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Interactive comment on "Optimising methodology for determining the effect of ocean acidification on bacterial extracellular enzymes" *by* T. J. Burrell et al.

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Thank you very much for your comments, the following text contains replies to your questions.

General comments "The study suggests two main actions for optimization, i.e. addition of buffer solution to the fluorogenic model substrate in order to stabilize the sample pH and the application of gas-permeable-silicon tubing for introducing CO2 into seawater."

To clarify, the paper suggests four main actions; in addition to the two above we also recommend pH stabilisation of fluorophores, and also determining the effect of pH on enzyme activity over short timescales (hours) (Section 2.4).

"I am not convinced that the results of this study represent an 'optimal procedure', or can be generalized as 'best practice' approach."

The paper describes potential improvements to the methodology for measuring bacterial enzyme activity under different pH, but does not aim to present an optimal procedure or best practice. To clarify this the title of the article has been changed to: "Assessing methodology for determining the effect of ocean acidification on bacterial extracellular enzyme activity".

"The enzyme tests conducted in this study differ largely from previously published studies, and methods often applied for ocean acidification studies, e.g. addition of small volumes of high CO2 seawater, or addition of CO2-supersaturated seawater and addition of bicarbonate were not evaluated."

The enzyme tests do not differ significantly from previously published studies, and some of the ocean acidification techniques assessed in our paper have been used in published enzyme studies (Grossart et al, 2006; Piontek et al, 2010). The aim of our paper was not to assess the impact of all different ocean acidification techniques - the EPOCA Ocean Acidification Best Practice identifies at least 6 different approaches to acidification – but to focus on the techniques that have been used for smaller volume experiments. There are some published papers that compare acidification techniques, but these also do not consider all potential approaches, and primarily focus on phytoplankton (Chen and Durbin 1994; Hurd et al, 2009; Shi et al, 2009; Hoppe et al, 2011) rather than bacteria. The value of our paper is that there have been no previous comparison of the effects of different acidification techniques on bacteria & enzyme activities.

"On the other hand, much of the information and conclusions given in this study have been published before or are well known, so that the amount of new information may be insufficient to justify a standalone publication."

Our paper provides new information that is not published elsewhere. Although exist-

ing literature has highlighted the effect of pH on fluorophore fluorescence (Piontek et al. 2010, 2013; Endres et al. 2013, 2014), these papers do not detail how this was determined or the magnitude of this effect. Similarly, few papers examining the effect of ocean acidification on enzyme activity consider the effect of fluorescent substrate addition on seawater pH (Hoppe 1993). In addition, as mentioned above, there have been no comparisons published on the effects of different acidification techniques on bacteria and enzyme activities. We believe this new information justifies a standalone publication.

General comments: "Was the MUF and MCA calibration done with pH adjusted standard solutions?"

Yes, both MUF and MCA calibration curves were determined at pH 7.8 and 8.1. This has now been clarified in Section 2.4 and 2.5.

"This is how previous studies that investigated ocean acidification effects on enzyme kinetics accounted for the – well known- effect of pH on these chemicals."

As some published results investigating the effect of high CO2 on enzyme kinetics do not mention this (Grossart et al., 2006; Engel et al. 2014), or if considered do not show how it was determined (Piontek et al., 2010; Endres et al., 2013), we feel it is necessary to document this issue.

"Were the tests to evaluate the need of buffering MCA and MUF solutions conducted with seawater?"

No, they were not conducted in seawater. Using 2-methoxyethanol lowered the pH of the stock solution (MUF pH 6.22 and MCA 6.58 at 18.6 °C respectively) and the small volume of seawater would not have been able to buffer this. Consequently, to ensure the fluorophore standards were at the specific treatment pH values, they were buffered in Tris.

"How relevant is the effect of the chemicals on seawater pH and the pH effect on the

fluorophores at the concentration range usually applied for investigations of enzyme kinetics (i.e. concentrations an order of magnitude lower than tested here)?"

This has now been clarified in Section 3.1. We show a significant pH effect on fluorophore fluorescence at both the typical concentration range when buffered with Tris, and also at high concentrations without buffer. Although existing studies mention a pH effect on fluorophore fluorescence, we quantify the scale of this effect, and provide details of how this was determined.

Specific comments:

5845, line 3-5: "Previous acidification studies have accounted for this pH effect by using standard solutions that have the same pH as the acidified seawater sample (e.g. Piontek et al. 2010), or tested the effect of pH on the substrate at applied, and more realistic range of substrate concentration (Endres et al. 2014). Were the substrates in this study calibrated against standard solutions at the respective pH?"

Yes, substrates in this study were calibrated against standard solutions at their respective treatment pH values, as now clarified in Sections 2.4 and 2.5. As we note above, existing research has acknowledged the effect of pH on fluorophore fluorescence, but few quantify the impact or detail how this was tested (only Piontek et al., 2013; Endres et al., 2014). Piontek et al (2010) buffer MUF but do not present data on the impact of pH on MUF-derived activity.

