Point-by-point response and associated relevant changes made

Author response to Reviewer #1
We thank the reviewer for the constructive comments on this manuscript. We have taken them on board and our responses to reviewer comments, including the modifications done to the manuscript, are detailed in the following:

REVIEWER COMMENT 1 by Referee #1:
General comment The article by Baltar et al. shows a strong relationship between the proportion of dissolved extracellular enzymatic activities and seasonal variations (temperature) occurring at a coastal site in the Baltic Sea. The topic of the manuscript is very important, and not fully understood yet. It is a clear and straightforward document that describes a valid experimental design.

Author response: We appreciate the positive comments of the reviewer

REVIEWER COMMENT 2 by Referee #1:
My only concern regards the lenght of the paper. In particular, the authors focus only on one important environmental factor: temperature. In order to make the article's conclusions sound (e.g. the potential effect of global warming on organic matter degradation performed by cell-free enzymes) some experimental manipulations could have been performed. Alternatively, the effect of factors other than temperature could have been considered (and eventually discarded if not significant). For these reasons I suggest to improve the analysis with other environmental factors (if available). Alternatively, the manuscript should be considered for a 'short note' type of paper. Since I'm aware that BG does not include 'short notes' as potential article types, an option could be the 'Ideas and perspectives' type, within which the article could perfectly fit in its current form with a slight modification of the title (just as a suggestion, it could become something like: "temperature affects the activity of cell-free extracellular enzymes: a seasonal case study in the Baltic Sea).

Author response: We have included other factors than temperature and light (e.g. nutrients, chlorophyll-a, salinity, DOC, bacterial abundance, bacterial production) in the analysis (new Table 1) and we have revised manuscript text accordingly (p.7, l.30 to p. 8, l. 2; p. 8, 10-15).
We appreciate the title suggested by the reviewer, and have modified the title inspired on the reviewer suggestion. It now reads: "Cell-free extracellular enzymatic activity is linked to seasonal temperature changes: a case study in the Baltic Sea".

REVIEWER COMMENT 3 by Referee #1:
Specific comments:
Page 1 line 27-29: This conclusive sentence is very strong: although potentially true it would deserve further investigations (see General comment)

Author response: We have rewritten this sentence to make it less strong and to include a suggestion for the need for further investigations in the revised version (p. 1, l. 28 to

p. 2, l. 2). The sentence now reads: " This might also suggest a potential effect of global warming on the hydrolysis of organic matter via a reduction of the contribution of of cell-free enzymes to the bulk hydrolytic activity, and call for the need of further research to confirm it."

REVIEWER COMMENT 4 by Referee #1:
Page 2 line 3: although heterotrophic prokaryotes are much of the story, I would not ascribe only to them the pivotal DOM reworking role, especially when EEA are involved (exoenzymes from cyanobacteria can be even more efficient). I'd be more cautious just saying 'prokary- otes'. Page 2 line 15: extra ')'

Author response: We have modified this accordingly, and the sentences now read (p. 2, l. 5): " Prokaryotes play a central role…". We have also deleted the extra ')'.

REVIEWER COMMENT 5 by Referee #1:
Page 3 line 25: "Temperature was measured on site" How? Was water collected and temperature measured on a aliquot by means of a thermometer? Anyhow this should be indicated.

Author response: We have included this information in the revised version (p. 4, l. 6-8), which reads as follows: " Temperature was measured on site through thermometer placed in the Ruttner sampler, and the water was transported to the laboratory in acid-washed Milli-Q-rinsed polycarbonate bottles within 1 h.".

REVIEWER COMMENT 6 by Referee #1:
Page 6 line 8: I am afraid this (temperature and light) is a little restrictive. pH is an important factor in enzyme activity, especially in extracellular ones. What is the pH variability in the 'low-salinity' high-chlorophyll Baltic sea? I suspect that in the sampling area pH variations might be broader that those tested in many ocean acidification experiments for which many references are available. Substrate concentration and composition also affect enzyme activity with or without links to the metabolic state of the source organism (Arnosti 2011) Catalytic elements have also been shown to drive (to some extent) hydrolysis rates of at least LAPase (Fukuda et al., 2000). These aspects (especially pH and substrate) should be mentioned.

