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Optimising methodology for determining the effect of ocean acidification on bacterial extracellular enzymes

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Abstract

To fully understand the impact of ocean acidification on biogeochemical cycles, the response of bacterial extracellular enzymes needs to be considered as they play a central role in the degradation and distribution of labile organic matter. This study investigates the methodology, and potential artefacts involved in determining the response of bacterial extracellular glucosidase and protease to ocean acidification. The effect of pH on artificial fluorophores and substrates was examined, as well as the impact of three different acidification methods. The results indicate that pH has a significant effect on the fluorescence of the artificial fluorophore 4-methylumbeliferone for glucosidase activity, and 7-amino-4-methylcoumarin for protease activity, while artificial aminopeptidase substrate alters the pH of seawater, confirming previous observations. Before use in ocean acidification research these enzyme assay components must be buffered in order to stabilise sample pH. Reduction of coastal seawater pH to 7.8 was shown to increase β-glucosidase activity rapidly (0.5 h), while no significant response was detected for leucine aminopeptidase, highlighting the need for short-term direct effects of pH on enzyme activities. Bubbling with CO_2 gas resulted in higher β-glucosidase activity when compared to acidification using gas-permeable silicon tubing and acidification with HCl. Although bubbling showed variable effects between two experiments conducted at different times of the year. In addition, bacterial cell numbers were 15–40 % higher with bubbling relative to seawater acidified with gas-permeable silicon tubing and HCl. Artefacts associated with bubbling may lead to the overestimation of extracellular enzyme activities, and interpretation of the impacts of ocean acidification on organic matter cycling.

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1 Introduction

Global atmospheric and oceanic conditions are changing rapidly relative to the pre-industrial period (IGBP-IOC-SCOR, 2013; IPCC, 2013). Average atmospheric CO_2 concentrations have increased from 280 ppmv in the mid 1700's to 391 ppmv in 2011 (IPCC, 2013), a 40 % increase since pre-industrial times. Atmospheric CO_2 is freely exchanged with the ocean at the ocean/atmosphere interface, and its absorption directly alters the oceans carbonate chemistry and reduces pH (Zeebe and Wolf-Gladrow, 2001). In the last three centuries ocean pH has declined from 8.2 to 8.1, and is predicted to decline to 7.8 by the year 2100, a rate that is unprecedented in the last 55 million years (IGBP-IOC-SCOR, 2013; IPCC, 2013). This decline in ocean pH and the associated change in carbonate chemistry is important because it will significantly impact biological metabolic reactions and influence carbon cycling in the ocean (Endo et al., 2013; Engel et al., 2014; Piontek et al., 2010; Riebesell et al., 2007). For this reason, researchers have investigated the sensitivity of a wide range of biotic and abiotic factors to the carbonate system and pH predicted in the future. Catabolic enzymes are pH sensitive (Tipton and Dixon, 1979; Piontek et al., 2013) and because bacterial extracellular enzymes are by definition free, they are directly susceptible to future changes in seawater pH (Cunha et al., 2010). Bacterial extracellular enzyme activity has been investigated in microbial ocean acidification (OA) studies (reviewed in Cunha et al., 2010) due to the role they play in the degradation of labile high molecular weight organic matter (Azam and Ammerman, 1984; Azam and Cho, 1987; Law, 1980; Münster, 1991) and the vertical flux of carbon to the deep ocean (Piontek et al., 2010; Riebesell and Tortell, 2011; Segschneider and Bendtsen, 2013). Proteins and carbohydrates constitute two of the most common labile organic substrates in the ocean (Benner, 2002; Benner et al., 1992; McCarthy et al., 1996), both of which are essential for cellular growth and repair (Azam et al., 1983; Simon and Azam, 1989). Two groups of extracellular enzymes commonly

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studied for their role in protein and carbohydrate degradation are aminopeptidases and glucosidases respectively.

Current research suggests that bacterial extracellular enzyme activities may increase under future OA conditions (Grossart et al., 2006; Maas et al., 2013; Piontek et al., 2010, 2013; Yague and Estevez, 1988). OA experiments which investigate bacterial extracellular enzyme activity typically examine responses over days to weeks, however enzymes may also respond directly to changes in pH on timescales of hours (Piontek et al., 2013). A direct short-term change in enzyme activity could result from a change in the ionisation state of the enzyme's component amino acids (Dixon, 1953), affecting the polar and non-polar intramolecular attractive and repulsive forces within an enzyme. A reduction in pH may also change the protein amino acid side chains through oxidation (Suzuki et al., 2010) and the exterior three dimensional structure of the enzyme's active sites (Duffy et al., 2002), both of which could lead to inhibition or altered substrate affinity. An indirect enzyme response observed on longer timescales of days may result from changes in intramolecular forces in the organic matter substrate, affecting its structural stability and structure (De Paolis and Kukkonen, 1997). Due to extracellular enzyme specificity, the substrate molecule may no longer physically fit the enzymes active sites, resulting in inhibited or reduced activity. Low pH conditions may also affect phytoplankton and bacterioplankton community composition (Endo et al., 2013; Engel et al., 2008; Riebesell, 2004; Witt et al., 2011), bacterial secondary production and cell numbers (Endres et al., 2014; Maas et al., 2013), and phytoplankton-derived organic exudation (Engel, 2002; Engel et al., 2014), all of which may influence the concentration and composition of high molecular weight organic substrate.

