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BIOLOGIA MARINA

Biodiversità dei procarioti nei sedimenti profondi della scarpata della Sardegna nord-occidentale

Prokaryotic diversity in deep-sea sediments of the North-Western Sardinia margin

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Summary

Abstract

The "Nurra Escarpment" (North-Western Sardinian slope) ranges from 600 m to 2400 m of depth. To date, data on the benthic prokaryotic diversity and its drivers in this area are still missing. In this study, the spatial patterns of abundance and diversity of benthic prokaryotic assemblages along this continental margin and the potential drivers influencing their distribution were explored.

The results of this study suggest that the area was overall environmentally homogeneous, a fact that was also reflected on the benthic prokaryotic diversity and community composition. Prokaryotic abundance significantly decreased with depth, but no significant changes were observed among stations located at the same depths. The distribution of the prokaryotic abundance was mainly driven by the amount of fresh material of photosynthetic present on the seafloor. Proteobacteria, Planctomycetota and Acidobacterota, dominated prokaryotic communities, whereas Crenarchaeota represented almost the entire archaeal assemblages. The composition of prokaryotic assemblages was mainly driven by temperature, chlorophyll-a and biopolymeric carbon, although a large fraction of the variance remained un-explained.

Further studies are needed to clarify the contribution of other biotic and abiotic factors in shaping microbial assemblage composition and their role in the functioning of the benthic microbial food web along continental margins.

Chapter 1 – Introduction

1.1 The Mediterranean Sea and its peculiarities

With an area of 2.5 million km^2 and an average depth of ca. 1,450 meters, the Mediterranean Sea is known as the largest and deepest semi-enclosed basin in the world (Coll et al., 2010). It covers the area between Europe and Africa and reaches the maximum depth of 5,200 m in the Hellenic Trench, off the Southern Greek coast. The basin is characterized by peculiar geological, geomorphological and hydrological features, which led to a very diverse marine biota and high levels of endemism. Moreover, its thermohaline circulation differs from the oceanic circulation, exhibiting strong seasonal and inter-annual variability as well as the presence of eddies and numerous meandering currents (Schroeder et al., 2012).

The present Mediterranean basin is the outcome of a long geological history that has shaped it through the rifting, spreading, collision and subduction of plates and microplates (Picotti et al., 2014). Two geologically different areas (i.e. Western and Eastern basins), characterized by different settings and age, could be identified due to the physical barrier created by the channel of Sicily (Sevastou et al., 2013). In addition, several biological features reflect both physical and geological separations, with the Western area characterized by a higher productivity - mainly concentrated in areas characterized by high continental inputs (i.e. the Gulf of Lions), whilst the Eastern one displaying a very low primary productivity (Tselepides et al., 2004; Danovaro et al., 2010).

A great variety of geomorphologic structures and habitats can be observed, such as submarine canyons, seamount, mud volcanoes, cold seeps, trenches, area of intense hydrotermalism, deep hypersaline anoxic basins (DHABs) and

deep-sea coral mounds. All of these structures represent unique habitats and host peculiar biological communities (Ramirez-Llodra et al., 2010). Another peculiarity of the Mediterranean basin lies in its deep waters thermal signature, which is 10°C higher than any oceanic system at comparable depth. Thus, also its abyssal temperatures are typically around 12.5-13°C and these temperatures seem to have a critical role in sustaining high rates of prokaryotic growth and activities (Santinelli et al., 2010; Luna et al., 2012).

Unfortunately, another important feature to be taken into account is the number and variety of anthropogenic threats and impacts that the Mediterranean Sea is subjected to, due to its geographical position and its role in human society; in fact, cities, tourism, maritime traffic and exploitation of abiotic and biotic resources have constantly grown and continuously stress the Mediterranean marine environment (Ramirez-Llodra et al., 2011). In particular, habitat fragmentation and loss, over-exploitation of living resources like fishes, waste disposals and chemical pollution, invasive species influx following the Suez Canal opening, shellfish transfer, ballast water, climate change-related phenomena such as acidification and tropicalization are only few of the most relevant threats originated by humankind (Ramirez-Llodra et al., 2011).

1.2 The open slope systems: a snapshot

The Mediterranean deep-sea floor includes areas characterized by complex sedimentological and structural features, such as, for example, continental slopes, which represent potential “hot-spots” of biodiversity (Danovaro et al., 2010). Slope systems can be subjected to complex phenomena such as dense water cascading, landslide and gravity flow events (Canals et al., 2006) and are

characterized by a mosaic of environmental conditions which can promote deep-sea biodiversity (Ramirez-Llodra et al., 2010; Levin & Sibuet 2012). In particular, multiple and interacting local ecological characteristics, environmental factors, topographic and sedimentary conditions in each specific slope environment act as drivers of diversity; this complexity may promote the settlement of a large number of species (Danovaro et al., 2010). Despite slope systems represent less than 20% of the world's oceans (Vanreusel et al., 2010), they represent key ecosystems from a biogeochemical, biological and ecological perspectives potentially hosting species that have still to be discovered (Mamouridis et al., 2011; Weaver & Gunn 2009). These systems can also be considered sources of diversity for both shelves and deeper basin thanks to radiation and dispersal processes, as assumed by Rex and colleagues in the so called "source-sink hypothesis" (Rex et al., 2005).

1.3 The deep Mediterranean Sea: the role of prokaryotes

A key component of marine life is represented by prokaryotes, which account for the largest fraction of biomass and biodiversity (Luna, 2015). Marine microbes are key component for the ecosystem functioning, since they drive biogeochemical cycles, regulate the gaseous composition of the atmosphere, influence the Earth's climate, recycle nutrients and organic carbon, and decompose chemical pollutants (Azam and Malfatti 2007; Luna 2015; Bell et al., 2005). Moreover, the discovery of their high metabolic activities and specific functional characteristics has led to conclude that deep-sea prokaryotes are crucial players in all ecosystems, both having a crucial role in marine food webs and nutrient cycle, as they are responsible

for recycling particulate organic matter that sinks down from the photic zone (Danovaro et al., 2008a; Giovannelli et al., 2013; Molari et al., 2013) and also by affecting the degradability of organic matter (Aller & Aller 1998), due to their ability to make exploitable by other organisms the sedimentary organic matter which would normally be undegradable, defined as refractory.

Given the importance of biochemical cycles, it is important to deepen the prokaryotic contribution to these cycles. In particular, with regard to the carbon cycle they carry out the breakdown of all organic matter originated from plants and animals and for this reason they are called decomposers. By doing that, they are also the major contributor to carbon dioxide's concentration of the atmosphere and to ammonium ions of the sediments, playing an important role in determining the composition of atmosphere and ocean.

Beside organic C reaching the deep-sea floor through particle sinking, deep-sea ecosystems can be sustained also by chemosynthetic production based on inorganic C assimilation (Molari et al., 2013; Jørgensen & Boetius 2007).

As regards nitrogen, it is the major component of proteins and nucleic acids and it is obtained from ammonia (NH_3), ammonium ions (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and nitrogen gas (N_2) through several processes. The largest pool of nitrogen available in the terrestrial ecosystem is gaseous nitrogen from the air, but this nitrogen is not usable by plants, which are primary producers. Thus, prokaryotes transform the atmospheric nitrogen (N_2) into more-readily available form producing ammonium (NH_4^+), which

could be used by plants or converted to other form by other prokaryotes. In addition, ammonia can be released during the decomposition of nitrogen-containing organic compounds, a process that is called ammonification and then catabolized anaerobically, yielding N_2 as final product. In alternative, a group of prokaryotes named nitrificators could perform the conversion of ammonium to nitrite (NO_{2^-}) and nitrate (NO_{3^-}). This process is carried out by genera *Nitrosomonas, Nitrobacter* and *Nitrospira*, whilst the reverse process consisting in the reduction of nitrate to gaseous compounds (i.e. N_2O, NO and N_2) is performed by denitrifying microorganisms (Gruber & Galloway 2008; Stein & Klotz 2016; Stüeken et al., 2016). In marine ecosystems nitrogen is considered one of the main limiting factors for phytoplankton production. Thus, there are well-established positive relationships among nitrogen and phosphorus flux, phytoplankton primary production (i.e. carbon cycle) and fisheries yield (Rabalais 2002; Ward, 1996).

Deep-sea sediments are food limited environments in which the abundance and distribution of benthic organisms are directly related to the amount and quality of food reaching the sediment surface (Danovaro et al., 2000). The study performed by Deming and Yager was the first to show a cells number decreasing with increasing water depth, suggesting that bacteria, like other benthic components, depend upon the Particulate Organic Carbon (POC) flux from the photic layer, which exponentially decreases with the increase of water depth (Deming & Yager 1992; Jørgensen & Boetius 2007). Such relationship between the flow of organic matter and microbial abundance and biomass has suggested that carbon quantity may be critical to their distribution and growth. Other studies provided evidence on

the importance of the quality of organic matter in influencing benthic bacterial standing stocks in the ocean seafloor. To this regards, Danovaro and co-authors (2000) have observed that in Cretan Sea bacterial abundance increased up to 3-fold in response to the increased amount of sedimentary proteins and CPE.

However, beside bottom up control, benthic deep-sea prokaryotes are also influenced by predatory processes, mainly dependent on viral infections (Danovaro et al., 2008b). Viruses, being capable of converting prokaryotic biomass into dissolved organic matter (DOC) through the cell's lysis, have an important effect on biochemical cycling, microbial loop dynamics and diversity (Wilhelm & Suttle 1999; Glud & Mathias, 2004; Fischer et al., 2007; Danovaro et al., 2008b; Corinaldesi et al., 2012).

Deep-sea prokaryotic dynamic thus depends from a complex array of factors including changes in the food supply from the photic layers (Ritzrau et al., 2001) and interaction with other benthic components (Danovaro et al., 2008).

Previous studies highlighted that the vertical input of organic carbon is insufficient to explain the high values of benthic prokaryotic production (Manini et al., 2002). Furthermore, the autochthonous production accounted only between 6% and 25% of the total carbon requirement, suggesting that other C, possibly through lateral advection processes, contribute to benthic metabolism.

All considered, we still have limited knowledge on benthic prokaryotic diversity in the Mediterranean Sea (Figure 1), but this is important for better understanding of ecosystem functioning.

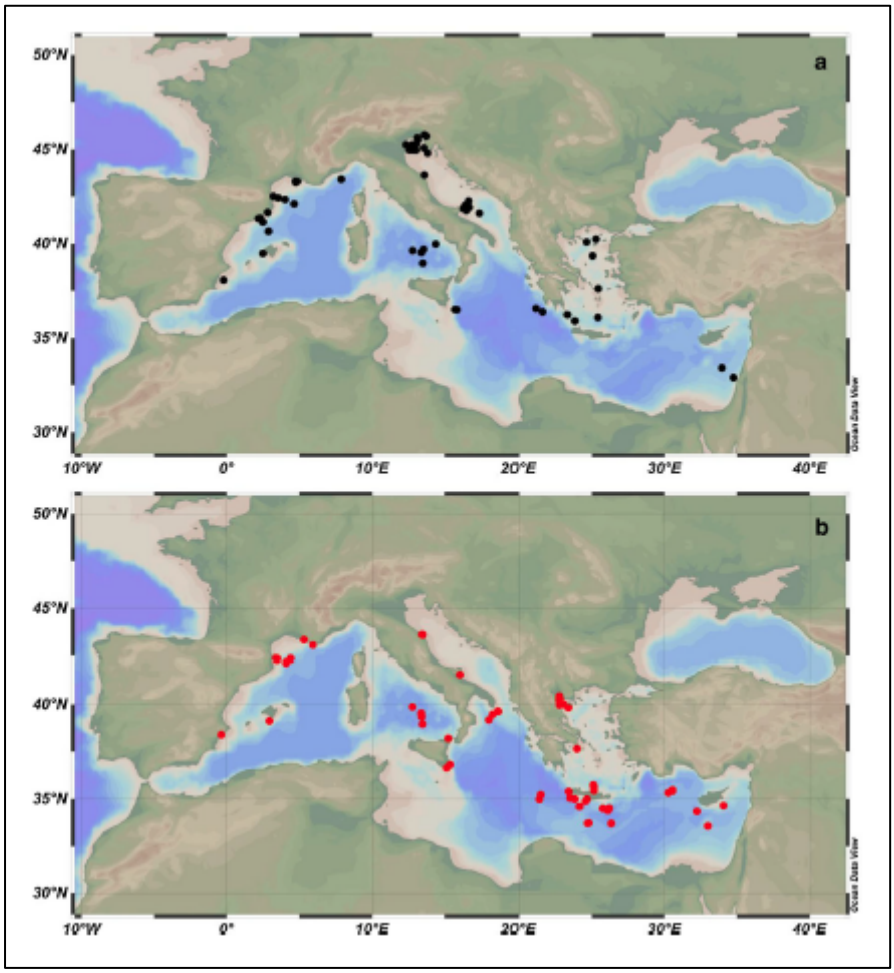

Figure 1: Map showing the location of Mediterranean sites where bacterial and/or archaeal diversity has been described using molecular tools, in the pelagic (a) and benthic (b) domain. Studies performed in coastal lagoons, DHABs and shallow vent areas have not been included. Figure from Luna et al., 2015.

