Response to Reviewers
bgd-8-1335-2011

Silvio Pantoja
Corresponding author

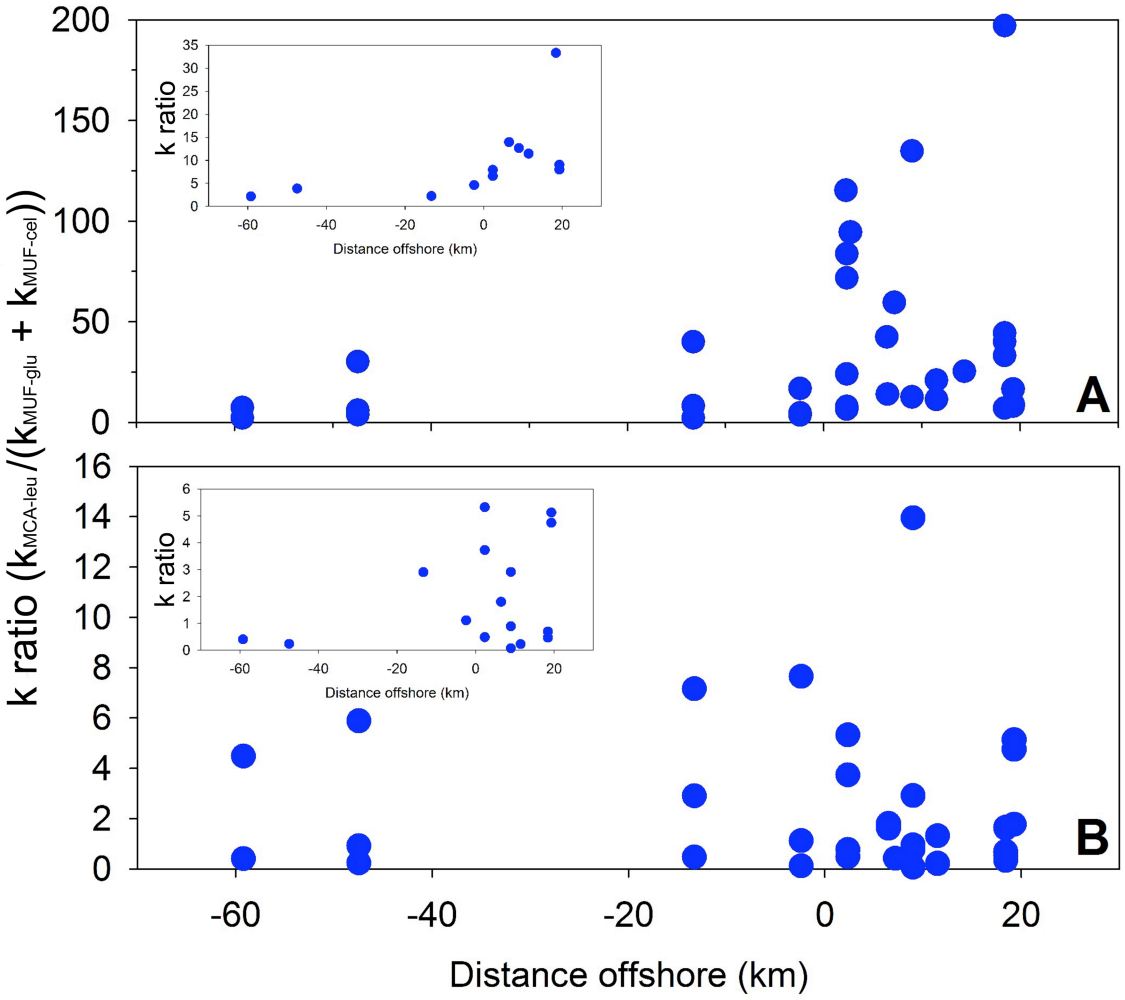
Reviewer 1

1. *I have strong reservations as to the validity of the hydrolysis potential (carbon budget) model… There are major points within this paper that I would like to see addressed before publishing regarding the methodology and interpretation of some of the data. However, I think the work Pantoja et al presented here can be of use to the aquatic science community and fits within the theme of Biogeosciences.*

