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Variable response to warming and ocean acidification by bacterial processes in different plankton communities

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ABSTRACT: Extracellular bacterial enzymes play an important role in the degradation of organic matter in the surface ocean but are sensitive to changes in pH and temperature. This study tested the individual and combined effects of lower pH (–0.3) and warming (+3°C) projected for the year 2100 on bacterial abundance, process rates and diversity in plankton communities of differing composition from 4 locations east of New Zealand. Variation was observed in magnitude and temporal response between the different communities during 5 to 6 day incubations. Leucine aminopeptidase activity showed the strongest response, with an increase in potential activity under low pH alone and in combination with elevated temperature in 3 of 4 incubations. Temperature had a greater effect on bacterial cell numbers and protein synthesis, with stronger responses in the elevated temperature and combined treatments. However, the most common interactive effect between temperature and pH was antagonistic, with lower bacterial secondary production in the combined treatment relative to elevated temperature, and lower leucine aminopeptidase activity in the combined treatment relative to low pH. These results highlight the variability of responses to and interactions of environmental drivers, and the importance of considering these in experimental studies and prognostic models of microbial responses to climate change.

KEY WORDS: Ocean acidification · Ocean warming · Interactive effects · Extracellular enzyme activity · Bacterial production · Bacterial diversity · Southwest Pacific

INTRODUCTION

Bacterial processes and carbon cycling in the surface ocean are intimately linked to the production and release of organic matter by phytoplankton (Bjørnsen 1988, Biddanda & Benner 1997, Azam & Malfatti 2007). Labile high molecular weight organic matter is primarily derived from plankton during bloom events and is a vital nutrient source for bacteria (Lancelot & Billen 1984, Benner 2002, Carlson 2002, Buchan et al. 2014). Phytoplankton-derived organic matter typically consists of a range of carbohydrates, proteins, amino acids and lipids (Benner 2002, Church 2008, Engel et al. 2011), the composition and concentration of which vary with time and community composition (Carlson 2002, Buchan et al. 2014, Engel et al. 2014). Consequently, changes in plankton abundance and diversity during a bloom, coupled with changes in nutrient status and organic matter, may lead to variation in bacterial processes and community composition (Buchan et al. 2014).

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Both free extracellular and attached ectoenzymes catalyse the degradation of high molecular weight organic matter and thus control an important step in the cycling of organic carbon and nutrients in the ocean (Billen et al. 1980, Chróst 1990, Cunha et al. 2010). It is well established that enzymes have both an optimal pH and temperature (Segel 1975, Tipton & Dixon 1979), and as these enzymes are active outside the cell, they are directly susceptible to variation in ocean pH and temperature (Cunha et al. 2010). Consequently, the projected reduction in ocean pH and increase in temperature (Collins et al. 2013) may have both direct and indirect effects on bacterial enzyme activity (Piontek et al. 2013). For example, a change in pH may directly alter the ionisation state of the enzyme's component amino acids (Dixon 1953) or indirectly alter enzyme production through changes in substrate composition and availability (Münster 1991) or changes in bacterial community composition (Endo et al. 2013). An increase in temperature will stimulate enzyme activity in accordance with the Arrhenius equation (Laidler 1984), with potential indirect increases in bacterial enzyme activity resulting from enhanced organic matter aggregation (Piontek et al. 2009). Temperature and pH are also important determinants of plankton community composition (Sommer et al. 2015), and as different plankton groups have differential effects on the availability and composition of organic carbon (Engel et al. 2014), future ocean acidification and warming may cause changes in organic substrate, altering bacterial community composition and the type of bacterial enzymes synthesised (Endo et al. 2013). These potential responses may have both positive and negative feedback on the ocean carbon cycle; for example, a change in organic matter degradation may alter the efficiency of the biological carbon pump (Wohlers et al. 2009, Piontek et al. 2010, Weinbauer et al. 2011), with subsequent impacts on carbon sequestration and higher trophic levels (Rossoll et al. 2012).

Although the individual effects of low pH and elevated temperature have been examined, research into the impact of multiple environmental drivers has been limited to date, and the potential for additive or antagonistic interaction in the future ocean remains unclear (Sala et al. 2015, Sommer et al. 2015, Gunderson et al. 2016, O'Brien et al. 2016). There has also been limited consideration of how phytoplankton community composition influences the response of bacterial processes to these drivers, both individually and in combination. Insight into the combined effect of low pH and elevated temperature, and the variation between different phytoplankton communities, is critical for robust modelling and projection of carbon and nutrient cycling in the future ocean. To address this shortcoming, the present study investigated the response of bacterial abundance, diversity, production and extracellular enzyme activity in Southwest Pacific surface waters to elevated temperature and low pH projected for 2100 to determine whether responses differ with plankton community type and biogeochemical status, and to assess the interactive effects of warming and ocean acidification.

MATERIALS AND METHODS

Sampling site description

Four perturbation incubations were completed during 2 research cruises in the austral summer of 2012 in waters east of New Zealand. Seawater was collected from phytoplankton blooms in 4 different locations, with each bloom located using satellite imagery and continuous surface measurements (Bell et al. 2015). Seawater for incubations 1 and 3 was collected from open ocean locations on the southern slope of the Chatham Rise; seawater for incubation 2 was collected further east; and seawater for incubation 4 was collected in the Southern Cook Strait, all from a depth of 10 m (Fig. 1). Seawater was collected using a Sea-Bird Electronics 32 Carousel Water Sampler fitted with 24 × 10 l external-spring Niskin-type bottles (Ocean Test Equipment Standard 10 BES). *In situ* water column parameters were monitored and depths acquired using a Sea-Bird Electronics 911 plus CTD attached to the carousel water sampler.

Experimental incubation design

Analysis of the Coupled Model Intercomparison Project Phase 5 model outputs (http://cmip-pcmdi.llnl.gov/cmip5/) for the Southwest Pacific around New Zealand indicates that under the business-as-usual carbon dioxide (CO_2) emission scenario (Representative Concentration Pathway 8.5 [RCP8.5]), surface ocean pH in the Chatham Rise region will decline to 7.77, while ocean temperatures are projected to increase by ~2.5°C by 2100 under the RCP8.5 scenario (Rickard et al. 2016). For each incubation, 3 perturbation treatments were compared to an unmodified seawater sample, the ambient control. The high temperature (HT) treatment consisted of ambient pH seawater with an elevated temperature (+3°C), the ocean acidification (OA) treatment

