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Assessing approaches to determine the effect of ocean acidification on bacterial processes

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Abstract

Bacterial extracellular enzymes play a significant role in the degradation of labile organic matter and nutrient availability in the open ocean. Although bacterial production and extracellular enzymes may be affected by ocean acidification, few studies to date have considered the methodology used to measure enzyme activity and bacterial processes. This study investigated the potential artefacts in determining the response of bacterial extracellular glucosidase and aminopeptidase to ocean acidification, and the relative effects of three different acidification techniques. Tests confirmed that the fluorescence of the artificial fluorophores was affected by pH, and that addition of MCA fluorescent substrate alters seawater pH. In experiments testing different acidification methods, bubbling with CO_2 gas mixtures resulted in higher β-glucosidase activity relative to acidification by their introduction via gas-permeable silicon tubing, or by acid addition (HCl). In addition, bacterial numbers were 15–40 % higher with bubbling relative to seawater acidified with gas-permeable silicon tubing and HCl. Bubbling may lead to overestimation of carbohydrate degradation and bacterial abundance, and consequently incorrect interpretation of the impacts of ocean acidification on organic matter cycling.

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1 Introduction

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 studied for their role in protein and carbohydrate degradation are aminopeptidases and glucosidases, respectively. Enzyme activity is sensitive to different environmental factors, and consequently degradation of proteins and carbohydrates will vary accordingly. Most enzymes are pH sensitive and have different pH optima (Tipton and Dixon, 1979, Piontek et al., 2013), and consequently a change in ocean pH may result in a decline or increase in activity of extracellular enzymes as these are directly exposed to the external seawater pH (Orsi and Tipton, 1979; Tipton and Dixon, 1979). Atmospheric CO_2 has increased by 40 % since the 18th century (IGBP-IOC-SCOR, 2013; IPCC, 2013), which is of concern as CO_2 freely exchanges with the ocean and directly alters ocean carbonate chemistry and pH. As a result ocean pH has declined from 8.2 to 8.1, with a continued decline to 7.8 predicted by the year 2100. This decline in ocean pH and the associated change in carbonate chemistry, referred to as ocean acidification (OA), will significantly impact 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 future changes in ocean pH and the carbonate system.

Bacterial extracellular enzyme activity has been investigated in OA studies (reviewed in Cunha et al., 2010) due to the important 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). 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). This may result from the direct effect of pH on the ionisation state of the enzyme's component amino acids (Dixon, 1953), or from indirect influences on longer timescales (Boominadhan et al., 2009). The latter may be arise in response to changes in the concentration and composition of high molecular weight organic substrate due to the effect of pH on phytoplankton and bacterioplankton community composition (Endo et al., 2013; Engel et al., 2008; Riebesell, 2004; Witt et al.,

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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).

Bacterial extracellular enzyme activity is regularly determined 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 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 (Cornwall and Hurd, 2015; 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) in a closed system (Emerson and Hedges, 2008); consequently although it is relatively simple to adjust pH using acid, the balance of carbonate species does not reflect the changes that will occur in response to increased CO_2 uptake unless corrected for by the addition of a base (Iglesias-Rodriguez et al., 2008; Riebesell et al., 2010). Another method for acidifying seawater is the use of CO_2-Air gas mixtures, which alter 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). Schulz et al. (2009) suggest that microbial 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. A review by Hurd et al. (2009) concluded that differences in carbonate chemistry arising from the use of different acidification methodologies can influence phytoplankton photosynthesis and growth rates, as well as particulate organic carbon production per cell, and so it is important to ensure changes in all carbonate system species reflect that projected from an increase in CO_2 (Cornwall and Hurd, 2015).

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In addition to the method of acidification, the mode of application also needs to be considered. A commonly used method 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; Mopper et al., 1995; Passow, 2012; Schuster and Herndl, 1995; Zhou et al., 1998), as well as microbial interactions (Kepkay and Johnson, 1989). This mechanical disturbance may be particularly exacerbated when bubbling is used in small-volume incubations at the laboratory/microcosm experimental scale (<20 litres). An alternative method of introducing CO_2 gas is by using gas-permeable tubing (Law et al., 2012; Hoffmann et al., 2013), which eliminates physical artefacts associated with bubbling whilst achieving realistic future carbonate chemistry. 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 comparison of the bacterial response to seawater acidified with acid and CO_2 gas aeration has been carried out. In addition, there are no published comparisons of CO_2 gas introduced through gas-permeable silicon tubing with bubbling to assess their suitability for OA research. Consequently the aims of the following study were two-fold; to determine the effect of pH on the sensitivity of fluorogenic substrates used bacterial enzyme analysis, and also to compare the response of bacterial processes to different approaches of acidification in small-volume incubations.