Bacterial extracellular enzyme activity is regularly determined by using artificial fluorogenic substrates. These substrates consist of a fluorescent moiety covalently linked to one or more natural monomer molecules (Arnosti, 2011; Kim and Hoppe, 1984). The molecule is non-fluorescent until it is hydrolysed by an extracellular enzyme, which triggers a fluorescent response, allowing it to be detected and quantified (Hoppe, 1993). The sensitivity of the analytical method to pH has been assessed in terrestrial

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soils (Malcolm, 1983; Niemi and Vepsäläinen, 2005), however limited information is available on how these components respond to a reduction in seawater pH (Piontek et al., 2013) . If pH does have a significant effect on the individual assay components, and this is not corrected, then calculated enzyme kinetics will under or overestimate the true activity rates.

Several methods are commonly used to artificially adjust seawater pH (reviewed in Riebesell et al., 2010). The simplest acidification method involves the addition of a strong acid (typically HCl). The acid decreases the sample pH through the formation of hydronium ions and modifies total alkalinity (TA), but does not alter dissolved inorganic carbon (DIC) (Emerson and Hedges, 2007); consequently although it is relatively simple to adjust pH using acid, the balance of carbonate species is incorrect (Iglesias-Rodriguez et al., 2008; Riebesell et al., 2010). Another common method to acidify seawater is by using CO_2-Air gas mixtures. which alters the seawater carbonate species in ratios predicted to occur from the uptake of atmospheric CO_2 under future scenarios (Gattuso and Lavigne, 2009; Riebesell et al., 2010; Rost et al., 2008; Schulz et al., 2009). A review (Hurd et al., 2009) concluded that the differences in carbonate chemistry arising from different acidification methodologies can influence phytoplankton photosynthesis and growth rates, as well as particulate organic carbon production per cell. Schulz et al. (2009) suggest that biological organisms are likely to respond to changes in carbonate species (e.g. CO_2, HCO_3^- or CO_3^{2-}), rather than changes in overall DIC or TA.

In addition to the method of acidification, the mode of application also needs to be considered. One mode of introducing CO_2-Air gas mixtures into seawater is by bubbling. This method is simple to implement and maintain for extended periods, however, the physical disturbance associated with bubbling CO_2 gas may influence coagulation of organic matter (Engel et al., 2004; Kepkay and Johnson, 1989; Zhou et al., 1998), as well as microbial interactions (Kepkay and Johnson, 1989). Independent header tanks can be used to minimise this, however the source seawater composition may change before introduction into the treatment due to gas-exchange

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and biological processes (Bockmon et al., 2013). An alternative method of introducing CO_2 gas is using gas-permeable silicon tubing (Law et al., 2012; Hoffmann et al., 2013). Although this method of pH adjustment is more time consuming, and provides additional surface area for biofilm attachment and growth, potential physical artefacts of bubbling are removed and realistic future carbonate chemistry is achieved. Previous research has been conducted comparing the effect of acid addition and CO_2 gas bubbling on phytoplankton growth, with no significant effect detected (Chen and Durbin, 1994; Hoppe et al., 2011; Shi et al., 2009). However, to date no research has been carried out directly comparing the bacterial response to seawater acidified with acid and CO_2 gas aeration. In addition there have been few published comparisons of CO_2 gas introduced through gas-permeable silicon tubing with bubbling to determine the most suitable acidification method for OA research. The following experiments were carried out to investigate the effect of pH on the individual components of enzyme assays, identify direct short-term (hour) extracellular enzyme response to changes in pH, and determine the optimal acidification technique.

2 Material and methods

2.1 pH determination

Sample pH was determined using a CX-505 laboratory multifunction meter (Elmetron) equipped with a platinum temperature integrated pH electrode (IJ44C-HT enhanced series; accuracy 0.002 pH units) which was regularly cleaned using potassium chloride reference electrolyte gel (RE45-Ionode). Electrode pH measurements were validated using a pH spectrophotometer with colourmetric determination using a thymol blue dye solution (Law et al., 2012; McGraw et al., 2010). Following recommendations in the European Project on Ocean Acidification (Riebesell et al., 2010), reported pH values in this research reflect the total hydrogen ion scale (pH_T).

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2.2 Extracellular enzyme activity