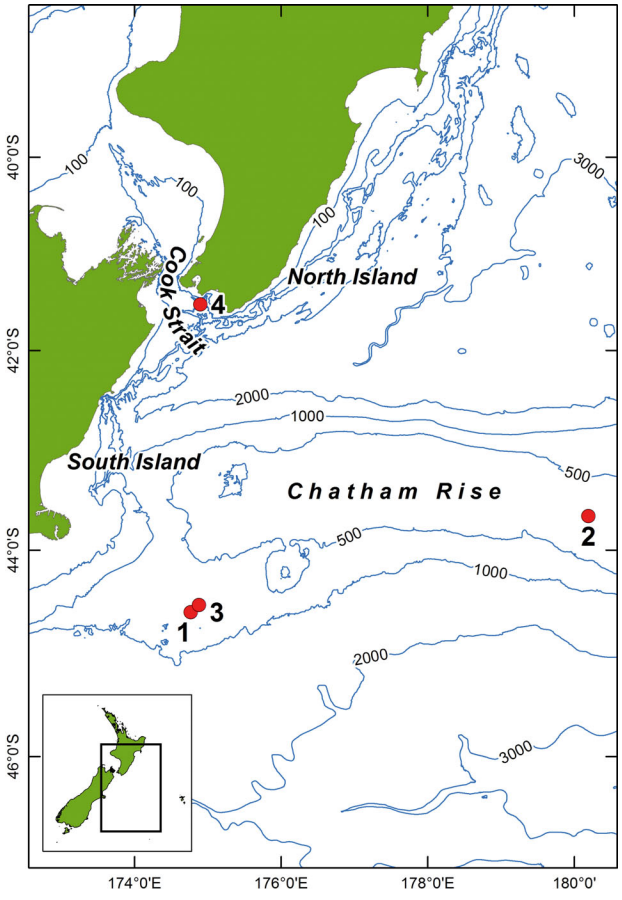

Fig. 1. Sampling sites off the eastern coast of the South Island of New Zealand and from Cook Strait at the base of the North Island. Numbered points correspond to individual incubations

consisted of artificially lowered pH seawater (total scale pH [pH_T] 7.8) at ambient seawater temperature, and the greenhouse (GH) treatment consisted of low pH seawater and elevated temperature at the same levels as the individual treatments. Each treatment and control were replicated in triplicate and maintained in acid-washed Milli-Q water pre-rinsed 4.3 l low-density polyethylene cubitainers (Thermo-Fisher Scientific).

To achieve the target pH value, different CO_2 gas mixtures were passed through gas-permeable tubing loops (Tygon tubing R-3603; inside diameter 1.6 mm; outside diameter 3.2 mm; Connect 2 Control) in each cubitainer, as in Law et al. (2012) and Hoffmann et al. (2013). Sample pH in the OA and GH treatments was lowered through the sequential application of 100% CO_2 for 25 min and then a 10% CO_2 gas mixture (in 20.8% oxygen [O_2] in nitrogen [N_2], BOC Gas) for 70 min. A CO_2 gas mixture of 742 to 750 µatm (also in 20.8% O_2 in N_2) was then continuously introduced through the tubing to maintain the target pH throughout the incubation, whereas the control and HT treatments received ambient external air delivered by an air pump. To eliminate gas exchange, there was no headspace in the cubitainers. Each cubitainer was housed in one of 2 identical Perspex incubation chambers, one of which was held at ambient surface water temperature, while the other was at ambient +3°C. The water sample within each cubitainer was continuously mixed using an external inflating diaphragm system connected to a time-controlled air pump. Photosynthetic light was maintained in each cubitainer by external fluorescent light banks (Philips TL-D 36W/840), with the light intensity at 10 m depth (approximately 10% of surface ocean photosynthetic radiation), reproduced using neutral-density polycarbonate screening. Manually adjustable mains timers ensured an automated diurnal 12 h light:12 h dark cycle, with black polythene applied to the external viewing windows to minimise inadvertent light exposure during the dark cycle. Incubations 1, 2 and 3 were each conducted over a 6 d period (144 h), while incubation 4 was restricted to 120 h. During each incubation, a range of biotic and abiotic parameters were sampled at 12 h intervals, with the cubitainers removed and inverted 3 times prior to sampling.

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 unit), which was regularly cleaned using potassium chloride reference electrolyte gel (RE45, Ionode). Electrode pH measurements were validated onboard using a pH spectrophotometer with colorimetric determination using a thymol blue dye solution (McGraw et al. 2010). Following recommendations in the European Project on Ocean Acidification best practises guide (Riebesell et al. 2010), reported pH values in the present study reflect the total hydrogen ion scale (pH_T).

Bacteria and phytoplankton cell numbers

Triplicate samples were collected in 2 ml Cryovials (Raylab) and frozen in liquid nitrogen (Hall et al. 2004) for up to 12 wk prior to analysis. Bacteria and phytoplankton cell numbers were determined by flow cytometry (FACSCalibur, Becton Dickinson), as described in Hall et al. (2004). Plankton bloom com-

munities were identified by their optical properties using satellite imagery and subsequent optical microscopy.

Bacterial secondary production

Potential bacterial secondary production (BSP) was measured using both ^{3}H-thymidine and ^{3}H-leucine of high specific activity (>80 Ci mmol^{-1}, SciMed) as indicators of DNA and protein synthesis, respectively, using the methodology of Knap et al. (1996) and Smith & Azam (1992). Each value was multiplied by a known carbon and protein constant sourced from Smith & Hall (1997) and Simon & Azam (1989), respectively.

Enzyme activities

The potential activities of 2 proteases, arginine aminopeptidase (AAP) and leucine aminopeptidase (LAP), and 2 glucosidases, α-glucosidase (AG) and β-glucosidase (BG), were quantified (V_{max}, nmol l^{-1} h^{-1}) using saturating substrate concentration and calibration curves, as described in Burrell et al. (2016). Briefly, artificial fluorogenic substrate was added to each seawater sample to give a final saturating assay concentration of 39 µM (Maas et al. 2013). A buffered 4-point calibration curve (0, 4, 40, 200 nM final concentration) was created using 4-methylumbelliferone for glucosidase activity, and a separate calibration curve (0, 40, 400, 4000 nM final concentration) was created using 7-amino-4-methylcoumarin for protease activity (Sigma-Aldrich). Each assay plate was read using a Modulus microplate reader (Turner Biosystems) at 365 nm excitation and 460 nm emission wavelength at ambient surface water temperature. Artificial fluorogenic compounds used to determine extracellular enzyme activity are affected by, or alter, seawater pH, and so buffering with 0.1 M Tris/HCl was used in each calibration curve and treatment to minimise this artefact (Burrell et al. 2016). Cell-specific rates were calculated by dividing the activity per litre by bacterial cell numbers per litre. Samples collected during incubations 1, 2 and 3 could not be analysed at sea, so they were frozen at −80°C and processed post cruise. As there may be a change in enzyme activity during storage and thawing (Chróst & Velimirov 1991, Bélanger et al. 1997, Mayer et al. 1997), enzyme activities are only compared within each experiment, as these experienced the same conditions, and there is no comparison of the magnitude of responses across experiments or with published activity rates. The ratio between LAP and BG activity was calculated for each treatment at 72 and 144 h. The total integrated LAP and BG activity at 72 and 144 h relative to the control was also used to compare treatment responses (Piontek et al. 2013). To investigate the temperature sensitivity of BG and LAP activity, Q_{10} factors were determined at 72 and 144 h using the following equation:

$$Q_{10} = (R_1/R_2)^{10/(T_1 - T_2)} \quad (1)$$

where R_1 and R_2 are total potential enzyme rate constants at temperatures T_1 and T_2 (°C) and $T_1 > T_2$ (Sherr & Sherr 1996).

