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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.

T. J. Burrell et al.

timbo.burrell@gmail.com

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

General and specific comments:

Page 5842 Line 18-20: "This is an incomplete sentence."

This has been addressed in a revised draft.

Page 5843 Line 16: "**ALL** enzymes are pH-sensitive!"

This has been addressed in a revised draft.

Line 17: "In my (and other researchers') opinion, it makes more sense to refer to any enzyme that is exposed to the extracellular medium as 'extracellular', whether or not it is attached to the cell membrane."

This has been addressed in a revised draft.

Page 5844 *Line 11-14: "The authors should delete the text starting with "affecting the polar..." and ending with "...or altered substrate affinity."

This has been addressed in a revised draft.

Line 16: "Again, the reference isn't as relevant as the authors suggest. De Paolis and Kukkonen is about binding of model pollutant compounds (pentachlorophenol and benzo(a)pyrene, both relatively small, aromatic compounds) to humic substances."

Thank you for the clarification, this has been removed and we instead refer to potential changes in enzyme production levels as an additional indirect enzyme response to changes in pH.

Line 16-18: "This statement requires a relevant reference - or, better, should be left out."

This has been addressed in a revised draft.

Page 5845 Line 9-10: "Well, it depends on the system, doesn't it? In an open system, adding acid will drive down DIC as CO2 equilibrates with the atmosphere."

Yes, it does depend on whether the system is open or closed to the atmosphere. This has been clarified in a revised draft.

Line 20: "Probably more accurate to say "respond to changes in carbonate species concentrations" and to put the species in brackets to indicate concentration."

This has been addressed in a revised draft.

Line 24-27: "This is an important point, and the authors have stated it clearly."

Page 5846 Line 15: "What about adjusting the pCO2 of the experimental treatment headspace, as in (for instance, I believe) Ries et al (2009) Geology 37(12): 1131-1134?"

There was no headspace in any treatment. In addition, adjusting pH via the headspace takes longer and/or requires greater mixing than the methods described. This has now been clarified in Section 2.4.

Page 5847Line 13: "Show the data used to determine that 40 uM are "optimal" in the supplementary data."

This concentration has also been used and published in Maas et al. (2013).

Line 23: "I'm not clear on how the authors actually calculated Vmax. There are two ways to do it: either to assume that substrate concentrations are much larger than Km (and that the enzyme kinetics do in fact approximate Michaelis-Menten kinetics, which may not always be true; c.f. Steen and Ziervogel (2012) Soil Biology and biochemistry) in which case one makes the approximation that the observed hydrolysis rate approximates Vmax. This is the most common procedure, although it is not really a calculation. The second method is to use the following equation: Vmax = (v0*(Km+S))/S, where v0 is the observed reaction rate and S is substrate concentration. Ie Hoppe 1993. What did the authors do?"

Vmax was approximated using a high saturating substrate concentration, which has been confirmed to exceed the Km values, and we assume the observed hydrolysis rate approximates Vmax. Therefore enzyme kinetics may not follow the Michaelis-Menten model.

Page 5849 *** "TRIS is a dangerous buffer for peptidase studies, because it contains an amine group which might interfere with peptidases' active sites. To avoid having to redo the entire experiment, the authors should compare the activity of coastal pepti-

dases at identical pH using TRIS and another buffer (borate is an option in seawater at around pH 8). The results of this manuscript can only be trusted if there is no major difference in activity between those two buffers."

Tris may affect enzyme activity (Baker and Prescort, 1983; Desmarais et al., 2002; Saishin et al., 2010) so to investigate any buffer effect tests were carried out using LAP and BG substrate (39 μM final concentration) buffered with a) 0.1M Tris, b) 4-2-hydroxymethyl-1-piperazineethanesulfonic acid (HEPES) and c) 3-(N-morpholino)propanesulfonic acid (MOPS) with pH adjusted to 8.1. Enzyme activity was determined in natural seawater (pH 8.18). There was no non-buffered control at pH 8.1, due to the acidic nature of the aminopeptidase substrate (non-buffered LAP substrate pH was 5.87), and the inability of seawater to effectively buffer this (as identified in Section 3.1).

MOPS was trialled as this has also been used as a buffer in studies of pH effects on enzymes (Piontek et al. 2010), while HEPES is a widely used laboratory buffer with a suitable pH range from 6.8-8.2 and pKa of 7.5 at 25°C. Borate buffers were not trialled because they have a bactericidal effect on microbial activity (Houlsby et al. 1986) and may enhance enzyme activity (Murakami et al., 2001).

Duplicate trials showed that LAP activity with Tris was 15-18 % higher than MOPS, while activity with HEPES was very low. Conversely BG activity in MOPS was significantly greater than with Tris and HEPES (see Table 2 attached).

Comparison of Tris with MOPS and HEPES buffers indicates, contrary to expectations, that Tris does not inhibit LAP activity but may have a minor stimulatory effect. However Tris/HCl buffer was selected for use in our experiments as the difference in LAP activity was < 20 % that of MOPS. The Tris/HCl buffer buffers between the pH range 7.8-9.0, has a pKa of 8.06 at 25°C (Biological Buffers, AppliChem, 2008) and is referred to as an "optimal buffer system" in Hoppe (1993, pg 429). Furthermore, as all treatments in each experiment use the same amount of Tris at each pH, our experiments are

internally consistent.

Page 5853 Section 3.1: "Data from this section should be represented as plots or tables rather than inline in text."

Table 1 has now been added.

Line 5-8: "The syntax here is confusing: it reads as if the authors are comparing pH to fluorescence."

This paragraph has since been rewritten in a revised draft.

Line 21: "The results here probably only apply to the HCl salts, as seen from the lack of change of pH on addition of the sugar substrates."

Yes, we note that these are acid salts on Pg 5853.

Page 5856 Line 1: change to "faster, more"

This had been corrected in the revised draft.

Page 5858 Line 16-20: "This is not a complete sentence."

This had been corrected in the revised draft.

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

Table 2. Effect of different buffers on BG and LAP activity in natural seawat ±SE)

Trial	Enzyme	0.1M Tris pH8.1	0.1M MOPS pH8.1	0.1M HEPES pH
1	BG	5.48 (±0.29)	39.26 (±2.04)	3.26 (±0.21)
2	BG	6.54 (±0.15)	23.88 (±0.63)	3.81 (±0.12)
1	LAP	51.54 (±2.32)	43.42 (±1.43)	0.96 (±0.05)
2	LAP	35.92 (±0.81)	29.34 (±1.08)	0.82 (±0.06)

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.*