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Effects of experimental seawater acidification on an estuarine plankton community

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ABSTRACT: The atmospheric CO_2 concentration is rising, and models predict that by the end of the century it will have increased to twice the amount seen at any given time during the last 15 million yr. This will cause a decrease in average surface water pH of 0.4, and planktonic protists will be among the organisms to be affected first by this change. We tested whether reduced pH (and increased free CO_2) would affect plankton communities over an incubation period of 14 d. In a laboratory microcosm setup using a natural plankton community from the Derwent River estuary, Australia, 2 treatments with reduced pH (8.0 and 7.7) were compared to an unaltered control of pH 8.3. An extreme pH 6.3 was included for comparison. Measured parameters included community photosynthesis, nutrient uptake and biomass build-up as well as enumeration of 25 protist taxa and quantitative HPLC of phytoplankton pigments. A major succession was seen during the 14 d, but no effects at all were found in pH treatments 8.0 and 7.7, whereas the extreme pH 6.3 clearly affected the community for all measured parameters. Thus, it is unlikely that the investigated plankton community would be significantly affected by a pH and CO_2 change as predicted for the 21st century. This has previously been found for other coastal plankton assemblages as well, and we suggest that high pH resilience is a necessity for protist species living in coastal waters with relatively large pH fluctuations.

KEY WORDS: Seawater acidification · pH · CO_2 · Climate change · Plankton · Pigment analysis

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INTRODUCTION

Estimates have put the atmospheric CO_2 concentration between 180 and 300 ppm during the past 420 000 yr of glacial and interglacial periods (Petit et al. 1999), and it has apparently not exceeded 300 ppm for the past 15 million yr (Pearson & Palmer 2000). Anthropogenic activities, including fossil fuel burning and deforestation, are now increasing the concentration at an unprecedented rate, and models predict a CO_2 concentration of 700 ppm or higher by the end of the century (Raven et al. 2005, IPCC 2007). Nearly a third of all CO_2 emitted during the last 250 yr has been taken up by the world's oceans, and this has caused an increase in seawater CO_2 and HCO_3^- and a corresponding drop in average seawater pH of ~0.1 (Sabine et al. 2004, Raven et al. 2005). If emissions continue as predicted, the average seawater pH will have dropped by an additional 0.3 to 0.4 units by the end of the century (Orr et al. 2005).

Such changes in seawater pH and CO_2 have been suggested to affect marine plankton communities in a number of ways, affecting photosynthetic rates, species composition and carbon cycling (Boyd & Doney 2002, Beardall & Raven 2004, Schippers et al. 2004). These assumptions are primarily based on the fact

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that the carboxylase reaction of algal RuBisCO is under-saturated at present day CO_2 concentrations and the understanding that seawater acidification will impede calcification processes of coccolithophorids and foraminifers (Beardall & Raven 2004, Riebesell 2004). Despite considerable scientific effort, particularly during the last 10 yr, these effects have not yet been unambiguously described, let alone understood to a level that allows global-scale inferences. Culture work has so far been restricted to a limited number of species, and the effects of seawater acidification have varied and are difficult to extrapolate to the field (Burkhardt et al. 2001, Morales et al. 2002, Rost et al. 2003, Iglesias-Rodriguez et al. 2008). Field and mesocosm work, on the other hand, is as close to *in situ* as we can get, but has so far not produced consistent results. Some experiments have shown no effects of acidification (Kim et al. 2006, Suffrian et al. 2008, Nielsen et al. 2010) whereas others have described a variety of effects (Tortell et al. 2002, Schulz et al. 2008, Feng et al. 2009). This inconsistency in community-based work is most likely caused by large functional diversity between plankton communities rather than methodological problems, meaning that it is not unlikely that different plankton communities will react differently to seawater acidification.

Thus, it is still largely unknown to what degree decreasing pH/increasing free CO_2 will affect species composition, primary production and mass transport in plankton communities. At our present state of knowledge, the best opportunity we have to overcome this lack of insight is to study many different plankton communities under future CO_2 and pH conditions. Only by investigating numerous plankton communities with different biological and physicochemical conditions will we be able to generalize on the future effects of seawater acidification and ultimately estimate the effects of predicted CO_2 changes on a global scale. The aim of this work is to investigate the effects of decreasing pH, and the associated increase in free CO_2, on a coastal plankton community. Coastal plankton communities are often highly productive, and they contribute significantly to the global primary production (Antoine et al. 1996, Field et al. 1998). They also differ from their oceanic counterparts in the relatively large pH variations sometimes observed. Coastal seawater pH has been shown to vary as much as from 7.5 to 9.6 (Macedo et al. 2001, Hansen 2002). Thus, the present study aims to investigate the response of a natural summer plankton community to seawater acidification in order to determine whether future lowered pH levels may reshape community composition and functioning.

MATERIALS AND METHODS

Seawater with a natural plankton community was collected from the surface layers of the Derwent River estuary, Tasmania (42° 53′ 12″ S, 147° 20′ 22″ E) in December 2007. On-site water temperature was 16.0 ± 0.5°C and salinity was 31.0 ± 1.0 (means ± SD). The water was immediately brought back to the laboratory, where it was filtered (250 µm mesh) to exclude larger metazoan grazers. Twelve polycarbonate bottles of 2.5 l were filled to capacity, and triplicates of 4 pH treatments were set up using 1.0 and 0.1 M HCl additions. The remaining water was filtered (0.2 µm) and stored in darkness at *in situ* temperature for later refilling. Seawater carbonate chemistry has most often been manipulated either by bubbling with gas mixtures with different concentrations of CO_2 or by acid/base additions. The first mimics atmospheric CO_2 changes more precisely, but the latter can be preferable when dealing with organisms that are sensitive to continuous bubbling (Rost et al. 2008). Here we chose the acid/base addition method because it is straightforward and based on the presence of a number of sensitive dinoflagellate and ciliate species. The pH levels of the 4 treatments were 8.3 (*in situ* pH), 8.0, 7.7 and 6.3. Treatments pH 8.0 and 7.7 were achieved immediately, whereas pH 6.3 was achieved in steps of 0.5 units at 12 h intervals. The pH 8.3 treatment worked as a control, and treatments 8.0 and 7.7 represented 2 different levels of increased atmospheric CO_2 which is likely to occur at some point during the 21st century. The pH 6.3 treatment is unrealistic in terms of anthropogenically derived CO_2 increases but served to test how an extreme pH would affect the communities. It also served to demonstrate if, and how well, the applied methods would pick up changes in the protist plankton community.

The 12 bottles were placed on a plankton wheel (1 rpm), in front of a cool white light source (100 ± 10 µmol photons m^{-2} s^{-1}, 16 h light : 8 h dark cycle), in a 16.0°C temperature controlled room. At each of the following samplings, pH was measured, appropriate subsamples were removed (see paragraph below), bottles were refilled with 0.2 µm filtered seawater (adjusted to treatment pH) from the site and pH was readjusted to treatment pH ± 0.02 using 0.1 M HCL and NaOH. The water used for refilling was 0.2 µm filtered immediately after collection and again just before use. Sampling was conducted every second day in accordance with the details provided below, and most of the parameters were measured only on Days 0, 4, 8 and 14. The experiment was terminated after 14 d. Samplings removed on average 280 ml

from the flasks, and a 10% dilution of the experiment thus occurred. The nutrient concentration in the water used for refilling was similar to that reported for the onset of the experiment (same water), so dilutions added moderate amounts of nutrients to the flasks. pH was measured every second day using an OxyGuard Handy pH-meter calibrated daily with pH 4.0 and 10.0 buffers (NBS scale).

Subsamples of 1.5 ml were removed for dissolved inorganic carbon (DIC) concentration determination 5 times during the 14 d experimental period. Samples were fixed with $HgCl_2$ and stored in glass flasks with serum stoppers in the lid, allowing no head space. Samples were stored in the dark at 5°C and later brought to Denmark for analysis. Concentrations of DIC were determined with an infrared gas analyser (IRGA) using a 2.00 mM bicarbonate standard as reference. Details of the specific setup have been described previously (Nielsen et al. 2007).

