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Effect of ocean acidification on bacterial abundance, activity and diversity in the Ross Sea, Antarctica

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ABSTRACT: Three ocean acidification experiments were conducted on water from the same location in the Ross Sea, Southern Ocean, to ascertain how surface-water mixed populations, including the microbial community, would respond to changes in pH (pH 7.80 and 7.65). Bacterial extracellular enzymes, abundances, thymidine uptake rate, the diversity of the active fraction of the bacterial community and phytoplankton diversity were measured in response to changes in pH. Bacterial abundance increased at lower pH, and the active fraction of the bacteria decreased, concurrently becoming less diverse within 8 d. However, as the active fraction of the bacterial community evolved, changes in bacterial extracellular enzyme rates occurred, with phosphatase, β-glucosidase and lipase activity increasing up to 2-fold in the acidified incubations. These results suggest that carbohydrates and lipids may be hydrolysed faster with more rapid regeneration of nutrients at lower pH. The changes observed in our experiments indicate that the bacteria in the Ross Sea adapt quickly to lower pH but that bacterial diversity will be lost. However, this loss of diversity did not adversely affect bacterial activity and in fact enhanced their ability to break down carbohydrates and lipids and recycle phosphate. These changes will alter the rate of carbon and phosphate regeneration, potentially accelerating decomposition in surface waters and short-circuiting the biological pump.

KEY WORDS: Ocean acidification · Bacterial abundance · Extracellular enzymes · Bacterial diversity · RNA-T-RFLP · Ross Sea

INTRODUCTION

Marine bacteria remineralise 75 to 95% of the available organic carbon in the upper oceans (Martin et al. 1987, Boyd et al. 1999), using either substrate-specific cell-bound ectoenzymes or by releasing extracellular enzymes. Microbes can only transport small molecules across their cell membrane (Weiss et al. 1991), and therefore, the production of small monomers by extracellular enzymes is essential for bacterial growth and nutrient recycling (Chróst & Rai 1993, Hoppe 1993, Arnosti 2003, 2011). However, extracellular enzymes are not buffered in the cell, and their activity is directly affected by changes in the ambient environment.

The world's oceans have absorbed about 30 to 40% of anthropogenically emitted CO_2 from the atmosphere since the beginning of the industrial era (Feely et al. 2004, Sabine et al. 2004). The uptake of this CO_2 alters the ocean's carbonate chemistry, and as a

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consequence, the pH is reduced in the upper ocean (Caldeira & Wickett 2003, Raven et al. 2005). Most enzymes are sensitive to temperature and pH (Tipton & Dixon 1979), and therefore, changes in surface ocean pH may influence their activity.

At present, little is known about how extracellular enzymes, and the bacteria that produce them, respond to changes in surface ocean pH (Liu et al. 2010, Joint et al. 2011). Changes in surface ocean pH are hypothesised to alter bacterial cell numbers (abundance), activity (carbon uptake rates and extracellular enzyme production) and community composition (Liu et al. 2010, Joint et al. 2011). Although total bacterial abundance changes due to pH have not been observed in ocean acidification experiments (Liu et al. 2010, Joint et al. 2011), bacterial abundance is known to alter in response to phytoplankton bloom dynamics (Rochelle-Newall et al. 2004, Grossart et al. 2006, Allgaier et al. 2008). Higher growth rates and abundance of bacteria attached to phytoplankton cells at low pH (700 ppm CO_2) have been observed (Grossart et al. 2006), whereas several other studies have found no significant differences in total bacterial abundances with increasing CO_2 concentrations (Yamada et al. 2010, Arnosti et al. 2011, Teira et al. 2012). The response of bacterial activity has also shown contradictory results. Coffin et al. (2004) and Allgaier et al. (2008) found no significant difference in bacterial cell-specific carbon uptake rates for free-living or attached bacteria under super- and sub-ambient CO_2 concentrations (350, 700 and 1050 ppm); this contradicts the results from Grossart et al. (2006), which show a pronounced effect on cell-specific carbon uptake rates of attached bacteria in 700 ppm CO_2.

Bacterial extracellular enzyme activities have been examined in a number of studies (Liu et al. 2010, Joint et al. 2011). Experiments using water from different marine systems demonstrated that protease and lipase appeared to be the most sensitive to pH changes, whereas β-, α-glucosidase and phosphatase showed very little change in activity (Yamada & Suzumura 2010). Grossart et al. (2006) observed that total protease activity was higher as a function of CO_2 concentration, as was glucosidase activity (both α and β), although the results were not statistically significant. Seawater samples and phytoplankton cultures showed higher rates of extracellular glucosidase activity under elevated CO_2, which significantly accelerated the degradation of polysaccharides, increasing the availability of glucose for bacterial uptake (Piontek et al. 2010). Arnosti et al. (2011) investigated the effect of CO_2 concentrations on the degradation of 4 common polysaccharides by extracellular enzymes and found that activity varied with both CO_2 concentration and the phase of the phytoplankton bloom (Arnosti et al. 2011). Although an increase in protease activity with decreasing pH has been consistent in most experiments, the response of bacterial activity to changes in CO_2 has been contradictory.

Few studies have investigated how the bacterial community composition changes with decreasing pH. Grossart et al. (2006), using denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene, reported that only minor differences were seen in the total bacterial community with respect to changing CO_2 levels. However, the bacterial community analysis was not differentiated into free-living or particle-associated bacteria, and changes in bacterial activity were only seen when the bacterial community was differentiated into free-living and attached, which may explain why no bacterial compositional changes were detected (Grossart et al. 2006). DGGE analysis by Arnosti et al. (2011) revealed that the composition and function of the bacterial community altered under different CO_2 levels, with a divergent microbial community developing in the 190 and 700 ppm CO_2 incubations that was distinct from the initial bacterial community. Such bacterial community shifts under increased CO_2 have also been shown in biofilms on corals (Meron et al. 2011, Witt et al. 2011).

These observed changes in bacterial community composition are not unexpected because bacterial composition is known to vary with phytoplankton species, growth and the physiological state (Grossart et al. 2005). Pinhassi et al. (2004) showed that quantitative and qualitative differences in phytoplankton species composition led to pronounced differences in bacterioplankton species composition (Pinhassi et al. 2004). Bacterial diversity has also been shown to change during the progression of a phytoplankton bloom as bacteria respond to the different substrates released by the phytoplankton (Eilers et al. 2000, Riemann et al. 2000, Tada et al. 2011).

The response of non-calcifying phytoplankton species composition to changes with increasing CO_2 is not as well characterised as that of calcifying species. Tortell et al. (2002) provided the first evidence that the relative abundance of non-calcifying species of phytoplankton changed with increasing CO_2. The phytoplankton communities became dominated by *Phaeocystis* sp. and diatoms, and diatoms became especially dominant at the highest CO_2 concentration (Tortell et al. 2002). Feng et al. (2009) reported that diatoms became dominant at the highest CO_2 concentration, using natural assemblages from the North Atlantic annual spring bloom.

CO_2-induced changes in bacterial activity may reflect a range of drivers from a direct effect on enzyme velocity to indirect effects such as changes in phytoplankton community composition.

Three separate experiments were carried out on water from the same water mass in the Ross Sea, Southern Ocean, to ascertain how surface-water mixed populations, including the microbial community, would respond temporally to changes in pH. Specifically, the experiments focused on how the bacterioplankton reacted to increasing CO_2 concentration in terms of abundance, activity and diversity (the active fraction of the bacterial community) and on changes in the phytoplankton community composition. These experiments are the first ones carried out in the Ross Sea, Southern Ocean, on natural assemblages that measured both bacterial diversity and activity and phytoplankton composition.