Author response: The reviewer is correct on pointing to those factors (pH and substrate) as potentially affecting bulk EEA. However, in that paragraph we are describing the potential factors explaining/affecting the patterns in "dissolved EEA" (that is the central point of the study), but not in the bulk/total EEA. We are not aware of studies indicating that substrate or pH affect the proportion of dissolved EEA in seawater, so we cannot add anything about it. Nevertheless, to improve the interpretation of factors potentially controlling the proportion of dissolved EEA, we have now included other measured variables as well (see response to reviewer comment 2), although we did not measure pH (pH *in situ* is extremely challenging to measure with the required accuracy for in situ relevance); but we hope the reviewer will agree that the strongest variations in pH would be caused by autotrophic and heterotrophic activity, wherefore our inclusion of phytoplankton biomass

[chlorophyll-a] and bacterial heterotrophic activity, which are linked to fluctuations in pH, have relevance).

REVIEWER COMMENT 7 by Referee #1:
Page 10 line 30: heterotrophic MARINE flagellates

Author response: Fixed now.

REVIEWER COMMENT 8 by Referee #1:
Figure 2, upper panel: error bars should be shown

Author response: We have now included the error bars in Figure 2.

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 the modifications done to the manuscript:

REVIEWER COMMENT 1 by Referee #2:
General comments: The authors attempt to address an interesting and important problem, with a time series of measurements of potential enzyme activities, including the fraction that is cell free. These measurements are a good start, but the data lack context, and the framework which the authors build does not adequately reflect current understanding of microbial community activities and dynamics.

Author response: Our main objective (and novel contribution) was to focus on the factors controlling the proportion of dissolved EEA, since there are many other reports in the literature on the factors controlling the bulk EEA. To increase the background and the depth of our analyses of the temporal patterns of the proportion of dissolved EEA, we have included several additional measured variables (concentration of nitrate, phosphate ammonium, chlorophyll-a and DOC, salinity, bacterial abundance, bacterial production) and built a new table (Table 2) showing these results (see response to reviewer 1 comment 2). We have also adjusted the framework according to the reviewer's comments (see below).

REVIEWER COMMENT 2 by Referee #2:
Are other data in addition to T and chl a available? Salinity, DOC, circulation, other physical or chemical data for this site? A multitude of factors can affect potential enzyme activities, as well as the fraction of activity that is cell-free. Are cell counts avail- able, or are there other studies done at the same location that provide a more in-depth background on the microbial ecology of the system? In particular, the Baltic has a very strong N-S salinity gradient that is linked with both compositional and functional changes in microbial communities (e.g. Dupont et al. (2014). "Functional tradeoffs underpin salinity-driven divergence in microbial community composition."

PLOS one 9: 89549). It would be helpful to have some sense of microbial dynamics at this site; the T and chl a data are not really a sufficient context.
In its current form, this manuscript lacks sufficient significance and quality and presen- tation; it needs a lot of work.

Author response: Yes, we have other data available, and we have included those in the revised version (new Table 1) (please see response to reviewer 1 comment 2 for details).

REVIEWER COMMENT 3 by Referee #2:
Specific comments: Pg 2 lines 11, 12 Note that Arnosti (2011) is cited improperly ("This is why the activity of extracellular enzymatic activity (EEA) has been recognized as the rate-limiting step in organic matter degradation (Arnosti, 2011)." Arnosti (2011) does not state that EEA is the rate limiting step: that reference points out that EEA is the initial step in organic matter degradation, and discusses this idea at great length. These statements are not the same!

Author response: We are sorry for this misunderstanding, we have changed "rate-limiting" for "initial" (p. 2, l. 13).

REVIEWER COMMENT 4 by Referee #2:
Lines 18, 19: earlier reports of cell-free activity, including Karner & Rassoulzadegan (1995), Obayashi & Suzuki (2008) and Keith & Arnosti (2001).
Note that one of the key points of the current ms - that enzyme activities may be decoupled from the producing organisms, and may contribute greatly to carbon cycling - is explicitly also discussed in Arnosti (2011), and summarized as one of the key points in that manuscript.

Author response: We have included those references (Karner & Rassoulzadegan (1995) and Obayashi & Suzuki (2008) and Keith & Arnosti (2001)) at the end of that statement (p. 2, l. 20-21). We have also included references of the previous papers where the decoupling of EEA from the producing organisms has been suggested before ((Arnosti, 2011) and Baltar et al., 2010) (p. 2, l. 25-26)).

