Response to referee 2 "Spatial patterns of biphasic ectoenzymatic kinetics related to biogeochemical properties in the Mediterranean Sea" by France Van Wambeke et al. ms BG-2020-253

Anonymous Referee #2 and our responses in blue

The paper "Spatial patterns of biphasic ectoenzymatic kinetics related to biogeochemical properties in the Mediterranean Sea." by France Van Wambeke et al., reports prokaryotic ectoenzymatic activity, abundance and heterotrophic production in the epipelagic and the upper part of the mesopelagic layers in the Mediterranean Sea. In this study, the Vm and Km of the 3 enzymes (alkaline phosphatase (AP), aminopeptidase (LAP) and β-glucosidase (βGLU)) were determined using 2 series of substrate concentration. The paper points out that the choice of substrate concentrations affects the results and their interpretation.

Strength points

The paper presents an impressive quantity of data on ectoenzymatic activity of 3 enzymes: alkaline phosphatase (AP), aminopeptidase (LAP) and β-glucosidase (βGLU).
The data are of good quality and I think they deserve publication. The methods are detailed and well described. This paper has the potential to be a reference work for future studies on enzymatic activity, since the authors showed that the use of different ranges of concentrations of substrates give different results and therefore lead to bias in the interpretation of the results. I think this is a very important point, since the measure of enzymatic activity is crucial to get insights into the main biogeochemical fluxes in the oceans. Often the published data are difficult to compare due to the different concentrations of substrate used leading to contrasting interpretations.

Weak points

The main shortcoming is the language, often due to language issues, I did not understand what the authors means and the paper is confusing in many points. There are also some grammatical mistakes and typos. I think that there are too many details in the results making them hard to follow. The discussion is not focused, there are too much information that confound the reader. My main recommendation is to deeply revise the English, to simplify the results and to rework the discussion, in order to make the paper easier to follow and to highlight its main message.

We thank the referee for the suggestions and helpful comments. He/she read deeply the manuscript, checking numbers cited, tables, figure legends and provided many recommendations for style corrections and reduction of the results section as well as reorganization of the discussion, that we used to improve the manuscript and redefine various aspects of the focus of the paper as suggested. In particular:

- we shortened the description of the water masses, cited references and modified Fig.2 and Fig.8
- we simplified the results section (removed citation of numbers, DVFs too much detailed, descriptions of Figures..)
- we re-organized the discussion focusing on the interpretations of biphasic kinetics

Specific comments are reported below.

Introduction

All the introduction would benefit of a deeply revision of the English. The text does not flow well and there are some grammatical issues making hard to read it
The revised ms have been corrected by a native English speaker.

L38-40, P1. "Most of the organic matter being in the state of high molecular weight material, its hydrolysis by ectoenzymes plays an important role in the degradation, utilization and mineralization processes in aquatic environments, but also in nutrients regeneration (Hoppe, 1983; Chróst, 1991)." I think there is a grammatical issue in this sentence.
The sentence was modified as:
'In aquatic environments the organic matter compounds available for bacterial utilization are dominated by high molecular-weight organic molecules. In order to be assimilated, first they need to be hydrolyzed into smaller sized molecules by ectoenzymes outside of the cell. This represents a limiting step in organic matter degradation, and in nutrient regeneration (Hoppe, 1983; Chróst, 1991).'

L47 Seldomly?
The sentence was modified as: 'Kinetic experiments are time-consuming and most studies reporting ectoenzymatic activity examine enzyme kinetic patterns using only one or two samples. A single concentration of a presumed saturating substrate is then used to determine the activity of all the samples.'

L49-51, P2. "Within these 5 studies for the concentration kinetic the minimum concentration used was 50 nM at the lowest, ..." I think there is a grammatical issue in this sentence.
The sentence was modified as:
'Only 5 studies used a range of substrate concentrations to determine the enzyme kinetics, of these the lowest concentration used was 50 nM, (the lower concentration in the set is typically between 1 and 5 µM), and the highest concentration used was 1200 µM (the range of the higher concentration in the set is 5 - 1200 µM, median 200 µM).'

