Biogeosciences Discuss., 11, 3489–3534, 2014
www.biogeosciences-discuss.net/11/3489/2014/
doi:10.5194/bgd-11-3489-2014

Biogeosciences Discussions
Open Access

Carbon cycling and phytoplankton responses within highly-replicated shipboard carbonate chemistry manipulation experiments conducted around Northwest European Shelf Seas

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Received: 22 January 2014 – Accepted: 31 January 2014 – Published: 3 March 2014

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Published by Copernicus Publications on behalf of the European Geosciences Union.

Abstract

The ongoing oceanic uptake of anthropogenic carbon dioxide (CO_2) is significantly altering the carbonate chemistry of seawater, a phenomenon referred to as ocean acidification. Experimental manipulations have been increasingly used to gauge how continued ocean acidification will potentially impact marine ecosystems and their associated biogeochemical cycles in the future; however, results amongst studies, particularly when performed on natural communities, are highly variable, which in part likely reflects inconsistencies in experimental approach. To investigate the potential for identification of more generic responses and greater experimentally reproducibility, we devised and implemented a series of highly replicated ($n = 8$), short term (2–4 days) multi-level (≥ 4 conditions) carbonate chemistry/nutrient manipulation experiments on a range of natural microbial communities sampled in Northwest European shelf seas. Carbonate chemistry manipulations and resulting biological responses were found to be highly reproducible within individual experiments and to a lesser extent between geographically different experiments. Statistically robust reproducible physiological responses of phytoplankton to increasing pCO_2, characterized by a suppression of net growth for small sized cells (< 10 µm), were observed in the majority of the experiments, irrespective of nutrient status. Remaining between-experiment variability was potentially linked to initial community structure and/or other site-specific environmental factors. Analysis of carbon cycling within the experiments revealed the expected increased sensitivity of carbonate chemistry to biological processes at higher pCO_2 and hence lower buffer capacity. The results thus emphasize how biological-chemical feedbacks may be altered in the future ocean.

1 Introduction

Since the beginning of the industrial period, the oceans have taken up around 25–33 % of anthropogenic CO_2 emissions (Ciais et al., 2013). This additional carbon increases

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the dissolved inorganic carbon (C_T) pool and causes changes in carbonate chemistry including an increase in proton concentration ($[H^+]$) (lowering of pH) in surface waters, which is widely termed "ocean acidification" (Caldeira and Wickett, 2003; The Royal Society, 2005). Such changes in carbonate chemistry have the potential to influence a range of biological processes (Riebesell and Tortell, 2011). For example, drops in pH and carbonate saturation state (i.e. when $\Omega < 1$), often appear to influence calcification (Orr et al., 2005; Fabry et al., 2008; Bednaršek et al., 2012; Kroeker et al., 2013), while photoautotrophic organisms are also potentially sensitive to increased availability of certain inorganic carbon species (Rost et al., 2008; Raven et al., 2011).

Experimental studies investigating the potential impact of ocean acidification on natural phytoplankton communities have generated ambiguous results, often failing to establish generic responses for key organisms or groups, or across communities. For example, primary production measured by ^{14}C fixation or the net production of particulate organic carbon (POC) has variously been shown to be enhanced (Riebesell et al., 2007; Egge et al., 2009; Engel et al., 2013; Silyakova et al., 2013), decreased (Riebesell et al., 2009; Zondervan et al., 2007), or not significantly influenced (Tortell et al., 2002; Delille et al., 2005) following experimental elevation of pCO_2. Such variability in response may be related to: differences in experimental design; the influence of other environmental factors; or differential sensitivities amongst species generating variability related to the natural composition of microbial communities. For example, with respect to C_T uptake and utilisation by phytoplankton, while the majority of taxa are able to regulate their carbon acquisition through use of carbon concentrating mechanisms (CCMs) (Raven and Johnston, 1991), the efficiency of the CCMs differs widely among species, between functional groups (Giordano et al., 2005) and potentially as a function of cell size (Wu et al., 2014).

All microbes regulate cellular acid-base balance in the presence of both active solute transport across cellular membranes and primary metabolism (Raven, 1970; Smith and Raven, 1979; Raven et al., 2011; Flynn et al., 2012). For phytoplankton in particular, diel variations in the balance between photosynthesis and respiration have the potential

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to drive large oscillations in proximal cell surface $[H^+]$ ($[H^+]^{prox}$) and pCO_2 (Flynn et al., 2012) with subsequent cumulative influences on the concentrations of carbonate chemistry species in bulk seawater, e.g. $[H^+]^{bulk}$. However, changes in both $[H^+]^{prox}$ and $[H^+]^{bulk}$ are buffered by the carbonate system (Egleston et al., 2010; Flynn et al., 2012). As the uptake of anthropogenic carbon by the oceans continues into the future, the ability for the carbonate system to resist changes in composition, referred to as buffer capacity, will decline (Egleton et al., 2010). Consequently, microbial processes will tend to drive larger magnitude diurnal through seasonal scale variability in both $[H^+]^{prox}$ and $[H^+]^{bulk}$ (Egleston et al., 2010; Flynn et al., 2012). While all microbes might thus be expected to experience larger ranges in the concentrations of carbonate chemistry species, both relative and absolute changes should vary with cell size, with larger cells, having a bigger diffusive boundary layer, expected to experience greater variability under both natural and altered conditions (Milligan et al., 2012; Flynn et al., 2012).

The majority of studies aimed at evaluating the effect of ocean acidification on phytoplankton has been performed on individual species (Gattuso and Hansson, 2011), based on single clones isolated from the field many years or decades earlier. Observed physiological responses in such experiments may not be fully representative of populations or natural communities, as a range of complex biological and environmental interactions may be absent (Riebesell and Tortell, 2011). Moreover, cell lines kept in culture may not even have retained the physiological characteristics of the original clones (Joint et al., 2011). Natural community perturbation experiments have the potential to provide a greater environmental relevance through investigation of the entire (microbial) ecosystem structure and function in an environment better approximating natural conditions (Tortell et al., 2002, 2008; Delille et al., 2005; Engel et al., 2005; Hare et al., 2007; Feng et al., 2009, 2010; Hopkinson et al., 2010; Lomas et al., 2012; Losh et al., 2012). However, interpreting the results of such field experiments can be complicated by the multiple biogeochemical feedbacks and food web interactions, which characterize responses to perturbation in any complex

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natural community (Rose et al., 2009; Krause et al., 2012; Brussaard et al., 2013). Furthermore, whilst laboratory experiments provide the opportunity for a high degree of replication and thus considerable statistical power, field approaches may have limited scope for replication, with geographical scales often reduced to one unique location (Table 1).

Timescale is also a concern in the interpretation of all ocean acidification research. The temporal scales applied in all field experiments to date (Table 1) are many orders of magnitude smaller than those which will characterize the ocean acidification process driven by slow uptake of anthropogenic CO_2 over many decades. The ocean acidification timescale will be comparable to many thousands of microbial generations, suggesting that evolutionary processes are highly likely to have an influence on system level responses (Collins and Bell, 2006; Lohbeck et al., 2012; Jin et al., 2013; Reusch and Boyd, 2013). Indeed, the studies performed to date over longer timescales indicate the potential influence of evolutionary adaptation to increased pCO_2 over modest (< 1.5 yr) periods (Lohbeck et al., 2012; Jin et al., 2013). Consequently, although experimentation on natural communities can potentially account for compositional changes, which are highly likely due to both interspecific and intraspecific variations in the plasticity of response (Schaum et al., 2013), they will struggle to account for adaptation occurring through decades of evolutionary processes.

The available experimental techniques for studying ocean acidification could thus all be considered imperfect (Havenhand et al., 2010) and extrapolation of results needs to be performed with great caution. Identification and mechanistic understanding of any generic robust ecophysiological sensitivity of differing microbial groups to changes in carbonate chemistry is thus crucial. In the current study we prioritized experimental replication and hence greater geographical and environmental coverage of the responses of natural upper ocean microbial communities to carbonate chemistry manipulation. Specifically, we designed and implemented a series of shipboard experiments focusing on the short timescale responses of multiple variables to imposed discrete changes in pCO_2 and other associated carbonate chemistry species.

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We investigated the impact of experimentally imposed shifts in carbonate chemistry on phytoplankton processes and subsequent carbon cycling including inorganic uptake and organic matters release, in Northwest European shelf seas within a series of experiments performed at five pCO_2 levels, alongside three additional experiments where both macronutrients and carbonate chemistry were simultaneously manipulated. Within the current manuscript, we describe the overall implementation of the experiments with explicit reference to current advice on best practice in ocean acidification research (Barry et al., 2010; Havenhand et al., 2010; LaRoche et al., 2010) and present some first order biogeochemical responses.

