Response to referee 1 "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 #1 and our responses in blue

General comments:

This article refers on the enzyme activity rates along the epipelagic and mesopelagic layers of a Mediterranean area, taking into consideration the variability of their kinetic properties in relation to the addition of different amounts of fluorogenic substrates. Particularly, two ranges are compared (0.025-1 µM with respect to 0.025-50 µM) to assess the degree of affinity between the enzyme and its substrate (in terms of Km Michaelis-Menten constant). I think that the subject of this manuscript falls within the aims of Biogeosciences, This paper presents a large dataset of enzyme kinetics in different regions of the Mediterranean; their interpretations and conclusions are quite good, the supplied figures are explicative and the list of references is consistent,

We are grateful to the referee 1 for reviewing the manuscript. We appreciate all his/her insightful comments and we acknowledge that they have encouraged us to simplify and improve the manuscript. Please find below a detailed answer to all the raised questions and comments

However the English language at some points is not very clear and could be improved.
The revised ms has been proofread by a native English speaker

Moreover, the structure of the Introduction could be improved as suggested in the Specific comments.
We reorganized the introduction, find below our reply to specific comments

However, the major doubt I have regarding its acceptance is that the Special Issue Atmospheric deposition in the low-nutrient-low-chlorophyll (LNLC) ocean- where it has been submitted - has a focus different from the Special Issue main theme and the subject of this manuscript (enzyme expression and its variability depending on the added substrate concentrations) refers on the effects of the atmospheric deposition or aerosol on marine processes in 4 lines of the discussion only (from 714 to 717). Therefore in my opinion this represent a too short and insufficient discussion to justify the inclusion of this manuscript in the Special Issue above reported.

The main reason why this manuscript has been submitted to the above cited Special Issue is that the presented data were obtained during the cruise PEACETIME. As stated in the description of the SI: "(…) It will present the results obtained during the PEACETIME (ProcEss studies at the Air-sEa Interface after dust deposition in the MEditerranean sea) cruise conducted in 2017 in the Mediterranean Sea, but the special issue is open to any submissions provided that the subject is consistent with the objectives defined above."
The other reason is that ectoenzymatic activities presented here are also the base of the discussion of 2 other papers fully related with the topic of the special issue: one has recently been submitted (Van Wambeke et al., bg-2020-411) and the other one is in preparation (Pulido-Villena et al., in prep). However, we shall leave this decision to the Editors of the special issue.

Van Wambeke, F., Taillandier V., Desboeufs, K., Pulido-Villena, E., Dinasquet, J., Engel, A., Marañón, E., Ridame, C., Guieu, C.: Influence of atmospheric deposition on biogeochemical cycles in an oligotrophic ocean system, Biogeosciences Discuss., https://doi.org/10.5194/bg-2020-411, in review, 2020.

Pulido-Villena, E., Van Wambeke, F., Desboeufs, K., Petrenko, A., Barrillon, S., Djaoudi, K., Doglioli, A., D'Ortenzio, F., Fu, Y., Gaillard, T., Guasco, S., Nunige, S., Raimbault, P., Taillandier, V., Triquet, S., Guieu, C. Phosphorus cycling in the upper waters of the Mediterranean Sea (Peacetime cruise): relative contribution of external and internal sources, in prep for Biogeosciences, special issue PEACETIME.

Specific comments:

line 20, the definition of the depth range of epi- and meso-pelagic layers should be indicated
Instead, we have specified the 4 depths sampled as follows:
'Ectoenzymatic activity, prokaryotic heterotrophic abundances and production were determined in the Mediterranean Sea. Sampling was carried out in the sub surface, the deep chlorophyll maximum layer, the core of the Levantine Intermediate waters and the deeper part of the mesopelagic layers.'

line 23, and throughout the text, I suggest to refer to low and high affinity enzymes (instead of affinity systems)
This is done

lines 28-29, please check this sentence, it remains without a conclusion (probably "although" should be deleted).
The sentence was modified as:
'The contribution of ectoenzymatic hydrolysis to the heterotrophic bacteria requirements was high in terms of N, but it was low in terms of C.'

lines 32 and successive: current and past interpretation of the three tested enzymes and their relative differences regarding the choice of added....;
We have rephrased the sentence to make it clearer:
'This study clearly highlights the bias in current and past interpretation for the kinetic parameters of the 3 enzymes according their fluorogenic substrate concentration sets.'

