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1 # Temperature and UV light affect the activity of marine cell-
2 # free enzymes

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8

9 **Abstract**

10 Microbial extracellular enzymatic activity (EEA) is the rate-limiting step in the degradation of organic matter in
11 the oceans. These extracellular enzymes exist in two forms, cell-bound which are attached to the microbial cell
12 wall, and cell-free which are completely free of the cell. Contrary to previous understanding, cell-free
13 extracellular enzymes make up a substantial proportion of the total marine EEA. Little is known about these
14 abundant cell-free enzymes, including what factors control their activity once they are away from their sites
15 (cells). Experiments were run to assess how cell-free enzymes (excluding microbes) respond to ultraviolet
16 radiation (UVR) and temperature manipulations, previously suggested as potential control factors for these
17 enzymes. The experiments were done with New Zealand coastal waters and the enzymes studied were alkaline
18 phosphatase [APase], β-glucosidase, [BGase], and leucine aminopeptidase, [LAPase]. Environmentally relevant
19 UVR (i.e., *in situ* UVR levels measured at our site) irradiances reduced cell-free enzyme activities up to 87%
20 when compared to controls, likely a consequence of photodegradation. This effect of UVR on cell-free enzymes
21 differed depending on the UVR fraction. Ambient levels of UV radiation (KJ) were shown to reduce the activity
22 of cell-free enzymes, for the first time. Elevated temperatures (15°C) increased the activity of cell-free enzymes
23 up to 53% when compared to controls (10°C), likely by enhancing the catalytic activity of the enzymes. Our
24 results suggest the importance of both UVR and temperature as control mechanisms for cell-free enzymes. Given
25 the projected warming ocean environment and the variable UVR light regime, it is possible there could be major
26 changes in the activity of cell-free EEA and their contribution to organic matter remineralization in the future.

27

28 ## 1 Introduction

29 Heterotrophic microbes are ubiquitous in the marine environment, recycling most of the organic matter available
30 in the oceans. The discovery of the microbial loop made clear that heterotrophic microbes are one of the most
31 important nutrient vectors in marine food webs (Azam and Cho, 1987; Azam et al., 1983). According to the size-
32 reactivity model, microbes selectively prefer high molecular weight dissolved organic matter (HMWDOM) due
33 to its superior nutritional value (Amon and Benner, 1996; Benner and Amon, 2015). The main obstacle for use

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34 of HMWDOM by microbes is that these compounds are generally too large to be transported across microbial
35 cell membranes. Enzymatic hydrolysis outside of the cell is required to break HMWDOM down to smaller size
36 fractions (<600 Daltons) before uptake can occur (Weiss et al., 1991). Thus, microbial extracellular enzymatic
37 activity (EEA) is the process that initiates the microbial loop (Arnosti, 2011; Hoppe et al., 2002), and is
38 recognised as the rate limiting step in the degradation of organic matter in the oceans (Hoppe, 1991). This key
39 role has led to extracellular enzymes being referred to as "gatekeepers of the carbon cycle" (Arnosti, 2011).

40 There are two forms of EEA; cell-bound, which are attached to the outside of the microbial cell wall or reside in
41 the periplasmic space, and; cell-free, which are completely free of the cell, suspended in the water column. Cell-
42 free enzymes can come from a variety of sources in the marine environment including the sloppy grazing
43 behaviour of protists (Bochdansky et al., 1995; Hoppe, 1991), microbial starvation (Chróst, 1991), the lysis of
44 cells by viruses (Kamer and Rassoulzadegan, 1995) and the direct release by microbes in response to the
45 detection of appropriate substrates (Alderkamp et al., 2007). Up until recently, research on extracellular enzymes
46 has been mostly on cell-bound enzymes, as they were considered to be the only abundant form (Hoppe, 1983;
47 Hoppe et al., 2002). This lead to a view that cell-bound extracellular enzymes were the only form of ecological
48 significance (Chróst and Rai, 1993; Rego et al., 1985). However, studies have now shown that the second form,
49 cell-free extracellular enzymes, can make up a substantial proportion of the total extracellular enzyme pool
50 (Allison et al., 2012; Baltar et al., 2013; Baltar et al., 2010; Baltar et al., 2016; Duhamel et al., 2010; Kamer and
51 Rassoulzadegan, 1995; Li et al., 1998). This has been a major conceptual shift for research in marine enzymatic
52 activity, generating new research questions about what controls cell-free enzymes in the marine environment and
53 how they function (Arnosti, 2011; Arnosti et al., 2014; Baltar et al., 2010; Baltar et al., 2016).