2 Methods

2.1 Bioassay set up

Shipboard incubation experiments were conducted on board the RRS *Discovery* as part of the cruise D366 (6 June–10 July 2011). Experimental locations are indicated in Fig. 1 and presented in Table 2, alongside the initial environmental conditions for each of the eight bioassay experiments performed: 5 multi-pCO_2 level manipulation experiments (E1–E5, hereafter termed main experiments) and 3 combined carbonate chemistry/macronutrient manipulation experiments, (E2b, E4b and E5b, hereafter termed additional experiments). On the day of the experimental set up, vertical profiles of temperature, salinity, oxygen, fluorescence, turbidity and irradiance were obtained in order to choose and characterize the depth of experimental water collection within the water column structure. Vertical profiles of temperature and chlorophyll fluorescence from the main experiments are presented in Fig. 2. Experiments were set up and run in three principal stages as detailed below.

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2.1.1 Water collection and filling

Water from within the surface mixed layer (< 20 m) containing the intact natural community was collected from a unique CTD cast. Once on-deck, the total seawater collected within the 24 × 20 L CTD Rosette OTE (Ocean Test Equipment) bottles (480 L) was dispensed from randomly assigned OTE bottles through silicon tubing amongst 72 × 4.5 L (E1–E5) or 24 × 1.25 L (E2b, E4b and E5b) acid-washed and Milli-Q rinsed clean clear polycarbonate bottles (Nalgene™). Sub-samples were collected simultaneously for time-zero (T_0) measurements of each of the variables to be measured over the subsequent time-course (Table 3).

2.1.2 Carbonate chemistry manipulation and nutrient additions

Subsamples at time-zero (T_0), taken directly from the CTD, were immediately measured for total alkalinity (A_T) and dissolved inorganic carbon (Table 2) and hence characterization of the carbonate chemistry system in seawater. Dissolved inorganic carbon was analyzed with an Apollo SciTech C_T analyzer (AS-C3), which uses a CO_2 infrared detector (LI-COR 7000). Total alkalinity was determined using a semi closed-cell titration (Dickson et al., 2007) within the Apollo SciTech's AS-ALK2 Alkalinity Titrator. For both C_T and A_T the precision was 0.1 % or better, with accuracy verified using Certified Reference Materials (A.G. Dickson, Scripps). The remaining variables of the carbonate system were calculated with the CO2SYS programme (version 1.05, Lewis and Wallace, 1998; Pierrot et al., 2006), using the constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987). Carbonate chemistry in the experimental bottles was subsequently manipulated using equimolar additions of strong acid (HCl, 1 mol L^{-1}) and HCO_3^- (1 mol L^{-1}). This approach constitutes one of three methods allowing accurate replication of on-going and future changes in seawater carbonate chemistry, namely an increase in C_T at constant A_T (Gattuso et al., 2010). The volumes of HCl and HCO_3^- required to adjust pCO_2 to the chosen target values (Ambient, 550, 750 and 1000 µatm) were calculated from the measured

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ambient state of the carbonate system in seawater using CO2SYS. In order to validate the carbonate chemistry manipulation, four additional bottles were adjusted to the experimental conditions and immediately sub-sampled and measured as manipulated T_0 values.

Additional experiments were supplemented with low levels of major macronutrients (nitrate (NO_3^-), silicic acid (dSi) and phosphate (PO_4^{3-})) under the ambient state of the carbonate system or manipulated towards a target pCO_2 of 750 µatm. Four nutrient conditions were run in triplicate: (1) control, (2) 2 µmol L^{-1} added NO_3^- and dSi, (3) 0.2 µmol L^{-1} added PO_4^{3-} and 2 µmol L^{-1} dSi, and (4) 2 µmol L^{-1} added NO_3^- and dSi and 0.2 µmol L^{-1} added PO_4^{3-} (hereafter control, +N, +P, +NP), with four independent bottles analysed for T_0 values.

2.1.3 Incubation

Microbial communities were incubated in a purposely-converted commercial refrigeration container located on the aft deck of the ship. Irradiance (100 µmol photons m^{-2} s^{-1}), was provided by daylight simulation LED panels (Powerpax, UK) over a 18/6 h light/dark cycle approximating the ambient photoperiod. Temperature was maintained at the in situ values (± < 1 °C) at the time of water collection (Table 2). For the 5 main experiments (E1–E5), incubations lasted for a total of 4 days (96 h) including a time point after 2 days (48 h), with separate incubation bottles being sacrificed at every sampling point. The additional experiments including inorganic nutrient addition (E2b, E4b and E5b) were run under the same temperature and light regime for a shorter incubation period of 48 h with a single sampling point at the end.

2.2 Measured variables

In order to provide the volume of water required for the measurement of the whole suite of sampled variables in the main experiments (Table 3), it was necessary to incubate 3 sets of triplicate bottles in parallel, i.e. for each time-point and treatment

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a total of 9 bottles were sacrificially sampled. The three parallel sets of triplicate bottles are hereafter referred to as "Group A, B and C". In order to provide a check on reproducibility between the groups of triplicates, and consequently provide a further measure of biological reproducibility within the whole experimental process, a range of variables with low volume requirements were sampled across all 3 sets. Thus total chlorophyll *a* (Chl *a*), macro-nutrients, carbonate chemistry variables, and community structure as measured by flow cytometry were analyzed on a total of 9 bottles, corresponding to 3 groups of triplicates for each treatment at each time-point. Methods for variables explicitly discussed herein are briefly described below. References for other methods are provided in Table 3.

Due to the limited seawater volume available within the additional experiments, fewer variables were measured, specifically: carbonate chemistry (C_T and A_T), macronutrients, total and size-fractionated Chl *a*, photosynthetic efficiency (F_v/F_m, FRRf), DMS/DMSP (Hopkins et al., 2014), primary production, calcite production and coccolithophore cell counts (see Poulton et al., 2014; Young et al., 2014).

2.3 Particulate organic carbon (POC)

Aliquots of 750 mL of seawater were filtered onto 25 mm glass fibre filters (GF/F, Fisher MF 300, pre-combusted at 400 °C) and oven dried at 60 °C for 8–12 h. Inorganic carbonates were removed from the filters by acidification with sulphurous acid [6 % w/v] under vacuum for 24–48 h (Verardo et al., 1990). The filters were then re-dried at 60 °C for 24 h, packaged in pre-combusted aluminium foil (Hilton et al., 1986) and analyzed on a Thermo Finnegan flash EA1112 elemental analyzer using acetanilide as the calibration standard.

2.4 Nutrients

Samples for macronutrients (nitrate (NO_3^-), silicic acid (dSi) and phosphate (PO_4^{3-})) were collected directly from each of the incubation bottles into a 25 mL polystyrene

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container and stored at 4 °C pending analysis within 12 h. The samples were run on a Skalar San+ Segmented Flow Autoanalyser using colorimetric techniques (Kirkwood, 1996) with the exception that the flow rate of the sample through the phosphate channel was increased to improve reproducibility and peak shape.

2.5 Total and size fractionated Chl *a*

Aliquots of 100 mL were sampled from incubation bottles and filtered onto 25 mm GF/F filters (Whatman, 0.7 µm pore size) or 10 µm pore size polycarbonate filters (Whatman) (to yield a total and > 10 µm size fraction, respectively and therefore by difference a < 10 µm size fraction). Filters were extracted into 6 mL 90 % HPLC-grade acetone overnight at 4 °C in the dark and fluorescence was then measured using a fluorometer (Turner Designs Trilogy) (Welschmeyer, 1994). Final Chl *a* concentrations were calibrated against dilutions of a solution of pure Chl *a* (Sigma, UK) in 90 % acetone with instrument drift further corrected by daily measurement of a solid fluorescence standard.

2.6 Variable chlorophyll fluorescence (F_v/F_m)

The photosynthetic physiology of natural communities was measured using a Fasttracka™ Mk II Fast Repetition Rate fluorometer (FRRf) integrated with a FastAct™ Laboratory system (Chelsea Technologies Group LTD, West Molesey, Surrey, UK). All samples were dark acclimated for 30 min and FRRf measurements were corrected for the blank effect using carefully prepared 0.2 µm filtrates for all experiments and time-points (Cullen and Davis, 2003). F_v/F_m was taken as an estimate of the apparent Photosystem II photochemical quantum efficiency (Kolber et al., 1998).

2.7 Primary production

Daily rates (dawn–dawn, 24 h) of total primary production (PP) and > 10 µm primary production were determined following Poulton et al. (2014) (see also Poulton et al.,

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2010). Water samples (70 mL volume, 3 lights) from the incubations were spiked with 15–40 µCi (total PP) or 3–8 µCi (> 10 µm PP) of ^{14}C-labelled sodium bicarbonate, and incubated for a further 24 h. Incubations were terminated by filtration through 25 mm 0.45 µm (total) or 25 mm 10 µm (> 10 µm) polycarbonate filters (Nuclepore™, US).

Organic carbon fixation was determined using the Micro-Diffusion Technique (MDT) (Poulton et al., 2010, 2014).