MATERIALS AND METHODS

Experimental set-up on board RV 'Tangaroa' in the Ross Sea, Antarctica

A conductivity-temperature-depth (CTD) instrument (Seabird Electronics SBE-911) and a rosette fitted with 24 Niskin bottles (10 l each) was used to collect 240 l of surface seawater for each experiment (1 to 3) at each site location in the Ross Sea area, Antarctica (Fig. 1).

The seawater was filtered through a 200 µm mesh to remove the large grazers and divided sequentially into six 20 l cubitainers, which were sealed without a headspace. These were incubated on deck in flow-through incubators at *in situ* temperature under 25% incident photosynthetically available radiation. Two of the cubitainers were left untreated and were classed as controls at ambient levels of CO_2, while in the remaining 4 treatments, the pH was adjusted using HCl (0.1 M HCl in 0.6 M NaCl). The initial conditions were determined from the measured pH on the National Bureau of Standards scale (pH_{NBS}) converted to pH_T (total scale, see Table 1), and alkalinity as estimated from the *in situ* temperature and salinity using the Lee et al (2006) algorithms. The target conditions of pH 7.80 (~700 µatm CO_2) and pH 7.65 (~1000 µatm) were attained by the addition of HCl (0.1 M HCl in 0.6 M NaCl). pH_{NBS} was measured after acid addition and mixing, and also at the end of the experiment in each duplicate incubation using a

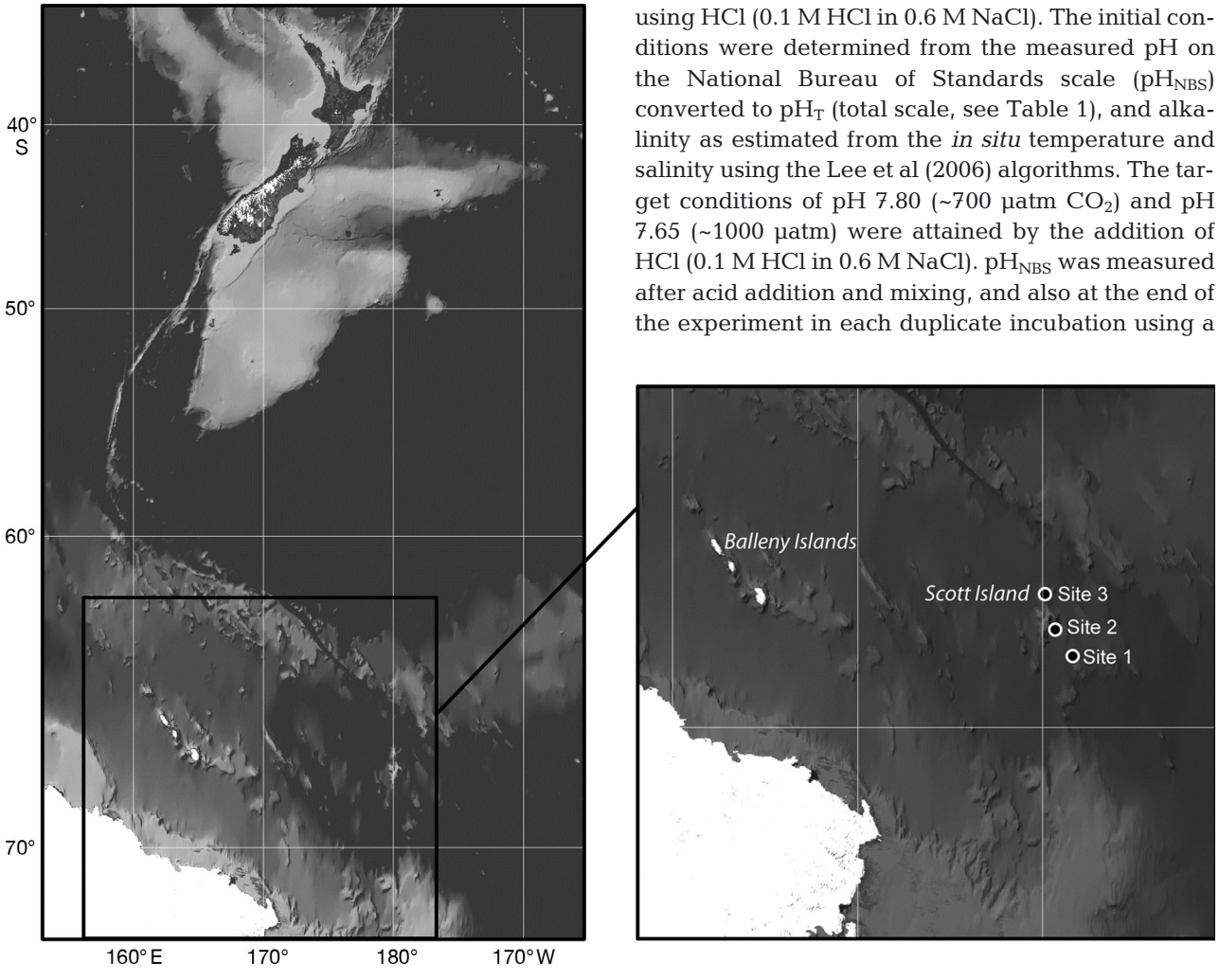

Fig. 1. Sites where ocean acidification experiments were conducted in the Ross Sea, Southern Ocean, Antarctica

calibrated pH probe (826 pH mobile and 827 pH Lab, Metrohm AG, pH precision ± 0.003). Each treatment was conducted in duplicate cubitainers, denoted A and B. Alkalinity for each control was confirmed by subsequent measurements of samples collected on Day 0 in duplicate for each experiment. The initial and final pH_T and XCO_2 (the mole fraction of CO_2 in air) were calculated (Table 1) using the CO_2 speciation calculator software (Hunter 2007). Adjustment of the carbonate system by acid addition results in a decrease in alkalinity but no change in dissolved inorganic carbon, whereas bubbling with a high CO_2 mixture has the reverse effect on these 2 parameters (Riebesell et al. 2010). Although bubbling is more representative of ocean-atmosphere exchange of CO_2 and its effect upon the carbonate system, we were primarily interested in the influence of changes in H^+ on enzyme velocity and also wanted to avoid any artefactual effects of turbulence on particles and bacterial activity. At the start of the experiment (Day 0), the starting water was sampled in duplicate, and then each cubitainer was sampled on Days 3, 5 and 8 during the on-board incubation.

Bacterial and phytoplankton cell numbers

Samples for analysis of bacterial and phytoplankton numbers were collected in duplicate from each cubitainer in the experiments and were frozen immediately at −80°C (Hall et al. 2004) for up to 10 wk prior to analysis. Bacterial numbers were determined using flow cytometry techniques, using the DNA stain SybrGreenII™ (Invitrogen) and incubating in the dark for 10 min before analysis. Prior to analysis, 50 µl of BD Trucount™ (BD Biosciences) beads was added to each sample as a tracer. Samples were run on low flow on a FACScan with CellQuest v3.1 software (BD Biosciences). The sheath fluid was 0.1 µm filtered seawater, and the sample volume analysed was calculated using the BD Trucount™ bead numbers (Lebaron et al. 1998, Hall et al. 2004). Phytoplankton numbers were analysed immediately after thawing on the FACScan flow cytometer using the natural fluorescence of chlorophyll *a* (chl *a*), with 0.1 µm filtered seawater as sheath fluid. BD Trucount™ beads were again used to measure the sample volume analysed.