The main criticism of the Reviewer, and coincides to that of Reviewer #2, is related to the fact that we use high concentrations of our model substrates therefore our rates cannot be regarded as actual but apparent rates. We agree with both reviewers that this is a handicap for the interpretation and we have decided to removed that conclusion from the paper, and compared the change in activity during winter and spring-summer as a measure of increase production between the two periods that coincides with the increase in photosynthetic production.

2. *This manuscript does not analyze the regulation or expression of hydrolysis activity in the area, but rather provides a spatially explicit, quantitative description of activity. However, there is limited discussion as to what relationship activity may have to other biogeochemical parameters (oxygen, chlorophyll, etc).*

Even though we had performed those analyses, we omitted them in the original version of the ms. We have now added the results of the significant relationships found among activity and chlorophyll and distance from the coast in the new version. Based on that comment, we detected a change in the relative activity of MCA and MUF substrates in the river and marine environments (a new figure summarizes those results that will be incorporated in the new version. Please see below).



Specific comments

3. *I am wondering if there was a specific reason MUF-glu and MUF-cel were used instead of a nonglucose substrate (like a lipid) to extend the idea of carbon degradation further. Are there any known differences in the hydrolytic activity between single glucose and multiple glucose substrates? This paper almost always lumped the two together and the data is very similar between the two.*

This a very good point to consider, that was incorporated in the new version of the paper. We used MUF-glu, MUF-cel and MCA-leu with the aims of representing organic matter of different reactivity and different models of extracellular enzymes (glucosidase, glucanase, and aminopeptidase, respectively). The coastal ecosystem off central-southern Chile undergoes upwelling of nutrient-rich deep waters during spring and summer, and freshwater from rivers during winter (Sobarzo et al., 2007), which could result in organic matter of different reactivity. Thus, labile marine organic matter was represented as the protein model MCA-leu, and the more refractory pool associated to river input as the cellulose model molecules MUF-glu

and MUF-cel. Considering that the microbial community responds to the variability in the quality of substrates, we used these synthetic molecules to study the spatial and temporal changes in extracellular hydrolysis of this coastal ecosystem.

Carbohydrate polymers as cellulose are complex molecules that require the participation of enzymatic complex to be degraded. In the case of cellulose, the endo and exoglucanases are responsible to cleave internal and terminal sites in the cellulose chain releasing oligosaccharides of different sizes and cellobiose or glucose, respectively and glucosidases acts on disaccharides to release glucose monomers (Lynd et al. 2002, Microbiol Mol Biol Rev 66: 506–577). Our results showing a similar trend in the activity of the enzymes glucanase and glucosidase suggest that aquatic microorganism are able to efficiently degrade cellulose polymers and release glucose available to be used as substrate for heterotrophic microbes.

4. *P1342-L4-11. P1342 L4□11: I would like to see a more detailed description of the enzyme methods. I am assuming the enzyme activity measurements were made immediately after taking the water samples (if that isn't the case, the conditions of the treatment need to be specified). Was anything done to keep the samples at in situ temperature during the incubation?*

A more detailed description is presented in the Method section of the new version. Water samples were collected with Niskin oceanographic bottles, and 1L subsamples were immediately removed and placed in acid-clean carboys until arrival in the laboratory (10 h later). Carboys with water samples were darkened and kept at 4-7^{o}C on board using water baths with icepacks or a refrigerator.