Initially and on Days 4, 8 and 14, subsamples of 50 ml were removed and 0.2 µm filtered for determination of concentrations of inorganic phosphorus, ammonium, nitrate, nitrite and silicate. Samples were stored at –18°C until analysis. Silicate was quantified as the molybdate reactive fraction using an Aquakem discrete analyser. Inorganic phosphorous, ammonium, nitrate and nitrite were all quantified by the APHA 4500 method (APHA 2005).

Rapid light curves (RLCs) were attained every second day from a 20 ml subsample using pulse amplitude modulated (PAM) fluorometry. All fluorescence measurements were performed using a water PAM fluorometer (Walz), zeroed with filtered seawater, and samples were dark adapted for 30 min prior to measuring. Measurements were always conducted at midday to avoid differences due to diurnal cycles. RLCs were run under software control (WinControl, Walz) with 8 actinic light levels in addition to the initial quasi darkness (0, 67, 101, 148, 230, 342, 488, 682 and 1134 µmol photons m^{-2} s^{-1}). Fluorescence yield was measured with a weak measuring light (<0.15 µmol photons m^{-2} s^{-1}) that gives a background fluorescence (F_0) but is too weak to induce photochemical reactions in Photosystem (PS) II. Chlorophyll fluorescence was detected at wavelengths >710 nm. Actinic light was provided by blue-light emitting diodes (LED) and each light level was provided for 10 s. At each light level, relative electron transport rate (rETR) was calculated as rETR = PAR × $\Delta F/F_m'$ (where F_m' is maximum fluorescence) and plotted against photosynthetically active radiation (PAR). No photoinhibition was observed, and thus light-response curves were fitted to the simplified model of Harrison & Platt (1986) using the 'Regression Wizard' of Sigma Plot 10.0:

$$P = P_{max}\left(1 - e^{-\frac{\alpha E_d}{P_{max}}}\right) \quad (1)$$

where P is rETR, P_{max} is the photosynthetic capacity at saturating light, α is the initial slope and E_d is the downwelling irradiance (PAR). The PAR-intensity above which saturation becomes dominant, E_k, was calculated as α/P_{max}. Maximum quantum yield of PSII was calculated as $F_v/F_m = (F_m - F_0)/F_m$ (where F_v is the variable fluorescence). The parameter F_v/F_m has often been used as a general stress indicator of phototrophic organisms (Berges et al. 1996, Olaizola et al. 1996, Ralph & Gademann 2005). It denotes the proportion of energy that is transferred through the photochemical reactions of PSII, and thus is indicative of the balance between light harvesting and the subsequent quenching reactions. Although deviations can occur (Flameling & Kromkamp 1998), a linear correlation between oxygen evolution and electron transport rate of PSII has been found in microalgae, and thus rETR is a good proxy for relative photosynthesis under most conditions (Kolber & Falkowski 1993, Hartig et al. 1998). The photochemical quenching parameter q_P, and the non-photochemical quenching parameters q_N and NPQ were also measured. These each denote a potentially interesting aspect of fluorescence quenching. For a comprehensive description see Schreiber (2004).

For quantification of particulate organic carbon (POC), 200 ml water from each flask was filtered onto a pre-burned (600°C) 25 mm Whatman GF/F filter under gentle vacuum. Filters were dried at 80°C for 24 h, folded, wrapped in aluminium foil and stored at –70°C. This was done initially and on Days 4, 8 and 14. The analysis for POC was conducted using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser.

During the 14 d period, subsamples of 130 ml were removed 4 times from each of the 12 flasks for enumeration of plankton taxa by inverted microscopy. Samples were fixed with acidic Lugol's iodine (final concentration 1%) and stored cold and dark until examination. For enumeration, 50 ml Utermöhl sedimentation chamber were used, and cells were allowed 24 h of settling time, after which samples were examined on a Zeiss Axiovert A35 inverted microscope (40–400× magnification). Based on an initial inspection, 25 quantitatively important protist taxa were selected for enumeration. Also, the dimensions of at least 10 cells of each taxa were measured, and cell volumes were calculated through simple vol-

umetric calculations in accordance with Thomsen (1992). Volumes were converted to carbon values following equations given by Menden-Deuer & Lessard (2000), except for the dinoflagellate *Noctiluca scintillans*, for which an average of 3 literature values was used (Kiørboe & Titelman 1998, Tada et al. 2000, Umani et al. 2004).

Initially and on Days 4, 8 and 14 of the experiment, 200 ml subsamples were removed from each flask and filtered through 25 mm GF/F filters under slight vacuum for phytoplankton pigment analysis. Filters were folded twice and gently pressed between tissue paper to remove excess water. Each filter was wrapped in aluminium foil and immediately stored at −70°C. The whole process was carried out in a low-light environment to avoid pigment breakdown. At the end of the experiment, phytoplankton photo pigments were quantified following the method of Wright et al. (2010). In short, filters were extracted for 1 h (at −18°C) in a mixture of dimethylformamide and methanol, whereafter 0.7 mm Zirconia beads were added and the cryotubes were shaken for 20 s at 4800 cycles min^{-1}. Particulate matter was removed by centrifugation and extracts were analysed by HPLC. Pigments were identified by their retention time and absorption spectra, and quantified against standards using the internal standard method (Mantoura & Repeta 1997). Phytoplankton group abundances were estimated using the algorithm CHEMTAX (Mackey et al. 1996). Plankton groups included were prasinophytes, chlorophytes, cryptophytes, haptophytes, pelagophytes, cyanobacteria, diatoms, *Pseudo-nitzschia*-like diatoms and 2 classes of dinoflagellates (peridinin and fucoxanthin/19′-hexanoyloxyfucoxanthin containing ones, respectively).

Silicon uptake was calculated as the reduction in the concentration of free silicon between each measurement. For each of the enumerated species, diatom carbon build-up was also calculated from one count to the next. The total diatom carbon build-up was calculated as the sum of carbon build-up from each diatom species. However, only species with a positive carbon build-up rate during each period were included in order to account only for positive growth. This allowed carbon build-up and silicon uptake to be compared.

Data on DIC, free CO_2, nutrients, PAM fluorometry based parameters P_{max}, α, E_k, F_v/F_m and quenching parameters, POC, cell concentrations and pigment based phytoplankton group abundances were analyzed using a 1-way ANOVA with repeated measurements, and Tukey's test for pair-wise comparisons. All statistical analyses were performed in SAS 9.1.3 with the significance level set to 0.05.

Due to possible difficulties with the conservation of DIC samples at the pH 6.3 treatment, an additional experiment was set up months after the termination of the first experiment. With the aim of measuring DIC immediately, instead of relying on conserved material, we set up a replicate of the pH 6.3 treatment.

The experiment was a close copy of the first one in terms of bottles, mixing, irradiance, temperature, pH adjustment, and sampling intervals and volumes. The only parameters recorded, however, were pH and DIC. The DIC samples were taken using 20 ml scintillation vials allowing no head space, and immediately (<15 min) analyzed using the same IRGA setup as previously described. Of course the plankton community would not have been the same, but this was not crucial since this experiment was designed only to describe the physical DIC out-gassing.