line 35. I suggest to change into: were different depending on activity estimates were derived from high or low concentrations of substrates were used;
The sentence was modified as:
'In particular, aminopeptidase/βglucosidase ratios, and some depth trends, were different depending on whether activities estimates were derived from high or low concentrations of the fluorogenic substrates.'

line 42, is a subject This is done

line 51, for the enzyme kinetics (instead of concentration kinetic), the minimum substrate concentration. This is done

The Introduction should be re-organized, since on line 60 the authors talk about multiphasic kinetics, and they again return on the concept of biphasic kinetic on line 69 and
also on lines 76-77, in a logical order it would be better to move lines 69 and 76 close

to line 60 to discuss about multiphasic kinetics Again, sentence on lines 88-90 could be moved close to lines 80-83 (both talking about the enzyme assay);
This part of the introduction has been re-organized following the referee comments.

line 59, within a single species This is done

line 81, prior incubation of the sample with the substrate This is done

line 92, especially regarding the origin This is done

line 97, interaction among different enzymes This is done

line 100, since in the Abstract the acronyms of the enzymes are reported, please use them for aminopeptidase and phosphatase;
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 section 2.1.

line 104, the kinetics of three enzymes targeting This is done

line 118, in this manuscript (in full) This is done

line 130, epipelagic (0-250), what reference do the authors used to select these depth ranges? please include it;
The sentence was modified as:
'Generally, at least 3 casts were conducted at each short station. One focused on the first 250 meters and the second one on the whole water column….. The third cast…'

line 142, remove comma after rosette This is done

lines 182, 186, 324 , NO3 (3 in small character)
We assumed this is just a matter of the choice of the abbreviation term. To be formerly correct we should use nitrate, or NO_3^-. For simplicity to avoid abundant superscripts and subscripts in the ms, we decided to abbreviate nitrate as NO3. To be clear for the reader we modified the sentence in sub-section 2.2 as:
'Nitrate (abbreviated as NO3),…'

line 182,Within epipelagic, nutrient (DIP and NO3) depleted layers;
The sentence was modified as:
'Within the epipelagic surface layers, DIP and NO3 were determined using the liquid waveguide capillary cell method (LWCC)…'

line 182, LWCC in full (liquid waveguide capillary cells); This is done

line 202, Two replicates per each TCHO sample; This is done

line 214, acetonitrile as solvent B was used; This is done

line 250, The amounts of the products MCA and MUF, released by LAP.....; This is done

line 255, were produced instead of dispatched; This is done

line 260, The hydrolysis rate was calculated; This is done

line 279, Vm1 and Km1; This is done

line 290, Results are reported as means ± standard errors; This is done

line 290 correlations among variables; This is done

line 296, remove (the) , changing seasonal pattern; This is done

line 308) were different from the EMDW water mass being less salty and colder; This is done

line 310, please include a reference for the identification of water masses (according to their physical-chemical properties);
The sub-section 3.1 was rewritten as:
'The physical properties at the stations sampled, presented in the temperature-salinity diagrams (Fig. 2), show pronounced longitudinal variation which is in agreement with the thermohaline circulation features of the Mediterranean Sea (see Introduction). The deep waters, formed by two separate internal convection cells, have distinct properties in the Eastern basin (station ION, 13.43°C temperature, 38.73 salinity) and the Western basin (the other stations, 12.91°C temperature, 38.48 salinity). The deep layer samples 'mdw' were collected within or in the top of deep waters (grey dots, Figure 2). The intermediate layer samples 'liw' were collected in the vicinity of the salinity maxima (red dots, Fig. 2), which is used to identify the LIW cores (e.g., Wust, 1961). Salinity maxima in the LIW core are particularly pronounced in the presence of fresher and lighter waters of Atlantic origin above; this feature is progressively relaxed eastward. LIW properties decrease from ION, the closest station from their source, to the westernmost stations of the Algerian Basin, which is concurrent with their westward spreading and progressive dilution. In this springtime period, the productive layer was stratified with the apparition of a seasonal thermocline. This interface separated the warm surface waters with the cool waters of Atlantic origin in which the DCM developed. As a consequence, the two sample types collected in the productive layer ('surf" in blue dots and 'dcm' in green dots, Fig. 2), have their thermohaline properties remain similar in salinity, but clearly differentiated in temperature. For sake of clarity, the stations are ranked with respect to these longitudinal variations, in the following order: ST10, FAST, ST1, ST2, ST3, ST4, ST5, TYR, ST6 and ION.'