Phytoplankton identification

Water samples (500 ml) collected from Days 0 and 8 were preserved with 10 ml of 1% acidified Lugol's iodine solution. Samples were stored in dim light until cell counting. Identification and enumeration of phytoplankton taxa in Lugol samples were made using a Nikon Diaphot-TMD inverted light microscope (Nikon) as described by Chang & Gall (1998) and Chang et al. (2003). Phytoplankton cells were broadly classified into diatoms, dinoflagellates, microflagellates and monads. The percentage of each of

Table 1. Carbonate chemistry measurements and calculated values for each experiment and treatment at the start and end of the experiments. pH_{NBS}: pH on the National Bureau of Standards scale; pH_T: total scale; XCO_2: the mole fraction of CO_2 in air

	Parameter	Expt 1		Expt 2		Expt 3	
		Initial	Final	Initial	Final	Initial	Final
	Experimental temperature	−1.74	−1.74	−1.60	−1.60	−1.08	−1.08
	Salinity	33.63	33.63	33.64	33.64	33.79	33.79
	Measured initial alkalinity (AT) (µmol kg^{-1})	2129		2126		2140	
Control	Measured pH_{NBS}	8.10	8.08	8.15	8.05	8.05	8.03
(~385 ppm)	Calculated AT using pH_{NBS} (µmol kg^{-1})	2129		2126		2296	
	Calculated pH_T[a]	8.06	8.02	8.11	8.00	8.02	8.00
	Calculated XCO_2	341	383	302	394	387	407
Treatment	Measured pH_{NBS}	7.79	7.84	7.78	7.81	7.80	7.79
(nominal 700 ppm)	Calculated AT using pH_{NBS} (µmol kg^{-1})	2041	2061	2019	2058	2067	2072
	Calculated pH_T[a]	7.75	7.78	7.74	7.76	7.76	7.75
	Calculated XCO_2	708	671	717	693	704	716
Treatment	Measured pH_{NBS}	7.70	7.71	7.62	7.46	7.64	7.67
(nominal 1000 ppm)	Calculated AT using pH_{NBS} (µmol kg^{-1})	2016	2027	1977	1965	2027	2040
	Calculated pH_T[a]	7.65	7.64	7.58	7.41	7.60	7.63
	Calculated XCO_2	878	915	1044	1540	1009	946

[a]Calculated using experimental temperature

these groups was calculated using the totals obtained by microscopic counting.

Chlorophyll *a* analysis

To assess changes in phytoplankton biomass during the experiments, samples for total chl *a* were filtered in duplicate under low light conditions, and the filters were frozen and stored at −80°C until analysis within 10 wk of collection. These samples were analysed using a standard acetone extraction and fluorometric analysis (APHA 1998).

Bacterial production

Bacterial production in each of the treatments was measured in triplicate using the tritiated thymidine method (Hall & Safi 2001). Each sample was incubated at the experimental temperature for 3 to 5 h, with no adjustment of pH before or during the incubation, with the samples being processed using a TCA precipitation and microcentrifuge method. Bacterial production rates for each incubation were calculated using a conversion factor of 2.4×10^{18} cells mol^{-1} thymidine incorporated and an estimate of 20×10^{-15} g C $cell^{-1}$ using the bacterial numbers measured by flow cytometry.

Extracellular enzyme activity

Extracellular enzyme assays were carried out in triplicate for each cubitainer on board. Extracellular enzyme activity in each sample was measured using either 4-methylumbelliferone (MUF) or 4-methylcoumarinyl-7-amide (MCA) fluorogenic substrates following the methods of Hoppe (1983, 1993). Protease, β-glucosidase, phosphatase, chitinase and lipase activities were measured using L-leucine-7-amino-4-methylcoumarin, 4-methylumbelliferyl β-D-glucopyranoside, 4-methylumbelliferyl phosphate, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide and 4-methylumbelliferyl oleate, respectively (all obtained from Sigma). All substrates and the 2 fluorophores (MUF and MCA) were dissolved in 1% 2-methoxyethanol (Sigma) to 1.6 mM for the substrates and 200 µmol l^{-1} for the fluorophores. Each sample was pipetted in triplicate into 96 black well plates (Nunc), and substrates were added to these samples to give a final concentration of 39 µM, which was determined to be the optimum concentration for calculating the maximum velocity of enzyme hydrolysis in these samples (data not shown). Control enzymes were added in duplicate to wells containing the corresponding substrate to check for activity. A range of concentrations (0 to 8 nmol) of either the MUF or MCA fluorophore was also measured so that the amount of fluorescence detected could be converted into nmol fluorophore. The 96 well plates were incubated in a fluorescent plate reader (Turner BioSystems) at 15°C for 6 h, and the fluorescence was measured (excitation 365 nm and emission 410 to 460 nm) every 5 min. The maximum velocity of enzyme hydrolysis was calculated for each sample and for each enzyme using the Michaelis-Menten equation in nmol fluorescence produced l^{-1} sample h^{-1}.

Bacterial diversity

Bacterial diversity prior to and 8 d after acidification was assessed using terminal restriction fragment length polymorphism (T-RFLP) (Osborn et al. 2000, Osborn & Smith 2005). The T-RFLP analysis was performed on total RNA (metabolically active bacteria) (Gentile et al. 2006) to give an indication of which bacterial populations were metabolically active. One litre of seawater (in duplicate) at the start of the experiment and 1 l of each of the incubations on Day 8 was filtered through a 0.22 µm polycarbonate filter (Millipore) and stored in RNA*later*™ (Ambion) at −80°C on board. The filter was removed from RNA*later*™ and cut in half aseptically, and the total RNA was extracted using the RNeasy mini kit (Qiagen) with several modifications. The filter was cut in pieces and placed in 'Lysing matrix E' tubes ('Fast DNA Spin kit for soil' [MP Biomedicals]) with 200 µl sterile phosphate-buffered saline containing acid-washed 0.1 mm glass beads and 600 µl of RLT buffer from the RNeasy kit. The tubes were secured in a Hybaid RiboLyser (Hybaid) and agitated for 30 s (setting 6), after which the tubes were cooled on ice for 90 s and the cycle was repeated twice. The tubes were centrifuged at $10\,000 \times g$ for 1 min, after which the supernatant was removed and an equal volume of freshly made 70% molecular biology grade ethanol (Sigma) was added and mixed. The RNA extraction was then continued from step 2 in the RNeasy mini kit instructions. The total RNA concentration was measured using RiboGreen™ (Invitrogen). A 100 ng aliquot of total RNA was converted to cDNA using random hexamers and the SuperScript III First-Strand Synthesis as per the manufacturer's instructions (Invitrogen). The cDNA concentrations

were measured using PicoGreen™ (Invitrogen). Bacterial community profiles were generated from the cDNA, and the 16S rRNA gene was amplified using iProof High-Fidelity DNA polymerase master mix system (Biorad). Reactions were prepared according to manufacturer's instructions using 1 µl of cDNA as a template in a 50 µl reaction with 2 µl of F27-6FAM (10 µM) and R1392-VIC (10 µM) (Lane 1991). The PCR conditions were as follows: denaturing at 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1.5 min, with a final extension at 72°C for 7 min. PCR reactions were run on a 1% agarose gel to verify the presence of a single amplicon of the correct size. PCR products were quantified using PicoGreen™, and 500 ng of each PCR product was digested using *Alu*I according to the manufacturer's instructions (Fermentas). The digestions were cleaned using the DNA Clean and Concentrate kit (Zymo) per the manufacturer's instructions and quantified again using PicoGreen™. These digestions were diluted to 12 ng μl^{-1}, and a 1.2 µl aliquot of each was sent to the Allan Wilson Centre Genotyping service (Massey University, New Zealand) for capillary separation on an ABI3730 Genetic Analyser (Applied Biosystems). Genotype files were analysed using GeneMapper software (v3.7) (Applied Biosystems) with the following settings: AFLP Analysis method (analysis range: 50 to 1400 base pairs, normalisation by sum of signal, bin width 1.0 base pair, name alleles using [absolute] labels threshold at 50%: 1 or 0; all other settings left as default). The resulting binary table of genotyping data was loaded into BioNumerics software (v5.10) (Applied Maths). The numbers of terminal restriction fragments (T-RFs) were calculated for each sample. The RFLP profiles were compared using BioNumerics software by creating a similarity matrix from selected samples using the simple band-matching coefficient, and dendrograms were resolved using the UPGMA algorithm in BioNumerics.

