Nature and kinetics of extracellular peptidases in subsurface sediments of the White Oak River estuary, NC

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Abstract

Microbial communities inhabiting subsurface sediments contain abundant heterotrophs, which oxidize organic matter to obtain carbon and energy. Subsurface sediments contain very low concentrations of bioavailable compounds, and it is not clear what fraction of sedimentary organic matter heterotrophs are able to access. To gain a more mechanistic understanding of heterotrophy in subsurface sediments of the White Oak River, NC, we examined the genetic potential for extracellular peptidase production encoded within metagenome-assembled genomes, and experimentally assayed the kinetics of a wide range of extracellular peptidases in bulk sediments. We identified genes coding for at least 15 classes of extracellular peptidases and observed enzyme-catalyzed hydrolysis of 11 different peptidase substrates. Potential activities (V_{max}) of extracellular peptidases decreased downcore, but cell-specific V_{max} was relatively constant and similar to values observed in seawater phytoplankton blooms. Half-saturation constants also decreased downcore, and the relative contribution of enzymes relevant to degraded organic matter increased with increasing depth. Together, these results suggest a subsurface community that accesses organic matter using mechanisms that are basically similar to those in surface environments, but which is adapted to the highly degraded organic matter that is present in the subsurface.

Introduction

Marine sediments are one of the largest microbial environments on earth (Kallmeyer *et al.*, 2012). Many sedimentary microbes appear to be heterotrophs, slowly metabolizing organic matter (Jørgensen and Marshall, 2016; Biddle *et al.*, 2006), but the mechanisms by which these heterotrophs access old, unreactive organic carbon remain poorly characterized.

In surface environments, where photosynthesis fuels carbon fixation, heterotrophic microorganisms gain energy from a combination of small molecules (<600-1000 Da), which can be taken up directly via general uptake porins (Benz and Bauer, 1988) and macromolecules, which must be broken down outside of the cell by extracellular enzymes. Because most freshly-produced organic matter is macromolecular and large molecules tend to be more bioavailable than small ones (Benner and Amon, 2015), the nature and activity of extracellular enzymes present in surface environments is a major control on the rate of microbial carbon oxidation in such environments.

It is not clear whether microbial extracellular enzymes play the same role in subsurface sediments. Extracellular peptidase activity has been identified in sapropels up to 389 cm below seafloor (cmbsf) in the eastern Mediterranean Sea (Coolen and Overmann, 2000; Coolen *et al.*, 2002), in sediment from 600-630 cmbsf in Aarhus Bay sediments (Lloyd *et al.*, 2013b) and in the interior of seafloor basalts at the Loihi seamount (Jacobson Meyers *et al.*, 2014). However, the nature and relative importance of extracellular enzymes in subsurface environments remains poorly constrained because these studies examined few samples, and only limited enzymatic classes and sample numbers were assayed. It is possible that, like heterotrophs in surface environments,

65 heterotrophs in subsurface environments mainly gain access to organic carbon via
66 extracellular enzymes. On the other hand, some of the unique aspects of subsurface
67 sediments suggest that extracellular enzymes might not be an effective strategy to obtain
68 carbon or energy. First, subsurface sediments contain markedly fewer bioavailable
69 compounds such as amino acids and sugars than do surface sediments (Burdige, 2007).
70 Therefore, these compounds may be insufficiently abundant to be viable heterotrophic
71 substrates. Second, in order for the production of extracellular enzymes to be part of a
72 viable metabolic strategy, each enzyme must, over its lifetime, provide the cell with at
73 least as much carbon or energy as was required to synthesize the enzyme (Vetter *et al.*,
74 1998; Allison, 2005; Schimel and Weintraub, 2003). In subsurface sediments, where
75 metabolic rates may be orders of magnitude slower at the surface, enzyme lifetimes
76 would need to be correspondingly longer to become 'profitable'. Since enzyme lifetimes
77 are finite, there must exist a community metabolic rate below which extracellular enzyme
78 lifetimes are too short to become profitable. Unfortunately that limit is difficult to
79 quantify because enzyme lifetimes in any environment are poorly constrained (e.g., Steen
80 and Arnosti, 2011).
81 It is also possible that extracellular enzymes are not the primary mechanism by
82 which heterotrophs access sedimentary organic matter. More exotic mechanisms, such as
83 abiotic liberation by reactive species formed from radioactive decay of naturally-present
84 radioisotopes (e.g. Blair *et al.*, 2007) are conceivable, but have not been demonstrated.
85 The goals of this work are to determine whether subsurface heterotrophic communities
86 make substantial use of extracellular enzymes to access organic matter, and if so, to
87 characterize the set of enzymes used.

88 We investigated genes for extracellular enzymes and the activities of
89 corresponding enzymes in sediments of the White Oak River, NC by estimating the
90 presence of peptidase families in available metagenomic sequencing data and
91 complementing this by measuring the hydrolysis rates of eleven potential peptidase
92 substrates. This site was chosen because the porewater geochemistry and microbiology of
93 these sediments has been well-characterized (Martens and Goldhaber, 1978; Kelley *et al.*,
94 1990; Baker *et al.*, 2015; Lazar *et al.*, 2016; Lloyd *et al.*, 2011) and because they contain
95 abundant Bathyarchaeota and Marine Benthic Group D archaea, which appear to be
96 capable of metabolizing detrital organic matter (Kubo *et al.*, 2012; Lloyd *et al.*, 2013b;
97 Meng *et al.*, 2014). We focused on peptidases because protein degradation appears to be
98 an important metabolism for some subsurface archaea (Lloyd *et al.*, 2013b) and because
99 peptidases were more active than other enzymes in similar environments (Coolen and
100 Overmann, 2000; Jacobson Meyers *et al.*, 2014). Because environmental samples contain
101 a wide range of distinct peptidases at variable activities (Obayashi and Suzuki, 2005;
102 Steen and Arnosti, 2013) we measured the hydrolysis of eleven different substrates that
103 may be hydrolyzed by structurally and genetically diverse extracellular peptidases. By
104 measuring potential activities (i.e., the capacity of the enzyme to catalyze hydrolysis if
105 substrate concentrations were not limiting) and substrate affinities of microbial
106 extracellular enzymes, we illuminated some of the mechanisms by which subsurface
107 heterotrophic communities access organic carbon.