Other references were also added to the introduction. The lines 110-114 in the introduction were modified to include new references:
'The distribution of biogeochemical properties below the productive zone is the result of large-scale dynamic transport systems associated with three distinct thermohaline circulation cells (Wust, 1961; Hopkins, 1978; The Mermex Group, 2011 and references therein). These open cell convey fresh and cool waters of Atlantic origin in the upper 150-200 m water layer extending into the eastern part of the Levantine Sea.The return branch is composed of warm, saline waters, the Levantine intermediate waters (LIW), which spreads over the whole Mediterranean Sea at a depth of 200-500 m (Kress et al., 2003; Malanotte-Rizzoli et al., 2003; Schroeder et al., 2020). In addition, two closed cells, internal to each Mediterranean sub-basin, are driven by deep water convection and spread below the LIW (e.g., Lascaratos et al., 1999; Testor et al., 2018).'

New references added:

Hopkins, T. S.: Physical processes in the Mediterranean Basin. Estuarine transport processes, B. Kjerfve, editor, University of South Carolina, 269-310, 1978.

Lascaratos, A., Roether, W., Nittis, K., and Klein, B.: Recent changes in deep water formation and spreading in the eastern Mediterranean Sea: a review, Prog. Oceanogr., 44, 5–36, 1999

Schroeder, K, Cozzi S, Belgacem, M, Borghini, M, Cantoni, C, Durante, S, Petrizzo, A, Poiana, A and Chiggiato, J.: Along-Path Evolution of Biogeochemical and Carbonate System Properties in the Intermediate Water of the Western Mediterranean. Front. Mar. Sci. 7:375. doi: 10.3389/fmars.2020.00375, 2020

Testor, P., Bosse, A., Houpert, L., Margirier, F., Mortier, L., Legoff, H., Dausse, D., Labaste, M., Kartensen, J., Hayes, D., Olita, A. Ribotti, A., Schroeder, K., Chiggiato, J., Onken, R., Heslop, R., Mourre, B., D'Ortenzio, F., Mayot, N., Lavigne, H., de Fommervault, O., Coppola, L., Prieur, L., Taillandier, V., Durrieu de Madron, X., Bourrin, F., Many, G., Damien, P., Estournel, C., Marsaleix, P., Taupier-Lepage, I., Raimbault, P., Waldman, R., Bouin, M-N., Giordani, H., Caniaux, G., Somot, S., Ducrocq, V. and Conan P.: Multiscale observations of deep convection in the northwestern Mediterranean Sea during winter 2012–2013 using multiple platforms. Journal of Geophysical Research: Oceans, 123, 1745–1776. https://doi.org/10.1002/ 2016JC012671, 2018.

The Mermex Group. Marine ecosystems' responses to climatic and anthropogenic forcings in the Mediterranean. Progress in Oceanography, 91(2), 97–166, doi: 10.1016/j.pocean.2011.02.003, 2011

Wust, G.: On the vertical circulation of the Mediterranean Sea. Journal of Geophysical Research, 66, 10, 3261-3271, 1961

line 327, The depth of dcm (use acronym); This is done

line 333, the mean values of DOC/DON, This is done

line 334, the mean values of TAA; This is done

line 337, decreased from epipelagic to deeper waters; This is done

line 338, contribution of TAA-N to DON which ranged from 3 to 9% at surf and dcm and from 1.6 to 4.6% at liw and mdw respectively;
This sentence was modified as:
'…this trend was confirmed by the ratio of TAA-N to DON (Fig. S1a) which decreased significantly with depth ($p < 0.001$).'

line 342, according to; This is done

line 344, varied among the stations; This is done

line345, the highest values were measured; This is done

line 352, were determined over highly variable trophic conditions; This is done

line 357, the finding that measurements at liw and mdw layers for GLU were below detection limits
The sentence was modified as:
'For 'liw' and 'mdw' layers computation of βGLU kinetics was impossible as only a few time series gave a significant linear increase of fluorescence with time, when adding 25 or 50 µM fluorogenic substrate.'

