UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di ricerca in Scienze della Terra e del Mare
Dipartimento di Scienze della Terra e del Mare
Settore Scientifico Disciplinare BIO/07-Ecologia

Investigations into the development and role of a Mediterranean intertidal bioconstruction for coastal conservation: the Vermetid Reef

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CICLO XXIX
ANNO CONSEGUIMENTO TITOLO 2017

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ABSTRACT

Investigations into the development and role of a Mediterranean intertidal bioconstruction for coastal conservation:the Vermetid Reef

Vermetid reefs are intertidal biogenic habitats created by a dense aggregation of mollusks, frequently cemented by calcareous algae, and are typical of sub-tropical and warm–temperate rocky shores. These bioconstructions are valuable key-habitats of the coastal zones, increasing their productivity and biological value. In the Mediterranean, the main vermetid reef builders belong to the genus *Dendropoma* and are associated to encrusting coralline red algae. These organisms are ecosystem engineers protected under international European Legislation, although vermetid reef conservation is limited by a lack of biological and ecological knowledge. The two-way interactions between biota and the physical environment have not been previously studied for these reefs, further limiting understanding of the ecological value and function of this habitat. Phases of the *Dendropoma* life cycle are also understudied, making the key-processes during the reef development unclear. In an integrated coastal zone management framework, all of this knowledge is fundamental to strengthen vermetid reef conservation and management in the Mediterranean.

This thesis aims to further understanding of traits of the *Dendropoma* reef system, undertaking novel themes of research, and by using an interdisciplinary approach.

The research project has two main goals:

1) To describe which contribution the reef provide in preserving rock substrate from physical alteration
2) To understand which cues may promote *Dendropoma* reef formation

A set of laboratory and field experiments and observations has been used to fulfill these aims. The study was conducted in Sicily, central Mediterranean, where the main reef builderspecies is *Dendropoma cristatum* (Biondi, 1857), associated with the coralline algae*Neogoniolithon brassica-florida* (Harvey) Setchell & Mason 1943.

In detail, topic 1 aims to describe the bio-protective role of the *Dendropoma* encrustation on the underneath rock substrate. Bioprotection is described as the contribution that biological layers give to the conservation of the substratum they colonize, by mediating the deteriorative action of other factors, such as physical, chemical and biological weathering. In this study, internal temperature variations and salt weathering have been considered as rock physical stressors that are particularly relevant in the coastal zone, and which may be mediated by the *Dendropoma* encrustation. Lab experiments and analysis have been employed to describe subsurface variations of temperature and salt content inside rock cores covered by live and dead vermetids *vs* bare rocks.

The experiments have demonstrated that the *Dendropoma* encrustation exerts a control on the factors responsible for rock weathering.

Topic 2 aims to detect which physical and biological factors may promote *D. cristatum* settlement and recruitment, with implications for the reef development.*D. cristatum* recruitment and settlement have been measured in the fieldand in different conditions.

A seasonal pattern of recruitment has been described for the Sicilian species *D. cristatum*, and the hydrodynamic regime has been showed do not affect this pattern within a range of Km. Aside, at a small spatial scale (from mm to cm), biogenic surface have been shown to affect the settlement dynamics of the crawling larvae of *Dendropoma cristatum*,more than physicalcomplexity of the substratum. In detail, biological cues provided by a layer of crustose coralline algae or by a microbial film,may positively affectsettlement dynamic, indicating suitable site for larvae attachment and having an influence on the early stages of *Dendropoma cristatum* development.

In conclusion, this research has contributed to the description of some ecological traits of the reef-builder *Dendropomacristatum*and provided a wider view of the two-way interactions between this ecosystem engineer and the surrounding physical and biological environment.

Within the perspective to improve *Dendropoma* reef management at Mediterranean scale, the gaining of information about the ecological functioning and role of this coastal systems may provide a valuable contribution.

TABLES OF CONTENTS

LIST OF FIGURES

CHAPTER 1.
The vermetid reef, an overview

1.2 Background

Marine bioconstructions are biogenic structures originated by the aggregation of organisms from one or more species which modify the environment theycolonize, originating a habitat with peculiar characteristics. These biogenic structures persist within the time and are the result of a complex multitude of physical and biological processes.

Organisms able to build bioconstructions are defined “habitat formers” and also “physical ecosystem engineers” or “foundation species”, because they physically and biologically modify the environment and are considered key-species which, directly or indirectly, regulate the availability of space and resources, as food or repairs and shelters, to other organisms (Jones, 1997).

This new structures represents a secondary substratum which controls the biotic component of an ecosystem, and also the nonliving one (Hastings *et al.*, 2006, figure 1), by binding the substrates, stabilising habitats and physically modifying the environment (Stallins, 2006; Naylor *et al.*, 2002).

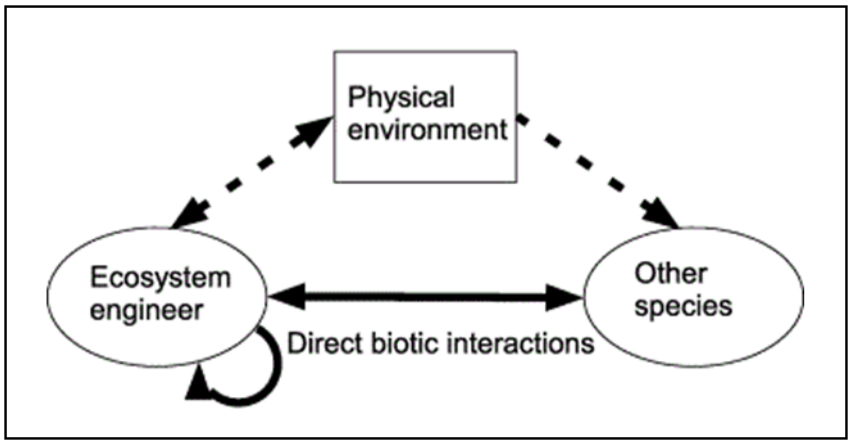

Figure 2, Schematic representation of the interactions between ecosystem engineers and the environment, from Hastings et al.*, 2007.*

When physical ecosystem engineers are able to precipitate hard structures, such as shells or skeletons, they commonly originate carbonatic surfaces called “reefs”. Reefs are biogenic constructions made by a single or a group of organisms, which progressively modify at local

scale the environment (Fägerstrom, 1987) and generate physical structures which contribute to the development of landforms, modifyingcoastal geomorphology and the habitat heterogeneity and structural complexity (Bianchi, 2001; Bell *et al.*, 1991; Butler, 1995).

Coral bioconstructions, mussel and oyster beds, polychaete aggregations and vermetid platforms, are common examples of biogenic reefs made bycalcium-carbonate secreting organisms. Thus, due to the characteristic carbonatic structure, reefs are highly resistant to physical disturbances and have greatspatiotemporalpersistence, representing wave-resistant structures which enhance the stability of the coastal environments (Safriel & Ben-Eliahu, 1991).

1.3 The Vermetir Reefs

Vermetid reefs are examples of intertidal biogenic habitats created by a dense aggregation of mollusks, frequently cemented by calcareous algae, and are typical of sub-tropical and warm–temperate rocky shores of the world,recorded from several locations, such as:Bermuda (Stephenson & Stephenson, 1954),Brazil (Van Andel & Laborel, 1964), New Zealand, the tropical Caribbean (Focke, 1977; Jones & Hunter 1995) and the Mediterranean (Milazzo *et.al.*, 2016). These bioconstructions are valuable key-habitats of the coastal zone, which affects coastal dynamic and productivity and improves its solidity and the biological value.

Figure 2, Example of a vermetid reef (NW Sicily)

In the Mediterranean the main building species of these constructions, are the gregarious gastropods belonging to the genus *Dendropoma* and encrusting coralline red algae from the genus *Neogoniolithon, Lithophyllum* and *Mesophyllum*. These biogenic structures provide protection from coastal erosion, regulate the transport of sediment, act as a carbon sink, provide habitat for fish and invertebrates of commercial and recreational interest, and greatly enhance the marine biodiversity (Chemello, 2009; Chemello & Silenzi, 2011). Because of its ecological relevance and vulnerability to anthropogenic pressures, the vermetid reef is considered as a"determinant habitat" for the Mediterranean (Relini, 2009), recently listed as threatened bioconstructions in the Mediterranean Red Data Book, and the reef-builders organisms are ecosystem engineers protected under international European Legislation.*Dendropoma* spp is listed in Annex II (Strictly Protected Fauna species) of the Berne Convention and in the Annex II (List of Endangered or Threatened Species) of the Protocol concerning Specially Protected Areas and Biological Diversity in the Mediterranean of the Barcelona Convention (1996).

Although this internationally recognized need of protection, vermetid reef conservation is limited by a lack of biological and ecological knowledge.

The two-way interactions between the biota and the physical environment have not been previously described for these reefs, further limiting to understand the ecological value and function of this habitat.Phases of the *Dendropoma* life cycle are also understudied, making the key-processes during the reef development unclear.

This Ph.D research aims to further understanding ecological and biological traits of the *Dendropoma* reef-system.

Prior to present the topics of this research, the goals and to illustrate the adopted methodological approach,a literature-based state of the art of the vermetid reef is following provided.

1.4 Mediterranean vermetid reefs, general characteristics and main morphologies

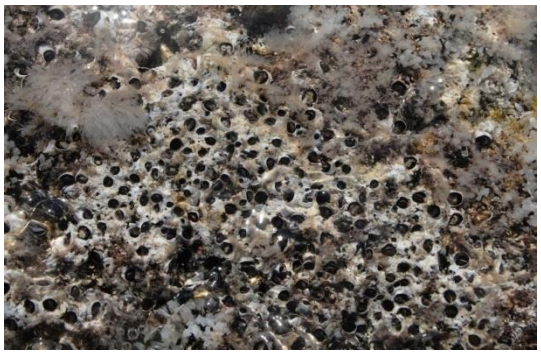

Figure 3, Close view of the Dendropoma spp. *encrustation. Shells are covered by crustose coralline algae.*

The vermetid reef,also known under the name “trottoir à vermets" (according to Molinier & Picard, 1953) or vermetid platform, are key-habitats of the Mediterranean warmest rocky coasts, with a pattern of distribution which caused local segregation and speciation events of the reef builder vermetidae (Calvo *et al.*, 2009; 2015).

The vermetid reefs develope on intertidal abrasion platforms and its morphology, horizontal extension and physical features vary according to the environmental conditions. Although the reef morphology represents the most impressive structure,also other kinds of smaller structures made by vermetid gastropods are present within the Mediterranean(Antonioli *et al.*, 1999):

the “crust”, which is a thin layer of *Dendropoma* spp. shells on a rocky substratum; the “ledge” (usually <1m wide and 20-30cm thick); the “mushroom-like pillar”, which seems a large vermetid rim on a thin base; the “micro-atoll”; “the island” (Safriel, 1966). The last three forms are resulting from differential erosive processes between the rock substrate and the vermetid bioconstruction (Antonioli *et al.*, 1999).

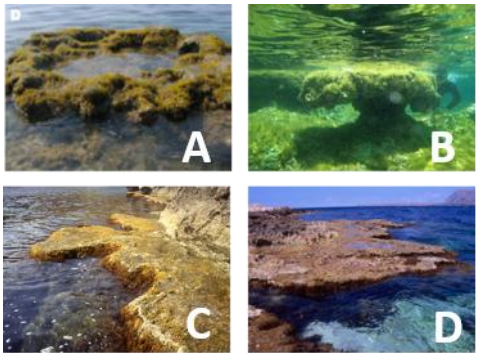

Figure 4, Different typologies of vermetid bioconstructions: A). The micro atoll, B) the mushroom-like pillar, C) the ledge and D) the true platform.

Moreover, the density of *Dendropoma*may vary among these morphologies, being much lower on crust and ledge structures.

The development of the vermetid reefs seems to be dependent on abiotic factors which affect their distribution at high spatial scale and their physical extent: the geological nature of the substratum, the exposure to waves and the coastal slope (Chemello *et al.*, 2000; Chemello & Silenzi, 2011).

- A strict relation between the geological nature of the rock substrate and the way the reef may develop best has been recognized by some authors (Chemello *et al.*, 2000, among others) and the rock typology and texture beneath these reefs are central factors for its build up. The more a rock substrate is subject to physical erosion, the higher is the width of a reef. The best rock type for the development of a vermetid reef is represented by eolianites and calcarenites (Chemello, 2009). Overall, progressively less suitable rocky substrates aredolomite, basaltic, granitic and flysch (but see Schiaparelli *et al.*, 2003). On these rock typologies, indeed, the formation of a true reef is not possible (Chemello, 2009) and the typical vermetid constructions is the layer or the crust.
- The slope of the shoreline is another physical factor which affects the size and shape of a vermetid reef. On flatter coasts (0° - 15°) these formations are less wide and appear as ledges or thin crusts. Underneath the upper intertidal notch of cliffs with a slope >40°,the reefs progressively show reduced thickness and width,being completely absent on slope >50°. On Sicilian carbonate substrates, for instance, Chemello *et al.* (2000) observed that *D. cristatum*larger reefs develop on shore with a slope range between 15° and 40°.
- Hydrodynamic conditions are known to play a relevant role inthe development of biogenic habitats as vermetid reefs. Hydrodynamics is an important environmental feature that influences the distribution of vermetids (Chemello & Silenzi, 2011; Hughes, 1985).Overall, Mediterranean vermetid platforms are found on moderately exposedshores,and are absent on the most exposed rocks,on too much sheltered coasts and on vertical rock substrates directly exposed to wave action (Chemello & Silenzi, 2011; Calvo *et al.*, 1998).

Dendropoma density, moreover, increases with hydrodynamic exposure (Azzopardi & Schembri, 1997).

The synergistic effects of these abiotic factors, thus, may strongly affect the size and shape of a vermetid bioconstruction and the density of the main reef-building species.

1.5 Physical structure of a vermetid reefs

A vermetid reef is characterized by a horizontal extension which includes a multitude of microhabitat with peculiar morphological, physical, ecological and biological features and is delimitated on the seaward and on the landward by an outer and an inner margins (Laborel, 1987; Chemello *et al.*, 2000).

Likely to other shallow biogenic habitats, such as oyster and mussel beds, the vermetid reef does change the morphology of a given coast and well developed external margins amplify often up to 3 times the coastal profile (Chemello *et al.*, 2000). Additionally, at low spatial scale this biologicalencrustation modifiesthe substratumcharacteristics, enhancing the presence of crevices, holes and irregularities that locally ameliorate the surface rugosity, increasing space and generating microclimatic conditions which are different from the surroundings.

Studies on the Sicilian vermetid reefs describe the outer edgeas the most biologically active side of the platform (Chemello *et al.*, 2000). It is generally made by a thick layer and highly articulated encrustation of *Dendropoma* spp. and *Neogoniolithon brassica-florida* which may exceed 50 cm in thickness. Below the outer margin, the vermetid reef is fringed by a subtidal belt made by the canopy-forming algae *Cystoseira amentacea* var. *stricta*, which growabove the wave-cut notch.

The inner edge develops more vertically, is less thick and characterized by a lower density of *Dendropoma* individuals and may be subjected to long times of emersion during low tide, desiccation and UV-irradiation which locally increase the physical stress. The density of *Dendropoma* aggregations is generally higher on the outer margin rather than the inner (Di Franco *et al.*, 2011).

The space included between these two edges is the *cuvette* (Molinier & Picard, 1953; Pérès & Picard, 1952) which extension represents the reef width and may host severalsubmerged pools and crevices. The cuvette may be defined as a “depression” which usually holds water on the flat during period of low tide and is covered by perennial canopy-forming brown algae and encrusting organisms of subtidal origin (Milazzo *et al.*, 2016).

Moreover, other organisms may participate to the edification of the reefs, supporting *Dendropoma* spp. and the red coralline algaein the process of bioconstruction: the foraminiferan*Miniacina miniacea*, the coralline algae *Lithophyllum byssoides*, *L. incrustans* and *Neogoniolithon mamillosum*, some encrusting bryozoans and the solitary vermetid *Vermetus*

*triquetrus*Bivona-Bernardi, 1832 (Safriel, 1974). On the other hand, a variety of bioerosive organisms, such as sponges, bivalves and sipunculid worms, have been especially found on the outer edge of the reef (Garcia-Raso *et al.*, 1992; Chemello, 2009).

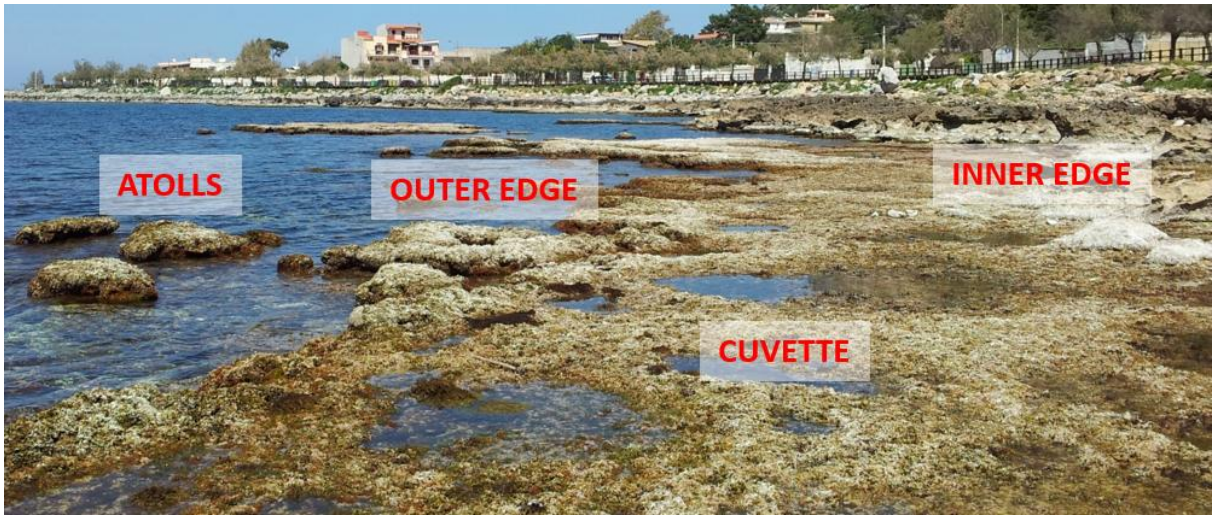

Figure5, Structure of a Sicilian vermetid reef.

1.6 Distribution of the vermetid reefs within the Mediterranean Sea and phylogenetic differentiation

The vermetid reefs are characteristic bioconstructions of the Southern sector of the Mediterranean Sea with a firm distribution which segregates these bioconstructions under the40°N of latitude and in water characterized by winter SST higher than 14°C (Chemello *et al.*, 2000).

However, over the last decades*Dendropoma* concretions have progressively expanded in the northernmost sectors, crossing this biogeographical range probably as a consequence of the climatic change (Milazzo *et al.*, 2016).

From the east to the west of the Mediterranean, largest vermetid formations have been recorded along the Israeli(Safriel, 1966; 1974; 1975; Galil, 2013), the Lebanese and the northern part of the Syrian coast, from Lattakia up to the Turkish border(Dalongeville, 1977;

Bitar & Bitar-Kouli, 1995a; 1995b; Dalongeville *et al.*, 1993; Al-Nimeh & Elassafin, 1996). Large reefs have also been described in southern Turkey and and Greece (Laborel, 1987; Bakır *et al.*, 2012; Ramos-Esplá *et al.*, 2007; Scaperrotta *et al.*, 2012; Kelletat, 1979; Naylor & Viles, 2002). Well structured vermetid platforms are also present in Algeria (Molinier & Picard, 1953; Pérès & Picard, 1952). In Malta vermetids mainly develop as small encrustation and reefs (Azzopardi, 1992; Azzopardi & Schembri, 1997). In Italy, most of the vermetid platforms are distributed in Northern Sicily, between Milazzo Cape (NE Sicily) and the Egadi islands (NW Sicily, Molinier & Picard, 1953; Chemello *et al.*, 2000). In Spain, vermetid reefs are present from Castellòn de la Plana (Valencia) to Càdiz (Andalusia), including the Balearic Islands and the Alboran Sea (Molinier & Picard, 1953; 1956; Templado*et al.*, 1992). The most developed formations are reported from the southeastern regions of Alicante, Murcia, and Almeria.

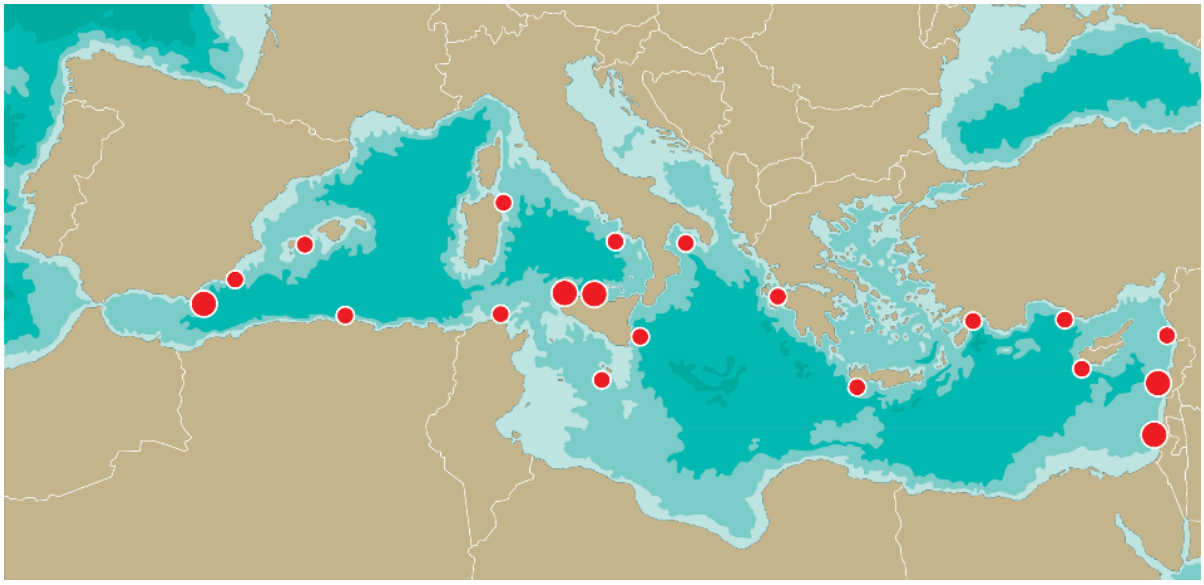

Figure 6, Mediterranean distribution of vermetid bioconstructions. The size of red dots reflects the overall dimension of the reef.

According to this wide range of distribution within the Mediterranean, recent genetic analyses revealed that the genus *Dendropoma,* which was supposed to have a unique species within the whole basin*, D. petraeum* -originally described along the NW Sicilian coast and supposed to be widely distributed throughout the Mediterranean Sea - comprises a complex of four cryptic species. The four lineages are geographically separated and roughly coincide with the sub-basins or sub-provinces within the Mediterranean Sea (Calvo *et al.*, 2009; Calvo *et al.*, 2015; Templado *et al.*, 2016).

These species have a separated geographical distribution, although they share similar intertidal habitats and are able to build reefs: *Dendropoma anguliferum*, in the Levantine Sea and a Ionian-Aegean lineage which lacks a detailed morphological description; *Dendropoma lebeche* in the Western Mediterranean and *Dendropomacristatum* in the central Mediterranean (Sicily and Malta Islands) with similar morphological characteristics(Templado *et al.*, 2016).

The extremely low dispersion ability as sessile organisms with little larval motility and the scarce population connectivity are probably the causes of these speciation events which involved the genus *Dendropoma*(Calvo *et al.*, 2009).

1.7 Description of *Dendropoma cristatum* (Biondi, 1859)

Basing on the most recent phylogenetic description of the *Dendropoma* genus within the Mediterranean (Templado *et al.*, 2016), *Dendropoma cristaum* is the species distributed along the Sicilian coast, which is the area of interest of this manuscript.

Here below, a description of this species.

A) Anatomy

Dendropoma cristatum (Biondi, 1859) has been recently proposed by Templado *et al.*, (2016) as the valid name to refer to the *Dendropoma* complex from the central Mediterranean (Sicily as the type locality), instead the old name *Dendropoma petraeum* (Monterosato, 1884). The first description of this mollusc has been provided by Biondi (1859), which reported about individuals up to 14mm as maximum diameter, although Templapo *et al.* (2015) reported up to 4-5 mm in maximum outside whorl diameter and specimens from Malta normally range between 3 and 4 mm (Azzopardi & Sghembri, 1997).

Figure 7, Dendropoma cristatum*, adult and juvenile individuals. Source: www.marinespecies.org*

Dendropoma cristatum is a prosobranch sessile gastropod with a tube-shaped shell closed at the extremity by a thick corneus operculum (Scaperrotta *et al.*, 2012), which allow to resist to prolonged emersions. The shell is characterised by a very rugose surface due to the densely imbricate lamellar folds that are produced during shell growth and by the presence of a crest along the dorsal side (Calvo *et al.*, 1998; Templado, 2016), absent on the larvae shells.

The body of adult specimens measure approximately 2 cm in length, while larvae are under 1mm of size. A wide buccal mass occupies the entire head cavity and opens through a vertical slit-like mouth to a short pre-buccal cavity with a pair of hemispherical bilateral mandibles, which line the lateral walls of the buccal mass (Templado *et al.*, 2015).

The mantle cavity is characterised by an anal orifice with a terminal dorso-ventral slit and its outer lip forms dorsallya long finger-like appendix which protrudes anteriorly.

The intestine is relatively longer, and posteriorly it borders the kidney, running for a short tract backwards parallel to the style sac before turning at a right angle to the right.

B) Biology and ecology

Despite phylogenetic differences, *Dendropoma* species share a very peculiar reproductive and developmental biology and feeding strategies. The main biological characteristics of these species are following reported.

Figure 8, Egg capsule brooded by females and containing a multitude of embryos. The scale bare indicates 400 µm.Source: Calvo et al., 2009.

All the species have separate sex, with no evident sexual dimorphism and sex are distinguishable only by the gonad observation.

Males areaphallicand their sperm is encapsulated in spermatophores which are released into the water and subsequentlytrapped by females through feeding sticky filaments. Sperm is held in the female mantel cavity until gonads maturation and internal fertilization occurs when the seawater starts to warm. Mature maleshave been foundbetween November-December and May-June, while ovaries appear at the beginning of the summer.Females brood their fertilised eggs in capsules held in the mantle cavity and metamorphosed larvae hatch inside the maternal shell. Embryos at different stages of development occurred in females from June to October. Females from the central Mediterranean have been observed to brooding up to 14 egg-capsules containing up to 25 eggs (Calvo *et al.*, 1998). Not fertilised eggs may be used as food source from other embryos and cannibalism may occur.

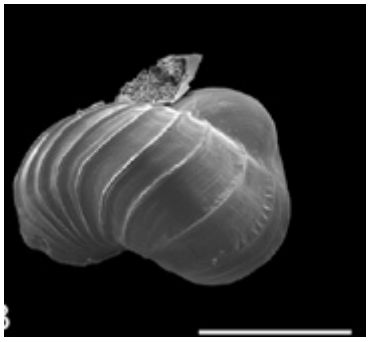

Figure 9, SEM image of D. cristatum *larvae. The scale bar is 400µ. Source: Templado* et al.*, 2015.*

Larval development is lecithotrophic and intracapsular without a pelagic stage. The inner yolk of the embryos is likely to be the main source of intracapsular nutrition andis enough to complete their development (Calvo *et al.*, 1998). After hatching, crawling juveniles typically

spend a few hours moving on the substratum with their foot and finding a suitable place to settle, very close to the maternal shell, where they immediately calcify a flat base to firmly attach to the substratum and start a sessile life. It seems that settlement is triggered by the coralline algae (Spotorno*et al.*, 2015), letting hypothesising the occurrence of intra-specific interaction.

Feeding strategies in *Dendropomacristatum* show great adaptation and may vary according to local environmental conditions and reef morphology.

The individuals forming intertidal platforms are mainly filter feeders, while those building 'crusts or 'ledge' reefs in the upper subtidal are rather shifted to mucus feeding (Schiaparelli *et al.*, 2006). Differential exposure to tides and wave energy, furthermore, can affect feeding rates and behaviours of *Dendropoma* species (Vizzini *et al.*, 2012). Besides enhancing food inputs, water turbulence and wave-pumping of water through the bioconstructions may also aid cementation and lithification processes and most authors recognise that such reefs grow best under exposed conditions, thus benefiting from wave action (Chemello & Silenzi, 2011).

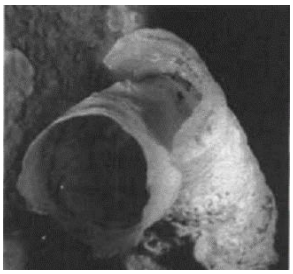

Figura 10, Example of Dendropoma *spp. feeding tube. Source: Schiapparelli, 1999.*

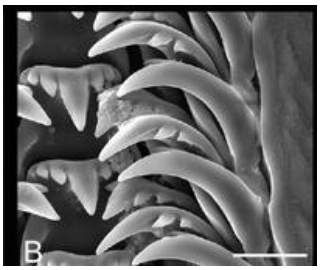

Figura 11, SEM immages of D. cristatum *radula. Source: Templado* et al.*, 2015.*

Also, grazing with the radula has been observed in *Dendropoma* species particularly in the larval stage (Calvo *et al.*, 1998).

1.8 Biological value of the vermetid reef

Due to the previously stated morphological complexity which multiplicates the availability of space for other organisms and to the simultaneous presence of microhabitats within the reef(mainly the inner and outer edges and the cuvette), coastal biodiversity highly increases on vermetid reef.The vermetid reef represents a habitat which amplify the intertidal space and the availability of resources in microtidal environment and are considered relevant hot-spots of biodiversity in the Mediterranean (Chemello *et al*., 2000). This complex structure positively influences the associated assemblages and makes the vermetid formations important in structuring the Mediterranean coastal ecosystems(Safriel & Ben-Eliahu, 1991).

In areas with very low tidal ranges on the Mediterranean coastline, indeed, space is a limited resource for intertidal species. Vermetid reefs contribute in different ways to widen the available ecological nichesfor a variety of benthic and fish assemblages: by limiting physical disturbances, by providing refuge from predation and critical nursery habitat, by affecting the strength of competitive interactions, or representing themselves important food resources (Goren & Galil, 2001; Consoli *et al.*, 2008; Chemello, 2009; Milazzo *et al*., 2016). A clear example of this reduction of competitive interactions due to the high availability of resourcesand spatial segregation was described for three congeneric and ecologically similar crabs associated to the reef: *Pachygrapsus marmoratus*(Fabricius, 1787)*, Pachygrapsus maurus* (Lucas, 1846) and *Pachygrapsus transversus* (Gibbes, 1850). Although all these species actively feeding on algal turfs in the cuvette, finding refuge from predators and shelter from adverse environmental conditions, an opposite trend of habitat use for the two most abundant crab species, *P. marmoratus* and *P. maurus* , has been found and the third species exhibited a low density (Milazzo *et al.*, 2016).

Moreover, empty *Dendropoma* shells, reef crevicesand holes retain sediment and organic matter for many invertebrates, such asmolluscs, polychaetes and crustaceans, which use these resources as food or as shelter for the cryptofauna, as the hermit crab *Calcinustubularis*.

Likewise other intertidal biogenic habitats, such as oyster and mussel beds, the physical structure of vermetid reefs reduce theenergy of waves and attenuate their physical impact on the shore. This allows an increasing number of species to be able to colonise suchintertidal habitat. Species richness of gastropods has been measured 1,6 fold higher in rocky shores with

vermetid reef than the surrounding environment, witha high number of gastropods' shell morphologies with different foot shapes (Milazzo *et al.*, 2016).

The algal assemblages associated with these reefs are composed by at least a hundred of macroalgal species distributed across the different habitats of the reef (Mannino, 1992). In addition to *Neogoniolithon brassica-florida*, whose central role is to cement the *Dendropoma* shells, and *Lithophyllum byssoides* – often forming cushion-like structures in the two margins of the platform – the most characteristics algae are the red algae of the so-called *Laurencia* complex, and the brown algae *Padina pavonica, Cystoseira* spp and *Dyctiota* spp, mainly distributed in the cuvette (Graziano *et al.*, 2009). Under anthropogenic disturbances, these species are often substituted by Corallinales ed Ulvales (Graziano *et al.*, 2007). Encrusting red algae, and sometimes *Halimeda tuna*,are dominant in those reefs where the *cuvette* is deeper (sometimes 0.5 m deep at low tide), tidal pools are present, or underneath the *Cystoseira* and *Dictyota* canopy. Below the outer rim, the narrow upper infralittoral fringe is often characterised by *Cystoseira*-dominated assemblages. In this lower border of the reef formation high-density belts *of Cystoseira compressa* and/or *Cystoseira amentacea* var. *stricta* are usually dominant.

The *Dendropoma-Neogolithon* aggregation and the macroalgal species associated with, host highly diverse zoobenthic assemblages. Undercanopyspecies are mostly present in the reef flat (*cuvette*) and in the upper infralittoral *Cystoseira* fringe.

At least 50 species of molluscshave been found in association with the vermetid reef (Pandolfo *et al.*, 1992a), and among these, the most abundant and highly distributed are:*Mytilaster minimus*, *Carditacalyculata*, *Lepidochitona corrugata*, *Onchidella celtica*e *Patella ulyssiponensis*.*Patella caerulea*, *Pisinnaglabrata*, *Eatoninacossurae*and *Barleeia unifasciata*(Pandolfo *et al.*,1992b) are typically found within the cuvettes. Along the internal edge, the non-indigenous species *Brachidontes pharaonis*is even more abundant(Milazzo *et al.*, 2009).

Among the Polychaetofauna, relevant species which dominate on the outer edge are: *Perinereiscultrifera*, *Platynereis dumeriliii*and *Lepidonotus clava*,*Syllisamica*and*Perinereis macropus*are typically found on the internal edge (Badalamenti *et al.*, 1998). *Palolasiciliensis*, *Lysidice collaris*and*Scoletomafunchalensis*are characteristic within the cuvettes.

Moreover, a comparison of the polychaete communities associated to vermetid rims in tropical and Mediterranean regions, revealed that the Red Sea species pool was 1.6 times larger than the Mediterranean one, but the Red Sea vermetid reefs were only 1.3 richer (90 *vs*. 70 polychate species) than the Mediterranean reefs (Safriel & Ben-Eliahu, 1991).

Also the fish assemblage associated to the Israeli and Italian vermetid reefs has been investigated. In Israel, a total of 36 fish species was recorded and represented the highest fish biodiversity reported in any habitat along the Mediterranean coast of Israel (Goren & Galil, 2001) with Gobids and blennids the most abundant families.More recently, the comparison of the fish assemblages of three shallow Mediterranean rocky habitats, the vermetid reef, the rocky-algal reef and the boulder field, revealed that, despite total number of species did not differ between habitats, the vermetid reefs showed the higher physical complexity supporting on average the highest values of fish density and species richness (Consoli *et al.*, 2008). Commercially relevant species such as *Diplodus* spp were more abundant around vermetid formations, letting hypothesized that reefs provide additional resources for adults and juveniles of many fish species.

1.9 Impacts which affect the vermetid reefs

Increasingly, anthropic activities affect the coastal environment and threaten the proper functioning of coastal ecosystems, matching with natural disturbances. Due to its intertidal position, vermetid reefs are subjected to a multitude of impacts and much research considered the response of this habitat to anthropogenic threats (Di Franco *et al*., 2011; Milazzo *et al.*, 2014; Graziano *et al.*, 2007).

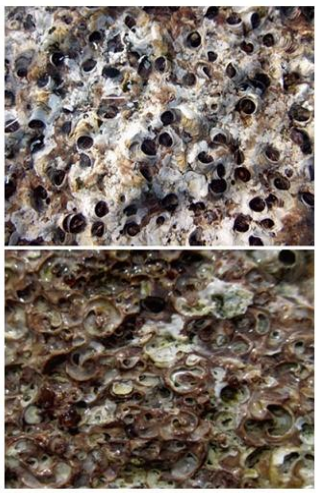

Figure 12, Close view of a vermetid encrustation in pristine condition (above) and strongly eroded (below). Source: Milazzo et al*., 2016.*

Under particular stress, the vermetid reef may arrest its growth. This is was occurred in the Levantine basin, specifically along the Israeli coast, where the outer rims of the vermetid reef have been eroded, as a result of the local extinction of *Dendropoma anguliferum* (Galil, 2013; Rilov, 2016).

