UNIVERSITÀ POLITECNICA DELLE MARCHE

Department of Life and Environmental Sciences
PhD course in Life and Environmental Sciences
Curriculum in Marine Biology and Ecology

Diversity of microbiomes associated with benthic invertebrates inhabiting Antarctic ecosystems

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XXXII cycle

AA 2018-2019

"Life did not take over the globe by combat, but by networking"

Lynn Margulis

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1. INTRODUCTION

1.1 The microbiome world: from human to marine organisms

Growing studies are highlighting that life could not persist without the profound impact of beneficial host-microbe interactions that underpin virtually every aspect of plant and animal biology, including human biology (McFall-Ngai et al. 2013; Petersen and Osvatic, 2018). The field of microbiome research, which aims to understanding how microbes drive health, development, functions and evolution of their hosts, has greatly expanded in the last years (Fig. 1).

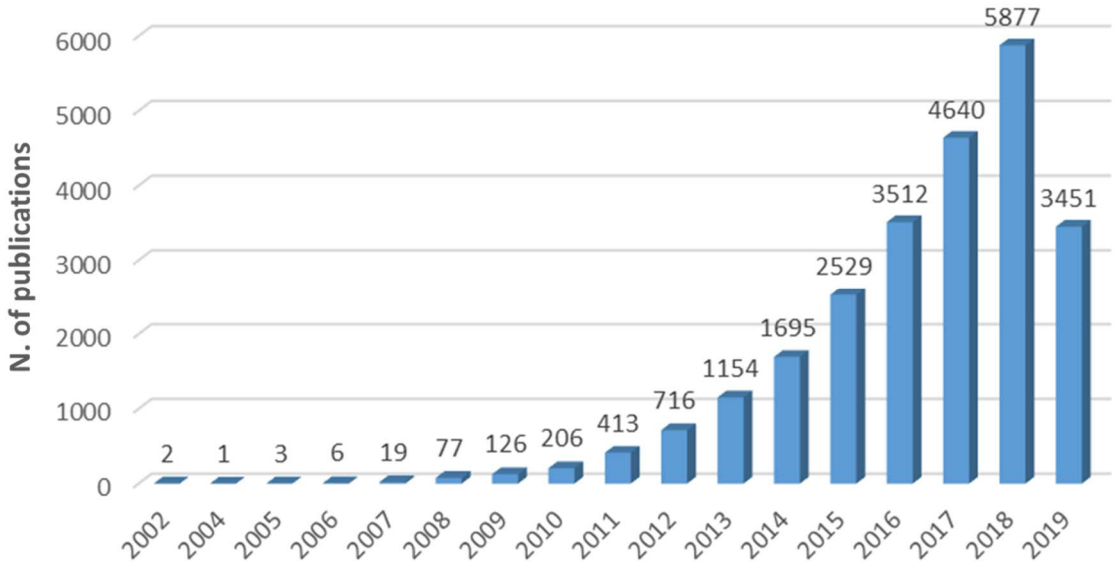

Figure 1: Graphical representation showing the exponential numbers of publications about "microbiome" from 2002 to date.

This is clearly also an exciting time for symbiosis research, as beneficial microbes have never had a more prominent position in biology, medicine and public interest. In fact, exactly in this time (2007) the American National Institutes of Health had funded the Human Microbiome Project with the mission of generating resources that would enable the comprehensive characterization of the human microbiome and analysis of its role in human health and disease (Turnbaugh et al. 2007; Human Microbiome Project consortium 2012a; 2012b; Lloyd-Price et al. 2016). From birth, a stable symbiotic relationship exists between human cells and microbiome, a huge array of commensal and symbiotic microorganisms

whose role in our life is indispensable and beneficial (Lederberg and McCray, 2001; Rojo et al. 2017). Due to its vast metabolic capacity, the microbiome has been also considered as an 'organ' of our body, able to evolve over time and adapts during the entire life of the host (Moya and Ferrer 2016; Yatsunenko et al. 2012; Ding and Schloss 2014; Goodrich et al.2016; Rojo et al. 2017). Recent studies in humans and laboratory animals have shown more extraordinary and deeper connections between these microscopic organisms and our body: some experiments on the microbiome through manipulation of diet, infection and exercise, suggested direct effects on cognition, including learning and memory (Davidson et al. 2018, Ley 2010; Cho and Blaser, 2012); some others, revealed a strong connection between microbiome and some diseases, demonstrating a role in preventing the growth of pathogens, contrasting the development of cancer and metabolizing toxins (Schwabe et al. 2013; Golombos et al. 2018; Zitvogel et al. 2017; Rojo et al. 2016). It has been seen that its composition is continuously exposed to factors that influence it dynamically: genetic background, age, stress, interaction with the environment (Bashan et al. 2016; Noecker et al. 2017; Tiihonen et al. 2008; Biagi et al. 2010; Walker et al. 2011; Wu et al. 2012; Konturek et al. 2011). Studies on human microbiome have revealed that individuals can vary remarkably in the microbes that occupy different habitats of the body (oral cavity, gut, skin, etc.) with strong niche specialization both within and among individuals (Chiarello et al. 2015; Gao et al. 2018; Paga´n-Jime´nez et al. 2019; Olsen et al.2019). Much of this diversity remains unexplained, although diet, environment, host genetics and early microbial exposure have all been implicated (Human Microbiome Project Consortium, 2012a). The exploding of research on human microbiome and the growing evidences of the key role of these microorganisms in the human life have opened several questions on how these associations influence the life of all the other organisms.

Marine organisms share the sea with a vast diversity of microorganisms, including protists, bacteria, archaea, fungi, and viruses which comprise millions of cells in each milliliter of the 1.3 billion km^3 of water comprising the oceans (Eakins and Sharman, 2010). The roles of these microorganisms in oxygen production, nutrient cycling, and organic matter degradation provide critical functions to the oceans and Earth (Arrigo, 2005; Falkowski et al., 2008). Microbes that take part of the animal's microbiome are the collection of those reside on or within the animal. Some of the microorganisms comprising the microbiomes of marine animals are thought to originate from this surrounding supply of seawater-associated cells, while other cells appear to have strict inheritance patterns, passed on through generations from the host (e.g., Nussbaumer et al., 2006, Sharp et al., 2007; Apprill, 2017).

The vast diversity of associated microorganisms are considered an essential part of host phenotype influencing their nutrient supplementation, methabolism, fitness, adaptation and ecological traits (Blaxter 1962; Douglas 1998; Brune and Ohkuma 2010; Aronson et al. 2017; McFall-Ngai, 2002; O'Hara and Shanahan, 2006; McFall-Ngai, 2007; Fraune and Bosch, 2010; Gilbert et al., 2012; Bordenstein and Theis, 2015; Deines et al., 2017). These host-associated microbial communities are generally very diverse and the processes that govern their composition are numerous and not well-understood yet (Adair et al. 2017).

Several studies carried out on corals, sea stars and sponges have highlighted a lower diversity in microbiomes than free living bacteria in the surrounding environment, but a higher contribution of each bacterial group to the whole community (Pantos et al. 2015; Jackson et al. 2018; Dunphy et al. 2019). In a recent investigation carried out on microbiomes associated with different species of deep-sea corals, diversified assemblages of bacteria were found in each species, suggested to represent the bacterial groups responsible for the different adaptative strategy present in the species (Rothig et al. 2017). Similar results were observed also in sponges, where, despite a high intraspecific variability, a different way of bacteria acquisition from surrounding sea water and specific bacterial groups stable over time were found among individuals of the same species (Turon et al. 2018).

A key role of host-specificity was seen also in another investigation on corals of Pollock et alii (2018), where they have found evidence of coral-microbe phylosymbiosis, in which coral microbiome composition and richness were closely related to coral phylogenetic history (Pollock et al. 2018).

Despite the strong effect of host species identity, significant variation in microbiome composition could be associated with environmental factors, such as geographic location, presence of contaminats or different avaibility of nutrients (Pantos, et al. 2015; van de Water et al. 2018; Griffith et al. 2019). A study carried out on ecto- and endobiotic associations of copepods showed a strong influence of the environmental conditions, especially the availability and type of trophic resources, in selecting different bacterial taxa of their microbiomes (Tang, 2005). Evidences of a key role of external environmental conditions in shaping taxonomic composition of microbiomes was seen also in a study carried out on the scleractinian coral *Seriatopora hystrix*, where the diversity of bacterial assemblages varied among different geographic locations but not among the different genotypes of corals (Pantos et al. 2015). Moreover, presence of different contaminants in the environment could

have a role in selecting specific metal-resistant bacteria in microbiomes, giving to their host the ability to survive, as it was seen in marine polychaetes (Neave et al. 2012).

Movement of microbes, both among host-associated communities and between host-associated and free-living communities, is likely to be key to maintaining microbiome diversity and reducing variation in microbiome composition among individuals (e.g. Moeller et al. 2016). Sometimes, a clear driver that shape the microbiome is really difficult to find: the nematode microbiome profiles demonstrated no correlation with the feeding morphology, phylogeny or morphological identity of the hosts, neither with the different ocean regions or marine habitat types considered (Schuelke et al. 2018).

In addition to all these factors, host-associated microbes simultaneously compete and cooperate with one another (De Boer et al., 2007). In fact, microbiomes can harbour active bacterial predators, which may protect the host by consuming potential pathogens or alter the microbiome structure and directly the host functionality, feeding other bacteria (Welsh et al. 2016).

Moreover, the origin of microbiome is an important factor that determines its diversity of microbiomes. Horizontally transmitted bacteria are acquired from the environment, or from other organisms anew by each host generation and evidences of this way of transmission were found in numerous organisms (copepods: Moisander et al. 2015; polychaetes: Nussbaumer, et al. 2006; Aida et al. 2008; Vijayan et al. 2019). Nevertheless, bacterial communities associated with the life stages of some marine invertebrates reveal that microbiomes may shift over time, but specific bacterial taxa, that represent the key of resilience and adaptation of hosts, remain stable across life stages (sponges: Fieth et al. 2016; corals: Lema et al. 2014; anemones: Mortzfeld et al. 2016). These are all examples of vertically transmitted microbiomes, often transferred through the female germ line and can create species-specific assemblages, completely different from free-living bacteria (Sharp et al. 2007; Bright et al. 2010). However, numerous evidences in which transmission can also be mixed, involving both vertical and horizontal transfers from the environment and intraspecific or interspecific host switching, exist (Bright et al. 2010).

Furthermore, the marine environment is changing at unprecedented rates due to global warming and the anthropogenic impact (Doney et al. 2012). This could alter the assemblage composition or abundance of marine microbes in the environment and lead to significant effects on host fitness and survival, changing the microbiomes associated with them. Despite studies on the microbiomes associated with marine organisms are growing, several questions

on the nature of these associations and the role that they have in the host's life and in the entire ecosystem remain still open.

1.2 Microbiomes associated with benthic Antarctic invertebrates

1.2.1 The Antarctic ecosystem

About 85% of the biosphere is permanently exposed to temperatures below 5°C throughout the year (Margesin et al. 2007). Although cold areas were previously considered to be uniform environments, recent investigations have highlighted that they include a variety of geological variations (e.g., different sediment textures, a mixture of ice and snow with different degrees of salinity, nutrients, and thermal values; Tytgat et al. 2016). Among all the cold habitats polar regions (Arctic and Antarctica) represent 14% of the total biosphere. In particular, the Southern Ocean region represents the 5.4% of the world's oceans and it is the major driver of global ocean circulation, playing a key role in interacting with the deep-water circulation in the Pacific, Atlantic and Indian oceans (Griffith, 2010).

Sea surface temperatures in the Southern Ocean range annually from -1.86° C to +0.3° C while benthic temperatures are typically very cold (< 1°C) and characterized by marked spatial and latitudinal variations (Dinniman et al. 2004; Griffith 2010; Lo Giudice, 2018). Desiccation, osmotic stress, ice-covering, changes in salinity and in nutrient availability, extreme seasonality in light conditions are among the main environmental stresses characterizing this environment (Pearce et al. 2012). All these factors, adding the presence of Antarctic Circumpolar Current and the Polar Front, have acted shaping the evolution and the distribution of marine invertebrates in Antarctic ecosystem (Thatje et al. 2005; Crame, 2013; Chown, 2015).

Our knowledge of the biodiversity of the Southern Ocean is largely determined by the relative inaccessibility of the region. Benthic sampling is largely restricted to the shelf and in the areas where the scientific bases were located (Griffith 2010). Despite this, ocean expeditions routinely bring up samples in which the majority of species are new to science (e. g. Brandt et al. 2007; Schiapparelli et al. 2013; Chown 2015). In fact, more than 8,000 marine species have been discovered here, but the number could be higher than we think (De Broyer and Dany, 2011). The 50-97% of the discovered species are endemic with different rates among classes (Bryozoa: Cyclostoma 47%, Cheilostoma 56%; Mollusca: Cephalopoda 54%, Bivalvia 43%, Gastropoda 74%, Pycnogona 55%, Ascidiacea 44%; Griffiths, 2009; Chown et al. 2015). These numbers are growing, thanks to modern molecular techniques

that have identified cryptic species and species complexes in almost every Antarctic group that has been studied, from polychaetes to bivalves, isopods and pycnogonids. Investigation of the high marine endemicity has revealed how a complex set of earth system processes has interacted to shape the evolution of the southern biota (Crame, 2013). Globally, the drivers of diversity have become increasingly well characterized, although controversy about their relative significance continues (Chown, 2015). Both benthic and pelagic communities tend to show a high degree of patchiness in both diversity and abundance. In particular, the benthos is the richest element of the food web in terms of numbers of species, but their roles and interactions are poorly known (Griffith, 2010). Benthic patterns, especially when analysed over a broad range of spatial scales, are determined by the combined effects of multiple physical, chemical and biological drivers. Conversely, at local scale, the spatial patterns of benthic communities have been likely shaped by non-measured environmental variables, as well as by intra-specific interactions, like migration and dispersal processes of different life stages, and by ecological interactions (Cornell and Harrison 2013; Schiapparelli 2014; Gutt et al. 2019).

The recurrent findings of new species and the hardly predictable patterns of biodiversity allow us to consider the Antarctic ecosystem as a hotspot of biodiversity (Chown, 2015). For that reason, the need for developing novel conservation strategies that explicitly consider multiscale variability and patchiness are strongly required (Gutt et al. 2019; Neal et al. 2018; Kennicutt 2014).

1.2.2 Host-microbiome associations

The established symbioses with numerous bacterial communities, allowing organisms to adapt in a broad range of habitat: from shallow and tropical to extreme environments (Goffredi 2010; Petersen et al. 2016). Even single bacterial species can display extensive phenotypic variability/heterogeneity that can enhance resilience to environmental changes and thus facilitate adaptation of the host (Raj and van Oudenaarden, 2008; Justice et al., 2008). Antarctica, as an extreme and isolated environment, offers a unique opportunity to study the peculiar interactions that are established between a benthic host and its symbionts and to evaluate the role of these symbionts in the development of adaptation strategies of the hosts (Lo Giudice et al. 2019). Bacteria-invertebrate associations in Antarctica have been rarely investigated and our current knowledge remains quite scarce and fragmentary (Lo Giudice and Rizzo, 2018). In fact, available investigations are limited to few organisms, as sponges (Webster et al. 2004; Rodriguéz-Marconi et al. 2015; Papaleo et al. 2012; Mangano

et al. 2009; Mangano et al. 2014; Mangano et al. 2018; Xin et al. 2011), the soft coral *Alcyonium antarcticum* (Webster et al. 2007), the sea-urchin *Sterechinus neumayeri* (González-Aravena,et al. 2016) and the oligochaete *Grania sp.* (Herrera et al. 2017). Despite this, findings of a key role of these associations in several aspects of the life of Antarctic organisms are already evident.

In 2004, Webster et al. in a pioneer study, explored the microbial communities of five species of Antarctic sponges, indicating that the bacterial communities were sponge-species related regardless of the sampling sites, with representatives of Gamma (e.g. genera *Vibrio* and *Alteromonas*) and Alpha Proteobacteria (mainly *Roseobacter spp*) and Bacteroidetes (mainly *Polaribacter spp.).* A recent investigation of Rodrigez-Marconi et alii (2015) corroborated these results, observing a host specificity in microbiomes composition. Moreover, they observed that sponges shared with the surrounding seawater only few bacterial phylotypes, highlighting the important role of sponges as a bacterial diversity reservoir. The presence of a specific core–microbiome was also identified in the soft coral *Alcyonium antarcticumis,* showing spatially stable bacterial groups across different sites characterized by an environmental impact gradient (Webster et al. 2007).

An important functional role was reported in the microbiomes associated with *S. neumayeri* from Maxwell Bay (King George Island, South Shetland Islands, González-Aravena et al. 2016). Isolates, predominantly affiliated to Gammaproteobacteria (with the genera *Pseudoalteromonas*, *Psychrobacter*, *Shewanella* and *Pseudomonas*), Flavobacteria and Actinobacteria, mainly resulted heavy metal- and antibiotic resistant. These results are in accordance with the only previous report existing on zinc tolerance in *S. neumayeri* (De Moreno et al. 1997), which showed high concentrations of zinc in the tissues. In the case of mercury and cadmium, bacteria have probably developed resistance through constant exposure to toxic compounds in the environment or accumulation in the host tissues (Truzzi et al. 2008, Mangano et al. 2014). A recent investigation carried out on the oligochaete *Grania* sp. corroborated this functional role of microbiomes, demonstrating the implication of bacteria living in the host's gut in producing extracellular proteases, esterases, amylases, cellulases and agarases, able to metabolize nutrients and favor the digestive capabilities of the host (Herrera et al. 2017). A potential contribution of host associated bacteria in marine biogeochemical cycles were found in several investigations, as the case of ammonia-oxidizers Nitrosomonadales and nitrifiers Methylophilales in Antarctic sponges, suggesting significant role of that microbiomes in nitrogen conversion (Rodriguéz-Marconi et al. 2015).

Furthermore, to contrast the colonization of their surfaces by unwanted microorganisms and little invertebrates, benthic organisms can adopt chemically mediated defensive strategies (McClintock et al. 2010; Peters et al. 2010). Interestingly, some recent reports suspect that a number of metabolites obtained from host invertebrates may be produced by their microbial symbionts (Thomas et al. 2010; Fuerst et al. 2014; Blockley et al.2017; Lidong et al. 2016). In fact, investigating the associations between bacteria and some Antarctic sponges, as *Isodictya setifera L. nobilis, A. joubini* and *H. verrucosa,* from the Ross Island and Terra Nova Bay, were screened several forms of antibiotic activity against opportunistic pathogens (Papaleo et al. 2012; Blockley et al.2017).

Moreover, some bacteria (the so called "cold-adapted bacteria") are able to produce Extracellular Polymeric Substances (EPS), biochemical compounds that represent a survival strategy to thrive with low temperatures, avoiding cell damage in environments such as the cold Polar regions (Lo Giudice and Rizzo, 2018). Recently, evidence of EPS-producing bacteria was found in three Antarctic sponges microbiomes from Terra Nova Bay, *Haliclonissa verrucosa, Hemigellius pilosus* and *Tedania charcoti* (Caruso et al. 2018).

Given the high number of marine invertebrates present in the Antarctica and the even greater number of associated microbes on and within each host, are without doubt necessary further investigations to better comprehend the biodiversity and nature of the intricated links between microbiomes and their hosts and the potentiality of these associations in the adaptation to this extreme environment.

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2. OBJECTIVES

The main objectives of this PhD project are:

1. Investigating the diversity of microbial assemblages associated with different marine benthic taxa inhabiting the Antarctic ecosystem to expand our knowledge on marine biodiversity.

2. Evaluating the diversity and putative functions of microbiomes associated with different body parts of a host (e.g., oral cavity, parapods, teguments, gut) inhabiting the Antarctic ecosystems.

3. Assessing environmental drivers (e.g., depth, trophic conditions, geographical locations) that may influence and select the taxonomic composition of microbiomes of marine benthic taxa.

4. Exploring the potential sources of microbes associated with benthic invertebrates by comparing diversity of the microbiomes and microbial communities inhabiting surrounding sediments.

3. UNRAVELLING ANTARCTIC POLYCHAETES DIVERSITY USING MORPHOLOGY AND MOLECULAR TOOLS

3.1 Introduction

High levels of diversity and endemisms are hosted in the cold and harsh environment of the Southern Ocean. To date, more than 8,000 marine species have been discovered here and 50-97% of them, including various groups such as sponges, tube worms, amphipods, mollusks, sea spiders, are endemic (De Broyer et al. 2011; 2014; Brandt et al., 2012; Kaiser et al., 2013; Chown et al. 2015). Different environmental factors, such as temperature, salinity, light, sea floor morphology, trophic conditions, idrodinamims, has acted as drivers in favoring marine organisms isolation and shaping much of the recent faunal distribution of Antarctic marine invertebrates (Thatje et al. 2005). In addition, the variation in size and extent of the continental ice-sheet during the glacial cycles has influenced the benthic community to take refuge on the shelf, and/or forcing a shift in their distribution along the continental shelf and slope (Clarke 2008). Ice could also play an important effect as a source of physical disturbance in Antarctic benthic marine ecosystems, removing dominant competitors, increasing habitat heterogeneity and sustaining a diverse group of scavengers (Arntz et al. 1994; Gutt and Piepenburg 2003). The combination of all these factors could have led to a genetic drift, resulting in genetically distinct populations or sister species: they accumulated differences that lead to reproductive isolation and possibly generating cryptic species (Chehida et al. 2019).

Given the high numbers of cryptic species complexes discovered in the Antarctic ecosystems, it has become clear that molecular taxonomy, in combination with traditional taxonomic methods, offers the best chance to better understand the evolutionary history of biological communities and, most of all, to avoid the risk of underestimating environmental richness and biodiversity (Grant et al. 2011; Brasier et al. 2016; Chehida et al. 2019). Proper identification of cryptic species may also be crucial for the detection of both invasive species and endemic ones, and thus have fundamental implications for conservation and management (Bickford et al. 2006). Surveys on cryptic speciation in Antarctica have been detected in several taxa (e.g., *gastropods*: Wilson et al. 2009, *pycnogonid*s: Krabbe et al.

2010; Collins et al. 2018; *isopods*: Havermans et al. 2010; *ostracods*: Brandão et al. 2010; *asteroids*: Janosik and Halanych 2010; Peck et al. 2018; *crinoids*: Wilson et al. 2007; Chehida et al. 2019; *ophiuroid*s: Hunter and Halanych 2008; Jossart et al. 2019; *nemerteans*: Mahon et al. 2010; *amphipods*: Hupalo et al. 2019). Despite several studies on cryptic species of polychaetes have been carried out around the world, there are still few surveys concerning the Antarctic ecosystems (Schüller 2011; Nygren 2014; Brasier et al. 2016).

Polychaetes are one of the dominant taxa in Antarctic benthic marine communities, where they represent more than 70% of the macrofauna (Gambi et al. 1997; Glover et al. 2008; Brasier et al. 2017). As discussed in a recent review by Nygren et alii, there is evidence to suggest that cryptic species are common among all polychaete families, making up a significant portion of their biodiversity (Nygren et al. 2014). In fact, their taxonomy is characterized by a high number of apparently cosmopolitan species, although investigations at the molecular level have revealed that many of these species are complexes of morphologically identical or almost identical cryptic species (Bleidorn et al. 2006; Barroso et al. 2010; Tomioka et al. 2016; Kongsrud et al. 2017, Schiapparelli et al. 2016; Blake et al. 2017; 2018).

One of the largest genetic investigation into the prevalence of cryptic polychaete species within the deep Antarctic benthos have uncovered cryptic diversity in 50% of the 15 morphospecies targeted, through the comparison of mitochondrial DNA sequences, as well as 10 previously overlooked morphospecies, increasing the total species richness in the sample by 233%. Moreover, they have observed a taxon-specific differences in phylogenetic outputs and genetic variation between and within potential cryptic species, that impede to find a universal rules for the detection of cryptic species within polychaetes, or normalization to expected number of species based on genetic data (Brasier et al. 2016).

Moreover, cryptic species of polychaetes could have different ranges of distribution within the Antarctic region. Most of them, probably due to the larval dispersal around the continent, aided by oceanographic currents, highlight a wide distribution (Linse et al. 2007; Brasier et al. 2017). Some others resulted to be present only in restricted areas, suggesting another method of evolution, for example differences in reproductive traits (Palumbi, 1994), responses to competition (Alizon et al., 2008) or predation (Wilson et al., 2013) or the under representative sampling. In fact, the estimation and the spatial distribution of the discovered species were strictly determined by the relative inaccessibility of the region and the large part of the human investigations were done in coastal zones and the areas closed to the scientific bases (Baird et al. 2011). Whatever or why cryptic species are more prevalent

within certain polychaete families, functional groups or within some specific environments or geographical areas remain still unknown.

In this study I have investigated the biodiversity of Antarctic polychaetas applying morphological and molecular approaches, comparing the taxonomic keys suitable for Antarctic polychaetes with sequence alignments of the 16S rDNA, 12S rDNA and COI mtDNA genes, with the aim of detecting the presence of cryptic species in our polychaetes samples.

3.2 Materials and methods

3.2.1 Study area and samples collection

Sampling was carried out during the XXXIII Italian Expedition in Antarctica at Terra Nova Bay (Ross Sea) in the framework of the Italian National Program of Antarctic Research (PNRA). Specimens were collected using a Van Veen Grab (31 x 58 cm). Three sampling areas were selected considering different trophic conditions (Tab.1; Fig.1): Adelie Cove (characterized by high organic input due to the presence of penguin assemblages), Rod Bay (characterized by possible anthropogenic impact due to the near Italian research base "Mario Zucchelli Station"), Central Bay (characterized by the absence any source of impact). In all areas, samples were collected at three different depths (25, 70 and 140 m). Polychaetes were separated sieving the sediment, then preserved in ethanol (95%) and stored at -20°C until the morphological identification and molecular analysis.

Table 1. Table listing the sampling stations organized by area, depths and geographic coordinates.

Area	Station	Depth (m)	Latitude	Longitude
Adelie Cove	St 9	25	-74,46.467	164,00.266
	St 8	70	-74,46.390	163,57.977
	St 11	140	-74,46.617	164,02.798
Rod Bay	St 5	25	-74,41.831	164,07.532
	St 10	70	-74,41.918	164,07.896
	St 6	140	-74,41.972	164,08.208
Central Bay	St 2	25	-74,43.037	164,06.908
	St 4	70	-74,43.078	164,07.757
	St 3	140	-74,43.101	164,08.399

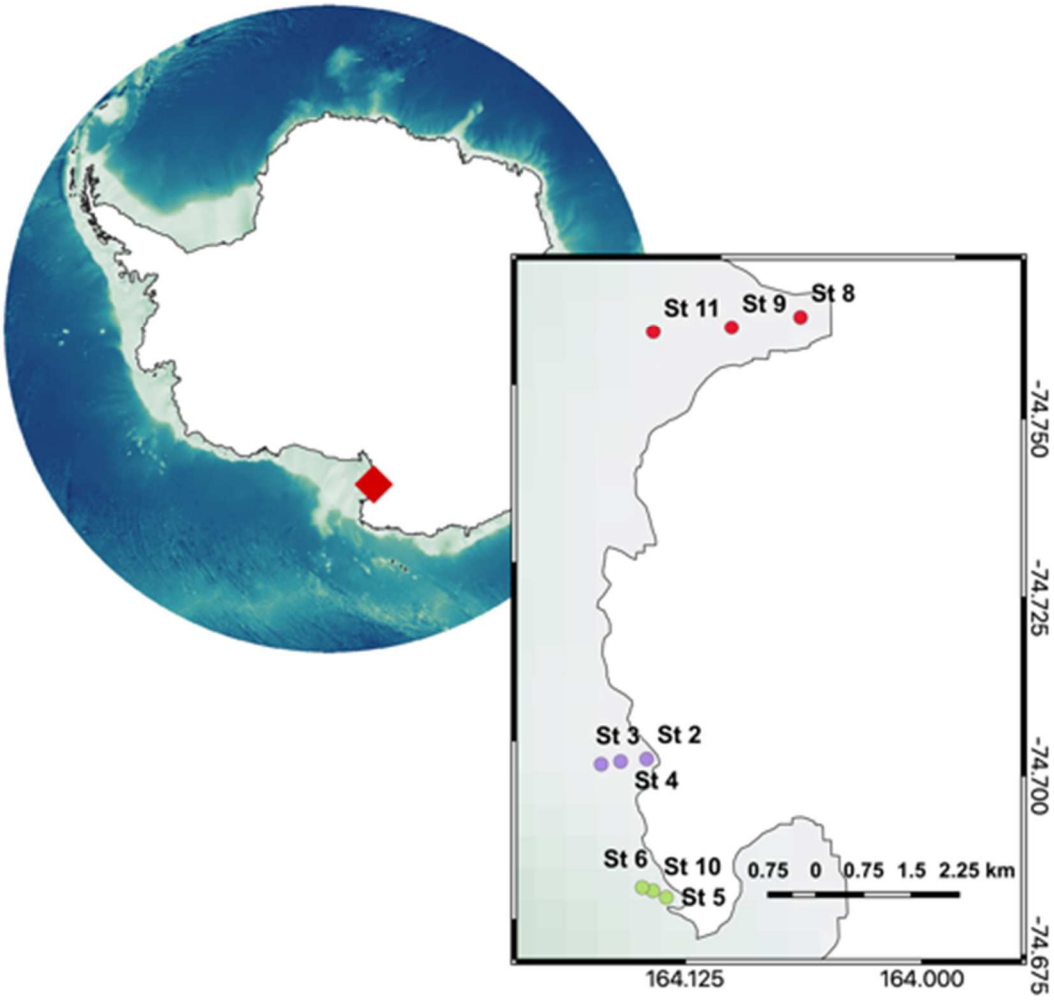

Figure 1. Map showing the three sampling areas where sediments and polychaetes were collected. The dots indicate the sampling stations.

3.2.2 Morphological identification of polychaetes species

Polychaetes specimens were identified from morphological characters with the help of two professional taxonomists, using specific indications for each Antarctic polychaetes species. In particular, the dichotomous keys described in Mackie, Andrew S.Y. (1987), Willey, A. (1902) and Blake J.A. (2017) were followed for the identification of *Leitoscoloplos geminus* (Mackie, 1987)*;* the dichotomous keys described in Saint-Joseph (1894), Hartman (1967), Hartmann-Schröder G. (1996) and Blake J.A. (2018) were followed for the identification of *Aphelochaeta palmeri* (Blake, 2018)*;* the dichotomous keys described in Hartman (1964; 1967; 1976) and Knox G.A. (1998) were followed for the identification of *Aglaophamus trissophyllus* (Grube, 1877).

3.2.3 Molecular identification of polychaetes species

Genomic DNA isolation

Total genomic DNA was extracted from a small portion of the tissue central section from all the specimens, using the Qiagen DNeasy Blood and Tissue Kit (Brasier et al. 2016) and following the manufacturer's instructions with an incubation with proteinase K at 56°C overnight. Genomic DNA quantification and quality control of the extraction, using 260/280 and 260/30 ratios, were performed with a NanoDrop ™ 1000 Spectrophotometer from Thermo Fisher Scientific.

Mitochondrial genes selection and PCR amplification settings

The phylogenetic relationship among our specimens was performed using three mitochondrial markers: the coding mitochondrial 16S and 12S rDNA genes were chosen for species discrimination (Vences et al. 2005a, 2005b; Li Yang et al. 2014) as they are often easier to obtain and, in the case of Antarctic invertebrates, most widely available (Grant & Linse, 2009). Moreover, also part of the mitochondrial protein-coding COI gene was chosen as it is the most widely accepted marker in the Barcode of Life Data System (BOLD). The COI gene is a suitable barcoding gene as it is fast evolving and exhibits a greater degree of genetic distance between than within species; it well performs in the group of polychaeta (Hebert et al. 2003; Brasier et al. 2016).

The amplification of 16S, 12S and COI genes was performed using these set of primers: 16SAnnF (5'-GCGGTATCCTGACCGTRCWAAGGTA-3') and 16SAnnR (5'-TCCTAAGCCAACATCGAGGTGCCAA-3') (342 bp; Sjolin et al. 2005), 12Slev (5'-GCCAGCAGCCGCGGTTA-3') and 12Sdes1 (5'-CCTACTTTGTTACGACTTAT-3'). (482-505 bp; Trontelj and Utevsky, 2005), LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (630 bp; Folmer et al. 1994), respectively. The reaction mixtures consisted of 5 µl of 5x My Taq Reaction Buffer (Bioline), 0,5 µl of each primer (20 µM), 0,5 µl of My Taq HS DNA Polymerase (5 U/µl concentration), 1µl of DNA template and a quantity of filtered and autoclaved Milli-Q water to reach a final volume of 25 µl. For some of the specimens, different DNA and primer concentrations were tested when the amplification process did not work efficiently.

The thermal cycling profiles consisted in an initial denaturation of 5 min (2 min for COI gene) at 95°C, followed by 35 cycles of 30 s at 95°C (94°C for COI gene), 30 s at 65°C (16S

gene) or at 50°C (12S gene) or at 48°C (COI gene), 45 s at 72°C, with a final extension of 10 min at 72°C. A touch-up PCR was performed for the COI gene in some samples in which the amplification did not work, using as annealing temperature 95°C for 3 min during the first 5-10 cycles and 55°C for the remaining 30-25 cycles.

DNA amplification product was checked with 1% agarose gel electrophoresis using 10.000x GelRed Nucleic Acid Stain (Biotium), 0,4 gr of agarose, 40 ml of TE Buffer for the gel preparation and 2 µl of 5x GelPilot DNA Loading Dye (Qiagen), 2 µl of GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) for the electrophoresis.

Sequencing of mitochondrial 12S, 16S and COI genes

PCR products were purified using Qiagen PCR Purification Kit or directly extracted from electrophoresis gel using Qiagen Gel Extraction Kit, when aspecific products were present. Then, sequencing was carried on using Sanger method (Sanger et al. 1977) on both stands at Molecular Facility of Stazione Zoologica Anton Dohrn using the same primers as in the PCR protocols, trough Applied Biosystems 3730 DNA Analyzer 48 capillaries (Life Technologies).

Phylogenetic and statistical analysis

The sequences obtained were analyzed using the software Geneious 7.1.9 (Kearse et al. 2012). The terminal section of the sequence including low-quality reading and primers were removed before assembling the two strands into consensus sequences. Multiple alignments for each marker were performed using MUSCLE algorithm (Edgar, 2004) in Alivew 1.26 (Larsson, 2014). All unique haplotype were confronted with sequences deposited in the public database GenBank (http://www.ncbi.nlm.nih.gov/genbank/) in order to validate the taxonomy of our specimens and to find other sequences belonging to close related species to used to build a phylogenetic relationship among Antarctic polychaetes. Searches were perfomed using BLAST verifying the significant similarity with other known sequences (https://blast.ncbi.nlm.nih.gov/). Results with a low percentage of coverage (< 90%) and with a low percent identity (< 95%) were not considered. Among all the sequences retrieved, the ones that could be aligned with the query sequences were selected as additional ingroup or outgroup to build a more complete phylogeny. Multiple alignments for each marker were performed using MUSCLE algorithm (Edgar, 2004) in Alivew 1.26 (Larsson, 2014). Phylogenetic analyses were conducted in MEGA X (Kumar et al. 2018) for all morphospecies investigated using the separate 16S, 12S and COI dataset. For each dataset,

the evolutionary history was inferred by using the Maximum Likelihood method applying the best-fit nucleotide substitution model: Hasegawa-Kishino-Yano (Hasegawa et al. 1985) for 16S and 12S and Tamura 3-parameter (Tamura, 1992) for the COI markers, respectively. Sequences were grouped in haplotypes using DNA Sequence Polymorphism (DNASP v6. 12.03) program (Rozas et al. 2017) and haplotype networks were built with PopArt (Leigh & Bryant, 2015), using the TCS network method (Clement et al. 2000). This graphical representation differs from a tree as it may show multiple connection to a single haplotype when there are alternative acceptable evolutionary paths. Finally, the Kimura-2-parameter model (K2P; Kimura, 1980; Kumar et al. 2018) was applied to compare our results with those present in literature (Hebert et al. 2004, Carr et al. 2011).

3.3 Results

3.3.1 Morphological identification

A total of 96 specimens were identified by classical taxonomic methods and attributed to 6 different species (Tab. 2). The selection of target species was done according to the numbers of specimens available and how the sampling was distributed in the different areas and depths (Fig. 2). Therefore, for this study it was possible to use 27 specimens identified as *Leitoscoloplos geminus* (Mackie, 1987), 31 specimens attributed to *Aphelocaeta palmeri* (Blake, 2018) and 27 specimens classified as *Aglaophamus trissophyllus* (Grube, 1877).
Moreover, the selected three species show different trophic strategy: the first two are burrowers and deposit feeders (Gambi et al. 1997; Van Colen et al. 2010) while the third is considered vagile carnivore, feeding on small invertebrates including mollusks, crustaceans, and other polychaetes (Fauchald & Jumars, 1979).

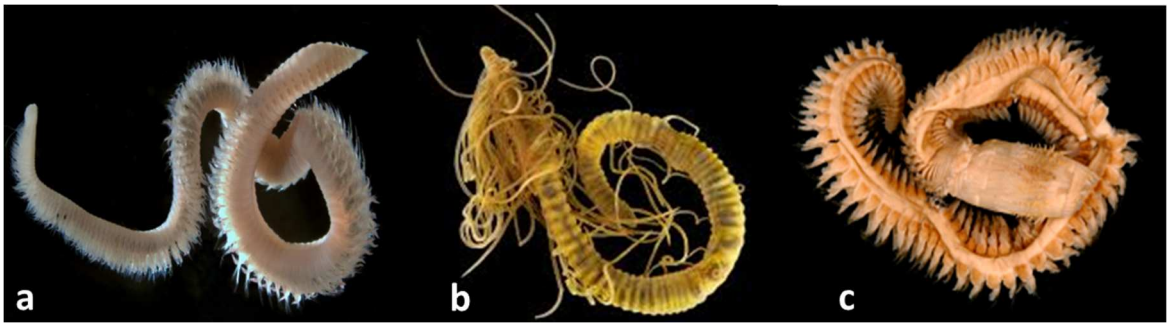

Figure 2. Pictures of Antarctic polychaetes chosen for the study; a) *Leitoscoloplos geminus* (Mackie, 1987), b) *Aphelochaeta palmeri* (Blake, 2018), c) *Aglaophamus trissophyllus* (Grube, 1877).

Table 2. List of all polychaetes identified by morphological analysis with the number of specimens caught in each sampling site. In bold the three species used in this study.

Species	Adelie Cove			Rod Bay			Central Bay		
	25 m	**70 m**	**140 m**	**25 m**	**70 m**	**140 m**	**25 m**	**70 m**	**140 m**
	St. 9	**St. 8**	**St. 11**	**St. 5**	**St. 10**	**St. 6**	**St. 2**	**St. 4**	**St. 3**
Leitoscoloplos geminus	3	3	3	3	3	3	3	3	3
Laonice cirrata									2
Capitella capitata		2				1			
Aphelochaeta palmeri		5	7					10	9
Levinsenia gracilis			1				1	1	3
Aglaophamus trissophyllus	3	3	3	3	3	3	3	3	3

3.3.2 Molecular and phylogenetic analyses

Primer sets used to amplify the mitochondrial 16S and 12S fragments have successfully amplified the fragments of genomic DNA of all *L. geminus* specimens. On the other hand, for this species, the primer pairs used to amplify the fragment of the COI gene did not provide a god quality PCR product in all specimens, despite applying changes in annealing temperatures and variable concentrations of $MgCl_2$ in the reactions. Good quality DNA sequences for the mitochondrial 16S, 12S and COI genes in *A. palmeri* were obtained for 23, 3 and 27 specimens, respectively. At last, DNA sequences for the mitochondrial 16S, 12S and COI genes in *A. trissophyllus* were obtained for 17, 20 and 25 specimens, respectively. The complete list of DNA sequences of 16S, 12S and COI obtained the sequencing for all specimens collected for the three species additional sequences downloaded from Genbank are reported in Table 1 SM. Moreover, the complete list of known sequences producing significant alignment with haplotypes on BLAST database. Additional sequences downloaded from Genbank are listed in Table 2SM.

Mitochondrial 16S rDNA gene

The alignment of 16S sequences and the resulting phylogenetic tree revealed that all the 27 sequences of *L. geminus* belong to a same haplotype (Tab. 3 SM). The 17 sequences of *A. trissophyllus* clustered including the *Aglaophamus* cf. *trissophyllus,* sequence available in GenBank (Table 2 SM), and are represented by 4 shared and 3 unique haplotypes (Tab. 3 SM), with a variability among haplotypes below 4 mutations (Tab.4 SM; Fig.5). Moreover, haplotypes are clearly separated in two clades with 100% bootstrap support (Fig. 2: yellow and red boxes). The 23 sequences of *A. palmeri* were identical and constitute a shared haplotype, that clustered with a sequence of *Aphelochaeta marioni*, available in GenBank (Table 2SM); the specimen 4ID, morphologically identified as *A. palmeri,* grouped separately, forming a unique haplotype, closed to a known sequence of *Chaetozone sp.* (Table 2SM). It differs from the *A. palmeri* shared haplotype for a total of 64 mutations (Tab. 4 SM; Fig. 5). An outgroup (*Sipunculus nudus*) was added to better visualize the phylogenetic distances among samples.

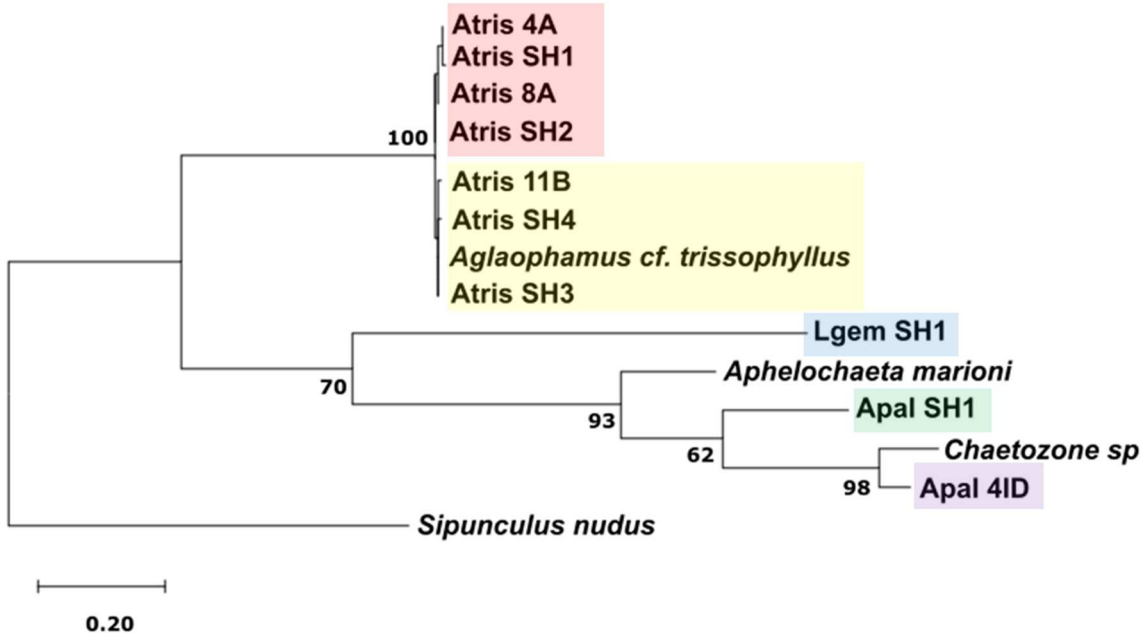

Figure 2. Phylogenetic tree of 16S rDNA mitochondrial gene.

Similar results were obtained by the haplotype network with four groups clearly separated: the shared haplotype of *L. geminus,* the group of *A. trissophyllus* with single and shared haplotypes described before, the shared haplotype and the 4ID specimen of *A. palmeri* (Fig. 5). The minimum distance between the *A. palmeri* group and the *A. trissophyllus* group is

111 nucleotide mutations; the minimum distance between the *A. trissophyllus* group and *L. geminus* group is 117 nucleotide mutations; finally, the minimum distance between the *A. palmeri* group and *L. geminus* group is 228 nucleotide mutations. Moreover, the different haplotypes found in the three species do not follow patterns related to different sampling areas and depths (Fig. 5; legend).

Mitochondrial 12S rDNA gene

The alignment of 12S sequences and the resulting phylogenetic tree revealed that all the 27 sequences of *L. geminus* were identical (Tab. 3SM). The 20 sequences of *A. trissophyllus* grouped into 5 shared and 6 unique haplotypes (Tab. 3SM), with a variability among each other's below 8 mutations (Tab. 5SM; Fig. 6). Moreover, also in the results of 12S marker there is a clear separation among haplotypes with 100% bootstrap support (Fig. 3: yellow and red boxes). The 3 sequences of 16S rDNA from *A. palmeri* grouped into one shared and one unique haplotype (Tab. 3SM) with a difference of 71 total mutations (Tab. 5SM; Fig. 6).

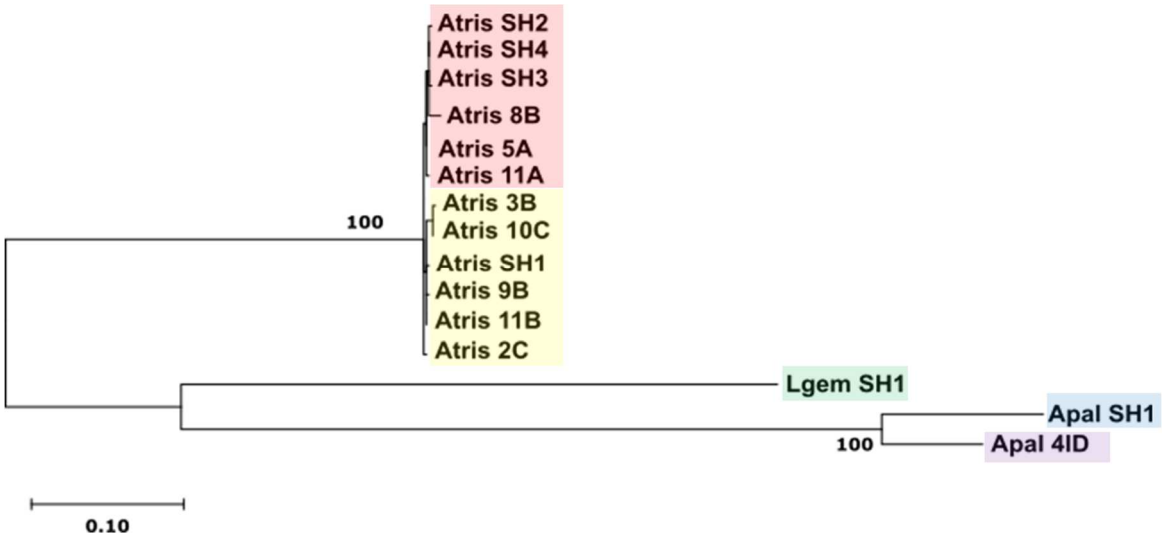

Figure 3. Phylogenetic tree of 12S rDNA mitochondrial gene.

Finally, four groups are clearly present and separated from the haplotype network: the shared haplotype of *L. geminus* (Lgem_SH1), the group of *A. trissophyllus* with single and shared haplotypes described before, the shared haplotype (Apal_SH1) and the 4ID specimen of *A. palmeri* (Fig. 6). The minimum distance between the *A. palmeri* group and the *A. trissophyllus* group is 188 nucleotide mutations; the minimum distance between the *A.*

trissophyllus group and *L. geminus* group is 163 nucleotide mutations; finally, the minimum distance between the *A. palmeri* group and *L. geminus* group is 351 nucleotide mutations. Moreover, the different haplotypes found in the three species do not follow patterns related to different sampling areas and depths (Fig. 6; legend).

Mitochondrial COI gene

All the combination of primers we had available has been tested and no COI sequences for *L. geminus* was successfully amplified. The 25 sequences of *A. trissophyllus* grouped in 5 shared and 12 unique haplotypes (Tab. 3SM), together with the sequence of *A. trissophyllus* from Genbank (Table 2SM), with a variability below 32 mutations (Tab. 6SM; Fig. 7). The grouping of COI haplotypes separates in two clades with high percentages of bootstrap support (Fig. 4: yellow and red boxes). This result supports the tree topology for the other molecular markers. The 27 sequences of COI from *A. palmeri* clustered in 3 shared and 6 unique haplotypes, that showed a variability of 147 total mutations (Tab. 6SM; Fig. 7). Three groups are clearly present and separated in the haplotype network: the group of *A. trissophyllus* with single and shared haplotypes described before, the shared and unique haplotypes of *A. palmeri* the 4ID specimen of *A. palmeri* (Fig.7). The minimum distance between the *A. palmeri* group and the *A. trissophyllus* group is 313 nucleotide mutations. Moreover, among the different haplotypes found in the three species a pattern that represent the different sampling areas and depths to which polycheates belong to is not identified (Fig. 7; legend).