2.8 Community composition

Phytoplankton community composition was assessed by a combination of flow cytometry (*Synechococcus*, pico-eukaryotes, nano-eukaryotes and heterotrophic nano-flagellates) and inverted light microscopy (microplankton: diatoms, ciliates and dinoflagellates) on water samples collected at the time of experimental water collection. Flow cytometry followed Zubkov et al. (2007) on paraformaldehyde fixed (0.1 % final concentration) and SYBr Green stained water samples using a Partec Cycflow Space Flow Cytometer (Partec UK). Cells were identified based on their light scattering properties, green fluorescence and phycoerythrin fluorescence. Inverted light microscopy followed Poulton et al. (2010) on preserved water samples (2 % final concentration of acidic Lugols solution) stored in 250 mL amber glass bottles and enumerated in 50 mL HydroBios setting chambers on a SP-95-I inverted microscope.

3 Results

3.1 Oceanographic setting

The five main bioassay experiments were set up and run along the cruise track at different geographical locations (Fig. 1) characterized by distinct environmental conditions (Table 2). The vertical profiles of temperature, chlorophyll fluorescence and nitrate illustrate the water column characteristics for each of the experiments at the time of their set up (Fig. 2). Water column conditions ranged from stratified (E1, E3

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and E5) to fully mixed (E2 and E4). The first experiment (E1) was characterized by high initial concentrations of nitrate (> 1 µM) and Chl *a* (> 3 µg L^{-1}) while the final (E5) was nutrient depleted (NO_3^- < 0.2 µM) with low Chl *a* (< 0.5 µg L^{-1}). The water column characteristics at E2 suggested a recent phytoplankton (diatom) bloom with high Chl *a* and depleted nutrients. Finally, E3 and E4 were set up in warmer waters and show similar initial environmental conditions with intermediate nitrate concentrations (between 0.5 and 1 µM). Initial phytoplankton community compositions, as determined by microscopy and flow cytometry were different between experiments (Table 4). Most phytoplankton communities were dominated by small cells (> 58 % < 10 µm Chl *a*), with E2 being the exception (80 % > 10 µm Chl *a*).

The additional experiments were set up in locations (Fig. 1) characterized by stratified water columns (results not shown) and low surface Chl *a* (< 0.8 µg L^{-1}) and intermediate (E2b) to low (E4b and E5b) nutrients (Table 2). Results obtained from size-fractionated Chl *a* suggest that the communities in the additional experiments were dominated by small cells < 10 µm (results not shown).

3.2 Carbonate chemistry shift and buffer capacity

The accuracy and precision of the carbonate chemistry manipulations is illustrated in Fig. 3. The achieved pCO_2 level was well matched to the target value at T_0 across all five experiments (Fig. 3a). Decreases in pCO_2 were subsequently observed at the 48 and 96 h time-points (Fig. 3b and c), which could largely be attributed to biological processes. However, differences in pCO_2 between target and measured initial values were more pronounced in the higher-pCO_2 treatments, likely reflecting the lower buffer capacity of the carbonate system at higher pCO_2 (see below). As expected, total alkalinity, remained stable across treatments and throughout the incubation period in the majority of experiments, except in E1, where an unexpected and unexplained difference in A_T values was observed between initial sampling (T_0) and all subsequent time-points (Fig. 3d). Consequently we do not consider the detailed carbon cycling within E1 further and treat all results from this experiment with caution.

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3.3 Experiment reproducibility

Standard deviations from the biological triplicates differed between the variables sampled, but were typically < 10 % of mean values. Moreover, the biological and chemical variables measured across the 3 parallel sets of triplicate bottles (Groups A, B and C) were highly comparable (Fig. 4). Specifically, carbonate system parameters, which responded to both the imposed environmental forcing and subsequent biological responses and feedbacks, were highly reproducible (Fig. 4a and b). In addition to observed consistent absolute magnitudes, observed changes over time in nitrate and total Chl *a*, representing indexes of bulk biological response both to the general enclosure of the community and the different imposed treatments, were also highly reproducible across all experiments (Fig. 4).

3.4 Carbon cycling and biological processes

Taken across all the experiments, net production or remineralisation of POC (ΔPOC) was strongly correlated with net changes in C_T(ΔC_T) ($r^2 = 0.62$, $p < 0.0001$, $n = 32$), in a manner, which was largely consistent with the former being the dominant driver of the latter (Fig. 5b, see also Fig. 6a). As previously indicated, variations in A_T observed through the experimental durations were less pronounced (Fig. 5). Consistent relationships were observed between ΔPOC and ΔC_T irrespective of the target pCO_2 condition (Fig. 6a). In contrast, calculated changes in pCO_2 and H^+ (ΔpCO_2 and ΔH^+) as a function of ΔPOC were much more pronounced under higher pCO_2 conditions (Fig. 6b and c). Variability in ΔpCO_2 and ΔH^+ as a function of ΔPOC thus progressively increased with higher target pCO_2 (Fig. 6), as would be expected following the reduction in buffer capacity which would result from the initial manipulation of the carbonate chemistry system (Egleston et al., 2010).

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3.5 Net autotrophic production and nutrient dynamics

The impact of carbonate chemistry manipulation on biogeochemical processes was assessed through observations of both biological (Chl *a*, F_v/F_m, organic matter production and community structure) and chemical variables (C_T, A_T and macronutrient concentrations). The overall nature and time-course of responses varied substantially between individual experiments (Figs. 7 and 8). For example, net declines in Chl *a* from initially high values were observed in E1 and E2, potentially indicating sampling within declining natural blooms. In contrast, net production was observed within at least some treatments for experiments E3–E5 (e.g., POC in Fig. 7 and < 10 µm Chl *a* in Fig. 8), which were initiated in warmer pico- and nanoplankton dominated waters (Table 4).

Despite considerable variability in overall dynamics, some underlying consistent responses of the natural phytoplankton communities to increasing pCO_2 were observed across many of the experiments (Figs. 7 and 8). Within E3–E5, increases in net phytoplankton (Chl *a*) biomass accumulation were observed over the first 48 h in the total and < 10 µm Chl *a* fractions under ambient conditions (Figs. 7 and 8) and were frequently associated with increased whole community macronutrient (nitrate) consumption (Fig. 7). However, within these experiments net production progressively reduced with increasing pCO_2, ultimately resulting in a switch to net loss of phytoplankton biomass (< 10 µm Chl *a*) and organic matter (POC) over 48 h in the 750 and 1000 µatm pCO_2 treatments in some cases (Figs. 7 and 8). Despite overall decreases in total Chl *a*, slightly larger declines within the high pCO_2 treatments over the first 48 h were also apparent in E1 (Fig. 7). Within E2, despite a lack of differences in total Chl *a* between treatments (Fig. 7), some indication of a similar sensitivity of the smaller size fraction (Fig. 8) and pico/nanoeukaryote numbers (Fig. 7) was also apparent. In contrast, the larger size fractions (> 10 µm Chl *a*) generally displayed less differential sensitivity to the imposed pCO_2 manipulation (Fig. 8). Although in 3 experiments (E2, E3 and E4) a significant increase of the > 10 µm chlorophyll under the two highest pCO_2 conditions could be observed by 96 h (Fig. 8).

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Consequently, despite overall differences in initial biomass, the relative response of the total and < 10 µm Chl *a* fractions and pico-nanoeukaryote numbers to increasing pCO_2 within E3–E5 was remarkably consistent (Figs. 7 and 8), displaying progressively larger relative responses as the difference between the initial condition and the manipulated state increased. For these 3 experiments, this progressive response could be best illustrated by considering the relative differences in the various autotrophic biomass indicators (Chl *a* and pico-nanoeukaryote numbers) between treatments as a function of the size of the imposed perturbation as indicated, for example, by the difference in $[H^+]$ concentration between the measurement point and the initial condition (ΔH^+) (Fig. 9). Statistically significant treatment effects (ANOVA, $p < 0.05$, Tukey-Kramer), dominated by the < 10 µm fraction (Fig. 9b and d) could be observed even under the lowest manipulation level (550 µatm), while no effect was observed for larger cells (Fig. 9c). Consideration of responses against the magnitude of the imposed chemical perturbation further allowed comparison with the approximate ranges of cell surface $[H^+]^{prox}$ likely encountered by cells over short times scales (i.e. h, days) under modern and 750 µatm pCO_2 (Flynn et al., 2012) (Fig. 9b and c). Thus, while perturbations were far in excess of likely $[H^+]^{prox}$ variability for the smaller size fractions (Fig. 9b), they were potentially comparable to those naturally encountered by the largest microbial size fractions.

