



D 3.3: Exploring tools to enhance the effectiveness of restoration actions under a changing ocean scenario

Marine Ecosystem Restoration in Changing European Seas MERCES

Grant agreement n. 689518

COORDINATOR: UNIVPM

LEAD BENEFICIARY: 2 - CSIC

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SUBMISSION DATE: 30/3/2019

DISSEMINATION LEVEL (e.g. Public)

PU	Public	
CO	CO Confidential, only for members of the consortium (including the Commission Services)	

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Introduction

The main goal of Deliverable 3.3. is to describe the activities seeking to enhance the effectiveness of active restoration actions face to the current environmental change and more particularly to warming. In fact, mounting evidences suggest that extreme climatic events are already severely impacting worldwide marine biodiversity (Hughes et al. 2018). In the last decades recurrent marine heat waves, “*periods of extreme warm sea surface temperature that persist from day to month and can extend up to thousands of kilometers*” (Frölicher & Laufkötter 2018), were reported worldwide including the European Seas where WP3 activities are carried out (Oliver et al. 2018). The occurrence of marine heat waves can alter the structure and functioning of entire ecosystems through a wide spectrum of processes such as onset of mass mortality events (MME), assemblages reconfigurations and shifts in species distributions (Smale et al. 2019). For instance, large scale MME in the North Western Mediterranean Basin impacting thousands of kilometers of coastal ecosystems was reported in 2003 (Garrahou et al. 2009). The warming can undermine the effectiveness of restoration activities implemented since for instance the transplants used in these actions can suffer mortality processes concomitant to the observed in natural populations. In fact, the marine heat wave recorded in the Catalan coast in 2017 resulted in a decrease on the survival of the transplants from the pilot action in the Medes Islands (WP3 report and Bensoussan et al. in press). In this context, the enhance the effectiveness of restorations is extremely urgent needed.

Interestingly, intensive field surveys conducted during the MMEs in the coralligenous species demonstrated that these events are characterized by their differential impacts at all the biological levels considered: among individuals, populations and species and even within colonies for colonial species such as octocorals (Garrahou et al. 2009). This suggests that individuals or populations within species may show different levels of tolerance to thermal stress. While the underlying eco-evolutionary processes explaining those differential responses remain to be fully characterized, the occurrence of warming-resistant individuals opens new perspective to enhance restoration actions.

In this context, the study of population-by-environment interactions (PEI), and particularly, the study of local adaptation, received increasing attention from theoretical and empirical perspectives (Ledoux et al. 2015, Alleaume-Benharira et al. 2006). While deciphering PEIs remain challenging, recent advances in sequencing technologies combined to experimental approaches, such as common garden experiments are promising tools to understand the impact of the shift in the regime of selection on biodiversity and to understand the differential responses to thermal stress reported from field surveys. Accordingly, the results of this integrative approach should allow the identification of potential resistant populations and/or colonies. These resistant colonies are of prime interest for active restoration actions such as transplantations. While active restoration actions are recognized as one of the most efficient tools to restore degraded tropical coral reefs impacted by bleaching, these technics remained to be adapted to the Mediterranean habitats impacted by MMEs.

On the other hand, a better understanding on the mechanisms enhancing the recovery rates could be used to improve the effectiveness of restoration actions. Within MERCES we explored how facilitation or positive interactions, i.e. encounters between organisms that benefit at least one of the participants and cause harm to neither, can support restoration actions. In our case, habitat forming species such as the gorgonians

growing in tree like colonies (up to 1 m in height) can alter the surrounding ambient owing to both physical and biological effects. These effects can be more or less detectable depending on the density of these colonies. In certain situations, by modifying abiotic conditions, they alter the variety of ecological niches available to other species, thus facilitating certain species and inhibiting others (Jones et al., 1994; Bruno et al., 2001). Facilitation mechanisms, according to the nature of the factors or processes involved, are divided into two main groups:

- Physical or environment-mediated mechanisms, including the buffering of environmental stress (e.g. temperature, desiccation, wave-forces, anoxic conditions) and the enhancement of suitable substrates (e.g. biogenic surfaces) or limiting resources (e.g. light, nutrients).
- Biological or interaction-mediated mechanisms, including the modification (lessening or increasing) by the benefactor of the rates or the efficiency of predation and grazing (Bulleri, 2009).

In terrestrial forests several environmental factors such as light, temperature and humidity are more stable during the day and during the year respect to outside, where wind, sun and rain can rapidly change (Allen et al. 1972). This effect for marine organisms has been documented in seagrass meadows (de Boer 2007), kelp forests (Gaylord et al. 2007), xenophyophore (Gooday et al 2017), garden sponges, anthozoan forests (Rossi et al., 2017) mussel and scallop beds (Cerrano et al. 2006), typically modify their own habitat increasing the fitness of associated species (Bruno and Bertness 2001).

The environmental conditions present inside these animal forests show a lower variability respect outside, affecting the structure of the associated benthic assemblages (Valisano et al., 2016, Ponti et al., 2018). For thi reason we design an experiment to assess if the presence of *P. clavata* can affect then settlement and growth of the bryozoan *Pentapora fascialis*, replicating the experiment in three different countries.

Within WP3 we explore two approaches to enhance the effectiveness of restoration actions i) Transregional thermotolerance experiment focused in unraveling the eco-evolutinonary processes to identify resistant populations and specimens to thermal stress to be used in restoration actions nts and ii) Transregional facilitation experiments to explore how to reduce recovery times through facilitation processes.

The transregional experimental approach adopted in MERCES WP· offered a novel and unique opportunity to investigate rarely explored environmental patterns and increase the inferential power of the obtained results. For the success of the implementation of the planned experiments, a remarkable coordination effort involving a 5 day field workshop organized by the CSIC and UB in the Medes Islands (Spain) (May 2017) and multiple teleconferences with the partners involved CoNISMa, UNIVPM, GAIA, PMF Zagreb.

Although the orginal plan for the Deliverable 3.3. was to work only in the Mediterranean coralligenous habitat, we expand the activities to the macroalgal forests dominated by *Cystoseira* spp. species. Likewise, we broaded the initial scope of the study both geographically and the analysis through the development of collaborations (see below).

i) Transregional thermotolerance experiments

Coralligenous habitats

We combined a trans-regional common garden experiment, involving individuals coming from five different Mediterranean regions, with whole genome sequencing and metagenomics analyses. We aimed to evaluate the role of eco-evolutionary processes (with a focus on local adaptation, genetic drift, acclimatization) on the differential responses of individuals and populations to thermal stress in the red gorgonian *Paramuricea clavata* species. Broadening the initial scope of the study, we also decided to characterize the spatial pattern of the microbiome (prokaryotes and micro-eukaryotes) associated to *P. clavata* and to test for a potential role of the microbiome in the differential responses. The final goal was to shed new light on the eco-evolutionary processes and environmental factors involved in the resistance to thermal stress, in view to identify potential donor populations for restoration actions.

Methods transregional thermotolerance experiment coralligenous

Study species:

The red gorgonian, *Paramuricea clavata* (Risso, 1826), is a habitat-forming octocoral with a key role in the structure and functioning of coralligenous habitats (Figure 1; Ballesteros 2006). Accordingly, *P. clavata* is of major interest for conservation and restoration purposes. Characterized by a patchy distribution mainly in the Western Mediterranean and in the Adriatic, this long-lived and gonochoric species reproduces annually and shows late sexual maturity (13 years of age) with a short larval phase (Linares et al. 2008). *P. clavata* was differentially impacted by past MMEs. For instance, during 2003 MME, the percentage of affected colonies (i.e. showing tissue necrosis) ranged from less than 5 up to more than 80% function of the considered populations (Garrabou et al. 2009). Within MERCES, the gorgonian *P. clavata* was one of the main species employed to fine-tune restoration protocols (reference to Deliverable 3.2.), implement pilot actions to scale up restoration activities and for other transregional experiments (See below Facilitation Experiment)

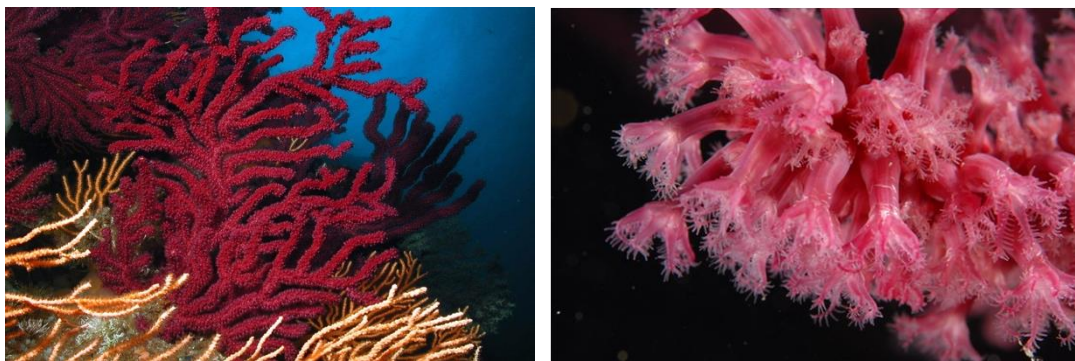


Figure 1: The red gorgonian, *Paramuricea clavata* (Risso, 1826) general view (left) and close-up on the polyps with penatulated tentacles.

Experimental set-up:

We carried out a common garden experiment in aquaria in which individuals from different origins were submitted to a common thermal stress in controlled conditions and their responses were assessed during the time of the experiment. We sampled 30 colonies coming from twelve populations located in five different regions (Southern Portugal, Catalonia, Corsica, Northern Italy and Croatia) separated by tens of meters to thousands of kilometers within the North and West Mediterranean and the Atlantic Ocean and inhabiting contrasting temperature regimes at the regional and local scales (Figure 2). We decided to broaden the panel of partners involved in the experiment. We developed a collaboration with the CCMAR team (Faro, Southern Portugal) in order to increase the number of environmental conditions of origin of the populations used in the experiment. Moreover, these populations experienced differential impact of MMEs from null (e.g. Croatia) to severe and repeated (e.g. Corsica) impacts.

It is noteworthy that setting up this experiment involved an important logistical effort shared among the different partners. Indeed, all the samples from the five regions were collected and sent alive and simultaneously at the ICM-CSIC experimental aquarium facilities in Barcelona (Spain). Field sampling was conducted by scuba-diving between the 16th and the 17th of September 2017 in each region by a local scientist team composed by three to five divers. The living samples (30 8-10 cm fragments of 30 different colonies by population) were collected, stabulated in a small aquarium system with filtration overnight and packed on the next morning in 2 liters bags of water (10 fragments per plastic bag) to be sent in polystyrene boxes (3 bags per box) on the 18th of September. The transport was conducted by Flying Sharks a company specialized in the transportation of living marine organisms. Transport duration was no more than 48 h in every case so that all the samples were in Barcelona on the 20th of September 2017.

