
SitexTreatment	1	0,1	7.43E+00	0,94	996
SitexTime	2	23,25	17,277	0,202	998
TreatmentxTime	3	16,067	11,939	0,316	999
SitexTreatmentxTime	1	0,9	6.69E+02	0,823	996

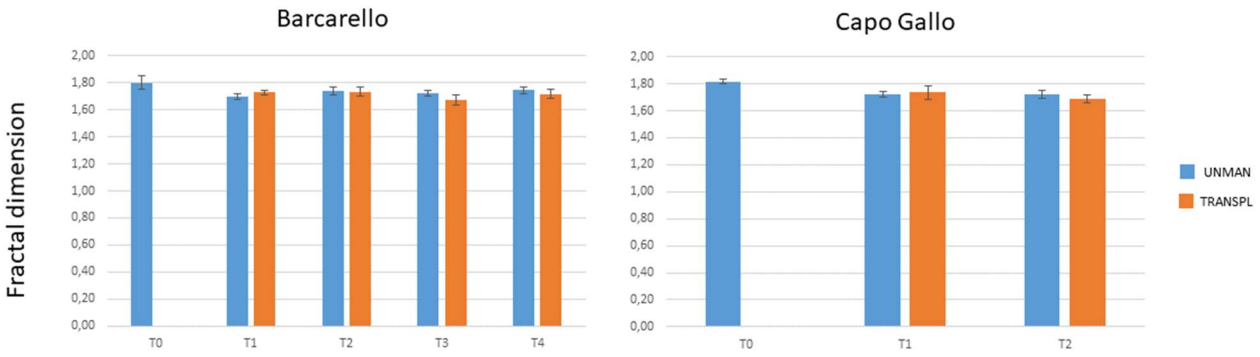

Figure S2 – Fractal dimension (mean ±SD) of C.amentacea transplanted and unmanipulated thalli for the two sites throughot the course of the experiment

Table S5 - PERMANOVA results testing the effects of the fixed and orthogonal factors Site, Treatment, and Time on the Fractal dimension of the thalli. Notes: a) Main test, b) Pair-wise tests. Significant differences are highlighted in bold font

PERMANOVA					
a) Main test					
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Site	1	1.93E+00	0,19772	0,616	995
Treatment	1	5.86E+01	60,083	**0,022**	996
Time	4	1.24E+02	12,728	**0,001**	999
SitexTreatment	1	9.93E+00	10,178	0,334	998
SitexTime	2	2.92E+01	29,927	0,063	999
TreatmentxTime	3	3.71E+01	38,052	**0,009**	999
SitexTreatmentxTime	1	6.32E-01	6.48E+02	0,811	996

b) Pair-wise tests				
Differences between treatments within times				
Time	**Treatment**	**t**	**P(perm)**	**perms**
T1	Unm vs Tran	16,159	0,103	998
T2	Unm vs Tran	13,508	0,18	996
T3	Unm vs Tran	27,671	**0,028**	120
T4	Unm vs Tran	1,587	0,135	123

Differences between times within treatments				
Treatment	**Time**	**t**	**P(perm)**	**perms**
Unmanipulated	T0, T1	72,212	**0,001**	998
	T0, T2	51,799	**0,001**	998
	T0, T3	38,153	**0,005**	995
	T0, T4	26,918	**0,021**	994
	T1, T2	18,125	0,091	999
	T1, T3	18,724	0,093	997
	T1, T4	3,218	**0,004**	996
	T2, T3	10,531	0,317	997

	T2, T4	0,20617	0,823	996
	T3, T4	16,185	0,165	110
Transplanted	T1, T2	12,519	0,243	997
	T1, T3	2,4	**0,04**	997
	T1, T4	0,55122	0,616	998
	T2, T3	28,027	**0,022**	998
	T2, T4	0,88483	0,402	989
	T3, T4	19,097	0,105	126