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), calibrated using Tris buffers (Cornwall and Hurd, 2015) and regularly cleaned using potassium chloride reference electrolyte gel (RE45-Ionode). Electrode pH measurements were validated using a pH spectrophotometer with colorimetric 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), pH values in this research reflect the total hydrogen ion scale (pH_T).

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122 **2.2 Extracellular enzyme activity**

123 The activity of two proteases was examined, with arginine aminopeptidase activity (AAP)
124 quantified using L-arginine-7-amido-4-methylcoumarin hydrochloride (Arg-MCA), and
125 leucine aminopeptidase activity (LAP) quantified using L-leucine-7-amido-4-methylcoumarin
126 hydrochloride (Leu-MCA). Two glucosidases were also examined; α-glucosidase activity
127 (AG) was quantified using 4-Methylumberlliferyl *α*-D-glucopyranoside (α-MUF), and *β*-
128 glucosidase activity (BG) was quantified using 4-Methylumberlliferyl *β*-D-glucopyranoside
129 (*β*-MUF, all from P212121 LLC, USA). Artificial fluorogenic substrate was added to each
130 seawater sample to give a final substrate assay concentration of 39 µM, which was determined
131 from independent tests to be the optimum concentration for calculating the maximum velocity
132 of enzyme hydrolysis in seawater samples (data not shown). A four point calibration curve (0,
133 4, 40, 200 nM final concentration) was created using 4-methylumbeliferone (MUF) for
134 glucosidase activity, with a separate calibration curve (0, 40, 400, 4000 nM final concentration)
135 created using 7-amino-4-methylcoumarin (MCA) for protease activity (Sigma-Aldrich).
136 UltraPure distilled water (Invitrogen™, Life Technologies) was used as a sample blank. Each
137 sample was assayed in triplicate using a single 96-microwell flat bottom black assay plate
138 (Nunc A/S), with a separate enzyme assay performed for glucosidase and protease activity.
139 Each assay plate was read at 5 min intervals for a minimum of 3 h using a Modulus microplate
140 reader (Turner Biosystems) at 365 nm excitation and 460 nm emission wavelength as in Burrell
141 et al., (2015). Incubation assay temperature matched the seawater temperature at the sampling
142 site. The potential for outgassing and associated increase in sample pH during the 3 h enzyme
143 assay was not tested. The maximum potential enzyme rate (V_{max}, nmol l^{-1} h^{-1}) was
144 approximated from the saturating substrate concentration of 39 µM. Triplicate V_{max}
145 approximations were averaged per sample. Cell-specific rates were calculated by dividing the
146 activity per litre by bacterial cell numbers per litre. The assay tests were carried out using
147 surface seawater collected from the south coast of Wellington, New Zealand (41°20'53.0"S,
148 174°45'54.0"E).

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2.3 Enzyme assays

2.3.1 The effect of pH on fluorophore fluorescence

The effect of pH on fluorophore fluorescence was investigated at both typical (Hoppe, 1983) and elevated fluorophore concentrations using two different buffer solutions, the organic solvent 2-methoxyethanol (Sigma-Aldrich) and 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). Each fluorophore was then diluted to 4000, 20000 and 40000 nM (referred to as high concentrations) at four pH values (8.2, 8.1, 7.9 and 7.8) in triplicate by addition of 0.1 N aqueous NaOH. The MUF and MCA fluorophore working standards made up in in 0.1 M Tris/HCl were prepared at pH 8.1 and 7.8 only, and also carried out at lower concentrations (MUF: 4, 40, 200 nM; MCA: 40, 400, 4000 nM).

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 using 1 % 2-methoxyethanol (Sigma-Aldrich). A time-zero reference pH was recorded for each seawater sample and, following the addition of each substrate at 39 µM final concentration, sample pH was recorded immediately and after 30 min. Each artificial fluorogenic substrate was run in triplicate at both pH values, and compared to controls without substrate addition at both pH levels.