RESULTS

The carbonate system and pH

Measured pH during the 14 d experimental period deviated only slightly from the designated pH treatment levels (Fig. 1A). Small changes in pH (~0.1 in 2 d) occurred due to photosynthetic carbon assimilation, but the 4 pH treatments were well separated throughout the experiment. The initial level of DIC was 2.1 ± 0.1 (mean ± SD) mmol l^{-1} and, for all treatments except pH 6.3, only small changes occurred during the experimental period (Fig. 1B). Flasks at pH 6.3 apparently lost DIC to the air, resulting in a rapid decline of total DIC to only half of the initial amount. This DIC loss at pH 6.3 was, however, most likely a result of sample storage, and the DIC and CO_2 concentrations reported for the pH 6.3 treatment are thus underestimates (see 'Discussion'). Under the assumption of steady-state, concentrations of free CO_2 were calculated from levels of pH, DIC, salinity and temperature using the equations of Mackereth et al. (1978) and dissocation constant (pK) values from Plath et al. (1980) (data not shown). Concentrations of free CO_2 in the different pH treatments were on average 11.3 ± 1.0 (pH 8.3), 21.6 ± 1.2 (pH 8.0) and 41.1 ± 2.2 (pH 7.7) µmol l^{-1}, and fluctuations were small owing to the small fluctuations in pH and DIC. All pH treatments were statistically significantly different from each other in CO_2 concentration (1-way ANOVA, $p < 0.0001$). Concentrations of free CO_2 were not calculated for the pH 6.3 treatment since values would be highly dependent upon the incorrect DIC concentrations for this pH treatment.

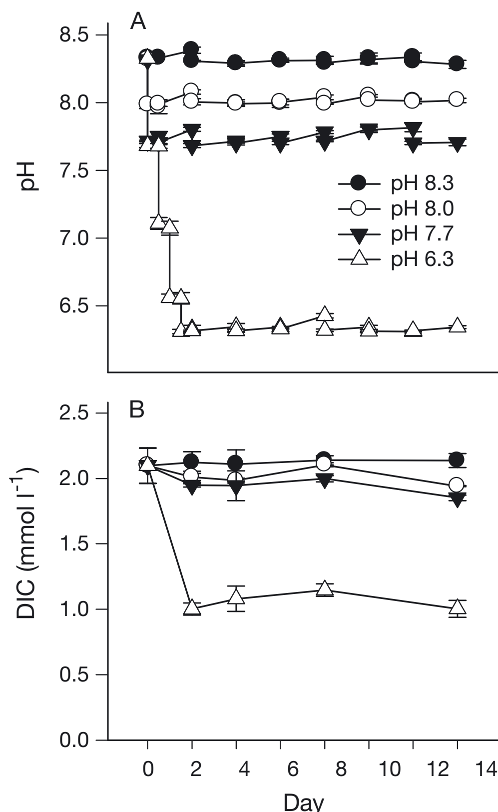

Fig. 1. Microcosm incubations. (A) Measured pH and (B) concentrations of dissolved inorganic carbon (DIC) in 4 pH treatments during 14 d incubations. Symbols and bars are means ± SD (n = 3)

Community changes between treatments at pH 8.3 to 7.7

Initial concentrations of inorganic phosphorus and ammonia were 0.51 ± 0.06 and 2.05 ± 0.12 (mean ± SD) µmol l^{-1}, respectively (Fig. 2A,B). These concentrations decreased over the 14 d experimental period to 0.17 ± 0.05 and to <1.0 µmol l^{-1}, respectively, in all pH treatments, with no statistically significant differences between treatments. Levels of nitrate and nitrite were below the detection limit (0.2 µmol l^{-1}) at all times (data not shown). Silicate was initially at a concentration of 11.2 ± 0.8 µmol l^{-1} (Fig. 2C). This decreased steadily throughout the 14 d period, and final concentrations were 2.2 ± 1.0, 2.8 ± 1.0 and 1.7 ± 1.0 µmol l^{-1} for the pH treatments 8.3, 8.0 and 7.7, respectively. No statistically significant differences were found between treatments (1-way ANOVA, p = 0.3).

There were no statistically significant differences found in PAM-derived P_{max} values either, although there was a non-significant trend towards higher P_{max} at higher pH from Days 4 to 10 (Fig. 3A) (Days 2 to 14, 1-way ANOVA, p = 0.09). However, even if this

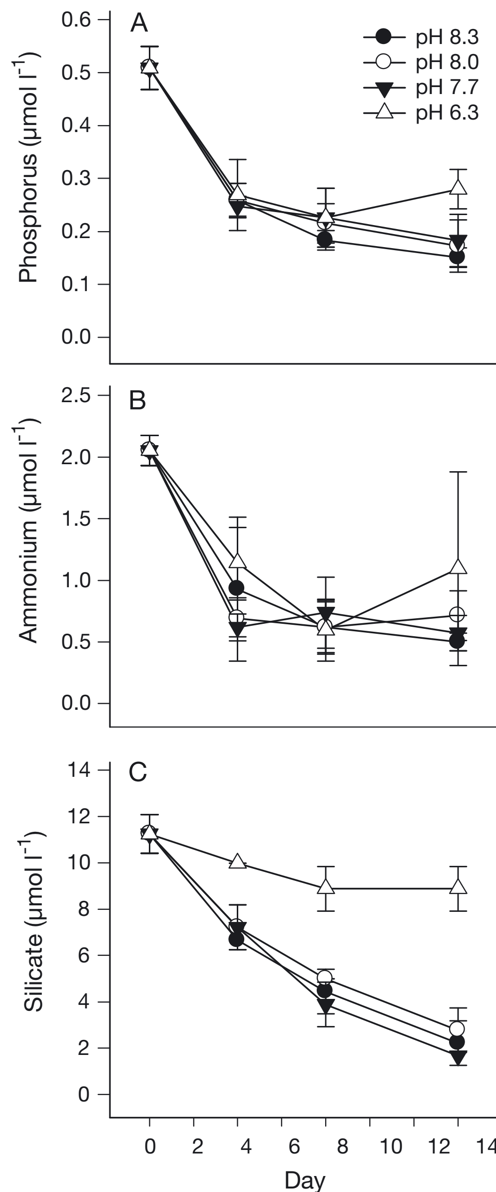

Fig. 2. Concentrations of inorganic nutrients of 4 pH treatments during 14 d microcosm incubations. (A) Inorganic phosphorus, (B) ammonium and (C) silicate. Symbols and bars are means ± SD (n = 3)

was indeed an actual difference between treatments, it is important to note that the difference leveled out again towards the end of the experiment and that it thus represents at most a temporary acclimation event. For the full experimental period, F_v/F_m values were 0.57 to 0.65, indicating continued good physiological 'health' (Fig. 3B) (Geider & LaRoche 1994, Greene et al. 1994, Boyd et al. 1999), and no statistically significant differences were found between pH treatments (Days 2 to 14, 1-way ANOVA, p = 0.81). The E_k of the initial plankton community was 260 ±

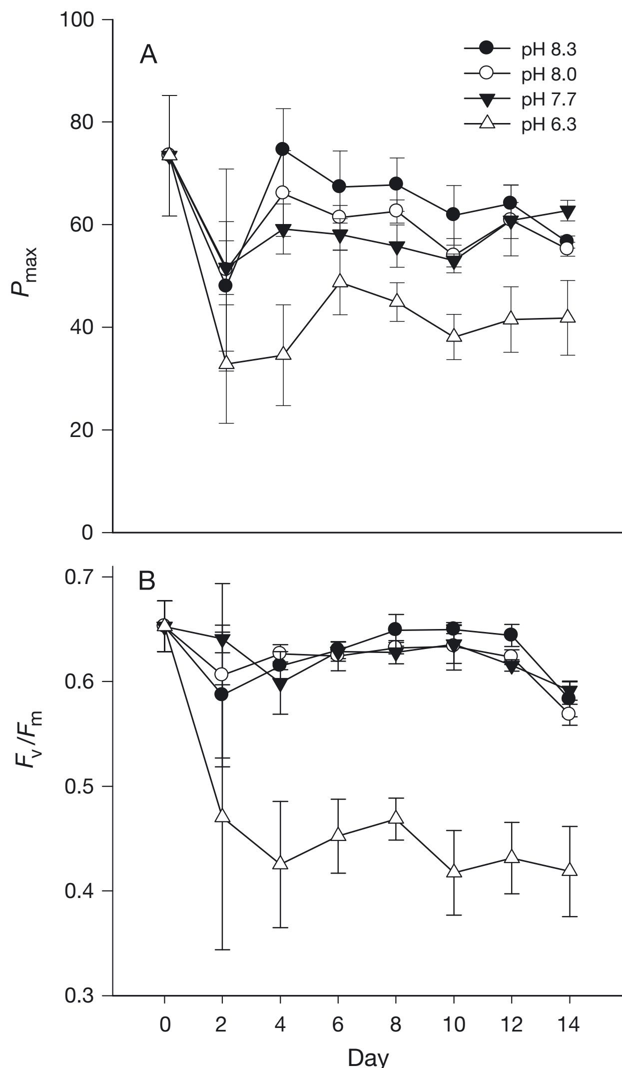

Fig. 3. Pulse amplitude-modulated photosynthesis parameters of the microcosm incubations. (A) Relative maximum photosynthetic rate (P_{max}) and (B) F_v/F_m of 4 pH treatments during 14 d incubations. Symbols and bars are means ± SD (n = 3)

40 (mean ± SD) µmol photons m^{-2} s^{-1}. Over the first 4 d this decreased similarly in all pH treatments to 146 ± 17 µmol photons m^{-2} s^{-1}, indicative of an acclimation to laboratory light conditions. The α was at first 0.29 ± 0.01, and this increased to 0.45 ± 0.03, with no statistically significant differences. Statistically significant differences were not found in the quenching parameters q_N, q_P and NPQ (E_k, α and quenching data not shown).

