Author's response

Dear Prof. Herndl,

Please find enclosed the revised version of our Baltar et al. manuscript entitled *"Temperature and UV light affect the activity of marine cell-free extracellular enzymes"* (bg-2017-29) intended as a Research Article in Biogeosciences.

Below, we detail our responses to each of the reviewers's comments, as well as the actions taken in response to the concerns they raised. We would like to acknowledge the support and insightful comments of the reviewers, which clearly helped improve the overall merit of the ms.

Thank you for your consideration of our manuscript, and we hope the manuscript will now be acceptable for publication in Biogeosciences.

Reviewer #1

General comments; The manuscript deals with a subject of utmost importance. Hydrolytic enzymes are essential for microorganisms to process DOM, and in the climate change situation the alteration of the environmental conditions will modify the hydrolysis rates of polymers, and consequently the functioning of the carbon cycle. In this respect, the analysis of the effect of UVR and temperature on the free hydrolytic enzymes becomes relevant and even necessary. The manuscript shows for the first time the reduction of the activity of the free enzymes by the UVR at environmental intensities and this is a substantial contribution. However, regarding the effect of temperature the aim of the study is unclear, the design of the experiments is confusing and needs to be explained or improved, and the analysis and interpretation of the results also requires significant changes. General problems are the high variability of data, the variability of the controls in the UVR experiments, as well as the erratic pattern of the variation in time of the measurements in the temperature experiments

Author response to Reviewer #1 general comment:

We thank the reviewer for the constructive comments on this manuscript. We have taken them on board and our responses to reviewer comments, including modifications to the manuscript, are detailed below. We hope the manuscript is clearer now and satisfies the reviewer.

REVIEWER COMMENT 1 by Referee #1:

The aim of this work is to study "the effects of temperature and UVR on the activity of three cell-free extracellular enzyme groups" (L80-81). However, the measurements of activity were made in the dark and in situ temperature. Samples were exposed to different UVR doses and at different temperatures for 6- 36 h but the hydrolytic activity was measured in the dark and in situ temperature with 3 hours of incubation (L152-153). The authors should explain why the activity was not measured under the same conditions as the samples. This experimental approach allows detecting changes in the molecular state of the enzymes when they are exposed to different doses of UVR and different temperatures, but the fact that samples return for 3 h to in situ

conditions makes it difficult to transfer the results to the ecosystem. Authors should explain the ecological sense of keeping the enzymes at 5◦C or 15◦C from 6 h to 36 h and then measuring the activity for 3 h at 10◦C. I think that this is an important point that should be clarified to facilitate the understanding of the manuscript.

Author response:
In terms of the measurements for the temperature experiments; the enzyme assays were incubated at each of the three-respective treatment temperatures, so they were measured under the same conditions as the treatments. Thank you for pointing this confusion out; this has now been amended in the text (p. 6, l.169)(p. 6, l.185-186).
The UVR experiments were all run at the *in situ* temp of 10 °C. When it came to the incubations for the enzyme assays, it was not clear the UVR dose would be consistent with the treatment level through the 96-well plates, so these were run in the dark, with the effect on EEA being the UVR treatment prior to the incubation. Moreover, it is not appropriate to run the EEA assay under UVR since this radiation can affect the substrates analogues used in the assay. This has also been clarified (p. 5, l.154-155).

REVIEWER COMMENT 2 by Referee #1:
2. L104. In my opinion the treatment of the material used in the experiments should not be called sterilization. I think that the term decontamination would be more appropriate since sterilization destroys all living cells, included spores.

Author response:
We agree with the reviewer, this has been modified accordingly (p. 4, l.123).

REVIEWER COMMENT 3 by Referee #1:
L107. The filtration process is critical because the filtration pressure can break the cells and release their contents, resulting in an enrichment of the filtrate. The authors claim that the filtration was gentle but, could the authors point out what they mean by gentle? What filtering pressure was used?

