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The abiotic formation of TEP under different ocean acidification scenarios

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ABSTRACT

In view of rising atmospheric CO_2 concentrations, the question if the marine biological carbon pump will increase or decrease in efficiency as ocean acidification progresses becomes central for predictions of future atmospheric pCO_2. Aggregation and sinking of aggregates contributes significantly to the flux of carbon to depths and changes in aggregation behavior will have far reaching consequences for the biological pump. The abundance and characteristics of transparent exopolymer particles, TEP, are central in regulating aggregation. We investigated the impact of ocean acidification on the abiotic formation of TEP from their precursors. Our results demonstrate that, contrary to earlier suggestions, ocean acidification as expected in the future ocean has no impact on the equilibrium conditions between TEP and their precursors. However, if the carbonate system is altered by adding acid, which does not simulate the future ocean carbonate system correctly, TEP concentration increases with decreasing pH, presumably due to changes in total alkalinity (TA). This implies that abiotic TEP formation is sensitive to changes in TA, but not pH. The discrepancy in results caused by different experimental approaches emphasizes the fact that acidification experiments do not mimic future conditions adequately and may even be misleading.

1. Introduction

As atmospheric CO_2 concentrations are continuing to increase, the question of how marine pelagic ecosystems will react to increasing ocean acidification becomes critical. Ocean acidification results in an increase in total dissolved inorganic carbon (DIC) and a concomitant decrease in pH (Wolf-Gladrow and Zeebe, 2001). The pH of the ocean is, however, highly variable in space and time, and it has been argued that the expected changes in pH will have little impact on non-calcifying marine microbes e.g. (Berge et al., 2010; Joint et al., 2010), although that has also been disputed (Liu et al., 2010). Even if the livelihood of microbes is not threatened, small, non life-threatening changes in the physiology of these organisms may result in altered production, modification and exudation of organic matter which on timescales longer than that of individual organisms may cause shifts in marine biochemical cycling of elements. Thus, while ocean acidification may or may not significantly impact the well being of phytoplankton or bacteria themselves, these organisms may mediate appreciable shifts in biochemical cycling due to ocean acidification.

In this context, potential changes to the functioning of the biological soft tissue pump are of central importance, because of its direct feed-backs to atmospheric CO_2 concentrations. Both positive (Gehlen et al., 2007; Riebesell et al., 2007) or negative (Mari, 2008) feed-backs to atmospheric CO_2 due to the reaction of the biological pump to ocean acidification have been postulated. A large fraction of the particulate carbon carried to depths via the biological pump sinks as aggregates. The formation of such rapidly sinking aggregates depends largely on the presence of transparent exopolymer particles (TEP) (Alldredge et al., 1993). In fact, the aggregation state (measured as total aggregated volume) of diatoms depends linearly on TEP concentration (Gaerdes et al., 2010). Changes in TEP production or stickiness are therefore thought to result in a change in the efficiency of the biological pump (Jackson, 1995; Logan et al., 1995).

TEP are formed by a variety of pathways, but in marine pelagic systems, TEP are predominately formed abiotically from dissolved polymers released by phytoplankton and bacteria (Passow, 2000, 2002a). Such exopolymers slough off of cell surfaces as nanofibers (Leppard, 1984) which self assemble to colloidal sized nanogels (Chin et al., 1998), which coalesce to form larger gels and porous networks, many of which become visible as μm to mm sized TEP (Verdugo et al., 2004; Verdugo and Santschi, 2010a). Qualitative or quantitative changes in the release of polymers by organisms, as well as changes in the abiotic formation of TEP from such polymers would both potentially impact TEP concentration and consequently the efficiency of the biological carbon pump.

In this paper we present results from experiments investigating the effect of ocean acidification on the abiotic formation of TEP. Potential impacts of ocean acidification on the release of TEP precursors by organisms will be investigated elsewhere. The detailed chemical composition of TEP is not well characterized, but highly surface-active polysaccharides, which are enriched in deoxysugars (fucose

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and rhamnose) and covalently bound sulfate (Mopper et al., 1995; Zhou et al., 1998) make an important contribution. Based on theoretical considerations and the work of Mari (2008), which showed that a decrease in the pH resulted in an increase in abiotic TEP formation, we formulated the null hypothesis that the abiotic formation of TEP would increase under future carbonate system scenarios. We designed a series of experiments to test this hypothesis.

2. Methods

2.1. Design of experiments

Five experiments (experiments 1–5) were conducted to investigate the effect of future and pre-industrial marine carbonate chemistry on abiotic TEP concentration (future ocean experiments), where the dissolved inorganic carbon (DIC) and the pH was manipulated (Table 1). A methodological experiment (M1) tested the impact of the perturbations that are required to simulate PAST carbonate conditions on TEP production (Table 1). Additional three experiments (A1–A3) were designed to test the effect of acid addition (perturbation of alkalinity and pH) on TEP concentration (acid addition experiments, Table 2). All experiments were conducted in rolling tanks at 14 °C in the dark.

The five future ocean experiments all followed the same design but differed in the experimental solution: At the onset the carbonate system in the experimental solution was perturbed as needed for the different treatments and TA (total alkalinity), pH and TEP concentration determined initially and after 24 hours incubation. In experiments 3 and 4 DIC (dissolved inorganic carbon) was additionally analyzed to over-determine the carbonate chemistry. Three to five different treatments with two replicate tanks each were measured per experiment. The different treatments mimicked ambient, past and different future carbonate scenarios. An artificial TEP solution, generated by addition of Gum Xanthan and Alginic Acid to 0.2 prefiltered seawater (FSW) was used as the sample solution for experiments 1 and 2. Experiments 3 and 4, respectively, were conducted with 10 μm pre-filtered natural seawater and 10 μm pre-filtered sea water first incubated in 20 L bottles (bottle blooms). Experiment 5 was carried out with a 10 μm filtrate of a semi-continuous, dilute batch culture of the diatom *Thalassiosira weissflogii* (Table 1).

