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 growth and extracellular glucosidase and aminopeptidase activity to ocean acidification, and the relative effects of three different acidification techniques. Tests confirmed that the observed effect of pH on fluorescence of artificial fluorophores, and the influence of the MCA fluorescent substrate on seawater sample pH, were both overcome by the use of Tris buffer. In experiments testing different acidification methods, bubbling with CO_2 gas mixtures resulted in higher β-glucosidase activity and 15–40 % higher bacterial abundance, relative to acidification via gas-permeable silicon tubing and acid addition (HCl). Bubbling may stimulate carbohydrate degradation and bacterial growth, leading to the incorrect interpretation of the impacts of ocean acidification on organic matter cycling.

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). Labile substrate availability is limited by bacterial enzyme-driven hydrolysis of high molecular weight organic material (Azam and Cho, 1987; Munster, 1991). Two groups of bacterial extracellular enzymes (attached or released into surrounding water) commonly studied for their role in protein and carbohydrate degradation are aminopeptidases and glucosidases, respectively. The activity of individual enzymes are responsive to changes in environmental factors, and so overall glucosidase and peptidase activities will have different pH optima (Tipton and Dixon, 1979; Piontek et al., 2013). 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 18^{th} 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 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 potentially altering enzyme production (Boominadhan et al., 2009). Examples of the latter include 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., 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 different methods have been used to artificially adjust seawater pH in experimental systems (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).

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 difference 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 mixtures 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 identify any artefacts associated with the use of fluorogenic substrates in extracellular enzyme analysis, and also to compare the response of bacterial processes to different methods 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

123 determination using a thymol blue dye solution (Law et al., 2012; McGraw et al., 2010).
124 Following recommendations in the European Project on Ocean Acidification (Riebesell et al.,
125 2010), pH values in this research reflect the total hydrogen ion scale (pH_T).

126

127 **2.2 Extracellular enzyme activity**

128 The activity of two proteases was examined, with arginine aminopeptidase activity (AAP)
129 quantified using L-arginine-7-amido-4-methylcoumarin hydrochloride (Arg-MCA), and
130 leucine aminopeptidase activity (LAP) quantified using L-leucine-7-amido-4-
131 methylcoumarin hydrochloride (Leu-MCA). Two glucosidases were also examined; α-
132 glucosidase activity (AG) was quantified using 4-Methylumberlliferyl *α*-D-glucopyranoside
133 (α-MUF), and *β*-glucosidase activity (BG) was quantified using 4-Methylumberlliferyl *β*-D-
134 glucopyranoside (*β*-MUF, all from P212121 LLC, USA). Artificial fluorogenic substrate was
135 added to each seawater sample to give a final substrate assay concentration of 39 µM, which
136 was determined from independent tests to be the optimum concentration for calculating the
137 maximum velocity of enzyme hydrolysis in seawater samples (data not shown). A four point
138 calibration curve (0, 4, 40, 200 nM final concentration) was created at both pH 7.8 and 8.1
139 using 4-methylumbeliferone (MUF) for glucosidase activity, with a separate calibration curve
140 (0, 40, 400, 4000 nM final concentration) created using 7-amino-4-methylcoumarin (MCA)
141 for protease activity (Sigma-Aldrich). UltraPure distilled water (Invitrogen™, Life
142 Technologies) was used as a sample blank. Each sample was assayed in triplicate using a
143 single 96-microwell flat bottom black assay plate (Nunc A/S), with a separate enzyme assay
144 performed for glucosidase and protease activity. Each assay plate was read at 5 min intervals
145 for a minimum of 3 h using a Modulus microplate reader (Turner Biosystems) at 365 nm
146 excitation and 460 nm emission wavelength as in Burrell et al., (2015). Incubation assay
147 temperature matched the seawater temperature at the sampling site. The potential for
148 outgassing and associated increase in sample pH during the 3 h enzyme assay was not tested.
149 The maximum potential enzyme rate (V_{max}, nmol l^{-1} h^{-1}) was approximated from the
150 saturating substrate concentration of 39 µM. Triplicate V_{max} approximations were averaged
151 per sample. Cell-specific rates were calculated by dividing the activity per litre by bacterial
152 cell numbers per litre. The assay tests were carried out using surface seawater collected from
153 the south coast of Wellington, New Zealand (41°20’53.0”S, 174°45’54.0”E).