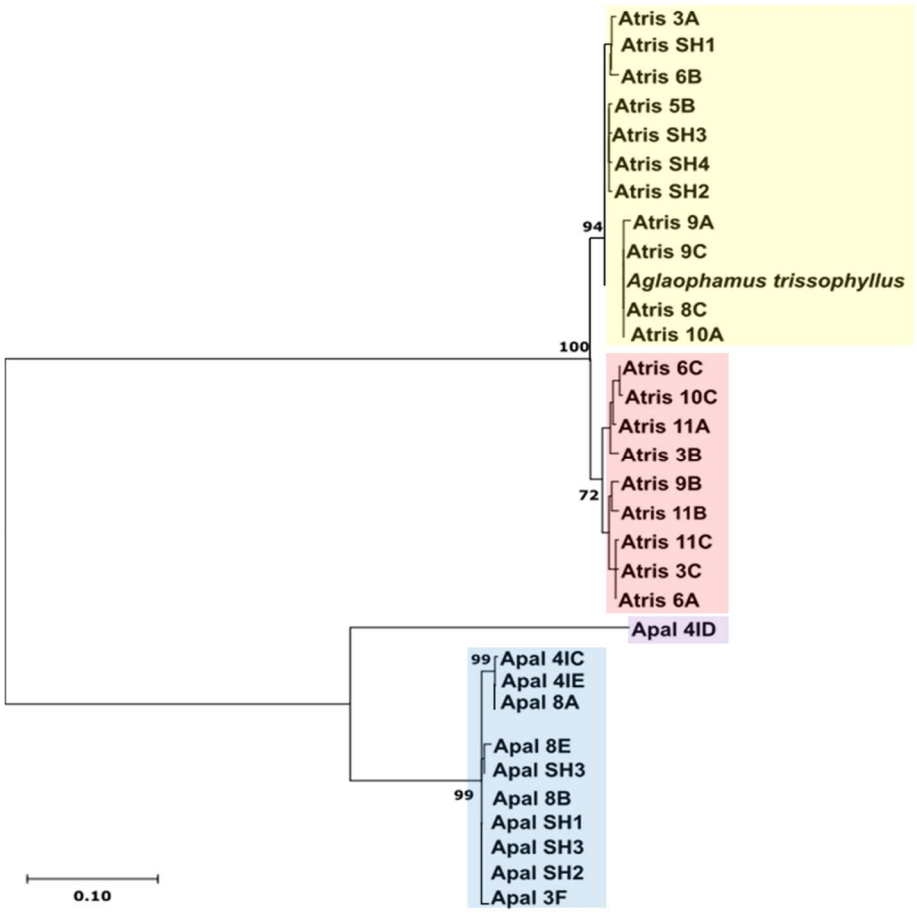

Figure 4. Phylogenetic tree of COI mitochondrial gene.

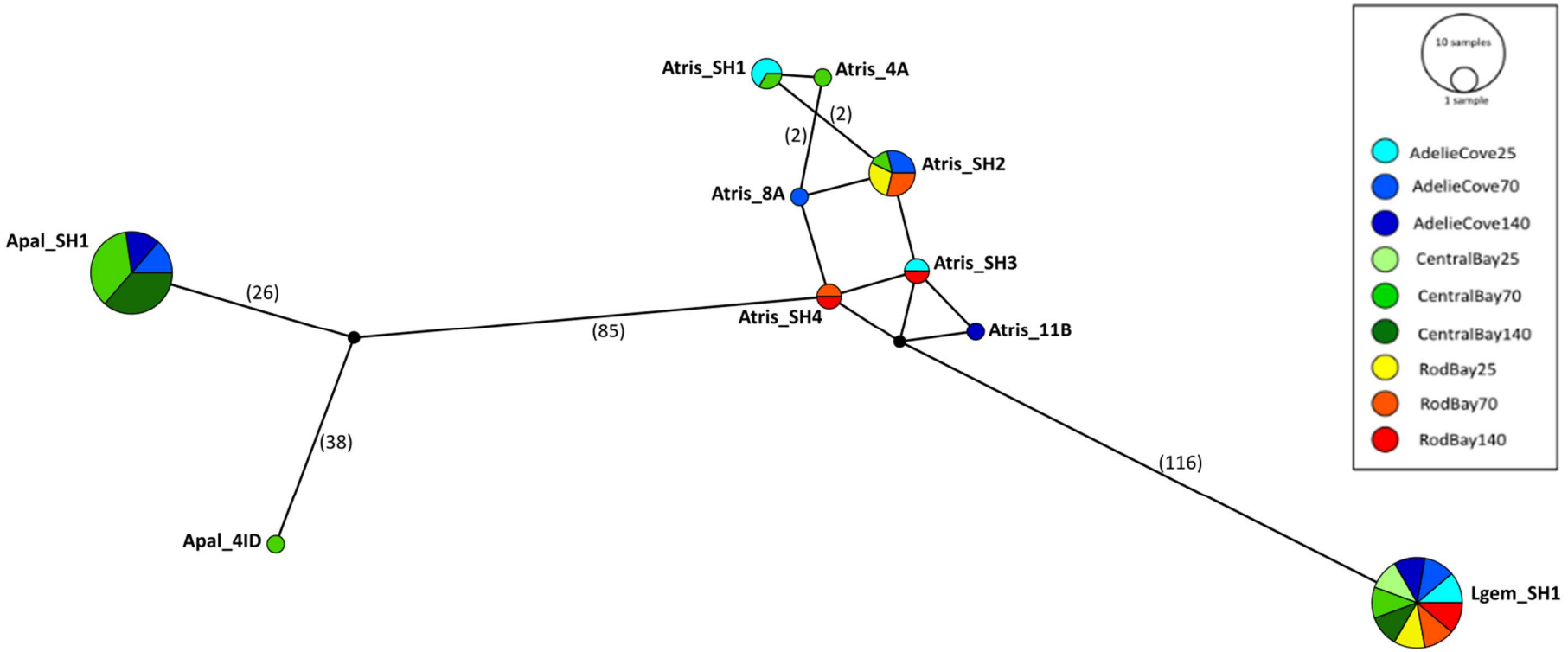

Figure 5. Haplotype network of 16S rDNA mitochondrial gene. Numbers inside the brackets: number of mutations; black nodes: hypothetical haplotypes present in the evolution pathway between the haplotypes identified; haplotype circle size: proportional to the number of individuals sharing the same sequence.

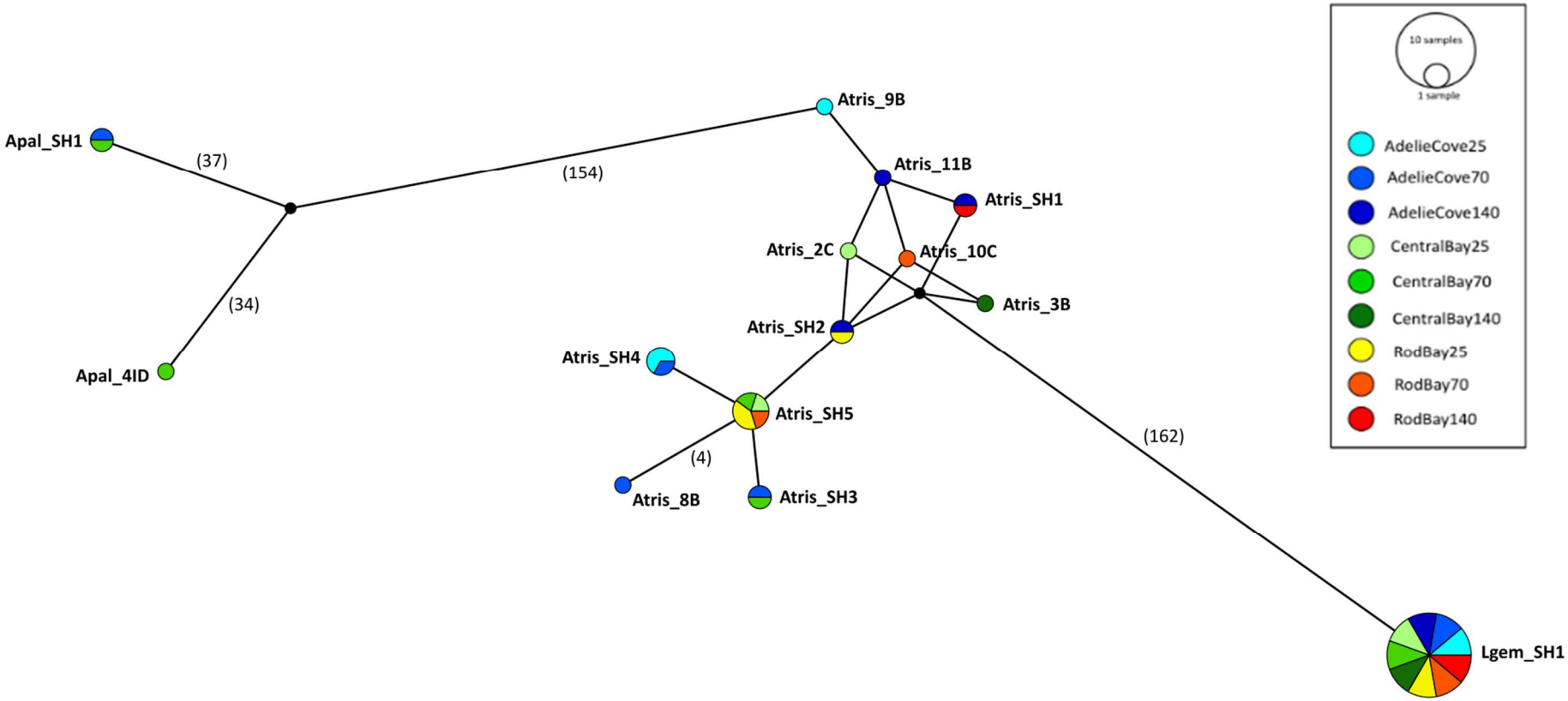

Figure 6. Haplotype network of 12S rDNA mitochondrial gene color coded by sampling sites. Numbers inside the brackets: number of mutations; black nodes: hypothetical haplotypes present in the evolution pathway between the haplotypes identified; haplotype circle size: proportional to the number of individuals sharing the same sequence.

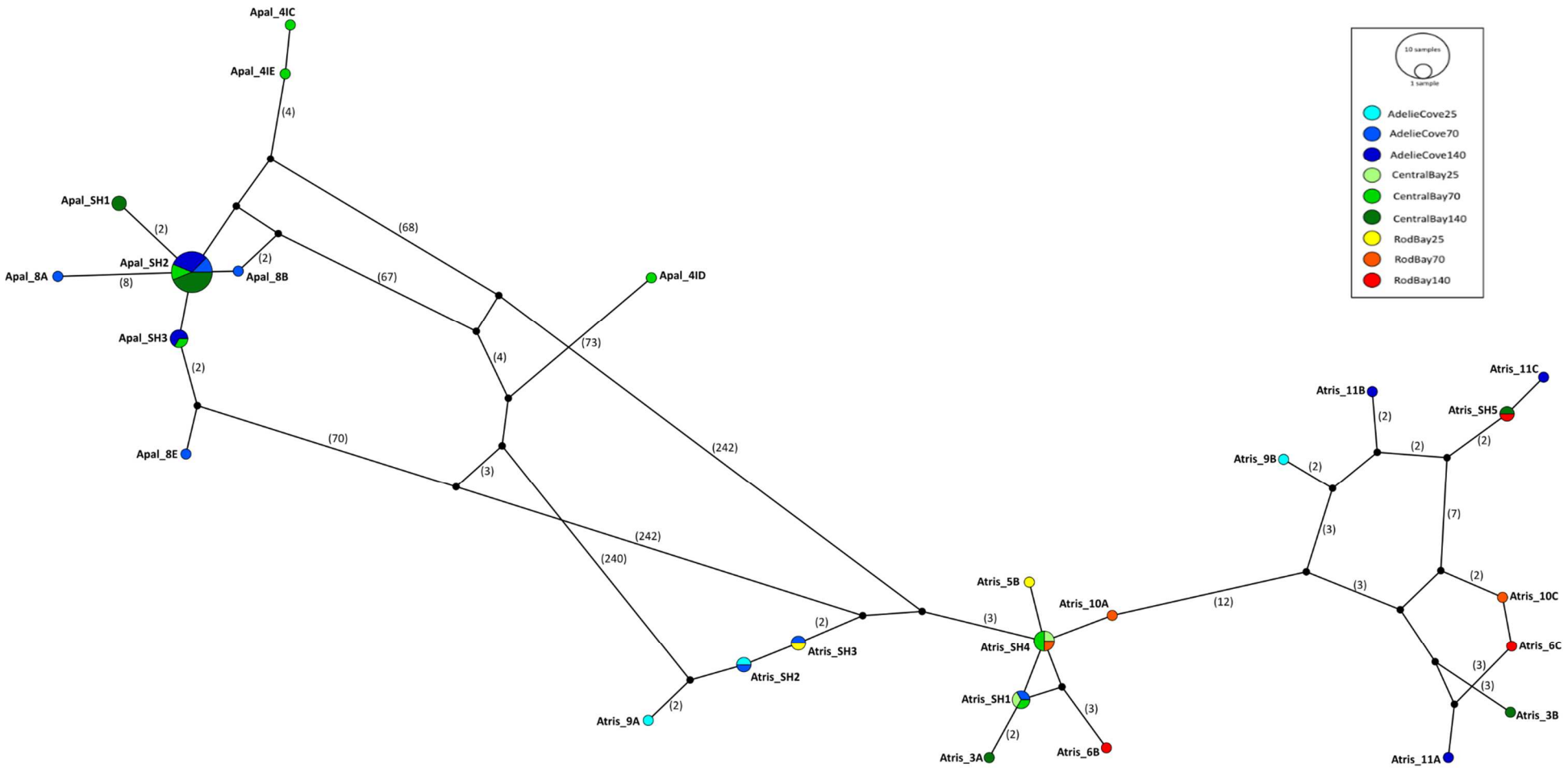

Figure 7. Haplotype network of COI mitochondrial gene. Numbers inside the brackets: number of mutations; black nodes: hypothetical haplotypes present in the evolution pathway between the haplotypes identified; haplotype circle size: proportional to the number of individuals sharing the same sequence.

3.4 Discussion

Research on cryptic species has increased exponentially over the past two decades, fueled in large part by the increasing availability of DNA sequences (Bickford et al. 2006). Cryptic species are found on all major branches of the tree of life and probably represent a significant portion of undiscovered biodiversity (Jörger et al. 2013; Pante, et al. 2015; Loxdale et al. 2016). In fact, because speciation is not always accompanied by morphological change, the true number of biological species is likely to be greater than the current tally of nominal species, most of which are delineated on purely morphological grounds (Bickford et al. 2006). It has been often predicted that cryptic species result from, and are more abundant, among those that live in extreme environments where harsh abiotic factors would favor a stabilizing selection and lead to morphological stasis (Borda et al. 2013).

In this study, the identification by means of classical taxonomic analyses revealed three different species identified as *Leitoscoloplos geminus* (Mackie, 1987), *Aglaophamus trissophyllus* (Grube, 1877) and *Aphelochaeta palmeri* (Blake, 2018).

The genetic characterization of mitochondrial 16S and 12S sequences of *Leitoscoloplos geminus*, clearly supported such results since all the specimens clustered together regardless different depth and different sampling area. Unfortunately, the analysis of COI gene characterized by a faster mutation rate than 16S and 12S do not give results in this species, therefore we cannot completely rule out the presence of cryptic species.

Similarly, previous studies revealed that 16S marker produced the more conserved diversity results and, without the comparison with COI, cryptic species would not have been identified (Brasier et al. 2016). However, other studies reported that 16S marker was more effective in defining the phylogeny despite its slower evolutionary rates (Ruta et al. 2007). So far, the distribution of *L. geminus* appears to be limited to the Southern Ocean: it has been reported from Adelie Land, Wilkes Land, Victoria Land, Ross Sea, King George Island and between Bellingshausen Sea and Gerlache Strait and was not observed presence of cryptic species within this group until now (de Souza Barbosa et al. 2010; Paiva et al. 2015; Blake 2017).

The species *Aphelochaeta marioni* is among the most enigmatic and difficult to identify of all cirratulids, because of the lack of obvious variability in setal morphology (Blake, 2018). St. Joseph (1984) firstly described *Aphelochaeta marioni* as *Heterocirrus marioni*. This species was originally described from the sub-Antarctic Kerguelen Islands in the Indian Ocean and has been reported widely from Antarctica by numerous authors (usually as *Tharyx*

cincinnatus or *Aphelochaeta cincinnata*). Moreover, the species *Aphelochaeta marioni* is characterized by a lecithotrophic and benthic larval stage and considered to be a cosmopolitan species, widespread distributed: e.g. from Mediterranean (La Porta et al. 2005) to Atlantic Ocean and Arctic (Elias and Rivero 2009; Kędra et al. 2011). Some investigations highlighted the presence of *A. marioni* specimens even at over 1000 m depth (Detinova, 1985), some others revealed that they have a different distribution depth on a scale of only hundreds of meters (Nygren et al. 2005, 2010; Bleidorn et al. 2006; Luttikhuizen and Dekker 2010). Due to errors in the original descriptions or misinterpretation of the published descriptions, each of the three species cited above have been misconstrued in the Antarctic ecological literature (Blake, 2018).The *A. marioni* is more probably belonging to a species complex and that its real distribution could be more limited.

A recent investigation done by Blake on the Cirratulidae family polychaetes in Antarctica have tried to solve this controversy, going deeper in the morphological identification of the genus *Aphelochaeta* and highlighting new dichotomous keys, and it has found new species in science (Blake, 2018).

The specimens used in this investigation were morphologically classified as *Aphelocaeta palmeri*, according to this new finding (Blake, 2018). The genetic analysis with all the three mitochondrial genes has shown the same results: all the specimens belong to a same group except for the specimen 4ID, that cluster a part. In fact, both the 16S and 12S genes show a same shared haplotype for all the individuals and a different haplotype for the specimen 4ID. Moreover, the COI gene, as a fast-evolving marker, highlights more haplotypes than the other markers, but comparing the nucleotide mutations among the individuals is clear the same result. In fact, the distance between the specimen 4ID and the other individuals morphologically identified as *Aphelocaeta palmeri* is higher and comparable with a distance that there could be present between different species or different genus (Brasier et al. 2016). We consider the specimen 4ID, morphological identified as *A. palmeri*, as belonging to another species. On the other hand, to confirm that the specimen 4ID is a cryptic species a secondary morphological examination after sequencing should be needed, to prevent false positive results and an overestimation of 'true' cryptic diversity, due to a primary misidentification (De Salle et al. 2005). Unfortunately, the limited size of the 4ID specimen and the several uses of the tissue for the DNA extraction do not allow us a second morphological identification.

Otherwise, using the principle that the genetic variation between species (interspecific) is greater than the genetic variation within species (intraspecific) Hebert and colleagues

outlined a rule of thumb to identifying a cryptic species: a minimum of 10 times the average intraspecific variation between clade differences has been suggested as considering a species a cryptic species (Hebert et al. 2004). This method was used to identify provisional species in a major polychaete barcoding project (Carr et al. 2011). Using this method, the specimen 4ID has enough distances to be considered cryptic species: in fact, it shows 147 nucleotide mutations against an average of intraspecific variation of 4.4, more than 33 times higher. Moreover, using the same principle (Hebert et al. 2003), where two or more species are distinct, there should be a lack of overlap between intraspecific and interspecific sequence variation, commonly referred to as the 'barcoding gap' (Meyer & Paulay, 2005). Potential cryptic species were identified based on phylogenetic analysis based on Kimura-2-Parameter distance (K2P; Kimura, 1980; Kumar et al. 2018), comparing interspecific and intraspecific genetic variation and the existence of a 'barcoding gap' once clades were determined. When the interspecific and intraspecific variation of the corresponding COI was measured there was no clear barcoding gap, although the average interspecific distance was greater than the intraspecific distances calculated in *A. trissophyllus* and in *A. palmeri*, 2.97 compared with 0.025 and 0.004, respectively (Fig. 8a).

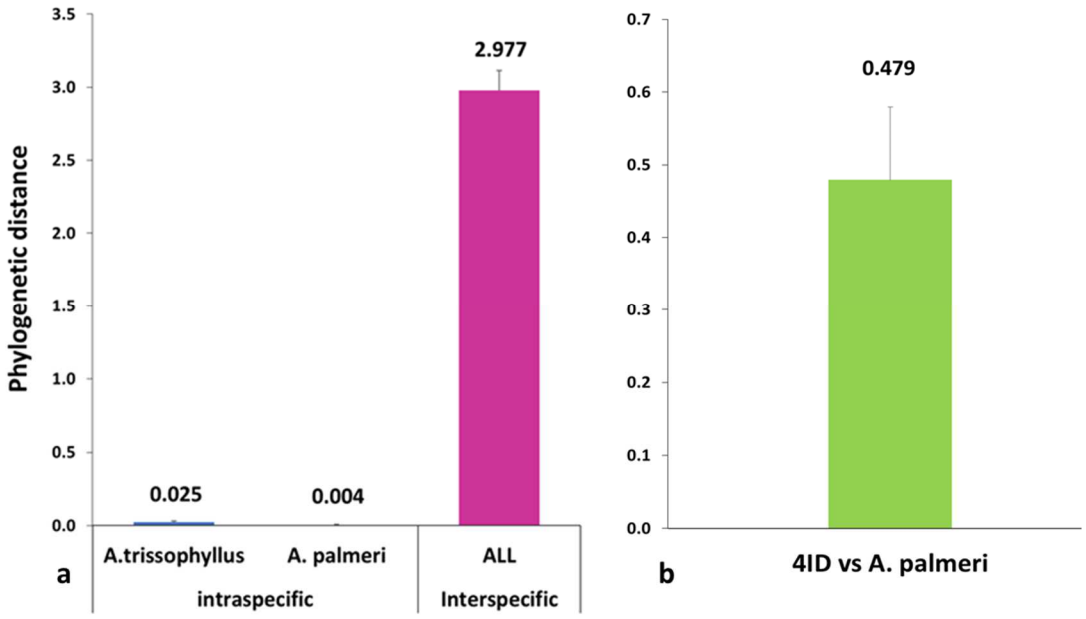

Figure 8. Graphs representing phylogenetic distance calculating with Kimura-2-parameter method on COI gene (a) within specimens belong to the *A. trissophyllus* and *A. palmeri* species (intraspecific distance) and among specimens belong to different species (interspecific distance); (b) between 4ID specimen and the *A. palmeri* group.

The phylogenetic distance existing between the specimen 4ID and the *A. palmeri* group is 0,49, a value that is lower than the interspecific but higher than the intraspecific phylogenetic distances measured (Fig. 8b). Moreover, these results indicate again that our ability to apply strict rules to the identification of cryptic species within polychaetes is limited. A lack of evidence for a global DNA barcoding gap in Annelida was also recorded in Kvist who evaluated over 70 million pairwise genetic comparisons using the Automated Barcoding Gap Discovery software (Puillandre et al. 2012; Kvist, 2016).

Finally, the molecular analyses revealed variability among the individuals morphologically identified as *Aglaophamus trissophyllus.* Each mitochondrial gene grouped individuals in different shared and single haplotypes and clustered them in two separated clades, similar in each marker used. For a better discussion of these results we have assigned them at clade A and clade B as it is shown in Table 5.

Table 5. List of specimens clustered in Clade A and Clade B for each mitochondrial gene. The specimens highlighted in blue are the ones that showed differences in the clustering.

16S		**12S**		**COI**	
Clade A	**Clade B**	**Clade A**	**Clade B**	**Clade A**	**Clade B**
Atris_4A	Atris_6A	Atris_2B	Atris_2C	Atris_2B	Atris_3B
Atris_4B	Atris_6C	Atris_4A	Atris_3B	Atris_2C	Atris_3C
Atris_4C	Atris_9B	Atris_4C	Atris_6A	Atris_3A	Atris_6A
Atris_5A	Atris_10C	Atris_5A	Atris_9B	Atris_4A	Atris_6C
Atris_5C	Atris_11B	Atris_5B	Atris_10C	Atris_4B	Atris_9B
Atris_8A		Atris_5C	Atris_11B	Atris_4C	**Atris_10A**
Atris_8B		Atris_8A	Atris_11C	Atris_5A	Atris_10C
Atris_8C		Atris_8B		Atris_5B	**Atris_11A**
Atris_9A		Atris_8C		Atris_6B	Atris_11B
Atris_9C		Atris_9A		Atris_8A	Atris_11C
Atris_10A		Atris_9C		Atris_8B	
Atris_10B		**Atris_10A**		Atris_8C	
		Atris_11A		Atris_9A	
				Atris_9C	
				Atris_10B	

A different grouping is found in the samples Atris_10A and Atris_11A, assigned at clade B with the COI gene and at clade A with 12S and partially with 16S. Despite this variability among individuals was expected with the COI gene, same results obtained with the more conservative and slower evolutionary rates of 16S and 12S mitochondrial genes underlines the possible presence of different species (Brasier et al. 2016).

Presence of this species is documented both in different parts of West Antarctica seas (e.g. Amundsen Sea, Scotia Arc, Weddell Sea) between 100 to 3500 m (Brasier et al. 2016; de Souza Barbosa et al. 2010; Pabis & Sicinski, 2012) and in South part as Terra Nova Bay, showing a wide depth distribution, occurring in soft bottoms from 16 up to 1000 m (Cantone et al. 2000; Heimeier et al., 2010). How this species and its distribution were established and maintained is most probably a result of several complex biophysical interactions over geological time. One of the factors that could have contributed is the larval dispersion. In fact, *A. trissophyllus* is characterized by pelagic larvae (Heimeier et al., 2010; Gallego et al., 2014); currents could carry particles or "larvae" between Antarctic regions in both directions around the continent.

In a recent investigation Brasier et al. demonstrated that the *Aglaophamus trissophyllus* is one of the Antarctic polychaetes in which a fast rate of genetic modification is taking place, showing the presence of both species complex, for a high intraspecific variation and morphological uncertainty (Orensanz, 1990), and cryptic species (Brasier et al. 2016). Despite we already use the best-fit phylogenetic model to describe the results, we analyzed again the sequences of *A. trissophyllus* with the Kamura-2-parameter (K2P; Kimura, 1980; Kumar et al. 2018) model to compare our results with those that Brasier obtained in his study. The Kimura-2-parameter model has evidenced the same distances among samples that the other models we used for the phylogenetic tree and haplotype networks (Hasegawa-Kishino-Yano model for 16S and 12S markers, Tamura 3-parameter model for the COI marker). The K2P distances among specimens inside and between the clades were shown in Table 6; besides K2P percentages of the 16S and COI mitochondrial genes used for the comparison with Brasier et alii. (2016) results, also the K2P distance of 12S were added for completeness. The K2P percentages measured among individuals belonged to the same clade are comparable with those Brasier and colleagues found among individuals assigned at a same group, both for the 16S and the COI genes. Moreover, comparing the K2P percentages between clade A and clade B both in 16S and in COI, we can notice that our values are lower than the values found among groups considered cryptic species by Brasier and colleagues but are in the same range of values between clades that they have considered complex

species. For that reason, we can consider the specimens assigned to the clade A and clade B as belonged to two different species complexes of *A. trissophyllus.*

Table 6. Phylogenetic distance of Kimura-2-parameter obtained inside and between the two clades.

	16S		12S		COI	
	Min	**Max**	**Min**	**Max**	**Min**	**Max**
Clade A	0.34	1.02	0.38	1.94	0.26	3.81
Clade B	0.34	1.02	0.38	1.15	0.26	3.83
Between Clade A and B	0.34	1.73	0.38	3.18	4.70	7.50

3.5 Conclusion

There are nearly 800 species records of polychaetes within the Register of Antarctic Marine Species (RAMS), which have been documented in Antarctic waters (Schuller, 2014). The combined factors of undersampling, undescribed species and cryptic species suggest that true Antarctic species diversity for polychaetes will be far in excess of this figure. DNA barcoding provides an effective approach for the rapid evaluation of species richness in groups where many species await description (Carr et al. 2011).

The results of our investigation provide further support to the assertion of the high variability and huge hidden diversity of Antarctic ecosystem. The presence of a potential cryptic species in *Aphelochaeta palmeri* and of two groups of species complexes in *Aglaophamus trissophyllus* highlighted the presence of multiple processes that are acting and contributing to widen biodiversity. The low number of specimens available at the different depths and different areas do not allow us to understand what are the potential pressures that are playing a role in driving the diversity. In fact, since the ability to apply a unique method to the identification of cryptic species within polychaetes is not possible yet since large, comprehensive genetic studies on species boundaries in polychaetes are rare and since these investigations merely concern a small fraction of all morphospecies of polychaetes, we are probably seeing the tip of the iceberg (Knowlton 1993, Westheide et al. 2003, Nygren, 2014). Other factors remain to be investigated for an exhaustive description and understanding of the diversity, biogeography and functionality of Antarctic marine fauna. Such data could be of the utmost importance for effective research-driven ecosystem-based management of the rapidly changing Antarctic marine ecosystem.

3.6 References

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3.7 Supplementary material

Table 1SM. List of sequences obtained for *Leitoscoloplos geminus* (Lgem), *Aphelochaeta palmeri* (Apal) and *Aglaophamus trissophyllus* (Atris). ST=Station, ID=individual, Y=yes and N=no

			Leitoscoloplos geminus				***Aphelocaeta palmeri***				***Aglaophamus trissophyllus***			
Area	**Depht**	**ST**	**ID**	**16S**	**12S**	**COI**	**ID**	**16S**	**12S**	**COI**	**ID**	**16S**	**12S**	**COI**
Adelie Cove	**25 m**	**9**	Lgem_9I	**Y**	**Y**	**N**					Atris_9A	**Y**	**Y**	**Y**
			Lgem_9II	**Y**	**Y**	**N**					Atris_9B	**Y**	**Y**	**Y**
			Lgem_9III	**Y**	**Y**	**N**					Atris_9C	**Y**	**Y**	**Y**
	70 m	**8**	Lgem_8I	**Y**	**Y**	**N**	Apal_8A	**Y**	**N**	**Y**	Atris_8A	**Y**	**Y**	**Y**
			Lgem_8II	**Y**	**Y**	**N**	Apal_8B	**N**	**N**	**Y**	Atris_8B	**Y**	**Y**	**Y**
			Lgem_8III	**Y**	**Y**	**N**	Apal_8C	**Y**	**Y**	**Y**	Atris_8C	**Y**	**Y**	**Y**
							Apal_8D	**Y**	**N**	**Y**				
							Apal_8E	**N**	**N**	**Y**				
	140 m	**11**	Lgem_11I	**Y**	**Y**	**Y**	Apal_11A	**N**	**N**	**Y**	Atris_11A	**N**	**Y**	**Y**
			Lgem_11II	**Y**	**Y**	**Y**	Apal_11B	**N**	**N**	**Y**	Atris_11B	**Y**	**Y**	**Y**
			Lgem_11III	**Y**	**Y**	**N**	Apal_11C	**Y**	**N**	**Y**	Atris_11C	**N**	**Y**	**Y**

							Apal_11D	**Y**	**N**	**Y**			
							Apal_11E	**Y**	**N**	**Y**			
							Apal_11F	**N**	**N**	**Y**			
							Apal_11G	**N**	**N**	**Y**			
Rod Bay	**25 m**	**5**	Lgem_5I	**Y**	**Y**	**Y**					Atris_5A	**Y**	**Y**	**Y**
			Lgem_5II	**Y**	**Y**	**N**					Atris_5B	**N**	**Y**	**Y**
			Lgem_5III	**Y**	**Y**	**N**					Atris_5C	**Y**	**Y**	**N**
	70 m	**10**	Lgem_10I	**Y**	**Y**	**N**					Atris_10A	**Y**	**Y**	**Y**
			Lgem_10II	**Y**	**Y**	**N**					Atris_10B	**Y**	**N**	**Y**
			Lgem_10III	**Y**	**Y**	**N**					Atris_10C	**Y**	**Y**	**Y**
	140 m	**6**	Lgem_6I	**Y**	**Y**	**N**					Atris_6A	**Y**	**Y**	**Y**
			Lgem_6II	**Y**	**Y**	**N**					Atris_6B	**N**	**N**	**Y**
			Lgem_6III	**Y**	**Y**	**N**					Atris_6C	**Y**	**N**	**Y**
Central Bay	**25 m**	**2**	Lgem_2I	**Y**	**Y**	**N**					Atris_2A	**N**	**N**	**N**
			Lgem_2II	**Y**	**Y**	**N**					Atris_2B	**N**	**Y**	**Y**
			Lgem_2III	**Y**	**Y**	**N**					Atris_2C	**N**	**Y**	**Y**
	70 m	**4**	Lgem_4I	**Y**	**Y**	**N**	Apal_4IB	**Y**	**N**	**Y**	Atris_4A	**Y**	**Y**	**Y**
			Lgem_4II	**Y**	**Y**	**N**	Apal_4IC	**Y**	**N**	**Y**	Atris_4B	**Y**	**N**	**Y**
			Lgem_4III	**Y**	**Y**	**N**	Apal_4ID	**Y**	**Y**	**Y**	Atris_4C	**Y**	**Y**	**Y**
							Apal_4IE	**Y**	**Y**	**Y**				
							Apal_4A	**Y**	**N**	**N**				
							Apal_4B	**N**	**N**	**N**				
							Apal_4C	**Y**	**N**	**Y**				
							Apal_4D	**Y**	**N**	**N**				
							Apal_4E	**Y**	**N**	**Y**				
							Apal_4F	**Y**	**N**	**N**				
	140 m	**3**	Lgem_3I	**Y**	**Y**	**N**	Apal_3IF	**Y**	**N**	**Y**	Atris_3A	**N**	**N**	**Y**
			Lgem_3II	**Y**	**Y**	**Y**	Apal_3IIIG	**Y**	**N**	**Y**	Atris_3B	**N**	**Y**	**Y**
			Lgem_3III	**Y**	**Y**	**N**	Apal_3A	**Y**	**N**	**Y**	Atris_3C	**N**	**N**	**Y**
							Apal_3B	**Y**	**N**	**Y**				
							Apal_3C	**Y**	**N**	**Y**				
							Apal_3D	**Y**	**N**	**Y**				
							Apal_3E	**N**	**N**	**Y**				
							Apal_3F	**Y**	**N**	**Y**				
							Apal_3G	**Y**	**N**	**Y**				

Table 2 SM. List of additional sequences retrieved from genbank using BLAST tool.

Marker	Haplotype	Description	Query cover (%)	Percent identity (%)	Accession number
16S	Lgem_SH1	Leitoscoloplos robustus	100	80.52	FJ612466.1
		Orbinia latreillii	100	80.84	AY961084.1
		Leodamas tribulosus	100	80.79	FJ612459.1
	Apal_4ID	Chaetozone sp.	100	87.62	KX867186.1
		Chaetozone sp.	100	86.97	KX867187.1
		Cirratulidae sp.	89	83.04	FJ944510.1
	Apal_SH1	Chaetozone sp.	97	83.39	KX867185.1
		Aphelochaeta marioni	96	81.79	DQ779602.1
		Cirratulidae sp.	96	78.33	FJ944510.1
	Atris_4A, Atris_SH1, Atris_SH2, Atris_SH3, Atris_SH4, Atris_8A, Atris_11B	Aglaophamus cf. trissophyllus	100	98-99	KX867144.1
		Aglaophamus sp.	100	95-98	KX867147.1
12S	Lgem_SH1	Orbinia latreillii	100	82.92	AY961084.1
		Phascolosoma sp.	8	100	KX814447.1
	Apal_4ID	Cirriformia cf. tentaculata	28	82.5	NC_037390.1
		Urechis unicinctus	27	82.8	EF656365.1
	Apal_SH1	Lima pacifica galapagensis	26	84.35	GQ166548.1
		Phascolosoma esculenta	13	93.1	MG873458.1
	Atris_8B, Atris_SH1, Atris_SH3, Atris_SH4, Atris_SH5, Atris_9B, Atris_11B, Atris_10C, Atris_3B	Nephtys sp.	85- 90	80.11-86.83	KY972376.1
		Goniada jaonica	75-82	72.21-79.59	KP867019.1
		Lepidonotus sp.	72-83	74.31-78.8	KY753831.1
	Atris_SH2	Phreodrilidae sp.	86	75.52	KM206342.1
		Helcion pectunculus	23	83.33	AF058176.1
COI	Apal_SH1	Capitella neoaciculata	51	83.12	KX121840.1
		Georissa niahensis	66	79.21	MH03388.1
	Apal_SH2, Apal_4IC, Apal_4IE, Apal_8A	Culicoides huffi	83-85	71.69-73.96	KT307818.1
		Capitella neoaciculata	47-58	74.15-77.68	KX121857.1
		Caenestheriella cf.packardi	88-90	72.6-73.55	KJ705937.1
	Apal_8B	Brachionus angularis	68	86.05	JX216501.1
		Lecane luna	87	80.00	JX216718.1
	Atris_6B, Atris_SH1, Atris_9A, Atris_SH2, Atris_SH3, Atris_SH4, Atris_10A, Atris_3A, Atris_5B, Atris_11A, Atris_9B, Atris_3B, Atris_6C, Atris_10C, Atris_11B, Atris_11C, Atris_SH5	Aglaophamus trissophyllus	96-99	98.51-99.27	KX867389.1
		Cf. Nephtyidae	98-100	87.9-94.06	GU227129.1
		Aglaophamus cf. trissophyllus	91-99	94.35-97.81	KX867381.1

Table 3 SM. List of single and shared haplotypes identified with the three mitochondrial genes.

Markers	Haplotypes	Samples included
16S	Lgem_SH1	All 27 individuals
	Apal_SH1	Apal_3A, Apal_3B, Apal_3C, Apal_3D, Apal_3F, Apal_3G, Apal_3IF,
	Apal_4ID	Apal_4ID
	Atris_SH1	Atris_4B, Atris_9A, Atris_9C
	Atris_SH2	Atris_4C, Atris_5A, Atris_5C, Atris_8B, Atris_8C, Atris_10A, Atris_10B
	Atris_SH3	Atris_6A, Atris_9B
	Atris_SH4	Atris_6C, Atris_10C
	Atris_4A	Atris_4A
	Atris_8A	Atris_8A
	Atris_11B	Atris_11B
	KX867144.1	*Aglaophamus* cf. *trissophyllus* (NCBI)
	KC686657.1	*Aphelochaeta marioni* (NCBI)
	KX867190.1	*Chaetozone sp.* (NCBI)
	EU260120.1	*Sipunculs nudus* (NCBI)
12S	Lgem_SH1	All 27 individuals
	Apal_SH1	Apal_4IE, Apal_8C
	Apal_4ID	Apal_4ID
	Atris_SH1	Atris_11C, Atris_6A
	Atris_SH2	Atris_5A, Atris_11A
	Atris_SH3	Atris_4A, Atris_8A
	Atris_SH4	Atris_9C, Atris_8C, Atris_9A
	Atris_SH5	Atris_2B, Atris_5B, Atris_10A, Atris_4C, Atris_5C
	Atris_9B	Atris_9B
	Atris_11B	Atris_11B
	Atris_3B	Atris_3B
	Atris_10C	Atris_10C
	Atris_2C	Atris_2C
	Atris_8B	Atris_8B
COI	Apal_SH1	Apal_3D, Apal_3A
	Apal_SH2	Apal_8D, Apal_11B, Apal_3B, Apal_11G, Apal_11D, Apal_3E,
	Apal_4ID	Apal_4ID
	Apal_4IC	Apal_4IC
	Apal_4IE	Apal_4IE
	Apal_8A	Apal_8A
	Apal_8B	Apal_8B
	Apal_8E	Apal_8E
	Apal_SH3	Apal_11C, Apal_11A, Apal_4C
	Atris_SH1	Atris_8A, Atris_4A, Atris_2C

	Atris_SH2	Atris_8C, Atris_9C
	Atris_SH3	Atris_8B, Atris_5A
	Atris_SH4	Atris_10B, Atris_4B, Atris_4C, Atris_2B
	Atris_SH5	Atris_3C, Atris_6A
	Atris_6B	Atris_6B
	Atris_6C	Atris_6C
	Atris_10C	Atris_10C
	Atris_11B	Atris_11B
	Atris_11C	Atris_11C
	Atris_3B	Atris_3B
	Atris_9B	Atris_9B
	Atris_11A	Atris_11A
	Atris_5B	Atris_5B
	Atris_3A	Atris_3A
	Atris_10A	Atris_10A
	Atris_2B	Atris_2B
	Atris_4C	Atris_4C
	Atris_4B	Atris_4B
	Atris_10B	Atris_10B
	Atris_5A	Atris_5A
	Atris_8B	Atris_8B
	Atris_9C	Atris_9C
	Atris_8C	Atris_8C
	Atris_9A	Atris_9A
	Atris_2C	Atris_2C
	Atris_4A	Atris_4A
	Atris_8A	Atris_8A

Table 4 SM. Estimates of evolutionary divergence between 16S sequences, using the Maximum Composite Likelihood model and the gamma distribution as the rate variation model. Samples 1: Lgem_SH1; 2: *Sipunculus nudus*; 3: Atris_4A; 4: Atris_SH1; 5: Atris_11B; 6: Aglaophamus cf. trissophillus; 7: Atris_SH3; 8: Atris_SH2; 9: Atris_8A; 10: Atris_SH4; 11: Chaetozone sp. 12: Apal_4ID; 13: Aphelochaeta marioni; 14: Apal_SH1.

	1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***	***13***	***14***
1														
2	106.73													
3	70.79	86.79												
4	70.87	86.67	0.00											
5	70.70	86.70	0.02	0.01										

6	69.86	86.59	0.02	0.01	0.00								
7	70.70	86.59	0.01	0.01	0.00	0.00							
8	70.79	86.59	0.01	0.01	0.01	0.00	0.00						
9	70.70	86.70	0.01	0.01	0.01	0.01	0.01	0.00					
10	70.61	86.70	0.01	0.01	0.01	0.00	0.00	0.01	0.00				
11	63.41	85.87	94.65	94.76	94.62	94.62	94.62	94.62	94.51	94.51			
12	75.50	96.88	83.53	83.62	83.70	85.80	83.70	83.62	83.53	83.60	0.22		
13	75.99	107.46	75.94	75.85	75.85	75.85	75.85	75.76	75.86	75.94	48.65	45.14	
14	86.64	112.60	99.72	99.83	99.63	102.54	99.63	99.70	99.58	99.51	34.40	36.42	32.01

Table 5 SM. Estimates of evolutionary divergence between 12S sequences, using the Maximum Composite Likelihood model and the gamma distribution as the rate variation model. Samples: 1: Apal_4ID; 2: Apal_SH1; 3: Lgem_SH1; 4: Atris_8B; 5: Atris_SH1; 6: Atris_9B; 7: Atris_11B; 8: Atris_SH2; 9: Atris_SH3; 10: Atris_SH4; 11: Atris_SH5 12: Atris_3B; 13:Atris_10C; 14: Apal_SH1.

	1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***	***13***	***14***
1														
2	2.52													
3	73.35	68.99												
4	36.20	33.69	37.62											
5	35.73	33.58	34.94	0.03										
6	36.92	34.66	36.37	0.03	0.01									
7	36.98	34.72	36.30	0.03	0.00	0.00								
8	37.10	34.61	36.18	0.02	0.01	0.01	0.01							
9	37.22	34.61	36.30	0.02	0.02	0.02	0.02	0.01						
10	38.61	35.85	37.68	0.02	0.02	0.02	0.02	0.01	0.01					
11	37.22	34.61	36.30	0.02	0.02	0.02	0.01	0.00	0.00	0.00				
12	37.16	34.61	36.24	0.03	0.01	0.01	0.01	0.01	0.02	0.02	0.01			
13	37.10	34.61	36.30	0.02	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.00		
14	36.98	34.72	36.18	0.02	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.01	

Table 6 SM: Estimates of evolutionary divergence between COI sequences, using the Tamura 3-parameter model and the gamma distribution as the rate variation model. Samples: 1: Atris_9A; 2: Atris_SH2; 3: Atris_9C; 4: Atris_8C; 5: Atris_6B; 6: Atris_3A; 7: Apal_SH1; 8: Atris_10A; 9: Atris_5B; 10: Atris_SH4; 11: Atris_SH3; 12: Atris_3B; 13: Atris_11A; 14: Atris_6C; 15: Atris_10C; 16: Atris_1C; 17: Atris_3C; 18: Atris_6A; 19: Atris_9B; 20: Atris_11B; 21: Apal_4ID; 22: Apal_8A; 23: Apal_4IC; 24: Apal_4IE; 25: Apal_8E; 26: Apal_SH1; 27: Apal_8B; 28: Apal_SH3; 39: Apal_SH2; 30: Apal_3F.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
1																														
2	0.01																													
3	0.01	0.00																												
4	0.01	0.00	0.00																											
5	0.03	0.02	0.02	0.02																										
6	0.02	0.02	0.02	0.02	0.01																									
7	0.02	0.01	0.01	0.01	0.01	0.00																								
8	0.02	0.01	0.01	0.01	0.01	0.01	0.00																							
9	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.00																						
10	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00																					
11	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00																				
12	0.05	0.04	0.04	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.04																			
13	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.01																		
14	0.05	0.05	0.04	0.04	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.01	0.01																	
15	0.05	0.04	0.04	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.04	0.02	0.01	0.00																
16	0.05	0.05	0.05	0.05	0.04	0.05	0.04	0.04	0.04	0.04	0.04	0.03	0.02	0.02	0.02															
17	0.05	0.05	0.05	0.05	0.04	0.05	0.04	0.04	0.04	0.04	0.04	0.03	0.02	0.02	0.02	0.00														
18	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.02	0.02	0.02	0.00	0.00													
19	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.04	0.04	0.03	0.02	0.02	0.02	0.02	0.01	0.01	0.01												
20	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.02	0.03	0.02	0.01	0.01	0.01	0.01											

21	2.26	2.22	2.28	2.28	2.23	2.20	2.15	2.25	2.25	2.20	2.20	2.19	2.18	2.39	2.33	2.36	2.35	2.30	2.28	2.15												
22	1.77	1.78	1.78	1.78	1.82	1.76	1.80	1.80	1.80	1.76	1.76	1.69	1.65	1.78	1.75	1.80	1.79	1.76	1.71	1.72	0.47											
23	1.72	1.72	1.72	1.72	1.76	1.71	1.74	1.74	1.74	1.71	1.71	1.67	1.60	1.72	1.69	1.74	1.73	1.70	1.66	1.67	0.48	0.03										
24	1.69	1.70	1.70	1.70	1.74	1.68	1.71	1.71	1.71	1.69	1.68	1.65	1.57	1.70	1.67	1.71	1.71	1.68	1.64	1.65	0.47	0.03	0.00									
25	1.78	1.78	1.79	1.79	1.83	1.77	1.80	1.80	1.80	1.77	1.77	1.69	1.65	1.79	1.75	1.80	1.80	1.76	1.71	1.73	0.48	0.02	0.02	0.02								
26	1.75	1.76	1.76	1.76	1.80	1.74	1.78	1.78	1.78	1.75	1.74	1.67	1.63	1.76	1.73	1.78	1.77	1.74	1.69	1.70	0.47	0.02	0.02	0.01	0.01							
27	1.71	1.72	1.72	1.72	1.76	1.71	1.74	1.74	1.74	1.71	1.71	1.63	1.59	1.72	1.69	1.74	1.73	1.70	1.66	1.67	0.45	0.02	0.01	0.01	0.01	0.01						
28	1.70	1.70	1.71	1.71	1.75	1.69	1.72	1.72	1.72	1.69	1.69	1.62	1.58	1.71	1.68	1.72	1.72	1.68	1.64	1.65	0.46	0.02	0.01	0.01	0.01	0.01	0.00					
29	1.71	1.72	1.72	1.72	1.76	1.70	1.74	1.74	1.74	1.71	1.70	1.63	1.59	1.72	1.69	1.74	1.73	1.70	1.65	1.66	0.45	0.01	0.01	0.01	0.01	0.00	0.00	0.00				
30	1.71	1.72	1.72	1.72	1.76	1.70	1.74	1.74	1.74	1.71	1.70	1.63	1.59	1.72	1.69	1.74	1.73	1.70	1.65	1.66	0.45	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00			

4. EXPLORING THE DIVERSITY OF MICROBIOMES ASSOCIATED WITH ANTARCTIC POLYCHAETES FROM DIFFERENT ENVIRONMENTAL SETTINGS

4.1 Introduction

Emerging evidence from invertebrate organisms' investigations has underlined the evolutionary and ecological significance of microbiome assemblages (Bourne et al., 2016; Schuelke et al. 2018). These host-associated microbial communities are generally very diverse and the processes that govern their composition are not well-understood (Adair et al. 2017). In fact, the composition of microbial communities associated with marine organisms is characterized by seemingly contradictory features: strong selection for specific taxa by the host, but substantial variability among hosts and over time within one host (Spor et al. 2011; Reveillaud et al. 2014; Adair et al 2017).

A study carried out on ecto- and endobiotic associations of copepods showed a strong influence of the environmental conditions, especially the availability and type of trophic resources, in selecting different bacterial taxa of their microbiomes (Tang, 2005). Evidences of a key role of external environmental conditions in shaping taxonomic composition of microbiomes was seen also in a study carried out on the scleractinian coral *Seriatopora hystrix*, where the diversity of bacterial assemblages varied among different geographic locations but not among the different genotypes of corals (Pantos et al. 2015).