By the end of the experimental time-courses, whole community uptake had frequently fully removed ambient nitrate, likely resulting in subsequent secondary biological responses to nutrient depletion (Fig. 7). In many cases these apparent secondary responses cascaded through the system at different times across different treatments, reflecting any initial influence of the pCO_2 manipulation on the net biomass uptake and nutrient drawdown (Fig. 7); i.e. reduced growth/biomass accumulation with elevated pCO_2 frequently resulted in slower macronutrient depletion. This nutrient starvation feedback effect was perhaps most evident within E4, where the depletion of nitrate at different times within the different treatments was always accompanied by a reduction in the apparent photochemical efficiency of photosystem II (F_v/F_m), as

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frequently accompanies nutrient starvation (Suggett, 2011), alongside a subsequent cessation in net biomass accumulation (Figs. 7 and 8). Overall, the presence of secondary feedback effects, despite the short duration of our experimental protocol, clearly illustrates the potential difficulties in differentiating direct from indirect effects over progressively increasing timescales in ocean acidification experiments. For example, treatment effects observable at 48 h had often collapsed (E3), or even reversed in sense (E4), by 96 h (Figs. 7 and 8), likely reflecting the dominance of nutrient depletion in the majority of the main experiments by that stage. Such potentially confounding influence of nutrient exhaustion will likely occur in any natural system and frequently necessitates additional system perturbation via nutrient amendment in longer-term experiments (Riebesell et al., 2013). However, for the current study, reproducible responses characterised by a reduction of net growth by the smaller phytoplankton size fractions were observed within experiments having relatively high (e.g. E4) and low (e.g. E5) starting macronutrient concentrations (Table 2).

Evidence for phytoplankton nutrient (co-)limitation under ambient conditions was apparent in two of the three combined nutrient addition-carbonate chemistry manipulation experiments (Fig. 10). Specifically, within both the experiments initiated in relatively low ambient nutrient ($< 0.3\,\mu M\ NO_3^-$) waters (E4b and E5b), the addition of NO_3^-, either alone (+N) or in combination with PO_4^{3-} (+NP), resulted in increased productivity and phytoplankton biomass. In contrast, there was no apparent nutrient response within E2b, which was initiated in waters containing relatively high ambient macronutrients (Table 2). Importantly, although addition of potentially (co-) limiting macronutrients (+N or +NP) increased community biomass and productivity in two of the experiments, both overall productivity and $< 10\,\mu m$ Chl *a* concentrations fraction were significantly altered by manipulation of the carbonate chemistry system. Such alterations could be observed under both ambient and experimentally induced high nutrient conditions across all these additional factorial experiments (Fig. 10). In contrast, despite also responding to nutrient amendment in E4b and E5b, the larger phytoplankton were once again less sensitive to pCO_2 with reduced (E2b) or

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insignificant (E4b and E5b) pCO_2 treatment effects observed for the > 10 µm Chl *a* concentration (Fig. 10). Overall results of the additional combined nutrient-carbonate chemistry manipulation experiments (Fig. 10), were thus qualitatively consistent with the results from the main experiments, particularly E3–E5 (Figs. 7 and 8).

4 Discussion

4.1 Performance of experimental method

The approach adopted here differed in a number of respects from many previous field-based experimental studies examining the potential effects of ocean acidification on phytoplankton ecophysiological processes and subsequent biogeochemical cycling. In contrast to many studies (e.g. Hare et al., 2007; Feng et al., 2009; Lomas et al., 2012; Riebesell et al., 2013), with the exception of the additional experiments (Fig. 10), we largely investigated natural communities without the supplementary addition of nutrients. The resulting necessary restriction on experimental duration was thus traded off against the incubations being performed at realistic natural nutrient levels. The restricted experimental durations also allowed more experiments to be performed over the period of the cruise, facilitating a better consideration of the so-called "sampling universe" (Ridgwell et al., 2009). Consequently, we could assess the responses of intact microbial communities sampled from eight geographical locations, representing a significant increase on even the most extensive prior studies (e.g., Hopkinson et al., 2010), allowing us to assess the generality of any observed responses.

The enhanced spatio-temporal scale coupled to high statistical power allowed rigorous assessment of within and between experiment reproducibility for multiple variables. The high reproducibility of within experiment observations indicated robust and repeatable biogeochemical responses both to the overall containment of the natural community and to the carbonate chemistry manipulations performed (Figs. 3 and 4). Moreover, the large suite of variables measured (up to 39, Table 3), provides the

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potential to investigate primary and secondary responses of complex natural microbial communities and resultant effects on biogeochemical cycling.

4.2 Well-constrained carbon cycling

In the majority of cases (i.e. excluding E1), the adopted carbonate chemistry manipulation allowed us to successfully increase total C_T without changes in A_T, as is predicted to occur as a result of on-going ocean acidification (e.g. Orr et al., 2005). Small A_T variations observed in the experiments over the time course (Fig. 5) were potentially attributable to nitrate uptake or carbonate mineral precipitation/dissolution (Cross et al., 2013). Specific to calcite, coccolithophores were a consistent, although relatively minor, component of phytoplankton communities collected through the experiments (Young et al., 2014), with calcite production (CP) significantly increasing in response to nutrient addition under ambient pCO_2 only (Poulton et al., 2014).

Irrespective of the treatments applied to the diverse microbial communities, changes in C_T (ΔC_T) were strongly correlated to the net production or remineralisation of POC (Fig. 6). Remaining deviation between C_T drawdown and POC accumulation could potentially be the result of a release of dissolved organic carbon (DOC) and/or formation of transparent exopolymer particles (TEP) as suggested in previous studies (Antia et al., 1963; Sambrotto et al., 1993; Riebesell et al., 2007). Within the current experiments, no significant treatment-dependent changes in total DOC accumulation could be observed, although TEP did vary as a function of experimental treatment within some experiments (MacGilchrist et al., 2014).

4.3 Size related physiological responses

Significant responses of phytoplankton to changes in carbonate chemistry were observed in all the eight experiments performed, although the magnitude of treatment effects was considerably reduced in E1 and E2 (Fig. 7). For the three main experiments where the community was dominated by $< 10\,\mu m$ cells (E3–E5), bulk community

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variables (Figs. 7 and 8) demonstrated a clear sensitivity to rapid changes in $[H^+]$ and pCO_2 ($\Delta[H^+]$ and ΔpCO_2), with a relative decrease in net biomass accumulation which scaled with increasing manipulation away from the ambient condition (Fig. 9). In addition, size fractionated responses within all the strongly responding experiments confirmed that the small size fraction (< 10 µm Chl *a*) was both the most sensitive to the imposed carbonate chemistry perturbation and was largely responsible for the observed bulk responses (Figs. 7–10). In contrast, experiments initiated within communities rich in large celled taxa (E1 and E2), displayed weaker responses.

It is not possible to unequivocally relate the responses observed consistently across the majority of experiments to a specific physiological mechanism. However, we suggest that the observed enhanced sensitivity of small-celled phytoplankton to the imposed rapid shifts in carbonate chemistry would be consistent with cell size specific differences in levels of adaptation to naturally experienced fluctuations in carbonate chemistry species within the environment (Flynn et al., 2012). The variability in $[H^+]^{prox}$ (or indeed other related carbonate chemistry variables such as $pCO_2{}^{prox}$ or CO_2^{2-prox}) experienced by phytoplankton should scale with phytoplankton cell size (Flynn et al., 2012), with smaller celled taxa hence expected to encounter relatively restricted $\Delta[H^+]^{prox}$ compared to larger cells or aggregates (Flynn et al., 2012). Such variability in $[H^+]^{prox}$ might impact cell physiology in a number of ways, for example, influencing nutrient transport and internal pH regulation (Milligan, 2012).

Consequently, consistent with our observations (Fig. 9), we suggest that smaller celled taxa might be expected to have a higher sensitivity to our experimentally induced perturbations of $[H^+]^{bulk}$ (and hence $[H^+]^{prox}$), which were likely outside the naturally experienced range of $[H^+]^{prox}$. Such variation in $[H^+]^{bulk}/[H^+]^{prox}$ could thus result in short-term detrimental effects on cellular processes and hence ultimately overall growth. In contrast, larger cells will naturally experience, and thus presumably be better adapted to, rapid changes in $[H^+]^{prox}$ (Flynn et al., 2012). Hence we might expect larger celled phytoplankton to be more capable of dealing with an imposed rapid experimental manipulation of $[H^+]^{bulk}$ without a major direct influence on cellular processes, again

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consistent with our observations (Figs. 8 and 9). Indeed, we note that modelled natural diel ranges of variability in $[H^+]^{prox}$ for the largest size classes (Flynn et al., 2012), although only indicative of extremes, are reasonably comparable in magnitude to our experimental $\Delta[H^+]^{bulk}$ (Fig. 9).

In addition to the initially imposed $\Delta[H^+]^{bulk}$, as expected (Delille et al., 2005; Riebesell et al., 2007; Egleston et al., 2010; Hopkins et al., 2010), the buffer capacity decreased as a function of increasing pCO_2 (Fig. 6). Decreased buffering would then result in increased diel and longer term $\Delta[H^+]^{prox}$ (and ΔpCO_2^{prox}) as well as $\Delta[H^+]^{bulk}$ (and ΔpCO_2^{bulk}) (Flynn et al., 2012), potentially further disadvantaging any taxa without a high pre-existing adaptation to such variability in the higher pCO_2 treatments. Overall, we thus argue that our results are consistent with the suggestion of Flynn et al. (2012), that size-dependent differential susceptibility to changes in $[H^+]$ might need to be considered in the design of experiments to investigate ocean acidification, interpretation of the results of such experiments and potentially in prediction of community structure responses to ongoing and future anthropogenic forcing (Milligan, 2012).