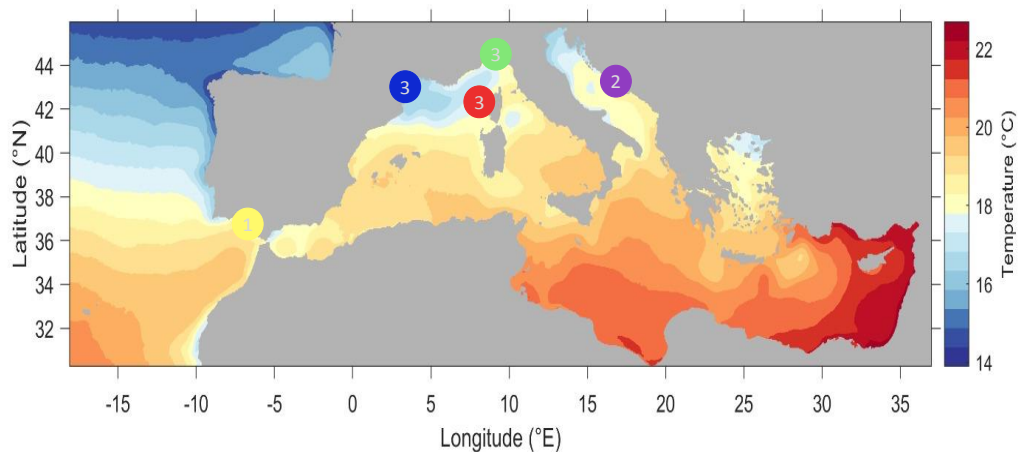


Figure 2. Map of the different localities sampled for the common garden experiment. Numbers within circles correspond to the number of sampled populations in each locality. This sampling was conducted by partners of the MERCES project (DiSVA-UNIVPM for Italian samples, PMF-ZAGREB for Croatian samples, CSIC and UB for Catalan and Corsican samples) with the collaboration of CCMAR (for Portuguese samples). Mediterranean Mean SST Generated using E.U. Copernicus Marine Service Information.

The experiment involved two aquarium sets: Control and Treatment. In the Control set, seawater temperature was maintained at 16-18°C during the whole experiment (Figure 3). In the Treatment set, the heat stress consisted of a stepwise temperature augmentation from 18°C to 25°C during 2-3 days. Once the 25°C temperature (i.e.

critical temperature for this species; Crisci et al. 2017) was reached, the condition was kept constant for 25 days.

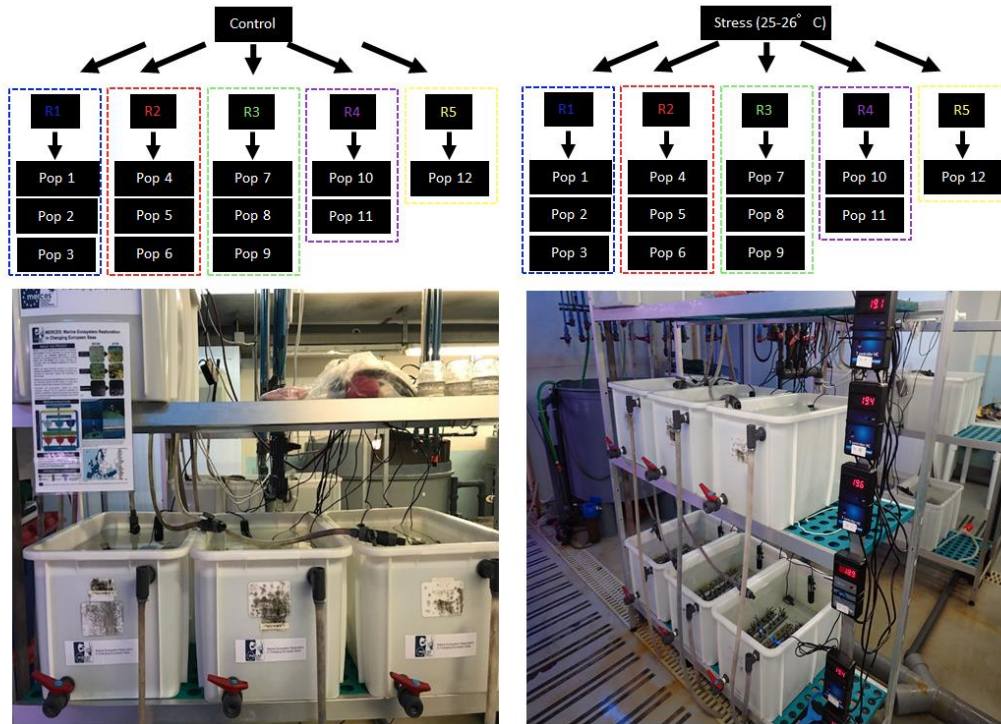


Figure 3: Design of the experimental set-up and photo of the installations at the ICM. Note that the colors used in the experimental set-up correspond to the colors used in the Figure 2.

Response variable:

The response variable was the level of tissue necrosis per individual, which was visually monitored daily in the Treatment and Control tanks. From these observations, the percentage of affected colonies (i.e., the proportion of colonies displaying tissue necrosis > 0%) and mean necrosis values per day were computed to assess the response to thermal stress.

*Whole genome sequencing analyses of *P. clavata*:*

In order to look for putative genetic factor(s) and to estimate the relative impact of eco-evolutionary processes involved in the differential response to thermal stress, we conducted a whole genome sequencing analysis. As a preliminary step, we generated the whole genome sequence of one individual of *P. clavata* coming from the Catalan coast. This *de novo* whole genome assembly was done following a hybrid strategy combining the data obtained from Illumina and Oxford Nanopore Technologies sequencing. RNA-seq from three different individuals coming from three different populations and submitted to control and treatment conditions are ongoing to annotate the genome. This strategy allows covering a large amount of the genes expressed during thermal stress. In parallel, we used the data on the response variable to identify five resistant and five sensitive colonies per population (120 individuals in total), which will be re-sequenced at lower coverage (10x). These re-sequencing data will be used to look for genetic variant and putative locus involved in the differential responses to thermal stress.

The whole genome sequencing analyses are conducted in collaboration with the Centro Nacional de Analisis Genómico in Barcelona (CNAG; <https://www.cnag.crg.eu>). DNA and RNA extractions needed for the *de novo* whole genome assembly and re-sequencing steps were or are conducted at the ICM. The following molecular analyses are conducted by the CNAG. More particularly, quality check of the extractions, library preparations, Illumina and MinIon sequencing are conducted by the CNAG Sequencing Unit. The same is true for the bioinformatics analyses needed for the *de novo* whole genome assembly and annotations, which are conducted by the Genome Assembly and Annotation team. The data generated during the re-sequencing step will also be analysed in collaboration with the Population Genomics group.

Microbiome analyses of P. clavata:

In parallel, we conducted microbiome analyses. These analyses aimed to: i) characterize the temporal dynamic of microbiome diversity in colonies of *P. clavata* submitted to thermal stress; ii) to look for microbiome differences among sensitive and resistant individuals. The microbiome of the individuals used in the experiment will be sequenced using metagenomic approach. Amplicon sequencing was performed by the Integrated Microbiome Resource facility at the Centre for Comparative Genomics and Evolutionary Bioinformatics at Dalhousie University. Two distinct sets of amplicon analyses were conducted: one focused on associated eukaryote and one focused on procaryotes. In both cases, sequencing was performed on an Illumina MiSeq using a 300 bp paired-end read design. These microbiome analyses were not planned at the beginning of the project. Nevertheless, we decided in accordance with all the partners to make the most of the samples used in the experiment. Accordingly, we developed a collaboration with the Microbiologist team of the ICM (Drs. Ramon Massana and Javier Del Campo).

Results transregional thermotolerance experiment coralligenous

Experiment and response variable:

First signs of necrosis appeared at least in some colonies for all populations after only one-week exposure at 25 °C (Figure 4a). Despite the general patterns of sensitivity, significant differences in the survival probability were found among regions ($p < 0.001$; Figure 4c).

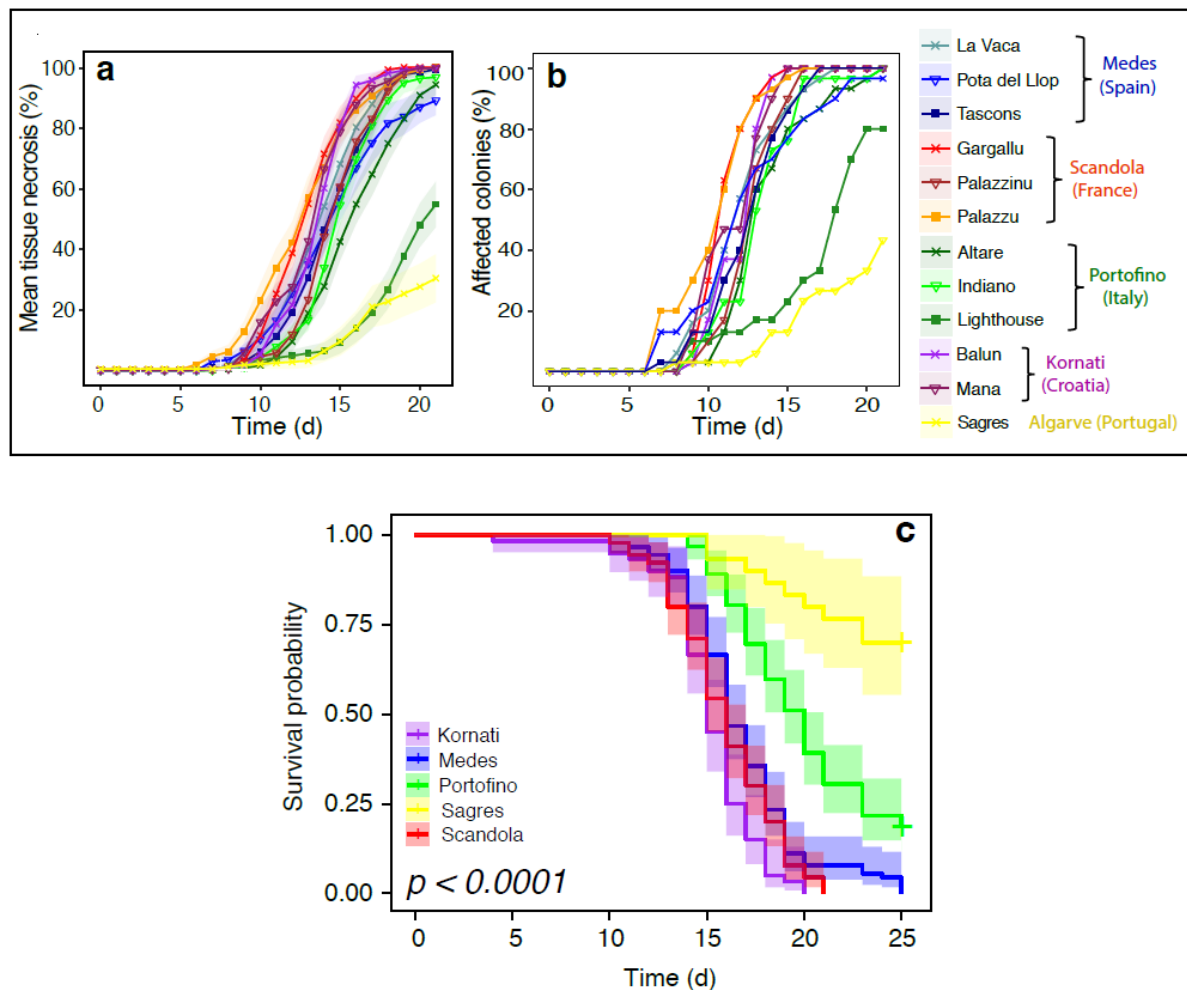


Figure 4: a) Mean percentage of tissue necrosis function of time for each of the 12 populations considered in the experiment; b) percentage of affected colonies function of time for each of the 12 populations considered in the experiment c) Survival probability function of time for each of the five regions considered in the experiment. The colors correspond to the colors used in the Figure 2.