Two proteases were examined; arginine aminopeptidase activity (AAP) was quantified using L-arginine-7-amido-4-methylcoumarin hydrochloride (Arg-MCA, P212121 LLC, USA), and leucine aminopeptidase activity (LAP) was quantified using L-leucine-7-amido-4-methylcoumarin hydrochloride (Leu-MCA, P212121 LLC, USA). Two glucosidases were also examined; α-glucosidase activity (AG) was quantified using 4-Methylumberlliferyl *a*-D-glucopyranoside (α-MUF, P212121 LLC, USA), and β-glucosidase activity (BG) was quantified using 4-Methylumberlliferyl β-D-glucopyranoside (β-MUF, P212121 LLC, USA). Artificial fluorogenic substrate was added to each seawater sample to give a final substrate assay concentration of 40 µM, which was determined to be the optimum concentration for calculating the maximum velocity of enzyme hydrolysis in these samples (data not shown). A four point calibration curve (0, 4, 40, 200 nM final concentration) was created using 4-methylumbeliferone (MUF) for glucosidase activity, and different calibration curve (0, 40, 400, 4000 nM final concentration) was created using 7-amino-4-methylcoumarin (MCA) for protease activity (Sigma-Aldrich). UltraPure distilled water (Invitrogen^{TM}, Life Technologies) was used as a sample blank. Each sample was assayed in triplicate using a single 96-microwell flat bottom black assay plate (Nunc A/S). A separate enzyme assay was performed for glucosidase and protease activity. Each assay plate was read at 5 min intervals for a minimum of 3 h using a Modulus microplate reader (Turner Biosystems) at 365 nm excitation and 460 nm emission wavelength. Incubated assay temperature matched the seawater temperature at the sampling site. The maximum enzyme rate (V_{max}, nmolL^{-1} h^{-1}) at substrate saturation (Rudolph and Fromm, 1979) was calculated using Michaelis–Menten kinetics (Tipton and Dixon, 1979). Triplicate V_{max} determinations were averaged per sample. Cell-specific rates were calculated by dividing the activity per litre by bacterial cell numbers per litre.

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2.3 Enzyme assays

The following assay tests were carried out using surface seawater collected from the south coast of Wellington, New Zealand (41°20′53.0″ S, 174°45′54.0″ E).

2.3.1 The effect of pH on fluorophore fluorescence

The effect of pH on fluorophore fluorescence diluted in two different solutions was investigated, the organic solvent 2-methoxyethanol (Sigma-Aldrich) as well as 0.1 M Tris/HCl. The pH of MUF and MCA fluorophore working standard (200 µM) diluted in 1 % 2-methoxyethanol (Sigma-Aldrich) was first recorded (pH 6.22 and 6.58 at 18.6 °C respectively) and used as controls. Each fluorophore was then diluted to 4000, 20000 and 40000 nM at four pH values (8.2, 8.1, 7.9 and 7.8) in triplicate by addition of 0.1 N aqueous NaOH. MUF and MCA fluorophores made to working standards using 0.1 M Tris/HCl were prepared at pH 8.1 and 7.8 only. Fluorophore pH tests diluted in 0.1 M Tris/HCl was carried at lower concentrations according to the four point calibration curves previously defined.

2.3.2 The effect of artificial fluorogenic substrate on seawater pH

Individual seawater samples were adjusted to pH 7.95 and 7.70 using 0.1 M HCl. All four artificial fluorogenic substrates previously described were made up to working standards of 1600 µM using 1 % 2-methoxyethanol (Sigma-Aldrich). A time-zero reference pH was recorded for each seawater sample then, following the addition of each substrate at working standard concentration, sample pH was recorded immediately, and after 30 min. Each artificial fluorogenic substrate was run in triplicate at each pH value, and compared to controls without substrate addition at both pH levels.

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2.3.3 Buffering artificial substrates

Duplicate trials were undertaken to determine if 0.1 M Tris/HCl could successfully buffer MCA substrate at working standard concentrations (1600 µM) when added to seawater of similar pH. Tris/HCl was used as a buffer as it has been used successfully with artificial fluorescent substrates in aquatic microbiology studies (Hoppe, 1993). Tris buffered Leu-MCA and Arg-MCA substrate working standards were made by diluting 500 µL of MCA substrate stock (16 mM) with 4 mL of 0.1 M Tris/HCl buffer. Duplicate Tris/MCA substrate solutions were adjusted to pH 8.1 and 7.8 by adding 10 % HCl and the pH of duplicate 10 mL aliquots of coastal seawater were also adjusted to pH 8.1 and 7.8. For each pH treatment, 250 µL of Tris/MCA substrate solution was added to 10 mL of seawater fixed at the corresponding pH. pH was recorded at room temperature using a pH electrode as described above.

2.4 Short-term acidification experiment

To investigate whether pH has a direct short-term effect on bacterial extracellular enzyme activity over a timescale of hours, coastal seawater was first filtered through a 15 µm filter and then a 1 µm inline cartridge filter. Two acidified treatments were created and incubated in acid-washed milli-Q water-rinsed 4.3 L low-density polyethylene (LDPE) cubitainers (ThermoFisher Scientific). The first treatment was acidified to pH 7.8 by adding 4.6 mL of 0.1 M HCl, and was referred to as the acid treatment. The second treatment, referred to as the CO_2 treatment was acidified by adding 120 mL of CO_2 saturated seawater (pH 5.97) to 4 L of ambient coastal seawater (pH 8.05) to achieve a final pH of 7.77. The CO_2 saturated seawater (pH 5.97) was produced by bubbling 10 % CO_2 gas (in 20.8 % O_2 in N_2, BOC Gas Ltd) into 500 mL of ambient seawater. After an initial 30 min equilibration period, BG and LAP activity were determined in both acidified treatments every 2.5 h for a total of 24 h, and compared to an ambient coastal seawater control in triplicate. DIC and alkalinity were also sampled

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3 h after acidification to confirm carbonate chemistry changes. Short-term temporal changes in pH were not monitored.