Coastal urbanization and the artificial sheltering of intertidal communities by the construction of jetties and marinas represent a great impact on vermetid reefs. Recently, Di Franco *et al.* (2011) demonstrated that the presence of a small marina in NW Sicily heavily affects the density of *D. cristatum*andthe cover of *N. brassica-florida*, when compared with near control locations.Although not causing local extirpation of the reef builder species, reductions in the gastropod density were specifically recorded in the outer rim of the vermetid reef and authors hypothesized that this was related to altered water flow regimes, increased water turbidity, along with nutrient enrichment and the accumulation of toxic compounds inside the marina

Recent work on the consequences of ocean acidification on vermetids, predicted that the higher levels of pCO_2expected to occur within this century, impair the *D. cristatum* (as *D. petraeum*)recruitment success, causing the dissolution of the recruits shells and altering their mineralogy (Milazzo *et al.*, 2014).Since the vermetid snails brood their young and the hatchlings crawl only a short distance before becoming a sessile individual, unless snails adaptation to ocean acidification does not occur, there are only slim chances for *Dendropoma*spp. to cope with this scenario of climate change.

Scant knowledge about the potential effects of sea-level rise on the vermetid reefs persists and research on the interacting effects of warming and acidification on vermetid reefs is presently

underway.Surely, the effects of ocean acidification and global warming will have an impact on the physiological performance, reproduction, calcification and recruitment rates of the reef-building species.

Sessile intertidal organisms are also sensible to human trampling and coastal zones subjected to high intensity of trampling show low specific diversity anddensity (Addessi, 1994). Reef vulnerability to human trampling depends mainly on the nature and morphology of marine organisms and on the level of human use. The presence of the vermetid platform on rocky shores may enhance human frequentation and related 'foot-traffic' on the shoreline. Encrusting forms like the coralline algae and the vermetids aggregations are resistant to this kind of disturbance showing no direct effects on their density and cover (Graziano *et al.*, 2007), although it is presently unclear if physical impacts of trampling may affect vermetid recruits survival and settlement success. In turn, the detrimental consequences of human trampling may be recorded on the macroalgae associated to the reef. Step on erect algae may cause a rapid decrease in algal cover, canopy and biomass, leading the community to a less structurally complex state dominated by low-profile and turfing-form algae (Milazzo *et al.*, 2004). This may have cascade effects on the community, which reduce the high levels of benthic and fish diversity associated to these reefs (Chemello *et al.*, 2000).

1.10 Conservation status of vermetid reefs within the Mediterrnean

Recognising their important role in the Mediterranean intertidal zone, *Dendropoma* spp. and some of the associated algae (as *Neogoniolithon brassica-florida*, *Lithophyllum byssoides* and *Cystoseira amentacea*) are included in the annexes of the Berna Convention, and in the Annex II (Endangered or Threatened Species) of the Protocol for Specially Protected Areas in the Mediterranean (SPAMI Protocol of the Barcelona Convention). *Dendropoma*has been also proposed to be included in the annexes II e IV of the Habitat directive (Chemello 2009) and thereef structures have been recently listed as threatened bioconstructions in the Mediterranean Red Data Book.

Despite this, only the 28.5 % of vermetid reefs at Mediterranean scale are protected and included within MPAs or coastal reserves, as showedby Chemello *et al.* (2014) and, at national level, the Spanish National Catalogue on Threatened Species and the Maltese "The Flora Fauna

and Natural Habitats Protection Regulations" are the only documents which, officially, suggest the need of protection of these reefs.This context of scarce conservation of a so relevant intertidal coastal key-habitat, strongly stress the need to extend action plans for vermetid reef protection and improve its management at the Mediterranean level.

However, accurate information on population connectivity and ecology, are essential points to improve the protection of this neglected coastal habitat and to develop a conservation strategy at a basin scale.

1.11 Conclusions and goals of the research

Healthy vermetid reefs locally promote the spatial heterogeneity of the intertidal zone, supplying shelter, food and refuges to a high number of different species, including fish and invertebrates of recreational and commercial interest, making them a key habitat in the Mediterranean intertidal zone and, when their physical structure is well conserved, serve as important wave breaks, preventing coastal erosion and other physical damagesand providing a fundamental contribution to the proper ecological functioning ofthe intertidal Mediterranean zones.

The ecological research on vermetid reefs has highly focused on the effect of pollution and human activities on the reefs, on their responses to the ongoing climate change and ocean acidificationand on the biological and genetic traits of these species.

The two-way interactions between biota and the physical environment have not been previously described for these reefs, further limiting understanding of the ecological value and function of this habitat. Phases of the *Dendropoma* life cycle are also understudied, making the key-processes during the reef development unclear.

To date scant information are available about the contribution the vermetid reef provides in offering protection to the underneath surface, mediating earth-surface processes and about the early steps of reef-formation.

Within this theoretical framework, the present research project has two main goals:

1) To describe which contribution the reef provide in preserving rock substrata from physical alteration

2) To understand which cues may promote the *Dendropoma* reef formation

In detail, topic 1 aims to describe the bio-protective role of the vermetid encrustation on the underneath rock substrata.

This topic includes two main research questions:

- Does the biological encrustation control the microclimatic conditions within the rock?
- Does the biological encrustation reduce the penetration of salt within the rock?

Microclimatic stress and salt crystallisation within the rock are known to be consistent causes of weathering of intertidal rocks, which damage their structure and cause their breakdown. Biological encrustations are able tomediate weatheringprocesses and often to reduce their action, contributing to the protection of the colonized substrata. This study detecs for the first time, the potential bioprotective of the *Dendropoma*aggregation, measuring its capacity to mediate weathering mechanisms on the underneath rock surface.

Topic 2 aims to detect if physical and biological factors may promote *Dendropoma* spp. settlement and recruitment and enhance the reef development.

This topic includes the following research questions:

- Which is the temporal pattern of recruitment of *Denropoma* (in Sicily) and how hydrodynamic and local physical conditions may affect this pattern?
- Which kind of substratum may induce *Dendropoma* settlement?
- Are biological cues driver of settlement?

Shedding light on these questions would be of relevance in the understanding of which factors crucially drive habitat selection by *Dendropoma* larvae and may affect the initial steps of reef formation.

The gaining of information about the ecological value and functioning and role of this Mediterranean coastal systems may provide tools to contribute to the management of this valuable natural resource, according to the principles expressed by the international environmental policy.

A set of laboratory and field experiments and observations has been used to fulfill these aims. All the field experiment were conducted in Sicily, central Mediterranean, from June 2014 to Dicember 2016. Within this area the main reef builder species is *Dendropomacristatum* (Biondi,1857), associated with the coralline algae *Neogoniolithon brassica-florida* (Harvey) Setchell & Mason 1943.

1.10 Time scale

According to the previously showed research goals and questions, over the three PhD years, the project has been approached as follow:

The topic 1 has been carried out between the second and the third year, by collection of samples from the field and mesocosm experiments performed at the School of Geography and the Environment of the University of Oxford.

Topic 2 was approached from the first year up to the third year, by mean of manipulative experiment in the field, aiming to measure the settlement behaviour of *Dendropoma* larvae.

This second aim of the research was based on a collaboration with the laboratory of Environmental Microbiology of the University of Palermo.

This research has been conducted with an interdisciplinary methodological approach, which allowed to fulfil the initial research questions, demonstrating the high value and necessity of integrated research for achieving useful outcomes.

CHAPTER 2.
Bioprotective role of a vermetid bioconstruction

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Bioprotective role of a vermetid bioconstruction

2.1 Introduction

Biogeomorphology and bioprotection

Substrate-coloniser interactions are not a one-way process and biological growth can modify surface properties by direct or not direct action.

Ecosystem engineers are organisms able to generate complex systems which modify landforms development and geomorphic processes (Butler, 1995).

The study of the interaction between the biological components of an environment, live or death, and its geomorphic features is a recent and increasing field of research which integrate ecology and geomorphology, and it is known under the name of "Biogeomorphology", (Viles, 1998a; Viles & Naylor, 2002).

This discipline is based on the premise that the distribution of species is often related to underlying geomorphological forms, while surface morphology may, in turn, be altered by organisms. Biogeomorphology aims to study the relevance of biogenic agents in geomorphological systems (Naylor, 2005).

The biogeomorphology explains that the organisms may influence the exogenic geomorphological processes in many ways and in different environments: through the removal of rock and sediment and by sculpting the rocks (bioerosion or bioweathering); through the construction of new space and secondary substrate (bioconstruction); through the direct or not direct control of the space they colonise (bioprotection or bio-stabilisation).

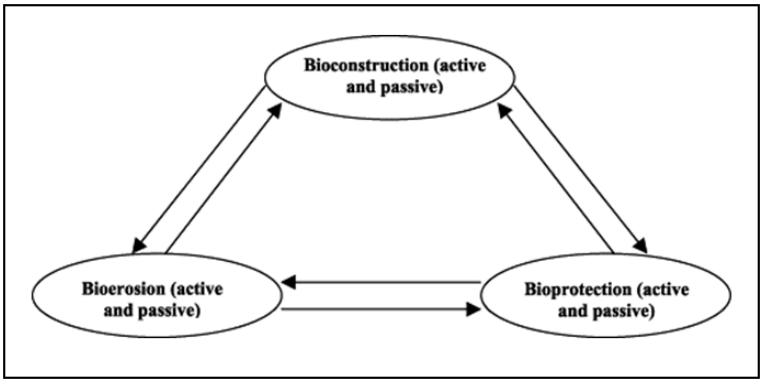

Figure 13, Diagram showing the whole set of biological impacts on geomorphological systems (from Naylor et al., 2002).

The interplay between these processes is complex and dynamic and the effect on the environment is the result of their mutual operation (as simultaneously or separately acting) and of the interaction with other geomorphological factors (i.e. substratum lithology, hardness).

Biogeomorphological studies have shown the role of lichens as weathering agents on limestone (Carter & Viles, 2004, 2003; Carter, 2002; Mottershead, 2000), salt marshes, coral reefs and mussel beds in buffering wave energy and storms against the coast (Ysebaert *et al.*, 2011; Lowe *et al.*, 2005; Moëller *et. al.*, 1999; King & Lester, 1995;), mangrove forests in stabilizing the shoreline (Badola & Hussain, 2005; Massel et al., 1999), microbial communities and marine invertebrates in eroding limestone surfaces (Naylor *et al.*, 2012; Viles *et al.*, 2000; Shneider & Torunski, 1983).

While bioerosion and bioconstruction have received a lot of attention in the biogeomorphological field, bioprotection is a more recent approach to the study of biologically mediated earth system processes, since its consequences are more cryptic and less visible.

Bioprotection is broadly defined as the direct or indirect contribution the organisms may give to the conservation of a colonised surface, by mediating the action of other earth surface processes (Naylor, 2005).

Bioprotective phenomena have been detected in the terrestrial and, more recently, in the marine environment and have been attributed to a wide range of organisms: terrestrial plants, lichens and turf layers, microbial film, canopy algae, barnacle and mussel coverages (Sternberg *et al.*, 2011; Coombes *et al.*, 2013; Coombes & Naylor, 2012; Coombes *et al.*, 2012; Gowell*et al.*, 2015; Carter & Viles, 2003, 2004). These organisms are able to generate persistent biological coverages and crusts – as live or alive layers- which interfere with substratum decay processes due to biological or physicochemical weathering.

The processes involved by bioprotection, are:

- Substratum stabilization:

A biological layer may entrap free-floating sediment and detritus and aid their stabilization in the substratum. For instance, rock debris may be stabilized by roots of plants which consolidate sloping surfaces. With this respect, ivy and turf have been demonstrated useful in the protection of soil.

- Control on substratum microclimate

A layer made by organisms may mediate exchanges of heat and humidity between the substratum beneath and the surrounding environment, buffering the extremes and mean values of sub-surface temperature and at the rock-air interface. The topographic complexity of a surface, at small scale, may also affect evaporative cooling which is responsible for theloss of water via evaporation and solute precipitation within the rock. These processes exert a control on the rock microclimate and crystallization of salt within the rock.

- Buffering against physical or biological erosion and mechanical stress

A biological coverage may protect the soil from physical disturbances, such as runoff, arresting or reducing the alteration and the loss of lithic material.
The biogenic layers also protect the substrates from the action of bioerosive organisms, such as grazers or borers (limpets, echinodermata, and endolithic cyanobacteria), reducing biodeterioration of the substrate.

Furthermore, the bioprotection hinders a suit of dynamics which produce the alteration of rock substrate and is known under the name of “weathering”. Weathering is described as a process which produces mechanical disintegration and chemical decomposition of rock materials(Hugget, 2007).
Consequences from weathering are: soil erosion; wetting and drying; salt weathering, bioturbation, rock disaggregation and decay.

In the marine environment, studies on bioprotection focus on the intertidal zone, because the intertidal is a unique environment of weathering, given the continuous tidal inundations, the abundance of moisture, salt crystallization, tidal and temperature excursions which rocky substrate are subjected to and desiccation during low tide times. Here, mechanical weathering

processes, such as wetting and drying, temperature variations, microclimatic fluctuations and salt crystallization, are responsible for rock weakness and breakdown, producing their decay and landscape modification (Sunamura, 1992; Coombes, 2014). Hence, the intertidal zone gives the opportunity to study several cases of bioprotection, although only a limited amount of scientific research has focussed on the contribution of bio-encrustations as bioprotective layers which preserve natural rocks from physical alteration (Naylor *et al.*, 2010; Naylor, 2005; Trenhaile, 2011).

Reefs are peculiar examples of bioencrustations and provide direct and indirect bioprotection as they represent biogenic shore platforms that might locally influence hydrodynamic, wave energy regimes, sediment availability and rock microclimatic conditions (Naylor & Viles, 2000; Naylor, 2001).

Moreover, lack of knowledge about these themes persists in the Mediterranean, where studies on bioprotection are not well represented and are a new field of research. Up to date, very few information are available about the relevance of biological colonization as natural buffers of weathering on the Mediterranean shores and vermetid encrustations have never been considered as a part of bioprotective dynamics of intertidal coasts.

Bioprotective potential of the vermetid bioconstruction

In the Mediterranean, vermetid bioconstructions are protected under international legislation (listed as threatened bioconstructions in the Mediterranean Red Data Book, UNEP-IUCN-GIS POSIDONIE, 1990 and in The European Red list of Habitats, 2016) due to their ecological relevance as key-habitat of the coastal zone. Although it is assumed that the substratum properties and characteristics are among the factors which, at small scale, can influence the development of this biological encrustations, it is not clear which influence the vermetid “layer” may exert on the underneath surface. Shedding light on these themes is a crucial point to increase knowledge about the ecosystem services provided by the vermetid reef, helping its conservation and showing new perspective for its management.

This research aims at establishing if the vermetid encrustation may act as a biological carbonatic layer which locally mediates weathering processes on the rock substrate itcolonizes. This would bea first proof of the bioprotective potential of these biogenic encrustations,which

shows a new and not previously considered ecological implication of this habitat for the conservation of Mediterranean intertidal ecosystems.

2.2 Aims of the research

The vermetid reef lies in the intertidal zone. This zone is mostly subjected to daily and seasonal variations inUV-radiation andtemperature,high evaporation rate, which also causessalt crystallization inside the rocks. In the central Mediterranean, during the summer, theintertidal temperature can easily reach more than 40° C in the daytime and less than 19° C during the night, with anaverage value of 26° C. The environment where the vermetid reef lies, therefore, is subjected to several weathering factors which produce the physical breakdown of the rocks and, therefore, coastal erosion.

This biological crustcan exceed some centimeters in thickness with avariable density of molluscs and crustose coralline algae coverage. Its tidal limit is the upper eu-littoral zone, reached in the inneredge of the reef, which is subjected to total emersion and submersion during the tide cycles.

Equally to other biological encrustations and biogenic habitats, vermetids are supposed to mediate the rock microclimatic conditions and to reduce, or alter, physical stressors onthe substrata, having implications on the bio-geomorphology of rocky shores and a potential role on the bio-protection of coastal systems.

In addition, the thickness of the biological encrustations and the density and the percentage of live vermetidsmight be relevant characteristics for their effect as bioprotective agents.

Liveand well-conserved biological layers, indeed, are supposed to be more efficient in mediating the physical processes on rocks (Coombes et al., 2012).

In this study, the factors considered as influenced by the vermetid encrustationare:

1) Rock internal temperature (i.e. subsurface temperature), given that the biogenic crust shelters the rock from direct irradiation and desiccation, which,as direct consequence,produce thermal stress within the rock substratum.
2) Subsurface crystallisationof salts, as result of a different heating and drying behaviour of rock under the vermetid encrustation and lower penetration of salt within the substratum, due to the thick vermetid coverage.

Why these two factors have been considered?

1) Thermal regimes can be responsible forrock breakdown as a consequence of cycles of expansion and contraction of minerals within the rocks in response to temperature fluctuations and peaks which induce stresses and decay within the rocks (e.g. Warke*et al.,*1998; Gómez-Heras*et al.,*2006), contributing to the progressive weakening of coastal shoreline.Expansion and contraction of coastal limestone, indeed, is advocatedasan important mechanism of decay and breakdown (Williams & Davies, 1987), especiallyin warm climates.
2) Salt crystallisation is a result of intense evaporation rate within rocks due to high temperature achievement and precipitation of salts. The formation of salt crystals in rock pores can exert pressures capable of gradually weakening and breaking off fragments of rock and causing the haloclastic breakdown of rock. This process iswell evident in the coastal zone where the abundance of mobile salts in solution and in the air, and the water evaporation rate during diurnal low tides and warmer weather are consistent.

The study of thebiological influences on these mechanisms of rock breakdownis stilllimited to a little range of encrusting organisms and represents a new field of research for the Mediterranean ecosystems. The potential role of the intertidalbioconstructions as bioprotective layers of rock substratamay give a great contribution to coastal protection and strength the necessity to preserve these habitats from loss of integrity.

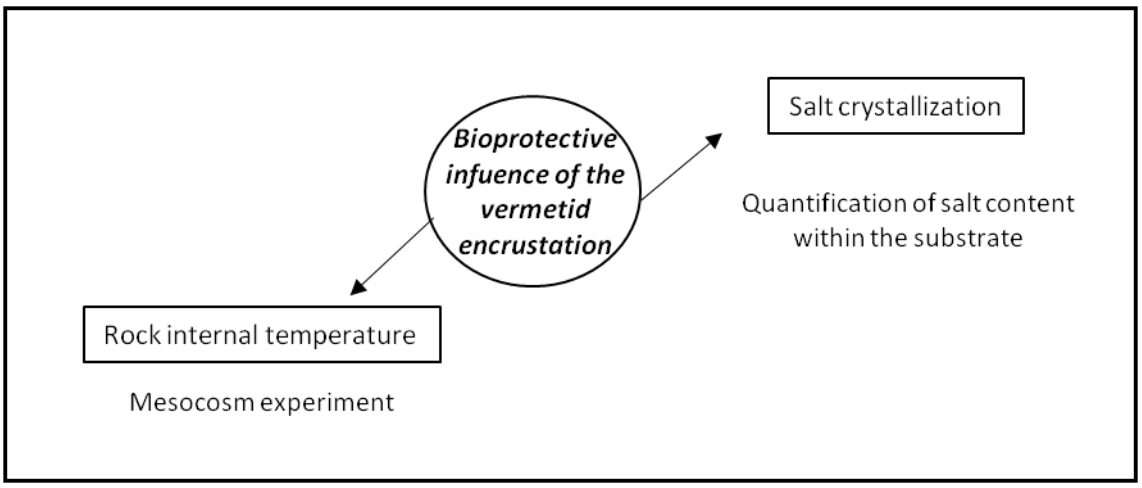

Figure 14, Implications of the vermetid encrustation on the bioprotection of the underneath substratum considered in this study.

Methods

Aiming to verify if the vermetid reef may influence rock temperature and salt crystallisation, laboratory experiments and observations werecarried out, measuring a number of variables and comparing them between biologically colonised and not colonised rock samples.

These experiment were carried out at the Oxford Rock Breakdown Laboratory (OxRBL), of the School of Geography and the Environment of the University of Oxford.

Sample preparation

The samples were rocky cores of 4 cm in diameter and height ranging between 3,5 and 5,5 cm(Figure 15), collected from a Sicilian vermetid reef (locality: Punta Raisi, N-W Sicily, described in chapter 3, pages 92 and 93) by a inox microcorer.

12 cores were collected in total: 5 of bare rock, 5 with a dead vermetid layer, 3 with a live vermetid layer.

Figure 15,Transversal view of a rocky core with the vermetid encrustation (A) and top view of colonised and bare rock cores (B).

Bare rock cores and dead vermetid cores were dried in an oven at 30°C for 2 days, while live vermetid cores were stored in containers with marine water and all the samples were shipped to the OxRBL of the University of Oxford.

Once arrived at the OxRBL the live vermetid cores were still alive and they were placed in tanks with artificial sea water and a water pump.

Rock internal temperature and salt crystallisation were analysed in different laboratory simulations and each simulation will be separately treated.

- How to monitor rock microclimate, salt crystallisation and their variation among sample typologies?

2.2 a)Internal rock temperaturemeasurement

The rock internal temperature of vermetid colonised and not colonised cores wasmonitored inmesocosm simulations, aiming to establish whether possible variations in rock thermal regimeare attributable to the presence of the vermetid crust.

Rock thermal behaviour and temperature fluctuations were monitored as indicators of thermic stress andpotential causes of rock weathering.

The experiment was carried out under simulated intertidal condition. The samples weresubjected to cycles ofsubmersion in tanks with artificial marine waterat chamber temperature (about 24°C) anddry phases in an environmental cabinet, which replicated diurnal low tide temperature and humidityvaluesfrom the *in situ* conditions. Considering that in the Mediterranean a tide cycle lasts around 12 hours, submersion and dry phases lasted respectively 6 hours each one.

Based on temperature and relative humidity field data collected between May and June and August and September 2015, the average highest climatic conditions (mentioned in the figure 16as “August”) and the average climaticconditions (mentioned in in the figure 16as “May”) during diurnal low tide phaseswere replicated in two different simulations (Figure 4):

1) August simulation: Temperature range: 28°-30°C and 72% of relative humidity (Rh%)
2) May simulation: Temperature range:20°-22°C and 76,5% of relative humidity

Diurnal low tide phases refers to low tides that occurred between 10 am and 16 pm, when sun radiation is highest.Furthermore, periods when maximum daytime insolation and low tides coincide (‘daytime dry phase’), are crucial for short-term fluctuations in rock internal microclimate and are of great interest for studies which focus on the weathering of coastal shores (Coombes, 2011a).

Rock internal temperature was recorded by using temperature probes allocated inside the samples.

3 samples of the 3 typologies (bare rocks cores, BR, dead vermetid cores, DV, and live vermetid cores, LV) have been used for this experiment. All the samples were coated with polyurethane varnish on all the faces except the upper side,to limit moisture movement through this one face, as occurs in natural conditions(Coombes, 2011a; Smith and McGreevy, 1982).On the bottom side of each sample, a hole (3mm of diameter) was drilled for the allocation of a temperature probe; bare rock samples were drilled up to 1 cm from the top surface and dead and live vermetid covered samples were drilled up to 1 cm from the rock-vermetid interface.

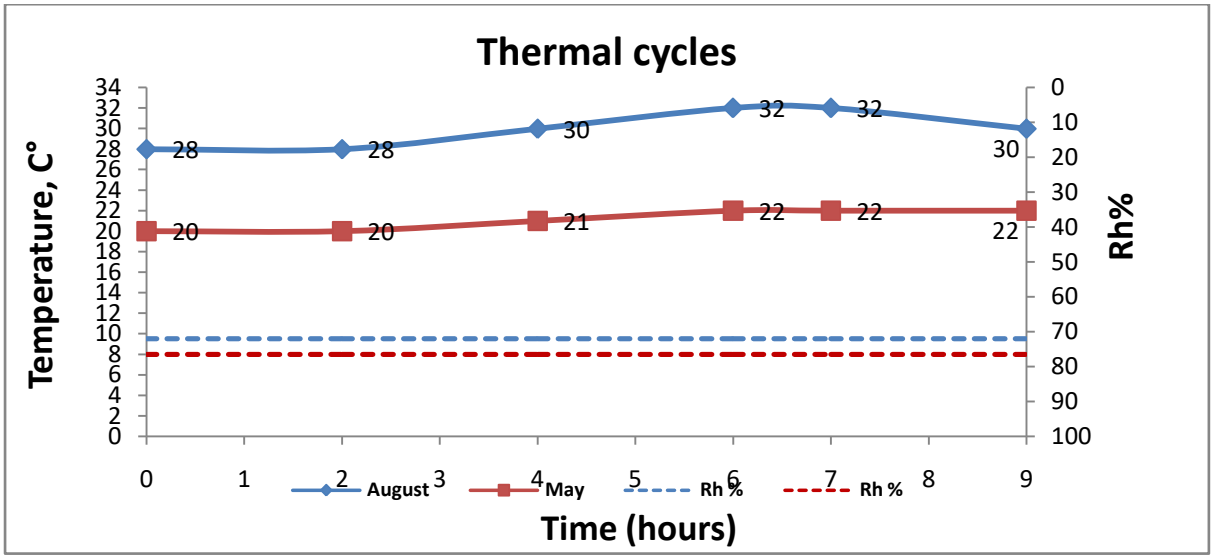

Figure 16, Temperature and humidity cycles during the low tide simulations. Rh%: Relative humidity.

In past studiesinternal rock temperature trends were found not consistently differ between 1 and 0,5 cm under the rocktop surface (Coombes *et al.*, 2012); therefore, in this study internal temperature wasmeasured only at one depth. For each sample, a continuous record of temperature over the dry phase and at intervals of 1 minute was measured. This allowed for comparison of rock microclimate among sample typologies.

Basing on the natural tide cycle, the samples were submerged in artificial sea water for 6 hours and then, they were subjected to a dry phase for other 6 hours, plus 3 additional hours at progressively lower temperature, simulating the beginning of the high tide.Once the programmed temperature and humidity settings became established, the samples were placed in the environmental cabinet, within a tray surrounded by polystyrene beads to concentrating thermal exchange through the upper face (the weathered face) and minimising edge effect on

the sides.A lamp was used to simulate rock direct insolation during the hours of maximum sun irradiation and it was switched off each 3 hours for 15 minutes to simulate possible cloudy passage.An additional temperature logger (iButton®) was placed in the environmental cabinet to check air temperature and humidity during the experiments. To avoid direct irradiation from the lamp, the iButton was covered with an aluminium foil. After each cycle, temperature data were downloaded from the loggers and compared.

Firstly, two simulations were performed:

1) Bare Rock *vs* Dead Vermetid (BR *vs* DV) under the August thermal cycle
2) Bare Rock *vs* Dead Vermetid under the May thermal cycle

Basing on the results of these two cycles and given the difficulty in maintaining live vermetid cores in good status for repeated cycles, just one simulation was run with the live vermetid cores:

3) Bare Rock *vs* Live Vermetid (BR vs LV) under the August thermal cycle

Each simulation has been repeated 5 times.

Figure 17, Core samples during a low tide simulation.

Expected results:

- Differences in the range of temperature experienced by different sample typologies
- Differences in the frequency and intensity of microclimatic fluctuations among biologically colonised and not colonised samples. The standard deviation of temperature values within ranges of 4 minutes was chosen as an indicator of temperature fluctuations.

2.2 b) Salt crystallisationmeasurement

In the intertidal zone, salts are among the primary agents in the breakdown of rocks which modify and damage stones producing cavities, surface scaling, deep cracking, surface powdering and micro-cracking, especially in porous materials.As well as mediating heat and moisture fluxes, an organic layer may alter the exchange of water, solutes and matter between the beneath substrate and the atmosphere.

This experiment aimed at quantifying the rock salt concentration (mg/L) through the ion chromatography analysis and at comparing salt content between samples with and without the vermetid encrustation.

Two bare rock cores and two dead vermetid cores were used for the experiment.Given that salt content within the rock is presumed not to differ under a live or dead biological layer, no cores with the live vermetid encrustation were used.
From the rock top surface of each sample, 2 holes were drilled (5 mm of diameter). On each hole 2 depth ranges were distinguished: 0–5mm, 5–10mm, and the produced dusts were separated between these 2 ranges. To avoid salt contamination between depths, each interval was drilled with a clean drill bit. On vermetid samples, the biological encrustation was drilled as well, but it was considered as a separatelayer, since it is not of interest to evaluate salt content within the rock and may be an additional source of salts.
Once collected, the powder samples were prepared for salt content analysis with the ion chromatography technique (using a Dionex IC DX500).

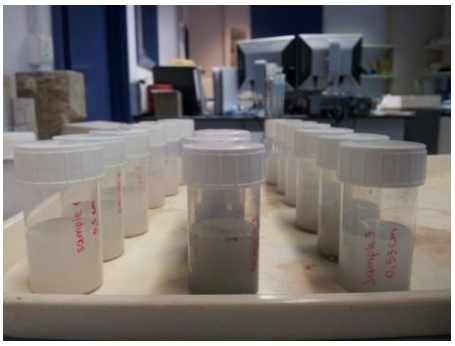

Figura 18, Rock powders preparation for Ion chromatography analysis.

This method is able to separate different chemical substances mixed in the same liquid and mobile phase, as a function of their affinity for a solid and stationary phase. The analysed samples consist of solutions of distillate water and rock powder, collected from drilling the rock blocks and prepared after different cycles of shaker and filtration to purify the samples. This analysis establishes not only the chemical elements dissolved in the samples but also their relative amount, expressed in mg/L.

EXPECTED RESULTS:

- Salt concentrations are expected to decrease with the depth into the samples according to the salt diffusion gradient
- The salt concentration is supposed to be lower under the biological encrustation
- Differences in salt depth profile between treatments are expected to occur

2.3 Results

a) Internal rock temperature measurement

The figures19, 20 and 21show the temperatures recorded 1 cm below the rock surface and under the rock-vermetid interface, alongside air temperature and relative humidity (Rh%).
The data reported in the graphs are the averagerock-internal temperaturesfor intervals of 4 minutes, recorded during the 5 low tide cycles replicated for each simulations (Br *vs* DV under August simulation; Br *vs* DV under May simulation; Br *vs* LV under August simulation). Data are averaged for samples typology and for each typology n=3.

On average, for all the three different simulations, the internal temperature is always lower within rock cores with a vermetid coverage, compared to bare rock cores.
Regard the August simulations (figure 19 and 21), this pattern is most pronounced after 3 hours from the beginning of the simulations (the warmest hours) until the end of the cycle, while in the May simulation differences among treatments are quite constant.
The standard deviation of the temperature values within intervals of 4 minutes was chosen as an indicator of short-termtemperature fluctuations and the results are reported in the figures 22, 23 and 24 Temperature oscillations are mostly pronounced within bare rock samples in both the August simulations (graphs 22 and 24). Under May thermic cycle the observed pattern is different and temperature fluctuations seem to be higher within vermetid covered samples.

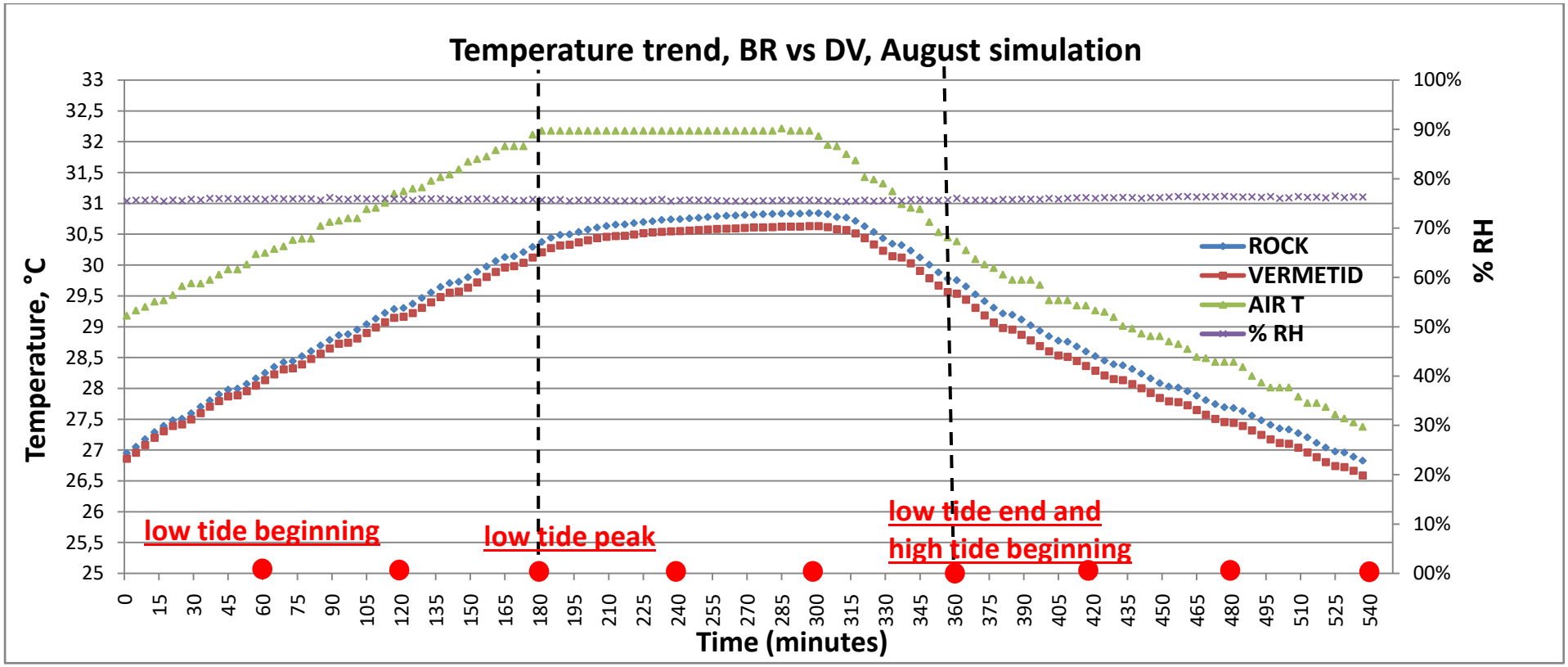

Figure 19, Comparison of the internal microclimate between bare rock cores (BR) and dead vermetid cores (DV) under low tide temperatures in August.

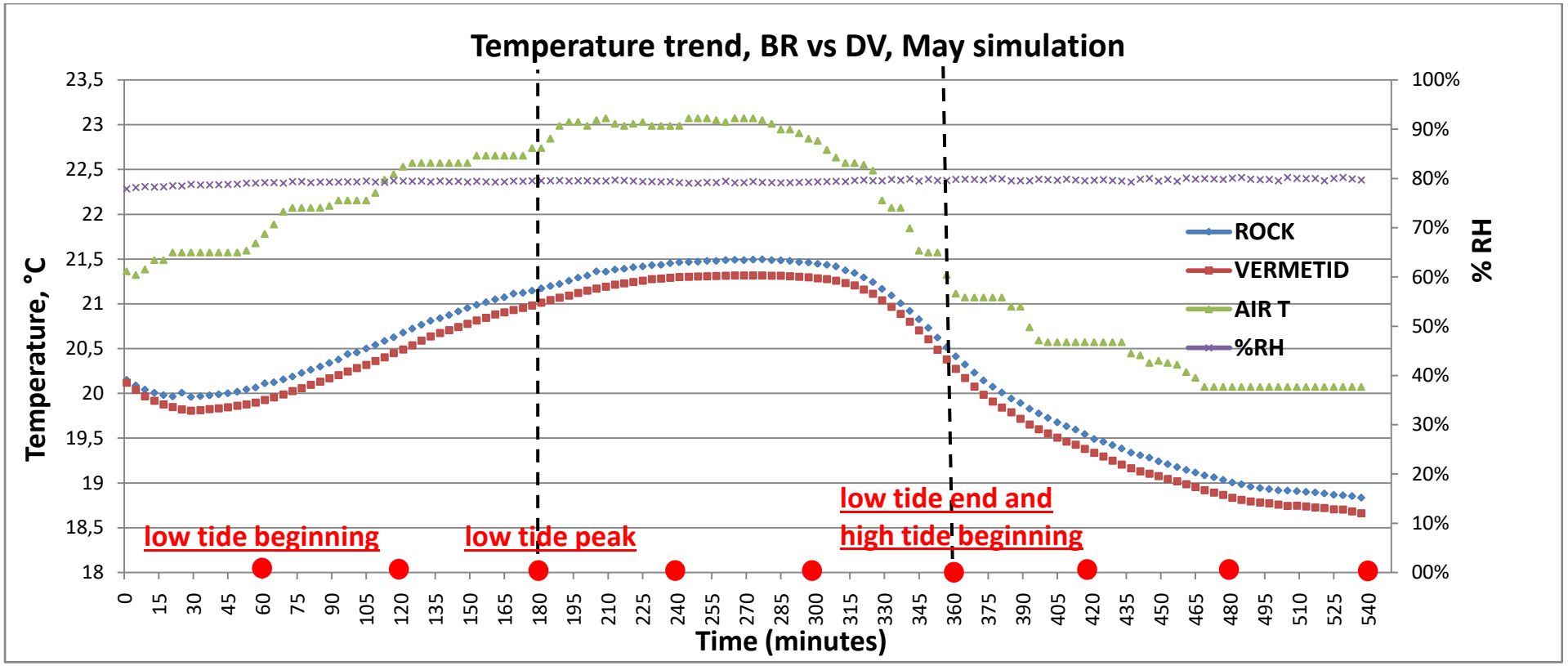

Figure 20, Comparison of the internal microclimate between bare rock cores (BR) and dead vermetid cores (DV) under low tide temperatures in May.