Author response:
We have removed the word gentle from the manuscript. Seawater was filtered using syringe filters as in previous works; we simply wanted to get across that the seawater was not forced through the filters in any way, but in hindsight, this is probably not necessary and was removed.

REVIEWER COMMENT 4 by Referee #1:
L148. In my experience the saturating concentrations are usually different in APase, BGase and LAPase, and frequently LAPase requires higher saturating concentrations than BGase and APase. The authors write that the concentration of substrate was established in previous kinetic experiments but should show some information about these experiments.
Both environmental factors, UVR and temperature, can affect to the kinetic parameters and this should be taken into account. For example, changes in temperature do not only affect to the hydrolysis rate but also can modify the affinity of the enzymes and therefore the saturating concentration, 100 μM can be saturating

at 10 ◦C, but no saturating at 5 ◦C. If molecules of enzymes are affected by UVR and temperatures, the kinetic parameters (Vmax and Km) will also be affected. Some kinetic experiments with different UVR doses and temperatures would also significantly improve the manuscript because would show modifications in the enzyme molecules

Author response:
We agree with the reviewer that the saturating concentrations can change, in fact in our preliminary saturation curves we did with water from the study site the saturating concentration was different for the different enzymes (i.e., around 83 µM, 57 µM and 39 µM for LAPase, than APase and BGase, respectively). We believed that in order to simplify confounding factors (because of all those different factors that the reviewer mention that can affect the saturating concentration) and with the aim to better compare the rates between the different enzymes, the best option was to use the same concentration for all the enzymes, which was saturating for all.
Nevertheless, we have included a statement mentioning about the potential influence of temperature and/or UV on the saturating concentration of the EEA (p. 6, l.178-181).

REVIEWER COMMENT 5 by Referee #1:
The introduction highlights the quantitative importance of the free enzymes and according to this it would be convenient to show, somewhere in the results, the percentage of total hydrolytic activity that dissolved enzymes represent in the analysed samples.

Author response:
We have included a new Table (Table 1) including the information requested.

REVIEWER COMMENT 6 by Referee #1:
It is not clear if figures correspond only to one UVR experiment (Fig 1) and one temperature experiment (Fig 2) or they show the average data of several experiments. If there are several experiments it would be more appropriate to show each experiment on one separate figure in order to reduce the standard errors. If data correspond to a single experiment it is not enough to reach any conclusion and the experiments must be repeated to find a common pattern.

Author response:
The data of those plots responds to one of each form of experiment. But we believe that the experiments were carefully well replicated (3 biological + 6 technical replicates per sample/treatment), statistically supported and worth publishing; a first stepping-stone towards more complicated and sophisticated experiments in the near future.

REVIEWER COMMENT 7 by Referee #1:
Regarding the discussion, the exposure of free enzymes to different doses of UVR and different temperatures during 6-36 h also provides information on the stability of enzymes under different conditions, but it is not discussed in the manuscript although

authors have experience on this issue. The authors detect differences between low and high dose of UVR and low and high temperature, but do not compare the evolution of the activity with time although there are important changes

Fig 1. For both BGase and LAPase controls varied between 12 and 36 h and both showed high standard errors. The controls should keep stable for 36 h unless the stability of enzymes is affected. In the case of APase and LAPase the activity of the controls increased from 12 h to 36 h, while for BGase decreased. Were the differences statistically significant? If so, how do you explain these changes?

In the case of BGase there was a general decrease from 12 to 36 h and the variability between replicates is so large that it possibly masks the effect of radiation, reason why there is not enough support to suggest that the effect of UVR is enzyme-specific and more experiments are required.

Author response:

The reviewer is right about the potential use of the experiment to learn more about the stability of the enzymes. However, the differences between the controls at 12h relative to 36h were not statistically significant for any of the enzymes.

Nevertheless, we have included a sentence in the text specifying that the temporal differences in the controls were not statistically significant (p. 7, l.214-215).