L66, P2. "among 44 isolated strains", strains of what? Bacteria?
Yes, this information was added

L95-97, P3. "The interaction between different enzymes has been largely studied in the Mediterranean Sea (Zaccone and Caruso, 2019) due to the particular role of this elemental stoichiometry." I do not understand this sentence. What do you mean with "interaction among enzymes"?
The sentence was modified as:
'Due to the particular role of this elemental stoichiometry,
the relative activities of different enzymes have been widely studied in the Mediterranean Sea (Zaccone and Caruso, 2019)'
The relative activities for instance the LAP/AP ratio, are often used as indexes of P deficiency and this is written as an example in the text of the ms.

How can it affect the C/N/P ratios of nutrients and organic matter?
If one enzyme releasing P or N shows higher activities than other types of ectoenzymes releasing only C, it is expected to see changes in the C/N/P ratios of the degraded organic matter. This will have also consequence on the fraction of N or P remineralized, as this fraction depends on the bacterial growth efficiency as well as on the relative differences in the C/N/P of organic matter relative to the C/N/P ratio of bacterial biomass.

L107-109, P3. “Our aim was to study the effects of the respective activities of the ectoenzymes in relation to the quality of the organic matter present, below the productive layer and above the deep Mediterranean waters” How was the quality of the organic matter investigated? I did not find anything about it in the paper.
We used C/N/P ratios of particulate and dissolved organic matter, TAA/DON and TCHO/DOC ratios

L104-107, P3. “In this study, we investigated in the Mediterranean Sea, the kinetics of three series of enzymes targeting proteins, phospho-mono esters and carbohydrates (aminopeptidase, alkaline phosphatase 105 and _-D -glucosidase respectively) in relation to the elemental stoichiometry of particulate and dissolved organic matter.” There is only 1 line (L333, P8) about stoichiometry data in the results and few lines (L572-573 and 592-593) in the discussion, so I think that this cannot be one of the main goals of the paper.
We agree. We have removed the last part of this sentence.

L106-107, P3. “We have paid particular attention to the use of a wide range of substrates concentrations to evaluate potential multiphasic kinetics.” and L116-117, P 3
“Finally, we discuss the biases in interpretation of past and current enzymatic kinetic, potentially induced by the reduced range of used substrates concentration.” I think that the use of different concentration ranges of substrate for the 3 enzymes leading to different results is the main message and I would focus the manuscript on it.
We agree and we have modified this part of the introduction accordingly.
The discussion has also been reorganized as suggested by the referee. In the revised version, the end of section 4.1 now introduces the new discussion plan as follows:
‘We have shown that the differences between the Km and Vm of the low and high affinity enzymes might change with the nature of the enzyme, with depth, and regionally. We will develop below the different interpretation that might emerge when discussing about i) the increase/decrease with depth ii) the use of enzymatic ratio as indicators of nutrient availability or DOM quality and iii) the estimates of *in situ* hydrolysis rates and their contribution to heterotrophic bacterial carbon or nitrogen demand.’

Accordingly, the titles of the discussion sections were modified as follows:
- 4.2 How the concentration set used affects ectoenzymatic kinetics trends with depth: possible links with access to particles,
- 4.3 How the concentration set used affects the interpretation of enzymatic properties as indicators of nutrient imbalance of DOM quality and stoichiometry.
- 4.4 How the concentration set used affects potential contribution of macromolecules hydrolysis to bacterial production

2. Materials and Methods
L143, P4. “Heterotrophic prokaryotic production (BP), heterotrophic prokaryotic abundances (BA)” I would use HPA and HPB as abbreviations.
I assume you want to write instead HPA and HPP. I recognize this would be clearer, but let me be an old generation scientist referring to the classical abbreviations BP and BA. This is just a matter of definition and both abbreviations have been properly defined in the text.
The sentence in section 2.1 was written as:
‘The water sampled with the conventional CTD-rosette was used for measuring heterotrophic bacterial production (BP, *sensus stricto* referring to heterotrophic prokaryotic production), heterotrophic bacterial abundances (BA, *sensus stricto* referring to heterotrophic prokaryotic abundances)…’

L147, P4. Replace ectoenzymatic activities by EEA, since the abbreviation is defined at L143-144. This is done as well as in other parts of the manuscript

L153, 154, 156 and 157, P4. I would use upper case letters for the abbreviations, in particular for DCM, LIW and MDW.
We prefer to keep lower case letters for the 4 layers and upper case letters for biological parameters.