5. *The 5 mL of water used in the assay will almost certainly be a higher temperature after a 6hr incubation on board, which would likely substantially increase the activity. The fluorescence of the standards (and fluorescence in general) is probably also temperature dependent, so a different calibration curve would be needed for each new batch of standard (or refrigeration prior to measurement to adjust everything to a standard temperature). Was anything done to the seawater the standards were made out of, like filtering out the biomass to prevent attenuation? What were the filter settings? Was the water for the standards taken fresh for every sample? Was a new batch of substrate made for each measurement? Dissolved in distilled water or filtered seawater?*

While the incubation system was being prepared back in the laboratory, carboys were kept in a cold chamber at 4-7^{o}C in the darkness. After addition of the substrate, flasks were incubated at ca. 10 ^{o}C under continuous agitation.

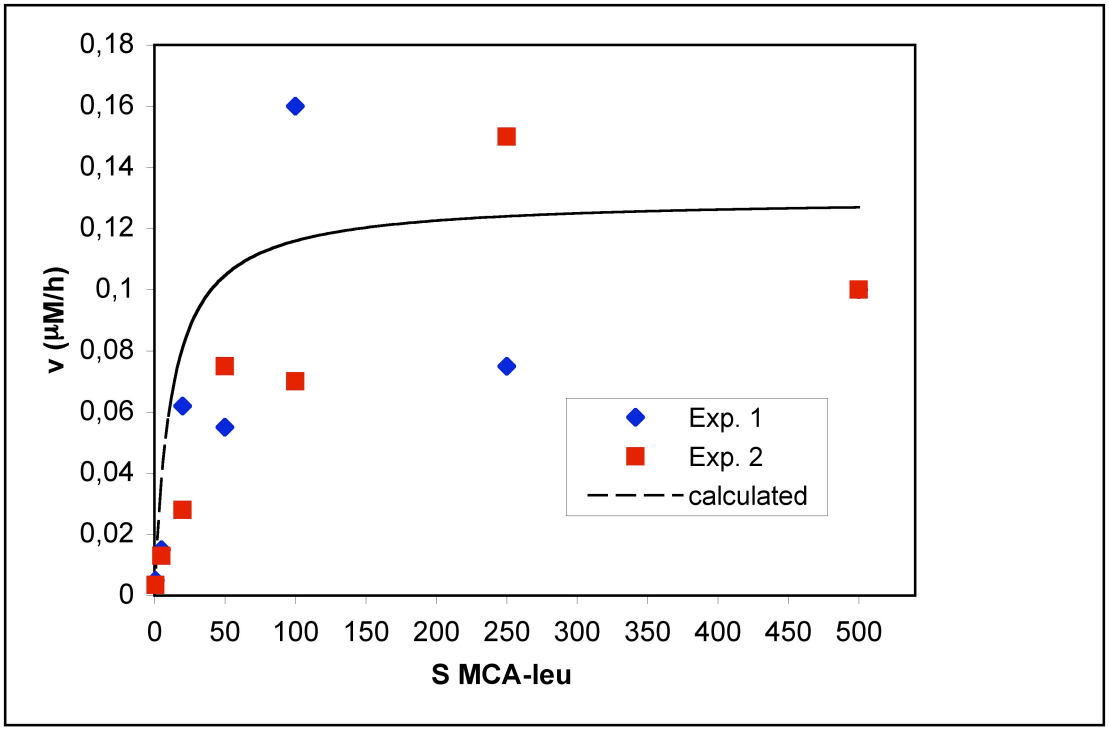
Standard curves were prepared with surface unfiltered seawater for each experiment. A one-point calibration was done at the beginning of the incubation using 0.5 μM MCA and the calibration curve at the end of every experiment with hydrolysis products MCA and MUF at concentrations ranging between 0.03 and 0.5 μM. The largest change in fluorescence before and after the experiment for the 0.5 μM MCA was 2%.