Bacterial diversity

The active fraction of bacterial diversity was assessed using the RNA of the 16S rRNA gene and terminal restriction fragment length polymorphism (RNA-T-RFLP), as previously described in Maas et al. (2013). The data were exported from GeneMapper in binary format and statistically analysed in PRIMER v.6.1.15 (PRIMER-E).

Ancillary biogeochemical measurements

Dissolved organic carbon (DOC) concentration was determined using the non-purgeable organic carbon method (American Society for Testing and Materials 1994), where 30 ml of sample solution was acidified with 250 µl of 2N HCl and sparged with carbon-free air. DOC samples were analysed using a total organic carbon analyser (TOC-VCSH, Shimadzu). Chl *a* detection followed the acidification method described in Strickland & Parsons (1968). Fluorescence was measured using a luminescent spectrometer (Perkin-Elmer LS55) fitted with a Xenon blue light source at 430 nm emission and 670 nm excitation. The chl *a* pigment was determined by subtracting the unacidified fluorescence reading from the acidified fluorescence reading. Resulting values were plotted against a 7-point linear calibration curve of known chl *a* concentration and fluorescence, with concentrations reported in micrograms per millilitre. Phosphate, nitrate, ammonium and silica concentrations were analysed simultaneously using an Astoria-Pacific segmented flow analyser following Astoria-Pacific International protocols (Rev. A6/00). Each analyte had a detection limit of 1 µg l^{-1}. Chl *a* and dissolved nutrient samples

were analysed using the FASPac II flow analyser software package.

Statistical analysis

Statistica v.10 (StatSoft) was used for basic graphics and descriptive statistics. Data were tested for normality and equality of variance prior to statistical analysis. Because of a small sample size, these assumptions were infrequently met, and data were log(x+1) transformed. ANOVA was performed at individual time points; standard hypothesis formulations were used, with the null hypothesis (H_0) mean = 0, and 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. The binary terminal restriction fragment (TRF) data were imported into the program PRIMER, and non-metric MDS plots were generated. A Bray-Curtis dissimilarity matrix was created, and a similarity profile routine (SIMPROF) was used to test for the presence of patterns between clusters that could have occurred by chance (Clarke et al. 2008). Clusters were created at a 5% significance level using 1000 random permutations. An MDS stress value <0.15 corresponds to a good agreement between multivariate matrices (Clarke 1993).

RESULTS

Surface water characteristics

The initial characteristics of the biogeochemistry and plankton community differed across the 4 sites (Table 1). Seawater collected for incubation 1 had the lowest temperature, salinity and pH and the highest dissolved nitrate concentration, and was dominated by dinoflagellates and flagellates (mainly Gymnodiniaceae, Oxytoxaceae and Ceratiaceae), with a minor diatom component (mainly Melosiraceae) (Table 1). Incubations 2 and 3 contained a similar mixed plankton community of coccolithophores, dinoflagellates and a range of microflagellates (silicoflagellates, cryptomonads and euglenoids), despite the very low nitrate concentration in incubation 2 (Table 1). Seawater collected for incubation 4 had the highest salinity, pH, dissolved silica and phosphate concentrations and chl *a* (Table 1), but phytoplankton composition was not determined. The RNA-T-RFLP data confirmed that the initial bacterial communities were significantly different between each incubation (SIMPROF; $p < 0.05$). Total glucosidase activity (BG and AG) was lower than aminopeptidase activity (AAP and LAP) (Table 1). Bacterial cell numbers and BSP protein synthesis were similar between each community, ranging from 5×10^5 to 1×10^6 cells ml^{-1}

Table 1. Characteristics of the surface seawater used in the 4 incubations at time zero (mean ± SE). pH_T: total scale pH; n/a: not available; BSP: bacterial secondary production; BG: β-glucosidase; AG: α-glucosidase; AAP: arginine aminopeptidase; LAP: leucine aminopeptidase; DOC: dissolved organic carbon; <LOD: below limit of detection; phytoplankton groups are indicated as the % of total phytoplankton biomass > 5 µm; n indicates the number of replicate measurements for each sample

Parameter	Incubation 1	Incubation 2	Incubation 3	Incubation 4
Location	44.61° N, 174.77° E	43.59° N, 180.53° E	44.54° N, 174.88° E	41.53° N, 174.90° E
Date collected (2012)	15 Feb	22 Feb	29 Feb	17 Mar
Temperature (°C)	11.8	15.8	14.5	14.2
Salinity (psu)	34.46	34.66	34.49	34.68
pH_T	8.00	8.13	8.07	8.16
Diatoms (% of total biomass)	9	5	1	n/a
Coccolithophores (% of total biomass)	3	40	47	n/a
Dinoflagellates (% of total biomass)	59	28	19	n/a
Microflagellates (% of total biomass)	29	27	33	n/a
Bacterial numbers (cells ml^{-1}, n = 3)	$5 \times 10^5 \pm 4.9 \times 10^3$	$1 \times 10^6 \pm 2.0 \times 10^4$	$6 \times 10^5 \pm 3.6 \times 10^3$	$5 \times 10^5 \pm 4.8 \times 10^3$
BSP DNA synthesis (µg C l^{-1} d^{-1}; n = 3)	1.56 ± 0.02	3.83 ± 0.03	3.82 ± 0.11	4.25 ± 0.19
BSP protein synthesis (µg C l^{-1} d^{-1}; n = 3)	1.22 ± 0.00	1.73 ± 0.02	1.19 ± 0.05	1.04 ± 0.03
BG (nmol l^{-1} h^{-1}; n = 2)	<LOD	0.04 ± 0.01	<LOD	0.13 ± 0.03
AG (nmol l^{-1} h^{-1}; n = 2)	<LOD	<LOD	<LOD	<LOD
AAP (nmol l^{-1} h^{-1}; n = 2)	<LOD	3.94 ± 0.37	7.29 ± 0.66	<LOD
LAP (nmol l^{-1} h^{-1}; n = 2)	0.84 ± 0.21	6.12 ± 0.75	2.67 ± 0.21	23.13 ± 3.48
Chl *a* (µg ml^{-1}; n = 6)	0.52 ± 0.16	0.54 ± 0.02	0.37 ± 0.01	1.59 ± 0.04
Nitrate (µmol l^{-1}; n = 3)	6.09 ± 0.65	0.04 ± 0.00	4.15 ± 0.22	3.29 ± 0.03
Phosphate (µmol l^{-1}; n = 3)	0.48 ± 0.02	0.15 ± 0.04	0.59 ± 0.15	1.64 ± 0.01
Silica (µmol l^{-1}; n = 3)	0.19 ± 0.03	0.17 ± 0.02	0.10 ± 0.05	1.78 ± 0.01
Ammonium (µmol l^{-1}; n = 3)	0.23 ± 0.05	0.37 ± 0.00	0.83 ± 0.16	0.22 ± 0.05
DOC (µg ml^{-1}; n = 2)	0.85 ± 0.02	23.43 ± 0.42	20.00 ± 0.31	0.84 ± 0.01

