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Effect of enhanced pCO_2 levels on the production of dissolved organic carbon and transparent exopolymer particles in short-term bioassay experiments

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Abstract. It has been proposed that increasing levels of pCO_2 in the surface ocean will lead to more partitioning of the organic carbon fixed by marine primary production into the dissolved rather than the particulate fraction. This process may result in enhanced accumulation of dissolved organic carbon (DOC) in the surface ocean and/or concurrent accumulation of transparent exopolymer particles (TEPs), with important implications for the functioning of the marine carbon cycle. We investigated this in shipboard bioassay experiments that considered the effect of four different pCO_2 scenarios (ambient, 550, 750 and 1000 µatm) on unamended natural phytoplankton communities from a range of locations in the northwest European shelf seas. The environmental settings, in terms of nutrient availability, phytoplankton community structure and growth conditions, varied considerably between locations. We did not observe any strong or consistent effect of pCO_2 on DOC production. There was a significant but highly variable effect of pCO_2 on the production of TEPs. In three of the five experiments, variation of TEP production between pCO_2 treatments was caused by the effect of pCO_2 on phytoplankton growth rather than a direct effect on TEP production. In one of the five experiments, there was evidence of enhanced TEP production at high pCO_2 (twice as much production over the 96 h incubation period in the 750 µatm treatment compared with the ambient treatment) independent of indirect effects, as hypothesised by previous studies. Our results suggest that the environmental setting of experiments (community structure, nutrient availability and occurrence of phytoplankton growth) is a key factor determining the TEP response to pCO_2 perturbations.

1 Introduction

Uptake of anthropogenic carbon dioxide (CO_2) is lowering the pH of the surface ocean (Doney et al., 2009). The effect that this will have on various components of the marine ecosystem is the subject of widespread research (Fabry et al., 2008; Hofmann et al., 2010; Wernberg et al., 2012). Of particular interest is the effect of ocean acidification on components of the marine carbon cycle, since there is the potential for both positive and negative feedbacks to rising atmospheric pCO_2 (Riebesell et al., 2007; Gehlen et al., 2011; Passow and Carlson, 2012). In this study, we focus on two elements of the marine carbon cycle: dissolved organic carbon (DOC) and transparent exopolymer particles (TEPs).

Dissolved organic carbon forms the largest oceanic reservoir of reduced carbon (Hansell et al., 2009). Typical DOC concentrations are 60 to 80 µM in surface waters of the open ocean and < 50 µM in the deep ocean (Hansell et al., 2009), while concentrations in coastal waters are enhanced by higher levels of primary production and inputs from rivers (Dafner and Wangersky, 2002). The biologically labile fraction of DOC is a substrate to heterotrophic microbial communities, who remineralise it to CO_2, and thereby make DOC an important component of the microbial carbon loop and marine carbon cycle (Hansell, 2013). The recalcitrant DOC

fraction that evades remineralisation is transported to the deep ocean through advection, making an important contribution to the biological carbon pump (Ducklow et al., 2001).

Transparent exopolymer particles are gel-like particles that form through coagulation of the polysaccharide fraction of dissolved organic matter (Passow, 2002). These particles have a "stickiness" that facilitates aggregation of other particles such as phytoplankton cells, forming large marine aggregates capable of sinking (Engel et al., 2004b). Furthermore, the elemental composition of TEPs is not constrained by stoichiometric ratios and can be rich in carbon (Passow, 2002), meaning that these sinking aggregates can also have a high carbon content. Consequently, through both their high carbon content and their role as a facilitator of export, TEPs make an important contribution to the biological carbon pump.

Dissolved organic carbon and TEPs are inherently linked and form key components of the marine carbon cycle and the biological carbon pump. Consequently, understanding their operation in a future high CO_2 world is central to predicting changes to the wider carbon cycle and determining possible feedbacks to rising atmospheric pCO_2.

Previous studies considering the effects of enhanced pCO_2 on DOC and/or TEPs have adopted a range of different approaches. We can broadly categorise them based on whether or not they stimulated growth by nutrient addition and whether they used a single phytoplankton species or a natural assemblage. With respect to DOC, a mesocosm study by Kim et al. (2011) observed $\sim 20\,\%$ more DOC production at high pCO_2 (900 µatm) compared to ambient levels in a natural phytoplankton community when growth was stimulated by nutrient addition. Yoshimura et al. (2010) and Yoshimura et al. (2013) found the opposite effect in bioassay experiments in the Sea of Okhotsk and sub-Arctic Pacific respectively, in which no nutrients were added. Yoshimura et al. (2010) observed significantly lower DOC accumulation in communities exposed to pCO_2 levels of > 480 µatm, while Yoshimura et al. (2013) measured consistently higher concentrations of DOC in the lowest pCO_2 treatment (300 µatm) over the first 10 days of their experiment. Engel et al. (2004a) stimulated growth (by nutrient addition) of the coccolithophore, *Emiliania huxleyi*, in mesocosms at three different levels of pCO_2 (190, 410 and 710 µatm) and found no significant difference in DOC accumulation with pCO_2 treatment. More recently, during a mesocosm experiment in the Arctic Ocean in which communities were exposed to pCO_2 levels between 170 and 1100 µatm, Engel et al. (2013) found that DOC production (measured by ^{14}C uptake) was greater at higher pCO_2 both before and after nutrient addition, while the accumulation of DOC was enhanced only before nutrients were added. In a similar experimental setup in the Baltic Sea, Engel et al. (2014) found no effect of increased CO_2 on DOC accumulation.

Experiments on TEPs have produced some more consistent results. Incubation experiments by Engel (2002) observed enhanced TEP production at higher pCO_2 in a natural phytoplankton assemblage in nitrate-limited waters. Similarly, the mesocosm study of Engel et al. (2004a) and growth stimulating batch culture experiments of Pedrotti et al. (2012) found that, after normalisation for variable levels of growth, more TEPs were produced by *E. huxleyi* and other coccolithophore species (*Calcidiscus leptoporus*, *Syracosphaera pulchra*) when they were exposed to pCO_2 levels of > 700 µatm. Riebesell et al. (2007) and Bellerby et al. (2008) noted enhanced non-stoichiometric carbon uptake at high pCO_2, which they infer to have resulted in enhanced TEP formation. Borchard and Engel (2012) directly measured extracellular release of organic carbon (using ^{14}C) as well as abundance of combined carbohydrates (precursors to TEP formation) in phosphorus-controlled chemostats with *E. huxleyi*, in which steady state growth was maintained by constant nutrient addition. They found that, under conditions of nutrient limitation, TEP production was significantly greater at greenhouse conditions (pCO_2 of 900 µatm, temperature of 18 °C) compared to ambient conditions (300 µatm, 14 °C) due to greater extracellular release of TEP precursors. The aforementioned study of Engel et al. (2014) also considered the effect of CO_2 on TEPs in nutrient-enriched mesocosm experiments in the Baltic Sea. During the peak of the bloom, TEP concentration was significantly greater at high pCO_2, and this appeared to facilitate higher levels of organic matter sedimentation.

