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### Authors

Mahmoudi, Nagissa

Hagen, Shane M

Hazen, Terry C

et al.

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

Nagissa Mahmoudi, Shane Hagen, Terry C. Hazen, Andrew D. Steen



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**25 Abstract**

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

**43 Keywords:**

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

46

## 47 **Introduction**

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

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

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

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

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

96

## 97 **1. Materials and Methods**

### 98 *2.1 Sample collection*

99 All sampling stations were located in the central Mediterranean Sea, off the coast of  
100 Libya (Fig. 1). Temperature, salinity, pH and oxygen concentrations at each station were  
101 measured ~ 25 meters from the seafloor (Table 1), using a MIDAS CTD + sensor array (Valeport  
102 Ltd, St. Peter's Quay, UK). Bottom water was collected in Niskin bottles at each station and  
103 stored at -20°C for analysis of inorganic nutrients (Techtmann et al., 2017). A total of 15  
104 sediment cores were collected at four different sampling stations during a research cruise in  
105 January 2014 using a Multicorer. The stations had water depths of 833 m, 1210 m, 1818 m and  
106 2226 m respectively. Bottom water temperature and salinity were consistent across stations and  
107 were approximately 14°C and 39 PSU, respectively. Likewise, concentrations of inorganic  
108 nutrients and dissolved oxygen were similar across sampling stations and reflected the nutrient-  
109 depleted waters associated with the Mediterranean Sea (Astraldi et al., 2002). Following  
110 collection, intact cores were sectioned and stored under ambient oxygen conditions at -20°C on  
111 ship. The frozen sediment cores were then transported to the University of Tennessee and stored  
112 at -80°C until analysis. The upper 0 to 2 cm of each sediment core was homogenized and used  
113 for this study.

### 114 *2.2 Measuring extracellular hydrolytic enzyme activity*

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

117 to measure the potential enzymatic activity of eight extracellular hydrolytic enzymes: three  
118 peptidases, four glycosidases, and one phosphomonoesterase (Table 2). Substrates were chosen  
119 based on their application in previous work and the metabolic function of the enzymes they assay  
120 (Bird et al., 2019; Steen et al., 2019a). Substrates used in this study were stored in the dark at -  
121 20°C until use.

122 2.75 g of wet sediment were blended with borate-buffered saline solution (pH=8.0, 200  
123 mM  $\text{Na}_2\text{B}_4\text{O}_7$ , 137 mM NaCl, 2.7 mM KCl) in a Waring blender for 1 minute to produce a  
124 sediment slurry for each sample. The osmolarity of this buffer was 1.20 osmoles per liter,  
125 comparable to seawater osmolarity of 1.11 osmoles per liter, suggesting that it was unlikely to  
126 cause cell rupture. A buffer-to-sediment ratio of 91 ml:2.75 g was used based on  
127 recommendations of Bell et al. (2013) and Schmidt (2016). Following blending, a small aliquot  
128 of sediment slurry was autoclaved on a liquid cycle for 60 minutes to produce abiotic “killed”  
129 controls.

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

140 Prior to each timepoint, the deep-well plates were centrifuged for 3 min at 3000 x g.  
141 Subsequently, 250µl of the resulting supernatant from each well was transferred to a 250µl 96  
142 well black bottom microplate. The deep-well plates were then re-sealed, inverted approximately  
143 50x and left to incubate at 21 °C on an orbital shaker at 0.3 Hz until subsequent readings were  
144 taken. In total, four readings were taken for each plate, at approximately 0, 2, 20, and 26-27 h.  
145 Fluorescence was measured using a Biotek Cytation 3 plate reader (excitation=360 nm,  
146 emission=440 nm, gain=50). Long timecourses were necessary given the low enzyme activities,  
147 and have been used in previous investigations of subsurface sediments (Bird et al., 2019). Linear  
148 patterns of fluorescence production as a function of time indicated that these incubation times did  
149 not introduce artifacts related to enzyme production, degradation, or microbial population  
150 changes.