1.3.1 Patterns of prokaryotic diversity in the deep-sea

Spatial patterns of prokaryotic diversity (e.g. richness) typically show higher values in coastal than offshore waters (Pommier et al., 2010; Quero & Luna 2014; reviewed in Luna 2015), likely as consequence of the higher availability of resources and hydrological processes occurring in coastal waters (e.g. inputs of organic and inorganic nutrients, higher water mixing). Overall, richness shows higher values in meso- and bathypelagic zones than in surface (Luna 2015). This phenomenon has been hypothesized to result

from the higher niche heterogeneity in deep layers, the prevalence of particle-attached *versus* free lifestyles, or from the relative stability of deep-sea environments, which in turn may be factors promoting diversity (Pommier et al., 2010). Early studies based on fingerprinting or sequencing of clones reported values of bacterial richness of up to about 100 OTUs (Operational Taxonomic Units) per gram of coastal sediments (Yakimov et al., 2005; Danovaro et al., 2006; Luna et al., 2013), and up to about 1,500 OTUs per gram in deep Mediterranean sediments (Danovaro et al., 2010 and references therein). In addition, Pommier et al., (2010) provided additional evidences that the number of OTU's using Sanger sequencing of clones is around 1000 per sample. More recent studies using Next Generation Sequencing techniques (i.e. pyrosequencing, Ion-Torrent or Illumina sequencing of the 16S rDNA gene amplicons) reported higher richness, with up to about 10,000 different OTUs observed in only 19 samples (Sevastou et al., 2013) and more than 5,000 OTUs in a single deep-sea sediment sample (Corinaldesi et al., 2014). Benthic archaeal richness has been reported to be generally lower than bacterial diversity both in coastal and in deep environments, ranging from less than five to a few tens of OTUs per sediment gram (Luna et al., 2009; Danovaro et al., 2014; Roman et al., 2019). In general, prokaryotic diversity in the deep Mediterranean Sea is high and comparable to values reported for other oceans (Zinger et al., 2011; Corinaldesi et al., 2019). In addition, other studies reported that bacterial and archaeal abundance in deep-sea ecosystems greatly increased from middle to high latitudes. Such findings should be considered for understanding of diversity pattern on global scale (Danovaro et al., 2016).

Studies on the taxonomic diversity of prokaryotic communities in deep-sea surface sediments of the Mediterranean Sea are scarcer than those performed in pelagic environments (Luna, 2015). Using molecular approaches, investigations on prokaryotic diversity have been carried out in the Eastern basin (Polymenakou et al., 2005a, 2005b; Polymenakou et al., 2009; Sevastou et al., 2013; Corinaldesi et al., 2014), the Ionian (Luna et al., 2004) and the Tyrrhenian Sea (Danovaro et al., 2009b; Ettoumi et al., 2010). Phylogenetic analyses of deep benthic microbial communities have shown the dominance of Proteobacteria, characterized by a higher contribution of Gamma- and Delta- than Alpha-, Beta- and Epsilonproteobacteria (Ettoumi et al., 2010; Sevastou et al., 2013). In addition, other phyla characterizing benthic sediments were also found, such as Acidobacteria, Planctomycetes, Actinobacteria, Bacteroidetes, Verrucomicrobia, Chloroflexi, Nitrospirae, Firmicutes, Gemmatimonadetes, Lentisphaerae and Dictyoglomi, named in decreasing order of importance (Polymenakou et al., 2005a and 2005b; Polymenakou et al., 2009).

Phylogenetic studies of benthic archaeal assemblages in the deep Mediterranean are rare. According to Giovannelli and co-authors (Giovannelli et al., 2013) the archaeal deep-sea assemblages are dominated by Crenarchaeota MGI rather than Euryarchaeota, but detailed information on their taxonomic diversity is still largely lacking (Sevastou et al., 2013; Corinaldesi et al., 2014), prompting the need to carry out further studies to investigate the taxonomic and functional diversity of deep benthic Mediterranean archaea. Finally, multivariate analyses have shown that archaeal composition in deep-sea assemblages from Mediterranean

sediments differs from the Atlantic ones at comparable depths (Luna et al., 2009).

The knowledge gained so far suggests that prokaryotic assemblages of Mediterranean Sea are characterized by a high diversity, comparable or maybe even higher than the one observed in other oceans. Moreover, information acquired so far suggest the existence of both bacterial and archaeal ecotypes specifically adapted to unique hydrological, geological and geomorphological features of the Mediterranean basin (Luna 2015; Garczarek et al., 2007; Hugoni et al., 2013).

1.4 Objectives of this study

The "Nurra Escarpment", located in the North-Western Sardinian slope (western Mediterranean Sea), ranges from 600 m of the upper slope to 2400 m of depth in the lowest part of the slope. This area, object of a thorough exploration within the multidisciplinary project BIOFUN (Biodiversity and Ecosystem Functioning in contrasting southern European deep-sea environments: from viruses to megafauna), has been previously studied for the patterns and drivers of meio- and macrofauna diversity (Baldrighi et al., 2014; Schratzberger & Ingels 2018) for the identification of some bauxite deposits located in this area and their geological, geochemical and mineralogical features (Mameli et al., 2007) and also for finding seismic evidence of Messinian detrital deposits (Sage et al., 2005). However, to date, data on the benthic prokaryotic diversity and its drivers in this area are still missing.

The main objective of this study was to explore spatial patterns of abundance and diversity of benthic prokaryotic assemblages along the "Nurra Escarpment" and to identify potential drivers influencing their distribution. To do so, abundance, biomass, diversity and community structure of prokaryotes from 600 to 2400 m depth along with temperature, salinity, grain size, quantity and quality of food sources (i.e. organic matter sediment content) as potential drivers influencing their distribution were analyzed.

Chapter 2 – Materials and Methods

2.1 Study area

Samples were collected in October 2009 on board of the oceanographic vessel R/V Urania, within the framework of the multidisciplinary project BIOFUN (06-EuroDEEP-FP-005; 2007-2010). Samples were collected from the upper (600 m) to the lower parts of the slope (2400 m; Table 1) following a specific sampling design (see paragraph 2.2). The slope area under investigation lies in the southern part of Nurra Escarpment. While considering that, according to Kenyon and colleagues (Kenyon et al., 2002), the continental margin of western Sardinia is generally referred as steep with up to the 7° overall gradient for submarine slopes; such area is characterized by a slight slope in the upper part up to 900 m of depth, then the slope becomes steeper until its foot, which is located at 2400-2600 m.

The Sardinian western margin below 200 m depth is characterized by the presence of different water masses: from 200 to 2000 by the LIW (Levantine Intermediate Water) and TDW (Tyrrhenian Deep Water) and below 2000 m by the WMDW (Western Mediterranean Deep Water) flowing off Algeria and proceeding off the western coast of Sardinia with a potential temperature of 13.0°C and salinity around 38.44-38.48 PSU (Millot 1999).

Overall, hydrogeological characteristics of the water masses along the slope may be considered homogeneous, with a current speed estimation of ca. 2 cm/s (Leucher et al., 1995).

Table 1. List of stations with the relevant coordinates (i.e., “Latitude N” and “Longitude E”), depth and distance from A (i.e. “Position”).

Station	Latitude N	Longitude E	Depth (m)	Position (m)
600A	40°31.136'	07°47.026'	600	0
600B	40°31.260'	07°46.867’	600	200
600C	40°31.776'	07°46.316'	600	1000
900A	40°32.058'	07°42.878'	900	0
900B	40°32.428'	07°43.239'	900	900
900C	40°32.505'	07°43.222'	900	1000
1200A	40°32.310'	07°41.594'	1200	0
1200B	40°32.701'	07°41.880'	1200	800
1200C	40°32.775'	07°41.924'	1200	1000
1500A	40°32.449'	07°41.009'	1500	0
1500B	40°32.763'	07°41.013'	1500	600
1500C	40°32.981'	07°41.019'	1500	1000
1800A	40°32.658'	07°40.124'	1800	0
1800B	40°32.845'	07°40.377'	1800	500
1800C	40°33.055'	07°40.560'	1800	1000
2100A	40°32.758'	07°39.568'	2100	0
2100B	40°33.287'	07°39.472'	2100	900
2100C	40°33.310'	07°39.472'	2100	1000
2400A	40°32.983'	07°38.557'	2400	0
2400B	40°33.339'	07°38.703'	2400	700
2400C	40°33.458'	07°38.750'	2400	1000

2.2 Sampling strategy and samples collection

A hierarchical sampling design (Figure 2 B-C) was adopted, for a total of 21 sites, in order to investigate the spatial variability of microbial assemblages compared to the variability due to increasing water depth. Samples

have been collected every 300 m starting from 600 m to 2400 m, for a total of seven different depths and a length of the vertical transect of 16 km.

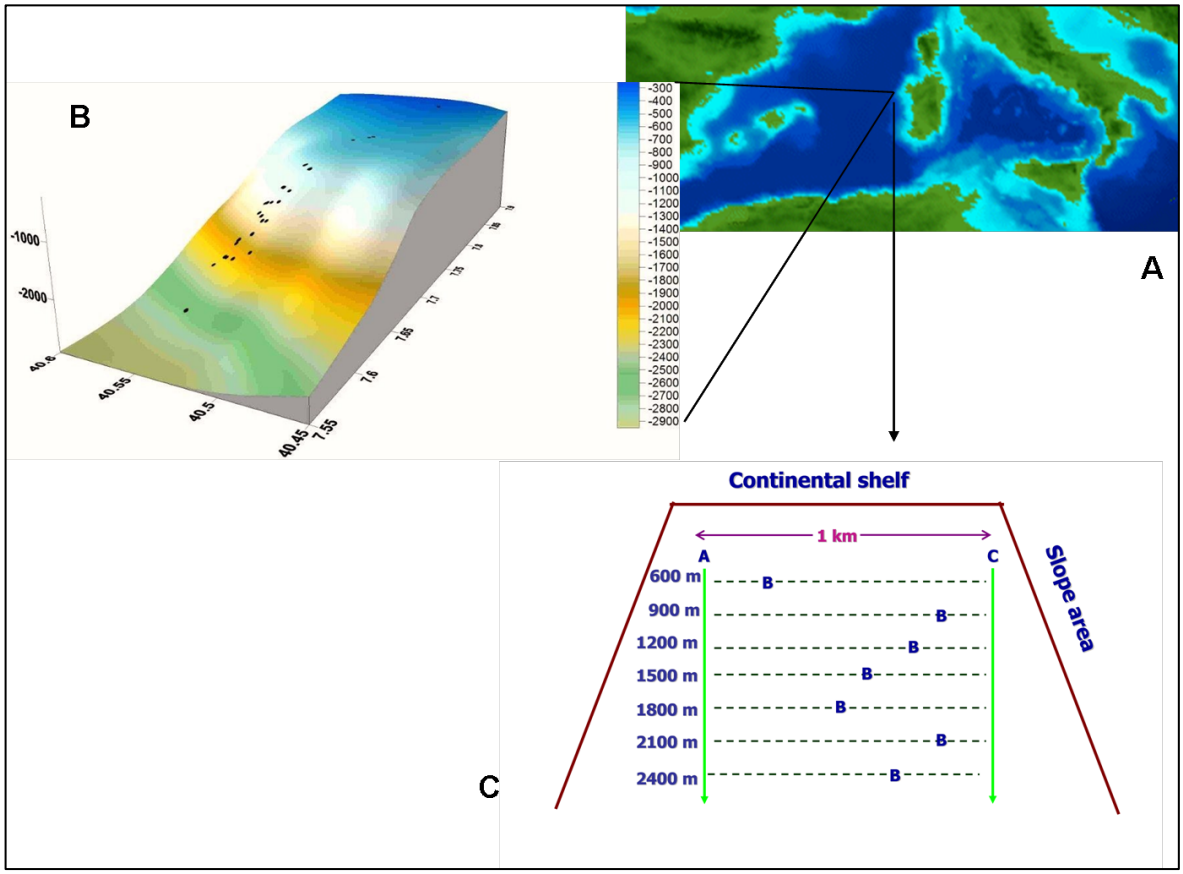

Figure 2: Location of the sampling site (a); bathymetry with box-corers deployments performed (back circles) (B) and sampling design (C) realized along the Sardinia slope.