line 365, please indicate how the CV coefficient of variation was calculated (mean/st. dev x100);
The definition of CV was added in the Material and Methods section 2.5 'statistics'

line 370 AP Vm50 and Vm1 mean values; This is done

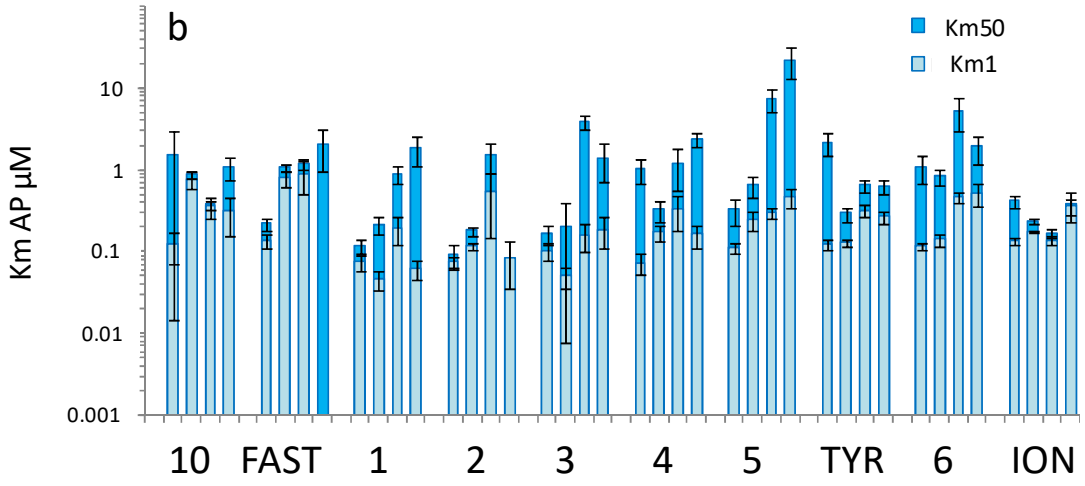
line 382, variable patterns (instead of inconsistent); This is done

line 390, their differences between two sets of concentrations (Vm50 and Vm1) were; This is done

line 394, with saturation rates occurring around 1 µM of added MUF-P (this repeats line 355);
The first sentence of the paragraph describing AP was modified as:
'AP was the enzyme for which Vm1 and Vm50 were the closest (averages of Vm50/Vm1 ratio for the whole data set was 1.6 ± 0.5) (Fig. 6a), showing that saturation rates occurred at 1 µM of added MUF-P (Fig. 3)'.
This was not repeated anymore in this paragraph.

line 403, about the declining trend; This is done

line 411, Km 50/Km1 ratio (Fig.6b) decreased with depth, so enzyme affinity for the substrate increased (please check this sentence);
Line 411 The sentence written is 'It was observed that the general trend was that Km50 increased more with depth (DVF > 0 at 7 stations, ranging from x2 to x29) than Km1 (DVF > 0 at 9 stations, ranging x1.9 to x3.8, see ST1 and ST5'
These statements are correct, but there was a mistake in Fig6b. Indeed we plotted 2 times the Fig 6a (VmAP) instead of Fig 6a (VmAP) and 6b (KmAP). We apologize for this mistake, the correct Figure 6b is presented below and in accordance with what was described line 411.



line 417, the turnover times were the shortest and the longest; This is done

line 439, the mean values (instead of medians);
Figure 7, to which line 439 refers to, represents Tukey box plots and, thus, the middle point is actually the median value of the dataset. This is why I talk about medians.

line 440 and in Tab. 3, gC cell (instead of bact) ; This is done

Tab. 2 caption: Microbial abundances, activity rates and kinetics measured at the 4 layers
The sentence was modified as:
'Heterotrophic bacterial abundances (BA), bacterial production (BP) and ectoenzyme kinetics for leucine aminopeptidase (LAP), β-glucosidase (βGLU) and alkaline phosphatase (AP) at the 4 layers studied for ectoenzymatic activities.'

first row, surface, dcm. liw. Mdw (remove layers and waters); This is done

Tab.3 caption: in deep waters (liw and mdw , This is done
per BP/LAP in nmolAA/nmolN (not C) and per BP/AP in nmolP/nmol P (not C);
The last 3 specific rates of this Table 3 are Vm of ectoenzymatic activities calculated per unit BP, BP expressed in carbon units.