and 1.04 to 1.73 µg C l^{-1} d^{-1}, respectively (Table 1), whereas DNA synthesis was lower in incubation 1 and higher in incubation 4 (Table 1).

Bacterial cell number and secondary production

The temporal trend in bacterial cell abundance differed between incubations, peaking in the first 36 h in incubations 1 and 2 and increasing throughout incubation 3 (Fig. 2). Bacterial cell numbers did not show a significant treatment response throughout incubation 1, in contrast to the other incubations. The elevated temperature treatments (HT and GH) initially showed slightly higher cell numbers than the control to 72 h in incubations 3 and 4 but then declined to significantly lower counts relative to the control and OA treatment ($p < 0.05$) by Day 5 (Fig. 2). Bacterial cell numbers in the OA treatment generally tracked the control, with stimulation at low pH only apparent during incubations 1 and 4 (2.1 and 1.4 times higher, respectively). Conversely, the HT and GH treatments showed similar numbers and temporal trends throughout all incubations, and there was no evidence of interaction between temperature and pH (Fig. 2), confirming temperature as the dominant determinant of bacterial abundance (Table 2).

Temperature also had a greater effect on the BSP response, with no significant difference between the control and OA treatment in all incubations (Fig. 3). The response of DNA synthesis generally mirrored that of bacterial cell numbers, with the HT and GH treatments peaking and declining earlier than the control and OA treatment in incubations 2 and 4. However, in contrast to bacterial abundance, DNA synthesis was higher in the HT treatment than the GH treatment in incubations 3 and 4, being 3-fold higher at the end of incubation 3, and indicative of an antagonistic interaction between temperature and pH (Fig. 3, Table 2). DNA synthesis was approximately 4 times higher than protein synthesis within each treatment (see Supplement 1 at www.int-res.com/articles/suppl/a079p049_supp.pdf). In contrast to DNA synthesis, protein synthesis was significantly higher in the HT treatment relative to the control at 144 h in incubation 1 as well as incubation 3 ($p < 0.05$).

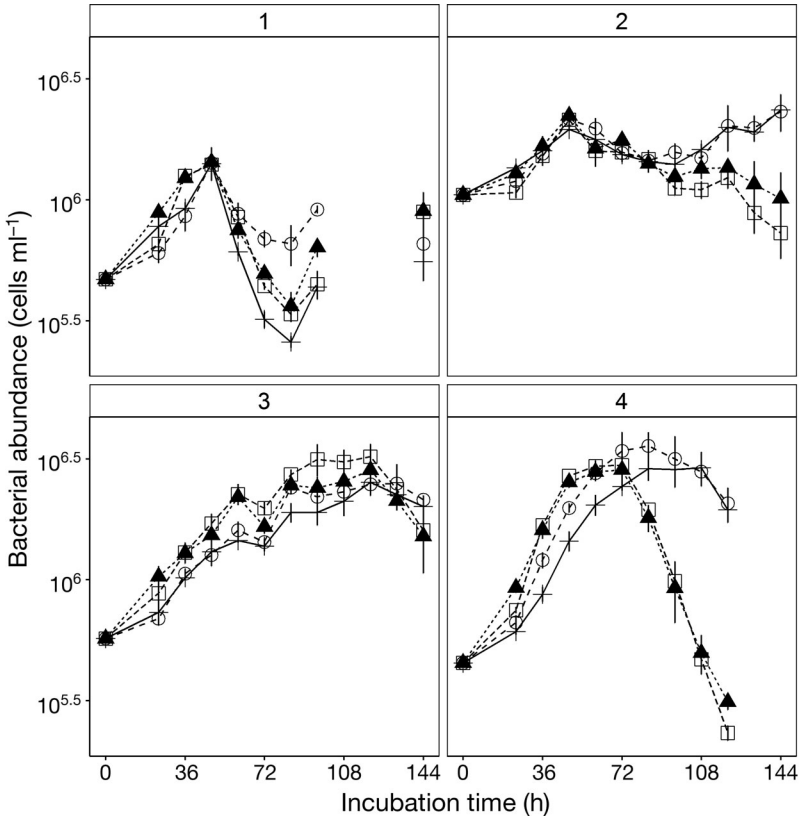

Fig. 2. Bacterial cell numbers (log scale, mean ± SE, n = 3) in incubations 1 to 4. Control: solid line black crosses (+); greenhouse treatment: dotted line black triangles (▲); high temperature treatment: long dashed line open squares (□); ocean acidification treatment: short dashed line open circles (○)

Extracellular enzyme activity

Bulk water BG and AG activity were low throughout incubation 1, with no detectable treatment response (Table 3). BG and AG activity were highest in the OA treatment during incubation 2 but declined in each treatment by the end of the incubation (data not shown). During incubations 3 and 4, BG and AG activity increased rapidly in the elevated temperature treatments; after 60 to 72 h, activity in the HT and GH treatments declined below the control and OA treatment and remained low for the duration of the incubation (data not shown). The positive response of BG activity to elevated temperature in incubations 3 and 4 was reflected in Q_{10} values ranging from 0.5 to 3.6 (Table 3).