Other studies carried out on corals highlighting, despite high levels of variability among individuals, a frequent harboring of species-specific associations and a natural "core microbiome" dependent from the host, that can greatly differ from those inhabiting the surrounding environment (Angulo-Preckler et al. 2015; Dunphy et al. 2019). Discovering a core microbiome (i.e., suite of members shared among microbial consortia from similar habitats; Shade et al. 2017) is important for understanding the stable, consistent components across complex microbial assemblages. Movement of microbes, both among host-associated communities and between host-associated and free-living communities, is likely to be key to maintaining microbiome diversity and reducing variation in microbiome composition among individuals (e.g. Moeller et al. 2016). Sometimes, a clear driver that shape the microbiome is really difficult to find: the nematode microbiome profiles demonstrated no correlation with the feeding morphology, phylogeny or morphological identity of the hosts,

neither with the different ocean region or marine habitat types considered (Schuelke et al. 2018).

Moreover, the source of microbiome is an important factor that determines the diversity of microbiomes. Horizontally transmitted bacteria are taken up from the environment anew by each host generation and evidences of this way of transmission were found in numerous organisms (copepods: Moisander et al. 2015; polychaetes: Nussbaumer, et al. 2006; Aida et al. 2008; Vijayan et al. 2019). Nevertheless, bacterial communities associated with the life stages of some marine invertebrates reveal that microbiomes may shift over time, but specific bacterial taxa, perhaps important for the health of the organism, persist across life stages (sponges: Fieth et al. 2016; corals: Lema et al. 2014; anemones: Mortzfeld et al. 2016). These are all examples of vertically transmitted microbiomes, often transferred through the female germ line and can create species-specific assemblages, completely different from free-living bacteria (Bright et al. 2010). These observations suggest that understanding how a host-associated community is assembled requires consideration of several factors, both deterministic and stochastic processes occurring at multiple and local scales (Adair et al. 2017). This could be particularly true in the Antarctic ecosystem, in which the drivers of diversity are unusual and not yet totally known (Chown et al. 2015). In fact, investigation of the high marine endemicity has revealed how a complex set of earth system processes, including glaciation, differential diversification and isolation, has interacted to shape the evolution of the southern biota (Crame, 2013). However, bacteria-invertebrate associations in Antarctica have been rarely investigated and our current knowledge remains quite scarce and fragmentary as it is limited to few organisms (e.g. sponges: Webster et al. 2004, Rodriguez- Marconi et al. 2015; anthozoans: Webster and Bourne 2007, Murray et al. 2016; oligochaetes: Herrera et al. 2017). Nevertheless, the available results suggested a functional role of microbiomes in metabolizing nutrients, in the heavy metal detoxification or in developing antibiotic resistance or adaptation strategy against the harsh temperature of Antarctica (González-Aravena et al. 2016; Herrera et al. 2017; Lo Giudice and Rizzo, 2018). More investigations are needed to understand the mechanisms that govern and shape the microbial associations and that favor the adaptations of marine organisms to environmental conditions and current climate change.

In this contest the specific aims of this work are: 1) to investigate the intraspecific and interspecific variability of biodiversity of microbiomes associated with two different species of polychaetes, *Leitoscoloplos geminus* and *Aphelocaeta palmeri*, collected in three areas of Terra Nova Bay, characterized by different environmental features (e.g., trophic conditions

and sediment characteristics); 2) to compare these microbiomes with the microbial assemblages of surrounding sediments to understand their origin; and 3) to verify if the environmental setting could have a role in shaping the taxonomic composition of microbiomes.

4.2 Materials and methods

4.2.1 Study area and samples collection

Sampling was carried out during the XXXIII Italian Expedition in Antarctica at Terra Nova Bay (Ross Sea) in the framework of the Italian National Program of Antarctic Research (PNRA). Three sampling areas were selected considering different trophic conditions (Tab.1; Fig.1): Adelie Cove (characterized by high organic input due to the presence of penguin assemblages), Rod Bay (characterized by possible anthropogenic impact due to the near Italian research base "Mario Zucchelli Station"), Central Bay characterized by the absence any source of impact).

In each area, samples were collected using a Van Veen Grab (31 x 58 cm) at three different depths (25, 70 and 140 m), with three independent deployments for each depth. Specimens were extracted, sieving the sediment, were preserved in ethanol (95%) and stored at -20°C. Samples of surrounding sediments were collected using plexiglass cores in the same areas and depths of polychaetes and stored at -20°C.

Table 1. Table listing the sampling stations organized by area, depths and geographic coordinates.

Area	Station	Depth (m)	Latitude	Longitude
Adelie Cove	St 9	25	-74,46.467	164,00.266
	St 8	70	-74,46.390	163,57.977
	St 11	140	-74,46.617	164,02.798
Rod Bay	St 5	25	-74,41.831	164,07.532
	St 10	70	-74,41.918	164,07.896
	St 6	140	-74,41.972	164,08.208
Central Bay	St 2	25	-74,43.037	164,06.908
	St 4	70	-74,43.078	164,07.757
	St 3	140	-74,43.101	164,08.399

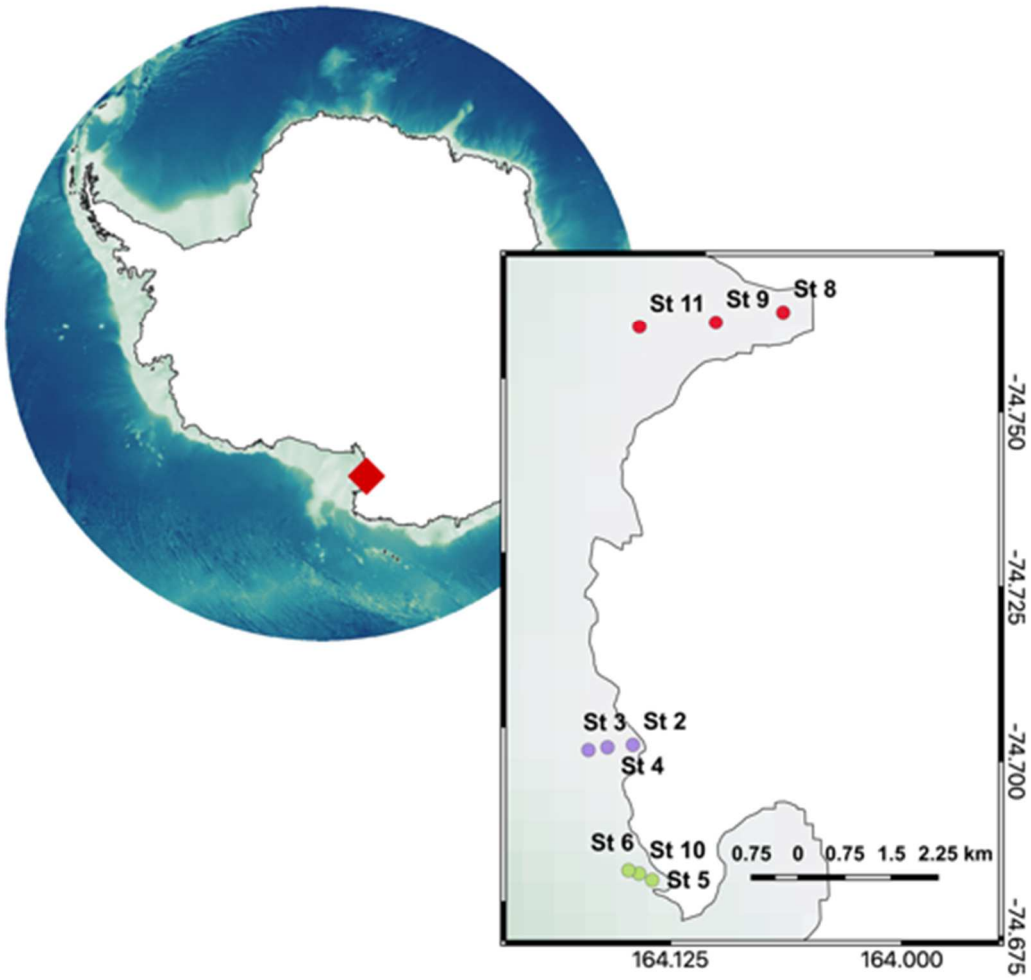


Figure 1. Map of the sampling area where polychaetes and sediments were collected. The dots represent the areas of sampling.

4.2.2. Morphological and molecular identification of polychaetes

Polychaetes selected for this study are a part of polychaetes investigated in Chapter 3. The morphological and molecular methods used for the identification have been described there. Specimens selected for this investigation are shown in Table 2 (the specimen 4ID, turned out to be a potential cryptic species from the results of Chapter 3, was excluded from this investigation).

Table 2. List of polychaetes chosen for this study.

Species	Adelie Cove			Rod Bay		Central Bay	
	25 m	70 m	140 m	25 m	140 m	70 m	140 m
	St. 9	St. 8	St. 11	St. 5	St. 6	St. 4	St. 3
Leitoscoloplos geminus	3		3	3	3		
Aphelochaeta palmeri		3	3			3	5

4.2.3 Extraction of DNA from polychaetes and sediments

The DNA of microbial assemblages was extracted from a whole-body 3 mm-long section of tissue from all polychaetes, using the Qiagen DNeasy Blood and Tissue Kit (Brasier et. 2016) and following the manufacturer's instructions with a modification (incubation with proteinase K at 56°C was extended overnight). Total DNA from the sediments was extracted using the PowerSoil DNA Isolation Kit, following a modified protocol (Danovaro, 2010): an initial treatment with a set of washing solutions and 10 min of incubation at 70°C was carried out in order to achieve a greater extraction efficiency. The washing solutions used are WS1 (50 mM Tris-HCl, pH 8.3; 200 mM NaCl; 5 mM Na2EDTA; 0.05% Triton X-100), WS2 (50 mM Tris-HCl, pH 8.3; 200 mM NaCl; 5mM Na2EDTA) and WS3 (10 mM Tris-HCl, pH 8.3; 0.1 mM Na2EDTA).

4.2.4 Amplification and sequencing of prokaryotic 16SrDNA

PCR amplification was performed on an approximately 550 bp fragment of the 16S rRNA genes, using the primer set Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') and Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') specific for bacteria (Herlemann et al., 2011). The reaction mixture used consisted of 37.5 µl of filtered and autoclaved Milli-Q water, 10 µl of 5x My Taq Reaction Buffer (Bioline), 0.25 µl of each primer (100 µM), 1 µl of My Taq HS DNA Polymerase (5 U/µl concentration), 1 µl of DNA extracted. The thermal cycling consisted in 2 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 53°C, 45 s at 72°C, with a final extension of 5 min at 72°C. Successful DNA amplification was verified by 1% agarose gel electrophoresis using 10.000x GelRed Nucleic Acid Stain (Biotium), 0,4 gr of agarose, 40 ml of TE Buffer for the gel preparation, and 2 µl of 5x

GelPilot DNA Loading Dye (Qiagen), 2 µl of GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) for the electrophoresis. The amplified DNA was sequenced on an Illumina MiSeq sequencer using the V3 technology (2x300 bp) with primers targeting Bacterial V4 region (Klindworth et al., 2013) at LGC Genomics.

4.2.5 Biochemical composition of sediment organic matter

Sediments were analyzed for total phytopigment, protein, carbohydrate and lipid contents according to Danovaro (2010). Chlorophyll-a and phaeopigments (used as proxies of primary organic material associated with primary producers) were analyzed fluorometrically (Lorenzen and Jeffrey, 1980) and total phytopigment contents defined as the sum of chlorophyll-a and phaeopigment concentrations. Total phytopigment contents were utilized as an estimate of the organic material of algal origin, including the living (chlorophyll-a) and senescent/detrital (i.e., phaeopigments) fractions (Pusceddu et al., 2009). Sediment phytopigment concentrations were converted into carbon equivalents using a mean value of 40 µgC phytopigment μg^{-1} (Pusceddu et al., 2009). Protein, carbohydrate and lipid contents were determined spectrophotometrically and their sum, once converted into C equivalents (using 0.49, 0.40 and 0.75 $mgCmg^{-1}$, respectively, as conversion factors), referred as the biopolymeric C (BPC; Pusceddu et al., 2000; Danovaro, 2010).

4.2.6 Bioinformatic and statistical analysis

Raw sequences were analyzed through the QIIME2 pipeline (version 2019.4; https://qiime2.org/). Paired-end sequence files loaded and sequences pairs analyzed by means of the DADA2 plugin (Callahan et al., 2016), which infers community composition in each sample by partitioning sequences according to the respective error models, thus filtering for erroneous reads and chimeras and resolving minimal variations between prokaryotic taxa. Paired sequences were then merged by the pipeline before producing an Amplicon Sequence Variant (ASV) table. From the ASV table obtained, each sample was subsampled to 700 sequences, thus obtaining a normalized ASV table. The subsampling depth was chosen as a compromise between the highest number of sequences that fully described the biodiversity of samples and the lowest loss of samples. Four specimens of *A. palmeri* from Central Bay at 140m (4IC, 4A) and at 70m (3IG, 3A), and two specimens of *A. palmeri* from Adelie Cove at 70m (8A) and 140m (11B) were discared because they were charcterized by < 700 sequences. The normalized ASV table was used for the calculation of

rarefaction curves and as input for the subsequent analyses, such as the determination of α and β diversity indices (Shannon and Evennes indices, Bray curtis dissimilarity and Unweighted Unifrac distance). To infer the taxonomic affiliation of ASVs, a taxonomic classifier was first trained on the SSU region amplified by the primers utilized in the present study on the SILVA reference database v132 (Quast et al. 2012); the classifier was then used on the ASVs identified (Bokulich et al. 2018). Significant differences in the richness and in the taxonomic composition of microbiomes were highlighted through a permutational analysis of variance (PERMANOVA), a classification-clustering based on the Bray Curtis similarity of transformed quantity data and a SIMPER analysis, all included in the PRIMER-E 6 software (Anderson et al., 2008). The construction of a heatmap (QIIME2 pipeline plug-in) based on taxonomic composition and phylogenetic distance of microbiomes were added to better investigate the differences in microbiomes. Finally, analysis of distance-based linear models (DistLM), included in the PRIMER-E 6 software, was used to investigate relationship among the environmental variables considered in this study (e.g. organic matter composition) and microbiomes composition to identify potential drivers shaping prokaryotic assemblage structures.

4.3 Results

4.3.1 α and β diversity of microbial assemblages associated with polychaetes

The rarefaction curves showed that, considering the same number of sequences for all the samples, all the curves of microbiomes associated with polychaetes reached a plateau (Fig.2). Total ASV richness was calculated from each sample and validated with statistical analysis (Fig.3; Tab. 1 SM). The number of ASVs varied from 14 to 59 in *Leitoscoloplos geminus* and from 11 to 56 in *Aphelocaeta palmeri*. In each sampling area the number of ASVs varies from 14 to 59, from 11 to 56, from 12 to 50 in Rod Bay, Central Bay and Adelie Cove, respectively. Non-significant differences in terms of ASV richness were found both among polychaetes and in relation to the different sampling areas. Polychaetes collected at 140m in Adelie Cove presented higher number of ASVs than those collected in the other stations.

Similar results were obtained considering the Shannon and the Evenness indices with high variability among specimens within the same species, between the two species and among the different sampling areas, with no significant differences (Fig.4).

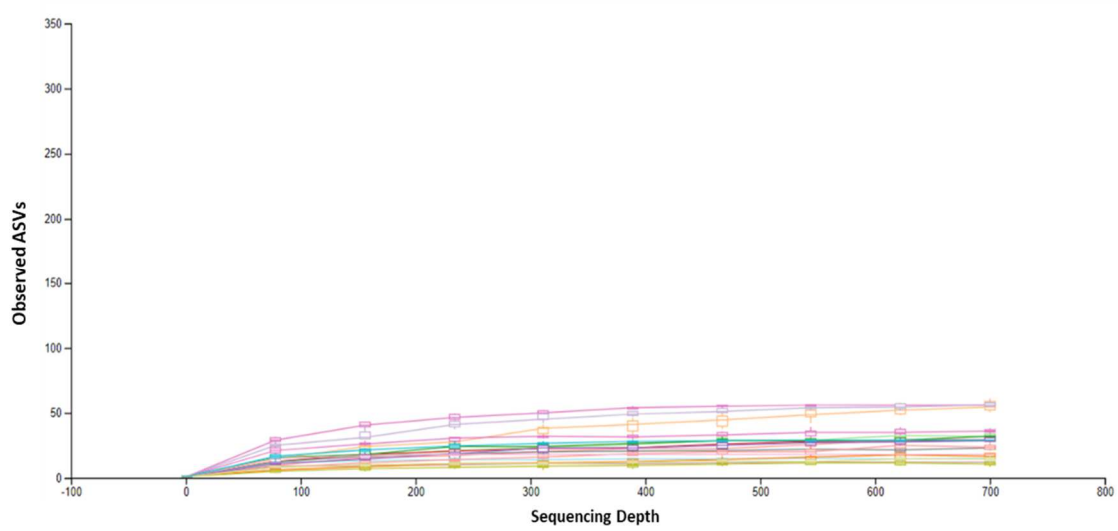


Figure 2. Rarefaction curve of microbiomes associated with polychaetes after a normalization of 700 sequences for each sample.

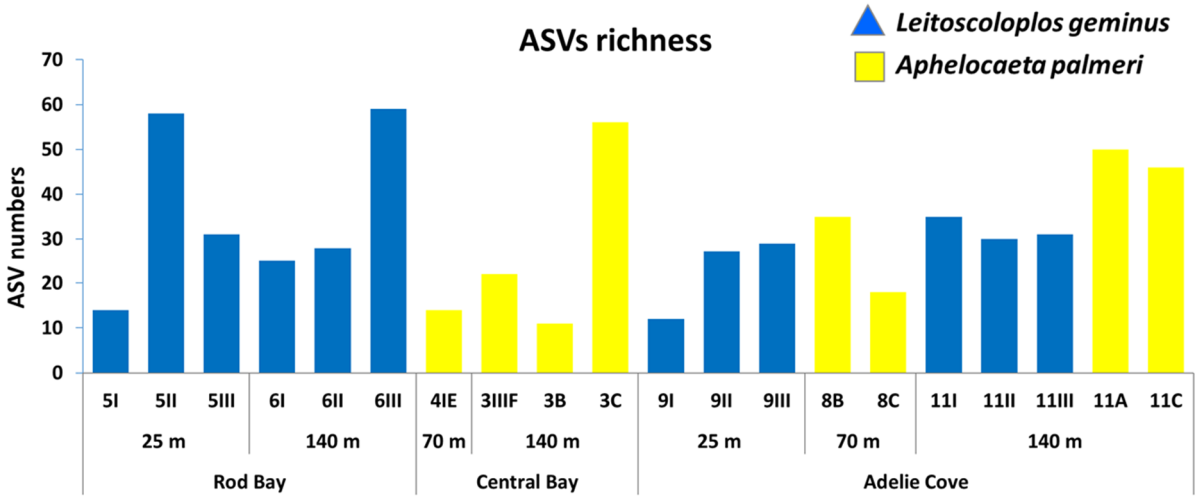


Figure 3. Number of ASVs observed in polychaetes in the three areas and at the three different depths considered. The blue and the yellow bars refer to *Leitoscoloplos geminus* and *Aphelocaeta palmeri* microbiomes, respectively.

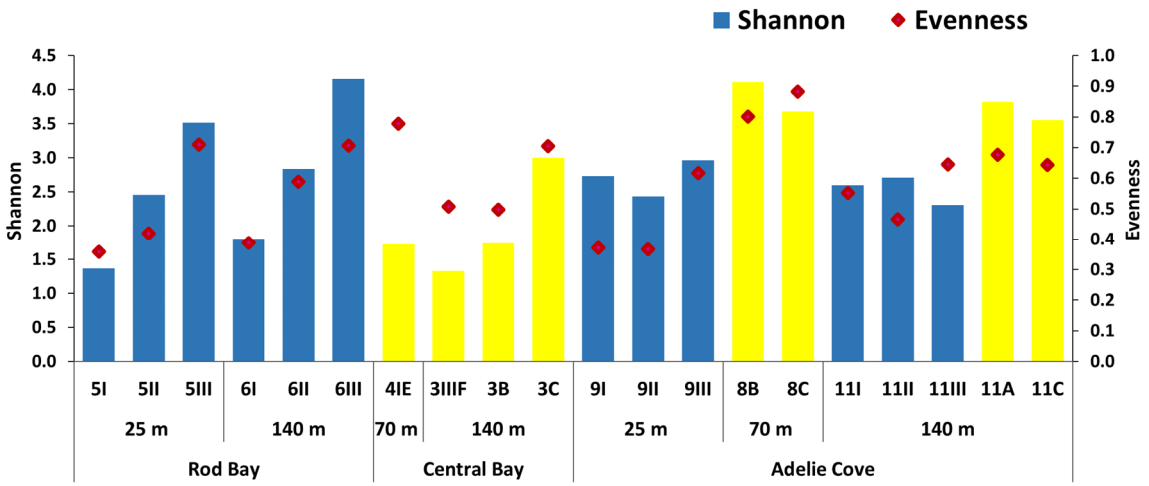
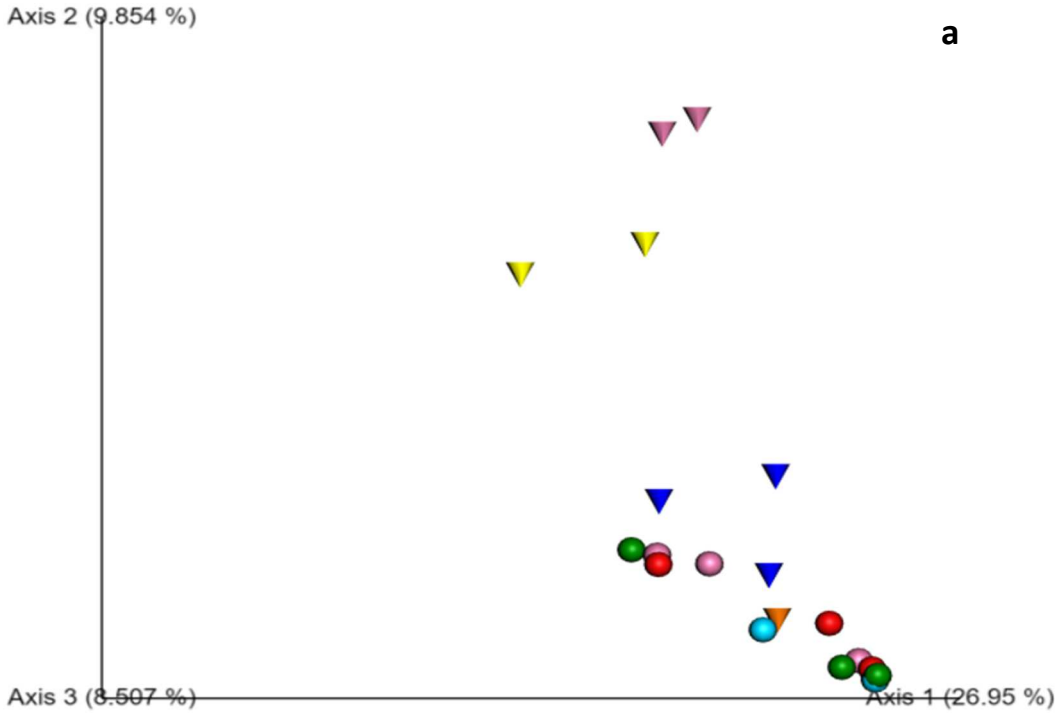


Figure 4. Shannon and evenness indices of microbial community associated with polychaetes in the three areas and at the three different depths considered. The blue and the yellow bars refer to *Leitoscoloplos geminus* and *Aphelocaeta palmeri* microbiomes, respectively.

The Beta diversity analysis revealed significant differences both in terms of composition and phylogenetic diversity of microbial communities between the two species of polychaetes. No significant differences were found among different sampling areas and different depths (Fig.5 a- b; Tab. 1 SM).



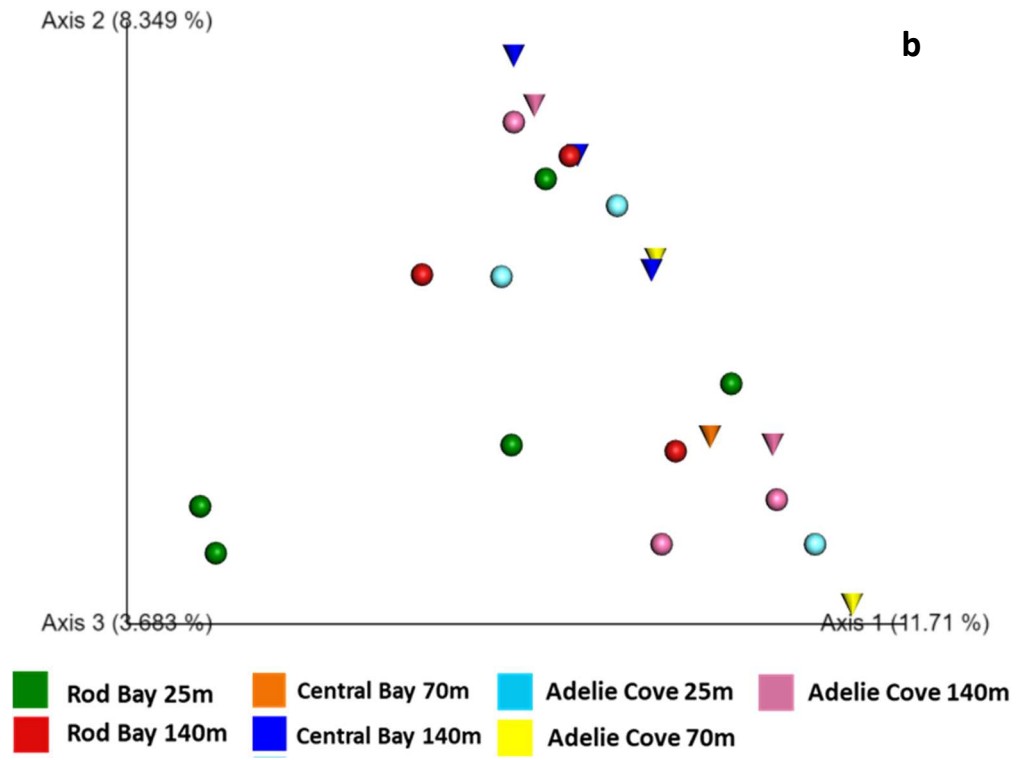


Figure 5. PCoA plot of Beta-diversity of microbiomes among polychaetes carried out on Bray Curtis dissimilarity (a) and unweighted UniFrac distance (b). *Spheres* referred to *L. geminus* individuals and *Cones* to *A. palmeri.*

4.3.2 Taxonomic composition of microbiomes associated with polychaetes from different environmental settings

Results of the analysis carried out on the taxonomic composition of microbiomes revealed that, among a total of 128 different families found only 3 are shared in all samples: the *Thermaceae, Bacillaceae* and the *Propionibacteriaceae* families. The *Thermacea* family, totally represented by bacteria belong to Meiothermus genus, is present with percentages ranging from 16% to 68% and from 2% to 68% in *Leitoscoloplos geminus* and *Aphelocaeta palmeri*, respectively. Percentages from 4% to 33% and from 4% to 25% of the *Bacillacea* family and from 0,2% to 2% and from 0,4% to 4% of *Propionibacteriaceae* family were found in *Leitoscoloplos geminus* and *Aphelocaeta palmeri*, respectively (Fig.6). These core families represent on average the 65% and the 48% of the total composition of microbiomes in *Leitoscoloplos geminus* and *Aphelocaeta palmeri*, respectively. Moreover, 9, 4 and 3 bacterial families are shared in all polychaetes collected at Rod Bay, Central Bay and Adelie Cove, respectively, representing their specific core microbiomes.

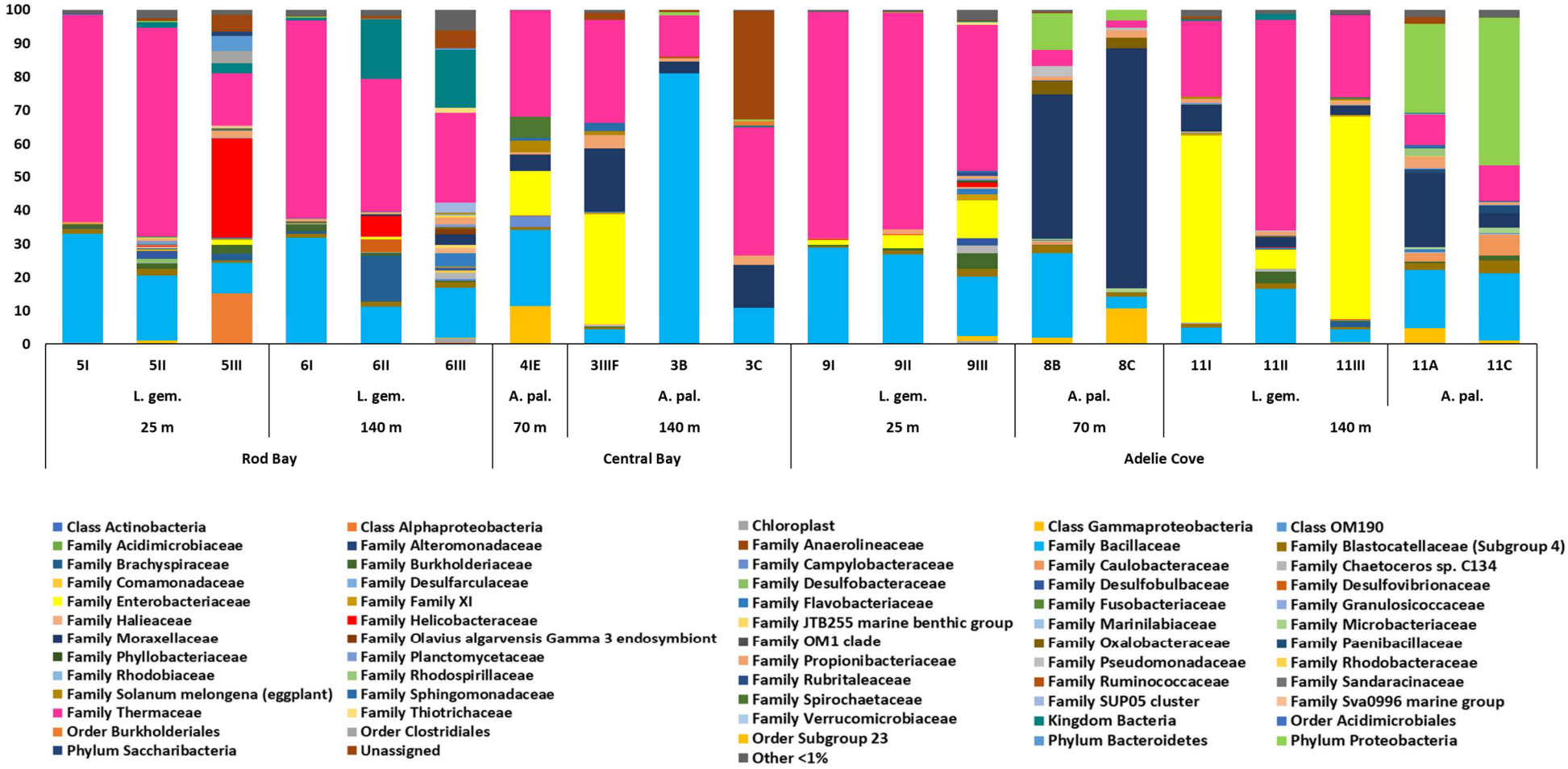


Figure 6. Taxonomic composition of microbiomes associated with polychaetes in the three areas and at different depths.

Microbiomes' composition in individuals of *Leitoscoloplos geminus*

The Simper analysis carried out on the taxonomic composition of microbiomes among *Leitoscoloplos geminus* individuals revealed a similarity of 63%, explained by the presence of a core microbiome that represent on average the 68% of the total microbiomes. The core microbiome is composed by 5 bacterial families: the *Thermacea* and *Bacillacea* families, describing the 94,5% of the core and the *Propionibacteriaceae, Burkholderiaceae* and *Blastocatellaceae* families describing the remaining 5,5 % (Fig.7).

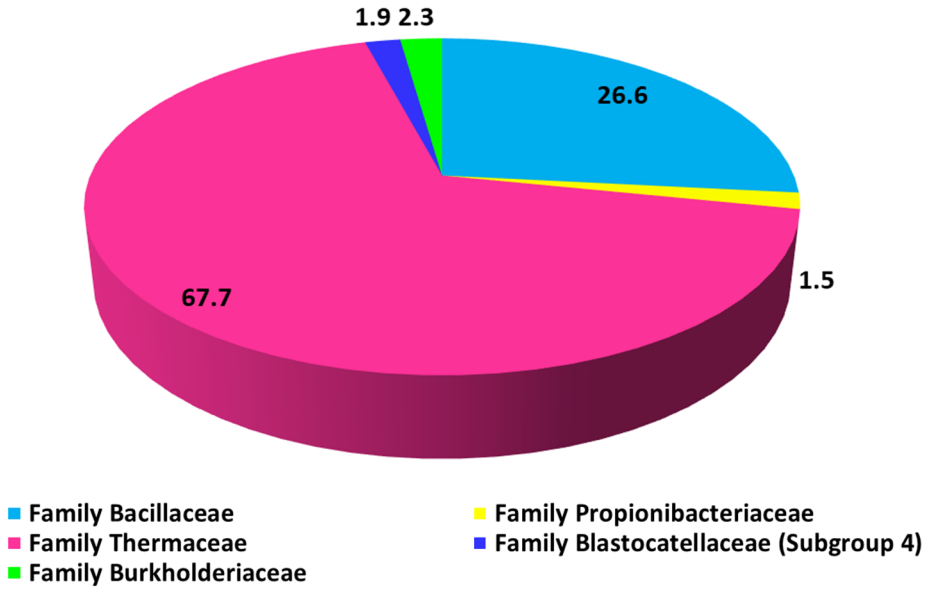


Figure 7. Taxonomic composition of the core microbiome of *Leitoscoloplos geminus*.

Cluster analysis revealed no similar patterns in the taxonomic composition among microbiomes associated with individuals collected in the same areas and at the same depths, with some exceptions, the two individuals collected at 25m (Lgem_9I, Lgem_9II) and 140m (Lgem_11I, Lgem_11III) of Adelie Cove (Fig.8). Same results were found also considering the taxonomic composition of microbiomes at ASV level.

Simper analysis revealed similarity among polychaetes collected at the same station of 58%, 61%, 71% and 72%, at 25m and 140m of Rod Bay and at 25m and 140m of Adelie Cove, respectively. Among a total of 128 bacterial families found in the specimens of *L. geminus* 16, 13, 7, 13 families compose the core microbiomes of specimens collected at 25 and 140m of Rod Bay and 25m and 140m of Adelie Cove, respectively (Figure 9 a – b – c - d).

Moreover, 20, 14, 5 and 7 bacterial families were exclusively found at 25m and 140m in Rod Bay and at 25m and 140m of Adelie Cove, respectively.

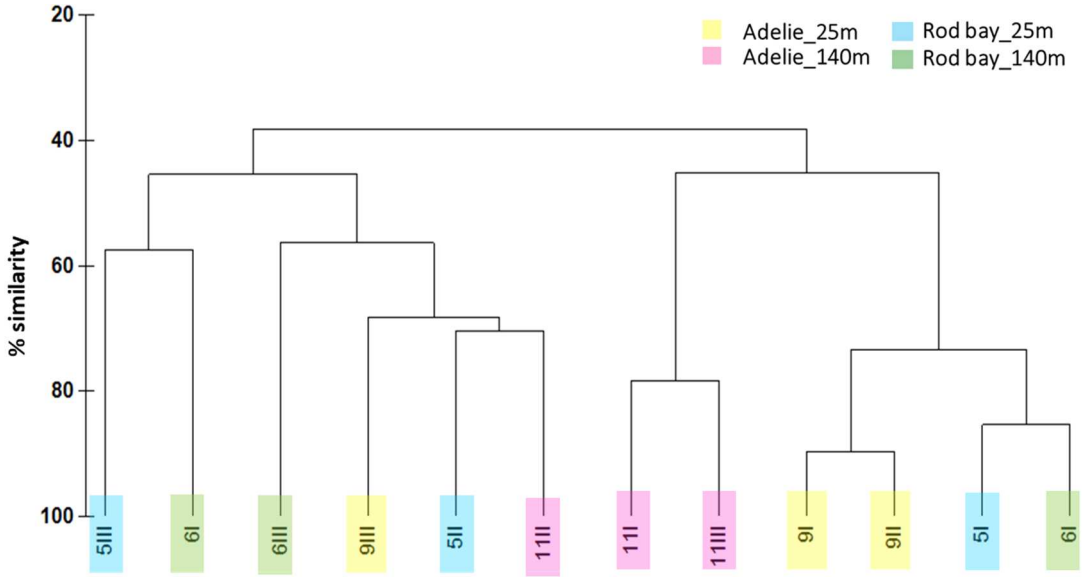


Figure 8. Cluster analysis comparing taxonomic composition of microbiomes associated with individuals of *Leitoscoloplos geminus* in the three areas and at different depths.

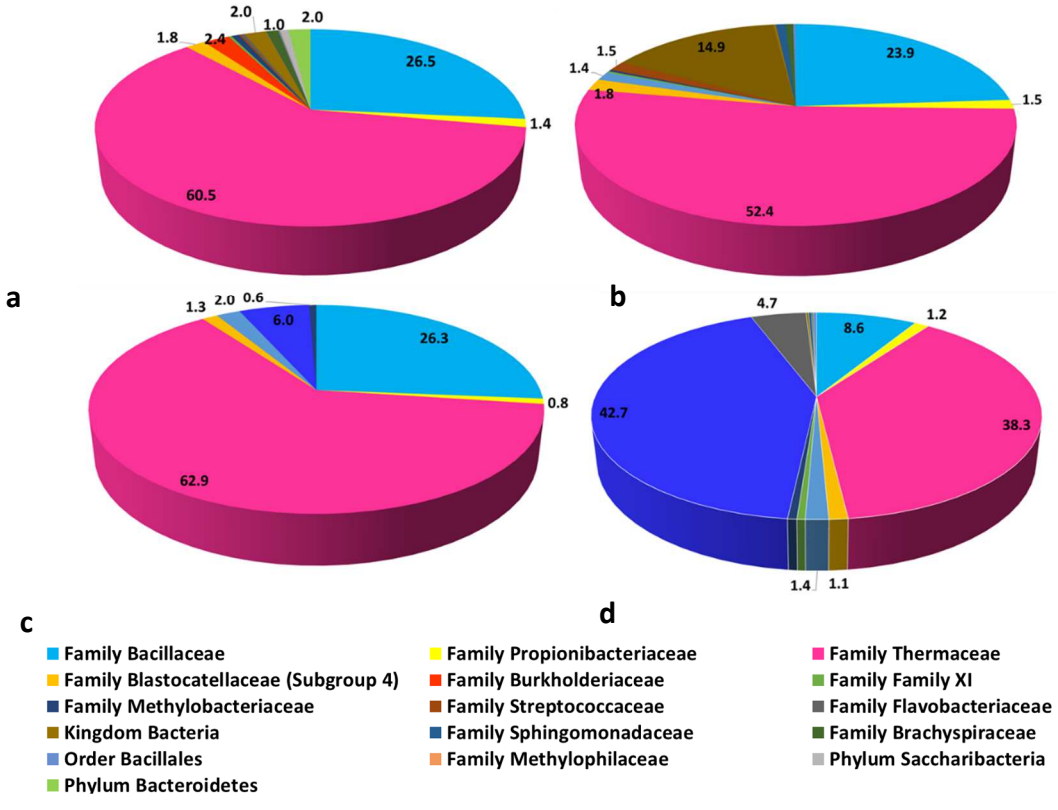


Figure 9. Taxonomic composition of the core microbiomes of *Leitoscoloplos geminus* collected at 25 m (a) and 140 m (b) of Rod Bay and at 25m (c) and 140 m (d) of Adelie Cove.

Microbiomes' composition in individuals of *Aphelochaeta palmeri*

The Simper analysis carried out on the taxonomic composition of microbiomes among *Aphelochaeta palmeri* individuals revealed a similarity of 55%, explained by the presence of a core microbiome that represent on average the 65% of the total microbiomes. The core microbiome is composed by 4 bacterial families: the *Thermacea*, *Bacillacea* and *Moraxellaceae* families, equally describing the 97% of the core and the *Propionibacteriaceae* family describing the remaining 3 % (Fig.10).

Cluster analysis carried out on the taxonomic composition of microbiomes associated with individuals belong to *Aphelochaeta palmeri* revealed similar pattern in specimens collected in the same area and at the same depth, except for the specimen 3IIF that clustered a part, showing a higher similarity with the individual belonging to the same area (Central Bay) but collected at 70 m (Fig.11). Same results were found also considering the taxonomic composition of microbiomes at ASV level.

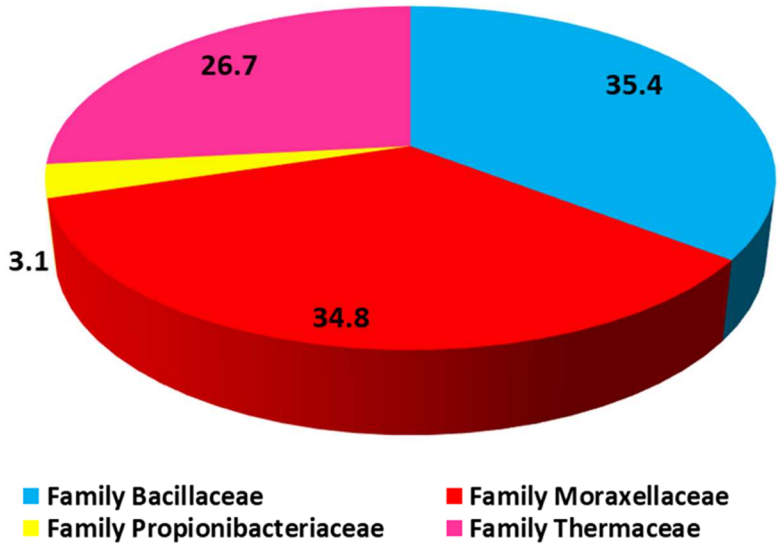


Figure 10 Taxonomic composition of the core microbiome of *Aphelocaeta palmeri.*

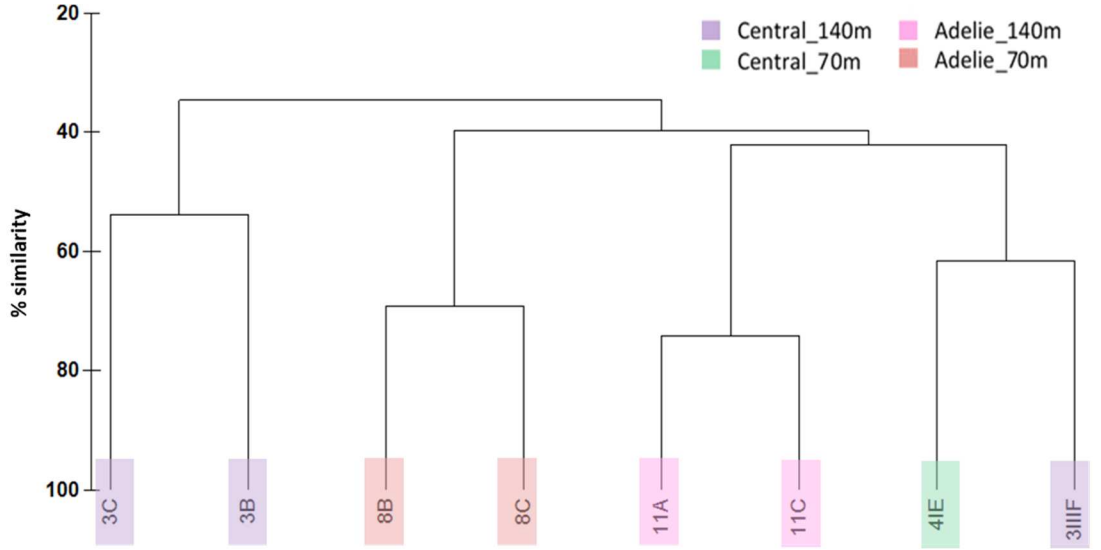


Figure 11. Representation of results of cluster analysis comparing taxonomic composition of microbiomes associated with individuals of *Aphelocaeta palmeri* in the three areas and at different depths.

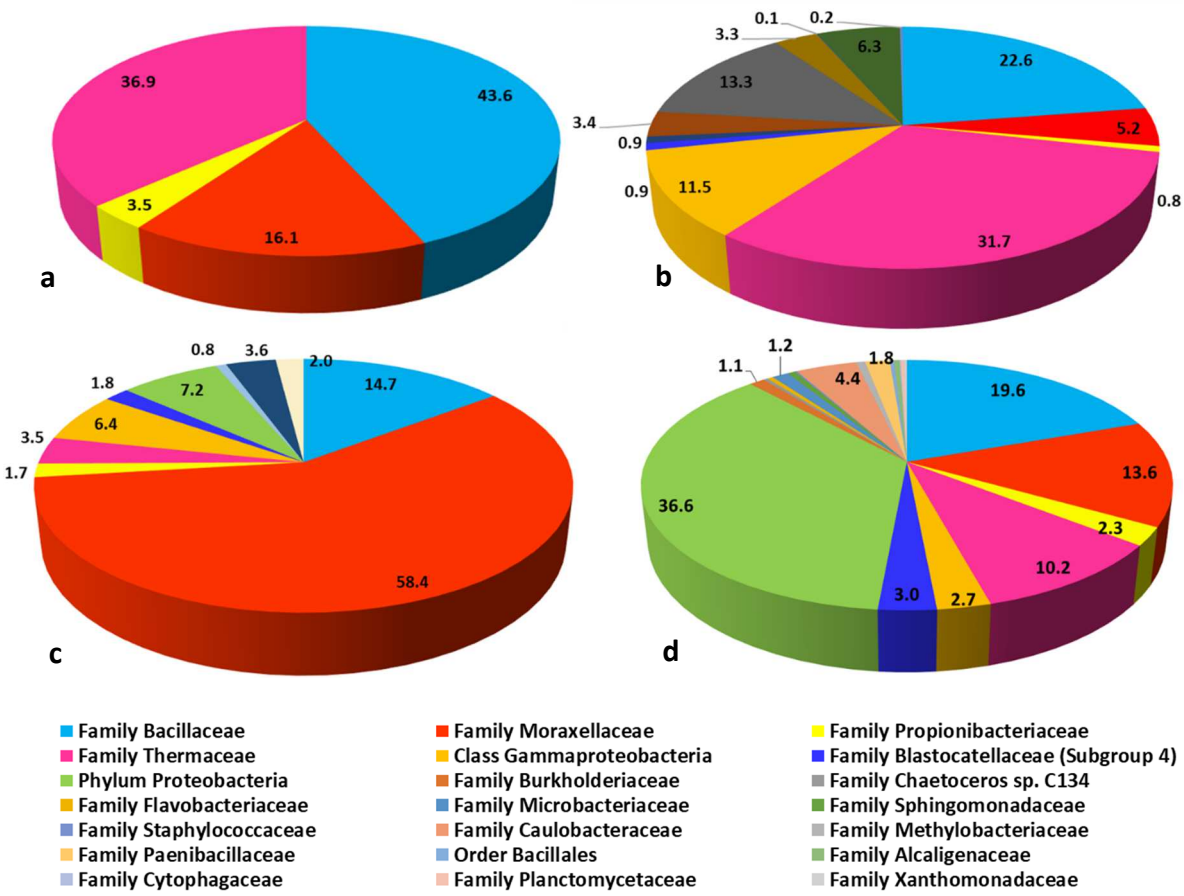


Figure 12. Taxonomic composition of the core microbiomes of *Aphelochaeta palmeri* collected at 70 m (a) and 140 m (b) of Central Bay and at 70 m (c) and 140 m (d) of Adelie Cove.

Simper analysis revealed similarity among polychaetes collected at the same station of 54%, 61%, 71% and 79%, at 70m and 140m of Central Bay and at 70m and 140m of Adelie Cove, respectively. Among the 59 families found in the specimens of *A. palmeri*, 4, 11, 10, 21 families compose the core microbiomes of polychaetes collected at 70 m and 140 m of Central Bay and at 70 m and 140 m of Adelie Cove, respectively (Figure 12 a – b – c - d).

Moreover, 7, 20, 3 and 4 bacterial families were exclusively found at 70m and 140m in Central Bay and at 70m and 140m of Adelie Cove, respectively.

Comparison between microbiomes associated with two species of polychaetes

Simper analysis carried out on the taxonomic composition of microbiomes among the two species of polychaetes revealed a similarity of 55%, explained by the presence of the core *Thermaceae, Bacillaceae* and *Propionibacteriaceae* families described above. Despite this core, significant differences in the taxonomic composition of microbiomes between the two species were found and evident also in the MDS analysis (Fig.13; Tab. 1 SM).