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

$$153 \quad v_0 = (V_{\max} \times [S]) / (K_m + [S]) \quad (\text{Eq. 1})$$

154 Hydrolysis rates were calculated as the slope of a linear least-squares regression of fluorescence  
155 versus elapsed time. Those slopes were normalized to the mass concentration of sediment in the  
156 sample and calibrated using standards of 7-amino-4-methylcoumarin (AMC) or  
157 4-methylumbelliferone (MUB) in sediment slurry, so that enzyme activities were expressed as a  
158 concentration fluorophore released per unit time per unit mass sediment. These standards were  
159 incubated alongside the samples, and a separate calibration curve was measured for each sample  
160 at each timepoint. No substantial sorption of the standard fluorophores to sediments were  
161 observed, and because the calibration curves were measured in sediment slurries, no separate  
162 correction for fluorescence quenching by the sample (as described in German et al., 2011) was

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

### 165 *2.3 Genomic DNA extraction and sequencing of bacterial and archaeal SSU genes*

166 Genomic DNA was extracted in triplicate from surface sediment (0-2 cm section) of each  
167 core using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA).  
168 Triplicate DNA extracts were subsequently further purified using the Genomic DNA Clean &  
169 Concentrator kit (Zymo Research, Irvine, CA, USA). DNA quality was assessed using a  
170 NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) by measuring ratios of optical  
171 absorption at 260/280 nm and 260/230 nm. The V4 region of the 16S rRNA gene were amplified  
172 in triplicate using Phusion DNA polymerase (Thermo Scientific, Waltham, MA) and primer pair  
173 515F and 806R (Caporaso et al., 2012), which amplifies both bacterial and archaeal genes. The  
174 reverse primers included a 12-bp barcode for multiplexing of samples during sequencing  
175 analysis. Following amplification, 16S libraries were prepared according to Mahmoudi et al.,  
176 (2015). Briefly, 16S amplicons were pooled together and analyzed by Bioanalyzer (Agilent  
177 Technologies) to assess quality and size of amplicons. Following dilution, libraries were  
178 subjected to quantitative-PCR (qPCR) to ensure accurate quantification of purified amplicons.  
179 16S libraries were sequenced using an Illumina MiSeq (San Diego, CA, USA) platform at the  
180 University of Tennessee.

### 181 *2.4 Sequence analysis*

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

186 0.005% of the total data set was removed to limit the effect of spurious OTUs on analysis  
187 (Bokulich et al., 2013; Navas-Molina et al., 2013). All analyses were carried out after pooling the  
188 technical replicates and rarefying the samples to the same sequencing depth (~15,000 sequences)  
189 using QIIME and R version 3.2.1 (R Core Team, 2016). Differences between sediment samples  
190 were assessed using non-metric multidimensional scaling (nMDS) on Bray-Curtis dissimilarity  
191 matrices. For nMDS, a stress function was used to assess the goodness-of-fit of the ordination.  
192 The Adonis implementation of PerMANOVA (non-parametric permutational multivariate  
193 analysis of variance; Anderson, 2001) was used to estimate the proportion of variation in  
194 microbial communities attributed to sampling location (i.e. water depth).

195

## 196 **2. Results**

### 197 *3.1 Extracellular hydrolytic enzyme activity*

198 Potential activities of extracellular enzymes in surface sediments varied as a function of  
199 substrate type and water depth (Fig. 2). In general, peptidases had greater potential activities than  
200 the esterase and glycosidase. Specifically, leucyl aminopeptidase and alkaline phosphatase were  
201 approximately 100 times more active than all other enzymes across all sediment samples.  
202 Cellobiase was observed to have the lowest potential activity, which was near or below the  
203 detection limit in all sediment samples. Extracellular enzyme activity appeared to decrease with  
204 increasing water depth for some enzymes but not for others. For example, leucyl aminopeptidase,  
205  $\beta$ -xylosidase and alkaline phosphatase activity was ~1.5 to 2.5 times higher for sediments  
206 collected from shallower water depths (833 and 1210 m) compared to deeper depths (1818 and  
207 2226 m). However, this was not the case for gingipain, cellobiase and  $\beta$ -N-

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

### 210 3.2 Microbial diversity and taxonomic composition

211 Illumina-based sequencing of 16S rRNA gene amplicons recovered a total of 7,671,636  
212 (2181 OTUs) 16S sequences with an average length of 253 bp. OTU rarefaction curves  
213 approached a saturation plateau and Good's coverage ranged from 98% to 99%, indicating that  
214 the rarified sequencing depth represented the majority of 16S rRNA sequences in each sample.  
215 Species richness and diversity indices were calculated for each sediment sample (Fig. 3, Table  
216 S1). Microbial diversity decreased with increasing water depth according to both the number of  
217 OTUs observed as well as the Shannon and Simpson's indices. Diversity was similar for  
218 sediment samples collected from water depths of 833 and 1210 m and decreased rapidly for  
219 sediment samples from 1818 and 2226 m. Similarly, Chao1 values revealed that species richness  
220 was significantly lower at deeper water depths of 1818 and 2226 m (T-test, P values < 0.05).