2.3.3 Buffering artificial substrates

Duplicate trials were undertaken to determine if 0.1 M Tris/HCl could successfully buffer MCA substrate at the working concentration (39 µM) when added to seawater of similar pH. Tris buffer contains an amine group which can affect peptidase activity (Baker and Prescort, 1983; Desmarais et al., 2002; Saishin et al., 2010), and so tests were carried out to compare the impact of different buffers. LAP activity was compared in seawater using LAP substrate (39 µM final concentration) buffered with 0.1 M Tris/HCl or 3-(N-morpholino)propanesulfonic acid (MOPS) with pH adjusted to 8.1. Enzyme activity was also determined in seawater (pH 8.18). A non-buffered LAP substrate addition was not included due to the acidic nature of the aminopeptidase substrate (non-buffered LAP substrate was pH 5.87). MOPS has been used as a buffer in studies of the effects of pH on enzymes (Piontek et al. 2010), and so was an

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appropriate comparison. Borate buffers were not trialled because they have a bactericidal effect on microbial activity (Houlsby et al., 1986). In two separate test experiments using coastal seawater Tris/HCl buffer did not inhibit LAP activity relative to MOPS but instead showed a minor stimulatory effect with 16-18% higher LAP activity (data not shown). Tris/HCl was selected for subsequent use as its optimal buffer range is pH 7.8-9.0, making it ideal for OA incubations, and it has a pKa of 8.06, so is appropriate for artificial fluorescent substrates (Hoppe, 1993).

Following the above tests, the following methodology was used for the seawater acidification tests. Tris buffered Leu-MCA and Arg-MCA substrate working standards were made by diluting 500 µl of MCA substrate stock (16 mM) with 4.5 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 was 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 Seawater acidification approach

The influence of acidification technique on biotic parameters was investigated 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 first filtered through a 15 µm filter and then a 1 µm inline cartridge filter. Three different methods were used to acidify seawater to that predicted by the end of the century (pH 7.80) (IPCC, 2013): (A) acid addition using 0.1 M HCl; (B) bubbling CO_2-Air gas mixture through an acid-washed aquarium airstone, and (P) CO_2-Air gas mixture introduced through gas-permeable silicon tubing (Tygon Tubing R-3603; ID 1.6 mm; OD 3.2 mm; Law et al, 2012). Treatment P was acidified to a pH of 7.8 by the sequential application of 100 % synthetically produced CO_2 gas for 25 min, followed by 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 within 3 h. Treatment B was acidified by bubbling seawater with 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 achieve the target pH 7.80. The volume of 0.1 M HCl required to acidify treatment A to pH 7.8 (2.0 ml - trial 1, 3.1 ml - trial

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2) was calculated based on the sample volume, DIC and alkalinity (pers. comm. Dr K. Currie, NIWA/University of Otago) using an algorithm from Dickson et al. (2007). To ensure a consistent rate of pH change across treatments, treatment B and A were adjusted to match that of the slower treatment P (150 min), with the pH of each sample verified using a pH electrode. Each treatment and an ambient seawater Control were then incubated in triplicate in acid-washed milli-Q water-rinsed 4.3 Litre low-density polyethylene (LDPE) cubitainers (ThermoFisher Scientific), without a headspace. No further pH adjustment took place during the 96 h incubation.

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 light (700 - 900 µE m^{-2} s^{-1}) was maintained in each cubitainer through external fluorescent light banks (Philips TLD 36 W/840); neutral density polycarbonate screening ensured light intensities were uniform between incubation chambers, while adjustable timers ensured an automated diurnal 12 h light/dark cycle. Mixing of water in each cubitainer 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 mixing. Cubitainers were also manually removed and inverted three times prior to each sampling. Time-zero sampling occurred after initial pH adjustment. Assay fluorophore and substrate standard solutions were adjusted to treatment pH.

2.4.1 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}.

2.4.2 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

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TCA precipitation and centrifugation methodology (Kirchman, 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.

2.4.3 Dissolved Inorganic Carbon and Total 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 ±5 µmol kg^{-1}, as determined by analysis of Certified Reference Material. Alkalinity samples were collected by filling a 1 liter 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 ±2 µmol kg^{-1}, also determined by analysis of Certified Reference Material.

