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Patterns in extracellular enzyme activity and microbial diversity in deep-sea Mediterranean sediments

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Abstract

Deep-sea sediments are populated by diverse microbial communities that derive their nutritional requirements from the degradation of organic matter. Extracellular hydrolytic enzymes play a key role in the survival of microbes by enabling them to access and degrade complex organic compounds that are found in seafloor sediments. Despite their importance, extracellular enzymatic activity is poorly characterized at water depths greater than a few hundred meters where physical properties, such as pressure and temperature, create a unique environment for influencing enzyme behavior. Here, we investigated microbial communities and enzyme activities in surface sediment collected at four sampling stations in the central Mediterranean Sea at water depths ranging from 800 to 2200 m. Fluorometric assays revealed that extracellular hydrolytic activity varied according to substrate type and water depth which suggests that the distributions of these enzymes within this basin are not homogenous. Furthermore, enzyme activities indicated substantial demand for phosphomonoesters and proteins, with measurable but much lower demand for polysaccharides. Barcoded amplicon sequencing of bacterial and archaeal SSU genes revealed that microbial communities varied across sampling stations and some groups displayed water-depth related trends. Our results demonstrate that heterotrophic capabilities of microbes in deep-sea Mediterranean sediments can differ substantially even within the same region.

Keywords:

deep-sea sediments; organic matter; biogeochemistry; extracellular enzymes; 16S rRNA; Mediterranean Sea

Introduction

The deep-sea floor covers approximately 65% of the Earth's surface and represents one of the largest organic carbon reservoirs on the planet. Organic carbon in seafloor sediments is derived from numerous sources including terrestrial material introduced via erosion and/or fluvial transport as well as material produced in the upper water column (Zonneveld et al., 2010). The latter is the predominant source and is produced by marine phytoplankton that reside in the euphotic zone. Only a fraction of this carbon (typically <1%) reaches the seafloor where it serves as the primary carbon and energy source for sedimentary microorganisms while the remainder is remineralized in the water column. While sinking to the seafloor, organic matter is continuously transformed by microbial and chemical processes (Karl et al., 1988). Consequently, the nutritive value of organic matter is thought to decrease with extended sinking times (Banse, 1990) such that readily degradable organic compounds are removed while less degradable, high molecular-weight (MW) compounds actually reach the seafloor. Thus, much of the organic matter in deep-sea sediments is chemically complex, macromolecular and more refractory in nature.

Seafloor sediments are populated by diverse taxa that belong to uncultivated bacterial and archaeal phylogenetic lineages (Biddle et al., 2008; Fry et al., 2008; Teske and Soresen, 2008; Lloyd et al., 2018). These microbial communities are primarily heterotrophic and derive their nutritional requirements from the degradation of organic matter that is deposited on the seafloor. In order to access complex organic matter, some microorganisms secrete hydrolytic enzymes to catalyze the degradation of complex polymeric compounds to smaller monomeric and oligomeric molecules which are can then be directly taken up by cells. These enzymes can be tethered to the cell membrane, adsorbed to sediment particles or freely dissolved in water (Arnosti, 2011). Extracellular hydrolytic enzymes are considered to be a controlling factor for the remineralization of organic carbon and a key step in the marine carbon cycle; however, our

knowledge of the distribution and activity of microbial enzymes in deep-sea environments is fragmentary. Most studies have focused on hydrolytic enzymes in coastal and near-surface environments (Coolen et al., 2002; Lloyd et al., 2013; Mahmoudi et al., 2017) with only a few studies examining enzymatic activities in surface sediments from deeper meso- and bathypelagic zones, where physical properties, such as pressure and temperature, can create a unique environment for influencing enzyme behavior (Boetius et al., 1994; Boetius et al., 2000; Dell'Anno et al., 2000; Nagata et al., 2010; Zaccone et al., 2012; Baltar et al., 2013).

