



UNIVERSITÀ POLITECNICA DELLE MARCHE

**DIPARTIMENTO DI SCIENZE DELLA VITA E DELL'AMBIENTE**

Corso di Dottorato XXXI ciclo  
BIOLOGIA ed ECOLOGIA MARINA

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Hidden lives: polychaetes inhabiting living and not-  
living substrata

*PhD candidate:*

**Lisa Pola**

*Supervisor:*

**Prof. ssa Barbara Calcinai**

Academic years 2015/2018



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*“.....to allow other eyes to enjoy  
the intrinsic beauty of symbiotic worms”*

*Martin & Britayev, 2018*



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# ABSTRACT

Polychaetes are known for their high level of diversity and for their ability to live in many ecosystems with different adaptive responses (Fauchald, 1977) and to constitute a high percentage of the benthic macrofauna. Most of them are usually free-living, but some establish several kinds of symbiotic relationships with other invertebrates, like Porifera and Cnidaria (Dales, 1957, Martin & Britayev, 1998; Gherardi *et al.*, 2001). While other can also live inside different kinds of substrates as important component of the boring communities (Hutchings, 2008).

The present PhD thesis is structured as follows:

i) *Polychaetes and Demosponges*

Sponges represent elevation habitat where organisms, belonging to different taxa, can find shelter, better solutions of feeding and nursery areas. There are many studies about symbiosis that involve sponges, polychaetes and other organisms in the coral reefs and deep sea; in the Mediterranean Sea, the macrofauna that lives associated with *Sarcotragus* spp. has been investigated by several authors principally for the Aegean Sea, while for the Italian waters the knowledge is scant and the last study was conducted about 40 years ago by Pansini (1980).

*Sarcotragus* spp. (Demospongiae: Irciniidae) are common in the Mediterranean Sea and are characterized by a massive shape, a firm and tough consistency and by a fibrous skeleton that makes it compressible.

This genus was reported to be more tolerant in cases of anomalies of the marine water temperature respect other sponges and can be a valid substrate also during climate change variations (Di Camillo *et al.*, 2013).

Two species, belonging to this genus, *S. foetidus* Schimdt, 1862 and *S. spinosulus* Schimdt, 1862, were investigated (Chapter 1). These species were collected respectively in Portofino Promontory (North Ligurian Sea) and in Ancona Promontory (North Adriatic Sea), bimonthly, for a total period of 18 months.

*S. foetidus* is a protected species included in the Barcelona Convention, living along the infralittoral and circalittoral rocky shore; it is characterized by great dimension (40-50 cm in diameter) and aquiferous system with large canals, the surface is typically irregular with big conules (2-3 mm).

*S. spinosulus* has reduced dimension of the aquiferous system (conules around 1-2 mm) and a regular surface in respect of the congeneric species and lives on the shallow waters of the infralittoral rocky shore.



- In order to evaluate any differences in the diversity and in the abundance of associated fauna in these two species of *Sarcotragus*, we collected one hundred specimens from November 2016 to March 2018, for a total period of 18 months. Moreover, volumes of these two sponges were also considered. Taxonomical analysis was conducted for the identification of the organisms at the lower, possible, taxonomic levels. Statistical analyses were conducted using Past 3.16 (Hammer *et al.*, 2001).

Morphological characteristics of the most common species *Ceratonereis (Composetia) costae* (Annelida: Nereididae) associated with *S. spinosulus* were analysed from July 2016 to July 2017. Data were elaborated using the programs ImageJ 1.51 j8 and Fisat II, Bhattacharya methods. New data, here reported, about the life cycle of this species support the functional role of the sponges as habitat-forming species.

ii) *Polychaetes and Octocorals*

Regarding octocorals, the study focused on the gorgonian *Paramuricea clavata* (Risso, 1826) which is known to be frequently associated with other organisms, even if very few data about the associations with polychaetes are available (Laubier, 1960; López *et al.*, 1996). In particular, the neglected association between the gorgonian *P. clavata* and the syllid *Haplosyllis chamaeleon* (Laubier, 1960) was the principal focus of the second part of this thesis.

Samples were collected at “Punta del Faro” and in other areas close to the Portofino Promontory (Ligurian Sea) and in other areas in the Adriatic and Tyrrhenian Sea. All the organisms were sorted, identified and counted and the morphological characters recorded. New data about the density and the frequency of this syllid, and of its reproductive period are reported.

Moreover, these data enlarge its breeding period, the geographic distribution and ascribe it, for the first time, to the Italian and Croatian marine fauna.

During the study of this symbiosis, samples of *P. clavata* were analysed in collaboration with the ICB (Istituto di Biochimica Molecolare), CNR of Pozzuoli (Napoli) in order to clarify the real nature of the symbiosis between this gorgonian and the polychaete *H. chamaeleon*. This study investigated the molecular composition of the gorgonian reporting a new molecular compound for the gorgonian and opening several research questions.

iii) *Boring Polychaetaes*

Erosion activity is largely diffused, but scant information is available for the Mediterranean Sea, also about the rate of the bioerosion processes (Calcinai *et al.*, 2011). Boring polychaetes live

inside calcareous substrates, both biogenic, as the corals' skeletons and the molluscs' shells, and rocks (Hutching, 1986; 2008). Most of the species involved in this process belong to the families Eunicidae, Cirratulidae and Spionidae. Many studies reported the negative effects that Polydorids (Annelida: Spionidae) have towards mollusc farms, where they are considered as a pest for the local production.

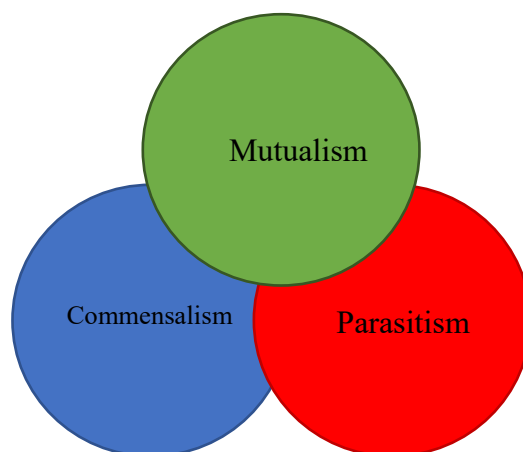
For this research, calcareous panels were soaked in two selected areas of the Ancona Promontory (Marina Dorica and Passetto) from June 2017 to June 2018 and, after January 2018, were collected bimonthly. For the sampling design and the analytical procedures, a modify protocol suggested by Tribollet *et al.* (2002) was followed. Species involved in bioerosion and the erosion rate of these were investigated and described in the last chapter.

## INTRODUCTION

Associations of organisms, that live together with different types of relationships, are common strategies of life in all the marine ecosystems; several examples are reported for the Antarctic Sea (Bavestrello *et al.*, 2000; Cerrano *et al.*, 2000a), deep sea (Buhl-Mortensen & Mortensen, 2004; Molodstova *et al.*, 2016), temperate areas (Martin & Britayev, 2018) and in the coral reefs, where these symbiotic relationships reach their maximal expression (Muscatine & Porter, 1977; Sheppard *et al.*, 2017). However, the Mediterranean Sea is a hot-spot of biodiversity (Ballesteros, 2006; Coll *et al.*, 2010; Ingrosso *et al.*, 2018), subjected to several anthropogenic impacts, as eutrophication and fishing activities (Bastari *et al.*, 2016; Costa *et al.*, 2018). Moreover, anomalies of the water temperature (Bianchi *et al.*, 2017; Longobardi *et al.*, 2017; Rivetti *et al.*, 2017), can lead to mortality events and biodiversity loss (Cerrano *et al.*, 2000b; Coll *et al.*, 2010; Di Camillo *et al.*, 2013; Turicchia *et al.*, 2018).

Multidisciplinary approaches are needed to better understand the complex relationships in marine organisms involved in symbiotic associations (Martin & Britayev, 2018). The chemistry of organic compounds and secondary metabolites can help understanding organism interactions and the role the surrounding ambient can play in the modulation of their expression, for example the chemical cues involved in host-symbiont recognition; the support of taxonomy is a fundamental step to clearly identify the actors involved in the different relationships.

First of all, it is also important to clarify that close associations between different species can be ascribed mainly to three categories: Mutualism (++); Parasitism (+-) and Commensalism (+0) with several examples of mixing within the different definitions (Figure I) (Dales, 1957). Despite the definition of these macro-groups of interactions, the actual possibilities are countless and can vary also in relation to internal (e.g. stress, age and health status of host/symbiont) and external factors (e.g. environmental characteristics), making difficult the correct positioning of peculiar interactions (Parmentier & Michet, 2013).

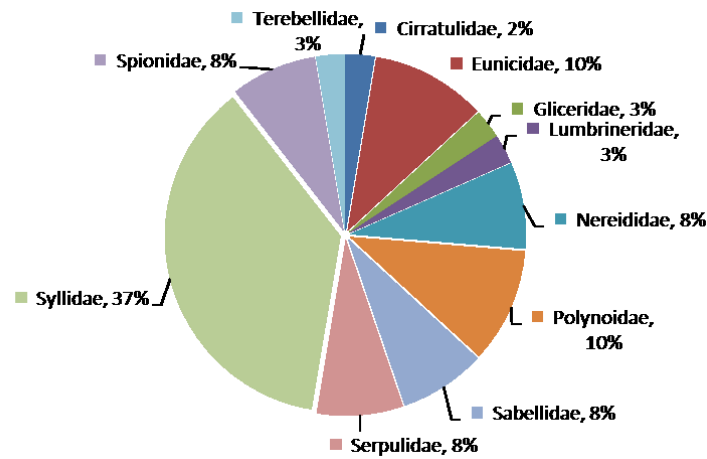


**Figure I** Types of symbioses.

The body plan of polychaetes requires very often peculiar structures of protection that can be built directly by the organism or can be obtained exploiting several kinds of substrates both living (developing different degrees of interaction with hosts) and not and in this case, substrates can be both soft and hard. In case of hard substrates, polychaetes can exploit natural crevices, but a particular adaptation can be described for boring polychaetes, able to erode calcareous substrates and create effective refuges. In the present work we considered the interactions between polychaetes and different biological substrata, focusing mainly on sponges and gorgonians, and polychaetes able to erode calcareous substrates.

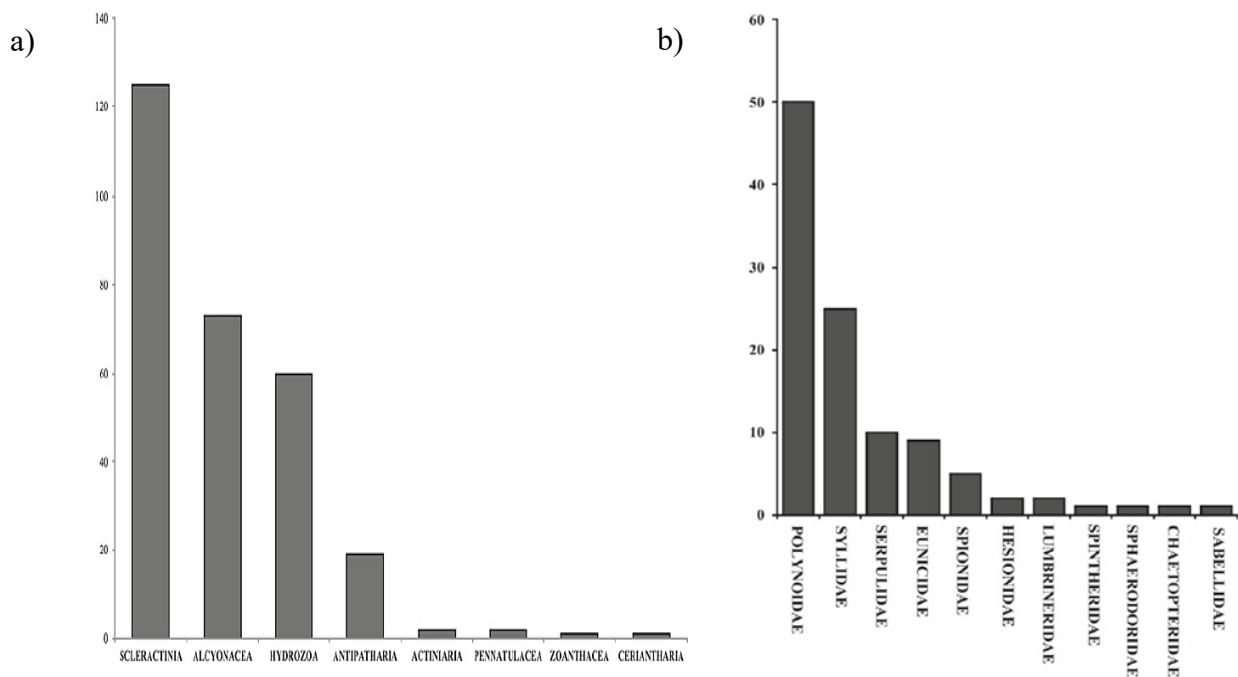
Polychaetes are one of the most common and diversified taxon in the marine ecosystems; despite most of them are free-living, crawling, burrowing, tube-dwelling (Fauchald, 1977), they can be engaged in symbioses and become also important members of the boring community. Symbiotic polychaetes have been recently updated to 618 species involved in 1626 relationships (Martin & Britayev, 2018). These authors report that 33 families are mainly commensal and Polynoidae and Syllidae present the highest number of reported symbiotic species, 171 and 114 respectively. The organisms hosting commensal polychaetes belong to several taxa, in particular, 137 Porifera are described to host 120 species of polychaetes while the phylum Cnidaria is involved in 329 associations with 279 species of polychaetes. The parasitic polychaetes (boring and not boring) are mainly ascribing to the family Spionidae (29 species). The hosts of this group of symbionts belong predominately to the order Decapoda and to the classes Gastropoda and Bivalvia (97, 84 and 84 species, respectively).

About polychaetes inhabiting demosponges, the 35% of the studies are from the Mediterranean Sea and only six concerned the Italian waters. In general terms, the family with higher percentage of association with sponges is Syllidae (37%) followed by Eunicidae (10%), Polynoidae (10%), Spionidae (8%), Nereididae (8%) and Sabellidae (8%) (Figure II).



**Figura II** Sponge-symbiont families of polychaete.

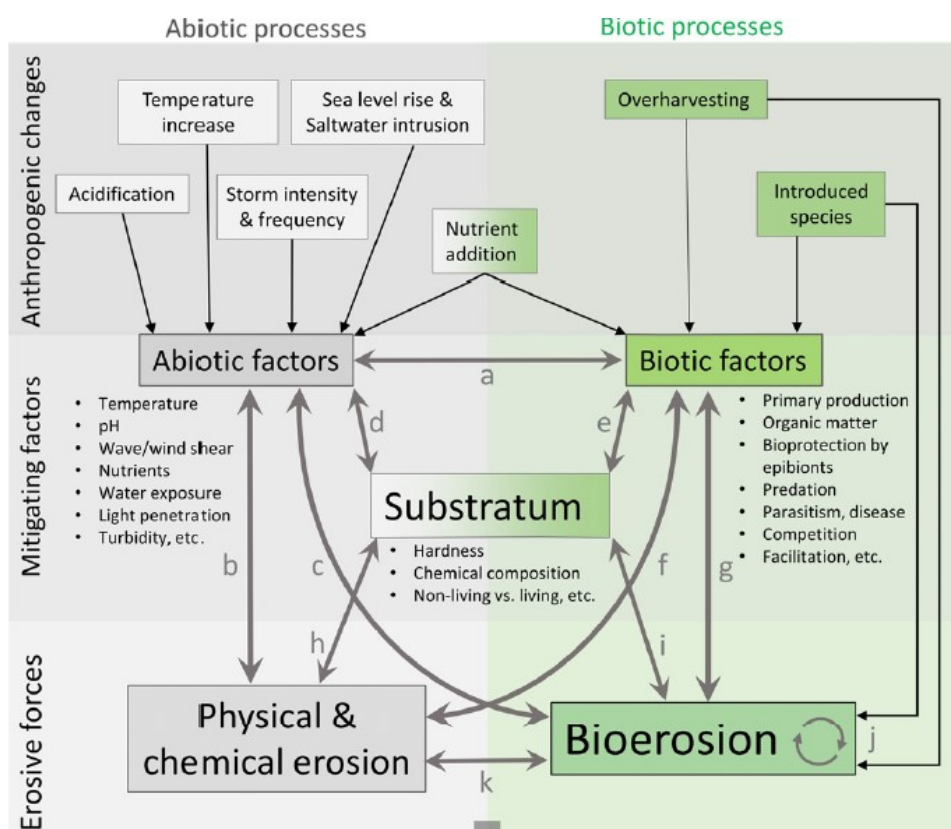
About polychaetes associated with Cnidaria, a recent update was given by Molodstova *et al.* (2016). In this review, authors describe 281 species of cnidarian hosts involved in 324 association, regarding principally skeleton-bearing taxa, as Scleractinia (Figure IIIa). The number of symbiotic polychaetes, here reported, is about 120 species, belonging to 10 different families that, as in the sponges, are mostly represented by Polynoidae and Syllidae (Figure IIIb).



**Figure III.** a) Number of cnidarian hosts; b) Number of symbiotic species each family. From Molodstova *et al.* (2016).

Several authors (Muscatine & Porter, 1977; Martin & Britayev, 1998; Buhl-Mortensen & Mortensen, 2004; Sheppard *et al.*, 2017) reported that these associations allow: i) the decreasing of the predator pressure; ii) refuges; iii) an additional opportunity of feeding and exploitation of the natural compounds; iv) the furnishing of nursery areas and “reproductive centers”.

Bioerosion is a widespread process and it is defined by Neumann (1966) as “the destruction and removal of consolidate mineral or lithic substrate by the direct action of organisms”, while Davidson *et al.* (2018) redefined this concept as “the process by which organisms remove, breakdown, dissolve or fractionate consolidate, hard persistent materials often into smaller constituent components, which are removed or displaced”. Moreover, Ekdale *et al.* (1984) describe as boring activities “all the processes of excavation of a hole in hard substrata by cutting across grains and cement, creating smooth wall; burrowing processes are defined as the activities that include the creation of a hole in a uncemented substratum, by shifting or moving sand grains”. The interaction of the multiple factors, abiotics and biotics, that are implicated in the bioerosion are multiple, as reported by Hutchings (2011), and they are summarized and illustrated by Davidson *et al.* (2018) (Figure IV).



**Figure IV.** Multiple interrelation between the several forces involved in bioerosion processes. From Davidson *et al.* (2018).

Schönberg & Wissack (2014) reported that marine bioerosion processes have been mainly investigated in the Pacific and Atlantic Seas but the Mediterranean Sea is quite represented (11%). These studies are principally conducted about faunistic aspects, while data on the bioerosion rates are scarce (Calcinai *et al.*, 2011; Färber *et al.*, 2015, 2016). Schönberg & Wissack (2014) reported also, that probably, due at their endolithic life style, bioeroders are generally ignored in researches about the biodiversity assessment. Porifera is the most common boring taxon, mainly studied in the Mediterranean Sea (Coll *et al.*, 2010) and in the tropical areas (Hutchings, 2011). Several families of polychaetes, for example Eunicidae and Spionidae, are recognized as the first macro-bores to colonize newly available substrata. They use chemical secretions, to dissolve, and/or typical modified chaetae (as the case of the of the 5<sup>th</sup> chaetiger of the Polydorids), to mechanically grind the substrate (Hutchings, 2008).

Data about boring species of polychaetes in Mediterranean Sea are scarce, and no data about erosion rates are available. A chapter of this thesis is a further step to the study of the bioerosion in the Mediterranean Sea.

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# AIMS

This thesis concerns three main topics: i) Polychaetes and Demosponges, ii) Polychaetes and Octocorals, and iii) Boring Polychaetes.

It is articulated around the following key research questions:

## *i) Polychaetes and Demosponges*

### **Chapter 1:**

- Are there any differences in the diversity and in the abundance of the fauna inhabiting two species of the genus *Sarcotragus* (Demospongiae, Irciniidae)?
- Is the volume of the sponges a key driver to explain the associated fauna abundance and diversity?

### **Chapter 2:**

- Which biometric features can be useful for the study of the nereidid *C. (C.) costae*?
- Are the sponges a functional habitat for this species?

## *ii) Polychaetes and Octocorals*

### **Chapter 3:**

- Which is the distribution, the seasonal trend and the reproduction of the neglected association between the gorgonian *Paramuricea clavata* and *Haplosyllis chamaeleon*?

### **Chapter 4:**

- Which is the molecular spectrum that characterized the gorgonian *P. clavata*?
- Are some compounds, typical of this gorgonian, present also in the associated polychaetes?

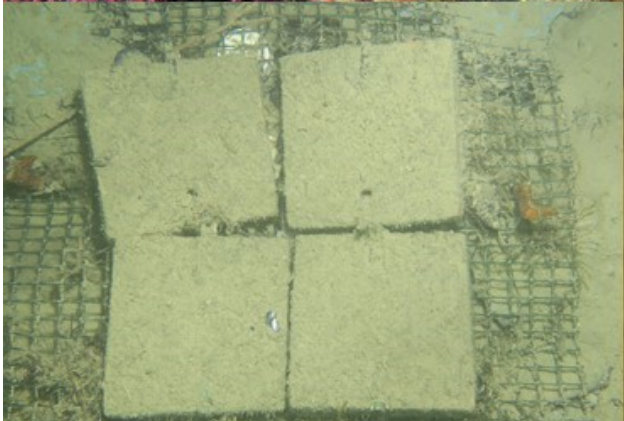
## *iii) Boring polychaetes*

### **Chapter 5:**

- Which are the most important species of polychaetes involved in the boring activity of calcareous substrates and which is their trend?
- Which is the rate of etching of polychaetes during an annual cycle?

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# Chapters



## *Chapter 1*

# **Macrofaunal assemblages inhabiting two Mediterranean species of *Sarcotragus* (Porifera, Demospongiae)**

### **Abstract**

Sponges are election habitat for many species of molluscs, worms, crustaceans and other marine organisms, which exploit them for food, shelter and nursery. Macrofaunal assemblages inhabiting two common species of keratose sponges, *S. spinosulus* and *S. foetidus*, were investigated from November 2016 to April 2018. Polychaeta, Crustacea, Mollusca, and Echinodermata, for a total of 182 taxa, were detected and the 60% of these are first recorded for the first time associated with sponges of the genus *Sarcotragus*. The assemblages inhabiting *S. spinosulus* and *S. foetidus* were different and varied in relation to the sampling period. In *S. spinosulus* 125 taxa were recorded and the abundance varied from a minimum of  $1.22 \pm 0.52$  ind/cm<sup>3</sup> in October 2017 to a maximum of  $6.21 \pm 4.07$  ind/cm<sup>3</sup> in January 2017; *S. foetidus* hosted 110 taxa with a minimum value of  $0.06 \pm 0.03$  ind/cm<sup>3</sup> in February 2018 and a maximum of  $0.43 \pm 16$  ind/cm<sup>3</sup> in October 2017. One-way ANOSIM test reported significant differences in the density of the associated fauna during the months for both the sponges. Our results suggest that the two species of *Sarcotragus* are characterized by a different composition of the associated macrofaunal communities and that *Sarcotragus* has an important ecological role as habitat-forming species enhancing local biodiversity.

*Keywords:* macrozoobenthos, Irciniidae, associations, Adriatic Sea, Ligurian Sea.



## 1.1 Introduction

Sponges are significant components of several benthic habitats as caves (Gerovasileiou & Voultsiadou, 2012, 2016), tropical (Rützler, 1978; Diaz & Rützler, 2001) and temperate (Cerrano *et al.*, 2001; Bertolino *et al.*, 2013) reefs. They are recognized as ecosystem engineers, performing important ecological roles (Wulff, 2006; Becerro, 2008) and establishing hot-spot for the marine biodiversity (Cerrano *et al.*, 2006). Marine sponges play multiple functional roles including bioerosion, substrate stabilization, benthic-pelagic coupling; they develop peculiar biochemical processes allowing symbiotic relationships with other invertebrates and enhancing local biodiversity (Bell, 2008). The complex structure of their aquiferous system, allows sponges to create numerous microhabitats, hosting many organisms (Cerrano *et al.*, 2006), and acting as “living hotels” (Gerovasileiou *et al.*, 2016). Moreover, sponges can reduce the predator pressure offering refuge, (Firth, 1976; Duarte & Nalesso, 1996), implement the source of food both in direct and indirect way (De Gooijet *et al.*, 2013), and may be nurseries for several species (Butler *et al.*, 1995; Stevely *et al.*, 2010).

There are several studies reporting examples of epibiosis and endobiosis between sponges (Firth, 1976; Amoreux *et al.*, 1980; Pansini & Daglio, 1980; Klitgaard, 1995; Duarte & Nalesso, 1996; Koukouras *et al.*, 1996; Rebeiro *et al.*, 2003; Gerovasileiou *et al.*, 2016) and microorganisms (Webster & Taylor, 2012), algae (Calcinai *et al.*, 2006; Di Camillo *et al.*, 2016), hydroids (Puce *et al.*, 2005), octocorals (Calcinai *et al.*, 2004; 2013) crustaceans (Thomas & Klebba, 2006; Yu *et al.*, 2017) and polychaetes (Pascual *et al.*, 1996; Sardà *et al.*, 2002; Musco & Giangrande, 2005; Lattig & Martin, 2011a, b). In the Mediterranean Sea, inquilinism is considered one of the principal types of symbiosis between demosponges and their associated organisms (Gheradi *et al.*, 2001; Fiore & Jutte, 2010), indeed the sponge surface and their aquiferous system can be used as a valid habitat for the benthic organisms (Westinga & Hoetjiez, 1981). Sponges of the family Irciniidae (Dictyocerida: Demospongiae) were studied in different areas of the Western Mediterranean Sea (Koukouras *et al.*, 1992); in particular, Koukouras *et al.* (1985), Ilan *et al.* (1994), Çinar & Ergen (1998), Çinar *et al.* (2002) and Pavludi *et al.* (2016) reported data about macrofaunal assemblages in species of the genus *Sarcotragus* Schmidt, 1862 living along the Aegean coasts. Macrofauna inhabiting *Sarcotragus muscarum* (= *Sarcotragus foetidus* Schmidt, 1862) is diversified and includes several taxa; polychaetes and crustaceans are the most abundant (Çinar *et al.*, 2002), and can reach the 90% of the associated fauna (Koukouras *et al.*, 1987). Similar results were reported by Koukouras *et al.* (1985) for the sponge *Sarcotragus fasciculatus* Pallas, 1766 where polychaetes and crustaceans are the 87% of the total abundance.

Polychaetes are known to be common among the fauna that lives in association with sponges (Rützler, 1975; Martin & Britayev, 1998) but, as reported by Gherardi *et al.* (2001), in some cases their biodiversity was principally linked to the habitat surrounding their hosts.

The large family Syllidae (Polychaeta, Errantia) is characterized by non-free-living forms and one of the most representative species, belonging to this family, is *Haplosyllis spongicola* (Grube, 1855). *H. spongicola* is also a typical symbiont of sponges described in several geographical areas (Martin & Britayev, 1998; López *et al.*, 2001); Licher (2000) summarized around 15 synonymies of this species and Martin *et al.* (2003) affirmed that is probably a sibling species complex.

The family Spionidae is frequent on several kinds of substrate, with species specialized to live on both mineral and living substrates (Dagli *et al.*, 2011), as the case of *Polydora colonia* Moore, 1907 a sponge-associated species (Martin & Britayev, 2018) that was signalled as invasive and cryptogenic and recorded for the first time in the Mediterranean Sea by Aguirre *et al.* (1986).

Crustaceans are the other principal taxon associated with Porifera, especially with Demospongiae (Crowe & Thomas, 2002; Riberio *et al.*, 2003), but also with Calcarea such as in the invasive *Paraleucilla magna* (Padua *et al.*, 2012). Some studies have shown how they may constitute the highest percentage of the associated fauna as in *Amphimedon viridis* (78%, Huang *et al.*, 2008), in *S. muscarum* (= *Sarcotragus foetidus*) (40%, Çinar *et al.*, 2002) and also in sponges living in peculiar system as caves (91%, *Aplysina aerophoba* and 65%, *Agelas oroides*, Gerovasileiou *et al.*, 2016). Examples of typical sponge-symbiotic crustaceans are the amphipods *Leucothoe spinicarpa* and *Colomastix pusilla*, and the isopod *Janira maculosa* (Gerovasileiou *et al.*, 2016). Few species feed on the tissue of the sponge, as *C. pusilla*, but the majority of these uses the opening and the channels of the sponges as refuges.

The bivalve *Hiatella arctica* and echinoderms as *Ophiothrix quinquemaculata* and *Ophiothrix fragilis* live frequently inside sponges as the keratose *S. foetidus*, as reported in Koukouras *et al.* (1985), Çinar *et al.* (2002) and Pavloudi *et al.* (2016).

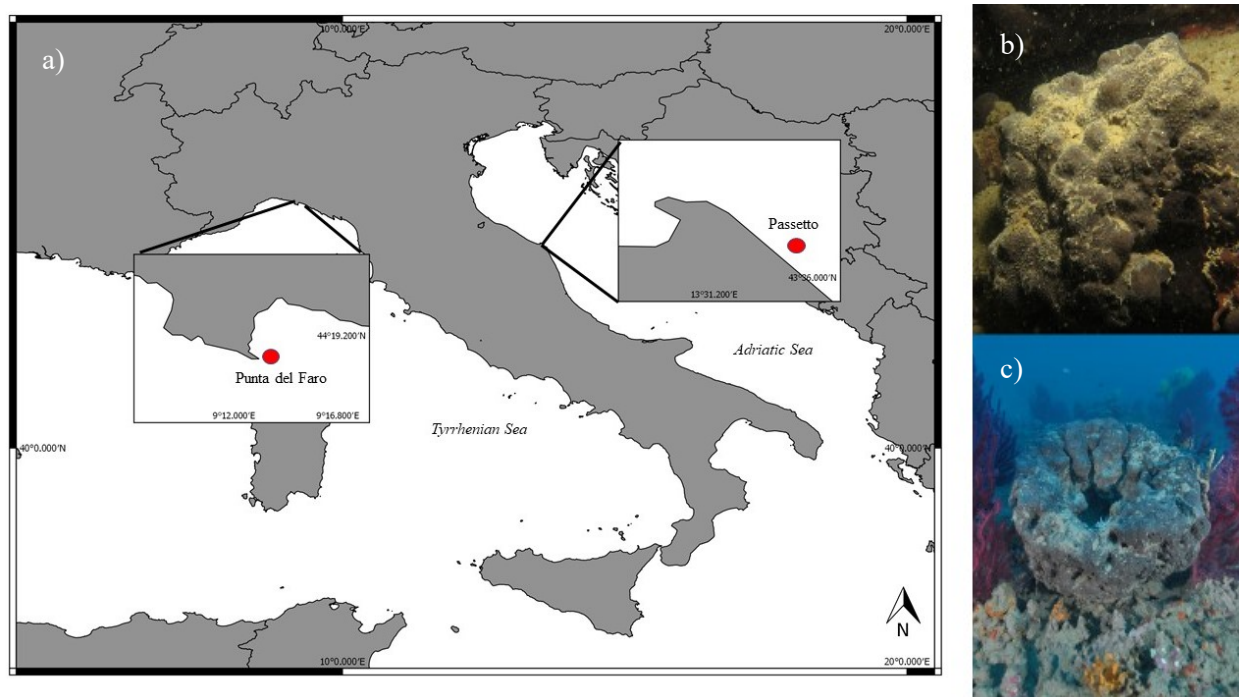
The aim of the present work is to identify and quantify, along a period of 18 months, the macrofaunal assemblages inhabiting the sponges *S. spinosulus* and *S. foetidus*, and to test the null hypothesis that polychaetes are the most abundant and diversified taxon hosted by the studied sponges.