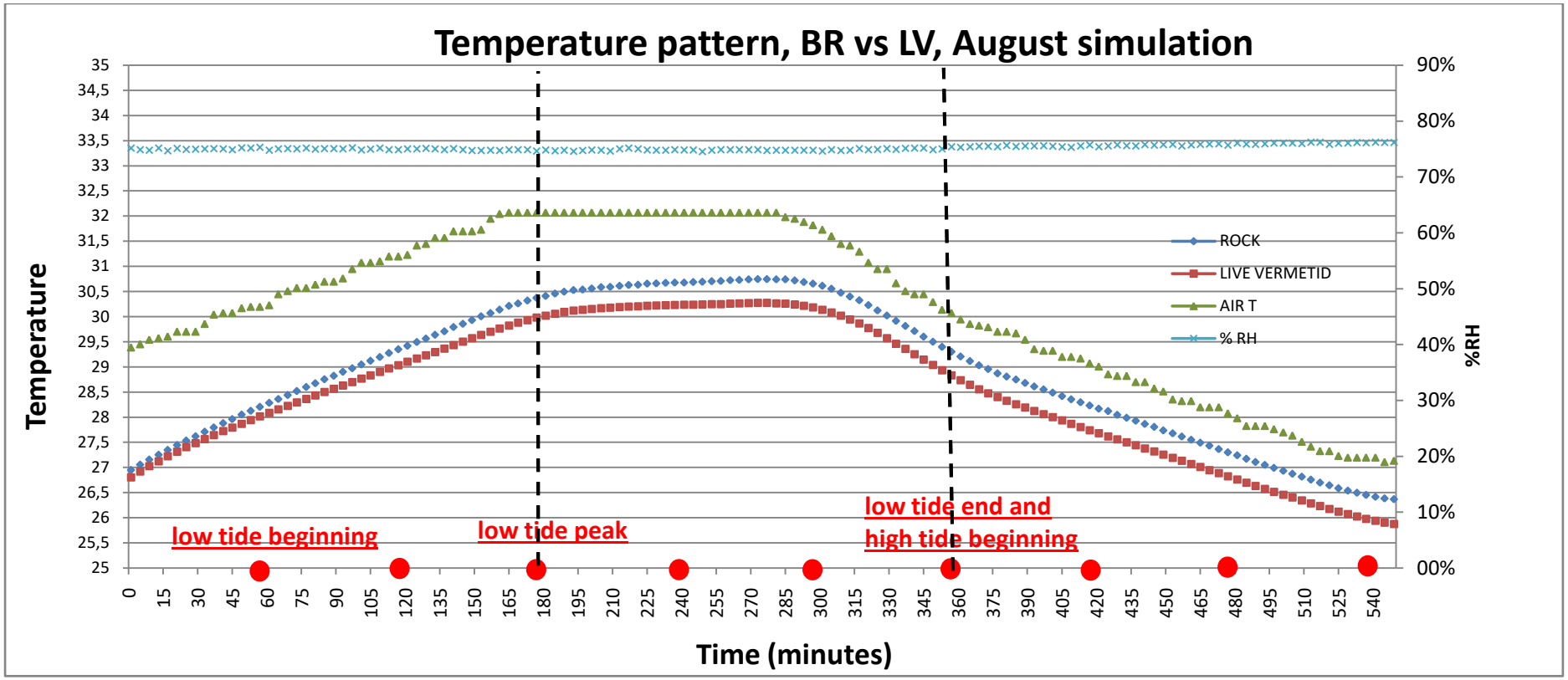

Figure 21, Comparison of the internal microclimate between bare rock cores (BR) and live vermetid cores (LV) under low tide temperatures in August.

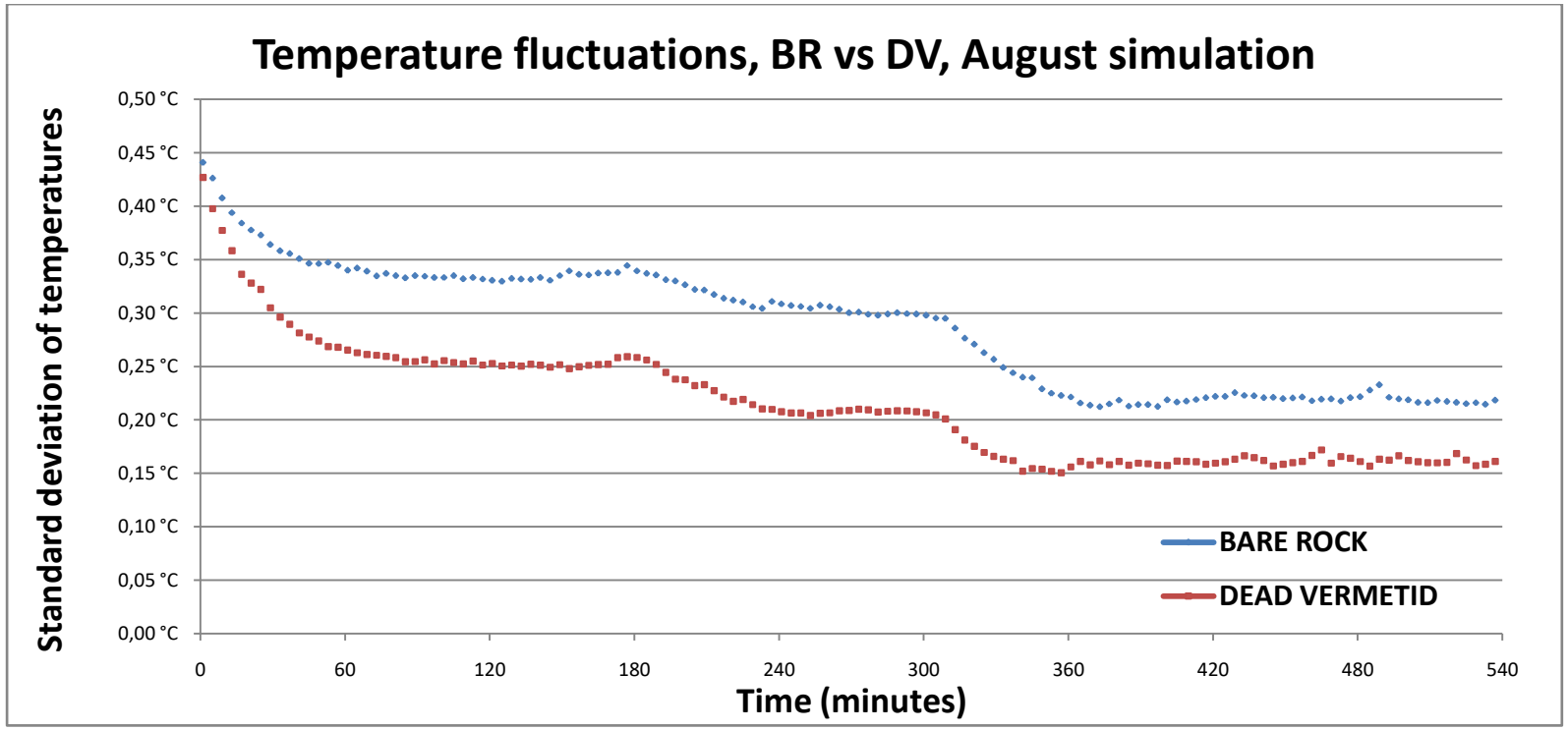

Figure 22, Comparison of the average internal thermic fluctuations between bare rock cores (BR) and dead vermetid cores (DV) over the August low tide simulation.

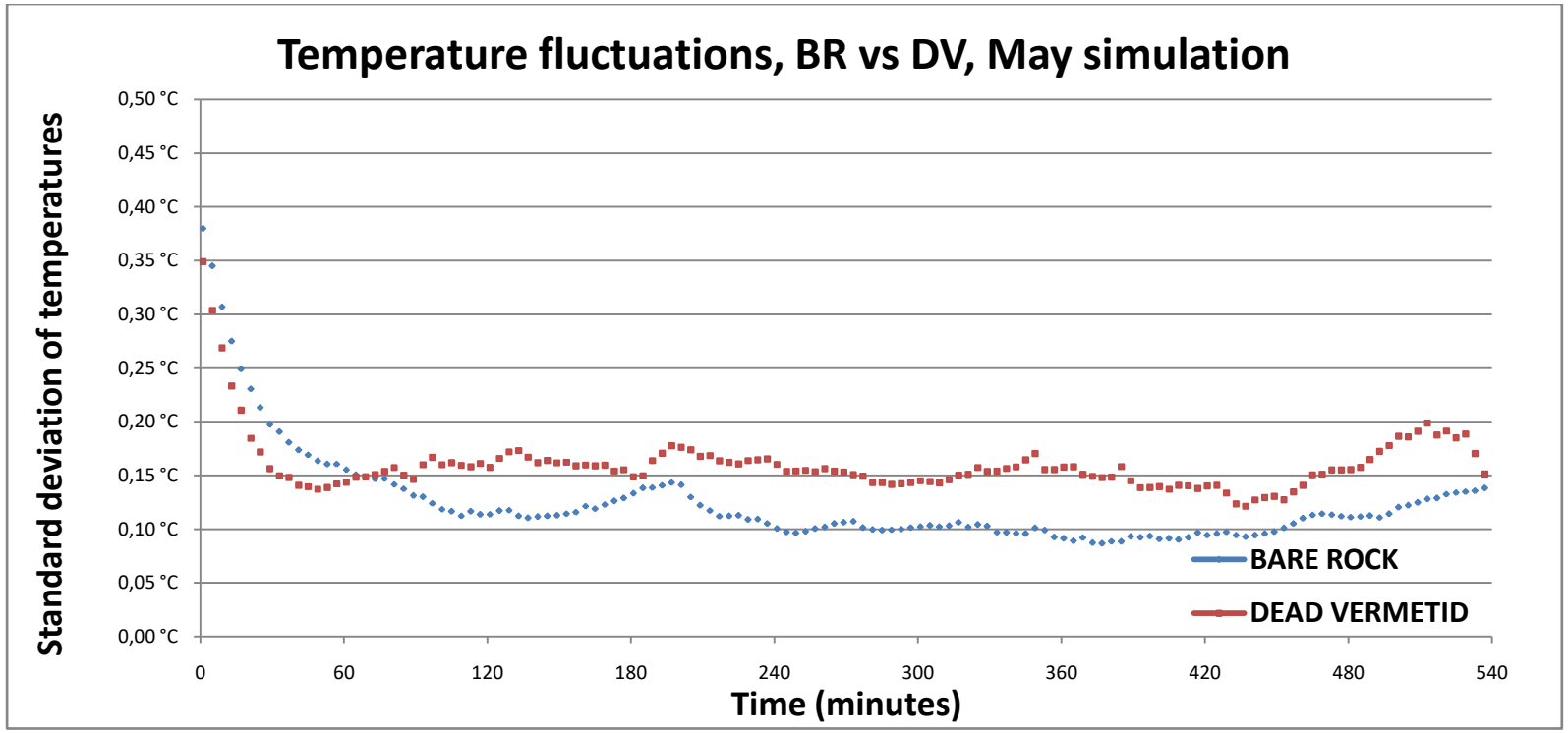

Figure 23, Comparison of the average internal thermic fluctuations between bare rock cores (BR) and dead vermetid cores (DV) over the May low tide simulation.

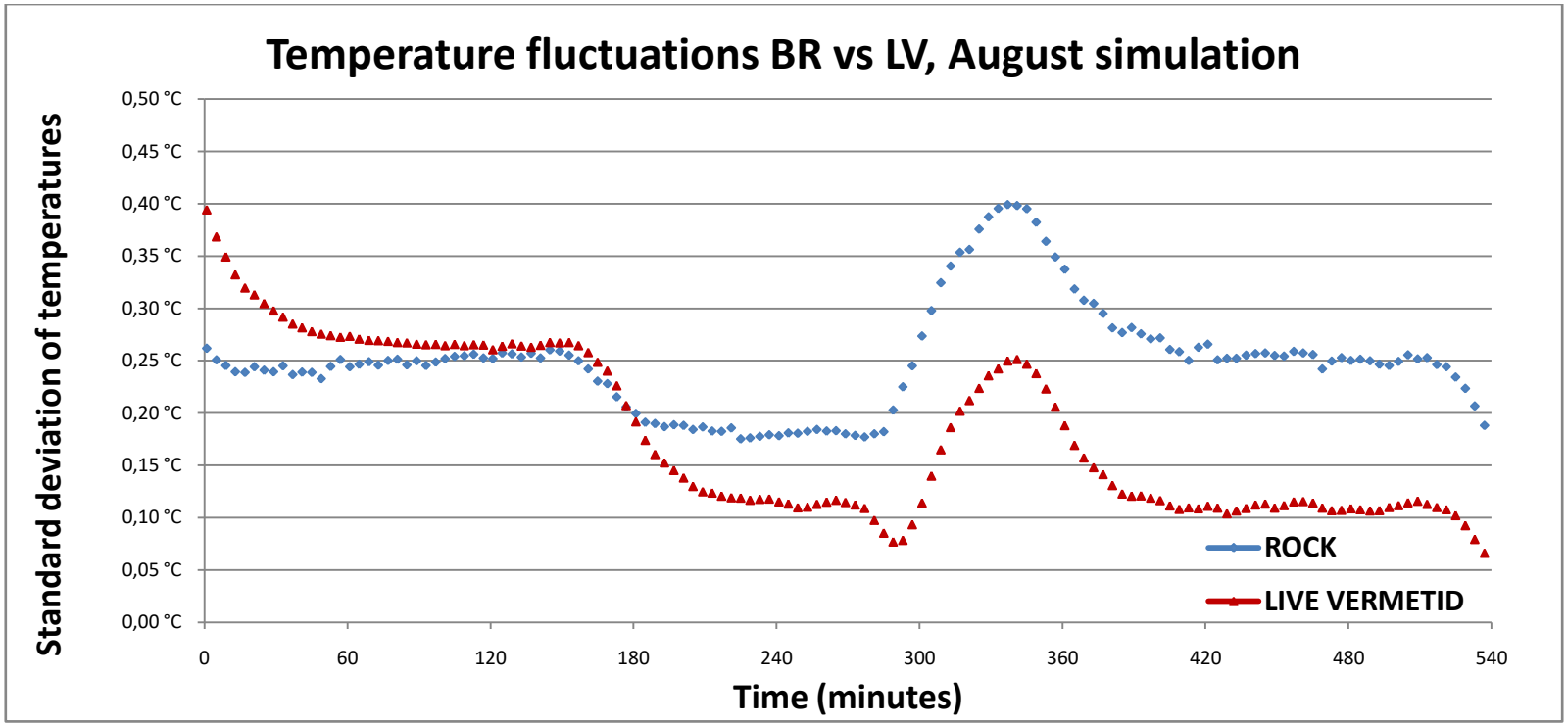

Figure 24, Comparison of the average internal thermic fluctuations between bare rock cores (BR) and live vermetid cores (LV) over the August low tide simulation.

Maximum, minimum and average temperature (±SD) for core typology and average differences among treatments during each simulation are reported in the table below.

	August				May			
	MAX T	**MIN T**	**AV.T (±SD)**	**Differences**	**MAX T**	**MIN T**	**AV.T (±SD)**	**Differences**
BR	30,8	26,8	29,2 (±1,28)	0,20 (±0,04)	21,5	18,8	20,36 (±0,88)	0,16 (±0,023)
DV	30,6	26,5	28,9 (±1,28)		21,3	18,65	20,20 (±0,88)	
BR	30,7	26,25	29,9(±1,35)	0,40 (±0,11)				
LV	30,3	25,8	28,6(±1,33)					

Figure 25, Table n. 1 with the average temperature values during each simulation.

The mean differences in subsurface temperatures between sample typologies are stronger in the August thermal cycles, especially in the comparison between bare rocks (BR) and live vermetid encrusted cores (LV): during the whole cycle, the temperature of bare rock cores is on average 0,40 °C (±0,11) higher.

b) Salt crystallization measurement

Chloride and sulphate werefound as the most representative ionswithin the samples, both for bare rock and for vermetid colonized cores and their concentration (mg/l) is showed in the following graphs. The graphs report the average concentrations for all the replicates of each treatment. For each treatment n=4.

For both the treatments chloride concentration is higher than sulphate (figure 26 and figure 28). The vermetid layer is confirmed to be an extra source of salts-particularly chloride-probably due to salt crystallisation on the shells during desiccation periods in low tide phases (see the figure 26 and 28). However, removing salt content retained from the vermetid layer, the overall salt content is lower within rock cores covered by vermetid (figure 27 and 29). This trend is confirmed both for chlorides and for sulphates.

The overall trend confirms a higher concentration of salts nearer the surfaces that were exposed to a source of salt (seawater).On average, subsurface chloride and sulphate mean contentsare lower within vermetid colonised cores (respectively 863,7 (± 109,8) *vs*927,2 (± 52,6) and 148,4 (± 22,1)*vs* 177,3 (± 10,4), see the figures 27 and 29) and for both the treatments salt ion concentration progressively decrease with the depth (figure32 and 33).

Ion concentrations varies with depth for both vermetid colonized and control cores, indicating progressively less penetration with the depth.

Total chloride amount from 0 to 0.5 cm of depth, however, wasstill higher for vermetid colonized samples (Figure 30), while from 0,5 to 1 cm under the rock surface it was lower within vermetid colonized cores.

Sulphate amount differed between treatments at each depth (graph 31) and differences are more pronounced over the depth.

However, variability between samples was generally high.

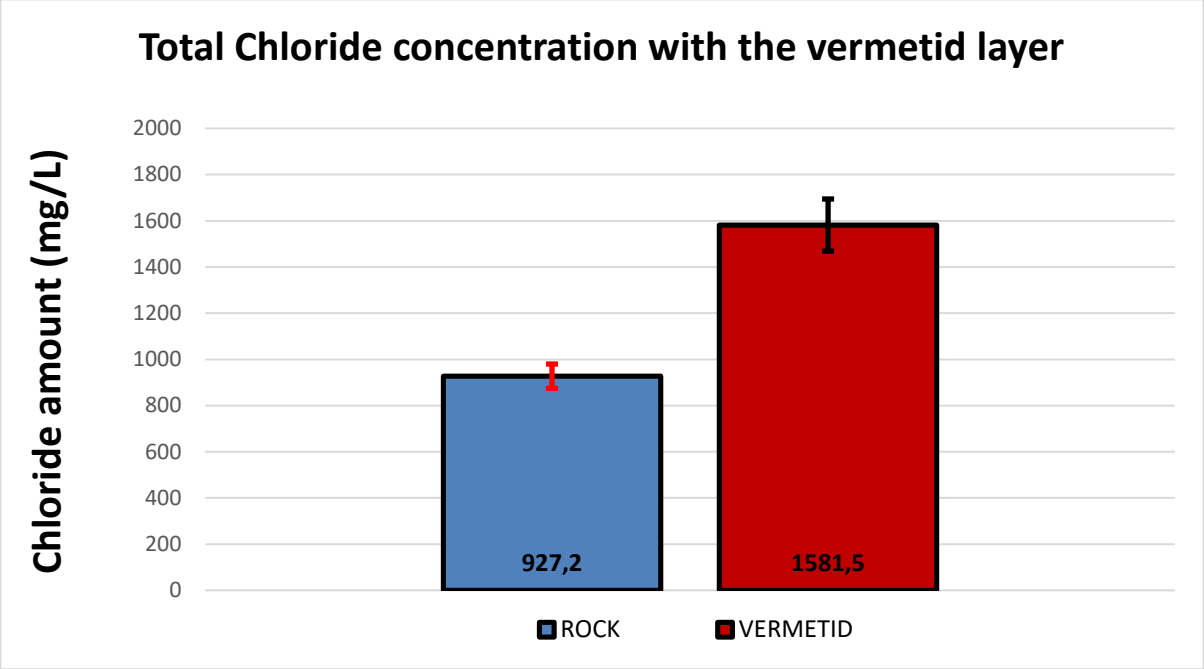

Figure 26, Cumulative chloride amount from the core top surface (±SE).

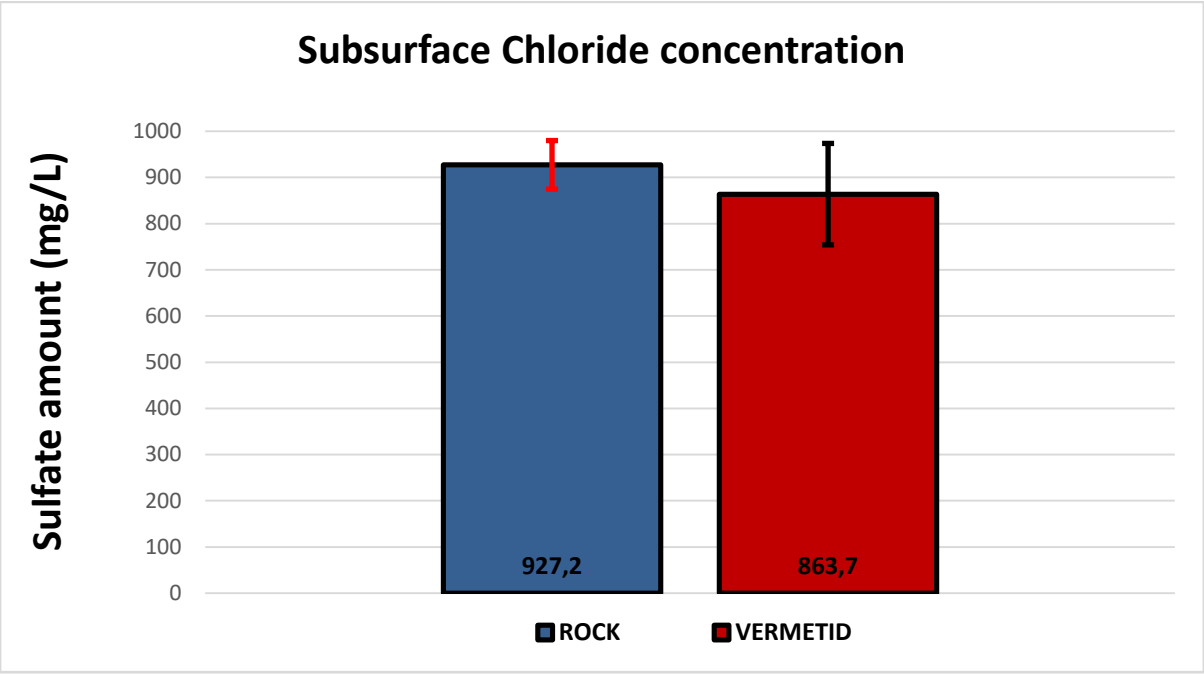

Figure 27, Cumulative subsurface chloride amount from the top surface of rock cores and under the vermetid layer (±SE).

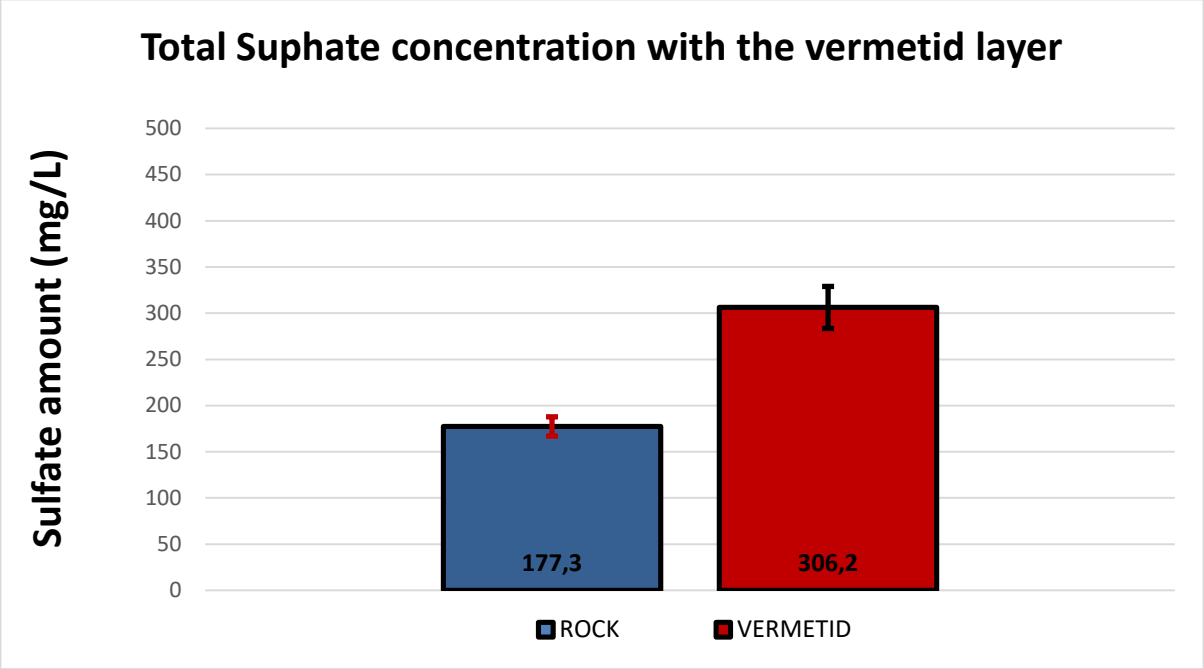

Figure 28, Cumulative sulphate amount from the core top surface (±SE).

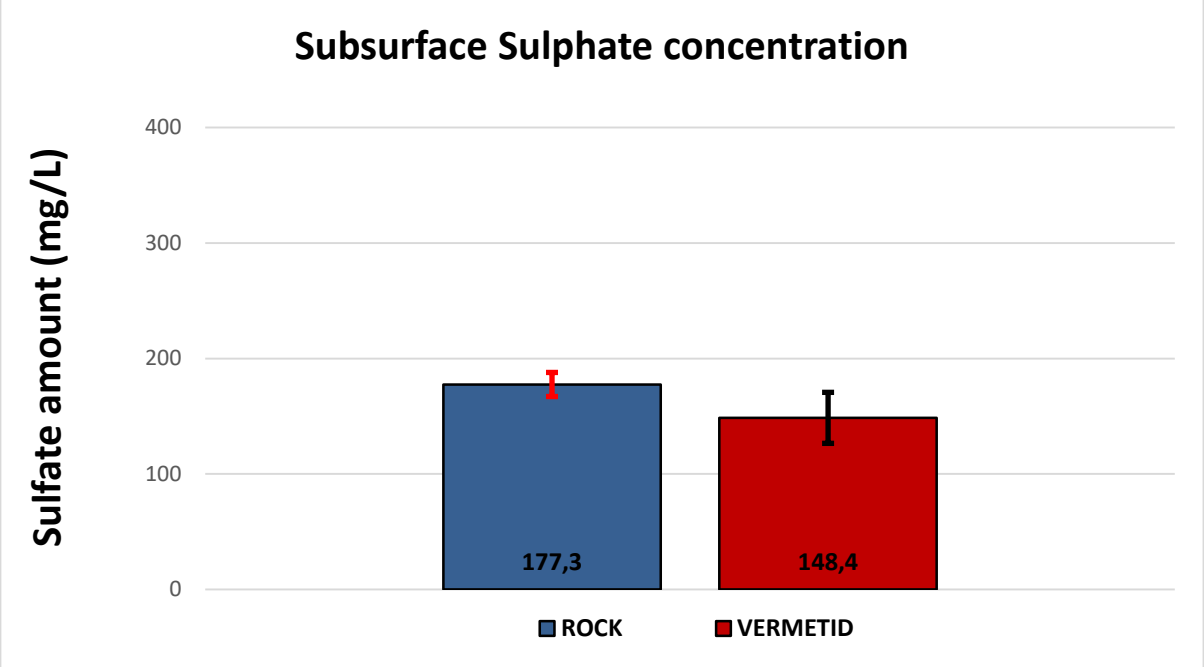

Figure 29, Cumulative subsurface sulphate amount from the top surface of rock cores and under the vermetid layer (±SE).

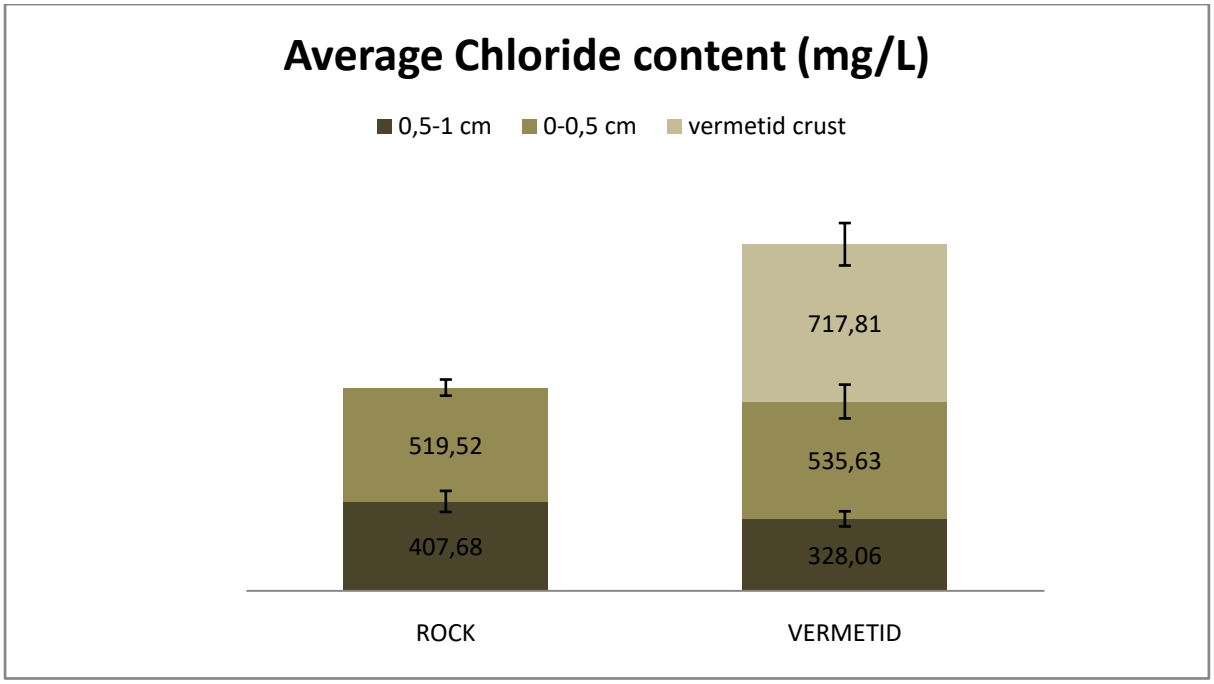

Figure 30, Comparison of the average chloride concentration (mg/L) at the considered ranges of depth (±SE).

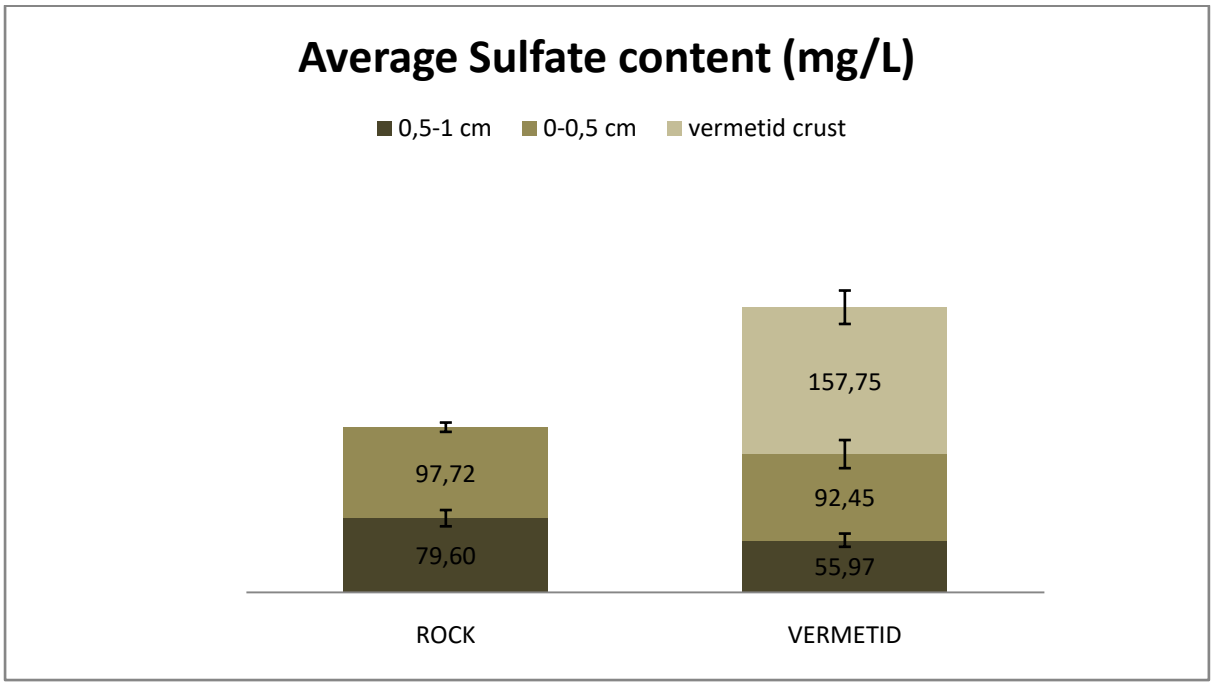

Figure 31, Comparison of the average sulphate concentration (mg/L) at the considered ranges of depth (±SE).

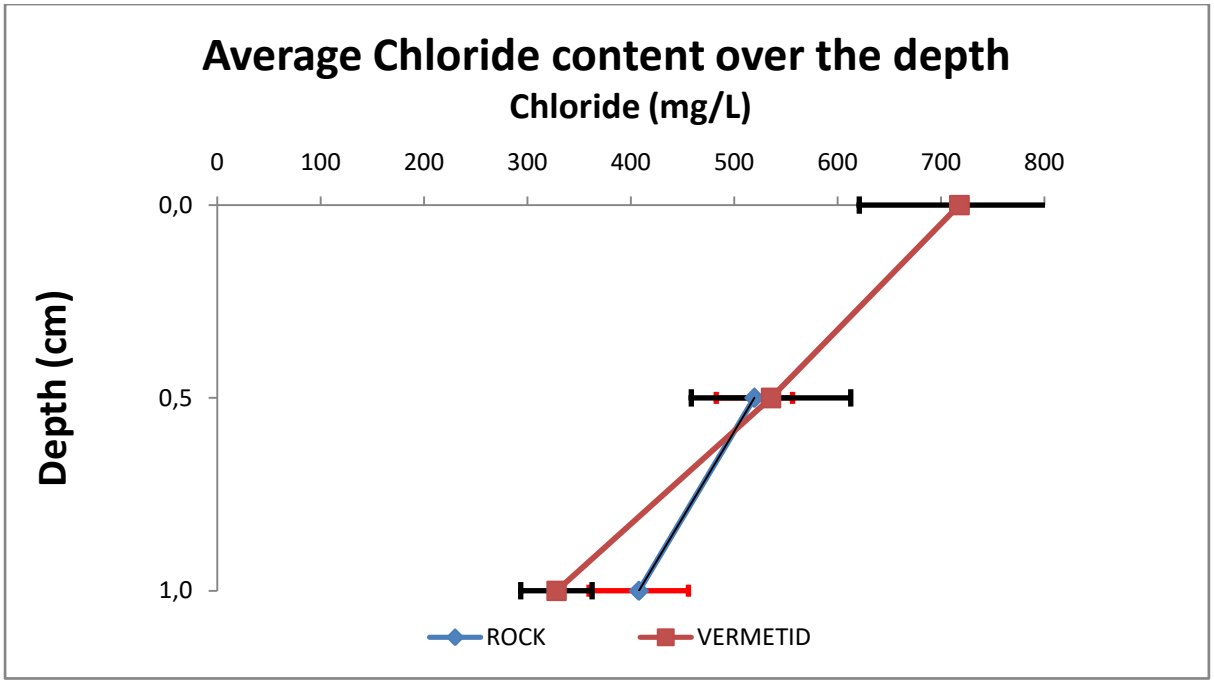

Figure 32, Chloride gradient over the depth (±SE).