REVIEWER COMMENT 5 by Referee #2:
Lines 31 forward: Steen & Arnosti (2011) investigated cell-free enzyme lifetime in Arctic surface waters

Author response: we have included that reference in that specific statement (p. 3, l. 2-3).

REVIEWER COMMENT 6 by Referee #2:
Pg. 3: Line 7, delete 'the' before 'cell-free' Line 8, delete 'what are' before 'the factors' Line 8, this thought is incomplete. Do you mean 'little is known about the factors that control changes in the proportion of total EEA that is dissolved' ? Line 9, move 'is needed' to the end of the sentence Line 11: move the information about the

specific enzyme activities assayed into another sentence following this one. Line 14: change ‘decipher what factors’ to ‘decipher the factors that control’ Line 16, change to ‘we hypothesized that there would be a strong link’, or something of this sort- need to improve the phrasing.

Author response: we have modified these points following exactly the reviewer’s suggestions (now on p. 3, l. 11-22).

REVIEWER COMMENT 7 by Referee #2:
Line 7 forward. This paragraph is not very clear – why will a long-term sampling strategy in particular help resolve the question of when and under what conditions cell-free EE is important? One could easily imagine that a detailed biochemical investigation of the nature and structure and dynamics of cell-free enzymes is what is needed (tho this of course is technically very challenging, and is a different question than the one examined here.) The whole paragraph needs more focus.
As it stands currently, this hypothesis is not very well phrased. At minimum, add another sentence or two: is this hypothesis based on rates of molecular motion, on rates of diffusion of enzymes and proteases, on an idea of the structural basis of enzymes that are produced under cold vs warm conditions, on kinetics of degradation/inactivation reactions?

Author response: We have now specified that despite the intrinsic effort required to carry out a long-term seasonal analysis on natural samples collected in the field, they are valuable to help shed light into this question because temperature strongly changes seasonally and field studies are usually more representative of what occurs in nature than experimental manipulations (p. 3, l. 11-15).
We have also added another sentence/s as suggested by the reviewer specifying that our hypothesis is based on the fact that higher lifetimes of EEAs have been found in cold compared to warm waters, suggesting that an overall low metabolic rate of microbes would result in higher percentages of dissolved EEA because the degradation of the enzymes is reduced under lower temperatures (p. 3, l. 24-28).

REVIEWER COMMENT 8 by Referee #2:
Pg. 4 Were killed controls (autoclaved water) subtracted from the measured values? Are other data (salinity, for example) available for this site? How variable are cell counts, or bacterial productivity, or DOM or POM or other biogeochemical parameters for this site?

Author response: Subsamples without substrate additions served as blanks to determine the background fluorescence of the samples. This is agreement with previous reports showing insignificant abiotic hydrolysis of the substrates (e.g. Hoppe HG (1993) Use of fluorogenic model substrates for extracellular enzyme activity (EEA) measurement of bacteria. Handbook of methods in aquatic microbial ecology: 423-431; Azúa I, Uanue M, Ayo B, Arrtolozaga I, Arrieta JM, et al. (2003) Influence of organic matter quality in the cleavage of polymers by marine bacterial communities. Journal of Plankton Research 25: 1451-1460; Unanue M, Ayo B, Agis M, Slezak D, Herndl GJ, et al. (1999) Ectoenzymatic activity and uptake of monomers in marine bacterioplankton described by a biphasic kinetic model. Microb

Ecol 37: 36-48). We have included this information in the revised version (see p. 5, l. 1-5).
We also included bacterial production and DOM data (as well as other variables) and comment on it in the revised version of the ms (see response to comment 2, and response to reviewer 1 comment 2 for details).

REVIEWER COMMENT 9 by Referee #2:
Pg 5: The data presentation and discussion on pg 5 is not very satisfactory. The BGase and LAPase plots do appear to track one another to some extent, but having the APase activity on the same plot and scale makes something of a mess. Try plotting with a secondary Y axis for APase. More critically, it is difficult to figure out what connections there might be: as the text says, there are sometimes –but not always- coincidental peaks in these different activities, but this pattern is not very strong.