## 1.2 Materials and Methods

Two species of sponges belonging to the genus *Sarcotragus* (Irciniidae: Demospongiae) were collected bimonthly, between November 2016 and April 2018, by scuba divers, in two stations along the Italian coast (Figure 1).

*S. spinosulus* Schmidt, 1862 is a widely distributed species present in the Mediterranean shallow waters; it can reach an average size of 15-20 cm diameter and its surface is uniformly covered by small conules. Samples of this sponge were collected at “Passetto” (Ancona Promontory, Adriatic Sea, 43°37'2.36"N; 13°32'6.55"E), at the depth of 5 and 7 m depth (Figure 1a, b).

*S. foetidus* Schmidt, 1862 reaches an average size of 40-50 cm in diameter and it is included in the Annex II of the Barcelona Convention (UNEP/MAP-SPA/RAC, 2018). Samples of this sponge were collected from the coralligenous substrate in “Punta del Faro” (Portofino Promontory, Ligurian Sea, 44°17'56.58"N; 9°13'8.13"E), at about 30 m depth (Figure 1a, c).



**Figure 1.** a) Map of the study areas; b) *S. spinosulus* (Ancona Promontory); c) *S. foetidus* (Portofino Promontory).

For each sponge species, a fragment was collected from 5 specimens and immediately transferred and closed into a plastic bag. The volume of each sponge portion was measured by water displacement. Associated fauna was extracted following the protocol adopted by Pansini (1970) and Pansini & Daglio (1980); then the collected samples were filtered through a sieve (mesh of 0.125 mm) and preserved in alcohol (70%). To remove all the organisms, eventually still present, each



sponge sample was cut by knife into small pieces (Pansini, 1970). All the sponge-associated fauna was sorted, counted and identified at the lower, possible taxonomic level.

Abundance of the identified macrofauna species inhabiting *Sarcotragus* spp. was calculated as number of individuals/sponge volume (cm<sup>3</sup>).

In both sites, data about water temperature during the sampling period were obtained by ARPA (<http://www.arpa.marche.it/>; <http://www.arpal.gov.it>) and Mareografico portal (ISPRA) (<https://www.mareografico.it/>).

### *Statistical analysis*

The community dissimilarities of the associated fauna, considering the temporal trend, was analysed by non-metric multi-dimensional scaling (nMDS). Differences in the macrobenthic assemblages inhabiting *S. spinosulus* and *S. foetidus* during the different sampling periods were compared, separately, using one-way ANOSIM test. Simper analysis (SIMilarity PERcentages) were conducted for the identification of the species that contributed to differentiate the months that results more dissimilar. Dominance and diversity analysis were performed considering the sampling periods, for the two species, by calculating Shannon-Wiener (H') and Equitability (J') indices. One-way ANOVA test and *post-hoc* Tukey's test were conducted for testing differences in the indices.

The relationship between number of taxa vs sponge volumes and density vs sponge volumes were estimated by linear regression analysis.

Statistical analyses were conducted using Past 3.16 (Hammer *et al.*, 2001).

### 1.3 Results

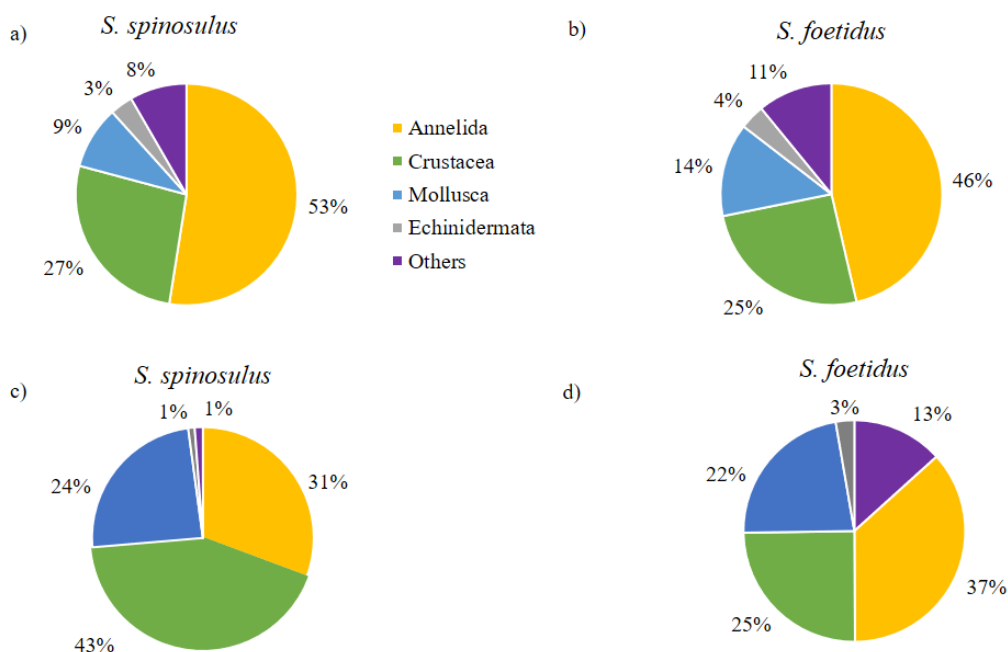
Considering the two species of *Sarcotragus*, 7892 specimens belonging to 182 taxa were identified but only 46 were common to both the sponges. 109 taxa were recorded for the first time as associated to species of this genus. The complete list of the taxa, their first record as symbionts of *Sarcotragus*, their feeding habits and presence during the studied period were reported in the Annex I.

In *S. spinosulus* 120 taxa were recorded and ascribed to Porifera (1), Cnidaria (2); Polychaeta (62), Sipunculida (1), Crustacea (32), Chelicerata (1), Mollusca (11), Echinodermata (4), Ectoprocta (2), Ascidiacea (3) and Nemertea (1). In *S. foetidus* 110 taxa were recorded and ascribed to: Porifera (1), Cnidaria (2); Polychaeta (49), Sipunculida (2), Crustacea (28), Chelicerata (4), Hexapoda (1), Mollusca (15), Echinodermata (4), Ectoprocta (1), Ascidiacea (2) and Nemertea (1).

In *S. spinosulus* the percentage of taxa was higher for Annelida (53%), followed by Crustacea (27%), Mollusca (9%), others (8%) and Echinodermata (3%) (Figure 2a).

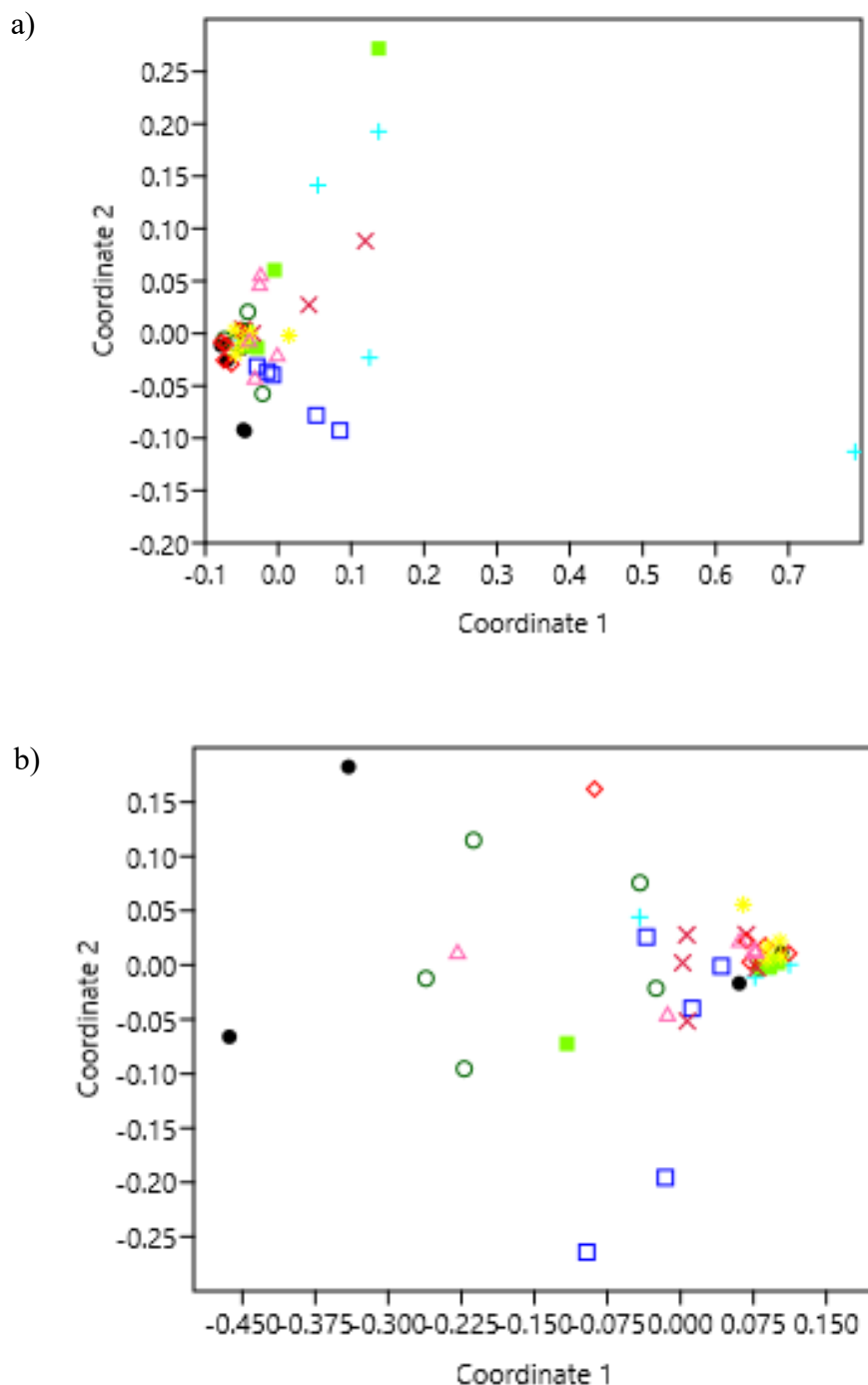
In *S. foetidus*, Annelida showed the highest percentage of taxa (46%), followed by Crustacea (25%), Mollusca (14%), others (11%) and Echinodermata (4%) (Figure 2b).

In *S. spinosulus* the most abundant taxon (as percentage of number of individuals/cm<sup>3</sup>) was Crustacea (43%) followed by Annelida (31%), Mollusca (24%), Echinodermata (1%) and other taxa (1%) (Figure 2c). In *S. foetidus*, the most abundant taxon was Annelida (37%), followed by Crustacea (25%), Mollusca (22%), other taxa (13%) and Echinodermata (3%) (Figure 2d).



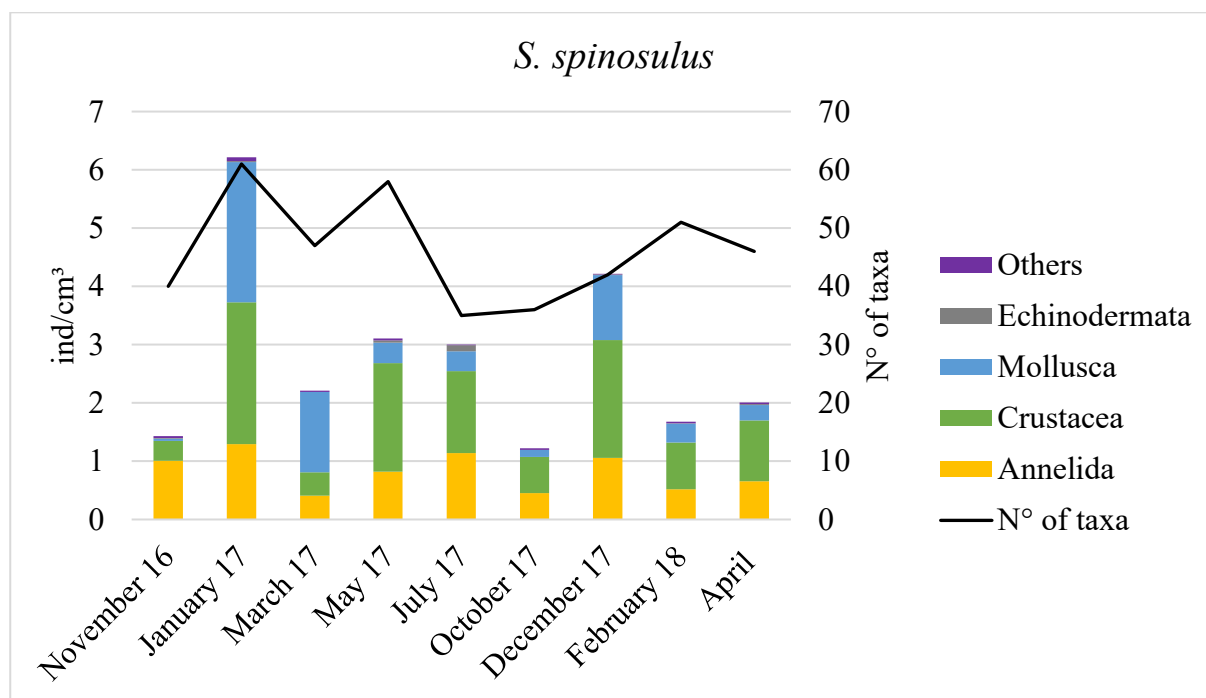
**Figure 2.** a) Percentages of the taxa associated with a) *S. spinosulus* and b) *S. foetidus* and percentage of number of individuals/cm<sup>3</sup> of each recorded taxon in c) *S. spinosulus* and d) *S. foetidus*.

The n-MDS analysis shows similarity of the abundances of the associated fauna (number of individuals/cm<sup>3</sup> sponge), during some of the sampling periods in *S. spinosulus* (Figure 3a) (stress=0.1364) and in *S. foetidus* (stress=0.0567) (Figure 3b), while others show higher distance (January '17, November '16, etc.).



**Figure 3.** n-MDS of the abundances of the assemblages inhabiting a) *S. spinosulus* and b) *S. foetidus*. Black dots November '16, light blue cross January '17, blue squares March '17, light green full squares May '17, rose cross June '17, dark green circles October '17, red lozenge December '17, yellow stars February '18, pink triangles April '18.

The density of the macrofauna inhabiting *S. spinosulus* changes between  $1.22 \pm 0.52$  ind/cm<sup>3</sup> in October '17 and  $6.21 \pm 4.07$  ind/cm<sup>3</sup> in January '17 while the number of associated taxa varied between 61 in January '17 and 35 in July '17 (Figure 4).



**Figure 4.** Density of *S. spinosulus* assemblages and number of taxa during the sampling months.

The non-parametric ANOSIM test reported clearly dissimilarity ( $p=0.0001$ ) between the abundances of the macrofauna associated to *S. spinosulus* during the months, as previously suggested by n-MDS.

Simper analysis identified that the bivalve *Mytilus galloprovincialis* Lamarck, 1819 (29.12%), the amphipod *Erichthonius brasiliensis* Dana, 1853 (15.61%), the tanaidacean *Chondrochelia savignyi* (Kroyer, 1842) (6.70%) and the barnacles *Acasta spongites* (Poli, 1791) (5.78%) are the species that contribute more to differentiate the assemblages in *S. spinosulus* comparing January '17 (higher value) vs. October '17 (lower value).

In *S. foetidus*, abundances varied between  $0.06 \pm 0.03$  ind/cm<sup>3</sup> in February '18 and  $0.43 \pm 16$  ind/cm<sup>3</sup> in October '17; during this month, the number of taxa was higher (61 taxa) and minimum in February '18 (19 taxa) (Figure 5).

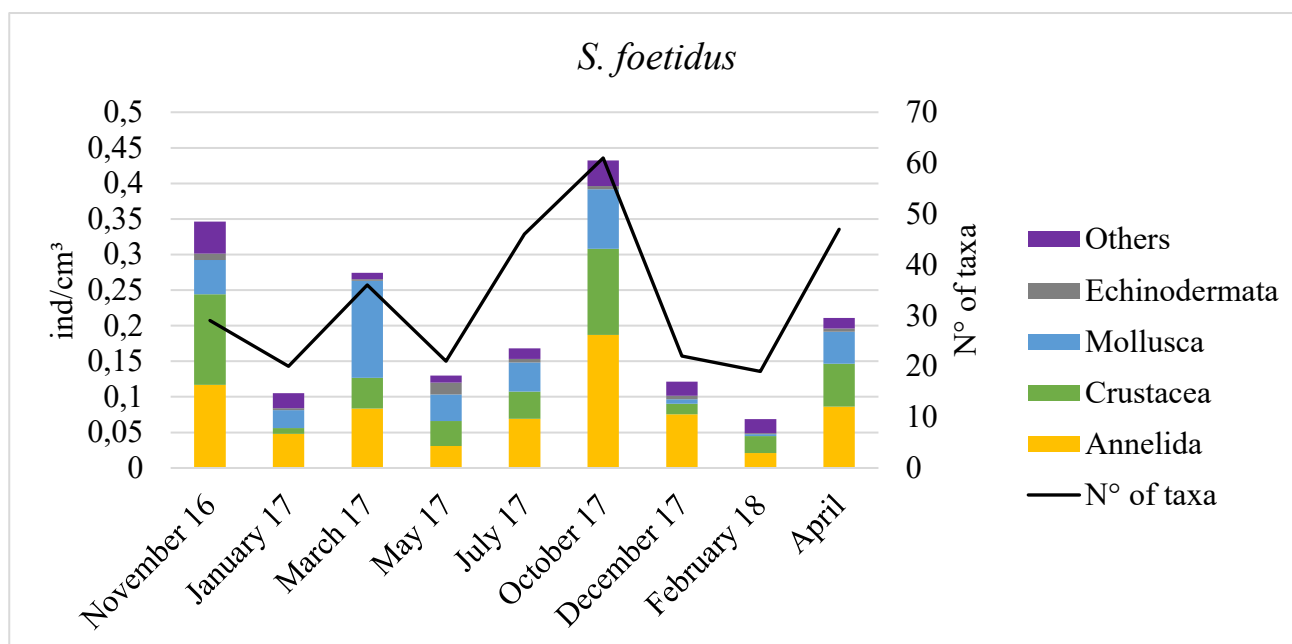


Figure 5. Density of *S. foetidus* assemblages and number of taxa compared among the months.

The partial dissimilarity showed by n-MDS test (Figure 4b) is supported by the ANOSIM test ( $p=0.0004$ ) that reported significant differences between the abundances of the macrofauna inhabiting *S. foetidus* among the sampled months. Simper analysis showed that mainly the bivalve *Hiatella arctica* (Linnaeus, 1767) (11.84%), the isopod *Janira maculosa* Leach, 1814 (7.78%), the polychaetes *Sphaerosyllis pirifera* Claparède, 1868 (6.56%) and *Polyophthalmus pictus* (Dujardin, 1839) (6.27%) affect the structure of the assemblages in *S. foetidus* comparing October '17 (highest value of diversity) vs. February '18 (lower value) (Figure 5).

In *S. spinosulus*, polychaetes showed maximum values in November '16 ( $5.043 \pm 0.702$  ind/cm<sup>3</sup>) January '17 ( $6.465 \pm 0.779$  ind/cm<sup>3</sup>) and July '17 ( $5.705 \pm 0.943$  ind/cm<sup>3</sup>) while remained quite constant during the other months. The species continuously present were *Micromaldane ornitochaeta* ( $0.62 \pm 0.72$  ind/cm<sup>3</sup> in July '17), *Ceratonereis (Composetia) costae* ( $0.03 \pm 0.028$  ind/cm<sup>3</sup> in July '17 and  $0.029 \pm 0.03$  ind/cm<sup>3</sup> in Feb '18), *Parasabella langheransi* ( $0.05 \pm 0.06$  ind/cm<sup>3</sup> in July '17 and  $0.023 \pm 0.025$  ind/cm<sup>3</sup> on Jan '17), *Exogone naidina* ( $0.40 \pm 0.26$  ind/cm<sup>3</sup> in Jan'17 and  $0.13$  ind/cm<sup>3</sup> in October and December '17 and February '18), *Syllis gracilis* ( $0.05 \pm 0.11$  ind/cm<sup>3</sup> in Nov'16) and *Sphaerosyllis pirifera* ( $0.12 \pm 0.18$  ind/cm<sup>3</sup> in Jan'17).

In *S. foetidus*, polychaetes were more abundant in October '17 ( $0.15 \pm 0.10$  ind/cm<sup>3</sup>) and in November '16 ( $0.11 \pm 0.14$  ind/cm<sup>3</sup>) while decreased in the other months, as in July '17 ( $0.06 \pm 0.03$  ind/cm<sup>3</sup>) and February 2018 ( $0.02 \pm 0.01$  ind/cm<sup>3</sup>). The nereid *Ceratonereis (Composetia) hircinicola* was recorded in each month and reached the highest density of  $0.022 \pm 0.023$  ind/cm<sup>3</sup> in October 2017.

In our samples, 32 species of Amphipods were also identified: 15 associated to *S. spinosulus* and 13 to *S. foetidus*, only 5 species were common to both the sponges (Annex I), as the case of the species *Stenothoe monoculoides* (Montagu, 1815). This amphipod was frequent and showed a similar seasonal trend during the year disappearing during the winter. In *S. spinosulus* this species reached the maximum abundance in March'17 ( $0.018 \pm 0.14$  ind/cm<sup>3</sup>), while in *S. foetidus* in May'17 ( $0.25 \pm 0.052$  ind/cm<sup>3</sup>). In *S. spinosulus*, *Gammaropsis maculata* was present during the whole studied period and reached the density of  $0.25 \pm 0.28$  ind/cm<sup>3</sup> in May'17; *Erichtonius brasiliensis* (Dana, 1853) (Annex II d,e,f) ( $0.85 \pm 0.57$  ind/cm<sup>3</sup> in January'17) was recorded only in November '16, January '17 and February '17; finally, the species *Elasmopus brasiliensis* (Dana, 1855) was common during all the period, reaching the value of  $0.16 \pm 0.30$  ind/cm<sup>3</sup> in January'17.

Specimens belonging to the superfamily Caprelloidea were found only in *S. foetidus*; in particular, *Phtisica marina* Slabber, 1769 was present, with similar abundances, during all the months (e. g.  $0.002 \pm 0.005$  ind/cm<sup>3</sup> in March '17 and  $0.003 \pm 0.006$  ind/cm<sup>3</sup> in April '18). The amphipods *Liljeborgia dellavallei* Stebbing, 1906 (Liljeborgiidae) and *Colomastix pusilla* Grube, 1861 were frequent and reached the higher value of abundance in May '17 ( $0.005 \pm 0.012$  ind/cm<sup>3</sup>) and in July '17 ( $0.01 \pm 0.009$  ind/cm<sup>3</sup>), respectively.

About bivalves, *Hiatella arctica* (Linnaeus, 1767) was present in both the sponges during all the period. In *S. spinosulus*, this species presented the higher value of abundance in February '18 ( $0.234 \pm 0.456$  ind/cm<sup>3</sup>) while in the remaining coldest months, the abundance was lower ( $0.0395 \pm 0.0361$  ind/cm<sup>3</sup> in November '16,  $0.02 \pm 0.027$  ind/cm<sup>3</sup> in January '17,  $0.019 \pm 0.015$  ind/cm<sup>3</sup> in Dec '17).

In *S. foetidus*, the abundance of *Hiatella arctica* was lower, indeed, the maximum and minimum values were  $0.023 \pm 0.019$  ind/cm<sup>3</sup> in July '17 and  $0.011 \pm 0.008$  ind/cm<sup>3</sup> in January '17, respectively.

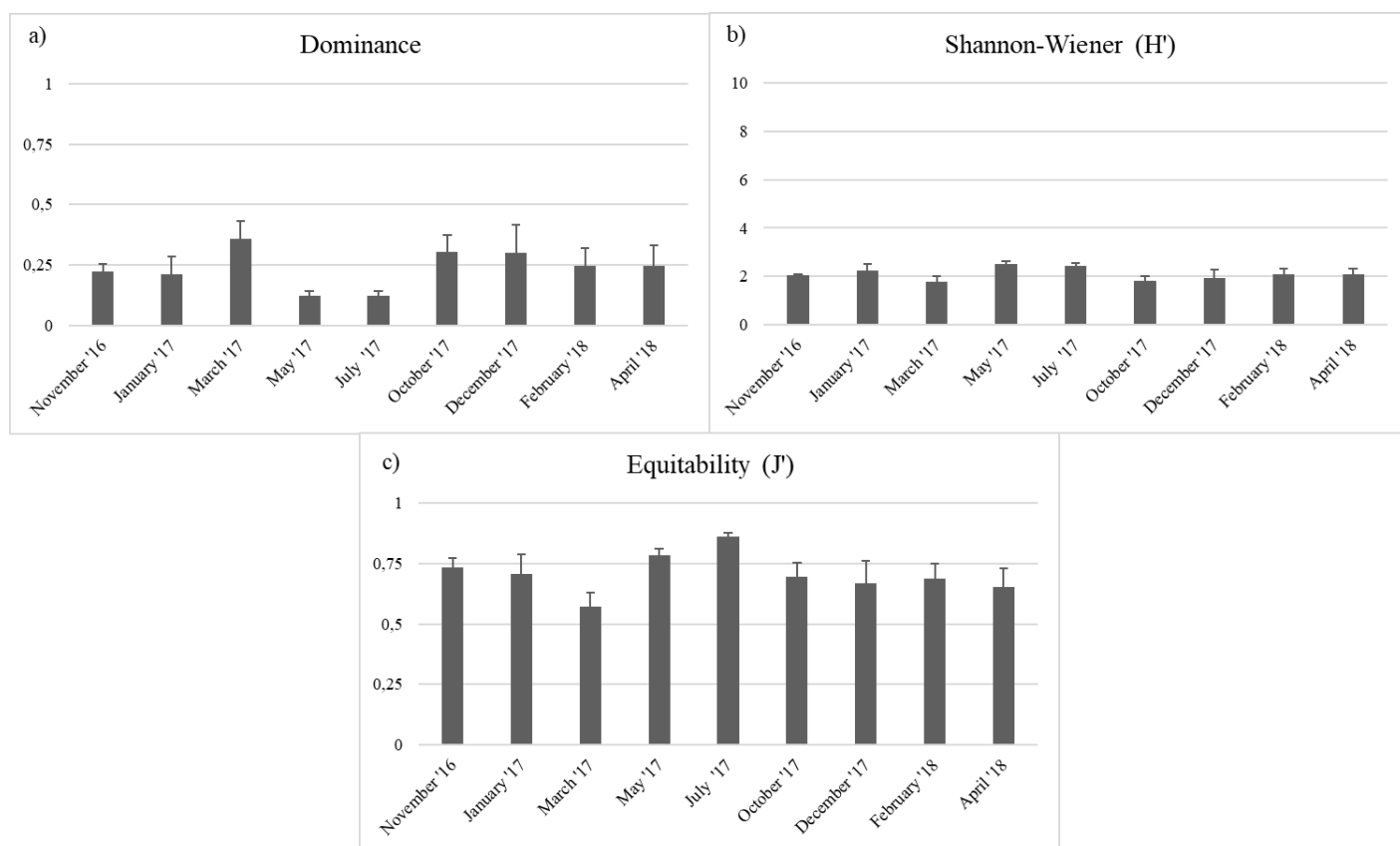
The species *Mytilus galloprovincialis* was recorded only in *S. spinosulus*. In this sponge the highest abundance was in January '17 ( $2.38 \pm 3.51$  ind/cm<sup>3</sup>) and it decreased during the following months (July '17  $0.25 \pm 0.10$  ind/cm<sup>3</sup>); the bivalve disappeared during the late summer and appeared again in February '18 ( $0.076 \pm 0.10$  ind/cm<sup>3</sup>).

Except for the species *Amphipolis squamata* (Delle Chiaje, 1828) (Echinodermata: Ophiuroidea), that was observed only associated with *S. spinosulus*, (highest value in July '17 ( $0.10 \pm 0.11$  ind/cm<sup>3</sup>), the other three species, *Amphiura chiajei* (Cantraine, 1835), *Ophiothrix quinque maculata* (Delle Chiaje, 1828) and *O. fragilis* (Abildgaard in O. F. Müller, 1789) were recorded in both the sponges. *A. chiajei* was principally associated to *S. foetidus*, where it was present during all the period and reaches the highest abundances in May '17 ( $0.01 \pm 0.02$  ind/cm<sup>3</sup>). Specimens associated

to *S. spinosulus*, showed a similar result in December '17 but they were present just in this month and in April '17 when *A. chiajei* was substituted by the species *O. fragilis* ( $0.012 \pm 0.012$  ind/cm<sup>3</sup>).

During this study, many specimens belonging to several taxa were observed with gametes or in their juvenile stage, as in the case of the polychaetes *Lysidice unicornis*, *Lysidice margaritacea*, *Exogone naidina*, *Ceratonereis (Composetia) costae*, *Ceratonereis (Composetia) hircinicola*; the amphipods *Erichthonius brasiliensis*, *Gammaropsis maculata*, *Leucothoe spinicarpa*; the isopod *Janira maculosa*; the barnacle *Acasta spongites* and the bivalve *Mytilus galloprovincialis*.