Statistical analysis

Statistical analysis (ANOVA with Fisher's least significant difference post hoc analysis) was carried out with Statistica (v8) StatSoft.

RESULTS

Biological and chemical parameters of the starting waters for each experiment are shown in Table 2. The temperature and salinity of the water at each site were very similar, although the phytoplankton community composition and some of the biological parameters varied slightly among sites (Table 2). Therefore, the results from each experiment are described separately below. The pH in each cubitainer had not altered significantly from the starting pH (maximum difference: −0.05) at the end of the 8 d period (Table 1), except for the controls and pH 7.65 incubations in Expt 2, which decreased in pH by 0.1 and 0.16, respectively (Table 1).

Chlorophyll *a*, phytoplankton numbers and identification

Expts 1 and 2 initially had similar chl *a* concentrations of 0.41 and 0.35 µg l^{-1}, but Expt 3 had only about half, 0.19 µg l^{-1} (Fig. 2). Chl *a* concentrations increased in the control and acidified incubations in all experiments, although at different rates, with a 2.6-fold increase in Expt 3, lower than in the other 2 experiments, a 6.5-fold increase in Expt 1 and a 9.3-fold increase in Expt 2. The trends in chl *a* concentrations in all incubations (control and acidified) were consistent with increases in phytoplankton cell numbers ($r = 0.73$ to 0.85, $p < 0.05$ for all comparisons across the 3 experiments). The influence of lower pH on phytoplankton growth rates varied, with higher

Table 2. Analysis of starting seawater for each experiment

Parameter	Expt 1	Expt 2	Expt 3
Date collected (2008)	1 Mar	2 Mar	7 Mar
Temperature (°C)	−1.74	−1.61	−1.08
Salinity	33.63	33.64	33.79
pH	8.10	8.15	8.05
Alkalinity (µmol kg^{-1})	2129	2126	2140
Chlorophyll *a* (µg l^{-1})	0.41	0.35	0.19
Total phytoplankton (cells ml^{-1})	6436	4929	3871
Diatoms (%)	12	32	11
Dinoflagellates (%)	3	4	4
Microflagellates (%)	43	29	17
Monads (%)	41	35	68
Bacteria ($\times 10^5$ cells ml^{-1})	2.8	2.1	1.4
Bacterial production (fg C $cell^{-1}$)	0.083	0.141	0.114
Protease (nmol l^{-1} h^{-1})	4.1	6.9	2.7
β-glucosidase (nmol l^{-1} h^{-1})	5.1	6.3	4.3
Chitinase (nmol l^{-1} h^{-1})	19.0	21.5	13.5
Phosphatase (nmol l^{-1} h^{-1})	73.0	76.6	145.7
Lipase (nmol l^{-1} h^{-1})	2.6	1.5	1.8

rates in the acidified incubations in Expts 1 and 3 but lower than the control in Expt 2 (Table 3). Phytoplankton groups changed in the experiments (Table 3), although changes were not statistically significant between the controls and the treatments on Day 8 due to large variations between duplicate cubitainers. Expt 1 initially had approximately equal numbers of microflagellates and monads, with only 12% diatoms, whereas diatoms dominated after 8 d at 66% in the control and 92% in pH 7.65 (Table 3). Phytoplankton cells at the start of Expt 2 consisted of nearly equal numbers of diatoms, microflagellates and monads, with greater dominance by diatoms in the control (85%) than in the acidified incubations (51 to 66%) by Day 8. Expt 3 was dominated at the start by monad cells, whereas by Day 8, diatoms had increased in both control (45%) and acidified incubations (33 to 62%).

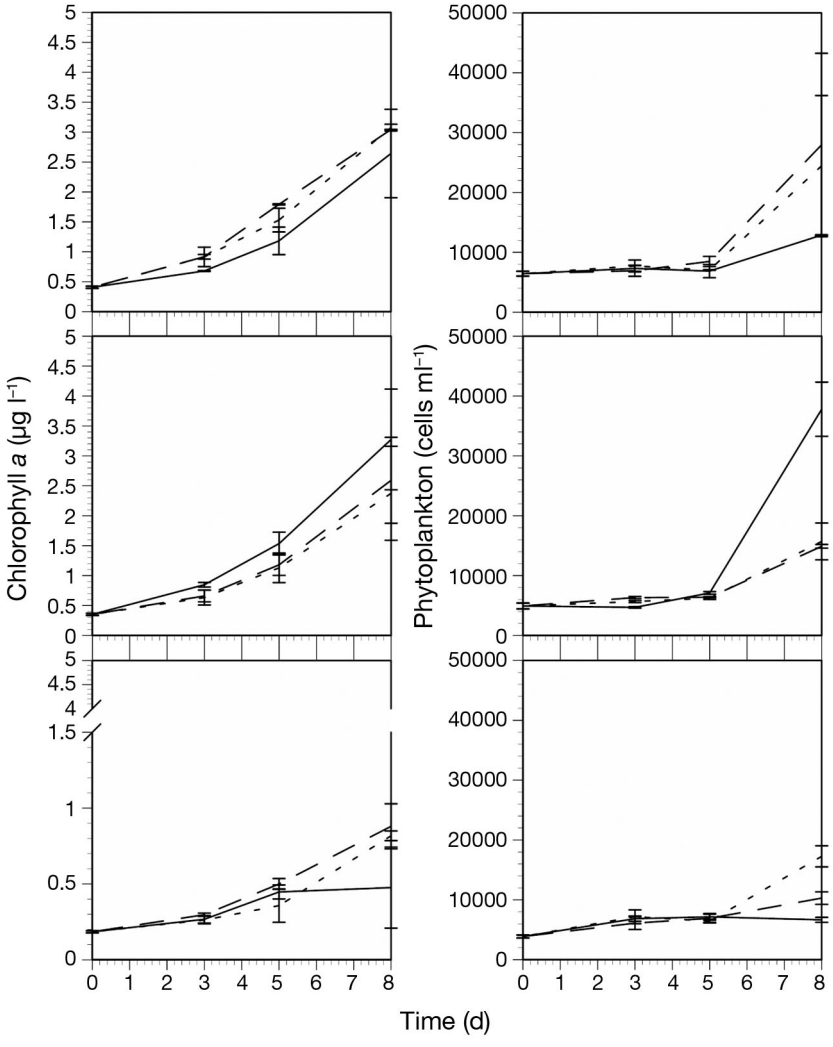

Fig. 2. Chlorophyll *a* concentrations and phytoplankton cell numbers in the control (solid line), pH 7.80 (long-dashed line) and pH 7.65 (short-dashed line) incubations over an 8 d period. Top panels: Expt 1; middle panels: Expt 2; bottom panels: Expt 3. Error bars = 1 SD

Bacterial numbers

Bacterial numbers in Expt 1 increased by up to 4-fold in the acidified incubations over 8 d but did not increase in the control incubations (Fig. 3), whereas in Expt 2, bacterial numbers increased by at least 4-fold in all incubations. In Expt 3, there was a doubling in bacterial numbers in the acidified incubations, whereas numbers declined slightly in the control by Day 8. The results of the 3 experiments indicated that bacteria were able to tolerate and grow in the lower pH conditions, although generally a 3 d delay in growth was seen in all incubations (Fig. 3). Although the trends differed among the 3 experiments, bacterial numbers showed an increase in all low pH incubations relative to the initial water, whereas the control incubations showed variable increases and decreases across the 3 experiments.