2.5 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. Data was log(x+1) transformed due to the small sample size at each sampling point. Standard hypothesis formulations were used for each Analysis of Variance (ANOVA), the null hypothesis (H_o) was $\mu = 0$. The significance level of each test was $p \leq 0.05$. If H_o was rejected, a Tukey's HSD post-hoc analysis test was run to identify individual variable responses.

3 Results and discussion

3.1 Enzyme assay methodology

MUF and MCA fluorescence was lower at pH 7.8 relative to pH 8.1, as previously reported in soils (Niemi and Vepsäläinen, 2005). The fluorescence of the unbuffered MUF 2-methoxyethanol at 40000 nM was 20 % higher at pH 8.1 than at pH 7.8 (t-test, $p < 0.05$), while MUF Tris buffered fluorescence at 200 nM was 15 % higher at pH 8.1 (t-test, $p < 0.05$; Table 1). MCA 2-methoxyethanol fluorescence at 40000 nM was 4 % higher at pH 8.1 than

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fluorescence at pH 7.8 (t-test, $p < 0.05$), while MCA Tris buffered fluorescence at 200 nM was 9 % higher at pH 8.1 than at pH 7.8 (t-test, $p < 0.05$; Table 1). These results confirm that pH has a significant effect on MUF and MCA fluorescence at both high and typical working concentrations, and so fluorophore calibrations should be carried out at the same pH as the sample.

Although there is awareness of the effect of pH on fluorophore fluorescence (Piontek et al., 2013; Endres et al., 2014), few studies consider the effect of fluorescent substrate addition on seawater pH. Due to the basicity of the MCA amino group, fluorescence intensity is less affected by pH and it has been suggested that buffering is not required (Piontek et al., 2013; Endres et al., 2014), whereas buffering of MUF has been reported (Piontek et al., 2010; 2013, Endres et al., 2013). 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 mins after addition when compared to time-zero pH (one-way ANOVA, $p < 0.05$). As both MCA substrates are hydrochloride salts, addition resulted in a significant pH change, as previously reported by Hoppe (1993). In 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 reduced 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, no statistically significant change in pH was recorded immediately following, or 30 mins after, addition of either α-MUF or β-MUF substrate to seawater at pH 7.95 or 7.70, indicating that these are neutral compounds. However, to eliminate possible bias, MUF substrates were also buffered using Tris/HCl.

3.2 Seawater acidification

Having established that the analytical procedures for determining extracellular enzyme activity are affected by, and alter pH, the influence of acidification technique was then considered in two separate trials in different seasons. Overall, the experiments showed that different acidification techniques had significant effects on BG and LAP activity in both trials (Fig. 1), while the response of AG and AAP activity was variable with no consistent treatment response relative to the Control (data not shown). Overall, BG and AG activity declined from time-zero

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to 96 hrs in the Control and treatments in trial 1, but were both significantly higher in the treatments relative to the Control from time-zero to 72 h, with BG activity approximately three-fold higher than AG activity (data not shown). 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 a direct effect of acidification (Piontek et al., 2013). Cell-specific BG activity was highest in treatment B from 24 h to 72 h by at least 14 % relative to treatment A and P (Fig. 1). In contrast to trial 1, cell-specific BG activity increased significantly throughout trial 2 (repeated measures ANOVA, $p < 0.05$). The opposing temporal trends between trials may signify seasonal differences in the response of glucosidase to OA, potentially reflecting differences in microbial community composition (Endo et al., 2013) or substrate availability (Morris and Foster, 1971). There was no significant difference in BG activity between treatments at time-zero in trial 2 (one-way ANOVA, $p > 0.05$) (Fig. 2), and BG activity was again highest in treatment B from 48 h, with activity at least 18 % higher relative to treatment P and A (Fig. 1). Bulk water LAP and AAP activity varied between treatments for trials 1 and 2. For example, both LAP and AAP activity were highest in treatment P throughout trial 1, whereas LAP activity was highest in treatment B from 72 h to 96 h in trial 2 (data not shown). Although cell-specific LAP activity showed evidence of a response to acidification, this was not significant in either trial (Fig. 1).

