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1 # Cell-free extracellular enzymatic activity is linked to
2 # seasonal temperature changes in the Baltic Sea

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10

11 ## Abstract

12 Extracellular enzymatic activities (EEA) are a crucial step on the degradation of organic
13 matter. Dissolved (cell-free) extracellular enzymes in seawater can make up a significant
14 contribution of the bulk EEA. However, the factors controlling the proportion of dissolved
15 EEA in the marine environment remain unknown. Here we studied the seasonal changes in
16 the proportion of dissolved relative to total EEA (of alkaline phosphatase [APase], β-
17 glucosidase, [BGase], and leucine aminopeptidase, [LAPase]), in the Baltic Sea for 18
18 months. The proportion of dissolved EEA ranged between 37-100%, 0-100%, 34-100% for
19 APase, BGase and LAPase, respectively. A consistent seasonal pattern in the proportion of
20 dissolved EEA was found among all the studied enzymes, with values up to 100% during
21 winter and <40% during summer. A significant negative relation was found between the
22 proportion of dissolved EEA and temperature, indicating that temperature might be a critical
23 factor controlling the proportion of dissolved relative to total EEA in marine environments.
24 Our results suggest a strong decoupling of hydrolysis rates from microbial dynamics in cold
25 waters. This implies that under cold conditions, cell-free enzymes can contribute to substrate
26 availability at large distances from the producing cell, increasing the dissociation between the
27 hydrolysis of organic compounds and the actual microbes producing the enzymes. This also
28 indicates that global warming could come to affect the hydrolysis of organic matter by
29 reducing the hydrolytic activity of cell-free enzymes.

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1 Introduction

Heterotrophic prokaryotes play a central role in the marine biogeochemical cycles by transforming dissolved organic matter (DOM) into living particulate organic matter (Azam and Cho, 1987). These organisms preferentially consume high molecular weight DOM, as explained by the DOM size-reactivity model (Amon and Benner, 1996; Benner and Amon, 2015). However, since only molecules <600 Da can be directly transported across the prokaryotic cell membrane (Weiss et al., 1991), heterotrophic prokaryotes need to use extracellular enzymes (EE) for hydrolyzing high molecular weight DOM into low molecular weight compounds suitable for uptake. This is why the activity of extracellular enzymatic activity (EEA) has been recognized as the rate-limiting step in organic matter degradation (Arnosti, 2011).

EEA in aquatic environments can be cell-associated (i.e., EEs attached to the cell wall or in the periplasmatic space), or dissolved (i.e., cell-free) in the surrounding waters (Hoppe et al., 2002)). Until recently, most EEA in the marine environment was believed to be associated to cells (Hoppe, 1983; Hoppe et al., 2002) leading to the perception that only cell-associated EEs were of ecological significance (Chrost and Rai, 1993; Rego et al., 1985; Someville and Billen, 1983). However, recent reports suggested a major contribution of dissolved EEA to the total oceanic EEA pool (Baltar et al., 2010; Baltar et al., 2013; Duhamel et al., 2010). This high proportion of cell-free EEA is important because it can decouple hydrolysis rates of organic material from microbial dynamics; this is, a high proportion of dissolved EEA could indicate a greater importance of the history of the water mass than of the actual processes occurring at the time of sampling (Baltar et al., 2013; Karner and Rassoulzadegan, 1995).