Author response: We have followed the suggestion by the reviewer and redone Figure 2 by plotting with a secondary Y-axis for APase. Now it is much clearer to see the strong connection (including coincidental peaks) that exists between all the different EEAs. We have also included a correlation analysis of the different bulk EEAs, explaining better their relations (p. 6, l. 7-12).

REVIEWER COMMENT 10 by Referee #2:
Moreover, the authors assert that BGase and LAPase show relative pro- tein/polysaccharide degradation ratios. Note that more recent work has demonstrated that this perspective is likely limited. Multiple investigations of a range of peptidases substrates (see in particular the work of Obayashi & Suzuki Obayashi, Y. and S. Suzuki (2005). "Proteolytic enzymes in coastal surface seawater: Significant activity of en- dopeptidases and exopeptidases." Limnol. Oceanogr. 50: 722-726. Obayashi, Y. and S. Suzuki (2008). "Occurrence of exo- and endopeptidases in dissolved and partic- ulate fractions of coastal seawater." Aq. Microb. Ecol. 50: 231-237. Bong, C. W., Y. Obayashi and S. Suzuki (2013). "Succession of protease activity in seawater and bacterial isolates during starvation in a mesocosm experiment." Aq. Microb. Ecol. 69: 33-46) which demonstrates that LAPase activity levels vary in a manner not indicative of other peptidase activities. See also the recent work of Steen et al. (2015; "Sub- strate specificity of aquatic extracellular peptidases assessed by competitive inhibition assays using synthetic substrates" AME 75: 271-281), who investigate the specificity of LAPase-hydrolyzing enzymes, and comment that "In some studies, Vmax for Leu-AMC hydrolysis is interpreted as a quantitative proxy of the total peptidolytic potential of a community (Kellogg et al. 2011). The results presented here show that approach to be invalid." Furthermore, the work of Arnosti (2011), which is cited in the ms, discusses the issue of the broad and variable spectrum of polysaccharide hydrolases present in the ocean; measurements of BGase do not present a complete or representative picture.

Author response: We have included a discussion about the limitations behind the BGase:LAPase, including all the citations referred to by the reviewer (p. 6, l. 18-26).

REVIEWER COMMENT 11 by Referee #2:
Note in addition that the peaks in BGase/LAPase ratios (which are not all that convinc- ing..this plot shows a general increasing trend over the timecourse of the study, and the single large peak is driven by 2 measurements of BGase activity) do not in fact coincide with the chl a max, since the peak chl a in Fig 1B occurs in ca August and then in May, whereas Fig 2b shows peaks in June and August.

Author response: In the study area, there is always an increase in chlorophyll found between April-May (diatom and dinoflagellate spring bloom) and July-September (cyanobacterial summer bloom), as shown for example in our previous paper (Lindh et al. 2015, Disentangling seasonal bacterioplankton population dynamics by high-frequency sampling. Environmental Microbiology: 17 (7), 2459–2476). The peaks in BGase:LAPase ratio are not exactly at the time of the blooms but are following just after the blooms (with a time lag). This lag makes sense because first comes primary productivity and then heterotrophic degradation, but they do not usually occur exactly at the same time. We have done our best to explain this better in the revised version of the ms (p. 6, l. 12-16).

REVIEWER COMMENT 12 by Referee #2:
The comments on lines 19-22 on different phytoplankton blooms and enzyme activities is simply not very convincing: APase activity might be directly connected to phytoplank- ton, given the presence of APase in some phytoplankton, but the BGase and LAPase connection would be more distant, given that heterotrophic prokaryotes are the sources of these enzymes.
The discussion of all of these data in fact leave the reader with the impression that the authors don't quite know what to do with this long time series. More digging in the literature, discussion with other colleagues who have data from this site might help, but this is currently a very weak part of the manuscript.

Author response: We partly agree with the reviewer suggesting that Apase might be directly connected to phytoplankton but much that the connection between phytoplankton and BGase and LAPAse would be less evident due to the presence of APase in some phytoplankton. However, we also think that different groups of phytoplankton can release different types of organic carbon compounds, which would likely select for different bacterioplankton groups/enzymes (Pinhassi et al. 2004 Changes in Bacterioplankton Composition under Different Phytoplankton Regimens, 70 (11), 6753-6766), further suggesting a potential link between phytoplankton groups and enzyme activities. In the revised version of the manuscript we now attempt to clearly explained better this potential link between phytoplankton community structure and changes in EEA (p. 6, l. 26-32).