At each depth, three stations set at a distance < or equal to 1 km have been sampled and named A, B and C. Station A and C were permanently positioned 1 km apart at all depths, unlike the position of B that changed randomly at each depth between A and C. This sampling design accounted for two factors: the 7 levels of depth that were considered as fixed factor and the 3 different position (A, B and C) of the stations, considered a factor nested in depth.

From each of the 21 stations sampled along the slope, independent replicates of undisturbed sediment samples (three for each environmental and biological parameter) were collected using a box-corer (Ø 32cm).

For the analysis of prokaryotic abundance and biomass and diversity, grain size, biochemical composition of organic matter, sub-samples from each box-corer were collected through plexiglass liners (Ø 3,6cm). The first 0-1 centimeters of each sample was frozen at -20°C for microbial, chlorophyll-a, phaeopigments and organic matter content analyses; it has been demonstrated that the analysis of the top cm represented a useful proxy of the sediment trophic status (Pusceddu et al., 2009; 2010).

2.3 Grain size, potential food sources and prokaryotes standing stock analysis

2.3.1 Grain size

Sub-samples of sediment were sieved using a 63 μm sieve and the obtained two fractions (sand > 63μm and mud < 63μm) were dried in the oven at 60°C. After 24-48h, both fractions were weighed and expressed as percentage of the total dry weight.

2.3.2 Phytopigment contents

Chlorophyll-a and phaeopigment analysis were carried out according to Lorenzen and Jeffrey (1980). Pigments were extracted from an aliquot of the top 1cm of sediment samples and carried out adding 5 ml of 90% acetone after the sample had been placed in a tube containing about 0.1g of magnesium carbonate (in order to avoid the decomposition of chlorophyll). Then the tubes were subjected to an ultrasonic bath for 3 minutes (a process named sonication) and stored in the dark for 12 hours at a temperature of 4°C. The extracts, centrifuged at 2500 rpm for 10 minutes, were quantified fluorometrically to

estimate chlorophyll-a and phaeopigments, after acidification with 200 ml 0.1N HCl. The concentrations of chlorophyll-a and phaeopigments were summed to obtain total phytopigments (CPE). Concentrations of total phytopigments were converted into C equivalents using 40 as a conversion factor (de Jonge, 1980) and expressed as mgC/g.

2.3.3 Quantity and biochemical composition of organic matter

Carbohydrate (CHO), protein (PRT) and lipid (LIP) content in the top 1cm of sediment were determined according to standard techniques (Danovaro 2010). Their concentrations were converted into C equivalents using the conversion factors: 0.40, 0.49 and 0.75 µgC/µg, respectively, and normalized to sediment dry weight after desiccation at 60°C for 24 hours. Biopolymeric organic C (BPC) was calculated as the sum of the C equivalents of protein, lipid and carbohydrate (Fabiano et al., 1995). Finally, in order to investigate the nutritional quality of the organic matter found in sediments, the contribution of phytopigment Carbon (CCPE) and protein Carbon (CPRT) contents to BPC concentration, together with the values of the protein to carbohydrate ratio (PRT/CHO) were calculated (Pusceddu et al., 2010).

2.3.4 Prokaryotic abundance and biomass

The total prokaryotic number (TPN) in the sediments was determined by epifluorescence microscopy following the staining technique with Acridine orange previously described (Luna et al., 2002). Prokaryotic biovolume was converted into C equivalent according to Fry (1990).

2.4DNA extraction

Prokaryotic DNA was extracted from 1 gram of sediment using the Powersoil DNA Isolation Kit (Qiagen, formerly Mobio Laboratories Inc, California) according to the protocol provided by the manufacturer, with slight modifications to increase the yield and quality of DNA:

- The first consisted in the addition of two more vortex phases at maximum speed for 2 minutes, each of which was preceded by an incubation at 70°C for 5 minutes, which aims at increasing the effectiveness of the lytic phase, during which the cells break to release DNA from the sample;
- The second modification concerns the washing with C5 solution, aimed at removing contaminants and other organic compounds typically co-extracted with DNA from sediment samples.

This protocol has been previously provided and is based on the combined action of different treatments in order to extract the DNA. Firstly, the breaking of cells was performed with three different methods:

1. Mechanical method, by the action of small spheres known as “microbeads” contained in sterile tubes in which sediments had to be inserted for the extraction;
2. Chemical method, by the action of C1 solution, a mix of several reagents (for example SDS) which facilitated the dissolution of the components of both cellular membrane and wall;

3. Physical method, through the usage of high temperatures in order to break the bonds between the different components of cellular membrane and wall.

Subsequently, the DNA released by cells was purified using C2 and C3 solutions, which contained reagents with the ability of precipitating proteins and other macro-molecular compounds released together with DNA during the breakage of cellular membrane and wall. Afterwards, the purification was carried out through the action of a centrifuge, to which followed the addition to the purified solution containing DNA of another solution (C4). C4 solution has the capability to promote the binding of the DNA with a filter membrane (A spin filter D), through which the solution was supposed to pass. The aim of this phase was retaining the DNA upon the membrane, while all other components were discharged. Once the DNA had been bonded to the membrane, the final step consisted in washing it with C5 solution, in which ethyl alcohol was included, allowing the elimination of further organic contaminants still present in the sample. Finally, solution C6 (a 10ml solution Tris pH 8) was used to elute the DNA through the filter by releasing it into a sterile tube. After these numerous passages, DNA concentration in the extracts was determined by the Nanodrop Nucleic Acid Quantification (Thermo Fisher Scientific) instrument, for the measurement of double strand DNA (dsdna).

Following the detailed DNA extraction protocol performed using the Qiagen DNeasy PowerSoil kit:

- Transfer 1g of sediment to Powerbead tubes and vortex for a few seconds;

- Add 60µl of C1 solution and alternate 2 minutes of vortex at maximum speed and 5 minutes of water bath at 70°C twice, ending with a third vortex phase for 2 minutes;
- Centrifuge for 30 seconds at 10,000 g, then transfer 500µl of supernatant to new sterile tube and add 250µl of C2 solution;
- Centrifuge for 1 minute at 10,000 g, then transfer 600µl supernatant and add 200µl C3 solution;
- Incubate for 5 minutes at 4°C in the refrigerator, then centrifuge for 1 minute at 10,000 g, transfer 750µl of supernatant to a new tube and add 1,200µl of C4 solution;
- Transfer the whole volume into an Eppendorf tube equipped with a filtering membrane (named "spin filter") and centrifuge for 1 minute to 10,000 g (3 relocations are required to filter the entire volume of 2ml);
- Discard fluids, but store the membrane to which the DNA is bound, and add 500µl of C5 solution;
- Centrifuge for 30 seconds at 10,000 g, then centrifuge another time for 1 minute without adding any solution;
- Transfer the filter to a sterile Eppendorf and add 100µl of C6 solution, to elute the DNA from the membrane;
- Centrifuge at 10,000 g for 30 seconds; discard the filter and store the Eppendorf containing the DNA at temperatures between -20°C and -80°C.

The sediment did not require any preparation other than the collection of about 1g from the sample. In order to extract the contents of each filter couple within a single Eppendorf, the 100 µl extracted from the first filter were taken and transferred to another Spin Filter and used for the last centrifugation step of the latter.

2.5 DNA sequencing and bioinformatic analyses

High-throughput sequencing (HTS) library preparation was performed using the Illumina Nextera protocol. Illumina Miseq sequencing (2×300 bp paired-end sequencing, V3 chemistry) analyses were carried out on the hypervariable V3 and V4 regions of the 16S rRNA gene by amplifying using the 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 906R (5'-CCGTCAATTCMTTTGAGTTT-3') universal bacterial primers (Jolivet et al., 2004). Raw sequences were pre-cleaned with Cutadapt v1.15 (Martin 2011) by clipping synthetic primers and sequencing adapters and removing sequences shorter than 280 bp. All sequences were imported in R and analyzed with the DADA2 package (Callahan et al., 2016).

Following the package instructions, sequences' quality was manually inspected and a maximum length cutoff was set to 490 bp. After truncation, sequences with an expected error higher than 2 nucleotides per 100 bp were discarded. Samples were pooled to estimate sequencing error rates and the convergence of the parametric error model was achieved using a random subset of 1M sequences. The amplicon sequence variants (ASVs) inference was performed on the dereplicated sequences after pooling all samples together to reduce possible biases due to low sampling depths. Prokaryotic taxonomy was assigned using SILVA (Quast et al., 2012) with a minimum similarity threshold of 86.5%. Cleaned sequences were deposited in the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under the accession numbers SRP134151.

2.6 Statistical analysis

Considering the positions of A, B and C stations as random and nested in the fixed factor depth, a two-way analysis of variance (ANOVA) was performed in order to assess spatial variability of environmental features and biological components. Then, a Student–Newman–Keuls (SNK) post-hoc comparison test (at α=0.05) was applied in order to ascertain at which depth or position values are significantly different. The homogeneity of variance of the entire dataset was tested using a Cochran's test and data were log(x+1) transformed when necessary. To those datasets for which the transformation returns heterogeneous variances, a more conservative level of significance was applied, in accordance to Underwood (1991). All ANOVA, SNK and homogeneity of variance tests were conducted using the STATISTICA 8.0 software package.

The ASV table produced with *dada2* was imported and analyzed in RStudio (RStudio Team, 2015). The ASV table was normalized with a random subsampling to the second lowest number of sequences (n = 16,750) with the *rrarefy* function in the *vegan* package (Oksanen et al., 2019). Alpha diversity was analyzed by using the *estimateR* and *diversity* commands to calculate species richness (i.e. the number of ASVs), Shannon diversity index and Pielou's evenness in *vegan*.

Non-metric multidimensional scaling (nMDS) was performed using (i) Bray-Curtis dissimilarity matrix and (ii) Jaccard similarity matrix and calculated through the command *ordinate* and plotted with the *plot_ordination* command included in the *phyloseq* package.

Cluster analysis was performed using a Bray-Curtis dissimilarity matrix by using the *hclust* function in the *vegan* package.

Analysis of similarities (ANOSIM) was performed to test the presence of statistical differences in microbial taxonomic and species composition across the three transects as well as along the slope, through the command *anosim* included in the *vegan* package.

The *adonis* approach (*vegan* package) was applied to identify the explanatory environmental variables, i.e. those showing significant correlations with the community dissimilarity (Bray–Curtis index) matrix. The selected variables were used to perform Canonical Correspondence Analysis (CCA) using the *vegan* and *ggplot2* R packages.

Finally, a Mantel test (*mantel* function, *vegan* package) was used to detect spatial correlations in the relationship between the community composition and environmental data sets.