Also, few studies mention the potential effect of substrate addition to seawater pH. Endres et al (2014) identify no significant effect of pH on MCA fluorescence which is contrary to our results. The substrate concentration used in this study was 39 μM, (the 1600 μM previously stated referred to the substrate concentration before addition to the seawater sample). The saturating concentration was used by Maas et al (2013) and is within the range tested by Endres et al. (2013, 2014) (0-150 μM), Piontek et al. (2013) (1-200 μM) and Engel et al. (2014) (1-200 μM).

5845, line 6: "A widely used approach is the addition of small amounts of CO2 saturated seawater to acidify a sample. This approach is missing here."

As previously mentioned, there are a number of methods that can be used to acidify seawater (as reported in the EPOCA Best Practice); our aim wasn't to test all methods, but to compare ones that have been previously used in OA experiments on bacteria and enzymes for small-volume experiments. Most importantly, to the best of our knowledge, there have been no published comparisons of acidification effects on bacteria.

5846, line 4-5: "Was biofilm growth investigated after the incubations? Attached bacteria may have released enzymes as well."

No this was not investigated. Due to the short duration of each incubation ($\leq$ 96 hours), significant biofilm growth was not considered to be an influential factor. The use of permeable tubing in the seawater acidification methodology experiment (Section 3.3), would have resulted in negligible differences in surface area relative to the cubitainer surface area, and would not have increased the surface area for biofilm growth significantly.

5846, line 19-21: "This means that the plate was open and samples + substrate analogues were incubated in the plate reader for 3h? What about outgassing from the wells? Especially in the low pH treatments; was the pH development in the single wells controlled over time?"

Outgassing is a potential issue but was not tested and we are not aware of any published tests. Most published studies leave samples in the plate reader for at least 2 hours (Piontek et al., 2010; Maas et al., 2013; Endres et al., 2013, 2014), and do not adjust pH or prevent outgassing during this period.

5846, line 23-24: "Again, was the pH effect accounted for when calculating enzyme kinetics? If the standard solutions were not set to the applied pH, the calculations would be wrong! Were the enzyme rates given in Fig. 1 and 2 corrected for pH effects?"

Yes, pH was adjusted to the appropriate pH for each sample treatment and so was accounted for when calculating all enzyme kinetics. This has now been clarified in Sections 2.4 and 2.5.

5848: 2.3.1 "The effect of pH on fluorophore fluorescence diluted in two different solutions was investigated, the organic solvent 2-methoxyethanol (Sigma-Aldrich) as well as 0.1M Tris/HCl.' It is unclear if this test was conducted in seawater, or not, as this description only refers to Tris/HCl buffered solutions and methoxyethanol solutions at different pH. Please, clarify. Seawater itself is buffering. Thus, in order to conclude on the necessity to buffer seawater for enzyme kinetics, the tests need to be conducted with seawater."

The fluorophore fluorescence test was not conducted in seawater, as detailed in the previous response above. The aim of the fluorophore experiment was to investigate if fluororphore fluorescence was affected by pH; confirming this lead to fluorophores made up to specific treatment pH values by the use of a buffer. Although seawater is buffering, it would not have been able to buffer the methoxythanol fluorophore solution when added.

5848, line 18: "A substrate concentration of 1600μmol l-1 is much higher than usually applied in marine enzyme studies. Of course the effect of the substrate on the pH will depend of the substrate concentration. What is the effect at realistic ranges? A substrate concentration of 10-200 μmol L-1 is commonly found to be saturating in marine samples (e.g. Hoppe et al., 1983; Baltar et al., 2009; Piontek et al., 2013; Endres et al., 2014)."

The 1600μmol l-1 refers to the substrate concentration prior to seawater addition. The text has now been corrected in Sections 2.2, 2.3.2, and 2.3.3 to clarify that the final substrate concentration was 39 μM, well within the typical range mentioned above.

5849, paragraph 2.3.3. "I am not sure, if I understood this test correctly. Was it tested if addition of a solution with a specific pH would affect a solution with the identical pH?"

Yes, this is correct. As it was shown that Leu-MCA and Arg-MCA substrates acidified seawater when added, the aim of this test was to ensure that Tris buffered substrates maintained the intended pH.

"What if the solution wasn't buffered; would mixing of two identical pH solutions create something different?"

This was not tested; if the solution was not buffered, addition of the MCA substrate would reduce the pH of the sample seawater (as determined in Section 2.3.2).

5849, line 10: "What was the final concentration of the Tris/MCA substrate?"

39 μM in 0.1M Tris.

5850: "Why was the method of addition of CO2 supersaturated water not included in longer incubation test to identify the best method for acidification studies?"

Please see comments above. The aim was not to compare all possible acidification techniques. We tested acidification methods used in other papers (Piontek et al. 2010) and focused on small-volume (eg. < 20 L) short-term (days) experiments, rather than longer (days-weeks) large-volume incubation's.

5850, lines 12-15 and 5856-85858: "The test of seawater acidification methodology is not conclusive. The applied methods do not only differ in the way of acidification, but also differ in the way of water treatment. Thus, in order to differentiate between the acidification effect and the treatment effect itself each treatment needs an appropriate control; i.e. bubbling with ambient air in addition to bubbling with high CO2 in order to differentiate between effect of bubbling and effect of CO2, a silicone tube with ambient seawater pH in addition to the one with high CO2 to account for the increased surface area by the tubes and to identify the true acidification effect."