Despite the different approaches of these studies, the generally accepted hypothesis is that, under high pCO_2 conditions, more of the organic carbon fixed by photosynthesis is channelled into the dissolved fraction and released from the cells. This leads to a greater standing stock of TEPs as the released matter coagulates into particulates, while the effect on the standing stock of DOC is less certain. While giving insights into likely mechanisms, these conclusions were mostly drawn from idealised (nutrient addition, single phytoplankton species) and/or isolated (single natural assemblage) experiments. Consequently, as the authors highlighted, their results may not be representative of the natural world response or applicable on a wide scale.

In the present work, we tested the conclusions of these previous studies in unamended (no nutrient addition) natural ecosystems from a range of locations in the European shelf seas and in a range of environmental settings. As such, to the extent that is possible in manipulation experiments, the results reflect a real-world community response to enhanced pCO_2 conditions and offer insight into the spatial variability of this response. The aim of this study is to investigate whether the production of DOC and/or TEP is enhanced at high pCO_2, and whether the environmental settings of the different experiments influence this relationship.

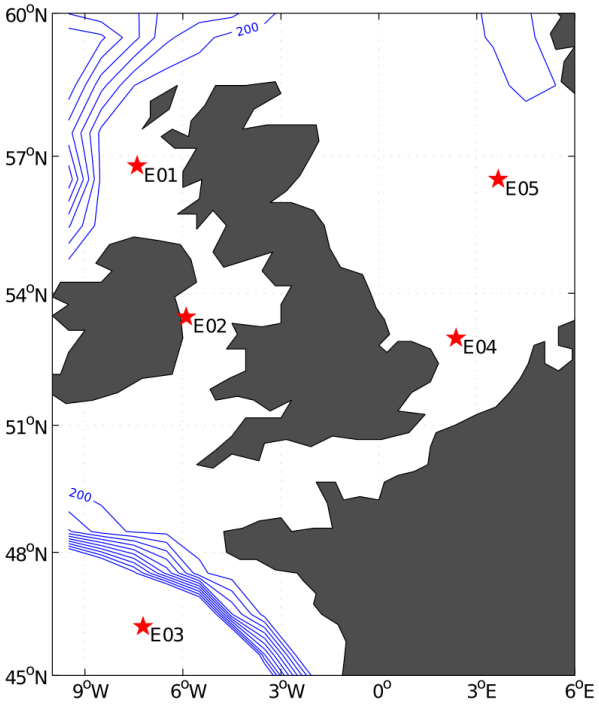

Figure 1. Map of study area showing locations of bioassay experiments as red stars. Blue lines are 200 m depth contours.

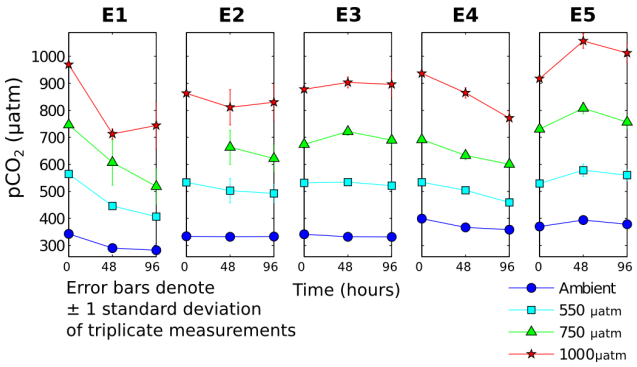

Figure 2. Evolution of pCO_2 in five bioassay experiments across three time points for all pCO_2 treatments. Legend shows the colours and symbols used to denote the different pCO_2 treatments.

2 Methods

2.1 Bioassay set up

Five shipboard bioassay experiments were performed during a cruise in northwest European shelf seas in June and July, 2011. Locations are presented in Fig. 1. An overview of the methodology is presented here and more details of the sampling and incubation procedures are provided in Richier et al. (2014b). All data are available from the British Oceanographic Data Centre (Richier et al., 2014a).

Surface seawater ($\sim$ 5 m depth) was collected before dawn from one CTD cast (24 $\times$ 20 L Ocean Test Equipment (OTE) bottles) and dispensed from randomly assigned OTE bottles through silicon tubing into 72 $\times$ 4.5 L acid-washed polycarbonate bottles (Nalgene). The bioassay experiments were carried out in a purpose-built incubator, which maintained in situ temperatures from the time of sampling and provided controlled light levels through daylight simulation LED panels (100 µE m^{-2} s^{-1}) on an 18/6 h light/dark cycle. Each bioassay ran for four days, with measurements taken at the start and after 48 and 96 h. Prior to sample collection, the bioassay bottles were gently agitated to ensure a vertically homogeneous repartition of all quantities to be measured. Following sampling, the incubation bottles were sacrificed and hence not further incubated.

For each of the five experiments, carbonate chemistry in the seawater was artificially manipulated to achieve three different target pCO_2 levels (550, 750 and 1000 µatm), in addition to an "Ambient" treatment without manipulation, through the equimolar addition of $NaHCO_3^-$ and HCl (Gattuso et al., 2010). The volumes of $NaHCO_3^-$ and HCl required to achieve the target pCO_2 levels were determined from the initial total alkalinity (A_T) and dissolved inorganic carbon (C_T) measurements in the sample seawater, using CO2SYS in Matlab and the equilibrium constants of the carbonate system from Mehrbach et al. (1973), refitted by Dickson and Millero (1987). Sampling and analyses for A_T and C_T were made following the sampling procedures described by Dickson et al. (2007); full details are provided in Richier et al. (2014b). The effectiveness of the manipulation was immediately verified by subsequent measurements. Initial pCO_2 values and the evolution throughout the experiments is presented in Fig. 2. The actual initial pCO_2 levels achieved by the manipulation were close to, but not exactly, the target levels (more information is provided in Richier et al., 2014b).