The total organic carbon value derived from cell counts was initially 222 ± 28 (mean ± SD) µg C l^{-1} (Fig. 4A). In all pH treatments of 8.3 to 7.7 this increased to 649 ± 18 µg C l^{-1} on Day 4, and over the next 8 d decreased slowly to 384 ± 27 µg C l^{-1}. On Day 14, no statistically significant differences were found (1-way ANOVA, p = 0.68).

At the onset of the experiment, the total POC concentration was 447 ± 100 µg C l^{-1} (Fig. 4B). In the first 4 d, this increased to 826 ± 76, 797 ± 82 and 862 ± 185 µg C l^{-1} respectively for the 3 pH treatments 8.3, 8.0 and 7.7, with no statistically significant differences (1-way ANOVA, p = 0.82). From Day 4 to the end of the experiment, levels of total POC were stable. The discrepancy between the total POC measurements and the cellular carbon values was approximately constant and most likely accounts for the detritus pool and/or nano- and picoplankton not seen under the microscope. Additionally, a small proportion (ca. <10%) of the discrepancy could be ascribed to residue inorganic carbon. By inverted microscopy, 25 protist taxa were enumerated (Box 1), but less than half of these are presented individually (see Figs. 5 to 7). Initially, the protist community was dominated by a diverse assemblage of dinoflagellates including

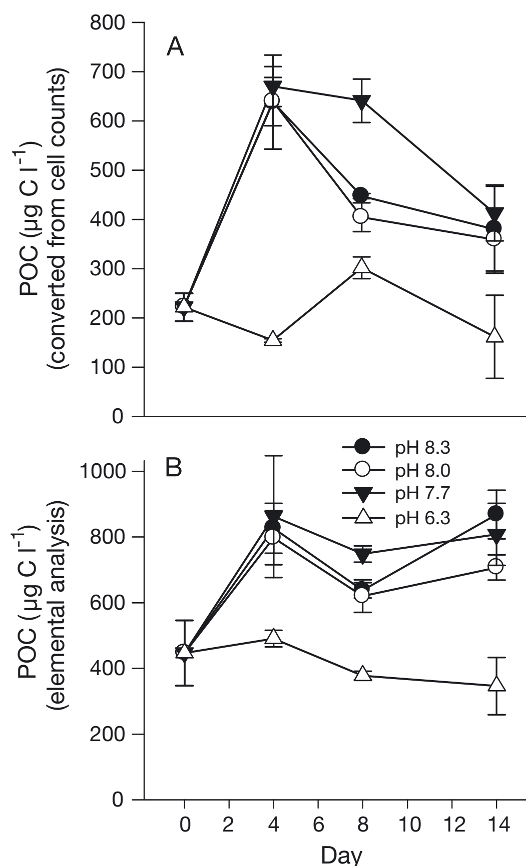

Fig. 4. Microcosm incubations. (A) Total cellular carbon calculated from microscopy cell counts and (B) measured total particulate organic carbon (POC) of 4 pH treatments during 14 d incubations. Symbols and bars are means ± SD (n = 3). Discrepancy between (A) and (B) of ~200 µg C l^{-1} is ascribed to the pool of detritus

Box 1. Complete list of taxa enumerated during the 14 d seawater acidification experiment from the Derwent River estuary. *Taxa presented individually in Figs. 5 to 7. Data on the remaining taxa are not presented, most often due to low cell concentrations close to the detection limit

Diatoms
Chaetoceros spp.*
*Dactyliosolen fragilissimus**
*Ceratoneis closterium**
Pseudonitzschia delicatissima
Rhizosolenia setigera
*Skeletonema pseudocostatum**
Thallasionema frauenfeldii
Chrysophytes
*Pseudopedinella elastica/pyriformis**
Ciliates
*Lohmaniella oviformis**
Cryptophytes
Teleaulax acuta
Dinoflagellates
Ceratium furca
Ceratium fusus
Ceratium tripos
Dinophysis spp.
Fragilidinium sp.*
Gymnodinium spp.*
Gyrodinium lachryma
Karlodinium spp.
Noctiluca scintillans
Prorocentrum gracile
*Protoceratium reticulatum**
*Protoperidinium cf. pellucidum**
Protoperidinium depressum
Euglenophytes
Eutreptiella spp.
Prasinophytes
Pyramimonas sp.*

Ceratium tripos, *C. fusus*, *C. furca*, *Fragilidium subglobosum*, *Dinophysis acuminata*, *D. acuta*, *Gymnodinium* spp. and the large heterotrophic species *Noctiluca scintillans* and also by the diatom *Dactyliosolen fragilissimus* (not all shown). A succession and a major shift in protist abundances occurred during the following 14 d, and especially significant was the appearance, and subsequent dominance, of the diatom *Skeletonema pseudocostatum* (Fig. 5A). Other diatom species also increased extensively in abundance during the period, and *D. fragilissimus* even managed to go through almost a full bloom cycle with both an increase and population decline

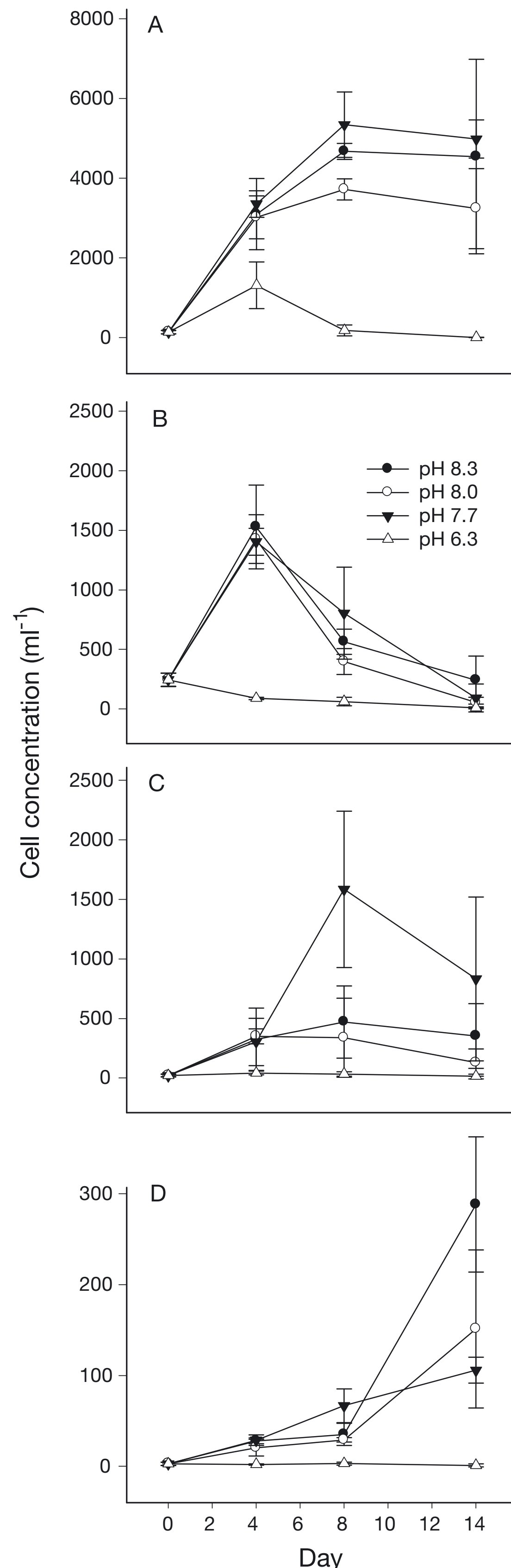

Fig. 5. Cell numbers of different diatom species during 14 d incubations at 4 different pH treatments. (A) *Skeletonema pseudocostatum*, (B) *Dactyliosolen fragilissimus*, (C) *Chaetoceros* spp. and (D) *Ceratoneis closterium*. Symbols and bars are means ± SD (n = 3)