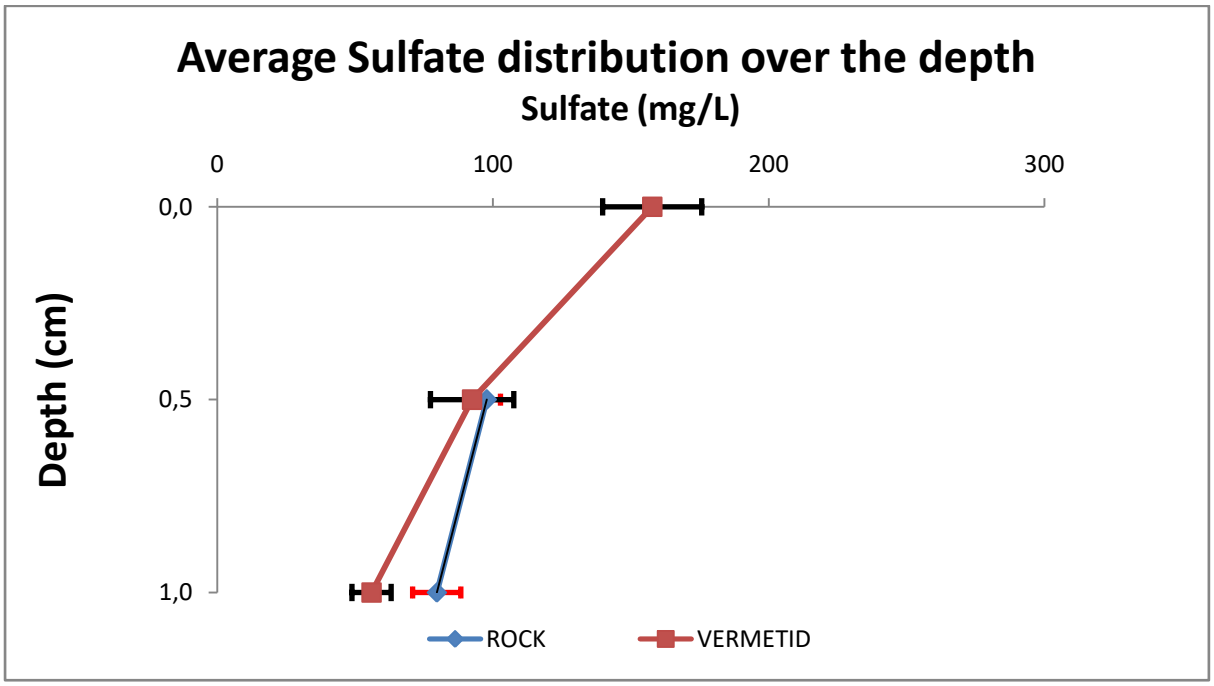

Figure 33, Sulphate gradient over the depth (±SE).

2.4 Discussions

Regard the internal rock temperature measurement, for all the three different simulations the subsurface rock temperature was always lower within cores with a vermetid coverage compared to bare rocks.This pattern wasmore pronounced for the August simulations(Figure 19 and 21), which also showed reducedshort-term temperature oscillations and lower peaks of temperature under the vermetid layer (figure 22 and 24).

Hence, at the detected depth and during the simulated low tide conditions, the vermetid encrustation exerted a control on internal rock microclimate. Under highest atmospheric temperature, this biogenic carbonatic layer was able to reduce temperature fluctuations within the substratum, as occurred during the August simulations.

Moreover, in the case of a "live" vermetid encrustationtheseobserved dynamics were enhanced, probably due tothe "soft"and living biological material within the mollusc shells which has a different thermic capacity compared to the mineral material of the empty shells.

These measures were intended as indicators of internal mechanical stresses, reflecting thermal extremes and the magnitude and rate of thermal cycling within the rock.

In natural coastal systems these processes are presumed to act similarly and to be enhanced by the continuity of the biological encrustation which should reduce edge effects and ensure the higher stability of subsurface microclimatic conditions.

The observed patterns are comparable to those Coombes *et al.* (2012) have shown for limestone colonised by barnacles (*Chtamalus* spp.) in simulated tidal cycles. The barnacle encrustation has been found to have an effect on the maintenance of temperature stability within rock. The average differences of temperature between samples covered by barnacle and bare sample were on average 0,66°C, almost equivalent to the 0,40°C (±0,11) measured between the live *Dendropoma* encrustation and the bare rock.

These effects, moreover, might be enhanced by the thickness of the vermetid encrustation. The *Dendropoma* layer was thicker no more than 1cm on the cores used for the experiment, but in the field the bioconstruction can reach more tens of cm on rock surfaces, isolating much more the substratum from the surrounding environment and sheltering it from direct sun irradiation and other disturbances. Basing on this experiment it is not possible to hypothesize an effect of the thickness of the biological layer on rock internal temperature

patterns. However, this could be additionally investigated, as well as the influence of the vermetid encrustation on other processes of rock weathering (such as the biological influences on the erosion of the substratum, which is a consistent cause of weathering on intertidal environment, especially on limestone rocks).

Regard to the salt crystallization measurement, the overall trend confirmed a higher concentrations of salts nearer the surfaces that was exposed to a source of salt (seawater)(Figure 30 and 31)anda gradient of absorption, indicating progressively less salt penetration with depth (figures 32 and 33).

The additional amount of salt provided by the vermetid layer was not maintained through the depth and should not have influences on the subsurface weathering processes. This is supported bythe differences in salt depth profiles between the treatments and at 1 cm of depth lowest salt concentrations were found within vermetid colonised cores (figures 30 and 31).

The high error standard associated with the average measurements for both the treatments, reflected the variability between replicates, probably due to the mineralogical dis-homogeneity of limestone rock. However, ion chromatography analysis found a general trend for lower ion concentrations within rocks where the vermetid and cca encrustation is present.

Therefore, the vermetid encrustation, alongside to influence rock internal microclimate, may reduce the penetration of deteriorative salt ions within rock substrata, with additional possible consequences for rock decay. The reduction of salt penetration and crystallisation may result not only from the presence of the organic layer but may be additionally due to the control onthe evaporation rate and humidity conditionswithin rocks colonised by vermetid. Thus, the bioconstruction contribute to the bio-protection of the rock surface by multiple processes and interaction with the colonised substratum.

Other investigations have shown a reduction of chloride ion penetration into intertidal natural and artificial substrata, once colonised by marine epibiota (Kawabata *et al.,* 2012; Maruya *et al.,* 2003; Coombes *et al.,*2016). Thus, sessile invertebrate with hard and persistent skeletons and high density on the substratum, such as the case of a *Dendropoma* encrustation, may originate an organic crust which buffers the penetration of salts into the colonised rock from the surrounding environment.

Moreover, this process of biological protection of the substratum from salt crystals formation may help rock integrity, especially on limestone intertidal coasts, which are highly prone to salt weathering, due to continuous cycles of inundation and desiccation.

It could be interesting to investigate salt extinction within the rocks, by considering further depth ranges and further investigations would be of interest to detect other faces of the vermetid-rock systems, such as microscopic analysis of the vermetid-substratum interface, aiming to examine if rock physical characteristics (presence of salt crystals, rock porosity, depth of the weathered zone) may differ between colonised and bare rock.

CHAPTER 3.
Early development of *Dendropoma cristatum*

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Early development of *Dendropoma cristatum*

3.1 Introduction

Settlement and recruitment dynamics

The processes which lead to the colonization of substrata by larval invertebrates are among the most important in determining the ecology of marine communities (Pawlik, 1992). Settlement and recruitment are recognized as crucial processes of these dynamics.

Settlement is the ability of larvae to reach a site suitable for their survival and recruit in adults (Harrington *et al.*, 2004). Itis a very crucial phase of the life cycle of certain aquatic organisms, with strong implications on population and community structures. For the majority of the marine organisms, the choice of the settlement site is not a random process, but several dynamics, directly or not, interact and bring propagules toward certain positions rather than others. Various factors may drive this process, operating at a variety of spatial and temporal scales (Connell, 1985; Sponaugle *et al.*, 2002). Overall, larval supply from adults, the transport of propagules by current or other means, inter- and intra-specific interactions and local factors associated to the substratum are the main processes that drive the settlement dynamics.The prevalence of one factor instead of another depends on larval ecology, on the propagules dispersion ability and possibility, on the establishment of species–specific relationships and on local conditions.

Overall, passive deposition of larvae and active substratum selection act on different scales: larvae passively accumulate and are deposited under the influence of hydrodynamical processes operating at large spatial scales (tens of meter or km), while active substratum selection occurs only at much smaller scales (mm/m) (Butman, 1987).

In some cases, the capacity of larvae to detect the surrounding environment and to reject sites as potential hazards for their survival and metamorphosis is of primary importance.It has become increasingly recognized that larvae within the size range of µm or mm, have well-developedsensorysystems that allow them to behaviorally respond to environmental conditions in ways that may impact their dispersal (Forward & Tankersley, 2001; Queiroga & Blanton, 2005). After that larvae perceive environmental cues indicating “right spots”

viaexternal chemoreceptors, transduce the cues into internal processes with neuronal or hormonal processes, and proceed through behavioral and developmental changes that bring to settlement and culminate into thetransformation into juveniles (Hadfield, 2011).

Substratum selection from larvae and implications on recruitment performance

For marine sessile organisms, choosing a site favorable for their survival and reproduction is extremely important (Hadfield & Paul, 2001), given that the attachment and the calcification to a surface are irreversible processes and there is no chance of relocation after metamorphosis onto a substrate (Tamburri *et al.*, 2008). Thus, active substratum selection by larvae can help minimize the chances of choosing unsuitable habitats for their development.

In the size-range of millimeters and centimeters typical of many intertidal benthic invertebrates, larval settlement is highly affected by substratum characteristics and chemical cues. What constitutes a site suitable for settlement involves the features of the surface, such as its structural complexity and composition, and the presence of biological stimuli. Thus, the larval choice results from a combination of environmental and biological factors (Wethey, 1984).

For gregarious organisms (such as polychaete, oysters, barnacles) is often hypothesized, and well documented, a biologically induced settlement (Hadfield &Paul, 2001). The nature of the biological signal that promotes settlement may be waterborne or strictly associated to the substratum. In this last case, extremely important is the contact between the larvae and the substratum, by mean of sensory organs (i.e. antennulae for cyprid larvae, apical sensory organ, ASO, for veligers, Hadfield *et al.*, 2000). Thus, the larvae of gregarious species respond to site-specific cues associated with conspecific occurrence, secondary substrata presence, such as encrustation of coralline algae and microbial film development (Crisp, 1974; Pawlik, 1992). These species-specific interactions became relevant for habitat-forming species which generate biogenic secondary substrata.

Larval settlement in responses to chemical signals associated to conspecific adults has been observed for dense and persistent aggregations, such as oyster reefs (Bayne, 1969; Tamburri *et al.*, 2007, 1996). Settlement and metamorphosis in many scleractinian coralsis known to

be positively related to living crustose coralline algae (Morse *et al.*, 1988, 1996, Heyward & Negri 1999), probably because they offer a potentially favorable attachment site which can facilitate the survival of planula larvae to reproductive maturity. The same result has been observed for a Brazilian reef builder Vermetidae (Spotorno *et al.*, 2015).

The occurrence of a microbial film mayalso influence the settlement performance of other reef-builders species, such as polychaete and mussels and of encrusting barnacles (Satuito *et al.*, 1995; Shimeta *et al.*, 2012; Thompson *et. al.*, 1998).

Thus, settlement performance may affect larval survival and exert a control on recruitment mechanism, which is the rate at which juveniles of a species join the adult population (Pineda, 2000). For benthic sessile organisms which develop onto hard substrata, the recruitment success depends on the survivorship rate of settlers up to they reach the reproductive ability. Recruitment is also strongly influenced by pre-settlement factors, if the reproductive out-put of a species is low and post-settlement factors - such as intra- and inter-specific competition or predation - when the reproductive out-put is high (McQuaid & Phillips, 2006).

Pre- and post-settlement factors, therefore, indirectly exert an important influence on the adult population, that, in turn, is the primary element that controls larval pool.

Hence, in order to fully understand the relative importance of one factor rather than another and to predict spatial and temporal dynamics of a species, it is important to integrate both the studies on the ecology of adults and larvae.

Much research in ecology focuses on settlement and recruitment dynamics, in order to study their consequences on population andcommunitystructure (Rognstad & Hilbish, 2014; McQuaid & Phyllips, 2006; McQuaid & Lindsay, 2000). In a wider view,the understanding of these mechanisms is crucial to provide robust predictions about ecosystem functioning and solving issues as the management and the conservation of marine habitats, and to predict how they may response to environmental change and stress.

Despite the growing consensus that larval behavior plays an important role in driving the species dispersal pattern, having important consequences on settlement and recruitment mechanisms, the behavioural traits of most species are poorly characterized or have been examined under laboratory settings that may not accurately reflect conditions experienced by larvae in the field (Pineda *et al.*, 2009). Few studies have been conducted to describe *in*

situ larval behavior during settlement and recruitment choices, due to the difficulty to observe larvae and juveniles in the field and to evaluate their behavior(Spotorno*et al.*, 2015; Thompson *et al.*, 1998; Bell *et al.*, 2015). In turn, this experimental approach rarely considers the multitude of environmental conditions which larvae experience in the field, suggesting that larval choices observed in the laboratory may be a subset of what larvae are capable in the field (Pineda *et al.*, 2009). As a consequence, this approach may mask some factors which act on settlement dynamics and introduce sources of stress on larval behavior, reducing their competence for settlement and providing unrealistic measurements (Chia *et al.*, 1984).

Early development of Dendropoma spp. *reefs*

In the case of biogenic habitats, larval settlement and recruitment are crucial mechanisms which have implications for the formation and stability of the biological construction, ensuring population and habitat persistence. High settlement results in high recruitment rates, unless there is a major disturbance, but if settlement is low, the population will be recruitment-limited and this will affect the development of the biogenic structure (Underwood *et al.*, 1983; Menge, 2000).

Regarding the *Dendropoma* spp reefs, the biogenic construction is a slow process that takes hundreds of years and several cross-scalar mechanisms have implications on its formation.

Dendropoma encrustations develop on intertidal rock platforms and the mineralogical composition of the substratum, hydrodynamic conditions, sea surface water temperature and coastal slope are environmental features which play a role in the reef establishment and distribution at wide spatial scale (Antonioli *et al.*, 1999; Chemello & Silenzi, 2011; Schiapparelli *et al.*, 2003). By contrast, on alocal scale, factors which influence the settlement rate and success of *Dedropoma*spp, have not been described.

The study of these dynamics needs to consider the ecology of *Dendropoma* spp. during the early phases of its life cycle.

Dendropoma larvae are lecithotrophic pediveligers and, once mature, hatch out from the ovariccapsula, leave the mother shells and crawl in the close space, choosing within hours or few days the suitable settlement site (Calvo *et al.*, 1998). The non-pelagic larval development and the use of own nutritional reserves are peculiar strategies restricted to a

narrow range of marine invertebrata (less than 20%, Pawlik, 1992) which reduce the dispersal ability of the species, but also the possibility to be predated or to meet lethal conditions. Lecitotrophic larvae, indeed, acquire the competence for settlement faster compared to pelagic larvae, probably due to the nutritional limitation, and once settled they quickly metamorphose in juveniles (Pawlik, 1992). Hughes (1979) assumed that *Dendropoma* juveniles disperse by crawling are restricted to distances less than 10 m. The retention of propagules within a restricted area would assure high reproductive fitness and restricted colonization dynamics (Johannesson, 1988).

In rare cases veligers may be released from the mantle cavity and swim in the water column for short periods before the attachment on the substratum (Lewis, 1960).

Hence, the absence of a true planktonic stage reduces the ability of dispersion of these species and local factors result fundamental for settlement dynamics and success.

The settlement and recruitment processes within the genus *Dendropoma* have been approached by some researches (Phillips & Shima, 2009; Calvo et al, 1998; Spotorno *et al.*, 2015; Hughes, 1978), given that both processes are of interest for detailing the species life history, but in most of the cases, these researches focused on the biological traits of these two mechanisms, disregarding both the implications of environmental factors on the processes and the behavioural traits of *Dendropoma* spp. larvae.

3.2 Aims of the research

This research aims to test:

a) Temporal pattern of recruitment of *D. cristatum*and possible implication of hydrodynamic conditions

b) Settlement success of *D. cristatum* on different typologies of substratum

The study of these topics has been approached by measurative and manipulative field experiments, carried out on vermetid reefs from different Sicilian localities.
This approach allowed to maintainunvaried the whole set of circumstances that larvae and recruits experience under natural conditions and to obtain realistic biological and ecological responses.

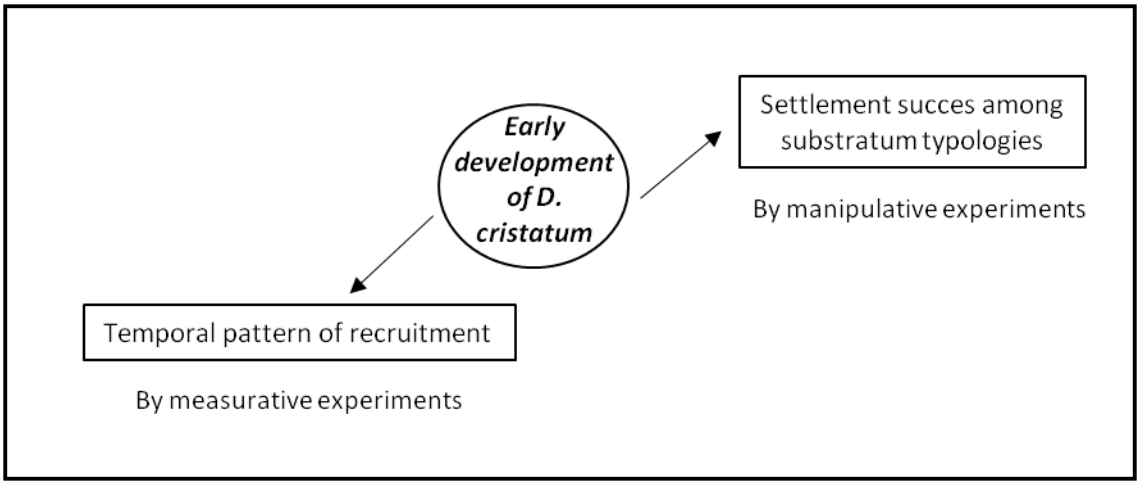

Figure 34, Scheme of the experiments discussed within the chapter 3.

3.3 Temporal pattern of recruitment of *Dendropoma cristatum* and possible implication of hydrodynamic conditions

Temporal dynamic of recruitment for the reef-builder *Dendropoma* spp. has never been described in the Central Mediterranean and the recruitment patterns within-reef microhabitats (the inner and outer edges) are still unexplored.

The inner and the outer edge represent, different microhabitats, subjected to peculiar levels of physical stress, due to the reduction of wave energy towards the inner edge of the reef, and different desiccation time during low tide and intensity of solar irradiation. These differences between the reef edges may have implications on the population structure, with consequences on settlement and recruitment dynamics.

Along the intertidal zone, solar radiation, desiccation time and oxygen deficiency are stressors which strongly affect the developmental rate of intertidal gastropods during the early stage of their life cycle (Przeslawski, 2005). Moreover, the inner and the outer edges have different morphological characteristics: *Dendropoma* forms an articulated biogenic layer on the external rim, with higher topographic complexity and thickness, while on the internal rim the bioconstruction develops as a smooth biological encrustation which covers the underneath rocks for a few of centimeters.

In addition, little is known about how local hydrodynamic regime may affect patterns of settlement and recruitment of species with benthic larval stages. The direction and strength of waves are known to play a relevant role in the establishment of biogenic habitats, and several authors have stated that the distribution of vermetid reefs is strongly related to local hydrodynamics. In the Mediterranean, *Dendropoma* reefs are well developed along moderately exposed rocky shores and are reduced or absent on most exposed and highly sheltered coasts (Antonioli *et al.*, 1999; Chemello & Silenzi 2011; Dieli *et al.*, 2001). Moreover, *Dendropoma* density tends to increase with hydrodynamic exposure (Azzopardi & Schembri, 1997).

The aim of this research was to describe the seasonal pattern of *Dendropoma cristatum* recruitment and to investigate possible variations within and between reefs, respectively due to the different physical conditions which occurs in the inner and outer reef edges and to the wave-exposure degree among differentreef. Potential relationships between wave exposure and reef width were also investigated.

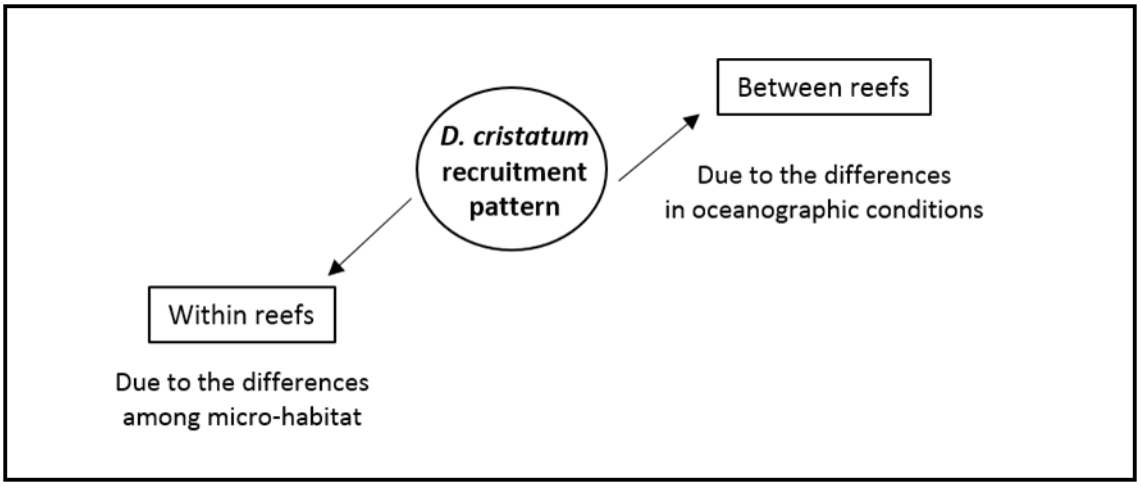

Figure 35, Schematic description of the experiment.

Methods

In order to describe the recruitment pattern of *D. cristatum*and possible variations due to local hydrological conditions, the number of recruits was recorded in the inner and outer edges of 3 Sicilian vermetid reefs between June and September 2014, at monthly intervals. Reef orientation varied between the three localities, considering different reef wave exposure.

- Study site

The study area was located within the Gulf of Cofano (N-E Sicily) (Figure 36). Vermetid reefs are abundant on this coast and develop on Mesozoic limestones and calcarenites of the early Pleistocene (Antonioli *et al.*, 1999). The sampling area extent is 8 km and three reefs with different degrees of wave exposure were chosen, in the following localities: Tonnara del Cofano (TDC), Scaru Zu Àsparu (SZA) and Isulidda (SLD), respectively on the northeast, northwest and west side of the gulf.

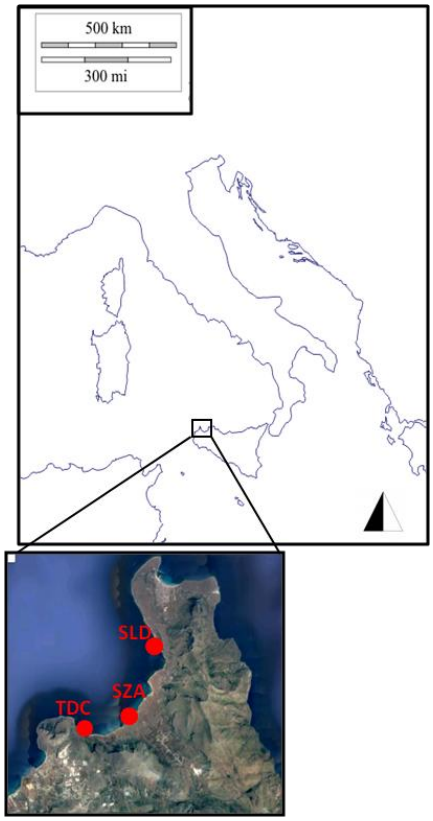

Figure 36, Study site, Gulf of Cofano. The red dots indicates the sampling stations.

- Wave exposure measures

Wave exposure for each locality was measured using the exposure index (Thomas 1986):

$$EI = W \times (F \text{ modified by } CS)$$

in which W is the wind energy, F is the fetch length in nautical miles (maximum of 100 nautical miles) and CSis the extent in nautical miles of water 10 m deep bordering the shore (used as the critical depth and adapted for the Gulf of Cofano).

Wind energy (Km h^{-1}) and direction were provided by the web sitewww.eurometeo.comandthe average data for each month were calculated.

The higher was the index, the greater was the exposure of the site (Thomas, 1986).

- Recruitment measures

Beside considering the density of *D. cristatum* recruits, this study took into account the number of living adults, given that in the case of benthic invertebrata with low dispersion ability recruitment is primarily determined by the population size and by the reproductive output of sexually mature individuals.

Data on recruit and adult abundance were collected by photo-sampling, an inexpensive methodology which does not have an impact on natural population. Sampling was conducted monthly, in calm water and under low tide conditions. Within each locality, 2 sites were selected (approximately 30 m from each other) and at each site, five photographic replicates were randomly allocated along the inner and the outer edges. Photos were taken within a 10 × 10 cm^2frame. For each locality, 20 photographic replicates were taken at each month, for a total of 240 replicates over 4 months. Subsequently, on each picture recruit and adult numbers were counted.

The distinction between recruits and adults was based on the shell size: for recruits, size ranged between 1 and 2 mm and the biggest exhibited a noticeable protruding scar at the protoconch-teleoconch boundary. The adult size ranged between 3 and 6mm and the shells were often covered by the coralline red algae (Calvo *et al.*, 1998; Milazzo *et al.*, 2014).The software used to estimate the size and the number of individuals was ImageJ.

The number of recruits was standardized against adult density (number of recruits/number of adults within 100 cm^2).

Furthermore, five measures of linear distance between the inner and the outer reef edges were collected at each site every 5 meters and reef width was estimated as the average value between the 5 measurements.

Data analysis

The relationship between reef width and the average EI was assessed with a linear regression analysis, with reefwidth as the dependent variable and EI as the independent variable.

A one-way univariate PerMANOVA (Permutational ANOVA/MANOVA) of adultabundance was conducted, In addition to this, the standardised number of recruits was analysed by univariate PerMANOVAusing a four-way design with time (Ti),fixed, four levels (June, July, August, and September); habitat(Ha), fixed and orthogonal, two levels (inner edge and outer edge); locality (Lo),fixed and orthogonal,three levels (TDC, SZA and SLD); and site (Si), random, two levels (nested in Lo). In both cases, PerMANOVA was based onEuclidean distance matrices with 9999 permutations. PERMANOVA was chosen because thismethod does not assume a normal distribution oferrors, allows for factorial designs, and accounts forinteraction effects (Anderson *et al.*, 2008). Theseanalyses were conducted using the Primer v. 6 statistical package with the PerMANOVA+ extension(PRIMER Ltd., Plymouth).

Results

For SZA the prevalent wind direction was north-west, whereas for TDC was north–northwest and for SLD was north-east.

Exposure index values and the average (± SD) reef width (m) are reported in the table below:

	EI	Reef width (m)
TDC	0,05 (±0,01)	6,02 (±0,6)
SZA	1,43 (±0,28)	11,47 (±0,63)
SLD	0,29 (±0,08)	3,19 (±0,19)

Figure 37, Table n. 2: Exposure index and the reef width (± SD) for the 3 reefs (TDC, SZA, SLD).

The highest reef extents were observed under the highest conditions of exposure to hydrodynamics and a significant positive correlation was found between the two variables ($r: 0.79$; $p < 0.001$).

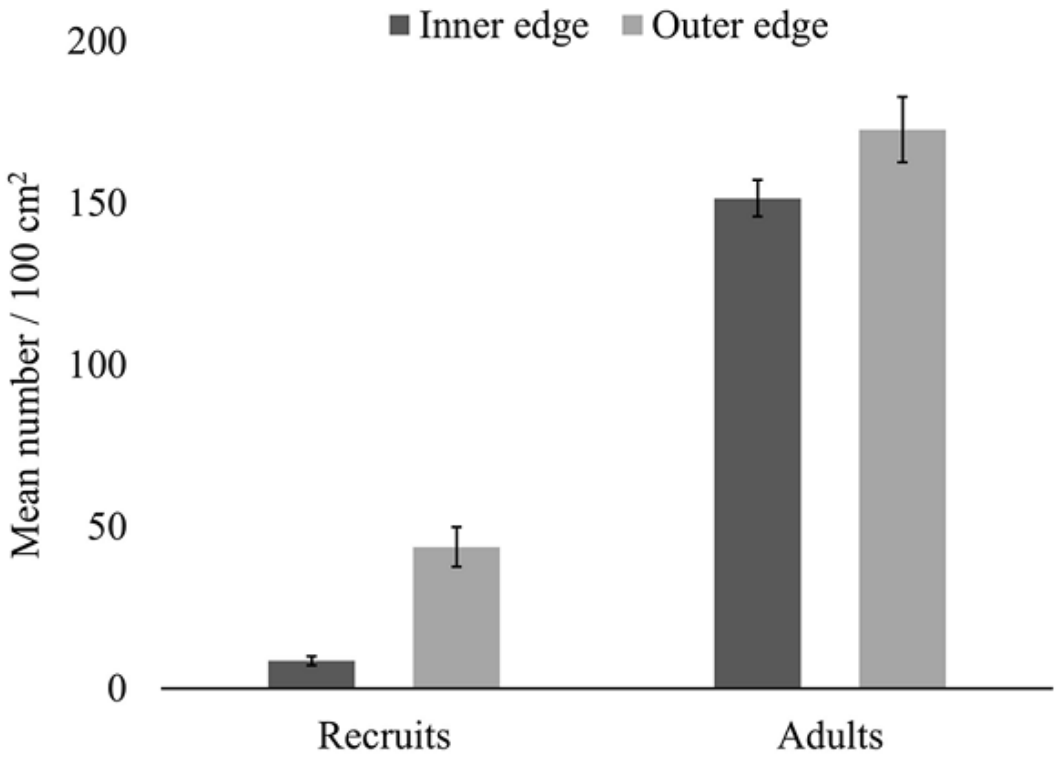

Figure 38, Average density of recruits and adult of D. cristatum within the reef microhabitats.

Overall, for all the considered reefs and during the whole sampling season (from June to September), the average number of adults did not differ between the inner and the outer edges. By contrast, the recruit number was on average significantly higher on the outer reef rims ($p \leq 0,05$) and this difference was confirmed for all the 3 localities. Therefore, no significant differences were found in the number of recruits and adults between reefs subjected to adifferent degree of wave exposure (SZA, TDC and SLD) and each reef showed the same temporal pattern of recruitment over the 4 months.

Recruitment pattern differed within reef edge: on the inner edge the average number of recruits did not vary over the four months (mean number of recruit/100 cm^2 ranges between 3,6 and 11,8figure 4, A). By contrast, on the outer edge a recruitment peak occurred in July and the average recruit number progressively decreasedthrough the breeding season (mean number of recruit/100 cm^2 ranged between 7 and 105, figure 4, B).

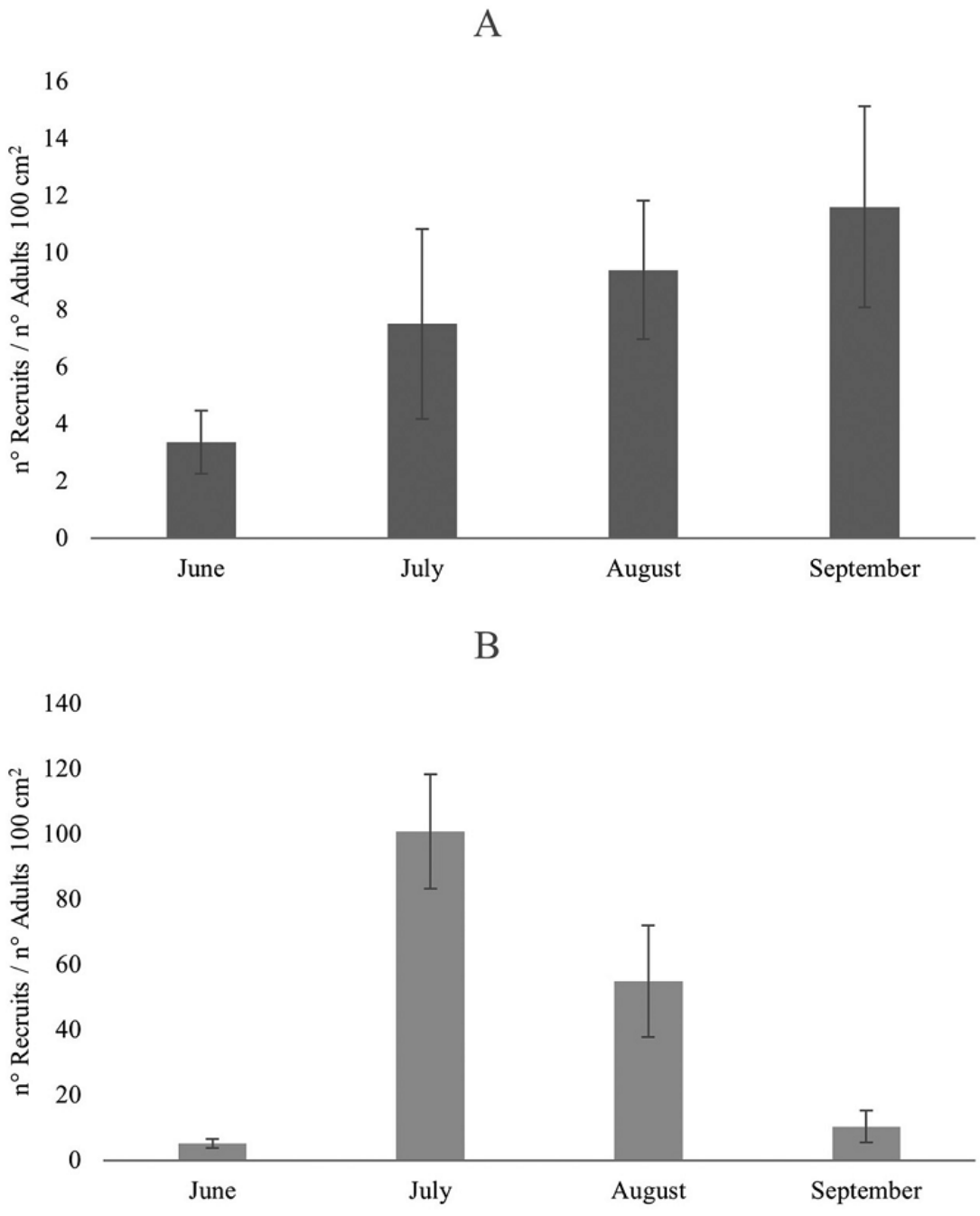

Figure 39, Average recruits density (±SD) along the inner (A) and the outer reef edge (B) for all the 3 localities and over the 4 months.

PerMANOVA analysis showed significant differences of recruit density for the fixed factors Habitat and Time. Hence, these differences were not constant over the 4 observation, but did vary.

Only for the outer edge the recruit density significantly varied among months and monthly differences in the recruitment pattern between both the edgewere significant just in July.

Source	df	MS	Pseudo-F	P(perm)	Pairwise comparison		
Ha	1	346.81	14.794	*	**Time**	**Habitat**	
Ti	3	191.71	10.125	**		Inner	Outer
Lo	2	70.467	2.9694	ns	June vs July	ns	*
Si (Lo)	3	23.731	3.2797	*	June vs Aug.	ns	*
Ha x Ti	3	183.88	9.4145	**	June vs Sept.	ns	ns
Ti x Lo	6	29.238	1.2472	ns	June vs Aug.	ns	*
Ha x Si (Lo)	3	23.443	2.2476	ns	July vs Sept.	ns	*
Ti x Si (Lo)	9	18.934	3.2399	*	Aug. vs Sept.	ns	ns
Ha x Ti x Lo	6	18.604	2.6168	**			
Ha x Ti x Si (Lo)	9	19.532	0.95251	ns	**Habitat**	**Time**	
Res	192	7.2357	2.6994	**	Inner vs Outer	June	ns
Total	239					July	*
						Aug.	ns
						Sept.	ns

Figure 40, Table n. 3: PerMANOVA analysis with 3 factors: "Ha", Habitat (inner vs outer edge); "Ti", Time (the 4 months); "Lo", Locality (SZA, TDC, SLD); "Si", Site (2 sites within each locality). Measured variable: n. of recruits 100 cm^2.

Discussions

This research provided first *in situ* data on the temporal recruitment dynamic of the reef-builder gastropod *Dendropomacristatum*.In all the considered localities the same spatial and temporal patterns of recruitment were observed: differences in the recruitment success occurred within reef microhabitats (i.e. external and internal rims) and on the external rim the recruitment dynamic showed a seasonal peak during the warmest month. However, in any month the number of living *D. cristatum* adults did not differ between reef edges and between localities. Within-reef environmental conditions affected the recruitment dynamic mostly than oceanographic features. Physical conditions which recruits are subjected to along the inner and the outer edges may have implications on the recruitment performance of this reef-builder species, while at the considered temporal and spatial setup the degree of exposure did not have an effect on the recruitment dynamic.

Two peculiar temporal patterns of recruitmentwere described for the internal and the external reef rims (see the figure39). The hypothesis is that these two patterns may depend on the diverse settlement success of larvae and post-settlement mortality of settlers between reef edges. The prolonged exposure to solar radiation during low tide time can have lethal consequences on *Dendropoma* crawling larvae and this effect is more likely to occur on the inner edge. Embryos of rocky intertidal species, indeed, may experience high rate of mortality particularly during summer when elevated UV radiation and high temperatures coincide with low tide cycles, which are conditions that presumably occur at the inner edge of the vermetid reefs (Russell & Phillips, 2009).