Different potential sources of cell-free EEA include direct EE release from cells in response to appropriate substrate (Alderkamp et al., 2007), to bacterial starvation (Albertson et al., 1990) to changes in cell permeability (Chrost, 1991), to viral lysis (Karner and Rassoulzadegan, 1995) and to protist grazing (Bochdansky et al., 1995). However, little is known about what happens once the enzymes are free in the marine environment, including information about their lifetimes. The few available studies on the EE lifetime, indicate a lifetime range between tens to hundreds of hours. Surface water EE lifetimes, when incubated at in situ temperature, ranged between >1 to 9 days (Bochdansky et al., 1995; Ziervogel and Arnosti, 2008; Ziervogel et al., 2010). However, EE lifetimes of surface waters were longer

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(up to 40 d) when incubated in the dark at 4°C than at the in situ conditions of light and temperature (Li et al., 1998). This is consistent with the only available study comparing cold deep versus warm surface waters EE lifetimes, where EE lifetimes were about one order of magnitude longer in the deep waters (Baltar et al., 2013). These results suggest that temperature could be a critical factor preserving the activity of cell-free EEA and thereby controlling the proportion of dissolved EEA in the marine environment.

Despite the importance and implications of the cell-free EEA in marine environments, little is known about what are the factors that control changes in the proportion of dissolved EEA. To resolve this question, a long temporal sampling strategy is needed that accounts for the long lifetime of EEs. Here we studied the temporal changes in the proportion of dissolved relative to total EEA (of glycolytic enzymes [β-glucosidase, BGase], a proteolytic enzyme [leucine aminopeptidase, LAPase]) and alkaline phosphatase [APase]), in a continuous biweekly sampling, with water from the Baltic Sea, for 18 months. We aimed to reveal the seasonal variability of dissolved EEA and to decipher what factors could control the proportion of dissolved relative to total EEA. Based on previous research suggesting longer EE lifetimes in cold environments, we hypothesized to find a strong link between temperature and the proportion of dissolved relative to total EEA, with lower proportions of dissolved EEA during warm periods (e.g. summer) than during cold periods (e.g. winter).

2 Materials and methods

2.1 Study site and sampling

Seawater from the Baltic Sea proper was collected twice weekly for almost 18 months, from March 22, 2012 to the August 15, 2013. Samples were taken at 2 m depth at the Linnaeus Microbial Observatory (LMO) (N 56°55.851, E 17°03.640), 10 km off the east coast of Öland, Sweden, using a Ruttner sampler. Temperature was measured on site, and the water was transported to the laboratory in acid-washed Milli-Q-rinsed polycarbonate bottles within 1 h.

2.2 Chlorophyll a concentration

Chlorophyll a (Chl *a*) concentration was analyzed following extraction using ethanol (Jespersen and Christoffersen, 1987).

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2.3 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 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 (FLUOstar – BMG Labtech) at excitation and emission wavelengths of 365 and 445 nm, respectively. Samples (300 µl) were incubated in the dark at *in situ* temperature 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 µm filtered sample water. A final substrate concentration of 31.2 µmol l^{-1} was used to measure BGase activities, 100 µmol l^{-1} for APase and 500 µmol l^{-1} for LAPase. These concentrations were previously determined as saturating substrate concentrations.

The total and the dissolved fraction of the EEA was distinguished as previously described (Baltar et al., 2010; Baltar et al., 2013). Briefly, raw seawater was used for total EEA; whereas for dissolved EEA, samples were gently filtered through a low protein-binding 0.2 µm Acrodisc Syringe filter (Pall) for dissolved EEA following the protocol of (Kim et al., 2007). The use of low protein-binding filters is important in this context since the adsorption of extracellular enzymes depends on the type of the filter material used for size fractionation (Obayashi and Suzuki, 2008). 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 replicate samples each.

2.4 Statistical analyses

The relations between variables were examined by means of correlation analysis computing Pearson pairwise statistics. Normality was checked with a Shapiro-Wilks test before Pearson correlations were calculated.

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3 Results and discussion

A clear seasonal pattern in temperature was observed, with lower and relatively stable temperatures during winter (3-4°C), and strong increases during spring-summer (up to 20°C), followed by a quick temperature drop in autumn (Fig. 1A). Chlorophyll-a concentration varied between 0.4 to 4.8 µg l^{-1}, with maximum peaks during the two types of blooms that typically occur in the Baltic (Lindh et al., 2015), the diatom and dinoflagellate spring bloom and the cyanobacterial summer bloom (Legrand et al., 2015) (Fig. 1B).