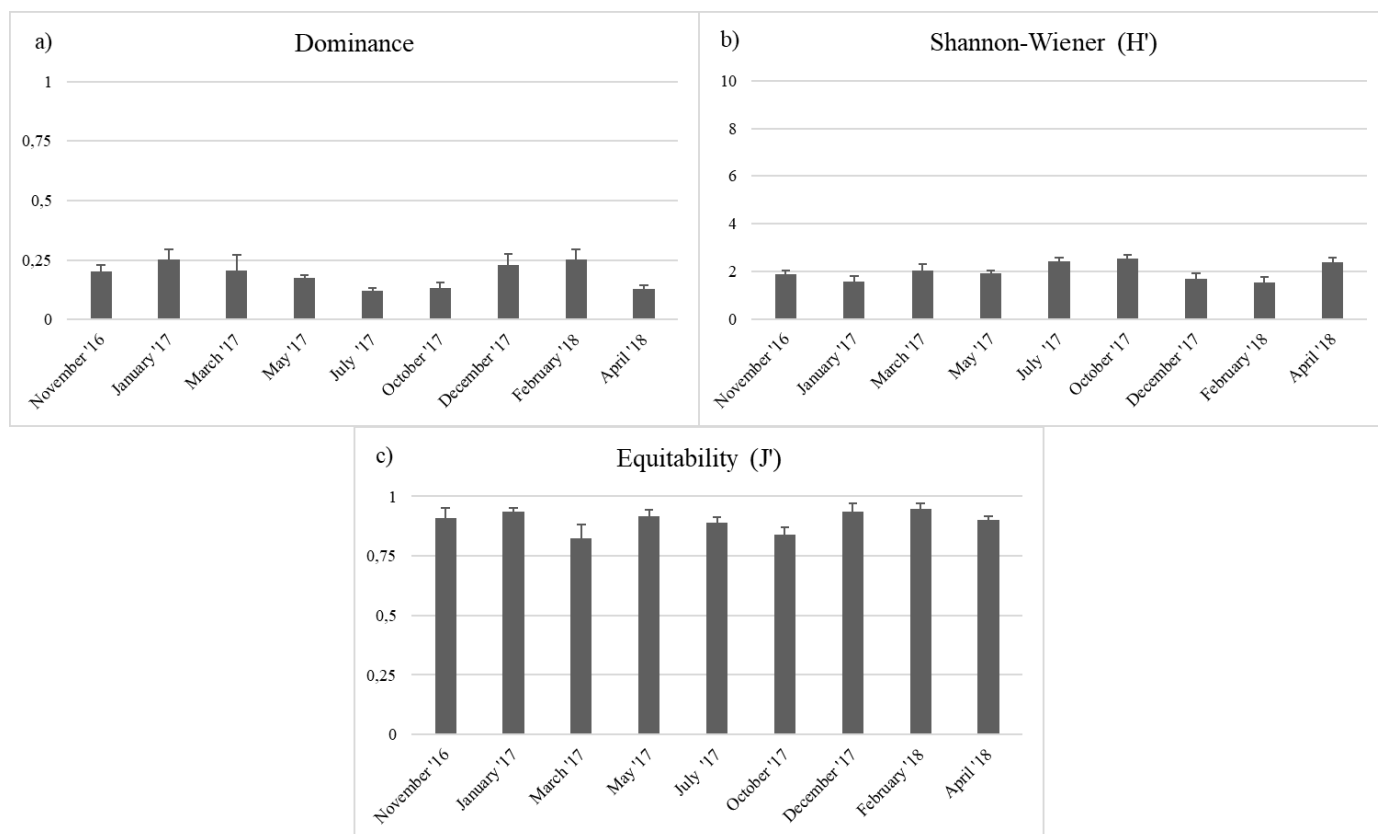
About *S. spinosulus* (Figure 6), dominance index presented low values comprised between  $0.122 \pm 0.019$  in July '17 and  $0.358 \pm 0.019$  in March '17 (Figure 6a), Shannon-Wiener index ( $H'$ ) was quite similar during the months (Figure 6b) and Pielou index ( $J'$ ) varied between  $0.573 \pm 0.057$  in March '17 and  $(0.861 \pm 0.015)$  July '17). No significant differences in indexes values resulted ( $p > 0.05$ ).



**Figure 6.** *S. spinosulus*: a) Dominance; b) Shannon-Wiener ( $H'$ ) index; c) Equitability ( $J'$ ) index.

In *S. foetidus* (Figure 7), dominance presents low values comprised between  $0.119 \pm 0.001$  in July '17 and  $0.252 \pm 0.044$  in February '18, Shannon-Wiener index ( $H'$ ) varied between  $1.539 \pm 0.209$  in February '18 and  $2.522 \pm 0.155$  in October '17, while Pielou index ( $J'$ ) had similar values during the different months. One-way ANOVA showed no significant differences in Dominance and Pielou

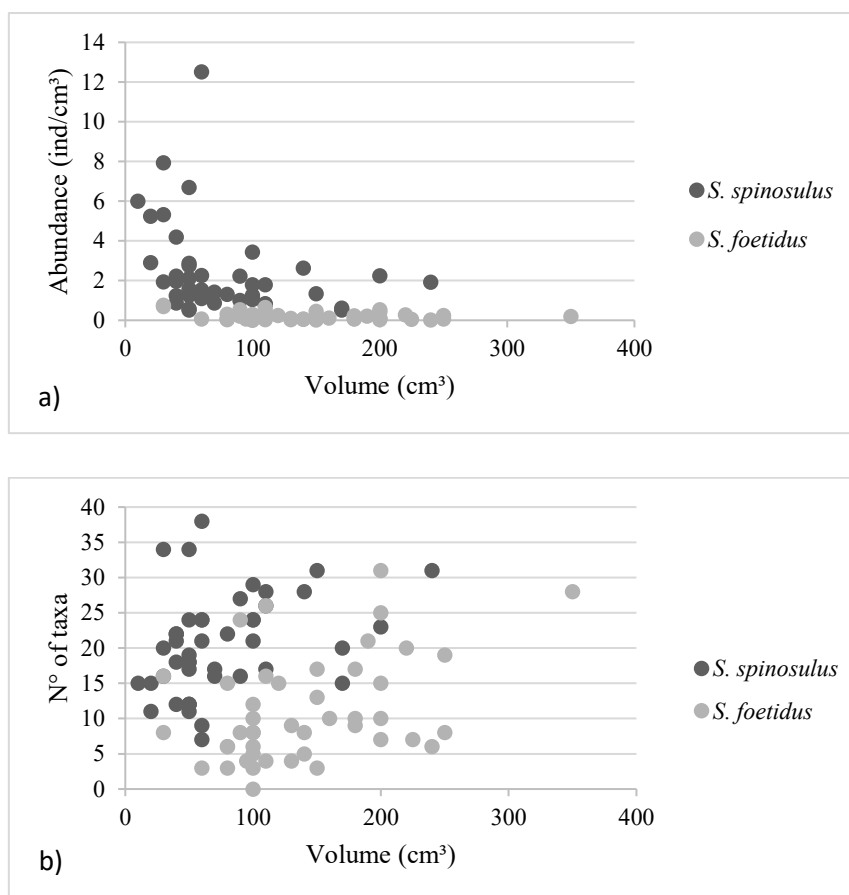
index values ( $p=0.002$ ), but significant differences in Shannon-Wiener index ( $p>0.05$ ). Post hoc pairwise comparisons, based on Tukey's test, showed differences between values of January '17 and October '17, between July '17 and February '18, and between October '17 and February '18.



**Figure 7.** *S. foetidus*: a) Dominance; b) Shannon-Wiener ( $H'$ ) index; c) Equitability ( $J'$ ) index.

The results of the linear regression analyses are shown in Figure 8a, b. The abundance of associated fauna and the number of taxa did not increase with the increasing of the volume in *S. spinosulus*,  $R^2 = 0.117$  and  $R^2 = 0.118$ , respectively. Also comparing the *S. foetidus* volume with the abundances and the presences no relationships were observed,  $R^2 = 0.041$  and  $R^2 = 0.163$ , respectively.





**Figure 8.** a) Relationship between the abundance of associated fauna in *S. spinosulus* and *S. foetidus* vs volume of the sponges; b) Relationship between the total number of taxa vs the volume of *S. spinosulus* and *S. foetidus*.

## 1.4 Discussion

Although macrofauna inhabiting *Sarcotragus* spp. has been already investigated in the last decades (Pansini, 1970, 1980, 1981; Koukouras *et al.*, 1985; Çinar *et al.*, 1998, 2002; and Pavloudi *et al.*, 2016) few data are available for *S. foetidus*, while *S. spinosulus* has never been studied. Here we give an update about *Sarcotragus* spp. associated species and about their trend along a period of 18 months.

As previously reported by other authors (Pansini, 1970; Koukouras *et al.*, 1985; Çinar *et al.*, 2002; and Pavloudi *et al.*, 2016), polychaetes and crustaceans are the most abundant and diversified taxa associated with *Sarcotragus* spp., followed by molluscs, echinoderms and other minor taxa. Regarding *S. spinosulus*, we confirm the results of Pansini (1970) and Pavloud *et al.* (2016) who found Arthropoda to be the most diversified phylum. Regarding *S. foetidus* our results agree with those of Çinar *et al.* (2002) who recorded 60% of the associated organisms as polychaetes.

In total, the density of the fauna inhabiting *S. foetidus* is comparable to that reported by the same authors; for example, Çinar *et al.* (2002) in *S. muscarum* (= *S. foetidus*) living along the Turkish coasts, evaluated a mean density of 0.22 ind/cm<sup>3</sup>, while Koukouras *et al.* (1985) for *S. muscarum* (= *S. foetidus*) and *S. fasciculatus*, along the Greek coast, values of 0.054 ind/cm<sup>3</sup> and 0.093 ind/cm<sup>3</sup>, respectively. Higher values were reported by Pansini (1970) for *Ircinia fasciculata* (= *S. fasciculatus*) along the Italian coasts (2.42 ind/cm<sup>3</sup>). On the contrary, all these published data are lower in respect with those calculated for *S. spinosulus* from Ancona promontory (up to 6.21 ind/cm<sup>3</sup>).

The high tropic inputs typical of the Adriatic Sea (Tagliapietra *et al.*, 2008) and the presence of several rivers, as the Misa and Esino, along the north coast of Ancona, supply nutrients and increase the turbidity of the marine water; moreover some rain spilling and also algal bloom (*Ostreopsis ovata*) in late summer (215.000 cel/L in October '17), also close to the sampling sites (ARPAM 2016, 2017), can have influenced the macrofaunal organisms living in this habitat and may be responsible for the highest general values registered in *S. spinosulus*. Whereas, the low value reported in *S. foetidus* can be ascribed to the oligotrophic characteristics of the Ligurian Sea (Misic & Fabiano, 2016; Di Carro *et al.*, 2018). Nevertheless, it is not easy to find the key factors that influence the macrofaunal living in these sponges, as reported by Pansini (1970). Several authors, for example, suggested that the photophilic environment surrounding the sponges contributes to increase the biodiversity and the abundance of the sponge-associated fauna, compared with bare rock habitats (Voultsiadou *et al.*, 1987; Gherardi *et al.*, 2001; Çinar *et al.*, 2002). Pansini (1970) reported a decrement of the density of the fauna associated with *S. fasciculatus*, *S. officinalis* and *P. ficiformis*, during the coldest months, while observed an increment of the density of the crustacean

and molluscs on late spring, and of polychaetes at the end of the summer; despite, the frequency of the species remained constant during the year. Similar results were also reported by Pavloud *et al.* (2016) that described differences, between the summer and the winter, in the fauna inhabiting *S. foetidus* collected in two areas of the North Aegean Sea. Our results confirm that the composition and the abundances of the sponge-associated fauna changes during the studied period (one-way ANOSIM test), with the highest values of abundance during the coldest months, in both the sponges. In some cases, the higher abundance is ascribable to the reproductive period of some species, such as *Mytilus galloprovincialis* and of *Erichthonius brasiliensis*. Along the Ancona-Promontory, *Mytilus* assemblages are very common and the spawning period that occurs in winter (January-February) (Da Ros *et al.*, 1985), explains the high density of juveniles of *M. galloprovincialis* in January on *S. spinosulus* surface. The filter activity of the sponge draws the larvae that find refuge around the oscules while the adults prefer the rocky substrate surrounding the sponges, where they are very common. The amphipod *E. brasiliensis* has been mainly detected in January '17, while it was quite absent in the rest of the sampled period. In this month, several juveniles, males and ovigerous females were collected, suggesting that this crustacean uses the sponge as nursery area. This ecological function is played by Porifera and studies about their importance in hosting juvenile forms and reproductive adults were conducted in several ecosystems. Examples have been reported for the Florida Keys where the abundance of the sponges influences the recruitment of the lobster *Palinurus argus* (Butler *et al.*, 1995; Stevely *et al.*, 2010), in the Caribbean Sea as supporter of the fish communities (Seeman *et al.*, 2018), while Gerovasileiou *et al.* (2016) stated as sponges, living in the entrance of several marine caves function as “reproductive centres” for several macrofaunal organisms as amphipods. We also observed other taxa using the sponges during their reproductive period, as the polychaetes *Caratonereis (Composetia)* spp., *Lysidice* spp. and other syllids; ovigerous females of the amphipod *Gammaropsis maculata* (Johnston, 1828) (Annex II g) and the isopod *Janira maculosa* Leach. 1814 (Annex II n, o, p).

In *S. spinosulus* the dominance and the biodiversity indices didn't differ, suggesting that the diversity does not change during the sampled period and no dominant species were present. Also, in *S. foetidus*, results suggest that prevailing taxa were not present (Dominance and Pielou indices), on the contrary the Shannon-Wiener index was significantly different comparing some months, as shows by the one-way ANOVA, in particular regarding the month of February 2018 that is characterized by lowest value of abundance and taxa. About Shannon-Wiener index, *S. spinosulus* and *S. foetidus* are characterized by lower value compared with those of Çinar *et al.* (2002) (value ranged from 1.09 to 4.35), on the contrary Pielou index was found to be highest in this study, both for *S. spinosulus* and *S. foetidus* (value ranged from 0.27 to 0.88, Çinar *et al.* (2002)).

Most of the associated taxa reported in the present paper for the sponge *S. spinosulus* are easily detectable in the typical North and Central Adriatic Sea assemblages (Cantone, 2003) and in coralligenous assemblages for *S. foetidus*. In this last sponge, few sponge's parasite species, as the polychaete *Haplosyllis spongicola* and the amphipods *C. pusilla* were recorded. As pointed out by López *et al.* (2001) in temperate waters, *H. spongicola* is hosted by sponges in very low density while in tropical areas it can be present in hundreds of individuals. In our specimens, *H. spongicola* was recorded at low density, up to 0.0125 ind/cm<sup>3</sup> in November '16. This aspect can be explained following Rützler (1976) and Alos *et al.* (1982), that suggested that sponges with aquiferous system characterized by bigger channels are able to host symbionts of bigger dimensions in respect to smaller sponges that host symbionts of reduced sizes. Nevertheless, in *S. spinosulus* we recorded both small juveniles and big adults as documented for some Eunicidae and Nereididae (see Chapter 2).

Other species, reported in this paper, such as the polychaetes *Exogone naidina* Örsted, 1845, *Syllis gracilis* Grube, 1840, *S. hyalina* Grube, 1863, *Ceratonereis (Composetia) costae* (Grube, 1840), *Lysidice collaris* Grube, 1870, *Lysidice ninetta* Audouin & H. Milne Edwards 1833 and *Lysidice unicornis* (Grube, 1840), the amphipods *Colomastix pusilla*, the tanaidacean *Condrochelia savignyi*, the isopod *Janira maculosa*, the bivalve *Hiatella arctica* and the ophiuroids *Ophiothrix fragilis* and *Ophiothrix quinquemaculata* have been already described as the most common *Sarcotragus*-symbionts (Koukouras *et al.*, 1985, 1992; Ilan *et al.*, 1994, Çinar & Ergen, 1998, Çinar *et al.*, 2002, Pansini & Daglio, 1980 and Pavludi *et al.*, 2016). Among polychaetes, Syllidae is the most diversified family and some species belonging to the genus *Exogone* are common endobionts (Martin & Britayev, 1998; Çinar & Ergen 1998) but live also on other substrates (Alös *et al.*, 1980). In this study, *Exogone naidina* was one of the most common taxon in *S. spinosulus*; conversely in *S. foetidus* this species was not present, while the congeneric *Exogone dispar* (Webster, 1879), typical of deeper water and supposed to eating the sponge tissue (Gherardi *et al.*, 2001) was present. Specimens of *Branchiosyllis exilis* (Gravier, 1900) were identified in the two species of sponges. This syllid is considered as sponge parasite (Martin & Britayev, 1998) and a lessepsian species, recorded in the Mediterranean Sea, along the Aegean coast by Çinar *et al.* (2005) on rocky bottom and sponges. First record of this species along the northern Adriatic Sea was reported by Mikac & Musco (2010) that collected specimens from Rovinj (Croatian coast) in habitat characterized also by the presence of sponges belong to the family Irciniidae. Several other polychaetes, belonging to other families, were recorded exclusively in *S. spinosulus*, such as *Micromaldanae ornitochaeta* Mesnil, 1897, that is considered rare and up today recorded only in the north Tyrrhenian and South Adriatic Seas, and *Polydora colonia* Moore, 1907, (Annex II s, t, u) a sponge's symbiont and

invasive species recorded only in the south Adriatic Sea (Occhipinti-Ambrogi *et al.*, 2011). These new records of *P. colonia* in the North Adriatic show how this species is enlarging its presence in the Mediterranean Sea. Another recorded alien species is the lessepsian eunicid *Lysidice collaris* (Kurth-Sahin & Çinar, 2017), (AnnexII v), already known for the Adriatic Sea, that with the congeneric, Mediterranean and endemic species *L. margaritacea*, was associated with *S. spinosulus*. These new findings of non-indigenous associated species confirm how studies about the macrofauna communities associated with Porifera can give an important contribute to the monitoring of the invasive species (Çinar *et al.*, 2002).

Regarding the family Nereididae, we observed two different species of the genus *Ceratonereis* (*Composetia*) associated with the two sponges; *Ceratonereis* (*Composetia*) *costae* was present in both the species, while the congeneric *C. (C.) hircinicola* (Eisig, 1870), that is a strict symbiont of keratose sponges of the Irciinidae family, was exclusively found in *S. foetidus*. This species is known in the North Adriatic Sea (Cantone, 2003) and is considered there quite common, but, surprising, not recorded in our specimens from Ancona promontory.

Crustacea was the most abundant taxon in the sponge *S. spinosulus* but second in term of diversity, while in *S. foetidus* was second for the abundance and the diversity. In both the sponges, most of the crustaceans were represented by amphipods. In *S. foetidus*, species that live in the circalittoral zone, as *Ampelisca ruffoi* Bellan-Santini & Kaim-Malka, 1977 and *Autonoe karamani* (Myers, 1976) were identified, while in *S. spinosulus* we recorded species of the infralittoral zone such as *Monocorophium sextonae* (Crawford, 1937) and *Gammaropsis maculata* (Johnston, 1828).

Although several colonies of epibionts, that are typical caprellid-hosting species, covered frequently the surface of the sponges, we recorded the caprellids *Phtisica marina* Slabber, 1798 and *Pseudoprotella phasma* in forma *typica* (Montagu, 1808) only associated with *S. foetidus*.

The obligate-symbiont barnacles *Acasta spongites* (Zintzen & Kerckhof, 2009) was recorded only in *S. spinosulus*, but not in *S. foetidus*, even if this is a common and typical symbiont of keratose sponges in the Mediterranean Sea. Our observations suggest that *A. spongites* does not occupy the oscules of the sponges, as reported by Kolosvary (1947) and Relini (1980), but it grows surrounded by the sponge tissue (Annex II a, b, c); the barnacles are completely covered except for a small opening where the cirri come out. Other authors reported the presence of other species of barnacles, as *Balanus perforatus* Brugiére, 1798, recorded in the sponge *S. foetidus* by Koukouras *et al.* (1985).

A typical bivalve living in sponges and crevices of the rocks is *Hiatella arctica* (Linnaeus, 1767); during the sampling period this species was always present, exploiting the lacunar tissue of the sponges. In these lacunae, we noticed an accumulation of soft sediments frequently hosting

polychaetes (Annex II q, r). The species *Modiolus barbatus* (Linnaeus, 1758) and *Musculus subpictus* (Cantraine, 1835) were exclusively found into the choanosome of *S. foetidus*, as reported also by Çinar *et al.* (2002). *M. galloprovincialis* exclusively recorded on *S. spinosulus* ectosome surrounding oscula, was recorded on this species also by Çinar *et al.* (2002).

Regression analysis showed no relationship between the sponge volumes and the total abundance of the associate community and the number of taxa. This result is in contrast with the majority of the data reported by other authors that described an increase of the associated fauna with the increasing of the dimensions of the sponges (Koukouras *et al.*, 1992; Gherardi *et al.*, 2001; Gerovasileiou *et al.*, 2016) and in particular, with those reported by Çinar *et al.* (2002) that analysed the sponge *S. muscarum* (= *S. foetidus*) collected along the Aegean coasts. Nevertheless, our results agree with those of Pansini (1970) that did not find any direct proportionality between the number of symbionts and the volume of their host *Ircinia fasciculata* (= *Sarcotragus fasciculatus* (Pallas, 1766)).

Concluding, the sampling period seem to influence more the abundance than the diversity of the sponge associated fauna, that is quite constant. *S. foetidus* hosts some specific sponge-associated and typical circalittoral taxa, moreover, the abundance of the macrofauna can be compared with the values present in literature.

This study allowed to partially refute the null hypothesis of considering polychaetes as the most abundant and diversified taxon in the studied sponges. Anyway, the support provided by these sponges to several macrofaunal communities, composed by species with different degrees of interactions, both in juvenile and adult stages, highlights the importance of sponges as key habitat enhancing local biodiversity.

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## ANNEX I

List of taxa, feeding habits and presence during the months in both the species of *Sarcotragus* spp. With \* we indicate a new record for *Sarcotragus* spp.; FF=Filter Feeders; SF=Suspension Feeders; DF= Deposit Feeders; H=Herbivorous; C=Carnivorous; P=Parasite and OM=Omnivorous.

Associated taxa		<i>Sarcotragus spinosulus</i>										<i>Sarcotragus foetidus</i>									
	Feeding habitus	November 16	January 17	March 17	May 17	July 17	October 17	December 17	February 18	April 18	November 16	January 17	March 17	May 17	July 17	October 17	December 17	February 18	April 18		
<b>PORIFERA</b>																					
<i>Sycon</i> sp.	FF																				
<i>Tedania anhelans</i> (Vio in Olivi, 1792)	* FF	■	■			■	■		■		■	■			■	■					
<b>CNIDARIA</b>																					
Campanularidae	* SF	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■		
<i>Cornularia cornucopiae</i> (Pallas, 1766)	* SF	■	■		■		■	■	■												
<i>Stolonifera</i> ind.	* SF									■											
<b>ANNELIDA</b>																					
<i>Harmothoe</i> sp.	*											■	■	■	■						
<i>Harmothoe extenuata</i> (Grube, 1840)			■																		
<i>Harmothoe imbricata</i> (Linnaeus, 1767)	*		■																		
<i>Harmothoe longisetis</i>	*											■									
<i>Harmothoe spinifera</i>															■	■	■	■	■		
Capitellidae ind.	*															■					
<i>Capitella capitata</i> (Fabricius, 1780)	DF			■															■		
<i>Heteromastus filiformis</i> (Claparède, 1864)	DF																		■		
<i>Notomastus latericeus</i> (Sars, 1851)	DF			■																	
<i>Chrysopetalum debile</i> (Grube, 1855)	C		■					■					■								
<i>Aphelochaeta marioni</i> (Saint-Joseph, 1894)	* DF																				
<i>Caulleriella alata</i> (Southern, 1914)	* DF		■						■												
<i>Caulleriella viridis</i> (Langerhans, 1881)	* DF		■																		
<i>Dodecaceria concharum</i> Örsted, 1843	DF	■			■			■													
<i>Protocirrinieris chrysoderma</i> (Claparède, 1868)	DF	■	■																		
Eunicidae ind.			■		■			■						■	■	■					
<i>Eunice vittata</i> (Delle	OM																				

Chiaje, 1828)																				
<i>Leodice antennata</i> Savigny in Lamarck, 1818	*	OM																		
<i>Lysidice collaris</i> Grube, 1870		OM																		
<i>Lysidice margaritacea</i>	*	OM																		
<i>Lysidice ninetta</i> Audouin & H Milne Edwards, 1833		OM																		
<i>Lysidice unicornis</i> (Grube, 1840)		OM																		
<i>Marphysa</i> sp.		OM																		
<i>Palola siciliensis</i> (Grube, 1840)		OM																		
<i>Glycera</i> sp.	*	C																		
<i>Lumbrineris</i> sp. Blainville, 1828																				
<i>Micromaldane</i> <i>ornithochaeta</i> Mesnil, 1897	*	DF																		
<i>Nephtys</i> sp. Cuvier, 1817	*																			
<i>Ceratonereis</i> ( <i>Composetia</i> ) ind.		H																		
<i>Ceratonereis</i> ( <i>Composetia</i> ) <i>costae</i> (Grube, 1840)		H																		
<i>Ceratonereis</i> ( <i>Composetia</i> ) <i>hircinicola</i> (Eisig, 1870)		P																		
<i>Nereis splendida</i> Grube, 1840	*																			
<i>Nereis lamellosa</i> Ehlers, 1868	*																			
<i>Nereis rava</i> Ehlers, 1868		H																		
<i>Platynereis coccinea</i> (Delle Chiaje, 1822)	*	H																		
<i>Arabella iricolor</i> (Montagu, 1804)		OM																		
<i>Polyophthalmus pictus</i> (Dujardin, 1839)		H/DF																		
<i>Phyllodoce</i> sp.	*	C																		
<i>Pseudomystides limbata</i> (Saint-Joseph, 1888)	*	H																		
<i>Pterocirrus</i> sp.	*																			
<i>Subadyte pellucida</i> (Ehlers, 1864)		C																		
Amphicorinae	*	FF																		
<i>Amphiglena</i> <i>mediterranea</i> (Leydig, 1851)	*	FF																		
<i>Bispira</i> sp.	*	FF																		
<i>Jasmineira elegans</i> Saint-Joseph, 1894	*	FF																		
<i>Parasabella</i> sp.	*	FF																		
<i>Parasabella langerhansi</i> (Knight-Jones, 1983)	*	FF																		

<i>Parasabella tommasi</i> (Giangrande, 1994)	*	FF																	
<i>Sabellaria spinulosa</i> (Leuckart, 1849)		FF																	
<i>Hydroides dianthus</i> (Verrill, 1873)	*	FF																	
<i>Hydroides dirampha</i> Mörch, 1863	*	FF																	
<i>Hydroides pseudouncinata</i> <i>pseudouncinata</i> Zibrowius, 1968		FF																	
<i>Serpula</i> ind.		FF																	
<i>Serpula concharum</i> Langerhans, 1880		FF																	
<i>Serpula vermicularis</i> Linnaeus, 1767		FF																	
<i>Spirobranchus triqueter</i> (Linnaeus, 1758)		FF																	
<i>Vermiliopsis striaticeps</i> (Grube, 1862)		FF																	
<i>Aspidosiphon</i> ( <i>Aspidosiphon</i> ) <i>muelleri</i> <i>muelleri</i> Diesing, 1851		FF																	
<i>Golfingia</i> sp. Lankester, 1885		DF																	
<i>Sphaerodorum gracilis</i> (Rathke, 1843)	*	DF																	
Spionidae ind.	*	DF																	
<i>Dipolydora armata</i> (Langerhans, 1880)		DF																	
<i>Dipolydora coeca</i> (Örsted, 1843)		DF																	
<i>Polydora agassizi</i> Claparède, 1869	*	DF																	
<i>Polydora ciliata</i> (Johnston, 1838)		DF																	
<i>Polydora colonia</i> Moore, 1907	*	DF																	
<i>Polydora hoplura</i> Claparède, 1868	*	DF																	
<i>Prionospio cirrifera</i> Wirén, 1883	*	DF																	
<i>Janua heterostropha</i> (Montagu, 1803)		FF																	
<i>Brania pusilla</i> (Dujardin, 1851)	*	H																	
<i>Branchiosyllis exilis</i> (Gravier, 1900)		C/P																	
<i>Exogone</i> ( <i>Syllis</i> ) <i>brevipes</i> (Claparède, 1864)	*	H																	
<i>Exogone dispar</i> (Webster, 1879)	*	H																	
<i>Exogone naidina</i> Örsted, 1845		H																	
<i>Haplosyllis spongicola</i> (Grube, 1855)		C/P																	
<i>Myrianida</i> sp.	*																		

<i>Odontosyllis</i> sp.	*	C																		
<i>Salvatoria clavata</i> (Claparède, 1863)	*	H																		
<i>Sphaerosyllis hystrix</i> Claparède, 1863	*	H																		
<i>Sphaerosyllis pirifera</i> Claparède, 1868	*	H																		
<i>Syllis</i> ind.		C																		
<i>Syllis armillaris</i> (O.F. Müller, 1776)	*	C																		
<i>Syllis gracilis</i> Grube, 1840		C																		
<i>Syllis hyalina</i> Grube, 1863		C																		
<i>Syllis variegata</i> Grube, 1860		C																		
<i>Amphitrite cirrata</i> Müller, 1776	*	FF																		
<i>Amphitritides gracilis</i> (Grube, 1860)	*	FF																		
<i>Terebellides stroemii</i> Sars, 1835	*	FF																		
<b>CRUSTACEA</b>																				
<i>Achaeus cranchi</i> Leach, 1817	*																			
<i>Brachiura</i> ind.	*																			
<i>Maya</i> sp.	*																			
<i>Micropipus</i> sp.	*																			
<i>Ampelisca ruffoi</i> Bellan- Santini & Kaim-Malka, 1977	*	OM																		
<i>Aora spinicornis</i> Afonso, 1976	*																			
<i>Colomastis pusilla</i> Grube, 1861		C																		
<i>Monocorophium acherusicum</i> (Costa, 1853)	*																			
<i>Cressa cristata</i> Myers, 1969	*																			
<i>Dexamine spinosa</i> (Montagu, 1813)	*																			
<i>Echinogammarus</i> sp.	*																			
<i>Elasmopus brasiliensis</i> (Dana, 1855)																				
<i>Erichthonius brasiliensis</i> (Dana, 1853)																				
<i>Erichthonius punctatus</i> (Spence Bate, 1857)	*																			
<i>Gammaropsis</i> sp. 1	*																			
<i>Gammaropsis</i> sp. 2	*																			
<i>Gammaropsis maculata</i> (Johnston, 1828)																				
<i>Gammaropsis palmata</i> (Stebbing & Robertson, 1891)	*																			
<i>Ischyrocerus</i>	*																			