within the experimental period (Fig. 5B–D). The increasing numbers of the diatom *Ceratoneis closterium* (=*Nitzschia closterium*) is evidence of a continued successional progression even at the end of the experiment. Of the 7 diatom taxa enumerated, none showed significant differences in cell abundances between pH treatments 8.3 to 7.7. For dinoflagellates, a species succession was also seen; species like *Protoperidinium cf. pellucidum* and *F. subglobosum* increased in abundance during the experiment, whereas others, e.g. *Gymnodinium* spp. and *Protoceratium reticulatum*, peaked and declined, and others again only declined, e.g. *N. scintillans* and *Ceratium* and *Dinophysis* spp. (Fig. 6). None of the enumerated dinoflagellates showed statistically significant differences between treatments. Although diatoms and dinoflagellates were the most dominant plankton classes, species of other groups were found as well: The ciliate *Lohmaniella oviformis* was continuously present at relatively low concentrations (<25 cells ml^{-1}), except on a single occasion where the concentration was as high as 120 cells ml^{-1} (Fig. 7A). An increase in the concentration of the prasinophyte *Pyramimonas* sp. was also noted throughout the experimental period, but with no statistically significant differences among pH treatments (Fig. 7B).

The initial chlorophyll *a* (chl *a*) concentration was 1.3 ± 0.3 (SD) µg l^{-1} (Fig. 8, left panel). According to the CHEMTAX analysis, several algal groups, including groups that were not identified by light microscopy, comprised this biomass. Dinoflagellates, cyanobacteria, and pelagophytes were the most important, but prasinophytes, haptophytes and chlorophytes were also present. Diatoms were found to represent only a very small part of the total chl *a* initially (<2%). However, it became the single most important group in all pH-treatments only 4 d later. Cyanobacteria disappeared altogether, but most other groups showed no significant succession during the first 4 d. Much smaller chl *a* concentrations were detected on Days 8 and 14 compared to Day 4. Diatoms were almost completely absent, and most other groups also decreased in biomass. Only minor differences in plankton group biomasses were found statistically significant, and this only occurred on single occasions.

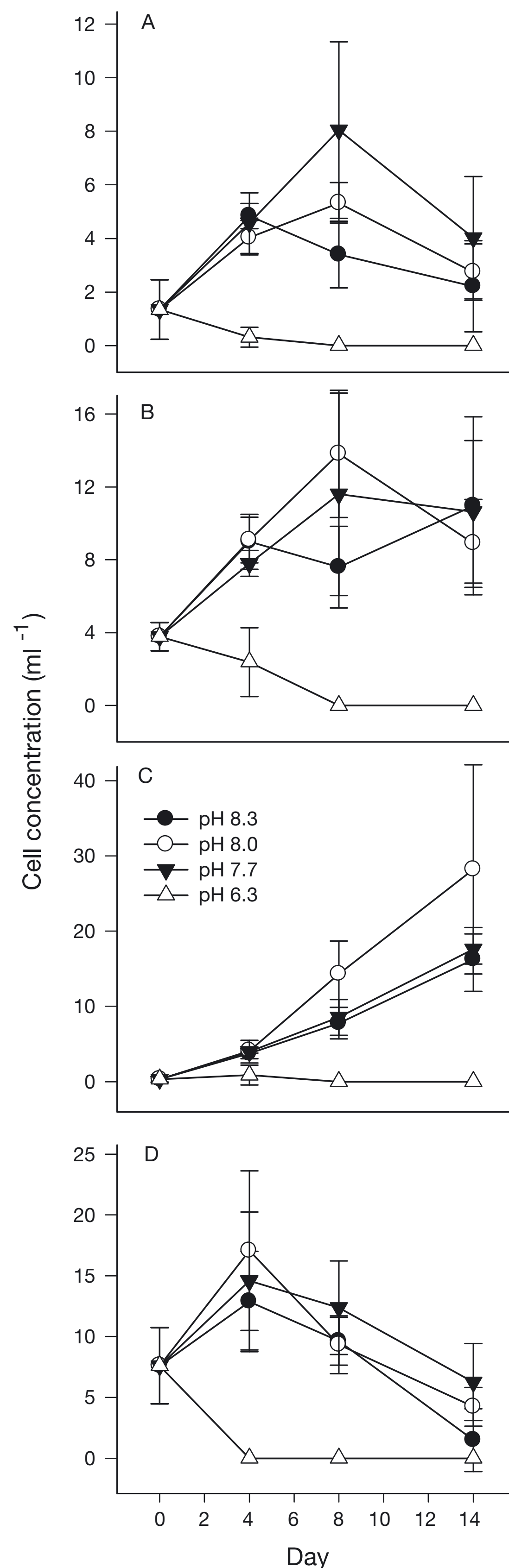

Fig. 6. Cell numbers of different dinoflagellate species during 14 d incubations at 4 different pH treatments. (A) *Protoceratium reticulatum*, (B) *Fragilidium* sp., (C) *Protoperidinium* cf. *pellucidum* and (D) *Gymnodinium* spp. Symbols and bars are means ± SD (n = 3)

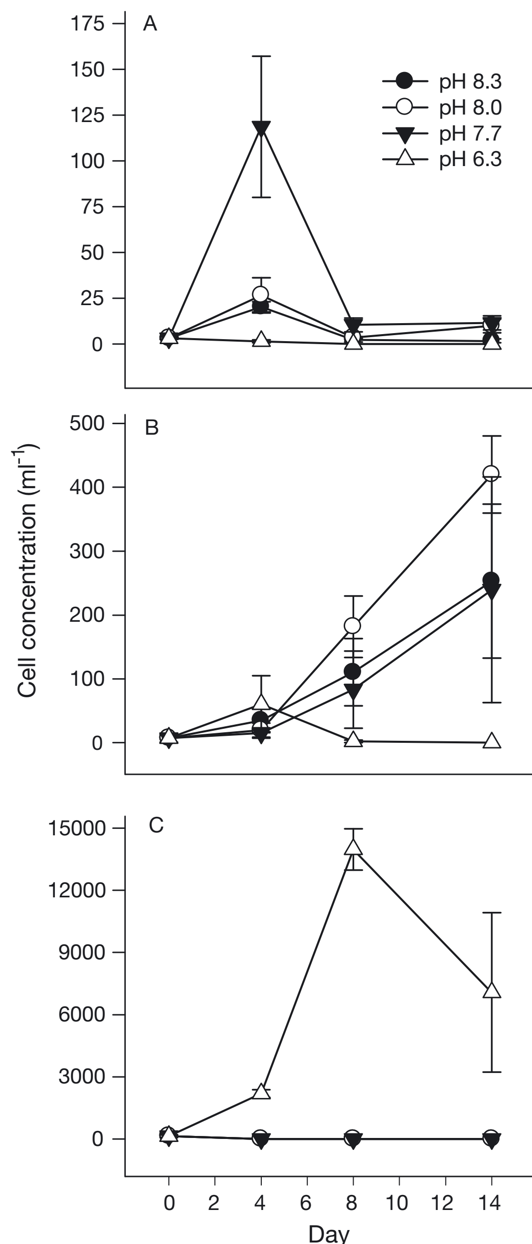

Fig. 7. Cell numbers of different protist species during 14 d incubations at 4 different pH treatments. (A) Ciliate *Lohmaniella oviformis*, (B) prasinophyte *Pyramimonas* sp. and (C) chrysophyte *Pseudopedinella elastica/pyriformis*. Symbols and bars are means ± SD (n = 3)

The decreasing chl *a* concentrations, co-occurring with increasing cell concentrations, caused a shift in the carbon to chl *a* ratios of the plankton community. Initially the ratio was 84:1, and this increased by an order of magnitude to 1000:1 on Days 8 and 14. Using carbon values from autotrophic species alone, the initial ratio was 50:1 and the values on Days 8 and 14 correspondingly lower.