The total (bulk) EEA of APase, LAPase and BGase followed a similar temporal pattern (Fig. 2A). APase was the EEA with the highest rates (ranging from 1.5-32 nmol l^{-1} h^{-1}), followed by LAPase (from 0.6-9.3 nmol l^{-1} h^{-1}) and BGase (from 0.1-21 nmol l^{-1} h^{-1}), indicating a potential significant P limitation in the Baltic Sea (Granéli et al., 1990; Hagström et al., 2001). The strongest peaks in BGase and LAPase co-occurred (June and August 2012, May and August 2013), together with some of the APase and the Chla-a peaks, suggesting a link between these different enzymes and the phytoplankton bloom. However, the BGase:LAPase ratio, indicative of the relative degradation of polysaccharides relative to proteinaceous material, peaked when the highest BGase and LAPase rates were observed, coinciding with Chl-a peaks. This is agreement with the results obtained in a recent seasonal study in the Adriatic Sea, where BGase prevailed over LAPase associated to phytoplankton blooms (Celussi and Del Negro, 2012). There was a tendency for higher EEA rates (and BGase:LAPase ratio) in 2013 as compared to 2012, likely explained by the reported interannual variability of phytoplankton communities linked to environmental conditions in the Baltic Sea (Kahru and Elmgren, 2014; Legrand et al., 2015).

The proportion of dissolved relative to total EEA ranged between 0-100%, where LAPase and APase showed a similar range (37-100% and 37-100%, respectively) and BGase showed the broadest range (0-100%) (Fig. 3). LAPase was the EEA with the lowest seasonal amplitude variability in the proportion of dissolved EEA, wheras BGase showed the largest seasonal variability. These values are within the same ranges reported in the surface coastal North Sea waters (Someville and Billen, 1983), Tokyo Bay (Hashimoto et al., 1985), Mediterranean Sea (Karner and Rassoulzadegan, 1995), Elbe estuary (Karrasch et al., 2003), Gulf of Mexico (Ziervogel and Arnosti, 2008; Ziervogel et al., 2010), North Pacific Subtropical Gyre (Duhamel et al., 2010) and the epipelagic to bathypelagic waters of the Atlantic (Baltar et al., 2010; Baltar et al., 2013).

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1 The proportion of dissolved EEA showed a clear seasonal pattern in our study (despite the
2 great variability in the bulk EEA rates), with higher values in winter and a pronounced
3 decrease in summer. This seasonal pattern in the % of dissolved EEA was conserved among
4 all the enzymes studied (APase, BGase and LAPase), suggesting that the main factors
5 regulating the proportion of dissolved EEA affect all enzymes equally, irrespectively of their
6 metabolic function. This advocates for some environmental factor rather than a biological
7 factor controlling the proportion of dissolved EEA. The most logical factors that could be
8 responsible for this seasonal pattern would be light and/or temperature. Inactivation of
9 extracellular enzymes by photochemical reactions have been found in biofilm microbiota
10 (Espeland and Wetzel, 2001). However, in laboratory experiments with Arctic surface waters,
11 the activity of cell-free EEs (APase and LAPase) were not affected by light (under full
12 spectrum natural sunlight), suggesting that photochemical reactions are not relevant pathway
13 for the decay of cell-free EE in seawater (Steen and Arnosti, 2011).

