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

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Abstract

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

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1 Introduction

Heterotrophic microbes are ubiquitous in the marine environment, recycling most of the organic matter available in the oceans. The discovery of the microbial loop made clear that heterotrophic microbes are one of the most important nutrient vectors in marine food webs (Azam et al., 1983;Azam and Cho, 1987). According to the size-reactivity model, microbes selectively prefer high molecular weight dissolved organic matter (HMWDOM) due to its superior nutritional value (Amon and Benner, 1996;Benner and Amon, 2015). The main obstacle for use of HMWDOM by microbes is that these compounds are generally too large to be transported across microbial cell membranes. Enzymatic hydrolysis outside of the cell is required to break HMWDOM down to smaller size fractions (<600 Daltons) before uptake can occur (Weiss et al., 1991). Thus, microbial extracellular enzymatic activity (EEA) is the process that initiates the microbial loop (Arnosti, 2011;Hoppe et al., 2002), and is recognised as the rate limiting step in the degradation of organic matter in the oceans (Hoppe, 1991). This key role has led to extracellular enzymes being referred to as "gatekeepers of the carbon cycle" (Arnosti, 2011).

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

controls cell-free enzymes in the marine environment and how they function (Arnosti, 2011;Arnosti et al., 2014;Baltar et al., 2010;Baltar et al., 2016).

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

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

94 Here we isolated the free extracellular enzymes from a coastal site and specifically studied the

95 effects of temperature and UVR on the activity of three cell-free extracellular enzyme groups;

96 alkaline phosphatase (APase), an enzyme used to acquire phosphorus from organic molecules;

97 β-glucosidase (BGase), a glycolytic enzyme that targets carbohydrates groups, and; leucine

98 aminopeptidase (LAPase), an enzyme associated with the degradation of proteins. UVR

99 treatments were hypothesised to reduce the activity of cell-free enzymes when compared to

100 dark controls by photodegradation, with a 'high UVR dose' treatments (including the entire

101 UV-B spectrum (280 to 320 nm) were hypothesized to have a stronger degradative effect on

102 cell-free enzymes than 'low UVR dose' treatments (which only include a fraction of the UV-

103 B spectrum, 280 to 305 nm). This was based on the reported effects of UV-B on microbes and

104 their metabolic rates including the total EEA (Herndl et al., 1993;Santos et al., 2012;Müller-

105 Niklas et al., 1995;Demers, 2001). Compared to ambient temperatures (10°C), cell-free

106 enzymes exposed to high temperatures (15°C) were hypothesised to be more active, and

107 *viceversa*, due to the general relationship between temperature and catalytic activity in

108 enzymes (Daniel and Danson, 2010, 2013). Experiments carried out here are the first to

109 directly test temperature effects on cell-free enzymes alone, and to directly test the effect of

110 UVR on cell-free enzymes in the Southern Hemisphere and under *in situ* measured

111 environmental-relevant UV-irradiances.

112

113 **2 Materials and methods**

114 **2.1 Study site, sampling and experiments preparation**

115 The experiments were conducted at the University of Otago's Portobello Marine Laboratory,

116 situated on the Otago Harbour, Dunedin, New Zealand (45.8281° S, 170.6399° E). Otago

117 Harbour is a tidal inlet which has an area of 46 km^2, consisting of two basins and with

118 extensive sediment flats (Grove and Probert, 1999;Heath, 1975). The laboratory is based on

119 the outer Otago harbour, which has waters similar in composition to coastal seawater, owing

120 to the rapid residence times for its waters exchanging with the open sea (Rainer, 1981;Grove

121 and Probert, 1999). Samples were taken from the second meter of the water column off the

122 marine laboratory's wharf that extends into a deep tidal channel. All sampling and laboratory

123 equipment used was prior decontaminated using triplicate rinses of 18 MΩ·cm high purity

124 water (Milli-Q^{TM}) water before and after soaking in 10% hydrochloric acid for >6 hours and

125   oven dried at 60°C. To separate the cell-free extracellular enzymes from the total

126   extracellular enzyme pool and the microbial community, samples were triple filtered through

127   low protein binding 0.22µm Acrodisc filters following published methods (Kim et al.,

128   2007;Baltar et al., 2010). 50 ml glass vials were filled with the 0.22 µm-filtered seawater for

129   use in experiments. Bacterial abundance was determined after both experiments by preserving

130   samples in glutaraldehyde and processing using SYBR Green nucleic acid stain with a BD

131   Accuri C6 flow cytometer (BD biosciences, USA). This was to ensure that no significant

132   bacterial growth occurred after filtering or during the incubation. Bacterial abundance was

133   reduced to less than 1% of the pre-filtered total and remained so during the 36-hour

134   incubations.

