Planktonic prokaryote and protist communities in a submarine canyon system in the Ligurian Sea (NW Mediterranean)

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Abbreviations:

AMA: Leucine aminopeptidase activity
AP: Alkaline phosphatase activity
BGLU: Beta-glucosidase activity
DIC: Dissolved Inorganic Carbon
DICfd: Dissolved inorganic carbon fixation rate: dissolved fraction
DICfp: Dissolved inorganic carbon fixation rate: particulate fraction
DICftot: Dissolved inorganic carbon fixation rate: total fraction
DOC: Dissolved Organic Carbon
HCP: Heterotrophic Carbon Production
LIP: Lipase activity
LIW: Levantine Intermediate Water
MAW: Modified Atlantic Water
PCD: Prokaryotic Carbon Demand
PCoA: Principal Coordinate Analysis
PN: Particulate Nitrogen
POC: Particulate Organic Carbon
dbRDA: distance-based redundancy analysis

WIW: Winter Intermediate Water

WMDW: Western Mediterranean Deep Water

Abstract

Submarine canyons are large geomorphological features that incise continental margins and act as highly dynamic conduits of sediments form shallow to the deep-sea regions. They are often regarded as biodiversity and biomass hotspots, as a consequence of the channelling mechanisms of organic matter from the continental shelf, but their role in influencing the plankton communities is still poorly known. We studied the ecology of deep-sea planktonic microbes in a submarine canyon system (Polcevera and Bisagno canyons) in the Ligurian Sea (NW Mediterranean Sea), aiming at understanding whether these large-scale incisions act as peculiar systems, with respect to the overlaying water and the adjacent uncarved slope, in terms of biogeochemical and biological features. In particular, we analysed microbial communities' composition by high-throughput sequencing of 16S and 18S rRNA genes and their metabolism (heterotrophic carbon production, dissolved inorganic carbon fixation, respiration and the activity of the exoenzymes leucine aminopeptidase, alkaline phosphatase, beta-glucosidase and lipase). Both prokaryotic and eukaryotic assemblages did not significantly vary inside the canyons, but they were rather shaped by the water masses dynamics in the area. The shallowest Modified Atlantic Waters, Levantine Intermediate Waters and Western Intermediate Waters hosted higher percentages of Alphaproteobacteria, Bacteroidetes and Dinophyta, while the deepest Western Mediterranean Deep Waters hosted more Gammaproteobacteria, Chloroflexi, Discoba and Fungi. Among the tested functional traits, only the activity of the exoenzyme leucine aminopeptidase seemed to respond, with enhanced rates, to a potential 'canyon effect'. However, local hotspots within the canyons characterised by high particulate matter load and high C:N ratio (interpreted as refractory material from sediment local resuspension) displayed pronounced degradation activities.

1. Introduction

The dark portion of the Ocean (i.e. > 200 m depth) represents the largest biome on Earth, comprising about 75% of the whole volume of the planet's seas. Despite this, it still is the least explored set of ecosystems in the planet, mostly due to the challenges in accessing those habitats and the need for high-tech dedicated sampling instruments (Danovaro et al., 2014). In fact, only recently the efforts in studying its ecological dynamics are shedding lights on our gaps in understanding its biogeochemistry (Burd et al., 2010). These gaps were mostly based on the unbalance between the amount of organic carbon reaching the deep sea though the sinking of particulate material from the surface ocean and the estimates of meso- and bathypelagic microbial activities, that could not be fuelled only by export (Burd et al., 2010). The discovery of pronounced bicarbonate fixation rates (e.g. Herndl et al., 2005; Celussi et al., 2017) and of slow sinking or buoyant organic particles (feeding heterotrophic metabolism; Baltar et al., 2009) are now at the base of researches on microbial dynamics in the deep water column. Although it could be regarded as a homogeneous environment, several features determine the dark ocean ecological inner variability. Among these, depth-related pressure gradients (Moesender et al., 2001; Celussi et al., 2009), deep water masses dynamics (i.e. oxygenation, advection and convection phenomena) (Celussi et al., 2010; Wilkins et al., 2013; Luna et al., 2016) and the variability in vertical fluxes of particulate organic carbon sinking to the bottom (Azam and Long, 2001) have the potential to structure microbial communities and their functioning.

The morphological features of the seafloor, that clearly determine the ecology of marine sediments (Zeppilli et al., 2016), are expected to have an impact on the overlying water column, especially in its deepest layers. For example, seamounts can alter the local hydrological features, by conveying deep water to shallower depth, potentially resulting in an upwelling of nutrients, or by modifying the fluxes of allochthonous material (retention mechanisms) (Clark et al., 2010 and references therein). Submarine canyons, incising the continental margins, generally act as major conduits of sediments and suspended material from shallow to deep sea regions (Puig et al., 2014), although up-canyon transport is not uncommon (Amaro et al., 2016). A great variability characterizes the ecology of these structures. They are often regarded as biodiversity and biomass hotspots, as a consequence of the channelling mechanisms of organic matter from the shelf (Polymenakou et al., 2008; Fernandez-Arcaya et al., 2017) and their marked seafloor heterogeneity (Pierdomenico et al., 2017). Nevertheless, even though several studies highlighted this aspect (Polymenakou et al., 2008 and references therein), there is still a general lack of consensus in the scientific literature (Amaro et al., 2016).

If these facets appear controversial when considering the canyon benthic systems, the knowledge on the 'canyon effect' on the overlying water column biota is even sparser. Canyons are prominent topographic features that modify the coastal circulation, and the intensification of both coast to ocean and vertical water transport within submarine canyons is expected to affect the dynamics of plankton ecosystems in the vicinity of canyons (Guerreiro et al. 2014). In two independent modelling studies, Skliris and Djenidi (2006) and Muñoz et al. (2017) showed that the upwelling of deep water rich in nutrients, channelled into canyons in the NW Mediterranean, can enhance photosynthetic primary production at surface. Gili et al. (1999) reported three undescribed species of Hydroidomedusae in sediment traps placed in the Foix submarine canyon (Catalan coast), and postulated the presence of an unusually prolific plankton community in the canyon, probably supported by the continuous flux of organic matter from the continental shelf. Guerreiro et al. (2014) reported local hotspots of coccolithophore and phytoplankton biomass in the Nazarè canyon (central Portuguese margin), potentially associated with perturbations of surface water circulation by the canyon. Misic and Fabiano (2006), while examining a deep-canyon system in the Ligurian Sea, found that microbial communities inside these structures display enhanced organic matter degradation rates, as a consequence of irregular resuspension of organic and inorganic materials within the bottom sediments next to canyon heads. As a matter of fact, the rapid and episodic flushing of canyons complicates the understanding of microbial communities' dynamics in their interior, and the question whether they can be considered as confined environments or fully embedded with the surrounding systems is still unanswered. This might depend on the morphological features of canyons, on the level of seawater mixing with upper-layers, and on their interplay with the local circulation system.

In this context, we sampled seawater from several stations located inside two submarine canyons in the Ligurian Sea (NW Mediterranean Sea), as well as in the adjacent continental slope. Our hypothesis was that the Bisagno and the Polcevera canyons act as peculiar systems, with respect to the overlaying water and the adjacent uncarved slope, in terms of biogeochemical and biological features. Since the main circulation in the area is perpendicular to the main axis of the canyons (Barth et al., 2005), the mixing with the overlaying water should be limited, and thus the effect of episodic flushing can be retained into the canyons (i.e. higher loads of organic matter and nutrients). We tested the potential canyon effect on planktonic microbial features, by investigating microbial diversity (by high-throughput sequencing of 16S and 18S rRNA genes of prokaryote and protist communities, respectively) and activities (heterotrophic carbon production, dissolved inorganic carbon fixation, respiration and the microbial degradation potential of polysaccharides, lipids, polypeptides and phosphorylated organic compounds).