REVIEWER COMMENT 8 by Referee #1:

Fig 2. This figure tries to represent the effect of temperature on the activity of the free enzymes but it does reflect the stability of the enzymes to different temperatures over 36 h. In the case of BGase again the variability of data makes any comparison difficult.

For LAPase the activity decreased after 12 h but increased after 24 h and again after 36 h at the three temperatures. The effect of temperature could be expected to be maintained or increased over time. Authors should try to explain these erratic tendencies.

Author response:

The higher variability of BGase was only found in the 6h time and in the 15°C, but not so much in the others. Moreover, the 10°C control of BGase was remarkably stable during all the length of the experiment (and showing low variability). So we believe that the data is good enough to allow for comparison.

The more dynamic/erratic pattern observed for LAPase might be related to potential changes in adsorption/desorption and binding/unbinding of proteins/amino acids. Nevertheless, we believe that the fact of always having a control at every time point accounts for potential changes in this and other confounding factors.

REVIEWER COMMENT 9 by Referee #1:

L230-234. I have some problems with this paragraph. The effect of UVR on APase is evident at 12 h and also for LAPase there are differences between doses at 12 h. Thus, the scales are not as different as the authors claim.

Author response:

We have deleted this paragraph.

Author response to Reviewer #2
We thank the reviewer for the constructive comments on this manuscript. We have taken them on board and our responses to reviewer comments, including modifications to the manuscript, are detailed in the following:

Reviewer #2
REVIEWER COMMENT 1 by Referee #2:
This is an interesting paper, but a bit overly simplistic and seems to miss much of its potential. The fact that enzymes are affected by UV should not be surprising (they are complex organic molecules and the literature is replete with photochemistry). What are the structures of these enzymes? Since the result is different, what's different about the structures of the enzymes that suggests differences in sensitivity to UVR?

Author response:
Although we agree with the reviewer that the effect of UVR on free enzymes could be expected, it had not been shown how marine produced cell-free enzymes were affected by UVR. We had a hypothesis based in fundamental theory and we applied that hypothesis to the marine environment, within a context where cell-free enzymes happen to be very important, and in an environment that happen to frequently fluctuate in temperature.
It is difficult to tell at this point what the differences could be due to exactly in terms of the structure of the enzymes. For that we would need to perform more sophisticated protein structural research which is far from our scope here. The reality is that the majority of marine cell-free enzymes are poorly characterized and understood. There is likely to be structural differences between the glycolytic and proteinous enzymes for example which could affect their relative sensitivity to UVR, but a claim such as this would be a postulation/speculation at this point.

REVIEWER COMMENT 2 by Referee #2:
Nowhere do the authors address whether the effect is on the enzyme or perhaps the substrate? What's the structure of the substrates, will they absorb UV?

Author response:
We are not sure whether the reviewer refers to the natural substrates in the seawater sample or the artificial substrates used in the EEA assay. In the first instance, it is a good point that UV could affect the substrate (e.g. proteins, carbohydrates, etc), so we have now included this possibility in the discussion (p. 7, l.216-217). . For the latter, it is not a problem as the incubated plates themselves (which were supplemented with the artificial substrates) were not exposed to UVR, thus the artificial substrates were not exposed to UVR directly.

REVIEWER COMMENT 3 by Referee #2:

The exposure methodology is unclear, the samples were placed in glass vials but were they irradiated through the glass (blocking much UV) or left open and irradiated from the top?

Author response:
They were left open and irradiated from the top. This has been clarified in the methods (p. 5, l.150-151).

REVIEWER COMMENT 4 by Referee #2:
Spectrum of the lab light source is very different from the spectrum found in seawater, lamps are a necessary evil, but a bit over simplification to say they had environmentally relevant irradiance. Why not do the incubations in situ in UV transparent containers (quartz, teflon, polyethylene?)