2.5 Seawater acidification methodology

The influence of acidification technique on selected biotic parameters was investigated in longer incubations (days), in two separate experiments conducted under controlled temperature conditions in late summer (May 2013 – trial 1) and in early spring (October 2013 – trial 2). Coastal seawater was collected and filtered as described above. Three different methods were used to acidify seawater to that predicted by the end of the century (pH 7.80) (IPCC, 2013), including acid addition using 0.1 M HCl (A), bubbling CO_2-Air gas mixture through an acid-washed aquarium airstone (B), and CO_2-Air gas mixture introduced through gas-permeable silicon tubing (Tygon Tubing R-3603; ID 1.6 mm; OD 3.2 mm; Connect 2 Control Ltd) (P). Each acidification treatment and an ambient seawater control were incubated in triplicate in the 4.3 L cubitainers as described above. No further artificial pH adjustment took place over the 96 h incubation.

Treatment P was acidified through the sequential application of 100 % synthetically produced CO_2 gas for 25 min, and 10 % CO_2 gas (in 20.8 % O_2 in N_2, BOC Gas Ltd) for 60 min at a flow rate of < 26 mL min^{-1}. The initial use of pure and 10 % CO_2 gas made it possible to reach the target pH 7.80 within 3 h. Treatment B was acidified by direct bubbling of 742 µatm CO_2 gas (in 20.95 % O_2 in N_2, BOC Gas Ltd) for 143 min at < 25 mL min^{-1} to reach the target pH 7.80. The volume of 0.1 M HCl required to acidify treatment A to pH 7.80 (2.0 mL – trial 1, 3.1 mL – trial 2) was calculated based on the sample volume, DIC and alkalinity (K. Currie, personal communication, NIWA/University of Otago, 2013) using an algorithm from (Dickson et al., 2007). To ensure a consistent rate of pH change, treatments B and A were adjusted to match that of the slower treatment P (150 min), with each sample pH verified using a pH electrode.

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Each cubitainer was housed in one of two identical perspex incubation chambers (1730 mm long, 450 mm high by 325 mm deep), set at in situ ambient seawater temperature (15.1 °C – trial 1, 15.5 °C – trial 2). Artificial photosynthetic light (700–900 $\mu E\,m^{-2}\,s^{-1}$) was maintained in each cubitainer through external fluorescent light banks (Philips TLD 36 W/840), neutral density polycarbonate screening (The Light Site Ltd) ensured light intensities were uniform between incubation chambers, while adjustable timers ensured an automated diurnal 12 h light/dark cycle. Mixing of water in the cubitainers was achieved using an inflating diaphragm positioned underneath each cubitainer, with the inflation and collapse of the diaphragm under the weight of the sample resulting in continual water displacement and mixing. Cubitainers were manually removed and inverted three times prior to each sampling. Time-zero sampling occurred after initial pH adjustment.

2.5.1 Bacteria and picoplankton cell numbers

Triplicate samples were collected in 2 mL Cryovials (Raylab Ltd) and frozen in liquid nitrogen (Hall et al., 2004) for up to 12 weeks prior to analysis. Bacterial cell numbers were determined by flow cytometry (FACSCalibur, Becton-Dickinson) following staining with SybrGreenII (Invitrogen) (Lebaron et al., 1998), and count events were normalised to volume using TruCount bead solution (BD Biosciences) (Button and Robertson, 1993). Total eukaryotic picoplankton numbers (< 2 µm) were determined by fluorescence of chlorophyll (wavelength 670 nm), phycoerythrin (585 nm), and phycourobilin (530 nm) as well as forward light-scatter providing an estimate of cell size. Final count values were recorded as cells mL^{-1}.

2.5.2 Bacterial secondary production

Potential bacterial secondary production (BSP) was measured using ^{3}H-leucine (^{3}H-Leu) of high specific activity (> 80 Ci $mmol^{-1}$, SciMed Ltd) in triplicate 1.7 mL samples. Following the TCA precipitation and centrifugation methodology (Kirchman,

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2001; Smith and Azam, 1992), ^{3}H-Leu incorporation was determined using a liquid scintillation counter (Tri-Carb 2910 TR) and converted to secondary production using a protein conversion factor (1.5 kg C mol^{-1} leucine) (Simon and Azam, 1989). Cell-specific rates were calculated by dividing the BSP rate by total bacterial cell numbers.

2.5.3 Dissolved inorganic carbon and alkalinity

Pre-combusted 12 mL sample DIC vials (Labco Ltd) were triple rinsed with sample seawater and filled, ensuring no air bubbles. One drop of saturated $HgCl_2$ was added to each DIC sample, with storage at room temperature. DIC was determined using evolved CO_2 gas after sample acidification on a Marianda AIRICA system, the accuracy of this method was estimated to be $\pm 5\,\mu mol\,kg^{-1}$, as determined by analysis of Certified Reference Material. Alkalinity samples were collected by filling a 1 L screw top bottle, and following the same sample preparation and storage procedures as DIC above. Samples were later analysed by potentiometric titration in a closed cell (Dickson et al., 2007) with an accuracy of $\pm 2\,\mu mol\,kg^{-1}$, as determined by analysis of Certified Reference Material.