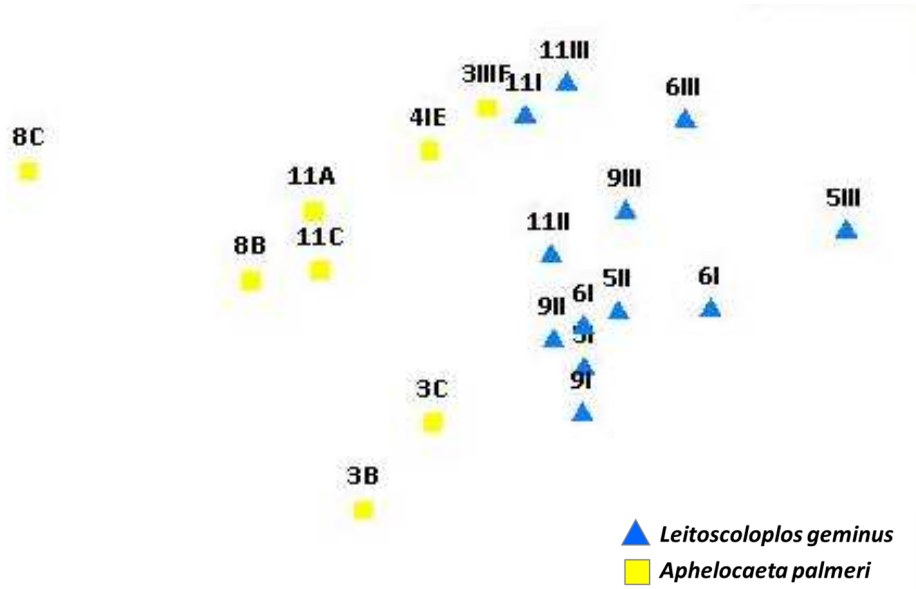


Figure 13. MDS analysis comparing taxonomic composition of microbiomes associated with the two species of polychaetes.

In fact, each species is characterized by different contributions of bacterial families: the *Thermaceae* family with the 46% and the 18%, the Enterobacteriaceae family with the 12%

and the 6%, the *Helicobacteraceae* family with the 3% and the 0.01% of the total microbiome in *L. geminus* and *A. palmeri,* respectively; *Moraxellaceae* family with 1.4% and the 23%, the *Proteobacteria* phylum with the 0.1% and 11% of the total microbiome in *L. geminus* and *A. palmeri,* respectively. Unassigned ASVs were found in the two species of polychaetes with different contributions, 1% in *L. geminus* and the 5% in *A. palmeri.* Exclusive bacterial families in each species of polychaetes were not found.

4.3.3 Environmental characteristics

Biochemical composition of sediment organic matter

Concentrations of total phytopigments vary between 15.3 ± 6.9 µg*g^{-1} and 126.9 ± 23.9 µg*g^{-1} in the sediments of Adelie Cove at 25 m and 70 m, respectively. No significant differences were found between Central Bay and Rod Bay, but are present within Adelie Cove among the three different depths and between Adelie Cove and the other two areas, The lowest and the highest values of total phytopigments concentration were found at 25 m and at 70 m of Adelie Cove, respectively.

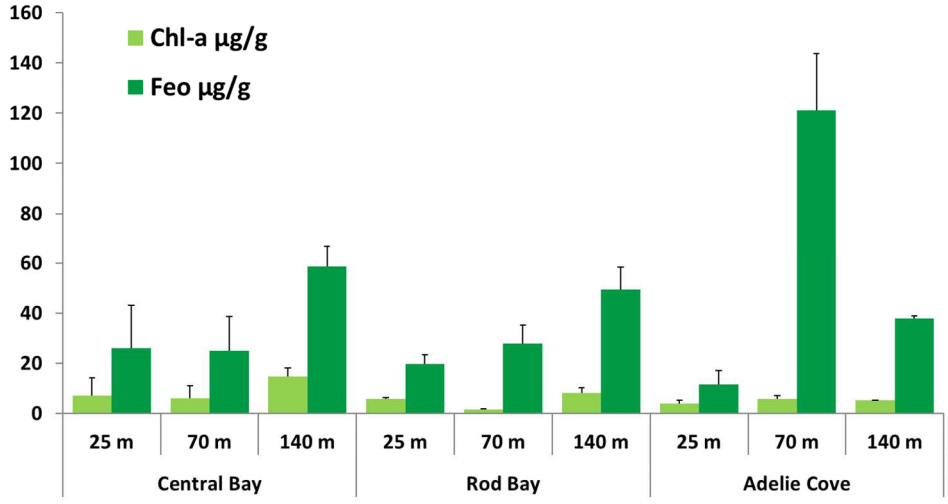


Figure 12. Chlorophylla-a (Chl-a) and pheopigments (Feo) concentrations (ug/g) measured in the three areas.

Concentrations of total proteins vary between 0.61±0.1 mg g^{-1} and 4.1± 1.3 mg g^{-1}, of total carbohydrates between 0.24±0.08 mg g^{-1} and 4.0±0.83 mg g^{-1} m and of total lipids vary between 0.15±0.04 mg g^{-1} and 1.6±0.4 mg g^{-1}, in the sediments of Adelie Cove at 25 m and

70 m, respectively. No significant differences are found between Central Bay and Rod Bay but are present in the concentrations among the three depths in Adelie Cove and between Adelie Cove with lower values at 25m and higher at 70m than those in other two areas (Tab. 1 SM).

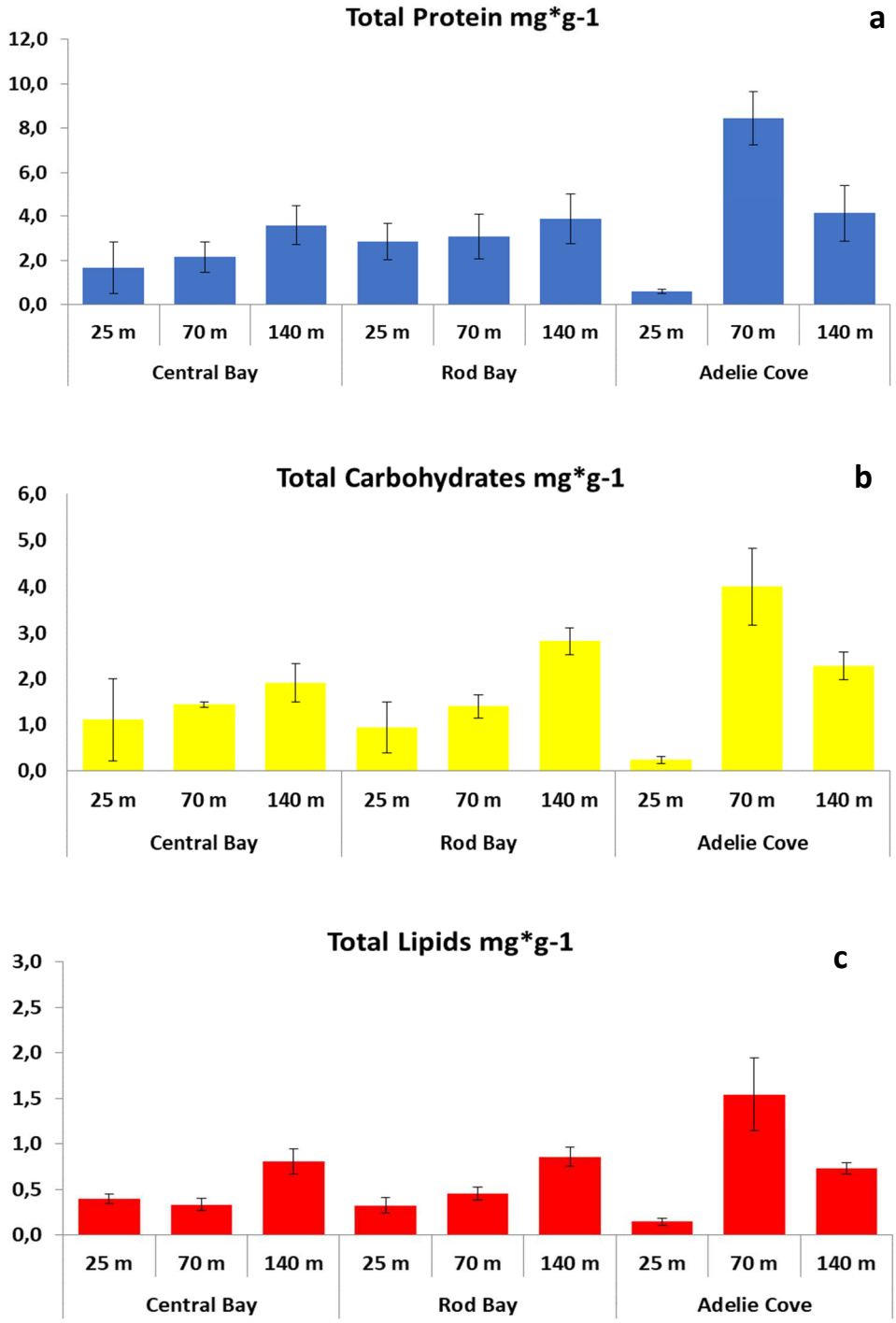
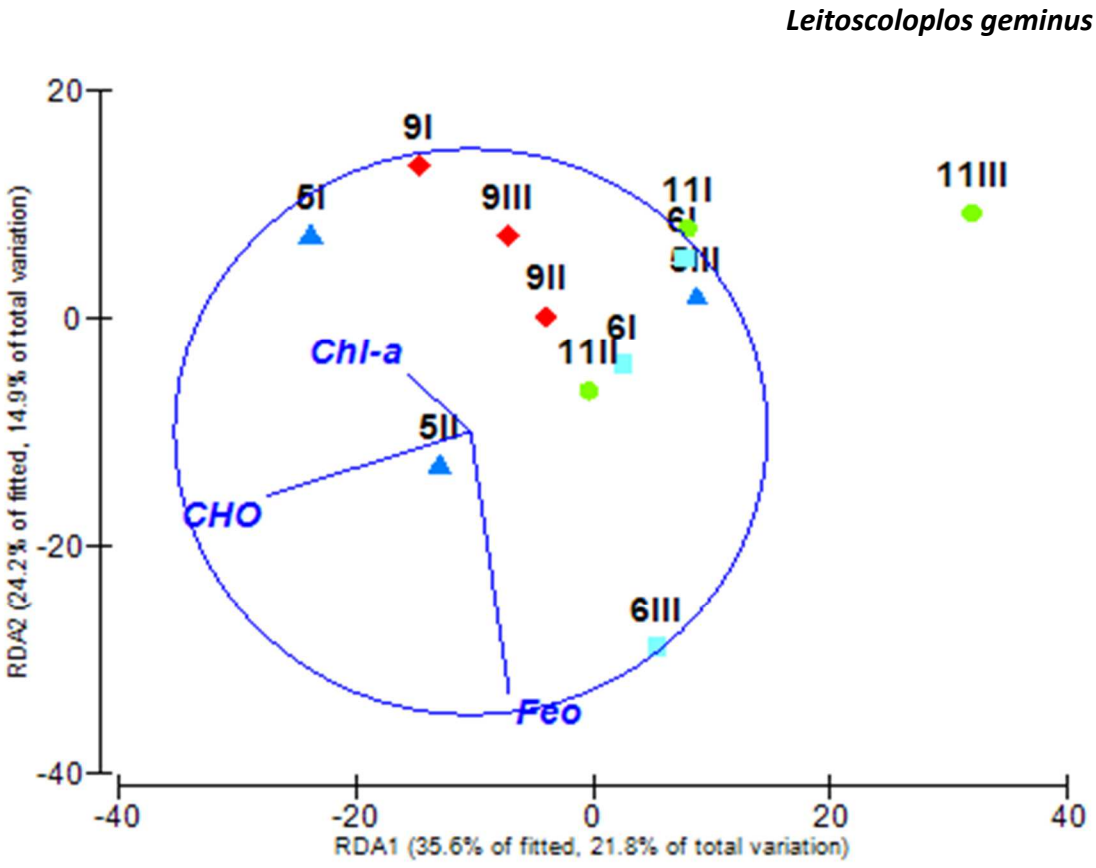


Figure 13. Total protein (a), carbohydrates (b), lipids (c) concentrations (mg/g) measured in the three areas.

Relationship between trophic conditions of the areas and microbiomes community structure

The DistLM analysis carried out on the taxonomic composition of microbiomes associated with polychaetes revealed a significant effect of the different trophic conditions in microbial assemblages associated in the individuals of *A. palmeri* (48% of the total variation explained). In particular, the chlorophylla-a, pheopigments and carbohydrates concentrations are the variables of the biochemical composition that drive this statistical significance, explaining the 26%, 18% and 4% of the total variation, respectively.

No significant role of trophic conditions in explaining the variation in the taxonomic composition of microbiomes in the individuals of *L. geminus.*



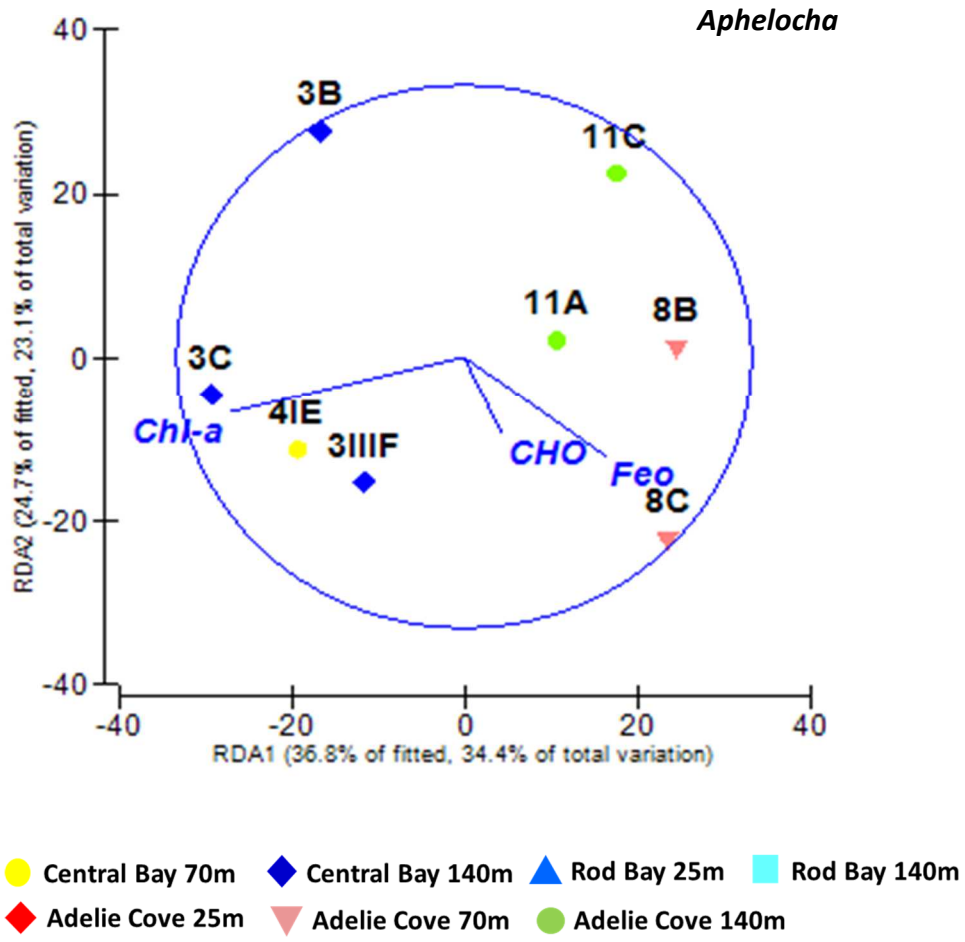


Figure 15. dbRDA on the Distlm analysis between the environmental factors and the taxonomic composition of microbiomes associated with polychaetes.

4.3.3. Comparison of microbiomes associated with polychaetes and those living in surrounding sediments

Significant differences were found between microbiomes of polychaetes and of surrounding sediments both in terms of ASV richness and taxonomic composition (Tab. 1 SM). In fact, the number of ASVs varies from 11 to 59 and from 110 to 299 in the microbiomes associated with polychaetes and surrounding sediments, respectively, highlighting a richness up to ten times higher in the sediments than in polychaetes (Fig.16). Higher number of ASVs were found in the sediments at 140m than at more shallow depths in each area, except for Adelie Cove in which the highest number was found at 70m.

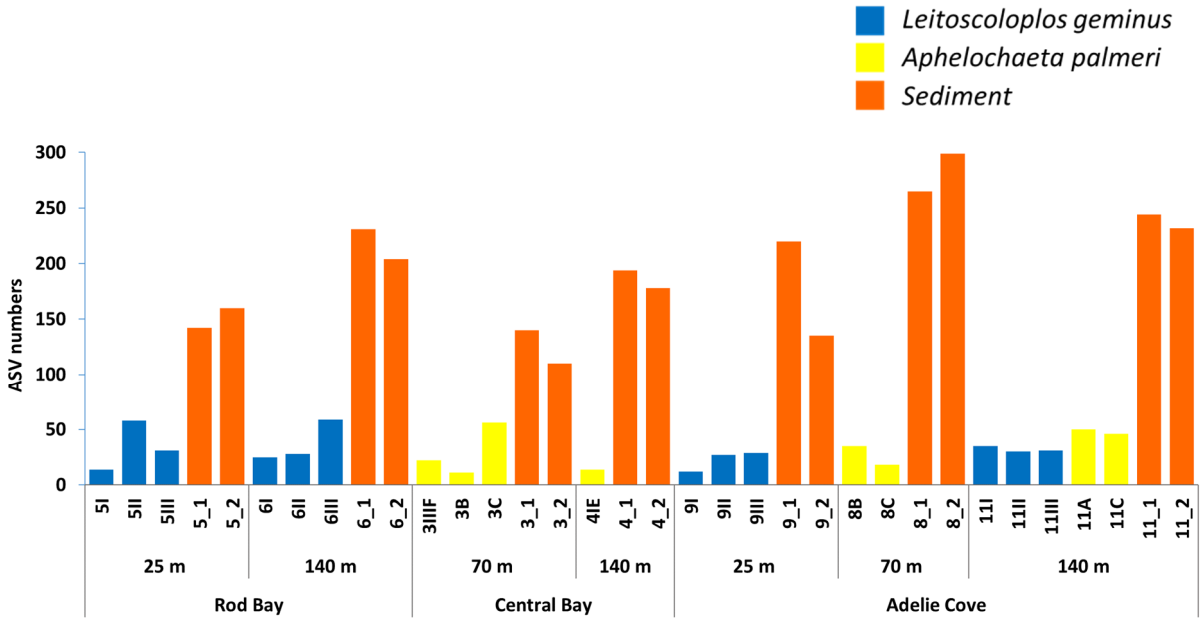


Figure 16. Number of ASVs observed in polychaetes and sediments in the three areas and at the three different depths considered.

Results of analysis on the β-diversity of microbial communities showed total dissimilarity between sediment and polychaetes, both in terms of composition and phylogenetic diversity (Fig.17 a- b; Tab. 1SM). In fact, among the 305 total families that described the taxonomic composition of microbiomes, no one is shared among all samples of sediments and polychaetes. Only 3 families are in common with the 80% of samples. These are the *Flavobacteriaceae*, *Propionibacteriaceae* and *Blastocatellaceae* families: the first is present in polychaetes with an average abundance of 0,4% against the 12% in sediments; the last two are present in the polychaetes with an average abundance of 1,4% and 1,3% against the 0,03% and the 0,04% in sediments, respectively (Fig. 18). Very low contribution of *Thermaceae*, *Bacillaceae* and *Propionibacteriaceae* families, the core families among polychaetes, was found to the bacterial assemblages in the sediment samples (< 0,1%). Moreover, simper analysis highligheted a 77% of dissimilarity between polychaetes and sediments in the taxonomic composition of microbiomes. The taxonomic composition of bacterial assemblages living in the sediments showed a similarity of 77% considering the same area and the 85% considering the same station.

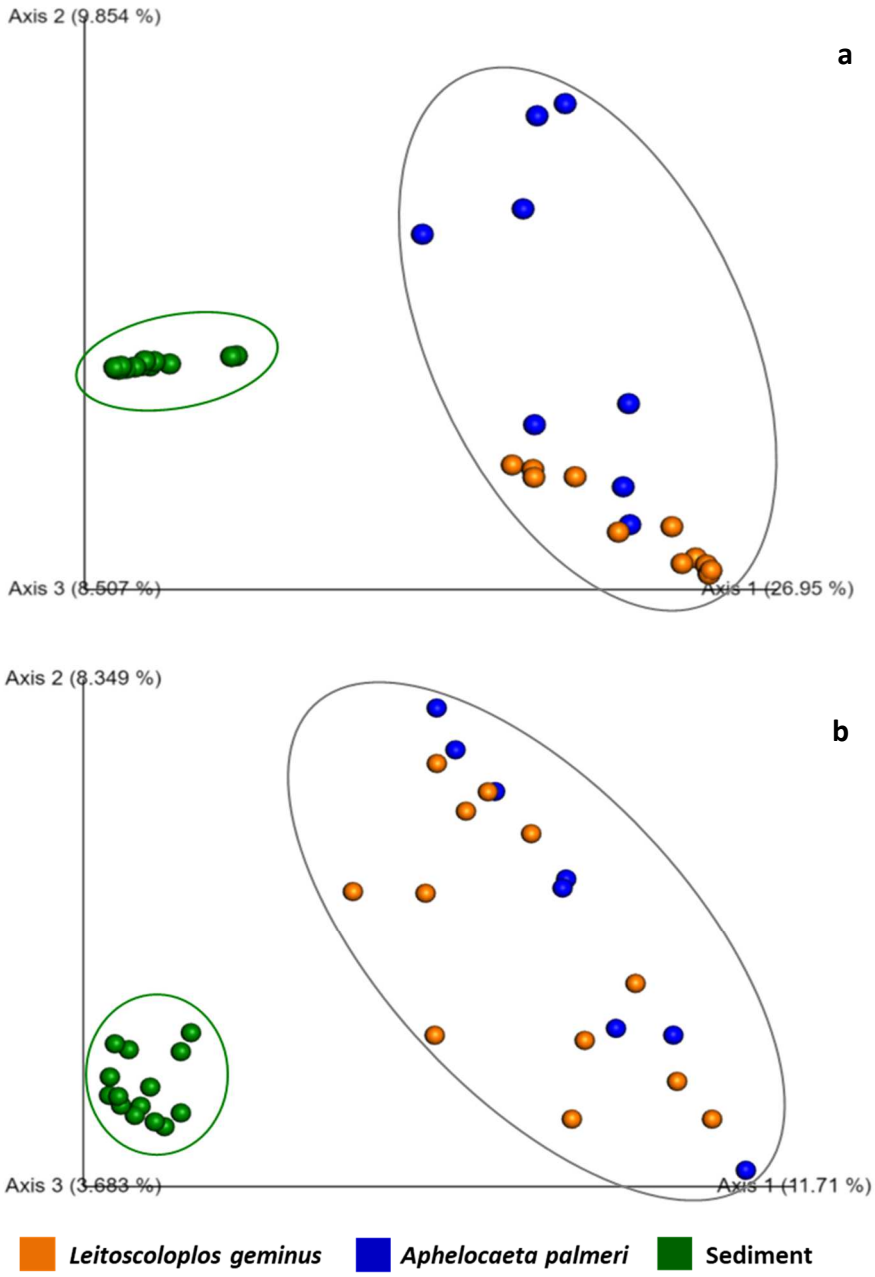


Figure17. PCoA plot of β-diversity among polychaetes and surrounding sediments microbiomes, measured by Bray Curtis dissimilarity (a) and unweighted Unifrac distance (b).

Results were further validated by the construction of a heatmap based on both the taxonomic composition and the phylogenetic distances of microbiomes associated with polychaetes and with surrounding sediments (Fig.19). The graduation of colors shows the differences in the frequency of each ASV in the samples. Polychaetes were characterized by the presence of ASVs that are completely absent or present with a low frequency in microbiomes associated with sediments and vice versa.

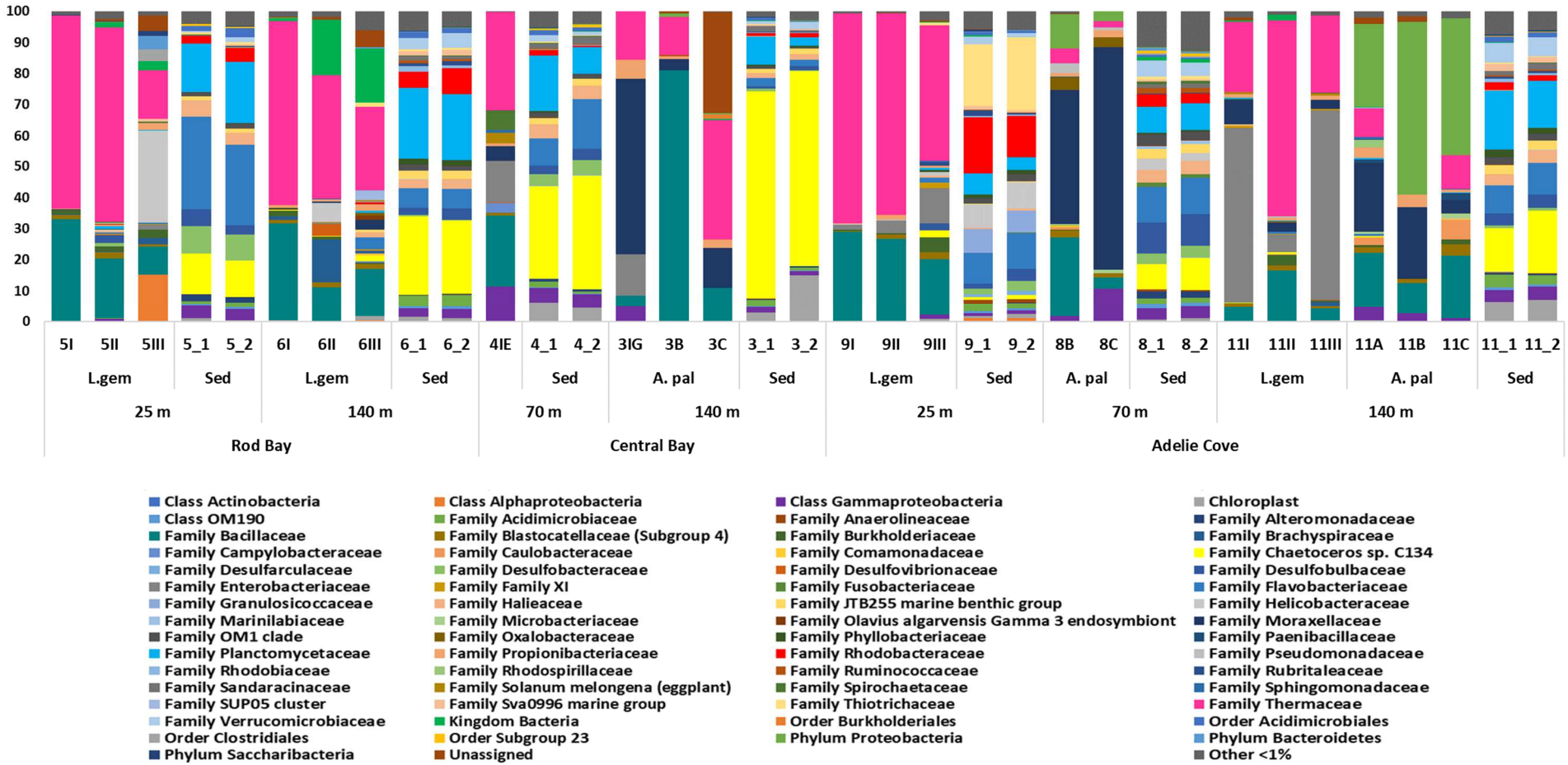


Figure 18. Taxonomic composition of microbiomes associated with polychaetes and sediments in the three areas and at different depths.

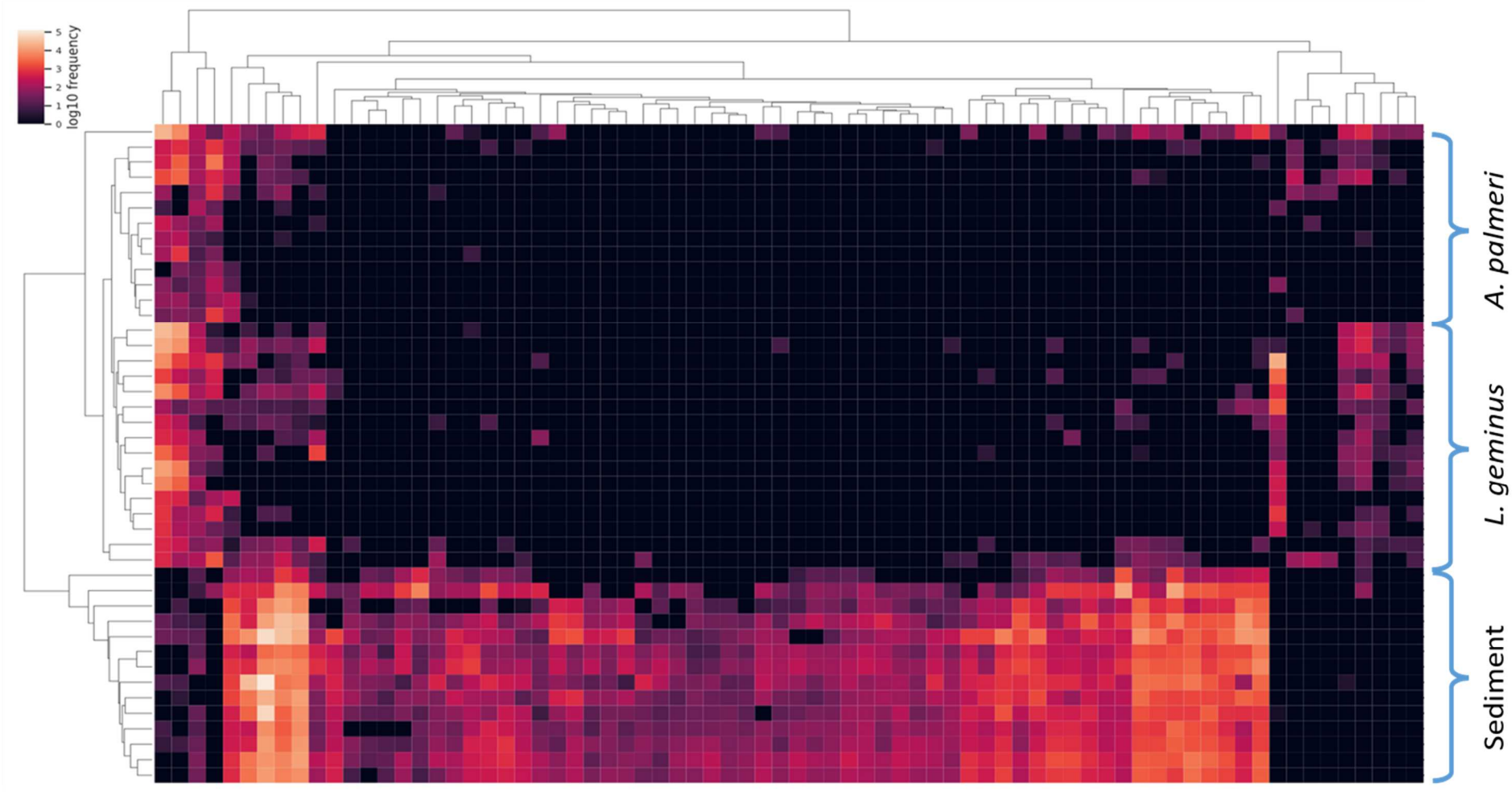


Figure 19. Heatmap showing the taxonomic composition of microbiomes associated with polychaetes and surrounding sediments. Each line represents a sample, each column represents a bacterial family. The graduation of colors shows the different frequencies of each bacterial family in the samples.

4.4 Discussion

4.4.1 Intra-specific and inter-specific biodiversity of microbiomes associated with polychaetes.

To date our knowledge about bacteria-invertebrate associations in Antarctic ecosystems is scarce and limited only to few organisms (Lo Giudice and Rizzo, 2018). Nevertheless, from these early studies there is a great deal of evidence about the important role of the microbiomes for their hosts (Pucciarelli et al. 2014; González-Aravena et al. 2016; Yarzábal, 2016). The only study about microbiomes associated with Antarctic annelids was carried out on the oligochaete *Grania sp.*, highlighting their functions in the digestive capability in metabolizing nutrients and underlining a mutualistic relationship between host and microbes (Herrera et al. 2017).

Few studies are present in literature on polychaetes microbiomes: low diversity have been found in microbiomes associated with marine polychaetes such as *Ophelina sp.* and *Neanthes glandicincta*, living in contaminated areas of Australian coasts and in *Riftia pachyptila* and *Osedax spp*., collected in hydrothermal vents, where, in this last were well characterized and recognized as sulfur-oxidizing bacteria (Li et al. 2009; Neave et al. 2012; Goffredi et al. 2007; Dubilier et al. 2008). A recent investigation on the polychaete *Hydroides elegans* has revealed a high intraspecific variability in the diversity of microbiomes among adults and over time but mostly composed of Gamma proteobacteria (Vivjian et al. 2019)

The present study represents the first investigation on the microbial assemblages associated with Antarctic polychaetes.

Results of our investigations highlighted a similar diversity of microbiomes, in terms of ASV richness between the two species of polychaetes and a higher percentage of unknown ASVs in the individuals of *A. palmeri* (5%) than in individuals of *L. geminus* (1%). The investigation on the taxonomic composition of microbiomes revealed a 55% of similarity between the two species. This resulted by the presence of three core bacterial families between the two species *Thermaceae* (mostly represented by *Meiothermus* genus), *Bacillaceae* and *Propionibacteriaceae*. Bacteria of the family *Thermaceae* are highly resistant to environmental hazards, also known as extremophiles, obligately oxidative and have been found worldwide but mostly in thermal environments such as terrestrial or marine hydrothermal areas (Albuquerque et al. 2014).

Similarly, the members of *Bacillaceae* family, have been detected in freshwater and marine ecosystems, in human and animal systems and in extreme environments (Xue et al. 2006). They are considered among the most robust bacteria on Earth, due to the ability to form endospores that provide high resistance to adverse conditions for a prolonged period (Mandic-Mulec et al. 2016). A benefit to the host could be also the presence in the microbiome of the family of *Propionibacteriaceae.* Numerous species belonging to this family show antimicrobial and antifungal abilities, that could increase their survival in the harsh Antarctic ecosystems (Bruggemann et al. 2004; Schwenninger et al. 2004; Stackebrandt et al. 2014). Bacteria of Firmicutes and Actinobacteria phyla, to which belong the *Bacillaceae* and *Propionibacteriaceae* families, were found in associations with Antarctic sponges, sea urchins and corals (Lo Giudice and Rizzo, 2018). The Deinococcus–Thermus phylum, to which belong the *Thermacea* family, was not found associated with organisms till now.

All the individuals of *L. geminus* show a 63% similarity in their microbiomes, explained by the presence of a core microbiome that represent on average the 68% of the total abundance of all individuals. The families that shape this core are *Thermaceae*, *Bacillaceae*, *Propionibacteriaceae, Burkholderiaceae* and *Blastocatellaceae* families. *Burkholderiaceae* family is characterized by saprophytic organisms, phytopathogens, opportunistic pathogens, as well as primary pathogens for humans and animals (Coenye et al.2014). *Blastocatellaceae* family could give an important contribution to health host. In fact, most of them can use complex proteinaceous compounds for growth and are able to degrade other complex carbon compounds (Pascual et al. 2018).

All the individuals of *A. palmeri* show a 55% similarity in their microbiomes, explained by the presence of a core microbiome that represent on average the 65% of the total abundance of all individuals. Families that take part of that core are *Thermaceae*, *Bacillaceae* and *Propionibacteriaceae* and *Moraxellaceae.* The family of *Moraxellaceae* contributes up to 50% to the total abundance in the *A. palmeri* microbiomes. These bacteria are usually distributed in a wide variety of environments, including natural cold salines. Some of them (i.e genus *Psychrobacter*) are psychrophilic bacteria and produce cold-adapted proteins and enzymes (Teixeira et al. 2014). They could play an important role in the adaptive strategy of this polychaetes to the harsh conditions of Antarctica. High similarity (from 54% to 79%) was also found among microbiomes of individuals of *A. palmeri* collected in the same areas and depths, characterized by the presence of the *Oxalobacteraceae* and *Pseudomonadaceae* families, exclusively found in specimens of *A. palmeri* collected at Adelie Cove at 70m,

Planctomycetaceae and *Xanthomonadaceae* families exclusively found at Adelie Cove at 140m and, finally, *Campylobacteraceae* and *Desulfovibrionaceae* families exclusively found at Central Bay at 70m. All these families have a key role in metabolism of a wide array of nutrients as sulfur compounds (Roquigny et al. 2017; Lastovica et al. 2014), in the oxidization of organic and nitrogen substrates (Kuever et al. 2014), in the formation of biofilms (Mhedbi-Hajri et al. 2011) and in activity against various pathogens (Baldani et al. 2014). Moreover, some bacterial families showed a high abundance only in some individuals. This could be the case of the *Enterobacteracea* and *Helicobacteraceae* families, that reach up the 60% and 30% of abundance, respectively, in some individuals, not united by sampling area, depth or species. Members of these families are pathogens and can be responsible for a wide spectrum of diseases (Octavia et al. 2014, Mitchell et al. 2014). Their sporadic presence can be due to changes in the healthy status and to a possible lowering of the immune defense of the hosts. In addition to the known functions, bacteria (i.e. some genera of the *Planctomycetaceae* family) can modify their genetic features, as well as their expressed genes, indicating that their functions and their abilities can be wider than we think and can evolve in time (Guo et al. 2014).

Finally, multiple evidences have reported the important role of the rare or transient bacterial taxa, that could have an influence disproportionate to their abundance in the microbiome (Reveillaud et al. 2014; Shade et al. 2014; Shi et al.2016). This could be the case of all the species found in our polychaetes with a percentage of abundance lower than 1%, and that represent the 90% of the total families found, both in *L. geminus* and *A. palmeri.* Further investigations are needed to clarify their importance on the whole microbiome.

4.4.2 Possible drivers of the diversity of microbiomes associated with polychaetes

Many factors can influence the composition and structure of microbiomes associated with organisms (Thomas et al. 2016). Understanding how ecological, evolutionary, biological and environmental processes mainly contribute to host-associated community assembly remains a challenge (Nemergut et al., 2013). Several studies have reported a strong connection between environmental factors and microbiome composition (Pantos et al. 2015; Adair et al. 2017). In fact, environmental drivers can shape the microbiome assemblages, selecting a range of bacteria with metabolic pathways, able to adapt to areas with different contamination levels, or at different seasons, temperatures or nutritional inputs (Kelly et al. 2014; Vezzulli et al. 2013; Pantos et al. 2015; Mosainder et al. 2015).

In this study the trophic conditions of the areas, where polychaetes lived, were tested as possible drivers of diversity of microbiomes. Polychaetes were collected in three areas: Adelie Cove, characterized by high organic inputs due to the presence of penguin assemblages, Rod Bay, characterized by anthropogenic impact due to the presence of the Italian Station "Mario Zucchelli" and Central Bay, considered as uncontaminated area, because far from possible source of impacts. Our results revealed that the sediments of Adelie Cove at 25m and at 70 m contained the lowest and the highest protein, carbohydrate, lipid and chlorophyll-a concentrations, respectively. High values of phytopigments were found also in the deepest sampling points of Central Bay and Rod Bay.

Our results revealed that microbiomes associated with individuals of *L. geminus* did not show similar taxonomic pattern in the same areas and at the same depths and that the trophic condition of the environment did not explain the variability in the diversity of their microbiomes. Therefore, it is possible hypothesize that many other factors might have a role in shaping the microbiome composition in *L. geminus* such as the diet, the size, the age or the healthy state of each individual, or a natural selection of bacteria, that could subjected to a resource competition, that can create colonization by the stronger and more adaptive ones (Reese et al. 2018; Adair et al. 2017; Yatsunenko et al. 2012; Ezenwa at al. 2012; Zhang et al. 2016; Welsh et al. 2016). All these conditions can create pattern of biodiversity in the microbiomes that do not reflect changes in environmental settings and that can be unique in each individual (Califf et al.2014).

Conversely, a similar microbiome composition was observed among individuals belonging to the species *A. palmeri* in the same areas and at the same depths. Moreover, the clorophylla-a and pheopigments concentrations, and in a minor part the carbohydrates' contents, resulted to be the main drivers that explain the 48% of variability in the taxonomic composition of microbiomes found among individuals of *A. palmeri.* A strong influence given by the nutritional quality of the sediments on the microbial assemblages living in sediments was largely demonstrated in Antarctica (Learman et al. 2016; Fabiano et al. 2000). This environmental selection could have happened also on the microbiomes associated with *A. palmeri*, favoring only bacteria suitable for living in these habitats and acting on the diversity of a part of microbiomes.

Nevertheless, the presence of a specific core microbiome in *L. geminus* and in *A. palmeri* that remain stable in all the individuals and in all areas considered, suggest that a wide spectrum of biotic and abiotic factors that remain to be identified yet are playing a role in shaping the diversity of microbiomes.

4.4.3 Source of microbiomes: comparison of the taxonomic composition of microbiome associated with polychaetes and with surrounding sediments

Colonization of hosts' tissues by bacterial symbionts can occur in different ways: horizontally, in which sets of bacteria are selected from the pool of micro-organisms that inhabit the surrounding habitats or vertically, in which bacteria were transmitted from one generation to the next (Bright et al. 2010; Kwan et al. 2017). A recent study, Vijayan and colleagues have investigated the source of the microbiome associated with the polychaete *Hydroides elegans*, during all the development stages, from birth to death, and concluded that the host lacks a vertically transmitted microbiome, and bacteria found at each stage came from a varying environment (Vijayan et al. 2019). From this point of view, the microbial community within an individual host can be thought as a local community colonized by all the microbes that the host encounters in its environment during its life (Adair et al. 2017).

The two species of polychaetes investigated in this study are burrowing deposit feeders; they live closed to the surrounding sediments and they feed on the organic particles present among the sediment grains. This would be helpful in order to investigate the origin of the microbiomes and assess the differences between the prokaryotic assemblage composition in the sediments and in the host. Our results show that the microbiomes of the polychaetes considered was very different from the prokaryotic assemblage composition in the sediments, both in terms of phylogenetic distance and taxonomic composition. biodiversity. Moreover, sediments showed higher values of alpha and beta diversity, highlighting a high biodiversity, most of it represented by families of bacteria with low number of sequences. In fact, only 18 among the 306 families found in sediments show an abundance higher than 1%. No one family was shared among prokaryotic assemblages associated with all the sediment samples and polychaetes. The most important core families of polychaetes (*Thermaceae*, *Bacillaceae* and *Moraxellaceae* families) are present in the 70% of the samples of sediments analyzed, but with a contribution lower than 0,005%. Similarly, the dominant bacterial families in the sediments (*Flavobacteriaceae*, *Chaetoceros sp. C134* and *Planctomycetaceae*

families with 12%, 23% and 13% of abundance, respectively) were found in the 80% of polychaetes, with a contribution lower than 0,4%. These findings suggest that only a very small portion of bacterial taxa are potentially transferred from the surrounding sediments to the polychaetaes and they represent the rare part of the whole microbiomes. Different conditions could occur within hosts, selecting different taxa from those live in the surrounding habitat. Bacteria of *Thermaceae*, *Bacillaceae and Moraxellaceae* families could have found in their host suitable niches for life and could have established stable associations with time over generations. Rodiguez-Marconi and colleagues have shown that only few bacterial phylotypes are in common between sponge microbiomes and seawater, highlighting a possible host specificity and suggesting the role played by microbiomes-host associations as a diversity reservoir in the Antarctic marine ecosystem (Rodríguez-Marconi et al. 2015). Moreover, our findings suggest the possibility that some bacterial groups associated with polychaetes can be transferred vertically, supported by the presence of three core families shared between the two species of polychaetes. Despite the vertical transmission of microbial symbionts is documented in many animal phyla, to verify that bacteria were transmitted over generations, investigations of microbiomes during the different life stages of these polychaetes are needed (Sharp et al.2012; Vijayan et al. 2019).

Multiple factors can have had a role in the resulting microbiomes associated with these Antarctic polychates: the microbiomes could be fueled by vertically transmitted microbes, and then shaped by the environmental factors (*A. palmeri*) or other drivers (*L. geminus*) creating specific patterns of diversity (Kwan et a. 2017).

4.5 Conclusions

This study provides new insights into the knowledge of the microbiome world, expanding information on the biodiversity of microbiomes associated with the Antarctic polychaetes, on the environmental drivers potentially responsible for shaping their taxonomic composition and on the origin of bacterial taxa belonging to the microbiomes. Despite the lower diversity of the microbial assemblages associated with polychaetes than the surrounding habitat, their high beta diversity contributes to increase the estimates of biodiversity of the area. Microbiomes associated with polychaetes can significantly change among individuals, indicating a high intraspecific variability, which can be driven by different environmental or biological factors. Moreover, each species of polychaetes

revealed a core, that represent an important part of the diversity of whole microbiome with a high contribution of families shared between the two species.

Despite the polychaetes are deposit feeders, bacteria that live associated with them are completely different from those living in the surrounding sediments, suggesting a potential vertical transmission or different adaptative conditions of the bacterial taxa. Microbiome-host associations can be considered as a reservoir of those bacteria that are not able to survive in the surrounding sediments.

Findings reported here also suggest that most of the identified bacteria have a fundamental role in the polychaetes' wellbeing, contributing in the metabolism of a wide array of inorganic and organic compounds, in the defense against pathogens and in the adaptation to the harsh temperatures of Antarctica. Nevertheless, a high portion of bacteria that take part of the microbiomes remain unknown, leaving opened several questions on their identity and roles and allowing us to suggest the presence of novel taxa.

Major future investigations should be conducted to better comprehend the biodiversity and nature of the intricated links between microbiomes and their hosts.

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4.7 Supplementary material

Table 1 SM. Results of PERMANOVA main test carried out on:

AVS richenss of microbiomes between *L. geminus* and *A. palmeri*

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	1	18.195	18.195	0.10908	0.798	992
Res	18	3002.6	166.81			
Total	19	3020.8				

AVS richenss of microbiomes among different areas and depths

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Area	2	367.56	183.78	1.3019	0.353	999
Depth(Area)	4	549.59	137.4	0.81495	0.546	999
Res	13	2191.7	168.59			
Total	19	3020.8				

Taxonomic composition of microbiomes between *L. geminus* and *A. palmeri*

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	1	5653.8	5653.8	5.1655	0.001	992
Res	18	19701	1094.5			
Total	19	25355				

Taxonomic composition of microbiomes among different areas and depths

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Area	2	25.204	12.602	2.70E-02	0.995	999
Depth(Area)	4	638.5	159.62	0.229	0.942	999
Res	26	18123	697.04			
Total	32	18804				

Biochemical composition of sediments in different areas and depth

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Area	2	21.721	10.86	0.51968	0.723	999
Depth	2	55.333	27.667	23.323	0.001	999
AreaxDepth	4	83.593	20.898	17.617	0.001	999
Res	18	21.353	1.1863			
Total	26	182				

ASV richness of microbiomes between polychaetes and sediments

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Source	1	15171	15171	131.07	0.001	999
Res	32	3703.8	115.74			
Total	33	18875				

Taxonomic composition of microbiomes between polychaetes and sediments

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Source	1	48149	48149	49.725	0.001	999
Res	32	30985	968.3			
Total	33	79134				

5. MICROBIOME ASSOCIATED WITH DIFFERENT BODY PARTS OF THE ANTARCTIC POLYCHAETE AGLAOPHAMUS TRISSOPHYLLUS: DIVERSITY AND FUNCTIONS

5.1 Introduction

All organisms host, on their outer surface and in several internal organs and tissues, highly diversified assemblages of microorganisms that play critical roles for the health of their hosts (Sekirov *et al.* 2010). Each organ of the body can harbour specific assemblages of bacteria, which are involved in a multitude of functions, including nutrient absorption, metabolism regulation, or defence against pathogen invasion (Paga´n-Jime´nez et al. 2019; Olsen et al.2019). Skin, and each part of the body in contact with the external world (e.g. tegument, parapods, coticules), represent a unique interface, influenced both by surrounding environment and host-associated factors (health state, mobility, excretion of wastes and mucus, and immune molecules secretion) (Byrd et al. 2018). Therefore, the surface of each individual is like "a patchy physical-chemical habitat" acting as a physical barrier to prevent the invasion of foreign pathogens and, at the same time, providing a home to the commensal microbiota (Grice and Segre 2011). Recent studies have highlighted that skin-associated bacterial communities are highly variable in terms of abundance and diversity, among body parts, and among individuals (Fierer et al. 2010; Chiarello et al. 2015). These inter-individual and intra-individual variations have been related to individual physiology (e.g. age, sex, health state, immune system) and local-scale parameters (e.g. pH, temperature, humidity), even if the specific impact of each of these drivers, and the underlying interactions at a microbial scale have not been systematically demonstrated (Chiarello et al. 2015; Byrd et al. 2018).

Among the human and animal microbiomes, the oral and gut ones represent the two-best studied to date (Pascoe et al. 2017; Franzosa et al. 2014). In human microbiome, an imbalance of oral microbial flora contributes to whole-body systematic diseases, including inflammatory, cardiovascular, nervous and endocrine systems diseases (Gao et al. 2018). Studies in animals have indicated that oral bacteria can translocate to the gut and change its microbiota and possibly immune defense (Olsen et al.2019). Gut microbiome represents a

complex ecosystem strictly linked with the entire organism (Guinane and Cotter, 2013; Kostic et al., 2013; Heintz-Buschart et al. 2018). It has been demonstrated that the gut microbiome shows a succession of different microbial communities, that changes rapidly during the different life stages (Yatsunenko et al. 2012; Jeffery et al. 2012).