In the methodological experiment (M1), which examined the impact of perturbations required to simulate PAST treatments on TEP formation, artificial TEP solution was added to 0.2 μm FSW either before (first treatment) or after (second treatment) carbonate system perturbations simulating PAST or AMBIENT conditions. To this end, 4 mL of an artificial TEP solution (8704 ± 2023 μg GXeq L^{-1}) was added into each kg of 0.2 μm pre-filtered seawater (TEP concentration: 43 ± 3 μg GXeq L^{-1}) from the Santa Barbara Channel (3 replicates per treatment). The amount of TEP added with the artificial TEP solution made up about 50% of TEP in the final samples, whereas the other half stemmed from the filtered seawater. PAST conditions were simulated by first adding 0.70 mL of 0.1 M HCl per kg FSW open to the atmosphere, stirring overnight and then adding 0.82 mL of 0.1 M NaOH per kg FSW in a closed system (no overhead space). The AMBIENT treatment was treated identically, except that no HCl or NaOH was added (Table 1).

In the acid addition experiment A1, TEP concentration was determined initially and again after the addition of 0.1, 0.3, 0.5, 0.7 or 0.9 mL of 0.1 N HCl L^{-1} and gentle mixing. The design of experiment A2 was identical to that of A1 except that samples were incubated overnight after acid addition, but kept in a closed system, e.g. without overhead space. Final TEP concentrations were determined next morning. Experiment A3 differed from experiment A1 in that NaOH was added to each treatment directly after the additions of HCl and mixing, about 20 minutes before measuring final TEP concentrations. Experiments A1 to A3 were all conducted with the 10 μm filtrate of bottle blooms (Table 2).

2.2. Sample solutions

As TEP are a chemically heterogeneous but underdetermined class of substances rich in acidic polysaccharides, we used a wide variety of TEP and TEP-precursors of differing origin for experiments (Tables 1 and 2). The artificial TEP solutions for future ocean experiments 1 and 2 were prepared same day by adding 1.4 mg Gum Xanthan (SIGMA) and 2.3 mg alginic acid (SIGMA) into 250 mL of 0.2 μm prefiltered seawater (FSW) and homogenizing the solution with a manual pistil homogenizer. The artificial TEP solution for experiment M1 was prepared same day by mixing 1.9 mg Gum Xanthan and 0.9 mg Alginic Acid into 250 mL of FSW and homogenizing. The resulting solutions were rich in TEP-precursors. Freshly collected seawater from the Santa Barbara Channel (34° 23.134′N 119° 50.823′W) collected just below the surface was gravity filtered through 10 μm before use for experiment 3 (Table 1). Bottle blooms were grown by

Table 1
Future ocean experiments and methodological experiment.

Experiment #	Substrate	Treatments
1	Artificial TEP in 0.2 μm FSW	PAST, AMBIENT, FUTURE
2	Artificial TEP in 0.2 μm FSW	AMBIENT, FUTURE1, FUTURE2, FUTURE3
3	Natural seawater <10 μm	PAST, AMBIENT, FUTURE1, FUTURE2, FUTURE3
4	Bottle bloom <10 μm	PAST, AMBIENT, FUTURE1, FUTURE2, FUTURE3
5	*T. weissfloggi* <10 μm	AMBIENT, FUTURE1, FUTURE2, FUTURE3
M1	0.2 μm FSW + artificial TEP	TEP added before/after perturbation[a]; PAST, AMBIENT

FSW: filtered sea water.
[a] Perturbations consisted of the addition of 0.7 mL kg^{-1} of 0.1 M HCl, overnight stirring and the subsequent addition of 0.818 mL kg^{-1} of 0.1 M NaOH for the PAST treatment, and of overnight stirring for AMBIENT treatments. Three replicates per treatment.

Table 2
Acid addition experiments.

Exp. #	Experiment	Substrate	treatments
A1	TEP measured 20 min after HCl addition	Bottle bloom <10 μm	Add 0.1–0.9 mL HCL
A2	TEP measured 12 hours after acid addition	Bottle bloom <10 μm	Add 0.1–0.9 mL HCL
A3	TEP measured 20 min after HCl and NaOH addition	Bottle bloom <10 μm	Add 0.1–0.9 mL HCL and NaOH

addition of nutrients (40 μM NO_3, 3.6 μM PO_3, 53 μM $Si(OH)_4$) to seawater collected from Goleta Pier (34° 24′ N, 119° 49′ W; experiments 4, A2, and A3) or off the coast of Santa Barbara (34° 23′ N 119° 50′ W; experiment A1). Bottle blooms were incubated in clear 20 L bottles at 14 °C, at 15–30 μE s^{-1} for 12:12 hours and bubbled with an aquarium pump during the light hours to keep the carbonate system stable. Bubbling for 24 hours was avoided as this decreased the pH below ambient. After 4–7 days, when a phytoplankton bloom had developed, it was gravity filtered through 10 μm and the filtrate used for experiments. Experiment 5 was conducted with the filtrate of a culture of *T. weissflogii* grown in a dilute, semi-continuous batch approach. Media was prepared from sterile filtered seawater and vitamins and trace metal solutions added as in F/2, but macronutrients additions were reduced: Only 59 μM nitrate, 3.6 μM phosphate, and 53.5 μM silicic acid were added. The culture was grown at 14 °C, at 30 μE s^{-1} with 12:12 light:dark cycle and diluted before it reached a cell density of 60,000 mL^{-1} therewith keeping the carbonate system within a natural range. TA and pH were monitored during the growth phase of bottle blooms and the culture to ensure that values remained within the range found naturally off California (Feely et al., 2008).

2.3. Carbonate chemistry perturbations

Increasing atmospheric CO_2 alters the partial pressure of CO_2 (pCO_2), the pH and the dissolved inorganic carbon (DIC), but not the total alkalinity (TA) of the surface ocean. This change may be experimentally mimicked by bubbling seawater with pCO_2 adjusted air, or by chemically altering the seawater (closed system approach) (Rost et al., 2008). As TEP are formed by bubbling we perturbed the carbonate system using the closed system approach. To mimic pre-industrial conditions (PAST) first 0.1 M HCL was added, the solution allowed to equilibrate overnight with the atmosphere to expel DIC, and then TA was adjusted back to its original conditions by adding appropriate amounts of 0.1 M NaOH in a closed system (no headspace) while keeping DIC constant. As the exchange with the atmosphere is slow, a full equilibration is best accomplished by slowly mixing the seawater plus acid solution overnight in a Fernbach flask (large surface area to volume) in a well ventilated room using a magnetic stirrer. We tested the effect of this stirring on TEP production and found no significant difference in TEP concentration in stirred and unstirred samples (*t*-test, $t(8)=3.36$, $p<0.01$, $r=0.23$). No perturbations were used for treatments reflecting present (AMBIENT) conditions. Conditions as expected in the future (FUTURE) were generated by adding appropriate amounts of 0.1 M HCl, 0.001 M Na_2CO_3 and 0.1 M $NaHCO_3$ in a closed system to prevent gas exchange (see Table 3 for examples). The respective volumes needed for additions were calculated using CO_2Sys. Measurements of pH and TA confirmed that our perturbations changed the system as expected and that changes reflected those anticipated in the future ocean. The treatments of the future ocean experiments (experiments 1–5) were adjusted in this manner. In contrast, in the acid addition experiments (experiments A1–A3) the addition of 0.1 M HCL caused pH and TA to decrease while DIC remained unaltered (closed system). Addition of 0.1 M HCL followed by equimolar amounts of 0.1 M NaCl (experiment A3) had no net effect on the carbonate system.