135   2.2 UVR experiments

136   To determine *in situ* UVR irradiance and environmentally appropriate treatments for

137   experiments, the attenuation of UVR was measured through the upper 2 m of the water

138   column on site using a LI-COR LI1800UW spectroradiometer (LI-COR biosciences, USA.

139   The spectroradiometer was factory calibrated using NIST traceable standards. Once this was

140   determined, artificial lighting was installed in a controlled temperature room, set to the

141   ambient seawater temperature (10°C). The lighting consisted of two FS20 UV-R lamps

142   (General Electric, Schenectady NY, USA) and a full spectrum Vita-Lite 72 (Duro-Test,

143   Philadelphia, PA, USA) lamp, suspended above the samples. These lights were height

144   adjusted to yield an irradiance of 3.03 W m^{-2} s^{-1} UVR, approximating UVR irradiances

145   measured in the field at 2 m depth (3.5 W m^{-2} s^{-1}). Schott WG and GG long pass filters (15

146   cm X 15 cm) with nominal cutoffs (50% T) in the UVB (280 nm, 305 nm) were placed above

147   the filtered cell-free enzyme seawater samples contained in glass vials, with either a 'high

148   dose' (<280nm, 3.03 W m^{-2} s^{-1}, 130.8 kJ) or a 'low dose' (<305nm, 0.42 W m^{-2}, 18.1 kJ) of

149   UVR. All light was blocked except that which passed directly through the long pass filters

150   onto the open glass vials, to avoid any effect of the glass on the UVR dose. Controls were

151   kept without light by wrapping the glass vials containing the filtered cell-free enzyme

152   seawater samples in several layers of aluminium foil, and were placed in the same controlled

153   temperature room. Readings of enzyme activity rates were taken of three replicates of each

154   treatment at 12 and 36 hours. UVR was not applied directly to the plate incubations, as it can

155   affect the fluorogenic substrate analogues used in the assays. Temperature inside the vials was

156   also monitored to ascertain that the samples were constantly kept at the desired temperature.

2.3 Temperature experiments

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

2.4 Extracellular enzymatic activities assays

We used the method for assessing extracellular enzymatic activity rates based on the hydrolysis of fluorogenic substrate analogues developed by Hoppe (1983). The fluorogenic substrates: 4-methylcoumarinyl-7-amide (MCA)-L-leucine-7-amido-4-methylcoumarin, 4-methylumbelliferyl (MUF)-β-D-glucoside and MUF-phosphate were used to assess the leucine aminopeptidase, β-glucosidase and alkaline phosphatase activities, respectively. Substrate concentrations of 100μM were used for each enzyme based on pre-established kinetics, tested in the lab. Although differences in UVR or temperature might affect the kinetic parameters, we decided to use the same concentration for all the enzymes (which was saturating at the *in situ* conditions) to allow for a better comparison and reduce cofounding factors. 96-well falcon microplates were filled with six replicates of each of the three fluorogenic substrates (10μl) and seawater (290μl) to make up 300μl reactions. Plates were read in a Spectramax M2 spectrofluorometer (Molecular Devices, USA), with excitation and emission wavelengths of 365 and 445nm, both before, and after 3 hour incubations. All incubations were performed in the dark with UVR incubations set to the *in situ* seawater temperature, and temperature incubations set to each respective treatment temperature. Six samples without substrate addition served as blanks in each plate to determine the background

188 fluorescence of the samples, which were used to correct the activity rates in the plate readings
189 before and after incubation.

190 **2.5 Statistical analyses**

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

197

198 **3 Results and Discussion**

199 **3.1 UVR experiments revealed photodegradation of cell-free enzymatic activities at**
200 **environmentally relevant levels**