REVIEWER COMMENT 13 by Referee #2:
Pg 6 The high fraction of dissolved activity is indeed an interesting observation. The authors need to discuss here some ideas about why this might be - certainly others have also at times high cell-free activities, but these activities are high for most of the annual cycles. Moreover, the changes in the cell-free activities for example between March and May, when the data spikes up and down with considerable frequency, would allow for some discussion of lifetimes (for activity to drop this much, some of

the en- zymes must either be removed, or they are advected away, and one is sampling a different patch of water; to evaluate this, more data on the study site and the dynamics of the water masses is needed.)

Author response: We think that the variability observed in March-June could also be related to the phytoplankton bloom (also observed as an increase variability in APase, and an increase in the BGase:LAPase ratio), and the rapid succession we observed in different phytoplankton taxa during those months in this study site ((Lindh et al. 2015, Disentangling seasonal bacterioplankton population dynamics by high-frequency sampling: Environmental Microbiology: 17 (7), 2459–2476). We have extended our discussion on this topic, including our best potential interpretation of the ups and downs observed in the proportion of dissolved EEA between March and May in the revised version (p. 7, l. 13-18), which we hope helps the reader interpret our data.

REVIEWER COMMENT 14 by Referee #2:
As others have doubtless pointed out, correlation is not causation: there is a statistical correlation between T and cell-free activity, but this is a long way from a coherent or plausible explanation. To what extent do cell counts, bacterial productivity, or microbial community composition change at this site on seasonal scales? What is the physical oceanography of this setting –what water masses are sampled? It is extremely likely that the nature and types of enzymes that hydrolyze leu-MCA, B-MUF, and alkaline phosphatase are likely quite different under different conditions of productivity, and with different seasons, all factors that are correlated with differences in microbial community composition and activity. See for example Arrieta & Herndl (2002; "Changes in β- glucosidase diversity during a coastal phytoplankton bloom." Limnol Oceanogr 47: 594−599). In any case, drawing parallels between community activities in the Arctic and the Baltic needs also to consider the differences between permanently cold and temperate environments, in terms of community composition and activities.
With all of these issues that should be addressed, extending the discussion here to questions of global warming is much too far a reach.

Author response: We agree with the reviewer that correlations are not evidence for causal relationships, but also think that we need to show when significant correlations were found. A significant correlation together with a sensible hypothesis supporting it (based on previous results) supports the utilization of significant correlations. We have now included in the revised version, among others, the data on cell counts and bacterial productivity (see response to reviewer comment 2), as well as included information on our previous study of the study region showing a detailed seasonal study of microbial community composition (Lindh et al. 2015).
Despite the fact that so many different factors can affect the types of enzymes present, a clear and robust seasonal pattern was observed in the % of dissolved EEA, which we consider gives even more value to this pattern observed in this study, and probably reinforces the idea that an integrating variable (like temperature) that affects many other factors might be the main responsible in the regulation of the dissolved enzymes. This is also supported by the fact that longer lifetimes have been reported in the Artic and deep Atlantic than surface waters, further indicating that the differences in temperature and all the associated changes that come with it (e.g. changes in heterotrophic rates, community composition, etc.), might be the main driver of the %

dissolved EEA. This is further supported now by our new analysis done which shows a significant negative correlation between the proportion of dissolved EEA (BGase and LAPase) and the heterotrophic metabolic activities (bacterial production). We have now included discussion on this topic (p. 8, l. 10-15)
If temperature is the variable most strongly affecting the proportion of dissolved EEA (as suggested by our results), we believe that at least mentioning a potential link with global warming is appropriate. Nevertheless, we have rewritten these statements (on the link to global warming), at the end of the abstract and at the end of the Discussion, to make it less strong (p. 1, l. 28 to p. 2, l. 2 and p. 8, l. 20-21).

END OF REVISION

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

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Abstract

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

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

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1 Introduction

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 initial step in organic matter degradation (Arnosti, 2011).