The higher number of recruit found in the outer edge may be also explained by higher reproductive output of adults in this habitat. This may be due to the more favourable environmental conditions that the species experience along the outer rim. Not-direct measures of wave motion collected in the fieldand personal field observations confirm that on the outer rim *Dendropoma* matches higher intensity of wave action and this increases the food availability (Vizzini *et al.*, 2012). Suspension feeder species, such as *Dendropoma*, commonly benefit from high water flow, as it keeps organic particles in suspension and increases their feeding efficiency (Westerbom & Jattu, 2006), having consequences on other functional processes.

Despite the observed differences in recruitment, the number of adult individuals did not vary between the two edges, showing a non-direct relationship between recruitment and adult abundance in a long-lived species such as *D. cristatum*. One hypothesis to explain this result is that the higher number of recruits along the outer edge experiences higher post-recruitment mortality and, over the time, the number of adults between reef edges achieves a balance. This higher rate of mortality could be due to density-dependent mechanisms of regulation which usually characterize the dynamic of benthic populations. However, to confirm this hypothesis, field data on post-recruitment processes need to be collected.

At the spatial scale considered in the study, the recruitment mechanism seems not to be correlated with the degree of wave exposure. A contrasting trend has been described by Azzopardi & Schembri (1997) which, along Maltese coasts, observed a positive correlation between the density of *D.* cf. *petraeum* and reef exposure. Regardless the Atlantic reef-builder *Dendropoma irregulare*, Spotorno *et al.* (2015) found highest settlement rate along moderately exposed shores, with a positive correlation between the settlement of the species and the exposure to waves. However, differences in the exposure index between the localities included in the Spotorno's study were stronger than the differences among localities within the Gulf of Cofano: E.I. ranged between 10,7 and 15,4 in the Spotorno's study and between 0,05 and 1,43 in the present study. Hence, reefs selected in this research are subjected to more moderate differences of wave exposure and this could be the reason explaining the non-correlation between *D. cristatum* recruitment rate and the exposure to wave action. Moreover, Arribas *et al.* (2014) argued that waves energy often enhance the supply of larvae, such as the case of mussel beds and other biogenic reefs, but species with direct development, instead of a pelagic larval stages, are probably less dependent on wave flow to attach on the substratum and recruit in adults.

Additional research is necessary to explain the observed recruitment pattern and within-reef differences. Moreover, given that local hydrodynamic regime does not affect the recruitment pattern of this reef-builder, at the considered spatial scale, and seeing the peculiarbenthic ecology of *Dendropoma* larvae, the research here reported will focus also on the interactions between *D. cristatum* larvae and substratum cues which may have implications on settlement and recruitment dynamics of this Mediterranean habitat engineer.

3.4 Settlement of *Dendropoma cristatum* on various typologies of substratum

Settlement cue for marine invertebrate larvae

On rocky substrata, differential rate of larval settlement appears highly affectingthe recruitmentand substratum cues are often responsible for habitat selection from larvae.

While hydrodynamic processes are responsible for larval supply on suitable substrata (Abelson & Denny, 1997; Butman *et al.*, 1988; Spotorno *et al.*, 2015), the larval behaviour in response to substratum cueing has also been demonstrated to be important in determining settlement patterns (Hadfield, 1986, 2011; Raimondi & Morse, 2000; Steller & Càceres, 2009). During settlement, propagules are subjected to a variety of environmental factors and respond to a multitude of stimuli. The mechanisms of settlement and recruitment of benthic invertebrata can be affected by abiotic factors related to the structural features of the substratum and by biotic factors related to the inhabiting organisms (Perkol-Finkel & Benayahu, 2007). Physical properties associated with the substratum are important and settlement preferences have been demonstrated for variations in the contour, texture and thermal capacity of substrata, grain size of sediment, and water flow close to the substratum. Surface topography, often measured as roughness in the ecological studies, is generally described as a physical substratum property which promotes settlement (such as for barnacle, Berntsson *et al.*, 2000a), by increasing surface complexity and the available surface for benthic larvae.

For most species, however, the importance of physical features of the substrata is secondary to their bio-chemical characteristics. Overall, there is a hierarchy of cues by which larvae select a favorable habitat and specific sites for settlement.

During the research of site-specific cues, larvae test the surface with their apical ends, looking for bio-chemical stimuli which trigger their attachment and the successive, not reversible, morphogenetic transformation into juveniles. (Barnes & Gonor, 1973; Nott, 1973; Weissburg & Zimmer-Faust, 1991) This is known as pre-settlement behavior, typical of larvae from many phyla of marine invertebrata. For veliger larvae the site of reception of these bio-chemical cues is the apical sensory organ (Hadfield, 2000).

Sources of bio-chemical settlement cues

Settlement cues are often defined frombio-chemical origin because a biogenic substratum provides a chemical signal which induces larvae to settle. A variety of biological sources may provide these cues and, based on the involved mechanisms, it is possible to distinguish between different typology of settlement.

- Gregarious settlement: when larvae settle in response to the presence of adults, juveniles, or recruits of the same species. This behavior has been reported for many phyla, especially for tube-dwelling polychaete, oysters, barnacles and other organisms which, grouped in mono-specific aggregations, produce settlement cues. For instance, arthropodina, which is a protein associated with the barnacle test, is known to induce cyprid settlement and also the antennular secretion which cyprids left when they explore a surface, promotes the attachment of new cyprids (Crisp & Meadows, 1963; Larman *et al.*, 1982; Crisp & Meadows, 1962; Clare *et al.*, 1994).
- Associative settlement: is defined as the settlement of larvae specifically upon other species (Crisp, 1974). Intraspecific epiphytic associations are common and well described within marine organisms (Morse, 1992). For instances, crustose coralline algae (CCA) are secondary substrates which may drive the settlement of many species of corals, polychaete, molluscs (Harrington *et al.*, 2004; Gee, 1965; Steller & Càceres-Martìnez, 2009) while other invertebrata may show settlement preferences for erect algae (De Viçose *et al.*, 2012; Pawlik, 1989; Boettcher *et al.*, 1996). Larval settlement on invertebrate from other specieshas also been reported, as a case of associative settlement (Hadfield & Paul, 2001). The nature of these interspecific interactions may be related to the physical structure of the biogenic substratum or to the production of peculiar compounds able to induce larval settlement behavior. In this last case, the compounds are directly produced by the biogenic substratum or may be associated to it by means of other organisms (such as the case of epibionta).
- Biofilm-associated settlement: Biofilms are complex 3-dimensional layers composed of microorganisms (bacteria, unicellular algae, fungi and protozoa) embedded in a self-produced extracellular polysaccharide substance (EPS) which develop on each submerged surface and modifies their physical and biological characteristics (Dobretsov, 2010). Biofilm are well known to promote the settlement of a wide range of marine organisms from many

phyla, both for their structure and surface complexity and for the production of metabolites which trigger larvae attachment on the surfaces (Shimeta *et al.*, 2012; Hadfield, 2011; Zardus *et al.*, 2008; Zhao & Quian, 2002; Thompson *et al.*, 1998).

Settlement dynamic of Dendropoma cristatum *larvae*

Differences in physical conditions may affect the recruitment rate of *D. cristatum* larvae between reef rims, although local hydrodynamic conditionsdo not seem to have an effect.

At the settlement scale, substratum properties may be involved in the site-choice by *Dendropomacristatum*. It is hypothesized that once competent for settlement, crawling larvae of *Dendropoma* spp. actively evaluate the surrounding microenvironment by using their rhinophores (as pre-settlement behavior).

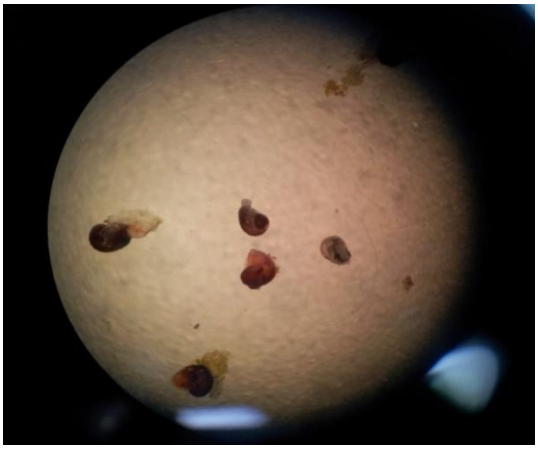

Figure 41, Crawling larvae of D. cristatum*. Magnification: 8x.*

To understand how the settlement dynamic occurs inside the genus *Dendropoma* and which factors crucially drive this process is of interest for detailing the species life history and the early stages of the reef-building dynamic. Moreover, benthic ecology for invertebrate larvae is not common and the settlement dynamic of these organisms is overall understudied.

This experiment aims to understand if the physical and bio-chemical substratum properties may influence *D. cristatum* settlement.

Settlement performance of *D. cristatum* larvae was measured onto different substratum typologies and in different field experiments, in order to distinguish between physical and bio-chemical induction of settlement.

This research aim is articulated in 2 experiments which compare the settlement rate of *D. cristatum* on:

a) Biogenic *vs* inorganic substrata
b) Living CCA *vs*non living CCA

Each condition has been tested in an independent experiment and each experiment will be treated in a separate section.

3.4 a) Biogenic *vs* inorganic substrata

Several studies focusing on settlement, compare the efficiency of settlement between inorganic, biogenic and artificial substrata. Biological responses are split in two categories: organisms which choose rock and baresubstrata, as not colonized surfaces and free from potential competitors (Bell *et al.*, 2015) and organisms which are advantaged by settling on secondary biogenic substrata.

In this experiment settlement rate of *Dendropoma cristatum*wasmeasured in the field and compared among 4 different typologies of substrata, placed on a reef:

1) Bare rock (limestone)
2) *D. cristatum* adult shells
3) Crustose Coralline Algae (CCA, mainly *Neogoniolithon brassica-florida*)
4) Non-toxic epoxy resin

The first 3 types of substratum are those which *Dendropoma cristatum* larvae found in their natural environment and where they settle on.

Methods

The settlement experiment was performed during September 2014, when anthropic presence along the shores was lower and, thus, constituted a less probable threat for the experiment success.

Chip surfaces of bare rocks, adult of *D. cristatum* and of CCA were collected with hammer and chisel from the area and were attached on the top surface of discs of forex (6,5 cm of diameter) using a water-resistant epoxy resin. 6 replicates of each substratum typology were arranged (limestone, *D. cristatum* adults, CCA, Epoxy resin) and were left for two days in a tank with marine water and a water pump, allowing the epoxy resin to completely dry out and to eliminate the potentially toxic components. Subsequently, artificial settlement

discs were bolted on the external rim of a vermetid platform, described as the most reproductively active portion of the reef (Franzitta et al., 2016). Within each site, 3 group of settlement surfaces were randomly placed within an area of 30 m and each group included one disc of each substratum typology. Settlement discs were left in the field for 20 days. After the field exposure, the number of *D. cristatum* settled on each typology of substratum was counted under a light microscope, given the small size of settlers (less than 1 mm).

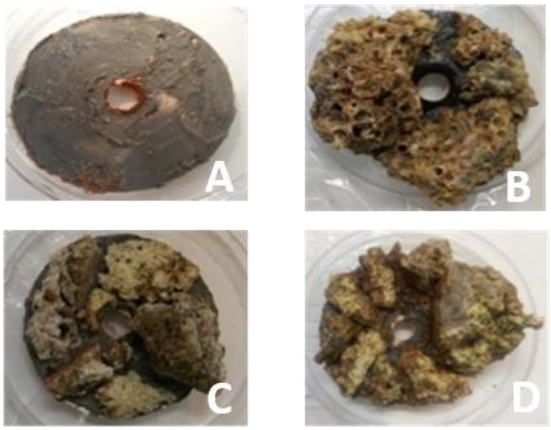

Figure 42, Examples of the artificial settlement discs. A: Epoxy resin; B: D. cristatum *adults; C: CCA; D: bare rock.*

Study site

The study area was located within the marine protected area of Capo Gallo-Isola dellefemmine, in the North-western coast of Palermo (Sicily, Figure 43). Here, the rocky shore is constituted by Mesozoic-Tertiary limestone formations and is characterized by well-developed vermetid reefs fringing the coast.

Due to the favourshore exposure to the dominant winds (from N-NW), reefs from this area are among the most representative vermetid construction of Sicily (Chemello *et al.*, 1990).

The settlement experiment was carried out in the locality named “Barcarello” (within theC-zone of the MPA) and was replicated in two sites within an area of 100 m.

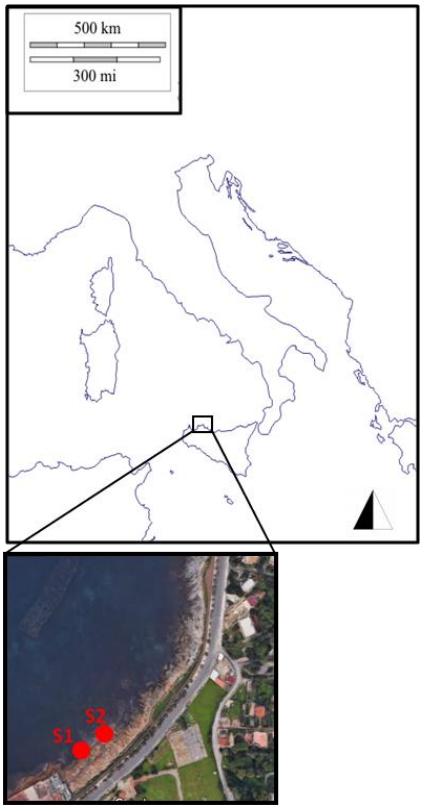

Figure 43, Study area, S1 and S2 indicate the two sites where the settlement experiment occurred.

Figure 44, Vermetid reef of Barcarello.

Data analysis

A univariate PerMANOVA on row data was performed to determine differences in the number of *D. cristatum* settlers between the 4 typologies of artificial settlement surfaces within both the sites. A 2-way design was used, with Substratum ("Su") fixed with 4 levels (CCA, *D. cristatum* adults, Bare rock and epoxy resin) and Site ("Si") random with 2 levels (site 1 and site 2). PERMANOVA was based on Euclidean distance matrix with 9999 permutations and was chosen because this method does not assume a normal distribution of errors, allows for factorial designs, and accounts for interaction effects (Anderson *et al.,* 2008).

A paired comparison between couples of levels within the factor Substratum completed the analysis. All the statistical analysis were performed on Primer v. 6.

Results

In total, 377 *Dendropoma cristatum* settlers were found on the settlement discs and the overall settlement rate for substratum typology is reported on the graph below (as % value):

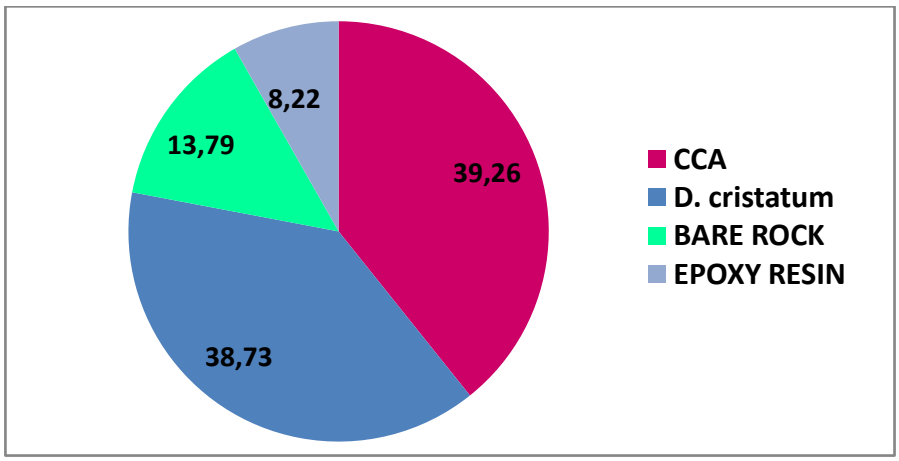

Figure 45, Percentage of settlers for substratum typology.

Within both the sites settlement rate is highest on biogenic substrata, compared to limestone bare rock and epoxy resin (graph below). The average number of settlers (±SD) was calculated for the total surface of each substratum typology in each site (165,83 cmq).

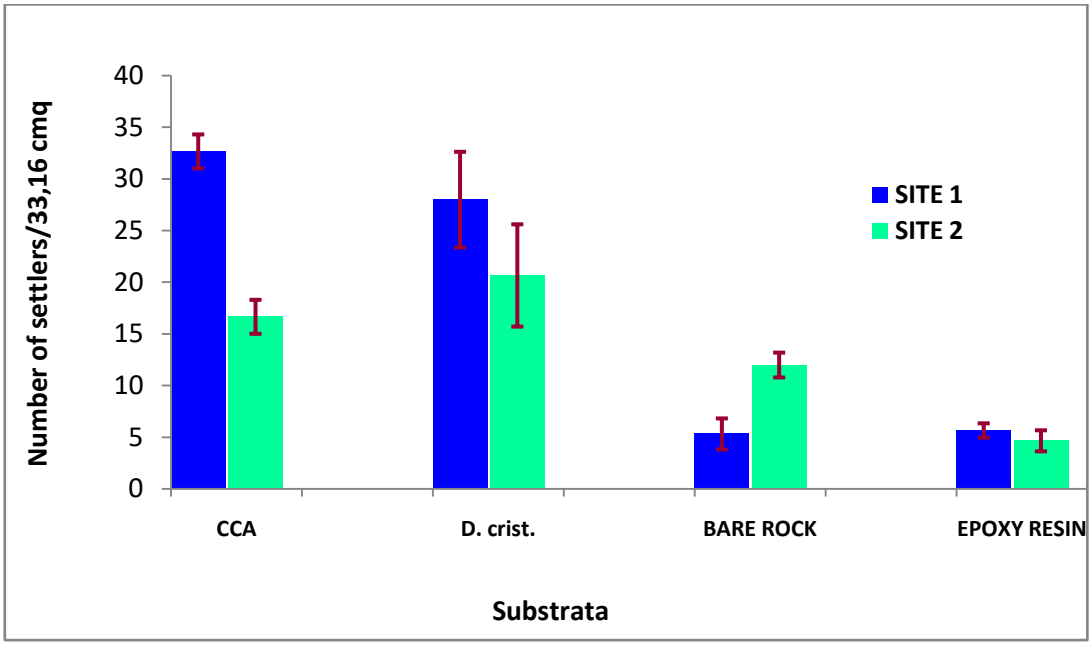

Figure 46, Average number of settlers (± SD) on each typology of settlement surface within site 1 and site 2.

The univariate PerMANOVA on settler densities showed significant differences for the factor Substratum and no differences among sites occurred. Pairwise test revealed that the number of settlers between biogenic (CCA and *D. cristatum* adults) and inorganic substrata (Bare rock and epoxy resin) is significantly different, but no difference between CCA and *D. cristatum* adults and between bare rock and epoxy resin were found.

Source	df	MS	Pseudo-F	Sign.	Pair-wise comparisons	
Su	3	11.028	11.619	***	**Substratum**	**Sign.**
Si	1	0.77273	0.81411	ns	CCA *vs* bare rock	***
SuxSi	3	2.3256	2.4502	ns	CCA *vs* e. resin	***
Res	16	0.94918			*D. crist. vs* bare rock	*
Total	23				*D. crist.vs* e. resin	*

*Figure 47, Table n. 4: Univariate PerMANOVA analysis (24 variables, not transformed data), showing changes in the number of settlers for substraum in relation to substratum typology (4 in total) and site (2 sites). The pairwise comparison for couples of levels within the factor Substratum is displayed on the right and only significant differences are shown. *, p≤0,05; **, p≤0,01; ***, p≤0,001; ns= not significant.*

Figure 48, D. cristatum *larvae settled on* D. cristatum *adult (left) and on CCA (right). Magnification: 10X.*

Discussions

Field studies to understand larval behavior of gregarious vermetidae and early stage of reef formation are few, although habitat selection by larvae is known to be relevant to post-settlement survival and adult distribution patterns (Mundy & Babcock, 1998, Baird *et al*., 2003).

This study analyzed the influence of the substratum typology on *Dendropoma cristatum* settlement and verified if the substratum properties may affect larvae during habitat selection process.In this first choice experiment, substratum preference for *Dendropoma cristatum* larvae was measured in the field, by using artificial settlement discs of 4 different materials. *Dendropoma cristatum* individuals found on experimental substrata were recognized as settlers and not as recruits because their morphological characteristics were not still retained those typical for recruits. Their shells sized less than 1 mm and the protruding scar at the protoconch-teleoconch boundary, typical of more developed juveniles, was always absent (Calvo *et al.*, 1998; Milazzo *et al.*, 2014; Franzitta *et al.*, 2016).

After 20 days, the numbers of settlers on both the biogenic substrata(crustose coralline algae) and *D. cristatum* shells, were more than double, compared to those found onto inorganic settlement discs (bare rock and epoxy resin). A significant preference for biogenic

surfaces was pointed out, even though differences between these 2 kind of biogenic substrates were not found. In addiction, pairwise comparison showed significant differences for the number of settlers between *D. cristatum* adults *vs* inorganic substrata and highly significant differences among CCA and inorganic substrata.

Hence, biogenic substrata significantly favor the settlement of *Dendropoma cristatum* in the field and both the sites confirmed the same trend. No interaction between sites and settling surfaces was found, indicating a clear effect of substratum on the choice of settlement substratum by *D. cristatum* larvae.In addiction, biogenic induction resulted relevant, but not fundamental for *D. cristatum* settlement, since settlers were also found on bare rock and on epoxy resin discs,although at verylow density.

The selection of settlement site is critical for the subsequent survival of benthic marine invertebrates, as this choice will largely determine the environmental conditions experienced by later life stages (Keough & Downes, 1982; Rodr'iguez et al., 1993). As discussed before, settlement preference for biogenic secondary substrateare widely diffused within the marine invertebrate. By contrast, some organisms may develop anti-settlement defence mechanisms, known as "allelopathy" (Maida *et al.*, 1995; Suzuki *et al.*, 1998) to inhibit or to remove newly settled organisms and to prevent epibiosi (Scardino *et al.*, 2003; Keats *et al.*, 1997; Wahl, 1998).

As showed, *D. cristatum* larvae are able to recognize and actively choose which substratum is suitable for their attachment and the substratum properties may drive larvae during habitat selection process.

This experiment showed that biogenic substrates positively affect the settlement choice of *D. cristatum* larvae, although it remain still not clear if this biological response is due to physical features of the preferred settlement surfaces or to substrate associated bio-chemicalstimuli which trigger larval attachment. In this last case, inter and intra-specific interactions among *D. cristatum* larvae and adult individuals and the coralline algae, mainly *Neogoniolithon brassica-florida*, may be hypothesized to exist and to be considered as akey-process during the reef development.

To address this question, more experiments need to be performed by comparing the settlement preference of *Dendropoma cristatum* on living and not living settlement surfaces.

As the highest significant differences in the settlement success were revealed in the comparison between coralline algae and inorganic substrate and that crustose coralline algaeare known to be implicated on the early stage of development of many marine invertebrates, the next experiment will concern the settlement rate of *D. cristatum* on living and not living crustose coralline algae.

3.4 b) Living CCA *vs* non-living CCA

Crustose coralline algae (CCA) are calcifying red macroalgae and are among the first colonisers of bare rocks in the euphotic marine zones (Dethier, 1994; Kaehler & Williams, 1997; Littler, 1972), which promote the establishment of complex assemblages (Airoldi, 2000a; Coleman, 2003; Maggi *et al.*, 2011). CCA are considered one of the first habitat engineers which organize the substratum for other organisms, originating complex layers able tomodify physical and biological features of hard substrata. These biogenic layers are known to affect the early stage of development of following colonists, providing a substratum more suitable for propagules of other species, compared to bare rocks, and by offering them favorable substrate and micro-habitat.

On rocky intertidal shores, the ecological role of CCA is widely described and address a crucial role in the maintenance of community structure and functioning (Fabricius *et al.*, 2015; Asnaghi *et al.*, 2015). Complex live surfaces, such as those found on coralline algae, provide a combination of cues that can influence settlement for a range of invertebrates (Walters & Wethey, 1996, Steinberg *et al.*, 2002). Corals, gastropods and bivalves, for instances, have been known to choose several species of CCA as settlement substrata and to improve their growth rate and survival when associated to CCA.

Regardless coastal bioconstructions, CCA are supposed to contribute to stabilize and consolidate the biogenic structure, by growing on and encrusting the bioconstructors (such as the case of scleractinian corals or vermetid reefs) and, additionally, represent a secondary substratum, which facilitate settlement and recruitment of the reef builders (Harrington, 2004; Morse *et al.*, 1988).

CCA are a heterogeneous substrate and may represent key-organisms in the establishment of biogenic habitats which promote larval settlement by multiple ways:

1) Increase of habitat complexity
2) Production of metabolites which attract larvae and enhance their fixation
3) Presence of associated epibionta which promote settlement

The previous field experiment showed that CCA are one of the biogenic surfaces preferred by *D. cristatum* larvae for their fixation to the substratum.This interesting finding needs to be deeply studied, aiming to identify the reason of this associative settlement mechanisms.

In this second field experiment, settlement success of *Dendropoma cristatum* was compared among living and not living CCA (mainly *Neogoniolithon brassica-florida*), aiming to distinguish between physical and bio-chemical induction of settlement, provided by CCA.

Study site

The experiment was performed during September 2016 in the locality of "Punta Raisi" (NW Sicily) and replicated within two sites, spaced apart 100 m.
Punta Raisi, approximately 30 km off west from Palermo, is located on a carbonatic sub-flat quaternary platform and hosts an airport and small residential areas.
Well developed vermetid reefs lie within this area. The coast is exposed to the dominant wind and during the winter is subjected to intense coastal storms and wave inundations for tens of m. The area is not protected and during the summer is moderately frequented by turists and fishers. Although the antropic presence, the vermetid reefs within this area do not show detrimental effect on their physical structure or associated assemblages. Hence, the adequate characteristics of this area, make Punta Raisi a site suitable for the experiment.

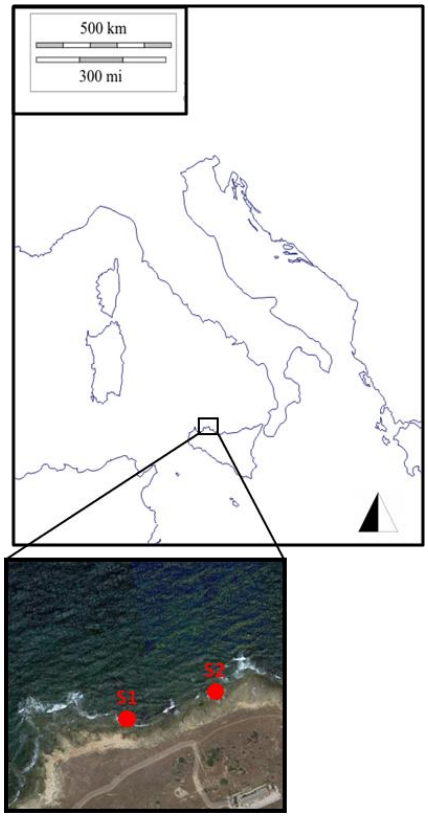

Figure 49 Study area, S1 and S2 indicate the two sites where the settlement experiment occurred.

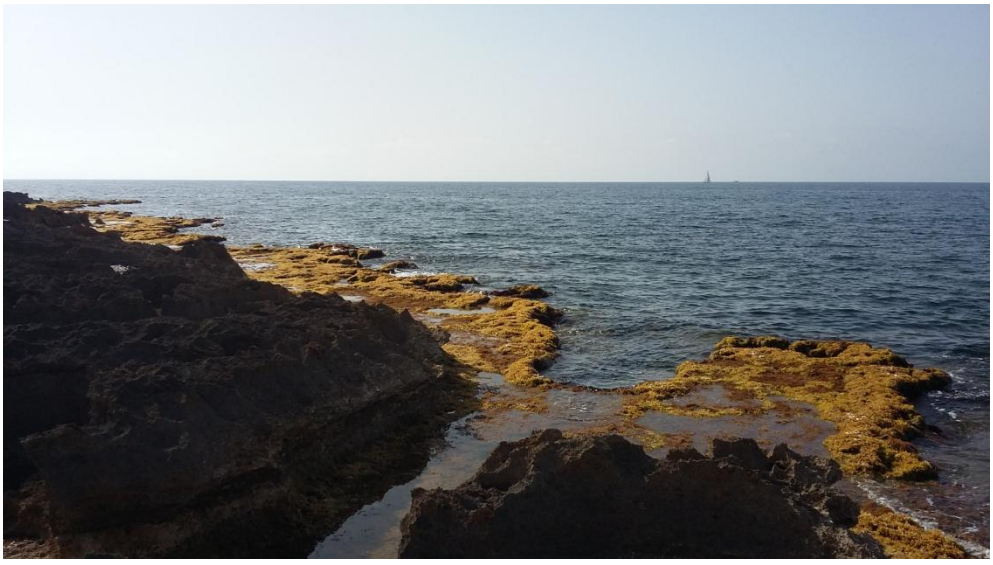

Figure 50, Vermetid reef of Punta Raisi.

Methods

- Settlement discs preparation

Settlement discs were preconditioned with a coverage of crustose coralline algae. A total of 32 forex discs (6,5 cm of diameter) were placed in the field in July 2015 in the locality of Punta Raisi(NW Sicily). To avoid CCA colonization, substratum roughness was increased by scraping the disc top-sides by sand paper. Discs were bolted on the internal rim of a vermetid reef and were distributed along 200m of coast. Discs were left on the reef inner edge because the highest coverage of crustose coralline algae there observed was supposed to facilitate CCA colonization process. Moreover, the recruitment rate of *D. cristatum* on the inner edge is lower (Franzitta *et al.*, 2016) and a reduced number of larvae are expected to settle on the discs during this preconditioning phase. During field exposure forex discs were periodically monitored and CCA coverage gradually increased. After 3 months a few number of *D. cristatum* recruits was found on the discs, but this juveniles were not able to persist longer. After 13 months all discs were collected (August 2016) and their top faces were well biologically colonized with a thin pink crusts of CCA covering at least the 70% of the discs. 24 of the most colonized discs were selected, were observed under the light microscope and *D. cristatum* individuals settled on them were removed. The half of these discs were oven-dried at 30°C for 48 hours, killing the CCA layer while the other half was stored in a tank with marine water and a water pump for 2 days.

- Field experiment:

Subsequently, all the discs with a living CCA encrustation and with a not living algal crust were bolted on the outer edge of a vermetid reef in the locality of Punta Raisi and the combination of 1 living CCA disc and 1 not living CCA disc was randomly repeated 6 times within the two sites. After 20 days the discs were collected and the number of *D. cristatum* settlers was counted under a light microscope.

Data analysis

An univariate PerMANOVA analysis on row data was performed to determine differences in the number of D. cristatum settlers between the living CCA and not living CCA. A 2-way design was used, with Substratum (“Su”) as fixed factor with 2 levels and Site (“Si”) as a 87

random factor with 2 levels (site 1 and site 2). PerMANOVA was based on the Euclidean distance matrix with 9999 permutations. All the statistical analysis were performed on Primer v. 6.

Figure 51, Example of a living CCA disc.

Figure 52, CCA discs placed in the field during the settlement experiment.

Results

Since forex discs were left in the field (July 2015), biological colonization on them was periodically monitored through the time. After 3 months of field exposure (October 2015), on average 0,66 (± 0,2 S.E.) juveniles of *D. cristatum* were attached on each discs, but at the following observation (January 2016) these settlers were not still fixed, probably because the bare forex was not an appropriatesubstratum to sustain *D. cristaum* juveniles. A thin and fragmented encrustation made of pink spots of CCA mixed to patches of recruit of barnacles was also present and covered between the 5-10% of the discs.

At the second observation (January 2016), CCA patches showed lateral growth and more shaped margins. Moreover, the number of CCA patches decreased.

In April 2016 the CCA covered between the 10 and the 50% of the disc surfaces and when the discswere collected (August 2016) this encrustation reached at least the 70%. At this time the CCA were the dominant biological encrustation with a thin calcareous pink crust.

D. cristatum larvae counted on these discs were on average 13,2 (± 1,57 S.E). The average number of settlers was calculated on a surface of 33,16 cmq.

Data on the biological colonisation of forex discs are reported in the table below (Table n. 5).

	CCA coverage on 33,16 cmq (%)	*D. cristatum* n./ 33,16 cmq (±SE)
1° Observation (October 2015)	5-15%	0,66 (± 0,2)
2° Observation (January 2016)	10-20%	0
3° Observation (April 2016)	10-50%	0
4° Observation (August 2016)	70- >90%	13,2 (± 1,57)

Figure 53, Table n. 5: temporal development of the biological encrustation on forex discs.

Overall, there was a higher settlement of *Dendropoma cristatum* on living CCA substrata: 230 larvae were found on them and 122 were on not living CCA.

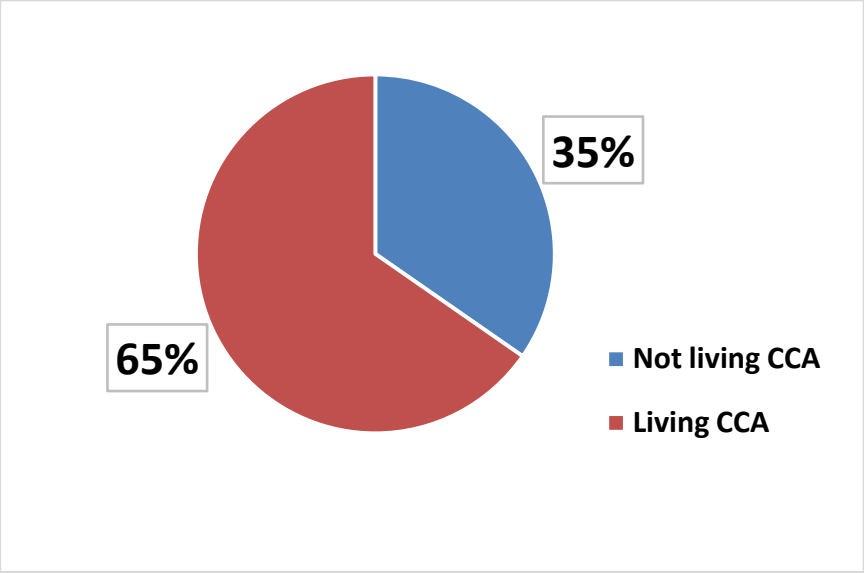

Figure 54, Percentage of settlers on living and not living CCA.

On average, 10,16 (±1,91 S.E.) and 25,38 settlers (±1,45 S.E.) were respectively counted on the not living CCA and on the living CCA discs. Settlement rate was 2,5 folds higher on living CCA.

Within both the sites the settlement rate was higher on living CCA, compared to not living CCA, with more pronounced differences between treatments within site 1 (Figure 55).

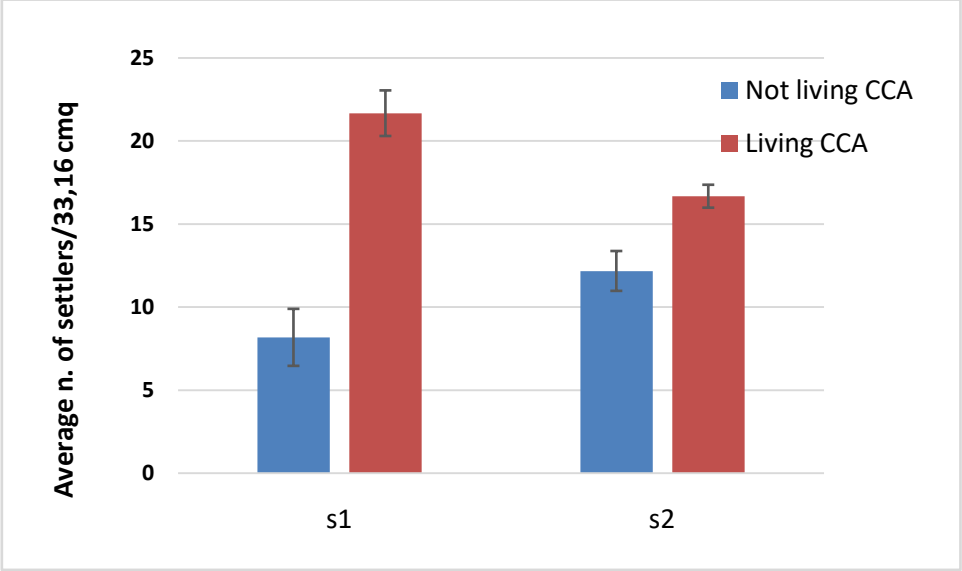

Figure 55, Average number of settlers on CCA discs within site 1 and site 2.