At the end of the experiment, 88 % of the total number of colonies showed 100% tissue necrosis (dead colonies) while only 5.6% (mainly from Portugal and one population from Italy: Lighthouse) remained without necrosis (Figure 4b).

The most resistant population was found in Portugal, with only 43% of colonies affected and an average of 30.3% of mean tissue necrosis after 3 weeks exposure at 25 °C. The second most resistant population, a population from Italy displayed 54.7% of mean tissue necrosis and 80 % of affected colonies over the same period. All other populations showed more than 80% of mean tissue necrosis after 3 weeks exposure at 25 °C. The most sensitive population was Palazzu from Corsica, which was affected after only 6 days and reached more than 80% of mean tissue necrosis after only 15 days, followed by a complete death of all its colonies in less than 3 weeks (Figure 4).

Despite the general patterns of sensitivity, significant differences in the survival probability were found among regions ($p < 0.001$; Figure 4).

From these results, we selected, in each population, five resistant individuals identified as the last five individuals reaching 50% of tissue necrosis and five sensitive colonies identified as the first 5 individuals reaching 50% of tissue necrosis (Fig. 5) to be re-sequenced.

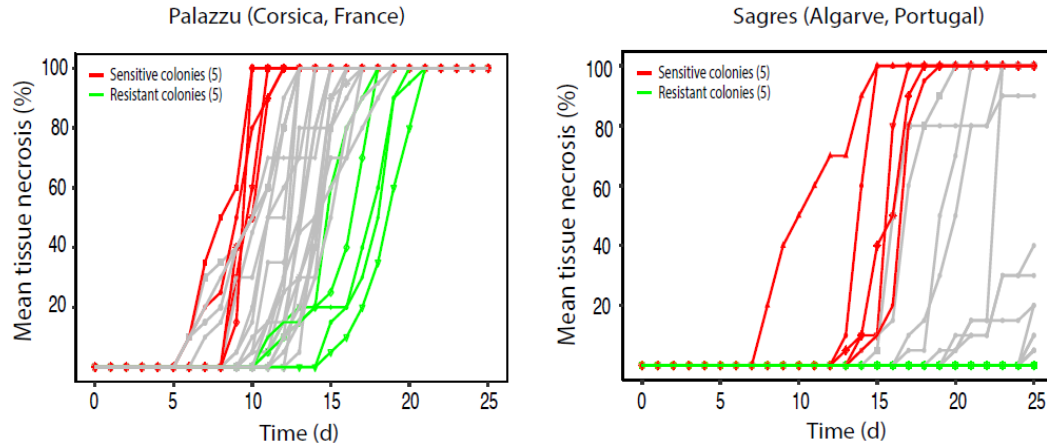


Figure 5: Percentage of necrosis for each individual used the two considered populations (Palazzu from Corsica and Portugal). In red are the five most sensitive individuals while the 5 more resistant individuals are shown in green. Those 10 individuals were chosen for the re-sequencing analyses.

Whole genome sequencing analyses of *P. clavata*:

De novo whole genome assembly:

While the genome-size estimated by Genomescope (Vurture et al. 2017) is close to 750 Mb, the resulting genome assembly of *P. clavata* is 760.25 Mb long. The contiguity is relatively low (contig N50 14.32 and scaffold N50 15.33 Kb). The gene completeness of this assembly was estimated with BUSCO v3.0.2 (Simão et al. 2015) identifying 76.2% of complete genes, 8.8% of fragmented genes and 15% of missing genes. From this assembly, the number of heterozygous single nucleotide variants (SNV) was estimated to more than 2×10^6 corresponding to a heterozygous SNV rate 3.8×10^{-3} SNVs/bp (i.e. we expect to find almost 4 SNPs per Kb). The assembly annotation is currently in progress. The annotated assembly should be obtained by the end of April 2019.

Re-sequencing of sensitive vs. resistant individuals:

The re-sequencing of the 120 individuals from the common garden experiment is on-going. We already extracted the DNA of all the individuals. The raw data of the re-sequencing are already available for 60 individuals (50%). The remaining data will be obtained in the coming two months. Populations genomics analyses to identify putative genomic regions involved in the differential responses should begin in the second half of 2019 in collaboration with the CNAG.

*Microbiome analyses of *P. clavata*:*

The microbiome analyses are still on-going. Based on our preliminary results, there is a difference in the prokaryotic communities among populations and among sensitive and resistant individuals to temperature increases. In all the individuals the bacterial communities are dominated by gammaproteobacteria and the coral associated

communities are clearly different from the surrounding water bacteria as well as the bacteria associated to their food source.

Moreover, we have observed a shift in the microeukaryotic communities when increasing the temperature.

Discussion transregional thermotolerance experiment coralligenous

Field observations following mass mortality events suggest that the capacity of the habitat forming octocoral, *Paramuricea clavata*, to deal with thermal stress may vary among individuals and populations. The results of the trans-regional common garden experiment conducted here confirmed previous field surveys. Indeed, the level of tissue necrosis varies significantly among individuals and populations. Interestingly, the population from Portugal and one population from Italy seem only marginally affected by the thermal stress.

Previous studies demonstrated that different factors may be involved in the differential responses to thermal stress in *P. clavata*. For instance, the physiological status of the colony was acknowledged as a central factor in the differential responses. Indeed, the impact of thermal stress was stronger in starved colonies compared to well-fed colonies (Coma et al. 2009). Then, the sexual maturity (adults vs. juveniles) and the sex (male vs. female) of the colonies play a significant role in the differential responses observed within population. Juveniles are more resistant compared to adults while female's reproduction is more impacted than male reproduction (Arizmendi-Meija et al. 2015). Focusing on eco-evolutionary processes, genetic drift has been suggested as a main driver of the differential responses, hampering potentially adaptation to local environmental conditions (Crisci et al. 2017). Nevertheless, some limitations of the approaches used in this study preclude definitive conclusions on the respective role of selected and neutral eco-evolutionary processes on the differential responses to thermal stress.

Previous studies characterizing the microbiome of *P. clavata* suggested a lack of geographic structure in the bacterial community inhabiting the species (La Rivière et al. 2013).

From the experiment, a first paper presenting the phenotypic results of the common garden experiment is already in preparation. While we are still gathering the re-sequencing and metagenomics data, the integrative approach developed in this work package will allow deep insights into the eco-evolutionary processes and environmental factors involved in the differential responses of organisms to climate change. Regarding the re-sequencing analyses, the high number of SNVs identified in the present study and their genotyping and comparison between resistant and vulnerable individuals from the experiment should allow the identification of genomic regions involved in the responses to thermal stress. In the meantime, we expect to improve our understanding of the processes driving the differential responses to thermal stress, to deepen our knowledge on the PEIs and on the evolutionary history in this species. Regarding the metagenomic approach, it seems that prokaryotic communities are different among the different populations used in the experiment. Interestingly, our results also suggested variations of the microbiome community with the duration of the thermal stress.

Overall, this experiment will refine our understanding of the processes underlying the resistance to thermal stress of colonies and populations of *P. clavata*. Accordingly, we should be able to improve the selection of source populations of *P. clavata* to enhance the effectiveness of restoration actions. Considering the central ecological role of *P. clavata* in the coralligenous, restoration actions will benefit the associated biodiversity.

Macroalgal habitats

In the last decades, several *Cystoseira* forests have gone missing from many coastal areas of the Mediterranean Sea (Cormaci and Furnari, 1999; Thibaut et al., 2005; Serio et al 2006; Blanfuné et al., 2016), and are being replaced by simpler and less productive communities dominated by opportunistic taxa (Benedetti-Cecchi and Cinelli, 1992; Thibaut et al., 2005). Consequently, with the exception of *C. compressa*, all species of the genus *Cystoseira* have been included in Annex II of the Barcelona Convention. Although marine heat waves have been related to large scale mass mortality events of several benthic species (Cerrano et al., 2000; Cerrano and Bavestrello, 2009; Garrabou et al., 2009), up to now there are still no evidences of regression of *Cystoseira* populations regarding abnormal high temperatures.

Cystoseira assemblages are amongst the most productive assemblages in the Mediterranean Sea. Concretely, *Cystoseira crinita* Dubi (Duby, 1830) is a perennial plant, endemic and distributed throughout the entire Mediterranean Sea. This species inhabits the upper sublittoral zones (0-1m depth) of semi-exposed and well illuminated areas (Sales & Ballesteros, 2009), where it can create very structured assemblages, hosting more than 200 species of flora and fauna (Sales and Ballesteros, 2010; Sales et al., 2012).

Methods transregional thermotolerance experiment macroalgal

In the Catalan coast (Spain), *C. crinita* assemblages are extremely rare and only in three locations it forms small populations of small patches of canopy (around few m²; Mariani et al., *in prep*). Density and size-class structure of these populations have been monitored since 2012. Moreover, *in situ* high-resolution (hourly records, $\pm 0.21^{\circ}\text{C}$ accuracy) temperature recorders (HOBO Water Temp Pro v2) were installed at the different locations and changed periodically.

After unusual high temperatures registered *in situ* during the summer of 2015 (Fig. 6), a population of *C. crinita* in NW Mediterranean revealed a general mortality of adult individuals. This fact was an evidence of the possible negative effects of high temperature on the viability of *C. crinita* populations, highlighting the increasing importance to understand the response of *Cystoseira* populations to thermal stress.

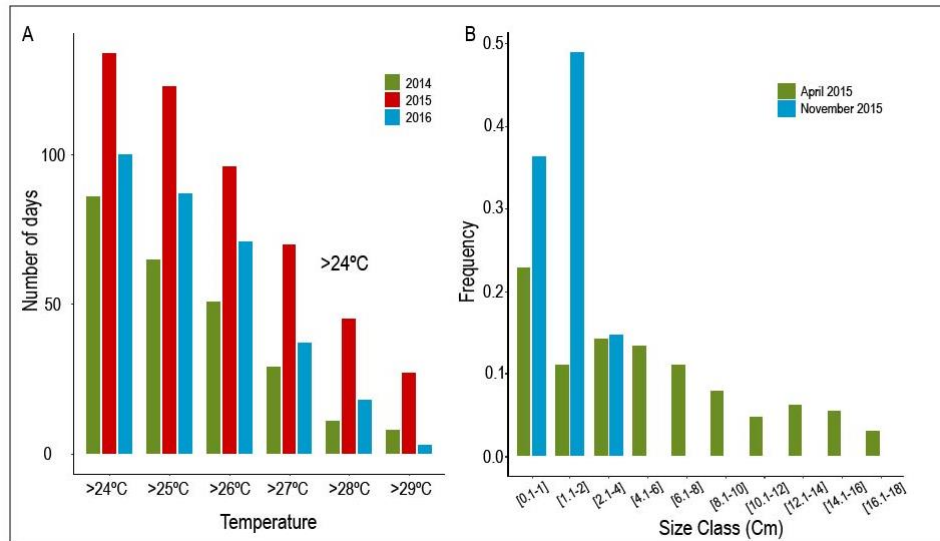


Figure 6. A) Total number of days per year (from June to October) in which maximum temperature reached the different thresholds, and B) size-class frequency of the population before (April 2015) and after (November 2015) the thermal anomaly.

