Warming and organic matter sources impact the proportion of dissolved to total activities in marine extracellular enzymatic rates

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1 **Summary**

2 Extracellular enzymatic activities (EEAs) are the rate-limiting step in the
3 degradation of organic matter. Extracellular enzymes can be found associated to cells
4 or dissolved in the surrounding water. The proportion of cell-free EEA constitutes in
5 many marine environments more than half of the total activity. This high proportion
6 causes an uncoupling between hydrolysis rates and the actual bacterial activity.
7 However, we do not know what factors control the proportion of dissolved relative to
8 total EEA, nor how this may change in the future ocean. To resolve this, we
9 performed laboratory experiments with water from the Great Barrier Reef (Australia)
10 to study the effects of temperature and dissolved organic matter sources on EEA and
11 the proportion of dissolved EEA. We found that warming increases the rates of
12 organic matter hydrolysis and reduces the proportion of dissolved relative to total
13 EEA. This suggests a potential increase of the coupling between organic matter
14 hydrolysis and heterotrophic activities with increasing ocean temperatures, although
15 strongly dependent on the organic matter substrates available. Our study suggests that
16 local differences in the organic matter composition in tropical coastal ecosystems will
17 strongly affect the proportion of dissolved EEA in response to ocean warming.

Introduction

Heterotrophic bacterioplankton (including bacteria and archaea) play a key role in the cycling of matter in the ocean by transforming dissolved organic matter (DOM) into living particulate organic matter and CO₂ (Azam & Cho 1987). From the bulk DOM, these microbes preferentially utilize the high molecular weight fraction (Amon & Benner 1996; Benner & Amon 2015). Since only small molecules (<600 Da) can be directly transported across the cell membrane (Weiss et al. 1991), heterotrophic bacterioplankton need to cleave the high molecular weight molecules into smaller pieces before they can be transported into and used by the cells. To do so, aquatic microbes rely on the use of extracellular enzymes. Thus, extracellular enzymatic activities (EEA) are considered to be a rate limiting step in the degradation of organic matter (Hoppe et al. 2002).

EEA in the marine environments can be found attached to the cell wall or periplasmic space (i.e., cell-associated), or dissolved (i.e., cell-free) in the surrounding waters (Hoppe et al. 2002). Although there has been the perception that only cell-associated extracellular enzymes were of ecological significance (Chrost & Rai 1993; Hoppe 1983; Hoppe et al. 2002; Rego et al. 1985; Someville & Billen 1983); evidences are starting to accumulate strongly supporting a major role of cell-free EEA in the marine environment (Allison et al. 2012; Baltar et al. 2010; Baltar et al. 2013; Baltar et al. 2016a; Duhamel et al. 2010; Karner & Rassoulzadegan 1995; Keith & Arnosti 2001; Obayashi & Suzuki 2008). A high proportion of dissolved relative to total EEA can cause a significant decoupling between the microbial processes occurring in a water mass and the actual cycling and/or hydrolysis of organic matter (Arnosti 2011; Baltar et al. 2010; Baltar et al. 2013; Karner & Rassoulzadegan 1995). These active cell-free enzymes away from their sites (cells) can condition

macromolecular DOM and organic surfaces for subsequent microbial growth (Hoppe 1984), suggesting a great potential ecological and biogeochemical importance of dissolved EEA, and emphasizing the need of further research on the factors controlling cell-free EEA.

Different potential sources of cell-free EEA have been suggested, including direct enzyme release from microbial cells in response to substrate, starvation, viral lysis, protist grazing and changes in cell permeability (Albertson et al. 1990; Alderkamp et al. 2007; Bochkansky et al. 1995; Chrost 1991; Karner & Rassoulzadegan 1995). The resulting contribution of dissolved relative to total EEA will therefore depend not only on the response of individual cells to stimuli but also on interactions between organisms and how the environment impacts the lifetime of these free enzymes. Recently, a study in the Baltic Sea showed that the proportion of dissolved EEA was inversely related to temperature (Baltar et al. 2016a). These authors suggested that a decreased heterotrophic activity during months with lower temperature allowed for a longer lifetime of cell-free enzymes, thereby increasing the proportion of dissolved relative to total EEA. Still, there are many factors, besides temperature fluctuating between seasons (e.g. water mass advection, irradiance, microbial communities composition, organic matter quality and quantity), which might also affect the proportion of dissolved EEA and are difficult to account for in a seasonal field study.