Bulk water LAP and AAP activity increased throughout each incubation and were generally highest in the OA treatment, as shown by the total integrated activity (Table 3). All incubations showed evidence of an antagonistic effect between temperature and

Table 2. Main driver and interactive effect for 4 measured variables from each incubation. Dom. (dominant driver): treatment that causes the maximum deviation in response from the control (whether positive or negative); this is identified where the treatment and control differed significantly on at least 3 sample time points during the 5 d experiments. Antag: greenhouse (GH) treatment response is lower than the response of the dominant driver. Add: GH treatment response is equal to the sum of the individual driver responses. The summary result is highlighted in **bold** where the dominant treatment or interaction occurred in at least 3 of the 4 incubations. Inter.: interaction; BSP: bacterial secondary production; BG: β-glucosidase; LAP: leucine aminopeptidase; Temp: temperature; OA: ocean acidification; HT: high temperature

Incubation	Bacterial abundance		BSP		Bulk BG		Bulk LAP		Cell-specific BG		Cell-specific LAP	
	Dom.	Inter.	Dom.	Inter.	Dom.	Inter.	Dom.	Inter.	Dom.	Inter.	Dom.	Inter.
1	OA						OA	Antag				
2	HT lowers		Temp		OA	Antag	OA	Antag	OA			
3	Temp		Temp	Antag	Temp	Antag	OA		Temp		OA	Antag
4	HT lowers		Temp	Antag	OA	Antag	OA	Antag	Temp	Add		
Summary	Temp		**Temp**	Antag	OA	**Antag**	**OA**	**Antag**	Temp			

pH, with lower LAP activity in the GH treatment relative to OA (Table 2, see also Supplement 2 at www.int-res.com/articles/suppl/a079p049_supp.pdf). Bulk water LAP activity in the HT and GH treatments in incubation 4 was lower than the control and OA treatment from 60 to 120 h (Supplement 2), in contrast to AAP activity, which increased in the HT and GH treatments throughout incubation 4 (data not shown). However, a positive LAP response to elevated temperature was evident, particularly in incubations 2 and 3 (1.6 to 1.9 times higher in the HT and GH treatments, respectively), and generated Q_{10} values which ranged from 0.2 to 31.8 (Table 3). Bulk water LAP activity was significantly higher than BG activity within each treatment and incubation ($p < 0.05$). Although there was significant variation in the LAP:BG ratio between treatments and incubations, the GH and OA treatments were generally higher than the control and HT treatment (Table 3).

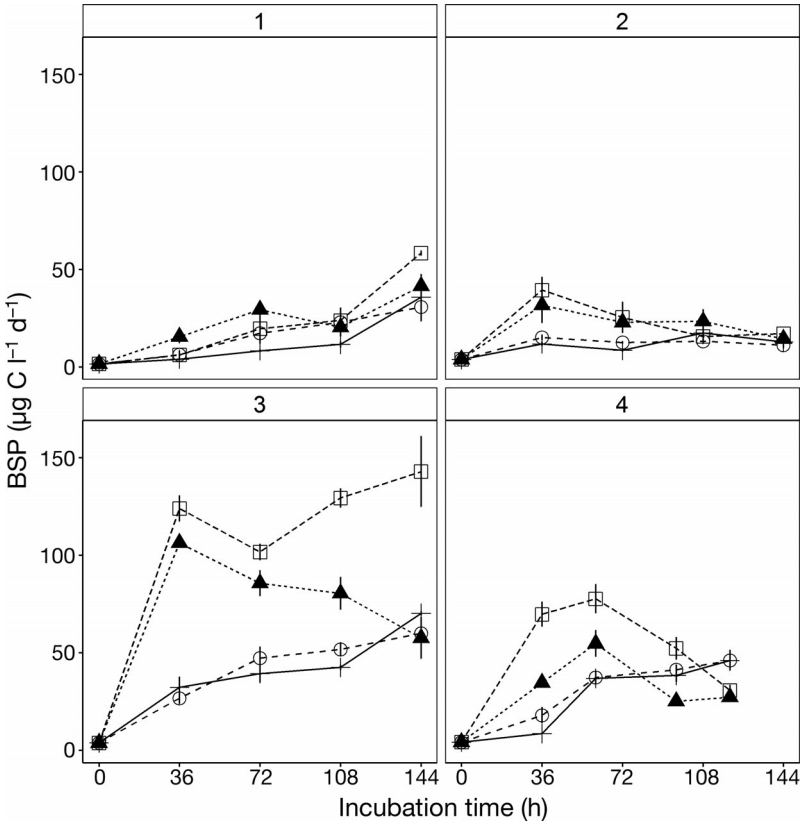

Fig. 3. Bacterial secondary production (BSP) (^{3}H-thymidine incorporation/DNA synthesis; mean ± SE, n = 3) in incubations 1 to 4. Control: solid line black crosses (+); greenhouse treatment: dotted line black triangles (▲); high temperature treatment: long dashed line open squares (□); ocean acidification treatment: short dashed line open circles (○)

Normalisation of potential enzyme activities to bacterial cell numbers showed that cell-specific AG activity was low and did not vary between treatments across all incubations (data not shown). Cell-specific BG activity was also low in incubations 1 to 3 (0.01 to 0.39 amol cell^{-1} h^{-1}), with variable temporal trends (Fig. 4). OA and GH treatments showed the highest cell-specific BG activity in incubation 2, whereas HT and GH had significantly higher activities from 84 h in incubation 3 and from 108 h in incubation 4. Cell-specific AAP activity increased throughout each incubation, with considerable variability in incubations 2 and 3 (data not shown). AAP activity was significantly higher in the OA treatment when compared to the control in incubations 1 and 3, whereas both elevated temperature treatments had a greater activity relative to the control from 108 h in incubation 4

Table 3. Bulk β-glucosidase (BG) and leucine aminopeptidase (LAP) Q_{10} values for both elevated temperature treatments (high temperature [HT] and greenhouse [GH]), LAP:BG activity ratios and integrated enzyme activities to sampling time relative to the control (C) at the middle and end incubation points (72 and 144 h in incubations 1, 2 and 3; 60 and 120 h in incubation 4). OA: ocean acidification treatment; na: no activity measured

Incubation	Time (h)	Q_{10} value				LAP:BG ratio				Total integrated activity (nmol l^{-1})					
		BG		LAP						BG			LAP		
		HT	GH	HT	GH	HT	GH	OA	C	HT	GH	OA	HT	GH	OA
1	72	na	na	2.3	29.3	na	na	na	na	0	0	0	85.6	277.6	396.9
	144	na	na	4.0	7.2	na	202.6	172.9	95.3	−0.3	0.4	4.3	363.7	734.5	1522.6
2	72	na	na	8.6	10.3	161.8	90.1	128.8	na	4.1	5.5	12.7	306.2	564.4	565.6
	144	na	0.1	0.5	0.4	na	479.5	na	327.5	4.5	5.3	18.9	317.6	573.3	1006.5
3	72	1.8	1.7	15.1	31.8	99.2	122.1	159.2	58.3	8.1	7.3	2.7	1104.2	1494.8	1774.4
	144	3.6	2.2	0.2	1.0	97.4	163.4	143.7	197.7	30.5	23.0	18.1	2582.3	4379.1	1774.4
4	60	0.8	2.0	0.5	0.9	80.7	74.3	73.5	92.2	50.7	95.8	69.6	5413.6	6768.9	8325.5
	120	na	0.5	na	0.9	60.3	98.3	100.3	81.6	97.3	13.5	164.8	11943.6	7561.0	11050.1