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 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 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 (supplementary material S1). 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).

Based on the buffer trials, 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 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. pH was monitored throughout each 96 h incubation (supplementary material S2 and S3), however no further pH adjustment took place.

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 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 litre 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 3.2 % higher at pH 8.1 (t-test, $p > 0.05$; Table 1). MCA 2-methoxyethanol fluorescence at 40000 nM was 25% higher at pH 8.1 than fluorescence at pH 7.8 (t-test, $p < 0.05$), while MCA Tris buffered fluorescence at 200 nM was 1.7 % higher at pH 8.1 than at pH 7.8 (t-test, $p > 0.05$; Table 1). 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 in seawater (Piontek et al., 2013; Endres et al., 2014), whereas buffering of MUF has been reported (Piontek et al., 2010; 2013, Endres et al., 2013). Our results confirm that pH has a significant effect on unbuffered MUF and MCA fluorescence and that 0.1 M Tris buffer minimises any pH effect at typical working concentration.

Although there is awareness of the effect of pH on fluorophore fluorescence (Mead et al., 1955; Piontek et al., 2013; Endres et al., 2014), few studies consider the effect of fluorescent substrate addition on seawater pH. 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 adding Tris buffered MCA substrate solutions adjusted to pH 7.8 and 8.1 to seawater at the same pH, the resulting 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 that of 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.

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 at select time points in both trials (Fig. 1), while the response of AG and AAP activity was variable with no consistent treatment response relative to the Control (supplementary material S4, S5, and S6). Overall, BG and AG activity declined from time-zero 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 at select time points, there was no consistent significant response throughout 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 x 10^3 to 2.6 x 10^3 cells ml^{-1}, trial 2 – 1.7 x 10^3 to 1.3 x 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 molecular weight substrate such as transparent exopolymer particles (Mopper et al., 1995; Passow, 2012; Schuster and Herndl, 1995; Zhou et al., 1998), which could explain the elevated cell-specific BG activity (Fig. 1).

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

4 Conclusions

Artificial fluorogenic substrates have been used to investigate bacterial extracellular enzyme activities in aquatic environments for decades (Hoppe, 1983; Somville and Billen, 1983). Although the technique has several limitations, including that the artificial fluorogenic

substrate may not represent the naturally occurring substrate (Chróst, 1989), so that the observed activity only represents potential hydrolysis (Arnosti, 1996; Unanue et al., 1999), the technique is rapid and easily applied in the field and most importantly, allows for a standardised method for comparison of results in different OA studies. This study confirmed that specific artificial fluorogenic substrates used to determine extracellular enzyme activity can alter sample pH, and consequently that buffering is required, particularly when used in OA research. Seawater acidification stimulated β-glucosidase activity as previously reported (Piontek et al., 2010; Burrell et al., 2015), but the use of different methodological approaches may generate variable results. Acid addition does not produce realistic seawater carbonate chemistry predicted in a future ocean (Riebesell et al., 2010), and bubbling with CO_2 gas has a significant effect on β-glucosidase activity and bacterial cell numbers, indicating artefacts associated with bubbling. It should be noted that these effects were observed in small-volume laboratory-scale experiments (< 10 litres), and may have less impact in larger-scale experiments. Although not all techniques previously used to artificially adjust seawater pH were trialled (Riebesell et al., 2010), the results presented here indicate that introducing CO_2-air gas mixtures using gas permeable-silicon tubing is an effective technique for investigating the response of bacterial processes to future OA conditions, that appears superior to alternatives methods. This approach should be considered for broader use in standardised protocols for ocean acidification (Riebesell et al., 2010; Cornwall and Hurd, 2015) to achieve robust meta-analyses and international inter-comparisons.