Despite the importance and implications of cell-free EEA in marine environments, little is known about the controlling factors and/or how this will change as a response to future ocean warming. To resolve this question, we performed controlled laboratory experiments, to test the effect of temperature and selected DOM sources on EEAs and the relative importance of cell-free relative to total EEA. We

studied the variation in EEAs of glycolytic enzymes [β -glucosidase, BGase], a proteolytic enzyme [leucine aminopeptidase, LAPase]) and alkaline phosphatase [APase]), and in the proportion of dissolved relative to total LAPase, with water from the Great Barrier Reef (GBR) supplemented with DOM extracted from mangroves, seagrass or glucose, relative to unammended controls. These experiments were performed at three different temperatures (*in situ*, -3°C, +3°C), aimed at capturing the ca. +3°C temperature increase expected to occur in ocean surface waters by the end of this century (Collins et al. 2013), without causing too much perturbation on DOM-microbial interactions, as previously done in higher latitude environments (Huete-Stauffer et al. 2016; Huete-Stauffer et al. 2015).

Based on the notion that different enzymes are used to hydrolyze different substrates (carbohydrates, proteins, etc.) we hypothesized that differences in organic matter sources would change EEA rates. Although no information was available on how the proportion of dissolved EEA would be affected by different organic matter substrates, we hypothesized that different DOM sources would also change the relative proportion of cell-free EEA due to differences in the heterotrophic response. Based on the reported positive effect of temperature increases on extracellular enzymatic activities (Hollibaugh & Azam 1983; Piontek et al. 2009; Piontek et al. 2010) we anticipated that warming would enhance the total EEA rates. Finally, we also hypothesized that increasing temperature would decrease the proportion of dissolved EEA due to an intensification of the heterotrophic metabolism and shorter lifetimes of EEA in warm compared to cold waters (Baltar et al. 2010; Baltar et al. 2016a). Also, higher EEA under high temperatures and subsequent less production and release of extracellular enzymes may be another reason for lower proportion of

cell-free EEA. This is the first study on the effect of temperature on the proportion of dissolved EEA under a controlled environmental setting.

Methods

Experimental setup

Surface seawater (5 m depth) from the Great Barrier Reef (GBR) off Townsville (Australia), was collected the 18th of October 2016 using a 25 L Acid cleaned Niskin bottle and brought back to the laboratory from a coastal site of 20-30 m depth / 4 km from the coast away from direct human influence (Table 1). One part of the seawater was filtered through pre-combusted (450°C for 4 h) GF/C filters (nominal pore size 1.2 µm) to establish a microbial culture to be used in all experiments. The other part of the seawater was gravity filtered through a dual-stage (0.8/0.2 µm) filter cartridge (Pall-Acropak Supor membrane), which had been pre-washed with Milli-Q (>10 L). The seawater was thereafter used both as the control treatment and to dilute the DOM obtained from a glucose solution, Mangrove and Seagrass leaves.

Fresh Mangrove (*Rhizophora stylosa*) and Seagrass leafs (*Halodule uninervis*) were collected in Cleveland Bay (19°13'05"S 146°55'19"E), Australia, brought back to the laboratory and rinsed thoroughly with 0.2 µm-prefiltered surface water. The Mangrove and Seagrass-derived DOM was thereafter extracted by adding approx. 25 g of wet leaves to a glass bottle containing 1 L of 0.22 µm-filtered seawater. After 24 h in the dark the water was filtered the water was filtered through a pre-combusted GF/C filter and, then, through a dual-stage filter cartridge (0.8/0.2 µm, Pall-Acropak Supor membrane) to isolate the DOM fraction. The dissolved organic carbon (DOC) of these solutions were then measured.

