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Interactive comment on "Cell-free extracellular enzymatic activity is linked to seasonal temperature changes in the Baltic Sea" *by* F. Baltar et al.

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

REVIEWER COMMENT 1 by Referee #2: General comments: The authors attempt to address an interesting and important prob- lem, 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 con- text, and the framework which the authors build does not adequately reflect current understanding of microbial community activities and dynamics.

Author response: We will increase the data context by including additional variables, and adjust the framework according to the reviewer's comments, in the revised version of the ms.

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 en- zyme 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 presentation; it needs a lot of work.

Author response: Yes, we have other data available, which we will include in the revised version (Salinity, DOC, etc.).

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, this will be corrected accordingly in the revised version.

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: Karner & Rassoulzadegan (1995) and Obayashi & Suzuki (2008) are cited in the manuscript, and we will also include Keith & Arnosti (2001) in the revised version. We will include a specific mention of the previous papers where the decoupling of EEA from the producing organisms has been suggested before, including Arnosti (2011) and Baltar et al. (2010).

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

Author response: we will include that reference in that specific statement as well in the revised version of the ms.

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 will modify this accordingly in the revised version.

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 will add another sentence/s as suggested by the reviewer. We will specify that our hypothesis is based on the fact that higher lifetimes of EEA have been found in cold compared to warm waters, suggesting that an overall low metabolic rates of microbes would be more prone to higher percentages of dissolved EEA because the degradation of the enzymes is reduced under lower temperatures. We will also specify that, despite the field long-term effort required, seasonal analysis are good 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.

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 did not include all the details of the methods because they have been already explained in previous works cited in the text, but we will include this information in the revised version. As mentioned in comment 2, we will also include bacterial production and DOM data and comment on it in the revised version of the ms.

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 will plot it using a secondary axis Y for APase as suggested, but we think that it is valuable to plot all the enzymes together because it is relevant to see how the biggest peaks in BGase and LAPase coincide with peaks in APase as well.

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 will discuss about the limitations behind the BGase:LAPase in the text, including the citations referred to by the reviewer.

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 increases in chlorophyll found between April-May (diatom and dinoflagellate spring bloom) and July-September (cyanobacterial summer bloom), as shown for example in our detailed previous paper (Lindh et al. 2015, Disentangling seasonal bacterioplankton population dynamics by high-frequency sampling: 17 (7), 2459–2476). The peaks in BGase:LAPase ratio are not exactly at the time of the blooms but are just following after the blooms (after a time lag). This lag makes sense because first comes primary productivity and then heterotrophic degradation, but not usually exactly at the same time. We will explain this better in the revised version of the ms.

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 probably did not explain the potential link between different phytoplankton blooms and enzyme activities sufficiently well. We partly agree with the reviewer suggesting that Apase might be directly connected to phytoplankton but much less BGase and LAPAse 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. The main aim of this study is to focus on the changes in the % of dissolved EEA, since this is the novel part of it, and there are many more studies on the bulk EEA. That is why we only tried to briefly describe what happens in the bulk EEA without going too much into detail just to set the scene before talking about the dynamics of the percentage of dissolved EEA. We will explain better the potential link between different phytoplankton groups and changes in the enzyme activities and in the revised version of the ms.

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 appreciate the suggestions by the reviewer about the changes observed between March and May. We think that besides what the reviewer suggests, 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 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: 17 (7), 2459–2476). We will extend our discussion on this topic in the revised version.

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) motivates statistical analyses the use of significant correlations. As mentioned in several previous comments, we will include in the revised version the data on cell counts and bacterial productivity, as well as include 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 observed in the % of dissolved EEA, what gives even more value to this pattern observed in this study, and probably reinforces the idea that an integrating parameter (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 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. If temperature is affecting the proportion of dissolved EEA we believe that at least mentioning a potential link with global warming is conceivable.

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