201 The proportion of cell-free EEA in the seawater at the time of sampling was 93.3%, 96.7 and
202 21.1% for APase, BGase and LAPase respectively (Table 1). UVR overall significantly
203 decreased cell-free APase when compared to dark controls ($p<0.001$, $F_{2,12}=15.85$, two-way
204 ANOVA) (Fig. 1a). Individual significant effects between treatments in APase were seen as a
205 significant decrease in activity in the low-dose treatment relative to the dark control at 12 h
206 ($p<0.05$, Tukey HSD), and between the dark control and both the high and low UV-dose
207 treatment at the 36-hour sampling point ($p<0.05$, Tukey HSD). BGase cell-free activity was
208 not significantly affected by UVR ($p=0.53$, $F_{2,12}=0.67$, two-way ANOVA). UVR had a
209 significant overall effect on LAPase, decreasing the cell-free activity when compared to dark
210 controls ($p<0.01$, $F_{2,12}=40.994$, two-way ANOVA) (Fig. 1c). Individual significant effects
211 were seen in LAPase, showing after 12 h a significant decrease in activity between the low
212 and high at 12 h ($p<0.01$, Tukey HSD), and after 36 h a gradual decrease from high to low
213 dose ($p<0.05$, Tukey HSD), and dark control to both low and high dose ($p<0.001$, Tukey
214 HSD). Changes observed in the controls of all the enzymes from 12 to 36 h were not
215 statistically significant ($p>0.05$, Tukey HSD).

216 Apart from the possibility that UVR treatments may have influenced the composition of the
217 seawater substrate itself, these experiments revealed a significant reduction in cell-free

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

Both APase and LAPase showed the strongest effect of UVR at the 36-hour sampling point, suggesting a UV-B dose-dependent response. LAPase also showed a gradual decrease in the effect between the low and high UVR treatments, which suggests the increase in UV-B irradiances also enhanced the degree of photodegradation. UV-B has been demonstrated to be a highly active part of the spectrum for degrading DNA in general (Sinha and Häder, 2002;Dahms and Lee, 2010), which is not only included in cells but is also an abundant component of the dissolved (extracellular) seawater fraction (Paul et al., 1987;Paul and David, 1989). Specific effects of UV-B on total extracellular enzymatic activities have been previously reported (Herndl et al., 1993;Santos et al., 2012;Demers, 2001;Müller-Niklas et al., 1995). However, it is important to distinguish these previous studies from the cell-free enzyme experiments performed here. Those previous studies tested the response of the entire microbial community, for total extracellular enzymatic activity, based on the assumption that UVR affects the organism (source of enzymes) directly. What is shown in this study is that UVR affects cell-free exclusively without the need to impact the source organism. The effects of UVR were different among the enzymes assessed, which may be of importance as some enzymes could be more impacted by UVR than others. For example, in this study, APase and LAPase were more affected by UVR than BGase, which could change the spectrum of extracellular enzyme activity in the surface of the ocean. The resulting higher BGase relative to APase or LAPase, could potentially condition macromolecular DOC composition by hydrolysing relatively less proteins than carbohydrates in response to UV. In turn, it is conceivable that any change in the enzyme spectrum due to variability in UVR light could cause a loss of productivity (e.g. due to a decrease in the inorganic P made available through APase activities), as the nutrients made available by extracellular enzymes may not be in suitable ratios for the effective growth of microbes (Arnosti et al., 2014;Häder et al., 2007).

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

The proportion of cell-free EEA in the original seawater at the time of sampling was 99.9%, 85.8 and 30.0% for APase, BGase and LAPase respectively (Table 1). Temperature significantly increased cell-free APase at the high temperature of $15^{o}C$ when compared to the ambient control of $10^{o}C$ ($p<0.01$, $F_{2,24}=11.57$, two-way ANOVA) (Fig. 2a). APase activity was significant increased, after 6 h, in the high relative to the low temperature ($p<0.001$, Tukey HSD), after 12 h between low and high temperature ($p<0.001$, Tukey HSD), and control and high treatments ($p<0.05$, Tukey HSD). Cell-free BGase showed a similar pattern of increased activity in response to higher temperature but it was not significant (Fig 2b). This lack of significant differences in cell-free BGase in response to temperature could be due to a relatively high variability in EEA among the high temperature ($15^{o}C$) treatments. LAPase significantly decreased in the low temperature treatment ($5^{o}C$), relative to the ambient control ($p<0.01$, $F_{2,24}=13.97$, two-way ANOVA) (Fig 2c). LAPase cell-free activity significantly increased between the low and high temperature treatments at the 6h and 12h time points ($p<0.05$, Tukey HSD). The temperature effect was dependent on time, finding significant effects after 6 and 12h, but not later for any of the studied enzymes.

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

282 the cell-free enzymatic activities. This also highlights the importance of scales when dealing
283 with microbial oceanographic processes.

284

285 **Conclusions**

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

304

305 **Acknowledgements**

306 We would like to thank the team of technicians out at Portobello Marine Laboratory, most
307 notably, Linda Groenewegen and Reuben Pooley. This research was supported by a
308 University of Otago Research Grant and a Rutherford Discovery Fellowship (Royal Society
309 of New Zealand) to FB. We would like to acknowledge the support and insightful comments
310 of the reviewers, which clearly helped improve the overall merit of the manuscript. The
311 authors declare that they have no conflict of interest.