Chapter 3 – Results

3.1 Study area and environmental parameters

Temperature (°C) and salinity (PSU) were measured at each depth using CTD SBE 911 plus probe mounted on a CTD-rosette system.

The slope area under investigation lies in the southern part of Nurra Escarpment, characterized by a slight slope in the upper part up to 900 m of depth, and a steeper slope until 2400-2600 m. Near-bottom temperature slightly decreased with depth from 13.7 to 13.2 °C; a similar result was observed for salinity, that ranged from 38.62 (600 m) to 38.46 (2400 m). On the contrary, oxygen content expressed in milliliter of oxygen per liter of water increased with depth ranging from 4,022 ml/L at 600m to 4,504 ml/L at 2400m (Table 2).

Table 2. Measured values of temperature (°C), salinity and dissolved oxygen at each sampled station.

Station	Temperature (°C)	Salinity (PSU)	Oxygen (ml/L)
600A	13.7	38.62	4.022
600B	13.7	38.62	4.022
600C	13.7	38.62	4.022
900A	13.4	38.57	4.108
900B	13.4	38.57	4.108
900C	13.4	38.57	4.108
1200A	13.3	38.5	4.260
1200B	13.3	38.5	4.260
1200C	13.3	38.5	4.260
1500A	13.2	38.47	4.309
1500B	13.2	38.47	4.309
1500C	13.2	38.47	4.309
1800A	13.4	38.53	4.440
1800B	13.4	38.53	4.440
1800C	13.4	38.53	4.440
2100A	13.2	38.46	4.473
2100B	13.2	38.46	4.473
2100C	13.2	38.46	4.473
2400A	13.2	38.46	4.504
2400B	13.2	38.46	4.504
2400C	13.2	38.46	4.504

3.2 Grain size, potential food sources and prokaryotes standing stock analysis

Results on grain size for each analyzed sediment sample are reported in Table 3.

Table 3. Grain size composition for each station.

Station	Sand (%)	Silt and clay (%)
600A	3.57	96.43
600B	17.41	82.59
600C	20.88	79.12
900A	12.3	87.7
900B	11,49	88.51
900C	10.51	89.49
1200A	6.27	93.73
1200B	7.86	92.14
1200C	9.8	90.2
1500A	9.05	90.95
1500B	12.29	87.71
1500C	10.44	89.56
1800A	8.37	91.63
1800B	19.8	80.2
1800C	14.18	85.82
2100A	10.54	89.46
2100B	6.43	93.57
2100C	31.06	68.94
2400A	9.51	90.49
2400B	10.26	89.74
2400C	36.19	63.81

Silt-clay fraction dominated the sediment at all investigated stations, with no significant changes both at stations located at similar depths and with increasing depth (ANOVA, n.s.). Similarly, no significant difference occurred among stations and depth for the sandy fraction (ANOVA, n.s.). However, sandy fraction showed lower values than silt-clay fraction at all depth and highest average values were reported at 2100 m and 2400 m.

Table 4 illustrates main parameters linked to phytopigments, namely sediment content of chlorophyll-a and phaeopigments, the sum of them (CPE) and IDP. Chlorophyll-a concentrations were very low ranging from 0.01±0.0 µg/g (2400C) to 0.14±0.07 µg/g (600A) and decreased with water depth. Significant higher values were observed at the shallowest station compared to all the other stations (SNK post-hoc, $p<0.01$). Phaeopigments concentrations ranged from 8.79±4.60 µg/g at the shallowest stations to 0.6±0.12 µg/g at the deepest stations. A significant decrease of CPE content was observed with increasing depth, due to the decreasing of phaeopigments in sediments. Significant differences of CPE concentrations were observed among shallower stations at 600m (SNK post-hoc, $p<0.01$ between A-B and A-C) (Table 4). Finally, IDP expresses the percentage of active photosynthetic pigments. This parameter ranges from 1.32±0.36 % (2100A) to 2.99±1.61 % (1800A) without showing any clear trend.

Table 4. Chlorophyll-a, phaeopigments and total phytopigment concentrations and IDP values in the investigated sediments.

Station	Chl-a (µg/g)	Phaeo (µg/g)	CPE (µg/g)	IDP (Chla/CPE*100)
600A	0.14 ± 0.07	8.79 ± 4.60	8.93 ± 4.66	1.64 ± 0.19
600B	0.02 ± 0.0	1.08 ± 0.16	1.09 ± 0.16	1.59 ± 0.34
600C	0.02 ± 0.0	1.21 ± 0.25	1.24 ± 0.25	1.92 ± 0.33
900A	0.05 ± 0.01	2.2 ± 0.44	2.25 ± 0.45	2.21 ± 0.34
900B	0.02 ± 0.01	1.11 ± 0.14	1.13 ± 0.13	1.79 ± 0.66
900C	0.02 ± 0.0	1.22 ± 0.26	1.24 ± 0.26	1.65 ± 0.15
1200A	0.04 ± 0.03	3.11 ± 3.43	3.15 ± 3.46	1.75 ± 0.57
1200B	0.01 ± 0.0	0.66 ± 0.22	0.67 ± 0.23	1.34 ± 0.04
1200C	0.01 ± 0.0	0.75 ± 0.12	0.76 ± 0.12	1.76 ± 0.10
1500A	0.02 ± 0.0	0.95 ± 0.21	0.98 ± 0.21	2.33 ± 0.74
1500B	0.02 ± 0.0	0.82 ± 0.17	0.83 ± 0.17	1.86 ± 0.32
1500C	0.01 ± 0.0	0.74 ± 0.05	0.76 ± 0.05	1.74 ± 0.28
1800A	0.03 ± 0.01	1.16 ± 0.63	1.19 ± 0.64	2.99 ± 1.61
1800B	0.02 ± 0.0	0.94 ± 0.35	0.96 ± 0.35	2.07 ± 0.61
1800C	0.02 ± 0.0	1.20 ± 0.96	1.22 ± 0.96	1.97 ± 0.93
2100A	0.01 ± 0.0	0.88 ± 0.16	0.90 ± 0.16	1.32 ± 0.36
2100B	0.04 ± 0.01	2.06 ± 1.16	2.10 ± 1.17	2.16 ± 0.58
2100C	0.02 ± 0.0	0.86 ± 0.19	0.88 ± 0.02	1.91 ± 0.13
2400A	0.02 ± 0.01	1.13 ± 0.12	1.15 ± 0.12	1.84 ± 0.48
2400B	0.02 ± 0.01	1.09 ± 0.10	1.11 ± 0.11	1.93 ± 0.73
2400C	0.01 ± 0.0	0.6 ± 0.12	0.61 ± 0.12	1.62 ± 0.69

Results on organic matter content are shown in Table 5. Protein (PRT) concentrations displayed a wide spatial variability with the highest values at 900m (station C) and 2100 m depth (station B; 0.98±0.32 mg/g and 1.19±0.21 mg/g, respectively), and lowest value at 600 m depth (B; 0.22±0.06 mg/g). No clear depth related patterns were observed. Carbohydrate (CHO)

concentrations generally decreased with increasing water depth, except for stations B at 2400 m, where the concentration of CHO was higher 1.80±0.48 mg/g. Lipids (LIP) concentrations did not show any clear spatial pattern, with the lowest value at 1500 m depth (B; 0.08±0.02 mg/g) and the highest value at 1800 m depth (A; 0.42±0.15 mg/g).

Biopolymeric carbon content displayed an irregular distribution with increasing water depth. Significantly higher values were observed at station B located at 2100 and 2400 m depth (1.38±0.20 mgC/g, and 1.37±0.29 mgC/g; ANOVA, $p<0.01$). Significant changes between stations at the same depth were reported at 2100 m (SNK post-hoc, $p<0.001$ among B-A and B-C) and 2400 m (SNK post-hoc, $p<0.001$ among B-A and B-C) depth.

The quality of organic matter is expressed with two different parameters: the protein to carbohydrate ratio (PRT/CHO) and the protein to biopolymeric organic carbon ratio (PRT/BPC). Values of the protein to carbohydrate ratio (PRT/CHO) displayed a wide spatial variability (Table 5). Values of stations C at 1500-1800 m and 2100 m depth different significantly from station A and B at the same depth (ANOVA, $p<0.01$). The PRT/BPC ratio ranged from 0.44±0.00 (Station B at 1800 m depth) to 1.19±0.12 (Station C at 2100 m depth) without showing any clear pattern both in relation to water depth and to the station position at the same depth.

Table 5. Quantity and biochemical composition of organic matter (CHO, PRT, LIP, BPC). Quality as PRT/CHO and PRT/BPC ratios.

Station	PRT (mg/g)	CHO (mg/g)	LIP (mg/g)	PRT/CHO	BPC (mgC/g)	PRT/BPC
600A	0.93 ± 0.12	0.75 ± 0.14	0.21 ± 0.04	1.26 ± 0.27	0.92 ± 0.12	1.02 ± 0.11
600B	0.22 ± 0.06	0.56 ± 0.17	0.18 ± 0.05	0.43 ± 0.22	0.47 ± 0.02	0.47 ± 0.12
600C	0.23 ± 0.05	0.27 ± 0.05	0.16 ± 0.05	0.87 ± 0.31	0.34 ± 0.03	0.68 ± 0.18
900A	0.78 ± 0.20	0.60 ± 0.09	0.36 ± 0.14	1.32 ± 0.29	0.90 ± 0.22	0.87 ± 0.01
900B	0.41 ± 0.05	0.93 ± 0.13	0.19 ± 0.01	0.44 ± 0.03	0.71 ± 0.07	0.57 ± 0.03
900C	0.98 ± 0.32	0.62 ± 0.14	0.23 ± 0.06	1.58 ± 0.37	0.90 ± 0.16	1.07 ± 0.19
1200A	0.50 ± 0.12	0.40 ± 0.07	0.10 ± 0.02	1.25 ± 0.25	0.48 ± 0.09	1.03 ± 0.08
1200B	0.40 ± 0.11	1.17 ± 0.12	0.11 ± 0.03	0.35 ± 0.10	0.74 ± 0.06	0.54 ± 0.11
1200C	0.82 ± 0.09	0.52 ± 0.17	0.25 ± 0.07	1.73 ± 0.62	0.79 ± 0.09	1.03 ± 0.04
1500A	0.77 ± 0.10	0.48 ± 0.15	0.14 ± 0.04	1.75 ± 0.85	0.67 ± 0.08	1.14 ± 0.15
1500B	0.43 ± 0.05	0.89 ± 0.26	0.08 ± 0.02	0.51 ± 0.15	0.63 ± 0.11	0.70 ± 0.14
1500C	0.74 ± 0.13	0.34 ± 0.13	0.23 ± 0.05	2.29 ± 0.54	0.67 ± 0.14	1.11 ± 0.08
1800A	0.32 ± 0.09	0.47 ± 0.10	0.42 ± 0.15	0.73 ± 0.30	0.66 ± 0.13	0.50 ± 0.17
1800B	0.36 ± 0.04	1.28 ± 0.16	0.17 ± 0.03	0.28 ± 0.01	0.82 ± 0.10	0.44 ± 0.00
1800C	0.65 ± 0.07	0.17 ± 0.06	0.23 ± 0.08	4.08 ± 1.11	0.56 ± 0.10	1.17 ± 0.12
2100A	0.52 ± 0.12	0.37 ± 0.06	0.27 ± 0.03	1.40 ± 0.21	0.61 ± 0.07	0.85 ± 0.12
2100B	1.19 ± 0.21	1.44 ± 0.38	0.30 ± 0.08	0.84 ± 0.09	1.38 ± 0.20	0.86 ± 0.03
2100C	0.80 ± 0.07	0.27 ± 0.10	0.23 ± 0.03	3.16 ± 1.02	0.68 ± 0.06	1.19 ± 0.12
2400A	0.46 ± 0.09	0.53 ± 0.11	0.20 ± 0.01	0.89 ± 0.32	0.59 ± 0.04	0.77 ± 0.14
2400B	0.91 ± 0.18	1.80 ± 0.48	0.27 ± 0.06	0.51 ± 0.05	1.37 ± 0.29	0.67 ± 0.01
2400C	0.47 ± 0.09	0.31 ± 0.12	0.18 ± 0.05	1.65 ± 0.51	0.44 ± 0.16	1.10 ± 0.19

3.3 Prokaryotic abundance and biomass

Results of the prokaryotic abundance and biomass are reported in Figure 3 and Table 6.