4.4 Biological-chemical feedbacks in the future ocean

Surface ocean carbonate chemistry is naturally subjected to considerable variability driven by multiple factors, including net photosynthesis and respiration from microbial activity (Joint et al., 2011; Patsch and Lorkowski, 2013). Simultaneously, multiple lines of research into the potential influence of ocean acidification on marine systems, including the evidence presented here, have revealed the potential for variations in different components of the carbonate chemistry system (e.g. pCO_2 level, carbonate ion and H^+ concentrations) to influence the biological activity of marine microbes (Liu et al., 2010; Riebesell and Tortell, 2011). Consequently, the biogeochemical dynamics of natural oceanic systems might be expected to be influenced by reciprocal interactions between carbonate chemistry and microbial activity.

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Accurate prediction of the overall microbial responses to altered carbonate chemistry in a future ocean are still hampered by the lack of clearly identifiable physiological responses that are consistent across multiple experimental studies and scales (Hofmann et al., 2010; Riebesell and Tortell, 2011; Wernberg et al., 2012). In contrast, as oceanic anthropogenic carbon uptake continues into the future, resulting changes in bulk carbonate chemistry, alongside the range of variability in the carbonate chemistry system which will result from any given biological process (Fig. 6), are reasonably well understood and predictable (Egleston et al., 2010). Thus, in addition to any direct microbial/biogeochemical responses to altered bulk values of carbonate chemistry parameters, the nature of any natural carbonate chemistry-biological feedbacks might be expected to alter into the future. Consequently, in addition to evaluating overall sensitivities of microbes to the state of the carbonate system (Joint et al., 2011) and assessing the potential of individual microbial strains and communities to adapt to ongoing change (Doney et al., 2009), future studies should perhaps pay more attention to identifying the significance of any existing natural feedbacks.

Recognition of such potential feedbacks also serves to further highlight that, as within any experimental study (Doney et al., 2009), extrapolation of the presented data to ongoing anthropogenic ocean acidification needs to be undertaken with care. The short timescale sensitivities to rapid carbonate chemistry manipulation we observed would not be expected to be directly translatable to the many orders of magnitude slower forcing that natural phytoplankton communities will encounter as a result of ocean acidification (Table 1) (Collins, 2011). Phytoplankton are characterized by differential plasticity to environmental forcing, likely including $[H^+]$ and pCO_2 (Schaum et al., 2012), alongside generation times which are short enough to potentially allow a degree of evolutionary adaptation to the slow anthropogenic build-up of CO_2 (Collins and Bell, 2004; Lohbeck et al., 2012; Reusch and Boyd, 2012). Consequently, any cell size specific sensitivity to variability in $[H^+]$ (or other carbonate species), as suggested by models (Flynn et al., 2012) and supported by our experiments (Figs. 8–10), might be expected to represent a further mechanism by which ocean acidification could drive

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slow changes in phytoplankton communities, through some combination of ecological and/or evolutionary processes (Milligan, 2012; Schaum et al., 2013).

5 Conclusions

Within the current study we observed phytoplankton responses to deliberate rapid changes in carbonate chemistry, using an experimental setup offering high replication and hence the potential for robust statistical analysis and reproducibility (Krause et al., 2012). Our study design thus facilitated sampling across a reasonably large, albeit still relatively constrained, geographical scale and range of environmental conditions. Despite variability in the phytoplankton responses across the different sites, a consistent trend was observed in the majority of experiments, which appeared to be driven by the suppressed activity of small phytoplanktonic cells following rapid H^+ (and/or pCO_2 changes). The observed responses were largely independent of initial or perturbed nutrient concentrations. Rapid increases in pCO_2 thus had a short-term negative influence on net phytoplankton production, which was progressive and reproducible, albeit with some degree of inter-experiment variability. Such increased sensitivity of small-celled phytoplankton groups to short term increases in pCO_2 is consistent with some theoretical considerations (Flynn et al., 2012). Variability in responses between experiments may then be speculated to relate to differences in initial community composition and/or size structure or potentially other environmental factors, including the initial state of the carbonate chemistry system and hence buffering capacity.

Acknowledgements. This work was funded under the UKOA programme via NERC grants NE/H017348/1 to T. Tyrrell, E. P. Achterberg and C. M. Moore, NE/H017097/1 to A. J. Poulton and M. V. Zubkov, and NE/H017062/1 to D. J. Suggett. We would like to thank the crew of the RRS *Discovery*, the technical staff from the National Marine Facilities, Mark Stinchcombe (NOC) for macronutrient data, Michelle Bennett (NOC) for Lugols count data, and Ross Holland (NOC) for flow cytometry data.

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Table 1. Environmental relevance vs. experimental power. For each approach, only an example of the study involving the longest incubation period is listed.

Approach	Experimental subjects	Time scale	Modification of initial conditions	Number of generations	Geographic scale	Processes	Replicates	References
Lab-culture	monospecies 1–6 genotypes	470 days ~ 1 yr	Aquil media f/2 media	682 500	– –	adaptation acclimation/ adaptation	3 5	Jin et al. (2013) Lohbeck et al. (2012)
In situ	communities (microcosm)	30 days	200 µm filtered, 0.2 µm seawater dilution	≈ 7–40*	1 site	acclimation	3	Hoppe et al. (2013)
	communities (mesocosm)	30 days	nutrient addition	3–15	1 site	acclimation	1	Schulz et al. (2012)
	ecosystems (observations)	> 10 yr	–	> 2000	global	acclimation/ adaptation		

*Spies et al. (1987)

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Table 2. Starting conditions in the bioassay experiments. Data for salinity and temperature was determined in situ with a CTD equipped with sensors. Average (±standard deviation) values are given when available.

Exp.	Lat. (°N)	Long. (°W/E)	C_T ($\mu mol\,kg^{-1}$)	A_T ($\mu mol\,kg^{-1}$)	Temperature (°C)	Salinity	Depth (m)	NO_3^- ($\mu mol\,L^{-1}$)	PO_4^{3-} ($\mu mol\,L^{-1}$)	dSi ($\mu mol\,L^{-1}$)	Chl *a* ($\mu g\,L^{-1}$)
1	56.79	−7.42	2091.83 (0.9)	2310.87 (2.3)	11.27	34.80	6	1.06 (0.1)	0.09 (0.0)	2.07 (0.2)	3.22 (0.0)
2	52.47	−5.90	2094.55 (0.9)	2322.23 (2.4)	11.77	34.44	5	0.28 (0.0)	0.14 (0.0)	0.45 (0.0)	3.51 (0.1)
2b	46.50	−7.22	2085.81	2345.59	15.02	35.67	< 10	0.94 (0.1)	0.07 (0.0)	1.15 (0.0)	0.55 (0.0)
3	46.20	−7.22	2083.80 (0.6)	2347.11 (3.6)	15.31	35.77	10	0.56 (0.0)	0.06 (0.0)	0.61 (0.0)	0.77 (0.0)
4	53.00	2.5	2085.49 (1.6)	2295.58 (0.4)	14.57	34.05	5	0.87 (0.1)	0.12 (0.0)	0.8 (0.0)	1.32 (0.0)
4b	57.77	4.5	2053.23	2291.88	13.09	34.80	< 10	0.3	0.0	0.3	0.48 (0.0)
5	56.50	3.66	2084.62 (1.5)	2310.82 (3.2)	13.86	34.99	12	0.26 (0.2)	0.05 (0.0)	0.07 (0.0)	0.25 (0.2)
5b	59.68	4.12	1997.22	2214.05	13.30	30.50	< 10	0.26 (0.0)	0.0	0.0	0.8 (0.0)

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Table 3. Biological and chemical variables measured in each of the main bioassay experiments.