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414

415 **Table 1.** Total, cell-free and proportion of cell-free relative to total extracellular enzymatic activity *in*

416 *situ* for the seawater collected for the UVR and Temperature experiments at the time of sampling.

417

	Total (nmol l^{-1} h^{-1})	Cell-free (nmol l^{-1} h^{-1})	% Cell-free (%)
UVR experiement			
APase	75.4	70.3	93.3
BGase	2.3	2.2	96.7
LAPase	35.4	7.5	21.1
Temperature experiment			
APase	121.9	121.8	99.9
BGase	3.2	2.7	85.8
LAPase	33.1	9.9	30.0

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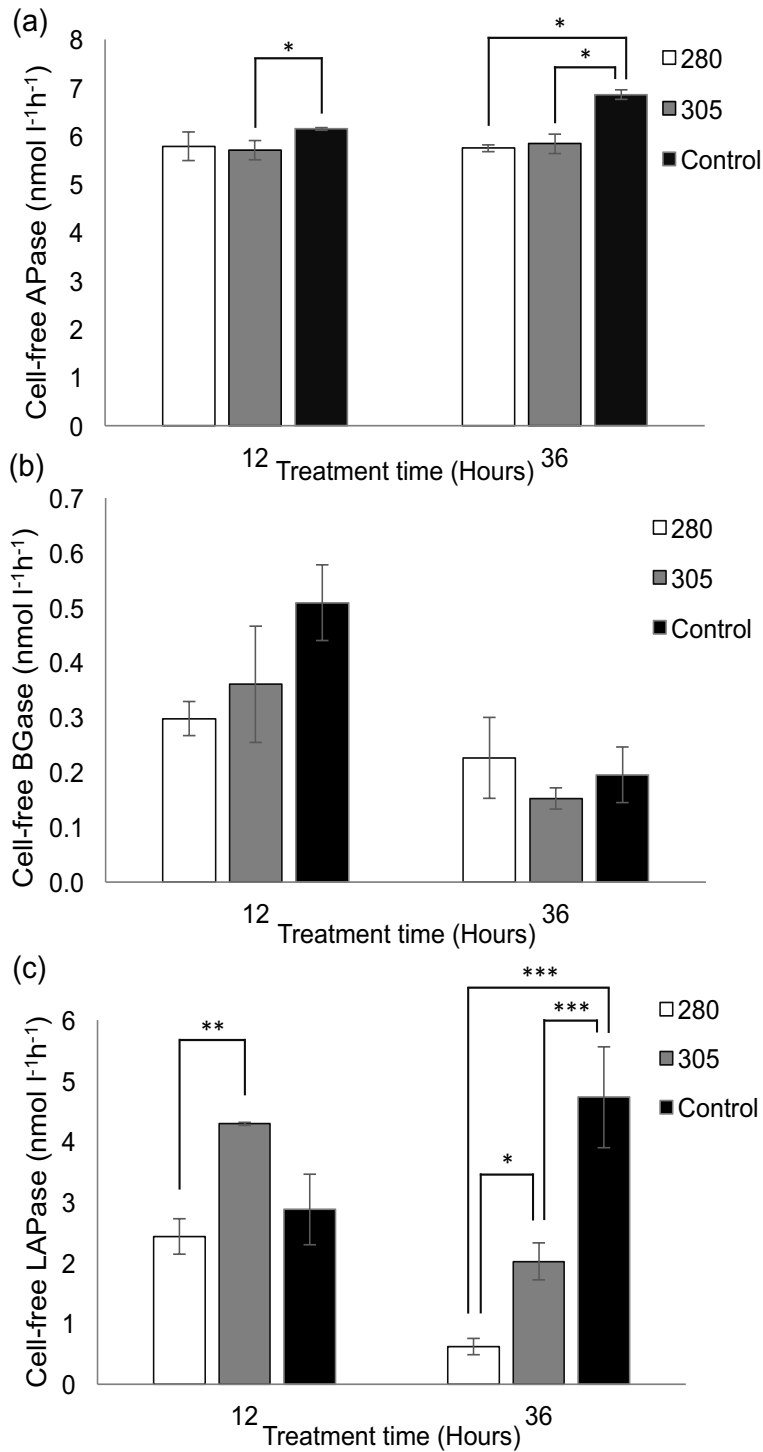
433 **Figure 1.** Results from UVR experiments showing the mean (±SE) cell-free extracellular enzyme
434 activity for alkaline phosphatase (a), beta-glucosidase (b), and leucine aminopeptidase (c), under a
435 high dose (of 280nm and above) and a low dose (of 305nm and above) in comparison to dark controls.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