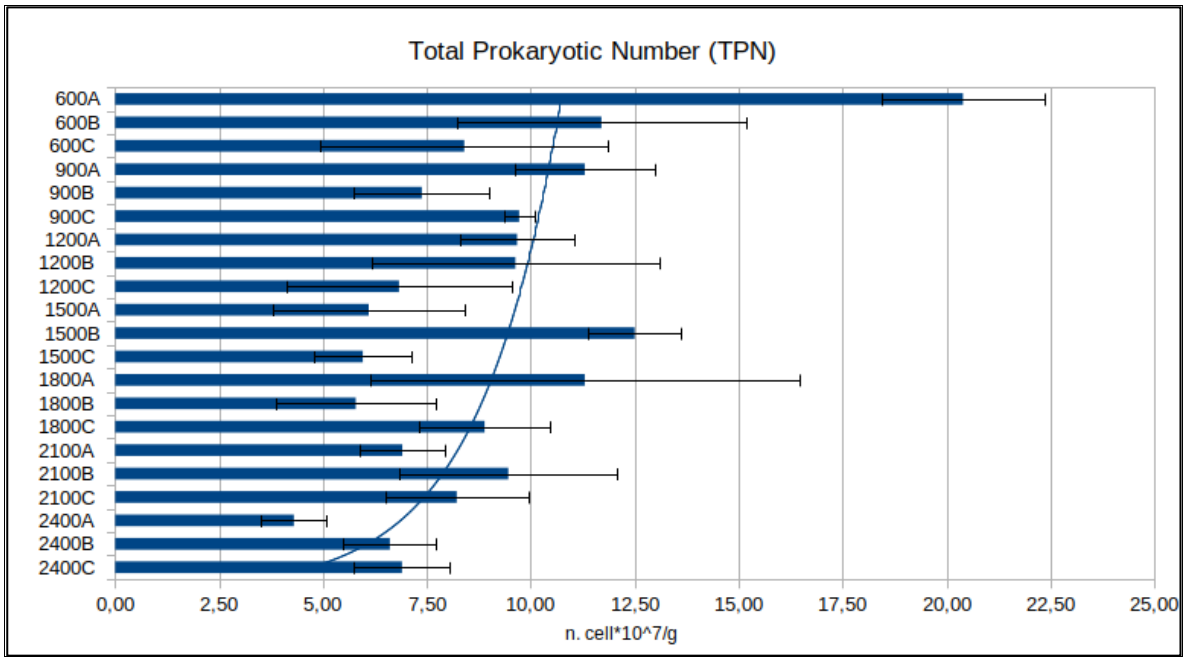

Figure 3: Barplot showing the total prokaryotic number (TPN, reported as number of cells x 10^7 per gram of sediment) at each investigated station.

Table 6. Results on the total prokaryotic biomass, reported as microgram (μg) of Carbon per gram of sediment and derived from the total prokaryotic number (Figure 5).

Station	TPB (μgC/g)
600A	4.08 ± 0.39
600B	2.34 ± 0.70
600C	1.68 ± 0.69
900A	2.26 ± 0.34
900B	1.48 ± 0.33
900C	1.94 ± 0.07
1200A	1.93 ± 0.27
1200B	1.93 ± 0.69
1200C	1.37 ± 0.54
1500A	1.22 ± 0.46
1500B	2.50 ± 0.22
1500C	1.19 ± 0.24
1800A	2.27 ± 1.03
1800B	1.16 ± 0.39
1800C	1.78 ± 0.31
2100A	1.38 ± 0.20
2100B	1.89 ± 0.52
2100C	1.64 ± 0.34
2400A	0.86 ± 0.16
2400B	1.32 ± 0.22
2400C	1.38 ± 0.23

Total prokaryotic abundance and biomass generally decreased with increasing water depth. Highest values of prokaryotic abundance were observed at station A at 600 m depth (20.40±1.97 cells*10^7/g), whilst lowest value at station A at 2400 m depth (4.30±0.79 cells*10^7/g; ANOVA, $p<0.01$).

Significant differences among prokaryotic abundance and biomass were only found among stations at 600 m depth (A-B and A-C; SNK post-hoc, $p<0.01$).

Total prokaryotic abundance, expressed as number of cells per 10^8, was positively and significantly related (R=0.62) to the total amount of phytopigments in the sediments (Chl-a + Phaeopigments; Figure 4) and to bottom water temperature (Figure 5).

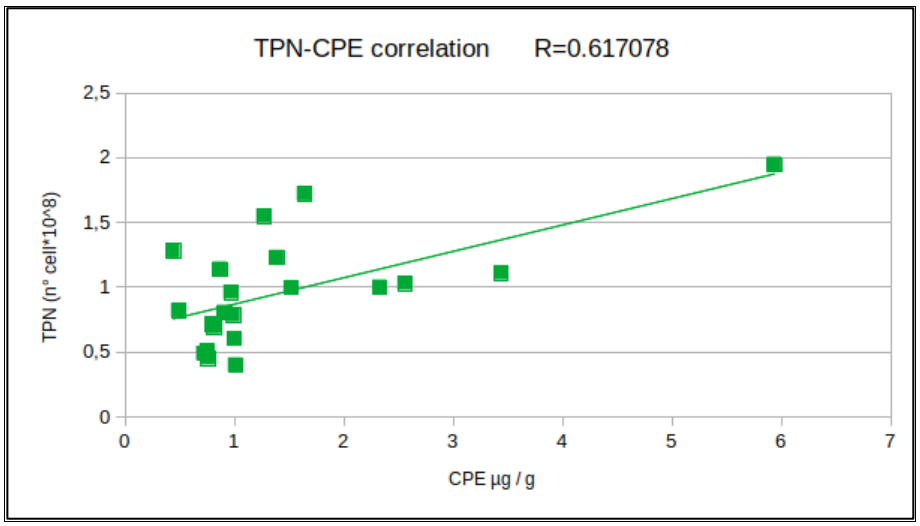

Figure 4: Correlation between TPN (n. of cell*10^8) and CPE (µg/g). Shown is also the R value.

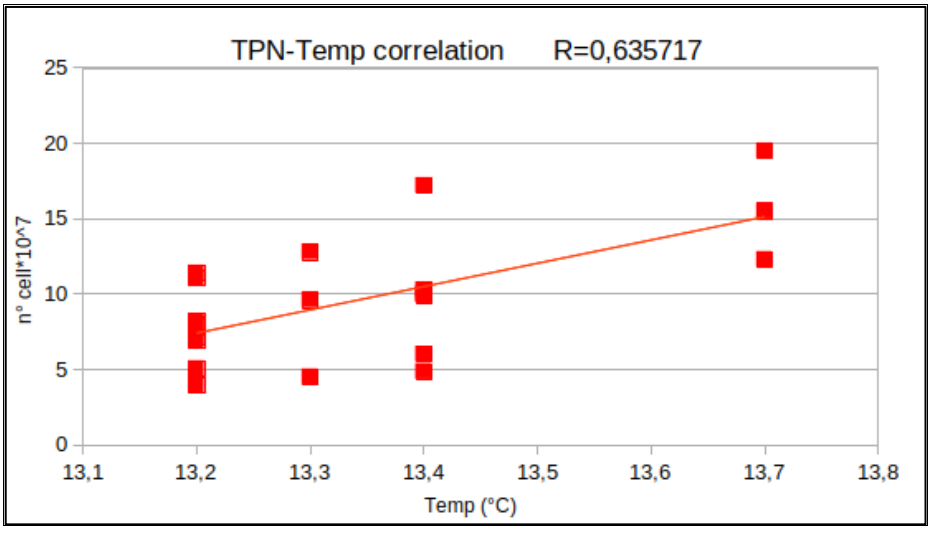

Figure 5: Correlation between TPN (n° cell*10^7) and bottom water temperature (°C). Shown is also the R value.

3.4 Prokaryotic diversity

Results showing the number of pre-processing and processed 16S rRNA gene sequences, as obtained by Illumina sequencing, are reported in detail in figure 6. Raw reads, as obtained after the sequencing, ranged from a minimum of 19,652 to a maximum of 64,954 reads, at 900A and at 1800C, respectively. Similarly, cleaned and processed reads count ranged from a minimum of 9,393 reads at station 900B to a maximum of 36,502 reads at station 2400A.

ASVs richness ranged from 1,271 at station 900B to 2,537 at station B at 600 m depth, with an average value of 2,121 ASV across the whole dataset (Figure 6). Shannon index ranged from 6.08 to 7.18 at stations A and B located at 600 m depth, respectively. Finally, evenness ranged from 0.80 at station B at 600 m to 0.93 at station B at 1500 m and stations C at 600 2100 m depth. No significant differences were found when comparing ASV richness, Shannon and Evenness values among depths and among transects (Kruskal-Wallis, n.s.).

In order to estimate the similarity between samples, nMDS analysis was performed both considering a presence-absence of ASVs (Figure 7, panel A) as well as their abundance (Figure 7, panel B). In both cases, the absence of a clear grouping of samples according to depth and transect was observed.

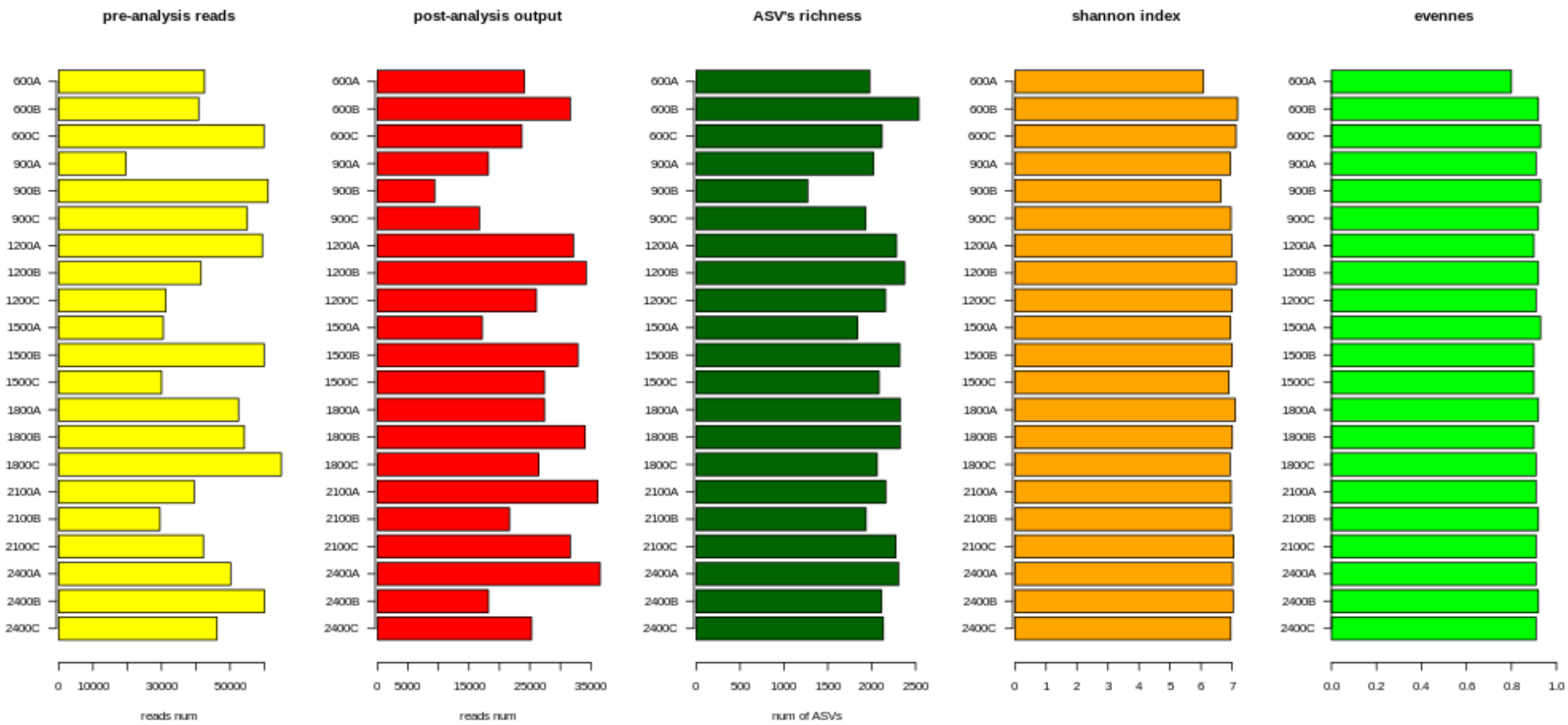

Figure 6: Summary graph showing (1) the number of raw reads before bioinformatic processing (yellow bars), (2) the numbers of reads after bioinformatic analyses (red bars), (3) ASV richness (i.e. number of ASVs counted in each sample) (dark green bars) after normalizing the ASV table at n=16,750 sequences, (4) Shannon index (orange bars) and (5) Pielou's evenness (light green bars).