Tab. 4, first row: surface, dcm, liw, mdw (remove layers and waters); This is done

Fig. 8, per cell V50 or V1 GLU?;
It is not Vm50 nor Vm1 as we could not make kinetics. This is why it is just written 'V'. This is explained in the legend:
'For β–glucosidase DVF, specific activities are based on the few detectable rates at high concentration (per cell Vβglu, yellow dots).'
For clarity we also separated Fig 8 in three sub-plots, see response to referee 2.

Fig. S1, include labels for a and b; This is done, as well as for Fig. S2

lines 442-443,please check this sentence: while the specific activities per unit cell decreased with depth, the activity per unit BP increased (but from 46-136 they change into 1-14), since in Figure 8 a decrease with depth instead of an increase is shown ;

- LAP per cell based on Vm1 decreased with depth (DVF 1.3-9.6, Table 3, Fig. 8),
- LAP per cell based on Vm50 increased with depth (Fig. 8)
- LAP per unit BP based on Vm1 increased with depth (DVF 0.09-0.76, Table 3)

The second sentence was modified as:
'While the specific LAPVm1 per unit cell decreased with depth, the specific LAPVm1 per unit BP increased with depth at all stations (Table 3, Fig. 8a).'

line 444, according to stations; This is done

line 449, increased with depth (include reference to Fig.8) ; This is done

A better title for Paragraph 3.5 should be Hydrolysis versus carbon/nitrogen demand;
We left the title unchanged. Indeed, in this sub-section, we estimate ectoenzymatic *in situ* hydrolysis rates (defined and calculated as in Material and Method section, last paragraph in sub section 2.4) but we do not compare them to carbon or nitrogen demand. This is done later on, in the discussion section.

line 452 higher than TAA concentrations (Table 2, Table S1); This is done

line 485, the differences between these two LAP systems could reach the differences?.(explain better this statement);
The sentence was modified as: 'The two LAP enzymatic systems observed in the water column could reach a difference as large as that found in the sediment (Tholosan et al., 1999), where large gradients of organic matter are found

lines 478 and 489, the difference between the two concentration sets of substrates (not two types of enzymes);
This sentence was removed

It sounds very strange that the <0.2 micron fraction exceeded the total enzyme activity! how do the authors explain this strange result?
It is probably an artifact of the filtration. A minimum of vacuum pressure is necessary and some cells can be broken during filtration, thus release of intracellular enzyme may occur. Anyway, as we simplified the results section by removing details, this part was reduced as follows:
'During the PEACETIME cruise we ran some size fractionation experiments in 'surf' and 'dcm' layers (results not shown). The contributions of the < 0.2 µm fraction to the bulk activity was on average 60 ± 34 % (n = 12) for AP, 25 ± 16 % (n = 12) for βGLU and 41 ± 16 % (n = 12) for LAP, confirming these trends in the Mediterranean Sea'

line 508, expression of enzyme activities instead of development; lines 507-509, re-write or delete this sentence, it is unclear;
The sentence was removed and this paragraph started by:
'LAP enzymatic systems showed, in opposition to AP, more differences and different trends with depth.'

line 529, This increases the importance of Km1 at low concentrations of substrate (is it right the meaning? just to avoid to repeat the risk of overestimation, already written in line 530); We agree, there is repetition, we removed the first sentence

line 530, difference in the response; This is done

please check the references: Siokou-Frangou et al. 2010 (not included in the reference list), Zaccone et al. (2010) not cited in the text; We modified the reference list accordingly.

line 555, remove for these authors; This is done

line 560, These authors suggested; This is done

line 604 (Limit of detection, not acronym); This is done

line 661 LLBN? please report in full; This is done

rewrite sentence on line 675; the direct influence on the determination of Km and Vm of the use of an appropriate set...; The sentence was modified as:
'Our results clearly showed the influence of the concentration set used to compute *in situ* hydrolysis rates'

lines 679,680, 682, 683, 694 instead of affinity system I suggest affinity enzyme; This is done

line 691 Lemée 2002 or 2012?; It is 2002

line 704, supported by peptides and polysaccharides hydrolysed by enzyme activities; This is done

line 705, please report BGE in full (no acronym has been reported before) This is done