<i>inexpectatus</i> Ruffo, 1959																				
<i>Autonoe karamani</i> (Myers, 1976)	*																			
<i>Lembos spiniventris</i> Della Valle, 1893	*																			
<i>Lembos websteri</i> Spence Bate, 1857																				
<i>Leucothoe spinicarpa</i> (Abildgaard, 1789)		C																		
<i>Liljeborgia dellavallei</i> Stebbing, 1906		C																		
<i>Liljeborgia psaltrica</i> Krapp-Schickel, 1975	*	C																		
<i>Lysianassa caesarea</i> Ruffo, 1987	*																			
<i>Maera grossimana</i> (Montagu, 1808)	*																			
<i>Megamphopus</i> sp.	*																			
<i>Monocorophium sextonae</i> (Crawford, 1937)	*																			
<i>Paracentromedon crenulatus</i> (Chevreux, 1900)	*																			
<i>Photis longicaudata</i> (Spence Bate & Westwood, 1862)	*																			
<i>Scopelocheirus hopei</i> (Costa in Hope, 1851)	*																			
<i>Stenothoe</i> sp.	*	OM																		
<i>Stenothoe monoculoides</i> (Montagu, 1815)	*	OM																		
<i>Phtisica marina</i> Slabber, 1769	*	OM																		
<i>Pseudoprotella phasma</i> forma <i>typica</i> (Montagu, 1804)	*																			
<i>Diastylis rugosa</i> Sars, 1865	*																			
<i>Anthura gracilis</i> (Montagu, 1808)	*																			
<i>Gnathia maxillaris</i> (Montagu, 1804)	*	DF																		
<i>Janira maculosa</i> Leach, 1814		DF																		
<i>Limnoria</i> sp.	*																			
<i>Uromunna petiti</i> (Amar, 1948)	*																			
<i>Heterotanais oerstedii</i> (Krøyer, 1842)	*	DF																		
<i>Chondrochelia savignyi</i> (Kroyer, 1842)		DF																		
<i>Tanais</i> sp.																				
<i>Alpheus dentipes</i> Guérin, 1832																				
<i>Eualus cranchii</i> (Leach, 1817 [in Leach, 1815-1875])	*																			



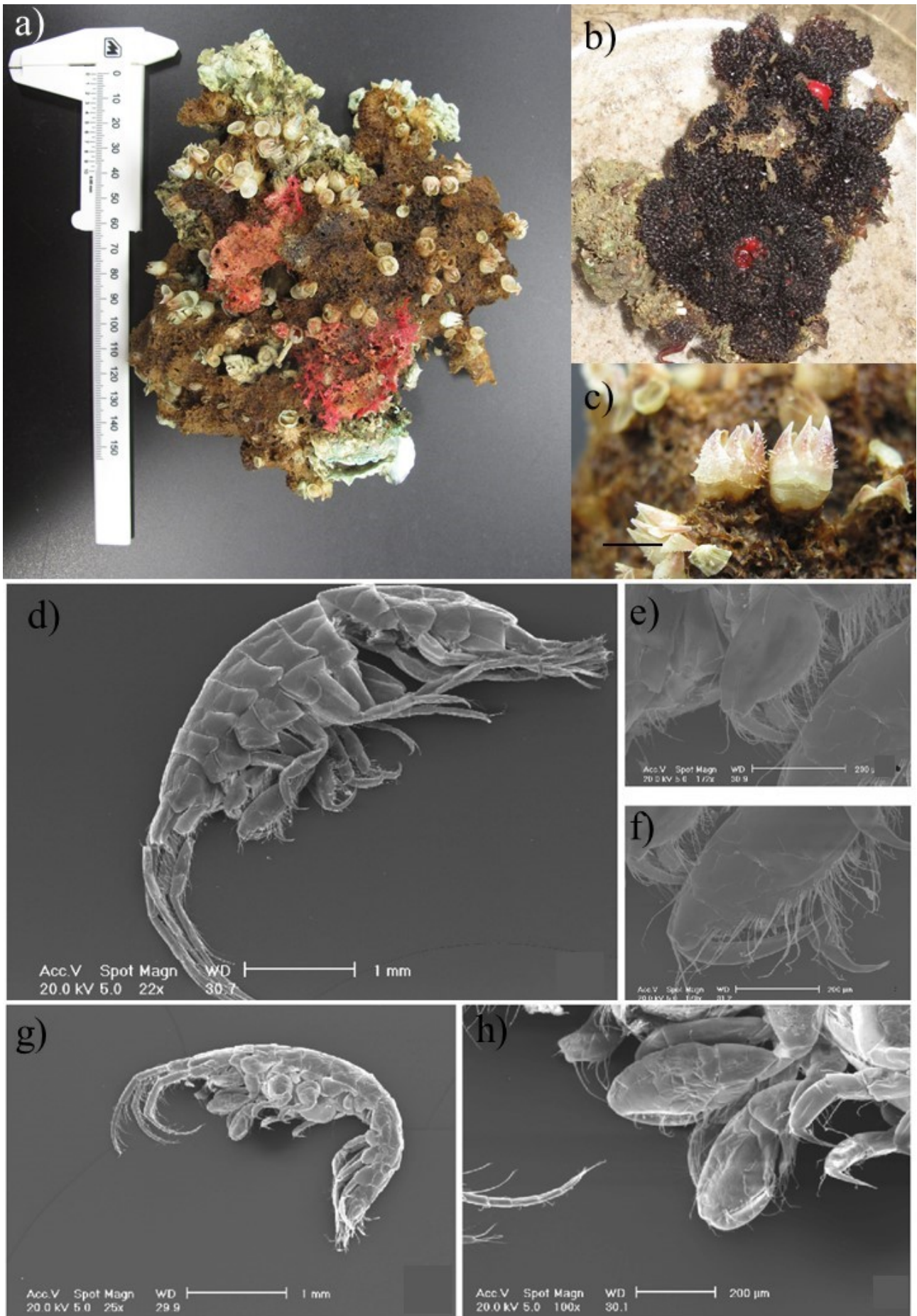
<i>Amphiura chiajei</i> Forbes, 1843		DF																		
<i>Ophiothrix quinquemaculata</i> (Delle Chiaje, 1828)		DF																		
<i>Ophiothrix fragilis</i> (Abildgaard in O.F. Müller, 1789)		DF																		
<b>ECTOPROCTA</b>																				
Ectoprocta ind.	*	SF																		
<i>Amathia lendigera</i> (Linnaeus, 1758)	*	SF																		
<b>ASCIDIACEA</b>																				
<i>Botryllus schlosseri</i> (Pallas, 1766)	*	FF																		
Ascidiacea ind.	*	FF																		
<i>Microcosmus vulgaris</i> Heller, 1877		FF																		
<b>NEMERTEA</b>																				
Nemertea ind.																				

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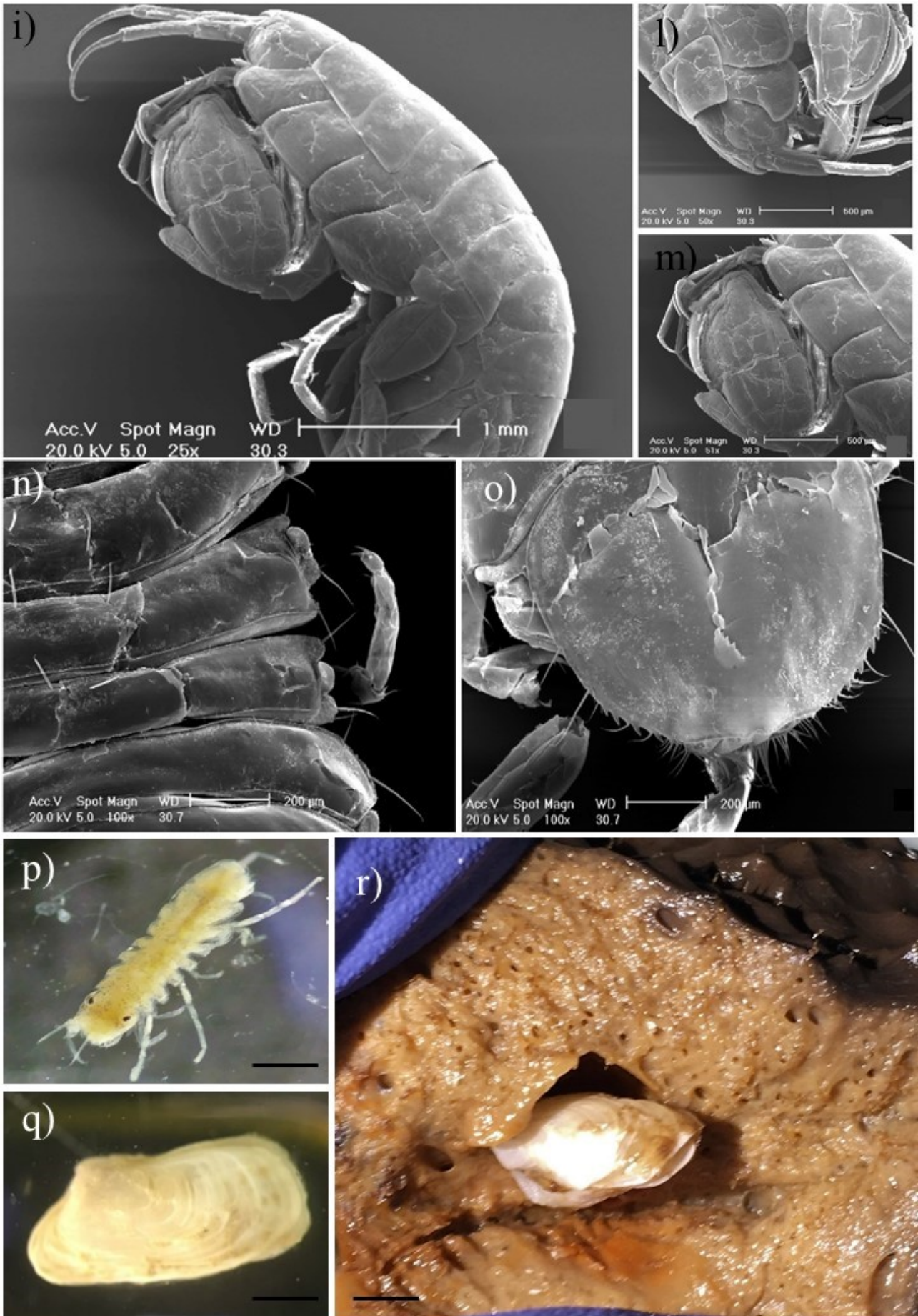
**ANNEX II**

a) Example of *S. spinosulus* infested by the barnacle *A. spongites* after treatment in Acid Chloride (37%); b) same sample before the treatment in Acid Chloride (37%); c) Two specimens of *A. spongites*; scale bar= 1 cm; d) Specimen of *Erichthonius brasiliensis*; e) Particular of the Gn1; f) Particular of the Gn2; g) Ovigerous female of *G. maculata*; h) Particular of the Gn1 and Gn2; i) Specimen of *L. spinicarpa*; l) Particular of the Gn1; m) Particular of the Gn2; n) segment of the species *J. maculosa*; o) Particular of the telson; p) Entire specimen of *J. maculosa*; q) Bivalve *H. arctica*; r) *H. arctica* inside a sponge; s) Tubes of the invasive spionid *P. colonia*; t) Particular of the modified chaetae of the 5<sup>th</sup> chaetiger; u) Particular of the modified chaetae of the last few chaetigera; v) The invasive eunicid *L. collaris* (SEM pictures); z) Example of ophiuroid on the *S. spinosulus* surface.

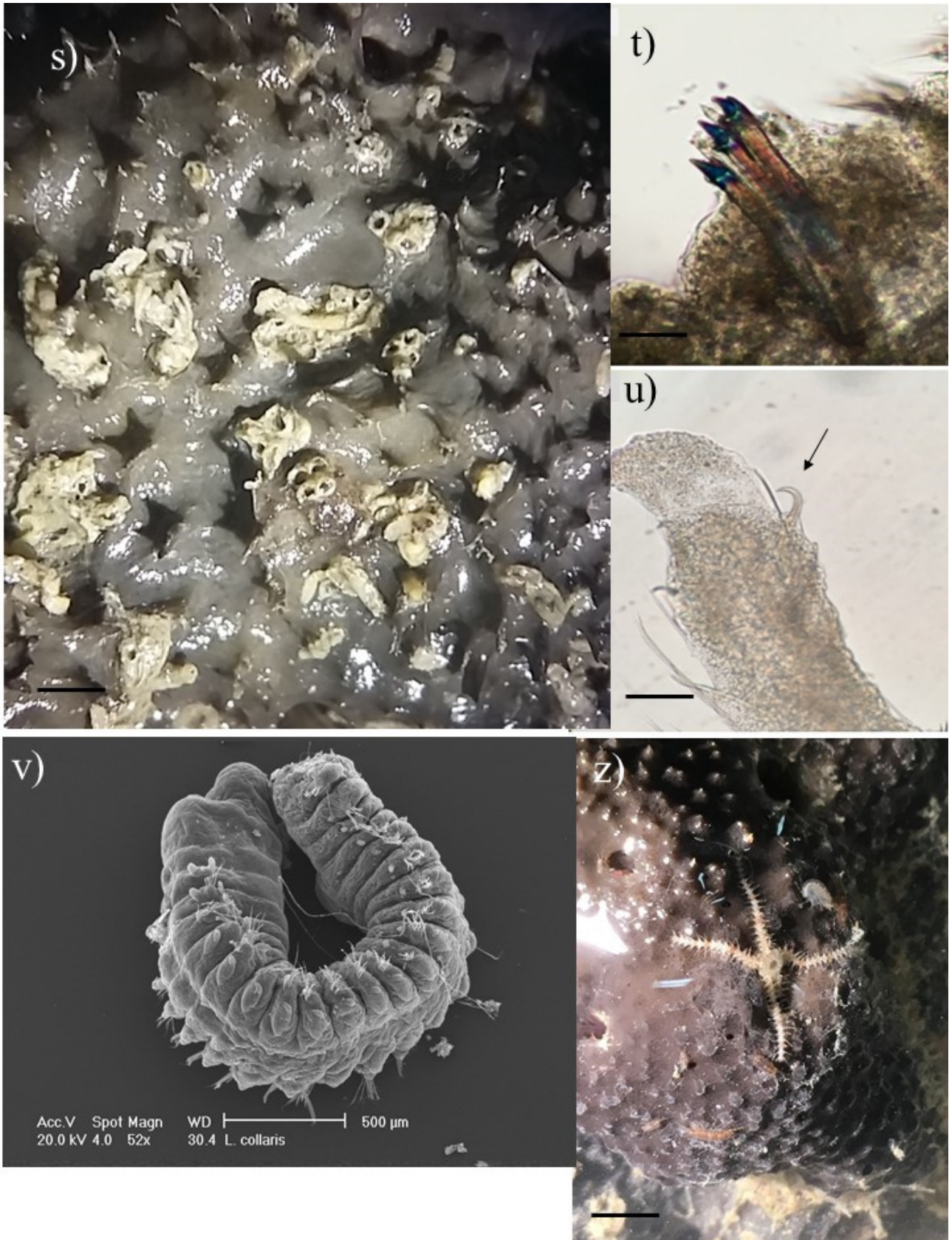
Scale bars: c= 1cm; p-s= 2 cm; t-u=0.5 mm; z=2cm.











## Chapter 2

# Morphometric analyses of *Ceratonereis (Composetia) costae* (Annelida: Nereididae) living with *Sarcotragus spinosulus* (Demospongiae: Irciniidae)

### Abstract

*Ceratonereis (Composetia) costae* (Grube, 1840) is a common and tolerant species reported for the Mediterranean Sea, Red Sea, North Atlantic Ocean and the South-Eastern African coast. A total of 31 specimens living with and inside *Sarcotragus spinosulus* Schmidt, 1862, were collected along the Ancona Promontory (North-Western Adriatic Sea), from July 2016 and July 2017. The width of the third and tenth chaetiger and the total length of the intact polychaetes were measured to evaluate the population structure using regression and correlation analyses; width of the head (3<sup>rd</sup> segment) resulted a statistical valid descriptor for morphometric analyses permitting to predict the length of broken specimens. The growth of *C. (C.) costae* seems to be both allometric and isometric.

Our results show that *S. spinosulus* hosts *C. (C.) costae* for all its life cycle, acting as a nursery for the polychaete, and update the data about the life cycle of *C. (C.) costae* and its habit supporting the role of the sponge in the ecosystem functioning.

*Keywords:* cohort, *S. spinosulus*, *C. (C.) costae*, Adriatic Sea.



## 2.1. Introduction

Nereididae Savigny, 1822 is one of the most common (Wilson, 2000) and diversified families of Annelida, with 540 species belonging to 43 genera (Hutchings *et al.*, 2000). For their diversity, frequency and abundance, nereidids were used as animal feed (Olive, 1994), baits for recreational fishing activities (Gambi *et al.*, 1994) and test organisms in research (Dean, 2008). The genus *Ceratonereis* Kinbergs, 1865 counts for 144 species, 59 of those belonging to the subgenus *Composetia* Hartmann-Schröder, 1985.

*C. (C.) costae* (Grube, 1840) is characterized by a subtriangular prostomium with smooth anterior margin, four pairs of peristomial slender and longer cirri, extending back to the 3<sup>rd</sup> and 7<sup>th</sup> chaetiger. Proboscis has conical paragnaths only present on the distal ring: I=0-1, II=7-8 in double row, III=3 in a triangle or lozenge; IV=10-12 in a cluster or trapezoid. Developed parapodial lobes are composed by two ligules, both in the notopodial and the neuropodial lobes, that become three from the 25<sup>th</sup> to the 30<sup>th</sup> chaetiger. This character and a similar composition of the arrangement of the paragnaths permit to differ this species from the congeneric *C. (C.) hircinicola* (Eisg, 1870).

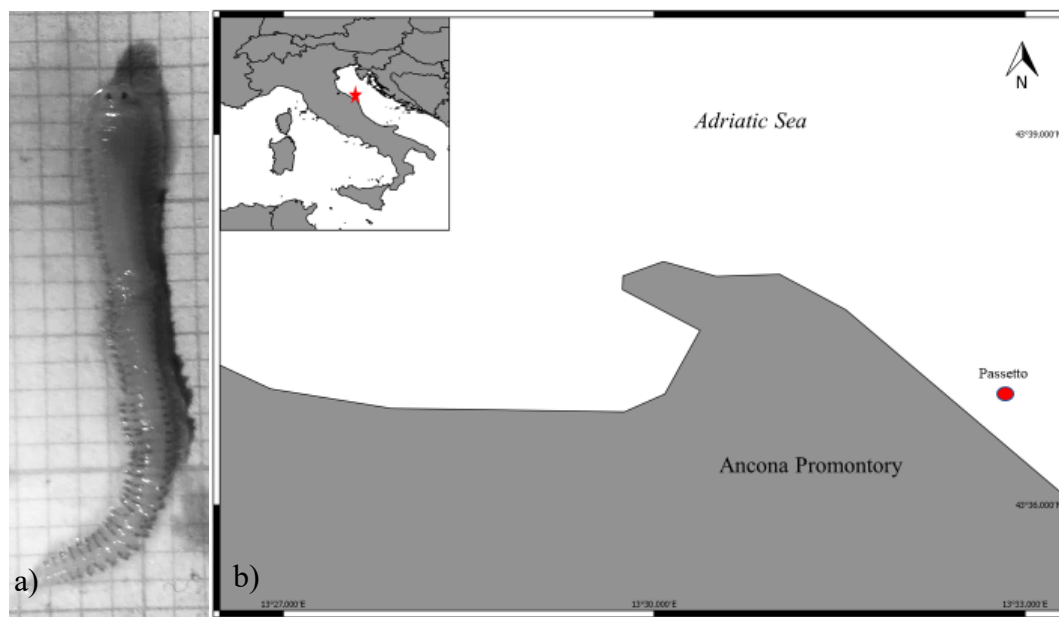
These two species are reported for the Italian waters (Castelli *et al.*, 2009) and for the Adriatic Sea (Cantone, 2003) but while *C. (C.) costae* lives freely also on rocky substrate, *C. (C.) hircinicola* is associated with keratose sponges. Nevertheless, *C. (C.) costae* was reported as one of the most frequent and abundant species associated to *Sarcotragus muscarum* (= *Sarcotragus foetidus* Schmidt, 1862) (Koukouras *et al.*, 1996; Çinar *et al.*, 1998, 2002; Pavloudi *et al.*, 2016).

The aim of this work is to clarify some aspects of *C. (C.) costae* biology and population structure, supporting the hypothesis of the functional role of *S. spinosulus* as habitat-forming species.

## 2.2. Materials and Methods

Sixty-one specimens of *Ceratonereis (Composetia) costae* (Figure 1a), living with the keratose sponge *Sarcotragus spinosulus* Schmidt, 1862 (Demospongiae: Irciniidae), were collected from July 2016 to July 2017, by scuba divers, along the “Passetto”, Ancona Promontory (Adriatic Sea) (43°37'2.36"N; 13°32'6.55"E) (Figure1b). Specimens were removed from the sponges, preserved in Ethanol 70° and identified. Densities were calculated considering the number of specimens and the volume of the sponges, evaluated with the water displacement; the non-parametric Krukall-Wallis test was conducted using Past 3.1 to compare the densities during the months.

Polychaetes were photographed and the length of the body and width of 3<sup>rd</sup> and 10<sup>th</sup> chaetiger were measured using the program ImageJ 1.51 j8 (Abramoff *et al.*, 2004).



**Figure 1.** a) Specimen of *C. (C.) costae*; b) Site of study.

Regression analysis was used to evaluate the relationship between the width of the 3<sup>rd</sup> chaetiger (Head width, HW) vs. the total length (TL), considered as dependent variable, and the width of 10<sup>th</sup> chaetiger (CW) vs. the total length (TL). Pearson correlation analysis was performed to validate these relationships among the biometrical measurements.

The total lengths obtained were used to analyse the size frequency using Bhattacharya's method by FiSAT II software (Gayanilo *et al.*, 2005). This method generates cohorts, assigning a separation index; values < 2 indicates not statistically supported cohorts that are therefore rejected (Gayanilo *et al.*, 2005).

The total lengths (TL) of all the recorded specimens of *C. (C.) costae* and their relative width (HW) were analysed by mean of non-hierarchical K-means cluster. This statistic method permits to split a set of multivariate data in a specific number of groups in base of their attributes. Groups were divided, based on dimensional variables, corresponding to mature individuals. Data were distributed in two groups thank to an iterative process that minimized the variance inside the groups and maximize that between the groups. Discriminant analysis was successively applied (Sampedro *et al.*, 1999).

For the study of the relative growth, we used the allometric equation (Huxley, 1950)  $y=axb$ . This was transformed in  $\ln(y)=\ln(a)+b \ln(x)$  where  $y$  is the dependent variable (HW),  $x$  is the independent variable (TL),  $a$  is the intercept on  $y$ -axis and  $b$  is the allometric coefficient of growth.

Growth was considered positively allometric when  $b > 1$ , negatively allometric when  $b < 1$  and isometric for  $b = 1$  (Silva *et al.*, 2017). T-student test was utilized for evaluate the isometric deviation.

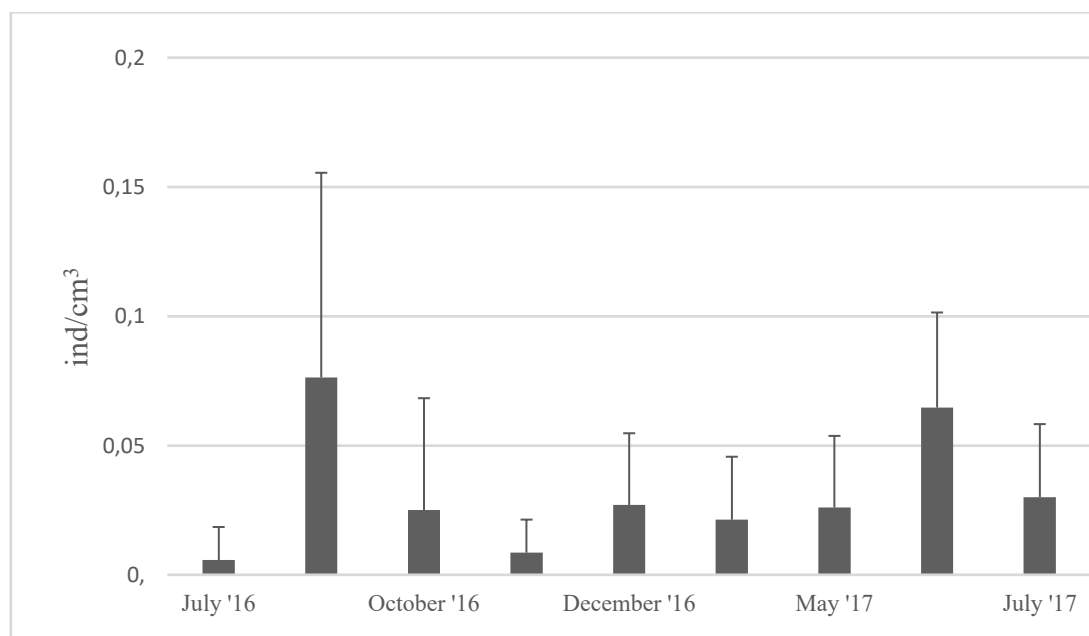
All biometric relationships were verified by a coefficient of determination ( $R^2$ ), while covariance analysis (ANCOVA) test ( $\alpha = 0.05$ ) was used to verify the presence of one or more regression lines for the empirical points estimated by ordinary least square (Sokal & Rolf, 1995). The size at maturity was determined by means of a logistic curve (estimated, using nonlinear regression, by the proportion of mature individuals for each 3 mm TL size class), indicating the size at which 50% of individuals reach sexual maturity (CL50) (Brown & Rothery, 1993).

Statistical analyses were conducted using Past 3.16 (Hammer *et al.*, 2001).

## 2.3. Results

In total 31 specimens of *Ceratonereis (Composetia) costae* were detected in 50 specimens of *Sarcotragus spinosulus*.

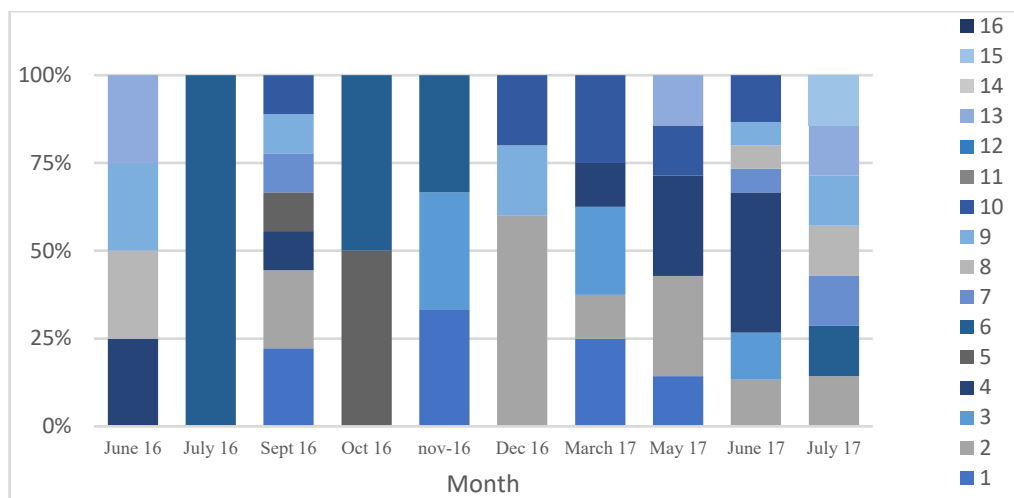
The polychaetes were recorded each month with maximum value of abundance in September '16 ( $0.076 \pm 0.079$  ind/cm<sup>3</sup>) and minimum value in July '16 ( $0.006 \pm 0.012$  ind/cm<sup>3</sup>) (Figure 2); Kuskall-Wallis test showed that the density of this species didn't vary significantly during the examined period ( $p = 0.1196$ ).



**Figure 2.** Densities of *C. (C.) costae* during the examined period.

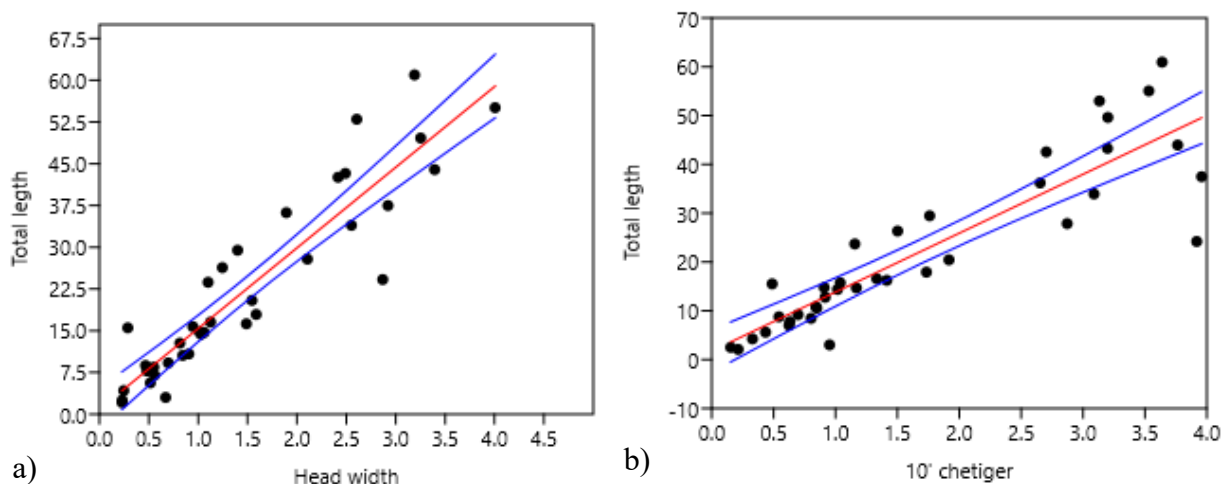
Polychaetes specimens belonged to the different size classes as showed in Figure 3. In September 2016, June 2017 and July 2017, nereidids were more diversified in length, while on July 2016,

October 2016, November 2016 and December 2016 polychaetes belonged to only three different size classes.



**Figure 3.** Relative percentage of the size classes distribution during the analysed months.

The relationship between body length and the considered parameters (width of the 3<sup>rd</sup> chaetiger HW and width of the 10<sup>th</sup> chaetiger CW) are presented in Figure 4 and in Table 1.



**Figure 4.** a) Linear regression between width of 3<sup>rd</sup> chaetiger and total length; b) Linear regression between width of 10<sup>th</sup> chaetiger and total length.

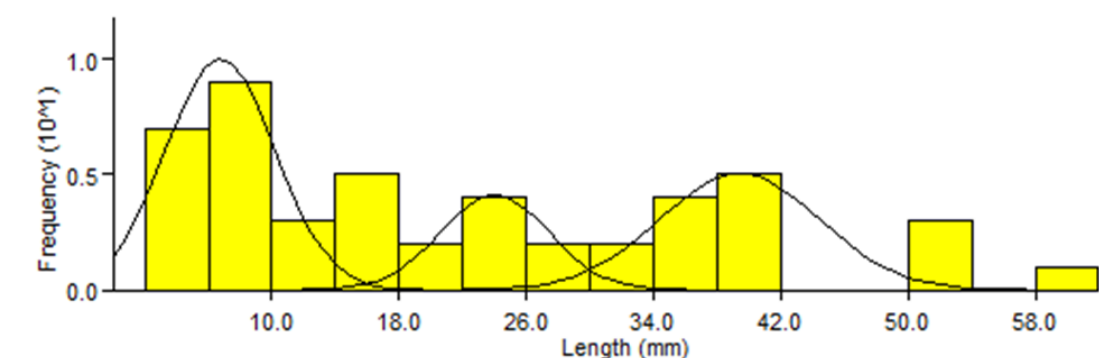
The relationships between the considered parameters were linear and the correlation scores were significantly high (Table 1) considering both HW and CW as independent parameters.