Table S6 – Checklist of macrofaunal community with the total number of individuals per taxa

Class	Taxa	Total N° individuals
Crustacea		**11387**
	Ampithoe ramondi	306
	Ampithoidae N.D.	81
	Apolochus sp.	61
	Caprella acanthifera	1257
	Caprella liparotensis	121
	Caprellidae N.D.	100
	Cumacea N.D.	1
	Cymodoce truncata	73
	Dynamene edwarsii	193
	Elasmopus pocillimanus	7
	Elasmopus rapax	158
	Gammaridae N.D.	1
	Gnathidae ND	1
	Hyale crassipens	24
	Hyale schmidtii	293
	Hyalidae ND	82
	Ischyrocerus inexpectatus	1040
	Ischyroceridae ND	5
	Janira sp.	19
	Ianiropsis sp.	17
	Jassa marmorata	33
	Leptochelia savignyi	27
	Maera inequipes	27
	Majidae N.D.	7
	Melitidae N.D.	3
	Parasinelobus chevreuxi	313
	Parhyale aquilina	6602
	Pereionotus testudo	33
	Podocerus variegatus	334
	Sphaeromatidae N.D.	2
	Stenothoe cavimana	220
	Zeuxo sp.	1
Mollusca		**572**
	Acanthochitona fascicularis	1
	Alvania cancellata	1

	Alvania discors	1
	Aplysiomorpha N.D.	1
	Barleeia unifasciata	24
	Brachidontes pharaonis	1
	Cardita caliculata	3
	Crisilla beniamina	1
	Crisilla semistriata	3
	Crisilla simulans	3
	Dendropoma sp.	2
	Eatonina cossurae	18
	Eubranchis sp.	1
	Fissurella nubecula	1
	Gasteropode N.D.	2
	Gibbula turbinoides	3
	Gibbula sp.	93
	Jujubinus sp.	2
	Lepidochitona caprearum	2
	Megalomphala azonus	6
	Mytilaster minimus	32
	Ocinebrina edwarsii	2
	Patella caerulea	2
	Patella sp.	1
	Pisania striata	4
	Rissoa guerinii	31
	Rissoa lia	1
	Rissoa similis	25
	Rissoa sp.	142
	Scissurella costata	9
	Setia amabilis	16
	Setia ambigua	1
	Setia maculata	19
	Setia sp.	54
	Sinezona cingulata	61
	Steromphala divaricata	2
	Tricolia tenuis	1
Polychaeta		**592**
	Autolytus sp.	7
	Euphrosine sp.	1
	Eusyllis assimilis	1
	Exogone naidina	3
	Exogone sp.	3
	Exogoninae N.D.	1
	Harmothoe sp.	5
	Lepidonotus clava	4
	Lumbrineris sp.	2
	Nereididae N.D.	8
	Nereis pulsatoria	1
	Nereis rava	1

	Pionosyllis pulligera	46
	Platynereis dumerilii	233
	Salvatoria clavata	2
	Salvatoria limbata	82
	Sphaerosyllis sp.	4
	Sphaerosyllis pirifera	11
	Syllis alternata	1
	Syllis gerlachi	46
	Syllis prolifera	118
	Syllis sp.	12