A recent work in mice revealed that some bacteria of the gut microbiome facilitates host life during cold exposure by promoting energy demand-driven regulation (Rosenberg and Zilber-Rosenberg, 2018). Similar results were found in the intestinal microbial communities of some Antarctic fishes, where groups of bacteria shared among different species were found to contribute to survival ability in the extreme temperatures (Song et al. 2016). Moreover, several investigations on holothurians gut have revealed that the position of bacteria in the intestinal tract can define their role: the anterior tract, usually, harbors diversified consortia of bacteria, shaped by environment, feeding habits and individual features; the posterior one, instead, is the most stable and indigenous part of microbiome, responsible to eliminate waste material from the body (Lau et al. 2002; Paga´n-Jime´nez et al. 2019).

Despite in all vertebrates the microbiome is highly partitioned across the body (e.g., gastrointestinal tract, skin, urogenital cavities, oral cavity) reflecting varying environmental conditions suitable for different microbial taxa, in invertebrates, however, often it lacks many of these differentiated body sites, so it is not clear how the microbiome is shaped in the organisms (Jackson et al.2018). For this reason, except for studies carried out on gut microbiomes (Becker et al., 2009; Gomez et al., 2012; Nguyen and Clarke, 2012; King et al., 2012; Gerdts et al., 2013; Chauhan et al., 2014; Heintz and Mair, 2014; Hakim et al. 2015) relatively little attention has been given to microbiomes associated with different tissues of marine invertebrates. A study of Jackson and colleagues, carried out on different species of sea stars, has investigated microbial communities among the pyloric caeca, gonads, coelomic fluid, and body wall: the microbiome appears to be anatomically partitioned and distinct from the microbial community of seawater. The most noticeable difference between anatomical sites was the greater relative abundance of *Spirochaetae* and *Tenericutes* found in hard tissues (gonads, pyloric caeca, and body wall) than in the coelomic fluid (Jackson et al.2018). Similar results were found by Aronson and colleagues, during an investigation of microbiomes associated with different anatomical parts of the gastropod *Rubyspira osteovata*, with a stable associated microbiome that showed lower community dissimilarity among individuals than either between different tissues or other snail species samples (Aronson et al. 2017).

No information about the functions and the diversity of microbiomes, how or if are they diversified in the different body parts of Antarctic organisms, are available in literature.
In this study we investigated the diversity, both in terms of richness and of taxonomic composition, and the putative functions of microbial communities associated with the Antarctic polychaetes *Aglaophamus trissophyllus*, to explore how microbiomes are portioned in four parts of the body (oral cavity, gut, tegument and parapods) of each individual. Moreover, a comparison between specimens of *A. trissophyllus* collected from different environmental settings was carried out to investigate the environmental factors shaping the composition of microbial assemblages. Finally, the potential origin of Antarctic polychaetes' microbiomes from the surrounding sediments was assessed through the comparison with the benthic microbial assemblages.

5.2 Materials and methods

5.2.1 Study area and samples collection

Sampling was carried out during the XXXIII Italian Expedition in Antarctica at Terra Nova Bay (Ross Sea) in the framework of the Italian National Program of Antarctic Research (PNRA). Specimens were collected using a Van Veen Grab (31 x 58 cm). Three sampling areas were selected considering different trophic conditions (Tab.1; Fig.1): Adelie Cove (characterized by high organic input due to the presence of penguin assemblages), Rod Bay (characterized by possible anthropogenic impact due to the near Italian research base "Mario Zucchelli Station"), Central Bay characterized by the absence any source of impact).
In all areas, samples were collected at 25 m and an added station at 140 m was selected in Adelie Cove. Specimens were extracted, sieving the sediment, were preserved in ethanol (95%) and stored at -20°C. Samples of surrounding sediments were collected using plexiglass cores in the same areas and stored at -20°C.

Table 2. Table listing the sampling stations organized by area, depths and geographic coordinates.

Area	Station	Depth (m)	Latitude	Longitude
Adelie Cove	St 9	25	-74,46.467	164,00.266
	St 11	140	-74,46.617	164,02.798
Rod Bay	St 5	25	-74,41.831	164,07.532
Central Bay	St 2	25	-74,43.037	164,06.908

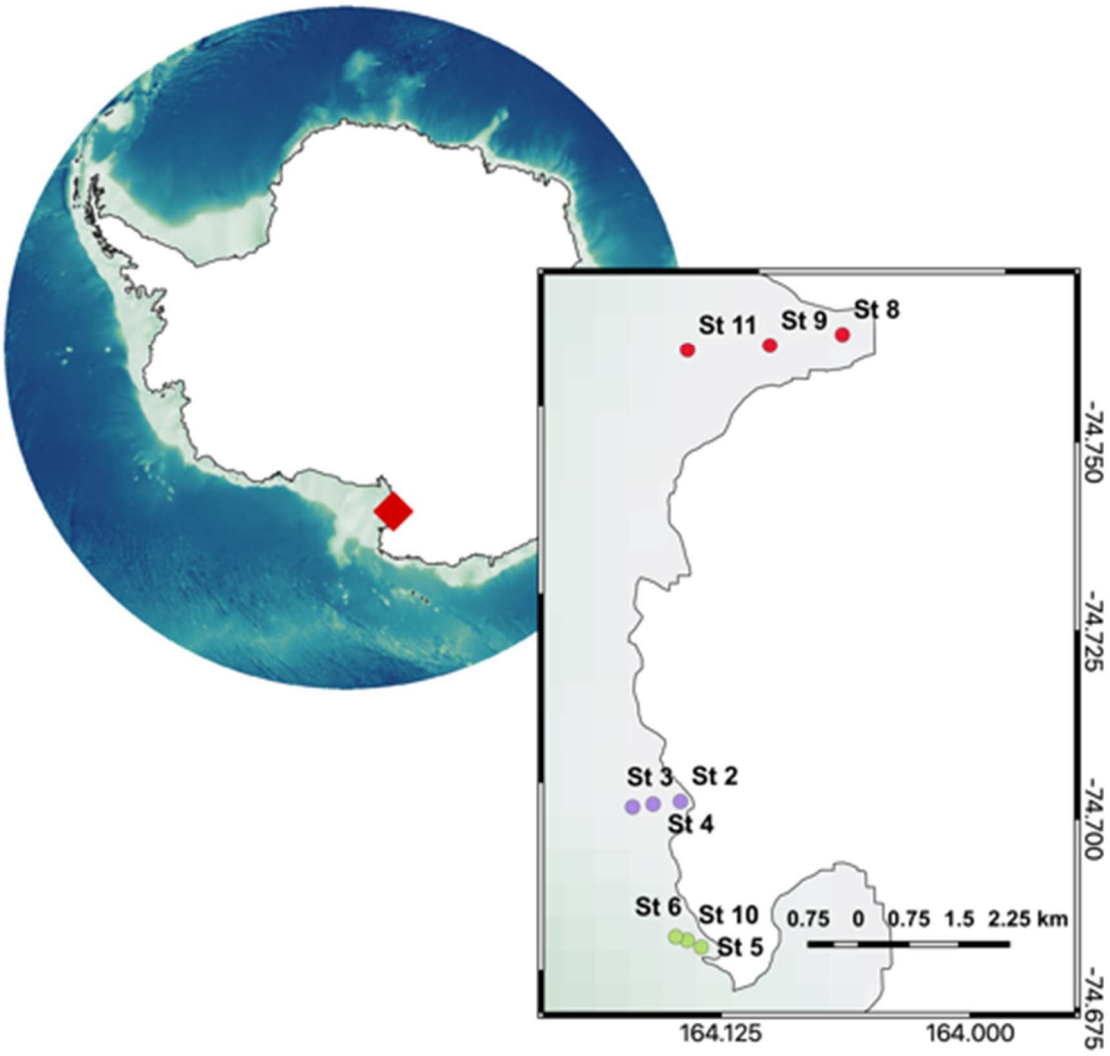


Figure 2. Map of the sampling area where polychaetes and sediments were collected. The dots represent the sampling sites.

5.2.2. Morphological and molecular identification of polychaetes

Specimens of *Aglaophamus trissophyllus* selected for this study are a part of polychaetes investigated in Chapter 3. The morphological and molecular methods used for the identification have been described there. The list of specimens used is shown in Table 2.

Table 2. List of tissues samples sectioned from specimens of *Aglaophamus trissophyllus*.

	Adelie Cove		**Rod Bay**	**Central Bay**
	25 m	**140 m**	**25 m**	**25 m**
Oral Cavity	9AB, 9BB, 9CB	11AB, 11BB, 11CB	5AB, 5BB, 5CB	2AB, 2BB, 2CB
Gut	9AG, 9BG, 9CG	11AG, 11BG, 11CG	5AG, 5BG, 5CG	2AG, 2BG, 2CG
Tegument	9AT, 9BT, 9CT	11AT, 11BT, 11CT	5AT, 5BT, 5CT	2AT, 2BT, 2CT
Parapods	9AP, 9BP, 9CP	11AP, 11BP, 11CP	5AP, 5BP, 5CP	2AP, 2BP, 2CP

5.2.3 Extraction of DNA from parts of the polychaetes' tissues

Each polychaetes was dissected, isolating four different body parts: oral cavity (OC), gut (G), parapods (P) and tegument (T). DNA of microbial assemblages was extracted from each body using the Qiagen DNeasy Blood and Tissue Kit (Brasier et. 2016) and following the manufacturer's instructions with a modification (incubation with proteinase K at 56°C was extended overnight). Total DNA from the sediments was extracted using the PowerSoil DNA Isolation Kit, following a modified protocol described in Chapter 4.

In this investigation, the microbiome associated with the whole-body of a polychaete was obtained summing all bacteria found in the four parts of the body of each polychaetes.

5.2.4 Amplification and sequencing of prokaryotic 16SrDNA

PCR amplification was performed on an approximately 550 bp fragment of the 16S rRNA genes, using the primer set Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') and Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') specific for bacteria (Herlemann et al., 2011). The reaction mixture used consisted of 37.5 µl of filtered and autoclaved Milli-Q water, 10 µl of 5x My Taq Reaction Buffer (Bioline), 0.25 µl of each primer (100 µM), 1 µl of My Taq HS DNA Polymerase (5 U/µl concentration), 1 µl of DNA extracted. The thermal cycling consisted in 2 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 53°C, 45

s at 72°C, with a final extension of 5 min at 72°C. Successful DNA amplification was verified by 1% agarose gel electrophoresis using 10.000x GelRed Nucleic Acid Stain (Biotium), 0,4 gr of agarose, 40 ml of TE Buffer for the gel preparation, and 2 µl of 5x GelPilot DNA Loading Dye (Qiagen), 2 µl of GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) for the electrophoresis. The amplified DNA was sequenced on an Illumina MiSeq sequencer using the V3 technology (2x300 bp) with primers targeting Bacterial V4 region (Klindworth et al., 2013) at LGC Genomics.

5.2.5 Bioinformatic and statistical analysis

Raw sequences were analyzed through the QIIME2 pipeline (version 2019.4; https://qiime2.org/). Paired-end sequence files were loaded, and sequence pairs analyzed by means of the DADA2 plugin (Callahan et al., 2016), which infers community composition in each sample by partitioning sequences according to the respective error models, thus filtering for erroneous reads and chimeras and resolving minimal variations between prokaryotic taxa. Paired sequences were then merged by the pipeline before producing an Amplicon Sequence Variant (ASV) table. From the ASV table obtained, each sample was subsampled to 700 sequences, thus obtaining a normalized ASV table. The subsampling depth was chosen as a compromise between the highest number of sequences that fully described the biodiversity of samples and the lowest loss of samples. The gut samples from *A. trissophyllus* at Adelie Cove (5BG and 5CG) were discarded because they were characterized by <700 sequences. The normalized ASV table was used for the calculation of rarefaction curves and as input for the subsequent analyses, such as the determination of α- and β-diversity (i.e. Shannon and Evennes indices, Bray curtis dissimilarity and Unweighted Unifrac distance). To infer the taxonomic affiliation of ASVs, a taxonomic classifier was first trained on the SSU region amplified by the primers utilized in the present study on the SILVA reference database v132 (Quast et al. 2012); the classifier was then used on the ASVs identified (Bokulich et al. 2018). To further predict the relevant potential functions of microbiomes a functional annotation using FAPROTAX database (Louca et al., 2016) was done. This database maps prokaryotic taxa to putative functions using information based on functional annotations of cultivated representatives. Significant differences (p-values <0.05) in the richness, in the taxonomic composition and in the putative functions of microbiomes were highlighted through a permutational analysis of variance (PERMANOVA) and Multi-Dimensional Scale (MDS) representations; similarities among the different groups were

evaluated by classification-clustering based on the Bray Curtis similarity of transformed quantity data with and the identification of the main responsible taxa describing the differences was done with SIMPER analysis, both included in the PRIMER-E 6 software (Anderson et al., 2008).

5.3 Results

5.3.1 α and β diversity of microbial assemblages associated with different parts of polychaetes.

The rarefaction curve showed that, considering the same number of sequences for all the samples, the curves reached a plateau in all the microbiomes of the body parts of the polychaetes (Fig. 2). Total ASV richness was calculated from each sample and validated with statistical analysis (Fig.3; Tab. 1 SM).

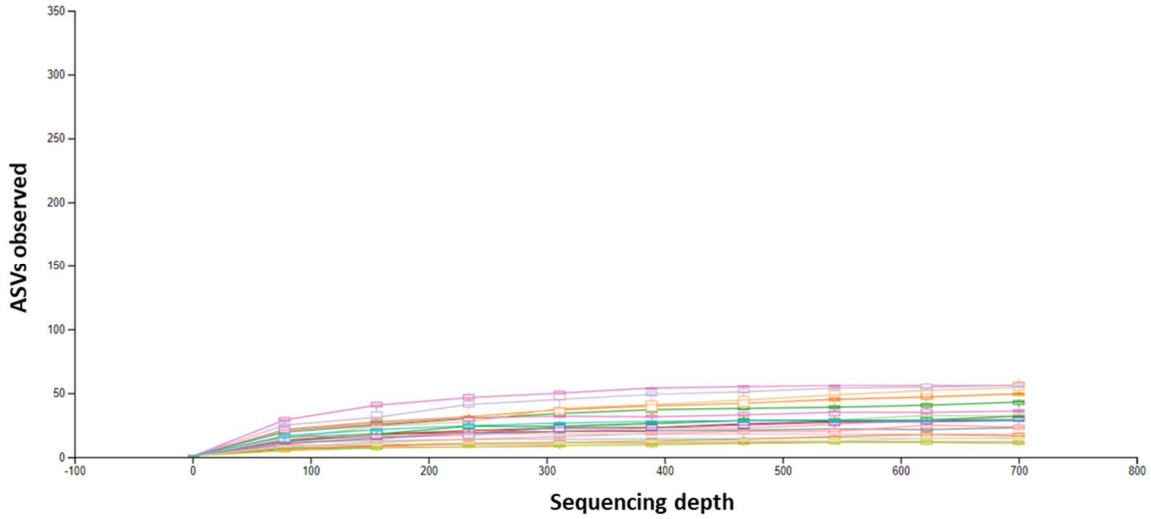


Figure 2. Rarefaction curve of samples after a normalization of 700 sequences for the different parts of the body of polychaetes.

The number of ASVs varied from 13 to 170 in oral cavity, from 14 to 88 in gut, from 14 to 129 in parapods and from 19 to 91 in tegument samples. The lowest value of ASVs richness was found in the oral cavity of a specimen collected at 25m in Rod Bay whereas the highest value in the oral cavity of specimens from the shallower sediments of Adelie Cove (5BB and 11AB). However, no significant differences were found in the number of ASVs obtained from the microbiomes associated to the different parts of the specimens' body (Tab. 1 SM). Considering the three areas investigated, the microbiomes of tissues of polychaetes collected at Central Bay showed a lower number of ASVs than in the other two areas. In fact, except

for the 2CG, the number of ASVs ranged from 14 to 34 in 2CP and 2BB samples, respectively.

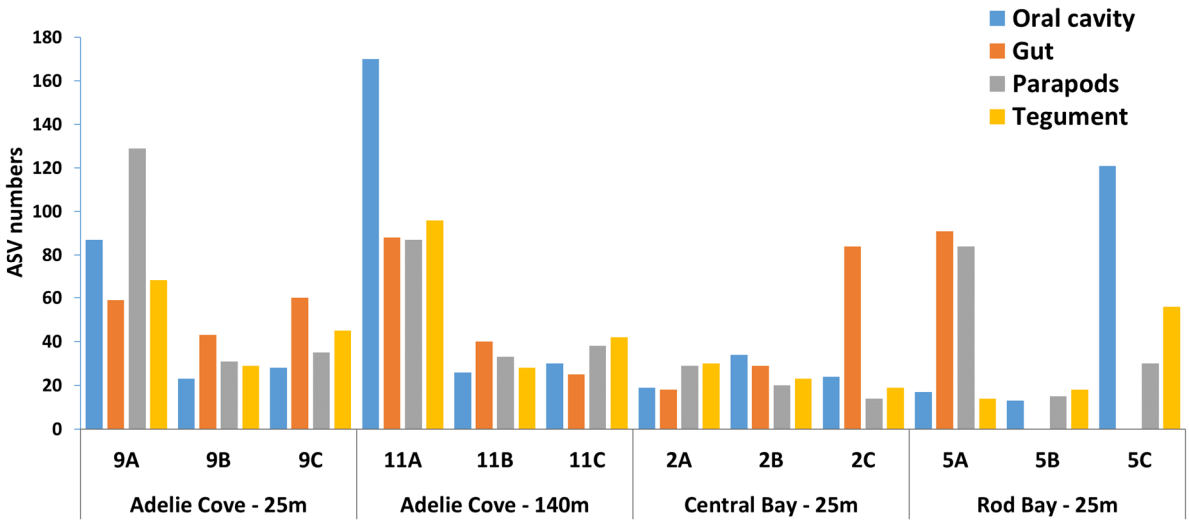


Figure 3. Numbers of ASV observed in different parts of *A. trissophyllus* specimens in the three areas and at the two different depths considered.

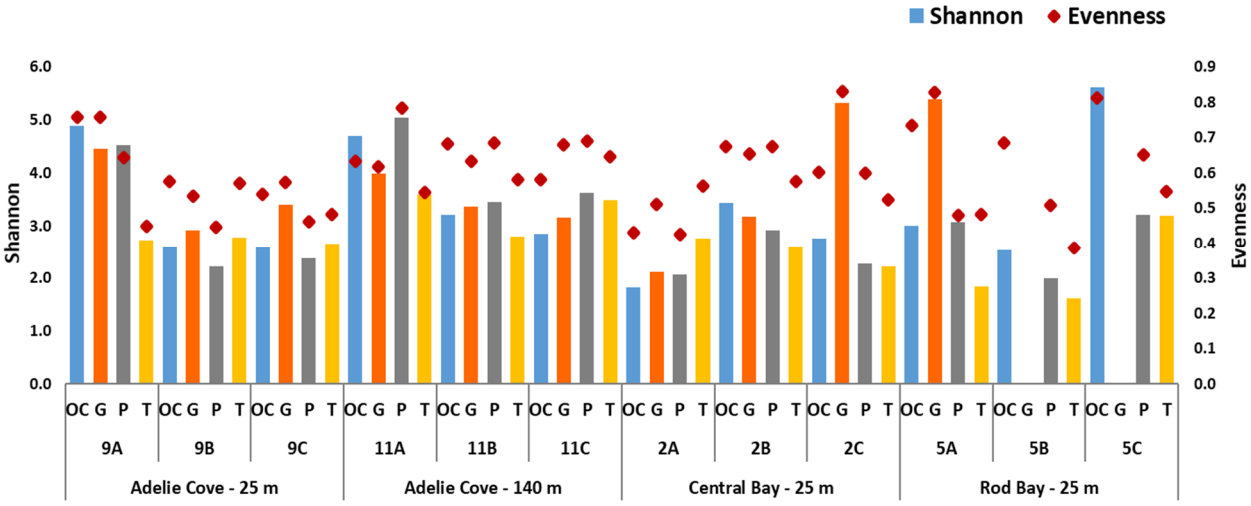


Figure 4. Shannon and evenness indices of microbial community associated with the different parts of the body of *A. trissophyllus* individuals.

High variability was found in Shannon and Evenness indexes among all samples, with no significant differences in alpha diversity neither among microbiomes associated with different parts of the body of *A. trissophyllus* specimens nor among different areas and depths considered (Fig.4).

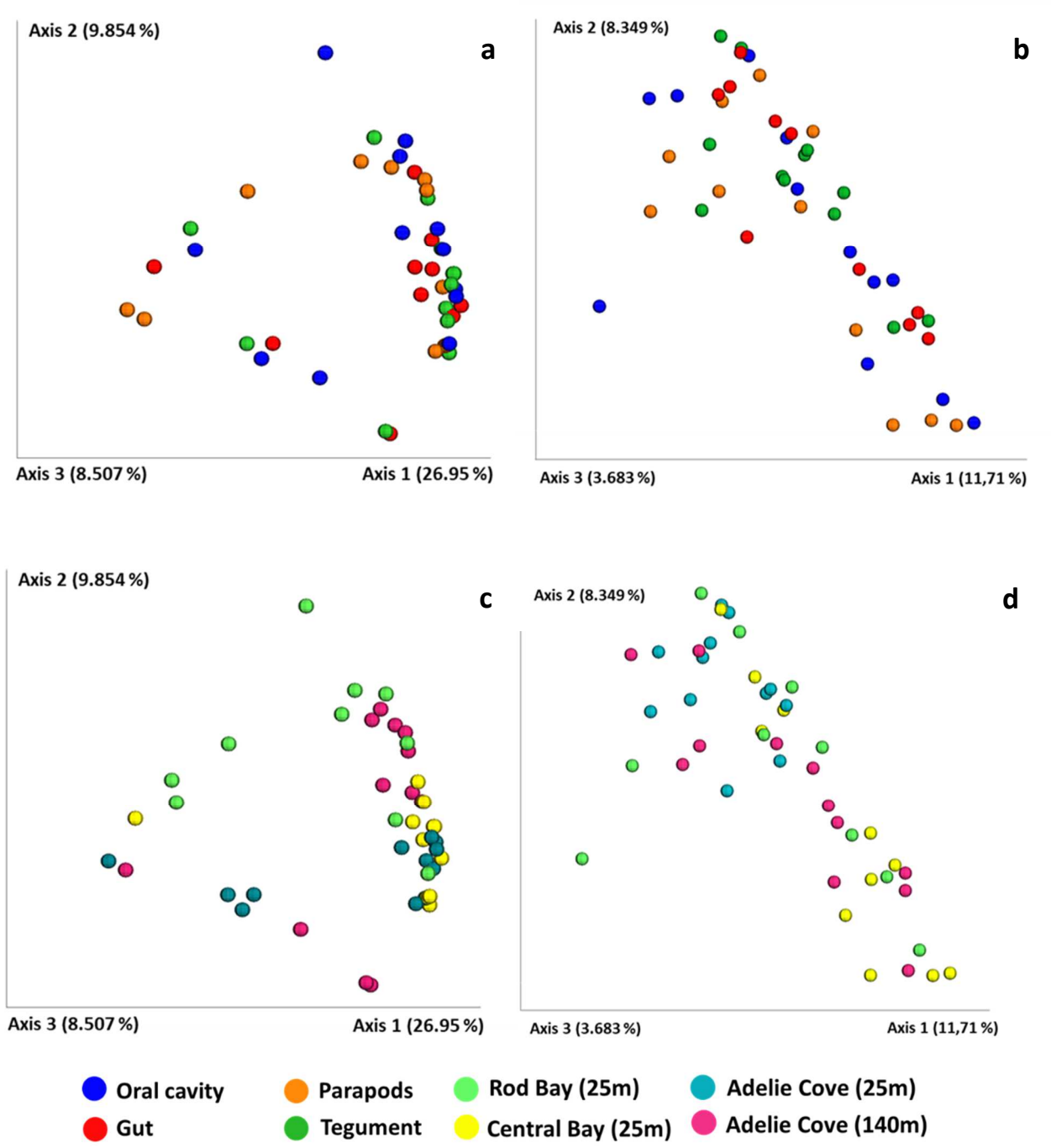


Figure 5. PCoA plot of Beta-diversity of microbiomes on Bray Curtis dissimilarity and unweighted UniFrac distance among different parts of the body of polychaetes (a-b) and among different areas and depths (c-d).

The Beta diversity analysis revealed no significant differences both in terms of composition and phylogenetic diversity of microbial communities between the different parts of the body

of polychaetes. No significant differences were found among different sampling areas and the two different depths in Adelie Cove (Fig.5 a- b; Tab. 1SM).

5.3.2 Microbiomes diversity associated with individuals of *A. trissophyllus*

Results of the analysis carried out on the taxonomic composition of microbiomes revealed that, among a total of 195 different families found only one was shared in all parts of the body of all individuals: the *Thermaceae* family The *Thermacea* family, totally represented by bacteria belong to Meiothermus genus, is present with percentages ranging from 2% to 75% in all samples (Fig. 6-7). Considering the "whole microbiome", despite the *Thermaceae* family, the *Burkholderiaceae, Blastocatellaceae, Propionibacteriaceae, Caulobacteraceae* families and *Proteobacteria* phylum were found in all individuals, with an average contribution of 4%, 2%, 1,4%, 0,6% and 3% respectively (Fig.8).

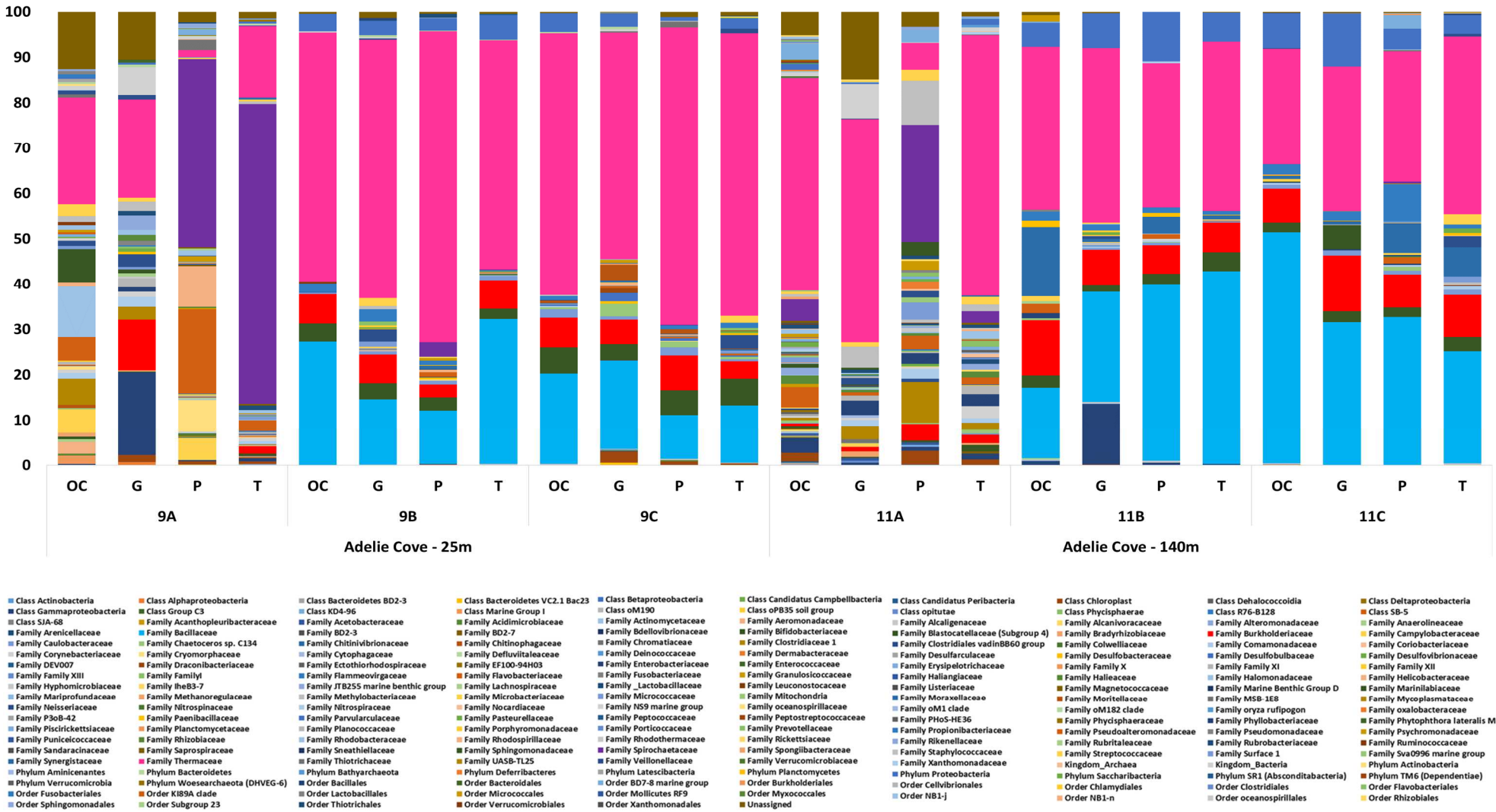


Figure 6. Taxonomic composition of microbiomes associated with different parts of the body of *A. trissophyllus* collected at Adelie Cove (25m and 140m).

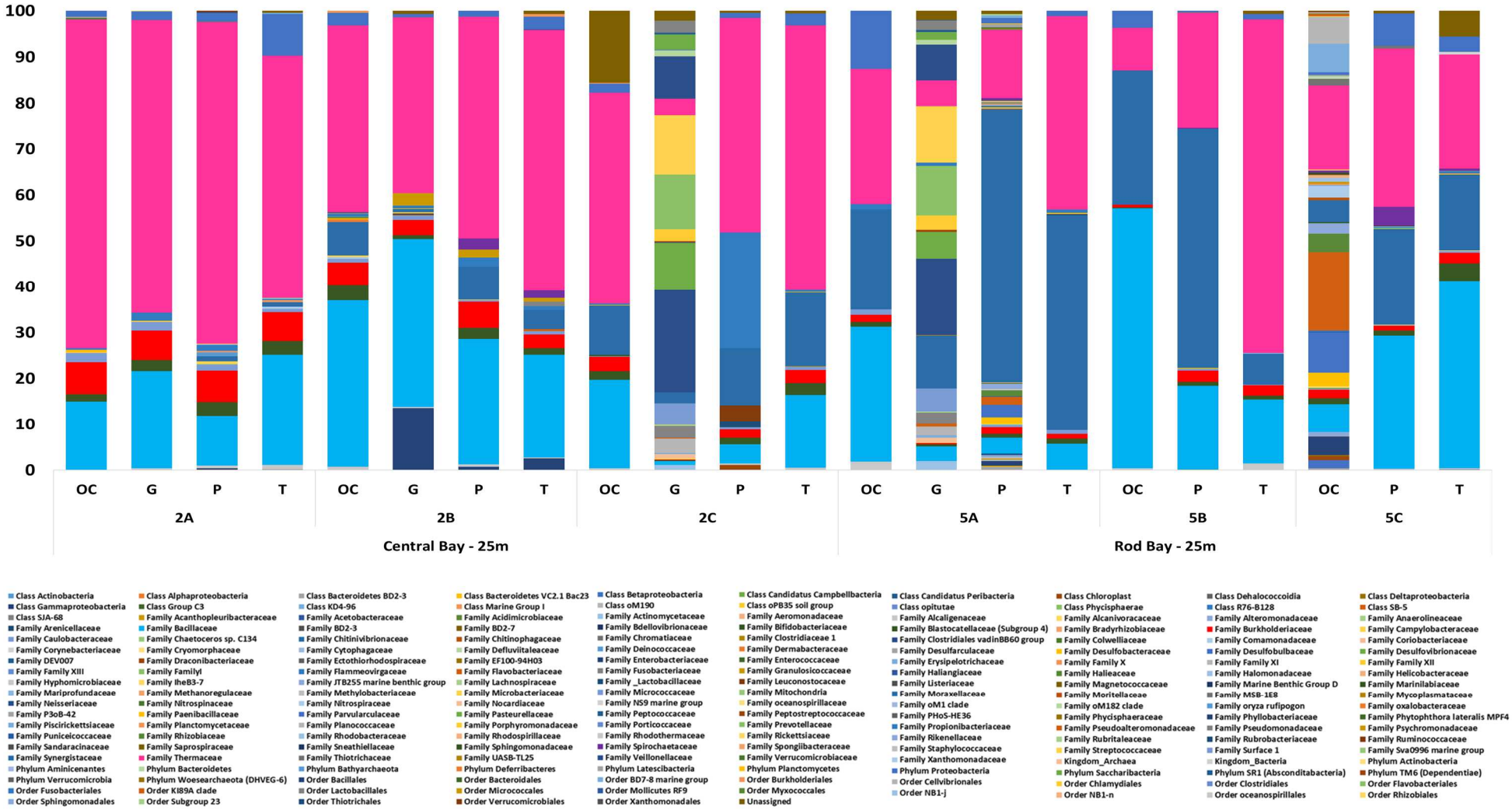


Figure 7. Taxonomic composition of microbiomes associated with different parts of the body of *A. trissophyllus* collected at Rod bay and Central Bay.

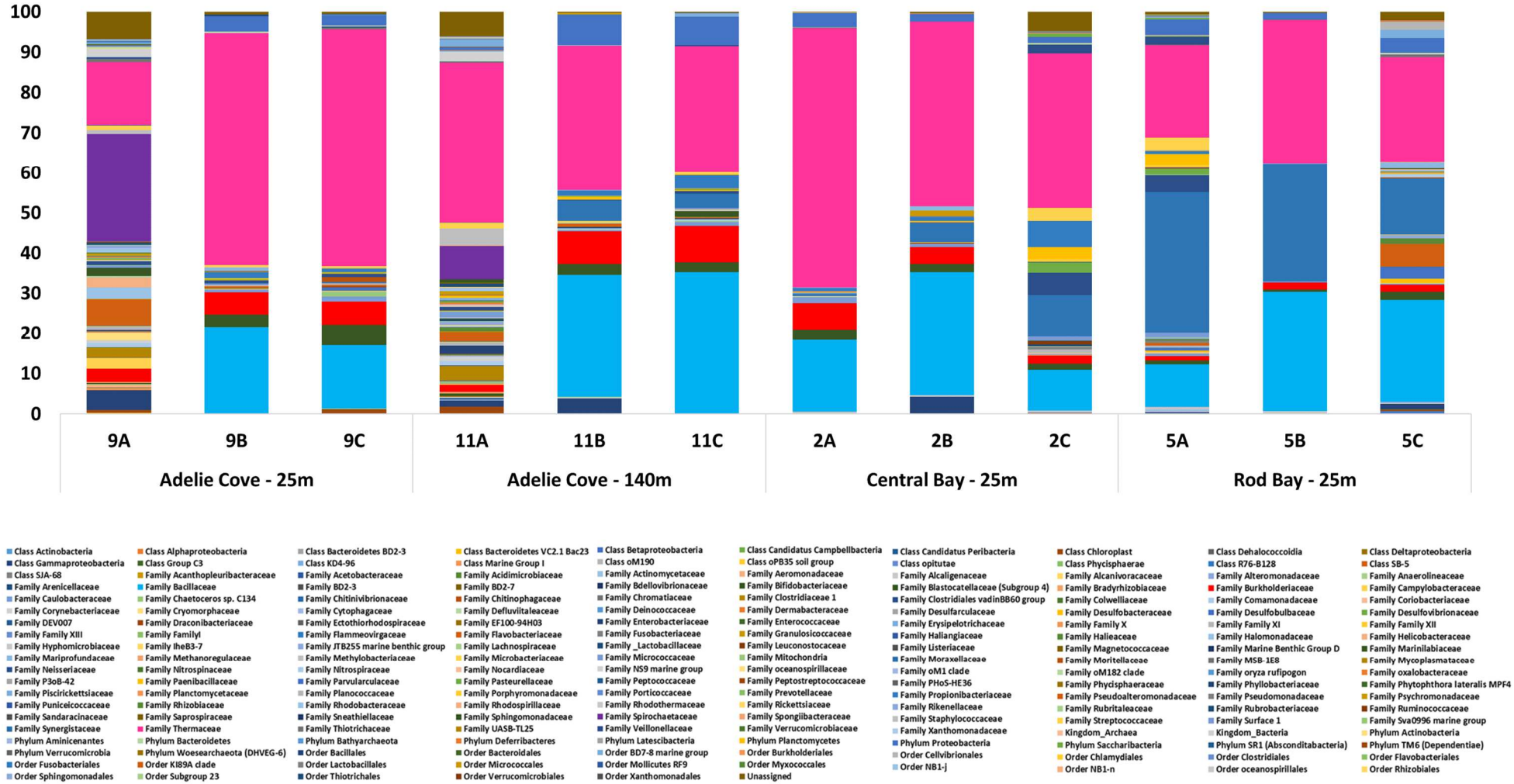


Figure 8. Taxonomic composition of microbiomes associated with individuals of *A. trissophyllus* collected at Adeliec Cove (25m and 140), Rod bay (25m) and Central Bay (25m).

Microbiome composition at the individual level

In Adelie Cove, the similarity among microbiomes from different parts of the body within each individual is around 60%, both at 25 m and 140m, except for the individual 9A and 11A, who's the similarity among the parts decreases to 20% (at 25m) and 40% (at 140m) (Fig. 9).

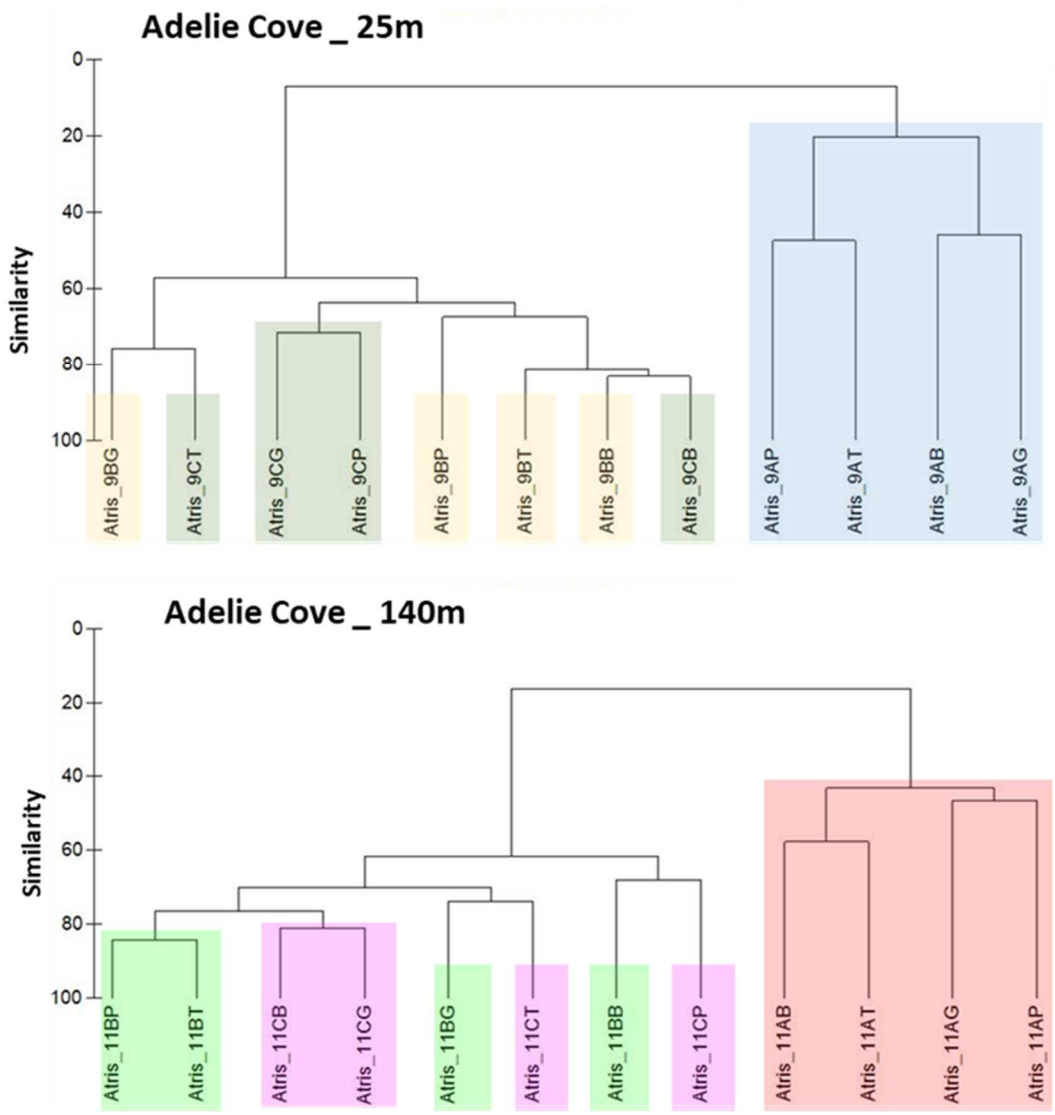


Figure 9. Cluster analysis carried out on the taxonomic composition of microbiomes associated with individuals of *A. trissophyllus* collected at Adelie Cove at 25m and at 140. Samples representing microbiomes collected in different parts of the same individual were highlighted with the same color.

Bacteria mostly responsible for differences in the individual 9A are those belong to *Spirochaetaceae* family, with a contribution to the whole assemblage of 60%, 40%, 11%

and 2% in tegument, parapods, oral cavity and gut, respectively. Bacteria belonging to *Burkholderiaceae* family and *Gammaproteobacteria* class are present in the gut with a contribution of 11% and 18%, respectively. On the other hand, the differences among the microbiomes of the body parts of the individual 11A are driven by ASVs affiliated with the *Spirochaetaceae* (that accounts for 26% in the parapods whereas, it not exceeds 4% in the other body parts) and *Thermaceae* (present in all parts for a fraction of 50% and only for 6% in the parapods) families and by unassigned ASVs (especially in the gut: 15%, which is more than 3 times higher than the other parts).

Microbiomes associated with polychaetes collected at Central Bay showed relatively high similarities among the different parts of the body of each individual (up to around 65%), except for the individual 2C, in which the taxonomic composition of microbiome associated with its gut had a similarity below 20% (Figure 10). In fact, the microbial community present in the gut is different from that of other parts, with a high percentage of *Neisseriaceae, Streptococcaceae*, *Prevotellaceae* and *Pasteurellaceae* families (22%, 13%, 12% and 10%, respectively) completely absent in the other parts; moreover, bacterial ASVs belonging to the *Thermacea* family, present in the other parts with a contribution of 47-57%, decrease down to 4% in the gut. Finally, *Propionibacteriaceae* family was represented in the parapods for a fraction of 25%, despite the negligible contribution in the other parts.

Finally, polychaetes collected at Rod Bay show similarities among microbiomes associated with the different parts of each individual of 60%, 30% and 20% in 5B, 5C and 5A, respectively (Figure 10). Bacterial ASVs mostly responsible for these differences in individual 5A, are those associated with tegument and parapods: such as bacteria belonging to *Neisseriaceae, Streptococcaceae*, *Prevotellaceae* and *Pasteurellaceae* families (17%, 12%, 11% and 6%, respectively) found in the tegument of the 5A but absent in the other parts; *Desulfobulbaceae* family, instead, was found only in the parapods (3%); moreover, *Moraxellaceae* and *Thermaceae* families are present in all the parts, but decreasing from 60% (parapods) to 11% (tegument) and from 42% (gut) to 6% (tegument), respectively. In the individual 5C, differences in taxonomic composition of microbiomes in the parts of its body are mostly driven by bacteria associated with oral cavity. In the oral cavity the *Flavobacteriaceae* and *Desulfobulbaceae* families, completely absent in the other parts, contributions of 17% and 3%, respectively, while, *Bacillaceae* and *Moraxellaceae* families show values of abundance four times lower than in the other parts.

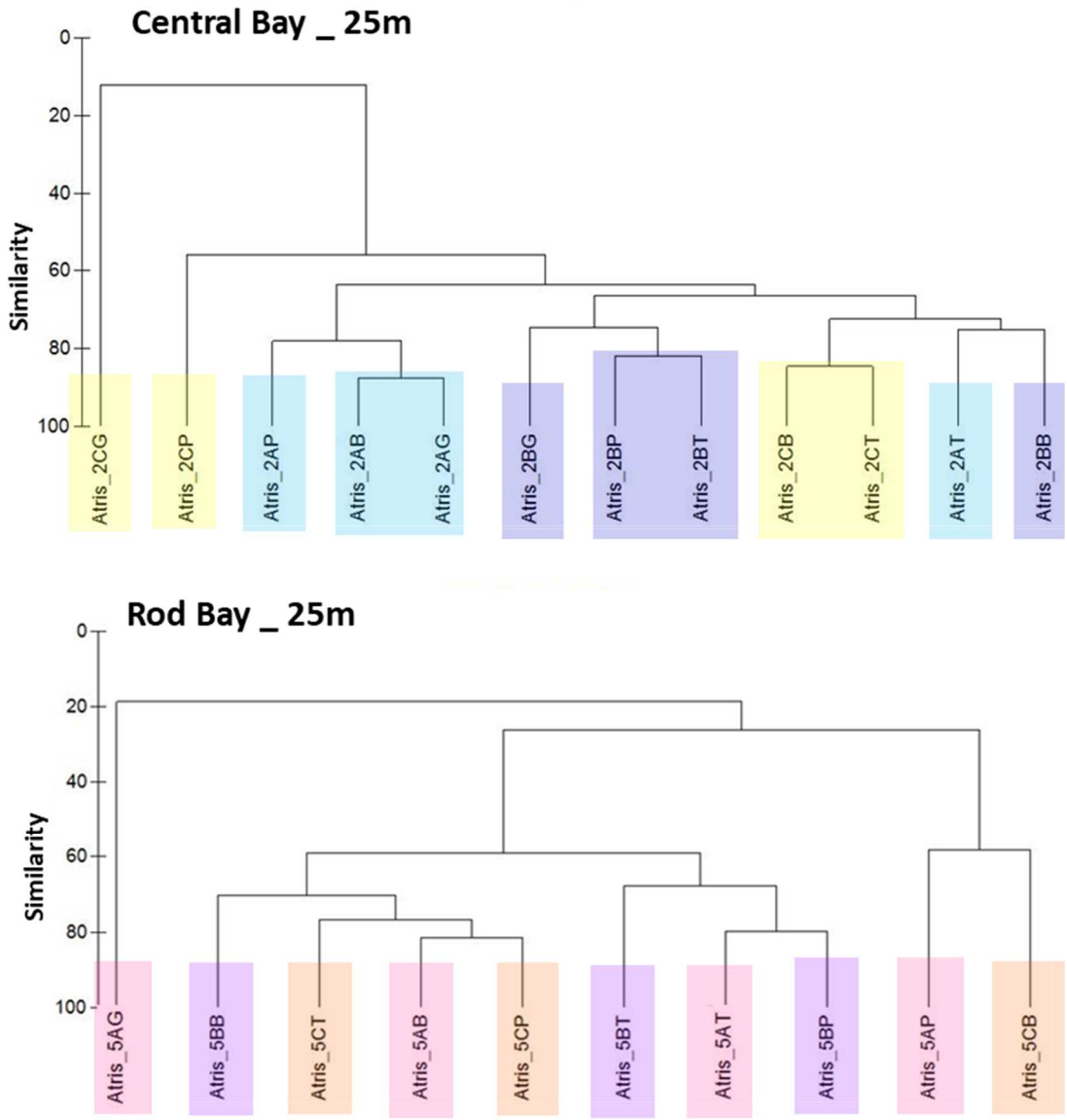


Figure 10. Cluster analysis carried out on the taxonomic composition of microbiomes associated with individuals of *A. trissophyllus* collected at Central Bay and at Rod Bay at 25m. Samples representing microbiomes collected in different parts of the same individual were highlighted with the same color.

Microbiomes composition associated with individuals collected in the same area

No significant differences were found in the "whole-body" microbiomes associated with polychaetes collected in the same areas and at the same depth (Tab. 1SM). In fact, microbiomes of polychaetes collected at Adelie Cove at 25 m showed a similarity of only 20%, driven by the individual 9A, which was characterized by a microbial assemblage completely different from the other two individuals (9B and 9C). Bacterial ASVs, which

mainly explained these differences, belonged to the *Spirochaetaceae* family, present in 9A individual with a percentage of 27% and absent in 9B and 9C specimens; and *Bacillaceae* family, present in 9B and 9C with a percentage of 21% and 16%, respectively and absent in 9A individual. The same result was found considering polychaetes collected at Adelie Cove at 140m, where the individual 11A clustered apart, showing a similarity of only 20% with the other two individuals. The bacterial ASVs that mainly explained these differences belonged to the *Spirochaetaceae* family, present in 11A with a percentage of 8% and absent in the 11B and 11C individuals; and the *Bacillaceae* family, present in 11B and 11C with a percentage of 30% and 35%, respectively and absent in 11A individual.