Bacterial production

Bacterial production rates increased over the first 5 d in all incubations, preceding the increase in bacterial abundance, which generally occurred after Day 5, and decreasing again to initial levels on Day 8 (Fig. 3). The bacterial production rates in Expts 1 and 2 on Days 3 and 5 were higher than in Expt 3. In Expt 1, there was a significant difference ($p < 0.05$) between the acidified incubations and controls on Day 3 only. In Expt 2, there was no significant difference between the acidified incubations and controls on Days 3 and 5, but there was a significant difference ($p < 0.05$) between the control and acidified incubations on Day 8, without a difference between the 2 pH treatments. In Expt 3, there was a significant difference ($p < 0.05$) between pH 7.65 and the control and pH 7.80 on Day 3 only. There was no difference between the pH 7.80 and control treatments. Overall, there

Table 3. Phytoplankton groups identified and counted microscopically (average % of total cells) in the starting water of each experiment and on Day 8 in each treatment and control, and the phytoplankton growth rate over the 8 d incubation period (d^{-1})

Phytoplankton group	Starting seawater	Control	pH 7.80	pH 7.65
Expt 1				
Diatoms	12	66	79	92
Dinoflagellates	3	1	0	0
Microflagellates	43	26	11	5
Monads	41	7	10	3
Phytoplankton growth rate		0.33	0.71	0.64
Expt 2				
Diatoms	32	85	66	51
Dinoflagellates	4	0	0	0
Microflagellates	29	8	30	38
Monads	35	7	3	11
Phytoplankton growth rate		0.95	0.53	0.56
Expt 3				
Diatoms	11	45	62	33
Dinoflagellates	4	2	1	0
Microflagellates	17	13	19	21
Monads	68	40	19	45
Phytoplankton growth rate		0.26	0.47	0.72

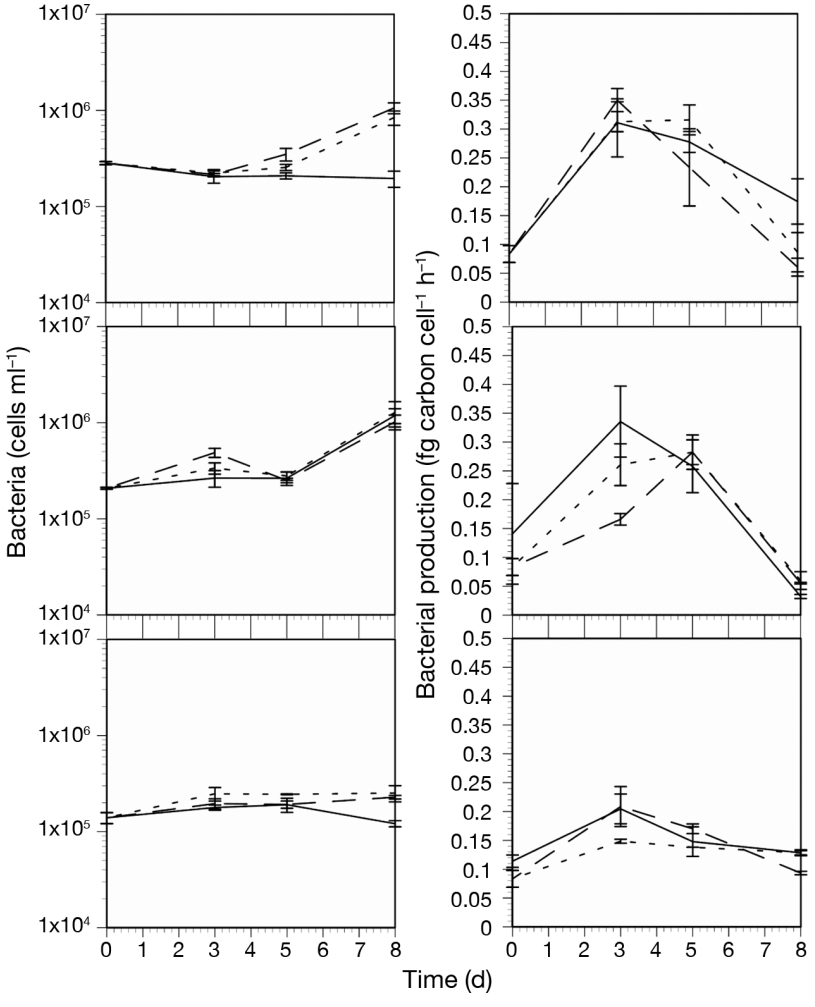

Fig. 3. Bacterial numbers and bacterial production in the control (solid line), pH 7.80 (long-dashed line) and pH 7.65 (short-dashed line) incubations over an 8 d period. Top panels: Expt 1; middle panels: Expt 2; bottom panels: Expt 3. Error bars = 1 SD

was no consistent response to the lower pH treatments for bacterial production.

Bacterial extracellular enzyme activity

In Expt 1, all extracellular enzyme activities increased relative to Day 0 over 8 d (Fig. 4, Tables 4 & 5). Total protease activity in the acidified incubations was not significantly different from the controls, whereas total β-glucosidase activity was significantly higher ($p < 0.05$) in the acidified incubations on Days 5 and 8 compared to the control. Total chitinase activity in Expt 1 increased throughout and was higher in the acidified incubations compared to the control (Fig. 4). This difference was significant on Day 5 between the control and pH 7.65 and on Day 8 between the control and both acidified incubations. Total phosphatase activity decreased by 25 to 33% on Days 3 and 5 but increased to 3-fold the initial values by Day 8. Total phosphatase was significantly higher ($p < 0.05$) in the acidified incubations relative to the controls by Day 8, and there was a significant difference between the lower pH treatments ($p < 0.05$), with the highest activity in pH 7.65. There was no difference between total lipase activity on Days 3 and 5 among all the incubations, although there was a significant difference ($p < 0.05$) between the control and acidified incubations on Day 8, with the highest lipase activity at pH 7.65.

Total enzyme activity in Expt 2 followed a different pattern from that observed in Expt 1, with maximum