6. *There is no mention of regressing enzyme activity against primary production or chl-a concentration and verifying if there is a relationship and/or if the relationship has seasonal variations. It would increase the strength of your claim for primary productivity driving enzyme activity if such a relationship could be shown or at least discussed. It's also relevant to the carbon budget model, since enzyme activity would then be expected to change in tandem with the phytoplankton population*

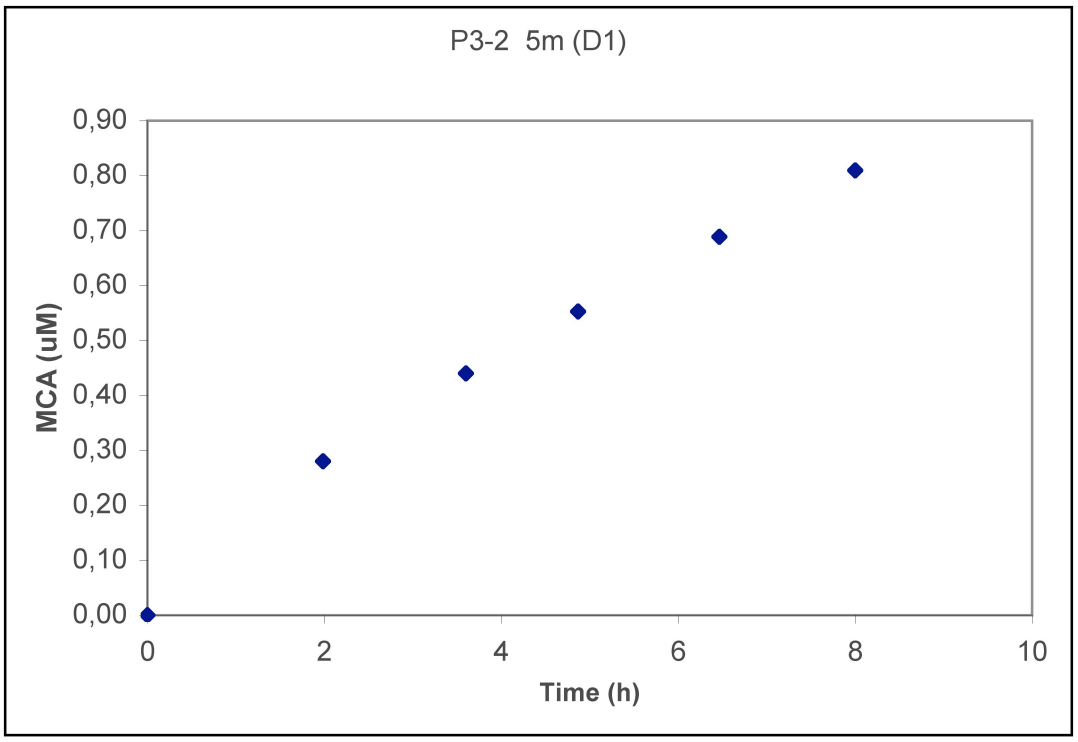
Point taken. Please see response to question #2 above.

7. *p1342 L10-19: 1) Especially for short incubations, the increase in product is usually close to linear; is there sufficient evidence from your incubations that a first-order model is better? Michaelis-Menton kinetics, which most hydrolytic enzymes seem to follow, are equivalent to the first-order model under conditions when [S]<Km, so I can see where that might come from, though it doesn't appear any kinetics were run for this region. For demonstration purposes, using the first-order model and the high peptidase hydrolysis rates (and back-calculating k from the activity and substrate concentration): C=10uM*e^(-0.0182 h-1*6h)=8.97 uM That's a difference of: 10 – 8.97 = 1.03 uM substrate used up over 6 hrs. If we plug the two concentrations into the model at the beginning of the time series, where the difference in substrate concentration has the largest effect: V(10 uM) = k[C0] = 0.0182 h-1 * 10uM = 182 nM h-1 V(8.97 uM) = 0.0182 h-1 * 8.97 uM = 163 nM h-1 If one uses a linear model with the rate given above (182 nM hr-1), then over 6 hrs we expect to see 1.09 uM produced, which is 60 nM more than the decay model predicts and an addition 10 nM per hour(average). That's not a large difference; it would be nice to see an example of the data showing the* curvature, since that's definitely different than the way most people calculate enzyme activity.