54 One of the many consequences of this discovery is that cell-free enzymes can be decoupled temporally and/or
55 spatially from the microbial community that produces them (Arnosti, 2011; Baltar et al., 2010; Baltar et al.,
56 2016), since cell-free enzymes have long residence times after they are released lasting up to several weeks
57 (Baltar et al., 2013; Steen and Arnosti, 2011). The activity of cell-free enzymes away from their sites (cells) can
58 condition macromolecular DOC and organic surfaces for subsequent microbial growth. This action at a distance
59 complicates discerning links between producing microbes and their enzymes expression, as cell-free enzymes
60 have the potential to contribute to the availability of nutrients at a great distance from the releasing cell (Arnosti,
61 2011; Baltar et al., 2010; Baltar et al., 2016). It has been suggested that the history of the water mass may be
62 more informative in understanding current cell-free enzyme activities than the *in situ* microbial community
63 present at the time of sampling (Arnosti, 2011; Baltar et al., 2013; Baltar et al., 2010; Baltar et al., 2016; Kamer
64 and Rassoulzadegan, 1995).

65 There is only a limited number published investigations into the dynamics of cell-free enzymes (Baltar et al.,
66 2013; Baltar et al., 2010; Baltar et al., 2016; Duhamel et al., 2010; Kamer and Rassoulzadegan, 1995; Kim et al.,
67 2007; Li et al., 1998; Steen and Arnosti, 2011). These papers provide good evidence of the importance of cell-
68 free enzymes in the marine environment, but the controls for cell-free enzymes (once separated from the
69 microbial cell) are poorly understood (Arnosti, 2011). Steen and Arnosti (2011) tested the effect of ultraviolet
70 radiation (UVR) on cell-free enzymes directly, finding that a reduction in cell-free enzyme activity only at
71 artificially high UVR doses (i.e., UV-B intensity 5–10 times higher than *in situ*), with natural illumination
72 showing no significant effects of photodegradation. One recent study by Baltar et al. (2016) in the Baltic Sea

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revealed strong correlations between seasonal temperature change and the proportion of cell-free to total EEA, suggesting seawater temperature and/or solar radiation as the most obvious abiotic mechanisms for the control of cell-free enzymatic activity. However, that was a field study of coastal waters, which includes the whole microbial community and many potential interactions and effects that can co-occur (e.g. production/consumption of free enzymes by microbes, variation in substrate concentration, etc.). Thus, to better understand the factors affecting marine free EEA we need to test the effect of environmental factors on free EEA under controlled conditions.

Here we isolated the free extracellular enzymes from a coastal site and specifically studied the effects of temperature and UVR on the activity of three cell-free extracellular enzyme groups; alkaline phosphatase (APase), an enzyme used to acquire phosphorus from organic molecules; β-glucosidase (BGase), a glycolytic enzyme that targets carbohydrates groups, and; leucine aminopeptidase (LAPase), an enzyme associated with the degradation of proteins. UVR treatments were hypothesised to reduce the activity of cell-free enzymes when compared to dark controls by photodegradation, with a 'high UVR dose' treatments (including the entire UV-B spectrum (280 to 320 nm) were hypothesized to have a stronger degradative effect on cell-free enzymes than 'low UVR dose' treatments (which only include a fraction of the UV-B spectrum, 280 to 305 nm). This was based on the reported effects of UV-B on microbes and their metabolic rates including the total EEA (Demers, 2001; Herndl et al., 1993; Müller-Niklas et al., 1995; Santos et al., 2012). Compared to ambient temperatures (10°C), cell-free enzymes exposed to high temperatures (15°C) were hypothesised to be more active, and *viceversa*, due to the general relationship between temperature and catalytic activity in enzymes (Daniel and Danson, 2010, 2013). Experiments carried out here are the first to directly test temperature effects on cell-free enzymes alone, and to directly test the effect of UVR on cell-free enzymes in the Southern Hemisphere and under *in situ* measured environmental-relevant UV-irradiances.