The Mediterranean Sea is an oligotrophic system characterized by nutrient-depleted waters and low levels of primary production. Typical ratios of nitrate to phosphate in the global ocean are ~16 (Karl et al., 1993), whereas those in the Mediterranean Sea range from 20 to 25 (Ribera d'Alcalà et al., 2003). In addition, elevated bottom water temperatures (12-13°C) and higher salinity (38-39) compared to other marine environments at similar depths and latitudes make deep sea sediments in the Mediterranean Sea unique. Here, we characterize the diversity and metabolism of microbial communities in Mediterranean Sea sediments and explore the extent to which water depth affects the distribution and activity of extracellular hydrolytic enzymes. Water depth influences hydrostatic pressure as well as the quality of organic matter available to seafloor microbes (Hedges et al., 2001; Lee et al., 2004). We collected sediments from four sampling stations in the central Mediterranean Sea with water depths ranging from 800 to 2200 meters and measured the enzymatic potential of eight extracellular hydrolytic enzymes. In addition, we applied barcoded amplicon sequencing of bacterial and archaeal SSU genes to evaluate potential linkages between taxonomic composition and diversity and water depth. The results revealed that extracellular hydrolytic activity varied according to substrate type and water depth which suggests that the distributions of these enzymes within this basin are not

homogenous. Furthermore, microbial community composition did not appear to predict the heterotrophic capabilities of sedimentary microbial communities.

1. Materials and Methods

2.1 Sample collection

All sampling stations were located in the central Mediterranean Sea, off the coast of Libya (Fig. 1). Temperature, salinity, pH and oxygen concentrations at each station were measured ~ 25 meters from the seafloor (Table 1), using a MIDAS CTD + sensor array (Valeport Ltd, St. Peter's Quay, UK). Bottom water was collected in Niskin bottles at each station and stored at -20°C for analysis of inorganic nutrients (Techtmann et al., 2017). A total of 15 sediment cores were collected at four different sampling stations during a research cruise in January 2014 using a Multicorer. The stations had water depths of 833 m, 1210 m, 1818 m and 2226 m respectively. Bottom water temperature and salinity were consistent across stations and were approximately 14°C and 39 PSU, respectively. Likewise, concentrations of inorganic nutrients and dissolved oxygen were similar across sampling stations and reflected the nutrient-depleted waters associated with the Mediterranean Sea (Astraldi et al., 2002). Following collection, intact cores were sectioned and stored under ambient oxygen conditions at -20°C on ship. The frozen sediment cores were then transported to the University of Tennessee and stored at -80°C until analysis. The upper 0 to 2 cm of each sediment core was homogenized and used for this study.

2.2 Measuring extracellular hydrolytic enzyme activity

Extracellular hydrolytic enzymes are grouped into distinct classes based on their physical structures and the types of biomolecules they hydrolyze. We used fluorogenic substrate proxies

to measure the potential enzymatic activity of eight extracellular hydrolytic enzymes: three peptidases, four glycosidases, and one phosphomonoesterase (Table 2). Substrates were chosen based on their application in previous work and the metabolic function of the enzymes they assay (Bird et al., 2019; Steen et al., 2019a). Substrates used in this study were stored in the dark at -20°C until use.

2.75 g of wet sediment were blended with borate-buffered saline solution (pH=8.0, 200 mM $Na_2B_4O_7$, 137 mM NaCl, 2.7 mM KCl) in a Waring blender for 1 minute to produce a sediment slurry for each sample. The osmolarity of this buffer was 1.20 osmoles per liter, comparable to seawater osmolarity of 1.11 osmoles per liter, suggesting that it was unlikely to cause cell rupture. A buffer-to-sediment ratio of 91 ml:2.75 g was used based on recommendations of Bell et al. (2013) and Schmidt (2016). Following blending, a small aliquot of sediment slurry was autoclaved on a liquid cycle for 60 minutes to produce abiotic "killed" controls.

Enzyme assays were performed according to a procedure modified from Bell et al (2013). Triplicate samples of the live slurries, killed slurries and buffer controls (containing no sediment) were then amended with enzyme substrates to final concentrations of 200 μM substrate (peptidase substrates) or 40 μM substrate (glycosylases), as follows: to each well of a 2-mL deep well plate we added 1.956 mL of sediment slurry, killed slurry, or buffer controls, 20 μl (peptidase substrates) or 4 μl (glycosylase substrates) of 20 mM substrate stock in 100% DMSO, and 24 μl (peptidase substrates) or 40 μl (glycosylase substrates) DMSO, so that DMSO concentrations would be identical for all treatments. The final DMSO concentration of 2.2% has previously been shown not to affect apparent enzyme kinetics (Steen et al., 2015). The deep-well plates were sealed and mixed by repeated inversion at the start of the incubation.