Samples for the analysis of DOC were filtered through prewashed (250 mL Milli-Q water) 0.2 μm filters (Pall, Supor membrane Disc Filter) and collected in pre-combusted (450°C, 12 hours) glass ampoules and preserved by adding 50 μL 25 % H_2PO_4 . DOC concentrations were measured by high temperature combustion (720°C) using a Shimadzu TOC-L carbon analyzer. Prior to analysis, CO_2 remaining in the acidified sample water was removed by sparging with O_2 carrier gas. Three to five replicate injections of 150 μL were performed per sample. Concentrations were determined by subtracting a Milli-Q blank and dividing by the slope of a daily standard curve made from potassium hydrogen phthalate and glycine. Using the deep ocean reference samples we obtained an average concentration of $43 \pm 1 \mu\text{mol L}^{-1}$, with the nominal DOC value provided by the reference laboratory (Prof. Hansell Lab) being are 41–44 $\mu\text{mol L}^{-1}$.

The DOM sources were thereafter added to different 20 l carboy's (seagrass, mangrove, glucose) to reach a DOC enrichment of ca. 40 $\mu\text{mol l}^{-1}$ (corresponds to the approximate seasonal build-up of in DOC in the GBR). Seagrass and Mangrove were used due to their known contribution of organic material to tropical waters which might change in response to anthropogenic perturbations (Duarte 2017). Glucose was selected for comparison as an easily degradable DOC source. The GF/C filtered microbial culture was thereafter added in a ratio of 1 part of microbial culture, to 9 parts of water (control, seagrass, mangrove, glucose).

The water was then distributed into acid-rinsed glass bottles (500 ml) and incubated at 3 different temperatures (*in situ*, -3°C, +3°C), with 3 replicate bottles being analysed for subsampling at Day 0, 1, 2, 3 and 4. The incubations were performed at the National Sea Simulator (SeaSim) of the Australian Institute of Marine Science (AIMS), which is able to keep the temperature constant within a 0.1°C range.

Measurement of Total and Dissolved extracellular enzymatic activity (EEA)

The hydrolysis of the fluorogenic substrate analogues 4-methylcoumarinyl-7-amide (MCA)-L-leucine-7-amido-4-methylcoumarin, 4-methylumbelliferyl (MUF)- β -D-glucoside and MUF-phosphate was analyzed to estimate potential activity rates of leucine aminopeptidase (LAPase), β -glucosidase (BGase), and alkaline phosphatase (APase), respectively (Hoppe 1983). The same procedure was followed as previously described (Baltar et al. 2010; Baltar et al. 2013; Baltar et al. 2009). Briefly, EEA was determined after substrate addition and incubation using a spectrofluorometer with a microwell plate reader (Biotek Cytation 3 Imaging Multi-Mode Reader) at excitation and emission wavelengths of 365 and 445 nm, respectively. Samples (300 μ l) were incubated in the dark at the corresponding temperature (*in situ*, -3°C, +3°C) for 1.5-3 h. The increase in fluorescence over time was transformed into hydrolysis activity using a standard curve established with different concentrations of the fluorochromes MUF and MCA added to 0.2 μ m filtered sample water. A final substrate concentration of 250 μ mol l⁻¹ was used to measure BGase activities, APase and LAPase.

The total and the dissolved fraction of the LAPase EEA were distinguished as previously described (Baltar et al. 2010; Baltar et al. 2013; Baltar et al. 2016a). Briefly, raw seawater was used for total EEA; whereas for dissolved EEA, samples were gently filtered through a low protein-binding 0.2 μ m Acrodisc Syringe filter (Pall) for dissolved EEA following the protocol of (Kim et al. 2007). In the present study, dissolved (cell-free) EEA is defined as the EEA recovered in the filtrate. Total and dissolved EEA were determined on six technical replicate samples per treatment and sampling point.