2. Methods

2.1 Study area

The Ligurian Sea is characterized by a general cyclonic circulation involving both the surface layer of Modified Atlantic Water (MAW) and the Levantine Intermediate Water (LIW) layer below. The system is generally described as oligotrophic. However, a permanent hydrological front leads to central upwelling and peripheral downwelling of water masses. In the former case, inorganic nutrients from LIW are provided to surface waters, stimulating primary production; in the latter, the freshly produced organic matter is allowed to sink, enriching the whole water column (Misic and Fabiano, 2006). Winter Intermediate Water (WIW) is a cooled and mixed MAW that reaches the buoyancy equilibrium between the MAW and the LIW layer, structuring into defined low-temperature thermohaline lenses (Gasparini et al., 1999). The bottom layer (> 1000 m) is generally occupied by Western Mediterranean Deep Water (WMDW), a dense water mass generated in the Gulf of Lions during winter (Barth et al., 2005).

The seafloor topography of the northern part of the Ligurian Sea presents a complex canyon system that incise the continental margin. Among the several canyons in the area, the Polcevera and the Bisagno are oriented in a NE-SW axis, present very steep slopes from the shelf break to about 1,500 m and coalesce in the Genoa Valley at about 2,000 m (Soulet et al., 2016).

2.2 Sampling and physico-chemical analyses

Fourteen stations in the Ligurian Sea (Fig. 1) were sampled between May 1st and May 6th 2013 (details on sampling stations are reported in Table S2). In the sampling design three transects were chosen: one in correspondence to the Bisagno canyon, one over the Polcevera canyon and one above the adjacent continental slope. Sampling stations were located according to the bottom depth in each transect, i.e. 200, 500, 1,000, 1,500 and, in the Bisagno and slope transects, also at 2,000 m. One further station (bottom depth 2,500 m) was located where the two canyons coalesce into the Genova Valley (Soulet et al., 2016). Seawater temperature, salinity and dissolved oxygen profiles were obtained by means of a SBE 9 Multiprobe. Samples were collected at depths of ca. 200, 500, 1,000, 1,500, 2,000 and 2,500 m with 12-L Niskin bottles mounted on a Rosette sampler.

Inorganic macronutrients, dissolved organic C (DOC) and particulate organic matter (C = POC; N = PN) concentrations were determined by standard procedures (Koroleff and Grasshof, 1983; Cauwet, 1994; Pella and Colombo, 1973). Detailed protocols are provided in the Supplementary Materials.

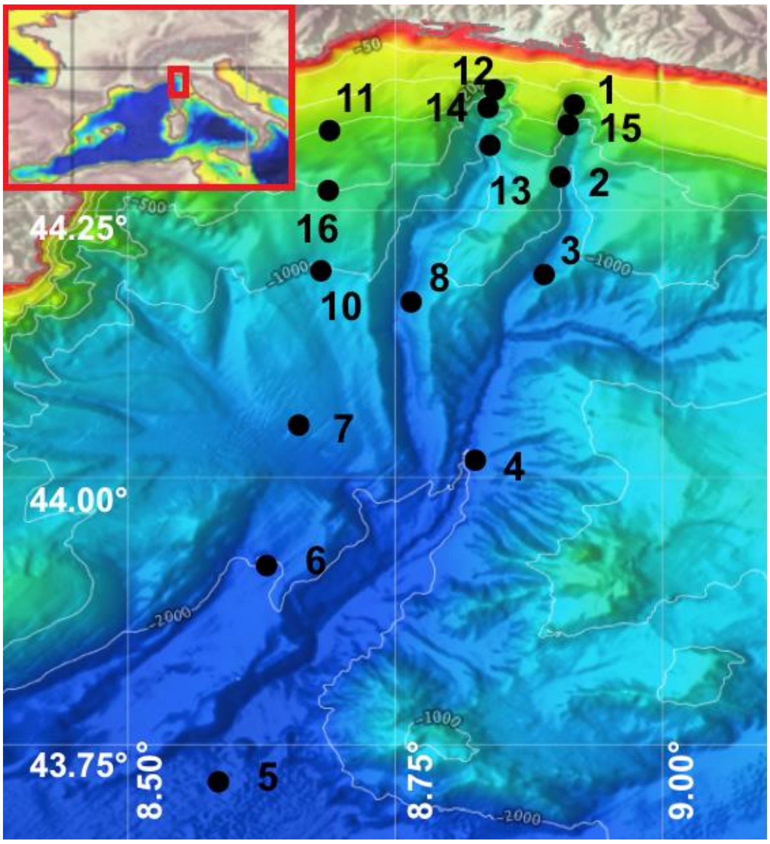

Fig. 1. Sampling stations in the Ligurian Sea (NW Mediterranean Sea). The stations are divided into three transect as follows: Slope stations 11 - 16 - 10 - 7 - 6 - 5; Polcevera canyon stations 12 - 14 - 13 - 8; Bisagno canyon stations 1 - 15 - 2 - 3 - 4. Latitude coordinates are in °N; longitude coordinates are in °E. The image has been created through the bathymetry web portal http://portal.emodnet-bathymetry.eu/. The sampling area is indicated by a red rectangle in the upper panel that shows the entire Western Mediterranean Sea.

2.3 Molecular analyses

Four and twenty L of seawater were collected at selected depth for prokaryotic and protist DNA analyses, respectively. Prokaryotic samples were directly filtered (≤ 100 mmHg) with a peristaltic pump onto 0.22 µm Cellulose Nitrate filters (Sartorius) while protist samples were pre-filtered through a 200 µm mesh to remove larger zooplankton and then filtered onto 2 µm pore size PCTE membrane (Sterlitech). All DNA samples were placed in sterile vials and frozen at -20 °C until molecular analyses.

2.3.1 DNA extraction and PCR amplification

Prokaryotic DNA was extracted from each filter using the PowerWater DNA Isolation Kit (MoBio Laboratories, California). We followed the manufacturer’s instructions with few modifications to increase the DNA yield and quality (see Supplementary Materials). The prokaryotic 16S rRNA gene was amplified using the universal prokaryotic primers 926F/1392R targeting the V6–V8 hypervariable regions of bacterial and archaeal 16S rRNA genes (926F: 5′-AAACTYAAAKGAATTGRCGG-3′ and 1392R: 5′-

ACGGGCGGTGTGTRC-3′) (Wilkins et al., 2013; Severin et al., 2016) . The amplicons were then sequenced by tag pyrosequencing, that was performed by Research and Testing Laboratory (Lubbock, USA) on a GS FLX+ platform (Roche, Branford, USA).

Protists DNA extraction and molecular analysis were performed as described in Zoccarato et al. (2016a). Briefly, DNA was extracted using PowerSoil DNA Isolation kit (MoBio) dissolving the membranes in chloroform and the 18S rDNA hypervariable region 9 (V9) was amplified using the pair pair 1391F (50-GTACACACCGCCCGTC-30) and EukB (50- TGATCCTTCTGCAGGTTCACCTAC-30) (Stoeck et al., 2010). For 3 random samples the extraction, amplification and sequencing steps were carried out in triplicate.