Materials and Methods

STUDY SITE

Samples were collected from Station H in the White Oak River Estuary, 34° 44.490' N, 77° 07.44' W, first described by Gruebel and Martens (1984). The White Oak River Estuary occupies a drowned river valley in the coastal plain of North Carolina. Station H is characterized by salinity in the range of 10 to 28 and water depth on the order of 2 m. The flux of ΣCO_2 across the sediment-water interface was 0.46 ± 0.02 mmol m^{-2} hr^{-1} (measured in May of 1987), primarily due to organic carbon oxidation via sulfate reduction, and the sediment accumulation rate averages 0.3 cm yr^{-1} (Kelley *et al.*, 1990). Total organic carbon content is approximately 5%. For this study, push cores of 40-85 cm were collected from Station H by swimmers on May 28, 2013, June 14, 2014, and October 22, 2014. In 2013, cores were transported to the nearby Institute of Marine Sciences (University of North Carolina) at Morehead City, where they were sectioned and processed for enzyme activities, porewater geochemistry, and cell counts within 6 hours of sample collection. Porewater sulfate in 2013 was depleted by 43.5 cm, and methane peaked at 79.5 cm (Fig. S1). In 2014, cores were transported on the day of sampling to the University of Tennessee, Knoxville, stored at 4 °C, and processed for enzyme activities the following day. Samples for metagenomic analysis were collected separately in October 2010 from three sites (sites 1, 2, and 3, as previously described by Baker et al (2015)), all of which are within 550 m of Station H.

128 METAGENOMIC ANALYSIS

129 To resolve the taxonomic distribution of extracellular peptidases we searched a
130 pre-existing White Oak River *de novo* assembled and binned metagenomic dataset (Table
131 S2; Baker *et al.*, 2015) for genes that were assigned extracellular peptidase functions.
132 These assignments were based on best matches to extracellular peptidases in KEGG,
133 pfam, and NCBI-nr (non-redundant) databases using the IMG annotation pipeline
134 (Markowitz et al., 2014). Genes were additionally screened for signal peptidase motifs
135 using the following programs: PrediSI setting the organism group to gram-negative
136 bacteria (Hiller *et al.*, 2004), PRED-Signal trained on archaea (Bagos *et al.*, 2009), the
137 standalone version of PSORT v.3.0 trained against archaea (Yu *et al.*, 2010), and
138 SignalIP 4.1 using gram-negative bacteria as the organism group (Petersen *et al.*, 2011).
139 All programs were used with default settings if not stated otherwise. Results are provided
140 in Supplementary Table 1.

141 In total, binned genomes from three different depth zones of White Oak River
142 sediments were examined. The sulfate-rich zone (SRZ) genomes were obtained from sites
143 2 and 3 core sections 8-12 and 8-10 cm, respectively. The sulfate-methane transitions
144 zone (SMTZ) genomes were recovered from site 2 and 3 and depths of 30-32 cm and 24-
145 28 cm. The methane-rich zone (MRZ) was from site 1 and 52-54 cm. Many of these
146 genes were binned to Bacteria (Baker et al. 2015) and Archaea community members
147 (Baker *et al.*, 2016; Lazar *et al.*, 2016; Seitz *et al.*, 2016). Taxonomic assignments of
148 peptidases identified in the community were based on this binning information. However,
149 since not all of the peptidases were binned, we used top matches to NCBI to identify the
150 unbinned genes. The majority of the Archaea present in the shallow (8-12 cm) sulfate-

151 rich zone were confidently binned, thus were used to determine the relative contributions
152 of archaeal extracellular peptidases (Fig 1b). A smaller proportion (68% of SRZ, 24% of
153 SMTZ, and 27% of MRZ) of the bacterial peptidase genes were confidently binned,
154 therefore, classification was based on top BLAST hits to NCBI. These classifications
155 were then refined using the bin assignments.

156 ENZYME ASSAYS

157 Enzyme assays were performed using different protocols in 2013 versus 2014. In
158 2013, enzyme assays were performed according to a protocol similar to the one described
159 in Lloyd et al (2013b). Cores were sectioned into 3 cm intervals. The following intervals
160 were selected for enzyme assays: 0-3 cm, 3-6 cm, 27-30 cm, 57-60 cm, and 81-83 cm.
161 Each section was homogenized, and approximately 0.5 ml wet sediment was transferred
162 into separate 5 ml amber glass serum vials, which had been pre-weighed and preloaded
163 with 4 ml anoxic artificial seawater (Sigma Sea Salts, salinity = 15, pH=7.5) Samples
164 were weighed again to determine the precise mass of wet sediment added, and then an
165 appropriate quantity of 20 mM peptidase substrate stock dissolved in DMSO was added,
166 up to 90 μL, for final substrate concentrations of 0, 25, 50, 75, 100, 200, or 300 μM.
167 Triplicate incubations with 400 μM Arg-AMC, Gly-AMC, Leu-AMC and Gly-Gly-Arg-
168 AMC were also created, but these were omitted for Ala-Ala-Phe-AMC and Boc-Phe-Val-
169 Arg-AMC because the latter two substrates are considerably more expensive. Each serum
170 vial was vortexed, briefly gassed with N_2 to remove oxygen introduced with the sample,
171 and approximately 1.3 ml slurry was immediately removed, transferred to a
172 microcentrifuge tube, and placed on ice to quench the reaction. The precise time of
173 quenching was recorded. This was centrifuged at 10,000 × *g* within approximately 15