Category	Parameter measured	References
Carbonate chemistry	C_T ($\mu mol\,kg^{-1}$)	Dickson et al. (2007)
	A_T ($\mu mol\,kg^{-1}$)	Dickson et al. (2007)
	pH (total scale)	Rerolle et al. (2012)
Nutrient concentrations	NO_3^- ($\mu mol\,L^{-1}$)	Kirkwood (1996)
	dSi ($\mu mol\,L^{-1}$)	Kirkwood (1996)
	PO_4^{3-} ($\mu mol\,L^{-1}$)	Kirkwood (1996)
Standing stock, composition, phytoplankton physiology	total Chl *a* ($mg\,m^{-3}$)	Welschmeyer (1994)
	> 10 µm Chl *a* ($mg\,m^{-3}$)	Welschmeyer (1994)
	F_v/F_m	Moore et al. (2006)
	sigma	Moore et al. (2006)
	τ	Moore et al. (2006)
Biological processes	Primary Production (mmol C $m^{-3}\,d^{-1}$)	Poulton et al. (2010, 2014)
	calcite production (µmol C $m^{-3}\,d^{-1}$)	Poulton et al. (2010, 2014)
	> 10 µm Primary production (mmol C $m^{-3}\,d^{-1}$)	Poulton et al. (2010, 2014)
Biogenic material	Bsi ($\mu mol\,Si\,L^{-1}$)	Poulton et al. (2006)
	PIC ($\mu mol\,C\,L^{-1}$)	Poulton et al. (2006)
Organic matters	POP ($\mu mol\,L^{-1}$)	Raimbault et al. (1999)
	POC ($\mu mol\,L^{-1}$)	Verardo et al. (1990)
	PON ($\mu mol\,L^{-1}$)	Verardo et al. (1990)
	DOC ($\mu mol\,L^{-1}$)	Spyres et al. (2000)
	DON ($\mu mol\,L^{-1}$)	Badr et al. (2003)
Community structure	Coccolithophores (cell mL^{-1})	Poulton et al. (2010)
	LNA bacteria (cell mL^{-1})	Marie et al. (1997)
	HNA bacteria (cell mL^{-1})	Marie et al. (1997)
	total bacteria (cell mL^{-1})	Marie et al. (1997)
	synechococcus (cell mL^{-1})	Zubkov et al. (2007)
	heterotrophic nanoflagellates (cell mL^{-1})	Zubkov et al. (2007)
	phototrophic nano and picoplankton (cell mL^{-1})	Zubkov et al. (2007)
	Diatoms, ciliates, dinoflagellates (cell mL^{-1})	Poulton et al. (2010)
Nitrogen cycle	NH_4 ($nmol\,L^{-1}$)	Clark et al. (2007, 2014)
	NH_{4reg} ($nmol\,L^{-1}\,h^{-1}$)	Clark et al. (2007, 2014)
	NH_{4ox}($nmol\,L^{-1}\,h^{-1}$)	Clark et al. (2007, 2014)
	N_2O ($nmol\,L^{-1}$)	Rees et al. (2011)
Others	TEP (µg. Xeq L-1)	Passow and Alldredge (1995)
	TN (µmol L-1)	Hilton et al. (1986)
	DMS ($nmol\,L^{-1}$)	Archer et al. (2013)
	Total DMSP ($nmol\,L^{-1}$)	Archer et al. (2013)
	Particulate DMSP ($nmol\,L^{-1}$)	Stefels et al. (2010)
	CH_4 ($nmol\,L^{-1}$)	Upstill-Goddard (1996)

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Table 4. Initial plankton community composition for each of the main experiments (plankton species counts, expressed as %'s for smaller plankton and concentrations for larger plankton). Average (± standard deviation) values are given when available.

Bioassay	< 10 µm Chl *a*	Plankton > 10 µm				Plankton < 10 µm	
		Diatoms	Dinoflagellates	Ciliates	Cryptophytes	Phototrophic nano and picoplankton	Synechococcus
	[%]	[cells mL^{-1}]					
E01	na	0.9 (0.2)	61.5 (23.8)	30.4 (47.2)	175.6 (264.9)	na	na
E02	20	44.3 (7.8)	39.7 (4.8)	1.2 (1.21)	5.0 (4.5)	1388 (85)	4407 (1407)
E03	58	3.2 (1.4)	57.2 (70.5)	6.3 (6.9)	2.6 (2.8)	2953 (518)	26 771 (1067)
E04	81	2.2 (0.5)	41.1 (21.4)	1.6 (2.1)	38.3 (20.6)	6688 (3847)	54 660 (34 139)
E05	77	2.5 (0.2)	20.9 (12.4)	7.3 (3.3)	6.7 (8.0)	6276 (4799)	149 844 (8083)

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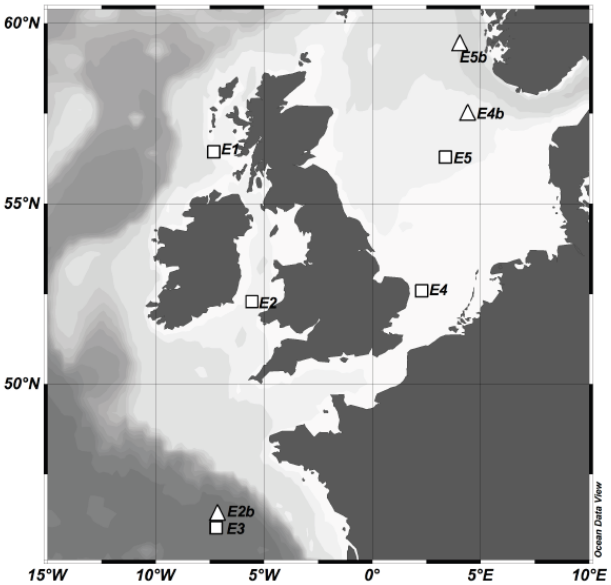

Fig. 1. Locations of the main (E1–E5) (square) and the additional (E2b, E4b and E5b) (triangle) bioassay experiments performed during D366 cruise in the NW European continental shelf seas.

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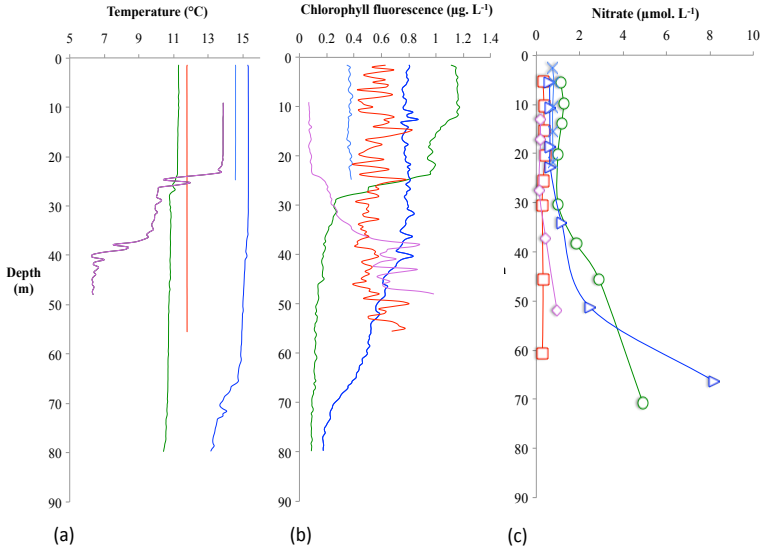

Fig. 2. Vertical profiles of **(a)** temperature (°C), **(b)** chlorophyll fluorescence ($\mu g L^{-1}$) and **(c)** nitrate ($\mu mol L^{-1}$) illustrating oceanographic setting of each of the five main bioassay experiments at the time of the set up. Temperature and fluorescence data were obtained from the CTD, and nitrate concentrations from discrete measurements of water column samples. Each colour/symbol corresponds to a bioassay: E1 (green, circles), E2 (red, squares), E3 (dark blue, triangles), E4 (light blue, cross) and E5 (purple, diamonds).

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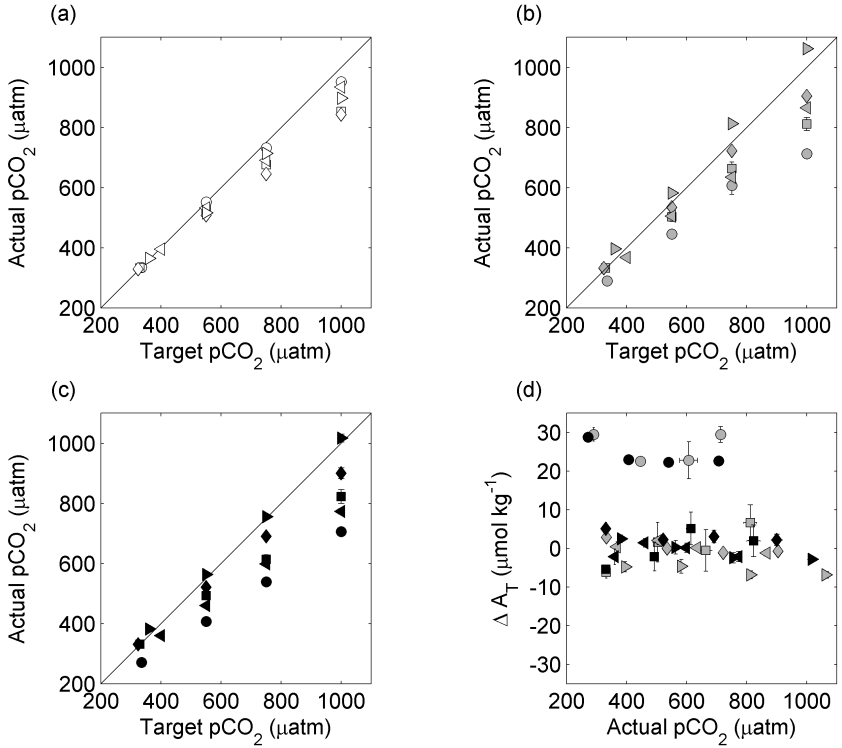

Fig. 3. Carbonate chemistry evolution in the main bioassays through the incubation period. Plots illustrate targeted vs. measured pCO_2 (µatm) at t_0**(a)**, 48 h **(b)** and 96 h**(c)** and difference in A_T between initial and subsequent time-points (ΔA_T) **(d)** in all the five main bioassay experiments. Open symbols **(a)** indicate initial conditions, with grey (**b** and **d**) and black (**c** and **d**) symbols indicating 48 h and 96 h time-points respectively. Symbol shapes indicate experiment: E1 (circles), E2 (squares), E3 (diamonds), E4 (left pointing triangles), E5 (right pointing triangles). Black lines in **(a–c)** indicate the 1 : 1 line. Plotted values are means ±1 se of biological triplicates.