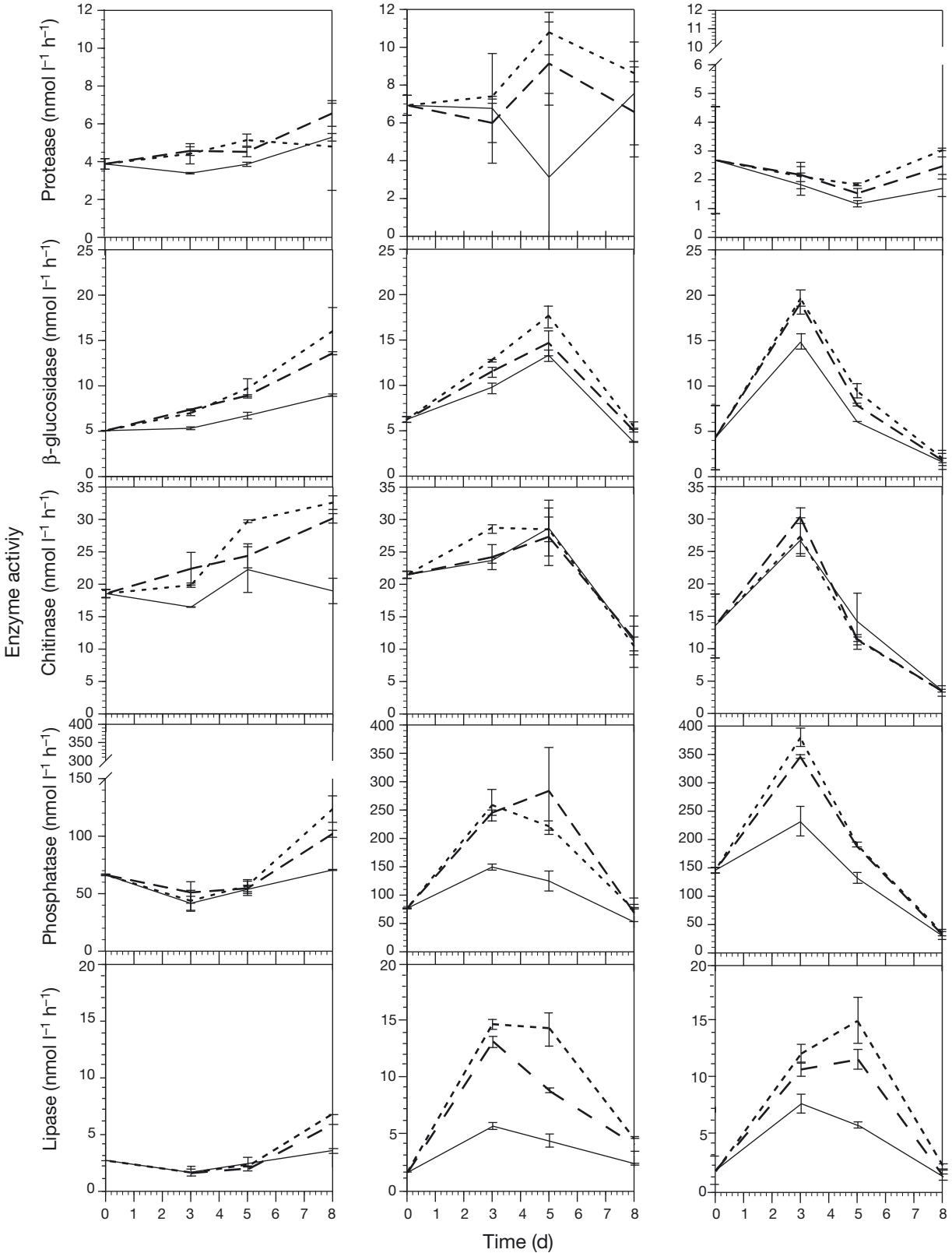

Fig. 4. Bacterial extracellular enzyme activity (mean ± SD) in each incubation of the control (solid line), pH 7.80 (long-dashed line) and pH 7.65 (short-dashed line) on Days 0, 3, 5 and 8 in Expt 1 (left panels), Expt 2 (centre) and Expt 3 (right panels)

Table 4. Bacterial extracellular enzyme comparisons (p-values) on each day and between the treatments and controls for each experiment. p-values in **bold**: $p < 0.05$; values in ***bold italics***: $p < 0.01$; not significant comparisons are not shown

			Expt 1						Expt 2						Expt 3					
			Day 3		Day 5		Day 8		Day 3		Day 5		Day 8		Day 3		Day 5		Day 8	
	pH		7.80	7.65	7.80	7.65	7.80	7.65	7.80	7.65	7.80	7.65	7.80	7.65	7.80	7.65	7.80	7.65	7.80	7.65
Protease	Day 5	Control									**0.029**	***0.009***						**0.034**		
		pH 7.80																		
	Day 8	Control																	**0.018**	***0.001***
β-glucosidase	Day 3	Control							**0.033**	***0.002***					***<0.01***	***<0.01***				
		pH 7.80																		
	Day 5	Control			**0.046**	**0.012**					***<0.01***						**0.039**	***0.002***		
		pH 7.80									***0.002***									
	Day 8	Control					***0.001***	***<0.01***					**0.036**							
		pH 7.80						**0.032**												
Chitinase	Day 3	Control	***0.008***																	
		pH 7.80																		
	Day 5	Control			***0.002***															
		pH 7.80			**0.014**															
	Day 8	Control					***<0.01***	***<0.01***												
Phosphatase	Day 3	Control							***0.008***	***0.004***					***<0.01***	***<0.01***				
		pH 7.80														**0.017**				
	Day 5	Control									***<0.01***	***0.008***						***0.001***	***0.001***	
	Day 8	Control					***0.001***	***<0.01***												
		pH 7.80						**0.013**												
Lipase	Day 3	Control							***<0.01***	***<0.01***					***0.008***	***0.001***				
		pH 7.80								**0.039**										
	Day 5	Control									***<0.01***	***<0.01***					***<0.01***	***<0.01***		
		pH 7.80										***<0.01***						***0.004***		
	Day 8	Control					***<0.01***	***<0.01***					**0.022**	***0.009***						
		pH 7.80						**0.013**												

activities observed on Days 3 or 5. Total protease activity was variable among the individual incubations but was significantly higher in the acidified incubations relative to the controls on Day 5 (Table 4). β-glucosidase was significantly higher ($p < 0.05$) in pH 7.65 and 7.80 on Day 3, relative to the control. Total chitinase activity was not significantly different on any day. Total phosphatase activity was significantly higher ($p < 0.01$) in the acidified incubations compared to the control on Days 3 and 5. Total lipase activity in the acidified incubations was significantly higher ($p < 0.05$ and $p < 0.01$) than in the controls across the 8 d period.

The temporal trend in Expt 3 was similar to that of Expt 2, with maximum enzyme activities on Days 3 or 5, except in the case of protease, which did not change significantly between Days 0 and 8 (Fig. 4, Table 4). Total protease activity was significantly greater on Days 5 and 8 in pH 7.65 ($p < 0.05$ and $p < 0.01$) and on Day 8 in pH 7.80 ($p < 0.05$) in Expt 3. Total β-glucosidase activity was significantly higher in the acidified incubations on Days 3 and 5 only in Expt 3. Total chitinase activity did not differ significantly in Expt 3. Both total phosphatase and lipase activity were significantly higher ($p < 0.01$) on Days 3 and 5 in the acidified incubations compared to the control in Expt 3.

There was a general shift in the timing of peak extracellular enzyme activity among experiments, with peak activity in both the control and acidified incubations occurring at a later stage on Day 8 in Expt 1 and on Days 3 to 5 in Expts 2 and 3 (Fig. 4, Table 4). The same trend is reflected in the deviation of extracellular enzyme activity between the control and acidified

Table 5. Summary of the changes in each individual parameter in the acidified incubations compared to the control incubations. ↑: parameter increased in the acidified incubations compared to the control; ↓: decrease; =: no change was observed

Parameter	Expt 1	Expt 2	Expt 3
Total phytoplankton numbers	↑	↓	↑
Chlorophyll *a*	↑ / =	=	↑ / =
Diatoms	↑	↓	= / ↑
Dinoflagellates	=	=	=
Microflagellates	↓	↑	=
Monads	=	↑ / ↓	=
Total bacterial numbers	↑	=	↑
Bacterial production	↓	=	= / ↓
Bacterial extracellular enzymes			
Phosphatase	↑	↑	↑
Chitinase	↑	=	=
β-glucosidase	↑	↑	↑
Lipase	↑	↑	↑
Protease	=	=	↑
Bacterial terminal restriction fragments (T-RFs)	↓	↓	= / ↓

incubations. The amount of total protease activity among the 3 experiments was higher in Expts 1 and 2 compared to Expt 3, and different trends within each experiment were observed. Total β-glucosidase activity peaked on different days in each experiment, as outlined above, but reached similar levels in all experiments and was significantly higher in the acidified incubations. Chitinase activity was only significantly different from the control in Expt 1 and was highest in the acidified incubations. Total phosphatase activity was lower in Expt 1 compared to Expts 2 and 3. Phosphatase activity was significantly higher in the acidified incubations compared to the controls in all the experiments. Lipase activity was generally significantly higher in the acidified incubations in all the experiments. Overall, the total enzyme activity for each of the different enzymes was significantly different ($p < 0.05$) among the experiments, indicating that the enzyme activity in each experiment (controls and acidified incubations) responded differently.