221 A total of 38 different phyla were detected across all sediment samples (Fig. S1) with  
222 *Proteobacteria* (50% of assigned reads on average) being the most abundant, followed by  
223 *Acidobacteria* (12%). Within *Proteobacteria*, the majority of sequences were assigned to the  
224 classes, *Gammaproteobacteria* (16%), *Alphaproteobacteria* (16%) and *Deltaproteobacteria*  
225 (11%) (Fig. S1a). *Gammaproteobacteria* dominated across all sediment samples and accounted  
226 for a greater proportion of sequences at deeper water depths of 1818 and 2226 m where it  
227 comprised of 24 to 49% of recovered reads. Within *Gammaproteobacteria*, the dominant orders  
228 were *Thiotrichales* (6%) and *Xanthomonadales* (7%). The relative abundance of  
229 *Xanthomonadales* varied with water depth and comprised a greater proportion of sequences at  
230 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

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

263         Since the majority of microbes in marine sediments have not yet been cultivated (Lloyd et  
264 al., 2018), it is difficult to determine the degree to which microbes in this environment are  
265 metabolically active. Leucyl aminopeptidase is a commonly measured hydrolytic enzyme  
266 secreted by heterotrophic microbes and has shown experimentally to be responsive to the  
267 addition of amino acids (Zeglin et al., 2007). Since amino acids and amino sugars contain both  
268 carbon and nitrogen, it is likely that this enzyme serves a dual role by allowing microbes to  
269 acquire both carbon and nutrients. Therefore, leucyl aminopeptidase has been used as an  
270 indicator of heterotrophic activity (Taylor et al., 2003). To date, only a handful of studies have  
271 reported the activity of extracellular enzymes in deep-sea environments (Coolen & Overmann,  
272 2000; Dell'Anno et al., 2000; Coolen et al., 2002). In the present study, the specific activities of  
273 L-leucyl aminopeptidase ranged from 2 to 6  $\mu\text{mol g}^{-1} \text{hr}^{-1}$  across all sediment samples. These  
274 rates are much higher than those observed in deep-sea basalt rocks collected from the Loihi  
275 Seamount in which L-leucyl aminopeptidase activity rates that were  $< 0.0007 \mu\text{mol g}^{-1} \text{hr}^{-1}$   
276 (Meyers et al., 2014). Similarly, the rates of leucyl aminopeptidase measured in the present study

277 were substantially higher than those observed in deep-sea surface sediments collected from a water  
278 depth of 2150 m in the eastern Mediterranean Sea (Coolen & Overmann, 2000), that is  
279 considered to be one of the most oligotrophic regions in the world (Psarra et al., 2000). Leucyl  
280 aminopeptidase activity in surface sediment from the eastern Mediterranean Sea was determined  
281 to be  $\sim 0.002 \mu\text{mol g}^{-1} \text{hr}^{-1}$  (Coolen & Overmann, 2000);  $\sim 3$  orders of magnitude lower than the  
282 activities reported here. Recent work by Bird et al., (2019) measured extracellular enzyme  
283 activity in Baltic Sea sediments collected during IODP Expedition 347 where the leucyl  
284 aminopeptidase activity peaked at  $\sim 17$  meters below seafloor (mbsf) and was observed to be  
285  $20,000 \mu\text{mol g}^{-1} \text{hr}^{-1}$ , significantly higher than those measured here. The Baltic Sea is a fairly  
286 eutrophic system, thus, it would be expected that these sediments are more energy-rich and  
287 would have higher activity rates of microbial activity compared to typical deep-sea sediments.

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

300 peptide bonds) was low relative to that of leucyl aminopeptidase, an exopeptidase which cleaves  
301 enzymes one residue at a time from the N terminus of proteins (Lloyd et al., 2013; Steen et al.,  
302 2016). Ratios of endopeptidase : exopeptidase activities are fairly stable within environments but  
303 highly variable among environments (Steen et al., 2013; Mullen et al., 2018). It is not clear what  
304 environmental parameters control these ratios.

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

322 Previous studies have found that the number of species that produce extracellular  
323 hydrolytic enzymes is a small fraction of the entire microbial community (Langenheder et al.,  
324 2006; Logue et al., 2016; Rivett and Bell, 2018). This indicates that the ability to produce these  
325 enzymes is a functionally dissimilar, rather than functionally redundant, trait such that changes in  
326 taxonomic composition will result in variations in hydrolytic activity. We observed that  
327 extracellular enzyme activity was fairly stable for sediment samples collected from the same  
328 water depth even though in most cases sediment samples from the same water depth did not  
329 closely cluster together based on their taxonomic similarity (Fig. S2). These results point to some  
330 degree of functional redundancy with respect to extracellular enzymes which is consistent with  
331 previous work that has characterized both the composition and enzymatic function of microbial  
332 communities (Wohl et al., 2004; Frossard et al., 2012; D'Ambrosio et al., 2014). Functional  
333 redundancy with respect to enzymatic capabilities occurs when several taxonomic groups have  
334 the ability to use a substrate such that the substrate capacity of a microbial community may be  
335 due to subtle changes among less dominant populations. The extent of functional redundancy  
336 appears to vary among microbial communities which makes it difficult to decipher the  
337 relationship between taxonomic composition and enzymatic capabilities.

