

1 **Cell-free extracellular enzymatic activity is linked to** 2 **seasonal temperature changes: a case study in the Baltic** 3 **Sea**

4
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11 12 **Abstract**

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

1 reduction of the contribution of of cell-free enzymes to the bulk hydrolytic activity, and call
2 for the need of further research to confirm it.

3

4 **1 Introduction**

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

14 EEA in aquatic environments can be cell-associated (i.e., EEs attached to the cell wall or in
15 the periplasmic space), or dissolved (i.e., cell-free) in the surrounding waters (Hoppe et al.,
16 2002). Until recently, most EEA in the marine environment was believed to be associated to
17 cells (Hoppe, 1983; Hoppe et al., 2002) leading to the perception that only cell-associated EEs
18 were of ecological significance (Chrost and Rai, 1993; Rego et al., 1985; Someville and
19 Billen, 1983). However, other reports suggested a major contribution of dissolved EEA to the
20 total oceanic EEA pool (Baltar et al., 2010; Baltar et al., 2013; Duhamel et al., 2010; Karner
21 and Rassoulzadegan, 1995; Keith and Arnosti, 2001; Obayashi and Suzuki, 2008a). This high
22 proportion of cell-free EEA is important because it can decouple hydrolysis rates of organic
23 material from microbial dynamics; this is, a high proportion of dissolved EEA could indicate
24 a greater importance of the history of the water mass than of the actual processes occurring at
25 the time of sampling (Arnosti, 2011; Baltar et al., 2010; Baltar et al., 2013; Karner and
26 Rassoulzadegan, 1995).

27 Different potential sources of cell-free EEA include direct EE release from cells in response
28 to appropriate substrate (Alderkamp et al., 2007), to bacterial starvation (Albertson et al.,
29 1990) to changes in cell permeability (Chrost, 1991), to viral lysis (Karner and
30 Rassoulzadegan, 1995) and to protist grazing (Bochdansky et al., 1995). However, little is
31 known about what happens once the enzymes are free in the marine environment, including
32 information about their lifetimes. The few available studies on the EE lifetime, indicate a

1 lifetime range between tens to hundreds of hours. Surface water EE lifetimes, when incubated
2 at in situ temperature, ranged between >1 to 9 days (Bochdansky et al., 1995; Steen and
3 Arnosti, 2011; Ziervogel and Arnosti, 2008; Ziervogel et al., 2010). However, EE lifetimes of
4 surface waters were longer (up to 40 d) when incubated in the dark at 4°C than at the in situ
5 conditions of light and temperature (Li et al., 1998). This is consistent with the only available
6 study comparing cold deep versus warm surface waters EE lifetimes, where EE lifetimes were
7 about one order of magnitude longer in the deep waters (Baltar et al., 2013). These results
8 suggest that temperature could be a critical factor preserving the activity of cell-free EEA and
9 thereby controlling the proportion of dissolved EEA in the marine environment.

10 Despite the importance and implications of cell-free EEA in marine environments, little is
11 known about the factors that control changes in the proportion of total EEA that is dissolved.
12 To resolve this question, a long temporal sampling strategy that accounts for the long lifetime
13 of EEs would be desirable, because this will include the strong seasonal changes, and because
14 a field study will be more representative of what occurs in nature than experimental
15 manipulations, particularly when looking at seasonal temporal scales. Here we studied the
16 temporal changes in the proportion of dissolved relative to total EEA, in a continuous
17 biweekly sampling, with water from the Baltic Sea, for 18 months. We aimed to reveal the
18 seasonal variability of dissolved EEA and to decipher the factors that control the proportion of
19 dissolved relative to total EEA (of glycolytic enzymes [β -glucosidase, BGase], a proteolytic
20 enzyme [leucine aminopeptidase, LAPase]) and alkaline phosphatase [APase]). Based on
21 previous research suggesting longer EE lifetimes in cold environments, we hypothesized that
22 there would be a strong link between temperature and the proportion of dissolved relative to
23 total EEA, with lower proportions of dissolved EEA during warm periods (e.g. summer) than
24 during cold periods (e.g. winter). This hypothesis is based on the previous evidences of higher
25 lifetimes of EEA in cold compared to warm waters (Baltar et al., 2010), suggesting that an
26 overall low metabolic rates of microbes would favor higher percentages of dissolved EEA
27 because the degradation of the enzymes (i.e., microbial heterotrophic activity) is reduced
28 under lower temperatures.