**Table 1:** Regression and correlation data using width of the 3<sup>rd</sup> chaetiger HW and width of 10<sup>th</sup> chaetiger CW, as independent variables.

Relationship:	r:	r2:	t:	p (uncorr.):	Permutation p:
HW vs TL	0.92187	0.84985	14.075	5.61E-16	0.0001
CW vs TL	0.89294	0.79735	11.735	1.10E-13	0.0001

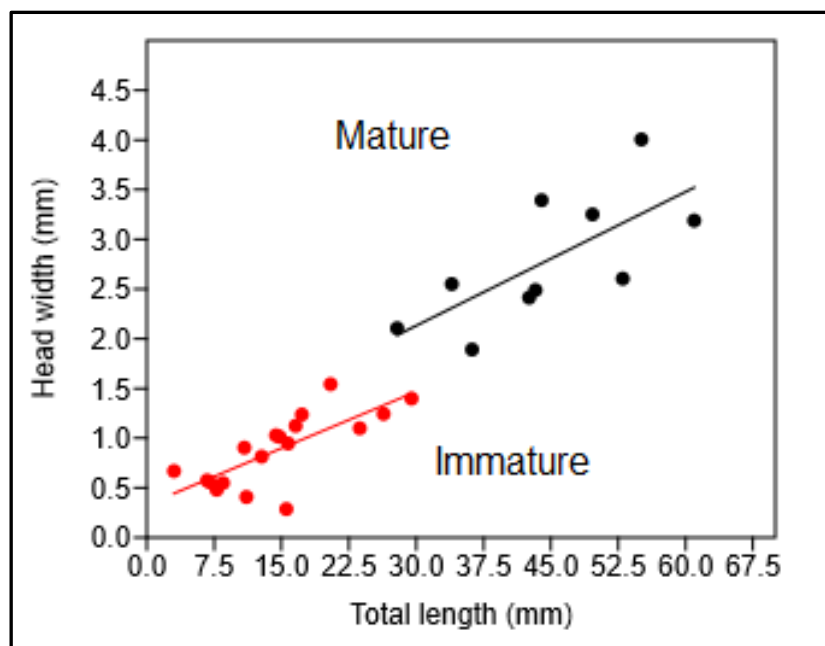
The relationship derived considering the width of 3<sup>rd</sup> chaetiger is the follow:  $TL (mm) = 14.482 \times HW + 0.92187$ .

Analysis evidences the presence of three cohorts (Figure 5): the first consists of young individuals without distinction of sex,  $6.74 \pm 3.44$  mm in length, on average; the second consists of youngs (males and females) and also of subadults specimens,  $24.01 \pm 3.39$  mm in length, on average, and the third cohort, mainly, of adults females,  $41.36 \pm 6.08$  mm in length on average.



**Figure 5.** Distribution of size frequency classes using the Bhattacharya Method (FisatII).

By means of a non-hierarchical K-means cluster and successive discriminant analyses, two groups of interest were identified. The morphometric relationship between the total length (TL) and the width of the 3<sup>rd</sup> chaetiger (HW) is shown in Figure 6, where a clear break in the data occurs at approximately 30 mm; this point of break corresponds to the beginning of the maturity stage. Immature and mature specimens were clearly showed in Figure 6.



**Figure 6.** Morphometric relationship between head width (HW) and total length (TL), showing two distinct groups in *C.(C.) costae* and the maturity stage.

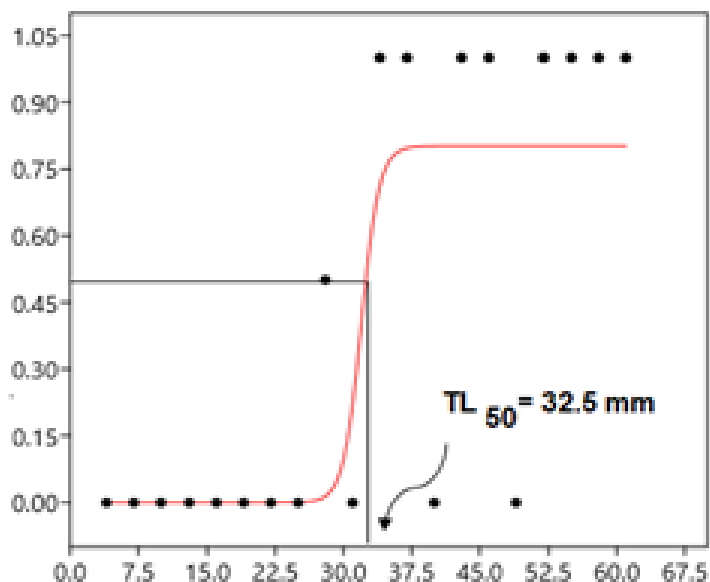
All linear regression parameters estimated for immature and mature individuals of *C.(C.) costae* by ordinary least square from log-transformed data are given in Table 2.

**Table 2:** Biometric analysis of the head width (Y) vs. total length (X) per immature and mature *C. costae*. N= number of specimens; SE = standard error;  $r^2$  = coefficient of determination; Al = allometric level, testing the slope against a standard of 1 [ $t(b = 1)$ ]; - = negative allometry; 0= isometry; ns = not significant; \*= $p < 0.02$ ; \*\*=  $p < 0.01$ ; F= Snedecor's F test values of the comparison between slope and intercept of immature and mature individuals.

Stage	N	Slope	SE	Intercept	$r^2$	$t(b = 1)$	AL	ANCOVA	
		(b)	(b)	(ln a)				F (b)	F (a)
Immature	18	0.476	0.168	-1.429	0.33*	3.11**	-	0.20 <sup>ns</sup>	8.43**
Mature	10	0.696	0.235	-1.625	0.52*	-1.29 <sup>ns</sup>	0		

Morphometric analyses showed that the immature individuals follow a negative allometric growth while the mature follow an isometric growth. Moreover, the covariance analyses (ANCOVA) suggested that the two slopes (b) were not significant different (Table 2).

The size at which 50% of individuals reaches sexual maturity, in relation to the morphometric variables considered (HW vs. TL), it is estimated to 32.5 mm TL (Figure 7).



**Figure 7.** Logistic maturity curve indicating the total length at which 50% of the individuals are considered reproductive. X-axis reports the total length of the body; Y-axis the correlation of the mature specimens.

## 2.4. Discussion

*C. (C.) costae* is known to be common in the Mediterranean Sea and to live on photophilous rocky bottom assemblages and *Posidonia oceanica* meadows (Gibbs, 1971; Ergen, 1976); it is also inquiline of several sponge species such as *Sarcotragus foetidus* (Çinar *et al.*, 2002; Pavloudi *et al.*, 2016) and *Geodia cydonium* (Gherardi & Giangrande, 2011) where it is frequent and dominant. Surprisingly, the congeneric *C. (C.) hircinicola*, considered as a symbiotic species commonly associated with *Sarcotragus* spp. was never detected, while we recorded *C. (C.) costae* every month suggesting a stable assemblage associated to *S. spinosulus* during all the year.

Sponges carry inside their aquiferous system POM and DOM that can be exploited by other organisms as filter and suspension feeders (De Goijet *et al.*, 2013, Alexander *et al.*, 2014). During the study a conspicuous quantity of mud and microalgae was observed; we can speculate that the accumulation of this material inside the aquiferous system, could sustain the feeding activities of this species that is characterized by an herbivorous habitus.

Individuals of *C. (C.) costae* belonged to three different cohorts; the first, principally composed by juvenile specimens (males and females); the second cohort consisted of bigger specimens (males and females), while in the third were composed mainly by females that reach longer size. The findings of individuals of different size classes all year round, and females with the coelomatic cavity full of mature eggs, suggest that *C. (C.) costae* finds in *S. spinosulus* an ideal habitat for the reproduction that, as reported by Durchon (1955), can happen in any period of the year. Moreover, the sponge represents also a refuge for small, young specimens present in high density in the sponge, as reported by the distribution of the different size classes and their frequency.

The pattern of growth seems to be different for the immature and mature organisms (respectively negative and positive allometry), but ANCOVA analysis showed that they are not statistically different. Polychaetes at the maturity grow rapidly in length, but the relationship between the two parameters, after maturity, remains constant. Isometric growth, as reported for other species (e.g. Hamdy *et al.*, 2014), reflects the importance of the anterior metamera in the growth.

Polychaetes have a soft body and the sampling, sorting and fixation often damage them. The morphological variable used to predict the total length of the body of *C. (C.) costae* were the 3<sup>rd</sup> chaetiger and the 10<sup>th</sup> chaetiger in relation to the total length of the body. The choice of the 3<sup>rd</sup> chaetiger as preferential parameter was driven by the derived statistically significant relationship, and because this part of the body, is a stable character, rarely damaged and not deformed during manipulation and fixation. Çinar *et al.* (2007) used the width of the 10<sup>th</sup> chaetiger to study assemblages of the lessepsian species *Pseudonereis anomala* Gravier, 1899, but other authors



indicate the peristomial width as a good growth estimator in nereidids (Cammen, 1980; Kent & Day, 1983).

Concluding, we can affirm that *S. spinosulus* is a habitat-forming species particularly important for *C. (C.) costae*, able to host entire subpopulations in each specimen, contributing to the dispersal and survival of this polychaete.

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## Chapter 3

# **Polychaetes and octocorals: first record of *Haplosyllis chamaeleon* Laubier, 1960 in the Italian waters and new traits on its natural and life history**

### **Abstract**

Gorgonians can create ideal habitats for many organisms, hosting both a complex and heterogeneous microbiome and several species with different levels of interactions. Some associations can be species-specific other can be more opportunistic. Syllidae (Annelida: Phyllodocida) are known for their high diversity and their plasticity allowing them to live in many different habitats showing peculiar adaptive responses and establishing many associations. Relationships between gorgonians and polychaetes are well known worldwide but in the Mediterranean Sea the strict symbiosis between the red gorgonian, *Paramuricea clavata*, Risso, 1826 and the syllid *Haplosyllis chamaeleon* Laubier, 1990 has been poorly documented and reported only for the Western Mediterranean Sea. The present paper enlarges the geographical distribution of this association for the North-Central Mediterranean Sea providing the first record of *H. chamaeleon* for the Italian and Croatian fauna. Moreover, insights into the biology and the life cycle of the species *H. chamaeleon* have been documented observing specimens from the Ligurian Sea.

*Keywords:* symbiosis, *P. clavata*, *H. chamaeleon*, Italian fauna.

### 3.1 Introduction

Symbiotic relationships are considered as long-term associations between organisms belonging to different taxa. According to the cost-benefit between host and symbiont (Margolis *et al.*, 1982) they can be reassumed in three types of association: parasitism (+/-), commensalism (+/0) and mutualism (+/+) (Dales, 1957). These strategies of life are common in the marine ecosystems all over the world, especially in complex habitats where competitive and cooperative interactions are more frequent as in the case of tropical and temperate bioconstructions.

In the Mediterranean Sea, the most complex and rich of species habitat is the coralligenous (Ingrosso *et al.*, 2018). Here big gorgonians can build, in case of high density of colonies, the so-called gorgonian forests (Cerrano *et al.*, 2010), that with their three-dimensional structure affects edaphic conditions (Valisano *et al.*, 2016) and the composition of benthic assemblages playing an ecological role similar to terrestrial forests and enhancing local biodiversity (Ponti *et al.*, 2014; 2018).

Cnidarians are frequently involved in different kinds of associations hosting organisms that can find refuge on them to complete part (Molodstova *et al.*, 2016) or the entire life cycle representing a valid substrate for food and/or reproduction (Goh & Chou, 1999). It has been recently reported how most of the symbioses involving this phylum are with polychaetes (Martin & Britayev, 2018) and almost the 25.97% of these relationships involve the order Alcyoniacea (Molodstova *et al.*, 2016). In particular, the subclass Octocorallia is known for its capacity to establish different kind of relationships with several organisms belonging to different taxa (e. g. Patton, 1972; Goh & Chou, 1999; Dias *et al.*, 2015), like sponges (Calcinai *et al.*, 2013), hydroids (Puce *et al.*, 2008; Seveso *et al.*, 2016; Pica *et al.*, 2017) and polychaetes (Barnich *et al.*, 2013; Britayev *et al.*, 2014; Carvalho *et al.*, 2014; Cùrdia *et al.*, 2015; Glasby & Watson 2001; Martin & Britayev, 1998; Martin *et al.*, 2002; Nygren *et al.*, 2010).

About symbiosis between octocorals and polychaetes, the family Polynoidae is the richest in term of species but the family Syllidae is quite common (Molodstova *et al.*, 2006). Syllids are characterized by a complex taxonomy and have many ecological strategies. Most species are free-living, but many others live in association with sponges, decapods and echinoderms (Martin & Britayev, 1998, 2018). Octocoral-associated syllids are not so numerous as for example the species *Imajimaea draculai*, a parasite of the pennatulacean *Funiculina quadrangularis* collected along the Swedish west coasts (Nygren & Pleijel, 2010). Other examples are principal represented by species belonging to the genus *Haplosyllis* Langerhans, 1879; these can assume different colorations and strategies of feeding in relation to the host (Hartmann-Schroder, 1993; Lattig & Martin, 2009), as the case of *H. villogorgicola* a kleptoparasite of the gorgonian *Villogorgia brebycoides* collected in

the Canary Islands and *H. anthogorgicola* a mutualistic symbiont of the gorgonian *Acanthogorgia bocki* from Japan; both these species were observed to modify the branches of the gorgonian building gall-like structure or verrucae, respectively (Martin *et al.*, 2002).

In the Mediterranean Sea, the purple/yellow gorgonian *Paramuricea clavata* (Risso, 1826) is considered an important component of the coralligenous assemblages (Linares *et al.*, 2008; Ponti *et al.*, 2014). This species is recognized as habitat-forming and well represents the concept of “animal forest” (Cerrano *et al.*, 2010; Ponti *et al.*, 2016; Valisano *et al.*, 2016; Rossi *et al.*, 2017). Many studies concerning the ecology, connectivity and reproduction have been conducted (Arizmendi-Mejía *et al.*, 2015; Mistri, 1995; Coma *et al.*, 1995a, 1995b; Cerrano *et al.*, 2005; Ponti *et al.*, 2016) also because in the past this species has been involved in mass mortality events implicating the death of gorgonians and of its correlated communities (Bavestrello *et al.*, 1994; Cerrano *et al.*, 2000; Huete- Stauffer *et al.*, 2011). Several taxa live in association with the genus *Paramuricea* Koelliker, 1865 in the Mediterranean Sea, as the hydroid *Ectopleura* sp., (Agassiz, 1982) (Bo *et al.*, 2011), the solenogaster *Anamenia gorgonophila* (Kowalevsky, 1880) (Mifsud *et al.*, 2008) , the pycnogonid *Callipallene spectrum* (Dohrn, 1881) and the nudibranch *Marionia blavillea* (Risso, 1818) (Ponti *et al.*, 2016), the amphipod *Balssia gasti* (Balss, 1921) (Mori *et al.*, 1995) and the syllid *Haplosyllis chamaeleon* (López *et al.*, 1996).

*Haplosyllis chamaeleon* Laubier, 1960 is known as a strict ectoparasite on the gorgonian *Paramuricea clavata* (Martin & Britayev, 1998). The symbiosis was firstly described by Laubier (1960) in Banyuls-sur-Mar (France) and successively by López *et al.* (1996) in the Chafarinas Islands. This symbiosis was principally recorded along the Catalanian coasts (Martin *et al.*, 2002), the Spanish Sea (Musco & Giangrande, 2005) and in the Alboran Sea (López *et al.*, 1996; Martin *et al.*, 2002). Biochemical signals are generally implicated in the recognition processes between the polychaete symbionts and their hosts (Martin & Britayev, 1998), even if the species *H. chamaeleon* is supposed to recognize the gorgonian *P. clavata* by a specific thigmotactic behaviour (Laubier, 1960). Recently this polychaete was re-described taking in account not only its morphology but also its ecology and its reproductive strategy (Martin *et al.*, 2002; Lattig & Martin, 2009). *H. chamaeleon* has been usually found on the apical part of the branches of colonies, where the polyps are more densely packed (Laubier, 1960; Martin *et al.*, 2002). The parasitic behaviour was correlated to the capacity of these polychaetes to partially hide inside the gastric cavity of the polyps, as reported by Laubier (1960) and Martin *et al.* (2002) that suggest also that this species probably eats the octocoral tissue. *H. chamaeleon* mimics exactly the coloration of its host, realising a perfect camouflage (Laubier, 1960; Martin *et al.*, 2002). Many aspects of the relationships

between *H. chamaeleon* and *P. clavata* are still unclear and need to be better investigated to clarify the reciprocal actual roles.

The aims of the present study are to update the geographic distribution of *H. chamaeleon* and evaluate its natural history and its reproductive cycle during an annual cycle.

### 3.2 Materials and Methods

To update the geographical distribution of *H. chamaeleon* in the Mediterranean Sea a bibliographic research was performed. Coordinates, host substrates and depth of samplings were recorded from the papers obtained by the web search engine “Google Scholar” and “Scopus”.

Specimens of *H. chamaeleon* were collected sampling apical branches of *P. clavata* in different sites: Croatia (North Adriatic Sea), Santa Teresa di Gallura (North Sardinia), Portofino Promontory (Ligurian Sea) and Costacuti shoals (Central Tyrrhenian Sea) as detailed in Table 1.

The references, coming from the bibliographic research, and the present new records (Table 1), were geo-referenced using the open-source program QGIS 2.4.0 ([www.qgis.org/it/site/](http://www.qgis.org/it/site/)).

**Table 1.** Summary of the records of *H. chamaeleon*.

Site	Latitude	Longitude	Host species	Depth (m)	Author	Year
Pontevedra (Spain)	42°30'58.10"	-8°56'16.28"	<i>P. grayii</i>	30	Latting & Martin	2009
Banyuls sur Mer (France)	42°28'49.32"	3° 8'47.48"	<i>P. clavata</i>	15	Laubier L.	1960
Cape of Creus (Catalonian Coast)	42°18'57.85"	3°19'2.70"	<i>P. clavata</i>	30	Alós C.	1988
El Barruls (Egyptian Coast)	31°41'52"	33°00'07"E	muddy-sand	20/50/100	Abd-Elnaby F. A.	2014
Mar de Alboran	35°51'2"	3°6'7"	Biocoenosis Red Coral	70-200	Baratech & San Martin	1987
Arafura Sea (Australia)	9°24'933'	134°18'619'E	Dragage of megabenthic organisms	89	Wilson	2006
Chafarinas Islands (Alboran Sea)	35°11'8.39"	-2°25'14.89"	<i>P. clavata</i>	23	López <i>et al.</i>	1996
Molunat (Croatia)	42°26'44.69"	18°26'11.32"	<i>P. clavata</i>	45	present work	2018
Portofino Promontory	44°18'0.61"	9°13'11.91"	<i>P. clavata</i>	40	present work	2018
Santa Teresa di Gallura	41°15'40.5''	9°12'22.7''	<i>P. clavata</i>	30	present work	2018
Costacuti shoals	41°23'49.06"	12°35'0.61"	<i>P. clavata</i>	42	present work	2018

Samples from the Portofino Promontory (Ligurian Sea) (44°17'56.58"N; 9°13'8.13"E) (Figure 1) were seasonally collected from May 2017 to June 2018 around 40 m depth, for a total of 204 apical branches of about 15 cm length, each one coming from different colonies.





**Figure 1.** Site of study.

All the gorgonian branches were cut with scissor and kept in plastic bags, transferred to the laboratory and observed *in vivo*. Under the stereomicroscope, gorgonians were maintained in cold sea water; pictures and videos were taken using Canon G16 and all the sides of them were investigated in order to locate the symbionts. After the observations, the majority of the samples were fixed in ethanol (96%), while few of these were fixed in glutaraldehyde (2.5 %) for histological analyses and electron microscopy (see details below). Sea-water was filtered through a sieve (mesh of 0.125 mm) to collect the symbionts detached from their hosts.

Polychaete specimens from Portofino, maintained associated to the gorgonian fragment at +4 °C for one night, were photographed, and then were carefully removed from their host and preserved in ethanol (96%). Polychaetes were identified under stereomicroscope, placed in slides for light microscope observations, and morphological measurements were taken following Lattig & Martin (2009). To confirm the taxonomic identification morphological characters of 40 specimens, collected along the Portofino Promontory, were compared with those reported by Martin *et al.* (2002) for the Cape de Creus assemblages.

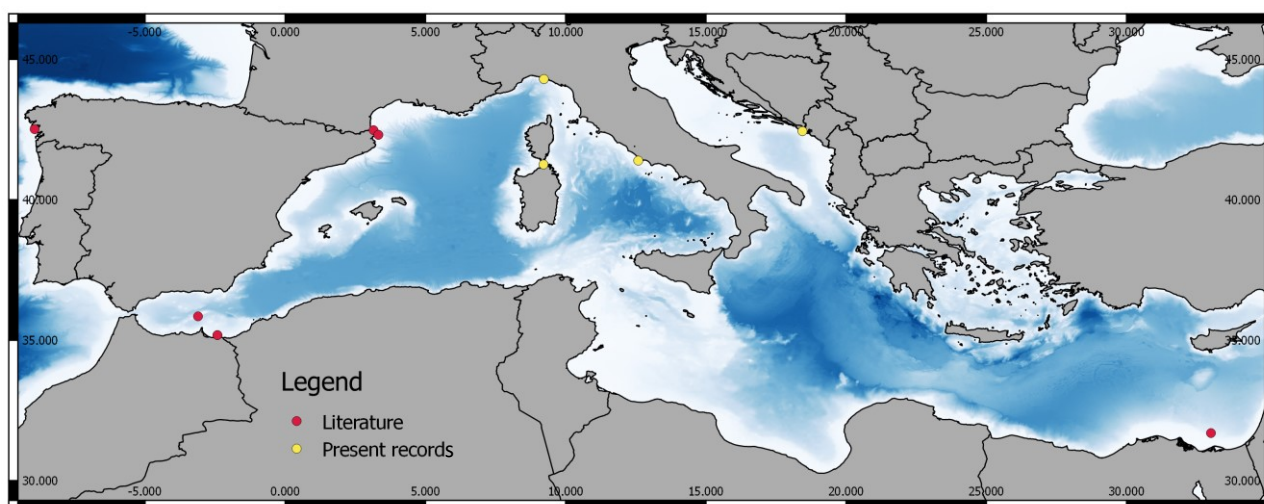
For scanning electron microscopy (SEM) analysis, the polychaetes were rinsed with distiller water, then dehydrated through a graded ethanol series (20%, 50%, 70%, 90%, 100%) and dried with

Hexamethyldisilazane 98% until completely evaporation. Finally, they were attached on stubs, coated with gold-platinum in a Balzer Union evaporator and examined under SEM Philips XL20. For the histological analysis, polychaetes were dehydrated through a graded ethanol series (20%, 50%, 70%, 90%, 100%) and then gradually included in resin (Technovit 8100) and attached on plastic stubs. Slices, 5  $\mu\text{m}$  thick, were cut with microtome Histo-line MRS3500, coloured by Toluidine Blue for 30sec, dried and closed with Eukitt glue. Twenty oocysts and spermatocysts of the polychaetes were measured under microscope using micrometric scale.

The percentage frequency of *H. chamaeleon* was estimated considering the ratio between number of gorgonians hosting polychaetes and the total number of gorgonian samples collected in each season. To estimate the density of the polychaetes (ind/cm), we counted the number of the individuals of *H. chamaeleon* and measured the total linear length (cm) of the sampled branches. Pictures of these were taken using Canon GS7x and the lengths of the branches were measured using the program “ImageJ 1.46r” (Abramoff *et al.*, 2004).

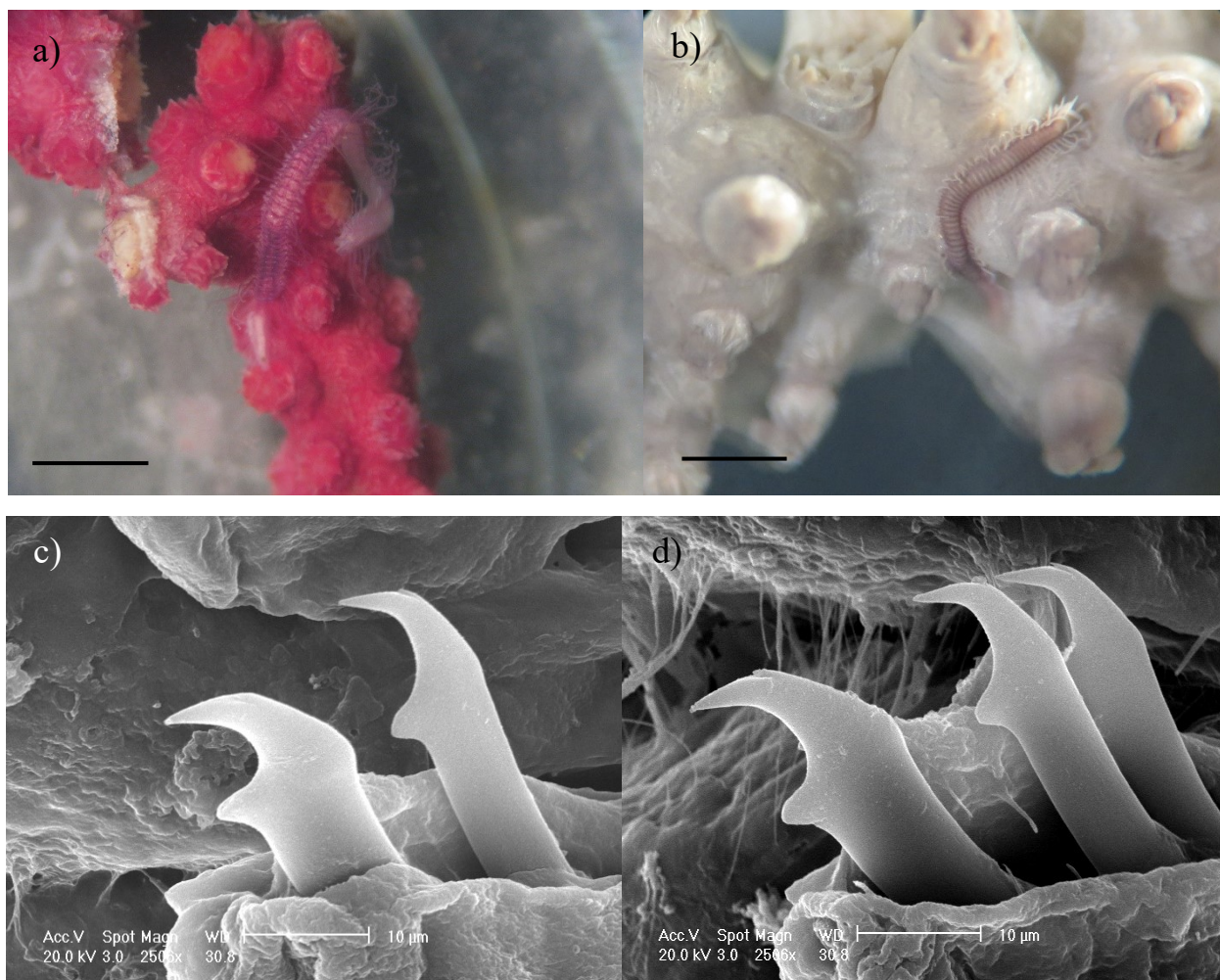
### 3.3 Results

Samples of *P. clavata* collected in Portofino Promontory, Santa Teresa di Gallura, Costacuti shoals and Molunat were infested by this syllid (Figure 2). This is the first record, of *Haplosyllis chamaeleon*, associated with the gorgonian *Paramuricea clavata*, for the Italian and Croatian fauna, extending the geographic distribution of this polychaete species in the Northern Mediterranean and Adriatic Sea.



**Figure 2.** Distribution of *H. chamaeleon*, red dots are from literature, yellow dots are the present records.

Specimens were associated both with yellow (from Costacuti Shoals) and red gorgonian morphotypes (from Portofino, Molunat and Tavolara Islands), and their colour fitted with that of the gorgonian colonies (Figure 3 a, b). The chaetae are simple and unidentate on the anterior parapodia (Figure 3c), and more hooded and clearly bidentate in the posterior parapodia (Figure 3d), as reported in López *et al.* (1986).



**Figure 3.** a) Specimen on a violet gorgonian branch; b) Specimen on a yellow gorgonian branch after preservation in ethanol (96°); c) Example of anterior chaetae (SEM picture); d) Example of posterior chaetae (SEM picture). Scale bars: a = 1 cm; b= 0.5 cm.

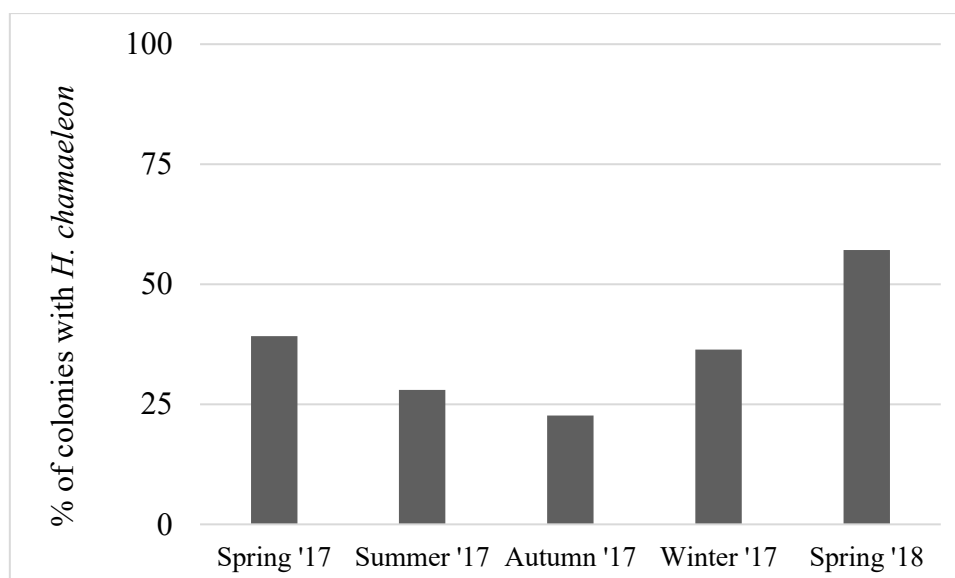
The comparison between morphological characters of 40 entire, well conserved specimens, from Portofino, and 11 specimens of *Haplosyllis chamaeleon* from Cape of Creus (North Spanish Sea), reported by Martin *et al.* (2002), are shown in Table 2.