Prior to each timepoint, the deep-well plates were centrifuged for 3 min at 3000 x g. Subsequently, 250µl of the resulting supernatant from each well was transferred to a 250µl 96 well black bottom microplate. The deep-well plates were then re-sealed, inverted approximately 50x and left to incubate at 21 °C on an orbital shaker at 0.3 Hz until subsequent readings were taken. In total, four readings were taken for each plate, at approximately 0, 2, 20, and 26-27 h. Fluorescence was measured using a Biotek Cytation 3 plate reader (excitation=360 nm, emission=440 nm, gain=50). Long timecourses were necessary given the low enzyme activities, and have been used in previous investigations of subsurface sediments (Bird et al., 2019). Linear patterns of fluorescence production as a function of time indicated that these incubation times did not introduce artifacts related to enzyme production, degradation, or microbial population changes.

Hydrolytic enzymes typically exhibit kinetics described by the Michaelis-Menten equation (German et al., 2011)

$$v_0=(V_{max} \times [S])/(K_m+[S]) \quad \text{(Eq. 1)}$$

Hydrolysis rates were calculated as the slope of a linear least-squares regression of fluorescence versus elapsed time. Those slopes were normalized to the mass concentration of sediment in the sample and calibrated using standards of 7-amino-4-methylcoumarin (AMC) or 4-methylumbelliferone (MUB) in sediment slurry, so that enzyme activities were expressed as a concentration fluorophore released per unit time per unit mass sediment. These standards were incubated alongside the samples, and a separate calibration curve was measured for each sample at each timepoint. No substantial sorption of the standard fluorophores to sediments were observed, and because the calibration curves were measured in sediment slurries, no separate correction for fluorescence quenching by the sample (as described in German et al., 2011) was

required. In any case, quenching was minimal, likely because the slurries were thin and sediments had low organic matter content, in contrast to Coolen et al. (2002).

2.3 Genomic DNA extraction and sequencing of bacterial and archaeal SSU genes

Genomic DNA was extracted in triplicate from surface sediment (0-2 cm section) of each core using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Triplicate DNA extracts were subsequently further purified using the Genomic DNA Clean & Concentrator kit (Zymo Research, Irvine, CA, USA). DNA quality was assessed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) by measuring ratios of optical absorption at 260/280 nm and 260/230 nm. The V4 region of the 16S rRNA gene were amplified in triplicate using Phusion DNA polymerase (Thermo Scientific, Waltham, MA) and primer pair 515F and 806R (Caporaso et al., 2012), which amplifies both bacterial and archaeal genes. The reverse primers included a 12-bp barcode for multiplexing of samples during sequencing analysis. Following amplification, 16S libraries were prepared according to Mahmoudi et al., (2015). Briefly, 16S amplicons were pooled together and analyzed by Bioanalyzer (Agilent Technologies) to assess quality and size of amplicons. Following dilution, libraries were subjected to quantitative-PCR (qPCR) to ensure accurate quantification of purified amplicons. 16S libraries were sequenced using an Illumina MiSeq (San Diego, CA, USA) platform at the University of Tennessee.

2.4 Sequence analysis

Sequence data was processed and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (version 1.9.1; Caporaso et al., 2010). Quality filtering and processing of paired-end reads was performed following Mahmoudi et al., (2017). Sequences were clustered into operational taxonomic units (OTUs) at 97% identity and any OTU that comprised less than

0.005% of the total data set was removed to limit the effect of spurious OTUs on analysis (Bokulich et al., 2013; Navas-Molina et al., 2013). All analyses were carried out after pooling the technical replicates and rarefying the samples to the same sequencing depth (~15,000 sequences) using QIIME and R version 3.2.1 (R Core Team, 2016). Differences between sediment samples were assessed using non-metric multidimensional scaling (nMDS) on Bray-Curtis dissimilarity matrices. For nMDS, a stress function was used to assess the goodness-of-fit of the ordination. The Adonis implementation of PerMANOVA (non-parametric permutational multivariate analysis of variance; Anderson, 2001) was used to estimate the proportion of variation in microbial communities attributed to sampling location (i.e. water depth).

2. Results

3.1 Extracellular hydrolytic enzyme activity

Potential activities of extracellular enzymes in surface sediments varied as a function of substrate type and water depth (Fig. 2). In general, peptidases had greater potential activities than the esterase and glycosidase. Specifically, leucyl aminopeptidase and alkaline phosphatase were approximately 100 times more active than all other enzymes across all sediment samples. Cellobiase was observed to have the lowest potential activity, which was near or below the detection limit in all sediment samples. Extracellular enzyme activity appeared to decrease with increasing water depth for some enzymes but not for others. For example, leucyl aminopeptidase, β-xylosidase and alkaline phosphatase activity was ~1.5 to 2.5 times higher for sediments collected from shallower water depths (833 and 1210 m) compared to deeper depths (1818 and 2226 m). However, this was not the case for gingipain, cellobiase and β-N-

acetylglucoasminidase, enzyme activities of which were similar across all sediment samples or slightly greater for samples collected from deeper depths, specifically 1818 and 2226 m.