174 minutes. The supernatant was transferred to a methacrylate cuvette and fluorescence was
175 measured with a Turner Biosystems TBS-380 fluorescence detector set to "UV"
176 (λ_{ex}=365-395 nm, λ_{em}=465-485 nm). Samples were then incubated at 16 °C,
177 approximately the in situ temperature, and the sampling procedure was repeated after
178 approximately 3 hours. The rate of fluorescence production was calculated as the increase
179 in fluorescence for each sample divided by the elapsed time between sample quenching.
180 Killed controls were made using homogenized, autoclaved sediments from 35-45 cmbsf.
181 However, we note that autoclaving does not destroy sediment enzymes because sorption
182 to mineral surfaces stabilizes enzyme structure, vastly increasing their ability to maintain
183 a functional conformation at high temperatures (Stursova and Sinsabaugh, 2008; Carter *et*
184 *al.*, 2007; Schmidt, 2016). We therefore used the autoclaved samples as a qualitative
185 control for the null hypothesis that enzymes were responsible for none of the observed
186 substrate hydrolysis, rather than as a quantitative method to distinguish enzymatic
187 substrate hydrolysis from potential abiotic effects. In some sediments, a large fraction of
188 fluorophore can sorb to particles, requiring a correction to observed fluorescence (Coolen
189 *et al.*, 2002; Coolen and Overmann, 2000), but we observed negligible sorption of
190 fluorophore to the White Oak River sediments.
191 In 2014, enzymes were assayed using a protocol based on the approach of Bell et
192 al (2013), which was designed for soil enzyme assays. In this approach, peptidase
193 substrates were mixed with sediment-buffer slurries in 2-mL wells of a deep-well plate.
194 These plates were periodically centrifuged and 250 µL aliquots of supernatant were
195 transferred into a black 96-well microplate. Fluorescence was read using a BioTek
196 Cytation 3 microplate reader (λ_{ex} = 360 nm, λ_{em} = 440 nm). Results from this method

proved considerably noisier than the single-cuvette method used in 2013, so kinetic parameters (V_{max} and K_m) were not calculated for these data. Nevertheless, results were qualitatively similar to those from 2013, and we have reported V_{max} from 2014 as v_0 measured at 400 μM substrate concentration, which was saturating. In June 2014, the following substrates were assayed: AAF-AMC, Arg-AMC, Boc-VPR-AMC, D-Phe-AMC, Gly-AMC, Leu-AMC, L-Phe-AMC, Orn-AMC, Z-Phe-Arg-AMC, and Z-Phe-Val-Arg-AMC. In October 2014, L-Phe-AMC, D-Phe-AMC, and Orn-AMC were assayed according to the same protocol in 3-cm core sections at 1.5, 4.5, 7.5, 10.5, 19.5, 22.5, 25.5, 28.5, 34.5, 37.5, 40.5, 43.5, 49.5, 52.5, 58.5, and 61.5 cmbsf.

GEOCHEMICAL AND MICROBIOLOGICAL MEASUREMENTS

Sediment porosity was measured by mass after drying at 80 °C, according to the equation

$$\phi = \frac{m_w/\rho_w}{m_w/\rho_w + \frac{m_d - S \times m_w/1000}{\rho_{ds}}}$$

Here, m_w represents mass lost after drying, ρ_w represents the density of pure water, m_d represents the mass of the dry sediment, S represents salinity in g kg^{-1}, and ρ_{ds} represents the density of dry sediment (assumed to be 2.5 g cm^{-3}). Porewater sulfate concentrations were measured using a Dionex Ion Chromatograph (Sunnyvale, CA) in porewater that was separated by centrifugation in 15 ml centrifuge tubes at 5000 × *g* for 5 minutes, filtered at 0.2 μm, and acidified with 10% HCl. Methane was measured using 3 ml sediment subsamples that were collected from a cutoff syringe, entering through the side

218 of a core section, immediately after core extrusion. Subsamples were deposited
219 immediately in a 20 ml serum vial containing 1 ml, 0.1 M KOH. These were immediately
220 stoppered and shaken to mix sediment with KOH. Methane was later measured by
221 injecting 500 µl of bottle headspace into a GC-FID (Agilent, Santa Clara, CA) using a
222 headspace equilibrium method (Lapham *et al.*, 2008).

223 CELL ENUMERATION

224 Cells were enumerated by direct microscopic counts. One mL of sediment was placed in
225 a 2-mL screw-cap tube with 500 µl of 3% paraformaldehyde in phosphate buffered saline
226 (PBS), in which it was incubated overnight before being centrifuged for 5 minutes at
227 3000 × *g*. The supernatant was removed and replaced with 500 µl of PBS, vortexed
228 briefly and centrifuged again at 3000 × *g*. The supernatant was subsequently removed and
229 replaced with a 1:1 PBS:ethanol solution. Sediments were then sonicated at 20% power
230 for 40 seconds to disaggregates cells from sediments and diluted 40-fold into PBS prior
231 to filtration onto a 0.2 µm polycarbonate filter (Fisher Scientific, Waltham, MA) and
232 mounted onto a slide. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and
233 enumerated by direct counts using a Leica Epifluorescence Microscope.
234