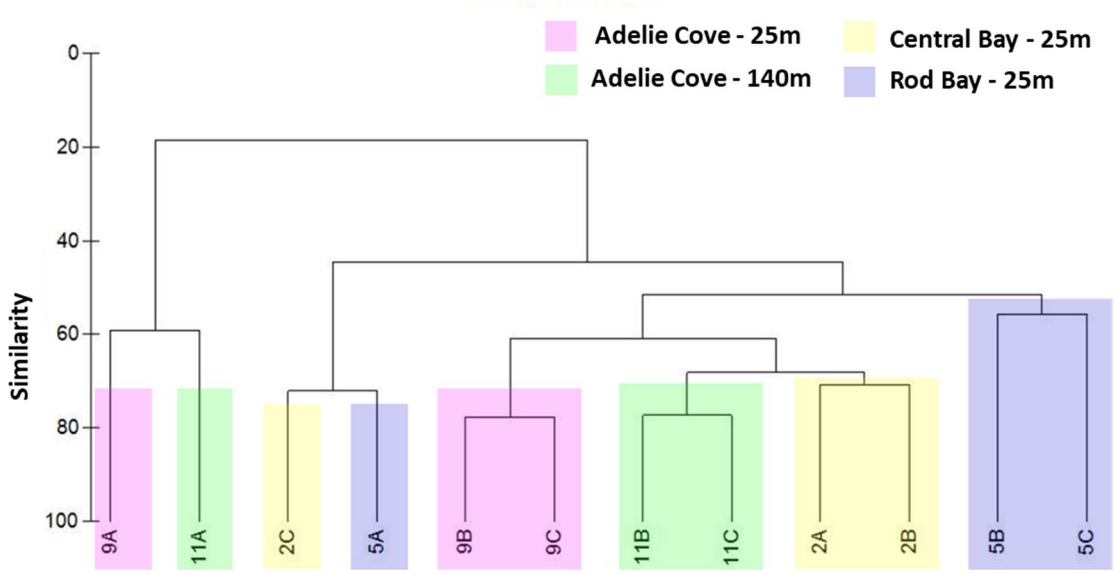


Figure 11. Cluster analysis carried out on the taxonomic composition of microbiomes associated with the whole-body of all individuals of *A. trissophyllus*.

Polychaetes collected at Central Bay showed a 45 % similarity in the community structures of their microbiomes, with the individual 2C that clustered far from the other two individuals. Differences were mainly explained by the exclusive presence of *Neisseriaceae* family in the 2C individual (6%) and by the contribution of the *Moraxellaceae* family, which in 2C individual was two and ten times higher than in 2A and 2B, respectively, and of *Bacillaceae* family (which in 2A and 2B was two and three times higher than in 2C, respectively). Finally, similar results were found in polychaetes collected at Rod Bay, in which the individual 5A clustered apart from the other two showing a similarity of ca. 45%. Families of bacteria that better explained these differences are: *Neisseriaceae* family, present

in 5A with a contribution of 4% and absent in 5B and 5C; *Bacillaceae* family, present in 5B and 5C with contributions three times higher than in 5A; *Moraxellaceae* family with a contribution of 35%, 30% and 14% in 5A, 5B and 5C, respectively.

Microbiomes composition associated with polychaetes collected in different areas

No significant differences in the taxonomic composition of microbiomes associated with the "whole-body" of polychaetes collected in different areas. The only differences were found between the two individuals 9A and 11A and the others (Fig. 12; Tab. 1SM).

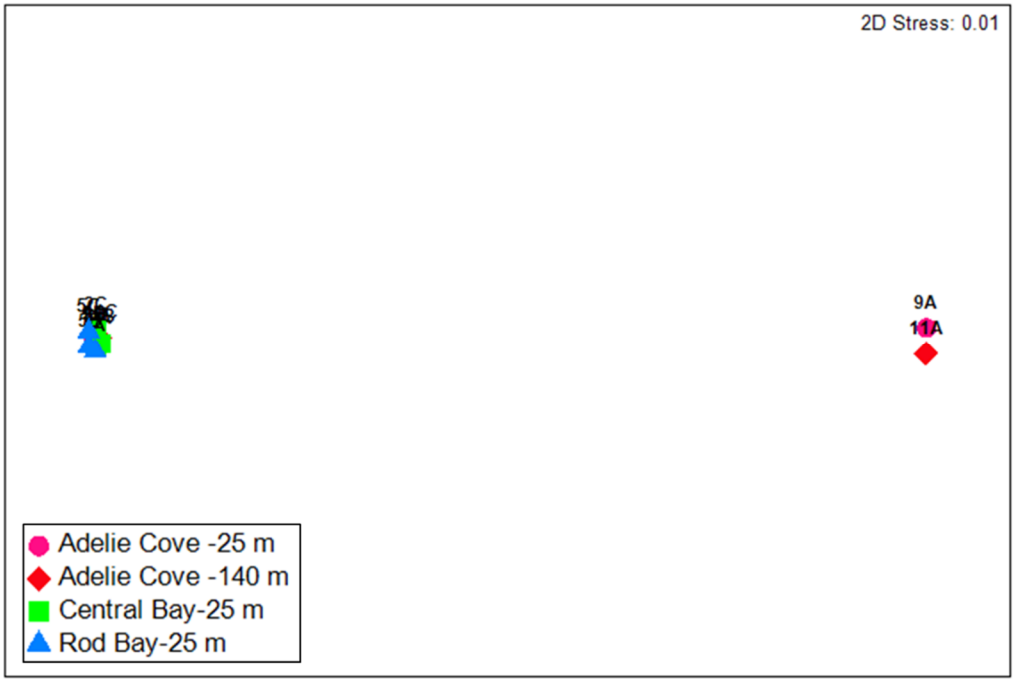


Figure 12. MDS on the taxonomic composition of microbiomes associated with "whole-body" microbiomes of individuals of *A. trissophyllus* collected at different areas and depths.

Among the 196 taxonomic affiliations totally found, six are present in all polychaetes: the *Thermaceae*, *Burkholderiaceae, Blastocatellaceae (Subgroup 4), Propionibacteriaceae* and *Caulobacteraceae* families and *Proteobacteria* phylum. Dissimilarities among the three areas reach the 50%, 58% and 46%, between Adelie Cove *vs* Central Bay, Adelie Cove *vs* Rod Bay and Central Bay *vs* Rod Bay, respectively and the differences are better explained by *Bacillaceae* family, present in all polychaetes, except in two individuals (9A and 11A)

collected of Adelie Cove, and *Moraxellaceae* family, that show percentage of abundance higher in Rod Bay (26%) than the other two areas (1% and 5%, in Adelie Cove and Central Bay, respectively).

Same results were found considering the taxonomic composition of microbiomes across the different parts of the body of polychaetes, with no significant differences in the three areas and between the two depths considered at Adelie Cove (Fig. 13).

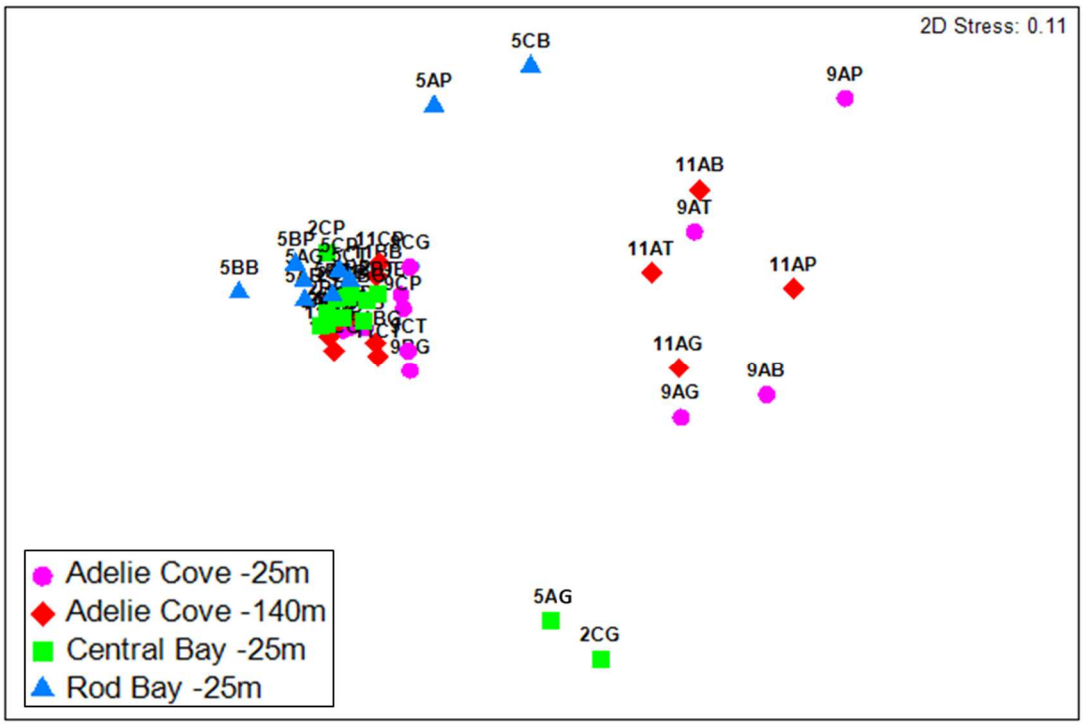


Figure 13. MDS on the taxonomic composition of microbiomes associated with the different parts of the body of *A. trissophyllus* collected at three different areas and different depths (B: oral cavity; G: gut; P: parapods; T: tegument).

Moreover, exclusive bacterial ASVs were found in each individual, with the highest numbers found in the individual 9A and 11A (20 and 32, respectively). The highest number of exclusive ASVs were found in the oral cavity and parapods (Fig. 11).

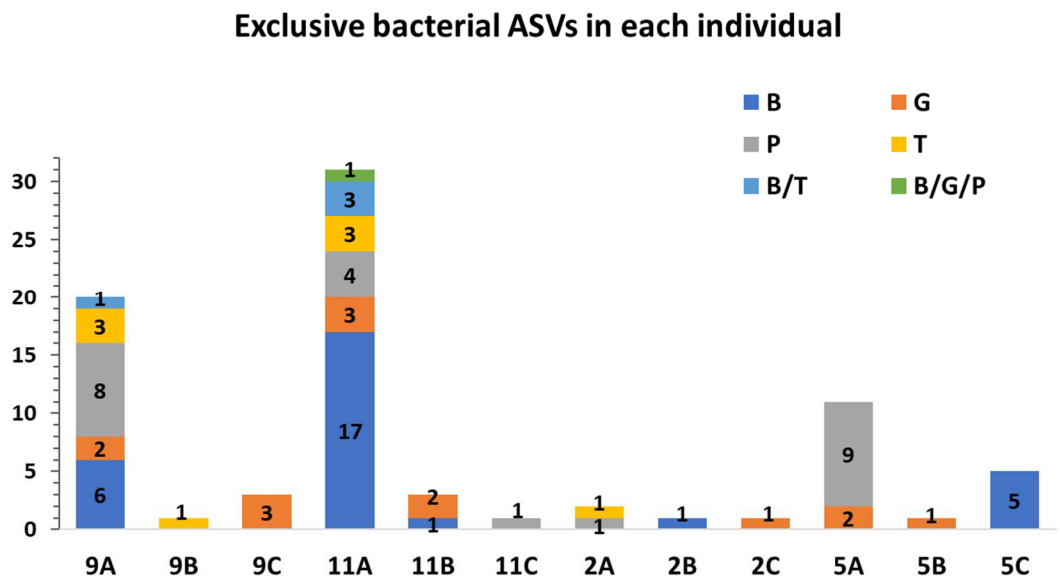


Figure 14. Numbers of exclusive bacterial ASVs in each individual and in which part of the body they were found (B: oral cavity; G: gut; P: parapods; T: tegument).

Microbiome composition associated with different parts of the body of all individuals of *A. trissophyllus*

No significant differences in the taxonomic composition of microbiomes were found among the different parts of the body of *A. trissophyllus* (Fig.15; Tab. 1SM). Simper analysis revealed that the similarities in the taxonomic composition of samples belonging to a same anatomic part varied from 46% to 53%.

A core microbiome was found in each part of the body. Families that composed the core microbiomes in oral cavity, gut, parapods and tegument, showed a contribution to the total assemblage of 44%, 47%, 37% and 55%, respectively (Figure 16 a – b – c - d). Beside of the *Thermaceae* family present in all the parts, the *Blastocatellaceae* family was found in all samples of the oral cavity, gut and tegument with from 3,4 to 4,7% contribution. Bacterial ASVs belonging to the *Burkholderiaceae* family were found in all samples of gut and tegument, with percentage of 11% and 7,5%, respectively. Bacteria belonging to *Protebacteria* phylum accounted for 8,6% and 6% to the core microbiomes of oral cavity and gut, respectively. Finally, on average, only in the tegument of polychaetes, 2% of bacterial ASVs was unassigned.

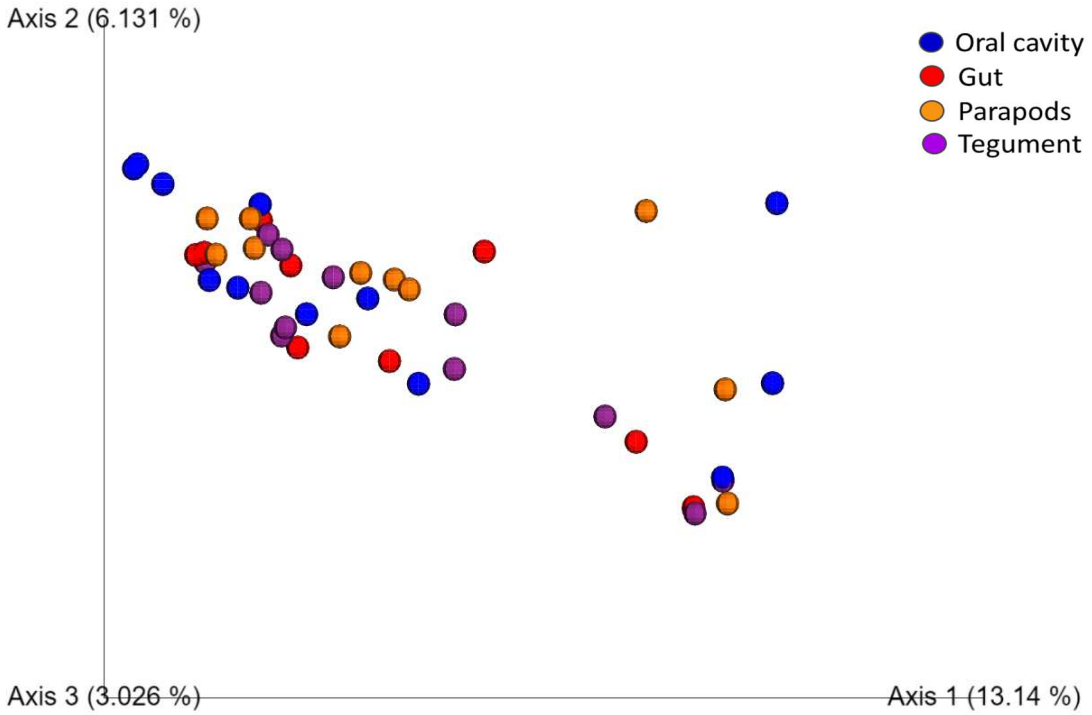


Figure 15. PCoA plot on the taxonomic composition of the different parts of the body of all individuals of *A. trissophyllus.*

Moreover, 2%, 1%, 0,6% and 0,3% of the bacterial families were found exclusively in the oral cavity, gut, parapods and tegument, respectively. The most important families exclusive of the oral cavity were *Nitrospiraceae* and *Ruminococcaceae* families and bacteria of *Cellvibrionales* order; *Rhizobiaceae* and *Rikenellaceae* families for the gut; *Rickettsiacea* family for the parapods and bacteria belonging to *Bathyarchaeota* phylum and *Synergistaceae* family for the tegument. Nevertheless, the contribution of the exclusive bacterial families in each body part did not exceed the 2% on the total microbiome.

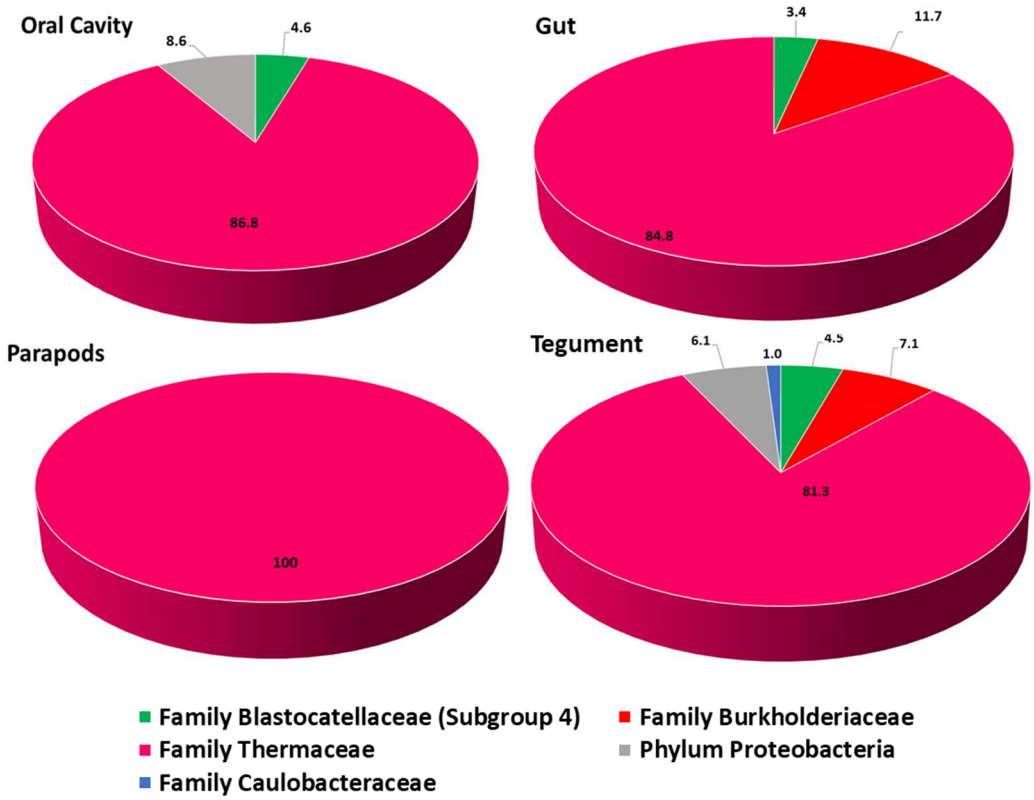


Figure 16. Taxonomic composition of the core microbiomes of oral cavity, gut, parapods and tegument of all individuals of *A. trissophyllus.*

5.3.3 Putative functions of microbiomes associated with the different body parts of *A. trissophyllus*

No significant differences were found in the putative functions of microbiomes associated with different parts of the body of individuals of *A. trissophyllus* collected at different trophic conditions and different depths (Fig. 18-19; Tab 1SM). The most important functions that were performed by bacteria in all the samples are the fermentation and the chemoheterotrophy, with a contribution from 1% (5AP) to 45% (11CB) and from 35% (11AB) to 88% (2AB), respectively. High percentages of bacteria able to degrade the hydrocarbon compounds were found in almost all samples (9BG, 2AB and 2AG excluded), with values from 0,1% (9AP) to 19% (5AG). Simper analysis revealed that samples belonging to the same part of the body showed a similarity in the putative functions of their microbiomes of ca. 70%, mostly explained by the groups of bacteria involved in fermentation, chemoheterotrophy, degradation of hydrocarbon compounds and bacteria recognized as parasites and human pathogens (Fig. 17). Dissimilarities among the different

parts of the body were mainly driven by bacteria involved in the respiration of nitrogen compounds, present in parapods, oral cavity, tegument and gut with different contributions (1%, 1,7%, 3% and 4,3% respectively), and bacteria responsible of the dark oxidation of sulfur compounds mainly found in oral cavity (2,5%) and in parapods (2,2%).

No significant differences in putative functions of microbiomes were found among polychaetes collected in different areas but significant differences were found between microbiomes at the two depths of Adelie Cove (Tab. 1SM): the percentages of dissimilarity among the areas varied from 25% and 35%, with the fermentation, degradation of hydrocarbon compounds and ureolysis as the main responsible groups of these differences.

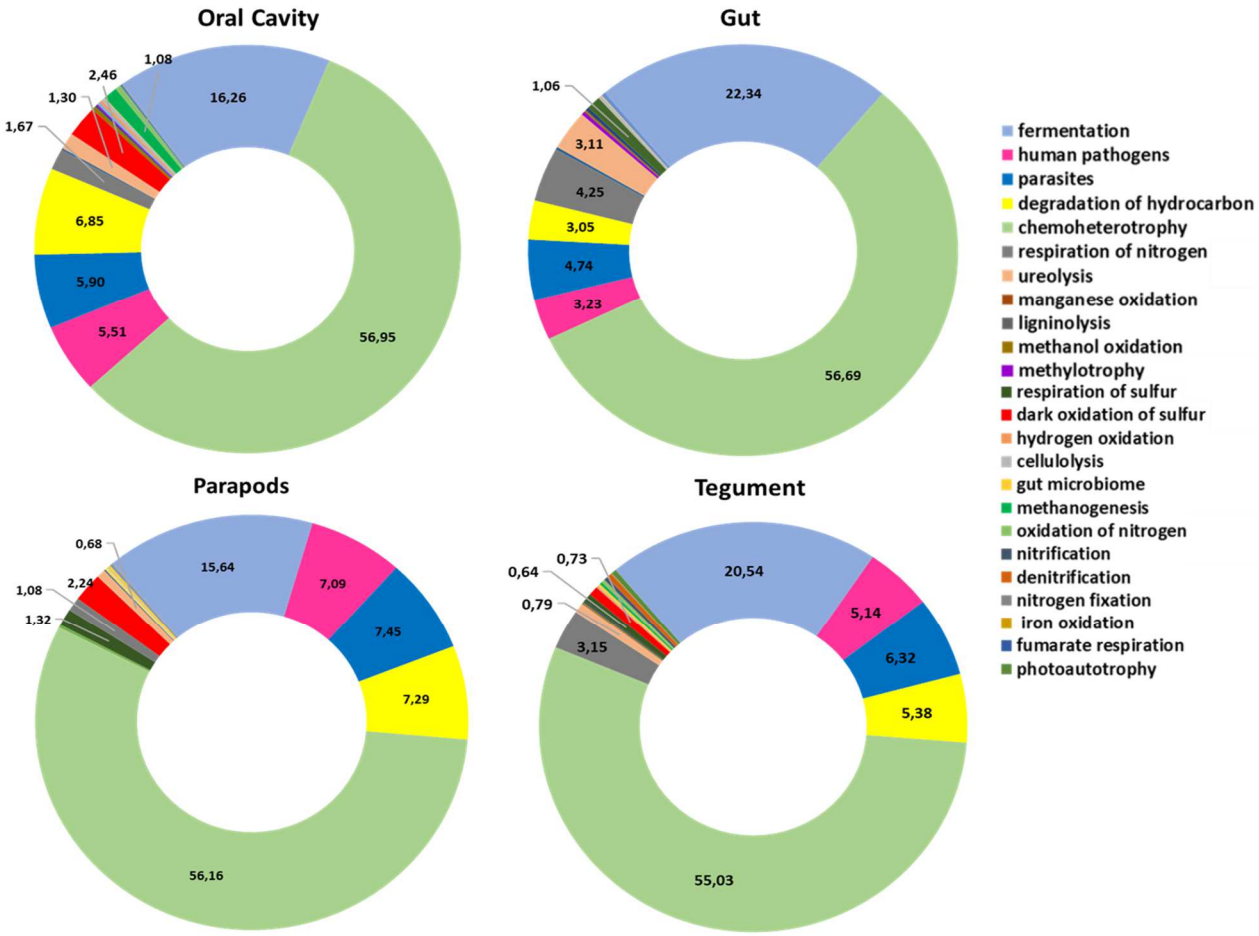


Figure 17. Putative functions developed by microbiomes associated with the different part of the body of individuals of *A. trissophyllus.*

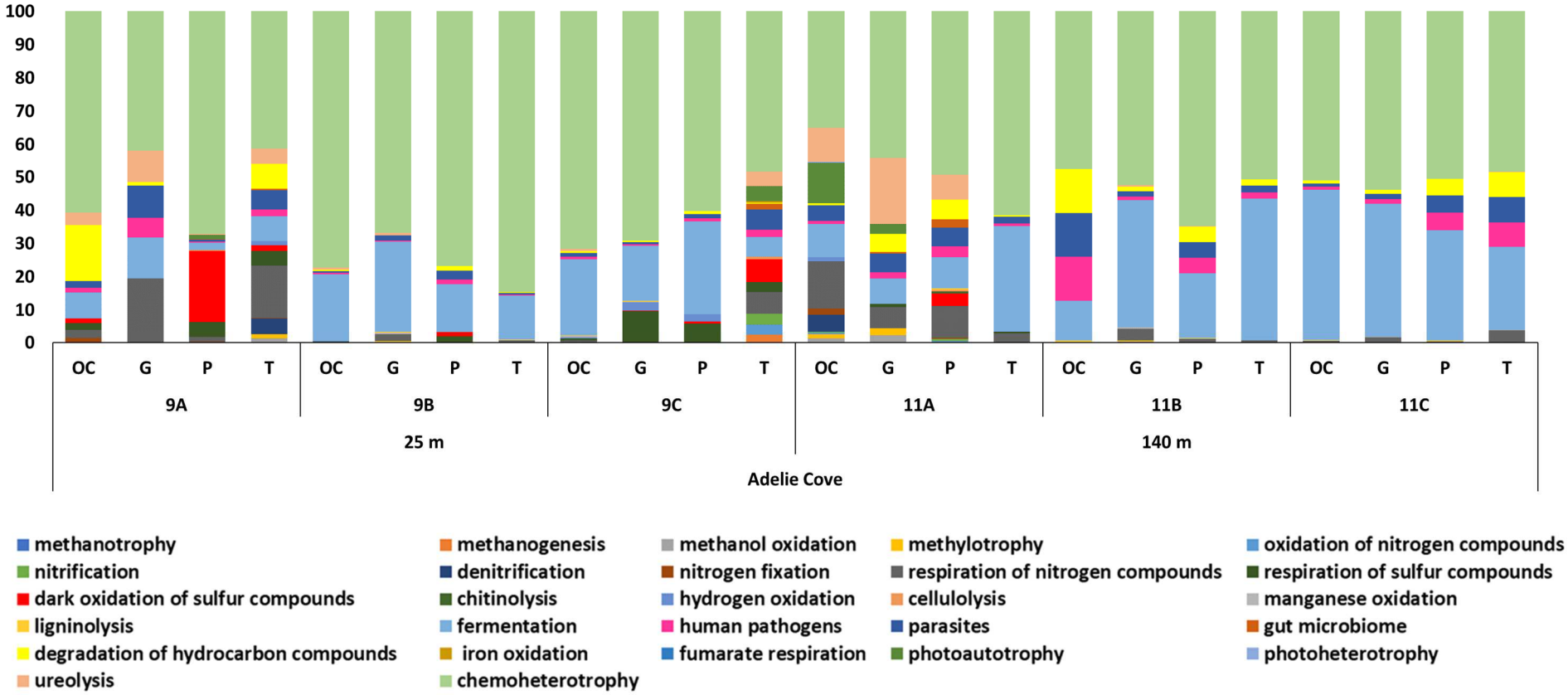


Figure 18. Putative functions of microbiomes associated with different parts of the body of *A. trissophyllus* collected at 25m and 140m at Adelie Cove (OC: oral cavity; G: gut; P: parapods; T: tegument).

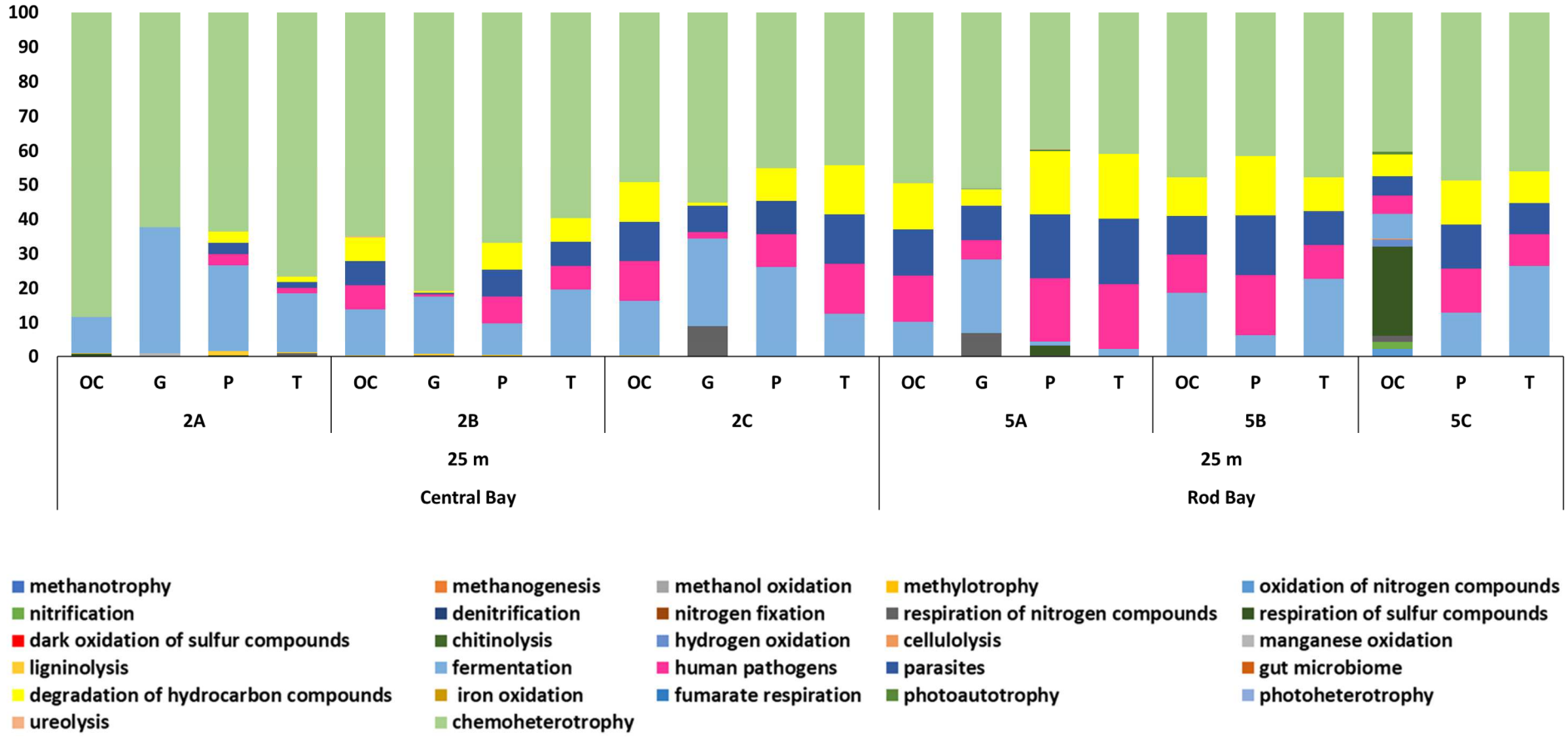


Figure 19. Putative functions of microbiomes associated with different parts of the body of *A. trissophyllus* collected at 25m at Rod bay and Central Bay.

5.3.5 Comparison between microbiomes associated with *A. trissophyllus* and bacteria living the surrounding sediments

Significant differences were found between microbiomes associated with polychaetes and surrounding sediments both in terms of ASV richness and taxonomic composition (Tab. 1SM). In particular, the analysis of the taxonomic composition at the family level in the microbiomes of surrounding sediments and polychaetes revealed that among 276 total families found, none was shared among all samples of sediments and polychaetes (Fig. 23). Only 3 families were in common with the 80% of the samples. These are the *Thiotrichaceae, Rhodobacteraceae* and *Chaetoceros sp. C134* families, present in the polychaetes with an average of abundance of 0,2% against the 5,7%, 6% and 12% in sediments, respectively. In fact, the dissimilarity among polychaetes and the corresponding surrounding sediment considering the whole-body microbiomes reached the 86%, explained by *Thermacea, Bacillacea and Chaetoceros sp. C134, Planctomycetaceae and Flavobacteraceae* families.

Cluster analysis carried out on the taxonomic composition of microbiomes associated with polychaetes and surrounding sediments highlighted the presence of three groups: most of the microbiomes of polychaetes (1) showed a total dissimilarity with those of the sediment (2), whereas the microbiomes of 9A and 11A individuals (3) were more similar to microbial assemblages of the sediments (Fig. 20).

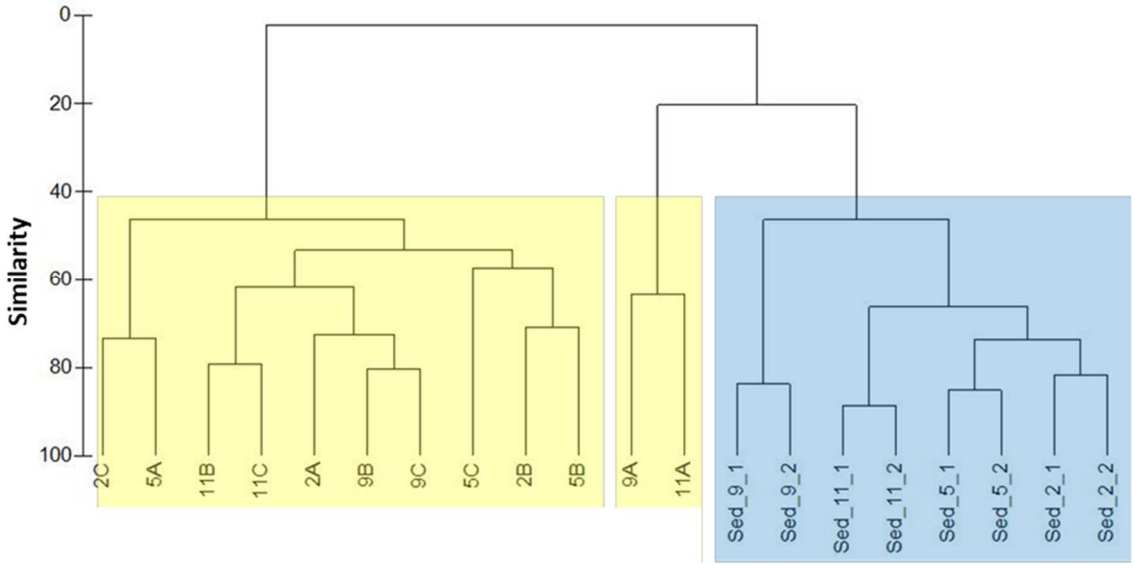


Figure 20. Results of a Cluster analysis carried out on the taxonomic composition of individuals of *A. trissophyllus* (yellow boxes*)* and sediments (blue box).

Despite the high dissimilarities between polychaetes and sediments, results of Cluster analysis revealed that microbiomes associated with oral cavities and parapods of the polychaetes were more similar with the microbial assemblages of the surrounding sediments than those associated with gut and tegument (Fig.21). In fact, the highest numbers of bacterial taxa shared with the surrounding sediments were present in the oral cavity and parapods (Fig. 22). Among these, 14, 4, 10, 1 bacterial families of the sediments were exclusively present in oral cavity, gut, parapods and tegument respectively. Nevertheless, bacterial families shared between these parts and sediments represent on avarage the 0,4% of the total assemblages.

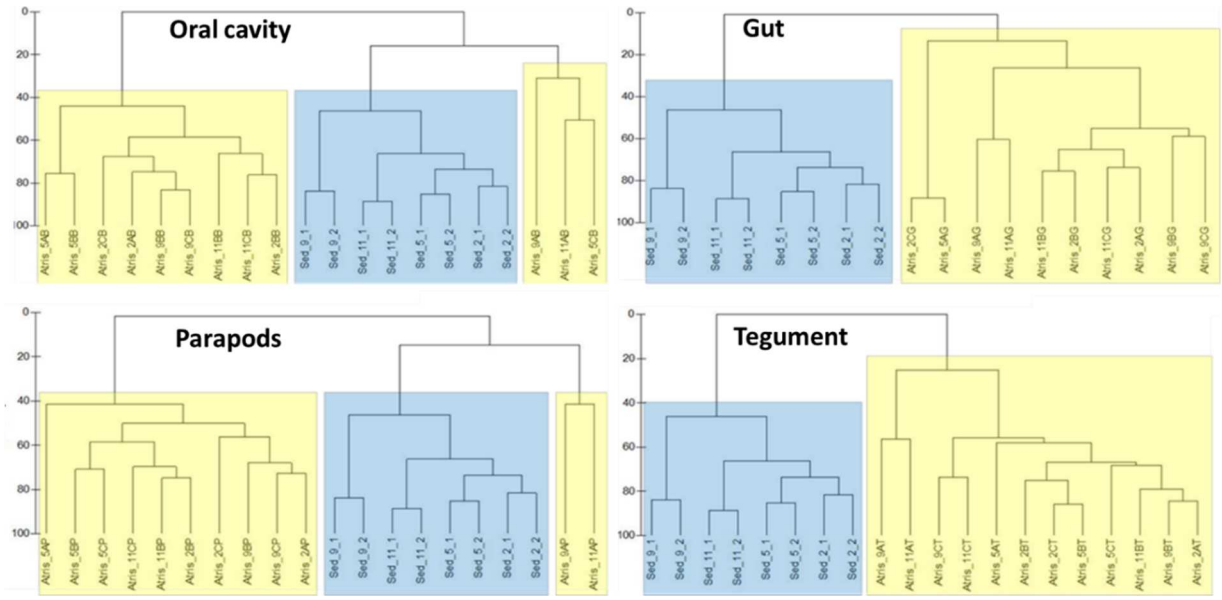


Figure 21. Results of the Cluster analysis carried out on the taxonomic composition of oral cavity, gut, parapods and tegument of *A. trissophyllus* (yellow boxes) and sediments (blue boxes).

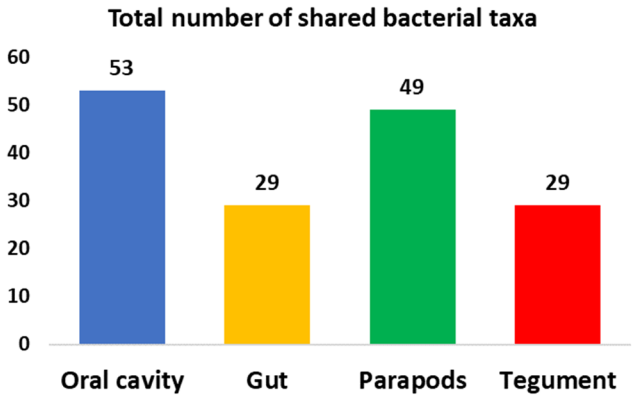


Figure 22. Total number of shared bacterial taxa between microbiomes associated with the different parts of polychaetes and the surrounding sediment.

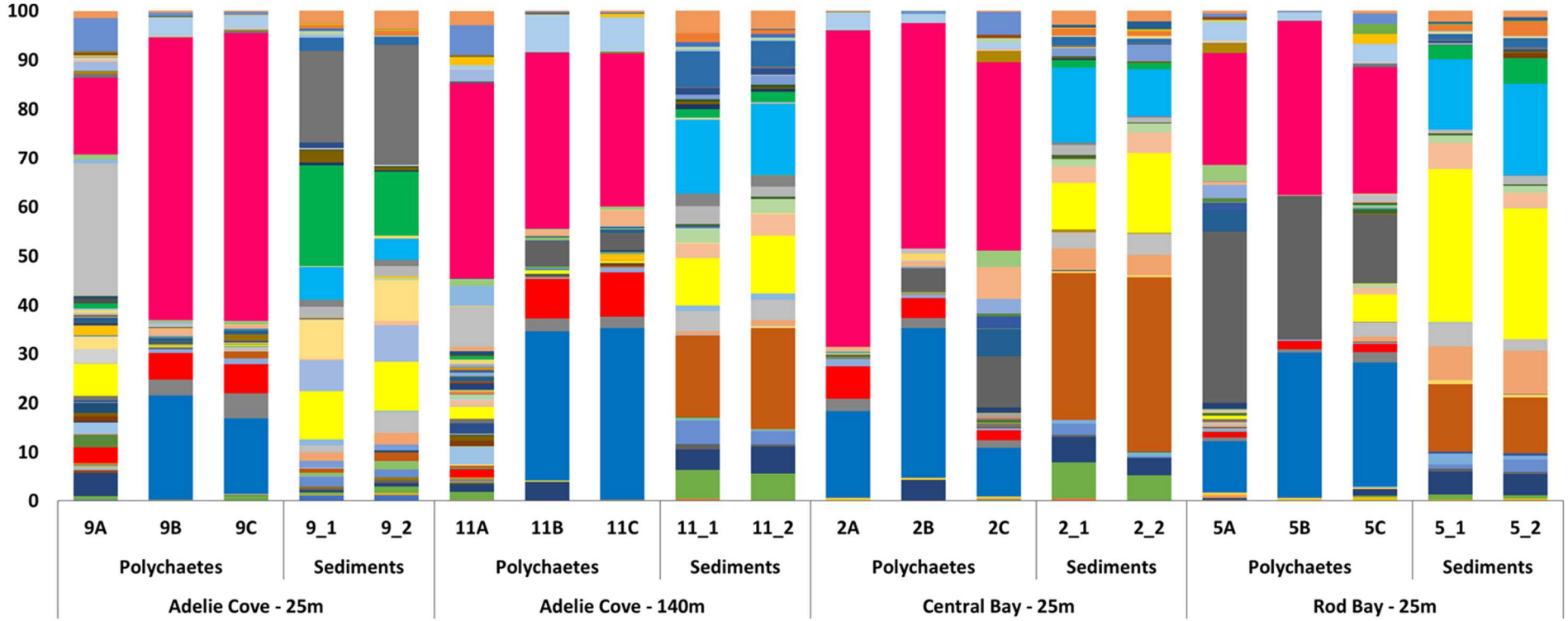


Figure 23. Taxonomic composition of microbiomes associated with the whole-body of the polychaetes and with surrounding sediments collected in the three areas of sampling.

5.4 Discussion

5.4.1 Bacterial assemblages in the different parts of the body of *A. trissophyllus* in the different environmental settings

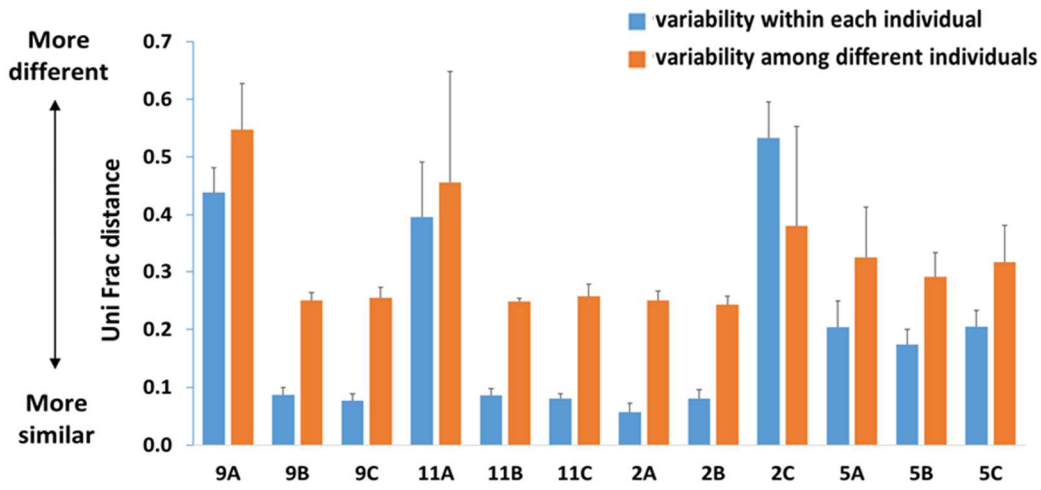
Microorganisms associated with metazoans have profound impacts on host health and development by altering the behavior, immunity, digestion and reproduction (Hadfield, 2011; Shin et al., 2011). These impacts can be mediated by microbes through a wide range of mechanisms and can differ in a host-tissue specific manner (Jackson et al. 2018). Numerous studies carried on human beings revealed partitioned microbiomes, characterized by different microbial communities depending on the part of the body (Huttenhower et al. 2012; Costea et al. 2017). Prior studies have focused on body "habitats" including the gut, skin and oral cavity and have revealed microbial communities that were highly variable both within and between individuals (Turnbaugh et al. 2009; Grice et al. 2009; Nasidze et al. 2009). In fact, the microbial habitats are not isolated from each other; each person comprises a complex, yet interconnected "landscape", consisting of many bodies "habitats" harboring microbiotas (Costello et al. 2009). On the other hand, the complexity and spatial organization of the microbiomes in non -human systems, especially in marine invertebrates, is little investigated for now, and some questions remain still opened.

In this study, we have investigated the diversity of microbiomes associated with 12 Antarctic polychaetes individuals, *Aglaophamus trissophyllus*, even comparing specific microbiomes of their different parts of the body (oral cavity, gut, parapods, tegument). We assessed differences and similarities among microbiomes from the intraindividual level (given by the comparison of microbiomes in the four body parts) to the intraspecific (interindividual) level, among the different specimens.

Within each individual, the similarities across microbiomes of oral cavity, gut, parapods, and tegument changed depending on the individual (from 15% to > 60%). Comparing the taxonomic composition of microbiomes of all the anatomic parts among different individuals, we found a core microbiome dominated by the *Thermaceae* family. Despite this core, results indicate the lack of a community structure that specifically described each anatomic part, indeed, the similarity of the microbiomes of the same part of the body among different individuals did not exceed the 10%. This results highlighted that the dissimilarity among microbiomes of the different body parts (oral cavity, gut, parapods, tegument) within one individual was lower than that between the same body parts (e.g., gut vs. gut vs. gut etc. or tegument vs. tegument vs tegument etc.) between different individuals. Conversely, recent findings on marine invertebrates highlighted the existence of a highly partitioned

microbiome in sea stars, with diversified microbial communities between the pyloric caeca, gonads, coelomic fluid and body wall of the animals. Differences were mostly driven by relative abundances of *Spirochaetae* and *Tenericutes* groups (Jackson et al. 2018).

To better evaluate the variation of microbiomes at the intraindividual and intraspecific (interindividual) levels we determined the Unifrac Distance (Figure 22).



Figur□22. M□a□valu□s of U□ifrac dista□c□calculat□d amo□g th□diff□r□□t parts of th□body withi□ □ach sp□cim□□(amo□g th□four body parts) a□d b□tw□□□that i□dividual a□d all th□oth□rs.

This analysis confirms a higher variation among microbiomes associated with different individuals of polychaetes than among different body parts of the same individual.

Similar results were obtained in several recent investigations, where the highest variability of microbiome was found at individual level (Huttenhower et al. 2012; Califf et al. 2014; Costea et al. 2018). Indeed, Costello and colleagues (2009) observed that the highest variability of microbiomes was found considering the different body parts in an individual. In particular, the differences were due to microbiomes associated with the oral cavity and gut, showing a completely unique microbiome, probably for the close link that these body parts can have with the environment (Costello et al 2009). Our results partially confirmed this, revealing that the variability in the microbiomes associated with the *A. trissophyllus* and the unicity of microbiome of each individual were due also to the presence of exclusive bacterial ASVs, that were especially found in the oral cavity and parapods parts. Considering the life habits of the *A. trissophyllus*, including its feeding and mobility strategies, the oral

cavity and the parapods could be the parts of the body more in contact with the surrounding environments. The higher values of microbiome variability among different specimens than within an individual sustains the concept of "personalized microbiome" that the human microbiome research is facing during these last years. Everyone harbors a specific microbiome, completely different from each other individual, that reflects personalized features and that can be driven and shaped by individual and often unknown factors (Califf et al. 2014; Gilbert et al. 2018). This concept can be also applied to our Antarctic polychaetes (particularly, in the individuals 9A and 11A), where microbial assemblages are so diversified among them, to make each polychaete different from each other. However, this is not a general rule as in the individual 2C, in which the intra-individual variability of the microbiomes was higher than the inter-individual one.

The dissimilarity observed among the microbiomes of different individual of *A. trissophyllus* could be also due to the different stability or temporal variability of each bacterial assemblage inhabiting organisms. In fact, important pathogens, environmental stressors and immune dysfunctions can lead to increased stochasticity in the microbiome (Sachs et al. 2011; Flor□□ □t al. 2015; Yilma□ □t al. 2015; Cas□y □t al. 2015). The presence of *Neisseriaceae, Streptococcaceae*, *Prevotellaceae* and *Pasteurellaceae* families in the gut of some polychaetes, increasing the intra-individual variability, could indicate altered conditions in the healthy status of that specimens (e.g., 2C, 5A). In fact, bacteria belong to these families are considered potential pathogens of humans and animals, responsible of serious diseases, or involved in the healthy status of the hosts (Wong et al. 2015; Lory et L. 2014; Rosenberg et al. 2014; Christensen et al. 2014). In particular, in human research the *Prevotellaceae* family is considered a biomarker for the Parkinson disease. Lower abundance of *Prevotellaceae* were found in affected patients and this low abundance was maintained during the disease progression.

The different environmental conditions of the sampling areas were tested as possible factors that shape the diversity of microbiomes. Polychaetes were collected in Adelie Cove, characterized by high organic input due to the presence of penguin assemblages, in Rod Bay, characterized by potential anthropogenic impact due to the presence of the Italian Station "Mario Zucchelli", and in Central Bay, considered as uncontaminated area, because far from possible source of anthropogenic impact. Moreover, to evaluate the potential effect of depth, in Adelie Cove we considered two different stations, located at 25 m and 140 m. The different trophic conditions were measured through the protein, carbohydrate, lipid and phytopigment concentrations in the sediments as already described in Chapter 4.