2.2 Nutrient and chlorophyll analysis

Analysis for micro-molar concentrations of nitrate + nitrite (hereafter nitrate) and phosphate were undertaken during the cruise using a segmented flow auto-analyser (Skalar San+) following methods described by Kirkwood (1996).

Both total and size-fractionated chlorophyll *a* (Chl *a*) concentrations were determined. Seawater was filtered through 25 mm diameter glass fibre filters (0.7 µM nominal pore size; Whatman GF/F) and 25 mm diameter polycarbonate filters (10 µM pore size; Nuclepore, Whatman) for total Chl *a* and the $>$ 10 µM size fraction (hereafter "$>$ 10 µM Chl *a*") respectively. The $<$ 10 µM size fraction (hereafter "$<$ 10 µM Chl *a*") was determined from the difference between total and $>$ 10 µm Chl *a* concentrations. All filters were extracted in 90 % acetone for 24 h, and Chl *a* was quantified by fluorometry (Turner Designs Trilogy fluorometer) following Welschmeyer (1994). Chlorophyll *a* concentrations

were calibrated against dilutions of a solution of pure Chl *a* (Sigma, U.K.) in 90 % acetone, with instrument drift further corrected by daily measurement of a solid fluorescence standard. No size fractionation was determined for the bioassay experiment E1.

2.3 Dissolved organic carbon and TEP measurements

For both DOC and TEPs, triplicate measurements were made for each pCO_2 treatment at each time point. For DOC, seawater was filtered using pre-combusted (450 °C, 4 h) glass fibre filters (0.7 µM nominal pore size; MF300, Fisher Scientific) to remove particulate carbon and most organisms. Samples were directly filtered into pre-combusted 25 mL glass ampoules and immediately acidified to pH < 2 using 40 µL 50 % HCl. The ampoules were sealed and stored at 4 °C. Onshore, the samples were analysed using a high temperature catalytic combustion technique (Shimadzu TOC-TDN; Spyres et al., 2000). The samples were sparged with high purity oxygen gas to remove C_T and combusted at 680 °C on a Pt catalyst to convert the DOC to CO_2, which was subsequently analysed using non-dispersive infrared detection. Acidified deep Sargasso Sea water, preserved in glass ampoules and provided by D. Hansell (University of Miami), served as a certified reference material. Our daily analysis of the reference material yielded a mean concentration of 42.7 ± 1.2 µM ($n = 64$), which was in good agreement with the certified value of 41–44 µM. Our analytical precision, based on the coefficient of variation (standard deviation/mean) of consecutive injections (typically 3–5 injections) of a single sample, was typically < 1%.

Samples of TEPs were collected by filtration of 200 mL of seawater through 25 mm diameter polycarbonate filters (0.45 µM pore-size, Sterlitech) at constant vacuum (200 mBar). The particles retained on the filters were stained with 500 µL of 0.02 % aqueous solution of Alcian blue in 0.06 % acetic acid (pH = 2.5). The dye was pre-filtered using a polycarbonate filter (0.2 µM pore-size; Sterlitech) before use. Stained filters were rinsed once with deionised water (Milli-Q, Millipore) and then transferred into 15 mL polypropylene centrifuge tubes (Fisher Scientific) and stored at −20 °C. Onshore, the amount of Alcian Blue adsorbed onto the filters was determined following a soak in 6 mL of 80 % sulfuric acid for 2 h and determination of the absorbance of the resulting solution at 787 nm (absorption maximum) using a spectrophotometer (U-1800, Hitachi). The amount of Alcian blue in the solution was directly related to the weight of the polysaccharide that was retained on the filter (Passow and Alldredge, 1995).

Staining of polysaccharides on coccolithophore cells has the potential to introduce error in the calculation of TEP concentrations (Engel et al., 2004a). The TEP filters were not examined microscopically to determine if staining of cells occurred. Engel et al. (2004a) calculated that the staining of *E. huxleyi* cells accounted for 2.59 ± 0.4 pg Xequiv cell^{-1} (pg Xanthan equivalent cell^{-1}). Adopting this value (following Engel et al., 2004a, 2009; Borchard and Engel, 2012), we calculated a potential error in TEP concentration of between 0 and 0.75 µg Xequiv L^{-1}, based on the abundance of coccolithophores in our experiments (between 0 and 250 000 cells L^{-1}) and assuming that they were predominantly *E. huxleyi* (Poulton et al., 2014). This is less than 1 % of the smallest measured TEP concentration, making this error negligible.

Elevated DOC concentrations were measured in just one of the three replicate bottles for each of the following experiments after 48 h: 750 µatm treatment of E1 and the 550 and 750 µatm treatment of E4. The measured value was considerably higher than in the other two replicates, causing a spike in the time evolution and an exceptionally large standard deviation at these time points (> 35 µM, compared to < 5 µM for all other experiments). The Grubbs test (Grubbs, 1969) was used to identify these elevated values as outliers at the 95 % confidence level. Given the susceptibility of DOC measurements to contamination (Spyres et al., 2000), and the fact that after 96 h DOC concentrations were back at more consistent values, we conclude that these single measurements were erroneous. Consequently, they were not included in the analysis.

2.4 Statistical analysis

For each variable, experiment and time point, a one-way ANOVA test was carried out to determine if mean concentrations were significantly different between treatments. Subsequently, the Tukey–Kramer test statistic was used to determine the significance of the difference between each treatment. In the results, quoted p values correspond to Tukey–Kramer test.

3 Results

3.1 Environmental settings and communities physiological response

Before considering the response of TEPs and DOC in the bioassay experiments, we describe the environmental conditions of each experiment, with respect to nutrient availability, phytoplankton size structure and phytoplankton growth. Those features that were found to be important for the interpretation of the DOC and TEP responses are highlighted here. For further results of the bioassay experiments, see Richier et al. (2014b).