235 GEOCHEMICAL MODELING

236 Organic carbon remineralization rates as a function of depth were estimated by
237 applying a multi-component reaction-transport model to depth distributions of sulfate and
238 methane concentration. The model is based on equations described in Boudreau (1996)
239 and includes only sulfate reduction and methane production due to lack of data regarding
240 oxic and suboxic processes. Thus the model is limited to depths greater than 4.5 cm

241 where sulfate reduction and methane production are the dominant processes, and
242 bioirrigation and bioturbation may be assumed to be negligible. The organic matter
243 remineralization rate is parameterized using the multi-G model first proposed by
244 Jørgensen (1978); a two-component model was sufficient to accurately simulate the
245 sulfate and methane data. For solutes, the upper boundary conditions were measured
246 values at 4.5 cm while the lower boundary conditions (200 cm) were set to zero-gradient.
247 The flux of reactive organic carbon to 4.5 cm was calculated from the sulfate flux across
248 the 4.5 cm horizon and an estimate of methane burial below the lower boundary (the
249 methane flux at the upper boundary was observed to be zero), with an assumed oxidation
250 state of reactive carbon of -0.7. The model contains four adjustable parameters that are
251 set to capture the major details of measured sulfate and methane data: first-order rate
252 constants for both fractions of the reactive carbon pool; the partitioning factor for both
253 fractions, and the rate constant for methane oxidation.

254 Results and Discussion

255 A total of 3739 genes encoding extracellular peptidases were identified among
256 metagenomes from the three depth zones examined, including 685 from SRZ, 1994 from
257 SMTZ, and 1060 from MRZ. Of the genes encoding for peptidases, 0-71% (depending on
258 class of peptidase, algorithm and sediment depth) contained a signal peptide and are
259 likely secreted by the SEC-dependent transport system (Supplementary Table 3). Among
260 the genes with signal peptidases, zinc carboxypeptidases, peptidases of class C25 and
261 genes of the clostripain family are most likely to be secreted. Alternative secretion
262 pathways, which do not rely on signal peptides and therefore would not be identified by

263 the algorithms we used, are also known to exist. These include Sec-independent secretion
264 systems (Bendtsen *et al.*, 2005) and release of cytoplasmic enzymes by cell lysis. The
265 relative importance of these pathways in sediments has not been assessed. At all three
266 depths, peptidases of class C25, belonging to the gingipain family, were the most
267 abundant extracellular peptidases (Fig 1a). Gingipains are endopeptidases (i.e., enzymes
268 that cleave proteins mid-chain rather than from the N- or C-termini) with strong
269 specificity for the residue arginine on the N-terminal side of the scissile bond (Rawlings
270 and Barrett, 1999). Bathyarchaeota, which are abundant in sediments of the White Oak
271 River, use extracellular gingipain (among other peptidases) to degrade detrital protein-
272 like organic matter (Kubo *et al.*, 2012; Lloyd *et al.*, 2013b). Genes annotated as encoding
273 extracellular methionine aminopeptidases and zinc carboxypeptidases were also
274 abundant. The composition of protein families was generally consistent with depth, but
275 genes for clostripain (another endopeptidase with preference for Arg N-terminal to the
276 scissile bond) and S24 peptidases (a regulatory peptidase involved in the SOS stress
277 response) were slightly enriched in the SMTZ.

278 In SRZ, Bacteria accounted for 61% of genes for extracellular peptidases for
279 which a lineage could be assigned, increasing to 69% in MRZ (Fig 1b). Archaeal
280 peptidases decreased from 39% in SRZ to 30% in MRZ, while Eukaryota accounted for
281 <1.5% at all depths. Consistent with this distribution, bacteria make up ca. 40-60% of
282 cells in White Oak River sediments as determined by CARD-FISH counts, with no
283 pronounced depth trend (Lloyd *et al.*, 2013a). Since the majority of the archaeal
284 peptidases in the SRZ were assigned to genomic bins, we were able to accurately classify
285 them. Interestingly, over half of the archaeal SRZ peptidases belong to marine benthic

286 group D (MBG-D) genomic bins (Fig 1c)(Lazar *et al.*, 2016). Additionally, a large
287 portion of the peptidases in the SRZ belong to newly described Archaea belonging to the
288 Asgard superphylum (Zaremba-Niedzwiedzka *et al.*) including Lokiarchaeota (Spang *et*
289 *al.*, 2015) and Thorarchaeota (Seitz *et al.*, 2016). Little is known about the ecological
290 roles of these novel Archaea. It has recently been shown that they contain metabolic
291 pathways for the degradation of proteins and acetogenesis (Seitz *et al.*, 2016). Sources of
292 bacterial peptidases varied less with depth than sources of archaeal peptidases, with
293 Proteobacteria, Bacteroidetes, and Firmicutes among the dominant phyla (Fig 1d). 13-
294 20% of extracellular peptidase genes belonged to bacteria but could not be assigned a
295 phylum, and 19-22 phyla contributed extracellular peptidases at each depth.
296 Deltaproteobacteria and Gammaproteobacteria dominated the SRZ community at the
297 White Oak River (Baker *et al.*, 2015), and accordingly the largest portion of the
298 extracellular peptidases also belong to them. These phylogenetic groups decrease with
299 depth in the SMTZ and MRZ, however, Deltaproteobacteria contribute a large portion of
300 extracellular peptidases downcore. Although these groups are commonly thought to rely
301 on sulfur and nitrogen respiration, these genomes were shown to contain metabolic
302 pathways for the degradation and fermentation of organic carbon (Baker *et al.*, 2015).
303 Additionally, several phyla thought to be involved in fermentation of detrital carbon
304 including *Clostridia*, *Bacteroidetes*, *Planctomycetes*, and the candidate phyla radiation
305 (CPR) constitute larger portions of the peptidases in the SMTZ and MRZ.
306 Bioinformatic tools are a powerful way to investigate the potential of microbial
307 communities to oxidize complex organic molecules, but these tools do not provide
308 information about the expression level of genes or the in situ activities of gene products