1 **2 Materials and methods**

2 **2.1 Study site and sampling**

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

9 **2.2 Biotic and abiotic environmental parameters**

10 Chlorophyll a (Chl *a*) concentration was analyzed following extraction using ethanol
11 (Jespersen and Christoffersen, 1987). Chlorophyll a (Chl *a*) concentration, dissolved inorganic
12 nutrients (NH_4^+ , NO_3^- , PO_4^{3-} and SiO_2) and were analysed following previously described
13 protocols (Jespersen and Christoffersen, 1987; Valderrama, 1981). Dissolved organic carbon
14 (DOC) was measured via high-temperature catalytic oxidation using a TOC-V detector
15 coupled to a TNM-1 unit (Shimadzu Corporation) (Pages and Gadel, 1990). Bacterial
16 abundance was determined using flow cytometry according to the protocol described in Del
17 Giorgio et al. (1996). Bacterial heterotrophic production was derived from ^3H -leucine
18 incorporation rates measured on quadruplicates and two controls according to Smith and
19 Azam (1992).

20 **2.3 Measurement of total and dissolved extracellular enzymatic activity** 21 **(EEA)**

22 The hydrolysis of the fluorogenic substrate analogues 4-methylcoumarinyl-7-amide (MCA)-
23 L-leucine-7-amido-4-methylcoumarin, 4-methylumbelliferyl (MUF)- β -D-glucoside and
24 MUF-phosphate was analyzed to estimate potential activity rates of leucine aminopeptidase
25 (LAPase), β -glucosidase (BGase), and alkaline phosphatase (APase), respectively (Hoppe,
26 1983). The procedure was followed as previously described (Baltar et al., 2010; Baltar et al.,
27 2013; Baltar et al., 2009). Briefly, EEA was determined after substrate addition and
28 incubation using a spectrofluorometer with a microwell plate reader (FLUOstar – BMG
29 Labtech) at excitation and emission wavelengths of 365 and 445 nm, respectively. Samples

1 (300 μ l) were incubated in the dark at *in situ* temperature for 1.5-3 h. Subsamples without
2 substrate additions served as blanks to determine the background fluorescence of the samples.
3 Previous experiments showed insignificant abiotic hydrolysis of the substrates (Azúa et al.,
4 2003; Hoppe, 1993; Unanue et al., 1999). The fluorescence obtained at the beginning and the
5 end of the incubation was corrected for the corresponding blank. The increase in fluorescence
6 over time was transformed into hydrolysis activity using a standard curve established with
7 different concentrations of the fluorochromes MUF and MCA added to 0.2 μ m filtered sample
8 water. A final substrate concentration of 31.2 μ mol l⁻¹ was used to measure BGase activities,
9 100 μ mol l⁻¹ for APase and 500 μ mol l⁻¹ for LAPase. These concentrations were previously
10 determined as saturating substrate concentrations.

11 The total and the dissolved fraction of the EEA was distinguished as previously described
12 (Baltar et al., 2010; Baltar et al., 2013). Briefly, raw seawater was used for total EEA;
13 whereas for dissolved EEA, samples were gently filtered through a low protein-binding 0.2
14 μ m Acrodisc Syringe filter (Pall) for dissolved EEA following the protocol of (Kim et al.,
15 2007). The use of low protein-binding filters is important in this context since the adsorption
16 of extracellular enzymes depends on the type of the filter material used for size fractionation
17 (Obayashi and Suzuki, 2008a). In the present study, dissolved (cell-free) EEA is defined as
18 the EEA recovered in the filtrate. Total and dissolved EEA were determined on six replicate
19 samples each.

20 **2.4 Statistical analyses**

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

24

25 **3 Results and discussion**

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

1 (April-May) and the cyanobacterial summer bloom (July-September) (Legrand et al., 2015)
2 (Fig. 1B).