2.3.2 Amplicon Sequencing Data Analysis

Raw sequences were pre-cleaned with Cutadapt v1.15 (Martin, 2011) clipping synthetic primers and sequencing adapters (matching error-tolerance of 1 error each 10 nucleotides) and removing sequences shorter that 430 bp and 100 bp for prokaryotic and protist datasets, respectively. Cleaned sequences were deposited in the European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena) under the accession numbers ###. All fastq files were imported in R and analysed with the workflow of the package DADA2 (Callahan et al., 2016) processing prokaryote and protist datasets independently. Sequences' quality was manually inspected and a maximum length cutoff was set to 490 bp and 135 bp for prokaryotes and protists, respectively. After truncation, sequences with an expected error higher than 2 nucleotides per 100 bp were discarded. Samples were polled to estimate sequencing error rates and the convergence of the parametric error model was achieved using a random subset of 1 M sequences. The sequence-variant (oligotype) inference was performed on the dereplicated sequences after pooling of all samples together to reduce possible biases due to low sampling depths. Prokaryotic taxonomy was assigned using SINA on-line aligner v1.2.11 (Pruesse et al., 2012) with a similarity thresholds of 85% and Silva classification was kept for downstream analyses. Protist taxonomy was determined performing a local alignment of the oligotypes against the PR2 reference database v4.7.2 (Guillou et al., 2013) by means of the function glsearch36 (FASTA suite available at http://faculty.virginia.edu/wrpearson/fasta/CURRENT/) (see Supplementary Materials for details).

2.4 Microbial activities

Heterotrophic carbon production (HCP) was determined with the ^{3}H-leucine method introduced by Kirchman et al. (1985). The activity in the samples was determined using a TRI-CARB 2900 TR Liquid Scintillation Analyzer, after following the microcentrifugation procedure described by Smith and Azam (1992). Carbon biomass production was estimated using the conversion factor 1.55 kgC mol^{-1} Leu assuming no isotope dilution (Kirchman and Ducklow, 1993).

Dark inorganic carbon fixation was estimated by following the uptake of $NaH^{14}CO_3$ (Herndl et al., 2005) by microbes collected at the deepest layer at each station. The amount of bicarbonate (per unit of volume and time) transformed into organic carbon was estimated both on the particulate and on the dissolved fraction, as detailed by Celussi et al (2017).

Respiration rates were calculated after the estimation of the activity of the electron transport system (ETS) in the deepest samples collected at each station, following the method by Packard and Williams (1981).

Exoenzymatic activities (beta-glucosidase activity = BGLU; alkaline phosphatase activity = AP; lipase activity = LIP; leucine aminopeptidase activity = AMA) were estimated by following the artificial fluorogenic substrate analogue method by Hoppe (1993), after evaluating the substrate saturating concentration in meso- and bathypelagic waters.

All analytical procedures are described in detail in the Supplementary Materials.

2.5 Statistical analyses

The Shapiro-Wilk test was used for assessing the normality of the datasets.

The evaluation of a potential ‘canyon effect’ (high concentration of nutrients and organic C - and response of the activities - due to canyon flushing, sediment resuspension, and limited mixing with the overlaying water layers) on biogeochemical variables and microbial activities was checked by means of the Wilcoxon matched pair test, as detailed in the Supplementary Materials.

Alpha-diversity was estimated calculating Species richness, Simpson’s diversity index and Pielou’s equitability index by mean of *specnumber* and *diversity* commands (package Vegan; Oksanen et al., 2008). For beta-diversity analyses, the differences in the sequencing depth among the samples were minimized by subsampling each sample with the *rrarefy* command (package Vegan) at 2,524 and 2,527 reads for prokaryotes and protists, respectively. The oligotype abundance values were log transformed to diminish the effect of the most abundant oligotypes and then Hellinger transformed (*decostand* function, package Vegan). The PCoAs were computed with the function *ordinate* (package Phyloseq; Oksanen et al., 2008) on the Bray-Curtis dissimilarity matrices (*vegdist* function, package Vegan).

We further explored the environmental drivers of the prokaryotic and protist community structures by using the distance-based redundancy analysis (dbRDA; *capscale* function, package Vegan). Before running the analysis, the environmental variables (depth, temperature, salinity, dissolved oxygen, ammonium, nitrate, phosphate, POC, PN and DOC concentrations) were normalized making the sum of squares equal to one. The ANOVA test was used to identity the best set of explanatory variables ($p < 0.05$).

The Indicator Species analysis (*multipatt* function, package indicspecies; Cáceres et al., 2010) was performed to identify sets of oligotypes significantly correlated with the grouping variable of interest. The results were visualized as networks by mean of the igraph R package (Csárdi and Nepusz, 2006). All the aforementioned analyses were performed using R version 3.4.3 (R Core Team, 2017).

3. Results and discussion

3.1 Environmental setting

The samples harvested for this study were collected inside four different water masses defined as follows: Western Mediterranean Deep Waters with a $\sigma_0 > 29.1$ kg m^{-3}, at depth ≥ 840 m; Levantine Intermediate Water (LIW) with σ_0 between 29.03 and 29.10 kg m^{-3} and relative θ and salinity maxima along the profile; Winter Intermediate Water (WIW) characterised by relative minima of θ (13.17 - 13.27 °C) and salinity (38.35 - 38.46), between 230 and 500 m; Modified Atlantic Water (MAW) that were warmer (θ = 13.43 - 13.52°C) and were located between 200 and 300 m. Unexpectedly, LIW, whose circulation was hypothesised to skip the canyons' interior, were found inside these submarine structures, at stations 2, 13, 14 and 15, around 500 m depth.

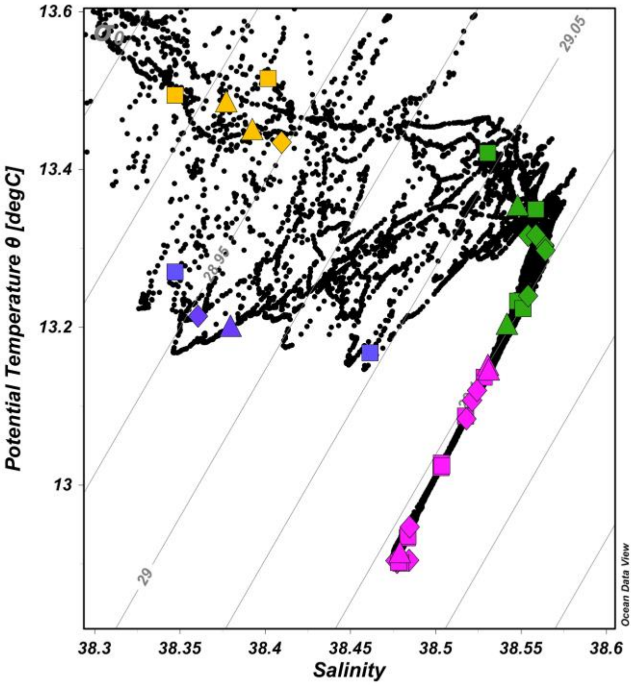

Fig. 2: θ-S diagram showing the thermohaline features of the sampling stations in the Ligurian Sea. Symbols refer to the sampling transect (diamond = Slope; triangle = Polcevera; square = Bisagno). Colors denote the different water masses (orange = MAW; blue = WIW; green = LIW; purple = WMDW).

The main biogeochemical features of the sampled area were typical for the deep Western Mediterranean Sea (Cauwet et al., 1997; Béthoux et al., 1998) displaying the following ranges: ammonia 0.29 - 1.73 µM, nitrate 0.03 - 9.61 µM, orthophosphate 0.05 - 0.45µM, dissolved organic carbon (DOC) 38.83 - 91-58 µM, particulate organic carbon (POC) 0.80 - 4.31 µM and Particulate Nitrogen (PN) 0.06 - 0.40 µM (Table S2; Fig. 3). The Wilcoxon matched pair test used to highlight significant differences between samples collected in canyons and those collected in the close slope transect did not show any significantly higher concentration of nutrients and organic carbon, inside the canyons. Therefore, we infer that the presence of waters affected by a recent canyon flushing phenomenon was unlikely. In fact, over the Polcevera and Bisagno river basins, in the period preceding our survey (January - April 2013) no major rainfall event had been occurring, even though the registered precipitation was slightly higher (ca. 550 mm) than climatological data (ca. 430 mm in the same months from 1961 to 2010; data from the Ligurian Environmental Protection Agency www.arpal.gov.it). Although not statistically significant, an accumulation of POC was observed at the bottom layer of both canyons (mean of all samples at the bottom depth in the Bisagno = 2.81 μM, in the Polcevera = 1.67 μM, in the slope transect = 1.16 μM; Fig. 3). The C:N ratio in particulate matter displayed an increasing trend along with depth, being on average = 9 (± 2, SD) between 200 and 500 m and = 12 (± 4, SD) below the 500 m isobath. In the bottom layer, C:N reached the highest values of 19, 15 and 24 in the Slope (sample collected within the Genoa Valley), the Polcevera and the Bisagno transects, respectively.