Figure 3 shows the time evolution of the nutrient (nitrate and phosphate) and Chl *a* concentrations (total and size-fractionated). The highest initial concentrations of nitrate (> 1 µM) and total Chl *a* (> 3 µg L^{-1}; no size-fractionation available) were observed in E1 (56°47.7′ N 7°24.3′ W, stratified water column) and these decreased rapidly throughout the time course in all treatments. There was no obvious

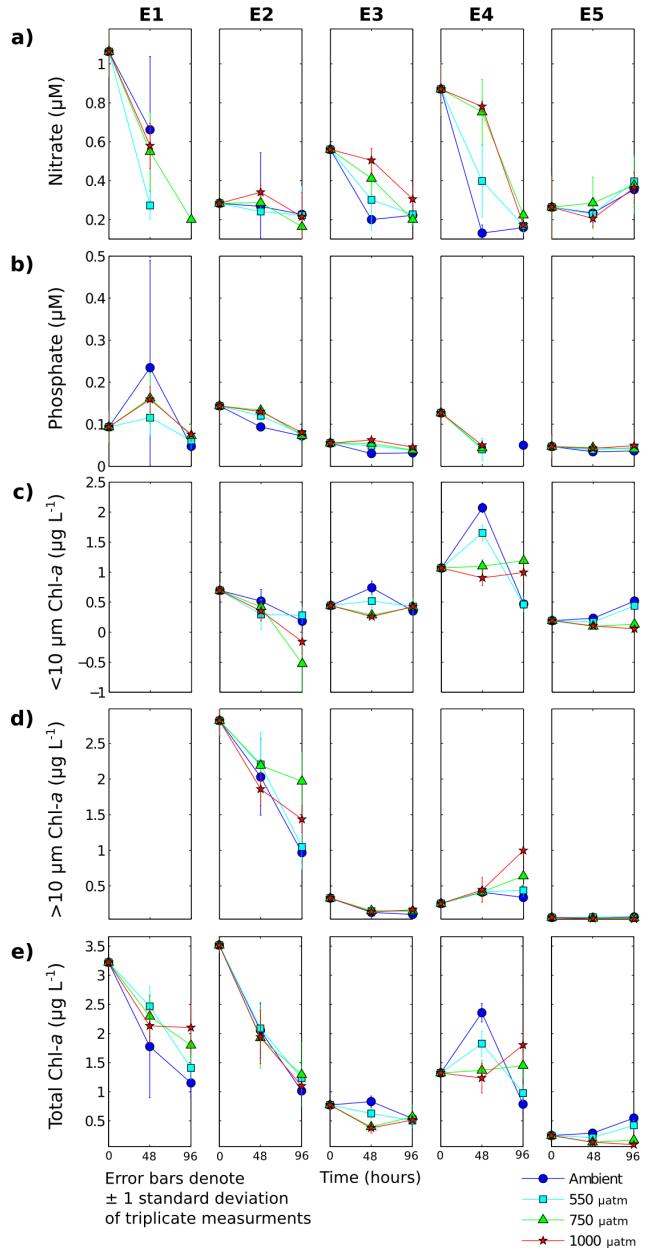

Figure 3. Evolution of environmental variables in five bioassay experiments across three time points for all pCO_2 treatments: **(a)** nitrate (μM), **(b)** phosphate (μM), **(c)** small size fraction (< 10 μM) chlorophyll *a* ($\mu g\,L^{-1}$), **(d)** large size fraction (> 10 μM) chlorophyll *a* ($\mu g\,L^{-1}$) and **(e)** total concentration of chlorophyll *a* ($\mu g\,L^{-1}$). Legend shows the colours and symbols used to denote the different pCO_2 treatments.

pattern of treatment dependence in the decline of these variables, except in the final concentration of Chl *a*, which was significantly higher in the 750 and 1000 μatm treatments than in the Ambient and 550 μatm treatments ($p < 0.01$).

In E2 (52°28.2′ N 5°54.1′ W, well-mixed water column), initially high levels of Chl *a* ($\sim 3.5\,\mu g\,L^{-1}$) and low nitrate concentration (~ 0.3 μM) were suggestive of a recent phytoplankton bloom that was reaching termination, possibly due to nitrate limitation. Chlorophyll *a* levels decreased through the time course, implying grazing and/or lysis of the phytoplankton that were present. In the 750 and 1000 μatm treatments at 96 h, there were higher concentrations of > 10 μm Chl *a* than of total Chl *a*, suggesting a filtration or measurement error. Except in the measurements corresponding to this possible error, there was no significant pattern of treatment dependence in the decline of Chl *a*. Nitrate and phosphate concentrations decreased overall, suggesting some continued utilisation despite no net growth.

Experiment E3 (46°12.1′ N 7°13.3′ W, stratified water column) had an initially enhanced concentration of nitrate (> 0.5 μM) but was depleted in phosphate (~ 0.05 μM). Over the first 48 h, significantly more nutrient utilisation was observed at lower pCO_2 ($p < 0.01$). Concurrently, an increase in < 10 μM Chl *a* – implying net growth of small-celled phytoplankton – was observed in the Ambient and 550 μatm treatments while a decrease occurred in the 750 or 1000 μatm treatments. This suggests that phytoplankton growth and nutrient uptake were initially suppressed in communities exposed to higher levels of pCO_2. Between 48 and 96 h, with nutrient concentrations now lower in the Ambient and 550 μatm treatments, further growth was suppressed and Chl *a* levels decreased. In contrast, there were sufficient remaining nutrients in the 750 and 1000μatm treatments to support net growth of small-celled phytoplankton through to the end of the incubation.

The pattern in the environmental conditions of E4 (52°59.7 N 2°29.8 E, well-mixed water column) was similar to that of E3, but the response was magnified due to the higher initial nutrient concentrations (nitrate ~ 0.8 μM, phosphate ~ 0.12 μM). As in E3, growth was suppressed at higher pCO_2 levels in the first 48 h leading to significantly less nitrate utilisation and net small-celled phytoplankton growth in the 750 and 1000 μatm treatments ($p < 0.01$). Subsequently, between 48 and 96 h, the depleted nitrate concentration suppressed growth in the Ambient and 550 treatments, leading to a decrease in Chl *a*, while net growth was observed in the 750 and 1000 treatments, consistent with E3. However, unlike in the previous experiment in which treatment dependent Chl *a* changes occurred within the small size fraction only, net growth in the higher pCO_2 treatments over this time step was predominantly of large-celled phytoplankton. Consequently, despite comparable utilisation of nutrients, significantly higher Chl *a* concentrations were measured at higher pCO_2 levels after 96 h ($p < 0.01$).