3 The total (bulk) EEA of APase, LAPase and BGase followed a similar temporal pattern (Fig.
4 2A). APase was the EEA with the highest rates (ranging from 1.5-32 nmol l⁻¹ h⁻¹), followed
5 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
6 potential significant P limitation in the Baltic Sea (Granéli et al., 1990; Hagström et al., 2001).
7 The strongest peaks in BGase and LAPase co-occurred (June and August 2012, May and
8 August 2013), and these enzymes were significantly correlated (Pearson's $r = 0.49$, $p=0.024$).
9 These peaks in BGase and LPase coincided with some of the APase and the Chla-a peaks,
10 suggesting a potential link between these different enzymes and the phytoplankton dynamics.
11 APase was significantly correlated to BGase (Pearson's $r = 0.53$, $p=0.013$) but not to LAPase
12 (Pearson's $r = 0.20$, $p=0.346$). The BGase:LAPase ratio, which have been generally suggested
13 to be indicative of the relative degradation of polysaccharides relative to proteinaceous
14 material, peaked when the highest BGase and LAPase rates were observed (May-June and
15 August-October), just following after the diatom/dinoflagellate spring bloom (April-May) and
16 the cyanobacterial summer blooms (July-September). This is agreement with the results
17 obtained in a recent seasonal study in the Adriatic Sea, where BGase prevailed over LAPase
18 associated to phytoplankton blooms (Celussi and Del Negro, 2012). Nevertheless, results
19 from other reports question the validity of the BGase:LPase ratio as an indicator of the
20 relative degradation of polysaccharides relative to proteins. Previous investigations of a range
21 of peptidases substrates (Bong et al., 2013; Obayashi and Suzuki, 2008b, 2005) have shown
22 that LAPase activity levels vary in a manner not indicative of other peptidase activities, and
23 other recent report suggested that LAPase should not be interpreted as a quantitative proxy of
24 the total peptidolytic potential of the community (Steen et al., 2015). Furthermore,
25 measurements of BGase do not present a complete or representative picture of the broad and
26 variable spectrum of polysaccharide hydrolases present in the ocean (Arnosti, 2011). There
27 was a tendency for higher EEA rates (and BGase:LAPase ratio) in 2013 as compared to 2012,
28 likely explained by the reported interannual variability of phytoplankton communities linked
29 to environmental conditions in the Baltic Sea (Kahru and Elmgren, 2014; Legrand et al.,
30 2015), since different phytoplankton groups can release diverse types of organic carbon
31 compounds, which would likely select for different bacterioplankton groups/enzymes
32 (Pinhassi et al., 2004).

1 The proportion of dissolved relative to total EEA ranged between 0-100%, where LAPase and
2 APase showed a similar range (37-100% and 37-100%, respectively) and BGase showed the
3 broadest range (0-100%) (Fig. 3). LAPase was the EEA with the lowest seasonal amplitude
4 variability in the proportion of dissolved EEA, whereas BGase showed the largest seasonal
5 variability. These values are within the same ranges reported in the surface coastal North Sea
6 waters (Someville and Billen, 1983), Tokyo Bay (Hashimoto et al., 1985), Mediterranean Sea
7 (Karner and Rassoulzadegan, 1995), Elbe estuary (Karrasch et al., 2003), Gulf of Mexico
8 (Ziervogel and Arnosti, 2008; Ziervogel et al., 2010), North Pacific Subtropical Gyre
9 (Duhamel et al., 2010) and the epipelagic to bathypelagic waters of the Atlantic (Baltar et al.,
10 2010; Baltar et al., 2013).

11 The proportion of dissolved EEA showed a clear seasonal pattern in our study (despite the
12 great variability in the bulk EEA rates), with higher values in winter and a pronounced
13 decrease in summer. Overimposed on this seasonal pattern, there were sometimes of the year
14 (March-May) when there were stronger fluctuations in the proportion of dissolved EEA (Fig.
15 3). This coincides with the phytoplankton bloom as indicated by the increases in Chl-a
16 concentration (Fig. 1B) and in APase (Fig. 2A); which is consistent with the rapid succession
17 observed by next generation sequencing in different phytoplankton (and bacterioplankton)
18 taxa during those months in this study site (Lindh et al., 2015).