Table 3
Example of chemical perturbation of the carbonate system.

Treatment	ΔpCO_2 (μatm)	0.1 M HCL (mL kg^{-1})[1]	0.1 M NaOH (mL kg^{-1})	0.1 M $NaHCO_3$ (mL kg^{-1})	0.001 M Na_2CO_3 (mL kg^{-1})
PAST	−100	0.570	0.665	NA	NA
PRESENT	NA	NA	NA	NA	NA
FUTURE 1	+260	0.546	NA	0.522	1.231
FUTURE 2	+520	0.898	NA	0.880	0.932

2.4. Analysis

TEP was measured colorimetrically in 5–6 replicates per tank by filtration onto 0.4 μm PC filters (Poretics) and subsequent staining with Alcian Blue following the procedure of Passow and Alldredge (Passow and Alldredge, 1995). The dye solution was calibrated using Gum Xanthan and TEP are expressed as Gum Xanthan equivalents per liter (GXeq L^{-1}). Operationally defined, TEP encompass particles retained on a 0.4 μm filter, whereas TEP-precursors pass this filter, but belong to the same substance class and may potentially form TEP. TEP thus are the particulate fraction of these substances that exist in a size continuum (Passow, 2002b). TEP determinations are semi-quantitative as the chemical composition of TEP varies, is complex and unknown, and variation between replicate measurements are high. The methodological coefficient of variation (standard deviation divided by average) between replicate measurements within each container in our experiments was usually ≤10%, always ≤20%. Standard deviations are calculated from replicate containers.

Chl. *a* was determined during growth of cultures and bottle blooms using a fluorometer (Turner 700) by filtration of samples onto 0.4 μm PC filters and soaking the filters in 90% acetone over night in the freezer (Strickland, 1972). *In vivo* fluorescence and quantum yield ($\varphi P_0 = F_v/F_m$), a measure for health of the phytoplankton, were also determined during the growth of bottle blooms and the culture using a cuvette AquaPen (Z985, Qubit System Inc.).

The carbonate system was monitored in the 5 future ocean experiments as well as during growth of bottle blooms and cultures by measuring pH and total alkalinity (TA). Samples were prepared and processed following both "Guides to Best Practices for Ocean CO_2 Measurements" (Dickson et al., 2007; Riebesell et al., 2010). Samples for pH were collected bubble free in 20 mL scintillation vials, those for TA in glass stoppered 125 mL borosilicate bottles. Both TA and pH were determined in the Hoffman laboratory at UCSB (Fangue et al., 2010). In brief, TA (μmol kg^{-1}) was measured within 4 hours after sampling by potentiometric titration (T50, Mettler Toledo) using certified acid titrant. The mass of the sample was recorded to 0.001 g and the temperature of the acid titrant and the sample kept within 0.1 °C. Total alkalinity was calculated following SOP3b using an excel spreadsheet. Salinity was determined from TA samples using a conductivity instrument (3100 Yellow Springs Instruments). The pH_T (total scale) was measured with a spectrophotometer (Shimadzu Biospec 16-1) using the indicator dye *m*-cresol purple (Sigma-Aldrich) within 1–2 hours after sampling. The temperature was held at 25 °C, and the absorbance measured at 730 nm, 578 nm, and 434 nm before and after dye addition (Clayton and Byrne, 1993; Fangue et al., 2010). The pH_T was calculated following SOP6b. The program CO_2Sys (Lewis and Wallace, 1998) was used to calculate the carbon chemistry form TA and pH_T. The dissociation constants *K*1 and *K*2 from Mehrbach et al., (1973) refit by Dickson and Millero(1987) were used and $KHSO_4$ according to Dickson. Measurements of certified reference material (TA = 2211.21 μmol kg^{-1}, $pH_T = 8.0929$) revealed high precision and accuracy: TA of 2210.7 ± 2.4 and a pH_T of 8.0931 ± 0.001 (Fangue et al., 2010). Samples for DIC (collected in 500 mL glass bottles) were poisoned with 112 μL of saturated mercuric chloride to stop microbial activity and DIC was determined using gas extraction and colorimetric titration with photometric endpoint detection (Johnson et al., 1985) in the laboratory of Azetsu-Scott, Bedford Institute of Oceanography, Canada. Any two of the main carbonate parameters (pH, TA, DIC, pCO_2) describe the carbonate system sufficiently and the others can be calculated. For simplicity we present results for pH_T and pCO_2 only. If not stated otherwise carbonate parameters are given for our experimental temperature (14 °C).

3. Results

3.1. Culture and bottle bloom development

The bottle blooms for experiments A1 to A3 were utilized after 2–4 days of incubation when chl. *a* concentrations had reached between 1 and 2 μg L^{-1} and F_v/F_m ranged between 0.4 and 0.5. Chlorophyll a concentration in the bottle bloom grown for experiment 4 increased from 0.5 μg L^{-1} to a maximum of 3.6 μg L^{-1} and had dropped to 2.0 μg L^{-1} at the start of the experiment. During the growth phase of all bottle blooms the pH_T ranged between 7.8 and 8.0.