You are absolutely right. We explored the kinetics of MCA and MUF substrates at the beginning of our study (the graph below shows the results for the MCA-leucine experiment). As we explained in answer to comment #1 above, we were in the linear zone below K_s (ca. 12 μM for MCA-leucine).

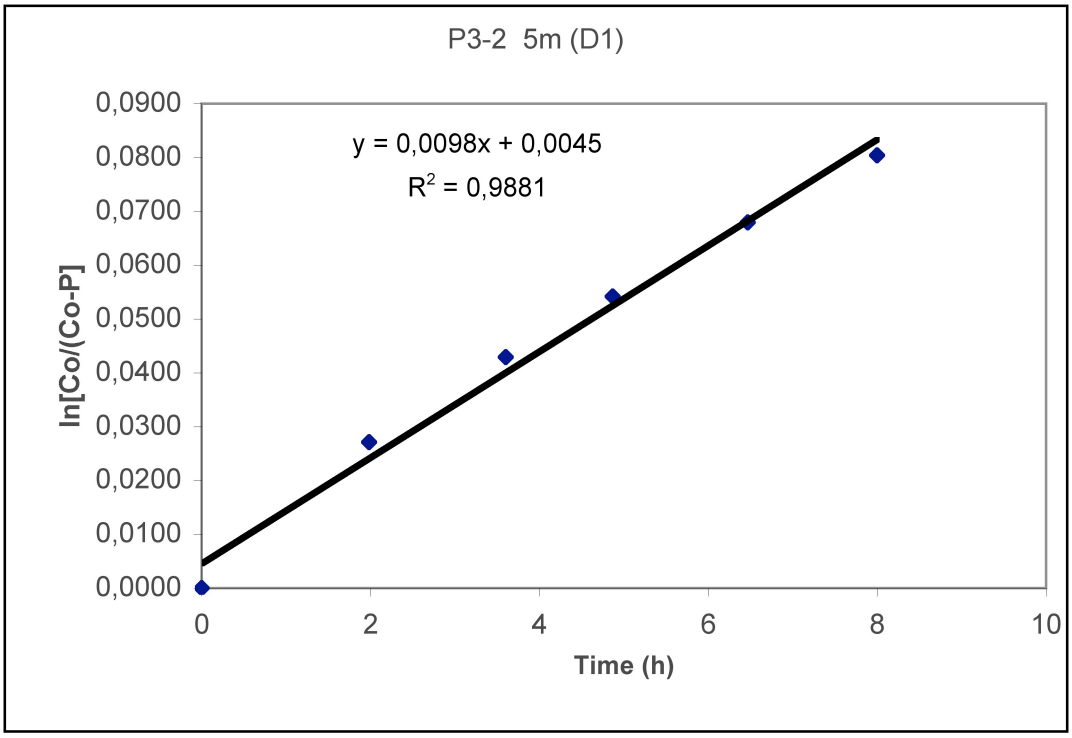


Our time course experiments lasted up to 8-9 hours. The figure below shows the production of MCA in an 8-h incubation experiment (PIMEX cruise #3, 5 m depth, 3 April 2008). Since substrate addition was 10 µM, our rate estimate only considers the initial part of the incubation (less than 10%, Pantoja and Lee 1994, Limnol. Oceanogr. 39, 1718-1726)



8. **p1342 L10-19: The rate constants are stated as being based on the first order equation: k = ln(C0/C0-P)/t, which is equivalent to C0=C*ekt. As written, this has the product exponentially increasing with time, which is probably not correct. From this I am assuming the original equation has C0 and C reversed from their usual positions in a first-order decay equation, and a missing negative sign on the kt term. Since the experiments measure the increase in fluorescent products, the equation would better reflect the increase in product and not the decrease in substrate concentration: P = C0(1-e-kt).*

It can be shown (Pantoja and Lee 1994, Limnol. Oceanogr. 39, 1718-1726) that $P = -C_0 \exp(-kt) + C_0$, where P is the product formed at time t, k is the first-order rate constant, and $C = C_0$ at t= 0. The rate constant k can be extracted from the slope of the plot $\ln [C_0/(C_0-P)]$ vs. time (please graph below).