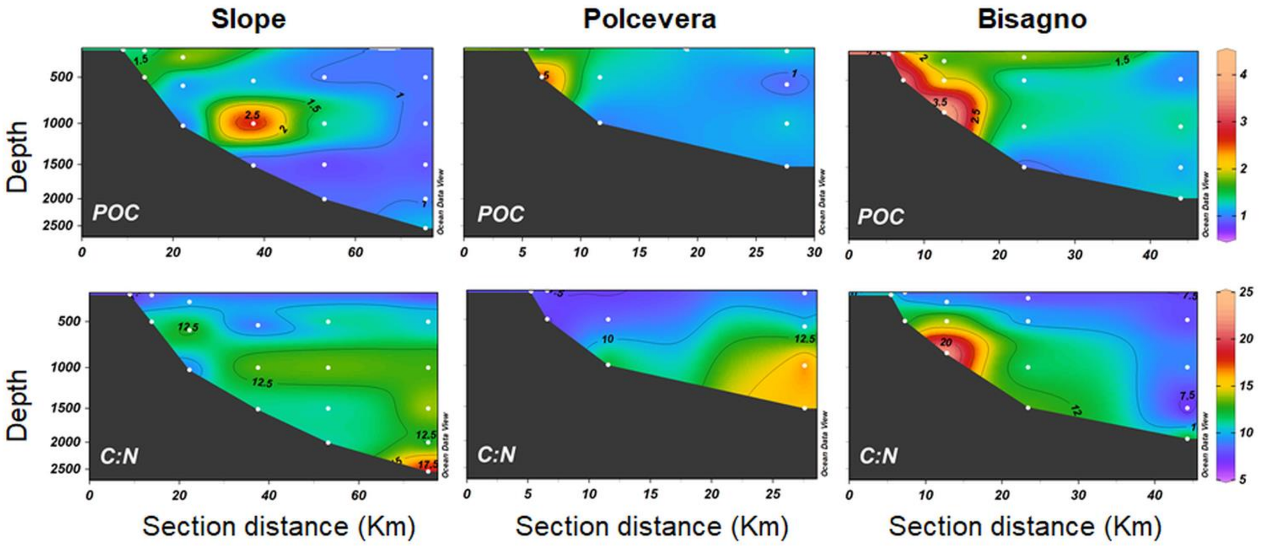

Fig. 3. Depth profiles of Particulate Organic Carbon (POC) concentration and C:N molar ratio in the particulate matter along the three transects in the Ligurian Sea. POC values are given in μM. The Y-axis is not linear (stretched to top) and depth is reported in m in the range 200 – 2,750. The image was drawn by means of the ODV software (Schlitzer, 2014)

3.2 Microbial diversity

In order to highlight a potential 'canyon effect' on microbial communities, two separate ordinations were performed for prokaryotes and protists. The PCoA analyses showed that prokaryotes did not segregate into in-canyon and out-canyon communities (one-way ANOSIM not significant) but they rather clustered according to the different water masses where they were collected (Fig. S1A). The analysis captured 41.8% of the total variation of prokaryotic community composition in the investigated stations. The water mass partitioning effect is statistically supported by the ANOSIM analysis (one-way ANOSIM; R = 0.56, $p < 0.001$) and the *post-hoc* pairwise test showed significant dissimilarities between WMDW and the other water masses (MAW, WIW, LIW) (all R > 0.58 and $p < 0.05$; Table S3). Alpha diversity estimates (Table S4) calculated per 'water mass' indicated that the most diverse was WMDW (804 ± 171, SD) followed by WIW

(714 ± 155, SD), LIW (682 ± 180, SD) and MAW (584 ± 46, SD), suggesting a general trend of increasing richness with increasing water depth, as already suggested by other authors (Severin et al., 2016; Mestre et al., 2017). The same analysis revealed that evenness was highest in LIW (0.856 ± 0.02, SD) followed by MAW (0.829 ± 0.03, SD), WMDW (0.821 ± 0.06, SD) and WIW (0.815 ± 0.01, SD).

A similar pattern emerged from the PCoA applied to protist communities (Fig. S1B), where the ANOSIM test for the 'canyon effect' was not significant, as opposed to the ANOSIM test for the 'water mass' effect (one-way ANOSIM; $R = 0.70$, $p < 0.001$). The *post-hoc* pairwise test revealed that all the water masses harboured communities that are significantly dissimilar among each other (all $R > 0.42$ and $p < 0.05$; Table S3), with the only exception being the pair LIW-MAW for which no significant dissimilarity was detected. Alpha diversity estimates for protists (Table S4) indicated that richness was higher in LIW (883 ± 112, SD), followed by MAW (847 ± 315, SD), WMDW (684 ± 250, SD) and WIW (621 ± 194, SD). Basing on the Simpson index, the protist diversity was highest in the MAW (0.985 ± 0.006, SD) followed by LIW (0.980 ± 0.006, SD), WIW (0.972 ± 0.005, SD) and WMDW (0.961 ± 0.032, SD).

To investigate the role of the environmental variables in shaping microbial communities' composition, we performed dbRDA analyses for prokaryotes and protists, constrained with those variables found to be significant for prokaryote and protist communities. The environmental drivers of the prokaryotic and protist community structure are shown in Figure 4 (panel A and B, respectively). Salinity and depth were found to be the most significantly correlated variables with the prokaryotes (Fig. 4A; $p < 0.05$ and $p < 0.001$, respectively), while temperature, salinity and depth was the set of environmental variables that best explained variations in the protist communities' structure (Fig. 4B; $p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively). The dbRDA ordinations showed that, for both prokaryotes and protists, the environmental parameters grouped the samples into separate clusters, where WMDW samples appeared to be separated from the other water masses. The outcomes of this analysis confirm and strengthen the ANOSIM results, since that best explanatory variables are the descriptors of water masses themselves and the concentrations of organic and inorganic nutrients appear to be a less important constraining factors in our dataset. Previous studies in deep pelagic environments have reported that prokaryotic communities are strongly structured by depth (Mestre et al., 2017) and by water masses (Agogué et al., 2011), suggesting that deep-water masses act as "bio-oceanographic islands" for prokaryotic plankton, and that dark ocean prokaryotic community composition is primed by the formation and the horizontal transport of water masses (Frank et al., 2016). Indeed, the same controlling mechanisms have recently been described also for deep sea protists (Pernice et al., 2015; Zoccarato et al., 2016b).