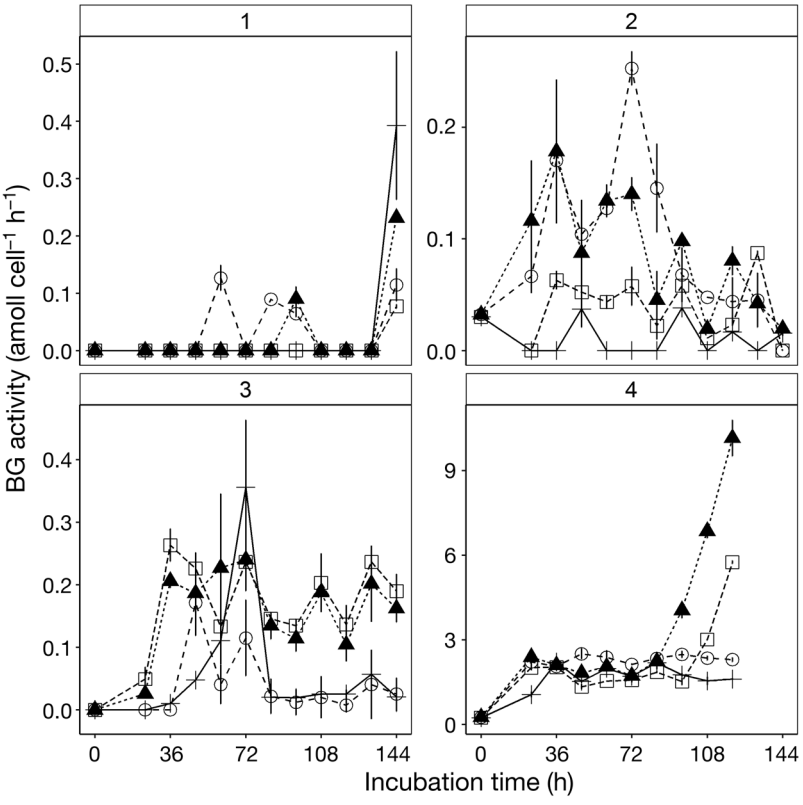

Fig. 4. Cell-specific β-glucosidase (BG) activity (mean ± SE, n = 3) throughout incubations 1 to 4. Control: solid line black crosses (+); greenhouse treatment: dotted line black triangles (▲); high temperature treatment: long dashed line open squares (□); ocean acidification treatment: short dashed line open circles (○)

(data not shown). Cell-specific LAP activity increased in all treatments throughout each incubation, except for the control in incubation 2 (Fig. 5). Cell-specific LAP activity was also higher in each treatment relative to the control throughout incubations 1 to 3, with the most significant response occurring in the OA treatment in incubation 3 (at least 2-fold higher than the control; Fig. 5). Although elevated temperature had a significant effect on both AAP and LAP activity, acidification had a greater effect in each incubation, with LAP activity 1.7 to 2.7 times higher than in the control, compared to 0.95 and 1.52 for the HT treatment. There was also evidence of an antagonistic interaction in the GH treatment relative to the OA response in incubation 3 and at select sampling points in incubation 4 (Table 2).

In summary, the most significant treatment responses were for LAP, which showed an increase in potential activity in 3 of 4 incubations in the OA and GH treatments (Table 3). Conversely, elevated temperature had a greater effect on bacterial cell numbers, DNA and protein synthesis, with the strongest responses in the HT and GH treatments (Table 2). However, the main interactive effect observed between temperature and pH was antagonistic, with a lower BSP in the GH treatment relative to HT and lower LAP activity in the GH treatment relative to OA in incubations 3 and 4 (Table 2).

Bacterial community composition

The RNA-T-RFLP profiles at time zero and the final sampling point at 144 h were compared for each treatment. The number of TRFs increased in each treatment and control in incubations 1 and 2 (173–191 to 210–250) but changed little during incubations

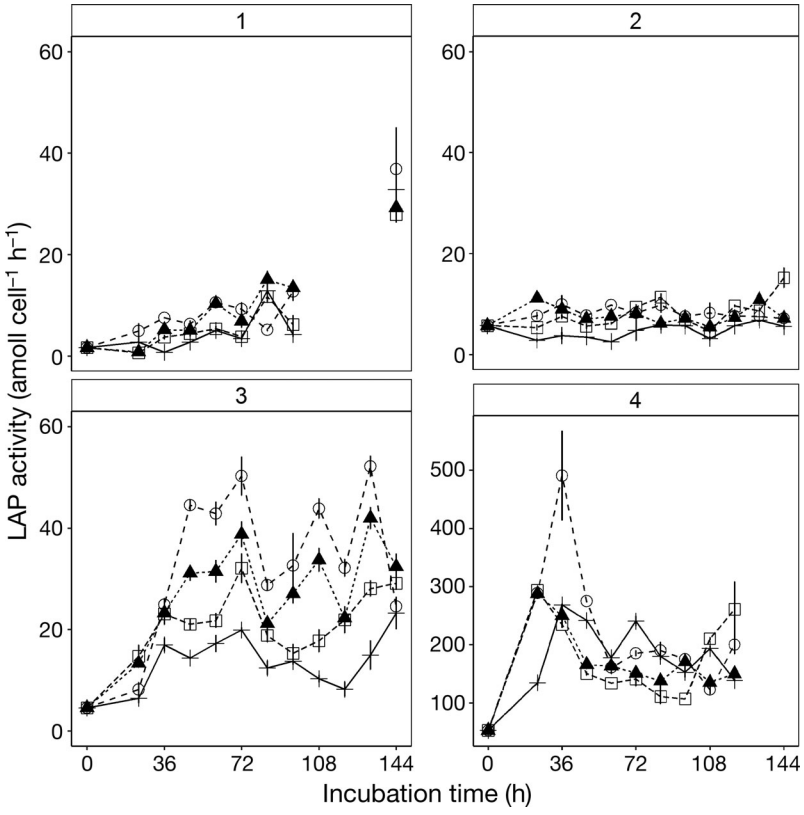

Fig. 5. Cell-specific leucine aminopeptidase (LAP) activity (mean ± SE, n = 3) throughout incubations 1 to 4. Control: solid line black crosses (+); greenhouse treatment: dotted line black triangles (▲); high temperature treatment: long dashed line open squares (□); ocean acidification treatment: short dashed line open circles (○). Missing data in incubation 1 from 96 to 132 h resulted from equipment failure

3 and 4 (197 to 202). Despite a change in bacterial community diversity (Fig. 6), total TRF counts were not significantly different between treatments in any incubation at 144 h ($p > 0.05$). Although no significant treatment effect was detected from the metabolically active fraction, some trends were apparent by the end of the incubations (Fig. 6). For example, warming showed a greater effect on community diversity at the final sampling point, with incubation 4 showing cluster separation of HT and GH treatments from the OA treatment and the control (Fig. 6). After 144 h in incubation 2, the OA treatment was separate from the HT and GH treatments but did not form a discrete cluster (Fig. 6).