438
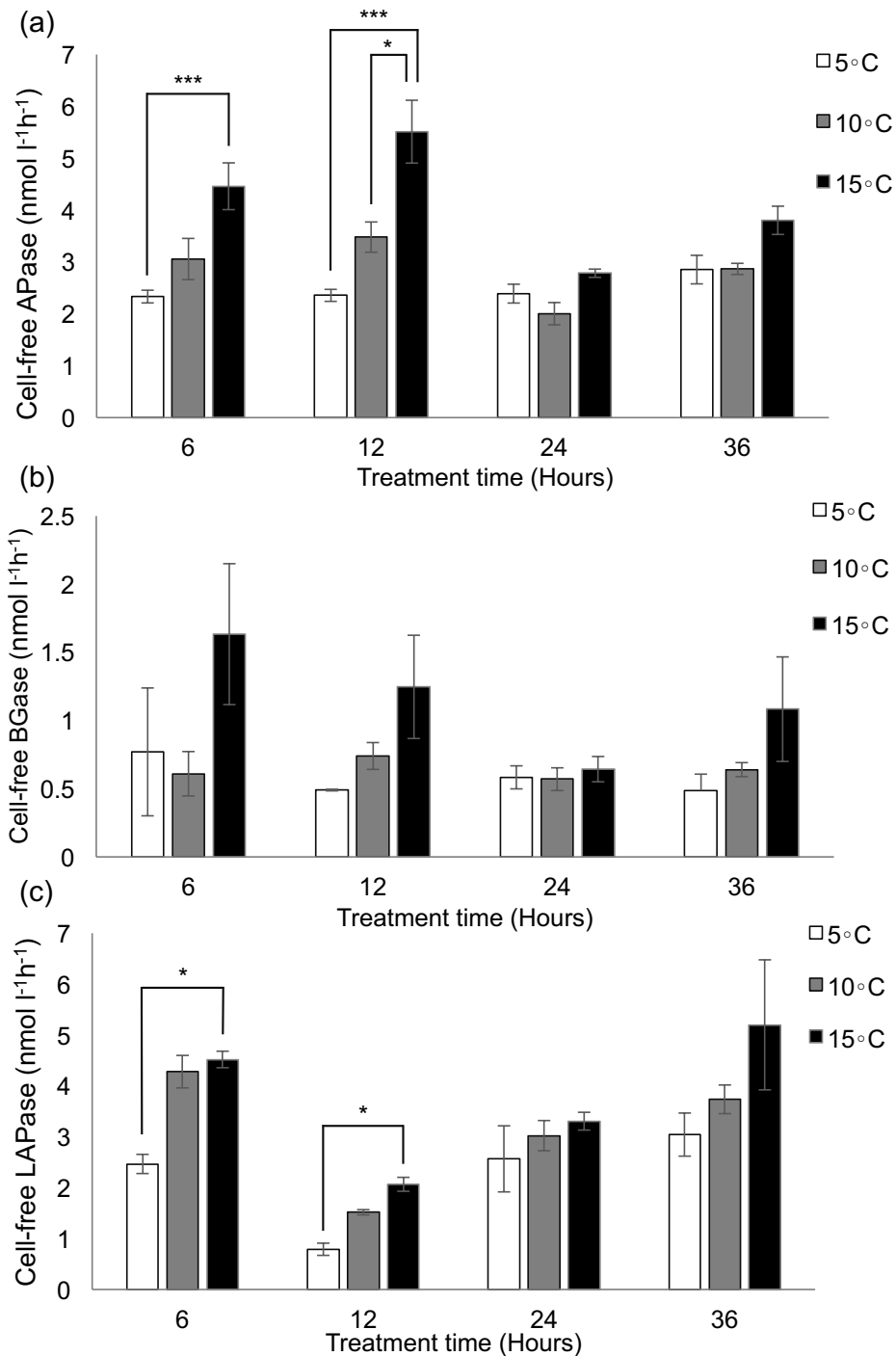
Figure 2. Results from temperature modification experiments showing the mean (±SE) cell-free extracellular enzyme activity for alkaline phosphatase (a), beta-glucosidase (b), and leucine aminopeptidase (c), under a high (15°C) and a low temperature (5°C) treatments in comparison to ambient controls (10°C). Asterisks above graphs represent individual significant effects between treatments in post hoc Tukey test (*<0.05, **<0.01, ***<0.001) (N=3).