The univariate PerMANOVA analysis on settler densities showed significant differences of settlement for the factor "Substratum" (Living CCA *vs* not living CCA) and no differences among sites occurred. The observed differences and the preference for *Dendropoma* larvae to attach on living CCA confirmed that in situ settlement is significantly improved by live coralline induction.

Source	df	MS	Pseudo-F	P(perm)
su	1	16.31	7.0423	*
si	1	0.22685	9.79E-02	ns
suxsi	1	2.8784	1.2428	ns
Res	20	2.316		
Total	23			

*Figure 56, Tablen. 6: Univariate PerMANOVA analysis (24 variables, not transformed data), showing changes in the number of settlers in relation to living and not living CCA. *, p≤0,05; **, p≤0,01; ***, p≤0,001; ns= not significant.*

Discussions

This study showed that settlement of *Dendropoma cristatum* is enhanced on living CCA substrata.

Surfaces made by living coralline algae attracted larvae and improved their settlement rate up to 2,5 fold respect to not living CCA substrates, although *D. cristatum*does not exclusively required living coralline algae for its settlement. Thus, living CCA may increase *D. cristatum* success at the initial stages of its life cycle. This result is similar to that Spotorno *et al.* (2015) reported for the Brazilian reef-builder *Dendropoma irregulare* (d'Orbigny, 1842). Reproductive biology of *D. irregulare* and larval behavior are similar to those described for *D. cristatum,* even thoughthe Brazilian vermetid is reproductively active for throughout the year (Lewis, 1960).Settlement mechanism of this tropical species was studied in the field and was significantly higher on substrate with live CCA, especially on moderately exposed shores (Spotorno et al., 2015).

Therefore, the presence of living encrusting red algae is supposed to be substantial in enhancing vermetid settlement, although the mechanism of this intra-specific interaction has not been described.

Living coralline algal surfaces offer a potentially favourable attachment site for disparate marine invertebrate taxa and this has been attributed to a variety of factors, including surface chemistry of these new secondary substrata, presence of photosynthetic pigments, proteins and associated epibionts (Morse & Morse, 1984; Stoner *et al.*, 1996; Daume *et al.*, 1999a). Thus, the surface cues associated with the living coralline and their greater surface complexity are important in enhancing larval settlement. Moreover, CCA have major impacts on the life cycle of many molluscs and their presence is often capable of altering community structures (Williams *et al.*, 2008), highlighting their importance on benthic community composition and successful molluscs recruitment.

In the case of *D. cristatum,* the preference to settle on living, rather than not livingCCA encrustations, indicates that biological or chemical properties of live CCA are more effective in inducing the settlement of this gregarious gastropod. The settlement responses of *D. cristatum* onto living coralline surfaces may be due to a complex synergy of cues that may include the coralline surfaces or other associated organisms.One of the most obvious features of CCA layers related to settlement dynamics is the highest surface

complexityrelative to bare rocks. Increased surface complexity is an important factor enhancing larval settlement of many benthic invertebrates, providing protection from predation and enhancing local availability of food resources (Crisp 1974, 1976; Brand *et al.*, 1980). Living and well conserved CCA layers are more able to preserve their structure and 3-dimensionality compared to not living coverage, ensuring the availability of resources for associated organisms. Moreover, living CCA surfaces can enhance the survival of settlers, by excluding other competitors and by providing protection from turf algae and the sediment entrapped therewithin (Babcock & Mundy, 1996, Ruiz-Zarate *et al.*, 2000).

Additionally, a surface cue associated with living coralline algae that has been shown to induce larval settlement in various gastropod species and that could contribute to enhance the settlement of *D. cristatum*is an unidentified peptide mimic of the common neurotransmitter, the α-amino butyric acid (GABA) (Morse & Morse, 1984; Daume *et al.*, 1999a; Searcy-Bernal *et al.*, 1991).

Another potential stimulus provided by living CCA is the presence of associated microbial films. Microbial films develop on various marine substrata, including live corallines and have also been shown to positively influence larval settlement for many invertebrates (Morse *et al.*, 1988, Daume *et al.*, 1999b, Huggett *et al.*, 2006).

This study provided further evidences that vermetid settlement occurs in response to substratum cues and additional investigations need to further characterize the main stimulus/stimuli which contributes to enhancing *Dendropoma* spp. settlement. Coralline cue found on living CCA, indeed, is currently the only biological factor tested as promoter of settlement for *Dendropoma* spp. larvae (Spotorno *et al.*, 2015; Huges 1978, 1979).

The presence of bacterial films could be an additional important substratum cue which may be involved in the settlement dynamic of the Mediterranean reef-builder *Dendropoma cristatum*.Further investigations conducted in this research will focus on the microbial film as settlement cue for *Dendropoma cristatum*.

CHAPTER 4.
Implications of the microbial film into the settlement of *Dendropoma cristatum*

CHAPTER 4.

Implications of the microbial film into the settlement of *Dendropoma cristatum*

4.1 Introduction

Ecological role of the microbial communities in marine systems

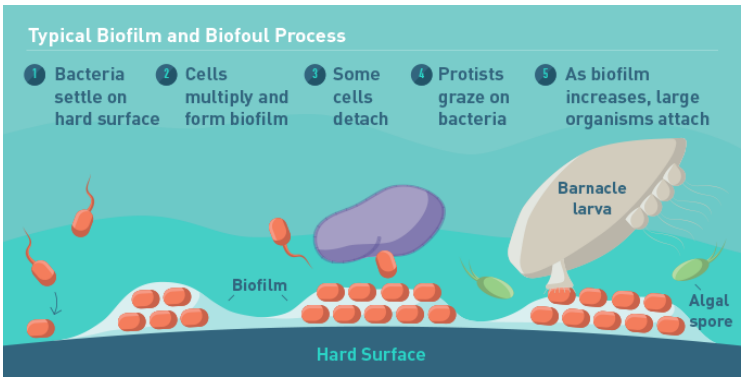

Figure 57, Scheme of the biofilm development.

The microbial film is a complex assemblage of microorganisms (bacteria, diatoms, fungi and protozoa) embedded in a self-produced extracellular polymeric substance, called EPS(Hadfield & Paul, 2001). Microbial films develop on all wet surfaces within few hours of immersion and increases in density and structural complexity over the time (Donlan, 2002).

A biofilm starts with microfouling (bacterial) colonization and its complexity increases as long as the substratum remains immersed. An organic matrix made up of proteins and carbohydrates develops rapidly on the substratum and enhances the fixation of additional pioneer bacteria, followed by microalgae (mainly diatoms), sponges and ciliate protists (Toupoint et al., 2012).

This biological interface between the substratum and the water column can influence settlement and succession by benthic invertebrates and macroalgae (Wahl, 1989),playing a key-rolein habitat selection by marine biota(Thompson *et al.*, 1998). Biofilms are precursors of complex and well-structured communities and have a relevant influence on the ecology of benthic communities. The interactions of the macrobiota with these microbial films lead, within days and weeks, to the attachment and growth of algaeand invertebrates, as their structure makes surfaces more apt to larval settlement and survival,

as many research state (Caribbean corals, Sneed *etal.,*2015; oysters, Tamburri *et al.*, 2008;barnacles, Thompson *et al.*, 1998; polychaetes, Hadfield, 2011 and Zardus *et al.*, 2008). The link between the biofilm presence and biota settlement is complex and include a multitude of processes. Firstly, biofilms are secondary biological substrate which modify physical and chemical properties of natural surfaces, at fine-scale, preparing the substratum for settlement of other organisms and providing additional resources for larvae and propagules. Biofilms may also modulate physical surface properties, such as wettability andfine-scale texture (Crisp & Ryland, 1960; Gray *et al.*, 2002), with possible consequences on larval pre-settlement behavior. Biofilms may provide chemical settlement cues, due to the production of metabolites, proteins and antibiotic compounds which advantage the settlement of some species rather than others and, finally, may increase the strength of larval attachment and their capacity to persist on a substratum under turbulent hydrodynamic conditions, typical of exposed intertidal shores. Biofilms may also affect post-settlement dynamics of macroinvertebrate from a variety of taxa, by reducing their mortality rate and improving the recruitment success.

Studies showed that these effects vary with general factors such as biofilm age, location, kind of the substrate and season of development, but often neither the biofilm organisms responsible for the influences on settlement (e.g. Todd & Keough 1994, Keough & Raimondi 1995, Wieczorek et al. 1996) nor the mechanisms involved in the increase of settlement rateare totally identified.

4.2 Aims of the research

This fourth chapter focuses on the microbial film as an additionalbiological cue which can stimulate *Dendropomacristatum*settlement.

The likely influences of the microbial community on the settlement and recruitment processes of the reef-builder *Dendropoma* spp. has never been detected before. This research aims to test if a mature and structured biofilm is a settlement cue for larvae of *Dendropomacristatum*and if settlers density increases on aged biofilms.

Larval settlement of *D. cristatum*on limestone cubes covered by biofilms of different age (and composition) was compared in the field during two separate experiments.

Additionally,the description of the microbiota community implicated into the settlement of *D. cristatum* and geneticcharacterization of the microbial assemblages will be showed in the chapter n.5.

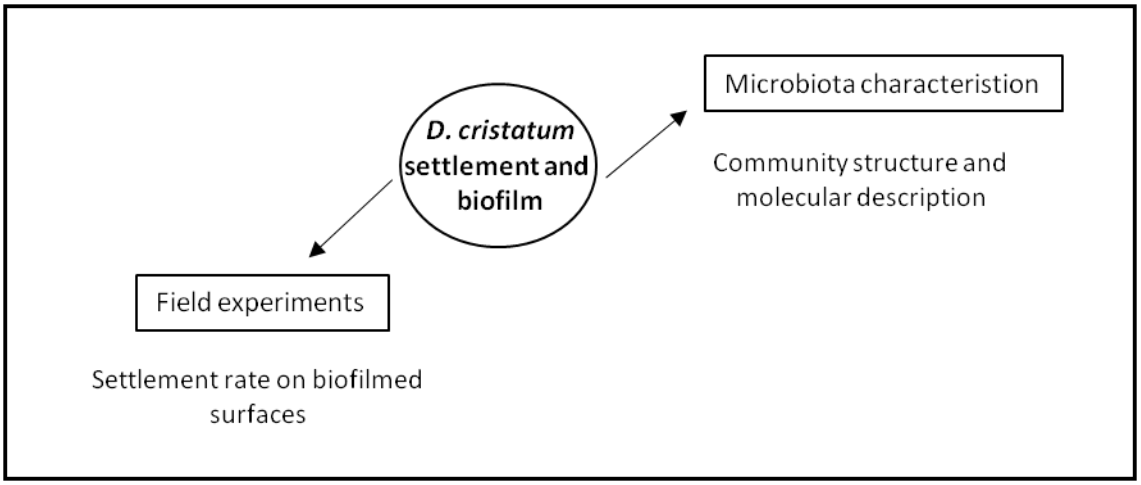

Figure 58, Schematic view of the approach to the study of the interactions between D. cristatum*settlement and the biofilm.*

The settlement rate of *Dendropomacristatum* was measured in the field on limestone cubes covered by biofilms with different characteristics.

Two experiments were performed:

a) in the first experiment (4.2 a)) the settlement rate was compared among biofilms at increasing maturity *vs* sterile substrata
b) in the second experiment (4.2 b)) the settlement rate of *D. cristatum* was compared among biofilms of different maturity and composition.

4.2 a) Settlement rate on biofilms at increasing maturity *vs* sterile substrata

This first experiment was carried out in the locality of Barcarello within the marine protected area of Capo Gallo-Isola delle Femmine (described at page 84 and 85).
In September 2015 sterilized limestone blocks of 5x5x2 cm were fixed on the external rim of a vermetid reef for 24 days, allowing the microbial film to colonize the cubes. During this pre-conditioning time, the cubes were wrapped in tulle fabric, avoiding *Dendropoma* larvae and other invertebrates to settle on them. The cubes were subsequently collected, handling them with sterile gloves to avoid biofilm contamination, the tulle was removed and were observed under the light microscope. No settlers of *Dendropoma* and other molluscs were found on the cubes.Pre-conditioned limestone blocks were randomly reallocated in two sites of the same reef, spaced apart 40 m, and coupled with an equal number of new sterile limestone cubes. The combination of 2 biofilmed blocks and two sterile blocks was repeated 5 times within both the sites.
The experiment was articulated in 3 temporal replicates within a 16-day period, as reported in the figure 59. For each temporal replicate one observation was done, to measure the density of *D. cristatum* larvae on biofilmed (preconditioned cubes) and control cubes (sterile cubes). Each observation was performed 4 days (4-d) later the beginning of each temporal replicate, allowing *D. cristatum* settlement to occur. At each observation control cubes were replaced by new sterile cubes. The sampling scheme is reported in the table below.

	Field experiment		
Pre-conditioning time	1° temporal replicate	2° temporal replicate	3° temporal replicate
24 days	28-days *vs* 0-days	32-days *vs* 0-days	40-days *vs* 0-days

Time (16 days)

Figure 59, Table n. 7: different experimental steps. After a period of pre-conditioning, biofilmed cubes were used for the settlement experiment which lasted 16 days. The number of settlers of D. criastatum was measured 3 times.

Figure 60, Biofilmed cube (left) vs *control cube (right) collected after the experiment.*

At each observation, the number of new settlers on biofilmed cubes was calculated as the total number of settlers minus the number of settlers recorded at the previous observation. Hence, just the number of new settlers at each observation was considered.

Subsequently, four chip surface for each biofilm treatment were collected and prepared for SEM observations (Hill & Hawkins, 1990), aiming to detect the biofilm structure.

Figure 61, The arrows indicate two settlers of D. cristatum *attached to the limestone cube. Magnification: 8x.*

Data analysis

After square root transformation, data were ordinated basing on the Euclidean Distance and analyzed with Univariated PerManova. Substrate type ("Su") and Time ("Ti") were treated as fixed factors, respectively with 2 and 3 levels (Biofilmed *vs* Control and T1 *vs* T2 *vs* T3); Site("Si") was a random factor with 2 levels (S1 and S2). The analysiswas performed on Primer v.6.

Results

Dendropoma cristatum settlement varied among treatments, being greatest on biofilmed cubes. Overal, 709 settlers were counted on all cubes, with the following spatial distribution: 195 in site 1 and 514 in site 2.The 76,44% of these settlers was found on biofilmed cubes and the 23,55% was counted on the control cubes (Fig. 62).

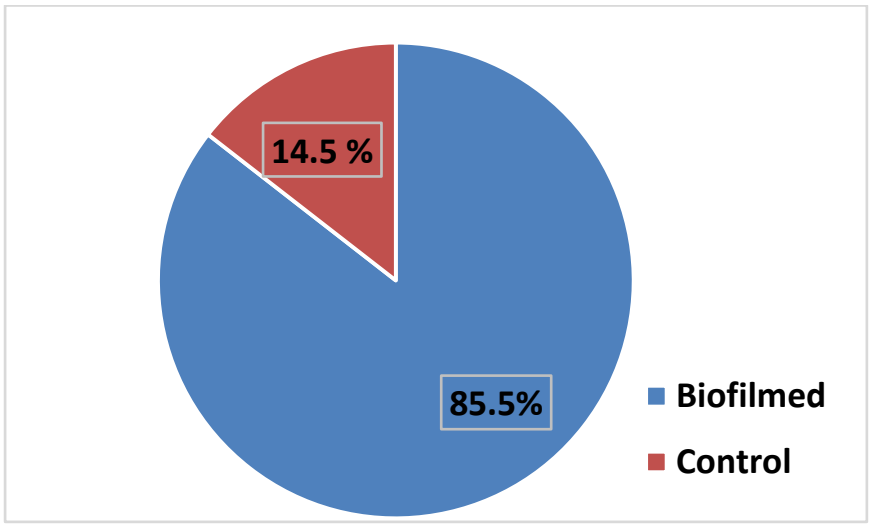

Figure 62, Overall percentage of D. cristatum *settlement on both the treatments.*

Hence, larvae showed a settlement choice for biofilmed surfaces and this preference was confirmed byall the 3 temporal observations (Fig. 63).

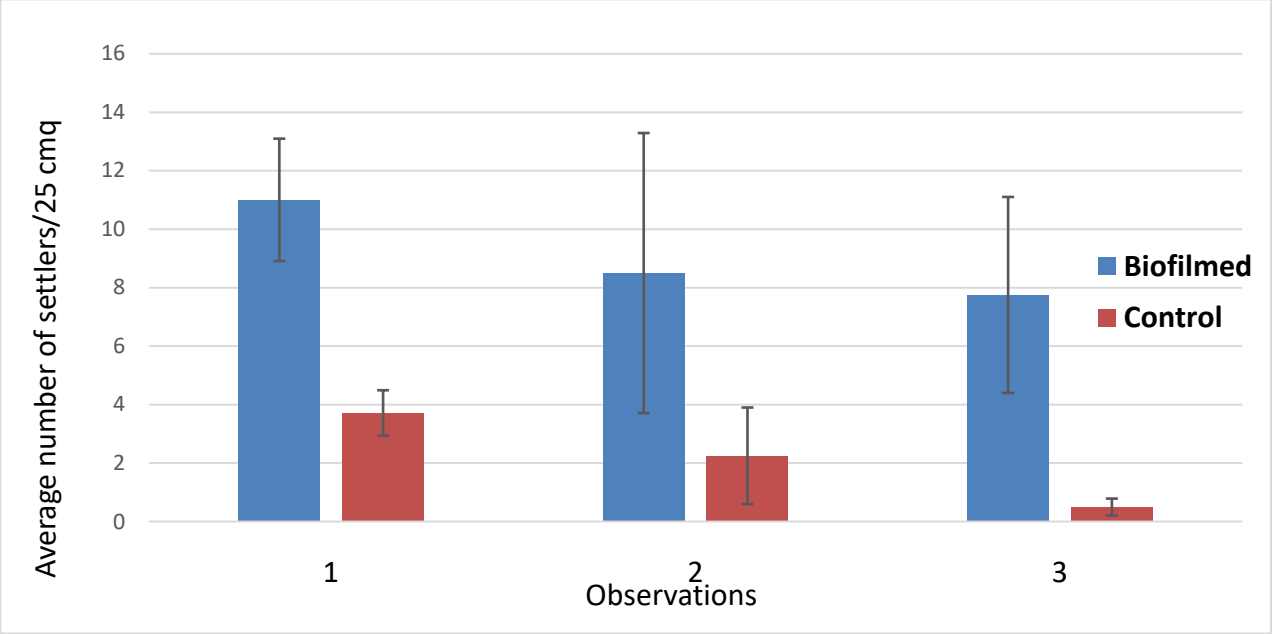

Figure 63, Comparison of the average number of settlers (±SD) among treatments.

However, settlement success on biofilmed limestone cubes did fluctuate over the time, while it was almost the same on control cubes (Figure 64).

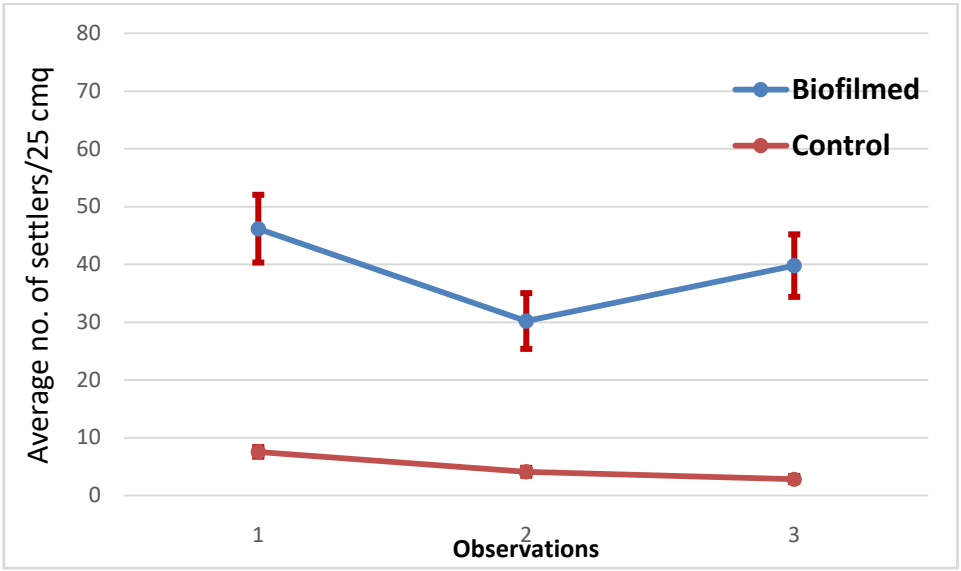

Figure 64, Temporal trend of the settlement success between treatments.

Within both sites the higher larval density was recorded on biofilmed cubes, although the average number of settlers was greater in site 2 (Figure 65, A and B).

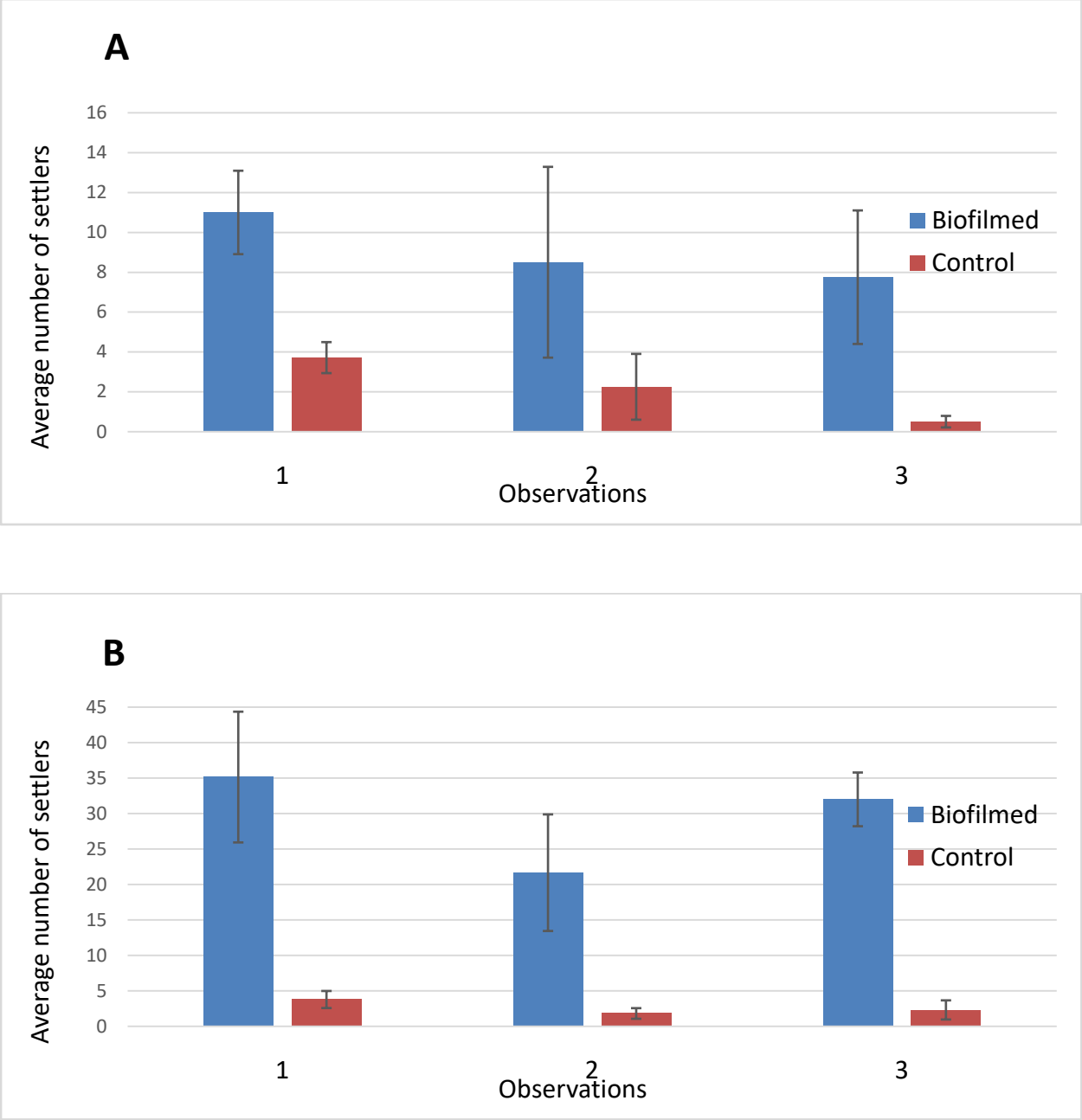

Figure 65, Average number of settlers (± SE) in site 1 (A) and site 2 (B), over the 3 observations.

Comparing settlement success among treatments, the number of settlers on cubes with a biofilm 28, 32 and 40 days old was respectively 6, 7 and 14 fold higher than that measured on control blocks (Fig. 66). The higher was the biofilm maturity the more it did attract *D. cristatum* larvae and enhanced their settlement. Within both the sites *D. cristatum* settlement was positively correlated to the biofilm maturity (r= 0,88 for site 1 and 0,95, for site 2,Fig. 67).

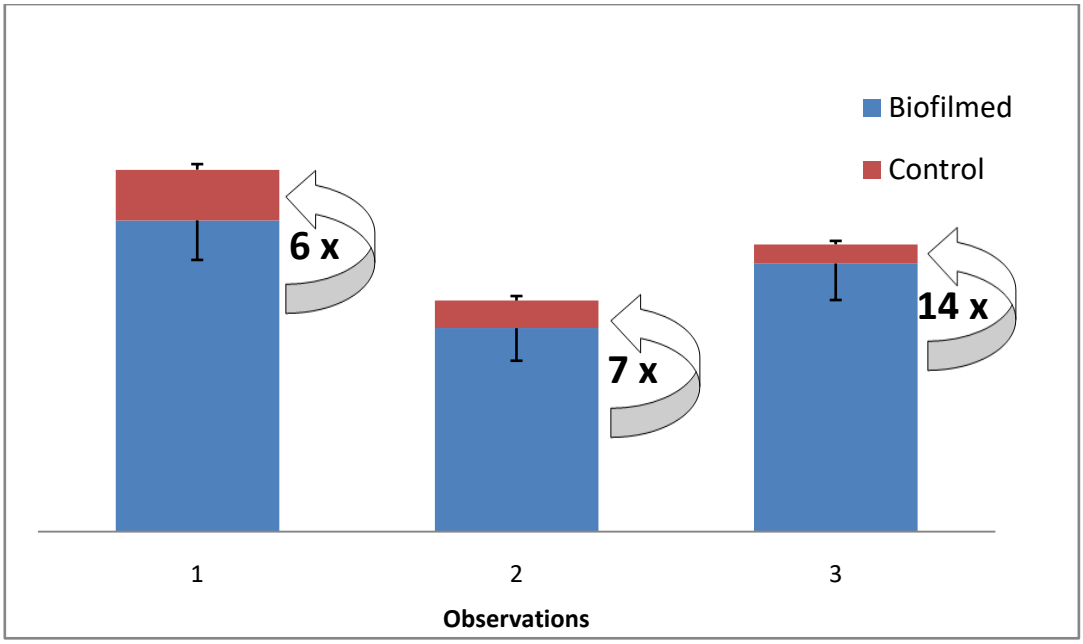

Figure 66, Comparison of the settlement rate among treatments and at increasing biofilm maturity.

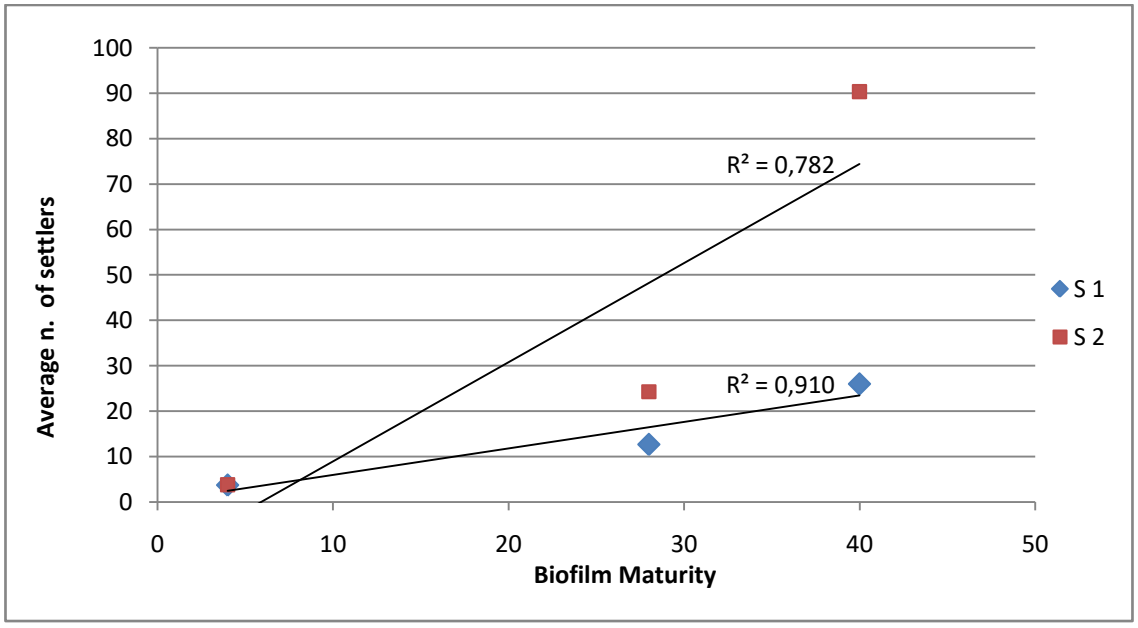

Figure 67, Linear regression between biofilm maturity and average number of settlers in sites 1 and site 2.

Permutational MANOVA revealed significant differences in the density of *D. cristatum* settlers among substratum treatments and study sites.

Source	df	MS	Pseudo-F	P(perm)
Sub	1	9.2909	36.663	**
Si	1	4.1655	48.665	*
Ti	2	0.13848	1.6487	ns
BixSi	1	6.49E-02	0.52026	ns
BixTi	2	0.19348	1.5675	ns
SixTi	2	8.40E-02	0.30315	ns
BixSixTi	2	0.12343	0.44546	ns
Res	47	0.27709		
Total	58			

*Figure 68, Tablen. 8: PerMANOVA analysis. The analysis includes 3 factors: "Sub", Substratum, (Biofilmed vs Control); "Si", Site (Site 1 and Site 2); "Ti", Time (T1 vs T2 vs T3). Measured variable: n. of recruits on 25 cm^2. *= p≤0,05; **= p≤0,01; ***= p≤0,001; ns= not significant.*

Scanning electron microscope was used to detect the structure of the biofilm on limestone cubes and its physical composition. Some bacteria,diatoms, cyanobacteria and severalunidentified organisms embedded within a well developed EPS were observed.

On biofilmed cubes the structure ofthe biofilm was patchy, including alsonon-colonizedareas.

Bacteria were rarely grouped andbiofilm was not exclusively done by prokaryotes. A variety of diatoms was observed, also making colonies rather than assingle cells.

The pictures confirmed the presence of bacteria within the biofilm, although organisms of higher size were mostly represented.

On control cubes, only few pionering organisms were observed (Figure 69).

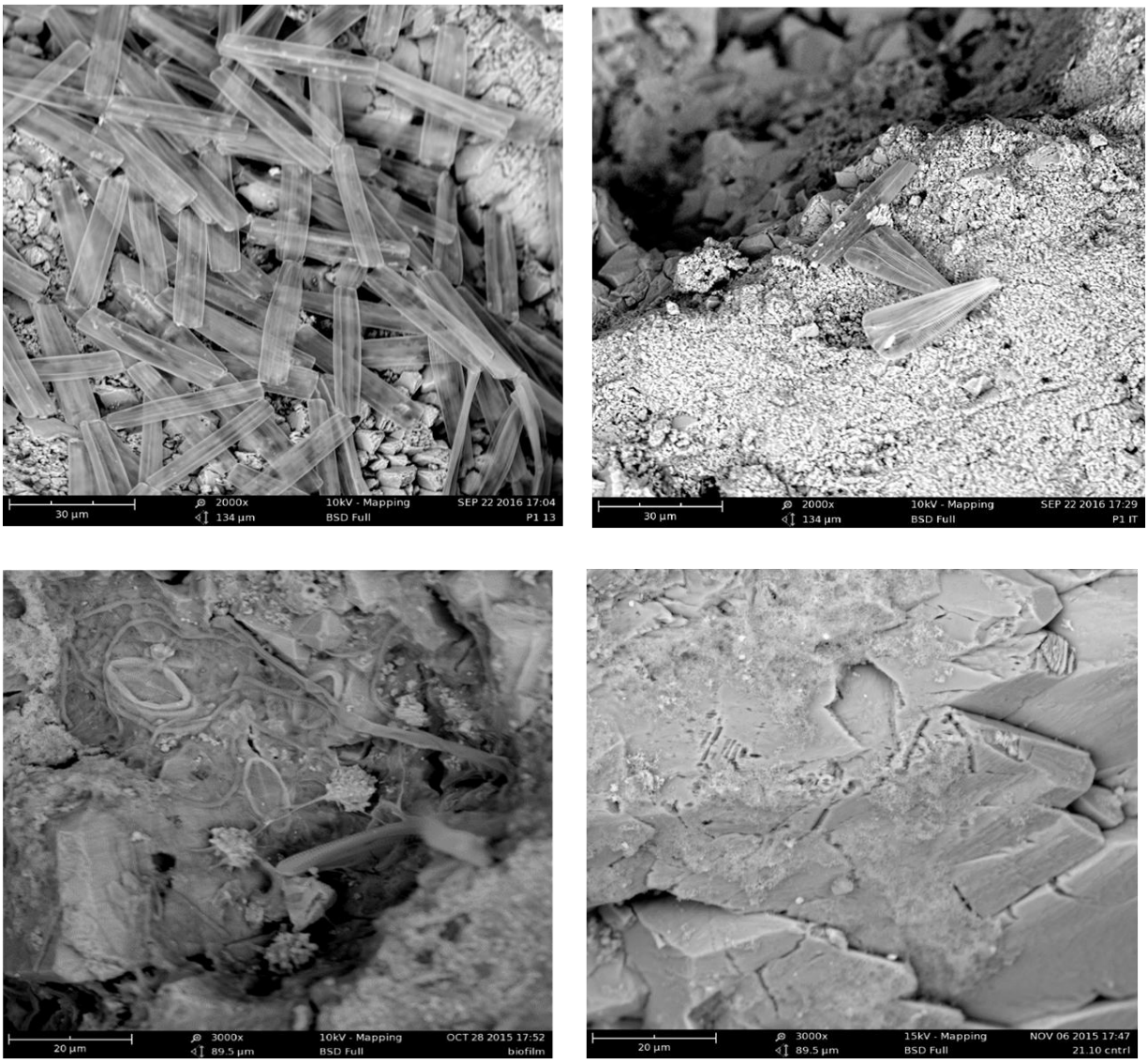

Figure 69, SEM images of biofilmed cubes (left) and control cubes (right). Magnification: 3000X.

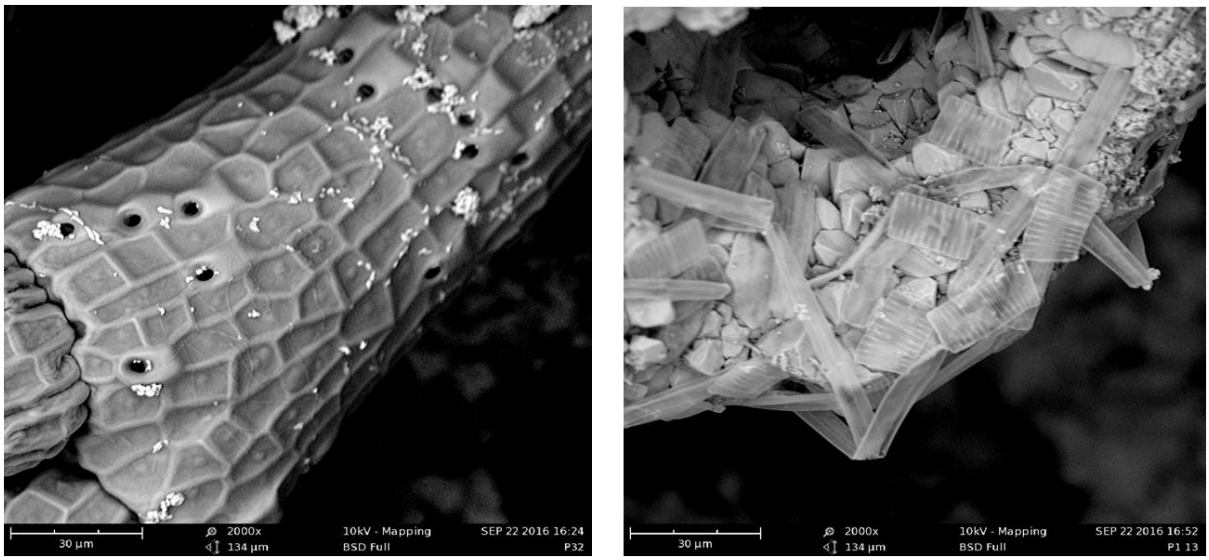

Figure 70, Bacterial cells (left) and diatoms with different morphologies (right) on biofilmed cubes. Magnification: 3000X.