We found that trophic conditions of the investigated areas where polychaetes were collected did not have any effect in shaping the taxonomic composition of microbiomes. In fact, the taxonomic composition of microbiomes of polychaetes collected in different areas did not show significant differences. Therefore, it is possible to hypothesize that other environmental and biological factors can have a role in shaping the bacterial assemblages (Reese et al. 2018; Adair et al. 2017; Yatsunenko et al. 2012; Ezenwa at al. 2012).

5.4.2 The potential role of microbiomes associated with *A. trissophyllus*

The community structure of microbiomes is related to the number and type of microbes present, whereas the functions to the metabolic activities and end products that result from their activity (Nicholson et al., 2012). Studies on human gut microbiomes highlighted that, although bacterial composition varies widely among different individuals, the distribution of functional genes of the microbiome is constant, suggesting that key functions in the gut could be carried out by different microbes (Human Microbiome Project Consortium, 2012; Backhad et al. 2012). Biodiversity is an important factor that influences the functioning of a system: studies have demonstrated that higher microbial diversity increases resistance and resilience of microbial processes and favors functionality under selective stress (Wittebolle et al. 2009; Mendes et al. 2015).

Our results highlighted that the microbial assemblages present in each part of the polychaetes covered a similar functional pattern, not diversified in the different anatomic parts: the highest percentages of bacterial ASVs found in polychaetes are potentially involved in the fermentation and chemoheterotrophy, followed by bacterial ASVs responsible of the degradation of hydrocarbon compounds, suggesting an important fraction of commensalist and symbiotic bacteria. Presence also of some parasites was observed.

Similar results were obtained in a recent work carried out on microbiomes associated with the gut of Capitella capitata, with the most functional classes involved in the chemoheterotrophy. Moreover, they have found high fractions of bacterial ASVs potentially involved in hydrocarbon and aromatic compounds degradations, that increase with increasing pollutant's concentrations in the environment (Hochstein et al. 2019). Higher percentages of bacterial ASVs possibly involved in hydrocarbon degradations in polychaetes collected at Rod Bay than in other sampling areas suggest a relatively high level of contamination, probably for the anthropogenic impact due to the near Italian research base "Mario Zucchelli Station". In fact, a recent study highlighted episodes of heavy metals

bioaccumulation in the tissues of *A. trissophyllus*, in same Antarctic areas, closed to research stations and human activities (Trevizani et al. 2016). The presence of functional classes involved in the respiration of nitrogen compounds and in the dark oxidation and respiration of sulfur compounds mostly in polychaetes collected at Adelie Cove could be explained by the high levels of proteins, carbohydrates and phytopigments that characterized the sediments of this area. Similar functional classes were found in highly productive environments, where bacterial assemblages control the turnover of organic carbon and the cycling of nitrogen and sulfur, as the estuaries (Baker et al. 2015). Moreover, bacterial ASVs involved in sulfur respiration were found in the human guts, where hydrogen sulfide is produced, increasing resistance to antibiotics and protecting the organisms from reactive oxygen species, suggesting an important role of these bacteria in the health and functioning of the host (Barton et al. 2017). A relatively high richness of functional groups was found in the individuals 9A and 11A, where high percentages of bacterial ASVs potentially involved in ureolysis were found. A recent work in amphibians showed that nitrogen liberated by ureolytic bacteria is incorporated into the biosynthetic compounds needed to restore body conditions at or before a hibernal emergence (Wiebler et al. 2018). In fact, since the microbiome can react more rapidly to environmental changes, it has been suggested that it can facilitate the adaptation of the host (Song et al. 2016). Information about this process driven by microbiomes in Antarctic organisms is limited but increasing evidence in last years, shows an important role of microbiomes in metabolizing nutrients and heavy metals (Rosenber et al. 2016; Lo Giudice 2015; González-Aravena et al. 2016; Rodrigez-Marconi et al. 2015). Further investigation is required to go deeper in the knowledge of this important associations.

5.4.3 The origin of the microbiome associated with *Aglaophamus trissophyllus*

Processes that regulate the formation of the microbiomes are largely unexplored for most species of organisms. Two modalities of transmission are known: a horizontal way, in which bacteria are selected from whole assemblages inhabiting surrounding environments or a vertical way, in which bacteria are transmitted in the host from the earlier generation (Bright et al. 2010; Kwan et al. 2017). Between these two extremes, a range of microbiomes composed by both exclusive bacteria inherited and taxa acquired from environment could be possible (Kwan et al. 2017). A recent study carried out on microbiomes associated with the polychaete *Hydroides elegans* during all the development stages has demonstrated that

dominant bacteria found on the body are not exclusive of the host, but may be transients because use the worm as a "convenient habitat" or may vary depending on environmental pressures (Vijayan et al. 2019).

The polychaete selected for this investigation, *Aglaophamus trissophyllus*, represents one of the dominant polychaetes in benthic marine communities of Antarctica (Brasier et al. 2016, 2017). It is a vagile carnivore, feeding on small invertebrates including mollusks, crustaceans and other polychaetes (Fauchald & Jumars, 1979) and moving among the grains of the sediments, which can represent a vector of bacteria to different parts of the body.

Our results have shown a high dissimilarity between the microbiomes associated with the polychaetes and bacteria inhabiting the surrounding sediments, both considering the taxonomic composition and the phylogenetic distance. This finding was coherent with the results obtained for the other two species of Antarctic polychaetes (Chapter 4). Among all the families found, none was shared among all the samples of sediments and polychaetes. The most important core families of polychaetes (*Thermaceae*, *Bacillaceae* and *Moraxellaceae* families) are present in sediments with a percentage of abundance lower than 0.005%. Our findings open the possibility of a vertical transmission of microbiomes, probably inherited by the earlier generation and shaped by different factors in each individual. Similar results have been obtained in recent studies carried out on sponges, tardigrades and sea stars, showing a unique microbiome associated with the organisms, completely different from the surrounding environment, probably present from the birth and shaped during the life (Sharp et al.2012; Rodríguez-Marconi et al. 2015; Vecchi et al. 2018; Jackson et al. 2018). Moreover, despite the dissimilarity among microbiomes of polychaetes with the surrounding sediments, some exchanges of bacteria were potentially present and varied among the different parts of the body. In fact, microbiomes associated with the oral cavities and parapods of the *A. trissophyllus* had a number of bacterial taxa in common with sediments two times higher than those of the other two parts. Oral cavities and parapods are directly in contact with the surrounding sediments, making possible an interchange of bacteria, the first ones probably during feeding activities or through the sensorial antennas, the second ones during the movements. Skin-associated bacterial communities, indeed, are highly variable between body parts, creating a patchy habitat, where commensal, pathogens and symbiotic bacteria from the environment could find ideal niches (Fierer et al. 2010; Chiarello et al. 2015). Different factors can play a role in shaping these patches, as the individual physiology, local-scale parameters, environmental stressors and immune

dysfunctions, that can lead to increased stochasticity in the microbiome, favoring the exchange with the environment more in one area than another (Zaneveld et al. 2017).

5.5 Conclusion

Investigations in biodiversity of microbiomes along the landscape of the body parts are well studied in human microbiomes, but little is known in the other organisms. This study represents the first investigation on diversity and functions of microbiomes associated with the different parts of the body of the Antarctic polychaetes, *Aglaophamus trissophyllus*. Our results revealed that the taxonomic composition of microbiomes is dissimilar at the inter-individual level, and possibly explained by the presence of exclusive bacterial taxa of everyone, mostly from the oral cavity and parapods (38 and 29% of the total exclusive bacterial ASVs). Despite such a dissimilarity, a core microbiome, dominated by the *Thermacea, Burkholderiaceae* and *Blastocatellaceae (Subgroup 4)* families, was found. The differences among microbiomes among different individuals were not explained by the different trophic conditions of the habitat. Other environmental and/or biological factors could act as main drivers in shaping bacterial assemblage. At the intra-individual level, microbiomes were not partitioned among the different anatomic parts of the body, so that we could not identify a specific microbiome for each different part/tissue of the animal. Also the main putative bacterial functions (chemoheterotrophy, fermentation, degradation of hydrocarbon compounds) in the microbiomes of the polychaetes were similar in all the parts of the body but some differences were potentially driven by the specific habitat. Indeed, polychaetes living in Adelie Cove, had a higher richness of functions, as ureolysis and dark oxidation and respiration of sulfur compounds. Despite *A. trissophyllus* lives in contact with the sediments, its microbiome is completely different from that in surrounding sediments, opening the possibility to a vertical transmission of bacterial taxa. However, oral cavities and parapods are amongst the body parts of the animals, which can mostly exchange bacteria with the environment during feeding activities and motility. Further studies are required to corroborate or better understand the source of microbiomes in the Antarctic polychaetes and the main factors driving the differences among microbiomes at the intraspecific level.

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5.7 Supplementary material

Table 1 SM. Results of PERMANOVA main test carried out on:

ASV richness of microbiomes among different body parts

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Parts	3	497.6	165.87	0.64395	0.596	998
Res	42	10818	257.58			
Total	45	11316				

ASV richness of microbiomes among different areas and depths

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Area	2	1873.1	936.55	45.149	0.004	999
Depth(Area)	1	6.5157	6.5157	2.90E-02	0.94	998
Res	42	9436.4	224.68			
Total	45	11316				

Taxonomic composition of microbiomes among different body parts

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Parts	3	4335.7	1445.2	0.94749	0.494	999
Res	42	64063	1525.3			
Total	45	68399				

Taxonomic composition of microbiomes among different areas

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Area	2	9766.3	4883.2	2.5592	0.07	999
Depth(Area)	1	1945.2	1945.2	1.4133	0.192	998
Res	42	57808	1376.4			
Total	45	69519				

Taxonomic composition of whole-body microbiome among individuals

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Individual(Area)	2	3324.2	1662.1	1.4271	0.153	953
Res	9	10481.8				
Total	11	13806				

Putative functions of microbiomes among different body parts

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Parts	3	1273	424.34	0.77995	0.692	998
Res	42	22851	544.06			
Total	45	24124				

Putative functions of microbiomes among different areas and depths

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Area	2	5706	2853	2.037	0.213	999
Depth(Area)	1	1470.2	1470.2	3.6435	0.005	997
Res	42	16947	403.51			
Total	45	24124				

ASV richness of microbiomes between polychaetes and sediments

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
source	1	9070.3	9070.3	40.893	0.001	999
Res	52	11534	221.81			
Total	53	20604				

Taxonomic composition of microbiomes between polychaetes and sediments

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
source	1	42838	42838	31.32	0.001	998
Res	52	71124	1367.8			
Total	53	1.14E+05				

6. DIVERSITY AND FUNCTIONS OF THE MICROBIOME ASSOCIATED WITH THE SEA STAR ODONTASTER VALIDUS IN DIFFERENT GEOGRAPHIC LOCATIONS OF THE ANTARCTIC OCEAN

6.1 Introduction

Antarctic ecosystems have been characterized by major events on a wide range of time scales such as the formation of the Antarctic Polar Front in repeated glacial cycles during the past million years, thus influencing genetic connectivity of fauna and producing a unique, but incredibly diverse marine community (Janosik et al. 2010). A recent investigation, carried out on the evolutionary history of the *Odontaster* species in the Southern Ocean, in terms of dispersal ability and population connectivity, has revealed that *Odontaster Validus* (Koehler, 1906) is geographically isolated from Antarctic and sub-Antarctic waters (Janosik et al. 2011), despite it is characterized by a planktotrophic mode of development with capability for vast dispersal. *O. validus* is likely currently restricted to Antarctic waters perhaps due to the combination of physiological constraints and physical barriers encircling the Antarctic continent and the sub-Antarctic islands. In particular, the Antarctic Polar Front may have facilitated speciation, acting as a barrier between the two geographic regions, and restricting the north–south exchange of organisms (Clarke et al. 2005; Janosik et al. 2011).

Animal–bacterial symbioses are a ubiquitous feature of life in the sea for diverse vertebrate and invertebrate taxa and are widespread amongst the five echinoderm classes (Zilber-Rosenberg and Rosenberg 2008; Gilbert et al. 2012; McFall-Ngai et al. 2013; Carrier et al. 2018). Many echinoderms, including sea stars, have subcuticular bacteria localized in the lumen between epidermal cells and the outer cuticle (Burnett et al. 1997; Lawrence et al. 2010; Hoj et al. 2018). This relationship is common, existing in about 60% of echinoderms studied so far and it appears to be related to host classification, in most cases at the family level (McKenzie et al. 1998; Hoj et al. 2018). Those bacteria are surrounded by an extensive network of sea star's epidermal microvilli, an arrangement that may facilitate transfer of nutrients from the bacteria to the host's cells (Roberts et al. 1991; Lawrence et al. 2010). In several species of echinoderms, sub-cuticolar bacteria were recognized as Alpha and Gamma proteobacteria, in particular among members of the families *Phyllobacteriaceae* and

Rhizobiaceae, bacteria usually involved in nitrogen fixation, and members of the order Chromatiales, usually involved in sulfur-oxidation. (Lawrence et al. 2010). Lesser and Walker investigated the importance of bacteria for the nutrition of brittle stars and found that a high rate of amino-acid uptake by the host may be mediated by its subcuticular bacteria, which could account for up to 5 % of the brittle star energy needs (Lesser and Walker 1992; Galac 2016). Implications in nutrition were found also in the microbiomes associated with sea-star larvae, that change in their bacterial community structures potentially to aid in acclimating to a variable feeding regime (Carrier and Reitzel 2017, 2018). In a recent investigation carried out on the coelomic fluid of different sea stars inhabiting coastal environments, a high individual variability of microbiomes with several unknown taxa was found, suggesting a role of these associations as a reservoir for unique microorganisms including potentially pathogenic and/or symbiotic bacteria. Moreover, different potential drivers were observed in shaping the taxonomic compositions including genetic background, diet, age, stress and environmental factors (e.g. temperature; Nakagawa et al. 2017). Moreover, Jackson and collegues showing the presence of a specific core microbiome, stable among the individuals of different species and completely different with microbial communities inhabiting surrounding seawater (Jackson et al. 2018).

Significant changes in microbiome composition could be associated with environmental factors, such as geographic location. Examples of the importance of this driver were found in different microbiomes of marine invertebrates as corals or sponges (Pantos, et al. 2015; van de Water et al. 2018; Griffith et al. 2019). In a recent investigation carried out on corals, it has been observed that despite a common core microbiome stable in all individuals of the same species was identified, the associated microbial communities showed biogeographical differences (Rubio-Portillo et al. 2018). Studies of microbial associations in Antarctic echinoderm are scarce. Nevertheless, literature analysis carried out on the sea-urchin *Sterechinus neumayeri*, highlighted microbiomes similar to those present in environment, with the presence of multi-antibiotic and metal resistant bacterial groups, isolated in the coelomic fluids (González-Aravena et al. 2016). Only one investigation on microbiomes associated to Antarctic sea stars is available and was carried out during the study of an outbreak affecting echinoderms and consisting of an ulcerative epidermal disease affecting ~10% of the population of the keystone predator *Odontaster validus* (Nunez-Pons et al. 2018). A direct relationship between bacteria and the development of epidermal lesions was not found and the microbiome of healthy sea stars was more consistent across individuals

than in diseased specimens, with the presence of a “core” mostly represented by the Actionbacteria class (Nunez-Pons et al. 2018).

In this contest the specific aims of this work are: 1) to investigate the diversity and putative functions of microbiomes associated with the sea star *O. validus* collected in different basins of the Antarctic Ocean; 2) to assess if different geographic locations of Antarctic Oceans can determine changes in taxonomic composition of the sea star’s microbiomes; and 3) to explore the origin of bacterial assemblages associated with the sea stars comparing them with the assemblages from surrounding sediments.

6.2 Materials and methods

6.2.1 Study area and samples collection

Sampling was carried out during the Antarctic expedition ACTIQUIM-4 in the South Shetland Islands (Weddell Sea) and during the XXXIII Italian Expedition in Antarctica at Terra Nova Bay (Ross Sea) in the framework of the Italian National Program of Antarctic Research (PNRA). Five sampling areas were selected: Port Foster’s bay, located in the Weddell Sea and Amourphus Glacier, Punta Calizza, Spiaggetta Tethys Bay and Adelie Cove, located in the Ross Sea (Fig.1). The different areas, chosen for this investigation, are in two different basins of the Antarctic continent. Port Fosters’ Bay is located in the center of Deception Island, in the Weddell Sea, in the north of Antarctic Peninsula. It’s a dynamic environment with a history of volcanic eruptions, characterized by strong tidal currents variations (Smith et al. 2003; Vidal et al. 2011). Amorphous Glacier, Punta Calizza, Thetys Bay and Adelie Cove are four sites of the Ross Sea area, located in the south part of Antarctica. At present, the Ross Sea is considered to be the most productive region and the most species-rich areas of the Southern Ocean and a biodiversity "hotspot" due to its heterogeneous habitats (Smith et al. 2012). Due to the presence of large, deep reaching cyclonic gyres, the Weddell and Ross gyres, can present diversified features and endemic species (Orsi et al., 1993; Jacobs et al., 2002; Carter et al. 2008, Chown et al. 2015).

In each area, individuals of *Odontaster validus* (Koehler, 1906) were collected by scuba diving at 25m of water depth. Specimens were preserved in ethanol (95%) and stored at -20°C. Samples of surrounding sediments were collected in the areas located in Ross sea using plexiglass cores and stored at -20°C.

Table 1. Table listing the sampling stations organized by area, depths and geographic coordinates.

Geographic location	Area	Latitude	Longitude
Weddell Sea	Port Foster's Bay	62° 58' 22.19" S	60° 38' 59.99" W
Ross Sea	Amorphous Glacier	74°41,237' S	164°02,183' E
	Punta Calizza	74°40,545' S	164°04,095' E
	Spiaggetta Tethys Bay	74°42.068' S	164°02,514' E
	Adelie Cove	74°46,467' S	164°00,266' E

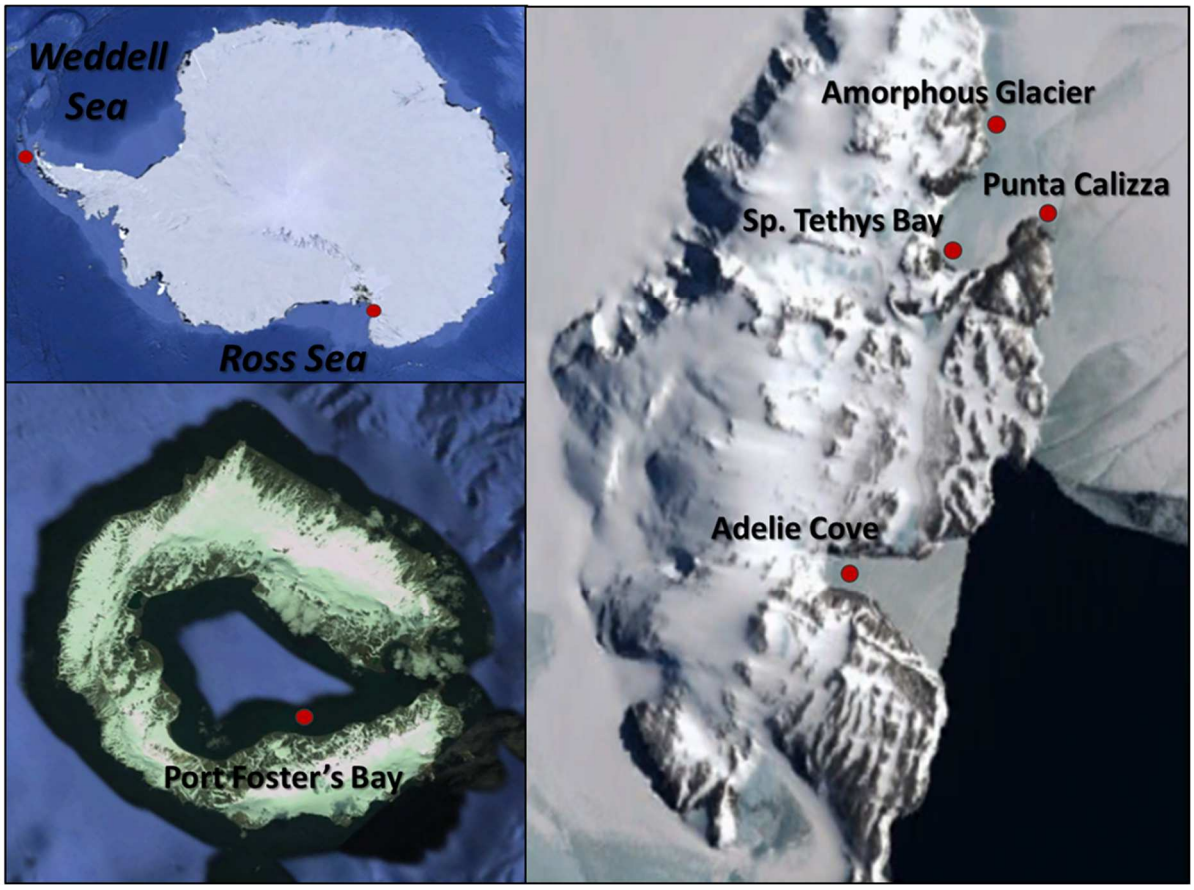


Figure 1. Map of the sampling areas where individuals of *O. validus* and sediments were collected.

6.2.2. Morphological and molecular identification of individuals of *O. validus*

Individuals of *Odontaster validus* were identified from morphological characters with the help of expert taxonomists and using the dichotomous keys. Key references and synopses used for the identification within the different classes of echinoderms were: Ludwig (1903), Koehler (1917), Clark (1962, 1963), Clark and Downey (1992), and Presler and Figielska (1997) and the recent investigation of Janosik (2010, 2011).

The molecular identification of individuals was performed using three mitochondrial markers: the coding mitochondrial 12S rDNA genes and part of the mitochondrial protein-coding COI gene (Vences et al. 2005a, 2005b; Li Yang et al. 2014). The amplification of 12S and COI genes was performed using the same set of primers and the same thermal cycles described in Chapter 3. Sequencing was carried on using Sanger method (Sanger et al. 1977) on both strands at Molecular Facility of Stazione Zoologica Anton Dohrn using the same set of primers used for the amplification, trough Applied Biosystems 3730 DNA Analyzer 48 capillaries (Life Technologies). The sequences obtained were analyzed using the software Geneious 7.1.9 (Kearse et al. 2012). The terminal section of the sequence including low-quality reading and primers were removed before assembling the two strands into consensus sequences. Multiple alignments for each marker were performed using MUSCLE algorithm (Edgar, 2004) in Alivew 1.26 (Larsson, 2014). Additional sequences to build a more complete phylogeny were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Phylogenetic analyses were conducted in MEGA X (Kumar et al. 2018) for all morphospecies investigated using the separate 12S and COI dataset. For each dataset, the evolutionary history was inferred by using the Maximum Likelihood method applying the best-fit nucleotide substitution model: Hasegawa-Kishino-Yano (Hasegawa et al. 1985) 12S and Tamura 3-parameter (Tamura, 1992) for the COI markers, respectively. Sequences were grouped in haplotypes using DNA Sequence Polymorphism (DNASP v6. 12.03) program (Rozas et al. 2017) and haplotype networks were built with PopArt (Leigh & Bryant, 2015), using the TCS network method (Clement et al. 2000).

Table 2. List of individuals of *Odonstaster validus* selected for this study.

Geographic location	Area	Individuals
Weddell Sea	Port Foster's Bay	70, 71, 72, 73, 74, 75, 76, 77, 78, 79
Ross Sea	Amorphous Glacier	AG1, AG2, AG3, AG4, AG5
	Punta Calizza	CAL1, CAL2, CAL3, CAL4, CAL5, CAL6
	Sp. Tethys Bay	SP1, SP2
	Adelie Cove	AC1, AC2, AC3, AC4, AC5

6.2.3 Extraction of DNA from seastars and sediments

The DNA of microbial assemblages was extracted from a whole-body 3 mm-long section of tissue from all seastars, using the Qiagen DNeasy Blood and Tissue Kit (Brasier et. 2016) and following the manufacturer's instructions with a modification (incubation with proteinase K at 56°C was extended overnight). Total DNA from the sediments was extracted using the PowerSoil DNA Isolation Kit, following a modified protocol (Danovaro, 2010): an initial treatment with a set of washing solutions and 10 min of incubation at 70°C was carried out in order to achieve a greater extraction efficiency. The washing solutions used are WS1 (50 mM Tris-HCl, pH 8.3; 200 mM NaCl; 5 mM Na2EDTA; 0.05% Triton X-100), WS2 (50 mM Tris-HCl, pH 8.3; 200 mM NaCl; 5mM Na2EDTA) and WS3 (10 mM Tris-HCl, pH 8.3; 0.1 mM Na2EDTA).

6.2.4 Amplification and sequencing of prokaryotic 16SrDNA

PCR amplification was performed on an approximately 550 bp fragment of the 16S rRNA genes, using the primer set Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') and Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') specific for bacteria (Herlemann et al., 2011). The reaction mixture used consisted of 37.5 µl of filtered and autoclaved Milli-Q water, 10 µl of 5x My Taq Reaction Buffer (Bioline), 0.25 µl of each primer (100 µM), 1 µl of My Taq HS DNA Polymerase (5 U/µl concentration), 1 µl of DNA extracted. The thermal cycling consisted in 2 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 53°C, 45 s at 72°C, with a final extension of 5 min at 72°C. Successful DNA amplification was verified by 1% agarose gel electrophoresis using 10.000x GelRed Nucleic Acid Stain

(Biotium), 0,4 gr of agarose, 40 ml of TE Buffer for the gel preparation, and 2 µl of 5x GelPilot DNA Loading Dye (Qiagen), 2 µl of GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) for the electrophoresis. The amplified DNA was sequenced on an Illumina MiSeq sequencer using the V3 technology (2x300 bp) with primers targeting Bacterial V4 region (Klindworth et al., 2013) at LGC Genomics.

6.2.5 Bioinformatic and statistical analysis

Raw sequences were analyzed through the QIIME2 pipeline (version 2019.4; https://qiime2.org/). Paired-end sequence files were loaded, and sequence pairs analyzed by means of the DADA2 plugin (Callahan et al., 2016), which infers community composition in each sample by partitioning sequences according to the respective error models, thus filtering for erroneous reads and chimeras and resolving minimal variations between prokaryotic taxa. Paired sequences were then merged by the pipeline before producing an Amplicon Sequence Variant (ASV) table. From the ASV table obtained, each sample was subsampled to 1400 sequences, thus obtaining a normalized ASV table. The subsampling depth was chosen as a compromise between the highest number of sequences that fully described the biodiversity of samples and the lowest loss of samples. Two specimens from Port Foster's Bay (74, 76), one specimen of Amorphous Glacier (AG3) and two specimens of Punta Calizza (CAL1, CAL5) were discarded because they were characterized by < 1400 sequences. The normalized ASV table was used for the calculation of rarefaction curves and as input for the subsequent analyses, such as the determination of α and β diversity indices (Shannon and Evennes indices, Bray curtis dissimilarity and Unweighted Unifrac distance). To infer the taxonomic affiliation of ASVs, a taxonomic classifier was first trained on the SSU region amplified by the primers utilized in the present study on the SILVA reference database v132 (Quast et al. 2012); the classifier was then used on the ASVs identified (Bokulich et al. 2018). To further predict the relevant potential functions of microbiomes a functional annotation using FAPROTAX database (Louca et al., 2016) was done. This database maps prokaryotic taxa to putative functions using information based on functional annotations of cultivated representatives. Significant differences (p-values <0.05) in the richness, in the taxonomic composition and in the putative functions of microbiomes were highlighted through a permutational analysis of variance (PERMANOVA) and Multi-Dimensional Scale (MDS) representations; similarities among the different groups were evaluated by classification-clustering based on the Bray Curtis similarity of transformed quantity data with and the identification of the main responsible taxa describing the

differences was done with SIMPER analysis, both included in the PRIMER-E 6 software (Anderson et al., 2008).

6.3 Results

6.3.1 Molecular and intraspecific analyses on *O. validus* individuals

Primer sets used to amplify the mitochondrial 12S successfully amplified the fragments of genomic DNA of all *O. validus* specimens. However, the primer pairs used to amplify the fragment of the COI gene provided good quality PCR products in only 6 specimens, despite applying changes in annealing temperatures and variable concentrations of $MgCl_2$ in the reactions. Good quality DNA sequences for the mitochondrial 12S in *O. validus* were obtained for 22 specimens. The complete list of DNA sequences of 12S and COI for the three species are reported in Tab. 1 SM.

The alignment of 12S sequences and the resulting phylogenetic tree revealed that the 23 sequences of *O. Validus* grouped into 2 shared and 5 unique haplotypes (Tab. 2 SM), with a variability among each other's below 7 mutations (Fig. 2 -4a).

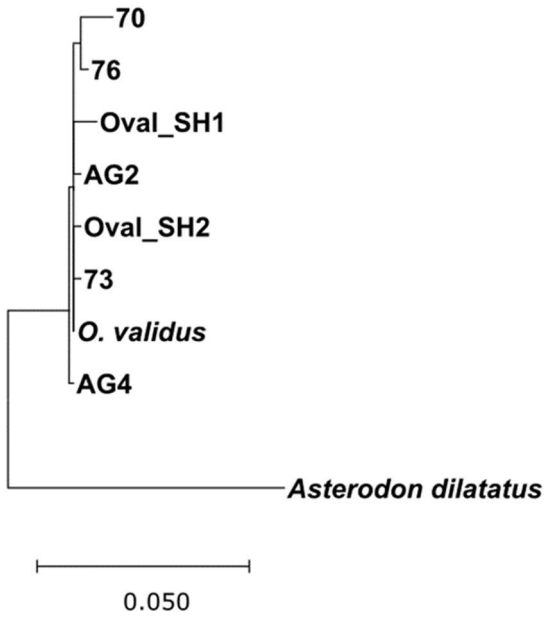


Figure 2. Phylogenetic tree of 12S DNA mitochondrial gene.

Similar results were obtained with the alignment of COI sequences, where the resulting phylogenetic tree revealed that the 6 sequences of *O. validus* grouped into 1 shared and 5

unique haplotypes (Tab. 2SM), with a high variability among each other (from 1 to 179 mutations) (Fig. 3 -4b). A unique group is present in the haplotype network of 12S mitochondrial gene and the different haplotypes found do not follow patterns related to the different sampling areas (Fig.3). In the haplotype network of COI mitochondrial gene two groups are evident, separating individuals of *O. validus* collected at Punta Calizza (Ross Sea) from those are collected at Port Foster's Bay (Weddell Sea). The number of nucleotide mutations among individuals of the same area (19 in Punta Calizza and from 1 to 7 in Port Foster's Bay) are lower than those are found between the two areas (more than 155) (Fig.5).

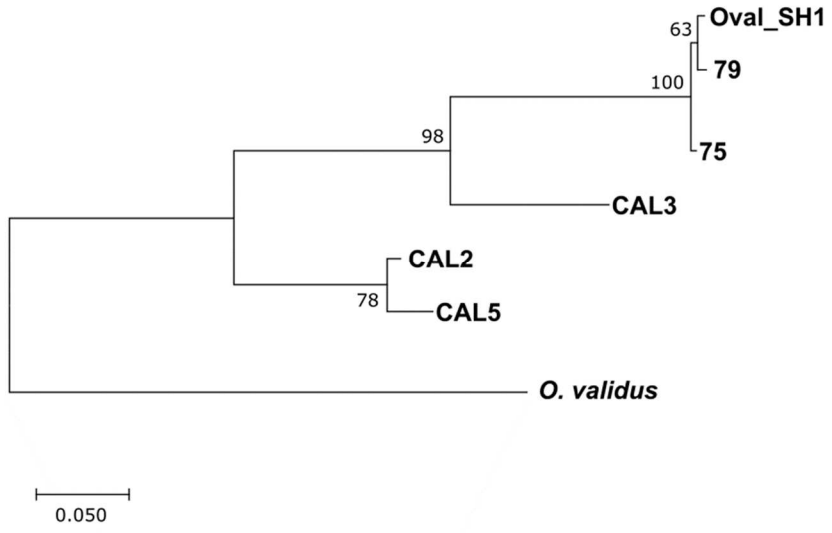
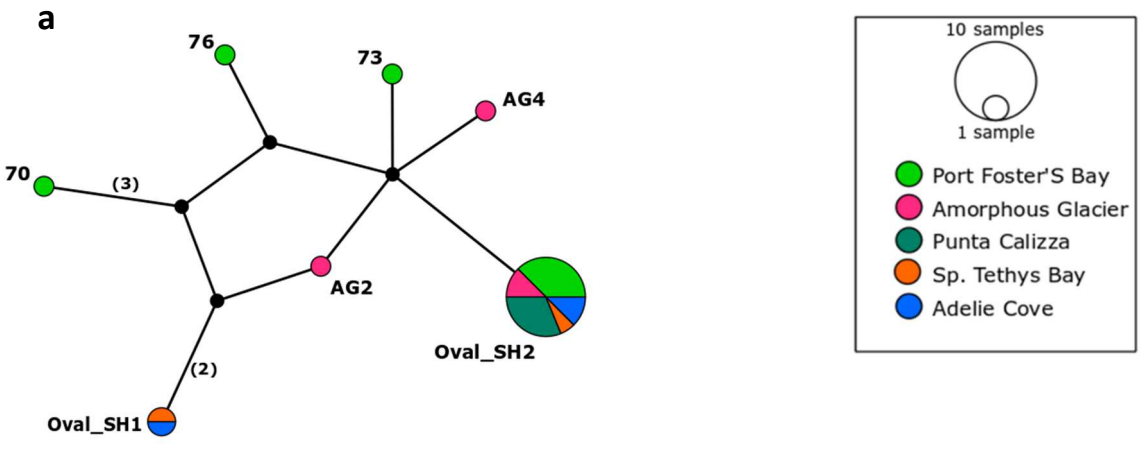


Figure 3. Phylogenetic tree of COI DNA mitochondrial gene.



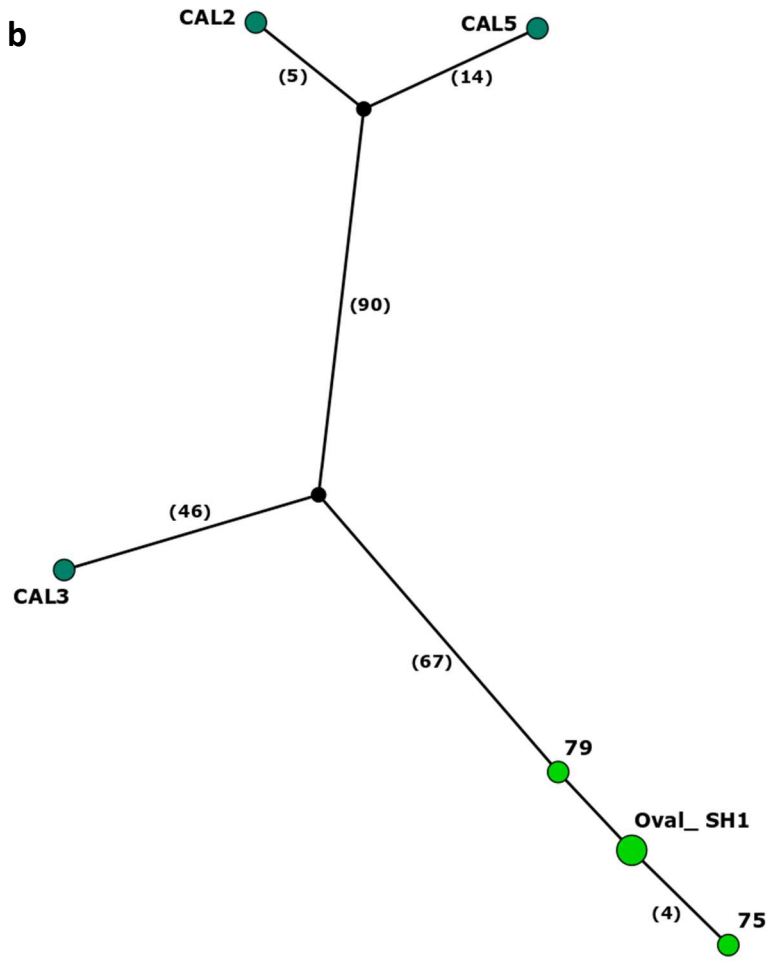


Figure 4. Haplotype network on 12S (a) and COI (b) rDNA mitochondrial genes. Numbers inside the brackets: number of mutations; black nodes: hypothetical haplotypes present in the evolution pathway between the haplotypes identified; haplotype circle size: proportional to the number of individuals sharing the same sequence.

6.3.2 α- and β-diversity of microbial assemblages associated with *O. validus*

The rarefaction curves show that all the curves of microbiomes associated with sea stars reached a plateau (Fig.5). Total ASV richness calculated for each sample varied from 10 to 147 (Fig.6). In each sampling area the number of ASVs varied from 10 to 25, from 86 to 105, from 24 to 81, from 48 to148 and from 28 to 52 in Port Foster's Bay, Amorphous Glacier, Punta Calizza, Sp. Tethys Bay and Adelie Cove, respectively. Significant differences in terms of ASV richness were found among individuals of *O. validus* collected in the different areas, with the lowest average number of ASVs found in Port Foster's Bay and the highest in Amorphous Glacier (Fig.6; Tab. 3SM). The Shannon and the Evenness

indices showed low values of richness in the individuals of Port Foster's Bay and a high variability in the other sampling areas (Fig.7).

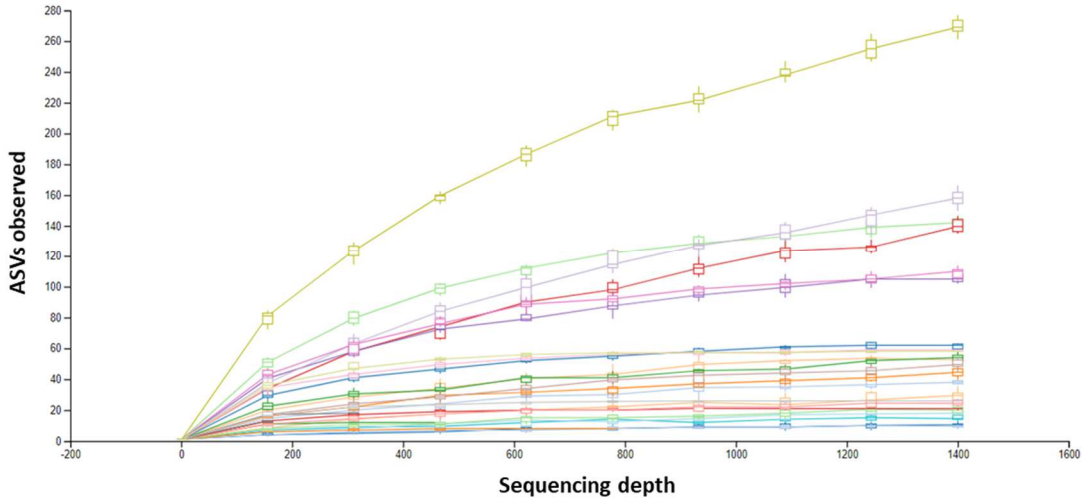


Fig. 5. Rarefaction curve of microbiomes associated with individuals of *O. validus* after a normalization of 1400 sequences for each sample.

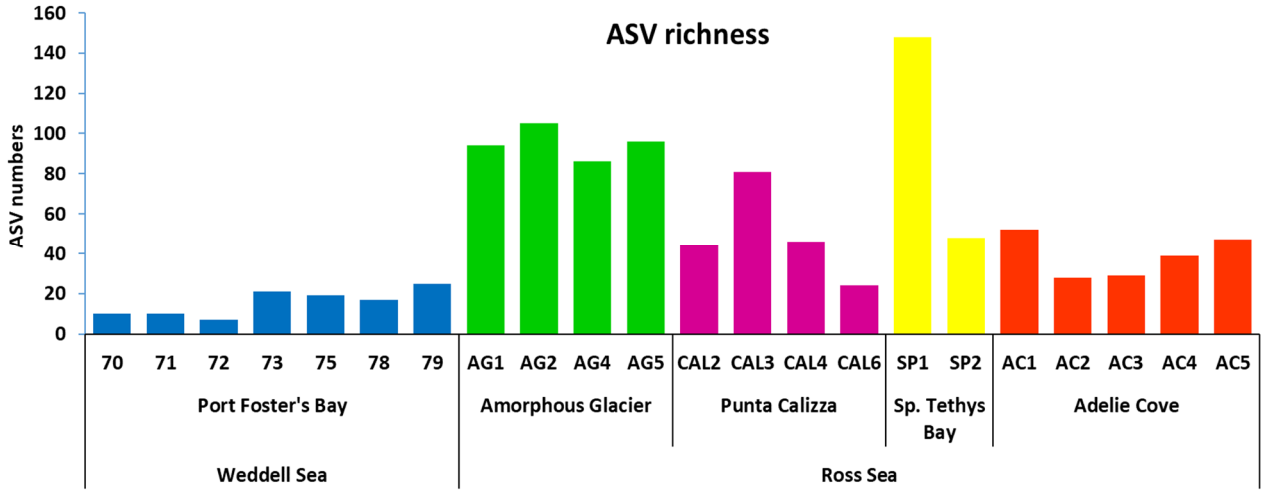


Figure 6. Number of ASVs observed in individuals of *O. validus* in the five sampling areas.

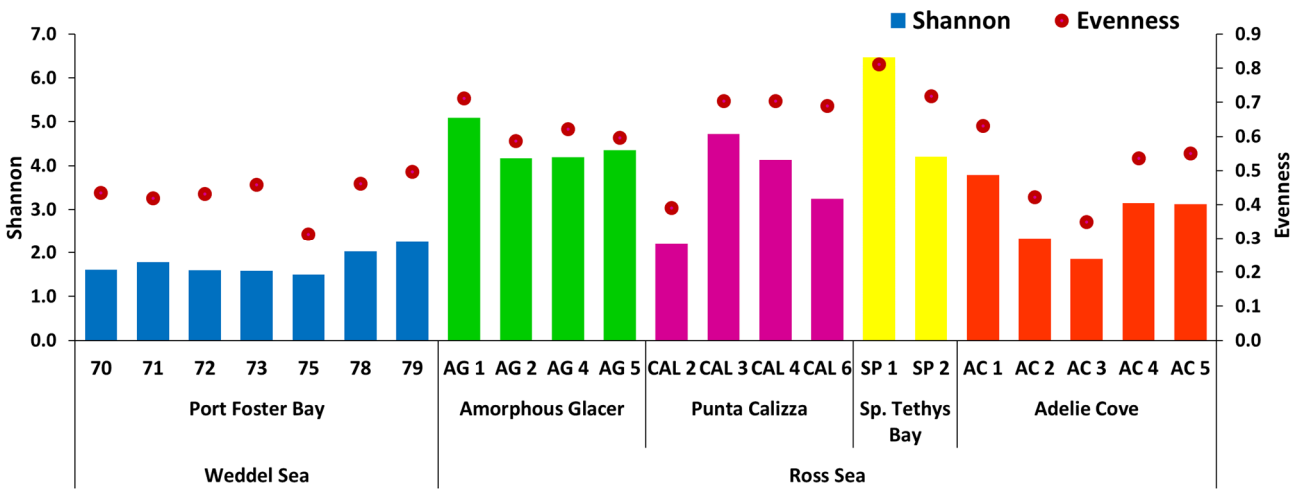
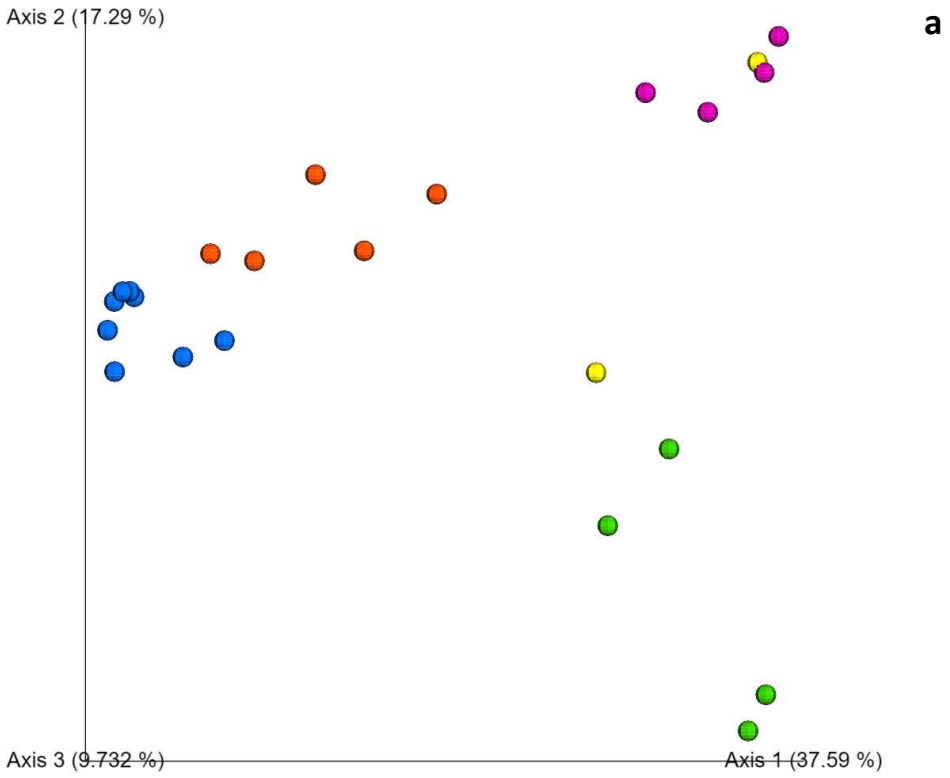


Figure 7. Shannon and evenness indices of microbial community associated with individuals of *O. validus* in the five sampling areas.

The Beta diversity analysis revealed significant differences among the microbiomes of individuals of *O. validus* collected in each area both in terms of composition and phylogenetic diversity (Fig.8 a- b; Tab. 3SM).



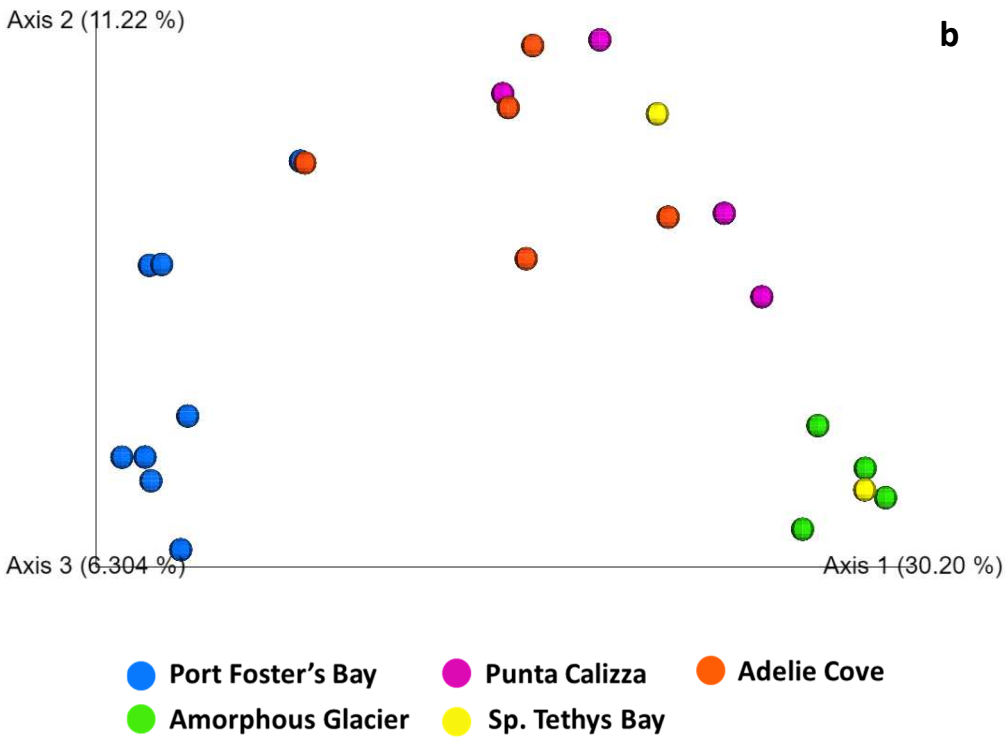


Figure 8. PCoA plot of Beta-diversity of microbiomes among individuals of *O. validus* carried out on Bray Curtis dissimilarity (a) and unweighted UniFrac distance (b).

6.3.3 Taxonomic composition of microbiomes associated with *O. validus* in different sampling areas

Results of the analysis carried out on the taxonomic composition of microbiomes reveal that, in a total of 156 different families, only one is shared in all samples: the *Rhodobacteracea*. The *Rhodobacteracea* family, represented by bacteria mostly belonging to the Sulfitobacter and Roseobacter genera, was found with percentages ranging from 86% to 99%, from 4% to 28%, from 1% to 27%, from 11% to 24% and from 49% to 88% in Port Foster's Bay, Amorphous Glacier, Punta Calizza, Sp. Tethys Bay and Adelie Cove, respectively (Fig.9). This core family represented on average the 95%, 14%, 13%, 17% 71% of the total bacterial assemblage in the individuals collected at Port Foster's Bay, Amorphous Glacier, Punta Calizza, Sp. Tethys Bay and Adelie Cove, respectively.

Moreover, 1, 26, 10, 25 and 8 bacterial families were shared among all polychaetes collected at Port Foster's Bay, Amorphous Glacier, Punta Calizza, Sp. Tethys Bay and Adelie Cove, respectively, representing their specific core microbiomes.