The ratio of silicon uptake to cumulative diatom carbon build-up was 7.39 ± 0.20 during the initial 4 d. From Days 4 to 8 and 8 to 14, the ratio was 5.44 ± 0.35 and 4.12 ± 0.34, respectively.

The extreme pH 6.3

Initially, the extreme pH 6.3 treatment experienced an uptake of inorganic phosphorus and ammonium similar to the one seen in the other treatments. However, at Day 14 a little less inorganic phosphorus had been taken up compared to pH treatments 7.7 to 8.3 (Day 14, 1-way ANOVA, p = 0.02) (Fig. 2A). Throughout the 14 d experiment, less silicate was taken up at pH 6.3 (Day 14, 1-way ANOVA, $p < 0.001$) (Fig. 2C), and it had continuously lower values of both P_{max} (Days 2 to 14, 1-way ANOVA, p = 0.001) and F_v/F_m (Days 2 to 14, 1-way ANOVA, $p < 0.001$) (Fig. 3). It also had a lower amount of cellular carbon (Days 4 to 14, 1-way ANOVA, p = 0.013) and total POC (Days 4 to 14, 1-way ANOVA, $p < 0.001$) (Fig. 4), and 24 of the 25 enumerated species died during the experimental period (Figs. 5 to 7). Only the chrysophyte *Pseudopedinella elastica/pyriformis*, was able to grow at this very low pH, where it reached cell concentrations of >13 000 ml^{-1} (Fig. 7C). The pigment analysis confirmed this dominance of chrysophytes at pH 6.3, and it, like all other methods used, indicated profound community changes at treatment pH 6.3.

Additional DIC experiment at pH 6.3

The replicate pH 6.3 experiment showed a rather different development in DIC concentration during the 14 d of incubation compared to the first experiment (Fig. 9). Here, the DIC concentration decreased by only 0.28 mmol l^{-1}, from 2.20 to 1.92 mmol l^{-1}, during the 14 d. Also, the rate of decrease was steady throughout the period compared to the initial drastic drop of >1.0 mmol l^{-1} found in the first experiment.

DISCUSSION

Effects of predicted 21st century acidification

The aim of the study was to test whether predicted seawater acidification over the next decade would affect primary production, biomass, and species composition of a coastal plankton community. Despite relatively low nutrient concentrations, the plankton

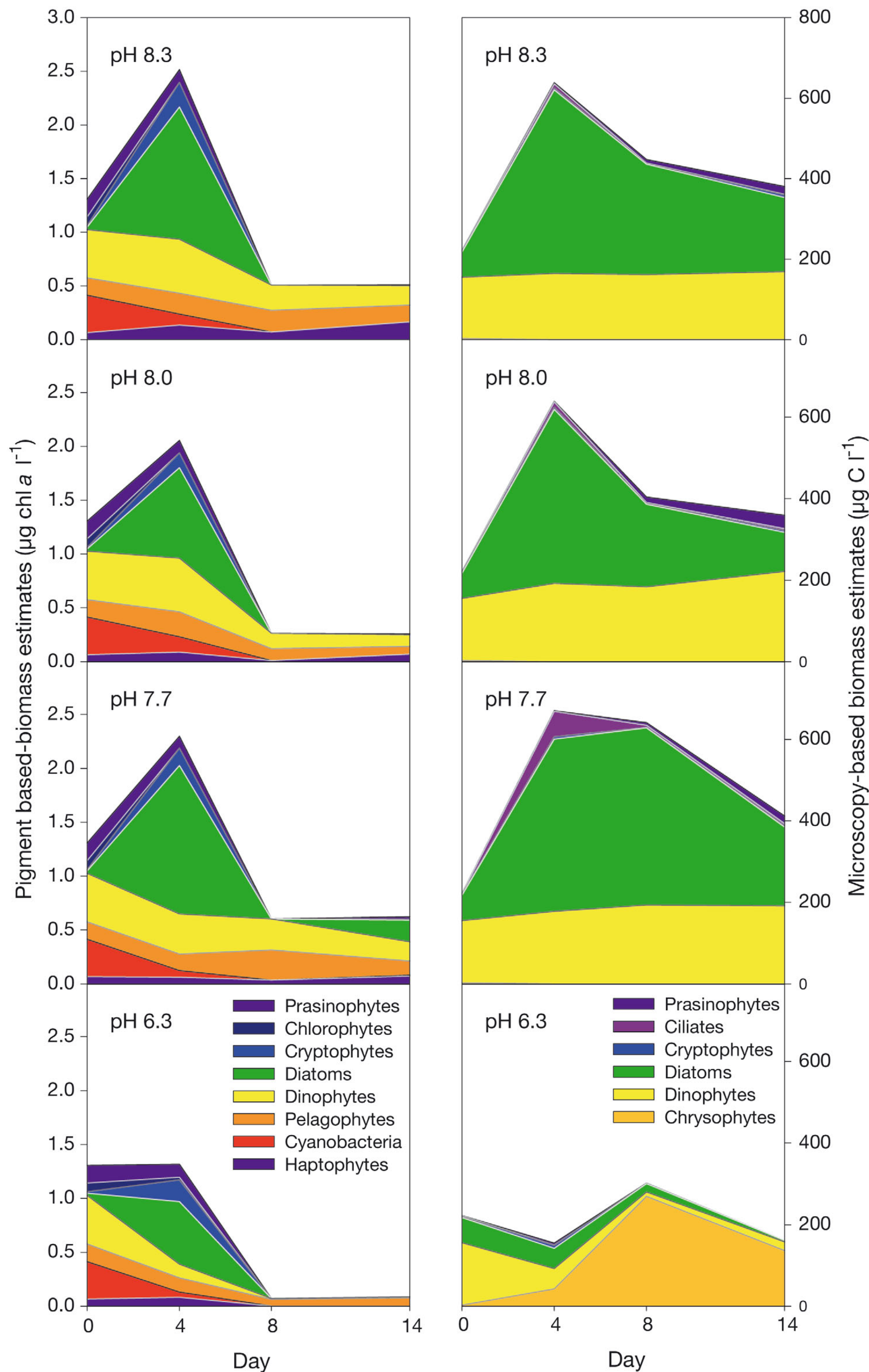

Fig. 8. Biomass estimates of 14 d microcosm incubations at 4 different pH treatments (8.3, 8.0, 7.7 and 6.3). Pigment and CHEMTAX-based biomass estimates in chlorophyll *a* (chl *a*) (left panel) and values calculated from microscopy cell counts measured in carbon (right panel). Average values (n = 3). Note: protist groups and colour codes differ between methods

community did experience a major species succession and biomass development, with continued good F_v/F_m values (except pH 6.3), during the 14 d incubation. This establishes the experiments as ecologically relevant, with continued 'healthy' and proliferating protist communities. Still, nutrient uptake and the photosynthetic parameters P_{max} and F_v/F_m were all unaffected by pH treatments 8.3 to 7.7 — treatments that match the predicted 21st century changes in CO_2 and pH. Also, cellular carbon and total POC