The aim of the Seawater Acidification Methodology experiment (Section 2.5) was to compare responses between acidification techniques that have been used in ocean acidification experiments. Whereas addition of a bubbling control might have provided

additional information, it was not required as the permeable membrane treatment is effectively the control for the Bubbling treatment. Both treatments lower pH by exposing seawater to gas of the same CO2 composition, with the only difference being that the membrane treatment does not generate mechanical or physical effects. It would be interesting to distinguish the individual effects of bubbling and acidification; however our aim was to determine whether different techniques of adjusting pH to the same level resulted in the same response.

"I don't think that bubbles in seawater can be regarded as an 'artefact'. Imagine a surface ocean without bubbles! Hence, it would be a mistake to generalize the conclusion that bubbling with higher pCO2 air is an inferior methodology compared to the silicone tubing approach (there are no silicone tubes in the ocean)."

Bubbles are a feature of the surface ocean; however in the small volume experiments described here (and used in other published ocean acidification experiments) the relative intensity of bubbling is greater, and the contact between gas bubbles and the water (and its constituents) is considerably more than in the surface ocean. In general, planktonic communities do not experience such continuous bubbling in the surface ocean, apart from perhaps near the surface during storm events. Certain phytoplankton groups are known to be sensitive to high turbulence (Shi et al., 2009; Barton et al., 2014). The permeable tubing provides a method of adjusting pH without mechanical disturbance that may influence the response to a change in pH.

"What this simply shows is the need for a proper control; the effect of high CO2 can also be identified in a bubbled assay when compared to a bubbled assay with ambient air. Otherwise one compares apples and oranges."

As we note it would have been interesting to also compare with air in a bubbling experiment; however the aim of our experiments was to determine whether bubbling influences the response to high CO2. Comparison with the diffusion-based method using permeable tubing suggests this is the case. It is also unclear to us how the reviewer's

suggestion would distinguish the additive or synergistic effects of bubbling (which may enhance cell lysis and release of dissolved organic matter) with acidification, and so the comparison of air and CO2 mix bubbling is not straightforward.

5851, lines 14-16: "Were the cells fixed before freezing, i.e. with GDA?"

No cells were not fixed, methodology followed Hall et al. (2013) and Maas et al. (2013).

5853, paragraph 3.1.: "The results given in this section are difficult to follow and cannot be evaluated. The full dataset is not provided (no figure or table). This is critical since the study concludes that buffering with Tris/HCl is necessary. In the text, it is unclear what results are compared, e.g. non-buffered MUF at 40000 nM with Tris buffered MUF at 200nM?"

Although the non-buffered (1% 2-methoxyethanol) and buffered (0.1M Tris) trials were run at different concentrations, we do not directly compare between them. The aim of the experiment was to identify if pH had an effect on the intensity of fluorescence. This has now been clarified in Section 3.1.

"Also, in the method section it says that MUF fluorescence was determined at 4000, 20000 and 40000 nM, but not at 200nM?"

Fluorophore pH tests (Section 2.3.1) were determined at both high and low concentration ranges. Four point calibration curves were carried out using MUF and MCA fluorophores diluted in 0.1 M Tris/HCl at lower concentrations, according to the previously defined in Section 2.2, including at 200nM. Tris buffer results at 200nM are now available in Table 1.

"However, as far as I understood the pH effect becomes smaller with decreasing MUF and MCA fluorescence. So, is there a real need for optimizing this method under naturally low deliberation of MUF and MCA?"

Our results show that pH has a significant effect at low realistic fluorophore concentrations (MUF 0-200 nM, MCA 0-4000 nM), this has since been clarified in Section

3.1.

"Another factor that also has to be considered is a potential effect of the buffer on microbial activity. Was this tested?"

No this was not investigated. Further reading suggests that some buffers can influence microbial activities, for instance, borate buffers have a bactericide affect (Houlsby et al. 1986). However, our experiments are internally consistent as both controls and pH adjusted treatments received the same amount of Tris buffer, and so any effect would have been equal.

5854: "The authors speculate about enzyme efficiencies and substrate affinities. As km and Vmax should be available from the enzyme kinetics performed, this could be directly tested (rather than speculated)."

Vmax was determined from the slope of the graph using 1st order Michaelis-Menten kinetics. This Vmax is a sample Vmax, as the samples will contain a mixture of enzymes, this is a potential enzyme rate which allows us to compare between samples. We do not calculate Km as we only measure activity at a single, saturating substrate concentration from mixed populations of enzymes.

Interactive comment on Biogeosciences Discuss., 12, 5841, 2015.

Table 1. Mean fluorophore fluorescence at different pH values (n=3, ±SE)

	Concentration (nM)	*Fluorophore*	*pH 8.1*	*pH 7.8*
0.1M Tris	*200*	*MUF*	*1621.44 (±3.43)*	*1373.3*
		MCA	*14948.90 (±2.52)*	*13626.*