309 and annotation algorithms often fail to identify the precise function of hydrolases,
310 particularly in deeply-branching lineages (e.g., Michalska *et al.*, 2015). Thus, we
311 measured the potential activities of a wide range of extracellular peptidases in the WOR
312 sediments. In 2013 (when the assay protocol used was more sensitive), all six peptidase
313 substrates tested were hydrolyzed faster in untreated sediments than in autoclaved
314 controls (Fig 2, Fig S2). Kinetics of substrate hydrolysis were qualitatively consistent
315 with the Michaelis-Menten rate law,
316
317 $v_0 = (V_{max} + [S])/(K_m + [S])$,
318
319 which is characteristic of purified hydrolases as well as mixtures of isofunctional
320 enzymes in environmental samples (Steen *et al.*, 2015; Sinsabaugh *et al.*, 2014).
321 Together, these lines of evidence show that the observed substrate hydrolysis was due to
322 extracellular peptidases rather than abiotic factors. Combining data from all three
323 sampling dates, unambiguous hydrolysis of eleven different peptidase substrates was
324 observed.
325 The diverse substrates used in this study were apparently cleaved by a wide
326 variety of peptidases, including aminopeptidases (peptidases that cleave an N-terminal
327 amino acid from a protein) and endopeptidases (peptidases that cleave internal peptide
328 bonds). Peptide bonds adjacent to a diverse set of amino acid residues were cleaved,
329 including glycine (the smallest amino acid), phenylalanine (among the largest amino
330 acids), arginine (positively charged at porewater pH) and leucine (uncharged,
331 hydrophobic). Individual extracellular peptidases can often accept a fairly broad range of

substrates, and sometimes a substrate may primarily be hydrolyzed by an enzyme that exhibits maximal activity towards a different substrate. For instance, in pelagic samples from Bogue Sound, an estuary in North Carolina, the substrate Leu-AMC, which is putatively a substrate for leucine aminopeptidase, was hydrolyzed more by arginine aminopeptidase than by leucine aminopeptidase (Steen *et al.*, 2015). Nevertheless, the large diversity of substrates that were hydrolyzed in this study suggests a diverse set of extracellular peptidases present in sediments, as has previously been observed in pelagic samples (Obayashi and Suzuki, 2008, 2005; Steen and Arnosti, 2013).

Bulk potential enzyme activities (V_{max} values) decreased with increasing depth (Fig 3a). However, no trend was evident in V_{max} values expressed per cell (Fig 3b), and V_{max} expressed relative to bulk organic carbon oxidation rate increased downcore by more than two orders of magnitude (Figs 3c-d). V_{max} per cell was approximately 100-200 amol cell^{-1} hr^{-1} throughout the core, comparable to previous measurements made in a surface sediments (2-100 amol cell^{-1} hr^{-1}) and surface seawater (mostly less than 100 amol cell^{-1} hr^{-1}, but with some measurements up to 10 nmol cell^{-1} hr^{-1}) (Vetter and Deming, 1994). Modeled organic carbon oxidation due to sulfate reduction and methane production decreased from 9315 µmol C (g wet sediment)$^{-1}$ hr^{-1} at 4.5 cmbsf (the top of the model domain) to 18 µmol C (g wet sediment^{-1}) hr^{-1} at 82.5 cmbsf, a decrease of a factor of 518. The sum of V_{max} of all peptidases measured in 2013 decreased from 94.7 µmol g sed^{-1} hr^{-1} at 1.5 cmbsf to 12.8 µmol g sed^{-1} hr^{-1} at 82.5 cmbsf depth, a decrease of a factor of 7.4. The value of the V_{max}:OC oxidation rate ratio is sensitive to the precise set of enzyme included in the sum (it is likely that some peptidases exist in the sediment that were not measured, and that some promiscuous enzymes are double-counted because they

355 hydrolyze more than one substrate), but the trend is clear: as sediment depth increased,
356 the potential activity of extracellular peptidases increased faster than the actual rate of
357 organic carbon oxidation. V_{max} is a proxy for the concentration of enzymes in an
358 environment, so the observed increase in V_{max} relative to OC oxidation rate suggests that
359 subsurface microbial communities produced similar quantities of enzyme per cell as
360 surface communities, but that those enzymes returned less bioavailable organic matter,
361 presumably due to lower substrate concentrations.

362 In the sense of bulk activities, therefore, subsurface heterotrophic communities in
363 WOR seem to be similar their surface counterparts in terms of reliance on extracellular
364 enzymes to access organic matter, although metabolisms are slower in the subsurface.
365 However, there are indications from enzyme kinetics and activities of specific enzymes
366 that subsurface communities are specialized for their environment. Peptidase K_m values
367 decreased with increasing depth (Fig 4, Fig S3, ANCOVA, $F(5, 22)=4.44$, $p < 0.05$).
368 When substrate concentrations are considerably less than K_m, as they likely are in
369 subsurface sediments, *in situ* substrate hydrolysis rates are controlled more by K_m than by
370 V_{max} (Steen and Ziervogel, 2012; Cornish-Bowden, 2012), so decreasing K_m values may
371 be an adaptive response to low bioavailable substrate concentrations in subsurface
372 sediments (Sinsabaugh *et al.*, 2014).