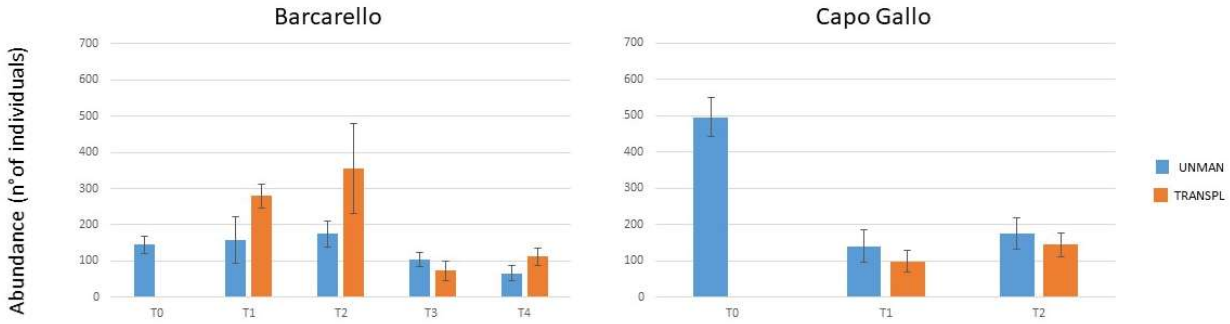

Figure S3 – Mean values (±SE) of total abundance of epifauna associated to Cystoseira amentacea *in the two sites for the different treatments (Unmanipulated vs Transplanted)*

Table S7 - PERMANOVA results testing the differences in the abundance of macrofaunal individuals in relation to the factors Site, Treatment, and Time. Notes: a) Main test, b) Pair-wise tests. Significant differences are highlighted in bold font

PERMANOVA					
a) Main Test					
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Site	1	54,742	0,23686	0,735	999
Treatment	1	53,126	0,22987	0,727	999
Time	4	1211,7	52,431	**0,002**	999
SitexTreatment	1	1169,2	50,588	**0,029**	999
SitexTime	2	818,59	3,542	**0,024**	999
TreatmentxTime	3	532,57	23,044	0,061	998
SitexTreatmentxTime	1	200,78	0,86877	0,378	999

b) Pair-wise tests				
Differences between sites within treatments				
Treatment	**Sites**	**t**	**P(perm)**	**perms**
Unmanipulated	BAR vs CAPG	18,591	0,071	999
Transplanted	BAR vs CAPG	2,993	**0,003**	999

Differences between sites within times				
Time	**Sites**	**t**	**P(perm)**	**perms**

T0	BAR vs CAPG	64,356	**0,012**	126
T1	BAR vs CAPG	1,797	0,08	999
T2	BAR vs CAPG	1,205	0,25	996

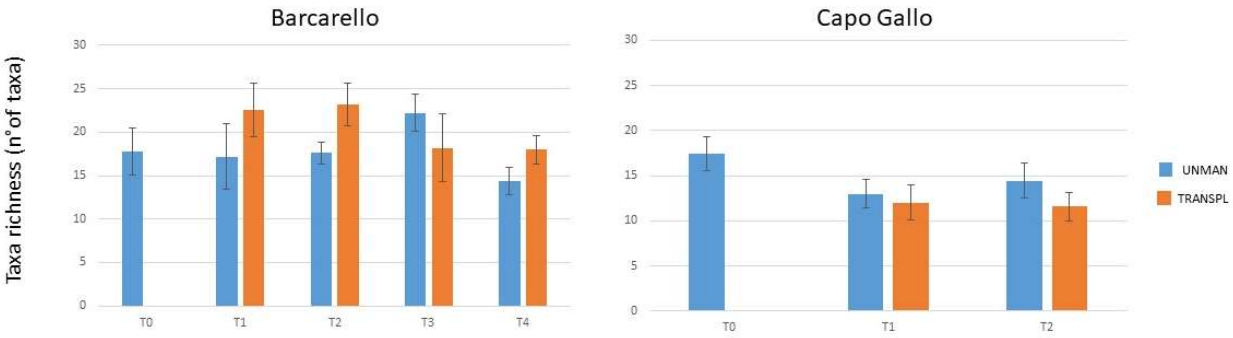

Figure S4 - Mean values (±SE) of taxa richness of epifauna associated to C. amentacea *in the two sites for the different treatments (Unmanipulated vs Transplanted)*

Table S8 - PERMANOVA results testing the differences in the macrofaunal taxa richness in relation to the factors Site, Treatment, and Time. Notes: a) Main test, b) Pair-wise tests. Significant differences are highlighted in bold font