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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, other 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; Karner and Rassoulzadegan, 1995; Keith and Arnosti, 2001; Obayashi and Suzuki, 2008a). 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 (Arnosti, 2011; Baltar et al., 2010; Baltar et al., 2013; Karner and Rassoulzadegan, 1995).

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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; Steen and Arnosti, 2011; Ziervogel and Arnosti, 2008; Ziervogel et al., 2010). However, EE lifetimes of surface waters were longer (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 cell-free EEA in marine environments, little is known about the factors that control changes in the proportion of total EEA that is dissolved. To resolve this question, a long temporal sampling strategy that accounts for the long lifetime of EEs would be desirable, because this will include the strong seasonal changes, and because a field study will be more representative of what occurs in nature than experimental manipulations, particularly when looking at seasonal temporal scales. Here we studied the temporal changes in the proportion of dissolved relative to total EEA, 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 the factors that control the proportion of dissolved relative to total EEA (of glycolytic enzymes [β-glucosidase, BGase], a proteolytic enzyme [leucine aminopeptidase, LAPase]) and alkaline phosphatase [APase]). Based on previous research suggesting longer EE lifetimes in cold environments, we hypothesized that there would be 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). This hypothesis is based on the previous evidences of higher lifetimes of EEA in cold compared to warm waters (Baltar et al., 2010), suggesting that an overall low metabolic rates of microbes would favor higher percentages of dissolved EEA because the degradation of the enzymes (i.e., microbial heterotrophic activity) is reduced under lower temperatures.

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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 through thermometer placed in the Ruttner sampler, and the water was transported to the laboratory in acid-washed Milli-Q-rinsed polycarbonate bottles within 1 h.

2.2 Biotic and abiotic environmental parameters

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

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

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(300 µl) were incubated in the dark at *in situ* temperature for 1.5-3 h. Subsamples without substrate additions served as blanks to determine the background fluorescence of the samples. Previous experiments showed insignificant abiotic hydrolysis of the substrates (Azúa et al., 2003; Hoppe, 1993; Unanue et al., 1999). The fluorescence obtained at the beginning and the end of the incubation was corrected for the corresponding blank. 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, 2008a). 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.

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

(April-May) and the cyanobacterial summer bloom (July-September) (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), and these enzymes were significantly correlated (Pearson's r = 0.49, p=0.024). These peaks in BGase and LPase coincided with some of the APase and the Chla-a peaks, suggesting a potential link between these different enzymes and the phytoplankton dynamics. APase was significantly correlated to BGase (Pearson's r = 0.53, p=0.013) but not to LAPase (Pearson's r = 0.20, p=0.346). The BGase:LAPase ratio, which have been generally suggested to be indicative of the relative degradation of polysaccharides relative to proteinaceous material, peaked when the highest BGase and LAPase rates were observed (May-June and August-October), just following after the diatom/dinoflagellate spring bloom (April-May) and the cyanobacterial summer blooms (July-September). 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). Nevertheless, results from other reports question the validity of the BGase:LPase ratio as an indicator of the relative degradation of polysaccharides relative to proteins. Previous investigations of a range of peptidases substrates (Bong et al., 2013; Obayashi and Suzuki, 2008b, 2005) have shown that LAPase activity levels vary in a manner not indicative of other peptidase activities, and other recent report suggested that LAPase should not be interpreted as a quantitative proxy of the total peptidolytic potential of the community (Steen et al., 2015). Furthermore, measurements of BGase do not present a complete or representative picture of the broad and variable spectrum of polysaccharide hydrolases present in the ocean (Arnosti, 2011). 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), since different phytoplankton groups can release diverse types of organic carbon compounds, which would likely select for different bacterioplankton groups/enzymes (Pinhassi et al., 2004).

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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).

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

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

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

Acknowledgements

We acknowledge Anders Månsson, Emil Fridolfsson, Kristofer Bergström, Saraladevi Muthusamy, Markus Lindh, Mireia Bertos Fortis, Oscar Nordahl for their never-ending energy to carry out field sampling and Sabina Arnautovic and Emmelie Nilsson for their skillful technical assistance in the processing of samples. This research was supported by grants from the European Science Foundation EuroEEFG project MOCA and The Swedish Research Council (VR) to JP, and the Strategic Marine Environmental Research program ECOCHANGE funded by the Swedish Research Council (Formas) to JP and CL. FB was supported by a University of Otago Research Grant. We would like to acknowledge the support and insightful comments of the reviewers, which clearly helped improve the overall merit of the manuscript.