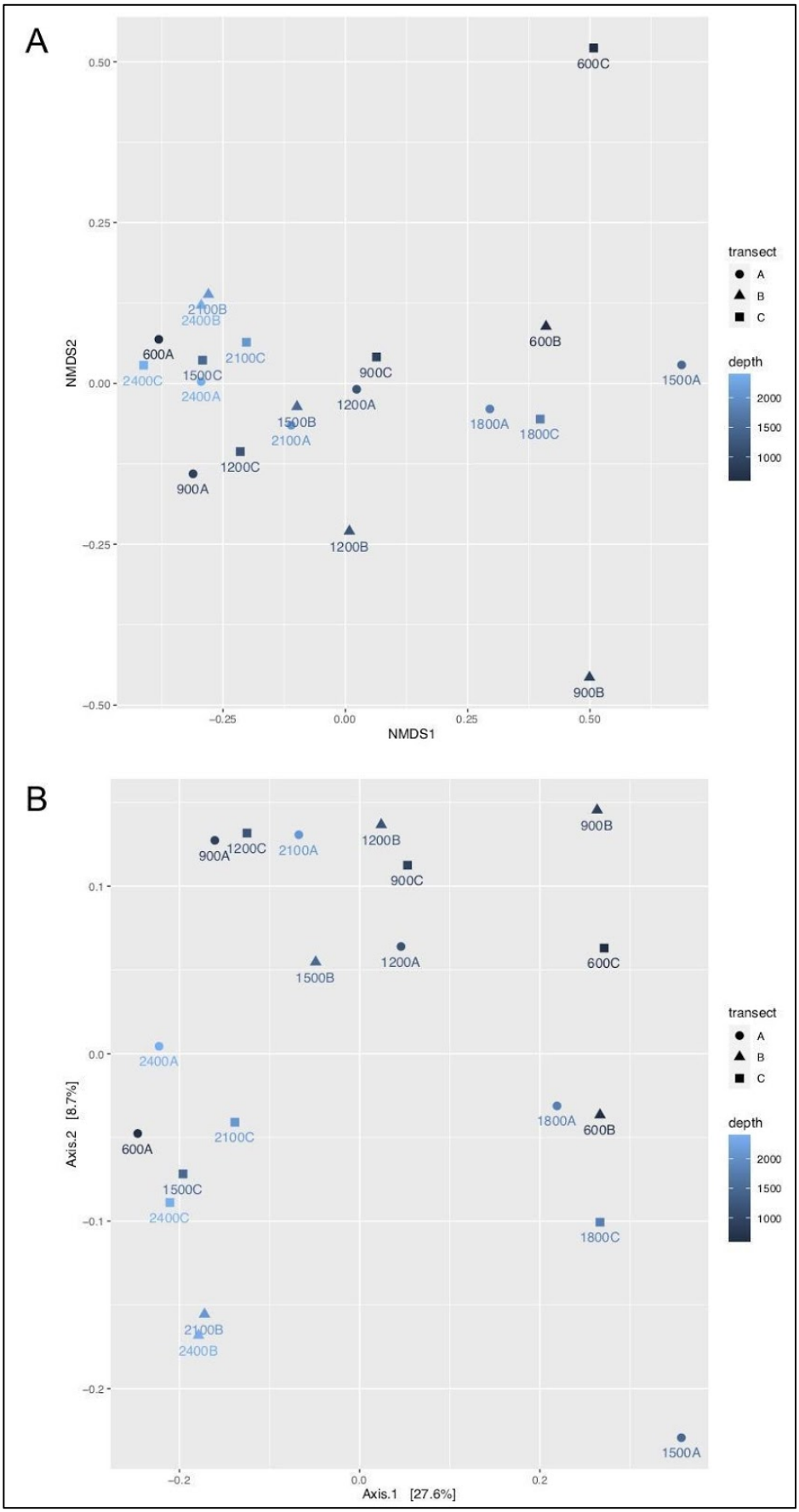

Figure 7: Non-metric Multi-Dimensional Scaling (nMDS) using (a) a matrix of dissimilarity calculated on presence/absence data (Jaccard) and (b) a dissimilarity matrix based on Bray-Curtis.

In order to get a deeper insight into the similarity of the analyzed prokaryotic communities, we also performed a hierarchical clustering based on Bray-Curtis matrix. Such analysis highlighted a higher dissimilarity of prokaryotic communities at stations 600C and 900B.

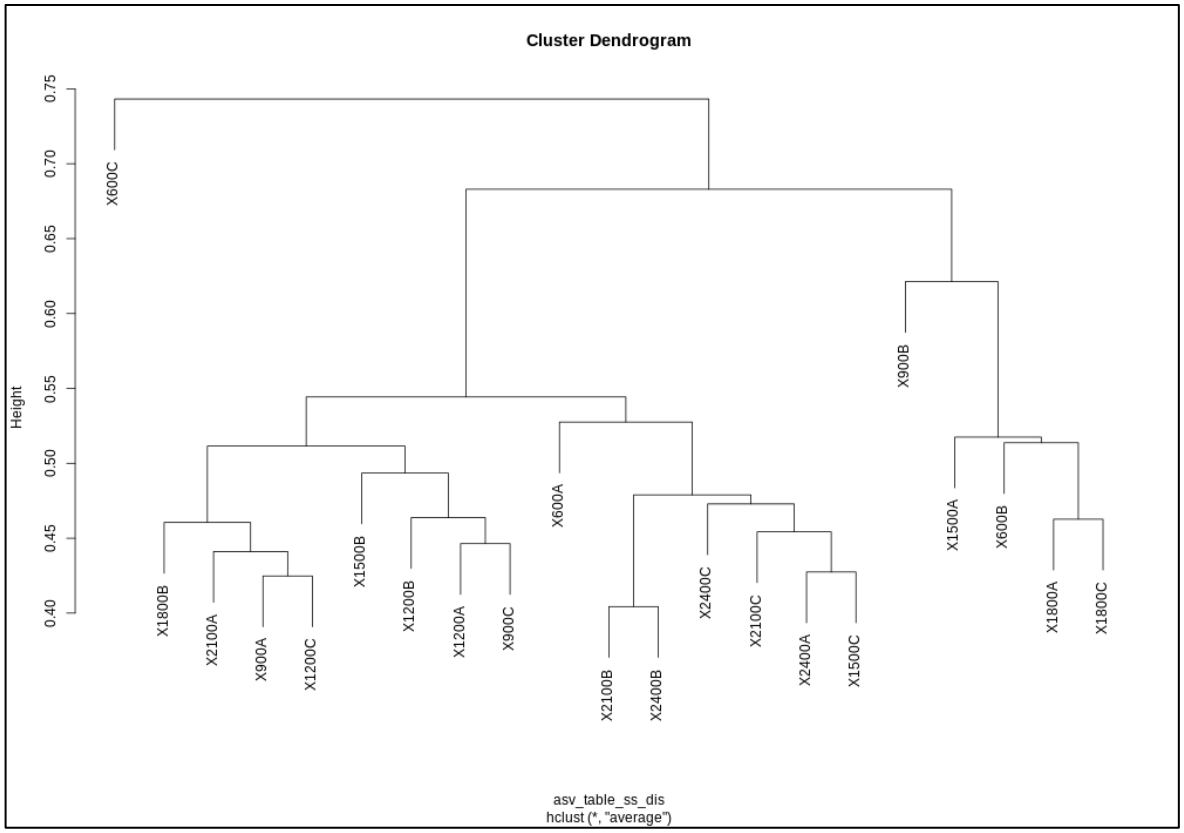

Figura 8: Dendrogram showing the similarity among prokaryotic community composition of each analyzed sample. The dendrogram was built using a hierarchical approach after a Bray-Curtis dissimilarity matrix was calculated.

Statistical analyses (i.e. ANOSIM) indicated the lack of significant differences among samples in relation to water depth (R=0.089, P=0.159) or sampling position at the same depth (R=0.097, P=0.997) (Figure 9 and 10).

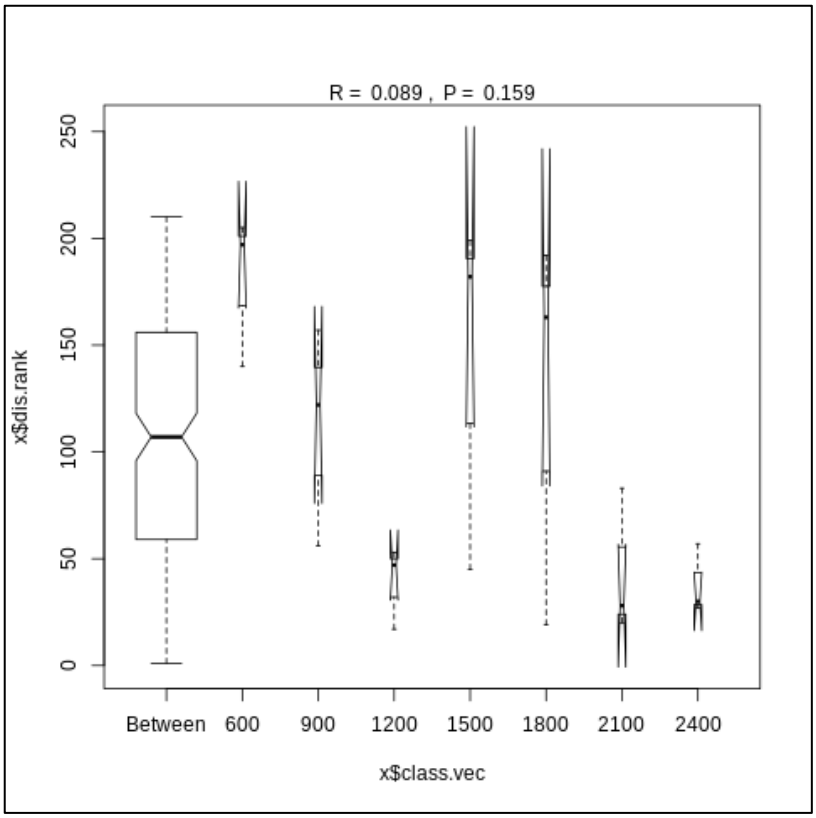

Figure 9: Graphical results of ANOSIM using "depth" as grouping factor.