2.6 Statistical analysis

Statistica v.10 (StatSoft Inc., USA) was used for basic graphics and descriptive statistics. Data was tested for normality and equality of variance prior to statistical analysis. Due to the small sample size at each sampling point, these assumptions were infrequently met and data was $\log(x+1)$ transformed. Standard hypothesis formulations were used for each ANOVA, the null hypothesis (H_0) was $\mu = 0$. The significance level of each test was $p \leq 0.05$. If H_0 was rejected, a Tukey's HSD post-hoc analysis test was run to identify individual variable responses.

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3 Results and discussion

3.1 Enzyme assay methodology optimisation

In both MUF and MCA working standard tests in 1 % 2-methoxyethanol (Sigma-Aldrich) and 0.1 M Tris, fluorescence declined in response to acidification, similar to that observed in soils (Niemi and Vepsäläinen, 2005). The non-adjusted pH in the 2-methoxyethanol solution was 6.22, however the MUF fluorescence at 40000 nM was twelve times higher at pH 7.8 (205493 RFU, $n = 3$) (t test, $p < 0.05$). The MUF fluorescence at pH 8.1 (269647 RFU, $n = 3$) was also 20 % higher than fluorescence at pH 7.8 (t test, $p < 0.05$). In contrast, MUF fluorescence made up in Tris at 200 nM was only 3.2 % higher at pH 8.1 (1604.24 RFU, $n = 3$) than fluorescence at pH 7.8 (1553.18 RFU, $n = 3$) and not statistically different (t test, $p > 0.05$). The MCA fluorescence made in 2-methoxyethanol at 40 000 nM was 25 % higher at pH 8.2 (126 265 RFU, $n = 3$) than fluorescence at pH 7.8 (94 030 RFU, $n = 3$) (t test, $p < 0.05$). While MCA fluorescence made up in Tris at 200 nM was 1.7 % higher at pH 8.1 (13 653.69 RFU, $n = 3$) than fluorescence at pH 7.8 (13 420.72 RFU, $n = 3$) and not statistically different (t test, $p > 0.05$). Because the intensity of fluorescence varies when made in 2-methoxyethanol, the fluorophores require buffering to accurately measure fluorescence at different pH levels.

Immediately following the addition of non-buffered Leu-MCA or Arg-MCA substrate to seawater at pH 7.95 or 7.70, pH decreased by at least 0.05 units for each substrate and remained significantly lower 30 min after addition when compared to time-zero pH (one-way ANOVA, $p < 0.05$). As both MCA substrates are hydrochloride salts, addition at working dilution resulted in a significant pH change, as previously reported by Hoppe (1993). Following tests of Tris buffered MCA substrate solutions adjusted to seawater pH 7.8 and 8.1, pH change ranged from 0.003 to 0.03 units (± 0.001 SE). As the addition of buffer solution minimised the pH change, both MCA substrates and fluorophores were subsequently produced using 0.1 M Tris/HCl, with pH adjusted to the respective experimental treatments and control. In contrast to MCA substrate, no

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statistically significant change in pH was recorded immediately following, or 30 min after the addition of either α-MUF or β-MUF substrate to sample pH 7.95 or 7.70 relative to respective controls, indicating that these are neutral compounds. However, to eliminate any possible bias, MUF substrates were also buffered using Tris/HCl.

3.2 Short-term acidification experiment

Bacterial extracellular enzymes are known to have different pH optima, and so a change in pH relative to the enzymes optimal pH may result in a decline or increase in activity (Orsi and Tipton, 1979; Tipton and Dixon, 1979). Disentangling a direct short-term effect of OA on the enzyme from indirect effects associated with biological responses can be challenging (Yoshimura et al., 2013). In the 24 h experiment using Tris/HCl buffered assay reagents, BG activity was four times higher in both the acid treatment and CO_2 treatment relative to the control at 0.5 h (one-way ANOVA, $p < 0.01$) (Fig. 1), indicating a short-term pH effect. BG activity continued to increase in the CO_2 treatment relative to the acid treatment and control to 5.5 h, however this response was not maintained beyond 6 h (Fig. 1). BG activity in the acid treatment was significantly higher relative to the control at 5.5 and 8 h (one-way ANOVA, $p < 0.05$) (Fig. 1).

This immediate but short lived BG response could reflect feedback inhibition (Berg et al., 2002; Boethling, 1975). As the amount of low molecular weight product accumulates, further enzyme synthesis is not required and BG activity declines. Alternatively, the initial increase in BG activity in both acidified treatments may have exhausted available labile substrate, the subsequent decline in activity may reflect the hydrolysis of semi-labile substrate. The short-term increase in BG activity could also reflect a change in the charge of key residues in the enzymes' active sites, potentially altering their tertiary and quaternary structure and increasing substrate attraction and active site accessibility (Dixon, 1953). Sample alkalinity was significantly lower in the acid treatment (2166 µmol kg^{-1}) when compared to the control at 3 h (2282 µmol kg^{-1}, one-way ANOVA, $p < 0.01$) and the CO_2 treatment (2282 µmol kg^{-1}, one-way ANOVA, $p < 0.01$). While DIC was significantly higher in the CO_2 treatment