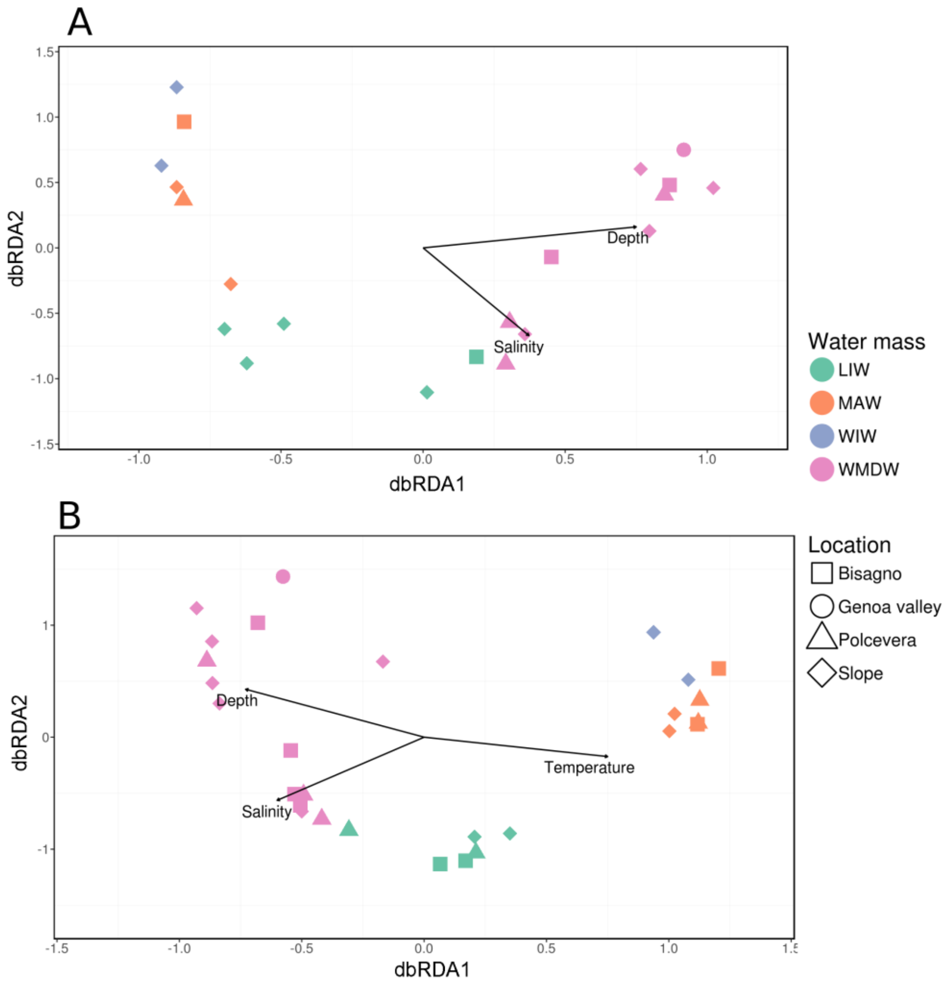

Fig. 4: Relationships between environmental variables and (A) prokaryotic and (B) protist community composition. For prokaryotes, the dbRDA axes explain a cumulative 33.5% of the variance in community structure. For protists, the dbRDA axis explains a cumulative 26.8 % of the variance in community structure.

The analysis of community composition for prokaryotes revealed the cumulative number of 1,630 prokaryotic oligotypes across all stations. Communities were dominated by Bacteria (range 86.8-99.6%) rather than by Archaea (range 0.08-12.1%). At the phylum level, prokaryoplankton was overall dominated by Proteobacteria (classes Alpha- and Gammaproteobacteria), Actinobacteria and Bacteroidetes phyla, accounting cumulatively for an average 77% of the sequences within each water sample (Figure S2A). Overall, an increasing trend in the relative abundance of Chloroflexi and Planctomycetes with depth was observed. A similar pattern was observed for Thaumarchaeota, a trend that has been previously reported for Archaea in the Mediterranean Sea (Korlević et al., 2015). At the order level (Figure S2C), the most abundant prokaryotic orders were SAR11 clade (range 9.3 – 46.2%) and Alteromonadales (average 14.9%, with a peak of 60.04% at station 8 at 1,000 mt), despite these latter were not ubiquitous in our dataset. SAR86 clade, Flavobacteriales and Thiomicrospirales showed decreasing relative abundance when moving toward the deepest stations, while SAR202 showed an opposite trend. Unassigned oligotypes, as well as those oligotypes that never exceeded 1 % of relative abundance in any sample (cumulatively grouped as “Others”), ranged from 19.6 to 45.7%.

The Indicator Species analysis provided a further insight into the distribution of unique and shared oligotypes among different water masses (Fig. 5A). WMDW and WIW harboured the highest sets of unique oligotypes (n=26 and n=14, respectively) (Fig. 5A). WIW unique oligotypes mainly belonged to SAR11 clade while, in the WMDW, the unique oligotypes belonged mainly to the Alteromonadales order (n=3), and to the SAR202 (n=4) and SAR11 (n=2) clades (Fig. 5C). A different pattern emerged for LIW and MAW which, on the other hand, harboured almost no unique oligotypes (only n=1 and n=3, respectively), that belonged to Nitriliruptorales (within the group ‘Others’) for LIW, and Fibrobacterales, Opitutales and one unclassified for MAWD. These two water masses showed high numbers of oligotypes shared with the other water masses: MAW shared 38 oligotypes with WIW (mostly Flavobacteriales and SAR11, accounting for 27.3 % of nodes; Fig. 5C), and 154 oligotypes with WIW and LIW together (Flavobacteriales, SAR11 and SAR86, accounting for 58.4 % of nodes). LIW, in addition to those oligotypes shared with MAW and WIW, showed another set of shared oligotypes with the WMDW (n=27 with 46.2 % of SAR11).

Similarly to our findings, Severin et al. (2016) reported, in the NW Western Mediterranean Sea, the dominance of Alphaproteobacteria, and especially SAR11, in different water masses (including LIW and WMDW) collected down to 2,000 mt. Other studies in the Mediterranean Sea similarly reported that members of the alphaproteobacterial clade SAR11 dominated the communities over a one-year study period (Korlevic et al., 2015). However, contrariously to this study, we observed a high percentage of Alteromonadales (mostly represented by members of the genera *Pseudoalteromonas* and *Alteromonas*) in some stations, with peaks up to 60.1% (at station 8 at 1,000 m), which were not observed in the study by Severin et al. (2016). This high percentage of Alteromonadales in the deep sea is not unexpected, since several studies, carried out using different techniques, have reported that members of the genus *Alteromonas* are typically abundant in the deep sea (Fuhrman and Davis, 1997; Baker et al., 2013; Lekunberri et al., 2013). In our dataset, the relative abundance of *Alteromonadales* was significantly and positively related with the concentration of DOC (r = 0.5872, $p < 0.01$), suggesting that the availability of dissolved organic resources is a key determinant for these bacteria in the deep sea.

The analysis of community composition for protists detected an overall number of 2,746 oligotypes. The communities were dominated by Dinophyta, Radiolaria and Discobia (Fig. S2B) that accounted together from 52.4% to 86.9% of sequences in each sample. As general trend, Dinophyta showed a decrease in relative abundance with depth, while Radiolaria and Discobia displayed an opposite trend. Other relevant groups were represented by Apicomplexa and Stramenopiles (accounting on average for 6.1% and 13.4% in samples above 300 m and 500 m, respectively), while were nearly absent in the deeper samples (average relative abundance of 2.0% and 5.8%, respectively). Fungi were nearly absent above 800 m, but represented on average 10.8% of sequences in deeper samples, with peaks of 27.3% and 33.3% at station 2 (843 m) and station 4 (1,945 m). At the class level (Fig. S2D) most of the phyla/division described above were predominantly represented by few classes: Dino-Group-I & II and unclassified Dinophyceae for Dinophyta; Collodaria, unclassified RAD-A and Spumellarida for Radiolaria; Diplonemea for Discobia, Gregarines for Apicomplexa, Pirsonia and Bacillariophyta unclassified for the Stramenopiles and Pezizomycotina (Ascomicota) for Fungi.