3.2 Microbial diversity and taxonomic composition

Illumina-based sequencing of 16S rRNA gene amplicons recovered a total of 7,671,636 (2181 OTUs) 16S sequences with an average length of 253 bp. OTU rarefaction curves approached a saturation plateau and Good's coverage ranged from 98% to 99%, indicating that the rarified sequencing depth represented the majority of 16S rRNA sequences in each sample. Species richness and diversity indices were calculated for each sediment sample (Fig. 3, Table S1). Microbial diversity decreased with increasing water depth according to both the number of OTUs observed as well as the Shannon and Simpson's indices. Diversity was similar for sediment samples collected from water depths of 833 and 1210 m and decreased rapidly for sediment samples from 1818 and 2226 m. Similarly, Chao1 values revealed that species richness was significantly lower at deeper water depths of 1818 and 2226 m (T-test, P values < 0.05).

A total of 38 different phyla were detected across all sediment samples (Fig. S1) with *Proteobacteria* (50% of assigned reads on average) being the most abundant, followed by *Acidobacteria* (12%). Within *Proteobacteria*, the majority of sequences were assigned to the classes, *Gammaproteobacteria* (16%), *Alphaproteobacteria* (16%) and *Deltaproteobacteria* (11%) (Fig. S1a). *Gammaproteobacteria* dominated across all sediment samples and accounted for a greater proportion of sequences at deeper water depths of 1818 and 2226 m where it comprised of 24 to 49% of recovered reads. Within Gammaproteobacteria, the dominant orders were *Thiotrichales* (6%) and *Xanthomonadales* (7%). The relative abundance of *Xanthomonadales* varied with water depth and comprised a greater proportion of sequences at deeper depths, it accounted for 4% (on average) of assigned reads at 833 and 1210 m and 17% of

231 reads at 1818 and 2226 m. Depth-related trends were also observed for *Betaproteobacteria*;
232 specifically the relative abundance of *Burkholderiales* increased with water depth such that they
233 comprised of 14% of all reads at 2226 m, and less than ~6% of reads at the other three sampling
234 locations. Archaeal sequences accounted for 9% of all reads on average, with almost all of these
235 reads belonging to *Crenarchaeota* (8%), specifically *Thaumarchaeota* (8%). The relative
236 abundance of *Thaumarchaeota* varied with water depth and was found to be lower in sediments
237 collected deeper water depths (4 – 9%) compared to those from shallower water depths (7 –
238 12%).

239 NMDS analysis based on the Bray-Curtis distance matrix showed that sediment samples
240 collected from a water depth of 1210 m showed strong clustering while sediment samples from
241 the collected from the other three sampling locations had a dispersed distribution, highlighting
242 the heterogeneity at these sites (Fig. S2). Similarly, ADONIS analysis confirmed that sampling
243 location (R^2=0.71, p=0.001, strata=location) affected the observed variation among microbial
244 communities.

245

246 **3. Discussion**

247 Little is known about the adaptations that allow for the growth and activity of microbes in
248 the deep sea (Dell'Anno et al., 2000; Luna et al., 2004, 2012; Giovannelli et al., 2013).
249 Extracellular hydrolytic enzymes play a key role in the survival of microbes by enabling them to
250 access and degrade complex organic compounds as a source of carbon and energy (Arnosti,
251 2011). The degradation of organic matter also serves as the primary source of organic nitrogen
252 that is available to heterotrophic microbes, mainly in the form of amino acids (Cowie & Hedges,
253 1994; Vandewiele et al., 2009). Extracellular enzymes are "expensive" for microbes to produce

in terms of carbon, nitrogen and energy expenditure (Vetter et al., 1998; Allison, 2005); all of which are in low supply in energy-limited systems such as deep sea sediments underlying oligotrophic waters (LaRowe & Amend, 2015; Bradley et al., 2018). In the deep-sea sediments measured here, potential enzyme activities indicated substantial demand for phosphomonoesters (alkaline phosphatase) and proteins (L-leucyl aminopeptidase, D-phenylalanyl aminopeptidase, gingipain), with measurable but much lower demand for polysaccharides. These results are consistent with the the severe phosphate limitation observed in the Mediterranean Sea (Krom et al., 1991; Thingstad et al., 2005) and indicate that sedimentary microbes have adapted to cope with the limited amounts of organic resources in this system.