In E5 (56°30.3′ N 3°39.5′ E, stratified water column), Chl *a* levels were initially low ($\sim 0.2\,\mu g\,L^{-1}$, all < 10 μm Chl *a*), as were nitrate and phosphate concentrations (~ 0.25 and ~ 0.05 μM respectively). In the first 48 h, there was a similar response in Chl *a* to that of E3 and E4, with significantly less net growth at higher pCO_2 ($p < 0.05$). A treatment-dependent response was not observed in the

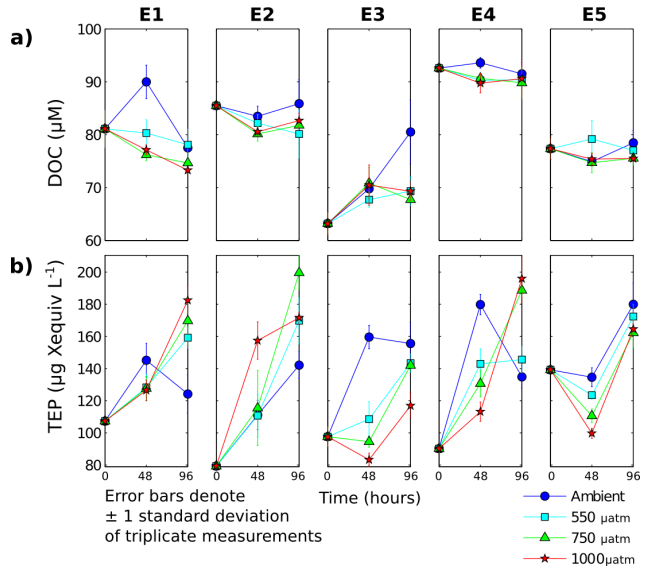

Figure 4. Evolution of **(a)** dissolved organic carbon (DOC; µM) and **(b)** transparent exopolymer particles (TEPs; µg Xequiv L^{-1}) in five bioassay experiments across three time points for all pCO_2 treatments. Legend shows the colours and symbols used to denote the different pCO_2 treatments.

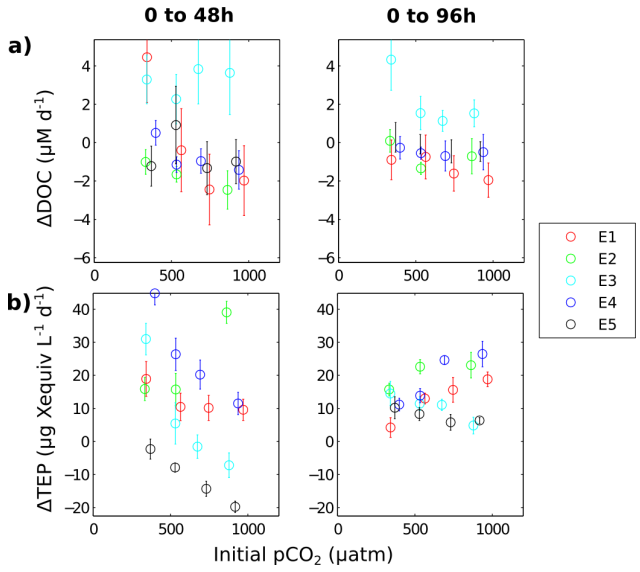

Figure 5. Relationship between the initial pCO_2 of each experiment (actual values) and the production per day of **(a)** dissolved organic carbon (ΔDOC; µM d^{-1}) and **(b)** transparent exopolymer particles (ΔTEP; µg Xequiv L^{-1} d^{-1}) for the time steps 0 to 48 h (left) and 0 to 96 h (right).

nutrient concentrations. Between 48 and 96 h, levels of < 10 µM Chl *a* continued to diverge, with further net growth in the Ambient and 550 treatments and a sustained decrease of Chl *a* in the 750 and 1000 treatments.

3.2 Response of DOC to pCO_2 perturbation

Figure 4a shows the time evolution of DOC concentrations in the bioassay experiments. The extent to which differences in concentrations between treatments were statistically significant is presented in Table 1. Initial DOC concentrations ranged between ~ 60 µM in E3 and ~ 90 µM in E4. Throughout the experiments, DOC concentrations varied by ± 10 µM but only rarely showed statistically significant differences between treatments.

Notably, on each occasion that significant differences between treatments were observed, it was due to a higher concentration of DOC in the Ambient treatment in comparison to that at higher pCO_2. This suggests that DOC production (or lack of DOC breakdown) was favoured most frequently in the Ambient pCO_2 treatment. This is reinforced by Fig. 5a, which shows the rate of DOC production/breakdown across each time step (0 to 48 h and 0 to 96 h) against the initial pCO_2 value (actual values rather than target pCO_2 levels). Although the response was highly variable, a lower pCO_2 level appeared to favour DOC production (or inhibit DOC breakdown) after 48 h in E1, E2 and E4. Between 0 and 96 h there is an indication of a moderate decreasing trend of production against initial pCO_2 in all experiments, although the signal is very small.

3.3 Response of TEPs to pCO_2 perturbation

Figure 4b shows the time evolution of TEP concentrations in the bioassay experiments. The extent to which differences in concentrations between treatments were statistically significant is presented in Table 1. For all experiments, initial concentrations of TEPs were between 80 and 140 µg Xequiv L^{-1} (µg Xanthan equivalent L^{-1}), and increased after the first time step in all experiments except E5 and the high pCO_2 treatments of E3. In contrast to DOC, a statistically significant difference between treatments was observed in all experiments (Table 1).

In E1, TEP concentrations increased throughout the incubation period in all except the Ambient treatment, in which concentrations decreased between 48 and 96 h to reach a final concentration that was significantly lower than those of the higher pCO_2 treatments ($p < 0.01$). Experiment E2 displayed some of the largest changes in TEP concentrations over the 96 h period, increasing from the lowest initial concentration (80 µg Xequiv L^{-1}) to some of the highest (200 µg Xequiv L^{-1} in the 750 treatment). After 96 h, final concentrations were significantly greater in the higher pCO_2 treatments, with twice as much TEPs produced over the incubation period in the 750 µatm treatment compared to the Ambient treatment. Stabilisation of the TEP concentration after 48 h in the 1000 µatm treatment meant that it was not statistically distinct from the 550 µatm treatment. Similarities in the treatment-dependent response of experiments E3 and E4, as observed in the environmental conditions, were also present in TEP concentrations. After 48 h, production of TEPs was

Table 1. Summary of statistically significant differences between treatments for DOC (top) and TEPs (bottom). Upward pointing arrows signify a positive correlation between pCO_2 and DOC/TEP production (more DOC/TEPs in the higher pCO_2 treatment) and downward pointing arrows signify a negative correlation (more DOC/TEPs in the lower pCO_2 treatment). Single-headed and two-headed arrows signify statistical significance at the 95 % and 99 % confidence level respectively (using the Tukey–Kramer test statistic); "–" signifies that treatments were not significantly different at the 95 % confidence level.