2 Materials and methods

2.1 Study site, sampling and experiments preparation

The experiments were conducted at the University of Otago's Portobello Marine Laboratory, situated on the Otago Harbour, Dunedin, New Zealand (45.8281° S, 170.6399° E). Otago Harbour is a tidal inlet which has an area of 46 km^2, consisting of two basins and with extensive sediment flats (Grove and Probert, 1999; Heath, 1975). The laboratory is based on the outer Otago harbour, which has waters similar in composition to coastal seawater, owing to the rapid residence times for its waters exchanging with the open sea (Grove and Probert, 1999; Rainer, 1981). Samples were taken from the second meter of the water column off the marine laboratory's wharf that extends into a deep tidal channel. All sampling and laboratory equipment used was prior sterilised by triplicate rinses of 18 MΩ·cm high purity water (Milli-Q^{TM}) water before and after soaking in 10% hydrochloric acid for >6 hours and oven dried at 60°C. To separate the cell-free extracellular enzymes from the total extracellular enzyme pool and the microbial community, samples were gently triple filtered through low protein binding 0.22µm Acrodisc filters following published methods (Baltar et al., 2010; Kim et al., 2007). 50 ml glass vials were filled with the 0.22 µm-filtered seawater for use in experiments. Bacterial abundance was determined

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110 after both experiments by preserving samples in glutaraldehyde and processing using SYBR Green nucleic acid
111 stain with a BD Accuri C6 flow cytometer (BD biosciences, USA). This was to ensure that no significant
112 bacterial growth occurred after filtering or during the incubation. Bacterial abundance was reduced to less than
113 1% of the pre-filtered total and remained so during the 36-hour incubations.

114 **2.2 UVR experiments**

115 To determine *in situ* UVR irradiance and environmentally appropriate treatments for experiments, the
116 attenuation of UVR was measured through the upper 2 m of the water column on site using a LI-COR
117 LI1800UW spectroradiometer (LI-COR biosciences, USA. The spectroradiometer was factory calibrated using
118 NIST traceable standards. Once this was determined, artificial lighting was installed in a controlled temperature
119 room, set to the ambient seawater temperature (10°C). The lighting consisted of two FS20 UV-R lamps (General
120 Electric, Schenectady NY, USA) and a full spectrum Vita-Lite 72 (Duro-Test, Philadelphia, PA, USA) lamp,
121 suspended above the samples. These lights were height adjusted to yield an irradiance of 3.03 W m^{-2} s^{-1} UVR,
122 approximating UVR irradiances measured in the field at 2 m depth (3.5 W m^{-2} s^{-1}). Schott WG and GG long
123 pass filters (15 cm X 15 cm) with nominal cutoffs (50% T) in the UVB (280 nm, 305 nm) were placed over the
124 filtered cell-free enzyme seawater samples contained in glass vials, with either a 'high dose' (<280nm, 3.03 W
125 m^{-2} s^{-1}, 130.8 kJ) or a 'low dose' (<305nm, 0.42 W m^{-2}, 18.1 kJ) of UVR. All light was blocked except that
126 which passed directly through the long pass filters. Controls were kept without light by wrapping the glass vials
127 containing the filtered cell-free enzyme seawater samples in several layers of aluminium foil, and were placed in
128 the same controlled temperature room. Readings of enzyme activity rates were taken of three replicates of each
129 treatment at 12 and 36 hours. Temperature inside the vials was also monitored to ascertain that the samples were
130 constantly kept at the desired temperature.

131 **2.3 Temperature experiments**

132 For the temperature experiments we utilised a large graded heat block system (see Lamare et al. (2014) for
133 design specifications). This heat block allowed for up to 15 replicate samples to be exposed to constant
134 temperature treatments over time. The heat blocks were tested five times a day for three days in advance with
135 blank samples to ensure the heat blocks were calibrated accurately; the variation in temperature was within
136 0.5°C of the target temperatures (i.e., 5, 10, and 15°C) in all measurements. These temperatures were selected
137 because 5 to 15 °C is the annual range of temperature in the sampling site, and 10°C was the *in situ* temperature
138 at the time of sampling (unpublished data). All treatments were kept in the dark by wrapping the glass vials
139 containing the filtered cell-free enzyme seawater samples in several layers of aluminium foil. Readings of
140 enzyme activity rates were taken of three replicates of each treatment were at 6, 12, 24 and 36 hours. When
141 incubating these samples, each was put into a separate incubator which was set to the treatment temperature so to
142 avoid confounding the temperature treatments.