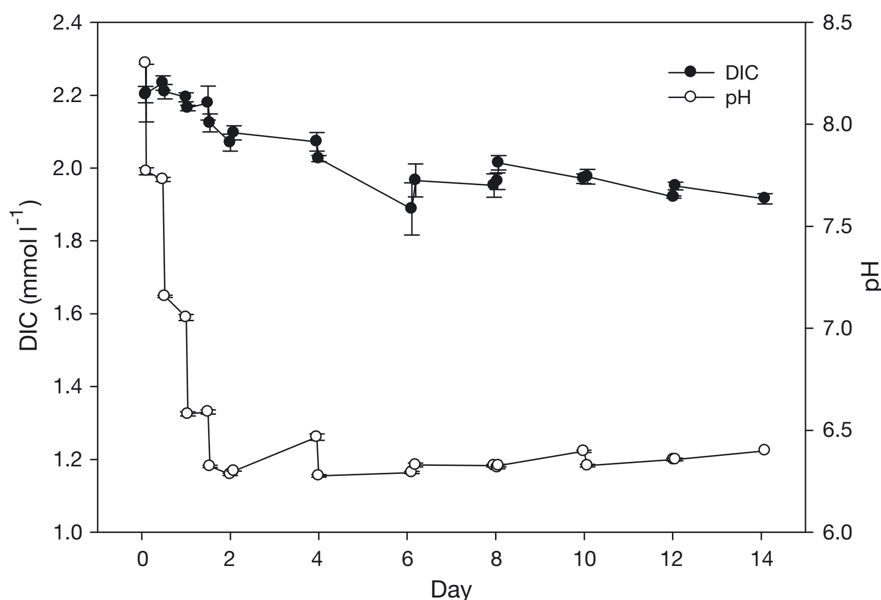

Fig. 9. Development in dissolved inorganic carbon (DIC) during the additional 14 d experiment at pH 6.3 using natural seawater. Experiment showed that DIC decrease seen at this low pH in the first experiment was an artefact of sample storage and not a decrease during the experiment. The first experiment thus underestimated DIC and CO_2 concentrations by a factor of ~2. Symbols and bars are means ± SD (n = 3)

were both completely unaffected by pH treatment within this range, and the same was true for the succession of all 25 enumerated protist species. Phytoplankton pigment analysis did not show effects of pH either, and the investigated plankton community was thus, in all ways, resilient to pH changes between 8.3 and 7.7, equivalent to the predicted changes for the next decade.

On the other hand, the pH 6.3 treatment completely altered the plankton community in nutrient uptake, photosynthesis and carbon build-up as well as in species- and phytoplankton group composition. This is hardly controversial since pH 6.3 is well below the natural range, but it demonstrates that pH can affect protist plankton communities directly and that the experiments were able to detect these changes through a number of different measurements.

Here, acid addition under stable DIC was applied to simulate future seawater acidification. It is not the perfect way to simulate seawater uptake of CO_2, since it slightly underestimates the amount of DIC (Rost et al. 2008). It is impossible to say, however, if this might have influenced the results of the experiment, but most protist species are generally not considered DIC limited (Burkhardt et al. 2001, Rost et al. 2003, Trimborn et al. 2008). The exception could be species without an effective CO_2-concentrating mechanism (CCM), but for those, CO_2, and not HCO_3^-, is the most important.

The fact that a pH decrease of 0.6 units did not affect the investigated plankton community is in apparent contradiction to the current views of protist physiology. Phototrophic species that do not have a CCM should benefit directly from the increased CO_2 concentration found at decreasing pH (Riebesell 2004). For species that have a CCM, the activity could be reduced at increased CO_2 due to the alleviated CO_2 shortage, thereby freeing energy to other processes, including growth (Beardall & Raven 2004, Riebesell 2004). Also, pH may affect cells more directly than through CCMs: Seawater pH affects intra-cellular pH and this in turn has the potential to influence the rate and functioning of enzymatic reactions and ultimately growth rates (Nimer et al. 1994, Suffrian et al. 2011). Thus, phototrophic as well as heterotrophic protists are both potentially affected by seawater acidification directly.

Despite this, others have also found no or very limited changes in phytoplankton communities in response to 21st century predicted changes in pH and CO_2, and it would seem that many coastal plankton communities are impervious to such changes (Kim et al. 2006, Riebesell et al. 2007, Nielsen et al. 2010). Supporting this, culture experiments with 8 coastal phytoplankton species recently showed unaltered growth- and productivity rates at pH from 7.1 to 8.5 (free CO_2 from 3 to 150 µmol l^{-1}), and a literature survey revealed that out of 26 species studied, only *Emiliania huxleyi* is possibly affected at pH 7.8 compared to pH 8.1–8.2 (Berge et al. 2010). Thus, there seems to be a discrepancy between the often hypothesized effects of altered seawater pH (Beardall & Raven 2004, Riebesell 2004, Trimborn et al. 2008) and the actual results of many micro/meso-cosm based studies — at least when concerning coastal plankton assemblages and species (Kim et al. 2006, Riebesell et al. 2007, Suffrian et al. 2008). While we acknowledge that the results could be different for more sensitive oceanic

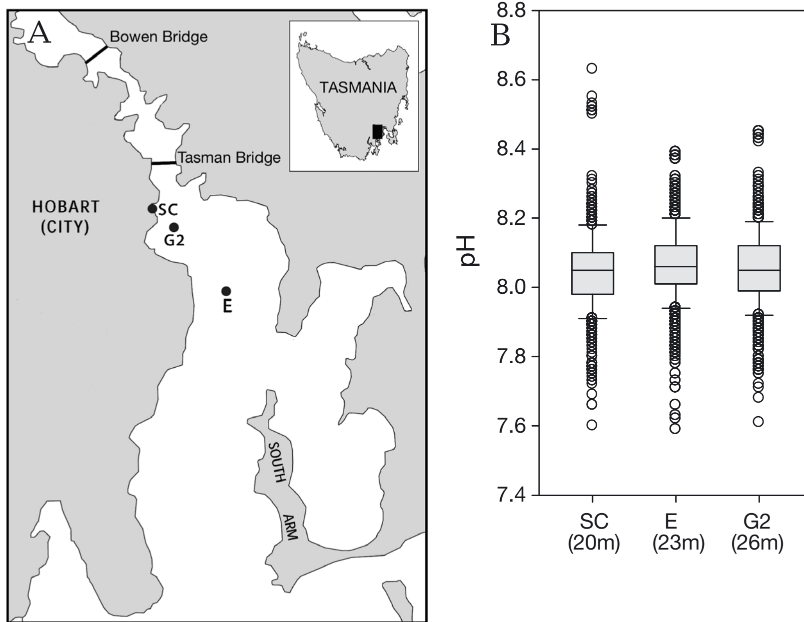

Fig. 10. *In situ* measurement from 3 different stations (SC, E, G2) in the Derwent River estuary, Tasmania, Australia. Data includes values from all water depths collected approximately once a month from January 2005 to June 2011 by the Derwent Estuary Program. pH-meters were calibrated before use and inter-calibrated yearly. (A) Collection sites in the estuary. The plankton community used in the present study was collected close to Stn SC. (B) Box plots of pH from the 3 stations showing the median values (line), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers), and remaining data as individual circles. The maximum water depth is given on the *x*-axis. The 12 values with pH >8.5 at SC are from different depths on the same day. It was not possible to determine whether these observations were correct or were an artefact of, for example, a poorly calibrated pH-meter. Data courtesy of The Derwent Estuary Program Norske Skog Nyrstar, www.derwentestuary.org.au/

species and ecotypes, we suggest that coastal plankton species show this broad level of pH-tolerance because pH in coastal waters often fluctuates as a result of respiratory and photosynthetic processes and due to hydrographical events. Seasonal, and even diurnal, fluctuations in coastal seawater pH have been shown to encompass 7.5 to 9.6 (Macedo et al. 2001, Hansen 2002). Concordantly, pH measurements from different stations in the Derwent estuary confirm that pH at the studied site varies significantly (Fig. 10). Stations close to shore as well as those further from land experience pH variations from 7.6 to 8.4. Thus, the protist community at the present site is annually subjected to pH fluctuations encompassing the predicted average pH from now until the year 2100. High pH has been shown to affect protist growth and survival, but most species do well even at pH 9 (Pedersen & Hansen 2003), and a pH decrease of half a unit should thus represent only a minor challenge to the majority of protist species.