The Indicator Species analysis showed that each water mass harboured a set of unique oligotypes (n = 43 for WIW, n = 41 for MAW, n = 12 for WDMW and n = 35 for LIW) (Fig. 5B) although there were 3 large sets of oligotypes that were shared between WIW and MAW (n = 97), between MAW and LIW (n = 28), and between LIW and WDMW (n = 47). Also, there was a prominent set of oligotypes shared among MAW-WIW-LIW (n = 75). The overlay of the taxonomic composition (Fig. 5D) revealed that the unique and shared oligotypes correlated to the WDMW mainly belonged to the order Diplonemea (59.7 % of nodes); the LIW was principally correlated with Diplonemea, Dino-Group-I & II (52.0 % of nodes) while MAW and WIW were correlated to Dino-Group-I & II, unclassified Dinophyceae and Bacillariophyta unclassified (57.3 % and 50.2 % of nodes, respectively). The taxonomic signature of this latter group is characterised by the (potential) phototrophic nature of these taxa. Since WIW are formed after the winter cooling of MAW, it is not surprising that even though they are relatively deep (230 - 500m), they keep their surface-water biological signatures while spreading south-westward, to the central Western Mediterranean (Gasparini et al., 1999), as previously observed in the evolution of other dense water masses (Luna et al., 2016; Zoccarato et al., 2016b).

The major phyla / divisions of protists that emerged in this study are those typical of meso- and bathypelagic zones (Pernice et al., 2015; Zoccarato et al, 2016b). The group Dinophyta dominated all communities above 500 m being a major player in the communities of MAW and WIW and to a minor extent also in the LIW. Dinophyta relative abundance decreased progressively with increasing depth while other groups such as Discobia and Fungi took over the deep communities of LIW and, especially, of WDMW. The taxon Dinophyta was mainly composed by the orders Dino-Group-I & II, which are know to make up the majority of dinoflagellates sequences in marine environments and which can play important ecological roles as parasites of fish and ciliates (Group-I; Guillou et al., 2008) and as parasites of other dinoflagellates (Group-II; Siano et al., 2011). Dinophyta also included an important fraction of unclassified Dinophyceae, however any ecological inference at this taxonomic level would be worthless due to the extreme heterogeneity of lifestyles found within this taxon (Le Bescot et al., 2016). The decreased abundance of Dinophyta with depth might thus be connected with the decreasing availability of suitable hosts as the abundance and biomass of the microzooplankton (which includes ciliates and dinoflagellates) was also reported to decrease with depth in this study area (Diociaiuti et al., this issue, submitted). The increasing trend of Discobia it is a likely adaptation of the protist community to one of the still available source of food, bacteria. Indeed, Discobia in our data are highly represented by the order Diplonemea, heterotrophic flagellates which include several bacterivorous organisms and are usually found in deep waters (Lara et al., 2009). Fungal sequences have already been reported to be abundant in global oceans (Pernice et al., 2015) and, although their ecological role in marine ecosystem hasn't been fully elucidated, Fungi are known for their capability of degrading recalcitrant organic matter (Clipson et al., 2006), potentially playing key roles within the microbial loop (Grossart and Rojas-Jimenez, 2016); in a recent study Bochdansky et al. (2017) showed how Fungi can equal bacterial biomass on marine snow (i.e. POC) suggesting that their ecology in deep sea can no longer be neglected. The dominance of the Fungi, especially at the bottom of station 2 and 4 (LIW and WDMW, respectively), has therefore intriguing implications because they could boost the microbial communities participating in the turnover of recalcitrant organic matter in the dissolved or particulate phase. In the present study, the most represented clade of Fungi is Pezizomycotina which consists of marine filamentous Fungi (Jebaraj et al., 2012). The most abundant oligotype within this order scored 100% of identity with several reference sequences of the different fungal genus (i.e. *Capronia*, *Fonsecaea*, *Phialophora*, *Cladophialophora*, *Exophiala*) capable of degrading recalcitrant and complex molecules such as polycyclic hydrocarbons and lignin (Fritsche, and Hofrichter, 2004; Hara and Uchiyama, 2012). As further remark of fungal relevance in deep ecosystems, fungal nodes were almost absent in the network analysis (Fig. 5D) as the related oligotypes were present in nearly all water masses (at least in low percentage of relative abundance) and they were therefore discarded from the indicator species analysis.

Within the Radiolaria division, the most represented order of was Collodaria. This taxon is widely distributed across global ocean from epipelagic to bathypelagic realms (Pernice et al., 2015; Zoccarato et al., 2016b; Biard et al., 2017) and all described species bears intracellular algal symbionts (see Biard et al., 2017) thus making their ecological role in the aphotic zones an intriguing aspect. In any case, it has to be kept in mind that it is a colony forming taxon and therefore the detected high relative abundance might be overestimated by the presence of colonies. The absence of strict phototrophic protists (except for possible mixotrophs such as unclassified Dinophyta) below 500 m indicates a limited export of POC from the surface to the deep water layers, as could be expected due to the low chlorophyll *a* concentration values in the upper water column (Diociauti et al., this issue, submitted). It has been shown that in areas of pronounced export of particulate matter, intact phytoplankton cell can be found at depth ~1,000 m, and signatures of both 16S rRNA genes from chloroplasts (e.g. Celussi et al., 2009; Luna et al., 2016) and 18S rRNA genes from phototrophs (Zoccarato et al., 2016b) can represent an important (even dominating) fraction of the entire amplicon sequence sets.