During its growth, the culture of *T. weissflogii* was diluted 6 times with fresh media to ensure that the carbonate system should not change excessively while exponential growth prevailed and enough biomass was generated. During the 15 day growth period after the 6 day grow-up phase cell concentrations ranged between 40,000 and 60,000 cells mL^{-1}, the growth rate was $\mu = 0.48 \pm 0.04\ d^{-1}$ and F_v/F_m remained between 0.6 and 0.8, indicating healthy exponential growth during the whole period. The pH_T varied between 8.10 and 8.36, thus remaining well within the limits of natural phytoplankton blooms, where the pH easily increases by 0.3 and reaches a pH of >8.8 in the open ocean and >9.5 in estuaries (Joint et al., 2010).

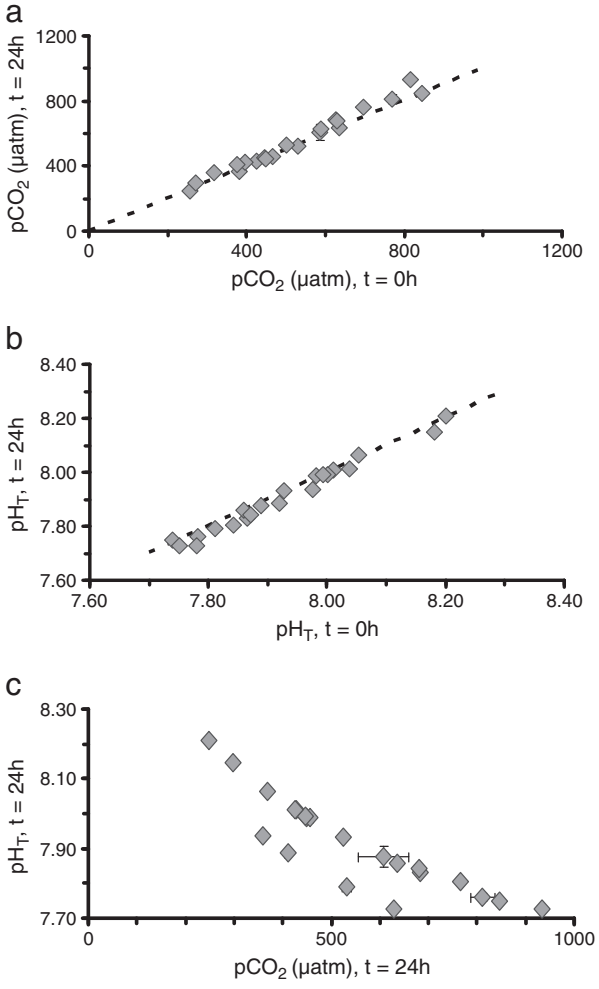

Fig. 1. The carbonate chemistry during the five future ocean experiments: changes in (a) pCO_2 and (b) pH_T during the 24 hour incubations and (c) pH_T vs. pCO_2 data after 24 hour incubation. Values are averages from replicate tanks and standard deviations are depicted by the error bars, which are usually smaller than the symbol.

3.2. Carbonate chemistry during future ocean experiments

Ambient pCO_2 and pH of surface seawater off the coast of California is highly variable (Feely et al., 2008) and pCO_2 and pH_T values of the AMBIENT treatments ranged from 316 to 477 μatm, and from 8.05 to 7.98, respectively, reflecting this *in situ* variability. PAST treatments ranged from a pCO_2 of 255 μatm and a pH_T of 8.20 to a pCO_2 of 270 μatm and a pH_T of 8.18. FUTURE treatments had a $pCO_2 \leq 850$ - μatm and a $pH_T \geq 7.74$. Changes in pCO_2 and pH during the 24 hours incubations were minimal in all cases, and reflected microbial respiration (Fig. 1a and b). At higher pCO_2 (future treatments) the observed shifts during the incubation period were slightly larger, as the buffering system weakens with increasing pCO_2. The pH_T vs. pCO_2 relationship of all treatments of the five future ocean experiments are depicted in Fig. 1c. The lower pH for similar pCO_2 stems from the experiment with the phytoplankton culture.

3.3. Over-determination of the carbonate chemistry

During experiments 3 and 4 we over-determined the carbonate system by additionally measuring DIC in one replicate of each treatment. Thus we have 15 independent samples, where pH_T, TA and DIC were determined simultaneously. We calculated pCO_2 using all three possible combinations, e.g. from pH_T and TA, from DIC and pH_T and from DIC and TA. Fig. 2 shows that pCO_2 based on pH_T and TA fit almost perfectly with that calculated from DIC and TA. DIC and pH_T based calculations give slightly different pCO_2 values and the correlation coefficient with those calculated from TA and either pH_T or DIC was slightly lower.

3.4. Future ocean experiments

TEP concentrations in the five future ocean experiments differed by more than an order of magnitude from ~80 μg GXeq L^{-1} (experiment 3), to ~450 μg GXeq L^{-1} (experiment 4), and ~4350 μg GXeq L^{-1} (experiment 5; Table 4, Fig. 3). Large amounts of TEP formed during the incubation in all treatments of experiments 1 and 2, which were conducted with artificial TEP solution. This freshly made solution was rich in TEP-precursors which were not in equilibrium with TEP concentration. Equilibrium conditions between TEP and their precursors are established within hours to a day (unpublished). Initial TEP concentrations in experiments 1 and 2 were 41 and 13 μg GXeq L^{-1}, respectively and increased to 106 and 81 μg GXeq L^{-1}. While the increase in TEP concentration during the incubation was significant in all treatments, the between treatment differences in TEP concentrations were not significant (*t*-test, $t(2) = 9.93$, $p < 0.01$, $r < 2.63$) and no trend with pCO_2 was observed (Fig. 3a).

Net changes in TEP concentration during the incubation were negligible during the other three experiments (experiments 3, 4, and 5)

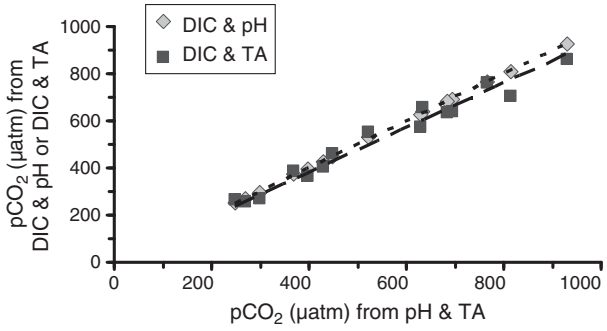

Fig. 2. Results of the over-determination of the carbonate system. Whereas pCO_2 calculated from pH_T and TA were identical to values calculated from DIC and TA ($y = 1.00x$, $r^2 = 1.00$) those calculated from DIC and pH_T differed ($y = 0.95x$, $r^2 = 0.97$).