Discussions

Relatively few authors have assessed the contribution of microbial films to the settlement of marine invertebrate on natural rock surfaces and in natural conditions (Thompson et al., 1998). Furthermore, studies regard the settlement dynamics and biofilm presence, have frequently been conducted using artificial substrata such as plastic dishes or glass microscope slides (e.g. Todd & Keough, 1994) which have different surface properties from those of natural substrate and this may considerably influence settlement patterns (Mihm et al., 1981; Wahl, 1989; Henschel et al., 1990). In this study, the high number of *Dendropoma cristatum* larvae settled on limestone cubes (709 in total within a period of 16 days) has demonstrated this material as suitable for the settlement of *D. cristatum* larvae. This allowed to evaluate*D. cristatum* settlement response on a substratum having mineralogical properties similar to the rock the larvae find in their habitat and to consider the observed larval behaviour as representatives of the natural *D. cristatum* settlement mechanism. Moreover, it is possible exclude that *D. cristatum* settlers were attracted by conspecific adults because they were not present on the limestone cubes. By contrast, the occurrence of previously settled larvae at the 2° and 3° observation does not exclude the influence of density-dependent mechanisms on the settlement rate, due to the gregarious

behaviour of this species. However, the proximity of conspecifics may be unimportant at a scale of a few centimetres (within the limestone cube) and at this level of habitat selection the presence of a microbial film should providesa greater stimuli (Thompson et al., 1998).

The settlement results clearly describe that larvae of *Dendropoma cristatum*choosed to settle on limestone blocks with a mature biofilm respect to control cubes and this preference is enhanced by biofilm maturity (see the figure 66). Moreover, settlement rate greatly differed among sites, being significantly higher in site 2 and showing a spatial variability of this biological process at a scale of less than 50 m and within the same reef. Although the occurrence of this site-scale difference, both the sites showed the same pattern of settlement and temporal trend (see thefigure 65).

D. cristatum larvae were capable to percept stimuli provided by some characteristic of the microbial community which had an increasingly effect on settlement dynamic according to the biofilm maturity.The number of settlers on 28- and 32-d biofilmed cubes was respectively 6 and 7 fold higher than those on the control cubes, and 14 fold higher on 40-day old biofilm (Figure 66). This could mean that a highly structured and developed biofilm is a resource that *D. cristatum* larvae needs for their settlement and during the early post-settlement phases.

Hence, habitat selection by crawling larvae of *D. cristatum* was influenced by the biofilm maturity, according to other studies which well documented that the settlement of a multitude of marine benthic invertebrate considerably enhances on highly developed microbial communities, such as barnacles, corals, polichaete, including also molluscs, such as oysters, limpets, mussels.Possibly, the differences in the composition of the microbial community among the temporal observations during which larvae density on limestone cubes was estimated, provided a cue which increasingly triggered settlement with the film maturity.

Microbiological study of the biofilm will be approached later in this manuscript, with a description of the community structure and metagenomic analysis of natural reef-associated biofilm and limestone cubes- associated biofilm.

In order to further understanding the role of the biofilm age in the settlement mechanism of *D. cristatum* and to strengthen conclusions about the improvement of settlement dynamic by the biofilm, in the following experiment the settlement rate of thevermetidaeis compared on a range of different film types.

4.2 b) Settlement rate of biofilms of different maturity and composition

Given the different settlement performance based on the biofilm maturity previously showed, in this experiment 6 different typology of biofilm were simultaneously tested as settlement cues for larvae of the Sicilian vermetidae*D. cristatum*.Biofilm treatments included in the experiment were: 0-d, 13-d, 23-d, 32-d old biofilm and two communities artificially cultured in the laboratory.The experiment was conducted in July 2016, at the locality of Punta Raisi (described at pages 92 and 93).

Methods

Limestone cubes (5x5x2 cm) were prepared as described in the previous experiment. The cubes were pre-conditioned for a different time, according to the biofilm treatment. Firstly, cubes for the 32-d old treatment were placed in the field. After 9 days and after 19 days cubes for the 23-d and 13-d old treatments were also deployed. All cubes were collected toghether and sorted under the microscope to check *D. cristatum* larvae or other mollusc presence on them.

Simultaneously, two bacterial community were prepared in the lab: a community cultured from a vermetid reef of Punta Raisi and a community composed by 4 isolates identified from this natural biofilm assemblage, by the sequencing the 16s gene: *Alteromonas genovensis, Vibrio* spp, *Cellulophaga lytica*, *Pseudoalteromonas* spp. These bacterial genus are knew to have implications in the settlement of benthic invertebrates. Both the communities were cultured on marine agar plates. For each community an inoculum was prepared by picking each community within 3 ml of marine broth and incubation at 30° overnight. Two culture broths were prepared by adding the inoculi in flasks with 500 ml of marine broth and 500 ml of autoclaved marine water. Limestone cubes were incubated in the culture broths for 48 h.

Overal, 96 limestone cubes were used for the experiment: for each biofilm treatment 16 replicates were equally distributed within two sites spaced apart approximately 100 m.

After 5 days all the cubes were collected and *D. cristatum* larvae settled on each cube was counted under the microscope. Unfortunetely, 7 cubes weredestroyed.

Subsequently, two chip surface for each biofilm treatment were collected and prepared for SEM observations(Hill & Hawkins, 1990), aiming to detect the biofilm structure.

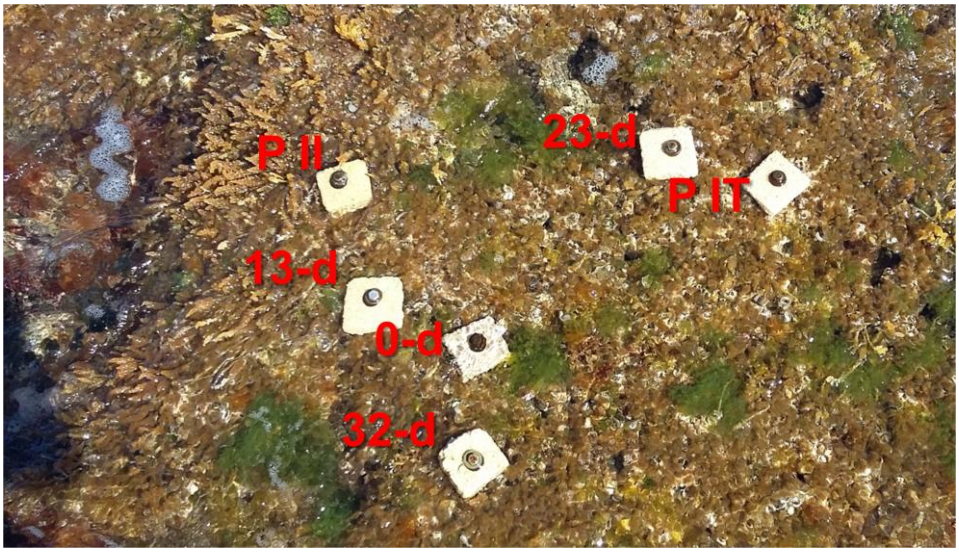

Figure 71, Complete set of biofilmed cube treatments during the settlement experiment.

Data analysis

Settlement data were not transformed for the statistical analysis and were ordinated basing on the Euclidean Distance, a dissimilarity coefficient appropriate for abundance data. Univariated PerMANOVA was performed with two factors: “Sub” (Substratum) treated as fixed factor with 6 levels: 32-d, 23-d, 13-d, 0-d, II and IT, and “Si” (Site), a random factor with 2 levels: S1 and S2. A posteriori pair-wise comparisons among all pairs of levels within factors that showed significant differences were also done.

Analysis were performed on Primer v.6.

Results

Overall, 433 *D. cristatum* settlers were counted on all the cubes, 223 in site 1 and 210 in site 2. The percentage settlement success varied according to the biofilm type and data are shown in the graph below:

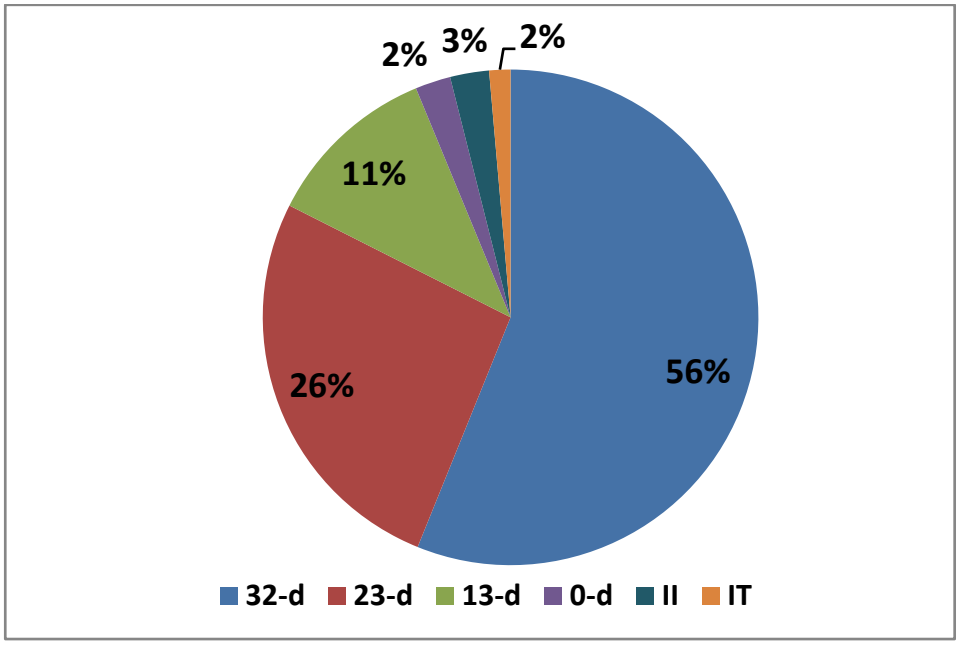

Figure 72, Percentage settlement success on biofilm types.

Settlement success increased with the maturity of the biofilms and artificially prepared biofilms did not promoted *D. cristatum* attachment, as well as the 0-d biofilm (Fig. 72).

The average number of settlers on each treatment did not vary among sites and the settlement rate progressively enhanced with the biofilm maturity (Fig. 73). In both the sites a positive significant correlation was found between density of *D. cristatum* larvae and biofilm maturity (Fig. 74).

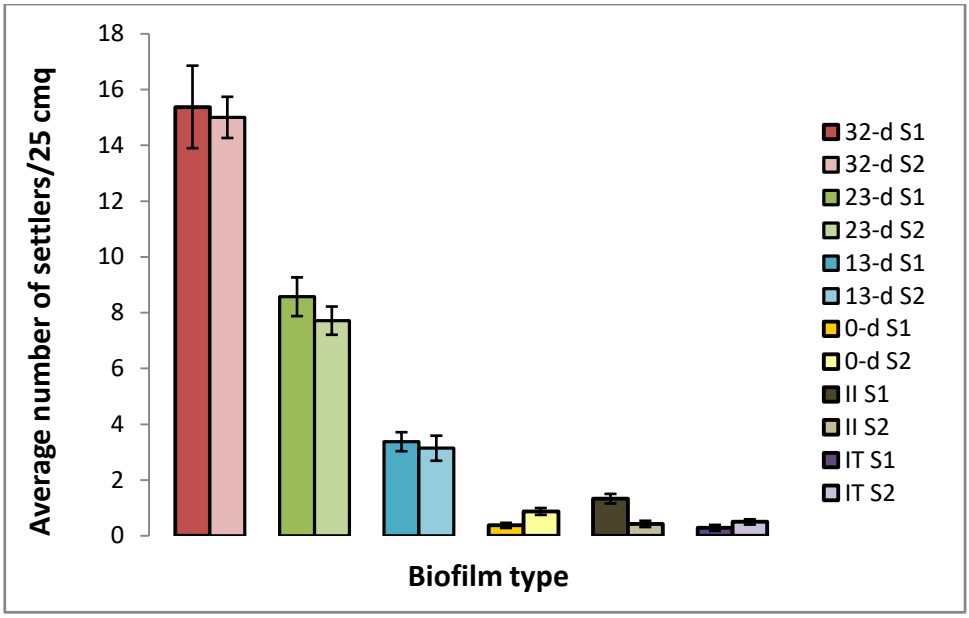

Figure 73, Average number of settlers (±SD) within both the experimental sites and for each tested treatment.

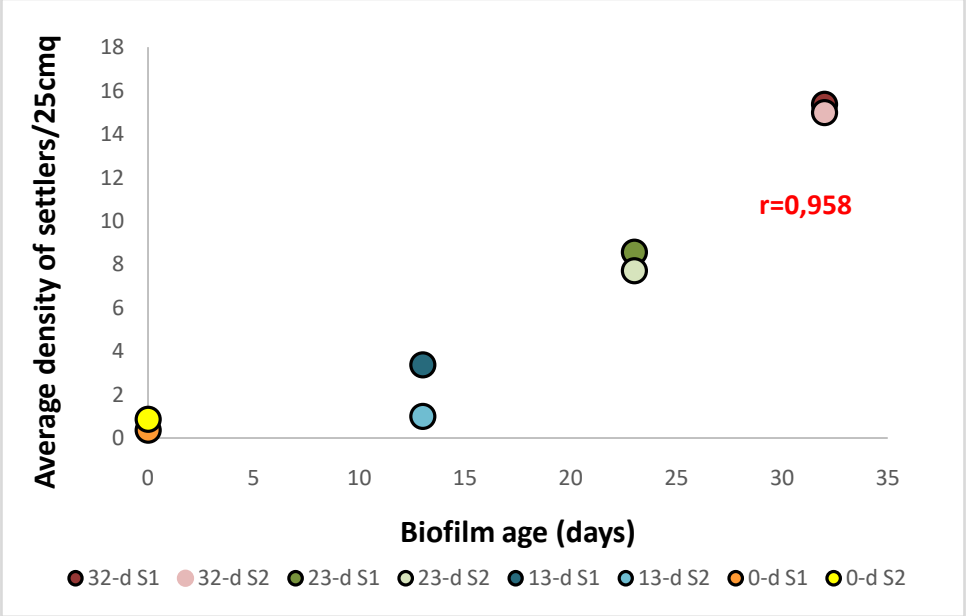

Figure 74, Correlation between the biofilm maturity and the average density of D. cristatum *settlers within both the sites.*

Permutational MANOVA showed highly significant differences among treatments within the factor Substratum, with a p value = 0.0005.

Differences among pairs of levels within the factor “Sub” were further analysed by pair-wise comparison and p values are reported in the table below.

Source	df	MS	Pseudo-F	P(perm)	Pair-wise comparisons	
Sub	5	510,72	60,203	***	**Substratum**	**Sign.**
Si	1	17,346	0,8303	0,3695	32-d vs 13-d	**
BixSi	5	8,4833	0,40606	0,8502	32-d vs 0-d	***
Res	77	20,892			32-d *vs* II	**
Total	88				32-d *vs* IT	***
					23-d *vs* 13-d	*
					23-d *vs* 0-d	***
					23-d *vs* II	***
					23-d *vs* IT	***
					13-d *vs* 0-d	**
					13-d *vs* IT	**

Figure 75, Table n 9: PerMANOVA analysis of the D. cristatum *settlement success for the factor “Sub” (Substratum, fixed with 6 levels) and “Si” (Site, random with 2 levels) and pair-wise comparison for pairs of levels within the factor substratum. *= p≤0,05; **= p≤0,01; ***= p≤0,001; ns= not significant.*

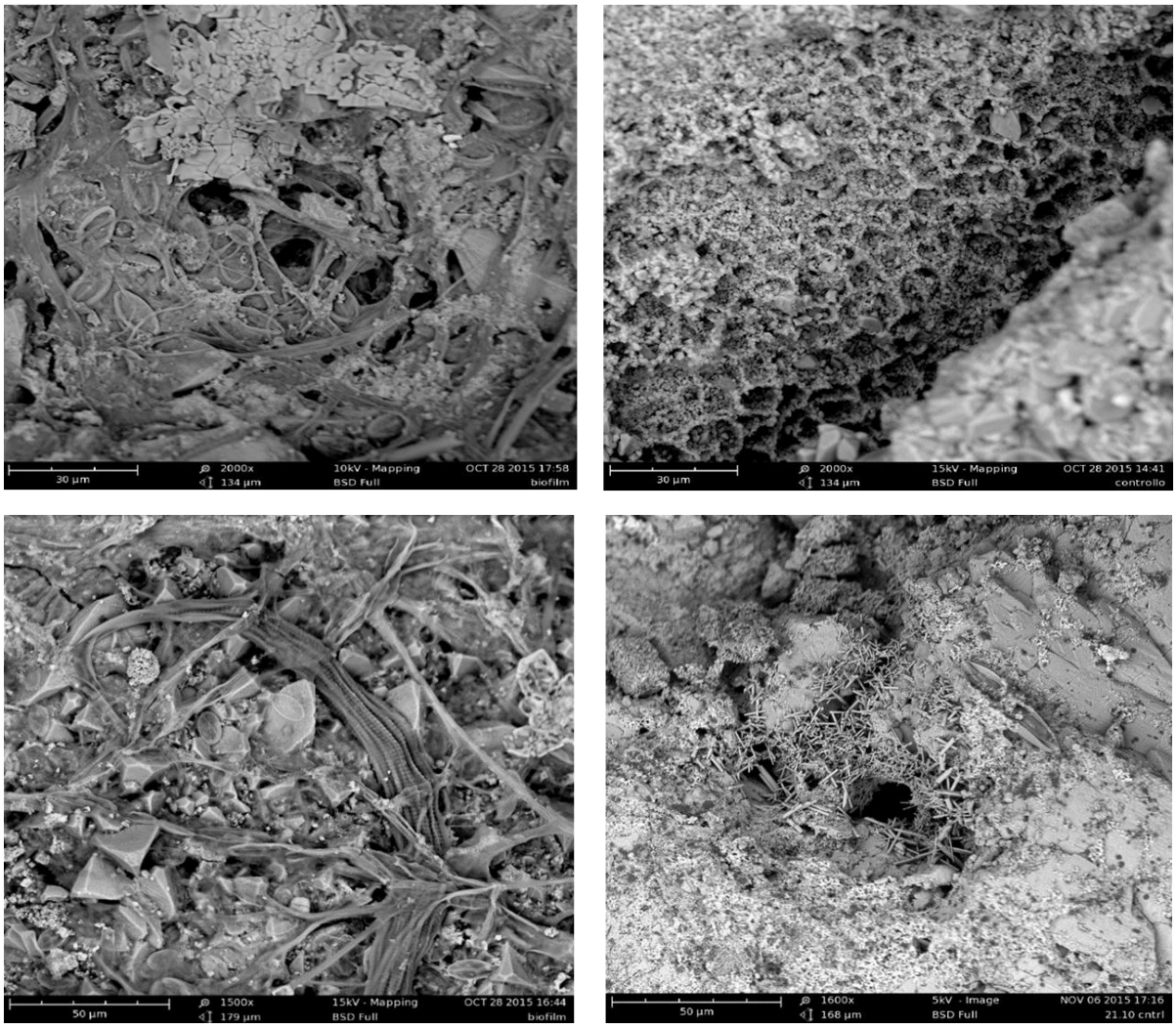

Figure 76, Scanning electron microscope immages of a 32-d (left) and 0-d biofilm (right) at 2000 x and 1500x of magnification, respectively above and below.

4.3 Discussions

Also in this second experiment, settlement dynamic of *Dendropoma cristatum* is strongly influenced by the biofilm occurence and maturity.

Settlement success is on average 5, 13 and 24 folds higher on 13-d, 23-d, 32-d biofilms compared to a 0-d biofilm and II and IT treatments. Settlement rate on lab cultured biofilms, II and IT, is probably so lower because the artificial biofilms did not have enough time to firmly develope a layer on the limeston cubes, as was showed by SEM immages. Moreover, both the sites showed similar numbers of larvae, indicating low variability of settlement at site-scale.

The settlement intensity of *D. cristatum*depended on biofilm characteristics related to its maturity. The influence of biofilmage on larval behaviour is reported to be quantitative since biofilm age is also linked to the densities of bacteria and diatoms (Rahim *et al.*, 2004; Bao *et al.*, 2007a). Microbial community composition, indeed, is known to changes over the time, with early biofilms less structured and reach in prokaryotes and diatoms and mature films made by diverse microbiota with dyatoms, cyanobacteria and CCA included within a well developed EPS which stabilise the biofilm.

This correlation between settlement success and biofilm maturity is consistent with findings from the research on the abalone *Haliotis australis* (Moss, 1999), which showed the highest settlement rate and post-settlement survival on diatom biofilms and mucous trail coated surfaces. Bacteria were demonstrated to be relevant source of food for the newly settled larvae of abalone and muco-polysaccharides were the initial energy source after their settlement (Kawamura & Takami 1995;Daume *et al.*, 1997; Kitting & Morse 1997). Moreover, abalone larvae have been observed to ingest extra-cellular algal secretions with their radula and *D. cristatum* larvae might do the same with their radula, once they have totally comsupted their yolk sources and before the development of the feeding tube.

An increase in settlement rate of *Mytilus edulis*with biofilm age was also found by Toupoint et al. (2012) and was explained by the quality of the biofilm, which could be an important source of food for settling larvae.

On the other hand, biofilms are known to produce waterborne or substratum associated compounds which interact with the settlement of benthic invertebrate. According to the study of Zhao *et al.* (2002) natural biofilm has a very rapid and strong effect on larval

settlement and metamorphosis of the limpet *Crepidula onyx* and the settlement cue seems to have a surfaced-associated characteristic.

Moreover, compounds released by the biofilm matrix may have a role in the deposition of biological cement that larvae produce for their attachment to the substratum.

SEM images provided the evidence that oldest biofilms had a higher 3-dimensionality and density of organisms, greatly modifying the characteristics of the limestone cubes,even though these features have not been quantified, The higher is the complexity of the microbial layer, the more it may attract the larvae, compared to biologically simpler substrata.Maybe a highly structured biofilm represents a most appropriate micro-habitat for *D. cristatum* larvae. The cell density of fouling communities, indeed, may serve as a cue which invertebrate larvae use to select habitat (Lau *et al.*, 2005).

Moreover, the microbial community structure is function of the biofilm age, since dominance in species (Wieczorek & Todd, 1997) and the metabolic activity change according to the temporal biofilm evolution (Satuito *et al.*, 1995). Since bacterial species are reported to influence larval responses (Ganesan *et al.*, 2010), changes in bacterial communities may modulate the intensity of *D. cristatum* settlement. This observation needs to be accompanied by molecular analysis of the biofilm, further considered in this manuscript.

CHAPTER 5.
Biofilm associated to the vermetid reef and limestone cubes: description

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Biofilm associated to the vermetid reef and limestone cubes: description

5.1 Introduction and aims

Microbial analysis wereperformed to detect the structure and the composition of the reef-associated biofilm, here described for the first time, and of the limestone cube-associated biofilm.

A combination of methods was used, in order to describe the microbial community by an integrated approach and aiming to detect the presence of prokaryotic species related to settlement process and to reveal differences and putative relationships among the assemblage structures between biofilms which showed a different success of *Dendropoma cristaum* settlement.

Microbial ecology studies the function, composition, structure and the ecological role of microbial communities, assemblages which are at the base of each ecosystem and have implication in a variety of processes and mechanisms, such as: energy flow regulation, biochemical cycling, ecological resilience of a system, nutrient availability, bioremediation, biological mechanisms (such as the case of the settlement of marine invertebrates).

The study of a microbial assemblage starts from three main aims: to detect which kind of microorganisms are present in the system; what these microorganisms do; how the activity of these microorganisms is related to the ecosystem functioning (Rastogi & Sani, 2011).

All of the approaches available for the analysis of microbiota structure and functioning have advantages and limitations and none provides complete access to the genetic and functionaldiversity of complex microbial communities. A combination of several techniques is the best way to investigate the diversity, functionality and ecology of microorganisms. With this aim, culture-based and culture-independent molecular techniques should be considered as complementary.

In this research,culture-based and culture-independent methods of analysis were adopted to describe the reef-associated biofilm, which is here described for the first time, while the limestone cube associated biofilms were described only by a culture-independent approach.

A) Culture-dependent approach

Culture-dependent techniques to describethe microbiota involve the cultivation of microbial strains on appropriate growth medium, designed to maximize the microbial growth and the isolationand phylogenetic characterization of the revealed microorganisms (Kirk *et al.*, 2004). This is a quite inexpensive approach, with the major limitation that >99% of the microorganisms in any environment observed through a microscope are not cultivable by standard culturing techniques (Hugenholtz, 2002).

However, this method may give useful information about the presence of ecologically relevant members of the microbial community and permits to cultivate under lab conditions those microorganisms of interest for the research and to further study their metabolic and physiological traits.

The identification and classification of cultured microorganisms are based on the sequencing of highly conservative genomic regions.

The 16S rDNA is a highlyconserved subunit of the prokaryotic ribosomes and it has been proposed as an “evolutionary clock”, which has led to the reconstruction of the tree of life (Woese, 1987). The nucleotidic sequence of this small subunit is a universal sequence among bacteria, having highly conservative regions (5’-3’), which include hypervariable regions.The sequencing of highly conservative regions allows the identification up to the level of generaand is used in the case of phylogenetic identification of isolated pure cultures.

Analysis of hypervariable regions bases on DNA sequence variations present in PCR-amplified 16S rRNA genes and Amplified ribosomal DNA restriction analysis (ARDRA) is one example among theses techniques.

In this analysis, the PCR product amplified from cultured DNA is generally digested with tetracutter restriction endonucleases (e.g., *Alu*I, and *Hae*III), and restricted fragments are resolved on agarose or polyacrylamide gels. Although ARDRA provides little or no information about the type of microorganisms present in the sample, the method is useful for rapid monitoring of microbial communities over the time, or to compare microbial diversity in response to changing environmental conditions(Rastogi & Sani, 2011). ARDRA is

also used for identifying the unique clones and estimating OTUs in environmental clone libraries based on restriction profiles of clones (Smit *et al.*, 1997).

B) Culture-independent approach

Given that more than the 99% of the microbes presentin the environment are known to be not culturable, the only application of culture –dependent techniques would be a highly reductive approach to describe the biofilm assemblages. Hence, in addition to culture-dependent approach, a combination of culture-independent techniquesis oftenadopted.
Culture-independent approaches include analyses of whole genomes or selected genes such as 16S and 18S rRNA (ribosomal RNA) for prokaryotes and eukaryotes, respectively.
A wide variety of molecular techniques has been developed for describing and characterizing the phylogenetic and functional diversity of microorganisms. Overall, these techniques have been classified into two major categories depending on their capability of revealing the microbial diversity structure and function: partial community analysis approaches and whole community analysis approaches.

In this chapter, a combination of both the approaches was chosen as the best way to characterize the composition of the different biofilm assemblages considered in the settlement experiments and the reef-associated biofilm.
Firstly, the community structure was detected by a genetic fingerprinting method and, after, themetagenomic analysis was performed.

The fingerprinting analysis and the metagenomic analysis performed in this study, are describedbelow.

- **The Fingerprinting analysis: ARISA technique**

Genetic fingerprinting analysis are microbiological techniques used to describe the prokaryotic phylotype richness and community composition in a rapid and accurate way. These techniques are increasingly utilized in ecological studies to estimate the number of bacterial taxa and to investigate spatial and temporal dynamics of both terrestrial and aquatic microbial assemblages (Luna *et al.*, 2004; Schwalbach *et al*., 2004; Winter *et*

al.,2004).Fingerprinting techniques involve DNA extraction of theenvironmental samples, amplification of a target usuallylocated within the ribosomal operon using PCR and electrophoreticanalysis to separate the mixture of fragments.

These techniques include DGGE/TTGE, SSCP, RAPD, ARDRA, T-RFLP, LH-PCR,RISA, and RAPD and produce a community fingerprint based on either sequencepolymorphism or length polymorphism.The "fingerprints" from different samples are compared using computer assisted cluster analysis by specific software packages (Rastogi & Sani, 2011).

The Terminal Restriction Fragment Length Polymorphism (T-RFLP) and the Automated Ribosomal Intergenic Spacer Analysis (ARISA),are recognised to be more sensitive thanother fingerprinting methods, such as the Denaturing Gradient Gel Electrophoresis (DGGE) or single-strand conformational polymorphismanalysis and are able to reveal the presence of less abundant taxa within the bacterial community (Hewson & Fuhrman, 2004).

Among the techniques used currently to assess the microbial diversity of aquatic communities (Dorigo et al., 2005), ARISA has proved to be a powerful tool, mostly for itssimplicity and rapidity (Danovaro et al., 2006; Hassenrück et al., 2015; Crosby & Criddle, 2003). Furthermore, the ARISA is a very effective and sensitive method for detecting differences betweenbacterial communities at various spatial scales (between- and within-sitevariability), and this makes this technique very suitable for the aim of this research.

ARISAis an improved version of RISA technique and is a rapid and reliable method to assess and compare the structureof microbial communities, especially useful at the fine spatial and temporal scales necessary in ecological studies.This method provides estimates of species diversity and community composition without the biasimposed by culture-based approaches. ARISA was first developed as anautomated version of the RISA technique on freshwaterbacterioplankton (Fisher & Triplett, 1999) and has been applied to various types of communities, such as soil samples (Ranjard *et al.*, 2001) and, more recently, aquatic biofilms (Lear et al., 2008, 2009). Due to automation, is one of the most convenient fingerprinting techniquesto analyze and compare large numbers of samples in a short time, especially if the analysis is performed by a capillary electrophoresis bioanalyzer, which enables ultrafast processing of numerous samples (Qu et al., 2009).

The method involves PCRamplification from total bacterial community DNA of the intergenicregion between the small (16S) and the large (23S) subunitrRNA genes in the rRNA operon, the ITS1 region, which is characterized by a significant variability in the length and

nucleotide sequence among different bacterial genotypes (Daffonchio et al., 2003; Fisher & Triplett, 1999). Both types of variation have been extensively used to distinguish bacterial strains and closely related species (Aubel et al., 1997; Maes et al., 1997; Scheinert et al., 1996; Jensen et al., 1993; Navarro et al., 1992). However, within the bacterial genome, the rRNA operon may be present in several copies (from 1 to 15), depending on the species (Klappenbach et al., 2001). These operons may have 16S-23S intergenic regions of different lengths and sequences. In this case, a single species will produce more peaks in the ARISA electropherogram, thus leading to an overestimation of the richness of bacterial taxa. In this regard, recent studies on marine bacteria have reported a low intragenomic heterogeneity among multiple rRNA operons from single organisms (Brown et al., 2005), suggesting that biases in the ITS analysis deriving from multiple operons may be negligible (Danovaro *et al.*, 2006).

The DNA amplification occurs by using fluorescently tagged oligonucleotideprimers, targeted to conserve regions in the 16S and 23S genes. The length heterogeneity of the intergenic spacer isexploited and the electrophoretic step issubsequently performed with an automated system, which provideslaser detection of fluorescent DNA fragments. ARISAPCRmay generate DNA fragments up to 1,400 bp in length (Borneman & Triplett, 1997). Discrimination of these larger size fragments represented a new application for the capillary electrophoresis system employed.

The obtained electropherograms allow quantification of the number, size, and relative abundance of the different members of the bacterial assemblages (Yannarell & Triplett, 2004).

- **The whole community analysis: Metagenomic technique**

Whole-community molecular analysisoffersa complete view of the genetic diversity compared to PCR-based molecular approaches that target only a single or few genes. Exploring microbial assemblages through the analysis of the whole communityis an integrated approach to understanding microbial ecology (Rastogi & Sani, 2011) and is crucially used to reveal the identity of uncultured microorganisms. These techniques attempt to analyze all the genetic information present within the DNA extracted from an

environmental sample andincludes: metagenomics, proteogenomics, metaproteomics, metatranscriptomics.

Metagenomics, defined as the culture-independent genomic analysis of an assemblage of microorganisms (Riesenfeld *et al.*, 2004),provides a direct identification of genetic material from environmental samples and does not rely on cultivation or prior knowledge of the microbial communities (Riesenfeld *et al.*, 2004).

In this study, the DNA characterization of the environmental microbiota was based on the amplification and sequencing by a large-scale sequencing technique, the Next-generation Sequencing (NGS)basing on Illumina platform, which gives a complete information of the phylogenetic composition and functional diversity of the considered microbial communities, at low cost and with high speed(Zwolinski, 2007).NGS sequencing considers hypervariable regions of 16S rRNA genes and offers two to threeorders of magnitude higher coverage of microbial diversity than typical Sangersequencing of a few hundred 16S rRNA gene clones. Genomic hypervariable regions between 100–350 bases are targeted and the nucleotidic sequences are recognized bypyrosequencing techniques, which has the advantage to analyze multiple environmentalsamples in a single run.

This approach was chosen to obtain a precise and global view of the phylogenetic diversity of the examined biofilms, aiming to reveal a list of all the components of each treatments and of the natural reef-associated biofilm and to consider which among the revealed prokaryotes may have promoted the higher rate of *D. cristatum* settlement, as responsible for larvae choice.

5.2 Methods

Biofilm assemblages associated to the reef and developed on the limestone cubes used for both the settlement experiment described in the chapter 4 were detected by culture-dependent and culture-independent approach. Biofilm samples are named with an acronyme which refers to the maturity of the biofilm (such as: 0-d, 13-d, 23-d, 32-d and 28-d, 32-d and 40-d), to the locality of origin (P is used for Punta Raisi and B for Barcarello) followed by a number which indicates the site within each locality (1 or 2).

a) Culture-dependent approach

Culture-based techniques were used as the first approach to describe the reef-associated biofilm.

The reef-associated biofilm from the locality of Punta Raisi was cultured on marine agar plates at room temperature for 48h. After two days a dense microbial growth occurred and 25 phenotypes were identified by microscope observation of the bacterial colonies. Colony identification was based on the phenotypical characteristics of each strain and each phenotype was isolated in pure culture.

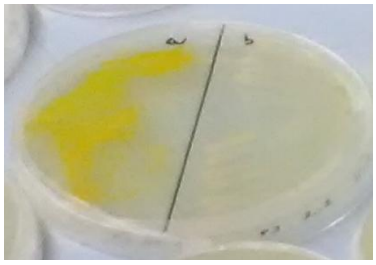

Figure 77, Marine agar plate with two different colonies (a and b) cultured from the vermetid reef.

The 16S rDNAs of the 25 strains wereamplified by PCR (Polymerase chain reaction) with Pr 27F (5'AGAGTTTGATCMTGGCTGAC3') and Pr 1492R (5'TACGGYTACCTTGTTACGACTT3') as a couple of primers.

PCR mixture (30µl) contained: Buffer OneTaq 1x; dNTPs 0,2 mM; 0,2 µM of Pr 27F and Pr 1492R; One Taq 0,025U/ µl.

PCR was performed with the program 1492R, as follows: one cycle consisting of 94° for 30 sec.; 30 cycles consisting of 94° for 30 sec, 50° for 1min, 68° for 1,5 min; one more cycle consisting of 68° for 5 min.

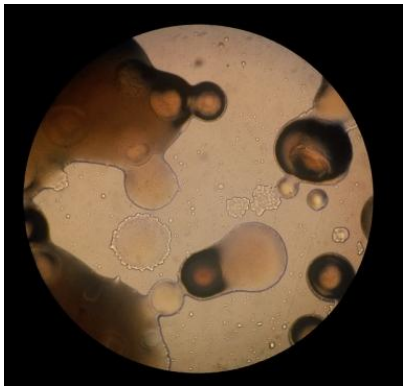

Figure 78, Bacterial colonies with different phenotypical characteristics under the microscope.

The PCR products were visualized by agarose gel electrophoresis (Sambrook et al., 1989) and wasfollowed by

the ARDRA analysis (Amplified Ribosomal DNA Restriction Analysis), with Afa1 (New England Biolabs) as restriction enzyme and electrophoresis in agarose gel 1,5% to compare the gene profiles among phenotypes.

The 25 profiles were grouped within 5 OTU (Operational Taxonomic Units) and one representative for each OTU was chosen for the sequencing.
Phylogenetic identification was made by comparing the determined sequences of each OTU to those provided by the databank BLAST n, through the programs Megablast and Ribosomal database Project. Both the programs showed assimilable results.
The quality and the length of the sequences allowed reaching an accurate identification, up to the species level.

b) Culture-independent approach

Both the ARISA fingerprinting analysis and the Metagenomic analysis are based on the analysis of DNA segment, hence, DNA extraction was necessary as thefirst step of the microbiological characterisation.