Author response:
We agree with the reviewer that *in-situ* experiments would be much closer to reality in terms of a UVR dose. Our aim was to have the greater number of conditions/factors as controlled as possible to avoid other confounding factors. These experiments are part of a series which will eventually test multi-stress patterns, including both UVR and temperature; temperature being much harder to control *in situ*.
We had already specified in the abstract that by "environmentally relevant irradiance" we mean that the authors tested and then used a dose level measured *in-situ* (p. 1, l.20-21).

REVIEWER COMMENT 5 by Referee #2:
Finally, the discussion misses some classic literature - there were numerous papers published in the 80's from John Paul's lab on extracellular nucleases (DNAse)

Author response: These papers from John Paul's lab have now been reviewed. Thank you for pointing these out; some of these references were added to the discussion (p. 8, l.231-233).

END OF REVISION

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

Blair Thomson[1], Christopher David Hepburn[1], Miles Lamare[1], Federico Baltar[1,2]

[1]Department of Marine Science, University of Otago, New Zealand

[2]NIWA/University of Otago Research Centre for Oceanography, Dunedin, New Zealand

Correspondence to: F. Baltar (federico.baltar@otago.ac.nz)

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.

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 and Cho, 1987; Azam et al., 1983). 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 (Allison et al., 2012; Baltar et al., 2013; Baltar et al., 2010; Baltar et al., 2016; 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).

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

76 There is only a limited number published investigations into the dynamics of cell-free
77 enzymes (Baltar et al., 2013; Baltar et al., 2010; Baltar et al., 2016; Duhamel et al., 2010;
78 Kamer and Rassoulzadegan, 1995; Kim et al., 2007; Li et al., 1998; Steen and Arnosti, 2011).
79 These papers provide good evidence of the importance of cell-free enzymes in the marine
80 environment, but the controls for cell-free enzymes (once separated from the microbial cell)
81 are poorly understood (Arnosti, 2011). Steen and Arnosti (2011) tested the effect of
82 ultraviolet radiation (UVR) on cell-free enzymes directly, finding that a reduction in cell-free
83 enzyme activity only at artificially high UVR doses (i.e., UV-B intensity 5–10 times higher
84 than *in situ*), with natural illumination showing no significant effects of photodegradation.
85 One recent study by Baltar et al. (2016) in the Baltic Sea revealed strong correlations between
86 seasonal temperature change and the proportion of cell-free to total EEA, suggesting seawater
87 temperature and/or solar radiation as the most obvious abiotic mechanisms for the control of
88 cell-free enzymatic activity. However, that was a field study of coastal waters, which includes
89 the whole microbial community and many potential interactions and effects that can co-occur
90 (e.g. production/consumption of free enzymes by microbes, variation in substrate
91 concentration, etc.). Thus, to better understand the factors affecting marine free EEA we need
92 to test the effect of environmental factors on free EEA under controlled conditions.

93 Here we isolated the free extracellular enzymes from a coastal site and specifically studied the
94 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~~ decontaminated using~~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

126 through low protein binding 0.22µm Acrodisc filters following published methods (Baltar et
127 al., 2010; Kim et al., 2007). 50 ml glass vials were filled with the 0.22 µm-filtered seawater
128 for use in experiments. Bacterial abundance was determined after both experiments by
129 preserving samples in glutaraldehyde and processing using SYBR Green nucleic acid stain
130 with a BD Accuri C6 flow cytometer (BD biosciences, USA). This was to ensure that no
131 significant bacterial growth occurred after filtering or during the incubation. Bacterial
132 abundance was reduced to less than 1% of the pre-filtered total and remained so during the
133 36-hour incubations.