DOC		A/550	A/750	A/1000	550/750	550/1000	750/1000
E01	48 h	↓	↓	↓	–	–	–
	96 h	–	–	–	–	–	–
E02	48 h	–	–	–	–	–	–
	96 h	–	–	–	–	–	–
E03	48 h	–	–	–	–	–	–
	96 h	↓	↓	↓	–	–	–
E04	48 h	–	–	↓	–	–	–
	96 h	–	–	–	–	–	–
E05	48 h	–	–	–	–	–	–
	96 h	–	–	–	–	–	–

TEP		A/550	A/750	A/1000	550/750	550/1000	750/1000
E01	48 h	–	–	–	–	–	–
	96 h	↑	↑	↑	–	–	–
E02	48 h	–	–	↑	–	↑	↑
	96 h	↑	↑	↑	↑	–	↓
E03	48 h	↓	↓	↓	–	↓	–
	96 h	–	–	↓	–	↓	↓
E04	48 h	↓	↓	↓	–	↓	–
	96 h	–	↑	↑	↑	↑	–
E05	48 h	↓	↓	↓	↓	↓	↓
	96 h	–	–	–	–	–	–

higher in the lower pCO_2 treatments. At this time point in both experiments, concentrations in the Ambient treatment were significantly greater than those in all other treatments ($p < 0.01$), while concentrations in the 550 µatm treatments were significantly greater than those in the 1000 µatm treatment ($p < 0.05$). Subsequently, between 48 and 96 h, concentrations in the Ambient and 550 µatm treatments stabilised or decreased (except in the 550 µatm treatment of E3), while those in the 750 and 1000 µatm treatments changed from decreasing to increasing (in E3) or increased at an enhanced rate (in E4). Notably, the final concentrations of TEPs showed a different treatment dependence in the two experiments: in E3, fewer TEPs were produced overall in the 1000 µatm treatment than in the others ($p < 0.05$), while in E4, more TEPs were produced in the 750 and 1000 µatm treatments than in the Ambient and 550 treatments ($p < 0.01$). In E5, there was initially a treatment-dependent decrease in TEP, leading to significantly higher concentrations at lower pCO_2 after 48 h ($p < 0.05$), consistent with experiments E3 and E4. Between 48 and 96 h, concentrations in all treatments increased at a fairly uniform rate.

Figure 5b shows the rate of TEP increase/decrease (per day) across each time step (0 to 48 h and 0 to 96 h) against the initial pCO_2 conditions of that experiment. It illustrates how the relationship varies between experiments and between time steps. Between 0 and 48 h, in E3, E4 and E5, greater TEP net production (or in the case of E5, less TEP net decrease) was observed in communities subjected to lower initial pCO_2. This was also the case in E1, but only between the lowest pCO_2 treatment and the rest. The opposite relationship was observed in E2, in which more TEP net production occurred at the highest pCO_2 level. Over 96 h, correlations between TEP production and initial pCO_2 were considerably less pronounced. Experiments E1, E2 and E4, exhibited a positive relationship, with more net TEP production at higher initial pCO_2. Experiments E3 and E5 maintained the same relationship as over the first 48 h, net production being lower at higher pCO_2.

4 Discussion

4.1 No suggestion of strong effect of pCO_2 on DOC

In the bioassay experiments, enhanced pCO_2 levels did not have a pronounced effect on the production of DOC, with statistically significant differences in DOC accumulation

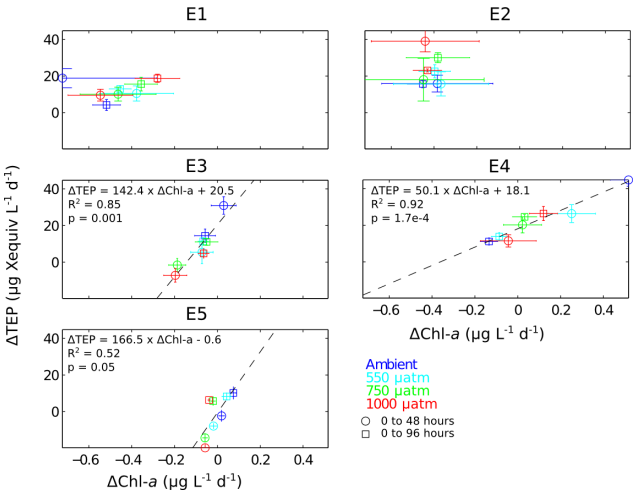

Figure 6. Relationship between transparent exopolymer particle production per day (ΔTEP; µg Xequiv L^{-1} d^{-1}) and total chlorophyll *a* production per day (ΔChl *a*; µG L^{-1} d^{-1}) for five bioassay experiments. The dashed line in E3, E4 and E5 is the result of a linear regression between these two variables and details of this analysis are also shown: regression equation, correlation (R^2) and *p* value. Regression analyses for E1 and E2 were not statistically robust.

between treatments being present at only 3 out of 10 time points across all experiments (Table 1). Engel et al. (2004a) and Engel et al. (2014) both found a similarly indistinguishable response for DOC in batch culture and mesocosm experiments respectively and hypothesised that loss of DOC, either through bacterial degradation or coagulation to TEP, occurred on timescales shorter than their measurement frequency, meaning that treatment-dependent changes in DOC concentration could be damped out between measurements. This may also be the case in our experiments, with bulk concentration measurements at 48 and 96 h being insufficient to identify possible treatment dependence of DOC production occurring on shorter timescales. Alternatively, the effect on DOC could just be small in comparison to the background concentration and thus difficult to detect.