14 Indeed, a significant negative correlation was found between the proportion of dissolved EEA
15 and temperature for APase (Pearson's r = -0.60, N = 22, p=0.0035)), BGase (Pearson's r = -
16 0.71, N = 22, p=0.0002) and LAPase (Pearson's r = -0.67, N = 22, p=0.0006). These results
17 are consistent with the negative effect of temperature on EE lifetimes found in an incubation
18 experiment with Red Sea surface water (Li et al., 1998), the extended life time of cell-free EE
19 in Arctic waters (Steen and Arnosti, 2011), and with the order of magnitude higher EE
20 lifetimes found in the deep as compared to the surface waters of the Atlantic (Baltar et al.,
21 2013). This suggests that low temperature preserves better (than warm temperature) the
22 constitutive activity of the cell-free enzymes, allowing them to remain active for longer
23 periods. This might be linked to a reduction in the metabolism of heterotrophic microbes that
24 would reduce the consumption/degradation rates of dissolved EEs. The higher proportion of
25 dissolved EEA during winter suggests that the decoupling of *in situ* hydrolysis rates from
26 actual microbial dynamics is stronger during winter (or in cold waters). Thus, these results
27 underpin the importance of considering this stronger cold-related decoupling when relating
28 EEA to other microbial processes. These results also suggest that, under the projected global
29 warming scenario, it is likely that the hydrolysis of organic matter due to cell-free EE might
30 be reduced due to a shorter lifetime of the EEs.

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4 Conclusions

Overall, the results of this study suggest that a relevant fraction of the total EEA measured in a particular environment can be due to free EEs, which might be a consequence of the substrate history of the water masses. Thus, advection of dissolved EEA might be a critical source of EEA at any given environment (Baltar et al., 2010; Baltar et al., 2013; Steen and Arnosti, 2011). Other factors might also allow for extended EE lifetimes during advection, like association of EEs to particles (Gianfreda and Scarfi, 1991; Naidja et al., 2000; Ziervogel et al., 2007), to exopolymeric matrix (Decho, 1990), and to detrital particle complexes (Nagata and Kirchman, 1992). Low temperature seems to be a critical factor favoring longer EE lifetimes and thereby higher proportions of dissolved relative to total EEA. This implies that under cold conditions, cell-free enzymes can contribute to substrate availability at large distances from the producing cell, potentiating the disconnection between the hydrolysis of organic compounds and the actual microbes producing the enzymes. Moreover, under warmer conditions, like those predicted to occur due to global warming, the hydrolysis of organic matter (i.e., rate limiting step in the degradation of organic matter) can be reduced due to a lower contribution of the cell-free EE hydrolysis.