Since the majority of microbes in marine sediments have not yet been cultivated (Lloyd et al., 2018), it is difficult to determine the degree to which microbes in this environment are metabolically active. Leucyl aminopeptidase is a commonly measured hydrolytic enzyme secreted by heterotrophic microbes and has shown experimentally to be responsive to the addition of amino acids (Zeglin et al., 2007). Since amino acids and amino sugars contain both carbon and nitrogen, it is likely that this enzyme serves a dual role by allowing microbes to acquire both carbon and nutrients. Therefore, leucyl aminopeptidase has been used as an indicator of heterotrophic activity (Taylor et al., 2003). To date, only a handful of studies have reported the activity of extracellular enzymes in deep-sea environments (Coolen & Overmann, 2000; Dell'Anno et al., 2000; Coolen et al., 2002). In the present study, the specific activities of L-leucyl aminopeptidase ranged from 2 to 6 μmol g^{-1} hr^{-1} across all sediment samples. These rates are much higher than those observed in deep-sea basalt rocks collected from the Loihi Seamount in which L-leucyl aminopeptidase activity rates that were < 0.0007 μmol g^{-1} hr^{-1} (Meyers et al., 2014). Similarly, the rates of leucyl aminopeptidase measured in the present study

were substantially higher those observed in deep-sea surface sediments collected from a water depth of 2150 m in the eastern Mediterranean Sea (Coolen & Overmann, 2000), that is considered to be one of the most oligotrophic regions in the world (Psarra et al., 2000). Leucyl aminopeptidase activity in surface sediment from the eastern Mediterranean Sea was determined to be ~ 0.002 µmol g^{-1} hr^{-1} (Coolen & Overmann, 2000); ~ 3 orders of magnitude lower than the activities reported here. Recent work by Bird et al., (2019) measured extracellular enzyme activity in Baltic Sea sediments collected during IODP Expedition 347 where the leucyl aminopeptidase activity peaked at ~17 meters below seafloor (mbsf) and was observed to be 20,000 µmol g^{-1} hr^{-1}, significantly higher than those measured here. The Baltic Sea is a fairly eutrophic system, thus, it would be expected that these sediments are more energy-rich and would have higher activity rates of microbial activity compared to typical deep-sea sediments.

Protein metabolism appears to be important for microbes in seafloor sediments (Lloyd et al., 2013) and peptidases have been found to be more active than other enzymes in deep-sea environments (Coolen & Overmann, 2000; Meyers et al., 2014). Extracellular peptidases are structurally and genetically diverse class of enzymes that can hydrolyze peptide bonds at variable rates. Previously, elevated ratios of D-aminopeptidases, such as D-phenylalanyl aminopeptidase, relative to L-aminopeptidases, has been observed in association with decrease in availability of bioavailable organic matter (Steen et al., 2019a). This does not appear to be the case here: while fluxes of bioavailable organic matter were likely to be lower in sediments collected from deeper water depths, the ratios of L-leucyl aminopeptidase to D-phenylalanine aminopeptidase did not significantly change among as a function of depth ($R^2 < 0.001$, $p = 0.97$, $n = 34$). These samples also differed from previously sampled “deep” environments in that the activity of gingipain, an endopeptidase (i.e., an enzyme that hydrolyzes proteins by catalyzing the cleavage of internal

peptide bonds) was low relative to that of leucyl aminopeptidase, an exopeptidase which cleaves enzymes one residue at a time from the N terminus of proteins (Lloyd et al., 2013; Steen et al., 2016). Ratios of endopeptidase : exopeptidase activities are fairly stable within environments but highly variable among environments (Steen et al., 2013; Mullen et al., 2018). It is not clear what environmental parameters control these ratios.

In the present study, microbial communities were dominated by *Proteobacteria* and specifically, by *Gammaproteobacteria*. This was particularly true for sediment samples from deeper water depths (1818 and 2226 m) where *Gammaproteobacteria* accounted for 24 to 50% of all assigned sequences. *Gammaproteobacteria* are one of the most abundant bacterial groups in marine sediments (Inagaki et al., 2003; Polymenakou et al., 2005; Ruff et al., 2013; Mahmoudi et al., 2013, 2015; Franco et al., 2017). Microbiological and genomic studies have demonstrated that many species within *Gammaproteobacteria* possess the genes needed to produce and secrete several different extracellular hydrolytic enzymes (Zimmerman et al., 2013; Steen et al., 2016; Mahmoudi et al., 2019). Thus, it is not surprising that the relative abundance of *Gammaproteobacteri*a is higher in seafloor sediments collected from deeper water depths that would be expected to receive less bioavailable compounds, more complex compounds, due to the extended sinking times of organic material from the surface. Likewise, we found that microbial diversity decreased with greater water depth which is consistent with the notion that seafloor sediments from shallower depths likely receive more labile and diverse organic substrates thereby supporting a higher diversity of microbes. Our results support previous observations that organic matter quality and quantity can determine shifts in microbial community structure (Luna et al., 2004; Mahmoudi et al., 2017).