Statistical analysis

The relation between temperature increase and the proportion of dissolved LAPase was examined by means of regression analysis between temperature and the proportion of dissolved LAPase on the final day of the experiment. A linear regression model was fitted to the data and the slope was presented as an indication of the relation between temperature and EEA. For calculating uncertainties in ratios, error propagation was taken into account; SDs were calculated using a formula for the propagation of error as described by (Bevington & Robinson 2003) as follows:

$$\Delta z / z = \sqrt{[(\Delta x)^2 / x] + [(\Delta y)^2 / y]}$$

where z is the percent abundance or the percent assimilation, which is equal to x divided by y, as described above. Δx and Δy are the SDs associated with x and y, respectively. Δz is the SD calculated for z. SDs were then converted to SEs for each calculation

Results and Discussion

In situ inoculum conditions

Leucine aminopeptidase (LAPase) was the most active extracellular enzymatic activity in the inoculum water used for this experiment (Table 1). LAPase was around 5- and 20-fold higher than alkaline phosphatase (APase) and β -glucosidase (BGase), respectively. This higher magnitude of the activity of LAPase as compared to APase and BGase is a recognized frequently observed characteristic in marine environments (Hoppe et al. 2002). LAPase was selected as the target EEA to study the dynamic of the proportion of dissolved relative to total EEA not only because LAPase is usually the most active EEA in seawater, as also found in this study, but also because the

proportion of dissolved APase and BGase have been shown to behave similarly to the proportion of dissolved LAPase over large spatial and temporal scales (Baltar et al. 2010; Baltar et al. 2013; Baltar et al. 2016a). The proportion of dissolved LAPase, sampled in October (austral spring), was $40.5 \pm 6\%$ in the inoculum water. That contribution is consistent with the 35-50% range of dissolved LAPase in the Baltic Sea during spring-summer (Baltar et al. 2016a), but lower than the 65-100% range observed during the rest of the year in the Baltic Sea and in the Atlantic Ocean (Baltar et al. 2010; Baltar et al. 2013).

Response of total EEA to different organic matter sources

The inoculum water was diluted 1:10 with 0.22 μm -filtered seawater (see Methods for more detail), causing the EEA rates to be below the detection limit at the beginning of the experiments. However, the EEAs were already detectable after day 1 (Fig. 1). APase was the EEA showing the fastest response among all the measured enzymes, particularly in the mangrove treatment (Fig 1). This faster and stronger response of APase to mangrove DOM compared with the other organic matter sources was sustained during the whole experiment. This enhancement in APase could be indicative of phosphorus limitation in the mangrove treatment since high APase relative to LAPase has been suggested as indicative of P limitation (Sala et al. 2001). Alternative, it is possible that this strong APase in the mangrove treatment could be due to a strong increase in heterotrophic rates in response to highly palatable DOM and/or linked to other processes such as cross-activation by nonpartner histidine kinases, and/or the activation of genes involved in the regulation of elemental balance during catabolic processes (Baltar et al. 2016b).

BGase remained relatively low during the whole experiment, with the exception of the mangrove treatment (Fig. 1). This higher BGase could be indicative of a carbohydrate rich material (typical of land plants) or it could simply be due to a higher heterotrophic growth rate. Although APase was the fastest EEA to react to mangrove DOM, LAPase was the EEA reaching the highest rates (Fig. 1), consistent with the highest LAPase found in the *in situ* inoculum water (Table 1). In contrast to BGase, LAPase was increased in all the DOM treatments and control during the length of the experiment, highlighting the generalist importance of this enzyme in the growth of heterotrophic microbes.

Dissolved relative to total LAPase in response to different organic matter sources

A consistent pattern was observed for total LAPase over time, increasing in all treatments (Fig. 2A). This increase in total LAPase was slightly higher in response to glucose than in the control, increasing from 0 to 34 and 17 nmol l⁻¹ h⁻¹, respectively. However, the strongest responses in total LAPase were found in the seagrass and mangrove DOM treatments, increasing from 0 to 119 and 396 nmol l⁻¹ h⁻¹, respectively. This stronger response observed in the seagrass and mangrove DOM treatments relative to the glucose one could be indicative of a more nutritious nature of the plant DOM containing not only carbohydrates but also other building blocks and cofactors such as aminoacids, metal-ions, and vitamins, etc.