134 **2.2 UVR experiments**

135 To determine *in situ* UVR irradiance and environmentally appropriate treatments for
136 experiments, the attenuation of UVR was measured through the upper 2 m of the water
137 column on site using a LI-COR LI1800UW spectroradiometer (LI-COR biosciences, USA.
138 The spectroradiometer was factory calibrated using NIST traceable standards. Once this was
139 determined, artificial lighting was installed in a controlled temperature room, set to the
140 ambient seawater temperature (10°C). The lighting consisted of two FS20 UV-R lamps
141 (General Electric, Schenectady NY, USA) and a full spectrum Vita-Lite 72 (Duro-Test,
142 Philadelphia, PA, USA) lamp, suspended above the samples. These lights were height
143 adjusted to yield an irradiance of 3.03 W m^{-2} s^{-1} UVR, approximating UVR irradiances
144 measured in the field at 2 m depth (3.5 W m^{-2} s^{-1}). Schott WG and GG long pass filters (15
145 cm X 15 cm) with nominal cutoffs (50% T) in the UVB (280 nm, 305 nm) were placed
146 above~~over~~ the filtered cell-free enzyme seawater samples contained in glass vials, with either
147 a 'high dose' (<280nm, 3.03 W m^{-2} s^{-1}, 130.8 kJ) or a 'low dose' (<305nm, 0.42 W m^{-2}, 18.1
148 kJ) of UVR. All light was blocked except that which passed directly through the long pass
149 filters onto the open glass vials, to avoid any effect of the glass on the UVR dose. Controls
150 were kept without light by wrapping the glass vials containing the filtered cell-free enzyme
151 seawater samples in several layers of aluminium foil, and were placed in the same controlled
152 temperature room. Readings of enzyme activity rates were taken of three replicates of each
153 treatment at 12 and 36 hours. UVR was not applied directly to the plate incubations, as it can
154 affect the fluorogenic substrate analogues used in the assays. Temperature inside the vials was
155 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 ~~were~~ 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~~and~~ ~~kept in incubators~~ set to the *in situ* seawater temperature,~~s~~ and ~~.~~temperature incubations set to each respective treatment temperature. Six samples without substrate addition served as blanks in each plate

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

2.5 Statistical analyses

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

3 Results and Discussion

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

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

Apart from the possibility that UVR treatments may have influenced the composition of the seawater substrate itself, t~~T~~hese 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 (Dahms and Lee, 2010; Sinha and Häder, 2002), 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). ~~with specific effects of UV-B on total extracellular enzymatic activities previously reported (Demers, 2001; Herndl et al., 1993; Müller-Niklas et al., 1995; Santos et al., 2012).~~ 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).

249 **3.2 Temperature experiments revealed enhanced catalytic activity of cell-free enzymes**

250 The proportion of cell-free EEA in the original seawater at the time of sampling was 99.9%,
251 85.8 and 30.0% for APase, BGase and LAPase respectively (Table 1). Temperature
252 significantly increased cell-free APase at the high temperature of 15°C when compared to the
253 ambient control of 10°C ($p<0.01$, $F_{2,24}=11.57$, two-way ANOVA) (Fig. 2a). APase activity
254 was significant increased, after 6 h, in the high relative to the low temperature ($p<0.001$,
255 Tukey HSD), after 12 h between low and high temperature ($p<0.001$, Tukey HSD), and
256 control and high treatments ($p<0.05$, Tukey HSD). Cell-free BGase showed a similar pattern
257 of increased activity in response to higher temperature but it was not significant (Fig 2b). This
258 lack of significant differences in cell-free BGase in response to temperature could be due to a
259 relatively high variability in EEA among the high temperature (15°C) treatments. LAPase
260 significantly decreased in the low temperature treatment (5°C), relative to the ambient control
261 ($p<0.01$, $F_{2,24}=13.97$, two-way ANOVA) (Fig 2c). LAPase cell-free activity significantly
262 increased between the low and high temperature treatments at the 6h and 12h time points
263 ($p<0.05$, Tukey HSD). The temperature effect was dependent on time, finding significant
264 effects after 6 and 12h, but not later for any of the studied enzymes.

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

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

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

Conclusions

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

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411

412

413 **Table 1.** Total, dissolved and proportion of dissolved relative to total extracellular enzymatic

414 activity *in situ* for the seawater collected for the UVR and Temperature experiments at the

415 time of sampling.