Bacterial diversity

The T-RFLP RNA profiles, which contain only the metabolically active fraction of the bacteria, were compared for each incubation between Days 0 and 8, and the total number of T-RFs in each profile was calculated. There was no significant difference in the number of T-RFs among the experiments on Day 0 ($p = 0.28$) (Fig. 5).

The number of T-RFs at the end of Expt 1 had increased from 35 ± 8 (±SD) to 58 ± 2 in the control and pH 7.80, with significantly lower number of T-RFs in pH 7.65 ($p < 0.05$), indicating that bacterial diversity decreased in the lowest pH. In Expt 2, the number of T-RFs had increased from 46 ± 4 to 51 ± 4 in the controls by Day 8 but decreased in pH 7.80 to 37 ± 1 ($p < 0.05$) and 43 ± 3 in pH 7.65 ($p = 0.078$, not significant). In Expt 3, bacterial T-RFs increased in all incubations, with no significant differences on Day 8. However, the number of T-RFs in pH 7.65 was lower compared to the control and pH 7.80.

Dendrograms were constructed using the bacterial community profile obtained from the T-RFLP RNA profiles (Fig. 6). In all the experiments, the control samples at the start of the experiment (labelled as the

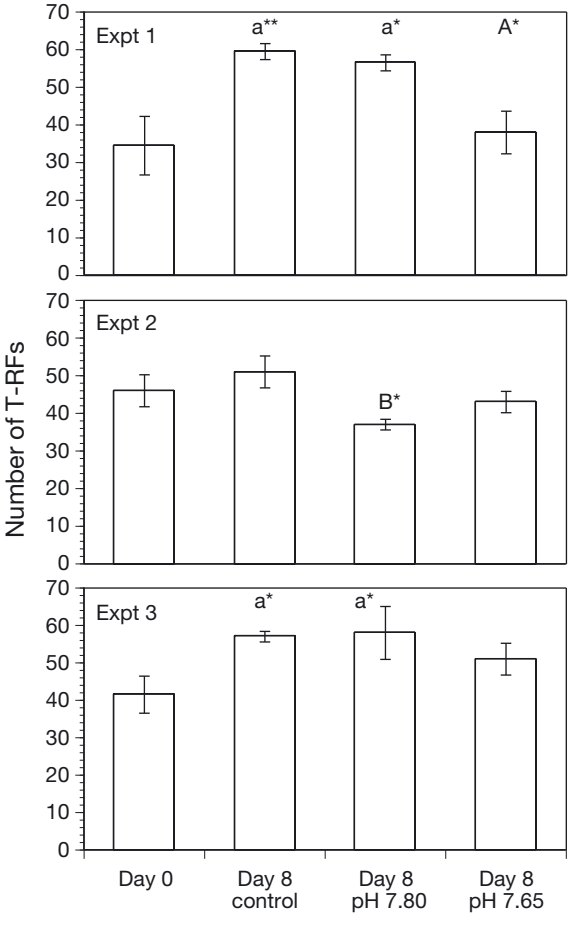

Fig. 5. Number of bacterial terminal restriction fragments (T-RFs) of the metabolically active bacteria in each of the incubations at the start (Day 0) and the end of the experiment (Day 8). Lowercase letters: comparison of Day 8 T-RF numbers in the incubations to control Day 0; significant results are indicated as $^{*}p < 0.05$, $^{**}p < 0.01$. Capital letters: comparison of T-RF numbers between Day 8 incubations; $^{*}p < 0.05$

Day 0 control) were most closely related to each other, with similarities between these 2 samples ranging from 91 to 93% (Fig. 6). In Expt 1, the acidified incubations on Day 8 at pH 7.65 (A) and (B) form 1 clade with ~94% similarity. The Day 8 control (B) is most similar to pH 7.80 (A) with ~92% similarity, forming 1 clade. This clade is joined by the Day 8 control (A) at 91% similarity. The Day 8 pH 7.80 (B) bacterial community profile is distinct from the other profiles and most closely related to the Day 0 controls (Fig. 6). In Expt 2, the bacterial profiles of the Day 8 pH 7.80 (B), pH 7.65 (A) and pH 7.80 (A) form a clade with 94% similarity. The other 3 bacterial community profiles are very different and do not form a single clade, but instead, the Day 8 control (B) is 92.5% similar to the acidified incubations clade, the Day 8 pH 7.65 (B) bacterial community is 90.5% similar to this clade, and the Day 8 control (A) profile is only 89.5% similar to the acidified incubation profiles (Fig. 6). In Expt 3, the Day 8 pH 7.80 (A and B) profiles form a single clade with 91.5% similarity between the profiles. The 2 Day 8 controls (A and B) form a single clade with 95% similarity. The 2 pH 7.65 profiles are different, with one pH 7.65 (A) being similar to the Day 8 controls (94.5%) and the other pH 7.65 (B) being distinct from the other profiles and having only 85.5% similarity with all the other profiles. The bacterial community profiling results showed that the bacterial community diversity was changing and that the communities in the acidified incubations had diverged from the Day 0 control samples and were more similar to each other than to the controls.

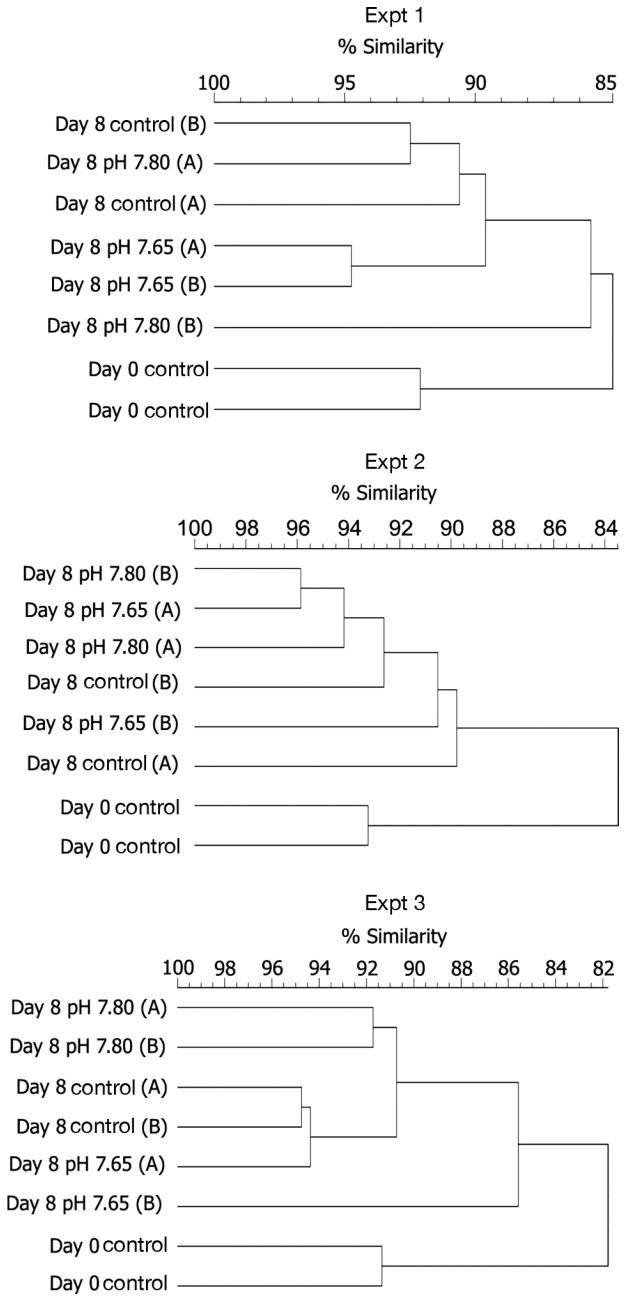

Fig. 6. Active bacterial community profiles (RNA) in each of the incubations at the end of the 8 d experiment and in the starting seawater (Day 0 controls). A and B denote the duplicate incubations

DISCUSSION

Although only minor differences were apparent in the physical and biogeochemical composition of the water in the 3 experiments, the phytoplankton communities differed (Table 2), potentially reflecting small-scale variability in the community composition. Alternatively, this may be due to temporal variation in community succession *in situ* between water collections for each experiment (Gall et al. 2001).