On each occasion where there was a statistically significant difference between treatments, this was due to a higher DOC concentration in the Ambient treatment (Table 1). Furthermore, Fig. 5a implies a small but negative correlation between pCO_2 and DOC in some experiments. This relationship is not consistent with recent mesocosm results in the Arctic (Engel et al., 2013). However, it agrees with the results of Yoshimura et al. (2010) and Yoshimura et al. (2013) in marginal seas around the sub-Arctic Pacific who noted that DOC accumulation was inhibited under high pCO_2 conditions. Yoshimura et al. (2010) suggested that this may be due to treatment-dependent changes in phytoplankton community structure, with less diatoms at high pCO_2 leading to a reduced DOC production. This link was not apparent in our experiments as we rather observed the opposite shift towards larger cells (e.g. diatoms) in the phytoplankton communities of interest (Richier et al., 2014b).

4.2 Significant but variable effect of pCO_2 on TEPs

The bioassay experiments showed that while TEP production was significantly affected by pCO_2 perturbations, the response was not consistent across experiments. The sign, consistency and magnitude of the relationship varied depending on the region in which the experiment was carried out and the time step across which the parameters were observed (Table 1). A strength of this study is the heterogeneous environmental conditions in which the several bioassay experiments were conducted. The variability of our results, therefore, indicates that these environmental conditions, and their evolution during the experiments, strongly affected the relationship between TEP production and pCO_2. Such variable relationships show that results from single experimental locations or culture experiments cannot easily be scaled to a general rule that applies equally across a diverse range of natural ecosystems.

In terms of methodology, our study is most similar to that of Engel (2002) in which carbonate chemistry was manipulated (to both past and future levels of pCO_2) in a natural phytoplankton assemblage with no nutrient addition. The study was carried out using seawater from two locations in the Baltic Sea and found that after 24 h, more TEP was produced at higher pCO_2 up to present day levels, but no effect was observed when pCO_2 was increased further. We did not determine the effect of reducing pCO_2 below Ambient levels. However, the heterogeneity of the responses we observed at increased pCO_2 clearly indicates that the relationship between pCO_2 and TEP production is more variable than observed by Engel (2002).

On the basis of previous studies, we hypothesised that TEP production would be enhanced at high pCO_2. We observed such a positive relationship on four occasions, but noted a negative relationship an equal number of times (Table 1). In the first instance, this suggests that the conclusions drawn from previous studies do not tell the whole story when the effects are measured in natural, unamended ecosystems. To gain better understanding of the effect of pCO_2 on TEP production, we must consider other processes influencing TEP concentrations in the different environmental settings of our experiments.

4.3 Net growth exhibits strong control on TEP variability

Batch cultures, mesocosm experiments and in situ measurements previously all found that TEP concentrations are closely correlated to chlorophyll during phytoplankton growth (Passow, 2002). In line with this, studies investigating the effect of pCO_2 on TEP production have noted that the

response is closely linked to variable levels of primary production (Engel et al., 2004a; Pedrotti et al., 2012). Figure 6 shows the relationship between the rate of TEP production and the rate of total Chl *a* production across all time steps in each experiment as well as the results of a linear regression between the two variables. A strong positive correlation was observed for experiments E3 and E4 with R^2 values of 0.85 and 0.92 respectively and the correlation was statistically significant in both cases ($p < 0.001$). This suggests that as Chl *a* increased, TEP concentrations also increased in a largely consistent manner irrespective of pCO_2 treatment. The relationship was maintained even when Chl *a* concentrations decreased, suggesting a tight coupling between these two variables during both production and degradation processes. The linear trend did not intersect at the origin in either experiment but rather TEP production was usually positive when Chl *a* production was zero or negative. This suggests that TEPs continued to increase after Chl *a* production ceased. This is consistent with the suggestion by Passow (2002) that TEPs continue to increase during the breakdown of a phytoplankton bloom. There was also a statistically robust positive correlation in experiment E5 ($R^2 = 0.52$, $p < 0.05$), although it was less strong than in either E3 or E4. While the relationship was also positive in E1 and E2, the correlation was not robust.

As a result of this close relationship between net growth and TEP production, we suggest that, in experiments E3, E4 and E5, the treatment dependence of TEP was due to the effect of pCO_2 on net phytoplankton growth, rather than a direct effect on TEP production. As noted in Sect. 3.1, growth was suppressed at higher pCO_2 during the first 48 h of E3, E4 and E5. Concurrently, fewer TEPs were produced (or in the case of E5, more TEPs were destroyed) at higher pCO_2 over this time period. Subsequently, between 48 and 96 h, nutrient availability became the dominant control on net growth in E3 and E4. Net growth was not sustained in the lower pCO_2 treatments and TEP concentrations decreased or stabilised accordingly, while net growth, having been previously delayed, was promoted in the higher pCO_2 treatments with concurrent delayed production of TEPs. Over the same time period in E5, net growth continued to be suppressed at high pCO_2, but a clear relation to TEP production was no longer apparent (Fig. 6). Despite this exception, we propose that it is the indirect effects of pCO_2 through growth that causes the observed treatment-dependent response of TEPs in experiments E3, E4 and E5.

The existence of this indirect effect on TEP variability makes it difficult to distinguish whether there is also an underlying direct effect of pCO_2 on TEP production in these experiments. In an experiment examining the effect of pCO_2 on TEP and DOC production by *Emiliania huxleyi*, Engel et al. (2004a) noted a close relationship between TEP concentration and cell abundance. They were able to normalise TEP production to account for this relationship and found a significant effect of pCO_2 on TEP production per cell. In the complex environment of our bioassay experiments, a similar normalisation is not trivial. We might consider how pCO_2 affected the relationship between TEP and Chl *a* production (e.g. perhaps more TEPs were produced per unit Chl *a* at higher pCO_2), but with only three time steps for each treatment in each experiment, there are not sufficient data to determine whether such an effect was present. Higher temporal resolution or a longer time series could provide further insight in future studies. Consequently, experiments E3, E4 and E5 do not support the hypothesis that TEP production was enhanced by a direct effect of high pCO_2, but rather suggest that it was primarily the effect of pCO_2 on phytoplankton growth that mediated the TEP response. Furthermore, this mediation frequently led to the opposite effect to that hypothesised, with significantly less TEP production at higher pCO_2 (Table 1).

During the latter half of E4, the remaining concentration of nutrients favours growth of large-celled phytoplankton, with significantly more growth observed at higher pCO_2 (Sect. 3.1, Fig. 3). Despite this change in community size structure, there was no change in the TEP to Chl *a* relationship (Fig. 6). Thus, in our experiments, there was no evidence for a further indirect effect of pCO_2 on TEP production, through its effect on community size structure.