Table 4
Final TEP concentrations in future ocean experiments and the methodological experiment.

Experiment/treatment	Avg. ± SD	*n*
Exp. 1		
PAST	131 ± 23	2
AMBIENT	98 ± 10	2
FUTURE	88 ± 3	2
Exp. 2		
AMBIENT	91 ± 18	2
FUTURE 1	74 ± 5	2
FUTURE 2	86 ± 10	2
FUTURE 3	73 ± 8	2
Exp. 3		
PAST	124 ± 18	2
AMBIENT	74 ± 4	2
FUTURE 1	60 ± 5	2
FUTURE 2	64 ± 4	2
FUTURE 3	78 ± 1	2
Exp. 4		
PAST	345 ± 3[a]	2
AMBIENT	354 ± 19	2
FUTURE 1	500 ± 6[a]	2
FUTURE 2	558 ± 33	2
FUTURE 3	531 ± 78	2
Exp. 5		
AMBIENT	4292 ± 38	2
FUTURE 1	4394 ± 23	2
FUTURE 2	4302 ± 142	2
FUTURE 3	4338 ± 52	2
M1		
TEP added before; PAST	75 ± 2.2	3
TEP added after; PAST	82 ± 6.8	3
TEP added before; AMBIENT	87 ± 35.3	3
TEP added after; AMBIENT	81 ± 4.0	3
PAST	79 ± 5.9	6
AMBIENT	84 ± 22.7[b]	6

n = Number of replicate containers.
[a] Statistically significant difference found between these two treatments. No other significant differences were found between treatments of any of these experiments.
[b] Even if the outlier of the third replicate container was ignored, there was no statistical significant difference between PAST and AMBIENT.

which were conducted with filtrate from natural seawater, a bottle bloom and a diatom culture, respectively. As in experiments 1 and 2, no between treatment differences were observable in these experiments (*t*-test, $t(2) = 9.93$, $p < 0.01$, $r < 9.24$), with one exception. In experiment 4 the TEP concentration in the PAST treatment was significantly different from that of FUTURE 1 (*t*-test, $t(2) = 9.93$, $p < 0.01$, $r = 31.37$). Chance alone can account for this outlier.

By normalizing TEP produced during the 24 hour incubation to initial TEP concentrations $((TEP_{t=24} - TEP_{t=0})/TEP_{t=0})$ data from all experiments can be compared directly (Fig. 4). Although experiments covered a large range of pCO_2 and TEP concentrations, there is no trend of TEP production with increasing pCO_2. No net TEP formation occurred under any of the carbonate scenarios of experiments 3 to 5, and TEP formation in experiments 1 and 2 was not a function of the carbonate system.

3.5. Methodological experiment

Experiment M1 investigated the impact of procedures necessary to simulate PAST conditions, e.g. the addition of first acid in an open system and 12 hours later base in a closed system, on TEP concentration. No significant difference in TEP concentration was found between PAST treatments where average TEP concentrations were 912 ± 75 μg GXeq L^{-1} ($n = 3$) when artificial TEP was added after the perturbation, and 835 ± 24 ($n = 3$) when TEP was added before the perturbation (Table 4). The two treatments of the AMBIENT samples also did not differ significantly. Moreover, no statistical difference was visible between TEP concentrations in PAST and AMBIENT treatments. Thus the perturbation procedure itself did not impact TEP concentration.

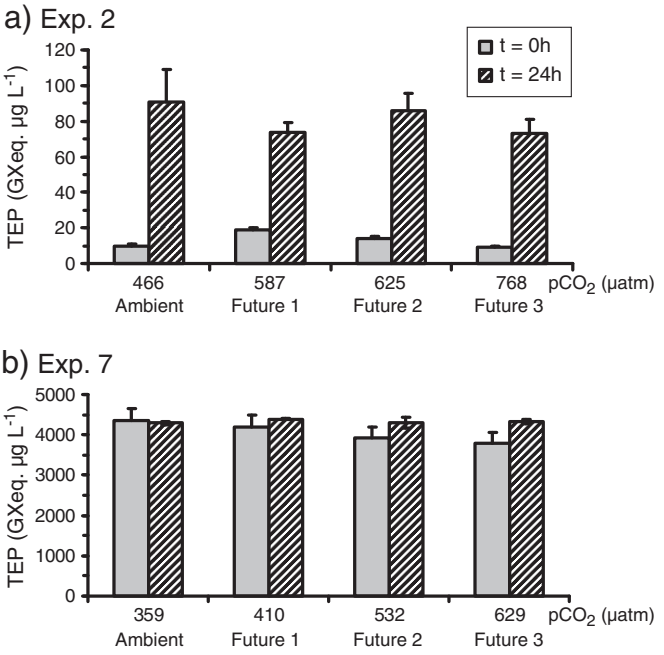

Fig. 3. Two examples of TEP production during future ocean experiments as a function of the carbonate system. Experiment 2 (a) was conducted with artificial TEP-precursors, whereas experiment 7 (b) was carried out with a TEP-rich filtrate from *Thalassiosira weissfloggii*.

3.6. Acid addition experiments

A second series of experiments was conducted to investigate TEP concentration as a function of acid addition. Results from experiment A2 where final TEP measurements were postponed for 12 hours after the addition of HCl did not differ from those of experiment A1, where TEP was measured directly after acid addition and mixing. In both experiments (A1 and A2) TEP concentrations were statistically significantly higher after addition of ≥0.3 mL of 0.1 M HCl (Fig. 5a and b, Table 5) compared to unaltered values. HCl addition of 0.1 mL L^{-1} also resulted in an statistical significant increase of TEP concentration compared to initial values in experiment A2, whereas the increase was not statistically significant in A1 (Table 5). In both experiments, HCl additions of >0.3 mL had no further impact; that is TEP concentration after addition of 0.3 mL HCl did not differ statistically from that after addition of 0.5, 0.7 or 0.9 mL HCL. In the third experiment (A3), where the addition of HCl was immediately followed by an addition of equimolar amounts of NaOH, no statistically significant

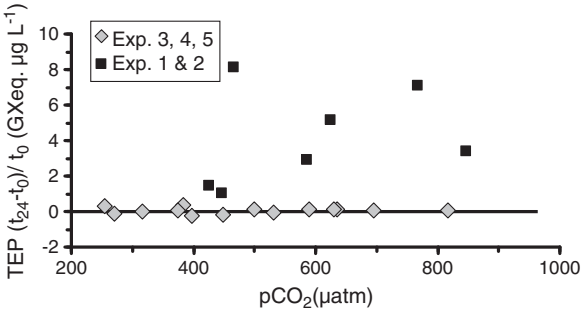

Fig. 4. Normalized TEP production for all five future ocean experiments plotted as a function of pCO_2.