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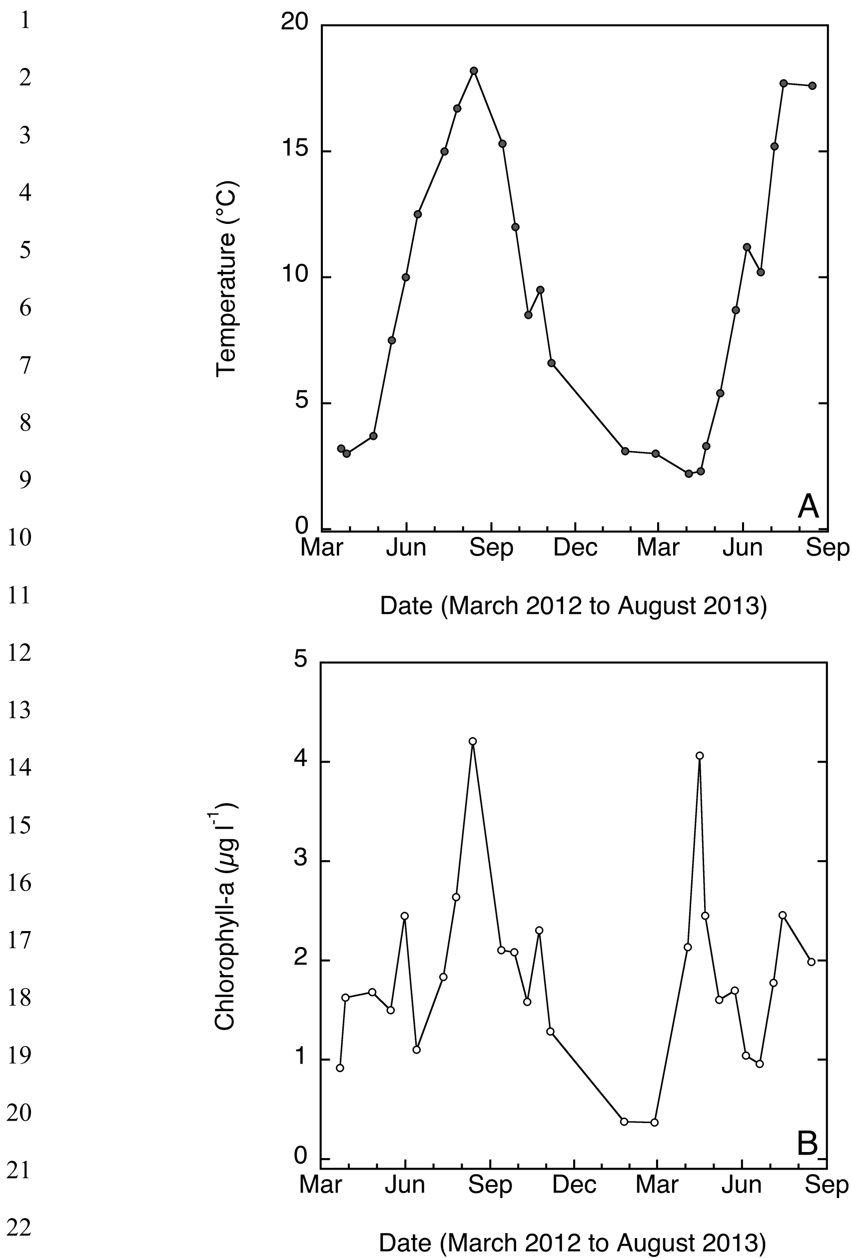

24 Figure 1. Temporal dynamics in (A) temperature and (B) chlorophyll-a concentrations, at the
25 Linnaeus Microbial Observatory (LMO, Baltic Sea) from March 2012 to August 2013

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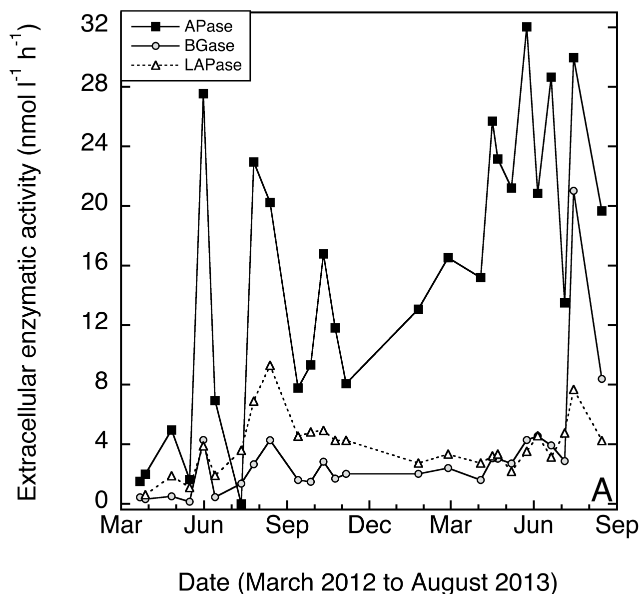