4.4 Environmental conditions impact the pCO_2 effect on TEPs

The different environmental conditions of our experiments influenced the response of TEP production to pCO_2 perturbations. We use this to consider the effect that these conditions, namely community structure, initial nutrient concentration and the timing of measurements relative to phytoplankton growth, had on our results.

Experiments E3, E4 and E5 initially exhibited a consistent trend in net growth and TEP production, distinct from that of E1 and E2, despite the incubation waters being sampled from different locations and with different initial nutrient concentrations. The phytoplankton communities of these experiments were predominantly from the small size fraction, suggesting that the initial inhibited growth at high pCO_2 that we observed, with concurrent inhibited TEP production, may be a general trend in ecosystems dominated by small-celled phytoplankton. Flow cytometry analysis of community structure also revealed that, over the first time step in these experiments, there was significantly less net growth of phytoplankton in the pico- and nano-size fractions at higher pCO_2. This is in contrast to previous investigations, which have found that pico- and nano-phytoplankton thrive at high pCO_2 (Paulino et al., 2008; Schulz et al., 2013), though not in all species (Meakin and Wyman, 2011). Other studies have observed this positive response (more growth at high pCO_2) in picophytoplankton only (Newbold et al., 2012), while some have found the opposite response (less growth at high pCO_2) in nanophytoplankton (Engel et al., 2008).

Evidently, the response to ocean acidification in these size fractions is highly variable, and dependant on species composition and environmental conditions.

While the trend in initial growth relative to pCO_2 was consistent between experiments E3, E4 and E5, the magnitude of the effect on TEPs and the manner in which these experiments subsequently evolved was not. Different initial nutrient concentrations in the different experiments led to contrasting net growth over the first 48 h, from very small in nutrient depleted E5 to pronounced in nutrient replete E4. In contrast, changes in TEPs over the initial 48 h of all of these experiments were of a similar magnitude, ranging between ~ 0 and $\sim 50\,\mu g\,Xequiv\,L^{-1}$ in each. This was most readily observed in the relationship between TEPs and Chl *a* production (Fig. 6) where the different slope gradients between experiments imply higher TEP production per unit increase in Chl *a* in E5 than in E4, with E3 being intermediate. Thus, while the relationship between pCO_2 and initial growth was of the same sign between these experiments, the resultant effect on the accumulation or degradation of TEPs was a function of the initial availability of nutrients, with higher TEP production relative to net growth when nutrients were depleted.

The environmental conditions of E2 suggest that it was initialised in the aftermath of a phytoplankton bloom. While Chl *a* decreased throughout the experiment, TEP concentrations increased from the lowest measured value in all of the experiments to some of the highest. This increase in TEPs as Chl *a* decreased could have been due to one of two processes: (i) continued coagulation of remnant dissolved particles produced during the preceding phytoplankton growth (noted to occur following phytoplankton blooms; Passow, 2002) or (ii) continued generation and exudation of organic carbon that, due to nutrient limitation, was not channelled into biomass (a process known as carbon overconsumption) and subsequently coagulated to TEPs (e.g. Mari et al., 2001; Engel et al., 2002; Schartau et al., 2007). The fact that TEP concentration was significantly greater at higher pCO_2 after both 48 and 96 h implies that one or both of these processes was enhanced at high pCO_2 during the time course of the experiment (unless the effect of high pCO_2 was to inhibit TEP breakdown). Since the decrease in Chl *a* (i.e. the breakdown of phytoplankton biomass) was largely treatment independent and the abiotic coagulation of TEPs is not affected by pCO_2 (Passow, 2012), it is unlikely that process (i) was influenced by pCO_2 in this experiment. On the other hand, there is considerable evidence suggesting that process (ii), i.e. extracellular release due to carbon overconsumption, is enhanced at high pCO_2 (Engel et al., 2004a; Riebesell et al., 2007; Borchard and Engel, 2012). We calculated carbon overconsumption (C^*) from the difference between the concurrent decreases in dissolved inorganic carbon and nitrate (applying a C : N ratio of 117:16; Anderson and Sarmiento, 1994):

$$C^* = |\Delta \mathrm{DIC}| - \frac{117}{16}|\Delta \mathrm{Nitrate}|. \quad (1)$$

At both time points more carbon was consumed than expected from the uptake of nitrate. Although an obvious treatment dependence was not determined, the occurrence of carbon overconsumption throughout this experiment suggests that process (ii) may have contributed to the treatment-dependent increase in TEP concentrations. This would suggest a direct enhancement of TEP production at high pCO_2 in this experiment. The environmental setting of E2 may be interpreted as mimicking post-bloom/nutrient-depleted conditions in nutrient-fertilised experiments (e.g. Engel et al., 2004a; Borchard and Engel, 2012; Engel et al., 2014). As in the present work, these experiments commonly showed an effect of pCO_2 following nutrient limitation. The fact that the results of E2 (in terms of the suggestion of a direct enhancement of TEP production at high pCO_2) are unique in our study, suggests that these may be the specific conditions under which an effect of pCO_2 is observed.

5 Conclusions

Through bioassay experiments in five different locations in northwest European shelf seas, we have considered the effect of high pCO_2 conditions on the production of DOC and TEPs in unamended natural ecosystems. We found no significant effect of pCO_2 on the accumulation of DOC, although there was a slight suggestion of a negative relationship. There was a significant but highly variable effect of pCO_2 on the accumulation of TEPs. In three of the five experiments, this effect could be largely explained by the impact of pCO_2 on phytoplankton growth, which was positively correlated to TEP production and which was initially inhibited at high pCO_2. In only one of the five experiments was there an enhancement of TEP production at high pCO_2, seemingly without indirect effects, possibly supporting the conclusions of previous studies.

Some of our experiments showed a similar pattern in initial responses, but the generally heterogeneous relationship between TEPs and pCO_2 treatment implies that the variable environmental conditions of the experiments were a strong determinant of responses. We found that phytoplankton community structure, initial nutrient concentration and the timing of measurements relative to phytoplankton growth, affect both TEP production and its treatment dependence. Consequently, the current study highlights how idealised and/or isolated experiments are likely to be insufficient for understanding the wider scale influence of ocean acidification on the production of DOC and TEPs. Future experiments must consider natural communities across a range of different initial environmental conditions in order to better understand the wider biogeochemical response to ongoing accumulation of anthropogenic carbon in the oceans.

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