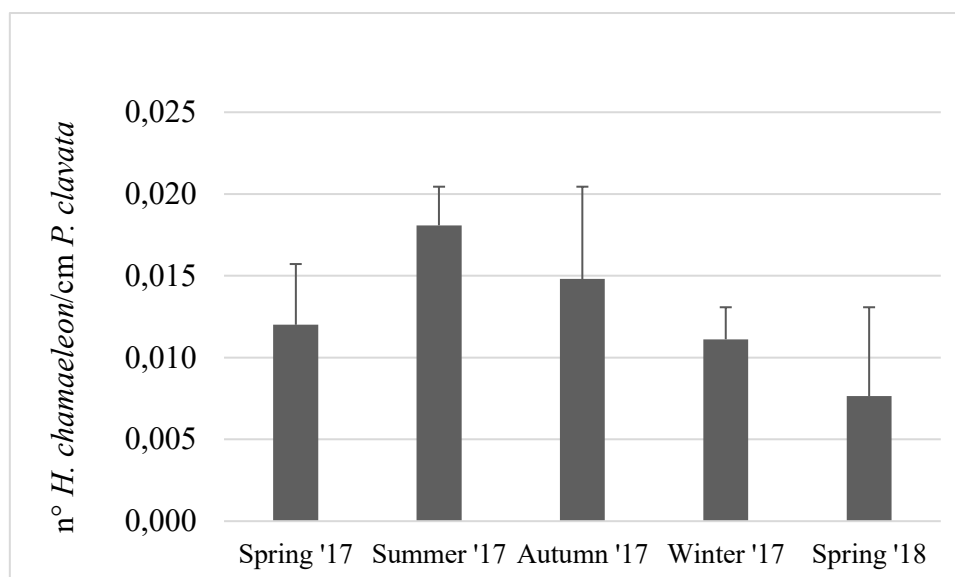
Specimens from Portofino reach higher maximal dimensions, but on average their sizes are comparable with those of Cape of Creus. The width of the body was measured without considering the parapodial lobes, according to Latting & Martin (2009), and showed higher values in respect with the Cape de Creus assemblages. The specimens from Portofino had longer pharynx and proventriculus in respect with those reported by Martin *et al.* (2002).

**Table 2.** PP: Present Paper; M: data reported by Martin *et al.* (2002).

	Number of chaetigers		Body length (mm)		Body width (mm)		Pharynx length (mm)		Proventriculus length (mm)	
	PP	M	PP	M	PP	M	PP	M	PP	M
<b>Max</b>	97	104	26.25	11.5	2	0.7	1.62	0.62	2.1	0.85
<b>Min</b>	28	38	5.12	3.87	0.37	0.42	0.25	0.3	0.5	0.36
<b>Avg ±</b>	66.6	74	8.98	8.7	1.01	0.55	0.76	0.41	1.06	0.58
<b>SD</b>	16.47	21.77	8.01	2.28	0.31	0.09	0.32	0.1	0.32	0.14

In total, 113 specimens of *Haplosyllis chamaeleon* were found on *P. clavata* living along the Portofino Promontory. The symbionts were not observed on every sampled branch and, when present, their number varied from 1 to 7 polychaetes/gorgonian's fragment. The frequency of infested gorgonians changed during the season and reached higher value during the Spring '18 (57.14%) and lower during the Autumn '17 (22.64%) (Figure 4). Variations about the density of these symbionts were observed, values were comprised between  $0.018 \pm 0.002$  ind/cm in Summer '17 and  $0.007 \pm 0.005$  ind/cm in Spring '18 (Figure 5).

**Figure 4.** Percentage of hosting colonies.

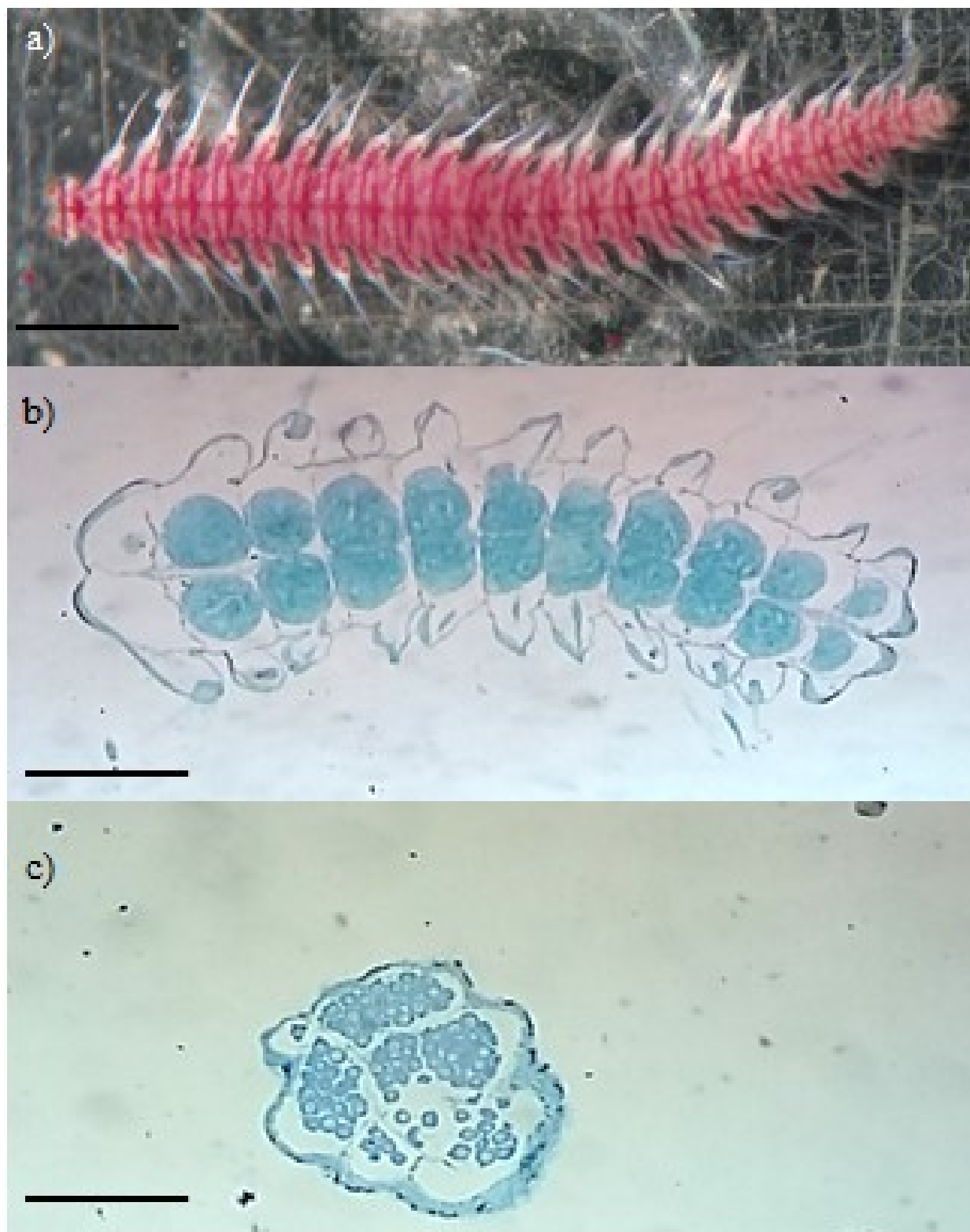


**Figure 5.** Densities during the seasons (n° *H. chamaeleon*/cm of gorgonians  $\pm$ sd).

Several reproductive, male and female stolons were detected associated with their parental individuals along all the observed periods.

As reported by Martin *et al.* (2002), at the beginning of the stolon formation, the posterior part of the body of the adult enlarges, becomes milky coloured and swimming chaetae develop. The stolon head is the last part to be formed, with the production, in the last adult segment, of two ventro-lateral protuberances; these are the joining segments between the progenitor and the stolon. When the stolon is ready, it presents a bilobed head, with two small antennae, four eyes, and long swimming chaetae (Figure 6a); the pigmentation was maintained as in the parental specimen. We observed the stolons during the release of the gametes, *via* the parapodial lobes, by mean of quick movements, in seawater and at room temperature.

Histological preparations with sperms and eggs are shown in Figure 6 b, c. Oocytes are on average  $50.75 \pm 4.94 \mu\text{m}$  in diameter, while spermatic cysts are  $211.5 \pm 44.34 \mu\text{m}$  in diameter.



**Figure 6.** a) Entire male stolon; b) Spermatic cysts; c) Oocytes. Scale bars: a= 1 mm; c-d= 100µm.

### 3.4 Discussion

The symbiotic relationship between *H. chamaeleon* and the gorgonian *P. clavata* has been described for the first time by Laubier (1960) in Banyuls sul Mar (France) and after this, rediscovered and better described by Alós *et al.* (1988) in Cape de Creus (Spain) and by López *et al.* (1996) in Chafarinas Islands (Alboran Sea, West Mediterranean). Up today, this association was reported in these areas of the western Mediterranean Sea only, (Figure 2) and more recently Lattig & Martin (2009) found *H. chamaeleon* associated with the gorgonian *Paramuricea grayii* along the Atlantic coast of Spain (Ria de Arousa, Pontevedra). Abd-Elnaby (2014) reported specimens on muddy bottom for the Egyptian waters and Wilson (2006) signalled this species also in the Arafura Sea, Australia. We think that this last record should be considered doubt, indeed, it is highly probable that the Australian species, *Trypanobia depressa* (Augener, 1913), firstly described as *Haplosyllis depressa* Augener, 1913, was confused with the Mediterranean species *Haplosyllis depressa* var. *chamaeleon*, now *H. chamaeleon*.

We had the opportunity to look for *H. chamaeleon* in different *P. clavata* populations expanding its areal of distribution. The new records here reported enlarge the geographic distribution of this elusive polychaete that likely retraces the distribution of its host (*P. clavata*), in both its morphotypes (violet/yellow).

A population of this species, that has been sampled for a period of one year, allowed us to furnish more details on its morphology and reproductive period.

To limit destructive sampling, we decided to collect only the distal fragments (15 cm long), from each gorgonian colony; this sampling method may have altered the results about *H. chamaeleon* density and presence; the specimens possibly present at the base or along the principal axis of the gorgonian were excluded from the sampling. However, as reported by some authors (Laubier, 1960; Martin *et al.*, 2002), *H. chamaeleon* is generally present on the apical parts of the gorgonian, where it probably can find better opportunity of feeding, due mostly to the highest number of polyps. We observed several polychaetes with the anterior part of the body inserted and coiled in the gastric cavity of the polyps. When disturbed these worms fasten crawl along the gorgonian axis while after the separation of their hosts tend to stay coiled and immobile, as observed also by Martin *et al.* (2002).

Although these polychaetes likely spend their entire life on the gorgonian, they do not inflict any injuries to the colonies. This is different from the cases of the species *Haplosyllis anthogorgicola* that inhabits galleries inside the coenenchym of its host, or from *Haplosyllis villogorgicola* Martin *et al.* (2002), that merges different gorgonian branches forming gall-like structures. Despite their different effects on the gorgonian skeletons, both these species were considered kleptoparasitic and



mutualistic because supposed to clean the gorgonian surface (Martin *et al.*, 2002). This aspect contrasts with the definition of parasitism, where the association clearly advantages one member but induces inconvenience in the other one (Dales, 1957) suggesting that the relationship between *H. chamaeleon* and *P. clavata* can probably be better ascribed as commensalism, as recently suggested by Martin & Britayev (2018). Even so, to clarify this aspect, studies about the biochemical composition of the species *P. clavata* and *H. chamaeleon* are needed.

In the studied samples, several branches hosted, at least, one syllid in Spring '18, while during Summer '17, although a lower number of branches were found infested, the abundance (ind/cm) was higher in respect to the other seasons. This is due to the presence of several specimens, up to seven, living on a single branch. Martin *et al.* (2002) reported that a single colony can be infested by ten worms; this result suggests that probably a colony of *P. clavata* can support a higher number of symbionts probably in relation to its dimension, age, health status and environmental conditions. Even though, Laubier (1960) indicates only May and June as reproductive period of *H. chamaeleon*, we observed stolons all year around, with sea temperature from 9°C to 25°C (<https://www.mareografico.it/>). This result suggests that other factors influence the reproduction of this species, as in *Typosyllus prolifera* (Krohon, 1852) that matures every month, following the semi-lunar cycle (Franke, 1999).

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## Chapter 4

# ***Paramuricea clavata* (Risso, 1826) and *Haplosyllis chamaeleon* Laubier, 1960: a step toward a better understanding of the chemical interaction between two partners?**

### **Abstract**

The Mediterranean gorgonian *Paramuricea clavata* (Risso, 1826) is a common species along the coralligenous habitat where it plays an important role as ecosystem-engineer. The neglected species *Haplosyllis chamaeleon* Laubier, 1960 (Annelida; Syllidae) lives associated with this gorgonian, but its presence in the Italian waters has been ignored up today. This worm is supposed to be a strict ectoparasite on the gorgonian, used as food source. In this study, the chemical composition of colonies, collected along the Portofino Promontory in April and in June 2018, was investigated for the identification of bioactive metabolites produced by this gorgonian as possible indicators of a trophic relationship with the polychaete. Associated polychaetes were analysed to verify the presence of the possible gorgonian products, eventually introduced with the diet. Results show a similar pattern of secondary metabolites in all colonies and inter-individual variability in the distribution of two furanosesquiterpenes: linderazulene, previously identified as the purple pigment of *P. clavata*, and isoactractylon, here reported for the first time in this species.

Results show that *H. chamaeleon* is characterized by the linderazulene and by a new compound, yet to be characterized, mainly distributed in the reproductive body region of the worms.

This first characterization tracks an important baseline for next studies on the ecological role of these compounds likely involved in the chemically-mediated interaction between these two species.

*Keywords:* *P. clavata*, *H. chamaeleon*, Ligurian Sea, linderazulene, isoactractylon.

## 4.1 Introduction

Most natural products isolated from the cnidarians came from members of the subclass Octocorallia (Ledoux & Antunes, 2018), which is considered one of the most rich sources of compounds of interest in the pharmaceutical sector. Accordingly, attempts to explore the taxonomic and geographical distribution of bioactive metabolites from octocorals especially aimed at the discovery of compounds that may be useful for developing new drugs (Leal *et al.*, 2012). Emphasis has been placed especially on bioactive metabolites occurring in unrelated and evolutionarily distant species, suggesting that they must have important biological functions by interacting with specific and conservative molecular targets (Tulp & Bohlin, 2005). For example, the Caribbean gorgonian *Plexaura homomalla* (Cnidaria: Gorgonacea) contains prostaglandins with the same configuration as mammalian prostaglandins, which have been found to play critical biological roles in almost all human tissues, while in the gorgonian they possibly function as chemical defence against predatory fish (Gerhart, 1984). As a further example, vidarabine (ara-A), an antiviral drug which is active against herpes simplex and varicella zoster viruses, has been derived in the 1950s from two nucleosides isolated from the Caribbean marine sponge *Tethya crypta* (Bergmann & Swift, 1951), and has been subsequently found in the Mediterranean gorgonian *Eunicella cavolini* (Cimino *et al.*, 1984). On the other hand, bioactive compounds from gorgonians are also known for their antifouling properties, thus for their ability to prevent the settlement of microorganisms, plants, and animals on solid substrate immersed in the marine environment. In this case, the actions of the natural products that make them interesting for industrial applications, obviously relate to the ability of the octocorals of inhibiting epibiosis pressure in natural conditions, keeping animal body surfaces free of fouling (Fusetani, 2011). In the Mediterranean Sea, some of the metabolites isolated from *Paramuricea clavata* (Risso, 1826), including both bioactive alkaloids and terpenoids, the non-toxic bufotenine and 1,3,7-trimethylisoguanine that showed significant antiadhesion activity against biofilm forming bacteria, while the linderazulene (**1**), considered the main pigment of the gorgonian and also reported for the Antarctic gorgonian *Acanthogorgia laxa*, recently showed good antifouling properties against macrofouling species (Patiño Cano *et al.*, 2018).

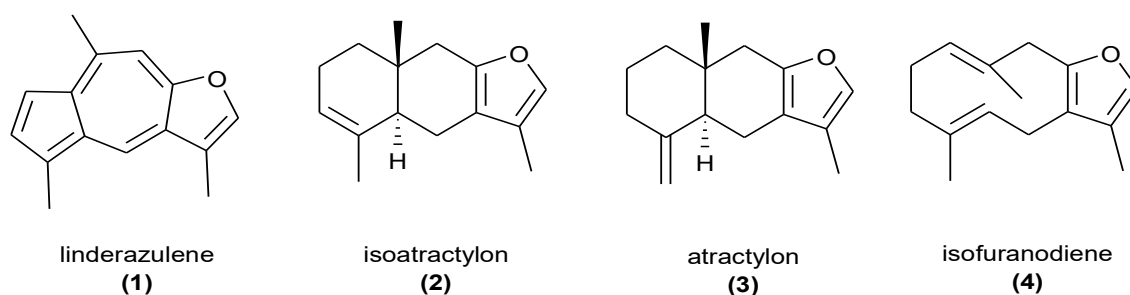
The availability of the above information on the antifouling potencies on the one hand and the paucity of data on other possible ecophysiological roles of the compounds isolated from *P. clavata*, is most likely due to the emphasis upon identifying bioactive compounds to be eventually incorporated in marine coatings to prevent the settlement of epizoic organisms on immersed artificial structures.

Contravening the widespread and human-centred perspective on natural products, especially moving towards possible industrial applications, in the present study we focus on the adaptive



significance of the bioactive metabolites produced by *P. clavata*, trying to provide new insights into the ecological role of chemical components of the gorgonian that provide protection from predators and cues to finding food, regulate physiological processes and inter- or intra-specific interactions, or maximize the reproductive success. Inter-individual variation in the secondary metabolite contents, possibly related to intrinsic (e.g. genetics, age, sex, diseases) or extrinsic (food availability, diet, stress conditions, presence of epibionts, exposition to predators and/or competitors) factors, have been thus investigated to achieve novel information that could help to hypothesize the ecophysiological roles played by the chemical components of *P. clavata*.

We individually analysed fragments from different colonies of *P. clavata* collected along the coast at Portofino (Ligurian Sea) and, unexpectedly, we found a similar pattern of secondary metabolites in all colonies except for the extreme inter-individual variability in the distribution of the two furanosesquiterpenes: linderazulene (**1**), previously identified as the purple pigment of *P. clavata*, and isoatractylon (**2**), a compounds that has escaped all previous chemical investigations on *P. clavata*. It is worth noting that **2** has been previously found in the Antarctic gorgonian *Dasystenella acanthina* (Gavagnin *et al.*, 2003), as well as in the Mediterranean octocoral *Maasella edwardsi* and in its specialist predator, the nudibranch *Tritonia striata* (Giordano *et al.*, 2017). Along with the closely related compounds **3** and **4**, isoatractylon (**2**) has been shown to mediate defensive strategies against generalist predators. However, compounds **2-3** have also been proposed to act as toxic weapons helping to capture dietary zooplanktonic crustaceans in *M. edwardsi* (Giordano *et al.*, 2017). This finding led us to propose, in a synthesis with previous studies on octocorals, a novel framework for future research projects aimed at shedding more light on the chemical ecology of *P. clavata*.



The obtained results are also discussed for their possible implications in the chemically-mediated host selection by symbiont polychaetes *Haplosyllis chamaeleon* Laubier, 1960 living in close association with *P. clavata*. Analysis conducted on the polychaete shows that it holds only the compound **1**, suggesting a possible selective accumulation of this molecule or its resistance to the

detoxification processes during worm digestion. Moreover, in the reproductive stolons we detected the presence of another compound, at the moment unidentified, probably associated to the gametes.

With this research, we provide the informational base for future studies aimed at clarifying the mechanisms allowing those associated animals to survive on their specific chemically-defended host.

## 4.2 Materials and Methods

### *Animals*

Apical branches of the gorgonian *P. clavata*, were sampled by scuba diving along the coast of the Liguria region (North-Western Italy) about 40 meters depth, at the dive site “Il Bigo” in Portofino. All the gorgonian samples were cut with a knife and put in plastic bags with seawater, then were transported to the laboratory in cooled seawater.

In order to better characterize the chemical composition of the species *P. clavata*, 16 colonies collected in June 2018 were analysed. These were kept alive after the sampling for the analyses and partially conserved at -80°C for possible future investigations.

Two gorgonians (V1 and V2) sampled in April '18 and their associated polychaetes, belong to the species *H. chamaeleon* Laubier, 1960, were successively investigated. To avoid the contamination of the gorgonian products, polychaetes were detached from the colonies, and kept alive for three days in separate bowls with seawater that was changed every day. Polychaetes have been sorted and analysed separately as it follows: two entire specimens (vial 1), fragments of bodies (vials 2, 3 and 4), juveniles (vial 5) and stolons (vial 6); the entire specimens were associated to the same colony (V2) while, due to the small dimension of the samples, other specimens, associated with both V1 and V2, were put together. Then, wet weight of worms in each vial was measured. Finally, gorgonians and polychaetes were conserved at -80°C.

### - *Gorgonians of June 2018 sampling*

#### *Extraction and comparisons by thin layer chromatography (TLC)*

Apical fragments of each of the 16 sampled colonies were separately extracted with acetone both by grinding and ultrasonic treatment to increase extraction efficiency. After acetone evaporation, the aqueous residues were subsequently partitioned with n-hexane. Each of the 16 n-hexane soluble portions of the acetone extract was analyzed by TLC by using petroleum ether/diethyl ether in different ratios as eluent, while Cerium sulfate and Ehrlich's reagents were used to detect the spots. TLC was performed on precoated silica gel plates (Merck Kieselgel 60 F254, 0.2 mm).

***Evaluation of inter-individual variations by gas chromatography-mass spectrometry (GC-MS)***

GC-MS analyses were performed on an ion trap mass spectrometer equipped with EI source (70 eV) (Polaris Q; ThermoScientific) coupled with a GC system (GCQ; ThermoScientific) with a 5% phenyl column (Trace TR-5, 30 m × 0.25 mm × 0.25 μm; ThermoScientific) and using helium as a gas carrier. Elution of volatile sesquiterpenoids required a temperature program starting at 120 °C for 3 min, followed by a 5 °C min<sup>-1</sup> gradient up to 220 °C, then 20 °C min<sup>-1</sup> up to 310 °C, holding for 5 min. Samples were directly injected (2 μL) in split (1:10) mode, with a blink window of 3 min, inlet temperature of 210 °C, transfer line set at 250 °C, and ion source temperature of 230 °C.

***Purification and identification of compound 2***

Silica gel column chromatography was performed using Merck Kieselgel 60 powder (0.063 mm to 0.200 mm) eluting with a gradient of petroleum ether/diethyl ether. The NMR spectrum was acquired in benzene-d<sub>6</sub> on a 400 MHz Bruker Avance III HD spectrometer equipped with a CryoProbe Prodigy, and the chemical shifts were reported in parts per million referenced to benzene-d<sub>6</sub> (δ 7.15).

***- Gorgonians and Polychaetes of April 2018 sampling******Extraction***

Apical fragment of each of the two gorgonians (V1 and V2) and polychaetes were separately extracted with acetone both by grinding and ultrasonic treatment to increase extraction efficiency. After acetone evaporation, the aqueous residues were subsequently partitioned with n-hexane.

***Evaluation of by gas chromatography-mass spectrometry (GC-MS)***

GC-MS analyses were performed on an ion trap mass spectrometer equipped with EI source (70 eV) (Polaris Q; ThermoScientific) coupled with a GC system (GCQ; ThermoScientific) with a 5% phenyl column (Trace TR-5, 30 m × 0.25 mm × 0.25 μm; ThermoScientific) and using helium as a gas carrier. Elution of volatile sesquiterpenoids required a temperature program starting at 120 °C for 3 min, followed by a 5 °C min<sup>-1</sup> gradient up to 220 °C, then 20 °C min<sup>-1</sup> up to 310 °C, holding for 5 min. Samples were directly injected (2 μL) in split (1:10) mode, with a blink window of 3 min, inlet temperature of 210 °C, transfer line set at 250 °C, and ion source temperature of 230 °C.

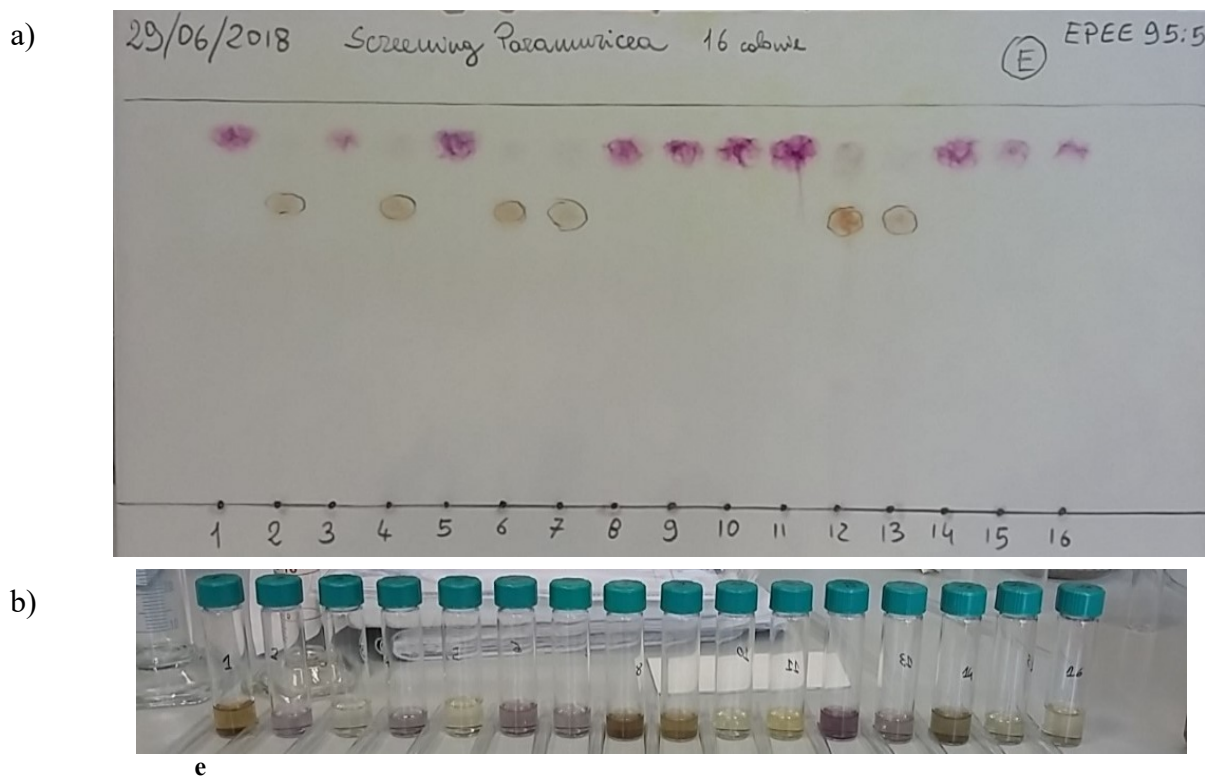
## 4.3 Results

### - *Gorgonians collected in June 2018*

#### *TLC comparisons*

TLC analysis performed by using petroleum ether/diethyl ether 95:5 as eluent revealed the presence of Ehrlich positive spots at  $R_f$  0.75 and  $R_f$  0.9, differentially distributed among the colonies (Figure 1a). Interestingly, Ehrlich positive spots at  $R_f$  0.75 were present in the extracts of colonies 2, 4, 6, 7, 12, 13, all showing a purple coloration under natural light (Figure 1b), suggestive of the presence of the purple pigment linderazulene (**1**), a furanosesquiterpene previously isolated from *P. clavata* (Imre *et al.*, 1981).

Conversely, extracts from colonies 1, 3, 5, 8-11, 14, 15, 16, showing the presence of a spot at  $R_f$  0.9 strongly reacting in violet after spraying with the Ehrlich's reagent, exhibited a yellowish to light brownish color.



**Figure 1.** a) Thin layer chromatography analysis of the *n*-hexane extracts from 16 different colonies of *P. clavata*. Eluent: petroleum ether/diethyl ether 95:5; Ehrlich's reagent. b) Vials containing *n*-hexane extracts from samples 1-16.

## GC-MS comparisons

GC-MS analyses (Figure 2) led us to confirm the presence of linderazulene (210  $m/z$ ) in the purplish extracts by comparison with the literature data (Imre *et al.*, 1981), while also indicating the presence of a different furanosesquiterpene with  $m/z$  value of 216 in all other extracts.