L156-157, P4. "and second sampled at 1000 m (the limit between meso and bathypelagic waters), except at 2 stations (FAST, 2500 m; ION, 3000 m) named 'mdw' "
Why did you select 1000 m as depth representative of deep waters and not a sample collected close to the bottom?
The first idea was to focus on the main aim of the PEACETIME cruise devoted to the impact of dust on primary production and associated fluxes. So, we focused mainly on surface and mesopelagic zones except at the 2 stations FAST and ION where it was possible to study deeper layers.

How do you think that the different sampling depth at station FAST and ION can affect the results? This should be discussed in section 4.2 and 4.3.
A sentence was added in sub-section 4.2 on this point:
'Note that for the deepest layers sampled (FAST: 2500 m and ION: 3000 m), results are also contrasting, specific AP decreases with depth at ION but increases at FAST.'

L192-193, P5. Please add the batch of the CRM you used for DOC analysis, its nominal and measured concentration.
We added the following sentence:
'The nominal and measured DOC concentrations of the two batches used in this study were 42-45 µM and 43-45 µM, for batch14-2014#07-14, and 42-45 µM and 42-49 µM, for batch17-2017 #04-17.'

L217, P5. "Bacterial production (BP, sensus stricto referring to prokaryotic heterotrophic production)", BP was already defined at L143, P4.
In the revised version, there are not anymore abbreviations neither in the abstract nor in the introduction and they are defined here in Material and Methods sub-section 2.1.

L225, P6. "On 9 occasions", do you mean replicates? Samples?
Samples. The sentence was modified as:
'On 9 occasions during the cruise transect….'

Results
The results are very heavy to read, in section 3.3 there are too many comparisons, too many details that are not relevant for the main message of the paper. I recommend to simplify this section and to avoid details not relevant for the main message of the paper.
We simplified the results section following the recommendation of the referee.

3.1 Hydrological situations.
The title should be changed, what does "Hydrological situations" mean? I suggest Physical properties

The sub-section 3.1 has been shortened following the general comment of the referee 1 with a focus on the physical properties of water masses in which the samples of enzymatic activity were collected.
The Figure 2 has been redrawn following the comment of the referee.

This section should cite some papers reporting the circulation of the Med Sea and the main physical properties of the water masses.
A short description and references on the thermohaline circulation have been added in the introduction. A reference on the identification of water masses from their physical properties has been added in this sub-section 3.1. (see response to referee 1)

L294-295, P7. "The sampled stations have basins and latitude characteristics that were superimposed on a changing the seasonal pattern", I do not understand this sentence.
This sentence has been removed in the revised manuscript.

L299, P7. "Modified Atlantic Waters (MAW) are characterized by low salinity below the seasonal thermocline" Do you mean above the seasonal thermocline?
The cruise was carried out during May-June 2017. At the period of the year (end of spring), the surface layer has been sufficiently warmed up by atmospheric fluxes to generate a seasonal thermocline. This interface separates the surface waters with the core waters of Atlantic origin: thermohaline properties remain similar in salinity, but warmer due to spring heating. MAW are located below the seasonal thermocline. This paragraph has been reformulated in this way.

L299-301, P7. "this property is stretched in the westernmost stations, then progressively relaxes on eastern station, revealing an eastward circulation in the Algerian Basin and a dispersion in the connected basins (northwestern Mediterranean, Tyrrhenian, and Ionian Seas)." And L303-305, P7. "This property is pronounced in the eastern stations and progressively lowered on the western stations, revealing an opposite circulation pattern to the MAW. " It is not possible to infer the water mass circulation from the T/S graphs. Please add references and rework the sentences.
We thank the referee for pointing out that the analysis of T/S diagrams is insufficient to infer circulation of water masses. This section has been simplified and a general description supported by references has been done in the Introduction.

L305-308, P7. Please add references.
This sub-section 3.1 was rewritten and references added as indicated above in response to referee 1.

L308-310, P8. Please add references.
This sub-section 3.1 was rewritten and references added as indicated above in response to referee 1.