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

	% 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**

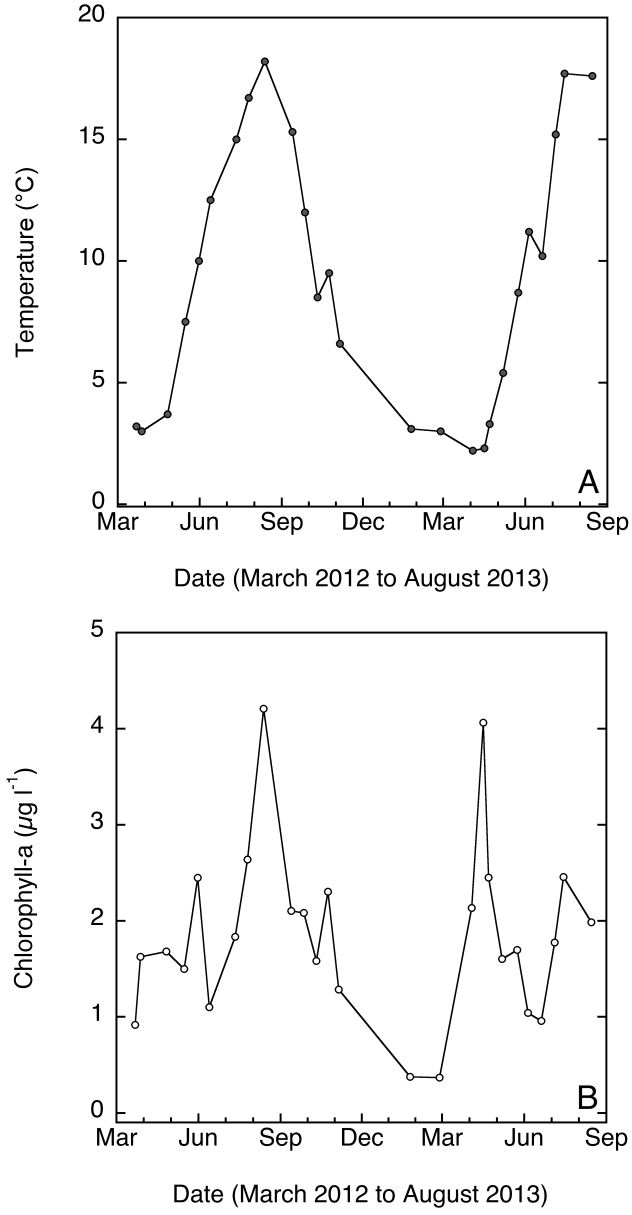

Figure 1. Temporal dynamics in (A) temperature and (B) chlorophyll-a concentrations, at the Linnaeus Microbial Observatory (LMO, Baltic Sea) from March 2012 to August 2013