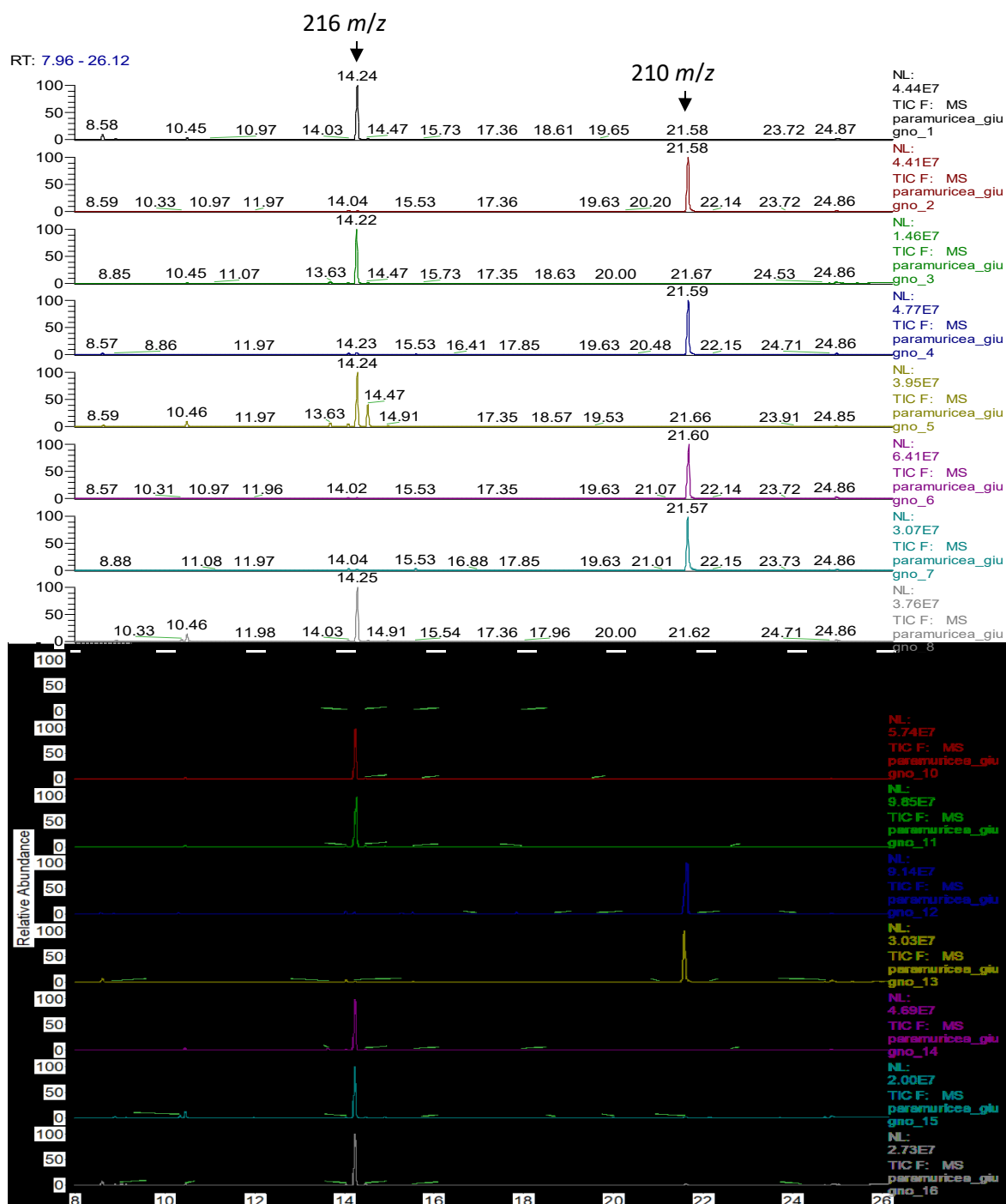
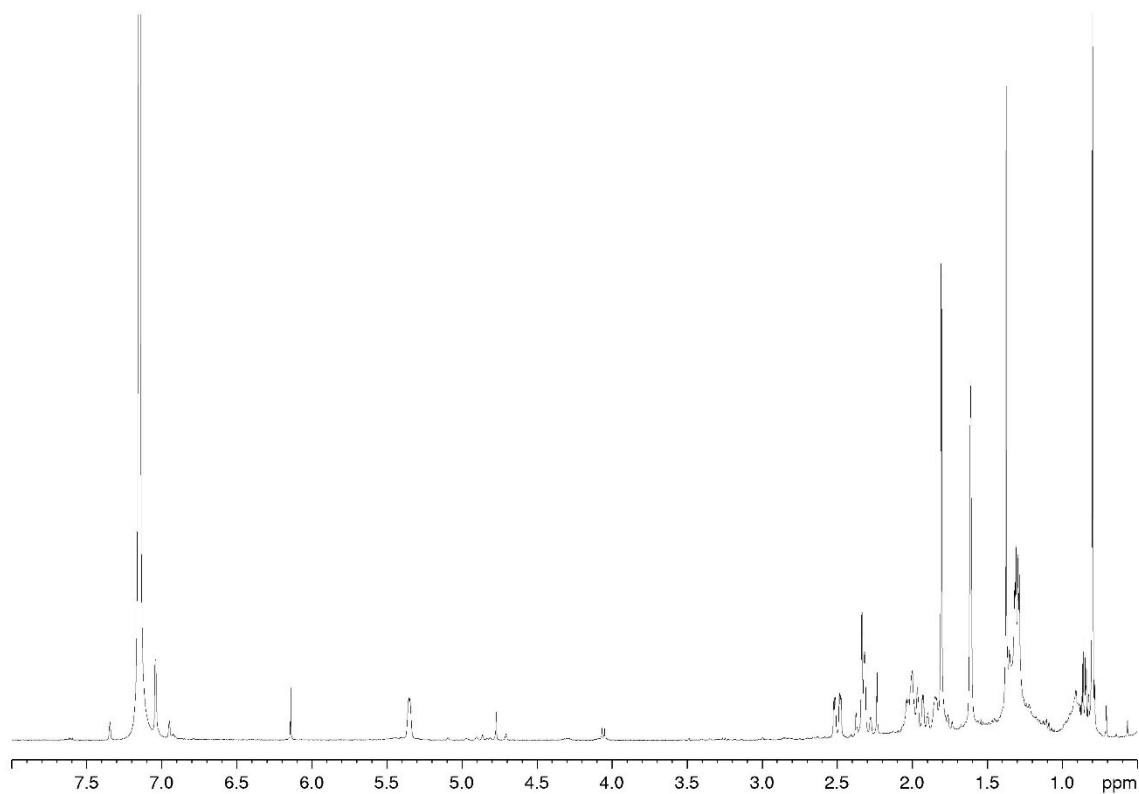


Figure 2. GC-MS profiles of sample 1-16.

### *Identification of isoatractylon*

The extract of a further sample of *P. clavata*, showing an Ehrlich positive spot at  $R_f$  0.9 when analyzed by TLC, was then fractionated by silica gel column chromatography to give purified compound **2**, identified by comparison of  $^1\text{H}$  NMR spectroscopic data (Figure 3) with the literature (Gavagnin *et al.*, 2003).



**Figure 3.**  $^1\text{H}$  NMR spectrum of isoatractylon (**4**) in  $\text{C}_6\text{D}_6$ .

- *Gorgonians and H. chamaeleon collected in April '18*

GC-MS analyses (Figure 4) led us to confirm the presence of linderazulene (210  $m/z$ ) in V1 and V2 and in all the polychaetes-samples. In the extract of the reproductive stolons, another furanosesquiterpene with  $m/z$  value of 232 was signalled.

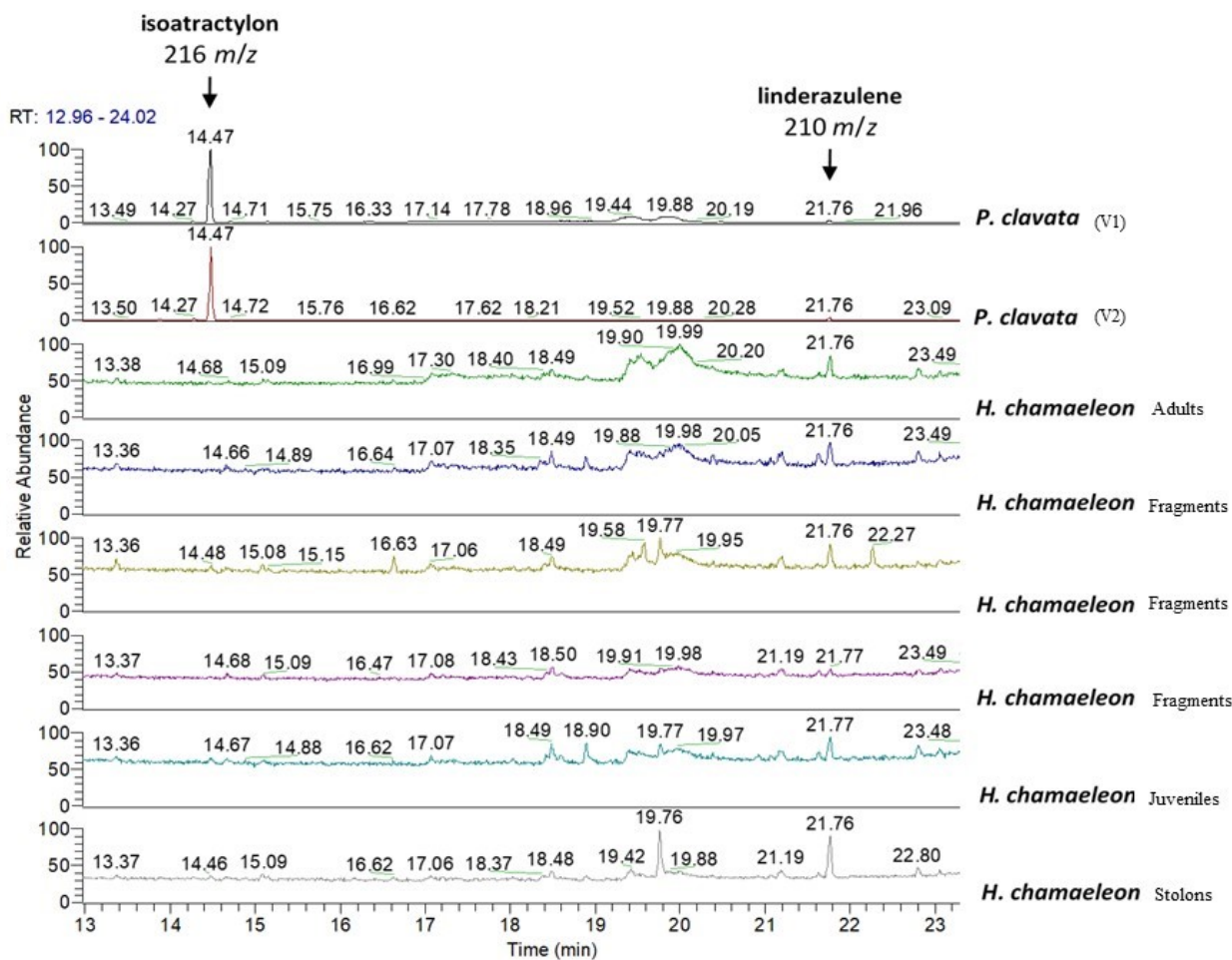


Figure 4. GC-MS profiles of the two *P. clavata* gorgonians and the polychaetes samples.

## 4.4 Discussion

In the marine environment, the three-dimensional structures of many sessile organisms, implement the complexity of the ecosystem providing new ecological niche, refuge from predators, nursery areas (Buhl-Mortensen & Mortensen, 2005; Cerrano *et al.*, 2010; Piazzini *et al.*, 2018) and affecting the environmental factors, as sedimentation and water circulation (Valisano *et al.*, 2016; Gori *et al.*, 2017). When these three-dimensional assemblages are built by benthic animals, the term “animal forest” (Rossi *et al.*, 2017) is commonly used and their role as ecosystem engineers is well documented (Ponti *et al.*, 2018), facilitating the interactions among several species. In particular, regarding gorgonians, Calcinaï *et al.* (2013) reported several species of Porifera living in close association with octocorals collected in the Indo-Pacific Ocean, while recently Molodstova *et al.* (2016) and Martin & Britayev (2018) update the number of polychaetes living in close association with cnidarians. The more complex the interactions, the more chemical communication between organisms takes on a fundamental ecological role.

Adding to the list of substances that have been found in *P. clavata*, we report here the first finding of the furanosesquiterpene isoatractylon (**2**), previously found in the Antarctic gorgonian *Dasystenella acanthina* (Gavagnin *et al.*, 2003), in the Mediterranean octocoral *Maasella edwardsi*, and in the nudibranch *Tritonia striata* that feed on *M. edwardsi* (Giordano *et al.*, 2017). The fact that the compound has never been previously isolated in *P. clavata* is possibly due to the occurrence of post collection oxidations or to the use of methanol as the extraction solvent. Furans are, in fact, susceptible to rapid transformations under natural or fluorescent light or in solution in methanol or chloroform. Linderazulene (**1**) also undergo oxidation to a five-membered keto-lactone under sunlight in ethanol (Alpertunga *et al.*, 1983). Instead, in the present work acetone and benzene were used for the extraction, while deuterated benzene (benzene-d<sub>6</sub>) was used to record the <sup>1</sup>H NMR spectrum of **2**.

It is worth noting that both compounds **1** and **2** occur in unrelated species belonging to marine invertebrates and plants possibly having an important biological function. It has been proposed, in fact, that “it is extremely improbable that in nature one particular compound is synthesized in totally unrelated species for no reason at all” (Tulp & Bohlin, 2005). The different terrestrial and marine sources of **1** and **2** are reported in table 1.

The table also reports sources of compounds **3** and **4** (closely related to **2**) for which a panel of biological activities have already been described in the literature suggesting that they interact with conservative molecular targets (Giordano *et al.*, 2017, and references therein).



**Table 1.** Marine (light blue) and terrestrial (light brown) sources of compounds **1-4**.

Linderazulene (1)	Isoatractylon (2)	Atractylon (3)	Isofuranodiene (4)
<i>Paramuricea clavata</i> (Anthozoa: Alcyonacea)	<i>Paramuricea clavata</i> (Anthozoa: Alcyonacea)	<i>Dasystenella acanthina</i> (Anthozoa: Alcyonacea)	<i>Dasystenella acanthina</i> (Anthozoa: Alcyonacea)
<i>Smyrniun perfoliatum</i> (Magnolopsida: Apiales)	<i>Dasystenella acanthina</i> (Anthozoa: Alcyonacea)	<i>Efflatounaria</i> and <i>Cespitularia</i> spp. (Anthozoa: Alcyonacea)	<i>Pseudopterogorgia</i> spp. (Anthozoa: Alcyonacea)
	<i>Maasella edwardsi</i> (Anthozoa: Alcyonacea)	<i>Maasella edwardsi</i> (Anthozoa: Alcyonacea)	<i>Pacificorgia pulchra</i> var. <i>exilis</i> (Anthozoa: Alcyonacea)
	<i>Tritonia striata</i> (Gastropoda: Nudibranchia)	<i>Tritonia striata</i> (Gastropoda: Nudibranchia)	<i>Efflatounaria</i> and <i>Cespitularia</i> spp. (Anthozoa: Alcyonacea)
		<i>Atractylodes</i> (= <i>Atractylis</i> ) spp. (Magnolopsida: Asterales)	<i>Stenogorgia</i> sp. (Anthozoa: Alcyonacea)
		<i>Podocarpus spicatus</i> (Pinopsida: Pinales)	<i>Maasella edwardsi</i> (Anthozoa: Alcyonacea)
		<i>Eugenia uniflora</i> (Magnolopsida: Myrtales)	<i>Tritonia striata</i> (Gastropoda: Nudibranchia)
		<i>Pinellia ternata</i> (Magnolopsida: Alismatales)	<i>Leminda millecra</i> (Gastropoda: Nudibranchia)
			<i>Chloranthus tianmushanensis</i> (Magnoliopsida: Chloranthales)
		<i>Commiphora</i> spp. (Magnoliopsida: Sapindales)	
		<i>Curcuma zedoaria</i> (Liliopsida: Zingiberales)	
		<i>Smyrniun</i> spp. (Magnolopsida: Apiales)	

TLC and GC-MS analysis also revealed a strong inter-individual variability in the levels of the two furanosesquiterpenes linderazulene (**1**) and isoatractylon (**2**). This finding suggests that the two compounds can play different ecophysiological roles in the octocoral. However, isoatractylon (**2**), along with compounds **3** and **4**, has already been shown to protect both the octocoral *M. edwardsi* and its specialist predator, the nudibranch gastropod *T. striata*, from potential predators, eliciting avoidance responses in the generalist shrimp *Palaemon elegans*. Although one can argue that **2** plays a defensive role also in *P. clavata*, it remains unclear if analogous feeding deterrent properties of **1** could compensate the extremely low levels of **2** in 37% of the studied colonies. In parallel, a possible role of both compounds **1** and **2** as toxic weapons helping to capture dietary zooplanktonic crustaceans should be also considered. However, the possible involvement of both compounds in defensive and alimentary strategies that are shared by all individuals cannot explain the inter-individual chemical variations. Conversely, it is possible — but the hypothesis deserves further investigation — that the two compounds are sex-specific, one of them acting as a sperm-attractant secreted from the oocytes. This would not be the first case of sperm chemotaxis in octocorals. For example, the terpene (-)-*epi*-thunbergol was identified as the natural sperm attractant in the eggs of *Lobophytum crassum* (Coll *et al.*, 1995), showing that sperm cells follow a concentration gradient

of the terpene secreted by the oocytes. Also polychaetes propose the involvement of specific compounds for the reproduction; indeed, the presence of the unidentified compound in the stolons, suggests a possible function of this in the recognition between the eggs and the spermatid cists after their release.

About Mediterranean octocorals, information regarding symbiont polychaetes are still scant. Some examples were reported by Martin *et al.* (2002) that describes for the first time the species *Haplosyllis villogorgiola* Martin *et al.*, 2000 inhabiting the gorgonian *Villogorgia bebrycoides* (Koch, 1887) and re-describes the species *Haplosyllis chamaeleon* Laubier, 1960 living on the branches of the red gorgonian *Paramuricea clavata* Risso, 1826 and the species *Haplosyllis anthogorgicola* Utinomi, 1956 living inside the coenenchym of the gorgonian *Anthogorgia bocki* Aurivillius, 1931. *P. clavata* colonies collected along the Portofino Promontory, (see chapter 3), host the syllid *Haplosyllis chamaeleon* that it is considered an obligate symbiont of this gorgonian. The colour of this worm exactly mimics the colour of its host, both in the yellow and in the violet morphotype, suggesting a similar biosynthetic way for the production of the body pigmentation or a probably accumulation of the gorgonian component, as the case of the compound **1** but not of the compound **2**. A study conducted on a sponge-associated syllid *Branchiosyllis oculata* Ehlers, 1887, Pawlik (1983) reports that after the consume of the sponges, some pigments were stored in the guts of the polychaetes, confirming the feeding activity of these but not the possible implication of the accumulation of the pigment in their body tissue, that it was not investigated.

This aspect opens several hypotheses (Table2): i) if the compound **1** is strictly related with the pigmentation, polychaetes can potentially exploit it for their camouflage; ii) if future investigations will confirm the deterrent property of this compound, as previously suggested, these polychaetes can selectively accumulate this molecule and employ it for dissuading attacks from predators, as described for nudibranchs; iii) the compound **1** is not digested, and the compound **2** is not assimilated by the polychaetes and for this not observed during the analysis and iiiii) the compartmentalization of some compounds (e. g. inside the polyps or in the coenenchyme), may influence their detection during the analyses, also in relation to the feeding behaviour of the symbionts. The question about feeding behaviour of the polychaetes is still open, and even if, they were observed many times with the anterior part of the body inside the gastric cavity of the polyps (Martin *et al.*, 2002; Chapter 4), no sign of feeding activities has been observed on the colonies surface.

In the present report, however, we show that linderazulene (**1**) cannot be considered the only pigment responsible for the violet color of *P. clavata*. Even colonies showing very low levels of **1**, in fact, did not show a different coloration. Further studies are thus needed to better understand the

chemical nature of the colour in both the symbiont and its host. On the other hand, given the toxicity and the antifouling properties of the furanosesquiterpenes **1** and **2** isolated in *P. clavata*, it remains to be fully elucidated how *H. chamaeleon* can survive on the gorgonian, and if it is protected from the toxic effects of terpenes by means of detoxification mechanisms. Finally, future research should also aim at assessing if *H. chamaeleon* feeds on *P. clavata* re-using dietary compounds **1** and **2** for its own defence, and if **1** and **2** act as chemosensory cues contributing to host recognition by the polychaete.

**Table 2.** Future investigations

Future investigation	Host ( <i>P. clavata</i> )	Symbiont ( <i>H. chamaeleon</i> )
Defence strategy	x	x
Feeding behaviour	x	x
Reproduction	x	x
Colour origin	x	x
Detox mechanism	x	x
Host-recognition		x

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## Chapter 5

# Rock-boring polydorids of the Central Adriatic Sea

### Abstract

Bioerosion was investigated along the Ancona Promontory, in the Central Adriatic Sea using artificial, calcareous panels. Two set of limestone panels were placed in a sheltered habitat (Marina Dorica harbour) and in open waters (Passetto) in June 2017 and collected bimonthly from January 2018 to July 2018. The spionid *Polydora ciliata* was the principal species involved in the bioerosion of these artificial, calcareous substrates. Erosion rate was estimated by measuring the typically 8-shaped openings and the eroded volumes obtained by resin casts of the holes. The density of *P. ciliata* (holes/cm<sup>2</sup>) is stable in Passetto but varied significantly among months of exposition in Marina Dorica and between the two sites.

The peculiar environmental conditions of the sheltered site trigger a faster colonization of boring polychaetes respect the dynamics recorded in open waters. In open waters a biogenic carpet made by the honeycomb polychaetes *Sabellaria spinulosa* covered the surface of the panels, compromising the attachment of the larvae.

*Keywords:* bioerosion, Adriatic Sea, polychaetes, limestone panels.

## 5.1 Introduction

Destruction of carbonate substrates due to organism activities, or bioerosion (Neumann, 1966), is a common process in several marine ecosystems of the world as temperate areas (Cerrano *et al.*, 2001a; Wisshak *et al.*, 2005, 2011; Schönberg *et al.*, 2014), coral reefs (Heins & Risk, 1975; Hutchings *et al.*, 1992, 2002, 2008, 2013; Pari *et al.*, 2002; Tribollet *et al.*, 2002) and even Antarctica (Cerrano *et al.*, 2001b). Rock-destroying organisms play a relevant role in the erosion of the coastlines (Naylor *et al.*, 2012), in producing sediments and in the carbonate cycle; they create complex habitats that can be harboured by several communities and can affect the ecosystem services (Davidson *et al.*, 2018). Moreover, the impact of the bioeroder communities can be influenced by several environmental factors, as salinity, acidification and nutrients, making the study of the bioerosion processes a present-day topic, considering also the influence of climate change (Schönberg *et al.* 2014, 2017a, b).

Schönberg *et al.* (2014) reported that only the 11% of the researches on bioerosion were conducted in the Mediterranean Sea and studies about the rates of bioerosion are still scant (Calcinai *et al.*, 2008, 2011; Färber *et al.*, 2015, 2016); in particular, no data are available about erosion activities and rate for polychaetes in the Mediterranean Sea.

Experimental studies for evaluating bioerosion rates are principally conducted using artificial blocks obtained by dead coral colonies (Tribollet *et al.*, 2002; Hutchings, 2008), rock carbonatic panels (Wisshak *et al.*, 2005), shells (Calcinai *et al.*, 2007), and wood (Reish *et al.*, 2018). To evaluate the erosion, it is fundamental to identify the agents responsible of these processes (Hutchings, 2011). Several organisms belonging to different taxa are involved in calcareous-boring activities and they can be microborers (Pica *et al.*, 2016) as cyanobacteria (Tribollet *et al.*, 2011a, 2011b;) and fungi (Gleason *et al.*, 2017), or macroborers as sponges (Bavestrello *et al.*, 1996, Calcinai *et al.*, 2008, 2011), molluscs (Schiaparelli *et al.*, 2005; Curin *et al.*, 2014; Peharda *et al.*, 2015), echinoderms (Belaústegui *et al.*, 2017) and polychaetes (Martin & Britayev 1998; Hutchings, 2008). The local pattern of currents influences the larval dispersion (Hutchings, 2002, Tribollet *et al.*, 2002) affecting the recruitment of boring organisms. The following successional stages are then related to the peculiarity of the borers life cycles and the achievement of a mature boring communities alter the substrate susceptibility (Hutchings, 2008). Polychaetes have been reported as the dominant borers on newly available, submerged substrates (Hutchings, 1992, 2011) and have been considered among major bioeroders with sponges and molluscs. Nevertheless, studies about values of bioerosion of polychaetes are not numerous and especially they concern coral reefs; in these areas, they are comprised between 0.104 kg/m<sup>2</sup> (Kiene & Hutchings, 1994), and 0.356 kg/m<sup>2</sup> (Hutchings, 2008) and are comparably lower in comparison to sponges (Pari *et al.*, 2002). Several



families of polychaetes are involved in bioerosion processes, as Eunicidae, Cirratulidae, Sabellidae and Spionidae. These worms exhibit a high range of feeding types but are in prevalence suspension feeders. Boring polychaetes use both chemical secretions and physical actions for dissolving the rocks and making their holes (Hutchings, 2008). Sabellidae for example, present developed glandular areas along the ventral part of the body, while in some species of *Polydora* the erosion of the carbonate substrate is possible thanks to peculiar modified chaetae on the 5<sup>th</sup> chaetiger, in addition to the production of chemicals compounds. Polydorids (Annelida: Spionidae) is one of the most studied boring group and this is principally due to the several damages that these polychaetes produce on oysters and abalone farms (Sato-Okoshi *et al.*, 2008; Walker, 2011). These polychaetes on the substrate are easily recognizable for their typical 8-shaped traces, due to two closed openings divided by a small mucus layer. In section the hole, where the worm lives, is U-shaped. Each 8-shaped trace corresponds to a single boring individual. Not all the genera belonging to this group have been recognized as boring species, but as reported by Sato-Okoshi (1999), polydorids can be divided in two different groups: boring and not boring. Sometimes it's no easy to collocate the species in the right group also because of the confusion in literature due to similar morphological characteristics shared by several species, (see for example Simon & Sato-Okoshi, 2015), and also for the presence of sibling species, as the case of the species-complex *P. ciliata* (Mustaquim, 1986, 1988; Manchenko & Radashevsky, 1998). Moreover, it may be also tricky distinguishing between nestlers and borers, but examining the species in situ, and the knowledge of the groups may assist in the study (Hutchings, 2008).

Considering the role that bioerosion plays in the marine habitat, in general, and the scanty knowledge of this phenomenon in the Mediterranean Sea, this study aims: i) to identify the pioneer, in particular polychaetes, bioerosive organisms occurring in the first stage of bioerosion along the Central Adriatic Sea; ii) to evaluate their rate of bioerosion activity during a period of one years.

## 5.2 Materials and Methods

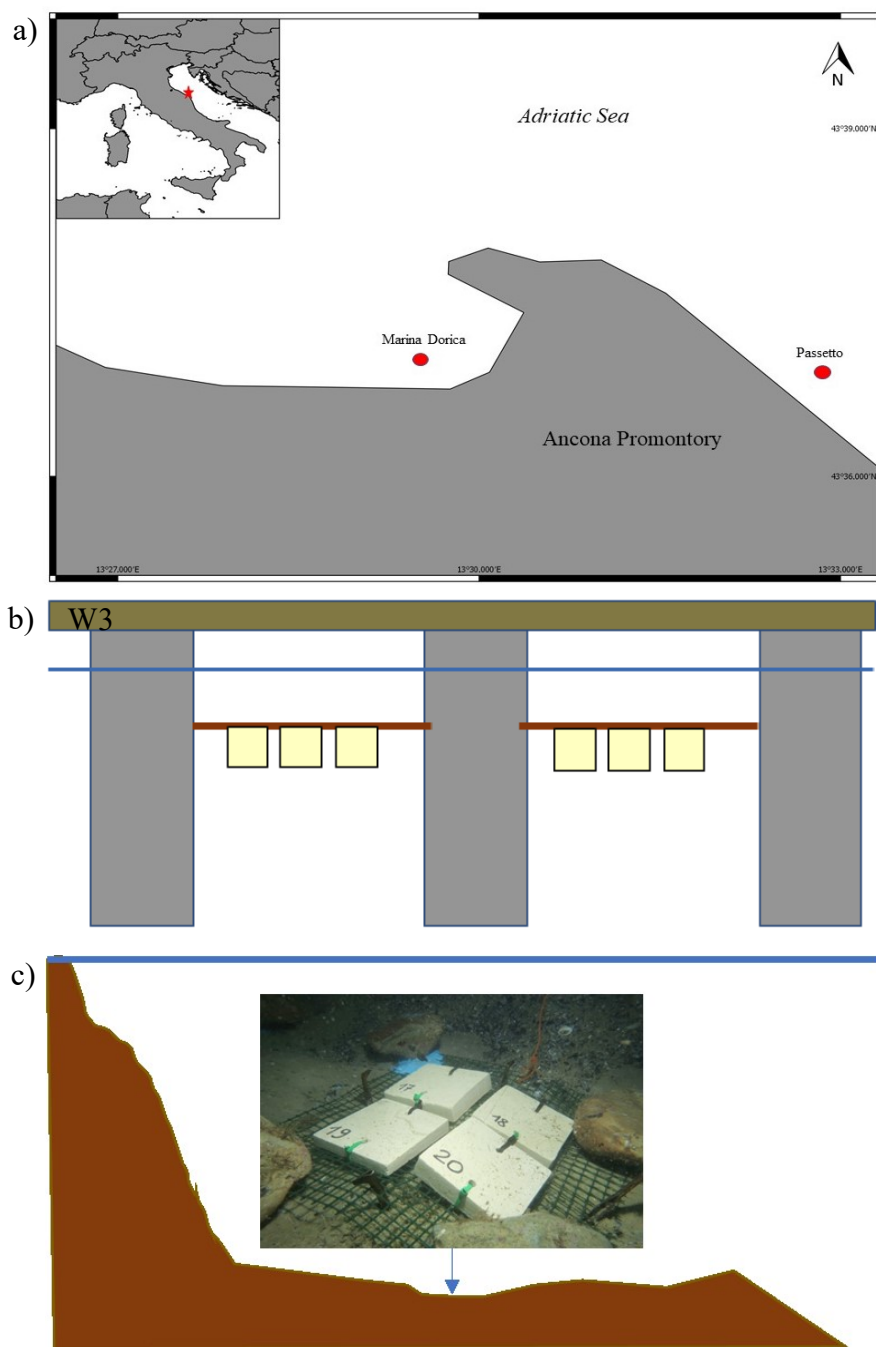
The experiment was conducted in a sheltered area (Marina Dorica) and in open sea (Passetto) (Figure 1a), located close to Ancona Promontory (Adriatic Sea, Italy); artificial panels, 10 x 10 x 4 cm in size, made of limestone, Leccese rock (Margiotta, 2006), were used for this purpose.

“Marina Dorica” (43°36’,65 N; 13°28’,91 E) is a recreational harbour in the northern-west coast of the Promontory. The bottom is characterized by muddy and sandy sediment and it is about 3 m deep. In this site, 6 sets of three calcareous panels (18 in total) were placed under a wharf at a depth of 2 m (Figure 1b). Panels were vertically positioned between the poles using a line and kept separated by plastic tubes 15 cm long. This wharf (W3) is in proximity of the canal port where the water exchange, with the open sea, is higher with respect of the internal zones.

“Passetto” is located in the southern coast (43°37’,041 N; 13°32’,150 N) and it is characterized by rocky bottom at a depth of about 10 m. This area hosts a quite high macrobenthic diversity with several species of sponges (Di Camillo *et al.*, 2016), cnidarians (Betti *et al.*, 2011), molluscs as *Mytilus galloprovincialis* and the honeycomb polychaetes *Sabellaria spinulosa*. In this habitat, many boring organisms are also present such as the sponge *Cliona adriatica* (Calcinai *et al.*, 2011), polychaetes as spionids and cirratulids and molluscs as *Rocellaria dubia* and *Pholas dactylus*.

Here, four sets of three panels (12 in total) were positioned at 8 m deep on a grid attached to the horizontal rocky substrate by epossidic plaster (SubcoatS, Veneziani Yachting) (Figure 1c).

In both sites, calcareous rocks panels were submerged from June 2017 to September 2018 and three panels were collected bimonthly from January ’18 to the end of the studied period. In January, for adverse weather conditions, the panels in Passetto were not sampled, and so the data collected in Marina Dorica, in this month, were not considered in the statistical analysis when the two sites were compared.