DISCUSSION

This study confirms the stimulatory effect of low pH and elevated temperature previously reported for extracellular enzyme activity and other bacterial processes (Piontek et al. 2009, 2013, 2014). Temperature was an important determinant of bacterial response, with cell abundance showing a rapid initial increase in response to elevated temperature in incubations 3 and 4, as previously observed (Piontek et al. 2009, Lindh et al. 2013), and DNA synthesis showing a more prolonged response to elevated temperature in incubations 1, 3 and 4 (Pomeroy & Wiebe 2001, Rivkin & Legendre 2001). This enhanced secondary production at elevated temperature reflects an increase in metabolic potential (Doney et al. 2012 and references therein), as highlighted by the Q_{10} values (Table 3). Although temperature has a significant effect on microbial production, the response is non-linear and may vary depending on additional factors such as community composition and substrate concentration (Pomeroy & Wiebe 2001). For instance, the decrease in bacterial cell numbers and BSP after 48 h in the elevated temperature treatments in incubations 2 to 4 (Figs. 2 & 3) may indicate a reduction in labile substrate availability in response to the increased initial growth rate. The timing of this switch from potential substrate-replete to substrate-depleted conditions in the elevated temperature treatments varied between incubations, potentially reflecting differences in community composition and bloom status (Table 1). Alternatively, this may be attributable to the onset of, or an increase in, viral lysis (Proctor & Fuhrman 1990, Danovaro et al. 2011) or predation by protists, as supported by the concurrent decline in phytoplankton abundance in incubations 2 and 4 (data not shown).

It is well established that an increase in temperature increases enzyme activity (Laidler 1984), with a positive glucosidase and protease response to warming reported for the Baltic (Piontek et al. 2009) and Arctic (Piontek et al. 2013, 2014). Piontek et al. (2009) hypothesised that particle aggregation may be enhanced at elevated temperature, which may increase substrate and attachment sites for bacteria and promote the development of different assemblages, as indicated in incubation 4 (Fig. 6). However, bacterial community composition was not significantly affected by warming and/or acidification over the

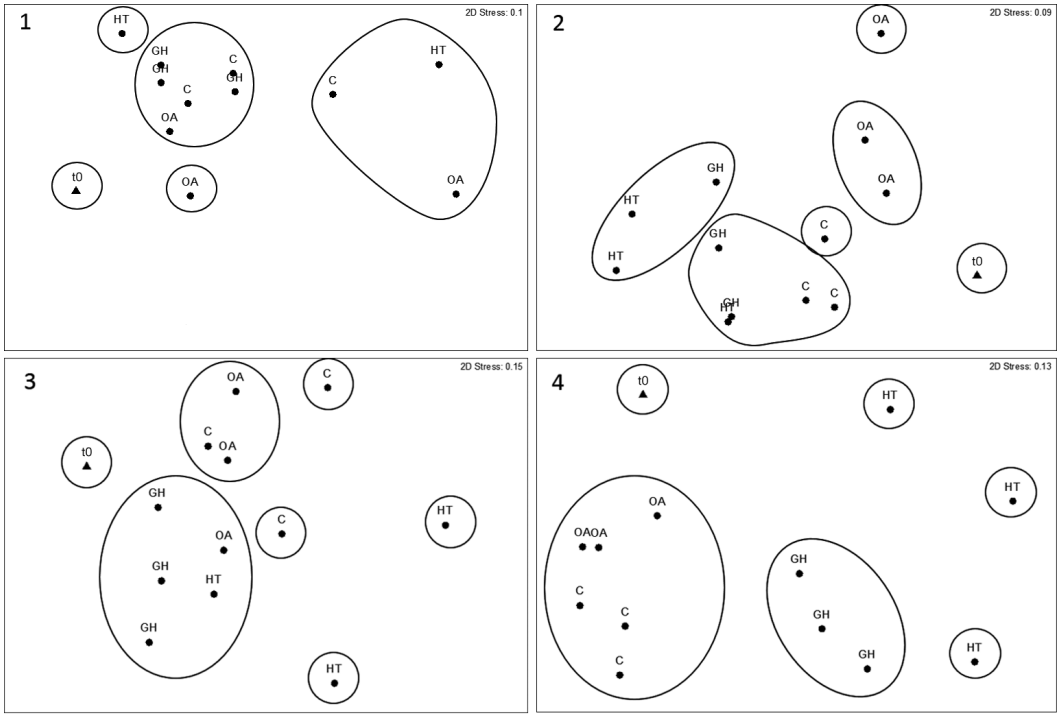

Fig. 6. Non-metric MDS plot created using Bray-Curtis similarity of bacterial community profiles (RNA terminal restriction fragment length polymorphism) generated by RNA analysis of the 16S rRNA gene for each treatment in incubations 1 to 4. Encircled clusters are based on a significant similarity profile routine analysis at 5% significance. ▲: time zero bulk seawater sample; ●: final sampling point at 144 h (or 120 h for incubation 4). C: control; GH: greenhouse treatment; HT: high temperature treatment; OA: ocean acidification treatment; t0: time zero

period of 144 h in the present study, although trends did begin to emerge in certain communities (Fig. 6). This is consistent with reports that lower pH has little effect on bacterial community composition (Newbold et al. 2012, Zhang et al. 2012) but contrasts with other studies (Lindh et al. 2013, Das & Mangwani 2015 and references therein). It is likely that the failure to detect a significant bacterial community change was due to the short duration of each incubation, as Lindh et al. (2013) reported a significant additive effect on bacterial diversity in Baltic seawater only after a 21 d incubation. Regardless, the results suggest that the influence of pH and temperature on organic matter type and availability, as discussed in the 'Introduction, will indirectly influence bacterial community composition.