L310-311, P8. "The core of LIW is characterized by lower oxygen content than its surrounding water masses, shallower (MAW) and deeper (WMDW and EMDW)". Looking at figure 2a, this observation is not always true, for example at stations ION and 6, LIW has higher oxygen than deep waters.
The referee is right. This correspondence stands for the Western Mediterranean Basin. In the Eastern Mediterranean, the oxygen minimum in LIW is local 'oxystad', its concentration

becomes larger than deep waters oxygen while getting closer to the region of LIW formation.
Note that only the Figure 2b (T/S diagrams) has been kept and redrawn in the revised version

L312-314 "We thus presented all the figures/tables in the order ST10, FAST, ST1, ST2, ST3, ST4, ST5, TYR, ST6 and ION, according to the expected circulation of the LIW (from the right to the left)." If you want to follow the LIW path, I think it is better to invert the order of the stations, since LIW move from ION to St.10.
We agree with the referee that this classification is misleading with respect to LIW circulation. We kept the longitudinal classification most commonly used in the literature and applicable also for the other sampled layers/water masses. We reformulated the sentence to specify the inverted way of LIW circulation and removed the link of causality between the choice of classification and LIW circulation.

3.2 Biogeochemical situation. I would replace situation with properties. This is done
In this section, the values of DOC, DON and DOP should be reported not only their change with depth.
The ranges of DOC, DON and DOP were cited in this paragraph as well as reference to Table S1.

Section 3.3. Ectoenzymatic activities – kinetic trends
This section is really heavy and in some parts there is not correspondence between the number in the text and in the tables. The authors should carefully rework this section deleting all the details that are not relevant and checking the correspondence between the number in the text and in the tables.
This sub-section was reworked and reduced. See response to comment 'L368-L374, P9' below for the correspondence between numbers in the text and in the Tables.

L352, P8. "The ectoenzymatic activities were determined using large trophic conditions", I do not understand this sentence.
The sentence was modified as: 'EEA were determined over highly variable trophic conditions and using a wide range of substrate concentrations ranging from 0.025 to 50 µM'

L368-L374, P9. Is this paragraph relevant? Looking at table 2, I find different numbers.
We agree with your remark and reduced the paragraph accordingly.
About the 'numbers', this is a mode of computation issue. We calculated 'mean of ratios' instead of 'ratios of means'. For instance, in Table 2, I agree that Vm1APmean / Vm1LAPmean is 5.3, not 6 as cited in this paragraph. Indeed we estimated more adequate to calculate individual Vm1AP/Vm1LAP at each station, and then to compute mean of the ratios. In this example, the 10 stations had a distribution of ratios as 1.61, 3.33, 2.33, 3.95, 1.16, 1.68, 3.67, 10.56, 7.36, and 25.49 which lead to an average mean of ratios of 6.1. Note that all 'ratios' cited in the text (Vm50/Vm1, DVF, Km50/Km1, Km/Vm…. are computed as means of ratios and not as ratio of means. This sentence was added in Material and Method section.

In addition, there was a typo error in the second part of this paragraph, as we presented LAP/βGLU ratios, not AP/βGLU ratios.
To finish with this referee comment, we agree that it is necessary to reduce all these descriptions and we have reduced this paragraph to:
'For each enzyme, the order of magnitude reached for Vmax was the same at the 'surf' and 'dcm' layers. In all layers, the highest mean Vm of the 10 stations were obtained for AP,

followed by LAP and then βGLU, whatever the range of tested concentrations (Vm50 or Vm1, Table 2).'

L376-380, P9. "For LAP (Fig. 4), Vm50 was on average 3 times higher than Vm1 in 'surf' and 'dcm' layers, but the differences between these two rates increased with depth (x9 in 'liw', x12 in 'mdw'). Vm50 decreased from epipelagic to mesopelagic waters by a factor of 8 on average, (ratio 'depth variation factor' – DVF), but by a factor x19 for Vm1 (Fig. 4a)." It is very hard to see these differences in Figure 4. I think you should also refer to Table 2.
Yes, we could refer to Table 2 but as we calculated means of ratios, they cannot be directly calculated from Table 2.

Looking at table 2 the number are different. As an example:
Vm50 was 10-times not 12 higher that Vm1 in mdw. "Vm50 decreased from epipelagic to mesopelagic waters by a factor of 8 on average,", if I consider dcm epipelagic and LIW mesopelagic Vm50 decreases by 4.6 times, if I consider surf as epipelagic the decrease is of 3.8, so I don't understand how the authors calculated a value of 8, the same for Vm1.
We computed means of ratios, not ratios of means.