The dissolved LAPase followed a similar pattern to the total LAPase, showing a stronger response in the mangrove DOM followed by the seagrass, glucose and control (Fig. 2B). However, in contrast to the total LAPase, the dissolved LAPase activities stopped increasing in most treatments (except mangrove) after day 3.

Contrary the proportion of dissolved LAPase showed a decreasing with time (Fig. 2C). This was more pronounced in the treatments with highest LAPase activities; decreasing from 95 to 6% and from 74 to 14% in the seagrass and in the mangrove treatment, respectively. This decrease contrasts the minor decrease in the glucose treatment (from 33 to 25%) and control (from 63 to 29%), where the proportion of dissolved LAPase was still >25% at the end of the experiment. These results suggest that changes in the sources of organic matter to tropical microbial bacterioplankton communities will not only change the total EEA rates and the spectrum of EEA (due to relative differences in the effect in different enzymes), but also the importance of dissolved relative to total EEA.

Dissolved relative to total LAPase in response to temperature

The proportion of dissolved relative to total LAPase decreased consistently with time in all treatments and temperatures (from 100 to 5%), with higher decreases in the M (from 74 to 5%) and S (from 100 to 4%) treatments, in turn characterized by higher initial values of the proportion (Fig. 3). However, experimental warming noticeably enhanced this decrease. Table 2 shows the effect of temperature on the final proportions (day 4) at each treatment. Our results suggest that regardless of the source of carbon, warming will likely result in a decrease in the fraction of dissolved EEA in tropical coastal waters. This analysis indicated that the proportion of dissolved LAPase dropped on average by 1.1 to 5% for every 1°C increase depending on the source of organic matter type supplied.

The results presented here are the first investigating the effect of temperature on the proportion of dissolved EEA under a controlled environmental setting. These results are consistent, with the seasonal relation observed between the proportion of

dissolved LAPase, BGase and APase and temperature in a field study in the Baltic Sea, where the proportion of dissolved relative to total EEA decreased during spring-summer (0-39%) and increased (up to 100%) during winter months (Baltar et al. 2016a). This seasonal pattern is also consistent with the observed increase in the proportion of dissolved LAPase from 65 to 100% from the epipelagic to the abyssopelagic layer of the Atlantic Ocean (Baltar et al. 2010), supporting the role of temperature as the environmental driver affecting this proportion.

The above mentioned findings are also consistent with the negative effect of temperature on the lifetime of extracellular enzymes (APase) in surface waters of the Red Sea (Li et al. 1998), the extended lifetime of cell-free LAPase, APase and BGase enzymes in Arctic waters (Steen & Arnosti 2011), and with the long lifetimes (of LAPase, APase and BGase) found in the deep as compared to the surface waters in the Atlantic Ocean (Baltar et al. 2013).

These results suggest that the proportion of dissolved LAPase is tightly connected to the heterotrophic activity level of the community, so that the higher the activity the lower the proportion of dissolved LAPase. This could be explained by two likely causes. One plausible explanation is that the decrease in the community heterotrophic rates would also mean a decrease in the degradation/consumption of free enzymes, allowing for extended lifetimes of free enzymes (Baltar et al. 2016a). This option gives a greater weight to temperature than to other factors, suggesting that the higher the temperature the shorter the lifetime of free enzymes and the proportion of dissolved EEA. An alternate explanation could be that actively growing microbes would rely more closely on cell-attached rather than cell-free EEA (i.e., produce more new enzymes which will be more frequently found, at least initially, attached to the cells), causing the proportion of dissolved EEA to decrease more strongly under

290 slower growth (conditions). This second option would also mean that the organic
291 matter substrates that most stimulate the heterotrophic activity of microbes will
292 thereby be the ones showing the largest decrease in the proportion of dissolved
293 relative to total LAPase. This second explanation becomes particularly important in
294 the context of this study, where most particles were initially removed by filtration,
295 since free-living microbes tend to show a tight hydrolysis-uptake coupling, whereas
296 particle-attached microbes tend to show a loose hydrolysis-uptake coupling producing
297 a lot of cell-free enzymes (Karner & Herndl 1992; Smith et al. 1992; Vetter et al.
298 1998). Thus, the absence (or strongly reduced number) of large particles and the
299 availability of substrate would favour a pronounced decrease in the proportion of
300 dissolved LAPase.