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

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

355 **Author Contributions**

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

360

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370

371

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- 550

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

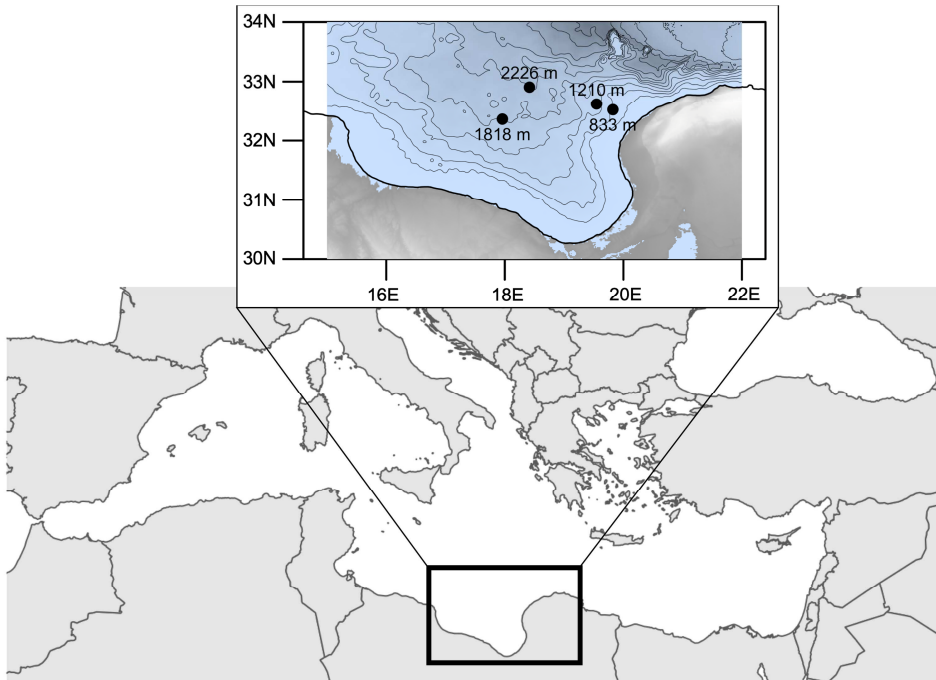
Water Depth (m)	Latitude	Longitude	Temp (°C)	Salinity (practical salinity units)	Oxygen Concentration (mg L <sup>-1</sup> )	Ammonia (µM)	Nitrate (µM)	Pressure (MPa)
833	32° 31' 22.720" N	19° 49' 24.961" E	13.7	38.7	5.9	0.04	5.10	8.37
1210	32° 37' 10.386" N	19° 32' 50.890" E	13.7	38.7	4.1	<0.02	4.80	12.16
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
$\beta$ -Glucosidase	4-MUB- $\beta$ -D-glucopyranoside	glycosidase	Carbon-acquire enzyme (cellulose degradation)
Cellobiase	4-MUB- $\beta$ -D-cellobioside	glycosidase	Carbon-acquiring enzyme (cellulose degradation)
$\beta$ -N-Acetylglucosaminidase	4-MUB N-acetyl $\beta$ -D-glucosaminide	glycosidase	Carbon-acquiring enzyme
$\beta$ -Xylosidase	4-MUB- $\beta$ -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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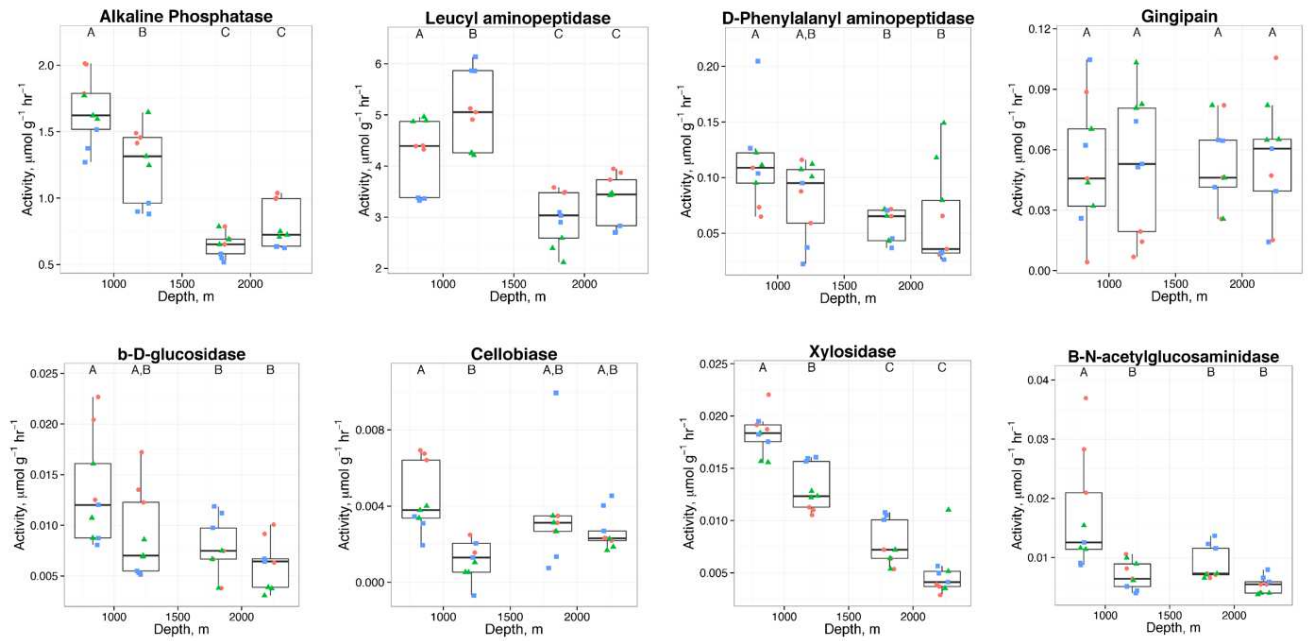