Previous studies have found that the number of species that produce extracellular hydrolytic enzymes is a small fraction of the entire microbial community (Langenheder et al., 2006; Logue et al., 2016; Rivett and Bell, 2018). This indicates that the ability to produce these enzymes is a functionally dissimilar, rather than functionally redundant, trait such that changes in taxonomic composition will result in variations in hydrolytic activity. We observed that extracellular enzyme activity was fairly stable for sediment samples collected from the same water depth even though in most cases sediment samples from the same water depth did not closely cluster together based on their taxonomic similarity (Fig. S2). These results point to some degree of functional redundancy with respect to extracellular enzymes which is consistent with previous work that has characterized both the composition and enzymatic function of microbial communities (Wohl et al., 2004; Frossard et al., 2012; D'Ambrosio et al., 2014). Functional redundancy with respect to enzymatic capabilities occurs when several taxonomic groups have the ability to use a substrate such that the substrate capacity of a microbial community may be due to subtle changes among less dominant populations. The extent of functional redundancy appears to vary among microbial communities which makes it is difficult to decipher the relationship between taxonomic composition and enzymatic capabilities.

Although the deep-sea sediments contain a substantial portion of microbial biomass, little is known about the metabolic activities and growth of microbes that inhabit these environments. Extracellular hydrolytic enzymes serve a critical function and enables microbes to access and degrade complex, macromolecular compounds to fulfill their nutritional requirements. Moreover, these enzymes play a critical role in recycling organic carbon compounds and nutrients in deep-sea sediments. The Mediterranean Sea is known for its unique physical characteristics including high salinity, elevated deep-water temperatures and low nutrient concentrations. Previous work

in Mediterranean waters has demonstrated that microbial activity varies both spatially and temporally and is reflected by a decreasing pattern moving from the Western to Eastern basin (Luna et al., 2012). In this study, we demonstrated that extracellular enzyme activity varied according to the substrate type and water depth across the central Mediterranean Sea; indicating that deep-sea Mediterranean sediments are far from steady state conditions and that the distribution of these enzymes are not homogenous. Moreover, our results show that the heterotrophic capabilities of microbes within this basin differ even within the same region. Future studies into the chemical nature of the organic carbon in these sediments as well as the precise mechanisms by which microbes access degraded organic compounds may further our understanding of microbial carbon cycling in deep-sea sediments.

Author Contributions

NM and ADS conceived the direction and design of the study, collectively mentored undergraduate student SH who carried out the extracellular hydrolytic enzyme assays, and analyzed the data. NM carried out the extraction, sequencing and analysis of 16S rRNA genes. NM and ADS led the writing and drafted the manuscript with contributions from SH and TCH.

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551 **Table 1.** Coordinates of sampling stations and environmental parameters of bottom waters

Water Depth (m)	Latitude	Longitude	Temp (ºC)	Salinity (practical salinity units)	Oxygen Concentration (mg L^{-1})	Ammonia (µM)	Nitrate (µM)	Pressure (MPa)
833	32° 31' 22.720" N	19° 49' 24.961" E	13.7	38.7	5.9	0.04	5.10	8.37
1210	32° 37' 10.386" N	19° 32' 50.890" E	13.7	38.7	4.1	<0.02	4.80	12.16
1818	32° 21' 45.906" N	17° 57' 31.874" E	13.8	38.7	6.0	<0.02	4.70	18.27
2226	32° 54' 22.509" N	18° 24' 57.618" E	13.8	38.7	6.1	0.10	4.60	22.37

552

553 **Table 2.** Enzymes and substrates used in the present study to measure extracellular enzyme
554 activity in Mediterranean Sea sediments