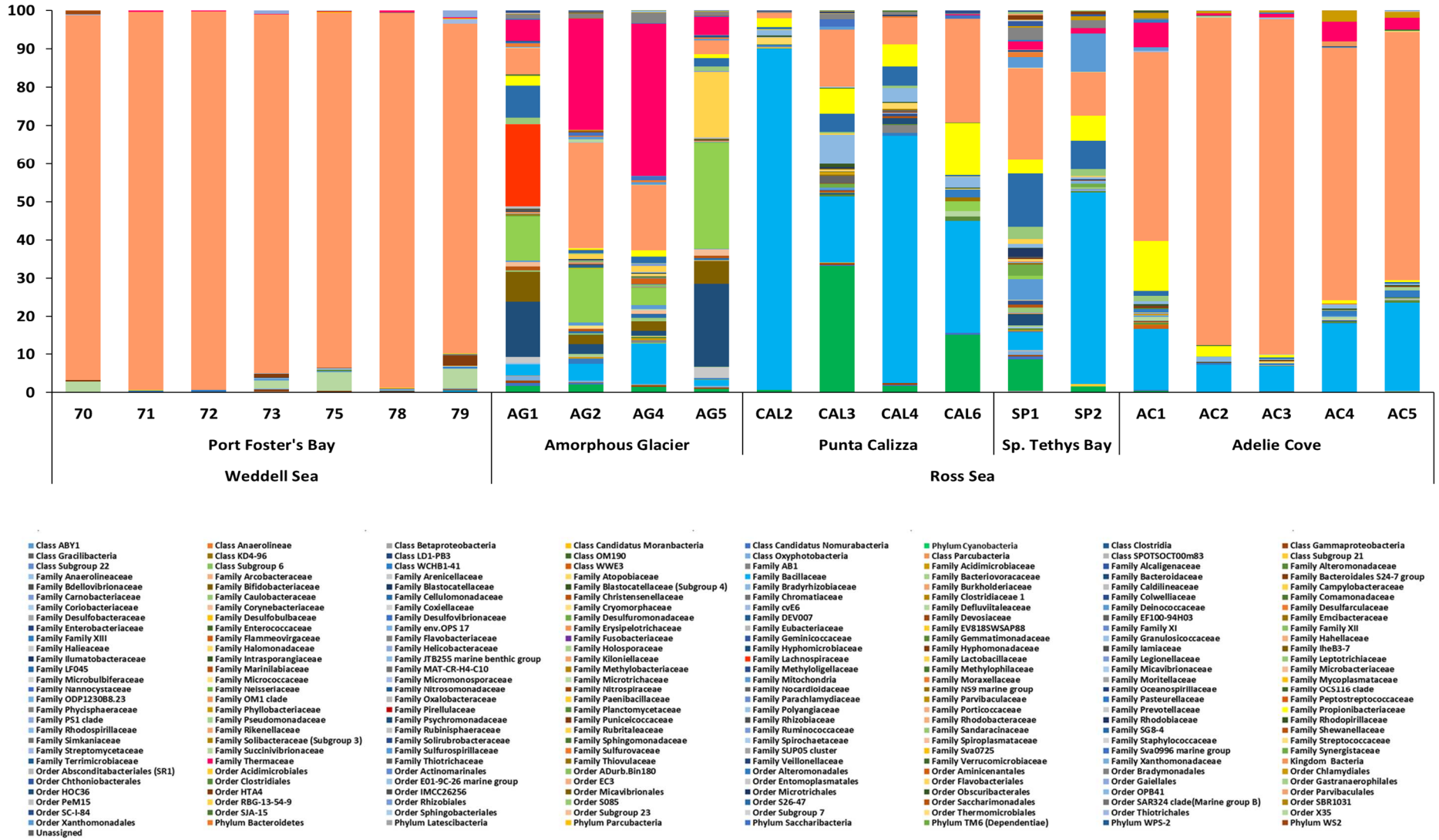


Figure 9. Taxonomic composition of microbiomes associated with individuals of *O. validus* in the five sampling areas.

Microbiomes' composition of individuals of *O. validus* in the two Antarctic basins

Significant differences were found in the microbiomes of individuals of *O. validus* collected in Weddell Sea and Ross Sea (Tab. 3SM). Simper analysis carried out on microbiomes of *O. validus* individuals collected in the areas of Ross Sea revealed a similarity of 45%, explained by the presence of a core microbiome that represented on average the 60% of the total microbiomes. The core microbiome was composed by 3 bacterial families: the *Rhodobacteracea* and *Bacillacea* families (55% and 38% of the core, respectively), and the *Propionibacteriaceae* families (7 %, Fig.10).

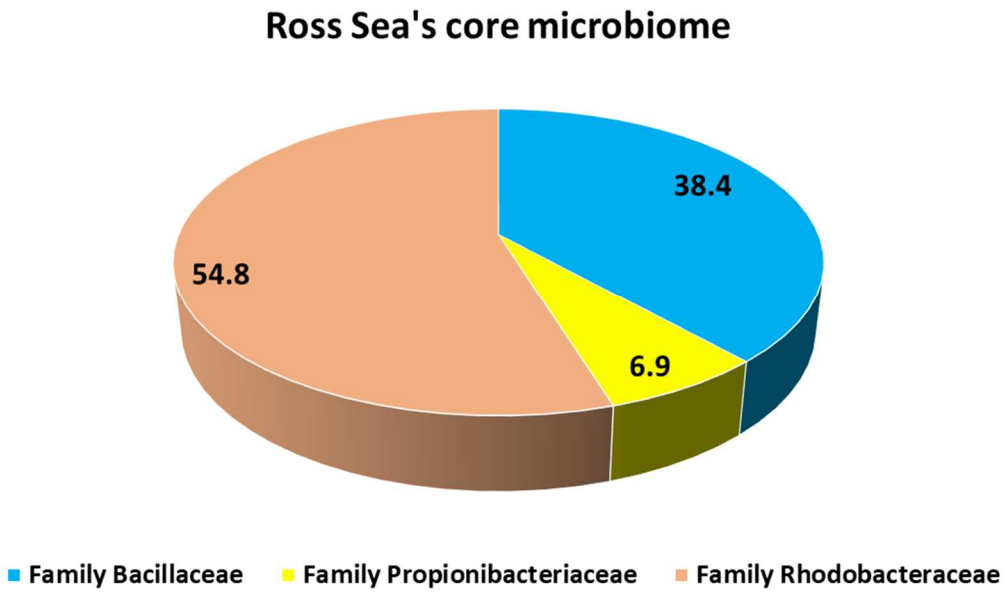


Figure 10. Taxonomic composition of the core microbiome of *O. validus* in Ross Sea.

Simper analysis carried out on the taxonomic composition of microbiomes of *O. validus* individuals collected in the area of Weddell Sea revealed a similarity of 67%, explained by the presence of a core family that represented on average the 95% of the total microbiome, the *Rhodobacteracea* family.

A 71% dissimilarity was found in the taxonomic composition of the microbiomes of individuals of *O. validus* from the two different geographical locations. Such a dissimilarity was mainly driven by the *Bacillaceae* and *Rhodobacteracea* families. 13 and 119 bacterial families were found exclusively in the area of Weddell Sea and in the areas of Ross Sea, respectively.

Microbiomes' composition of individuals of *O. validus* among the different

sampling areas.

Significant differences were found in the microbiomes of individuals of *O. validus* collected among the different areas (Tab. 3SM). The Simper analysis carried out on the taxonomic composition of microbiomes revealed a similarity of 67%, 62%, 56%, 57% and 65% among individuals of *O. validus* collected in Port Foster's Bay, Amorphous Glacier, Punta Calizza, Sp. Tethys Bay and Adelie Cove, respectively, explained by the presence of a core microbiome that represent on average from the 90% and 95% of the total microbiomes. Core microbiomes are composed by 1, 26, 10, 25 and 8 bacterial families in Port Foster's Bay, Amorphous Glacier, Punta Calizza, Sp. Tethys Bay and Adelie Cove, respectively (Fig.11).

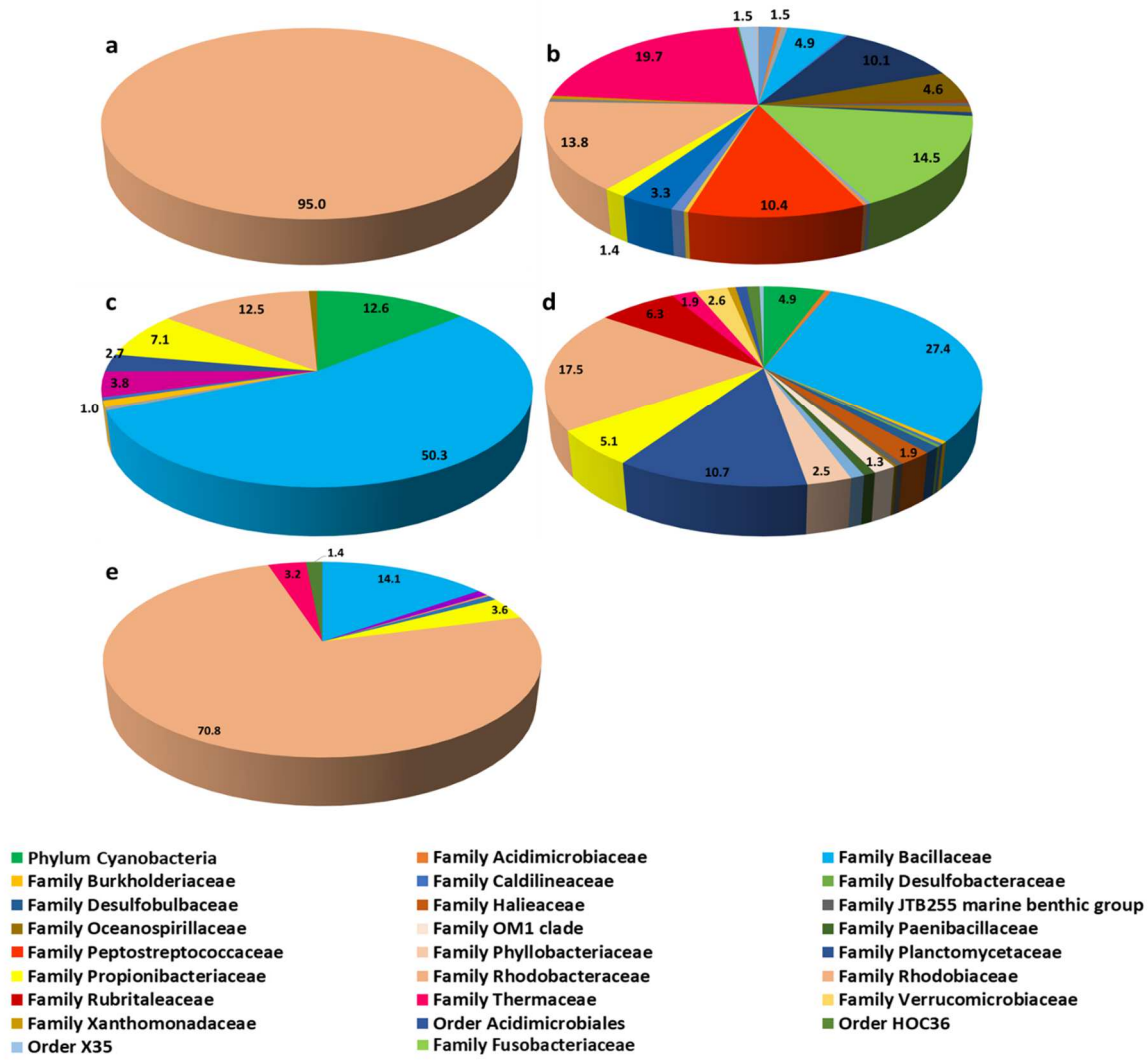


Figure 11. Taxonomic composition of the core microbiomes of *Odontaster validus* collected at Port Foster's Bay (a) Amorphous Glacier (b) Punta Calizza (c) Sp. Tethys Bay (d) and Adelie Cove (e).

Individuals of *O. validus* collected in the four areas of Ross Sea displayed 52%-68% dissimilarity among the taxonomic composition of their microbiomes from mainly driven by

the *Bacillaceae* family, present in the individuals of Weddell Sea with average abundance of 0,5% and *Rhodobacteracea* family. Microbiomes of individuals of Port Foster's Bay and all individuals collected in the other areas displayed a dissimilarity on average of 83%, except for the microbiomes of individuals of Adelie Cove. In fact, the lowest dissimilarity in microbiomes was found between Port Foster's Bay and Adelie Cove (50%) (Fig.12).

Moreover, 11, 25, 7, 8 and 18 bacterial families were found exclusively in the individuals collected at Port Foster's Bay, Amorphous Glacier, Punta Calizza, Sp. Tethys Bay and Adelie Cove, respectively.

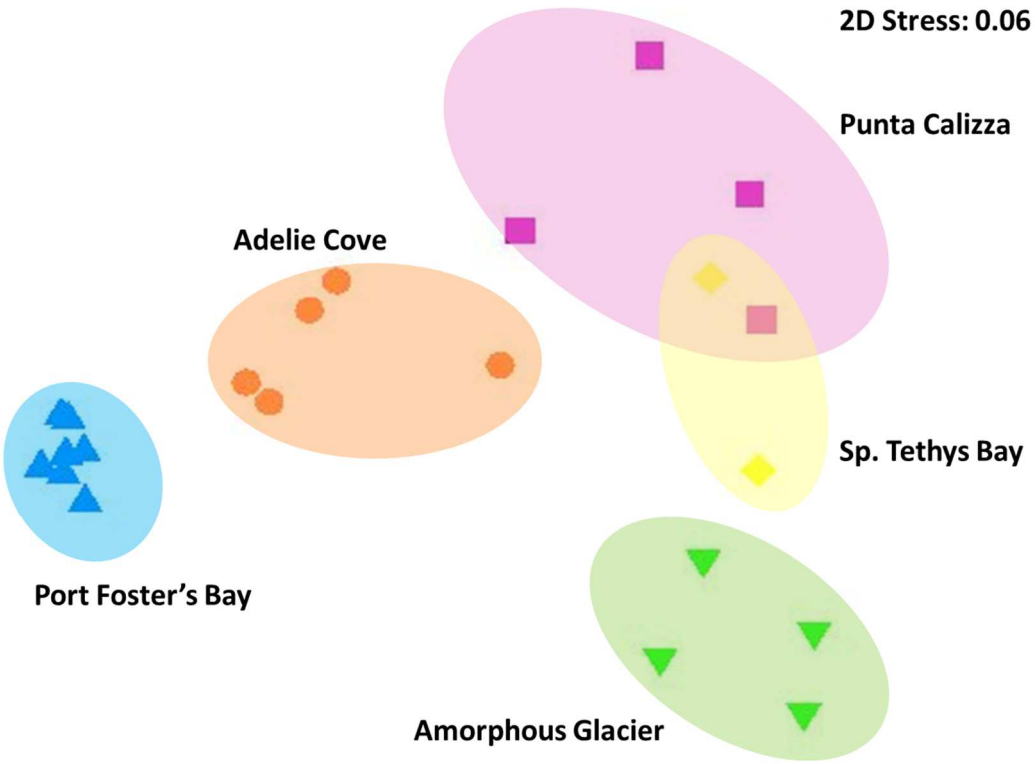


Figure 12. MDS analysis comparing taxonomic composition of microbiomes associated with the individuals of *O. validus* among the different sampling areas.

6.3.4. Putative functions of microbiomes associated with individuals of *Odontaster validus*

Results of the analysis carried out on the putative functions of microbiomes revealed that chemoheterotrophy and oxidation of sulfur compounds were the most represented functions in all the individuals, with percentages from 50% (AG 5) to 82% (77) and from 0,3% (CAL 2) to 43% (AC 3). High percentages of bacteria involved in fermentation were found in all samples except for individual 70, with a contribution from 0,1% (72, 73, 75) to 47% (CAL 2) (Fig. 13).

Significant differences were found in the putative functions of microbiomes associated with individuals of *O. validus* when the two geographic locations (Weddell Sea *vs* Ross Sea) and the five different areas were considered (Tab. 3SM).

Microbiomes of individuals of Port Foster's Bay and of all individuals collected in the other areas displayed a mean dissimilarity of 40%, except for the microbiomes of individuals of Adelie Cove, where the dissimilarity decreased to 22%. This result is explained by the high percentages of bacteria potentially involved in the oxidation of sulfur compounds present in Port Foster's Bay and Adelie Cove (on average 30%) against the low percentages in the other areas (on average 6%) and the percentages of bacteria involved in fermentation, present in Port Foster's Bay and Adelie Cove with an average contribution of 1% and 12% respectively, that increased to 33% in the other areas.

On the basis of this analysis percentages (on average <1%) of parasites were suggested in some individuals of Punta Calizza and Amorphous Glacier.

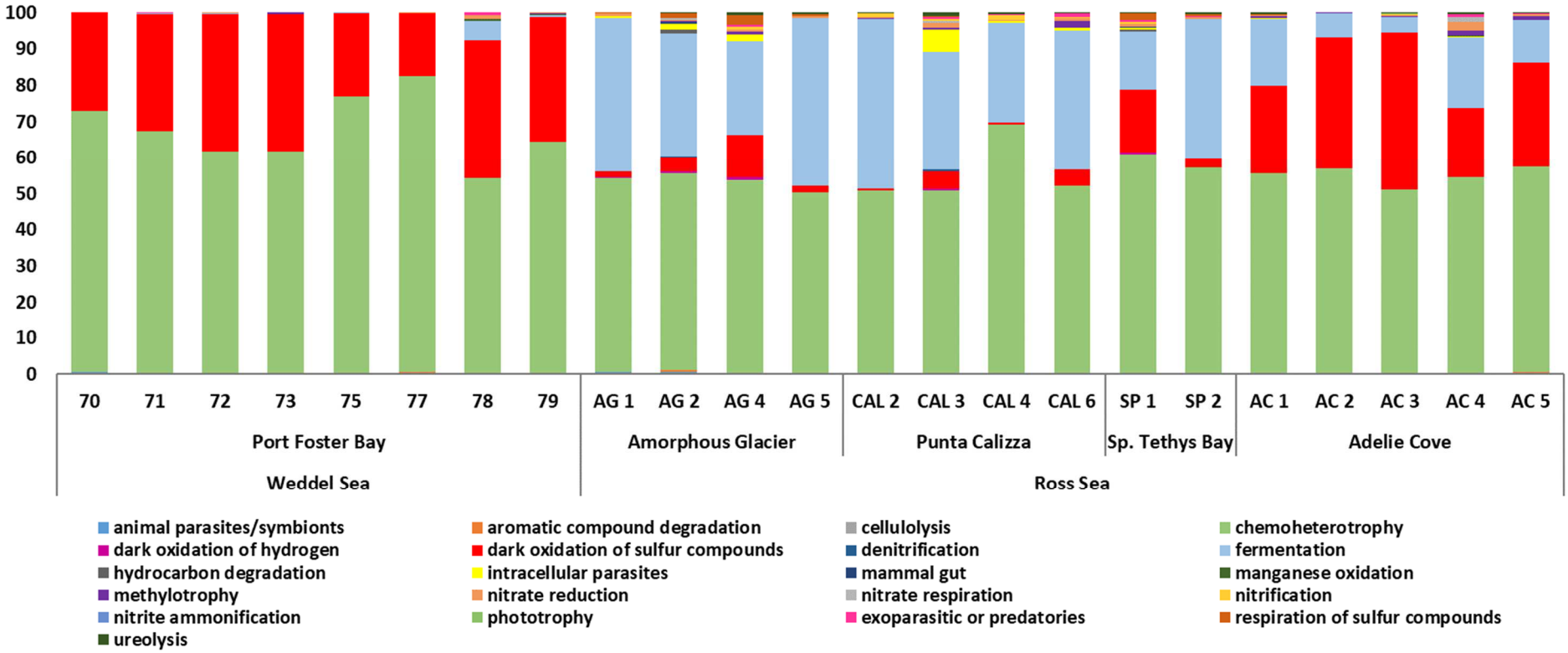


Figure 13. Putative functions of microbiomes associated with individuals of *Odontaster validus* collected in different benthic regions.

6.3.5. Comparison of microbiomes associated with *O. validus* in Ross Sea areas and those living in surrounding sediments

No significant differences were found between microbiomes associated with individuals of *Odontaster validus* and surrounding sediments in terms of ASVs richness. In fact, the number of ASVs varied from 24 to 148 and from 60 to 101 in the microbiomes associated with sea stars and surrounding sediments, respectively (Fig.14).

No significant differences were found in the ASV numbers among microbiomes of sediments collected in different areas.

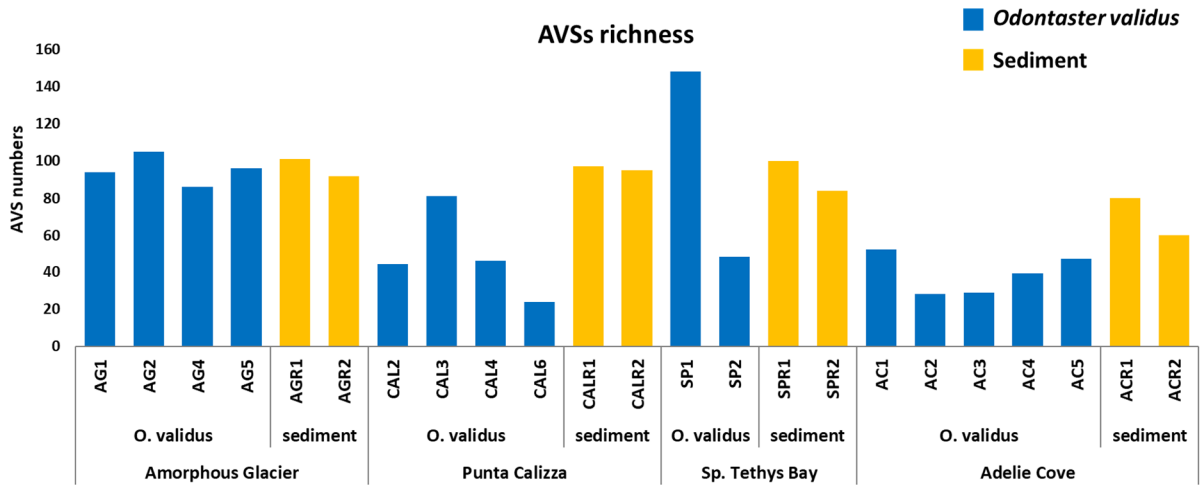


Figure 14. Number of ASVs observed in the sea stars and sediments in the four areas of Ross Sea location.

Nevertheless, significant differences were found between microbiomes associated with *O. validus* and with surrounding sediments in terms of taxonomic composition. This result is further validated by cluster analysis, that displayed two diversified groups between microbiomes associated with sea stars and surrounding sediments, with 85% dissimilarity (Fig. 15; Tab. 3SM).

In fact, among the 25 total families that described the taxonomic composition of all microbiomes, only one family was shared among all samples of sediments and sea stars, the *Rhodobacteraceae* family, showed an average contribution of 33% and 21% in the sea stars and sediments, respectively (Fig.17). Actually, considering the genus level, only the Roseobacter genus is present in both groups, with an average contribution of 40%. The Sulfitobacter genus, present in the sea stars with a contribution of 45%, is completely absent

in sediments. Viceversa, several different genera of *Rhodobacteracea* family were exclusively found in sediments (Fig.16).

Dissimilarities between sea stars and sediments were mostly driven by the *Bacillaceae* family, present only in the sea stars with an average contribution of 23% and by *Pirellulaceae* familyand the *Oxyphotobacteria* class, present only in the sediments with an average contribution of 12% and 19%, respectively. Presence of exclusive bacterial taxa were found both in the sea stars and in sediments (127 and 70, respectively).

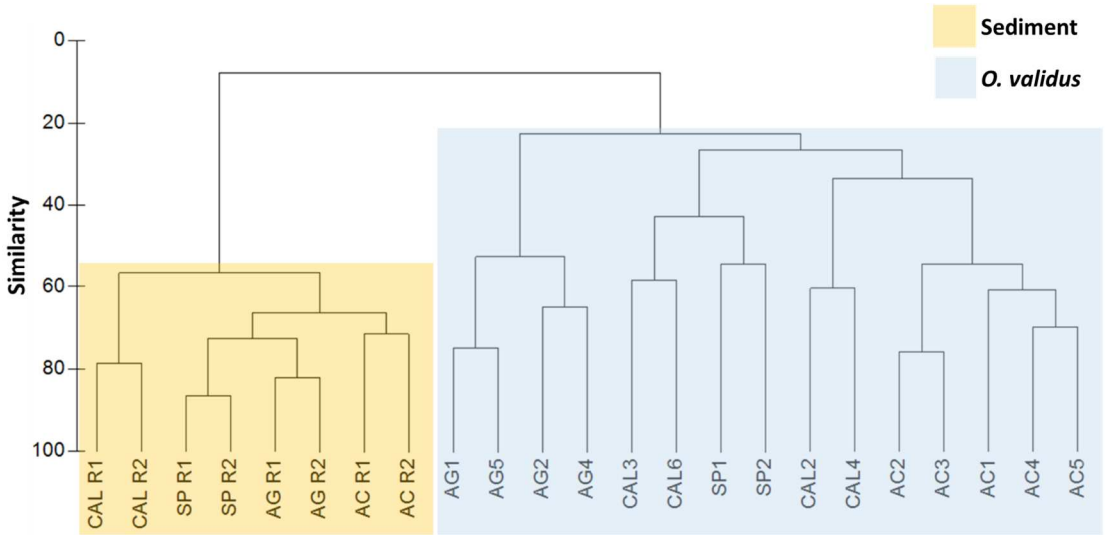


Figure 15. Graph of cluster analysis carried out on the taxonomic composition of microbiomes of *O. validus* and surrounding sediments.

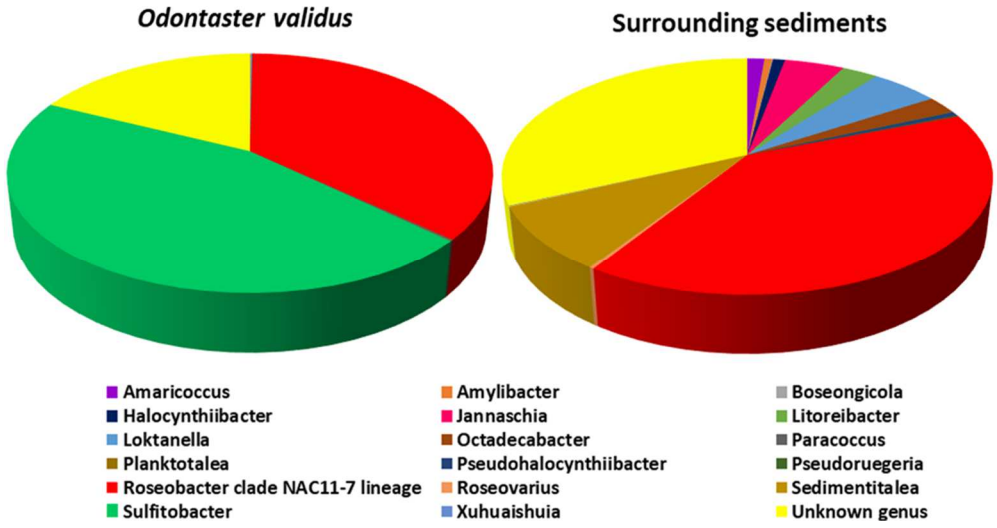


Figure 16. Genera of *Rhodobacteraceae* family present in microbiomes of sea stars and sediments.

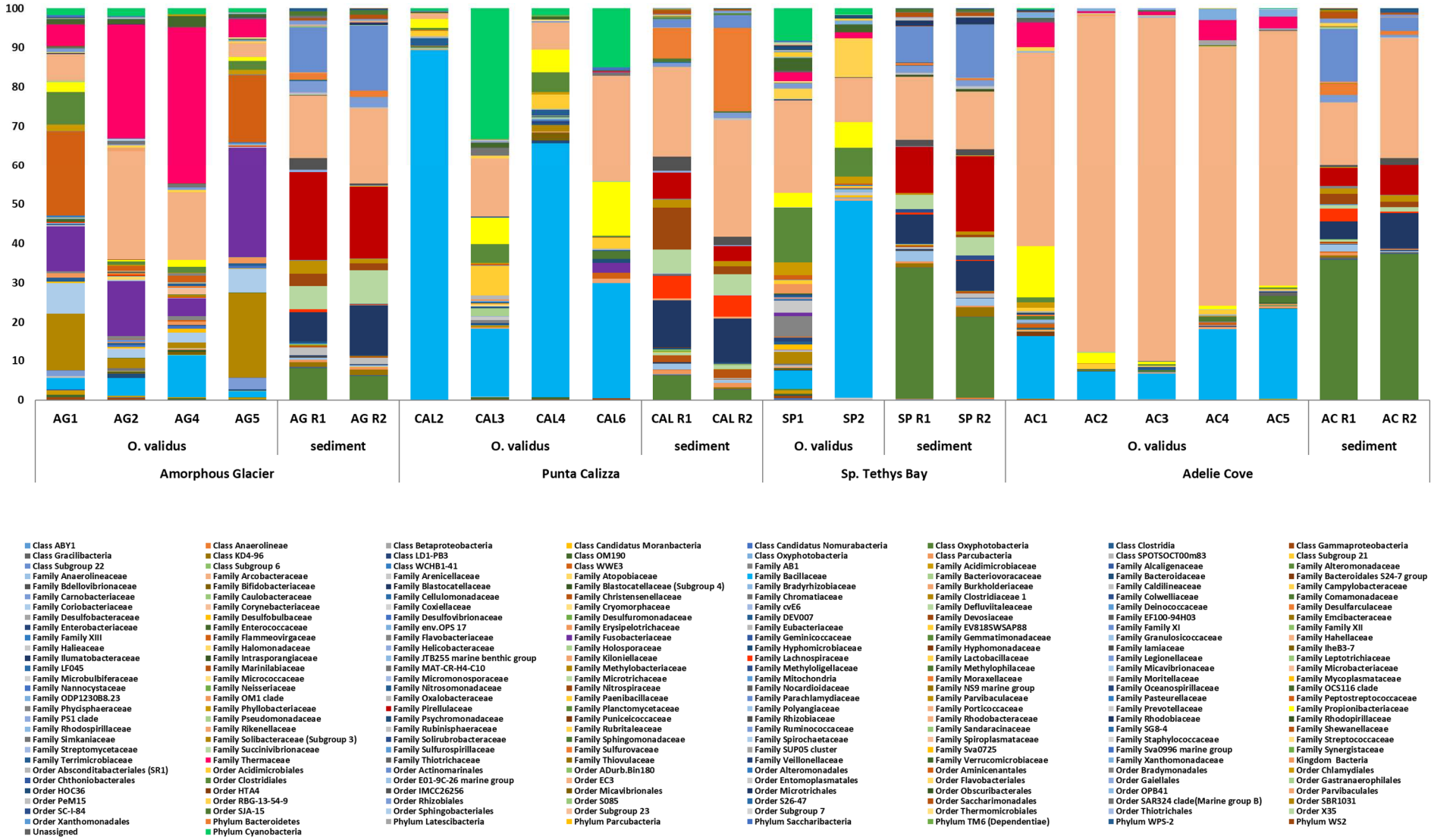


Figure 17. Taxonomic composition of microbiomes associated with *O. validus* and sediments in the four areas of Ross Sea.

6.4 Discussion

6.4.1 Molecular identification of individuals of *Odontaster validus*

The sea-star genus *Odontaster* is among the best-studied Antarctic invertebrate groups. Previous studies on its distribution have revealed a geographically isolated presence of *Odontaster validus* in Antarctic and sub-Antarctic waters, despite a highly extended pelagic larval phase, possibly lasting for 6 months or more, that increases the capability for vast dispersal (Peck and Prothero-Thomas 2002; Janosik et al. 2011).

In this study, the identification by means of classical taxonomic analyses identified all the individuals collected in the different Antarctic areas as *Odontaster validus* (Koehler, 1906). The genetic characterization of mitochondrial 12S sequences clearly supported such results since all the specimens clustered together regardless the different geographic locations. Nevertheless, the analysis of COI gene, characterized by a faster mutation rate than 12S, revealed differences among the individuals, identifying the presence of three clades; one including individuals from Port Fosters' Bay (Weddell Sea) and the other two clades including five individuals of Punta Calizza (Ross Sea). Despite this, all clades are closed to a known sequence of *O. validus* from Genbank. Recently, Janosik et al. (2011) using 16S and COI genetic markers obtained similar results, hypothesizing the presence of two new cryptic *Odontaster* species.: *O. pearsei* and *O. roseus,* previously classified as *O. validus.* Actually, after a detailed analysis of the morphology of the three species, they concluded the new species were not cryptic, but merely unrecognized and the species could be separated morphologically (Janosik et al. 2011). The variability in the morphological traits of this species was also investigated by Peck et al. (2018), that underlined the need to be careful when using reliable morphological criteria and to include the assessment of morphological variation when constructing taxonomic keys (Peck et al. 2018). The impossibility of a secondary morphological examination on our individuals of *O. validus* did not allow us to confirm the lack of potential false positives. Moreover, genetic differences among individuals of a same benthic species of distant locations, as our samples, do not necessarily indicate the presence of different populations or species. In fact, genetic diversity of individuals inhabiting areas within the two different geographic locations must be investigated, in order to better understand how genetic mutations are distributed and how to interpret our results.

6.4.2 Diversity and function of microbiomes of *Odontaster validus* from different

geographic locations

Several studies in literature have investigated the close relationship between bacteria and sea stars (Lawrence et al. 2010; Hoj et al. 2018; Carrier et al. 2018). These results suggest an important role of microbiomes in nitrogen fixation, sulfur-oxidation and in the adaptation processes to environmental changes (Lawrence et al. 2010, Galac 2016, Carrier and Reitzel 2017). The only study about microbiomes associated with Antarctic echinoderms was carried out on the sea urchin *S. neumayeri*, highlighting multi-antibiotic and metal resistant bacterial groups and a mutualistic relationship between host and microbes (González-Aravena et al. 2016).

Results of our investigations highlighted that the microbiomes associated with individuals of *O. validus* collected in Port Foster's Bay (Weddell Sea) were characterized by lower richness of bacterial ASVs than in the individuals of Ross Sea areas. In addition, in the Weddell Sea the taxonomic composition of microbiomes was dominated by three ASVs belonging to *Rhodobacteracea* family, that contributed up to 99% to the total composition. Conversely, individuals of *O. validus* collected in the four areas of Ross Sea were characterized by microbiomes with a high variability both in terms of ASVs richness and taxonomic composition and displayed a diversified core microbiome, specific for each area. Nevertheless, all the individuals of Ross Sea location were characterized by the presence of three core families: *Rhodobacteracea, Bacillacea* and *Propionibacteriaceae* families. The *Rhodobacteracea* family, mainly represented by the Sulfitobacter and Roseobacter genera, was found in all the individuals of *O. validus.* Bacteria belonging to the Sulfitobacter and Roseobacter genera were previously found associated with corals, anemones, sea stars and involved in the degradation of aromatic compounds and sulfur compounds, that potentially represent nutrient sources, structuring bacterial communities, with important consequences for the health of hosts (Ivanova et al. 2004; Raina et al. 2008; Du et al. 2010; Pujalte et al. 2014). Key role for the life of the host could be bacteria of *Bacillacea* and *Propionibacteriaceae* families too. Numerous species belonging to these families show antimicrobial and antifungal abilities and were found in associations with Antarctic sponges, sea urchins and corals (Bruggemann et al. 2004; Schwenninger et al. 2004; Stackebrandt et al. 2014; Lo Giudice et al. 2018). Similarities to our results, with a dominance of Alphaproteobacteria (class to which belong the *Rhodobacteracea* family) and the presence of Actinobacteria (class to which belong the *Propionibacteriaceae* family) and Firmicutes (class to which belong the *Bacillacea* family) were found also in recent investigations carried

out on microbiomes associated with numerous species of sea stars, including *O. validus* (Nunez-Pons et al. 2018, considering the healthy individuals; Hoj et al. 2018; Jackson et al. 2018).

Moreover, our results highlighted the highest dissimilarities (on average 83%) among microbiomes associated with individuals of *O. validus* collected at Port Foster's Bay (Weddell Sea) and in three areas of Ross Sea (Punta Calizza, Spiaggetta Tethys Bay and Amorphous Glacier), but the lowest dissimilarity (50%) between Port Foster's Bay (Weddell Sea) and Adelie Cove (Ross Sea), explained by the high percentages of bacteria of *Rhodobacteracea* family. The geographic location was identified among the main drivers of microbiomes' diversity in many investigations on marine organisms (Pantos, et al. 2015; Rubio-Portillo et al. 2018; van de Water et al. 2018; Griffith et al. 2019). Our results suggest that despite the geographic locations (Weddell Sea vs Ross Sea) could strongly shaped the taxonomic composition of microbiomes of *Odontaster validus*, so much that microbiomes could display high dissimilarities, this is not a general rule. In fact, the similarity between the microbiomes of individuals of *O. validus* collected at Port Foster's Bay and Adelie Cove suggest the presence of factors that could drive the taxonomic composition of microbiomes to the same extent. Port Fosters' Bay is an active flooded volcano in the center of Deception Island, subject to intense temperature fluctuations and tidal currents variation (Vidal et al. 2011). After the last eruption in 1970, the local benthos experienced remarkable recolonization of primarily algae and echinoderms, together with detritivore communities (Smith et al. 2003; Angulo-Preckler et al. 2018; Nunez-Pons et al. 2018). Its sediments were determined to be oxygenated and rich in organic matter (Cranmer et al. 2003; Sturz et al. 2003; Isla et al. 2006; Angulo-Preckler et al. 2017). High enrichment of organic matter was also found in Adelie Cove (see Chapter 4; Pusceddu et al. 2000), thus this could be the main factor shaping microbiomes in the individuals inhabiting the two areas. Similar results were found considering the putative functions of microbiomes. In fact, despite a consistent percentage of bacteria involved in chemoheterotrophy present in all samples (from 53% to 67%), microbial assemblages associated with *Odontaster validus* living in different geographic locations (Weddell Sea *vs* Ross Sea) showed different functional patterns, apart from Adelie Cove. In fact, high percentages of bacteria involved in the oxidation of sulfur compounds were found in the individuals inhabiting Port Fosters' Bay and Adelie Cove, corroborating the similarity between these two Antarctic areas. Overall, these results suggest that the presence of similar environmental or biological factors in both Antarctic basins, can

act as major drivers of sea-star microbiome compositions even overcoming the profound abiotic differences due to the geographic setting.

6.4.3 Origin of microbiomes associated with *Odontaster validus* in the Ross Sea

Processes that drive the formation of the microbiomes are largely unexplored for most species of marine organisms. Previous studies have revealed the potential presence of both the vertical (bacterial taxa were inherited through the generations) and horizontal (bacteria were acquired from the environment or other organisms) of microbiomes transmission in echinoderms (de Ridder et al. 2001).

The sea star selected for this investigation, *Odontaster validus*, represents one of the dominant echinoderms in benthic marine communities of Antarctica (Janosik et al. 2011; Angulo-Preckler et al. 2017; Peck et al. 2018). It is an active predator of sponges, gastropods, ostracods, shrimp, sea urchins, but it used to be also scavengers (necrophagous feeders) and detritivores, probably as the result of its high abundance and omnivorous feeding habits (McClinctock et al. 1994; Kidawa, 2009).

Our investigation has revealed a high dissimilarity between the microbiomes associated with the sea stars and those inhabiting the surrounding sediments. Moreover, despite sediments showed a richness similar to sea stars, values of alpha and beta diversity, highlighting a high biodiversity, most of it represented by families of bacteria with low number of sequences. In fact, only 18 among the 306 families found in sediments show an abundance higher than 1%. Among all the bacterial taxa found, only one is shared between the two groups, the Roseobacter genus within the *Rhodobacteracea* family. Roseobacter genus is one of the most numerically abundant marine lineages, especially in coastal and polar waters and sediments and it has been found involved in the global carbon cycle and in the degradation of aromatic and sulfur compounds (Buchan et al. 2005; Cunliffe et al. 2011). Members of this genus have been found to be free living, particle associated, or in commensal relationships with marine phytoplankton, invertebrates, and vertebrates (Ivanova et al. 2004; Buchan et al. 2005; Raina et al. 2008; Du et al. 2010; Cunliffe et al. 2011; Pujalte et al. 2014). In fact, microbial community within an individual host can be thought as a local community colonized by all the microbes that the host encounters in its environment during its life (Adair et al. 2017). The higher percentages of Roseobacter bacteria in the *O. validus* individuals than in the sediments could be due to a selection processes, during which bacteria, living in

the surrounding environment, could have found in the sea stars tissues an ideal niche for life. Similar results were found in a recent investigation on microbiomes associated with different species of sea stars, revealing high dissimilarity between the coelomic fluids microbiomes and the surrounding sea water, both in terms of richness and of biodiversity, suggesting a strong selection acting in the sea stars tissues against the presence of bacterial cells (Jackson et al. 2018).

Moreover, presence of several bacterial families exclusively in the *O. validus* tissues, but with different taxonomic patterns in the sampling areas, suggest a potential horizontal transmission of microbiomes, probably acquired by different preys or through different feeding habits that *O. validus* could have develop in the different areas.

6.5 Conclusion

This investigation provides new insights into the knowledge of the microbiomes associated with Antarctic invertebrates, expanding information on diversity, functions and origin of bacterial taxa belonging to the microbiomes.

In the present study, we found that the microbiomes associated with *O. validus* can significantly change in terms of richness and taxonomic composition among individuals collected in different Antarctic basins, although core taxa were observed. In fact, high similarities were found in the taxonomic composition and putative functions of microbiomes of individuals collected in a specific area of Ross Sea (Adelie Cove) and in Weddell Sea, suggesting the presence of similar environmental and/or biological drivers able to select specific microbiomes, stronger than the factor "geographic location".

Findings reported here also suggest that most of the identified bacteria could have a fundamental role in the sea-stars' wellbeing, potentially establishing commensalism and symbiotic relationships with their hosts and contributing in the metabolic pathways of a wide array of inorganic and organic compounds.

Since only one genus of bacteria (Roseobacter) was found both in *O. validus* and in surrounding sediments, we can suggest that host tissues are strongly "selective" for bacteria from sediments. Therefore, it is also possible to hypothesize a different origin of the microbiome *of O. validus* or snap changes in its compositions due to factors that remain still

unknown. Exclusive bacterial families in the *O. validus* tissues, with different taxonomic patterns in the different Antarctic basins, suggest a potential horizontal transmission of microbiomes, possibly acquired through different feeding habits that *O. validus* could have develop in the different areas.

Major future investigations should be conducted to better comprehend the biodiversity and nature of the intricated links between microbiomes and their hosts.

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6.7 Supplementary material:

Table SM1. List of sequences obtained for *Odontaster validus* ID=individual, Y=yes and N=no

	Area	ID	12S	COI
Weddell Sea	**Port Fosters' Bay**	**70**	Y	N
		71	Y	Y
		72	Y	Y
		73	Y	N
		74	Y	N
		75	N	Y
		76	Y	N

		77	Y	N
		78	Y	N
		79	Y	Y
Ross Sea	**Amorphous Glacier**	**AG1**	Y	N
		AG2	Y	N
		AG3	Y	N
		AG4	Y	N
		AG5	Y	N
	Punta Calizza	**CAL1**	Y	N
		CAL2	Y	Y
		CAL3	N	Y
		CAL4	Y	N
		CAL5	Y	Y
		CAL6	Y	N
	Sp. Tethys Bay	**SP1**	Y	N
		SP2	Y	N
	Adelie Cove	**AC1**	Y	N
		AC2	N	N
		AC3	Y	N
		AC4	Y	N
		AC5	Y	N

Table 2 SM. List of single and shared haplotypes identified with the two mitochondrial genes.

Markers	**Haplotypes**	**Samples included**
12S	**70**	70
	Oval_SH1	AC3, SP1
	76	76
	73	73
	AG2	AG2
	AC4	AC4
	AG4	AG4
	Oval_SH2	72, 78, CAL2, AC5, CAL6, AC1, AG5, 71, CAL4, 77, AG3, 74, SP2, 79, CAL1, CAL5
	O. validus	EF624444.1 (NCBI)

	A. dilatatus	DQ273732.1
COI	**Oval_SH1**	71, 72
	CAL2	CAL2
	CAL3	CAL3
	CAL5	CAL5
	79	79
	75	75
	O. validus	GU227092.1 (NCBI)

Table 3 SM. Results of PERMANOVA main test carried out on:

ASV richness of microbiomes between the two geographic locations

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Area	2	1819.2	909.59	17.968	0.001	997
Res	20	1012.5	50.623			
Total	22	2831.6				

ASV richness of microbiomes among the different areas

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Station	4	2135.8	533.95	13.813	0.001	999
Res	18	695.83	38.657			
Total	22	2831.6				

Taxonomic composition between the two geographic locations

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Area	2	17779	8889.6	6.8989	0.001	999
Res	20	25771	1288.6			
Total	22	43550				

Taxonomic composition among different areas

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Station	4	30348	7586.9	10.344	0.001	999
Res	18	13203	733.49			
Total	22	43550				

Putative functions of microbiomes between the two geographic locations

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
region	1	5206.9	5206.9	20.655	0.001	996
Res	21	5294	252.09			
Total	22	10501				

Putative functions of microbiomes among different areas

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
area	4	7742.2	1935.5	12.629	0.001	998
Res	18	2758.7	153.26			
Total	22	10501				

ASV richness of microbiomes between sea stars and sediments

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
source	1	618.2	618.2	5.034	0.043	997
Res	21	2578.9	122.81			
Total	22	3197.1				

Taxonomic composition of microbiomes between sea stars and sediments

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
source	1	26866	26866	20.594	0.001	997
Res	21	27396	1304.6			
Total	22	54262				

7. FINAL CONCLUSIONS

This thesis provides new insights on the biodiversity and potential role of microbiomes associated with the Antarctic invertebrates, on the drivers potentially responsible for shaping their taxonomic composition and on the origin of bacterial taxa that create these associations.

Microbiomes associated with Antarctic polychaetes can significantly change among individuals, indicating a **high level of intraspecific variability**; despite this, a core microbiome (i.e., **intraspecific core microbiome**), contributing for a significant fraction of the whole microbial assemblage, was found within each species of Antarctic polychaetes investigated, both in the deposit feeders (*L. geminus* and *A. palmeri*) and in the predators (*A. trissophyllus*). Core bacterial taxa identified in all polychaetes of the present research (i.e., **interspecific core microbiome**) was mostly represented by bacteria belonging to the *Meiothermus* genus. Further studies will be required to investigate the reason why these bacteria are shared among all the polychaetes and their functional role.

Microbiomes associated with the polychaete *A. trissophyllus* were **not partitioned among the different anatomic parts of its body**, so that we could not identify a specific microbiome for each different part/tissue of the animal. Also in this case, a high variability at the inter-individual level was detected, in part explained by the presence of exclusive bacterial taxa of each one, mostly coming from the oral cavity and parapods. Therefore, since these body parts are those which can mostly exchange bacteria with the surrounding environment during feeding activities and motility, it is possible to hypothesize that **benthic bacterial taxa can contribute to the intraspecific variability of the host' microbiomes**.

Multiple environmental (e.g. trophic conditions of the environment) and biological factors, at different extent can have a role in shaping microbiomes associated with Antarctic polychaetes.

Geographic location was identified as a factor influencing the taxonomic composition of microbiomes associated with the predator sea star (*O. validus*) collected in Weddell and Ross sea, but this is not a general rule. In fact, a high similarity was found between microbiomes of some individuals of the Ross Sea and those from Weddell Sea, suggesting also the presence of environmental and/or biological drivers in the two different Antarctic basins able to select similar microbiomes.

Despite the close links between polychaetes and sediments, due to the feeding and motility strategies, bacterial taxa that live associated with them are completely different from those living in the surrounding sediments, suggesting a **potential vertical transmission or different adaptative conditions of the bacterial taxa** and highlighting an **important contribution of microbiome-host associations as diversity reservoir** to the estimates of biodiversity of the area.

Only one genus (*Roseobacter* genus) of bacteria was shared among *O. validus* and surrounding habitat. Therefore, we can suggest that **host tissues are strongly "selective" for the growth of bacteria from sediments**. Therefore, it is also possible to hypothesize a different origin of the microbiome of *O. validus* or snap changes in its compositions due to factors that remain still unknown. Exclusive bacterial families in the *O. validus* tissues, with different taxonomic patterns in the different Antarctic basins, suggest a **potential horizontal transmission of microbiomes**, possibly acquired through different feeding habits that *O. validus* could have developed in the different areas.

Finally, findings reported here also suggest that most of the identified bacterial taxa can create **putative commensalist and/or symbiotic relationships with their hosts** and might have a **fundamental role in their wellbeing**, contributing to the metabolic pathways of a wide array of inorganic and organic compounds, in the **defense against pathogens** and in the **adaptation to the harsh temperatures of Antarctica**. Nevertheless, a portion of bacteria that take part of the microbiomes remains unknown, leaving open several questions on their identity and roles and indicating the **presence of novel taxa**.

Further studies should be conducted to better understand the nature of the intricated links between microbiomes and their hosts and their significant contribution in the adaptation to the harsh environment of Antarctica.