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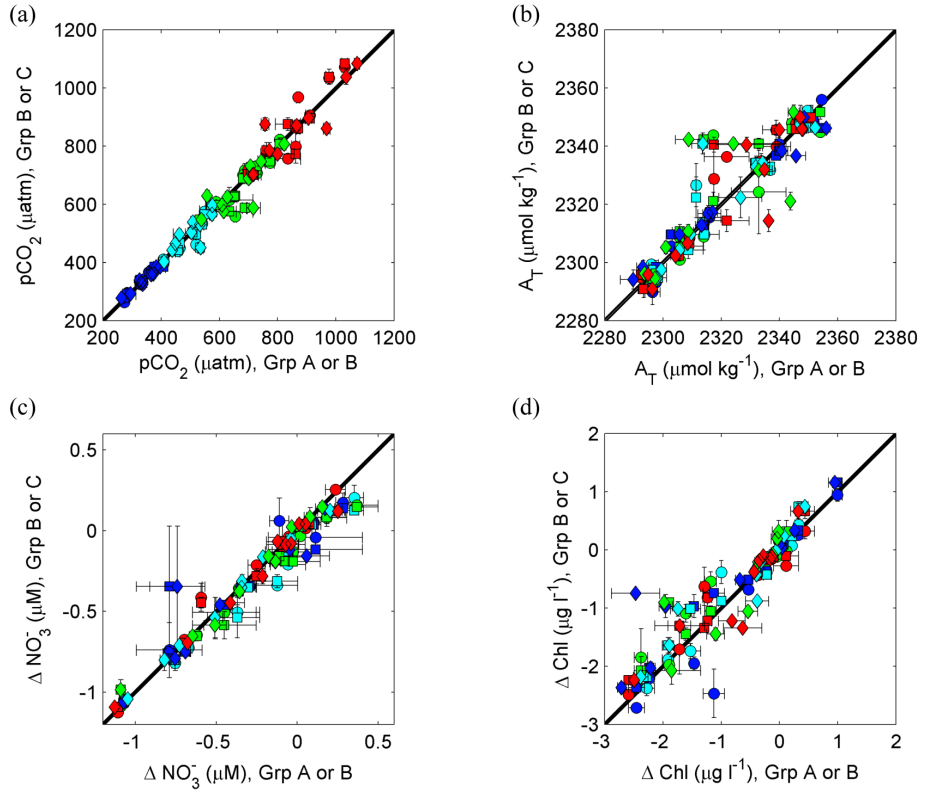

Fig. 4. Comparison of variables measured across the three parallel sets of triplicate bottles (grps. A, B and C) for all main experiments: pCO_2 (µatm) **(a)**, A_T (µmol kg^{-1}) **(b)** and nitrate (µmol L^{-1}) **(c)** and Chl *a* (µg L^{-1}) **(d)**. Plotted values are means ±1 se of triplicate bottles in all cases. Colour indicates target pCO_2 treatment (blue = ambient, cyan = 550 µatm, green = 750 µatm, red = 1000 µatm), with symbol shape indicating the following comparisons between groups: A vs. B (circles), A vs. C (squares) and B vs. C (diamonds).

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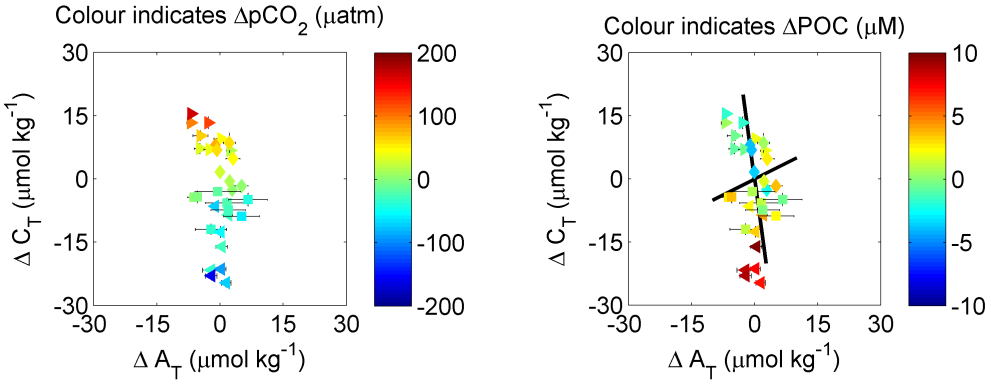

Fig. 5. Scatter plots of differences between initial condition and time point samples (Δ) for the main experiments excluding E1 (i.e. E2–E5). ΔC_T (μmol kg^{-1}) is plotted against ΔA_T (μmol kg^{-1}) with calculated changes in pCO_2 (ΔpCO_2) (μatm) and measured changes in POC (ΔPOC) (μmol L^{-1}) indicated by colours in the left and right panel respectively. Symbol shapes indicate experiments as in Fig. 3. Plotted values are means ±1 se.

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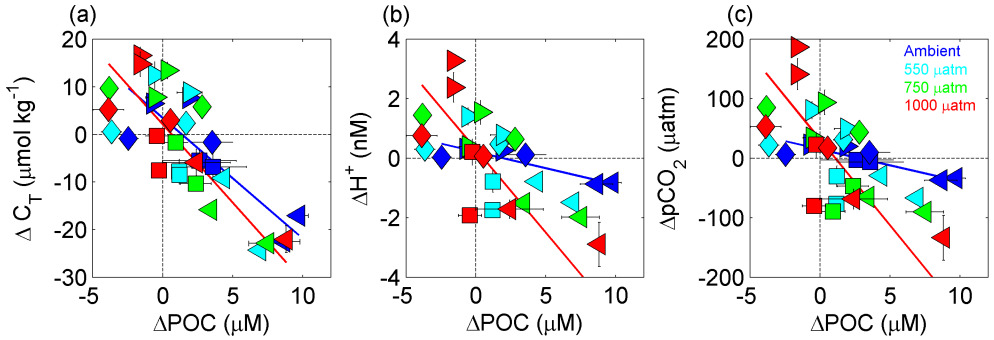

Fig. 6. Scatter plots of differences in carbonate chemistry parameters against differences in POC across all timepoints for the main experiments excluding E1 (i.e. E2–E5): ΔC_T (μmol kg^{-1}) **(a)**, ΔH^+(nmol L^{-1}) **(b)** and ΔpCO_2 (μatm) **(c)** are presented as a function of ΔPOC (μmol L^{-1}). Symbol colours indicate target pCO_2 treatment as in Fig. 4. Symbol shapes indicate the experiment number as in Fig. 3: E2 (squares), E3 (diamonds), E4 (left pointing triangle), E5 (right pointing triangle) with plotted values being means ±1 se of biological triplicates. Solid lines indicate model II linear regressions for the lowest (ambient = blue) and highest (1000 μatm = red) pCO_2 treatments. All regressions are significant ($p < 0.05$), with significant differences in slopes between the treatments presented in **(b)** and **(c)**. Regressions lines for intermediate pCO_2 treatments are omitted for clarity.

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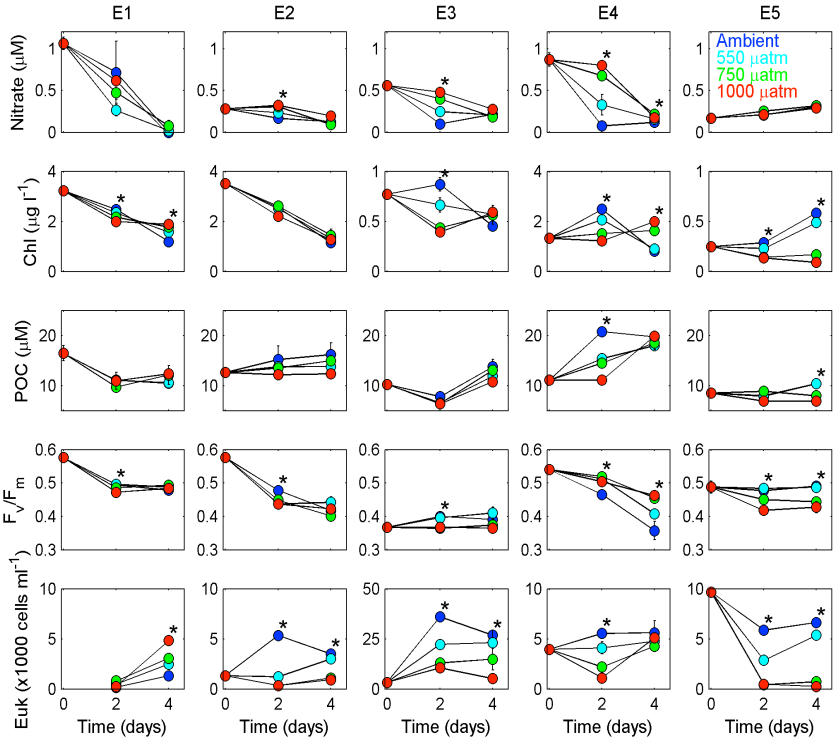

Fig. 7. Time series measurements of nitrate (µM), total Chl *a* ($\mu g L^{-1}$), particulate organic carbon (POC) (µM), photosynthetic efficiency (F_v/F_m, FRRf) and pico- and nanoeukaryote counts (cell $\times 1000\,mL^{-1}$) from the main experiments (E1–E5). Plotted values are means ±1 se, for biological triplicates. Where error bars are not visible, these are smaller than the symbol size. Note differences in scales for chlorophyll and cell count measurements. Observation of any statistically significant differences between treatments (1-way ANOVA, $p < 0.05$) for each variable and time point are indicated by "*" . The detailed results of subsequent Tukey–Kramer means comparison tests are omitted for clarity.