Enzyme	Substrate	Characterization	Function
β-Glucosidase	4-MUB-β-D-glucopyranoside	glycosidase	Carbon-acquire enzyme (cellulose degradation)
Cellobiase	4-MUB-β-D-cellobioside	glycosidase	Carbon-acquiring enzyme (cellulose degradation)
β-N-Acetylglucosaminidase	4-MUB N-acetyl β-D-glucosaminide	glycosidase	Carbon-acquiring enzyme
β-Xylosidase	4-MUB-β-D-xylopyranoside	glycosidase	C-acquiring enzyme (hemicellulose degradation)
Leucyl Aminopeptidase	L-leucine-4-AMC*HCl	exopeptidase	Nitrogen-acquiring enzyme (peptide degradation)
D-Phenylalanyl-Aminopeptidase	D-phenylalanine-AMC	exopeptidase	Nitrogen-acquiring enzyme (peptide degradation)
Gingipain	Z-Phenylalanine-Arginine-AMC	endopeptidase	Nitrogen-acquiring enzyme
Alkaline Phosphatase	4-MUB-phosphaste	esterase	Phosphorus-acquiring enzyme (phosphomonoester degradation)

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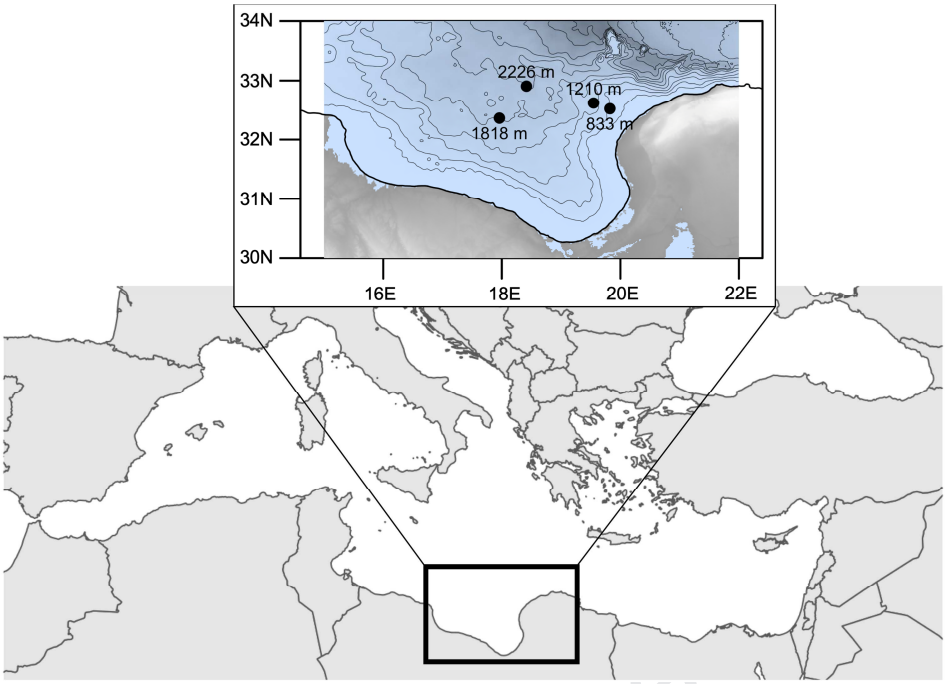

Figure 1. Map depicting sampling stations in the central Mediterranean Sea. Sediment samples were collected from four stations that ranged in water depth from 833 m to 2226 m (shown as black circle within the zoomed-in region).