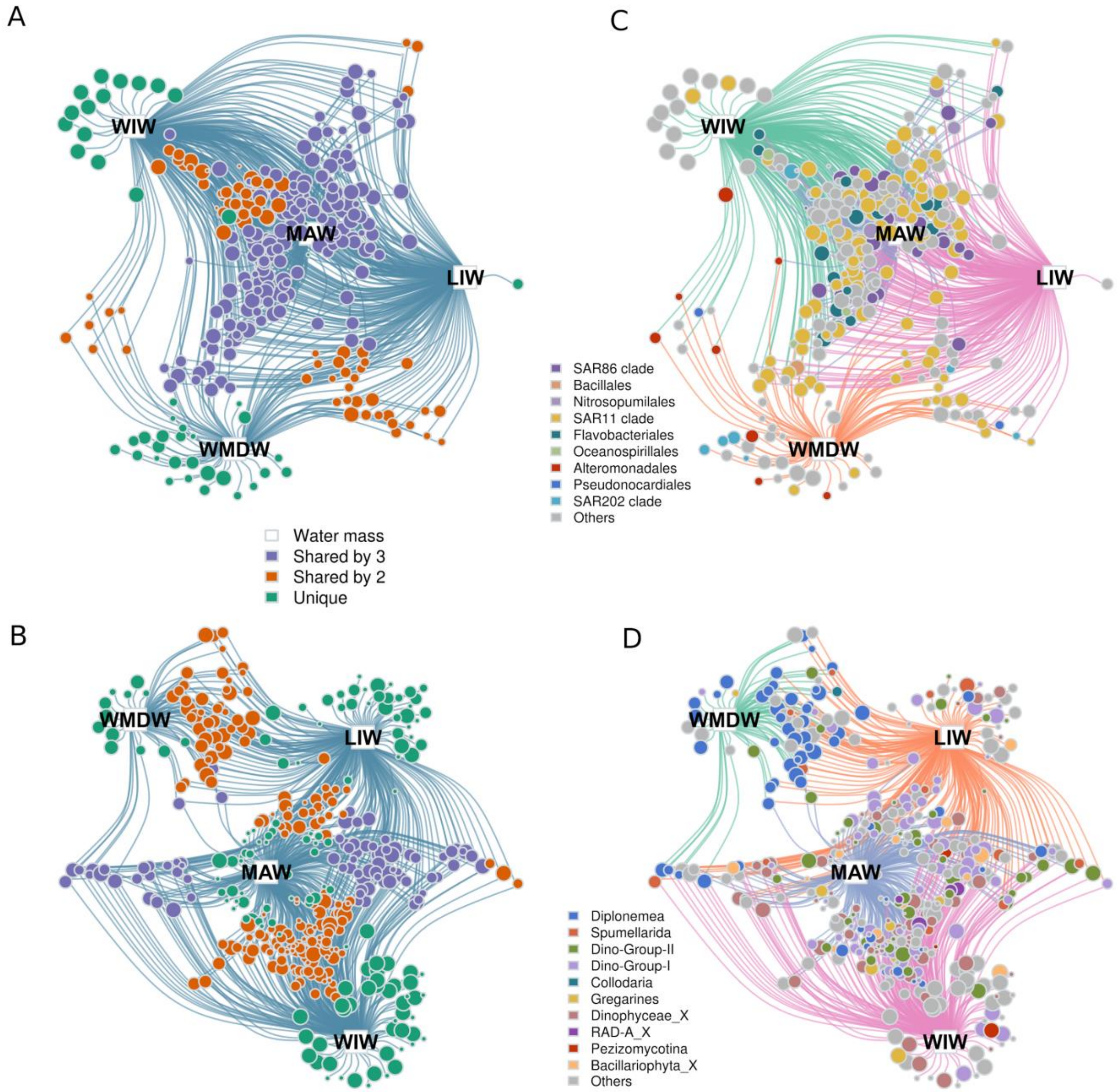

Fig. 5: Results of the Indicator Species analysis. The two networks on the left side of the panel show the correlation (i.e., presence of an edge) of unique and shared oligotypes (between 2 and 3 different water masses) of prokaryotic (A) and protist communities (B) with the water masses. The two networks on the right side of the panel highlight the taxonomic classification of the nodes that belonged to the most represented orders (i.e., those representing >1% of relative abundance in at least one sample) of prokaryotes (C) and protists (D). Non-dominant taxa (i.e., <1%) are reported as “Others”. The size of the nodes reflects the level of “fidelity” such that the largest nodes are those always present in all the samples of the respective water mass.

3.3 Microbial activities

The dissolved organic (HCP) and inorganic ($DICf_{tot}$) carbon utilization by microbes did not reveal different microbial metabolic strategies inside and outside the canyons. HCP data showed the classical depth-related gradient (Luna et al., 2012; Celussi et al., 2017), being on average 0.033 (± 0.028, SD) µgC $L^{-1}d^{-1}$ between 200 and 1,000m, and 0.010 (± 0.005, SD) µgC $L^{-1}d^{-1}$ below 1,000 m (Fig 6).

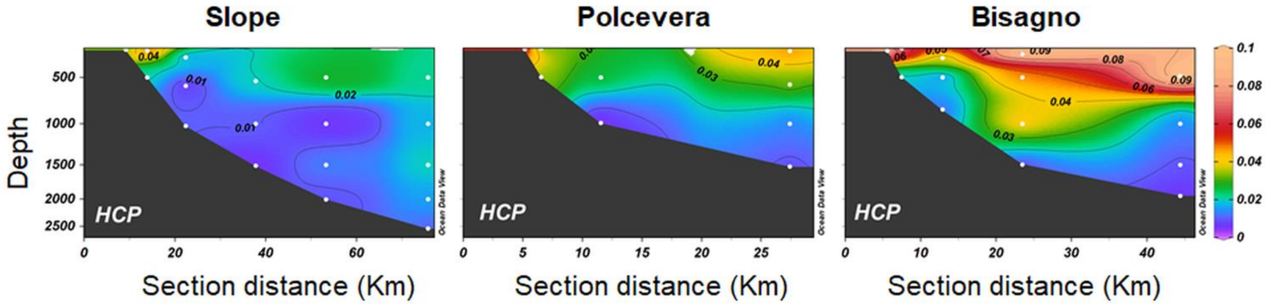

Fig. 6: Depth profiles of Heterotrophic Carbon Production (HCP) data along the three transects in the Ligurian Sea. Values are given in µgC $L^{-1}d^{-1}$. The Y-axis is not linear (stretched to top) and depth is reported in m in the range 200 - 2,750. The image was drawn by means of the ODV software (Schlitzer, 2014)

On the contrary, the uptake of DIC (measured only in the bottom layer of each station) was not dependent on the sampling depth, and ranged between 0.333 (± 0.001) and 2.880 (± 1.429) µgC $L^{-1}d^{-1}$ (Fig. 7). The DIC taken up by microbes can be utilized either as C source for chemosynthetic processes or can be channelled into heterotrophic pathways through anaplerotic reactions (Herndl et al., 2005; Yakimov et al., 2014). As reported previously, the amount of DIC utilized by Mediterranean deep-sea microbes is noteworthy, and can exceed both heterotrophic carbon production (Celussi et al., 2017; La Cono et al., 2018) and epipelagic photosynthetic carbon fixation (La Cono et al., 2018). Given the fast rates of DIC assimilation (if compared to HCP), we can speculate that in our study this form of C was utilized for chemosynthetic processes rather than anaplerosis (Celussi et al., 2017).

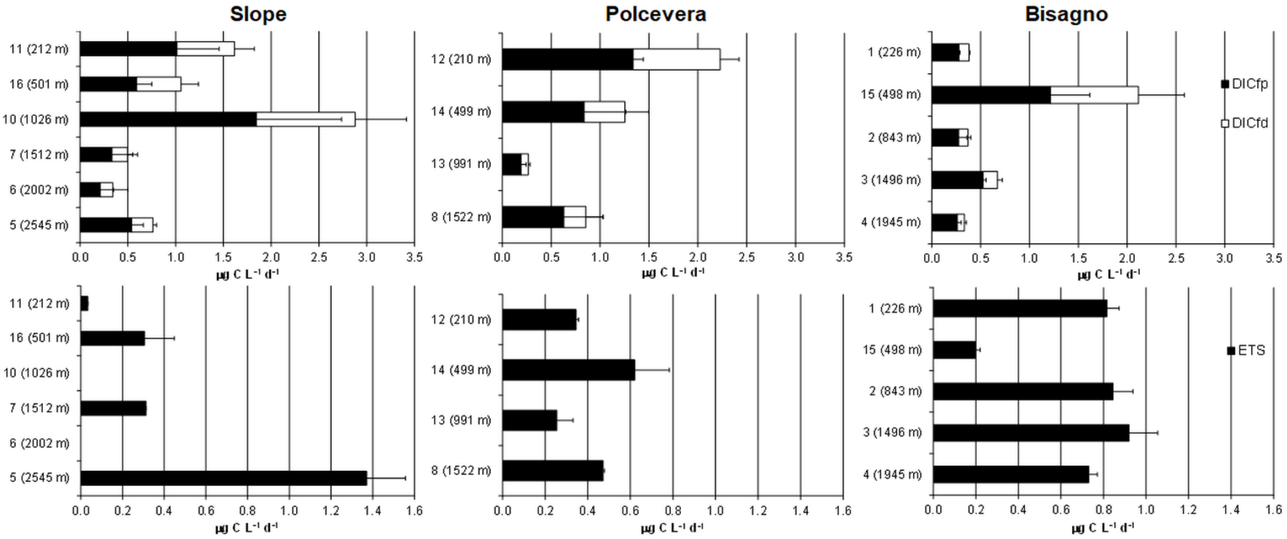

Fig. 7: Particulate and dissolved DIC fixation rates (DICf$_p$ and DICf$_d$, upper plots) and activity of the Electron Transport System (ETS, lower plots) at the bottom depth of stations located along the three transects in the Ligurian Sea. On the Y-axis the stations and depth (in brackets) are reported. Error bars indicate the standard deviation between two replicates.

Also the activity of the Electron Transport System did not significantly differ between inside and outside the canyons. This parameter, used to indirectly measure plankton respiration rates, ranged between 0.03 (± 0.00) and 1.37 (± 0.19) µgC L^{-1}d^{-1} and did not display a clear vertical gradient. By adopting the Respiration:ETS conversion factor of 0.68 (Arístegui et al., 2005) in order to provide data comparable to those presented by La Cono et al. (2018), we calculated the percent contribution of DIC utilization in sustaining the Prokaryotic Carbon Demand (PCD = HCP + Respiration). The computation provided data ranging from 0.6 to 31.7%. Even though our range is slightly lower than the one reported by La Cono et al. (2018) it still evidences how DIC utilization is a very important, previously unaccounted, process in sustaining this deep ecosystem.