The variable response of potential glucosidase and protease activity to lower pH and elevated temperature between the different phytoplankton communities may alternatively reflect the diversity of enzymes and their respective pH sensitivities. For example, Arrieta & Herndl (2002) detected up to 8 forms of BG at the peak of a coastal *Phaeocystis* phytoplankton bloom, and the results of the present study may reflect an assemblage of glucosidases and proteases with different pH and temperature optima in each community (Tipton & Dixon 1979, Das & Mangwani 2015). Different phytoplankton groups have differing CO_2 sensitivities (Rost et al. 2003), and may exude organic matter of differing composition and concentration depending on their physiological status (Buchan et al. 2014 and references therein). Consequently, the observed temporal variation in potential enzyme activity may reflect differences in phytoplankton community composition and status, which in turn may reflect nutrient availability (Piontek et al. 2014). This is potentially supported by Sala et al. (2015), who report that the response of AG and BG activity to OA was affected by seasonal changes in nutrient conditions in the Mediterranean Sea. The elevated nitrate and low temperature in incubation 1 indicates that this water was primarily of sub-Antarctic origin, whereas incubation 2 had the highest ambient temperature and ammonium and DOC concentrations and low nitrate, phosphate and silica concentrations (Table 1), indicative of post-bloom subtropical water dominated by heterotrophs (Buchan et al. 2014, Engel et al. 2014). Although from a similar location, incubation 3 seawater was collected 8 d after incubation 1 (Fig. 1), and the different phytoplankton com-

munity may reflect a transitional community composition within the same water body. Overall, the spatial variation in the response of bacterial processes to projected future conditions may reflect variations in plankton community composition and bloom status. As the latter are determined by factors such as nutrient and light availability, seasonal variability in response may also occur. Although direct relationships between nutrient status and phytoplankton community with bacterial responses were not identified, the results indicate that variability in these factors will alter the response to warming and lower pH.

Potential LAP activity was higher than BG activity in each treatment and control in all incubations (Table 3), indicating relatively higher nitrogen demand and enhanced protein hydrolysis. The LAP:BG ratio provides insight into the quality of the carbon pool, with a higher ratio indicating a preference for protein degradation and a lower ratio indicating a preference for carbohydrate degradation. The latter may then reflect lower protein availability or, alternatively, dissolved inorganic nitrogen replete conditions (Christian & Karl 1992, 1995). The observed LAP:BG ratio range (60 to 479) appears typical of temperate waters and exceeds values reported for oligotrophic equatorial waters (Christian & Karl 1995), the Southern Ocean (E. W. Maas et al. unpubl.) and acidified conditions in an Arctic fjord (Piontek et al. 2013), but were lower than in Antarctic waters (339 to 1052; Christian & Karl 1995). Temperature has been suggested as a determining factor for selective substrate degradation (Christian & Karl 1995); however, elevated temperature and lower pH did not significantly alter the LAP:BG ratio, and thus the preferential degradation of proteins over carbohydrates, in this study. The LAP:BG ratio may also reflect the relative bioavailability of carbohydrate substrates such as cellulose (Benner et al. 1992), which tends to be more recalcitrant than protein-based substrates (Arnosti 2003). Poretsky et al. (2010) reported variations in substrate transporter expression between coastal water bacterial species, and differences in carbohydrate degradation, and so the variable LAP:BG ratios may also reflect the differing bacterial community composition and bloom status in the 4 incubations.

Studies to date suggest that warming may be a stronger driver of the global pelagic system than acidification (Sommer et al. 2015), although the results of this study indicate a process dependence, with bacterial abundance and production being more sensitive to temperature while LAP activity shows a stronger response to low pH. The absence of a single dominant driver determining bacterial responses suggests a disconnect; although LAP activity is higher under lower pH, the bacteria do not appear to benefit from this in terms of an increase in productivity and abundance. Regardless, the increase in both drivers will be coincident in the future ocean, and understanding their combined effect is more critical for projections of future ecosystem status (Gunderson et al. 2016). Overall, there was no synergistic or additive effect observed, with 50% of responses in the GH treatments showing an antagonistic effect relative to the response to the dominant driver, whether temperature or pH (Table 2). This contrasts with reported observations summarised in recent meta-analyses, in which synergism is at least as dominant as antagonism, if not more so (Crain et al. 2008, Gunderson et al. 2016). Numerous factors can affect how a community responds to driver interactions, including diversity, trophic structure and growth rates (Crain et al. 2008), and therefore communities are dependent upon the *in situ* marine environment (Gunderson et al. 2016). BSP was strongly influenced by elevated temperature yet showed a significant antagonistic response in the GH treatment relative to the HT treatment in incubations 3 and 4 (Fig. 3, Table 2). The interaction of elevated temperature and low pH may have altered the bacterial response via one driver potentially counteracting the other (O'Brien et al. 2016). For example, the stimulation of secondary production under elevated temperature may be suppressed by low pH due to a reduction in low molecular weight substrate uptake by the cell (Mrozik et al. 2004, Thongbai et al. 2006). Conversely, the antagonistic effect in the GH treatment, relative to the OA treatment, for bulk water LAP activity, which was the dominant response in all incubations (Table 2, see also Supplement 2 at www.int-res.com/articles/suppl/a079p049_supp.pdf), may reflect indirect effects on enzyme production. For example, an increase in temperature may stimulate the activity and growth of heterotrophic protists (Sarmento et al. 2010 and references therein), with grazing on bacteria reducing bulk enzyme production and also BSP, as in the later stages of incubations 2 to 4 (Fig. 3).

The most consistent response observed was an increase in bulk water protein hydrolysis under lower pH, as previously reported (Grossart et al. 2006, Piontek et al. 2013, Endres et al. 2014). The resulting uncoupling of nitrogen (protein) remineralisation from carbon (glucose) arising from stimulation of LAP activity may alter the concentration and availability of dissolved inorganic and organic nitrogen in the future surface ocean. As phytoplankton and bacteria require dissolved inorganic nitrogen (Falkowski 2000,

Liu et al. 2010, Buchan et al. 2014), an increased turnover of proteins and amino acids in the future ocean may benefit surface ocean microbial communities, particularly in oligotrophic regions, whereas the potential reduction in particulate organic nitrogen export to deeper waters and corresponding increase in the carbon:nitrogen ratio may reduce the quality and lability of nutrients transferred to the mesopelagic and benthos, with possible cascade effects in the higher food web (Rossoll et al. 2012, Piontek et al. 2014). The projected warming may accelerate the heterotrophic microbial loop (Piontek et al. 2010, Passow & Carlson 2012, Endres et al. 2014), but potentially lead to a more rapid decline in bacterial numbers and secondary production as a result of substrate limitation or grazing. The resulting reduction in the export of high molecular weight macromolecules (Hopkinson & Vallino 2005) may also reduce the amount of carbon sequestered in the deep ocean (Wohlers et al. 2009, Piontek et al. 2010, Weinbauer et al. 2011), resulting in a positive feedback (Feng et al. 2009, Wohlers et al. 2009, Piontek et al. 2010) that may exacerbate acidification of the surface ocean. This research highlights the need to consider the effects of multiple climate drivers, their interactions and their variable responses to these factors in different plankton communities for robust prediction of the consequences of future global change on marine biogeochemical cycles.

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