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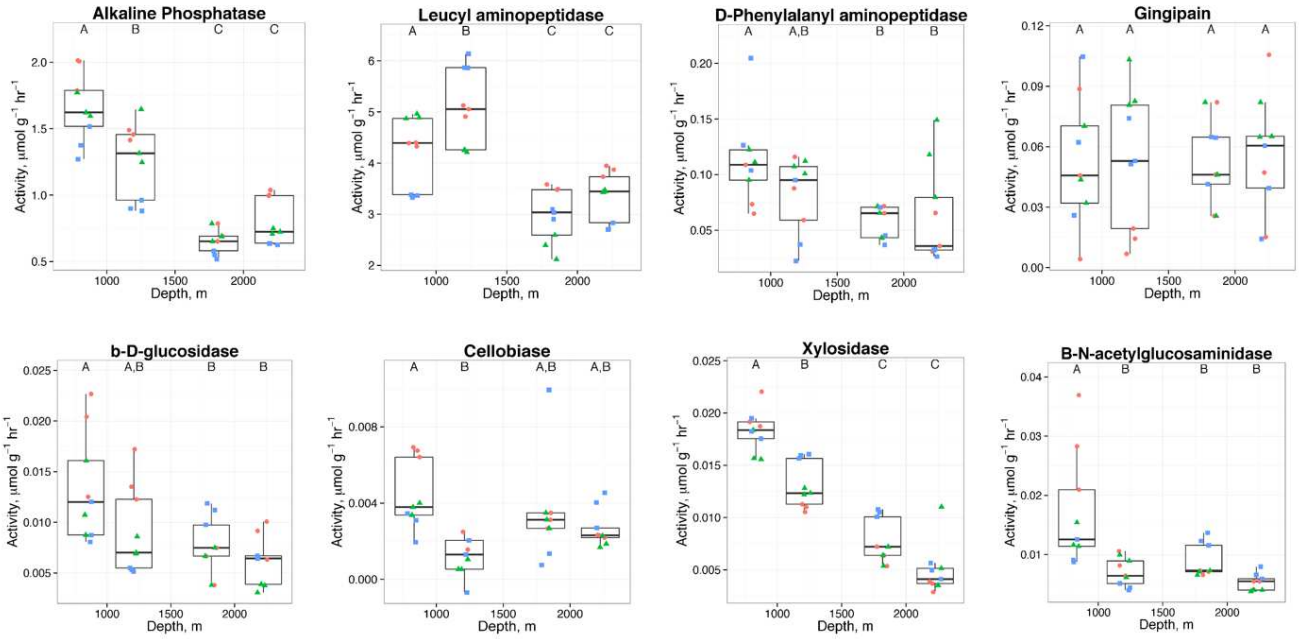

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563 **Figure 2.** Potential extracellular enzyme activities of observed in central Mediterran Sea
564 sediments. Activities were measured for eight different extracellular enzymes across four water
565 depths. Three technical replicates were each measured from three separate cores at each site.
566 Extracellular enzyme activities were expressed as micromole fluorophore per liter slurry per
567 hour.

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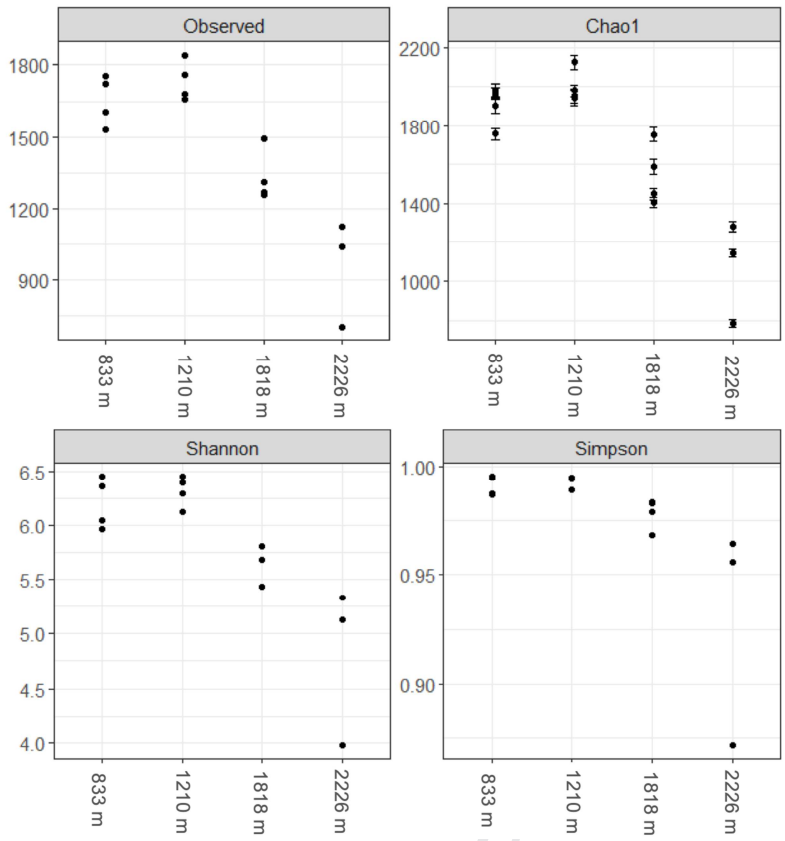

569

570 **Figure 3.** Estimates of alpha-diversity metrics for microbial communities in central
571 Mediterranean Sea sediment samples.

Highlights

Explored microbial communities in Mediterranean Sea sediments

Diversity decreased with greater water depth

Extracellular enzyme activity was not homogenous within this basin

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