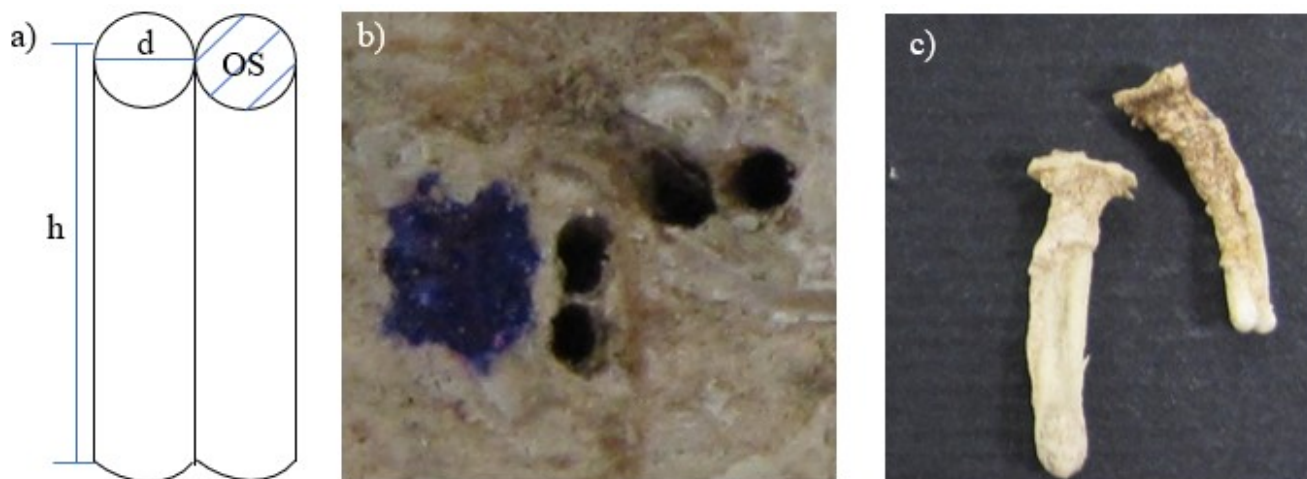
**Figure 1.** a) Ancona Promontory; b) Disposition of the panels under the wharf 3 (W3); c) Disposition of the panels in Passetto site.

When removed from the structure, panels were placed in separate plastic bags with seawater and, in the laboratory, all the encrusting organisms removed. The cleaning procedure did not affect the excavating worms and keeping them alive in sea water, allowed to better locate the holes and observe worms during the re-construction of their tubes. For the extraction of the worms (and in Passetto of the bivalves), magnesium chloride (7%) was added to the water, polychaetes and bivalves were push out of their holes with the help of a Pasteur pipette, fixed in alcohol solution (95%) and identified. For scanning electron microscopy (SEM) analyses, several specimens were

primary fixed in ethanol 20 %, then dehydrated through a graded ethanol series (50%, 70%, 90%, 100%) and dried with Hexamethyldisilazane 98% until complete evaporation. Finally, they were attached on stubs, sputtered with gold-platinum and observed under SEM Philips XL20.

In each panel, holes were assigned to the boring taxon, counted and for *Polydora* the diameter (d) of one of the two openings that constitute a single 8-shaped hole, were measured under stereomicroscope (Figure 2a, b); the surface of each openings (OS) was considered circular in shape and calculated with the formula  $OS=(d/2)^2\pi$ , where d was obtained as the average of measured diameters of all the openings, present in each panel, every month. Erosion made by bivalves was not considered in the analyses.

To assess the volume of carbonatic material removed by the polychaetes, a poly-uretanic resin (PU800) ([www.antichitàelsito.it](http://www.antichitàelsito.it)) was injected into the holes with a surgical syringe and let it dry. Then the panels were dissolved in Acid Chloride (37%) and the hardened resin casts removed. The resin casts reproduce worm excavations, i. e. two paired cylinders (Figure 2c); the total volume (TV) excavated by a single worm was calculated with the formula  $TV=2hOS$ , where OS is the area of a single opening, h is the height of the cast; the value was then multiplied by 2, considering that the holes have a paired cylinder shape. The value h was obtained by calculating the average height of 15 casts every month.



**Figure 2.** a) Scheme of the polydorid tube (d: diameter; h: height; OS: surface of a single opening); b) Example of Polydorid tube openings; c) Example of the resin casts.

The total volume excavated at the end of the sampling period, was used to calculate the maximal erosion rate of polychaetes as  $\text{mm}^3$  of excavated substrate per  $\text{cm}^2$  of substrate, per year<sup>-1</sup>.

To evaluate the weight (g) of the substrate eroded by polychaetes, the volumes removed by these, after one year of exposure, were multiplied by the specific gravity of the “Leccese” rock (2577  $\text{Kg/m}^3$ ) (<http://www.showroompietraleccese.it/>) and by the specific gravity of the micritic limestone

of the maiolica formation that characterizes the Conero substratum ( $2.5 \text{ g/cm}^3$ ) (Centamore & Micarelli, 1999; Calcinai *et al.*, 2011).

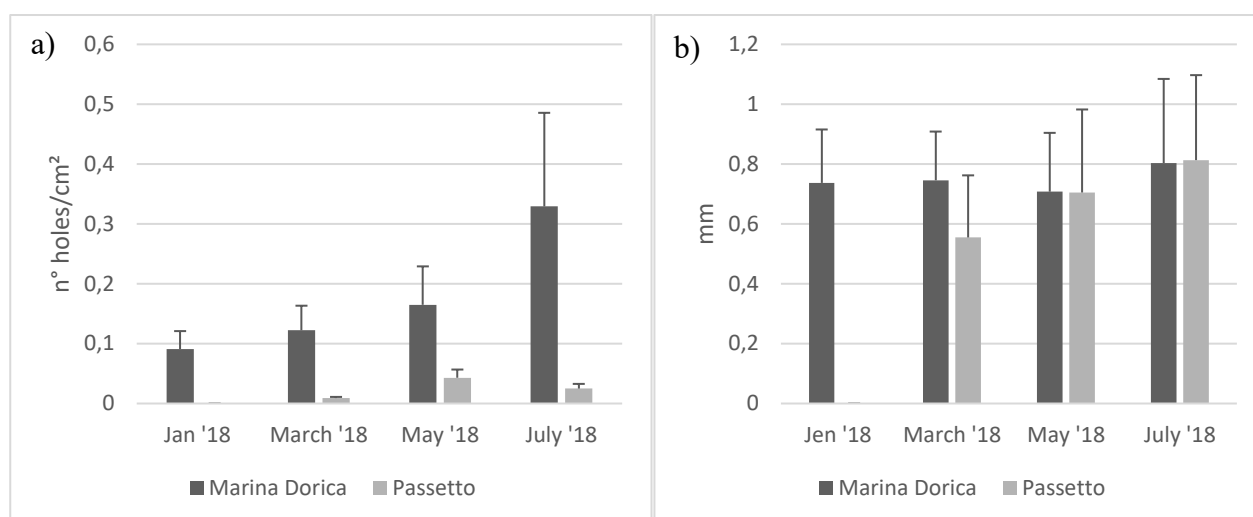
### *Statistical analyses*

One-way ANOVA test was conducted to evaluate differences of the density of the polychaetes during the experimental period in Marina Dorica and Passetto separately, and to compare the diameter of the openings and the height of the resin casts, considering the sampling period as the only variable. Two-way ANOVA test was conducted to compare the density of the polychaetes during the months March'18, May'18 and July'18 only, in the two study sites, because, due at the sampling failed in January'18 in the Passetto site; so, data about the panels collected in this month in Marina Dorica were not considered for this analysis.

All the statistical tests were conducted using PAST3.16 (Hammer *et al.*, 2001).

## **5.3 Results**

Boring species recorded into the experimental limestone panels placed in “Passetto” site, belonged to the species-complex *Polydora ciliata* (Annelida: Spionidae) (Walker, 2011) and also to the species *Rocellaria dubia* (Pennant, 1777) (Mollusca: Bivalvia). Panels collected in Marina Dorica were excavated by *P. ciliata* only. Evident signs of bioerosion made by *P. ciliata* have been present since the first sampling in January 2018, after six months of submersion, in both the sites. The boring bivalve *R. dubia* appeared only in the Passetto station in March 2018, after nine months. The density of the excavating worms in Marina Dorica during the different months (Figure 3a) is statistically different (one-way ANOVA,  $p < 0.05$ ) and ranges from  $0.09 \pm 0.02$  holes/cm<sup>2</sup> in January 2018 to  $0.32 \pm 0.15$  holes/cm<sup>2</sup> in July 2018; in particular, *post-hoc* pairwise comparison, based on Tukey's test, suggests that these values, recorded in these two months, significantly differentiate the assemblages. In Passetto, the density ranges from  $0.009 \pm 0.001$  holes/cm<sup>2</sup>, in March 2018, to  $0.04 \pm 0.01$  holes/cm<sup>2</sup>, in May 2018 (Figure 3a); one-way ANOVA does not show statistically differences comparing the months ( $p > 0.05$ ).



**Figure 3.** a) Density of the excavating worms, during the studied period; b) Sizes of the diameter of the openings in the studied period.

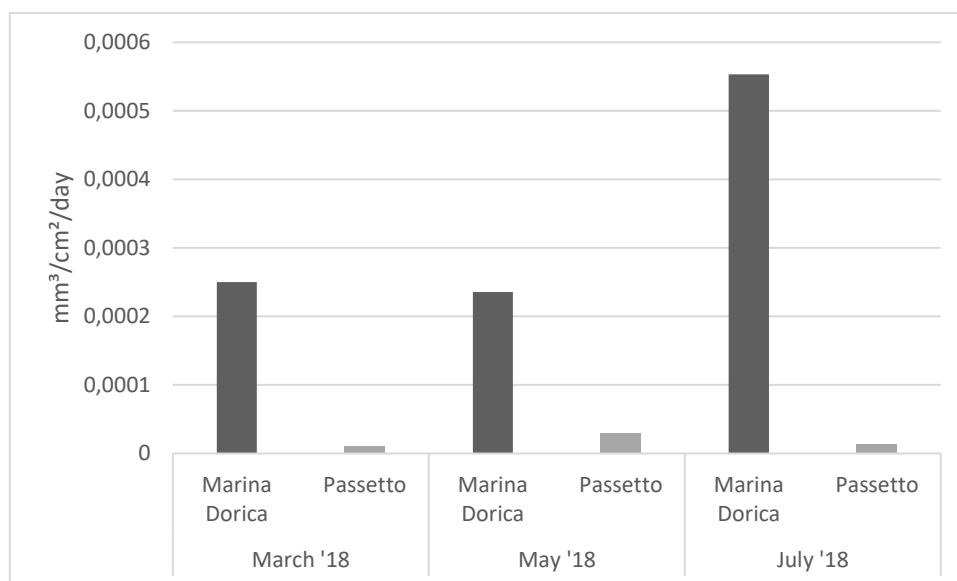
Two-way ANOVA was run only considering the months March'18, May'18 and July'18 (due to the failed sampling of the Passetto panels in January'18). Results revealed that the density of the polychaetes (holes/cm<sup>2</sup>) varied significantly between the sites but not among the months (Table 1); the interaction isn't statistically significant.

**Table 1.** Results of the two-way ANOVA comparing the different sites and the months of sampling.

Two-way ANOVA						
	Sum of sqrs	df	Mean square	F	p (same)	
sites:	0.164296	1	0.164296	32.38	0.000101	
months:	0.03355	2	0.016775	3.306	0.07183	
Interaction:	0.038359	2	0.019179	3.78	0.05332	
Within:	0.060887	12	0.005074			

Comparison of the size of diameters (mm) of the single openings is showed in Figure 3b. The largest values of these were recorded in July'18 for both the study stations, 0.80±0.28 mm in Marina Dorica and 0.81±0.28 in Passetto. The diameter of the openings does not change significantly during the months as also the measure of heights of the resin casts, as shown by the one-way ANOVA tests ( $p > 0.05$ ).

The rate of the bioerosion varied in Marina Dorica between 0.00024 mm<sup>3</sup>/cm<sup>2</sup>/day (March'18) and 0.00055 mm<sup>3</sup>/cm<sup>2</sup>/day (July '18), while in Passetto between 1.09829x10<sup>-5</sup> mm<sup>3</sup>/cm<sup>2</sup>/day (March'18) and 2.94345x10<sup>-5</sup> mm<sup>3</sup>/cm<sup>2</sup>/day (May'18) (Figure 4).

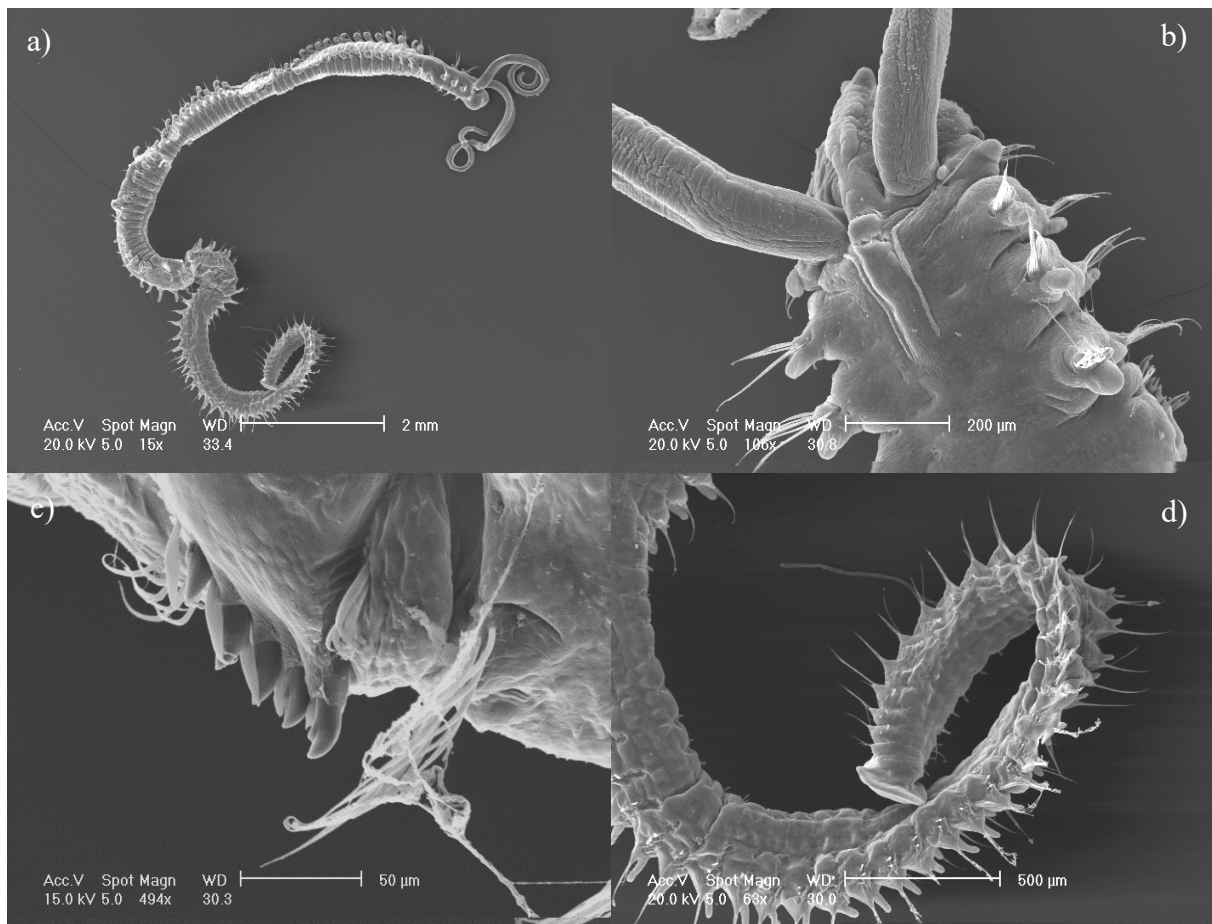


**Figure 4.** Values of the volume eroded (mm<sup>3</sup>) per day, per cm<sup>2</sup> of substrate.

In Marina Dorica, considering the highest value of erosion recorded in July'18 (0.00055 mm<sup>3</sup>/cm<sup>2</sup>/day), a total rate of erosion in one year was estimated in 0.202 mm<sup>3</sup>/cm<sup>2</sup>/year. While in Passetto, considering the highest value of erosion recorded in May '18 (2.94345x10<sup>-5</sup> mm<sup>3</sup>/cm<sup>2</sup>/day) the rate of erosion in one year was estimated in 0.0051 mm<sup>3</sup>/cm<sup>2</sup>/year.

Assuming that the specific gravity of the Lecce rock is 2577 Kg/m<sup>3</sup>, the weight of the substrate excavated by polychaetes per cm<sup>2</sup> of panel surface per year results 0.0005 g in Marina Dorica while in Passetto results 1.3201x10<sup>-5</sup> g. If we consider the specific gravity of the typical rock present along Ancona Promontory, (2.5 g/cm<sup>3</sup>), the value of the eroded volume is 0.005 g/cm<sup>2</sup>/year in Marina Dorica and 0.0001 g/cm<sup>2</sup>/year in Passetto.

The main borer, *Polydora ciliata* (Johnston, 1838), is illustrated in Figure 5. This species is characterized by the first chaetiger without notochaetae, bilobate prostomium, nuchal organ extending to the 3<sup>rd</sup> chaetiger in relation to the caruncle, modify chaetae of the 5<sup>th</sup> segment with a curved accessory tooth; only capillary chaetae are present in the posterior part of the body that finishes with an anal sucker.



**Figure 5.** a) Entire specimen of *P. ciliata*; b) Detail of the head, the caruncle and the nuchal organ; c) Detail of the modified chaetae on the 5<sup>th</sup> chaetiger; d) Detail of the posterior body and the anal shield.



## 5.4 Discussion

The aim of this study was to investigate which organisms were involved in bioerosion processes along the Ancona coast and to estimate the bioerosion rates of these. The North-Italian Adriatic coast is characterized almost entirely by sandy beaches and the Conero Promontory, where Ancona is located is, on the contrary, an important rocky site where benthic organisms, both epilithic and endolithic, as sponges and bivalves, colonize the hard substrata typical of this area (Calcinai *et al.*, 2009). Up today, only the erosion by the sponge family Clionaidae was studied in this peculiar ecosystem by Calcinai *et al.* (2011), and no research has been conducted on the erosion rate of boring polychaetes in the Mediterranean Sea.

For some borers, as the bivalve *Rocellaria dubia* (Pennant, 1777), and sponges, it is possible to estimate the erosion with non-destructive methods that permit to calculate the volume of the internal cavities by mean of the external openings (Schiaparelli *et al.*, 2005; Calcinai *et al.*, 2011). Unfortunately, for polydorids only destructive methods such as cracking the substrates can be used (Simon, 2011; Simon & Sato-Okishi, 2015; Radashevsky & Migotto, 2017; Radashevsky *et al.*, 2017). Here, for the first time, the volume eroded by polychaetes was directly calculated using the casts of the excavations. Considering that, as demonstrated by the statistical results, both diameters and heights of the excavations do not vary with the time of exposure (at least after 6 months), we suggest that it is possible to estimate the volume eroded by *Polydora ciliata* directly measuring the diameter of the openings, considering the erosion cavity as a double cylinder. The erosion pattern in this worm seems different in respect of other taxa as the bivalves *R. dubia* (Schiaparelli *et al.*, 2005), date mussels, or piddocks, where the erosion cavities and the superficial openings enlarge following the molluscs' growth.

This study shows that the first boring organism to appear, after six months of experiment, is the polydorid *Polydora ciliata* and after ten months of study, exclusively in Passetto, the bivalve *Rocellaria dubia* (Pennant, 1777) (Figure 6d). No evidence was recorded for boring sponges or echinoderms, that are widely considered as the principal taxa involved in boring processes both in the tropical and Mediterranean seas (Schönberg *et al.*, 2014). These organisms are known to appear late in the boring community, after worms that, in the coral reefs, are known to be pioneers (Hutchings *et al.*, 2002, 2008, 2013).

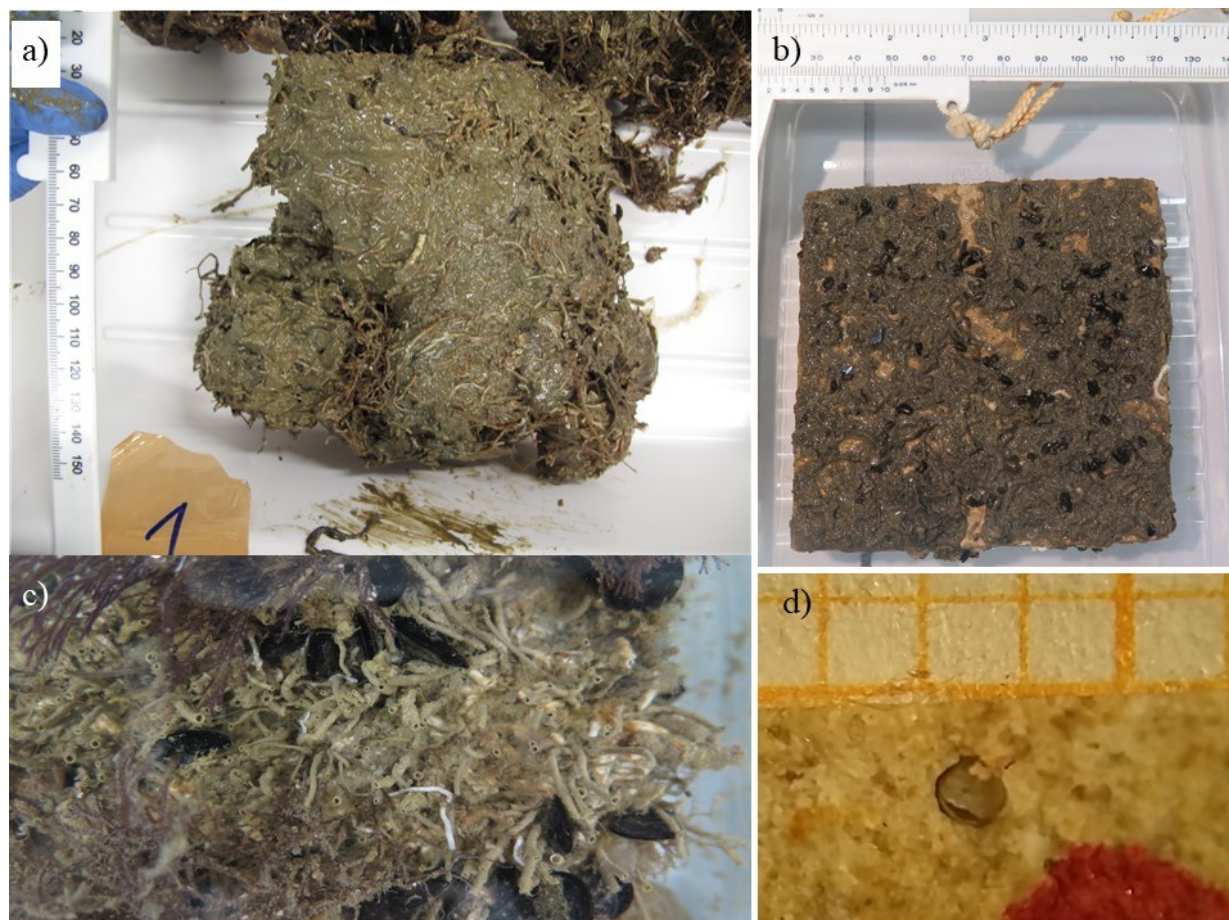
In this study, panels were sunk only for one year but, this experiment, although in the short term, highlights that also in the Mediterranean Sea, the first succession phase in the boring community is equivalent to that of the tropical areas (Hutchings *et al.*, 1992; Tribollet *et al.*, 2002).

Density of the species *P. ciliata* recorded in this study significantly changes among the sites, as shown by the two-way ANOVA, with the highest values recorded in Marina Dorica ( $0.32 \pm 0.15$

holes/cm<sup>2</sup> vs 0.04±0.01 holes/cm<sup>2</sup> in Passetto), and it varies with the time of exposure, as reported in other studies conducted in the tropical areas (Chazotte *et al.*, 1995; Hutching *et al.*, 2002; Hutchings *et al.*, 2013). Also, the volume eroded in July '18 was higher in Marina Dorica and, in particular, different in respect of that of January 2018, as showed by the one-way ANOVA test. This result is probably due to a new settlement of the species *Polydora ciliata* that, as reported by Blake (1969), is characterized by one or two reproductive events in a single season, usually in late spring. In fact, during the observation *in vivo*, done in May 2018, several breeding specimens were recorded. Anyway, the density of boring polychaetes, and the rate of bioerosion recorded by us are extremely lower in respect of the tropical areas. Hutching *et al.* (2002) analysed the distribution and the abundance of some coral-boring polydorids in several sites of the French Polynesia; they found that the density varied for example from 0.5±0.8 ind/100 cm<sup>-3</sup>, and 36.3±33.7 ind/100 cm<sup>-3</sup>; different values of density, but always high, were reported by Pari *et al.* (2002) (222.32 ind/100 cm<sup>-3</sup>) and more recently by Hutchings *et al.* (2013) for *Polydora* spp. (303.0±72.75 ind/100 cm<sup>-3</sup>). Tribollet *et al.* (2002) calculated for bivalves, sipunculans and polychaetes (designed as macroborers) rates of macrobioerosion between 0.01±0.01 and 0.09±0.15 Kg m<sup>-2</sup>year<sup>-1</sup> depending of the site; Pari *et al.*, (2002) reported 1.04±0.41 Kg m<sup>-2</sup>year<sup>-1</sup>, while Osorno *et al.* (2005) reported that polychaetes and sipunculans across the Great Barrier Reef eroded up to 0.356±0.243 kg/m<sup>2</sup>. Chazotte *et al.* (1995) reported an erosion rate of 2.15x10<sup>-3</sup> Kg m<sup>-2</sup>year<sup>-1</sup> for polychaetes in the French Polynesia. However, a comparison of our results with those reported in literature is difficult, because often the erosion rates are calculated without discriminating among the taxa, grouping polychaetes and sipunculans as “worms” and these and bivalves, generically, as macroborers; moreover, the differences about the density, and also the erosion rates may be due to the diverse kind of eroded substrates used in the experiments; in fact, it has been demonstrated that the bioerosion rate is higher in porous substrates (corals) in respect of denser material (Calcinai *et al.*, 2007). Besides, the bioerosion rates in the tropical exceeds the bioerosion in temperate and cold areas (Wisshak, 2006).

While in Marina Dorica, panels were covered by organisms typical of the fouling (Figure 6a, c), as ascidian, and soft tubes, made for example by amphipods, in Passetto, panels were covered by a thick and strong layer built by the polychaetes *Sabellaria spinulosa* (Leuckart, 1849) that paved the available surface (Figure 6b). This species is known as one of the most important ecosystem-engineer able to modify the biotic and physical dynamics (Gravina *et al.*, 2018). Probably, the settlement of the larvae of boring organisms, as *P. ciliata*, in Passetto was negatively influenced by these biocostructions, while the high concentration of mud, soft organisms and soft biogenic

structures on the panels positioned in the Marina Dorica does not hamper the establishing of the polydorids.



**Figure 6.** a) Panel collected in Marina Dorica in July 2018; b) Panel collected in Passetto in July 2018; c) Example of the long tubes of the *P. ciliata*; d) Example of juvenile of the bivalve *R. dubia*.

Differences about the boring community and the erosion rate in the two sites can be also explained considering that in habitats subjected to anthropogenic impacts, the decrease of the water quality, due to the increment of the suspended sediment and the consequent abundant food supply for suspension feeders, are factors that positively influence the composition of the initial macroborers communities and the rate of bioerosion (Sammarco & Risk, 1990, Hutchings *et al.*, 2002; Tribollet *et al.*, 2002; Le Grand & Fabricius, 2011, Schönberg *et al.*, 2014). However, we do not exclude that also the different placement of the panels in the two sites (due to the different environmental characteristic and logistics impediment) can have influenced the results.

If we consider the specific gravity of the rock commonly present along Ancona Promontory area, to calculate the weight of the eroded material, we get a higher value of erosion; this value is only indicative of the role of the boring polychaete *Polydora ciliata* in the area, as the data were obtained considering a different substratum, that can influence the erosion activity. Nevertheless, we suggest

that these data can be a useful baseline to monitor and extend researches about erosion in the Mediterranean Sea.

Finally, we report that, the first phase of bioerosion along the Ancona Promontory, are due to two principal taxa, polychaetes and bivalves. In particular, the presence of the species *P. ciliata*, its densities and its erosion rate changed in relation to the site and the time of exposition of the panels, suggesting that many factors, both abiotic and biotic, influence the bioerosion of the rocks in the North-Central Adriatic Sea.

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## CONCLUSION

Studies about the macrofaunal communities, their biodiversity and their annual trends were usually conducted by the sampling of unconsolidated sediments, as sand or mud, or, as the case of coralligenous, with the asportation of blocks or rocks. While macrofauna, and the polychaetes, living in association with other organisms were frequently analysed for the description of new species or for new symbioses. Instead, studies that evaluate the annual variation of the biodiversity and the abundances of already known associations are poorly investigated, as also the bioerosion processes in the Mediterranean Sea.

The results of this thesis highlight that:

*i) Polychaetes and Demosponges*

Different species of sponges, also belonging to the same genus, can host several kinds of macrofaunal assemblages. The research about *Sarcotragus* spp. highlights new taxa living associated with these sponges. Once again, sponges can be considered as “habitat-forming species” so with functional role as ecosystem engineers capable to provide habitat for many species.

*ii) Polychaetes and Octocorals*

These findings represent a new record for the North-Eastern Mediterranean Sea because this symbiosis has been reported for the Western Mediterranean basin. The analysis of the Ligurian specimens gave new information concerning the seasonal abundance (ind/cm), the frequency (% of findings) and the reproduction of this species. If this species feeds on the tissue of its host and/or utilizes its pigments for camouflage, as hypothesised in literature, remains an open question.

New information about the composition of the secondary metabolites in the gorgonian *P. clavata* are here reported, with the new finding of the Isoactractylon compounds, up today escaped all previous chemical investigations about this octocoral.

*iii) Boring polychaetes*

Bioerosion processes were common also along the Ancona Promontory. The spionid *Polydora ciliata* is the species mainly involved in the losses of rocky substrates. The habitat inside the touristic marina probably positively influences the settlement and the erosion of boring polychaetes, while sites more exposed to the current, as the case of Passetto, probably require longer time for the establishment of mature macrofaunal boring communities, that are composed by polychaetes (*P. ciliata*) and bivalves (*Rocellaria dubia*).

Future works could include: i) the investigation of the macrofaunal assemblages inhabiting other species of sponges, coming from other areas of the Mediterranean Sea; ii) genetic analysis of the species *H. chamaeleon* and other investigations about the molecular compounds that characterize the Octocorals in Mediterranean Sea in order to explore also their ecological functions; iii) implement the study of the bioerosion.

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