373 The trends in K_m warrant some discussion of how K_m values are measured and
374 interpreted. As with all environmental enzyme assays, our measurements relied on
375 measuring the rate of reaction of an artificial substrate that was added to the sample. The
376 sample also contained some quantity of natural substrate (i.e., proteins or peptides). This
377 naturally-present substrate can be viewed as a competitive inhibitor of the added artificial

378 substrate (Cornish-Bowden, 2012). As a consequence, the K_m we measure ($K_{m,app}$) is
379 actually the sum of the true enzyme K_m plus the concentration of *in situ* enzyme substrate
380 ([S_{is}]); $K_{m,app} = K_m + [S_{is}]$. It is therefore possible that the observed decrease in $K_{m,app}$
381 actually reflects a change in the concentration of natural peptidase substrates with depth,
382 rather than a change in substrate affinity of peptidases. [S_{is}] is extremely difficult to
383 measure (or even to define precisely) because it reflects the sum of the concentrations of
384 all of the individual molecules which can act as substrate for a given peptidase, weighted
385 by the degree to which the peptidase is capable of accessing each substrate. [S_{is}] almost
386 certainly decreased downcore, since a decrease in the concentration of enzymatically-
387 hydrolyzable, protein-like organic matter is a diagnostic feature of aged organic matter
388 (Amon *et al.*, 2001; Dauwe *et al.*, 1999). However, it is very unlikely that [S_{is}] could have
389 decreased enough to cause more than a negligible fraction of the observed decreases in
390 K_m, which were ranged from 110 μM (GGR-AMC) to 990 μM (AAF-AMC). Sediments
391 of Aarhus Bay, which are similar to those of the White Oak River in terms of organic
392 matter content and grain size, are characterized by porewater dissolved combined amino
393 acid concentrations in the range of 50-150 μM L^{-1} (Pedersen *et al.*, 2001). Only a fraction
394 of that dissolved protein-like material can act as substrate for any given peptidase. 10-
395 40% of total combined amino acids in sediments were accessible to a peptidase which
396 was specifically selected because of its ability to hydrolyze diverse proteins (Dauwe *et*
397 *al.*, 1999), and a smaller fraction may be available to the individual peptidases measured
398 in this study. Furthermore, K_m values were measured in a 9:1 buffer:sample slurry, so
399 [S_{is}] in the slurry would be one-tenth of *in situ* [S_{is}]. By multiplying assumed porewater
400 substrate concentrations (from Aarhus Bay) by the fraction that is enzymatically

401 hydrolysable and dividing by 10 to account for dilution in the slurry, we estimate that
402 changes in [S_{is}] could account for, at most, a 0.5-6 μM decrease in $K_{m,app}$. The remainder
403 of the 110-990 μM decrease must be due to changes in the true substrate affinity K_m,
404 indicating that subsurface enzymes hydrolyze low concentrations of protein considerably
405 more efficiently than surface enzymes.

406 The suite of specific peptidases that are active in deeper sediments also seems to
407 reflect adaptation to more degraded organic matter. In order to assess the degree to which
408 sedimentary extracellular peptidases target more recalcitrant organic matter, rather than
409 the extremely small pool of relatively labile organic matter, in 2014 we compared
410 potential activities of D-phenylalanyl aminopeptidase (D-PheAP) and ornithine
411 aminopeptidase (OrnAP) to those of L-phenylalanyl aminopeptidase (L-PheAP). D-
412 phenylalanine and ornithine are both markers for degraded organic matter. Most amino
413 acids are biosynthesized as L-stereoisomers. D-stereoisomers in sedimentary organic
414 matter can be produced via abiotic racemization of biomass (Bada and Schroeder, 1975;
415 Steen *et al.*, 2013) or by bacterial reprocessing of phytoplankton-derived OM (Pedersen
416 *et al.*, 2001; Kaiser and Benner, 2008; Lomstein *et al.*, 2006). Ornithine is non-
417 proteinogenic amino acid, which does not exist in high concentration in fresh biomass,
418 but which can be produced in sediments via deamination of arginine and therefore
419 indicates OM degradation (Hare, 1968; Lee and Cronin, 1984). L-phenylalanine is among
420 the most recalcitrant amino acids (Dauwe and Middelburg, 1998) but is more labile than
421 D-phenylalanine or ornithine, so we take L-PheAP activity as a marker of the
422 community's ability to access relatively fresh OM.

423 D-PheAP:L-PheAP and OrnAP:L-PheAP ratios increased significantly with depth
424 (Fig 5, D-PheAP:L-Phe-AP: $p<0.01$, $r^2=0.35$, n=16; OrnAP:L-PheAP: $p < 0.05$, $r^2=0.26$,
425 n=16), indicating that subsurface communities expressed peptidases that release amino
426 acids from relatively recalcitrant organic matter. These results do not indicate the actual
427 flux of organic matter to communities: V_{max} reflects a potential rate at saturating substrate
428 concentrations, not an *in situ* rate. Nevertheless, these results suggest that deeper
429 heterotrophic communities seek to access the larger pool of more recalcitrant organic
430 matter rather than the smaller pool of more labile organic matter.
431 In summary, genetic analysis and enzyme assays reveal that a diverse set of
432 extracellular peptidases are present throughout the top 83 cm of White Oak River estuary
433 sediments, and that these enzymes are produced by a wide range of Bacteria and Archaea.
434 Surface and subsurface communities express similar quantities of extracellular peptidase
435 per cell, but subsurface peptidases catalyze reactions much more slowly, likely because
436 concentrations of enzyme-labile substrates are much lower in the subsurface. This implies
437 selective pressure on subsurface communities to produce extracellular enzymes that
438 function more effectively in the presence of low concentrations of more-degraded organic
439 matter. Accordingly, subsurface enzymes showed higher affinity (lower K_m) than surface
440 enzymes, and the mix of subsurface enzymes shifted towards enzymes such as D-
441 phenylalanine aminopeptidase and ornithine aminopeptidase. These shifts are consistent
442 with community-level adaptation to heterotrophic communities to subsurface organic
443 matter, although we lack data as to whether that adaptation is caused by evolution of
444 surface communities as sediment is buried or selective survival of taxa that are more
445 capable of accessing degraded organic matter. Further analysis of the mechanisms by

which subsurface heterotrophs access sedimentary organic matter may yield continued insights into how heterotrophic microorganisms live in low-energy environments such as subsurface sediments.