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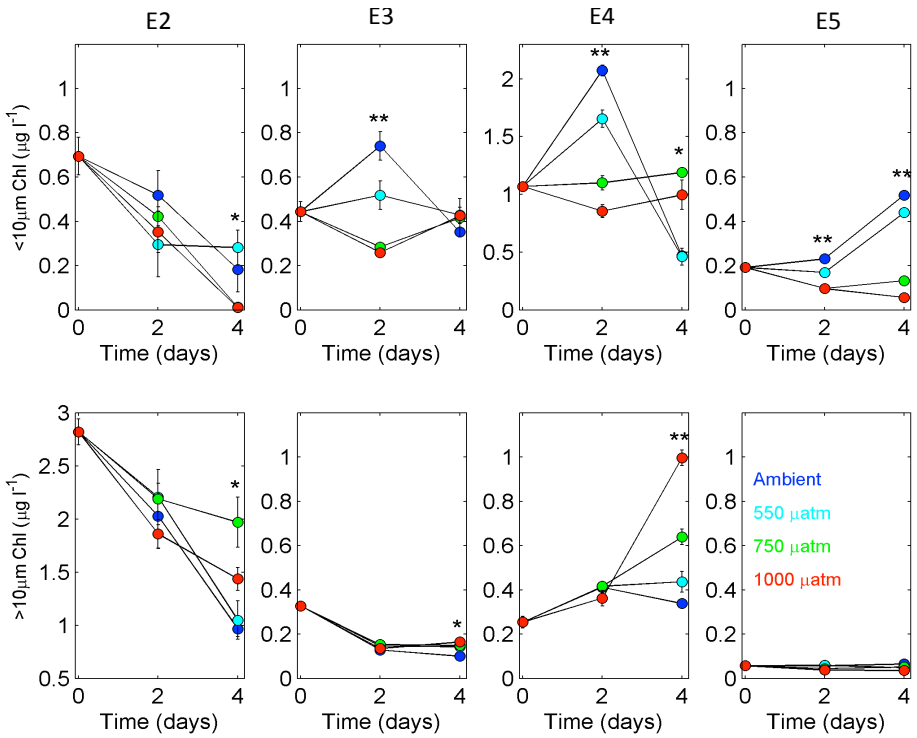

Fig. 8. Time series measurements of size fractionated Chl *a* (μL^{-1}) (< 10 µm and > 10 µm) from main experiments (E2–E5). At each time-point statistical differences between treatments were evaluated using 1-way ANOVA followed by a Tukey–Kramer means comparison test. Sets of measurements where at least one treatment was statistically different ($p < 0.05$) are indicated by "*", while sets where there is a clear progressive response as a function of treatment (i.e. where at least 3 groups could be distinguished between the 4 incubation conditions and where the effect consistently increased/decreased as a function of pCO_2/pH) are indicated "**".

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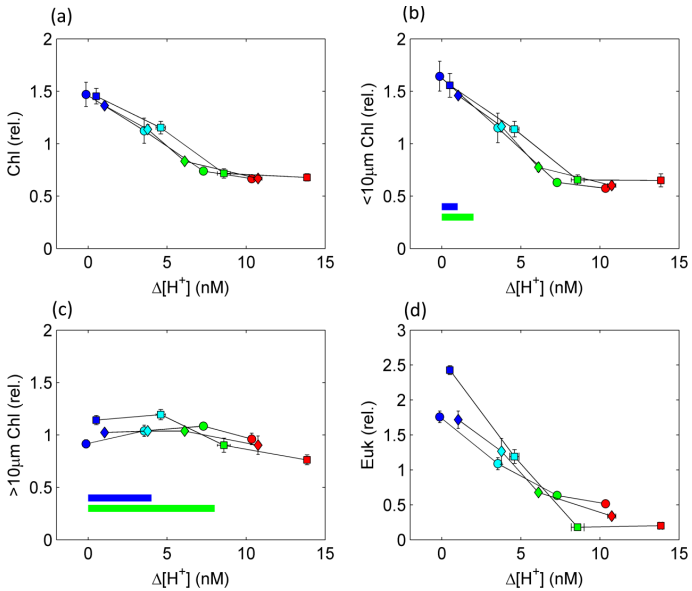

Fig. 9. Relative changes in total **(a)**, < 10 µm **(b)** and > 10 µm **(c)** size fractionated Chl *a* and **(d)** nano-picoeukaryotes counts as a function the change in $[H^+]$ after 48 h incubation in 3 (E3–E5) out of the 5 main bioassay experiments. Data for each variable are normalised to the mean value for this time-point and plotted against the difference in $[H^+]^{bulk}$ between the ambient incubation and the treatment. Values plotted are mean ±1 se for both axes. Solid bars in **(b)** and **(c)** indicate approximate ranges of cell surface ($[H^+]^{prox}$) which might be experienced by small and large cells over short timescales i.e. hours-days (see Kuhn and Raven, 2008; Flynn et al., 2012), under ambient modern conditions and at ~ 750 µatm (Flynn et al., 2012). Colours indicate conditions as in Fig. 7. Symbol shapes indicate experiments: E3 (circles), E4 (diamonds) and E5 (squares).

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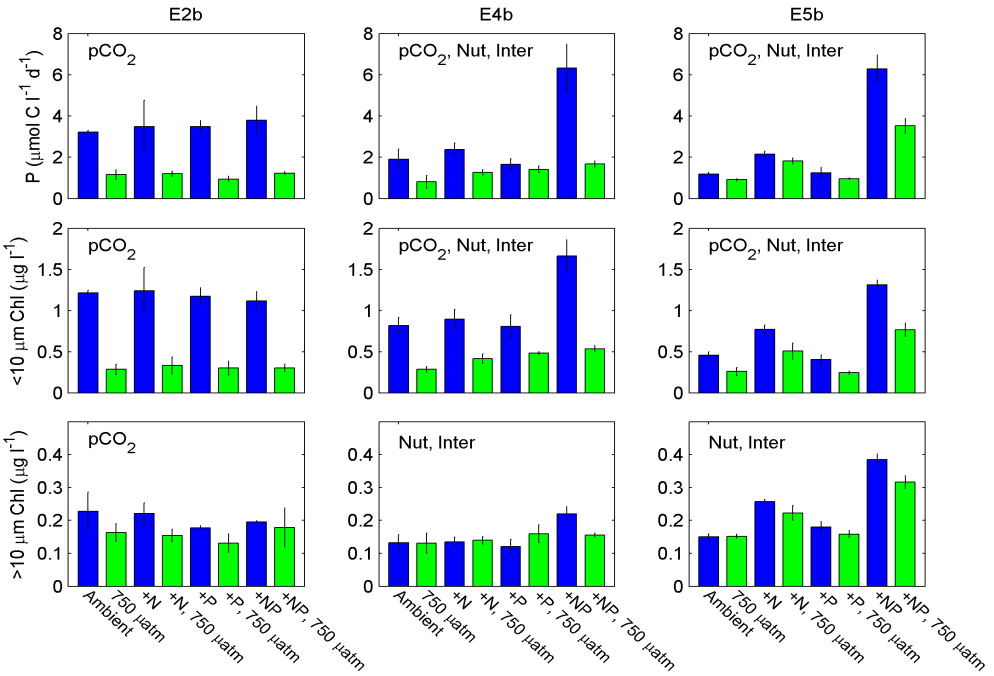

Fig. 10. Primary production (top), < 10 µm size fractionated Chl *a* (middle) and > 10 µm size fractionated Chl *a* (bottom) from the additional combined macronutrient and carbonate chemistry manipulation experiments (E2b, E4b and E5b). Data are presented as means ±1 sd of biological triplicates for each variable after 24 h incubation under ambient or 750 µatm pCO_2 (colours as in Fig. 7) and under either unamended or nutrient amended (+N, +P and +NP) conditions. Individual panels are annotated with the results of a 2-way ANOVA, with "pCO_2", "Nut" and "Inter" indicating significant ($p < 0.05$) differences between factors corresponding to the imposed perturbation of the carbonate system, macronutrients and an interaction between these factors respectively.

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