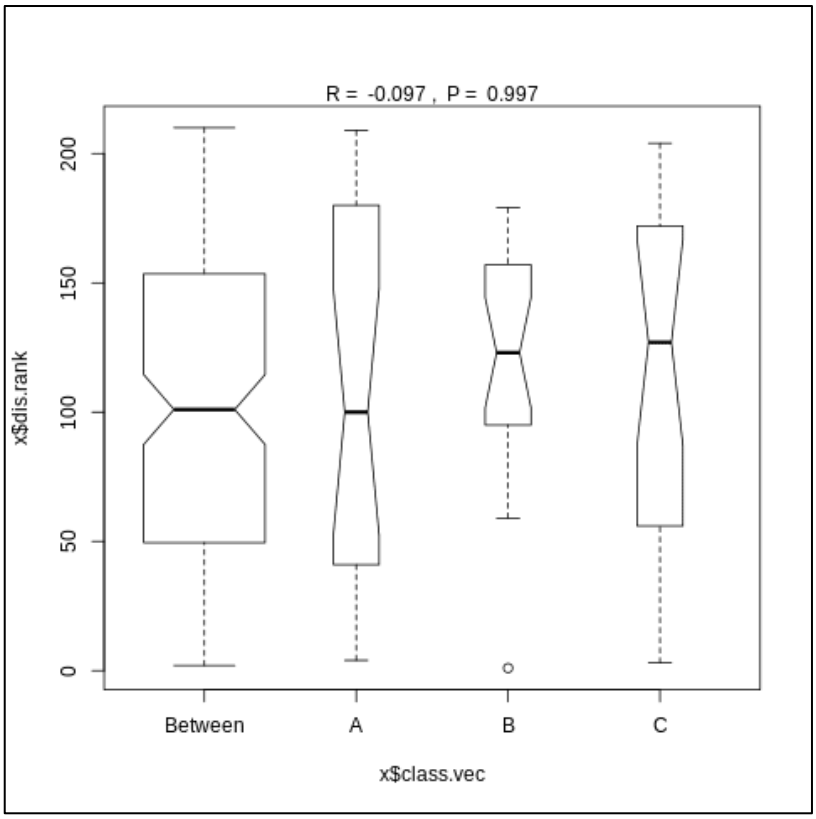

Figure 10: Graphical results of ANOSIM using "transect" as grouping factor.

The taxonomic composition of the benthic deep-sea prokaryotic communities was dominated by Proteobacteria phylum (19.1 ± 1.5 %), with Alpha- and Gammaproteobacteria being the two most represented proteobacterial classes (7.4 ± 1.5% and 11.7 ± 1.6%, respectively). Abundant phyla also included Planktomycetota (19.9 ± 6.2%) and Acidobacteriota (19.3 ± 3.3%). Creanarcheota and Bacteroidota were also present at relatively high percentages (8.9 ± 3.1 and 3.6 ± 1.0%, respectively).

Prokaryotic community composition resulted to be rather homogeneous at almost all depths and stations, with samples 600A, 900B and 1500A being slightly different from each other. In particular, we observed a peak of Planktomicetota at station 1500m, where their abundance reaches a value of 40%, and a relatively higher percentage of the same phylum at station 900B. Moreover, high relative abundance of Firmicutes (19.4%) was observed at station 600A.

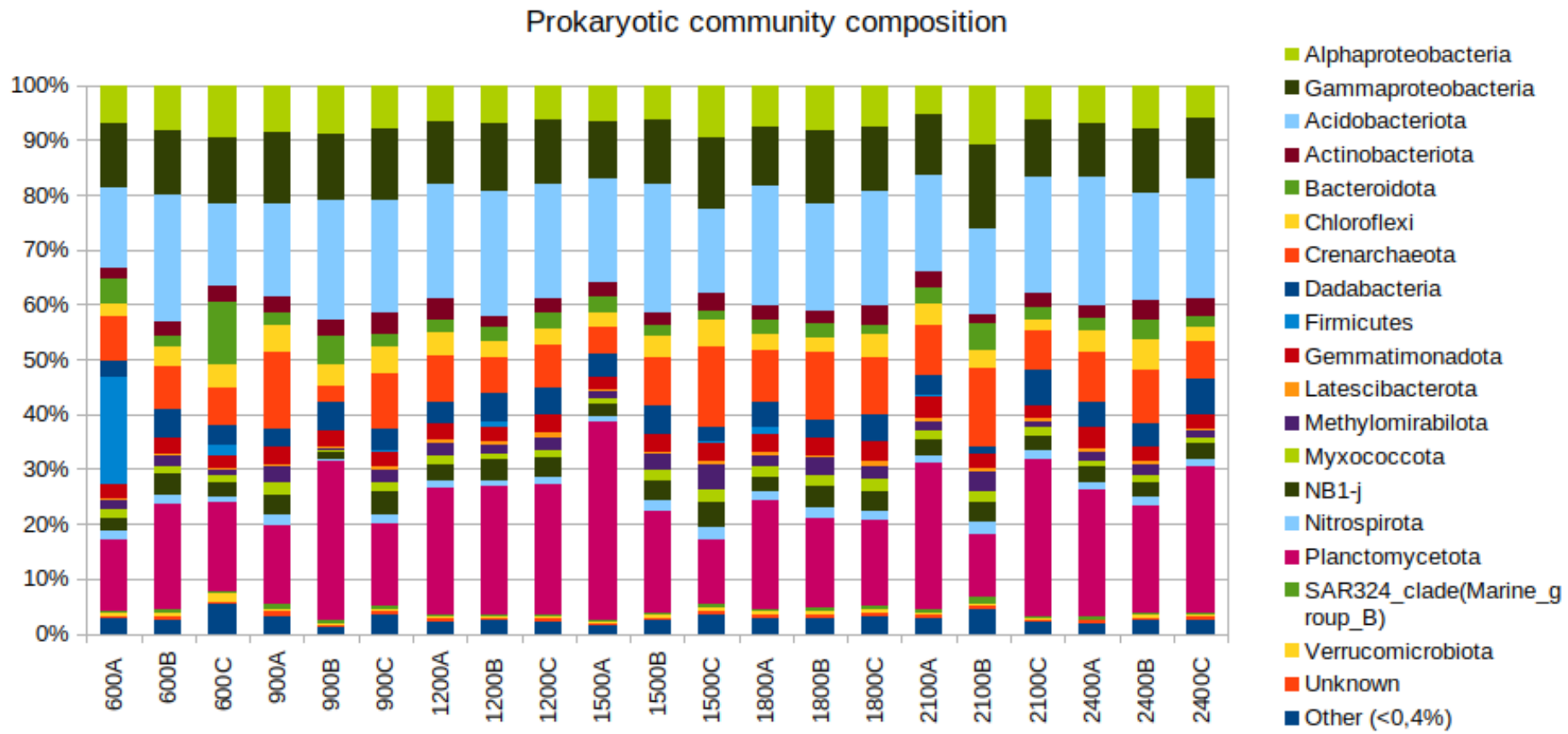

Figure 11: Prokaryotic community composition (as%) at the Phylum level (Class level for Proteobacteria only). Groups with an average relative abundance across all samples <1% were aggregated into the group reported as "Other". "Unknown" includes all those reads that did not match any known taxonomy.

Across the whole dataset, Archaea represented 2.9% (900B) to 16% (2100B) of the total prokaryotic community, with a total of 584 ASVs. Crenarchaeota was the most represented archaeal phylum, and crenarchaeal sequences were consistently found at all stations and depths, ranging from a minimum of 1.6% at station 900B to a maximum of about 14% of the total prokaryotic community at stations 1500B and 2100B (Figure 12). Other represented archaeal phyla were Nanoarchaeota (on average, 3.3% of the only archaeal community, from 0.36% at station 900B to 5.39% at station 1500B) and Thermoplasmatota (on average, 1.98% of the only archaeal community, from 0.4% at station 1500A to 5.5% at station 2100B) (Figure 12).

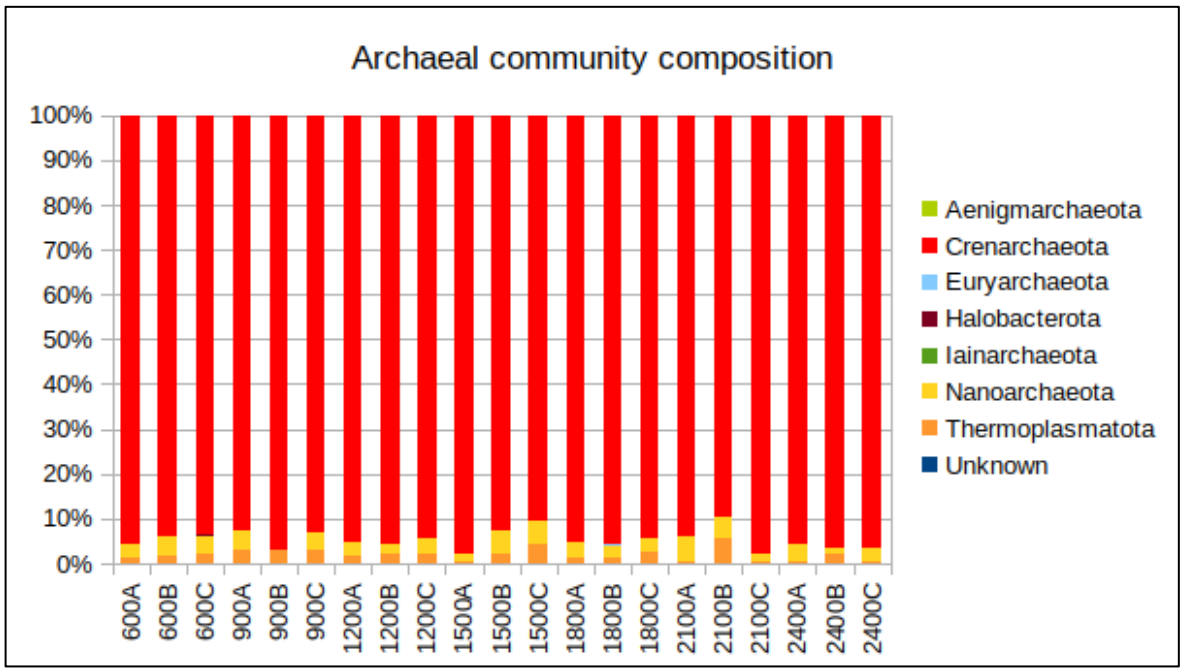

Figura 12: Benthic archaeal community composition at each sampled station.

Archaea to bacteria ratio showed a general increasing pattern with depth up to 2100 m (1.126 ± 0.061), with lowest ratio values recorded at 600 (0.086 ± 0.006) and 1200 m; however, a slight decrease was then observed at 2400 m (0.09 ± 0.019) (Figure 13).

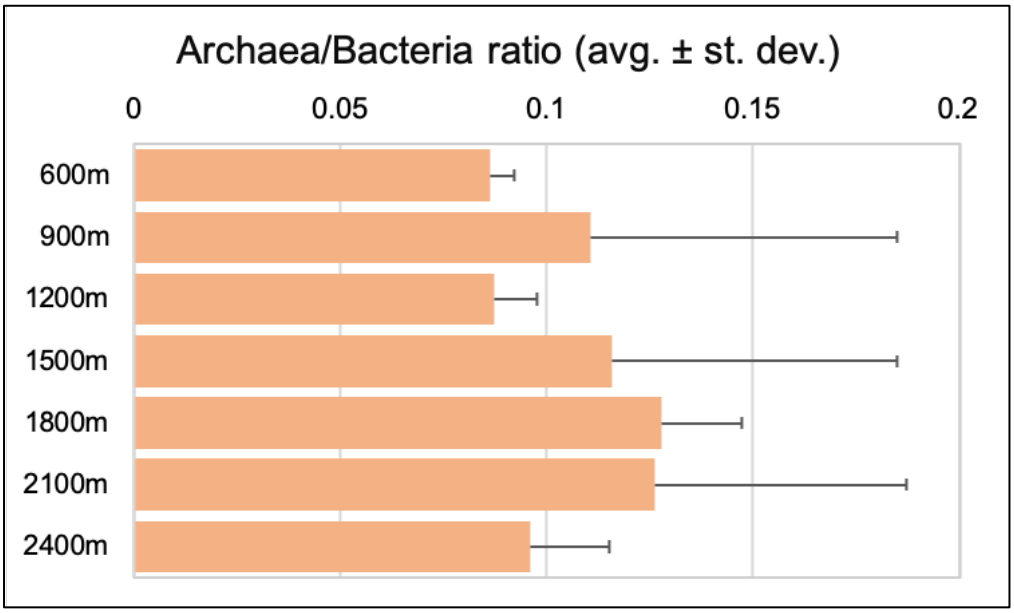

Figure 13: Archaea/Bacteria ratio calculated as the average of ratios among samples at the same depth ± standard deviation.