DNA extraction

Once the settlement experiments were concluded, limestone cubes were collected from the field and two pieces of the bioconstruction were also harvested by hammer and chisel from each experimental site within Punta Raisi and Barcarello. The whole set of samples was brought to the lab and to avoid contamination among biofilm typologies during the carriage, cubes were placed in separate containers with marine water according to the treatments.
After the number of *Dendropoma cristatum* larvae was recorded for each cube, biofilms were prepared for microbiological investigations. 0-d biofilmed cubes and II and IT treatments were excluded from these analysis, due to the bare settlement success, maybe due to poorly developed microbial layer.
The biofilms were scraped from the top face of each limestone block -where settlement was higher and microbial layer was mostly developed- with a sterile razor, by handling the cubes

with sterile lattice gloves and under a laminar flow cabinet (to avoid the contamination of the biological material).

The biological material was stored in sterile 2ml eppendorf at -20°C and all the replicates of each treatment were grouped within one tube, keeping sites separated.

DNA was extracted by using the FastDNA^{TM} SPIN Kit for Soil, suitable to separate DNA from rock matrix. The protocol includes the physical and chemical cellular lysis by using specific buffers, the DNA purification from the lysis solution and the elution of the genetic material. Once DNA was extracted, it also was quantified by nanodrop, to check that the DNA concentration and quality were appropriate for further analysis. The DNA concentration ranged among 141,02 and 5,27 ng/µl.

The obtained DNA was used for the fingerprinting analysis and metagenomic sequencing.

- **ARISA analysis**

For ARISA, extracted DNA was amplified using universal bacterial primers FW ITS (5'GTCGTAACAAGGTAGCCGTA) and RV ITS M13 (5'TGTAAAACGACGGCCAGTGCCAAGGCA-TCC), which amplify the ITS1 region in the rRNA operon plusca. 282 bases of the 16S and 23S rRNA (Hewson et al., 2004). Primer 23S-125R was fluorescentlylabeled with the fluorochrome FAM.

PCRs mixture (25µl) contained: buffer OneTaq standard (Biolabs) 1x, Buffer OneTaq GC (Biolabs) 0,5X, dNTP 0,2 mM, Primer FW ITS 0,4 µM, Primer RV Reub M13 0,3 µM, Primer RV M 13 FAM 0,3 µM, BSA (Biolabs) 0,1%, One Taq DNA polymerase (Biolabs) 0,025 U/µl.

PCR was performed with the following program: 94° for 30sec; 5 cycles of: 94° for 30 sec; 55° for 30 sec; 68° for 2 min; 35 cycles of: 94° for 30 sec; 50° for 30 sec; 68° for 2 min; one cycle at 68° for 5 min.

For each sample, about 20 ng of amplicons was mixed with 0,5 µl of internal size
Standard. Automated detection of ARISA fragments was carried out using the ABI
Prism 3100 Genetic Analyzer (Applied Biosystems)

ARISA fragments in the range between200 to 1,200 bp were determinedusing GeneScan analytical software version 2.02 (Applied Biosystems),and the results were analyzed by adopting of fluorescence among samples, eliminationof "shoulder" and nonreplicated peaks, and cutoff criterion.

5.3 Results

a) Culture-dependent approach

The comparison of the genetic profile of the 16S gene of the 25 phenotypes, evidenced 6 different profiles, showed below. Each profile indicates one different OTU (A, B, C, D, F and E) and M indicates the marker (100 bp).

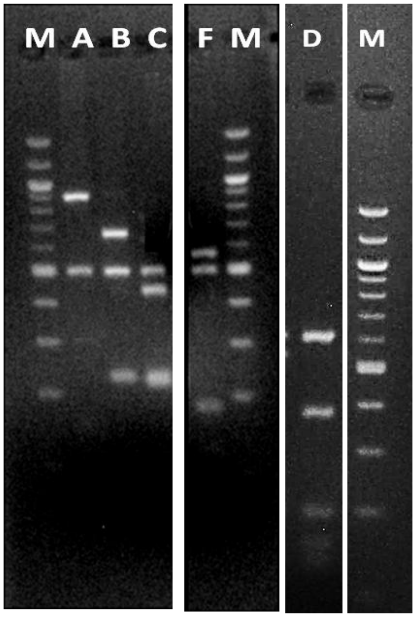

Figure 79, Electrophoretic analysis of the 16 S digestion products. M= marker (100 bp); A, B, C, F, D= OTU.

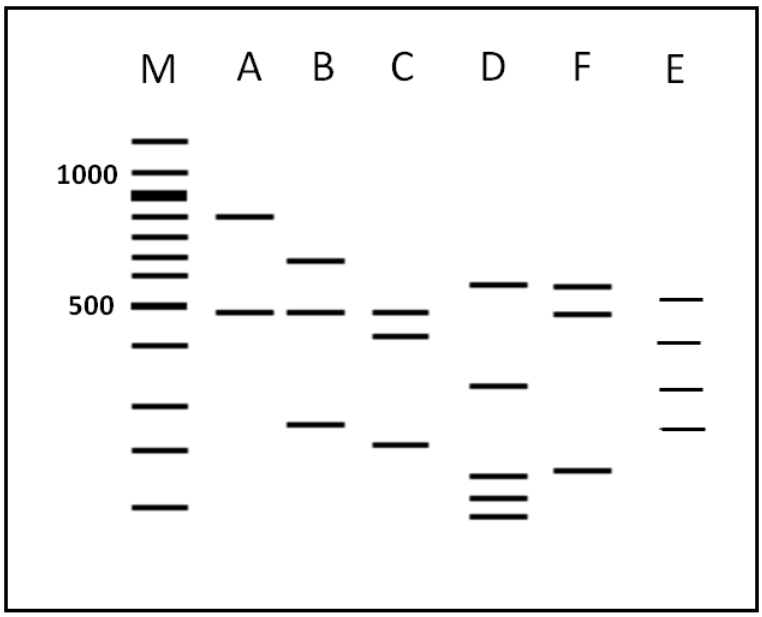

Figure 80, Scheme provided the electrophoretic analysis. M=Marker (100 bp); A, B, C, D, F, E,= OTU.

The sequencing of the 16S gene of each OTU, allowed the identification of the following species:

OTU A: *Alteromonas genovensis*

OTU B: *Vibrio aliginolyticus*

OTU C: *Vibrio gigantis*

OTU D: *Shewanella pneumatophori*

OTU F: *Pseudoalteromonas tetraodonis*

OTU E: *Cellulophaga lytica*

Otu	Reppresentative Isolate	n. isolate in the same Otu	Phylum	Class	Most closely related sequence	Bp	Id %	Accession n.
A	P2 2.2	3	Proteobacteria	Gammaproteobacteria	*Alteromonas genovensis*	1320	98	NR_042667.1
B	P8 4.1	4	Proteobacteria	Gammaproteobacteria	*Vibrio alginolyticus*	1370	99	NR_122060.1
C	P7 2.4b	7	Proteobacteria	Gammaproteobacteria	*Vibrio gigantis*	1319	99	NR_044079.1
D	P7 2.4a	1	Proteobacteria	Gammaproteobacteria	*Shewanella pneumatophori*	1002	99	NR_041292.1
E	P3 2.2	1	Bacteroidetes	Flavobacteriia	*Cellulophaga lytica*	1044	99	NR_074464.1
F	P5 2.3	12	Proteobacteria	Gammaproteobacteria	*Pseudoalteromonas tetraodonis*	1273	10	NR_114187.1

Figure 81, Table n 10: OTU description based on BLAST n Database rRNA_typestrains/prokaryotic_16S_ribosomal_RNA.

b) Culture-independent approach

The results of this section are underway, especially those of the metagenomic analysis. However, some of the results gained from the ARISA fingerprinting are showed below.

- **ARISA analysis**

The ARISA analysis provided the following electropherograms (Fig. 82).

Each panel (A, B, C, D), groups the biofilm assemblages from the same site. In theimage below, the considered nucleotide range is restricted between 200 and 780.

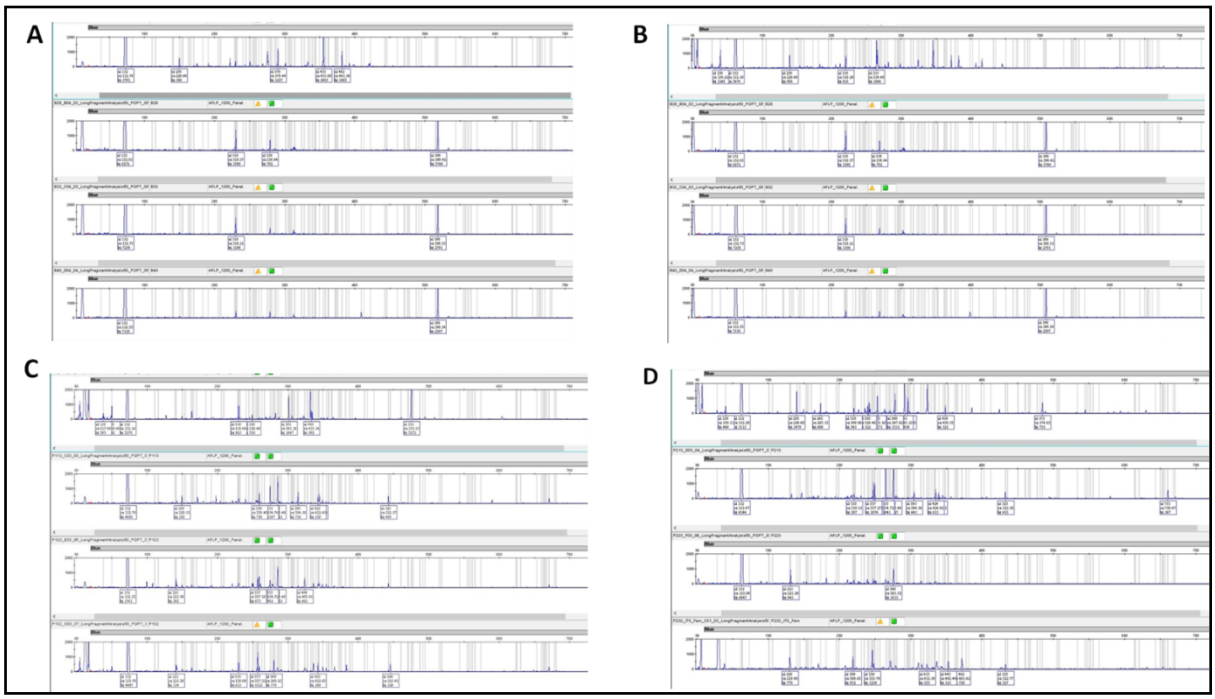

Figure 82 ARISA Electropherograms. Panel A: Barcarello S1 (sample order: B1, B28; B 32; B40); Panel B: Barcarello S2(sample order: B2, B28; B 32; B40); Panel C: Punta Raisi S1 (sample order: P1, P13, P 23, P 32); Panel D: Punta Raisi S2 (sample order: P2, P13, P 23, P 32).

The total number of revealed OTU was 58 and figure 83 shows the OTU distribution within each biofilm assemblage.

Bacterial richness ranged among 3 OTU within the sample 32-d B and 15 OTU within P 2 and the number of OTU within each sample is showed in the fig. 83.

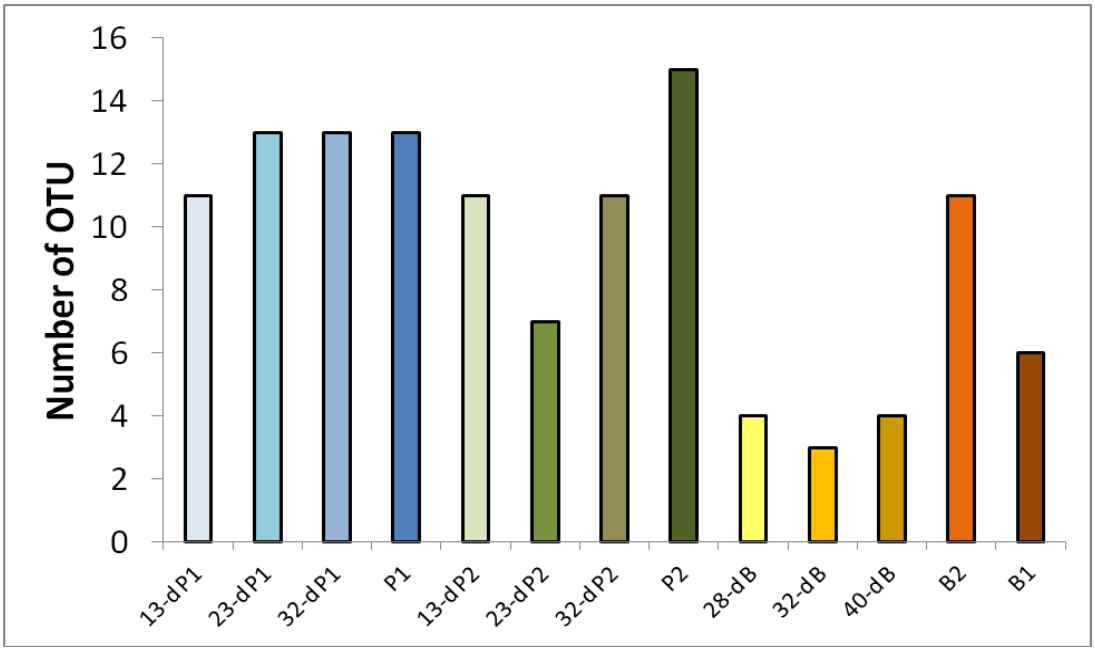

Figure 83, OTU richness for each assemblage. Blue bars indicate the samples from the site 1 of Punta Raisi, while the green bars indicate the samples from the site 2; orange bars indicate the samples from Barcarello. Different tonalities of blue, green and orange are used to distinguish the biofilm treatments.

The number of OTU was poorly correlated to the Biofilm maturity (r= 0,3). The higher number of OTU was not represented within the more mature assemblages.

The similarity in composition ofassemblages was detected by cluster analysis and the analysis of the principal components.

The cluster analysis showed a hierarchical grouping of the different assemblages, with the replicates of the same treatment quite close than the assemblages from different treatments. Reef-associated assemblages from Barcarello and Punta Raisi formed two close clades on the right of the cladogram.

Limestone-cube associated assemblages from Barcarello were in the middle of the cladogram, while the majority of limestone-cube associated assemblages from Punta Raisi formed a separate clade on the left of the cladogram.However, this pattern is not maintained for the sample P2 23, which showed more than the 20% of similaritywith the samples B1 and B2 (Figure 84).

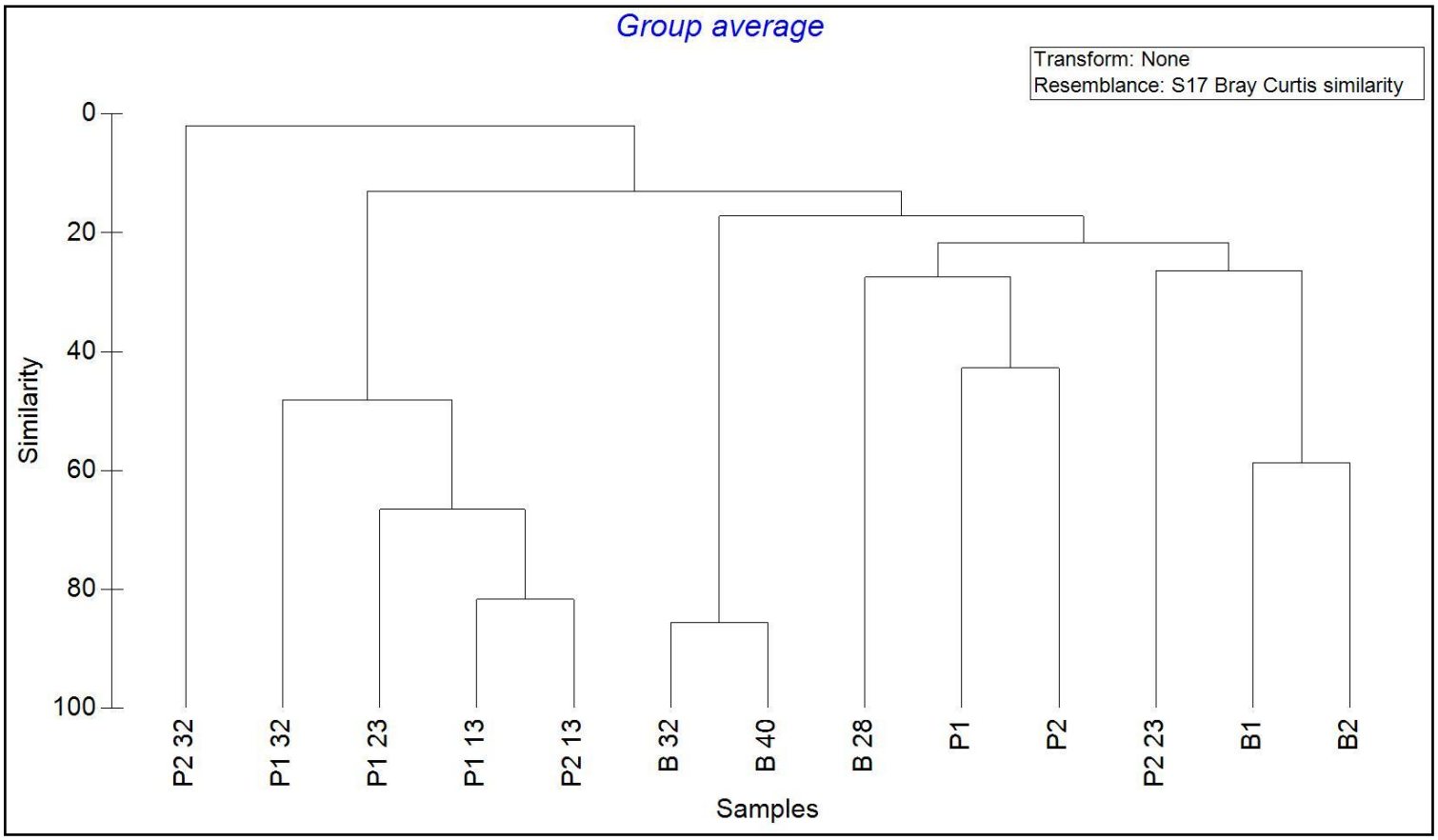

Figure 84, Cladogram showing the similarity in OTU composition among the compared assemblages.

The Principal component analysis (PCA)showed the similarity among samples based on the OTU they contained (fig. 85). The biofilm treatments were gruped within classes of maturity: “1” for the early biofilm (13-d for Punta Raisi and 28-d for Barcarello), “2” for the medium biofilm (23-d for Punta Raisi and 32-d biofilms for Barcarello), “3” for older biofilm (32-d for Punta Raisi and 40-d for Barcarello) and “n” refers to the reef-associated biofilms. P and B indicated the experimental localities (Punta Raisi and Barcarello).

Three groups were formed according to the classes of maturity: age classes 2 and 3 were close and may be considered a unique group; class 1 and n were spatially separated and formed other two groups.

The biofilm assemblages of the same class of maturitywere close because they had many OTU in common. Hence, the PCA analysis showed high similarity in OTU composition among biofilm of the same treatment.

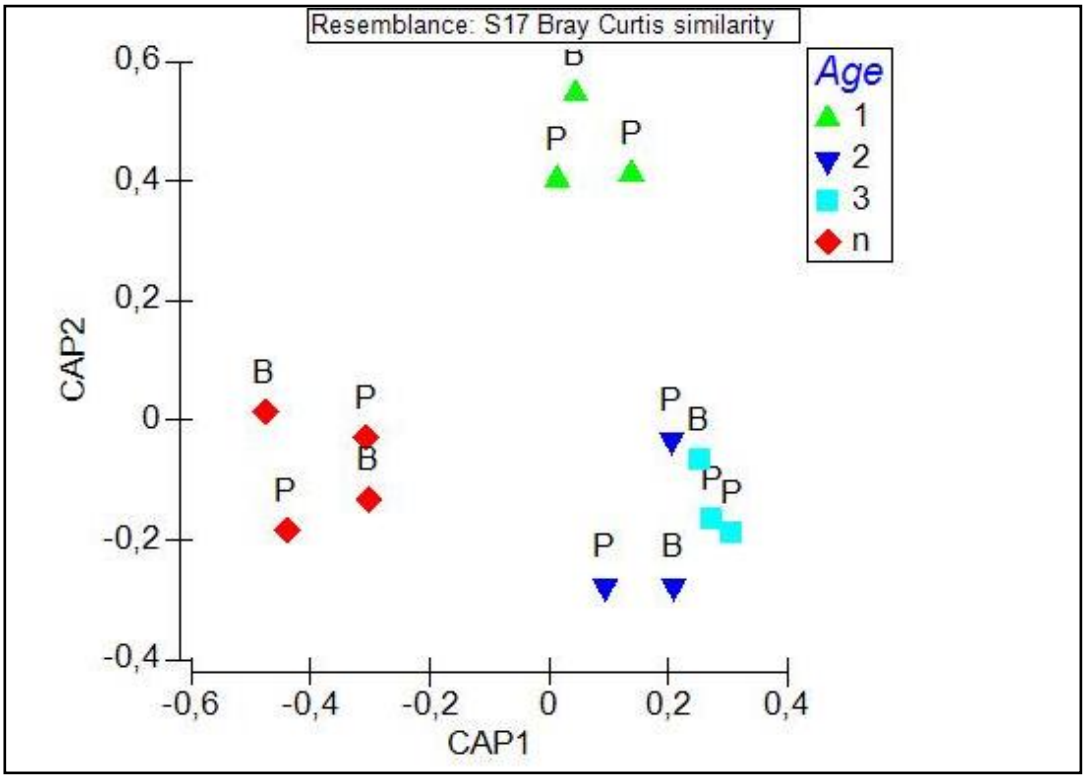

Figure 85, PCA analysis. P and B indicate the localities for each sample (P: Punta Raisi; B: Barcarello).

Permutational MANOVA was performed to detect differences regard the richness in species, among classes of maturity of the biofilm. Biofilm maturity, as in the PCA analysis, and specific richness is calculated as the total number of OTU within each sample.
Significant differences were revealed for the factor age. Within the factor age, differences among the treatments 2 *vs* n and 3 *vs* n were significant.

Source	df	MS	Pseudo-F	P(perm)	PAIR-WISE TESTS, term 'Age'	
Ag	3	3906.8	1.9107	*	Groups	P(perm)
Lo	1	8476.5	2.2114	***	1, 2	ns
Si(Lo)	1	3545.7	4.1824	ns	1, 3	ns
AgxLo	3	3328.3	1.6345	ns	1, n	ns
AgxSi(Lo)	3	1884.6	2.223	ns	2, 3	ns
Res	1	847.75			2, n	**
Total	12				3, n	*

*Figura 86 Table n. 11: Univariate PerMANOVA analysis with 3 factors: "Ag" (age classes), fixed with 4 levels (1, 2, 3, n); "Lo" (Locality), fixed with 2 levels (Punta Raisi and Barcarello); "Si" (Site), random and nested within the factor Lo. *= p≤0,05; **= p≤0,01; ***= p≤0,001; ns= not significant.*

- **Metagenomic analysis**

Basing on the similarity in taxa composition among biofilm treatments, provided by the ARISA fingerprinting (see the cladogram, fig. 84), for each locality one sample representative of each treatment was chosen for metagenomic analysis.
Metagenomic analysis of DNA samples is underway and, unfortunately, no results are here reported. However, a complete information about the prokaryotic composition of the analyzed biofilms is expected to be of relevance for further advances about the implication of bacteria on settlement dynamic of the reef-builder*D. cristatum.*

5.4 Discussion

In natural environments, the biofilm formation consists of a succession of processes, starting with the production of an organic layer made ofmolecules, such as amino acids, glycoproteins and humic materials, then advancing by the colonization of bacteria, diatoms, fungi and protozoans (Characklis & Cooksey 1983; Wahl 1989).As previously stated, successional changes that occur in association with the age of the biofilm affect larval settlement and metamorphosis of various species in varying manners. Also in the case of the vermetidae*D. cristatum, the*settlement is susceptible to the tested age treatments and is positively affected by the quantitative and qualitative changes which occur beside the biofilm maturity. However, older biofilms do not result in a higher richness in taxa, as fingerprinting analysis demonstrated in the present research (see the figure 83). Moreover, samples of the same age treatment show high similarity in OTU composition and are closely distributed in the cladogram, except the sample P2 23 that is closer to B1 and B2 (see the figure 84).

The effect of biofilm age on larvalsettlement may be attributed to quantitative and/or qualitative shifts (Todd & Keough 1994), e.g., differences in film densities, metabolic activities and/or compositions in relation to film age (Wieczorek & Todd 1997).

Bacterial and diatom densities, for instance, have been shown to facilitate the settlement and metamorphosis of cyprid larvae on the biofilm (e.g., Wieczorek *et al.*, 1995; Olivier *et al.,* 2000; Rahim *et al.*, 2004) and also changes in the metabolic activities occur within these films.

Bacteria are also key-mediators of reactive compounds between the substratum and the larvae (Satuito *et al.*, 1995; Bao *et al.*, 2007a) through the recognition of quorum sensing signals. Specific strains, including some *Pseudoalteromonas* spp., are known to stimulate invertebrate settlement (Huggett *et al.*, 2006; Hadfield, 2011).

16S gene sequencing of biofilm cultured from the field showed the presence of *Pseudoalteromonas* and *Alteromonas*spp. in the reef-associated microbial assemblage. These two species are common in marine biofilm and are recognized as potentially settlement inducers of various marine invertebrates, e.g. *Janua brasiliensis*(Kirchman *et al.*, 1982), *Crassostrea* spp. (Weiner, *et al.*, 1989; Fitt *et al.*, 1990).

Under laboratory conditions, *Pseudomonas-Alteromonas*group induced the settlement of*Mytilusgalloprovincialis*larvae (Satuito *et al*., 1997). This study reported that mussel larvae initiate settlement in response to extracellular products of *Pseudomonas-Alteromonas*bacterial cultures, tested in several dilutions of biofilm conditioned seawater. However, the active factor which induces larval settlement was not characterized.

Sneed et al. (2015) showed that *Pseudoalteromonas* induces complete settlement (attachment and metamorphosis) of Caribbean corals. In this study the activity of *Pseudoalteromonas* was attributed to the production of a single compound, tetrabromopyrrole (TBP), which has been shown previously to induce metamorphosis in Pacific acroporid corals and to induced larval settlement for two broadcast-spawning species (*Orbicella franksi* and *Acropora palmata*), indicating that this compound may have widespread importance among Caribbean coral species.

We do not know if in the vermetid reef-associated biofilm *Pseudoalteromonas* is able to produce TBP and which implication this compound may have on*Denpropoma cristatum* larvae.

Further investigations would need to identify potentially settlement cues from bacteria isolated from the biofilm and to advance some hypothesis about the effect of these metabolites on *D. cristatum* larval behavior.

Moreover, the films cultured in the laboratoty by mixing specific strains of bacteria associated to the *Dendropoma*-reef and tested in the settlement experiment of Punta Raisi, also included the *Pseudoalteromonal-Alteromonas* group. However, these artificial bacterial films did not induce larval attachment. Probably, it would have been necessary to test the formation of the biofilm on the settlement cubes prior to perform the settlement experiment, to be sure that the cultured bacteria were able to produce a biofilm on the limestone cubes and not simply to adhere to them.

At this step, we can only statethat the microbial associated signal responsible for cueing *D. cristatum* settlement is age-dependent.However, this is the first study considering bacterial communities in the settlement of the *Dendropoma* reef builders and other efforts are need to clarify the nature of this associative settlement mechanism.

With this respect, metagenomic analysis results will provide precious information about the presence of other potentially settlement implicated prokaryotes within the biofilm.

CHAPTER 6.
Conclusions of the research

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Conclusions of the research

This last chapter gives asummary of the research findings. The main conclusions of this research and recommendations for future work are followed reported.

The influence of a vermetid encrustation on rock deterioration processes, such as thermal degradation and salt weathering, is the first question which this research aimed to address.
The results from the laboratory simulations indicate that the *Dendropoma* encrustation buffers sub-surface microclimatic variations and reduces salt ingress within the substratum. The effect of these biological controls on weathering processes has not been directly assessed in this experiment, but is based on current knowledge and understanding of the weathering dynamics which act on natural intertidal systems.
Studies about the biological influences on rock weathering are often conducted in thelaboratory, given the complexity to monitor subsurface thermal conditions in the field (Gowel*et al.*, 2015; Coombes *et al.*, 2012, 2016). However, given the difficulty of introducing and maintaining living intertidal organisms under laboratory conditions, at this time the influence of a live biological encrustation on subsurfacethermal regimes has never been assessed. In this study, the internal rock temperature regime has been monitored under a live vermetid encrustation and the results sustain the hypothesis that an undamaged and well conserved *Dendropoma* layer could be of higher relevance in mediating the intensity of rock weathering processes compared to a dead or fragmented biological encrustation.
These findings introduce the concept of vermetids as agents of bioprotection and strength the ecological value of this Mediterranean intertidal habitat.The concept of bioprotection, moreover, sits well under the broader paradigm of ecosystem service provision (Bolund and Hunhammar, 1999; Costanza *et al.*, 1997) that should spur international environmental politics for attaining an effective protection of this Mediterranean temperate reef.
By contrast, in many areas of the Mediterranean is occurring a regression of the vermetid bioconstructions which is thought to be due to widespread environmental changes in recent decades (Galil,2013). Along the Israeli coast, for instance,the local extinction of *Dendropoma anguliferum*(Galil, 2013) led to a considerable erosion of the biogenic structure, up to

thelocal collapse of the entire structure, reducing the biological and physical coastal complexity and with an abrupt change in biodiversity.

Politics of conservation should also include the development of strategies toencourage vermetid colonization and the design of solutions to facilitate vermetid settlement and recruitment, in opposition to the reef decline, and to maintain reef persistence, integrity and ecological functions.

In the perspective of encourage the *Dendropoma*colonisation, the study of settlement and recruitment dynamic of this reef-builder gastropod was the second aim of the research and provided important findings to the current knowledge.

Up to date studies on the early stages of life cycle of the reef-building *Dendropoma* spp. have been mainly described from a biological perspective (Calvo *et al.*, 2009; Calvo & Templado, 2004; Calvo &Templado, 2005; Phillips & Shima, 2009). Studies on the ecology and behaviour of its larvae are rare and with difficulty are conducted with *in situ* experiments.

This research highlighted new features of the ecology of *D. cristatum,* with specific reference to the early development.

The experimental work described in chapter 3 has shown the within-reef variability of recruitment dynamic (experiment pages 61). This result is probably due to the local conditions and micro-habitats which both adults and larvae experience among the outer and the inner edges of the reef and that affect their reproductive performance and survival rate for larvae. At the same time, differences in recruitment were not dependent onhydrodynamic condition. *Dendropoma cristatum*is a species with direct development which do not experience pelagic transport of larvae. Due to this peculiar ecology, this species is probably less dependent on wave flow to attach on the substratum and recruit in adults, as suggested for other invertebrates with a similar ecology (Arribas *et al.*, 2014).

Larvae-substratum interactions have been approached in chapter 3, and 4, to understand which factors at site scale (within cm) drive the settlement of *D. cristatum*.

The *in situ* experiments on different artificial settlement surfaces showed that at the settlement stage, secondary biogenic substrata affect settlement rate more than physical features (experiment pages 72).

Habitat selection from crawling larvae of *Dendropoma cristatum*, is mainly affected by biological stimuli rather than the physical complexity of a substratum. This result is

confirmed by the high settlement success on living crustose coralline algae, compared to not living CCA (experiment page 83).

In chapter 4, the microbial film (biofilm), has been demonstrated to be another biological factor which positively affects the natural settlement of *D. cristatum* larvae (experiment page 95). Moreover, the settlement rate is positively correlatedd to the biofilm maturity, probably, due to its composition or complexity (experiment page 105). This point needs further attention, aiming to assess the occurrence of specie-specific interactions among the reef-builder and one or more members of the biofilm. With this respect, the metagenomic analysis will provide an overview of the composition of the microbial assemblages which will be matched to the settlement success data.

Hence, the existence of intra-specific interactions between the crawling larvae of *D. cristatum* and species able to generate biogenic secondary substrate drive the selection of sites suitable for settlement.

The considered biogenic substrata, the crustose coralline algaeand the biofilm, are supposed to provide chemical signals which induce larvae to settle and, in this perspective, bio-chemical cues resulted askey-factors in the settlement dynamic of *Dendropoma cristatum.* Metabolomic studies, moreover, could provide important information about the nature of these unidentified stimuli which trigger *D. cristatum* settlement.

These results highlight the capacity of active substratum selection by *D. cristatum* larvae. This characteristic behaviour is a crucial strategy to find a habitat suitable for a successful further development in sessile adults.

During the crawling phases, substratum stimuli mainly affect the habitat selection from larvae of *D. cristatum* and biological cues, such as living CCA and microbial film, strongly promote the settlement of this species.

Subsequently, local physical stress (such as the solar irradiation, the desiccation rate, the wave impact) regulate the development of settlers into recruits, and the abiotic factors became crucially relevant for the development into adults. The hydrodynamic does not seem to be involved in the described early processes of development.

Both biological and physical factors exert a control onthe development of *D. cristatum*, acting at different phases of its life cycle. At the settlement stage, the population dynamic is

regulated by biological factors, while at the recruitment stage, it is subjected to the action of local physical factors.

These findings are described for the Sicilian reef-builder *Dendropoma cristatum*, but, excluding the occurrence of particular local conditions, might be generalized to the rest of the Mediterranean *Dendropoma*spp.

These conclusions contribute to the description of the ecological traits of this Mediterranean reef-builder and provide a wider view of the two-way interactions between this ecosystem engineer and the surrounding physical and biological environment.

The reef contributes to the local attenuation of weathering causes on intertidal shores and this may concur at some extent - not still defined – to the conservation of the colonised substrate. This process may be considered as an additional ecosystem service provided by the *Dendropoma* encrustation which is added to the range of goods which a healthy vermetid reef provides: the natural buffering of waves and storms, preventing erosion and physical damages to the coasts; the local improvement of the spatial heterogeneity of the intertidal zone, supplying shelter, food, and home to a very high number of species, including fish and invertebrates of recreational and commercial interest.

The identification of the overall set of ecological goods provided by a natural system is a research priority and the lack of knowledge in this field is a real limit to the application of the principles of environmental management. Unfortunately, up to date the contribution of temperate vermetid reefs in providing such services has been less investigated than that of other functionally similar systems, such as tropical reefs (e.g., Moberg & Rönnbäck,2003). However, an important ecological value has been attributed to the *Dendropoma* constructions, recognized as habitats which critically structure the Mediterranean intertidal shores.With this respect, *Dendropoma* spp. and some of the associated algae (as *Neogoniolithon brassicaflorida*, *Lithophyllum byssoides*and *Cystoseira amentacea*) have been included in the annexes of the Berna Convention, and in the Annex II (Endangered or Threatened Species) of the Protocol for Specially Protected Areas in the Mediterranean (SPAMI Protocol of the Barcelona Convention). *Dendropoma*has been also proposed to be included in the annexes II and IV of the Habitat directive (Chemello,2009) and the reef structures have been listed as threatened bioconstructions in the European Red List of Habitats (Marine: Mediterranean Habitat). However, the Spanish National Catalogue on

Threatened Species and the Maltese “the Flora, Fauna and Natural Habitats Protection Regulations” are the only acts that officially suggest protection of these reefs at national level.

These ecological relevances clash with the ineffective conservation policy of the vermetid reef within the Mediterranean- only the 28.5 % of vermetid reefs in the Mediterranean are apparently protected by means of MPAs or coastal reserves, as summarized by Chemello et al. (2014) –This contrasting and complex frameworkoutlines the need to extend action plans for vermetid reef protection and management and to develop protocols for promotingvermetid reef restoration activities,as strategy to cope the local decline of the *Dendropoma* reefs and to favour their persistence. With this respect, accurate information on the ecology of the*Dendropoma*reef-building species are essential, especially regard the early stage of development(settlement and recruitment).This study provides first insights regarding the substratum characteristics which favour the settlement of *D. cristatum,*inducing the calcification of the crawling larvae to a site.

Hence, settlement process can be facilitated by improving the substratum biological characteristics (by mean of a living CCA encrustation, for instance) and by offering artificial substrates for spontaneous colonization.

In this study, limestone cubes have been demonstrated to be suitable to support a high number of settlers and to be colonized by *D. cristatum* larvae very quickly. The forex discs, although they are highly resistantto wave impact – due to the plasticity of this material- are not able to “accommodate” larvae unless a secondary substratum made by crustose coralline algae had not been prior established on them.

Further stages of study should consider the survivorship of the settled juveniles on these artificial surfaces and, within the perspective of recovery plans, should include the transplantation of this small *Dendropoma*-colonised “nuclei” to the depleted reefs, supporting the recolonization process and, thus, manipulating the early stages of natural succession.

Such an approach may be able to overcome the limits of natural recovery in reefs with low densities of adults or with restricted reproductive ability and fecundity, which impair the spontaneous recolonisation after a disturbance.

Further investigation towards this direction may give a valuable support to the management of this Mediterranean coastal key-habitat, in order to respond to the local current decline of

the reef ecosystems in some parts of the Mediterranean and to integrate vermetid reef protection with the valid bases provided by the ongoing experimental ecology research.

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