With the aim to shed new light on the eco-evolutionary processes and environmental factors involved in the differential responses to thermal stress, we conducted a preliminary trans-regional study, on the macroalgae *Cystoseira crinita*. These experiments were not originally planned in this subtask.

The common garden experiment performed with 4 populations located in the Western Mediterranean from different regions characterized by contrasted temperature conditions at the regional and local scales (Fig. 7).

Individuals of the different populations were submitted to thermal stress (under 26°C) in controlled conditions, and their performance response was evaluated during the time of the experiment, assessing the variation of wet-weight and the optimum quantum Yield. Specifically, 3 individuals of each population were cultured at 26°C for about 100 days.

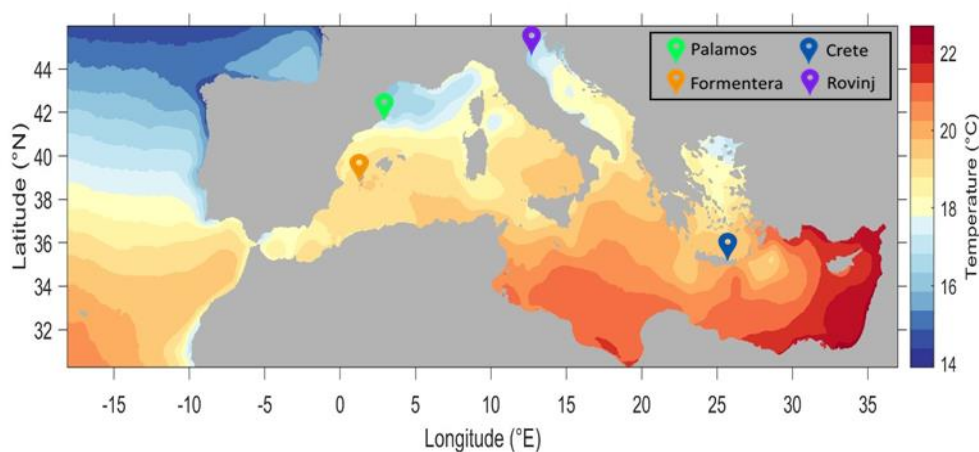


Figure 7. Annual mean sea surface temperatures (°C) are shown in the Mediterranean Sea map. The origin and the local thermal regimes of the selected populations for the experiment are also marked in the map.

Results and discussion of transregional thermotolerance experiment macroalgal

Aspect of individuals submitted to thermal stress during 100 days (Fig. 8). Photosynthetic efficiency did not show any difference among populations, however the variation of wet weight reveals a higher sensitivity to thermal stress for Palamos population (Fig. 9). These results indicate that thermal thresholds might be different among populations dwelling under contrasted thermal regimes.

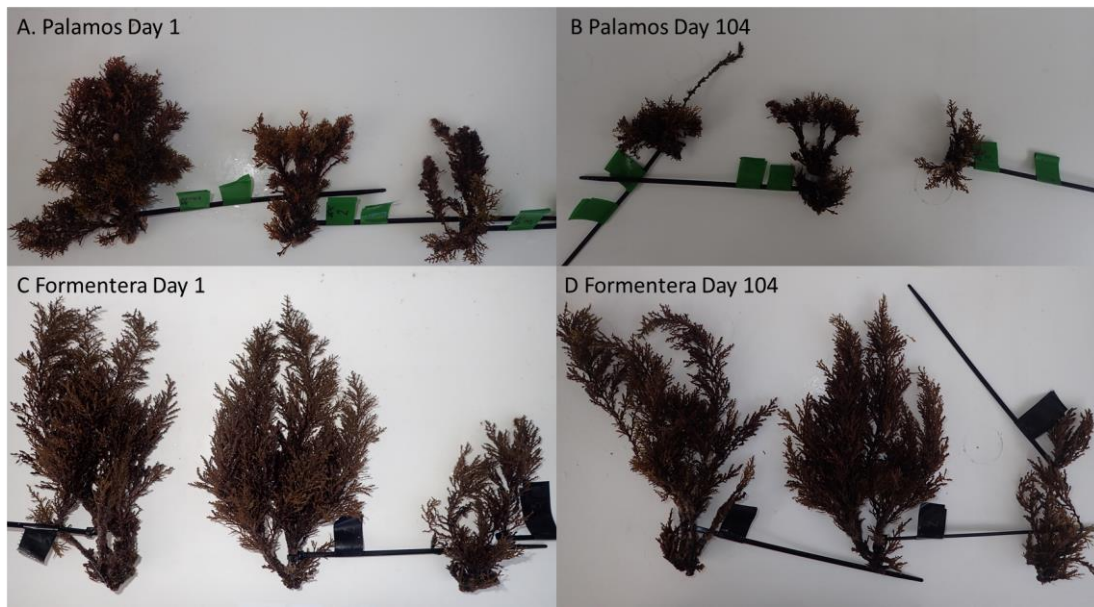


Figure 8. Individuals from Palamos (Catalan coast) and Formentera (Balearic Islands), submitted at 26°C, at the first and the last day of culture.

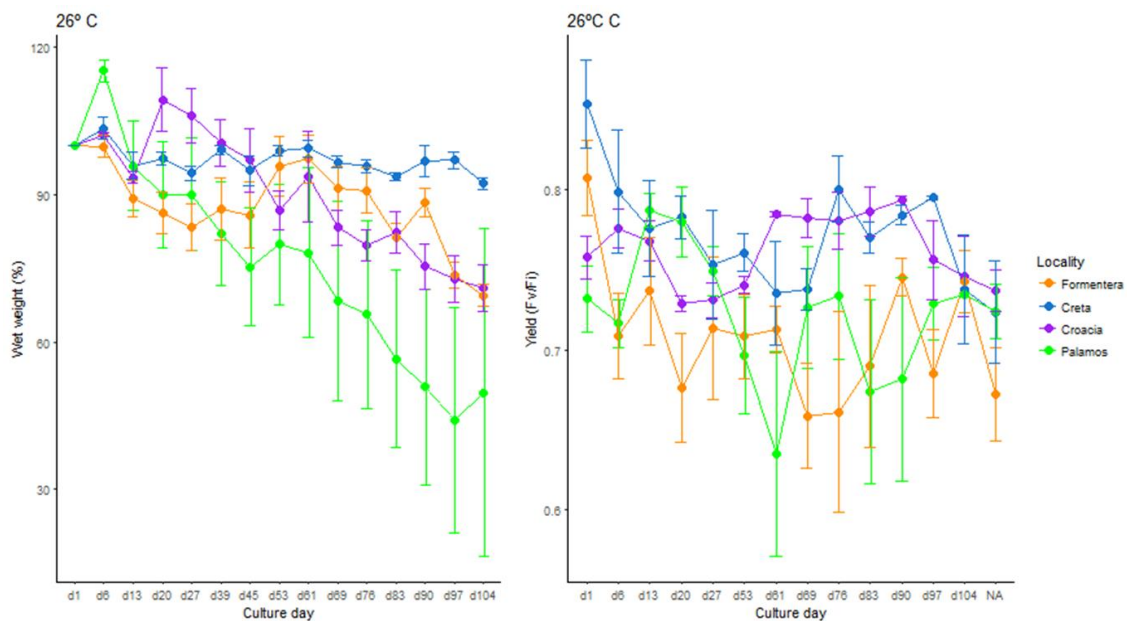


Figure 9. Variation of the wet weight (on the left) and the maximum quantum yield (on the right) during the experiment.

Although preliminary, the obtained results suggest a population-dependent response to thermal stress, opening a new research topic to identify thermoresistant donor populations to be used in the restoration actions of shallow macroalgal beds. However, further thermotolerance experiments, considering populations dwelling encompassing the temperature ranges experimented within the *C. crinita* geographic distribution conditions should be carried in order to test whether or not thermal history can shape the responses to thermal stress of these key species of macroalgal forests.

Gaining this information is highly relevant for conservation and restoration purposes. Knowing and characterizing the response to thermal stress of different populations, and therefore identifying which populations are more resistant or vulnerable to high temperatures is essential. This knowledge, could be crucial not only to adopt suitable conservation measures, but also to identify possible reservoir populations in future global warming scenarios and more resistant populations to use as donor populations for restoration plans

ii) Transregional facilitation experiments

Methods transregional facilitation experiment coralligenous

Experimental set-up:

In order to test whether erect habitat-forming species can facilitate the survival and growth of co-occurring encrusting and massive ones we carried out a manipulative field experiment using the red gorgonian *Paramuricea clavata* (a key structural species in the coralligenous habitat; Ballesteros 2006) and bryozoans (important coralligenous animal builders; Ballesteros 2006) as model organisms. Although our focus was primarily on bryozoans, we were additionally interested in the recruitment and succession of the entire early-stage sessile assemblage on experimentally set substrate inside and outside of the gorgonian forest. The experiment was replicated in Spain (Medes Islands Marine Protected Area (MPA); 42°2'N, 3°13'E), Italy (Portofino Promontory MPA; 40°48'N, 14°9'E) and Croatia (Cape Sokol, Krk Island, Natura 2000 site; 44°58'N, 14°49'E), thus encompassing 3 regions: the Northwestern Mediterranean, the Ligurian and the Adriatic Seas (Fig. 10). These sites host natural *P. clavata* populations and the experiment was set in the species upper bathymetric range: at 18 m depth in Medes Islands and 35 m depth in Portofino and Krk Island.