Although treatment B was only bubbled with gas mixtures for the pre-incubation period (143 mins), this had a greater effect on BG activity than in the other treatments, indicating potential artefacts associated with bubbling. Bubbling may have ruptured picoplankton cells or increased their susceptibility to viral lysis, 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 (trial 1 – 2.8×10^3 to 2.6×10^3 cells ml^{-1}, trial 2 – 1.7×10^3 to 1.3×10^3 cells ml^{-1}) in both trials (repeated measures ANOVA, $p < 0.01$). An increase in enzyme activity would theoretically increase the availability of low molecular weight organic substrate for bacterial assimilation, and 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. 2). An increase in bacterial abundance in response to bubbling has been previously 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

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334 molecular weight substrate such as transparent exopolymer particles (Mopper et al., 1995;
335 Passow, 2012; Schuster and Herndl, 1995; Zhou et al., 1998), which could explain the elevated
336 cell-specific BG activity (Fig. 1).

337 All acidification treatments had a significant negative effect on cell-specific BSP from 24 h to
338 48 h in trial 1 (one-way ANOVA, $p < 0.05$) (Fig. 3). During trial 2, cell-specific BSP was
339 significantly lower in treatments B and P when compared to the Control from 72 h to 96 h (one-
340 way ANOVA, $p < 0.05$), while BSP was twice as high in treatment A during this period (Fig.
341 3). Although a clear treatment response was not observed in either trial, the low cell-specific
342 BSP in treatment B relative to the Control and treatment A at 96 h in trial 2 was surprising as
343 enzyme activity and bacterial cell numbers were elevated. Existing literature also reports
344 variable BSP responses to acidified conditions. Arnosti et al., (2011) and Teira et al., (2012)
345 detected no significant BSP response, while Grossart et al., (2006) detected an increase, and
346 Maas et al., (2013) and Siu et al., (2014) recorded a decrease in BSP rates with increasing CO_2.
347 As the same response was not observed in trial 1, it is possible that additional indirect factors
348 such as bacterial community composition or substrate type may have influenced BSP under
349 OA conditions (Piontek et al., 2013).

350

351 ## 4 Conclusions

352 Artificial fluorogenic substrates have been used to investigate bacterial extracellular enzyme
353 activities in aquatic environments for decades (Hoppe, 1983; Somville and Billen, 1983).
354 Although the technique has several limitations, including that the artificial fluorogenic
355 substrate may not represent the naturally occurring substrate (Chróst, 1989), and so the
356 observed activity only represents potential hydrolysis (Arnosti, 1996; Unanue et al., 1999), the
357 technique is rapid and easily applied in the field and most importantly, allows for a standardised
358 method for comparison of results in different OA studies. This study confirmed that artificial
359 fluorogenic substrates used to determine extracellular enzyme activity are affected by, or alter,
360 pH, and so buffering is required particularly when used in OA research. Seawater acidification
361 stimulated β-glucosidase activity, but different methodological approaches can influence the
362 magnitude of this response. Simple acid addition does not produce realistic seawater carbonate
363 chemistry predicted in a future ocean (Riebesell et al., 2010), and bubbling with CO_2 gas has a
364 significant effect on β-glucosidase activity and bacterial cell numbers, indicating that there are

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365 artefacts associated with bubbling. It should be noted that these effects were observed in small-
366 volume laboratory-scale experiments, and may have less impact in larger-scale experiments.
367 Nevertheless, the results indicate that the most robust technique to investigate the response of
368 bacterial processes to future OA conditions is CO_2-Air gas mixtures introduced using gas
369 permeable-silicon tubing. This approach should be considered for broader use in standardised
370 protocols for ocean acidification (Riebesell et al., 2010; Cornwall and Hurd, 2015) to achieve
371 robust meta-analyses and international inter-comparisons.

372

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610 **Figure Legends**

611 Table 1. Mean fluorophore fluorescence at pH 8.1 ad 7.8 (RFU, n=3, ±SE).

	Concentration (nM)	*Fluorophore*	*pH 8.1*	*pH 7.8*
0.1M Tris	*200*	*MUF*	*1621.44 (±3.43)*	*1373.33 (±2.49)*
		MCA	*14948.90 (±2.52)*	*13626.54 (±2.52)*