PERMANOVA					
a) Main test					
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Site	1	858,6	13,374	**0,001**	997
Treatment	1	54,562	0,84991	0,393	999
Time	4	44,29	0,6899	0,581	999
SitexTreatment	1	309,33	48,184	**0,028**	998
SitexTime	2	27,565	0,42939	0,672	997
TreatmentxTime	3	117,37	18,283	0,153	999
SitexTreatmentxTime	1	23,365	3.64E+02	0,931	998

b) Pair-wise tests				
Differences between sites within treatments				
Treatment	**Sites**	**t**	**P(perm)**	**perms**
Unmanipulated	BAR vs CAPG	13,951	0,169	999
Transplanted	BAR vs CAPG	41,994	**0,001**	999

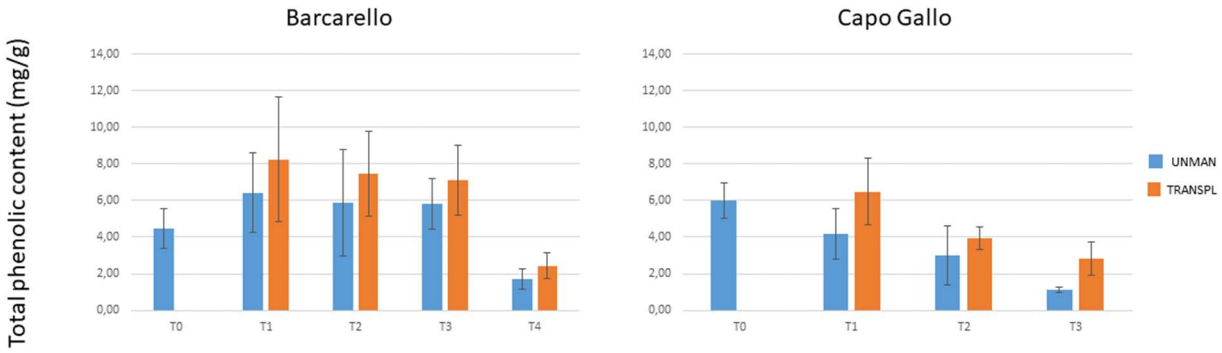

Figure S5 – Total phenolic content (mean±SD) of C.amentacea *transplanted and unmanipulated thalli for both the two sites during the course of the experiment. Values are expressed in mg/g*

Table S9 – PERMANOVA results testing the differences in the Total Phenolic Content in relation to the factors Site, Treatment, and Time. Notes: a) Main test, b) Pair-wise tests. Significant differences are highlighted in bold font

PERMANOVA					
a) Main test					
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Site	1	64,588	22,418	**0,001**	998
Treatment	1	27,23	94,513	**0,007**	998
Time	4	48,34	16,779	**0,001**	997
SitexTreatment	1	0,20095	6.97E+02	0,796	998
SitexTime	3	19,324	67,073	**0,002**	999
TreatmentxTime	3	0,53783	0,18668	0,904	998
SitexTreatmentxTime	2	0,83178	0,28871	0,745	999

b) Pair-wise tests				
Differences between sites within times				
Time	**Site**	**t**	**P(perm)**	**perms**
T0	BAR vs CAPG	22,791	0,052	126
T1	BAR vs CAPG	22,247	0,055	998
T2	BAR vs CAPG	34,772	**0,007**	999
T3	BAR vs CAPG	79,369	**0,001**	997

Differences between times within sites				
Site	**Time**	**t**	**P(perm)**	**perms**
Barcarello	T0, T1	15,078	0,168	997
	T0, T2	0,97861	0,334	995
	T0, T3	1,394	0,191	997
	T0, T4	53,258	**0,001**	997
	T1, T2	0,73253	0,463	997
	T1, T3	10,539	0,345	996
	T1, T4	5,853	**0,001**	998
	T2, T3	0,20453	0,837	994
	T2, T4	5,374	**0,001**	999
	T3, T4	77,769	**0,001**	998