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143 **2.4 Extracellular enzymatic activities assays**

144 We used the method for assessing extracellular enzymatic activity rates based on the hydrolysis of fluorogenic
145 substrate analogues developed by Hoppe (1983). The fluorogenic substrates: 4-methylcoumarinyl-7-amide
146 (MCA)-L-leucine-7-amido-4-methylcoumarin, 4-methylumbelliferyl (MUF)-β-D-glucoside and MUF-phosphate
147 were used to assess the leucine aminopeptidase, β-glucosidase and alkaline phosphatase activities, respectively.
148 Substrate concentrations of 100µM were used for each enzyme based on pre-established kinetics, tested in the
149 lab. 96-well falcon microplates were filled with six replicates of each of the three fluorogenic substrates (10µl)
150 and seawater (290µl) to make up 300µl reactions. Plates were read in a Spectramax M2 spectrofluorometer
151 (Molecular Devices, USA), with excitation and emission wavelengths of 365 and 445nm, both before, and after
152 3 hour incubations. All incubations were performed in the dark and kept in incubators set to *in situ* seawater
153 temperatures. Six samples without substrate addition served as blanks in each plate to determine the background
154 fluorescence of the samples, which were used to correct the activity rates in the plate readings before and after
155 incubation.

156 **2.5 Statistical analyses**

157 In all analyses, parametric assumptions were first checked using the Shapiro-Wilk test for normality and the
158 Levene's test for equal variance. Where appropriate, data was Log-transformed to meet normality assumptions
159 prior to analysis. Both experiments use two-way ANOVAs with an interaction term, with post hoc Tukey HSD
160 tests run to assess the individual significant effects between treatments. All analyses were run in the R software
161 environment (R Development Core Team, Austria).

162

163 **3 Results and Discussion**

164 **3.1 UVR experiments revealed photodegradation of cell-free enzymatic activities at environmentally**
165 **relevant levels**

166 UVR overall significantly decreased cell-free APase when compared to dark controls ($p<0.001$, $F_{2,12}=15.85$, two-
167 way ANOVA) (Fig. 1a). Individual significant effects between treatments in APase were seen as a significant
168 decrease in activity in the low-dose treatment relative to the dark control at 12 h ($p<0.05$, Tukey HSD), and
169 between the dark control and both the high and low UV-dose treatment at the 36-hour sampling point ($p<0.05$,
170 Tukey HSD). BGase cell-free activity was not significantly affected by UVR ($p=0.53$, $F_{2,12}=0.67$, two-way
171 ANOVA). UVR had a significant overall effect on LAPase, decreasing the cell-free activity when compared to
172 dark controls ($p<0.01$, $F_{2,12}=40.994$, two-way ANOVA) (Fig. 1c). Individual significant effects were seen in
173 LAPase, showing after 12 h a significant decrease in activity between the low and high at 12 h ($p<0.01$, Tukey
174 HSD), and after 36 h a gradual decrease from high to low dose ($p<0.05$, Tukey HSD), and dark control to both
175 low and high dose ($p<0.001$, Tukey HSD).

176 These experiments revealed a significant reduction in cell-free extracellular enzymatic activity for both APase
177 and LAPase in response to UVR, consistent with the predicted photodegradation; which was not evident for

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178 BGase. This was the first time that UVR has been demonstrated to reduce cell-free enzymatic activities at
179 environmentally relevant intensities. The only previous study (Steen and Arnosti, 2011) did show a reduction in
180 the cell-free extracellular enzymatic activity of APase and LAPase but only at artificially high UVR intensities
181 where UV-B was 5–10 times more intense from artificial lamps in the lab than outdoors. Interestingly, they
182 could not show significant UVR effects on BGase at any treatment level, which is consistent with the present
183 study.