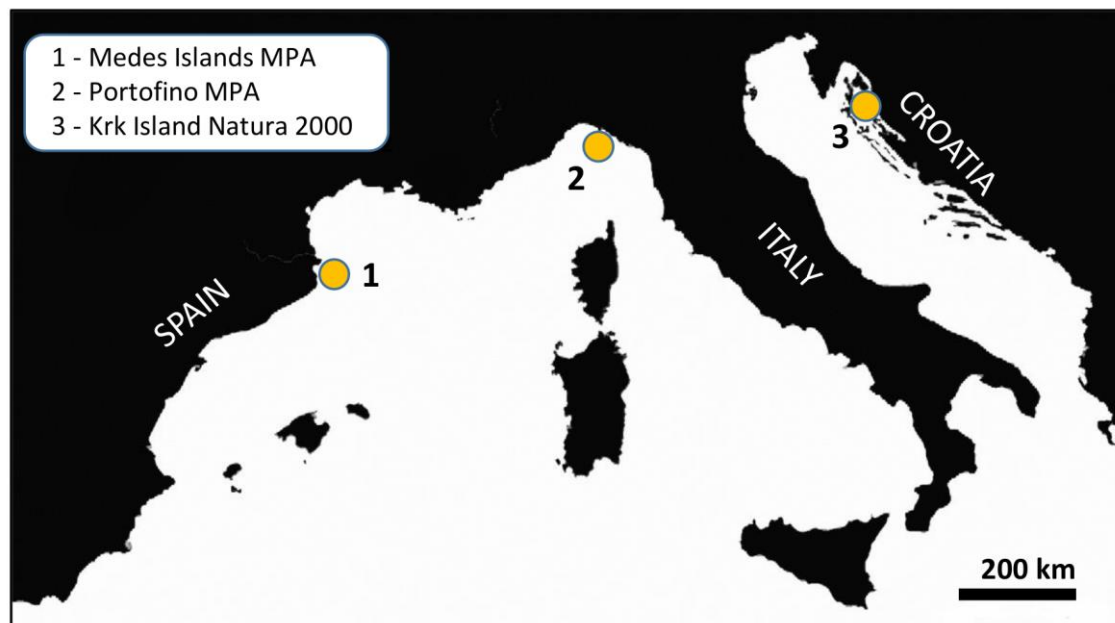


Figure 10: Map of study sites involved in the transregional facilitation experiment.

Following previous successful trials which indicated the suitability of plastic mesh as a substrate for bryozoan recruitment and growth (see Fig. 11), we used them in experimental treatments with and without gorgonian colonies (15 *P. clavata* fragments up to 20 cm in maximal height per experimental plot). Rubber bands (5 cm wide) were used to fix gorgonian transplants in the experiment (*P. clavata* forest treatment; PC) and additionally two types of controls were set: empty (plastic mesh only; C) and artefact one (plastic mesh + rubber bands; AC). There were 4 replicates per treatment and the size of each replicate plot was 0.25 m² (Fig. 12). The simulated density of gorgonians

was comparable to the maximum values observed in natural populations (Linares *et al.*, 2008). The experiment was initiated in May-July 2017 and followed until October 2018.



Figure. 11: Illustration of recruitment, survival and growth of bryozoan *Pentapora fascialis* over a period of 18 months on plastic meshes set within Medes Islands MPA.

Sampling and data analysis:

Photosampling was carried out seasonally, using the 25 x 25 cm quadrat to facilitate taxa identification (Fig. 12d). Images were analysed with PHOTOQUAD software (Trygonis & Sini, 2012), by superimposing a grid of 400 equal-sized squares (about 1 cm²). Organisms were identified to the lowest possible taxonomic level and the percentage cover of each taxon/category was calculated from the ratio of respective taxa cell counts and cumulative count of the experimentally set artificial substrate (plastic mesh or rubber).

A Bray-Curtis similarity matrix was constructed on square root-transformed percent cover data and non-metric multidimensional scaling (MDS) ordination was performed to visualize patterns of community similarities for each substrate separately (plastic mesh and rubber). Differences in early-stage sessile assemblage structures inside and outside (including both empty and artefact control) of gorgonian forest (Tr: fixed factor with 3 levels for mesh substrate and 2 levels for rubber substrate) and among sites (Si: fixed factor with 3 levels in fall 2017 and 2 levels in spring and fall 2018) were assessed by permutational multivariate analysis of variance (PERMANOVA) for each season: fall 2017 (October 2017), spring 2018 (April 2018) and fall 2018 (October 2018). When less than 999 unique values in the permutation distribution were available, asymptotical

Monte Carlo p-values were used instead of permutational p-values (values of $p < 0.05$ were considered significant). Significant interactions among main factors were investigated by post-hoc pair-wise tests. Univariate tests were run on square root transformed percent cover data using the Euclidean distance. SIMPER was used to assess the percentage contribution of each taxon to the average dissimilarity among sites. Statistical analyses were performed using PRIMER 6 with PERMANOVA+ add-on package (Clarke & Gorley, 2006 and references for respective procedures therein; Anderson *et al.*, 2008).

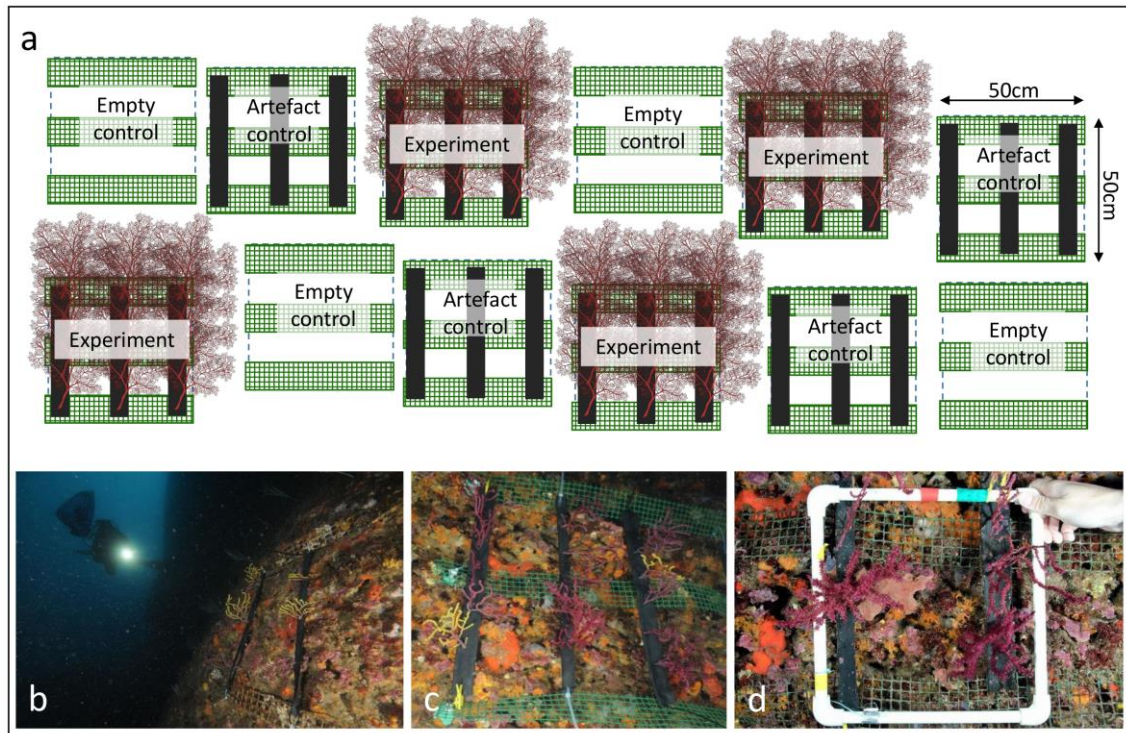


Figure 12: Experimental set-up: a) Facilitation experiment design, b) view of the *P. clavata* forest treatment replicate, c) use of rubber bands (5 cm wide) to fix gorgonian transplants in the experiment (15 colonies per replicate); d) photosampling assisted by the 25 x 25 cm quadrat to facilitate taxa identification

Although large effort has been invested by diving teams to set and fix experimental structures, which in cases of inadequately placed natural holes involved also drilling holes to set attachment points (plastic screws) to be fixed with epoxy putty, experiment did not last beyond first 4 months at all sites. Unfortunately, in the Medes MPA the shallower setup of the experiment made that the strong storms during winter 2017-2018 destroyed the experimental structures (Fig. 13a,b). In May 2018 the experiment was rebuilt (Fig. 13c), introducing modifications that could facilitate the maintenance of the structure despite storms. In particular, the replicates were built as sub-units to ensure better attachment of the entire structure to the substrate. This time the experiment had structurally resisted the winter storms, indicating that building small subunits was a better approach than the one used in the first attempt. However, a substantial amount of gorgonian transplants have detached from rubber bands (Fig. 13d), thus making it impossible to associate any change in recruitment or coverage to the effect of the gorgonian forest and causing the failure of the second experimental trial at this site.



Figure 13: Chronology of experimental trials in Medes MPA: a) and b) damaged experimental structure after heavy storms in winter 2017-2018 (assessed 8 months after experiment initiation); c) new experiment initiated in May 2018; d) loss of transplanted *P. clavata* colonies after storms during winter 2019 resulting in failure of the 2nd experiment.

Results transregional facilitation experiment coralligenous

Species richness

In total, 40 different taxa/categories recruited on the available artificial substrate within 15 months of experiment's deployment. Similar species richness was observed on mesh and rubber substrate at two sites: 15 and 12 in Medes, respectively, 14 and 15 in Portofino, respectively whereas on Krk Island almost double the number of species was observed on mesh substrate (21) in comparison to rubber (11). Algae were the most species rich benthic group at both substrates (Fig. 14).

There was no difference in species richness among experimental treatments per respective season and substrate (Fig. 14).

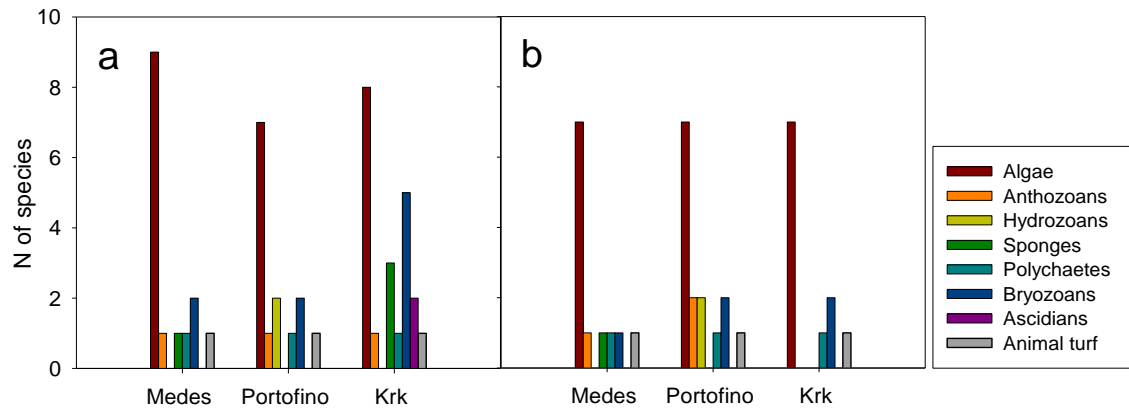


Figure 14: Species richness per site and the main benthic group: a) for the plastic mesh and b) the rubber.

The main taxa recruiting on the experimentally set substrate at least at one site were encrusting coralline algae, encrusting bryozoans, serpulid polychaetes and animal turf (Figs. 16 and 17).

Inter-site comparison

For both substrates, MDS clearly showed three distinct clusters in fall 2017 (Fig. 18a,e) and two in spring and fall 2018 (Fig. 9b-d,f,g), corresponding to sites which differed significantly in their early-stage sessile assemblages (Tab.1). For both substrates and all seasons, average dissimilarity between sites was $> 50\%$. In general, algae, especially crustose coralline algae, *Peyssonnelia* spp. and algal turf were more abundant in Medes, animal turf and brown algae of family Dictyotaceae were more abundant in Portofino whereas serpulid polychaetes and encrusting bryozoans, especially after spring 2018, were more abundant at Krk site (Figs. 16 and 17; SIMPER two-way analysis).