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(2152 µmol kg^{-1}) when compared to the control (2067 µmol kg^{-1}, one-way ANOVA, $p < 0.01$) and acid treatment (2066 µmol kg^{-1}, one-way ANOVA, $p < 0.01$). These changes were expected from carbonate system thermodynamic considerations and confirmed using a pCO_2 amendment spreadsheet based on an algorithm (Dickson et al., 2007). The discrepancy in carbonate system parameters from pH adjustment by acid and CO_2 gas also confirm those reported by others (Hurd et al., 2009; Riebesell et al., 2010). The elevated BG activity in the CO_2 treatment relative to the acid treatment indicates a potential methodological artefact that may result from differences in seawater carbonate chemistry and its effect on bacterial cell membrane permeability (Jacobs, 1940). In contrast, LAP activity was not significantly different from the control in either acidified treatment at 0.5 h, with no clear acidification treatment response detected up to 24 h (Fig. 1). Why a similar response was not observed for LAP activity is unknown, but may result from differences in protease size (Zhang, 2000) and their subsequent cellular release.

Using the same pH adjusted fluorogenic substrates, Piontek et al. (2013) also detected a short-term (6 h) enzyme response to acidification, cell-specific BG and LAP activity increased by a factor of 2.1 and 1.8 respectively at pH 7.79. The results suggest that pH 7.8 is closer to the optima of BG; while the current results suggest LAP may have a broader pH optimum and may be less sensitive to the changes in pH predicted for the end of the century. Importantly, soil enzymology findings support these observations and show that optimal aminopeptidase activity typically ranges from pH 7.5 or higher, while optimal glucosidase activity occurs at lower pH values (pH 5.5) (Niemi and Vepsäläinen, 2005). The contrasting short-term LAP pH response detected by Piontek et al. (2013) may reflect the presence of different proteases in a different coastal ecosystem. The short-term increase in BG in response to increased H^+ concentrations could have resulted from a change in the protein tertiary and quaternary structure, as these are dependent on charge-charge interactions (Applebury and Coleman, 1969). A change in H^+ concentration may also directly alter the charge of key residues in the enzymes' active sites, potentially increasing substrate attraction

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and active site accessibility (Dixon, 1953). This response would result in faster more efficient substrate transformation and turnover.

3.3 Seawater acidification methodology

Having established the immediate response of BG activity to acidification, there is a need to consider longer-term indirect effects, as well as establish the correct method of acidification. Overall, these experiments showed significant treatment effects on BG and LAP activity (Fig. 2), while AG and AAP activity was variable, with no significant treatment response observed relative to the control (data not shown). In trial 1, cell-specific BG activity was at least an order of magnitude higher in treatment B, P and A relative to the control at time-zero (one-way ANOVA, $p < 0.05$) (Fig. 2), which is consistent with the short-term acidification experiment in showing a strong direct effect of decreased pH on BG activity (Fig. 1). BG activity was highest in treatment B from 24 to 72 h by at least 14 % relative to treatment A and P (Fig. 2). In contrast to trial 1, cell-specific BG activity increased significantly throughout trial 2 (repeated measures ANOVA, $p < 0.05$). This opposing temporal trend may signify seasonal differences in the response of BG to OA, potentially reflecting differences in microbial community composition or substrate availability (Morris and Foster, 1971). Despite the different activity trend, there was no significant difference in BG activity between treatments at time-zero in trial 2 (one-way ANOVA, $p > 0.05$) (Fig. 2). BG activity was again highest in treatment B from 48 h, with activity at least 18 % higher relative to treatment P and A (Fig. 2). Although cell-specific LAP activity provides evidence of a response to acidification, there was no significant response observed in trial 1 or 2 (Fig. 2).

Although seawater in treatment B was only bubbled with CO_2-Air gas mixtures for the pre-incubation period (143 min), this had a greater effect on BG activity than the other treatments, indicating that there are artefacts associated with bubbling. The elevated BG activity relative to the control and other treatments may reflect the physical effect of bubbling. Time-zero picoplankton cell numbers were low in both trials (trial 1 – 6×10^3 cells mL^{-1}, trial 2 – 2×10^3 cells mL^{-1}), physical bubbling may have

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ruptured picoplankton cells or increased their susceptibility to viral lysing, leading to an increase in the release of labile organic carbohydrates. This is potentially supported by the decline in total eukaryotic picoplankton cell numbers in treatment B in both trials (repeated measures ANOVA, $p < 0.01$) (data not shown). An increase in enzyme activity would theoretically increase the availability of low molecular weight organic substrate for bacterial assimilation, which may explain the significant increase in bacterial cell numbers in treatment B relative to the control at 96 h in both trials (one-way ANOVA, $p < 0.05$) (Fig. 3).

An increase in bacterial abundance in response to bubbling was also reported by Kepkay and Johnson (1989) who suggested that surface DOC coagulation facilitated by bubbling resulted in increased respiration and bacterial numbers. It is possible that bubbling increased the abiotic coagulation of organic matter (Riley, 1963) and formation of high molecular weight substrate, which could explain the increase in cell-specific BG activity (Fig. 2).