184 Both APase and LAPase showed the strongest effect of UVR at the 36-hour sampling point, suggesting a UV-B
185 dose-dependent response. LAPase also showed a gradual decrease in the effect between the low and high UVR
186 treatments, which suggests the increase in UV-B irradiances also enhanced the degree of photodegradation. UV-
187 B has been demonstrated to be a highly active part of the spectrum for degrading DNA in general (Dahms and
188 Lee, 2010; Sinha and Häder, 2002), with specific effects of UV-B on total extracellular enzymatic activities
189 previously reported (Demers, 2001; Herndl et al., 1993; Müller-Niklas et al., 1995; Santos et al., 2012).
190 However, it is important to distinguish these previous studies from the cell-free enzyme experiments performed
191 here. Those previous studies tested the response of the entire microbial community, for total extracellular
192 enzymatic activity, based on the assumption that UVR affects the organism (source of enzymes) directly. What
193 is shown in this study is that UVR affects cell-free exclusively without the need to impact the source organism.
194 The effects of UVR were different among the enzymes assessed, which may be of importance as some enzymes
195 could be more impacted by UVR than others. For example, in this study, APase and LAPase were more affected
196 by UVR than BGase, which could change the spectrum of extracellular enzyme activity in the surface of the
197 ocean. The resulting higher BGase relative to APase or LAPase, could potentially condition macromolecular
198 DOC composition by hydrolysing relatively less proteins than carbohydrates in response to UV. In turn, it is
199 conceivable that any change in the enzyme spectrum due to variability in UVR light could cause a loss of
200 productivity (e.g. due to a decrease in the inorganic P made available through APase activities), as the nutrients
201 made available by extracellular enzymes may not be in suitable ratios for the effective growth of microbes
202 (Arnosti et al., 2014; Häder et al., 2007).

203 **3.2 Temperature experiments revealed enhanced catalytic activity of cell-free enzymes**

204 Temperature significantly increased cell-free APase at the high temperature of 15°C when compared to the
205 ambient control of 10°C ($p<0.01$, $F_{2,24}=11.57$, two-way ANOVA) (Fig. 2a). APase activity was significant
206 increased, after 6 h, in the high relative to the low temperature ($p<0.001$, Tukey HSD), after 12 h between low
207 and high temperature ($p<0.001$, Tukey HSD), and control and high treatments ($p<0.05$, Tukey HSD). Cell-free
208 BGase showed a similar pattern of increased activity in response to higher temperature but it was not significant
209 (Fig 2b). This lack of significant differences in cell-free BGase in response to temperature could be due to a
210 relatively high variability in EEA among the high temperature (15°C) treatments. LAPase significantly decreased
211 in the low temperature treatment (5°C), relative to the ambient control ($p<0.01$, $F_{2,24}=13.97$, two-way ANOVA)
212 (Fig 2c). LAPase cell-free activity significantly increased between the low and high temperature treatments at
213 the 6h and 12h time points ($p<0.05$, Tukey HSD). The temperature effect was dependent on time, finding
214 significant effects after 6 and 12h, but not later for any of the studied enzymes.

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215 The relationship found between temperature and cell-free activity is consistent with the general pattern of
216 increased catalytic activity of enzymes in relation to temperature (Daniel and Danson 2013). The positive
217 relationship between temperature and the activity of cell-free enzymes observed in this study is contrary to the
218 negative relationship between temperature and the proportion of cell-free relative to total EEA measured in a
219 seasonal field study in the Baltic Sea (Baltar et al., 2016). However, it is important to take into consideration the
220 fact that the study by Baltar et al. (2016) took place over a much longer temporal scale (1.5 years) and included
221 the whole microbial community; whereas in this study different factors were teased apart by focusing only on the
222 cell-free enzymes. This is supported by Baltar et al. (2016) where the proportion of cell-free relative to total EEA
223 was significantly negatively correlated to prokaryotic heterotrophic production, suggesting that the low
224 temperature preserves the constitutive activity of the cell-free enzymes better (than warm temperature) due to a
225 reduction in the metabolism of heterotrophic microbes that would reduce the consumption/degradation of
226 dissolved enzymes. The exclusion of heterotrophic microbes from our samples precluded this effect (i.e.,
227 heterotrophic degradation/consumption of free enzymes) of temperature from occurring, and allowed us to tease
228 apart the effect directly on the cell-free enzymatic activities. This also highlights the importance of scales when
229 dealing with microbial oceanographic processes.