19 This overall seasonal pattern in the % of dissolved EEA was conserved among all the
20 enzymes studied (APase, BGase and LAPase), suggesting that the main factors regulating the
21 proportion of dissolved EEA affect all enzymes equally, irrespectively of their metabolic
22 function. This advocates for some environmental factor rather than a biological factor
23 controlling the proportion of dissolved EEA. The most logical factors that could be
24 responsible for this seasonal pattern would be light and/or temperature. Inactivation of
25 extracellular enzymes by photochemical reactions have been found in biofilm microbiota
26 (Espeland and Wetzel, 2001). However, in laboratory experiments with Arctic surface waters,
27 the activity of cell-free EEs (APase and LAPase) were not affected by light (under full
28 spectrum natural sunlight), suggesting that photochemical reactions are not relevant pathway
29 for the decay of cell-free EE in seawater (Steen and Arnosti, 2011). Indeed, we found that
30 only temperature was significantly correlated to the proportion of dissolved EEA of the three
31 enzymes studied (APase (Pearson's $r = -0.60$, $p=0.0035$)), BGase (Pearson's $r = -0.73$,
32 $p=0.0002$) and LAPase (Pearson's $r = -0.68$, $p=0.0006$)) (Table 1). This strong correlation

1 between temperature and the proportion of dissolved EEA was always negative, suggesting
2 that lower temperatures favour the proportion of cell-free EEA. These results are consistent
3 with the negative effect of temperature on EE lifetimes found in an incubation experiment
4 with Red Sea surface water (Li et al., 1998), the extended life time of cell-free EE in Arctic
5 waters (Steen and Arnosti, 2011), and with the order of magnitude higher EE lifetimes found
6 in the deep as compared to the surface waters of the Atlantic (Baltar et al., 2013). This
7 suggests that low temperature preserves better (than warm temperature) the constitutive
8 activity of the cell-free enzymes, allowing them to remain active for longer periods. This
9 might be linked to a reduction in the metabolism of heterotrophic microbes that would reduce
10 the consumption/degradation rates of dissolved EEs. This hypothesis is supported by the
11 significant negative correlations found between the proportion of dissolved BGase and
12 LAPase and the bacterial heterotrophic production and DOC concentration (Table 1), which
13 suggest that the proportion of dissolved EEA (BGase and LAPase) is higher when the
14 microbial heterotrophic degrading activities are lower (during winter). This decreased
15 heterotrophic activity would facilitate the preservation or accumulation of dissolved EEA.
16 The higher proportion of dissolved EEA during winter suggests that the decoupling of *in situ*
17 hydrolysis rates from actual microbial dynamics is stronger during winter (or in cold waters).
18 Thus, these results underpin the importance of considering this stronger cold-related
19 decoupling when relating EEA to other microbial processes. These results also suggest that,
20 under the projected global warming scenario, it could be possible that the hydrolysis of
21 organic matter due to cell-free EE might be reduced due to a shorter lifetime of the EEs.

22

23 **4 Conclusions**

24 Overall, the results of this study suggest that a relevant fraction of the total EEA measured in
25 a particular environment can be due to free EEs, which might be a consequence of the
26 substrate history of the water masses. Thus, advection of dissolved EEA might be a critical
27 source of EEA at any given environment (Baltar et al., 2010; Baltar et al., 2013; Steen and
28 Arnosti, 2011). Other factors might also allow for extended EE lifetimes during advection,
29 like association of EEs to particles (Gianfreda and Scarfi, 1991; Naidja et al., 2000; Ziervogel
30 et al., 2007), to exopolymeric matrix (Decho, 1990), and to detrital particle complexes
31 (Nagata and Kirchman, 1992). Low temperature seems to be a critical factor favoring longer
32 EE lifetimes and thereby higher proportions of dissolved relative to total EEA. This implies

1 that under cold conditions, cell-free enzymes can contribute to substrate availability at large
2 distances from the producing cell, potentiating the disconnection between the hydrolysis of
3 organic compounds and the actual microbes producing the enzymes. Moreover, under warmer
4 conditions, like those predicted to occur due to global warming, the hydrolysis of organic
5 matter (i.e., rate limiting step in the degradation of organic matter) can be reduced due to a
6 lower contribution of the cell-free EE hydrolysis.

7

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1 Table 1. Pearson's correlation coefficient (r) between proportion of dissolved extracellular
 2 enzymatic activities (alkaline phosphatase [APase], β -glucosidase, [BGase], and leucine
 3 aminopeptidase [LAPase]) and Chlorophyll-a, salinity, inorganic nutrients, dissolved organic
 4 carbon (DOC), bacterial abundance (BA) and bacterial heterotrophic production (BP). Values
 5 of r are significant at $p < 0.05$ are highlighted in bold.