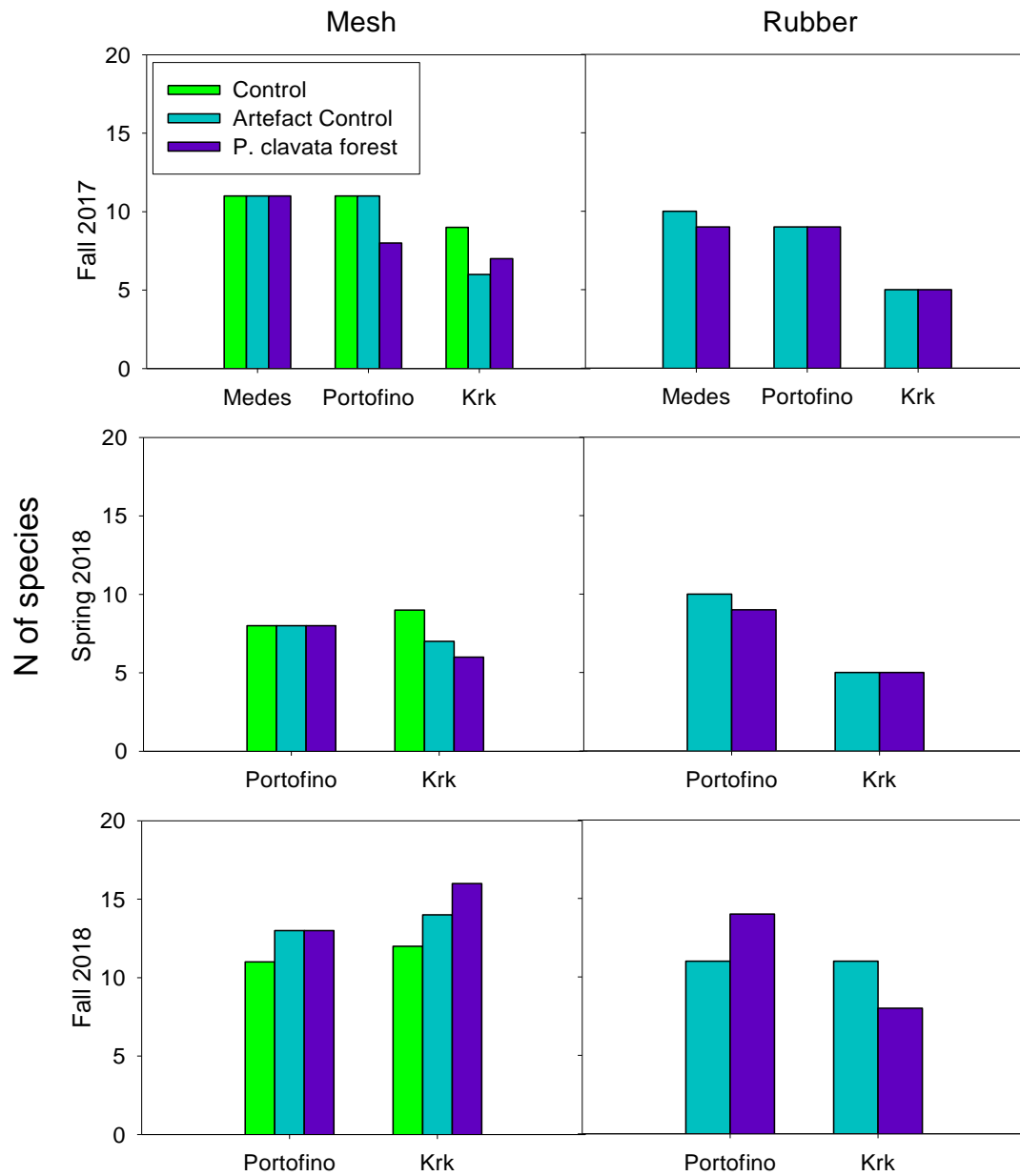


Figure 15: Species richness per site and treatment observed on both experimentally set substrates.

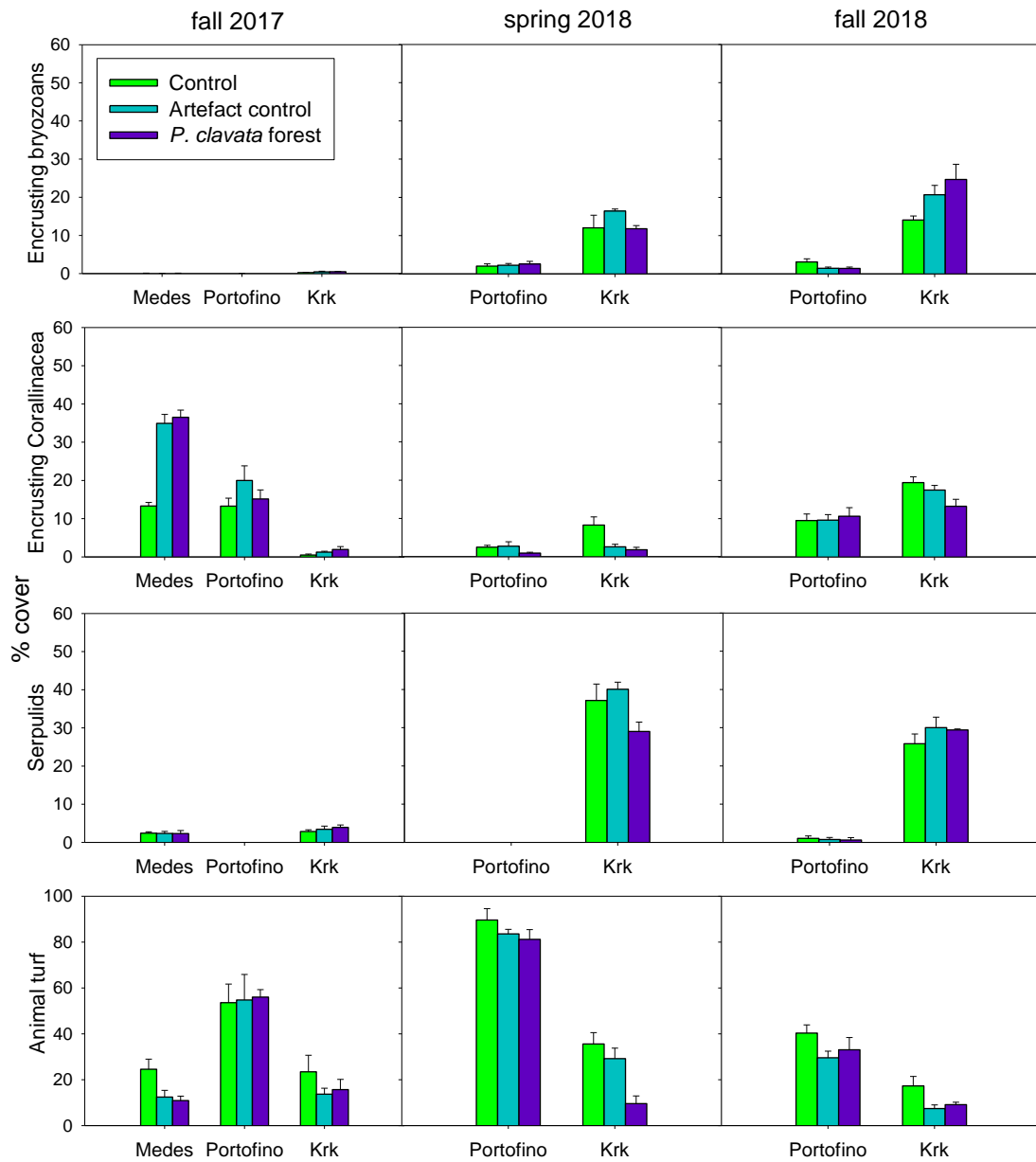


Figure 16: Percent cover (mean \pm SE) of the main taxa on the mesh substrate inside and outside of the *Paramuricea clavata* forest at different study sites. Note the difference in scaling for animal turf category.

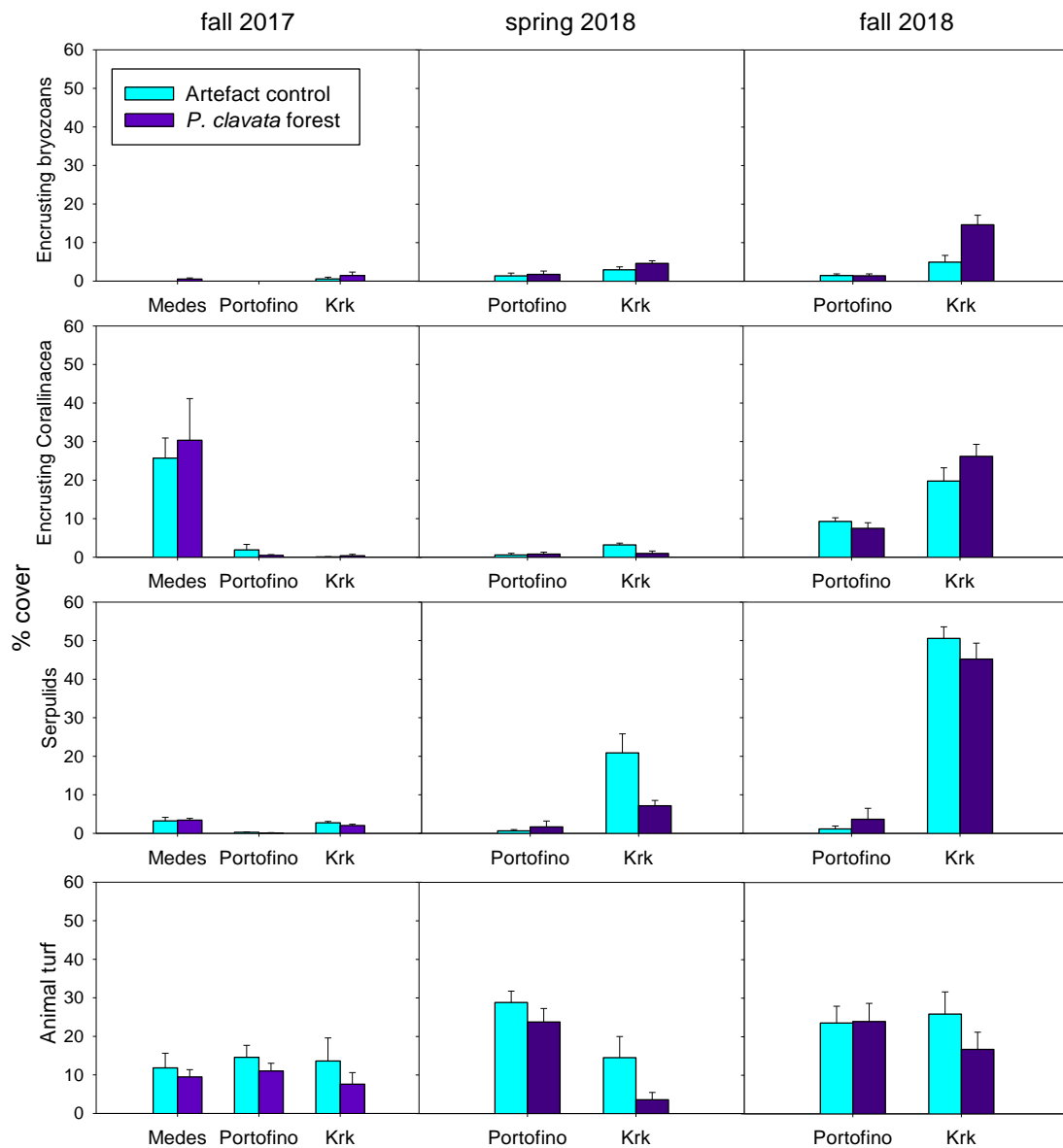


Figure 17: Percent cover (mean \pm SE) of the main taxa on the rubber substrate inside and outside of the *Paramuricea clavata* forest at different study sites.