Acknowledgements

We thank Michael Piehler for access to his laboratory facilities at the University of North Carolina Institute of Marine Sciences, the captain of the R/V Capricorn for sampling assistance, and Terry Hazen for use of lab equipment at the University of Tennessee. We thank Oliver Jeffers for reminding ADS that science is cool. Funding for KHM was provided by NSF grant DBI-1156644 to Steven W. Wilhelm. Funding for ADS was provided by NSF grant OCE-1431598 and a C-DEBI subaward. This work is C-DEBI Contribution number <<to be determined.>>

Conflict of interest

The authors declare no competing financial interests in relation to the work described.

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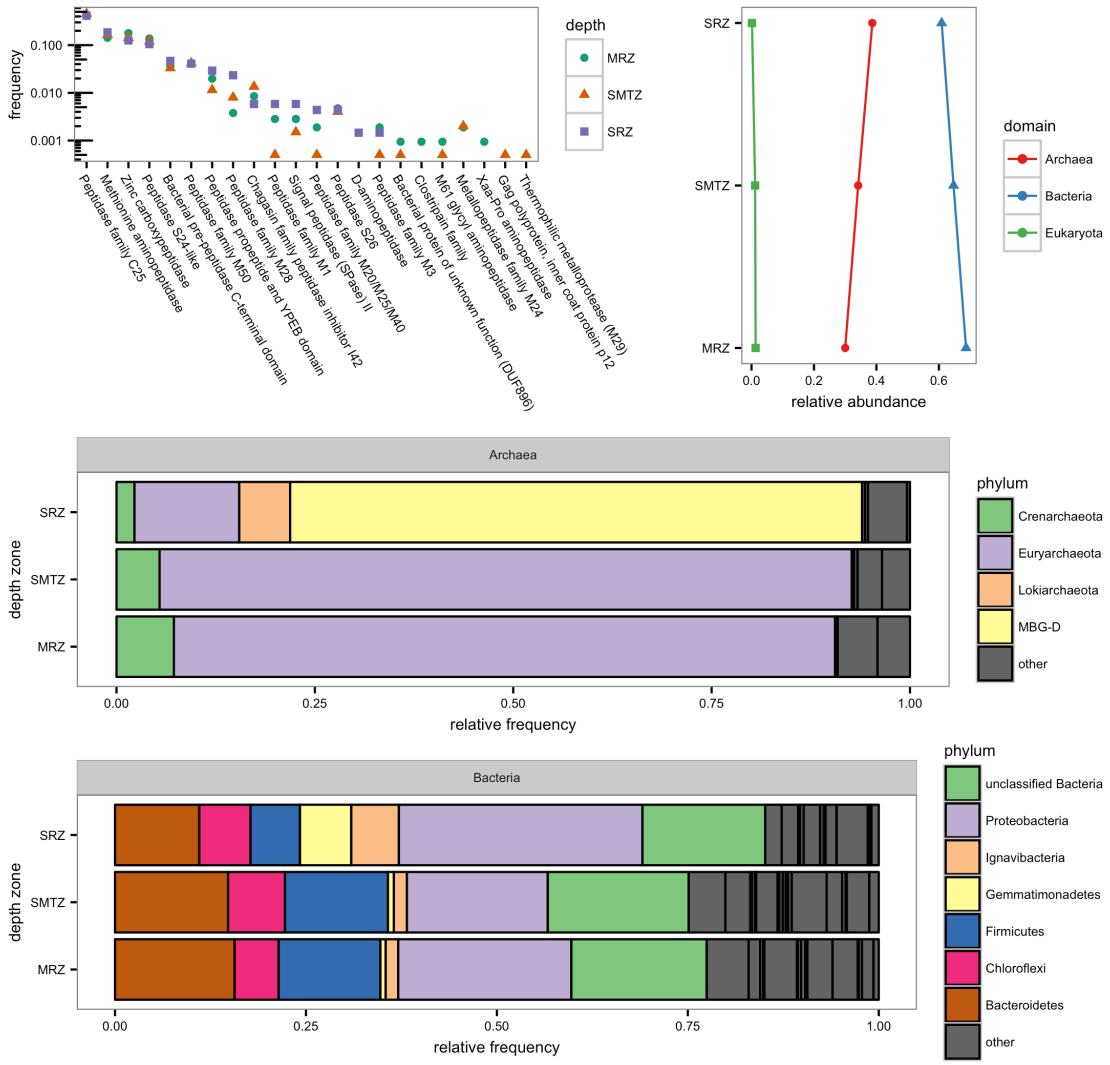
629 **Figure Legends**

630

631 Fig 1: (A) Frequency of genes for various classes of extracellular peptidases, relative to

632 all genes for extracellular peptidases; (b) sources of extracellular peptidases by domain

633 and depth; and sources of (c) Archaeal and (d) Bacterial peptidases by phylum and depth.



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637 Fig 2: Peptidase saturation curves, showing Michealis-Menten kinetics consistent with

638 enzymatic rather than abiotic substrate hydrolysis. Open triangles indicate autoclaved

639 controls.