The phytoplankton growth rates in our experiments ranged from 0.26 to 0.99 d^{-1}, which is similar to the natural maximal growth rates of Antarctic phytoplankton species (0.32 to 0.84 d^{-1}) (Sommer 1989, Pearce et al. 2008). Although our experiments were not conducted under trace metal-free conditions, our phytoplankton growth rates are consistent with reported growth rates in Southern Ocean CO_2 incubation experiments (Tortell et al. 2002). In addition, there was a clear difference in the growth rates between the acidified incubations and controls in all experiments. In 2 experiments (Expts 1 and 3), the growth rates were higher in the acidified incubations compared to the control, indicating that phytoplankton growth was stimulated by CO_2. In 2 out of 3 experiments (Expts 1 and 3), diatoms became more

dominant, although the shift was not significant, consistent with the findings of Tortell et al. (2002) and Feng et al. (2009).

Previous research has shown that bacteria and extracellular enzymes respond differently during the progression of a phytoplankton bloom, both *in situ* and during CO_2 incubation experiments (Eilers et al. 2000, Riemann et al. 2000, Arnosti et al. 2011, Tada et al. 2011). Total bacterial numbers increased in the acidified incubations in 2 of the 3 experiments (Expts 1 and 3) (Fig. 3, Table 5) compared to the control, in contrast to other studies (Rochelle-Newall et al. 2004, Grossart et al. 2006, Allgaier et al. 2008, Yamada & Suzumura 2010). Only one other study has shown increases in total bacterial abundance in response to CO_2, during the early phase of a phytoplankton bloom (Arnosti et al. 2011). In contrast, bacterial production (thymidine incorporation), which can be interpreted as a measure of bacterial growth, was lower in the acidified treatments than in the control incubations in 2 out of the 3 experiments (Expts 1 and 3), which was surprising because bacterial numbers increased and were higher in the acidified incubations (Table 5). Bacterial production did not appear to be coupled to bacterial numbers but instead preceded the increase in bacterial numbers, generally with a lag phase of ~5 d before an increase in numbers was observed (see Fig. 2). This is consistent with the findings of Ducklow et al. (1999), who reported a lag of up to 7 d for bacterial growth in the Ross Sea.

The active fraction of the bacteria in the incubations was changing, as measured by analysing the RNA of the 16S rRNA gene. The present work is the first ocean acidification study that has measured the response of the active fraction of the microbial community. The advantage of measuring the RNA fraction of the community is that the metabolically active part of the bacterial community responds quicker to changes in the environment than the DNA fraction (active and inactive) (Gentile et al. 2006). In nearly all the acidified incubations, the number of T-RFs decreased over the 8 d period, and the community composition diverged from that of the initial starting water. The communities in the acidified incubations became more similar to each other than the control incubations. These results are consistent with those of Witt et al. (2011) (using the same bacterial community profiling technique, but with DNA [active and inactive], for coral biofilm bacteria), who showed that the bacterial community became more specialised and had fewer OTUs under high CO_2. In contrast, Meron et al. (2011) showed that after a longer incubation period, the number of OTUs increased with decreasing pH in biofilms associated with corals. The only previous studies of bacterial community composition, using DNA and different techniques, showed only minor changes in response to CO_2 but that divergent communities were starting to establish in the high CO_2 incubations after 20 d (Grossart et al. 2006, Arnosti 2011). However, none of these bacterial composition studies analysed RNA, making it hard to compare their results to our data.

By analysing the active fraction of bacteria, we detected divergence of the bacterial community in the acidified incubations from the controls (and the initial water) after only 8 d, indicating that the bacterial community was able to adapt quickly. Fewer bacterial OTUs were active in high CO_2 conditions, suggesting that the bacterial community may become more specialised, with this reduction in diversity potentially having implications for the functional capability of the bacterial community under lower pH.

Bacterial extracellular enzymes in our studies generally increased in the acidified incubations, with slight differences among the experiments (Table 5). Phosphatase, β-glucosidase and lipase increased in all 3 experiments and were highly correlated to each other, perhaps not surprisingly as these enzymes are involved in the breakdown of phytoplankton and/or bacterial cell walls (Chróst & Rai 1993, Hoppe 2003). β-glucosidase activity has also previously been shown to increase with increasing CO_2 (Grossart et al. 2006, Piontek et al. 2010). Chitinase is associated with the breakdown of chitin, and potential sources of chitin include microzooplankton, phytoplankton, zooplankton and bacteria (Bassler et al. 1991, Riemann & Azam 2002). Chitinase activity was only elevated in Expt 1, but the activity was not correlated with bacteria or phytoplankton numbers in these experiments. Protease was only elevated in 1 experiment, in contrast to previous experiments that have shown protease activity to increase with increasing CO_2 concentrations (Grossart et al. 2006, Piontek et al. 2010). β-glucosidase activity was high in our experiments, indicating that carbohydrates were being broken down, perhaps explaining the low protease activity observed. Carbohydrates have a higher nutritional value and are preferentially degraded by marine bacteria (Christian & Karl 1995). In all 3 experiments, in the acidified incubations, the β-glucosidase activity was 1.3- to 1.6-fold higher than the control, indicating that carbohydrate hydrolysis had increased with decreasing pH. The phosphatase activity in the acidified incubations increased 1.5- to 1.7-fold in the acidified incubations, showing en-

hanced phosphate recycling. Lipase activity increased ~2.0-fold in the acidified incubations compared to the control. These changes indicate that carbohydrate and lipid hydrolysis as well as phosphate recycling will be enhanced at lower pH.

Bacterial abundance increased with lower pH, and concurrently, the active fraction of the bacteria responded within 8 d, resulting in a loss of diversity. The active fraction of the bacteria probably became more specialised, with a potential decrease in functionality. However, as the active fraction of the bacterial community evolved, changes in bacterial extracellular enzymes rates were apparent, with phosphatase, β-glucosidase and lipase activity increasing up to 2-fold. These results suggest that carbohydrates and lipids may be hydrolysed faster, providing nutrients for bacterial and/or phytoplankton growth, and inorganic phosphate recycling would be enhanced. The released inorganic phosphate would add to the inorganic phosphate pool and would be available for bacterial and phytoplankton growth. The changes observed in our experiments indicate that bacteria adapt quickly to lower pH but that bacterial diversity will be lost. However, this loss of diversity did not adversely affect overall bacterial activity, and in fact, the breakdown of carbohydrates and lipids and the recycling of phosphate was enhanced in the acidified incubations. Lower pH in Ross Sea waters in the future due to anthropogenic CO_2 uptake may then lead to the loss of bacterial diversity, increased bacterial numbers, increased breakdown of carbohydrates and lipids, increased phosphate recycling and changes in phytoplankton composition. Assuming that these experiments are representative of future change in the ocean, this shift will alter the way carbon and phosphate is recycled, potentially accelerating decomposition in surface waters and short-circuiting the biological pump (Piontek et al. 2010).

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