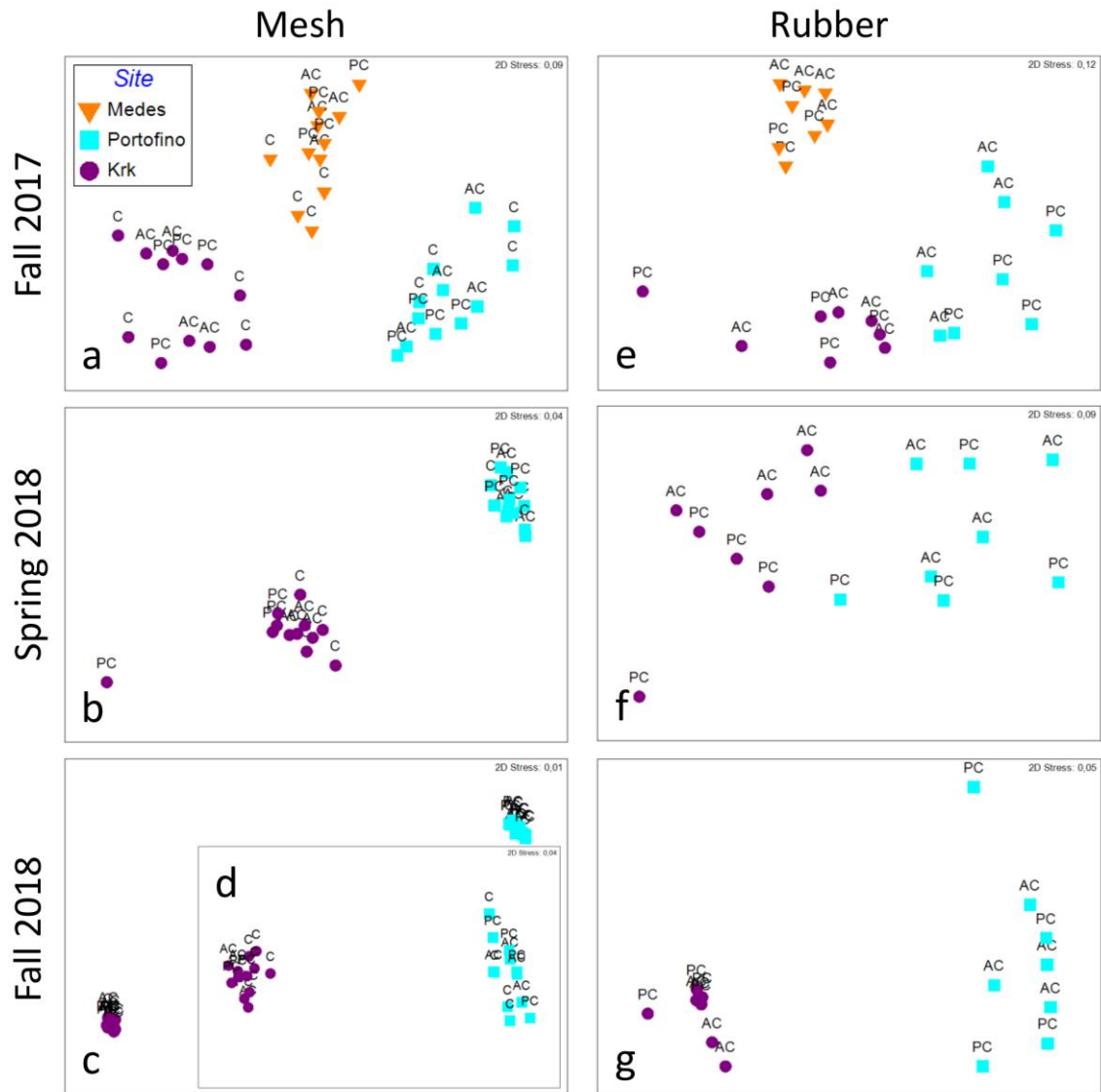


Figure 18: Non-metric multidimensional scaling (MDS) plots comparing early-stage sessile assemblages colonizing 2 different substrates (plastic mesh and rubber) inside and outside *Paramuricea clavata* forest among 3 sites belonging to different Mediterranean regions in three seasons: fall 2017, spring and fall 2018 based on Bray-Curtis dissimilarities of square root transformed percent cover data. Experimental treatments include: C = control, AC = Artefact control and PC = *Paramuricea clavata* forest. Note: insert d is a result of removing data for algae of family Dictyotaceae, recorded only in Portofino and causing data points clumping in panel c.

The effect of *P. clavata* forest

The effect of *P. clavata* forest on early-stage sessile assemblages depended on the site, time and substrate (Tab.1). In fall 2017 on mesh substrate, treatment effect on assemblage level was observed at Medes site, largely due to significantly lower abundance of crustose coralline algae outside the forest, though only in the (empty) control treatment (Fig. 16; Tab. S1a; pair-wise test; PC vs. C, $P=0.0002$; AC vs. C, $P=0.0001$; AC vs. PC, $P = 0.62$).

Table 1: Summary of PERMANOVA tests on the effects of *Paramuricea clavata* forest on the recruitment of sessile assemblages on two different substrates (plastic mesh or rubber bands) in different sites and different seasons (based on Bray-Curtis dissimilarities of square root transformed percent cover data). Si = Site, Tr = Treatment, P-F = Pseudo-F. For each analysis > 9900 unique permutations were available.

MESH						RUBBER				
AI) fall 2017						AII) fall 2017				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	2	28473	14236	40,98	0,0001	2	23058	11529	16,25	0,0001
Tr	2	911,35	455,68	1,31	0,2086	1	521,8	521,8	0,74	0,5771
SixTr	4	2198,6	549,66	1,58	0,0383	2	1249	624,5	0,88	0,5389
Res	27	9380,7	347,44			18	12775	709,7		
Total	35	40964				23	37604			
BI) spring 2018						BII) spring 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	16022	16022	99,11	0,0001	1	8222	8222	15,75	0,0004
Tr	2	765,09	382,55	2,37	0,0313	1	922,2	922,2	1,77	0,1798
SixTr	2	519,47	259,74	1,61	0,1385	1	1351	1351	2,59	0,0661
Res	18	2909,9	161,66			12	6263	521,9		
Total	23	20216				15	16758			
CI) fall 2018						CII) fall 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	16038	16038	66,51	0,0001	1	10458	10458	32,25	0,0003
Tr	2	705,23	352,62	1,46	0,1589	1	459,3	459,3	1,42	0,2503
SixTr	2	422,78	211,39	0,88	0,5655	1	251,9	251,9	0,78	0,5865
Res	18	4340,3	241,13			12	3892	324,3		
Total	23	21506				15	15061			

The recruitment of encrusting orange bryozoans notably increased after winter period on both substrates and their abundance (% cover) was significantly higher on Krk Island (reaching up to $24.69\% \pm 3.94$ SE and $14.63\% \pm 2.49$ SE in fall 2018 on mesh and rubber substrate, respectively) than in Portofino (reaching up to $3.10\% \pm 0.83$ SE and $1.48\% \pm 0.43$ SE on mesh and rubber substrate, respectively; Figs. 16, 17 and 19; Tab. 2). However, *P. clavata* forest effect on the encrusting bryozoan coverage was noted only in fall 2018 (Tab. 2) and only on Krk site. The effect there was clear on the rubber substrate, with significantly higher abundance inside the forest than outside (pair-wise test; AC vs. PC, $P=0.02$; Fig. 8), whereas on the mesh substrate abundance was significantly lower in empty controls but similar inside *P. clavata* forest and artefact control (pair-wise test; C vs. AC, $P=0.048$; C vs. PC, $P=0.031$; AC vs. PC, $P=0.448$; Fig. 16). The partial loss of experimentally set substrate in Medes Islands precluded the analysis of recruitment after winter on that site.

Besides the category of encrusting bryozoans that was the most abundant, single colonies or very low coverage of other bryozoan taxa have been also observed on the mesh substrate e.g. *Smittina cervicornis*/*Adeonella pallasii*, *Beania* spp., *Idmidronea* sp. on the Krk site, or *Sertella* spp. on Portofino site. Interestingly, erect bryozoan *Pentapora fascialis*, although present in study areas and preferentially settling on the mesh, as proven on the Medes site (Fig. 11), was not observed in this study.

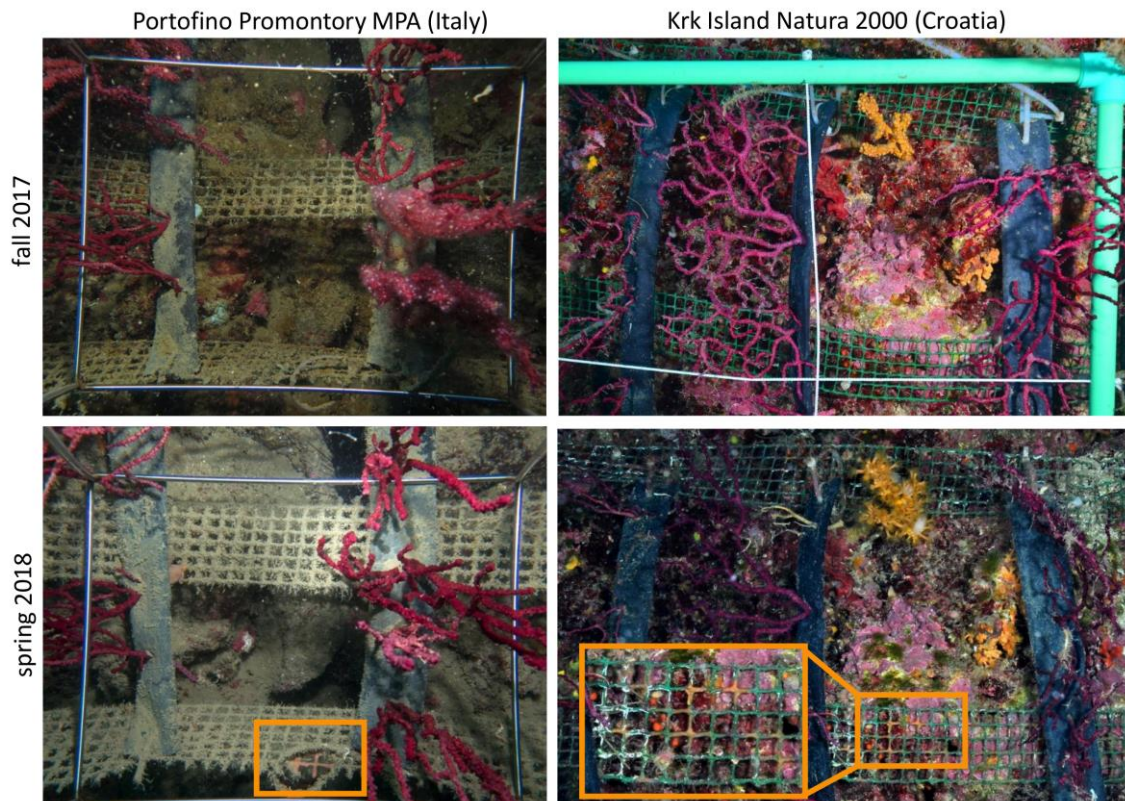


Figure 19: Illustration of the early stage sessile assemblage on experimentally set substrate (after 4 and 10 months, respectively) at two study sites and the bryozoan recruitment after winter period.