230 Moreover, the observed time dependence of the effect of temperature on cell-free enzymes (with effects
231 noticeable in short time scale of ≤12 h), together with the tendency for stronger UVR effect after 36 h than 12 h,
232 might suggest a potential different scale in the response of cell-free enzymatic activity to UVR and temperature,
233 where the catalytic effect of temperature occurs faster than the UVR photodegradation, but more research would
234 be required to confirm this hypothesis.

235

236 **Conclusions**

237 Overall, temperature and UVR were both demonstrated as potential control mechanisms for the activity of
238 marine cell-free enzymes, providing a baseline for future research. This is the first report revealing the effects of
239 photodegradation of cell-free enzymes at environmentally relevant levels of UVR, and the effects of enhanced
240 temperature on the catalytic activity of marine cell-free enzymes. Environmentally relevant UVR had a
241 significant photodegradative effect that might be enzyme-specific (affecting APase and LAPase but not BGase),
242 with the potential to alter not only the rates of cell-free EEA but also the spectrum of enzyme expression in the
243 seawater. Alteration of the cell-free EEA spectrum from UVR variability, could have ecological and
244 biogeochemical implications like the conditioning of macromolecular DOM (i.e., affecting DOM composition
245 by hydrolysing some DOM compounds more relative to others), and the change of the elemental ratio of some
246 nutrients (e.g., affecting the availability of inorganic P due to a change in APase activity), with implications for
247 productivity and nutrient cycling. Additionally, given the variable UVR light regime spatially and temporally
248 (i.e. the 150% increase in UV-B in polar regions during spring-time ozone depletion, Smith et al., 1992) and the
249 documented anthropogenic changes in ocean temperature (Chen et al., 2007), it is probable that the activity of
250 cell-free EEA and their contribution to organic matter remineralization might be affected in the future, if not
251 already.

252

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256 The authors declare that they have no conflict of interest.

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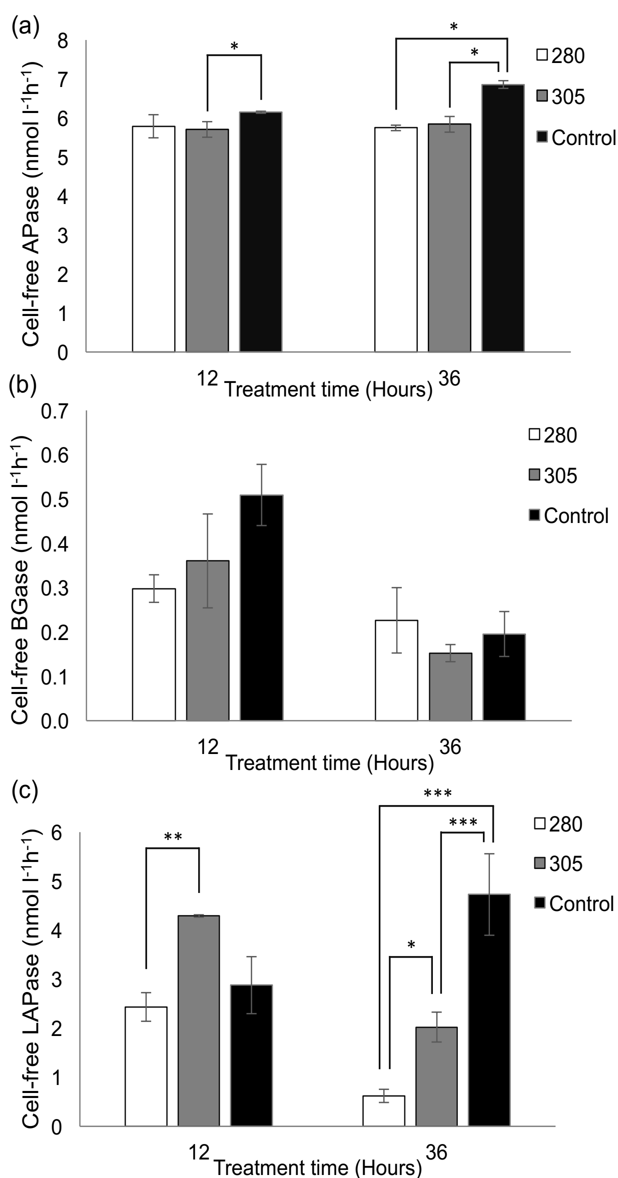