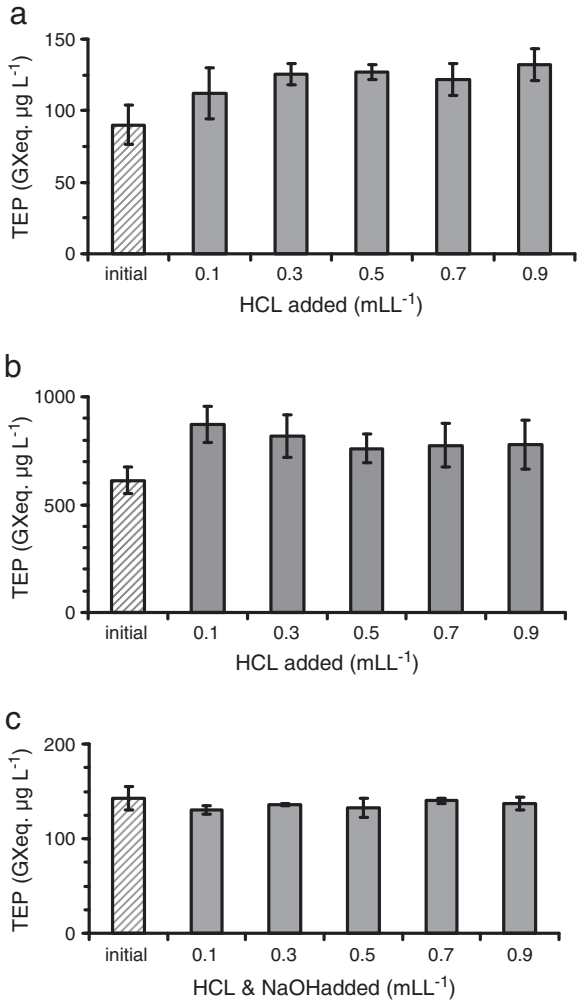

Fig. 5. TEP concentration in acid addition experiments as a function of acid added. HCl was added in experiment A1 (a) and A2 (b) and HCl followed by NaOH in experiment A3 (c).

change in TEP concentration was observable in any treatment (addition of 0.1 to 0.9 mL) compared to initial concentrations (Fig. 5c, Table 5).

4. Discussion

4.1. Perturbations of the carbonate system

Realistic experimental perturbations of the complete marine carbonate system are difficult and frequently introduce other unwanted artifacts into the system (Rost et al., 2008). Exchange between the overlaying air and surface water is too slow (Wolf-Gladrow and Zeebe, 2001) for water to adapt within a reasonable time frame for experiments. Most frequently seawater is bubbled with air adapted to target pCO_2 conditions to simulate pre-industrial or future conditions of the carbonate system of the ocean (Rost et al., 2008). However, bubbling itself impacts organic matter distribution, including TEP formation (Mopper et al., 1995; Schuster and Herndl, 1995; Zhou et al., 1998) as well as bacterial interaction with organic matter (Kepkay and Johnson ,1988; Kepkay and Johnson, 1989; Kepkay, 1994) and thus needs to be avoided when organic matter distribution is the focus of the experiments. Bubbling also impacts ecophysiological responses of phytoplankton (Hoppe et al., 2011) introducing an often unwanted variable. Therefore we used the closed system approach to chemically alter pH, HCO_3^- and CO_3^{2-} concentrations in such a way that the natural occurring changes were mimicked accurately, e.g. TA remained constant, whereas DIC, pCO_2 and pH shifted as appropriate. For preindustrial conditions, DIC was expelled by adding HCl and allowing the seawater to exchange with the atmosphere. By using seawater containers with a large surface area to volume ratio, gentle stirring overnight in a well ventilated room sufficed to reach equilibrium conditions. We ensured that this degree of stirring did not affect TEP concentrations. TA was then adjusted back to ambient by addition of NaOH in a closed system. FUTURE treatments were adjusted by additions of HCL, $NaHCO_3$ and $NaCO_3$ (Rost et al., 2008; Gattuso and Lavigne, 2010). The change in salinity due to the additions of HCL, $NaHCO_3$ and $NaCO_3$ for FUTURE and of HCl and NaOH for PAST treatments was negligible, but considered in carbonate system calculations.

Table 6 gives an example (from experiment 3) of the calculated target carbonate system parameters mimicking FUTURE and PAST conditions and the measured values after the respective perturbations. The addition of about 0.8 mL of 0.1 M HCL kg^{-1} and appropriate amounts of $NaHCO_3$ and $NaCO_3$ was needed for perturbations simulating FUTURE (649 µatm) conditions. In this scenario the pH_T decreased by 0.2 units to 7.85, whereas pCO_2 and DIC increased. Within the measurement accuracy, measured values were identical

Table 5
Results of *t*-tests for acid addition.