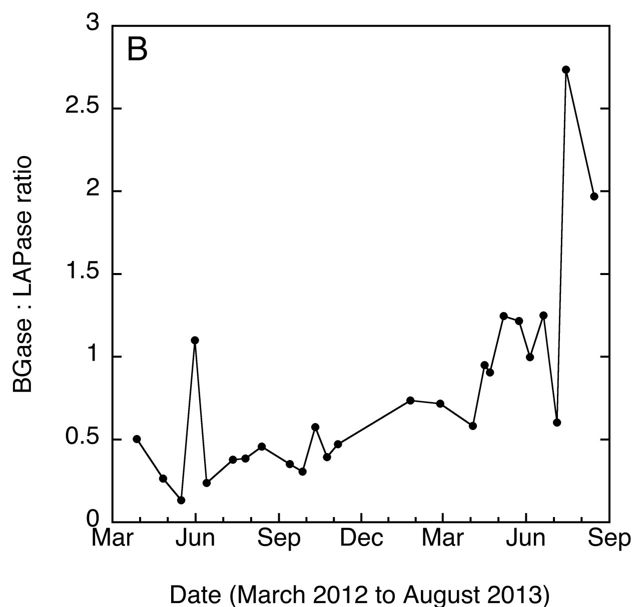

Figure 2. Temporal dynamics in (A) extracellular enzymatic activities of alkaline phosphatase (APase), β-glucosidase, (BGase), and leucine aminopeptidase, (LAPase) and), and (B) the BGase:LAPase ratio, at the Linnaeus Microbial Observatory (LMO, Baltic Sea) from March 2012 to August 2013.,

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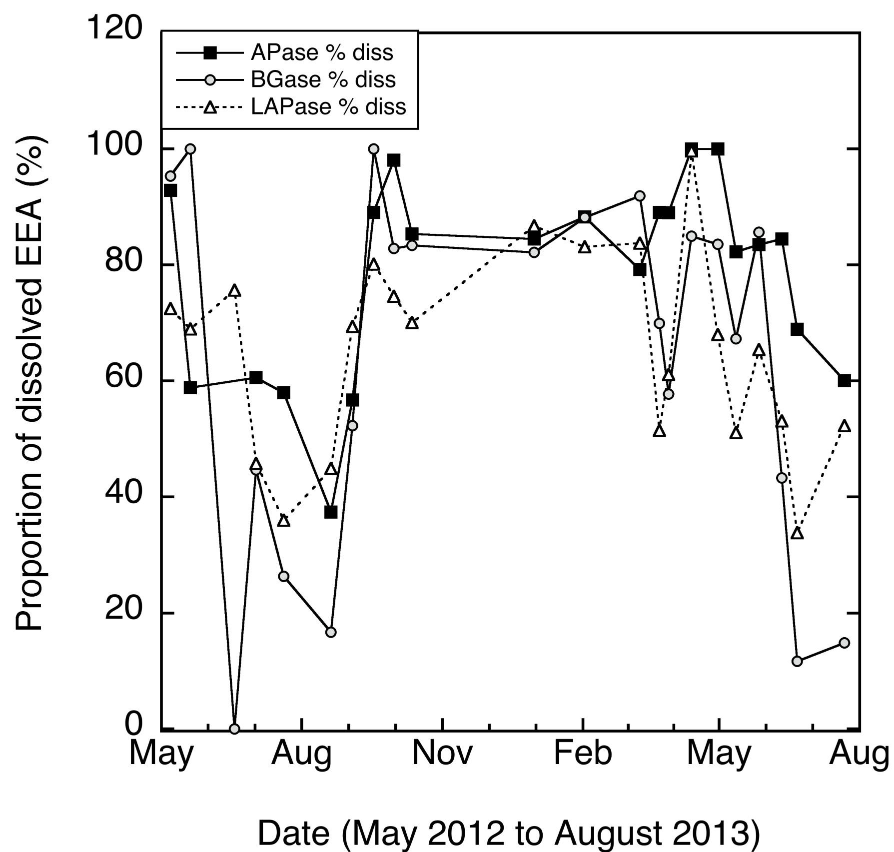

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4 Figure 3. Temporal dynamics in the proportion of dissolved relative to total extracellular
5 enzymatic activities (EEA) of alkaline phosphatase (APase), β-glucosidase, (BGase), and
6 leucine aminopeptidase, (LAPase), at the Linnaeus Microbial Observatory (LMO, Baltic Sea)
7 from May 2012 to August 2013.

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