359 **Figure 1.** Results from UVR experiments showing the mean (±SE) cell-free extracellular enzyme
360 activity for alkaline phosphatase (a), beta-glucosidase (b), and leucine aminopeptidase (c), under a
361 high dose (of 280nm and above) and a low dose (of 305nm and above) in comparison to dark controls.
362 Asterisks above graphs represent individual significant effects between treatments in post hoc Tukey
363 test (*<0.05, **<0.01, ***<0.001) (N=3).

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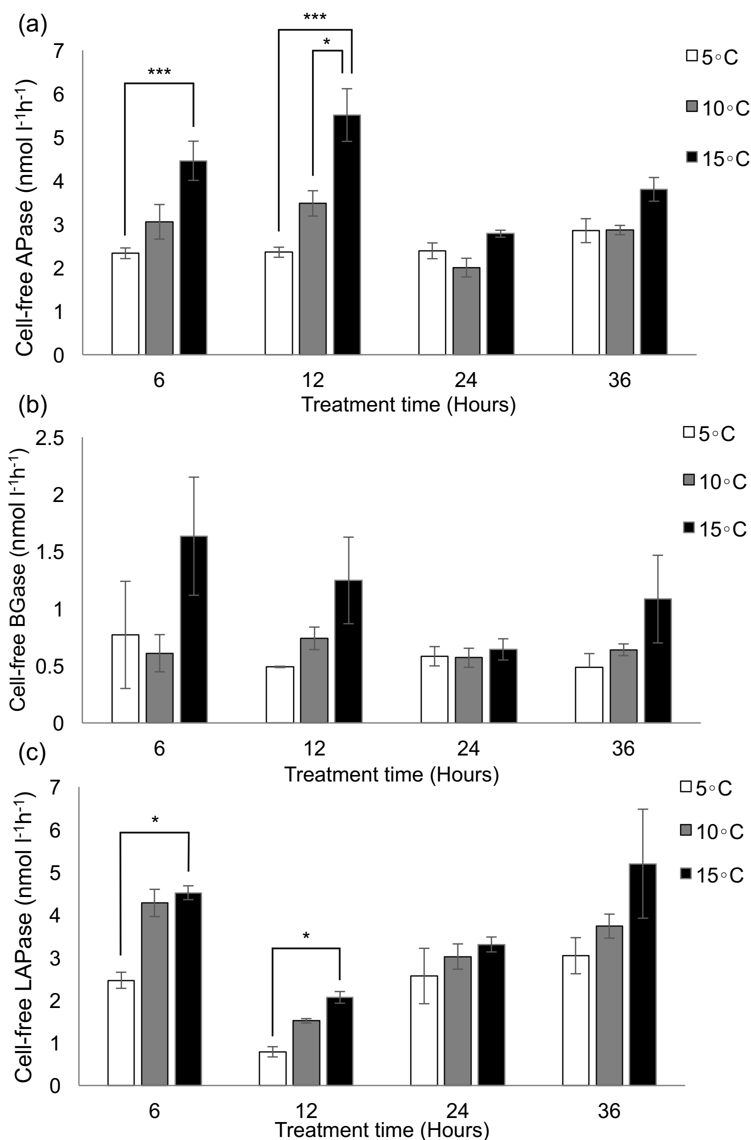

365 **Figure 2.** Results from temperature modification experiments showing the mean (±SE) cell-free
366 extracellular enzyme activity for alkaline phosphatase (a), beta-glucosidase (b), and leucine
367 aminopeptidase (c), under a high (15°C) and a low temperature (5°C) treatments in comparison to
368 ambient controls (10°C). Asterisks above graphs represent individual significant effects between
369 treatments in post hoc Tukey test (*<0.05, **<0.01, ***<0.001) (N=3).