Acknowledgements

This research was supported by a Marsden Fund Award from New Zealand Government funding, administered by the Royal Society of New Zealand to E. W. Maas and C. S. Law. We acknowledge assistance from Kim Currie, Debbie Hulston, Marieke van Kooten, Cara Mackle and Karen Thompson. We also thank John van der Sman for seawater supplied by the Victoria University Coastal Ecology Laboratory, Wellington.

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Figure Legends

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

	Concentration (nM)	Fluorophore	pH 8.1	pH 7.8
0.1M Tris	200	MUF	1604.24 (±17.86)	1553.18 (±38.41)
		MCA	13653.69 (±1518.05)	13420.72 (±2005.05)

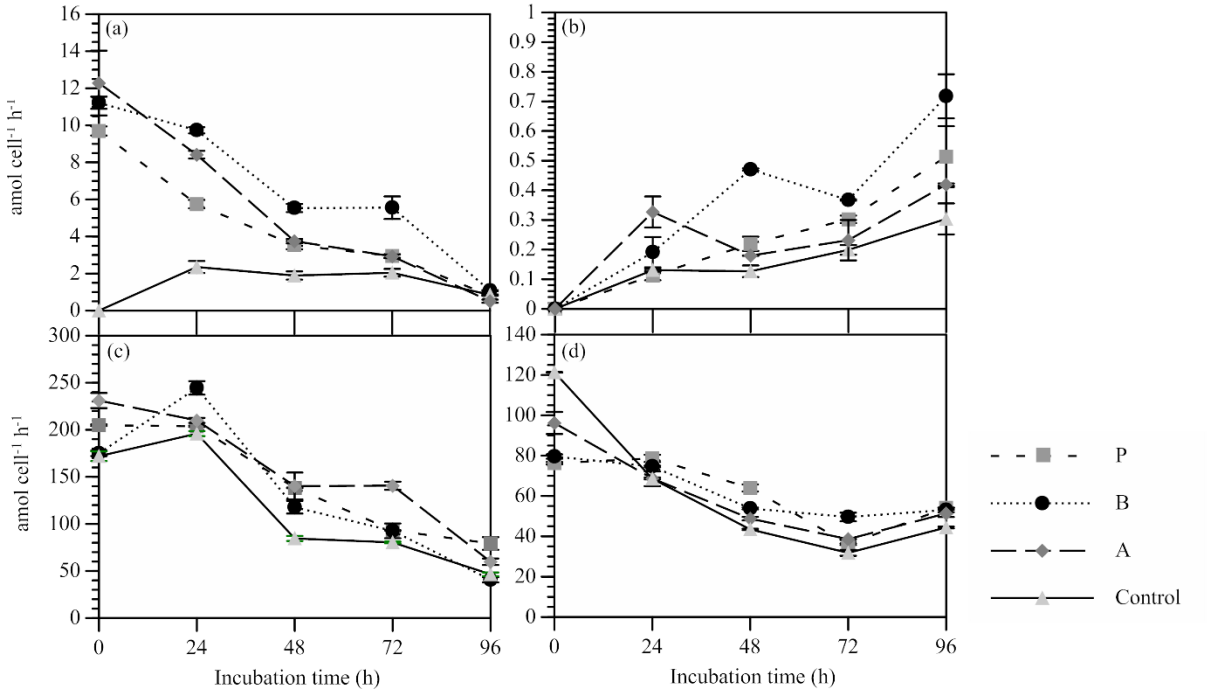
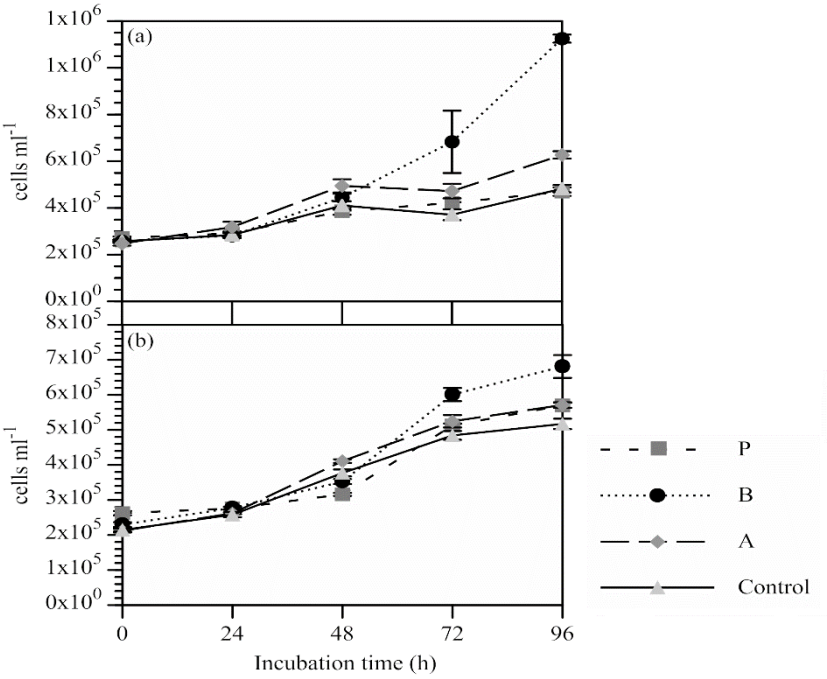