Each acidification treatment had a significant negative effect on cell-specific BSP from 24 to 48 h in trial 1 (one-way ANOVA, $p < 0.05$) (Fig. 4). During trial 2, cell-specific BSP was significantly lower in treatments B and P when compared to the control from 72 to 96 h (one-way ANOVA, $p < 0.05$), while BSP was twice as high in treatment A during this period (Fig. 4).

Although a clear treatment response was not observed in either trial, the low cell-specific BSP in treatment B relative to the control and treatment A at 96 h in trial 2 was surprising as enzyme activity and bacterial cell numbers were higher. Because this same response was not observed in trial 1, it is possible that additional indirect factors such as bacterial community composition or substrate type may also affect BSP under OA conditions (Piontek et al., 2013).

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4 Conclusions

Artifical fluorogenic substrates have been used to investigate bacterial extracellular enzyme activities in aquatic environments for several decades (Hoppe, 1983; Somville and Billen, 1983). Although the technique has several limitations including the possibility that the artificial fluorogenic substrate does not fully represent the naturally occurring substrate (Chrost, 1989), or that the observed activity only represents potential hydrolysis (Arnosti, 1996; Unanue et al., 1999); the technique is rapid, cheap, easily completed in the field and most importantly, allows for a standardised method for comparison of results in different OA studies. Using artificial fluorogenic compounds, this study has confirmed that key chemical components used to determine extracellular enzyme activity are affected by, or alter seawater pH, and buffering is therefore required when used in OA research. We determined that seawater acidification caused an immediate short-term stimulation of β-glucosidase activity, although this was not sustained beyond 6 h, and not detected in protease activity. The results suggest indirect effects arising from OA also influence β-glucosidase activity, but that different methodological approaches can influence the magnitude of this response. Because acid addition does not produce realistic seawater carbonate chemistry predicted in a future ocean (Riebesell et al., 2010), and bubbling with CO_2 gas was shown to have a significant effect on β-glucosidase activity and bacterial cell numbers, indicating that there are artefacts associated with bubbling. The results suggest that the least disruptive technique to investigate the response of ocean carbon cycling to future OA conditions is CO_2-Air gas mixtures introduced with gas permeable-silicon tubing. In order to accurately investigate the effects of OA on marine organisms and ecosystems around the world, researchers must establish robust and standardised protocols (Riebesell et al., 2010), only then can large scale meta-analyses and international research collaborations be achieved, furthering our understanding of the changing ocean.

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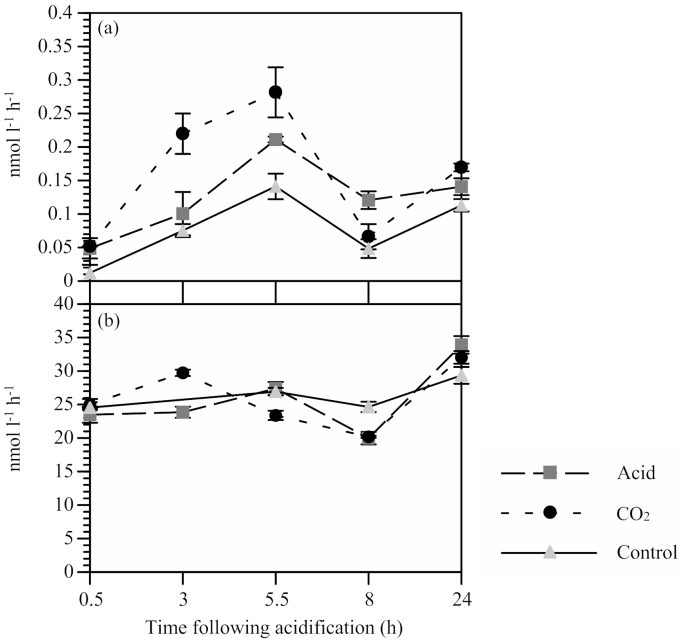

Figure 1. The short-term response of **(a)** BG and **(b)** LAP (mean ± SE, $n = 3$) to seawater acidified to pH 7.8 using 0.1 M HCl (Acid treatment) and CO_2 saturated seawater (CO_2 treatment), each compared to a non-acidified control at pH 8.05.