L383, P9. I think there is a typo SD10, SD2 and SD6 should be St10, St.2 and St.6. St. FAST and St.1 are missing, they also show Km50 of LAP lower at the dcm than in the surf.
Yes, we modified the sentence in the other way:
'Only 3 stations showed an increase at 'dcm' compared to 'surf' layers (ST3, ST4, ST5)'

L395-396, P9. Also here there is not correspondence among the numbers in the text and the numbers that I can calculate from the table. Please check.
We computed means of ratios, not ratios of means.

L410-411, P10. "Average Km50/Km1 ratio for _GLU was 320 and average Km1/Km50 ratio for LAP was 118." My calculation looking at table 4 indicate 240 instead of 320 and 79 instead of 118. Please check.
We computed means of ratios, not ratios of means.

L436, P10. I think you should cite Fig.7c,d not Fig.7a,b.
Yes, we modified the text accordingly

Discussion
The discussion is confounding, there are a lot of interesting ideas, but they are lost in the text. The discussion would strongly benefit of deeply revision of the English. I think that section 4.1 should be the focus of the paper, but I miss a conclusion. From your data do you suggest to use Vm1 or Vm50? Km1 or Km50? Or since they give different information does the use of one or the other depend on the goal of the work? From my understanding the use of a not-appropriate range of substrate determine bias in the results and in the interpretation and data, obtained using different range of substrate, are not comparable. I think these points should be better stressed in this section. I also think that the other sections should support this one, showing how the different ranges may affect the interpretation of trends with depth and regional variability.
We agree with referee 2 comments. This has been also suggested by reviewer 1, see our response above to 'L106-107, P3'

L481, P11. “The biphastic factor as defined in Tholosan et al (1999). “Please define it in the text to help the reader.
We changed the term of biphasic factor to biphasic indicator in order to use the same as in Tholosan et al. (1999).
The definition of the term was introduced in sub section 3.3 when we describe turnovertimes as:
‘We estimated the degree of difference between the kinetics based on low or high concentrations sets using the “biphasic indicator” as developed in Tholosan et al. (1999). This index tracks the difference between the initial slopes (Vm/Km) of Michaelis-Menten kinetics as (Vm1/Km1) / (Vm50/Km50).’

Then the term was also cited in the discussion sub section 4.1 where the sentence was modified as:
‘In our study the biphastic indicator (Km50/Vm50) / (Km1/Vm1) was used to determine the degree of difference between the two Michaelis-Menten LAP kinetics’.

L505-509, P12. Please rework, I do not understand this sentence.
The sentence was modified as:
‘Conversely to AP results, the higher differences between the 2 LAP enzymatic systems, suggest that microorganisms expressing LAP activity faced large gradients of protein concentrations and were adapted to pulsed inputs of particles.’

L535-539, P13. This paragraph should be moved in section 4.1.
The discussion section was re-organized as suggested by both referees

L569-571, P13. “With concentration kinetics ending at 50 µM of MUF-P, the specific activities of AP reached using per cell Vm50 or per cell Vm1 were not so different and their trend with depth were similar (Fig. 8). “ It is really hard to see these trends in Fig.8.
We modified the sentence as:
‘We could not conclude that there was a systematic increase of specific AP with depth. Specific AP decreased in 5 stations, increased in 3 stations and at the remaining stations specific Vm increased based on Vm50, but decreased based on Vm1 (Fig. 8b).

L572-573, P14. “whereas DOC/DOP ratio decreased (from 2200-2400 to 1500-1200), suggesting a preference for heterotrophic prokaryotes to use dissolved organic phosphorus as substrate of AP.” Usually DOC/DOP ratio increases with depth due to the preferential removal of P. Are these ratios calculated using data collected in this cruise? The removal of DOP by heterotrophic prokaryotes should increase the DOC/DOP so, this sentence has no sense to me.

We agree with the referee comment about an expected DOC/DOP increase with depth. Yes we measured DOP and DIP during this cruise however we have no DOP data at ST1, 2 and 10. In addition, DOP concentration at depth is on average 39 nM, and as it is obtained by a difference between TDP and DIP with average values around 350 - 400 nM, its estimate is subject to large errors. Thus we had only few data on DOC/DOP ratios at depth (6 in liw and 2 in mdw) associated with large errors. Instead of describing means per depth, which is subject to caution under such conditions, we examined in more detail variation with depth at all the stations. We found an increase of DOC/DOP ratio in liw or mdw layers in 2 cases (ST2 and ST4), a decrease in 4 cases (ST3, ST5, ST6 and ION), and the same order in one case

(TYR). Note that this observation is obtained from only 4 layers sampled along the profile, and such trend would be much easier based on a complete profile rather on only 4 data points.