Sample	Avg ± SD	*n*	Avg ± SD	*n*	*t*	Difference
A1						
Initial vs. 0.1 mL HCL addition	90 ± 14	5	112 ± 18	5	2.21	Not sig. diff.
Initial vs. 0.3 mL HCL addition	90 ± 14	5	125 ± 7	5	5.13	$P<0.01$
Initial vs. 0.5 mL HCL addition	90 ± 14	5	127 ± 5	5	5.68	$P<0.01$
Initial vs. 0.7 mL HCL addition	90 ± 14	5	122 ± 11	5	3.99	$P<0.01$
Initial vs. 0.9 mL HCL addition	90 ± 14	5	132 ± 11	5	5.39	$P<0.01$
A2						
Initial vs. 0.1 mL HCL addition	612 ± 62	5	870 ± 84	4	5.34	$P<0.01$
Initial vs. 0.3 mL HCL addition	612 ± 62	5	819 ± 98	4	3.87	$P<0.01$
Initial vs. 0.5 mL HCL addition	612 ± 62	5	760 ± 66	4	3.46	$P<0.05$
Initial vs. 0.7 mL HCL addition	612 ± 62	5	776 ± 101	4	3.01	$P<0.05$
Initial vs. 0.9 mL HCL addition	612 ± 62	5	777 ± 114	4	2.78	$P<0.05$
A3						
Initial vs. 0.1 mL HCL and NaOH addition	143 ± 12	5	130 ± 5	5	2.15	Not sig. diff.
Initial vs. 0.3 mL HCL and NaOH addition	143 ± 12	5	136 ± 1	5	1.36	Not sig. diff.
Initial vs. 0.5 mL HCL and NaOH addition	143 ± 12	5	133 ± 10	5	1.43	Not sig. diff.
Initial vs. 0.7 mL HCL and NaOH addition	143 ± 12	5	140 ± 3	5	0.50	Not sig. diff.
Initial vs. 0.9 mL HCL and NaOH addition	143 ± 12	5	137 ± 7	5	1.01	Not sig. diff.

Test results for additions of 0.1 or 0.3 mL vs. higher additions were all not significant in experiments A1, A2 and A3, but are not shown.

Table 6
Comparison of carbonate system perturbations in different types of manipulations: 14 °C, 32.8‰.

Treatment	pCO_2(µatm)	pH_T	TA (µmol kg^{-1})	DIC (µmol kg^{-1})	Required additions[a] mL kg^{-1}
AMBIENT, pH and TA measured	383	8.05	2211	2014	
Future ocean experiments					
FUTURE target	649	7.85	2211	**2095**	HCl: 0.81 $NaHCO_3$: 0.75 Na_2CO_3: 2.70
FUTURE measured after additions	–	7.86	2208	–	
PAST target	260	8.19	2211	**1937**	HCl: 0.70 NaOH: 0.83
PAST measured after additions	–	8.20	2212	–	
Acid addition experiments					
Closed system, calculated	571	7.89	**2141**	2014	HCL: 0.7
Other perturbations					
Acid addition, open system after equilibration[b], calculated	387	8.03	2127	1937	HCL: 0.7

Whereas pH changed similarly in both, DIC changed in future ocean experiments, while TA changed in acid addition experiments (in bold).
[a] HCL: 0.1 M; $NaHCO_3$: 0.1 M; Na_2CO_3: 0.001 M; NaOH: 0.1 M.
[b] Assumption: atm. pCO_2: 387 µatm.

with target values. Fig. 6 depicts the changes due to perturbations in the different carbonate parameters. Mimicking PAST conditions is a two step process, where first a similar (0.7 mL HCL kg^{-1}) amount of acid is added in an open system (exchange with atmosphere) resulting in an initial decrease in pH_T by 0.16 units to 7.89. During the equilibration with the atmosphere the pH_T went up, as DIC was released into the atmosphere reaching in our example a pH_T of 8.03 (Table 6). The subsequent addition of NaOH finished the perturbation by adjusting the TA back upward, increasing, in our example the pH_T to 8.2 (Fig. 6). The methodological experiment M1 was designed to determine if this 12 hour perturbation procedure for PAST conditions introduced unwanted artifacts in terms of abiotic TEP formation. No difference was found in TEP concentration whether TEP was added before or after the perturbation procedure was complete, indicating that any potential effects of individual steps of the perturbation on TEP concentration are fully reversible.

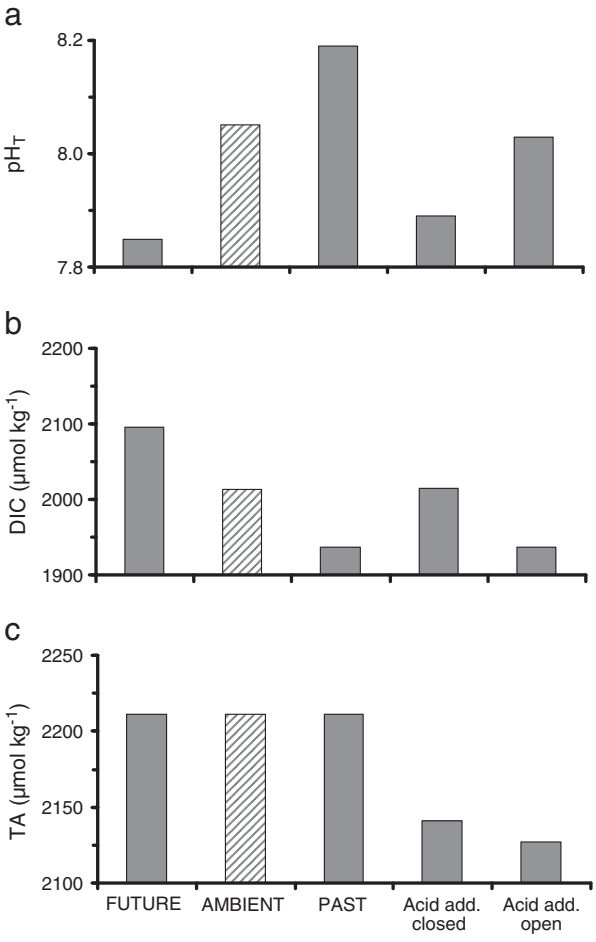

Fig. 6. Carbonate chemistry as a function of different types of perturbations. Whereas TA remained constant in all treatments of the future ocean experiment, TA changed appreciably in all acid addition experiments. Our acid addition experiments were conducted using the closed system approach, and consequently DIC remained unchanged compared to ambient conditions. If allowed to equilibrate DIC will decrease after acid addition.

Perturbations due to acid addition, as in our second set of experiments (A1–A3) alter the carbonate system in a different manner compared to the above described perturbations, although pH changes are comparable in both cases (Fig. 6). Addition of a strong acid in a closed system as in our second set of experiments reduces the pH and the TA without changing DIC. If the system is open to the atmosphere, equilibration will change the carbonate system further during the next hours to days, similar to the equilibration phase during a PAST perturbation (Fig. 6).

Over-determination of the carbonate system revealed the good quality of our carbonate system data. The slight discrepancy observed when DIC and pH rather than TA and either pH or DIC are used has been discussed elsewhere (Hoppe et al., 2010), and most likely represents the impact of organic acids that are not included in the TA calculations of CO_2Sys. This discrepancy has, however, little relevance for the interpretation of our experimental results.