The only other effect of *P. clavata* forest was observed for animal turf and serpulid polychaetes, but again it was site, time and substrate dependant (Figs. 16 and 17; sign. interaction Si x Tr, Tab. S1b,c). On Krk site, animal turf abundance on mesh substrate in spring 2018 was significantly lower inside the forest than outside (Fig. 7; Pair-wise tests: C vs. PC, $P = 0.015$; AC vs. PC, $P = 0.036$). Likewise, although not significantly, its abundance was generally lower inside *P. clavata* forest on the rubber substrate (Fig. 17, Tab. S1c). At the same study site, serpulid polychaetes were more abundant outside of *P. clavata* forest in spring 2018 (Fig. 17, Tab. S1b; Pair-wise test: AC vs PC, $P = 0.02$).

Table 2: Summary of PERMANOVA tests on the effects of *Paramuricea clavata* forest on the abundance of encrusting bryozoans settling on two different substrates (plastic mesh or rubber bands) in different sites and different seasons (based on square root transformed percent cover data and using Euclidian distance). Si = Site, Tr = Treatment, P-F = Pseudo-F. For each analysis > 9900 unique permutations were available.

MESH						RUBBER				
AI) fall 2017						AII) fall 2017				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	2	2,17	1,0854	33,8	0,0001	2	2,13	1,06	4,27	0,0259
Tr	2	0,04	2,23E-02	0,7	0,5105	1	0,62	0,62	2,49	0,1365
SixTr	4	0,07	1,79E-02	0,6	0,6961	2	0,31	0,16	0,62	0,547
Res	27	0,87	3,21E-02			18	4,49	0,25		
Total	35	3,15				23	7,55			
BI) spring 2018						BII) spring 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	27,58	27,58	89,15	0,0001	1	2,69	2,69	7,48	0,0204
Tr	2	0,73	0,36	1,18	0,3375	1	0,23	0,23	0,65	0,4287
SixTr	2	0,60	0,30	0,97	0,4108	1	0,20	0,20	0,57	0,4622
Res	18	5,57	0,31			12	4,32	0,36		
Total	23	34,47				15	7,44			
CI) fall 2018						CII) fall 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	55,69	55,69	228,1	0,0001	1	12,80	12,80	32,56	0,0002
Tr	2	0,41	0,20	0,8	0,4494	1	2,82	2,82	7,16	0,016
SixTr	2	3,26	1,63	6,7	0,0064	1	3,02	3,02	7,69	0,014
Res	18	4,39	0,24			12	4,72	0,39		
Total	23	63,75				15	23,36			

Interestingly, on Portofino site small portions of *P. clavata* tissue were observed to colonize rubber substrate to which colonies were attached (2.13% in fall 2017, 6%±1.45 in spring 2018 and 1.35 %±0.86 in fall 2018). In addition, invasive green algae *Caulerpa cylindracea* was observed there irrespective of substrate or treatment (with average cover <8%).

Discussion transregional facilitation experiment coralligenous

The *P. clavata* forest effects on the early-stage sessile assemblages within coralligenous habitat differed among taxa and were dependent on the site, time/season and substrate. Within the analysed period significant differences in taxa abundance inside and outside of the *P. clavata* forest were evident for encrusting bryozoans which were more abundant within the gorgonian forest after 15 months but only on rubber substrate and only on Krk Island Natura 2000 site. Moreover, on the same site abundance of animal turf and serpulid polychaetes on mesh substrate in spring 2018 was significantly lower inside the forest.

Similar effects were observed by Ponti *et al.* (2014, 2018) reporting *P. clavata* facilitation of encrusting bryozoans and lower abundance of serpulid polychaetes and turf inside the *P. clavata* forest. Interestingly, these authors observed *P. clavata* forest effects on encrusting bryozoans also in Portofino (where we observed no differences), but using a different substrate (PVC panels).

The pulse of bryozoan recruitment was recorded after winter in this study and plastic meshes have proven to be a suitable settlement substrate, at least on Krk Island (Adriatic Sea), where greater abundance of bryozoans was observed. Besides potential differences in larval supply and/or other local factors, lower bryozoan recruitment success observed in Portofino in this study may be the consequence of unavailable substrata, already occupied by competitive animal turf that dominated the sessile assemblage there, putatively preventing more successful bryozoan settlement in spring (Figs. 16, 17 and 19).

The newly recruited assemblages differed significantly among sites. Such an outcome is also in concordance with the study by Ponti *et al.* (2014), that revealed significant spatial variation of recruitment patterns between Portofino (Ligurian sea) and Tavolara (Thyrrhenian Sea), suggesting variability in larval supply, sedimentation, chemical-physical parameters and hydrodynamism as causes of observed site differences. Besides these factors, our work suggests that timing of the experiment, substrate availability at the time of recruitment as well as substrate type also may play a role.

It is clear that we are dealing with a complex system and that many factors may influence recruitment, survival and growth of benthic sessile organisms. These factors may overshadow or blur the effect of a habitat-forming erect species. Given the slow dynamics of coralligenous habitat (Teixidó *et al.* 2011), it is evident that what we have observed in 15 months is far from a mature assemblage developing on the experimentally set substrate. Since the effects of *P. clavata* forest may vary with time, there is definitely an interest to perform a longer term experiment of this sort. However, nowadays in the changing seas with different stressors acting upon model organisms and greater frequency of extreme weather conditions it becomes increasingly challenging to perform and maintain such experiments in the field.

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Supplementary material - transregional facilitation experiment coralligenous

Table S1: Summary of PERMANOVA tests on the effects of *Paramuricea clavata* forest on the abundance of the main sessile taxa settling on two different substrates (plastic mesh or rubber bands) in different sites and different seasons (based on square root transformed percent cover data and using Euclidian distance). Si = Site, Tr = Treatment, P-F = Pseudo-F. For each analysis > 9900 unique permutations were available.

A) Encrusting Corallinacea

MESH						RUBBER				
AI) fall 2017						AII) fall 2017				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	2	114,14	57,07	195,13	0,0001	2	114,26	57,13	50,62	0,0001
Tr	2	11,237	5,62	19,211	0,0001	1	0,00	5,18E-04	0,00	0,9836
SixTr	4	5,9284	1,48	5,0674	0,0039	2	0,59	0,294	0,26	0,7786
Res	27	7,8969	0,29			18	20,31	1,1285		
Total	35	139,21				23	135,16			
BI) spring 2018						BII) spring 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	1,54	1,54	4,24	0,0533	1	1,48	1,48	3,84	0,0745
Tr	2	5,04	2,52	6,93	0,0047	1	0,77	0,77	2,00	0,1772
SixTr	2	1,66	0,83	2,28	0,1298	1	1,70	1,70	4,40	0,06
Res	18	6,55	0,36			12	4,64	0,39		
Total	23	14,80				15	8,60			
CI) fall 2018						CII) fall 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	5,53	5,53	20,8	0,0003	1	14,03	14,03	38,50	0,0002
Tr	2	0,43	0,21	0,8	0,466	1	0,13	0,13	0,35	0,5671
SixTr	2	0,91	0,45	1,7	0,203	1	1,07	1,07	2,95	0,1083
Res	18	4,80	0,27			12	4,37	0,36		
Total	23	11,67				15	19,61			

B) Serpulids

MESH						RUBBER				
AI) fall 2017						AII) fall 2017				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	2	22,43	11,21	112,45	0,0001	2	10,00	5,00	47,58	0,0001
Tr	2	0,03	0,01	0,13	0,8829	1	0,13	0,13	1,21	0,2816
SixTr	4	0,17	0,04	0,42	0,8056	2	0,19	0,09	0,90	0,4222
Res	27	2,69	0,10			18	1,89	0,11		
Total	35	25,312				23	12,21			
BI) spring 2018						BII) spring 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	210,09	210,09	1472,10	0,0001	1	31,65	31,65	40,57	0,0005
Tr	2	0,96	0,48	3,37	0,0531	1	3,00	3,00	3,84	0,073
SixTr	2	0,96	0,48	3,37	0,055	1	3,92	3,92	5,03	0,0434
Res	18	2,57	0,14			12	9,36	0,78		
Total	23	214,58				15	47,94			
CI) fall 2018						CII) fall 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	141,40	141,40	345,11	0,0001	1	134,38	134,38	152,61	0,0002
Tr	2	0,23	0,12	0,28	0,7477	1	0,02	0,02	0,02	0,894
SixTr	2	0,55	0,27	0,67	0,5156	1	0,87	0,87	0,99	0,3539
Res	18	7,38	0,41			12	10,57	0,88		
Total	23	149,56				15	145,84			

C) Animal turf

MESH						RUBBER				
AI) fall 2017						AII) fall 2017				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	2	91,70	45,85	39,40	0,0001	2	1,71	0,86	0,59	0,5679
Tr	2	4,89	2,44	2,10	0,145	1	1,99	1,99	1,38	0,2563
SixTr	4	3,94	0,98	0,85	0,5105	2	0,58	0,29	0,20	0,8256
Res	27	31,42	1,16			18	26,06	1,45		
Total	35	131,93				23	30,34			
BI) spring 2018						BII) spring 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	124,32	124,32	141,62	0,0001	1	26,65	26,65	18,53	0,0008
Tr	2	14,99	7,49	8,54	0,0013	1	5,96	5,96	4,14	0,0654
SixTr	2	9,55	4,78	5,44	0,0066	1	1,98	1,98	1,38	0,255
Res	18	15,80	0,88			12	17,26	1,44		
Total	23	164,65				15	51,86			
CI) fall 2018						CII) fall 2018				
Source	df	SS	MS	P-F	P(perm)	df	SS	MS	P-F	P(perm)
Si	1	39,46	39,46	85,25	0,0001	1	0,46	0,46	0,40	0,5221
Tr	2	5,79	2,89	6,25	0,0073	1	1,01	1,01	0,89	0,3567
SixTr	2	0,29	0,14	0,31	0,7432	1	1,14	1,14	1,01	0,3352
Res	18	8,33	0,46			12	13,59	1,13		
Total	23	53,86				15	16,19			