Capo Gallo	T0, T1	21,478	**0,049**	997
	T0, T2	41,277	**0,002**	994
	T0, T3	99,382	**0,001**	994
	T1, T2	30,725	**0,01**	999
	T1, T3	64,701	**0,001**	997
	T2, T3	34,851	**0,006**	998

Table S10 - PERMANOVA results testing the differences in the FA profiles in relation to the factors Site, Treatment, and Time. Notes: a) Main test, b) Pair-wise tests. Significant differences are highlighted in bold font

PERMANOVA					
a) Main test					
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**	**perms**
Site	1	1.53E+02	18,687	**0,001**	999
Treatment	1	9.21E+01	11,27	**0,001**	998
Time	4	4.28E+02	52,346	**0,001**	999
SitexTreatment	1	1.99E+01	24,361	**0,042**	998
SitexTime	3	1.54E+01	18,847	**0,042**	999
TreatmentxTime	3	4.04E+01	49,407	**0,001**	997
SitexTreatmentxTime	1	3.00E+01	36,658	**0,009**	998

b) Pair-wise tests				
Differences between sites within treatments				
Treatment	**Site**	**t**	**P(perm)**	**perms**
Unmanipulated	BAR vs CAPG	29,933	**0,001**	999
Transplanted	BAR vs CAPG	48,919	**0,001**	999

Differences between sites within times				
Time	**Site**	**t**	**P(perm)**	**perms**
T0	BAR vs CAPG	0,69084	0,738	56
T1	BAR vs CAPG	45,975	**0,001**	998
T2	BAR vs CAPG	34,259	**0,001**	999
T3	BAR vs CAPG	24,847	**0,001**	996

Differences between treatments within times				
Time	**Treatment**	**t**	**P(perm)**	**perms**
T1	Unmanip vs Transpl	26,204	**0,001**	999
T2	Unmanip vs Transpl	18,701	**0,009**	998
T3	Unmanip vs Transpl	3,334	**0,001**	998
T4	Unmanip vs Transpl	22,757	**0,033**	56

Differences between treatment within sites and times

Site and time	Treatment	t	P(perm)	perms
Barcarello T1	Unmanip vs Transpl	15,038	0,085	126
Barcarello T2	Unmanip vs Transpl	11,937	0,227	126
Barcarello T3	Unmanip vs Transpl	33,048	**0,014**	126
Barcarello T4	Unmanip vs Transpl	22,757	**0,037**	56
Capo Gallo T1	Unmanip vs Transpl	2,597	**0,008**	126
Capo Gallo T2	Unmanip vs Transpl	25,162	**0,013**	126

Acknowledgements

I would like to sincerely thank all the people that contributed to the realization of my PhD project, for helping me in so many different ways.

Firstly, I'm deeply grateful to my supervisors Prof. Antonio Mazzola and Prof. Salvatrice Vizzini for the chance they gave me to work together, their precious advices, and for guiding and supporting me during these three years of PhD.

Thanks to all the people of LaBioMar, Cecilia, Andrea, Cristina, Geraldina, Elisa, Laura for all the technical (and emotional) support they gave me during this journey. And to Veronica: you were a fantastic roommate, the best companion to share this crazy adventure.

I'm really grateful to Prof. Isabel Sousa-Pinto for the opportunity she gave me to join her research group, one of the best and most useful working experiences in my life.

Thanks to all the group of the CIIMAR, especially to Itziar, Bianca, Joana, Rosa, Fernando, for all the time and the laughs that we shared. A special thanks goes to Tania, for being always supportive and present, more a friend than a supervisor.

I want to thank all the wonderful people that I met in Porto, you are too many to name you all! Just...I will be back, it's a promise.

Thanks to my family and close friends, for always believing in me and encouraging me, especially in hard times. And most of all thanks for the patience you had (...I'm sorry!)

Thank you all!