4.2. Abiotic TEP production and ocean acidification

Our results show that in contrast to our predictions abiotic TEP formation was not impacted by ocean acidification. Neither future nor pre-industrial conditions resulted in a change in abiotic TEP concentration in any of our experiments. Consequently we may assume that the equilibrium conditions between TEP and their precursors are not particularly sensitive to the degree of ocean acidification as expected in the near future. TEP, which are rich in acidic polysaccharides are a chemically heterogeneous group of substances which potentially may exhibit a wide range of characteristics. We used TEP and TEP-precursors from a variety of sources (seawater collected at different times, cultures, commercially available material) and at a wide range of concentrations giving us confidence that our results are valid for a large fraction of naturally occurring TEP at a range of concentrations. We conducted experiments with samples where TEP was not yet in equilibrium with their precursors, so that TEP concentration increased during the incubation time. We also conducted experiments where TEP was in equilibrium with their precursors at the start of the experiment, so that no net change in TEP

concentration was visible. As the abiotic TEP formation was not impacted in any of our experiments, and assuming that TEP formation in rolling tanks mimics TEP formation *in situ* reasonably well, we feel confident that abiotic TEP formation is not seriously impacted by ocean acidification as expected in the near future.

However, this result appears to contradict results from our second set of experiments, where the carbonate system was altered by the addition of HCl only. TEP concentration increased significantly with addition of acid, which also corroborates results of Mari (2008). Increased TEP concentration was observed both directly after acid addition (experiment A1) and after 12 hours (experiment A2), indicating a rapid and stable re-adjustment of the equilibrium conditions between TEP and their precursors after acid addition. The addition of HCl followed by equimolar amounts of NaOH (experiment A3), which had no net impact on the carbonate system, did not affect TEP concentration, suggesting that the change in TEP concentration after acid addition is a function of the carbonate chemistry and that the change is fully reversible.

Whereas TA remained constant and DIC changed in future ocean experiments (DIC perturbation) the reverse was true in acid addition experiments (TA perturbation) (Fig. 6), suggesting that changes in TA, not pH, which changed in both types of experiments, mediated the observed increase in abiotic TEP formation in acid addition experiments. TA may be defined as measuring the charges of the ions of weak acids and a lower TA would signify a reduction in the numbers of proton acceptors (Wolf-Gladrow and Zeebe, 2001). Concentrations of $CO_3^{2-}$and OH^-ions, which form strong complexes with divalent counter ions in natural seawater thus affecting their speciation (Millero et al., 2010) were higher under perturbations of our acid addition experiments compared to the future ocean experiments. In acid addition experiments concentrations of $OH^-$and CO_3^{2-}were 1.6 μmol kg^{-1} and 99.4 μmol kg^{-1} vs. 1.5 μmol kg^{-1} and 95.4 μmol kg^{-1} in future ocean experiments (14 °C, 32.8‰). The molecular basis regulating abiotic TEP formation is not understood well enough to explain the results of our two types of experiments satisfactorily. Precursors may associate to form TEP by hydrophobic bonding, electrostatic cross linking, or both. If TEP formation was based on Ca^{2+} cross linking (Alldredge et al., 1993; Chin et al., 1998), we suggest that either the Ca^{2+} activity increased in acid addition experiments or the protonation of polyanionic sites that get cross linked by Ca^{2+} changed, or both, therewith promoting TEP formation. An impact of TA on the binding efficiency of the Alcian Blue molecule, which has 4 binding sites (Passow, 2002b) to TEP is less likely because the increase of TEP concentration with a decrease in TA was observed both, with the colorimetric method (this study) and the microscopic method (Mari, 2008). Alternatively TEP formation may be the result of hydrophobic bonding (Ding et al., 2008, 2009; Verdugo and Santschi, 2010b). Lowering of the pH could make marine gels more hydrophobic by protonation of some weak acid sites, although the low isoelectric point of similar polysaccharides (e.g. ~3; Quigley et al., 2002; Xu et al., 2009) suggest that other fractions, e.g. protein might be affected more. Moreover, hydrophobic interactions are usually only weakly affected by shifts in protons or carbonates. The mechanistic understanding of changes in TEP formation due to changes in TA, but not pH *per se*, thus remain open for further investigations. Independent of the molecular formation mechanism, if the formation of TEP within rolling tanks is largely comparable to *in situ* TEP formation, our results imply that abiotic TEP formation will not be impacted by ocean acidification as expected in the future ocean.

The impact of ocean acidification on microorganisms or biochemical cycling is frequently investigated by the addition of a strong acid to seawater (Hansen, 2002; Mari, 2008; Thornton, 2009; Berge et al., 2010) as this procedure is easy and mimics the pH expected in the future well, although this procedure has also been criticized (Riebesell et al., 2010). A comparison between manipulations of alkalinity and pH by acid addition with perturbations of DIC and pH by bubbling with CO_2-adjusted air showed no noteworthy difference in growth or calcification rates of coccolithophores between both types of manipulations (Hoppe et al., 2011), implying that the logistically easier acid addition method was sufficient in those experiments. In contrast, our results that the equilibrium between TEP and their precursors is sensitive to changes in TA, but not in pH, emphasizes that acidification experiments cannot simulate future carbonate chemistry adequately. Acid addition does not mimic ocean acidification well enough to produce reliable results of complex biochemical interactions, and results from such experiments may in fact be misleading when applied to predict future conditions.

The suite of experiments presented here furnishes one puzzle piece towards addressing the question if ocean acidification will strengthen or weaken the efficiency of the biological pump. It has been postulated controversially that changes in TEP production will impact the efficiency of the biological pump (Riebesell et al., 2007; Mari, 2008). Our results address the abiotic formation rate of TEP, one of several essential steps leading to aggregate formation and sedimentation. However, the efficiency of the biological pump is determined by scores of processes, including primary and new production, food web structure, aggregation dynamics, sinking velocity and degradation rates, each of which might in part or in their totality be impacted by ocean acidification. Currently we do not have the knowledge to make predictions even on the direction of future changes in the efficiency of the biological pump. This is a serious deficiency as the biological pump plays a central role in moving carbon from the atmosphere to the ocean thus impacting atmospheric pCO_2 and therefore the climate considerably.

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