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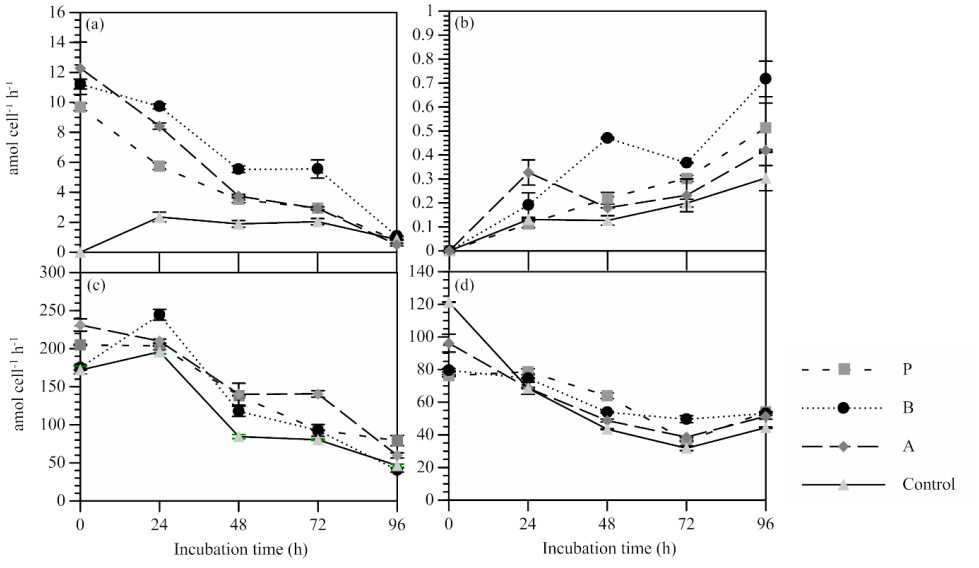

613

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

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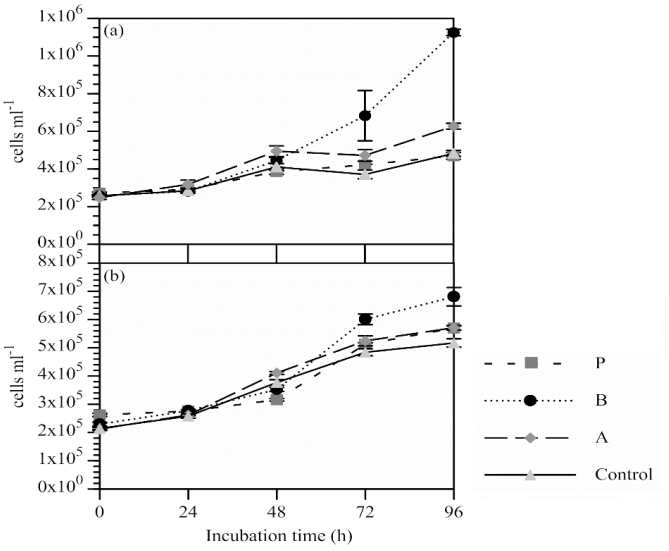
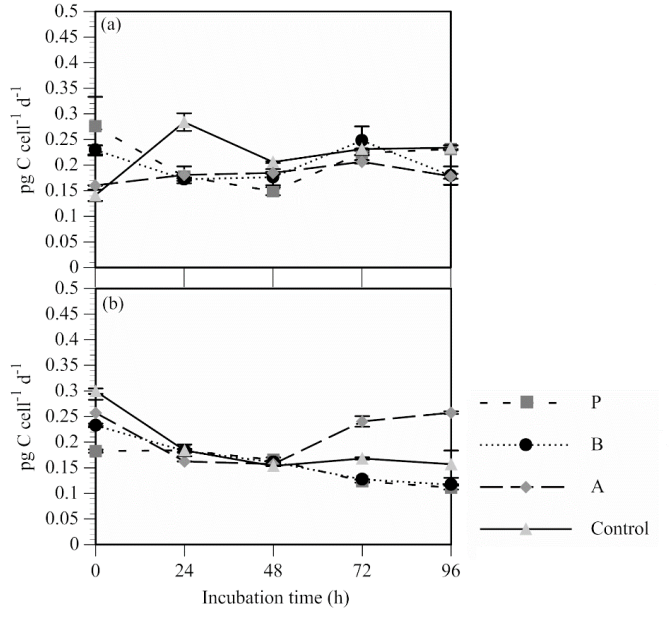


621

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



625

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