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557 **Figure 1.** Map depicting sampling stations in the central Mediterranean Sea. Sediment samples  
558 were collected from four stations that ranged in water depth from 833 m to 2226 m (shown as  
559 black circle within the zoomed-in region).

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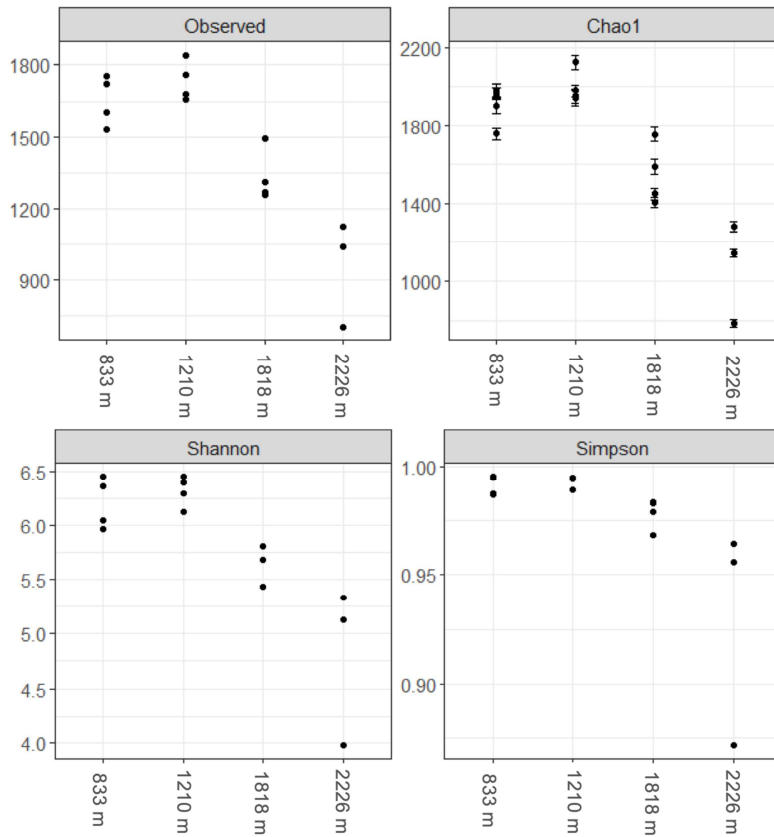
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**Figure 2.** Potential extracellular enzyme activities of observed in central Mediterranean Sea sediments. Activities were measured for eight different extracellular enzymes across four water depths. Three technical replicates were each measured from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

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**Figure 3.** Estimates of alpha-diversity metrics for microbial communities in central 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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