416

	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

417

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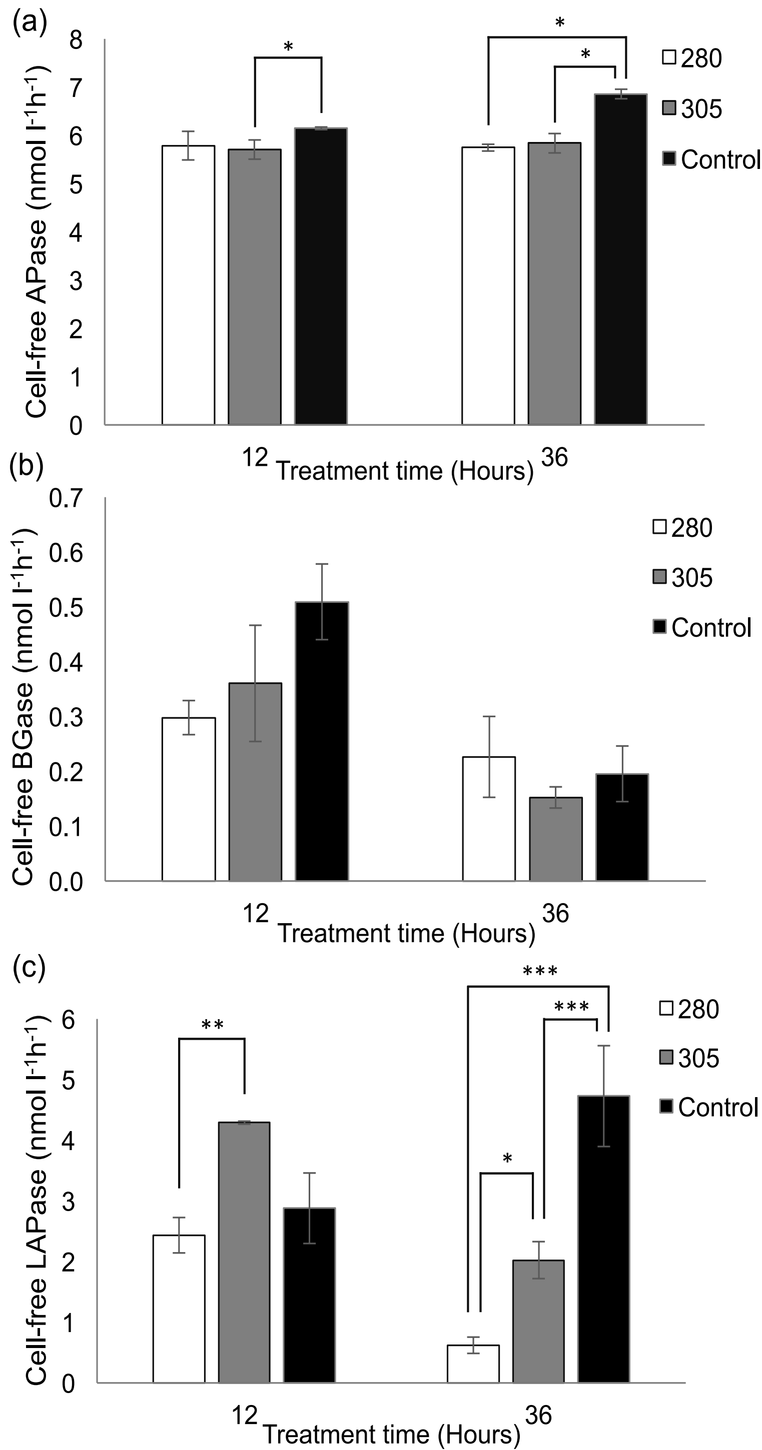
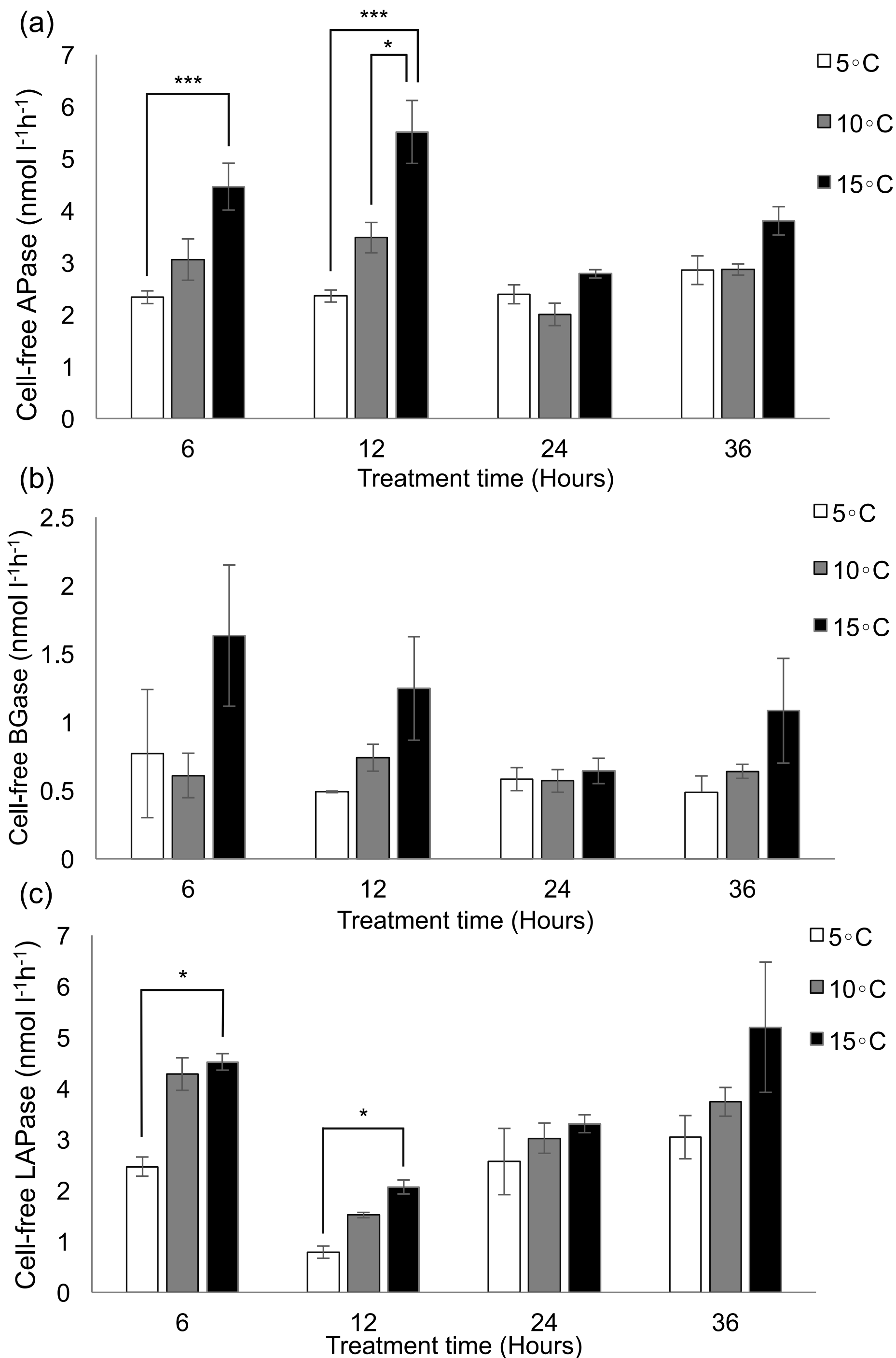


432 **Figure 1.** Results from UVR experiments showing the mean (±SE) cell-free extracellular enzyme
433 activity for alkaline phosphatase (a), beta-glucosidase (b), and leucine aminopeptidase (c), under a
434 high dose (of 280nm and above) and a low dose (of 305nm and above) in comparison to dark controls.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



437

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

443