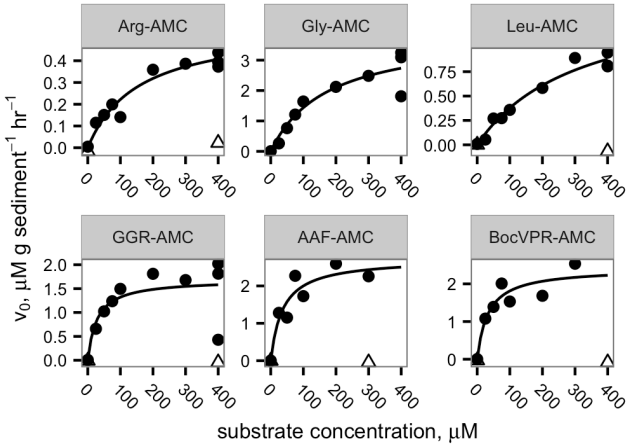

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Fig 3: Peptidase activities compared to microbial abundance and activity. Panel A: summed V_{max} of the six peptidases measured in 2013, expressed relative to the value at 1.5 cm. Error bars represent standard error of the estimate of the rate of fluorophore production. B: Summed V_{max} relative to cell abundance. Error bars represent error propagated from error of Vmax and standard deviation of cell counts. C: Modeled organic carbon oxidation rate. D: Summed V_{max} relative to organic carbon oxidation rate. Error bars represent standard error of the estimate of the rate of fluorophore production.

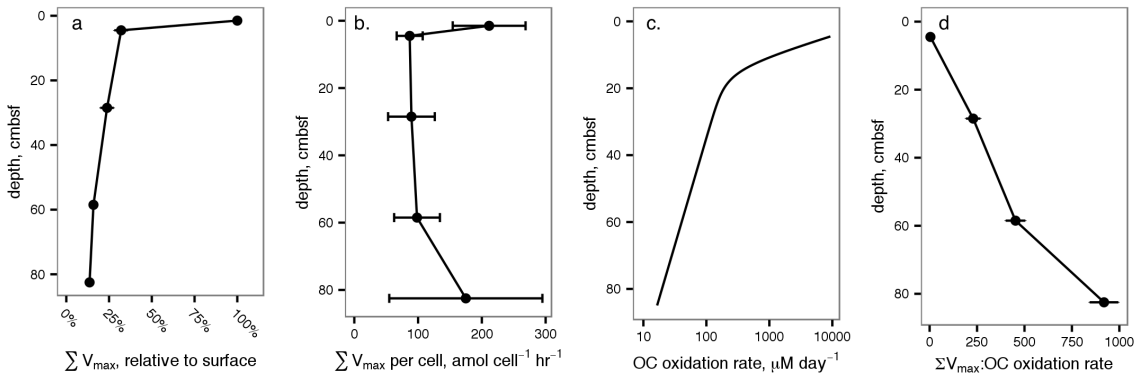

Fig 4: Estimated K_m values as a function of sediment depth. Colored lines indicate linear regressions for individual substrates, while the black line and shaded area represent a regression for all substrates taken together.

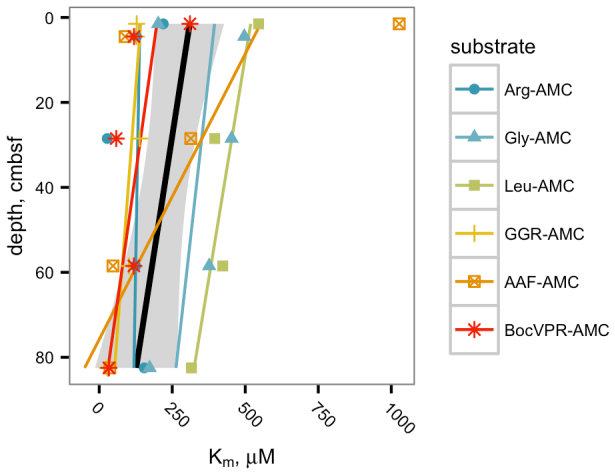
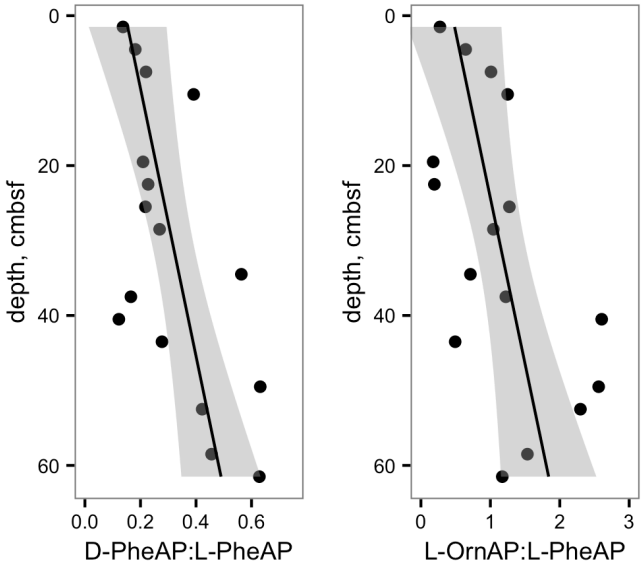
Fig 5: Left panel: Ratio of D-phenylalanyl aminopeptidase V_{max} to L-phenylalanyl aminopeptidase V_{max} versus depth. Right panel: Ratio of ornithyl aminopeptidase V_{max} to L-phenylalanyl aminopeptidase V_{max}. The shaded band indicates the 95% confidence interval of the fitted values.