However, when there is a decrease in average pH, there will also be a decrease in pH at extreme lows. The few yearly low pH events experienced e.g. at autumn mixing would have lower pH values than now, and this could potentially make them detrimental to plankton communities. Yearly lows of 7.5 have been reported previously, and with 21st century seawater acidification, such minima could approach 7.1 within the next 100 yr (Hansen 2002). Effects of extreme minimum pH have not been accounted for in the existing literature but could be a topic for future research.

Changes in carbon:chl *a* ratios during the incubations

Cellular carbon and chl *a* values did not develop synchronously during the 14 d, and a major shift in the ratio was seen towards the end of the experiment (Fig. 8). A comparable deviation has been found before in a similar independent experiment (Nielsen et al. 2010). Microscopic inspection of individual cells of the dominant diatom species *Skeletonema pseudocostatum* from the present experiment confirmed that cells were indeed alive prior to fixation (Fig. 11). Also, the molar ratio of successive diatom carbon build-up to silicon uptake from Day 0 to 4 was similar to the general ratio in diatoms of 7.7 (Brzezinski 1985). The decrease in this ratio later during the experiment indicates that carbon values were correct or even underestimated. Thus, nothing indicates that carbon values were overestimated e.g. due to counting of empty frustules.

Nutrient deficiency has previously been shown to affect the carbon:chl *a* ratio in diatoms (Laws & Bannister 1980), and this is most likely the explanation for the very low chl *a* values (compared to carbon biomass) found during the last 6 to 10 d of the experiment. Also, ratios of marker pigments to chl *a* are affected by nutrient deficiency (Goericke & Montoya 1998, Henriksen et al. 2002), and this may have affected analysis by CHEMTAX, which assumes that pigment:chl *a* ratios are constant for each taxonomic group. Alternatively, the deviations in carbon:chl *a*

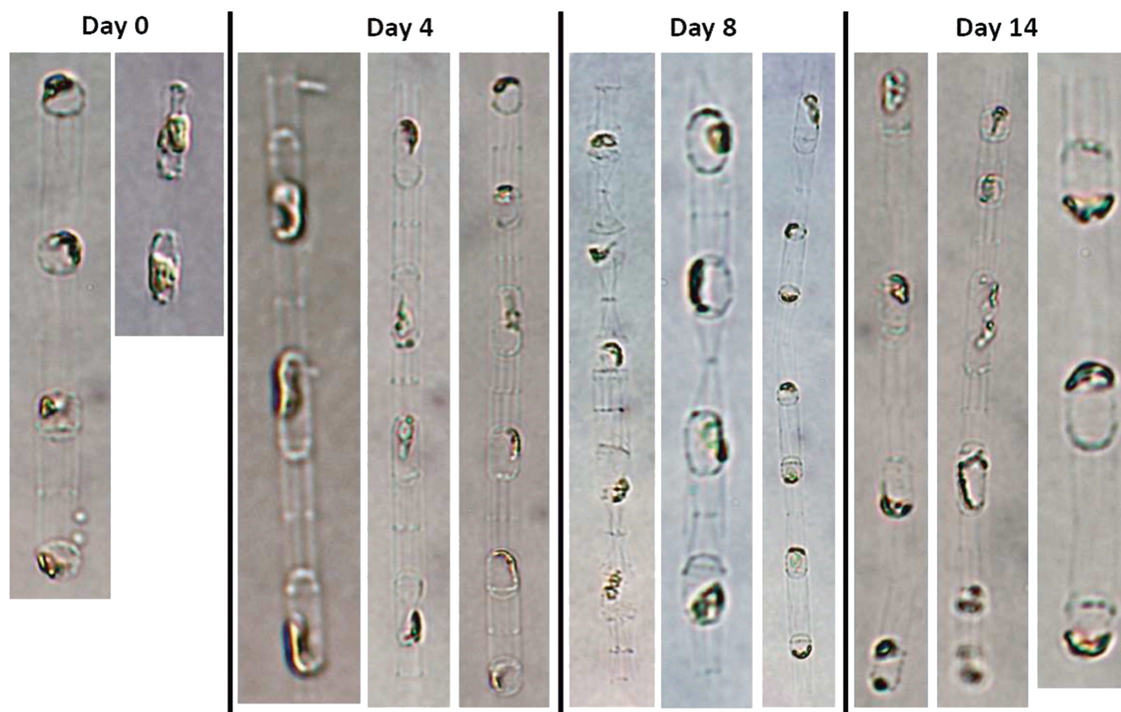

Fig. 11. Representative micrographs of the diatom *Skeletonema pseudocostatum* that dominated the microcosm incubations at pH 8.3, 8.0 and 7.7. Photos from Days 0, 4, 8 and 14, all from pH 8.3-treated incubations. Micrographs show that cells were alive prior to fixation

ratios may have been caused by the anomalous light regime offered in the laboratory (Geider 1987). Irrespective of the cause, the results indicate that care should be taken when using phytoplankton pigment data in mesocosm incubations. We advise that pigment analysis should always be accompanied by microscopy enumeration or a similar, automated method in order to avoid misleading results.

DIC and storage of samples

Data from the first experiment suggested that the DIC concentrations of the pH 6.3 treatment were most likely incorrect: Apparently, half the DIC disappeared from the incubated water during the first 4 d, but without any additional decrease in DIC during the remainder of the experiment. However, this would imply that the CO_2 concentration decreased rapidly from 765 ± 13 to 385 ± 36 µmol l^{-1} during the first 4 d, but afterwards remained stable for the remaining 8 d of the experiment, even though the concentration of free CO_2 was still ca. 30 times that of air-water equilibrium. This seems contradictive, and the additional DIC experiment confirmed that this was indeed not what had happened (Fig. 9). Here, DIC decreased only slightly during the experiment, when samples were of a larger volume and were analyzed immediately upon retrieval instead of being stored. This strongly suggests that the DIC decrease seen at pH 6.3 in the microcosm experiment was an artefact of sample storage before measuring. The samples would have been highly super-saturated with CO_2 (>700 µmol l^{-1}), and the small glass vials were apparently not as impermeable as believed. The small water volume would have given off excess CO_2, with an increase in pH as a result, until the CO_2 concentration was in equilibrium with that of the air. This explains why all pH 6.3 samples in the first experiment had very similar levels of DIC regardless of when they were taken.

Thus, both DIC and free CO_2 concentrations were severely underestimated in the microcosm experiment at pH 6.3. The second experiment suggests that both were in fact roughly twice that reported (thus 1.92 mmol l^{-1} and 716 µmol l^{-1}, respectively).

This is, however, mostly of interest when considering what caused the death of all except one protist species at this low pH. With this corrected DIC concentration at hand, only a direct pH effect seems plausible.

It is important to note that all this is of no or very little concern to the other 3 pH treatments (pH 8.3, 8.0 and 7.7), since these did not lose noteworthy amounts of DIC. Nevertheless, the finding that storage of low

pH samples may be difficult could be of importance when evaluating reported results, where stored DIC samples at low pH have been used (e.g. Berge et al. 2010, Nielsen et al. 2010). Those results may be biased in the same way. It is certainly something to consider for future DIC sampling and storage.

CONCLUSION AND PERSPECTIVES

The present study revealed an estuarine plankton community that was unaffected by changes in pH and CO_2 within the range expected for the next century. Resilient plankton communities have been found before, typically from coastal waters, and such communities could have this resilience because they regularly experience fluctuations in pH exceeding the 21st century predicted changes. We emphasise that oceanic plankton communities do not experience such pH fluctuations and thus may not have this broad level of pH tolerance. Studies in the near future will reveal if this is indeed the case.

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