6

	% APase		%BGase		%LAPase	
	r	p-value	r	p-value	r	p-value
Temp	-0.60	0.004	-0.73	0.001	-0.68	0.001
Chlorophyll-a	-0.16	0.499	-0.43	0.052	-0.57	0.006
Salinity	-0.22	0.330	0.18	0.443	0.42	0.059
Nitrate	0.21	0.372	0.34	0.132	0.46	0.034
Phosphate	0.38	0.088	0.47	0.030	0.51	0.017
Silicate	0.27	0.243	0.20	0.379	0.24	0.288
Ammonium	0.03	0.892	-0.07	0.758	-0.04	0.862
DOC	-0.18	0.433	-0.66	0.001	-0.65	0.001
BA	-0.32	0.157	-0.39	0.077	-0.16	0.500
BP	-0.38	0.092	-0.64	0.002	-0.63	0.002

7

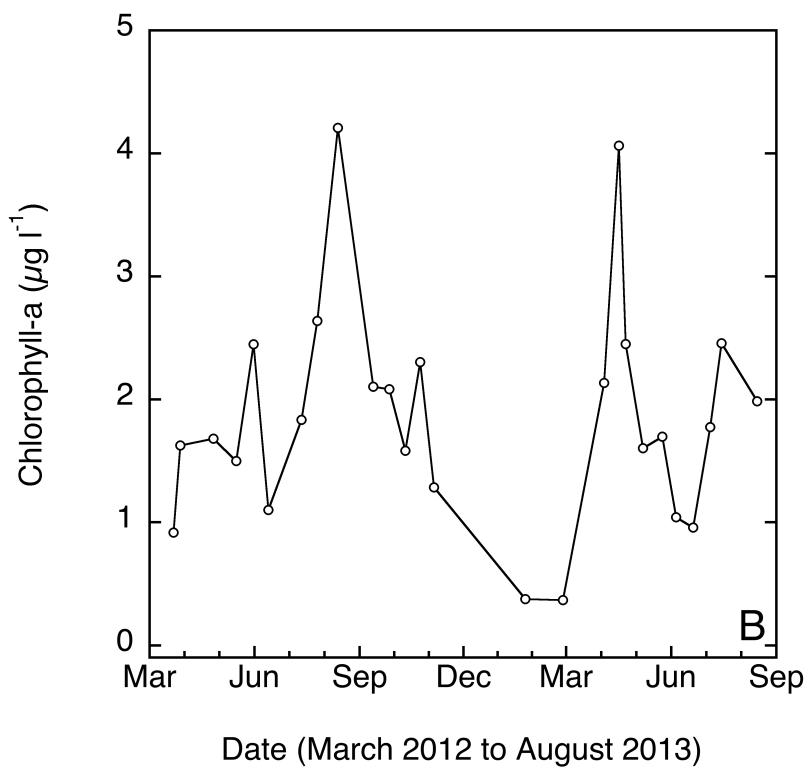
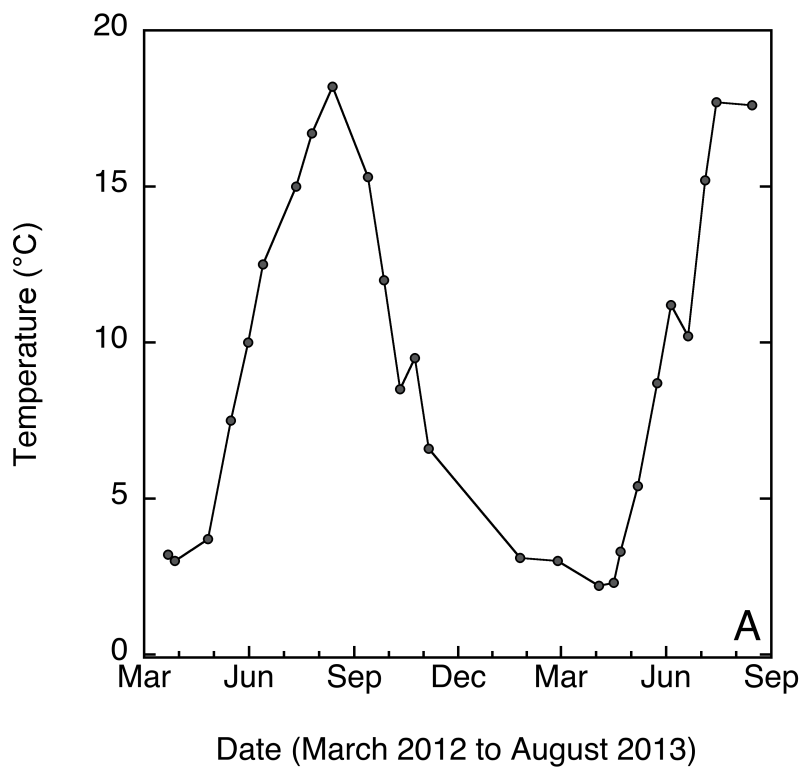
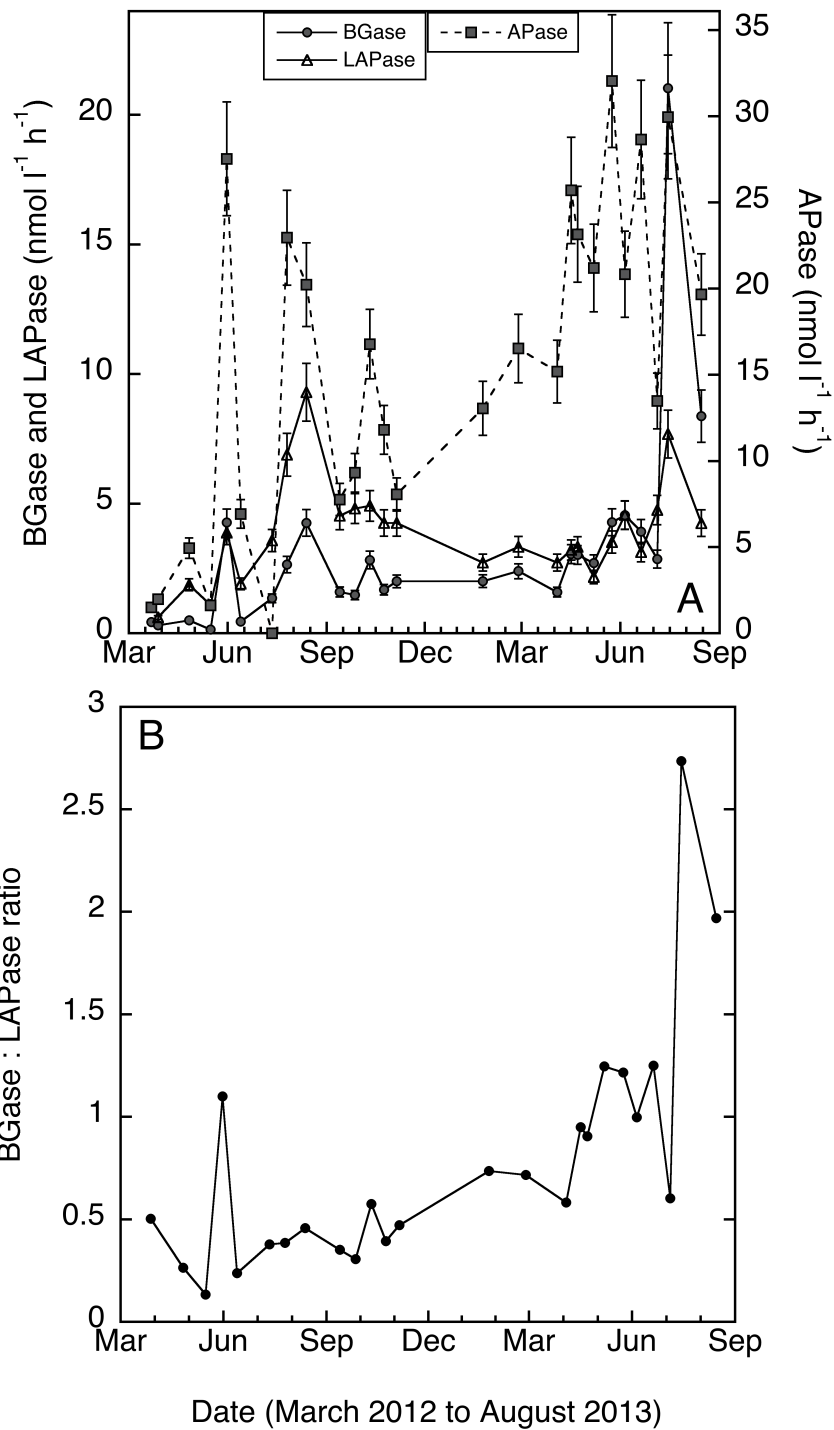
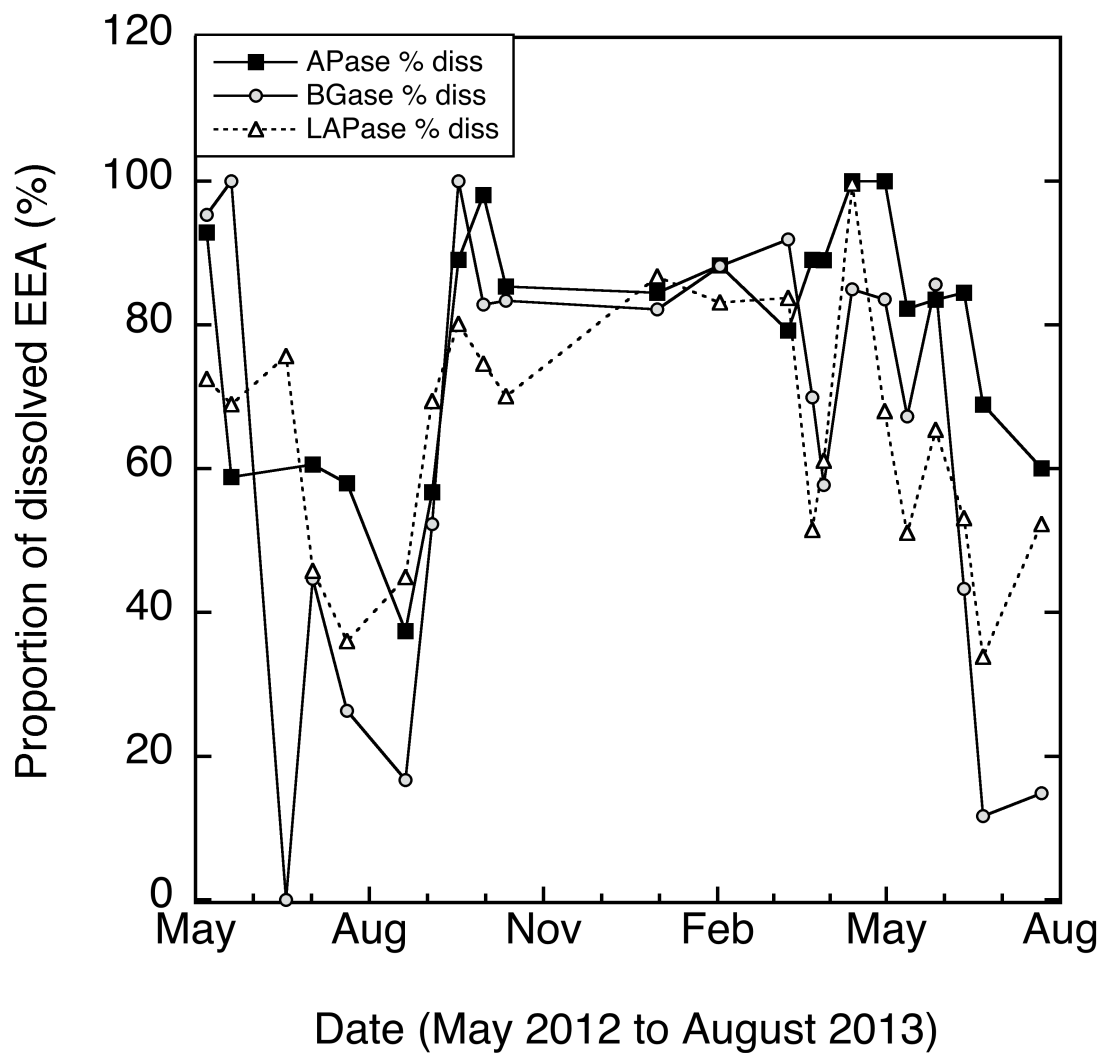


Figure 1. Temporal dynamics in (A) temperature and (B) chlorophyll-a concentrations, at the Linnaeus Microbial Observatory (LMO, Baltic Sea) from March 2012 to August 2013



23 Figure 2. Temporal dynamics in (A) extracellular enzymatic activities of alkaline phosphatase
 24 (APase), β -glucosidase, (BGase), and leucine aminopeptidase, (LAPase) and), and (B) the
 25 BGase:LAPase ratio, at the Linnaeus Microbial Observatory (LMO, Baltic Sea) from March
 26 2012 to August 2013.,

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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.

8