301 The results obtained in this study together with recent reports suggest that the
302 proportion of dissolved relative to total LAPase, and thereby the coupling between
303 hydrolysis of organic matter and other microbial heterotrophic processes (such as
304 production, respiration, etc.), depends not only on temperature but also on the organic
305 matter substrates available. Overall, the proportion of dissolved relative to total
306 LAPase will depend on the balance between “passive” accumulation of free enzymes
307 (controlled in turn by the balance between production and consumption of free
308 enzymes in response to changes in temperature and in community heterotrophic rates)
309 and “active” production of cell-attached enzymes in response to organic matter inputs.

311 **Conclusions**

312 To our knowledge this study is the first to determine the effect of temperature and
313 different DOM sources on the proportion of dissolved relative to total EEA. Our
314 results suggest that increased temperature will not only affect the total enzymatic

activity (increasing the hydrolysis of organic matter) but will also alter the structuring of EEA by reducing the proportion of dissolved relative to total EEA. Thus, these results suggest that the decoupling between the hydrolysis of the organic matter and the actual growth of heterotrophic microbes (as well as the magnitude of the conditioning of macromolecular DOC and organic surfaces for microbial growth by dissolved enzymes away from the source cells), will likely be reduced with increasing ocean temperatures. Moreover, this decrease will greatly depend on the organic matter substrates available, and therefore on present and future changes in the structure of tropical coastal ecosystems (i.e., health and cover of mangrove, seagrass, etc.).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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450

451 Table 1. Location, physicochemical characteristics and extracellular enzymatic
 452 activities of the inoculum water used to setup the experiment. APase: alkaline
 453 phosphatase, BGase: β -glucosidase, LAPase: leucine aminopeptidase.

454

Latitude (S)	19° 13' 6.60''
Longitude (E)	147° 8' 21.24''
Temperature (°C)	27.7°C
Salinity	35.5
APase (nmol l ⁻¹ h ⁻¹)	4.01
BGase (nmol l ⁻¹ h ⁻¹)	0.90
LAPase (nmol l ⁻¹ h ⁻¹)	19.29
Dissolved LAPase (nmol l ⁻¹ h ⁻¹)	7.81
Proportion of dissolved LAPase (%)	40.5
Bacterial abundance (cell ml ⁻¹)	4.18 x 10 ⁵
DOC (μmol l ⁻¹)	84

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457 Table 2. Linear regression model results for the proportion of dissolved LAPase
458 *versus* temperature on the final ratios (day 4) at each treatment.

459

	Slope (% °C ⁻¹)	SE	r ²	p	n
Control	-5.07	1.62	0.91	0.07	3
Glucose	-1.53	0.08	1.00	0.03	3
Mangrove	-2.48	0.18	0.99	0.02	3
Seagrass	-1.11	0.30	0.93	0.07	3

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464

Figure legends

Figure 1. Mean ($\pm$ SE) temporal variability of total extracellular enzymatic activities (APase: alkaline phosphatase, BGase: β -glucosidase, LAPase: leucine aminopeptidase) in the different dissolved organic matter treatments and controls. C: control, G: glucose, M: mangrove, S: seagrass, t1: day 1, t2: day 2, t3: day3, t4: day 4.

Figure 2. Mean ($\pm$ SE) temporal evolution of total LAPase (A), dissolved LAPase (B) and the proportion of dissolved relative to total LAPase (C) in response to the different dissolved organic matter treatments and controls. C: control, G: glucose, M: mangrove, S: seagrass.

Figure 3. Mean ($\pm$ SE) temperature effect on total LAPase (A-D) and the proportion of dissolved to total LAPase dissolved LAPase (E-H) during the incubations in the control (A, E) and in response to glucose (B, F), dissolved organic matter from mangrove (C, G) and from seagrass (D, H).

Total LAPase **Dissolved LAPase** **% Dissolved LAPase**