Figure 1. Cell-specific extracellular enzyme activity (mean ± SE, n=3) in response to seawater acidified with 0.1 M HCl (A), bubbled with CO_2-Air gas mixture (B) and CO_2-Air gas mixture introduced through gas-permeable silicon tubing (P). (a) BG activity in trial 1, (b) BG activity in trial 2, (c)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



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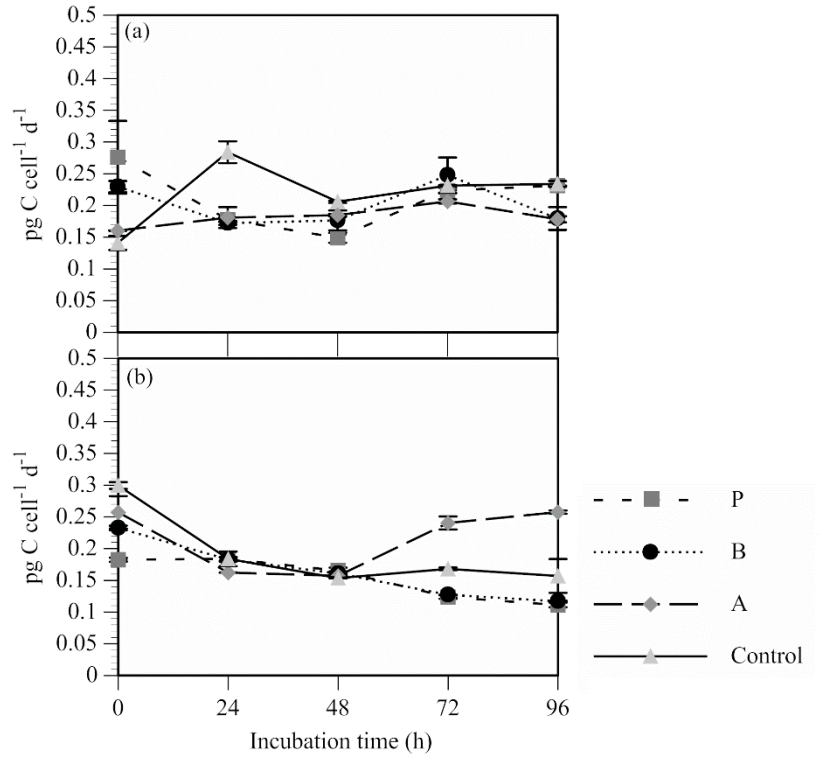
642 Figure 2. Bacterial cell numbers (mean ± SE, n=3) in response to seawater acidified with 0.1
643 M HCl (A), bubbled with CO_2-Air gas mixture (B) and CO_2-Air gas mixture introduced
644 through gas-permeable silicon tubing (P). (a) trial 1, (b) trial 2.



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