The rates of degradation of the major biopolymers displayed a typical decreasing gradient at increasing depths (Zaccone et al., 2003; Misic and Fabiano, 2006; Luna et al., 2012). Exoenzymatic activities were generally slow, with mean values of 1.23 (± 1.41, SD), 5.58 (± 14.42, SD), 2.17 (± 1.34, SD), 4.63 (± 2.09) nM h^{-1} for leucine aminopeptidase, alkaline phosphatase, beta-glucosidase and lipase, respectively (Fig. 8). However, some activity hotspots were detected, especially within the Bisagno canyon transect. At station 2, in fact, a relative maximum hydrolysis rate of leucine aminopeptidase (7.93 ± 0.16 nM h^{-1}), alkaline phosphatase (88.99 ± 0.26 nM h^{-1}) and beta-glucosidase (8.15 ± 0.59 nM h^{-1}) was measured at the bottom. Such a feature is in accordance to the results of Misic and Fabiano (2006) who reported irregular increases in the rates of polypeptide and polysaccharide degradation in the bottom layer of the canyon heads, in the same canyon system. Notwithstanding the presence of these ‘atypical’ pronounced rates at depth ~ 850 m, when considering all samples collected within canyons, the only hydrolytic activity that consistently responded to a potential ‘canyon effect’ was leucine aminopeptidase, as evidenced by the Wilcoxon matched pair test

(Bisagno vs Slope samples Z = 1.96, N = 8, $p < 0.05$; Polcevera vs Slope sample Z = 2.36, N = 7, $p < 0.05$). It is worth to note that at the sites where relatively fast potential degradation rates were measured, the C:N ratio within particulate organic matter was far above the Redfield's one (= 6.6), being up to 24 at the bottom of station 2. A high value of this ratio is indicative of the recalcitrant nature of organic matter, since N-containing compounds are preferentially consumed by degraders resulting in the accumulation of C (Jiao et al., 2010). Recalcitrant organic matter is difficult to be utilized by microbes and requires extensive degradation by specialized consortia for being finally oxidised (Carlson et al., 2011; Bochdansky et al., 2016). One more interesting finding about these 'hotspot' samples is the high relative abundance of Fungi (detected by 18S rRNA gene analyses) that might be (at least partially) responsible for recalcitrant organic matter degradation. Following the conclusions of Misic and Fabiano (2006) we can infer that this material derives from the local resuspension of particles from the seafloor, that consequently stimulates degradation efforts.

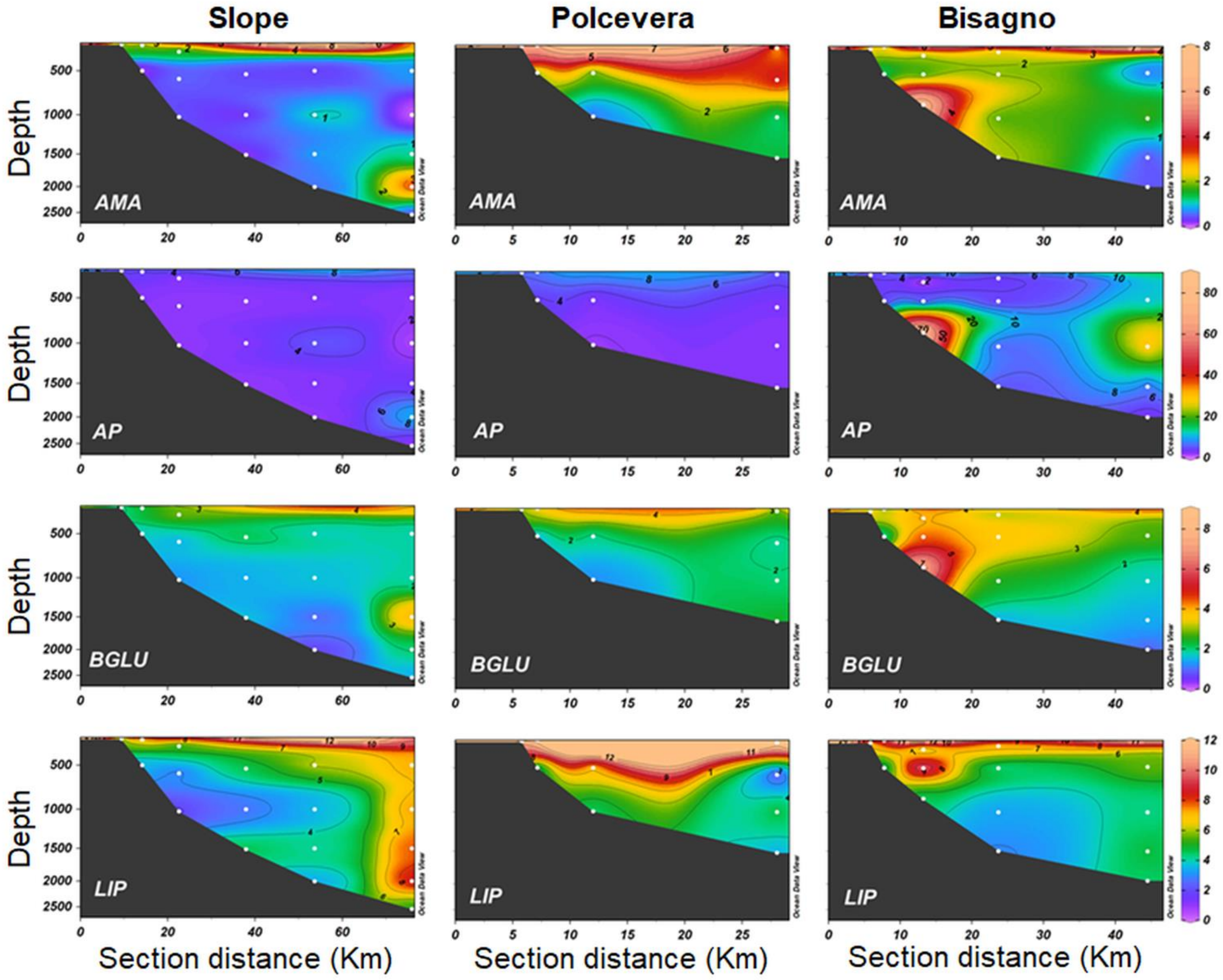

Fig. 8: Leucine aminopeptidase (AMA), Alkaline Phosphatase (AP), Beta-glucosidase (BGLU) and Lipase (LIP) activity rates measured along the Slope, the Polcevera and the Bisagno transects. The shallower depths

correspond to 200 m. All data are in nM h^{-1}. The Y-axis is not linear (stretched to top) and depth is reported in m. The image was drawn by means of the ODV software (Schlitzer, 2014)

4. Conclusions

Our overarching hypothesis was that the Bisagno and the Polcevera canyons act as peculiar systems in terms of biogeochemical and biological features in the pelagic environment. The main reason behind this idea was related to the main circulation in the area which is perpendicular to the main axis of the canyons, thus potentially limiting the mixing with the overlaying water and retaining the effect of episodic flushing (that can deliver high loads of organic matter and nutrients) inside the canyons. We confuted our hypothesis since no consistent difference was found when examining microbial assemblages' structures and their activity. We therefore conclude that other hydrodynamic processes (e.g. internal and/or tidal wave) contribute to the mixing of canyon and non-canyon waters. Such hydrological features are able to limit the persistency of flushing effects, even though in the proximity of canyon heads some morphological features (such as, for example, the steepness of canyons) can locally determine the accumulation and/or resuspension of refractory material, promoting microbially-mediated organic matter degradation.

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