Multivariate analyses identified temperature, chlorophyll-a and biopolymeric carbon as the only statistically significant variables ($p<0.05$ for temperature and $p<0.1$ for Chl-a and BPC) of the prokaryotic community pattern, and thus used to perform CCA analysis (Figure 14). CCA highlighted that the first two canonical axes explained about 16.5% of the total variance (Figure 14), and that chlorophyll-a and temperature contributed to a separation of samples at 600 m for the rest of samples. Prokaryotic communities below 900 m grouped together and overall driven by the sediment BPC content (Figure 14).

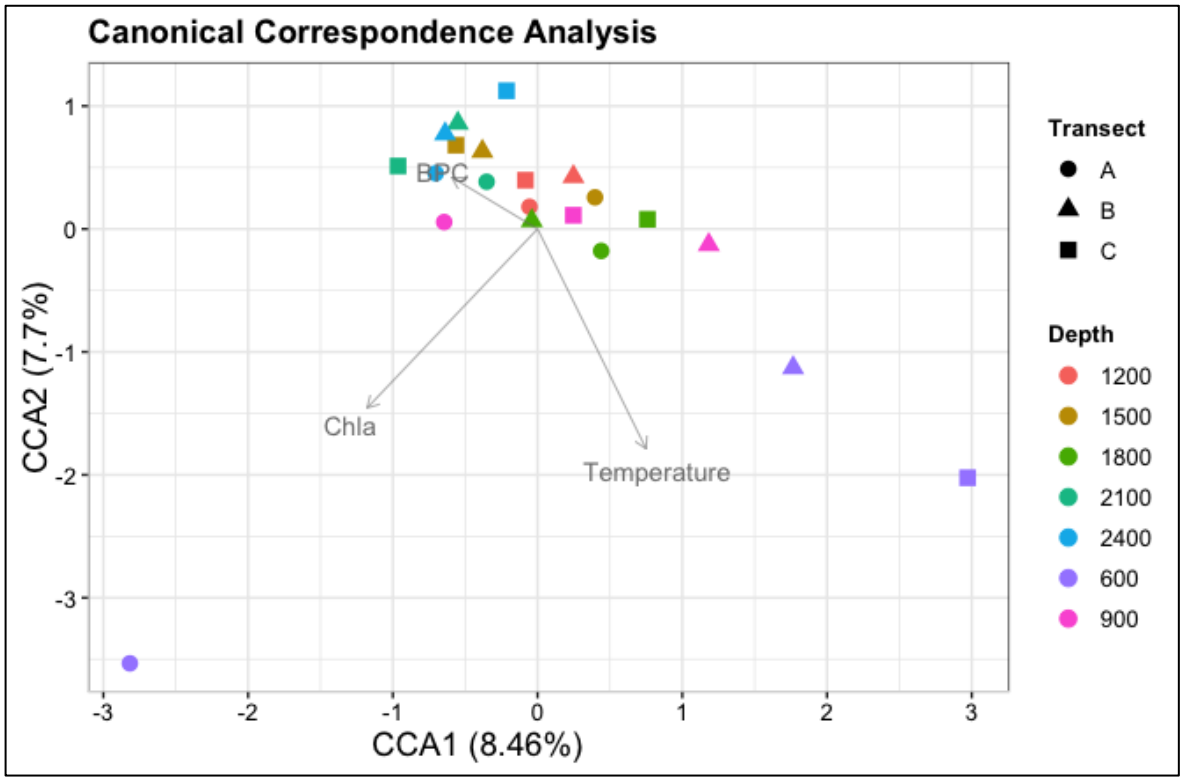

Figure 14: Canonical Correspondence Analysis (CCA) based on the normalized dataset, showing the biplot of environmental parameters resulted to be statistically significant from the *adonis* analysis ($p<0.1$). BPC: biopolymeric carbon; Chl a: chlorophyll a.

Chapter 4 – Discussion

Investigating spatial patterns of abundance and diversity of benthic prokaryotic assemblages in the deep sea and drivers influencing their distribution is of paramount importance for a better understanding of the ecosystem functioning and the potential consequences of climate-induced changes (Gaston & Blackburn 2008; Ferrier et al., 2004). Nevertheless, assessing the diversity of prokaryotes in the deep sea is challenging not only for logistic aspects related to sample collection (Danovaro et al., 2014; Celussi et al., 2020), but also because culturing techniques are not suitable for exploring their huge diversity.

To cope with it, "Next Generation Sequencing" (NGS) or "High-Throughput Sequencing" (HTS) techniques, including metabarcoding (i.e., HTS of target genes, such as the hypervariable regions of the 16S rDNA gene) and metagenomics (i.e., HTS of the genetic material included in a sample) can be used. Thanks to these tools and advanced bioinformatic pipelines, today it's possible to obtain more accurate richness estimates and identify abundant and rare taxa within the assemblages (Quero & Luna 2014; Luna et al., 2016; Celussi et al., 2018; Jia et al., 2018).

Here, for the first time, we investigated the abundance, diversity and assemblage composition of prokaryotic assemblages in the surface sediments of continental margin of the Sardinia.

It is widely recognized that prokaryotic abundance and biomass in the water column exponentially decreased with increasing water depth (Reinthaler et al., 2006; La Ferla et al., 2010). However, this pattern does not apply at benthic systems worldwide where prokaryotic standing stocks remain rather constant with increasing water depth (Rex et al., 2006; Danovaro et al., 2008).

Information available for the deep Mediterranean Sea revealed contrasting results: in some cases, a significant decrease with increasing water depth (Danovaro et al., 2000; Giovannelli et al., 2013) and in other cases no significant changes with increasing water depth (Gambi et al., 2017).

The results presented here indicate a clear depth related pattern of prokaryotic abundance in the Sardinia continental margin. Previous studies reported that primary productivity predictors (that is, chorophyll-a and phytopigments carbon) and water depth explained a large fraction of the variance of the distribution of benthic prokaryotic abundance and biomass in deep-sea sediment worldwide (Wei et al., 2010). Our findings corroborate these finding, highlighting a major role of the availability of fresh deposited organic matter of photosynthetic origin setting on the seafloor (i.e. total phytopigment concentrations) in influencing the distribution of prokaryotic standing stocks along the Sardinia continental margin.

Although a clear depth related pattern was observed, the prokaryotic abundance remained rather constant across stations located at the same depth suggesting rather homogenous environmental conditions of the continental margin at least at the spatial scale investigated in the present study.

The values of alpha-diversity of prokaryotic assemblages (in terms of ASV number) observed in the present study was high and similar to those previously reported in other benthic deep-sea ecosystem worldwide (Danovaro et al., 2010; Lins et al., 2016; Román et al., 2019). No significant changes were observed in ASV number either in relation with water depth or sampling site at the same depth. These results indicate that, conversely to what reported for

prokaryotic abundance, water depth has a minor role in controlling the richness of prokaryotic taxa along the Sardinia continental margin.

In this study, deep benthic prokaryotic assemblages were dominated by Proteobacteria (mainly Alpha- and Gammaproteobacteria), Planktomicetota and Acidobacteriota (for the latter two phyla, this is the reported names as in the Silva reference database). Despite early studies based on fingerprinting techniques have shown that deep benthic bacterial assemblages in the deep sea were widely dominated by (Gamma- and Delta-) Proteobacteria, with Planctomycetes and Acidobacteria being reported only among other common, but not dominant, benthic groups (Ettoumi et al., 2010; Sevastou et al., 2013); (reviewed in Luna, 2015), recent studies based on cloning or HTS approaches have re-evaluated assessed the quantitative relevance of these latter two phyla. In particular, a recent study performed on deep-sea sediments collected in submarine canyons and adjacent slopes of the Western Mediterranean Sea deep-canyons and adjacent slopes by Corinaldesi et al. (2019) showed that deep benthic Bacteria were mainly represented by Gammaproteobacteria, and that Planctomycetacia and Acidimicrobiia classes were also highly represented prokaryotic groups in all samples. Acidobacteria and Planctomycetes were also found to be abundant in surface sediments of submarine canyons from the Eastern Mediterranean Sea (Polymenakou et al., 2009) and in deep sea canyons off the Californian coast and Mexico Gulf, respectively (Goffredi & Orphan, 2010; Crespo-Medina et al., 2016; Harrison et al., 2018). Overall, Gammaproteobacteria, Acidobacteria and Planctomycetes are widespread in the dark ocean; currently, the ability for broad metabolic strategies, including heterotrophy and facultative autotrophy, have been hypothesized for some of

these taxa (Swan et al., 2014; Jasmin et al., 2017; Mußmann et al., 2017; Corinaldesi et al., 2019), suggesting a key role in the ecology of deep benthic deep-sea systems life.

Interestingly, some peculiar features, although not significant, were observed in the present study in terms of taxonomical contribution at station 600A, 600B, 900B and 1200B, where higher relative abundances of Firmicutes (particularly at station 600A) were detected. Firmicutes have been described as only minor components of deep benthic deep-sea prokaryotic communities in studies performed using molecular methods (Polymenakou et al., 2009; Danovaro et al., 2010), whereas they have been extensively reported as dominant in culture-dependent studies on deep sediments, especially in the Mediterranean Sea (Gärtner et al., 2011; da Silva et al., 2013; Polymenakou et al., 2015). In particular, Polymenakou et al. (2015) found that a single station was dominated by Firmicutes (around 24% of microbial sequences), while its average abundance in the remaining stations approached less than 2%. Despite an unclear explanation of such contribution was provided by the authors, has been hypothesized that the ability of members of this phylum in producing endospores and the great metabolic and physiological dversity are likely features facilitating their widespread distribution, and particularly in deep-sea sediments.

In the present study, no significant differences in the prokaryotic assemblage composition were observed neither in relation to water depth nor to sampling location at the same depth. Nevertheless, some of the stations, namely 600C, 900B and 1200B, appeared to be more dissimilar from the other

stations, suggesting that factors occurring at local scales can have an effect on prokaryotic assemblage composition.

In the present study most of the prokaryotic taxa were affiliated to Bacteria, with a minor contribution of Archaeal taxa. The higher relative abundance of Bacteria over Archaea has been repeatedly reported in numerous studies (Luna 2014, and reference therein; Bowman and McCuaig, 2003; Molari et al., 2013; Lipp et al., 2008; Tamburini et al., 2009; Giovannelli et al., 2013). Archaeal assemblages in the benthic deep-sea ecosystem investigated here was largely accounted by Crenarchaeota (about 90% of the total archaeal communities), in agreement with other studies performed on deep-sea sediments (Danovaro et al., 2009; Danovaro et al., 2016; Giovannelli et al., 2013). Crenarchaeota MG-I may have a major role in chemolithoautotrophy in deep-sea environments (Agogué et al., 2008; Yakimov et al., 2011; Agogué et al., 2008; Yakimov et al., 2011; Sintes et al., 2012) so that our findings lead to hypothesize that such component can contribute by inorganic carbon fixation to the overall functioning of the Sardinia continental margin investigated.

Factors influencing prokaryotic assemblage composition in deep-sea sediment are still largely unknown. Multivariate analysis carried out in the present study highlight that temperature, chlorophyll-a and biopolymeric carbon were variables significantly influencing prokaryotic assemblage composition. This can be relevant for a better understanding and prediction of the potential effects (i.e. temperature increase in deep waters and changes of the export of organic C from the surface to the deep interior) on benthic deep-sea prokaryotic assemblages induced by the present global climate changes.

However, the total fraction of the explained variance of the assemblage composition was rather low (ca. 17%), indicating that other abiotic or biotic factors can be relevant in shaping prokaryotic assemblage composition along the Sardinia margin.

Overall results of the present study expand our knowledge on the distribution of the abundance and diversity of prokaryotic assemblages in benthic ecosystems of the deep Mediterranean Sea and provide new insights on potential drivers influencing their distribution, thus contributing to a better comprehension of the ecology of this semi-enclosed system.

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