We added a sentence in the results section, sub section 3.2 as:
'Taking all 4 water layers, the mean values for the DOC/DON and DOC/DOP molar ratios were 14 ± 2 and 2112 ± 1644, respectively, with no significant trend with depth due to the variability within stations. Deep DOP was not sampled for at 3 stations and in addition DOP estimates are subject to large errors at depth (DIP is on average 10 times higher than DOP). We observed a DOC/DOP increase with depth at 4 stations, but a decrease at 2, and no trend at another.'

We modified the sub sub section 4.2 the discussion on this topic as:
'The particulate matter C/P ratio did not change with depth. However the variability in the trend with depth seen for specific AP was also observed with DOC/DOP ratio. We expected to see an increase with depth due to a preferential removal of P, however, it was not systematic.'

L574-587, P14. This paragraph should be moved in section 4.1.
The discussion section was re-organized as suggested by both reviewers

L595-611, P14. This paragraph is not clear to me.
This paragraph was reorganized and we hope it is clearer now

4.3 Regional variability
L613-615, P14. "In epipelagic waters, both AP maximum rates (Vm1, Vm50) significantly increased from the Algerian/Ligurian Basins to the Tyrrhenian Basin (t test, $p = 0.002$ and $p = 0.02$, respectively), and reached maximum values at ION.". In Figure 6 this pattern is not very clear.
Yes this is because the Fig 6 is in log scale, more adapted to compare AP rates between epipelagic and deep layers. The factor of increase between the 2 regions was about 3 (x3.3 for Vm50, x 2.6 for Vm1)
The sentence was modified as:
'In epipelagic waters, both AP maximum rates (Vm1, Vm50) significantly increased by approx 3 fold from the Algerian/Ligurian basins to the Tyrrhenian basin….'

L658-663, P15-16. This paragraph should be moved in section 4.1.
The discussion section was re-organized as suggested by both referees

L675-687, P16. This paragraph should be moved in section 4.1.
The discussion section was re-organized as suggested by both referees

Figure 2. Please enlarge the name of water masses. I think the value of this figure would strongly increase if you mark on the T/S graphs where the samples for enzymatic activity were collected. You could also add a zoom of intermediate and deep waters.
We followed the Referee's suggestion. The Figure 2 has been redrawn in the revised version with a zoom on the densest waters and the addition of samples marks as follows:

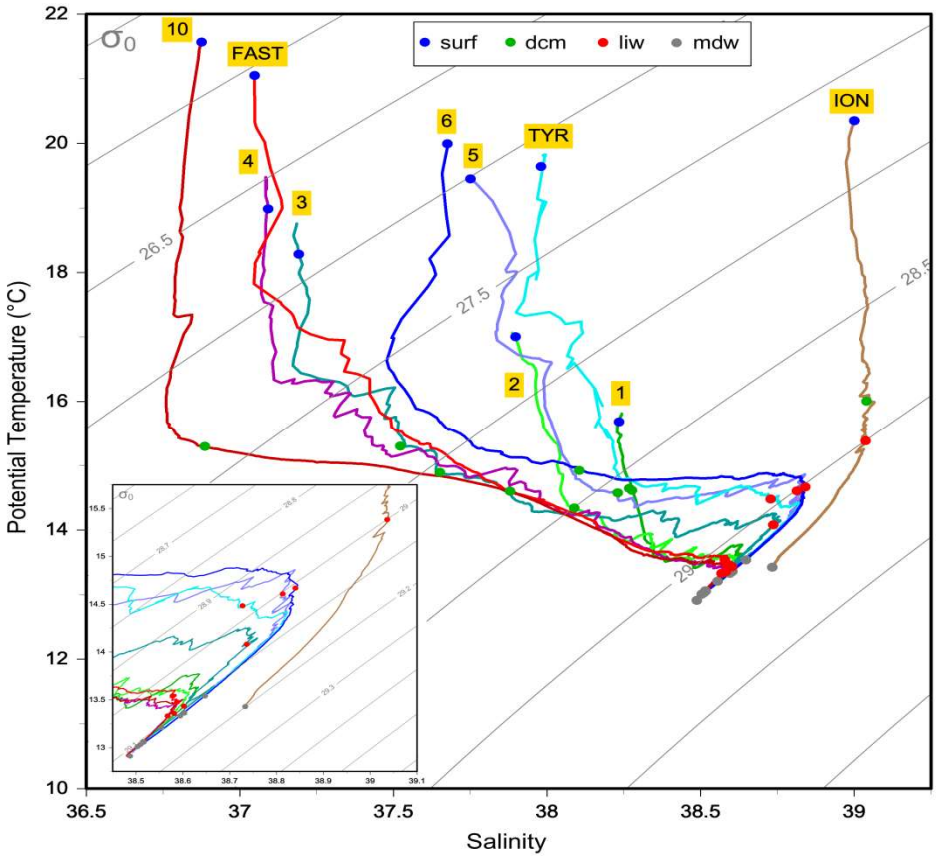

Figure 4-6. Please add the legend.
This is done

Figure 8. This figure is really confuse and hard to understand, I suggest to remove it.
We separated the different enzymes in separate plots as follows:

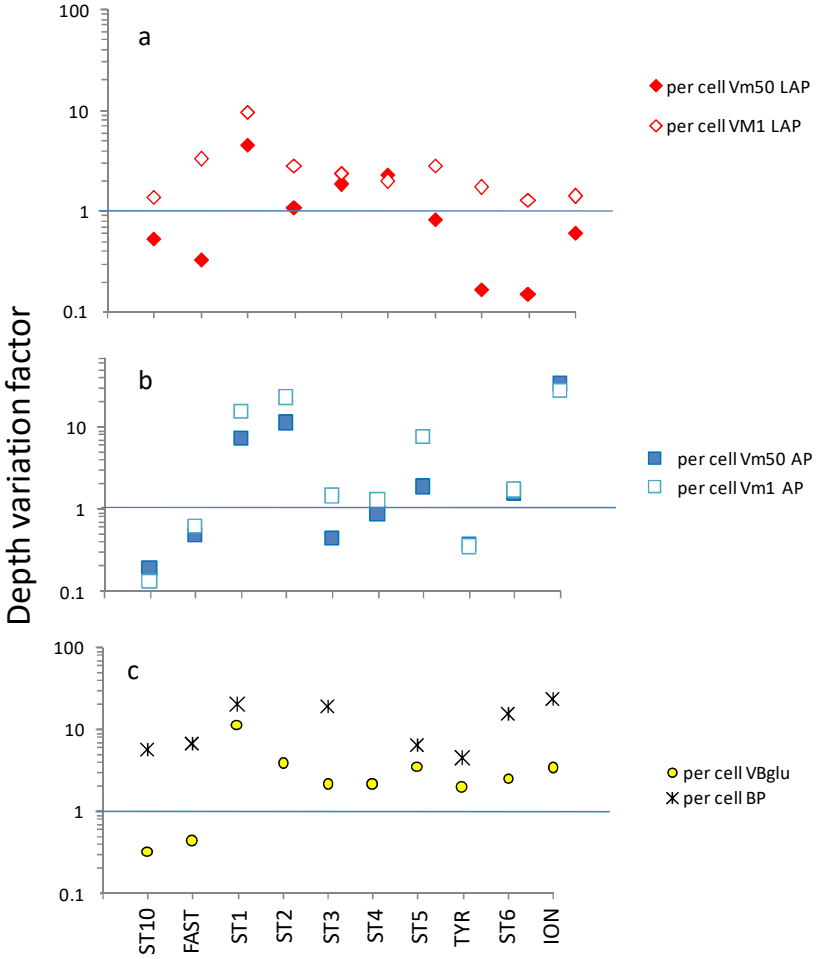

Figure 9 and 10. I suggest to report in the caption how bacterial nitrogen demand and bacterial carbon demand was calculated.
For Fig. 9 we modified the legend as:
'….and comparison to heterotrophic bacterial nitrogen demand, determined from BP assuming a biomass C/N molar ratio of 5 and no active excretion of nitrogen….'

For Fig. 10 we modified the legend as:
'…heterotrophic bacterial carbon demand (BCD, determined from BP assuming a BGE of 10%) in epipelagic ….'

Table1, Please check the title of the columns.
This has been done. Two blank missing (dcm depth, bottom depth) and the term 'layers' for the two last columns.

Table 2, line 6 column 5, I think the lines are inverted, if not please check the number in the text.
Yes mean sd and min max were inverted, we corrected.