In this example, using 6 data points for the estimate of the slope (k) gave us 0.0098 h^{-1} (≈ 0.01), similar to the value of 0.01 h^{-1} obtained using the first 4 hours of incubation.

9. *Figure 4, panel A, the datum furthest offshore (far right): There appears to be only 1 data point, but the average is much higher than the data point. In addition, the standard deviation range is much larger than the variability of the blue dot(s) for that point. Both suggest there is some missing data for this station.*

Thanks. We corrected the mistake in Figure 4.

10. *Also, I would rescale the bottom 4 panels; there's no data above 61 nM h-1, so no reason to keep all the data too close together. A short note in the caption could alert readers of the scale change*

Figures were corrected accordingly.

11. *Figure 6: The paper mentions a lack of a trend after the outflow was introduced in enzyme activity. While there isn't much data to really test such a hypothesis, I agree there doesn't seem to be much going on with surface water. However, all of the data points in the surface sediment are higher than all but one data point after the outflow is installed; could that be significant? The outflow is below the surface, at 30 m, which is also below the spring-summer thermocline. Many of the low surface values occur in the spring-summer when the thermocline is in place. Perhaps bacteria or substrate is trapped below preventing a surface signal but*

still present and able to create the sediment signal?

We agree with the observation of the reviewer and the text in the ms was changed to: The comparison of rates of hydrolysis of MCA-leu over time at a site near the outfall pipe did not show any difference in degradation attributable to the effect of the discharge from the secondary treatment of the pulp mill industry in the water column (Fig. 8A). On surface sediments, we observed an increase in extracellular enzymatic activity in the few measurements carried out after the outflow was introduced in this coastal area (Fig. 8B). Considering that the outfall pipe (30m depth) remain below the thermocline when the water column is highly stratified (spring and summer), we cannot rule out any possible effect of the outflow on surface sediments. However our results are preliminary but merit continuous long-term monitoring of the area.

12. *p1349 L22: The substrate concentration was 10 uM for MCA-Leu. However, the measured protein concentration was significantly less at 1-2 uM (and well below enzyme saturation). In both Michaelis-Menten kinetics and the first-order model used here, the initial hydrolysis rates are sensitive to substrate concentration. If we assume the substrate concentration is equal to the measured surface proteins (no free amino acids and all measured amino acids are the only valid substrates), but keeping the same k values (~0.0182 for MCA-Leu), the hydrolysis rate drops from go from 182 nM h-1 (10uM substrate) to 36.4 nM h-1, or an 80% drop. An 80% decrease in the integrated hydrolysis activity in the model puts primary production well ahead of hydrolysis substrate, opposite of the main argument of this section. Even ignoring in situ substrate concentrations, the error bars of primary production for both seasons are sufficient to equal the hydrolysis rates; I don't see how the numbers given in Fig 7 can be interpreted to say they definitively show a substrate deficit.*

You are correct in your interpretation. First of all, we acknowledged the apparent character of our rates (please see response to comment #1 above), and secondly, we changed the conclusion in the new version of the paper.

13. *Contrary to the induction model of these enzymes (expressed in order to scavenge nutrients), your data shows activity related to high substrate concentration. If the river is a major substrate transport pathway, then activity should be higher in regions of freshwater. Is this the case? For instance, if river flow is high in winter (more nutrients) but there is low biomass (no sun), then what does activity do? You would have to restrict the enzyme data to the surface waters within 6km of the shore instead of integrating through the water column like the reported data, but that should give a decent idea on how river flow may directly influence enzyme activity.*

After reading your suggestion, we tested surface hydrolysis data and we didn't detect such a pattern.