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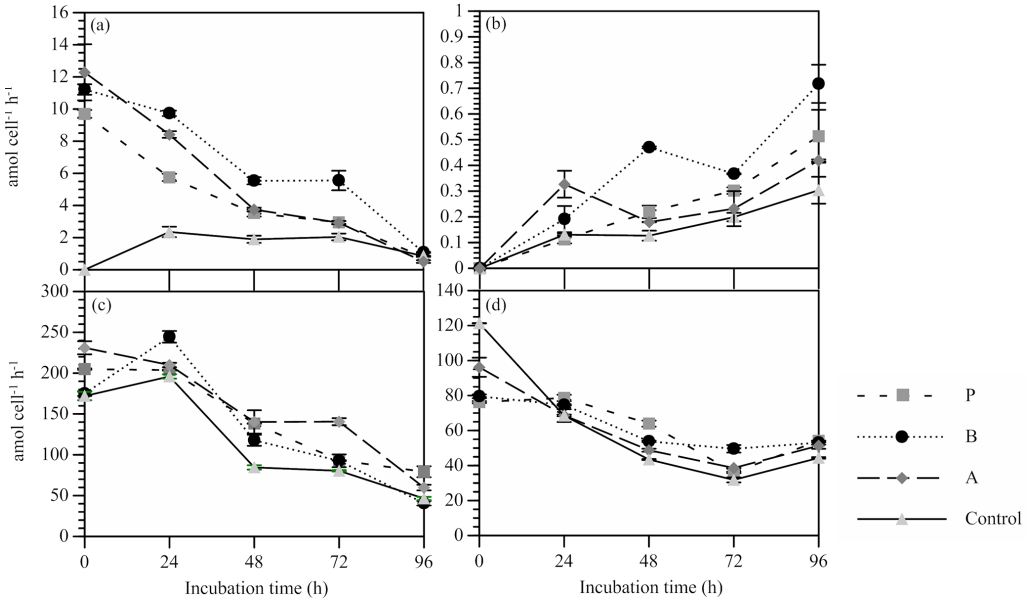

Figure 2. Cell-specific extracellular enzyme activity (mean ± SE, n = 3) in response to seawater acidified with 0.1 M HCl (A), bubbled with CO_2-Air gas mixture (B) and CO_2-Air gas mixture introduced through gas-permeable silicon tubing (P). **(a)** BG activity in trial 1, **(b)** BG activity in trial 2, **(c)** LAP activity in trial 1, **(d)** LAP activity in trial 2.

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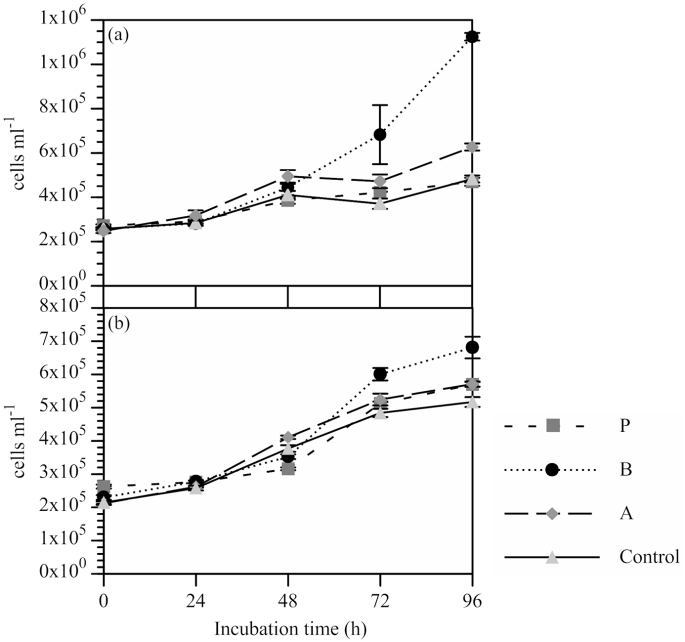

Figure 3. Bacterial cell numbers (mean ± SE, $n = 3$) in response to seawater acidified with 0.1 M HCl **(A)**, bubbled with CO_2-Air gas mixture **(B)** and CO_2-Air gas mixture introduced through gas-permeable silicon tubing (P). **(a)** trial 1, **(b)** trial 2.

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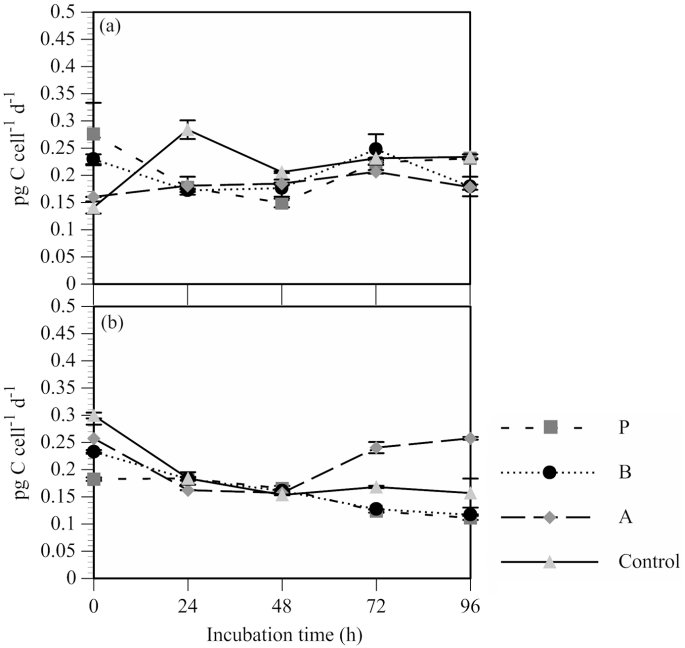

Figure 4. Cell-specific bacterial secondary production (mean ± SE, $n = 3$) in response to seawater acidified with 0.1 M HCl **(A)**, bubbled with CO_2-Air gas mixture **(B)** and CO_2-Air gas mixture introduced through gas-permeable silicon tubing (P). **(a)** trial 1, **(b)** trial 2.

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