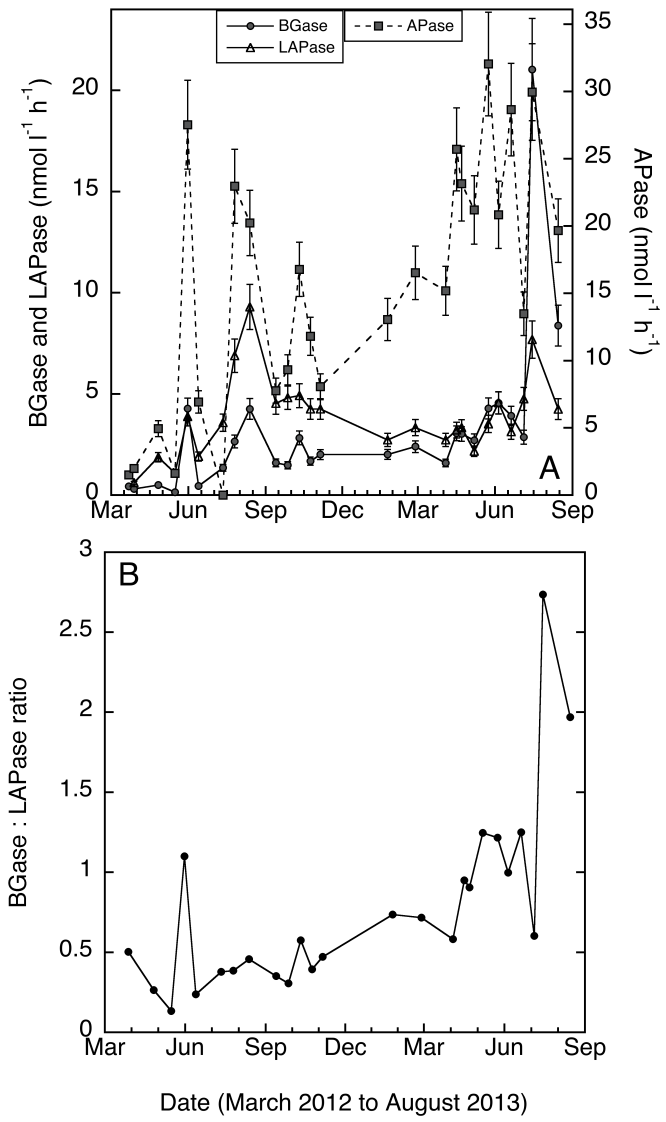

Figure 2. Temporal dynamics in (A) extracellular enzymatic activities of alkaline phosphatase (APase), β-glucosidase, (BGase), and leucine aminopeptidase, (LAPase) and), and (B) the BGase:LAPase ratio, at the Linnaeus Microbial Observatory (LMO, Baltic Sea) from March 2012 to August 2013.,

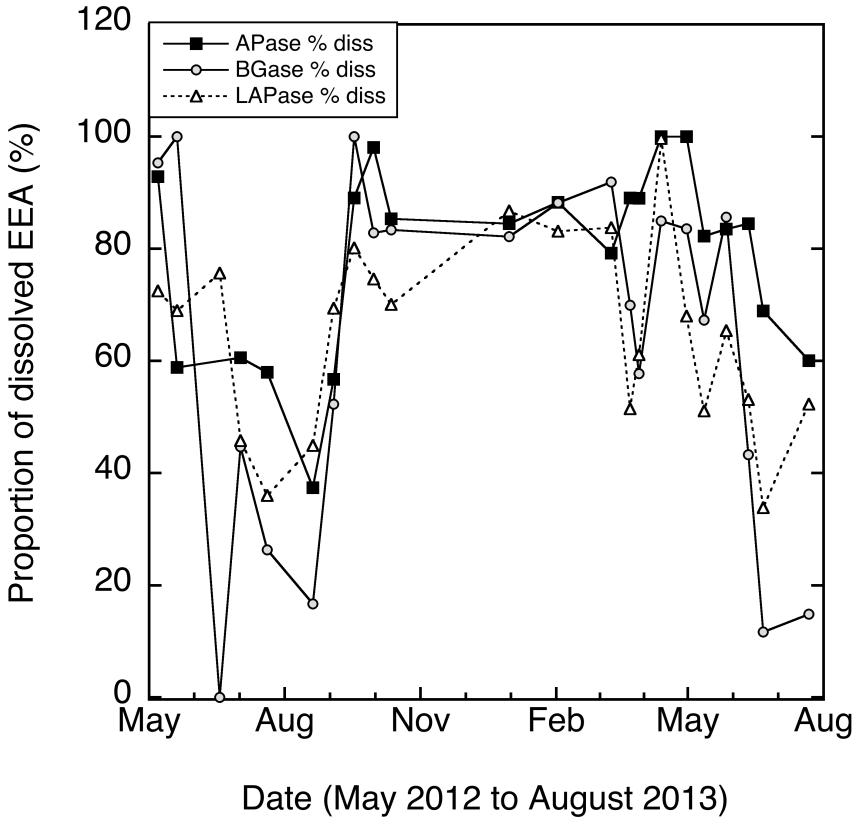

Figure 3. Temporal dynamics in the proportion of dissolved relative to total extracellular enzymatic activities (EEA) of alkaline phosphatase (APase), β-glucosidase, (BGase), and leucine aminopeptidase, (LAPase), at the Linnaeus Microbial Observatory (LMO, Baltic Sea) from May 2012 to August 2013.