All Technical Corrections were incorporated in the new version

Reviewer 2

14. *If the rates are potential rates they don't have any value in constructing a carbon budget and drawing conclusions about heterotrophy vs. autotrophy. Even if the rates could be used in this way, I don't think hydrolysis tells us much about this balance because you do not know the fate of the carbon that is being hydrolyzed, i.e., is it respired or transported offshore or incorporated into biomass that ends up in the sediments? If either of the latter two options occur, you would be using hydrolysis as an indicator of heterotrophy but the organic carbon is not completely remineralized.*

Rates were interpreted as indicator of heterotrophy. But in any case, we agree with the main criticism posed above (that coincides with Reviewer 1), and we removed that conclusion in the new version (please see response to comment #1 of Reviewer 1)

15. *Introduction first paragraph–the gap in knowledge of understanding transfers of carbon between the terrestrial and marine environments is an important one, but I don't feel that this paper resolves the issues very well.*

We are exploring the issue of disappearance of an important fraction of organic carbon in a coastal ecosystem. This is the context for which we are carrying out this research. I agree that we do not solve the problem, but the processing capacity of the microbial community is one of the factors hat is likely involved.

16. *Methods: p1341, line 5. The authors should clarify what nutrient measurements were made spectrophotometrically*

It was nitrate. Now mentioned in the new version

17. *1342, line 5-10–were these concentrations of substrate saturating to each of these enzyme activities? Also, were any time-course measurements made to insure that measurements were made in the linear portion of the activity measurements?*

Please see responses to both questions in #1 and #7 above. We also incorporated more detail on these issues in the method section of the new version.

18. *1342, line 15. As mentioned above, these rates are not 'actual' rates because you can not assume that there was 10 M of substrate for them to work on and you do not know that the model substrates actually behave the same way the in situ substrates do.*

Point taken. Please see response to comment #1 of Reviewer 1. We removed that conclusion in the new version of the ms.

19. 1342; sediment rates–it would be useful to the reader to be able to compare water column and sediment rates, i.e., the pelagic rates could be integrated.

Integrated rates in the water column were compared with those ones of surface sediments. Sediment hydrolysis rates of the sum of MCA and MUF substrates were 0.2 ± 0.1 and 0.2 ± 0.2 g C m^{-2} d^{-1} in winter and spring-summer, respectively. In the water column, integrated rates were higher, 0.7 ± 0.08 and 3.9 ± 0.3 g C m^{-2} d^{-1} in winter and spring-summer, respectively. These rates varied a bit from our former Figure 7 of the original version of the ms since now we included additional data (Please see letter to the Reviewer, and Methods section of the new version).

20. 1348, bottom of page. I agree that organic matter supply may not be the only thing impacting enzyme activity and as I mention above, I don't even think the organic matter supply is the most important one. One question comes to mind that is relevant here is whether these enzymes are inducible or not. I suspect that they are but it should be discussed because it is important to this conclusion.

As it was mentioned in the text, we are certain that organic matter input is not the only factor determining extracellular enzyme activity. Our results are consistent with other microbial processes such as secondary production and respiration that are enhanced by substrate availability during the productive season in this upwelling ecosystem (Quiñones et al. 2010 Carbon and Nutrient Fluxes in Continental Margins, The Humboldt Current System, Springer). In relation to that, we agree with the reviewer in considering that the synthesis of extracellular enzymes may be induced by higher substrate availability during peak photosynthesis.

21. Fig. 2: It's really hard to see much on these figures including the labels. I would suggest putting fewer panels on each page and making them much bigger.

We split Fig. 2 into two as suggested

22. Fig. 5: The legend must be wrong because it is labeled MCA-glu and MUF-glu and the MCA-glu is not discussed in the methods.

Corrected in the new version.