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Effects of high CO_2 and warming on a Baltic Sea microzooplankton community

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Global warming and ocean acidification are among the most important stressors for aquatic ecosystems in the future. To investigate their direct and indirect effects on a near-natural plankton community, a multiple-stressor approach is needed. Hence, we set up mesocosms in a full-factorial design to study the effects of both warming and high CO_2 on a Baltic Sea autumn plankton community, concentrating on the impacts on microzooplankton (MZP). MZP abundance, biomass, and species composition were analysed over the course of the experiment. We observed that warming led to a reduced time-lag between the phytoplankton bloom and an MZP biomass maximum. MZP showed a significantly higher growth rate and an earlier biomass peak in the warm treatments while the biomass maximum was not affected. Increased pCO_2 did not result in any significant effects on MZP biomass, growth rate, or species composition irrespective of the temperature, nor did we observe any significant interactions between CO_2 and temperature. We attribute this to the high tolerance of this estuarine plankton community to fluctuations in pCO_2, often resulting in CO_2 concentrations higher than the predicted end-of-century concentration for open oceans. In contrast, warming can be expected to directly affect MZP and strengthen its coupling with phytoplankton by enhancing its grazing pressure.

Keywords: CO_2, global warming, heterotrophic protists, mesocosm experiment, ocean acidification, protozooplankton.

Introduction

The concentration of CO_2 in the atmosphere has increased considerably in the last decades, from 280 ppm in pre-industrial times to currently ~400 ppm (Le Quéré *et al.*, 2013). By the end of this century, atmospheric concentrations are predicted to reach 1000 ppm (Collins *et al.*, 2013). Apart from the well-known greenhouse effect, a rise in CO_2 has a direct effect on the surface oceans. Acting as major sinks for CO_2, the increase in dissolved CO_2 in the surface waters results in a change in carbonate chemistry and a decrease in pH, termed ocean acidification (OA; Sabine *et al.*, 2004). On a global scale, pH values have already decreased by 0.1 units in the last 100 years (Hoegh-Guldberg and Bruno, 2010), but there are differences in the amount of CO_2 taken up by the oceans depending on the region (Sabine *et al.*, 2004). Linked to the predicted increase in CO_2, a further decrease in pH by up to 0.32 units by the end of the 21st century is likely (Ciais *et al.*, 2013).

OA is most problematic for organisms with skeletal calcium carbonate structures, especially molluscs, corals, and calcifying algae (Kroeker *et al.*, 2013). On the other hand, there are non-calcifying phytoplankton species that benefit from a higher availability of carbon enhancing their growth (Rost *et al.*, 2008; Low-Decarie *et al.*, 2014). Although a direct effect of a lowered pH on phytoplankton (Riebesell *et al.*, 2000a; Kim *et al.*, 2006) and zooplankton (Pedersen and Hansen, 2003; Mayor *et al.*, 2007; Cripps *et al.*, 2014) has been reported for some species, other studies point at only the indirect effects of OA, e.g. by changes in phytoplankton availability, quality, or changes in C : N : P ratios affecting higher levels (Iglesias-Rodriguez *et al.*, 2008; Suffrian *et al.*, 2008; Nielsen

et al., 2010; Aberle *et al.*, 2013). Therefore, several authors have argued for the necessity of community level experiments to understand whether and how biotic interactions dampen or amplify single-species responses (Joint *et al.*, 2011; Kroeker *et al.*, 2013; Rossoll *et al.*, 2013).

Microzooplankton (MZP) in the size range of 20–200 μm is a major phytoplankton consumer in planktonic foodwebs where it plays a vital role as intermediary between the microbial loop and higher trophic levels (Calbet and Landry, 2004; Calbet *et al.*, 2008). Owing to its high specific growth and grazing rates, MZP can have a strong impact on the biomass and species composition of phytoplankton communities, which can lead to dietary overlap and competition between MZP and mesozooplankton (Löder *et al.*, 2011). At the same time, higher trophic levels use MZP as a food source and can benefit from its ability to buffer nutritional imbalances especially at times when food quality of phytoplankton is low (Malzahn *et al.*, 2010).

On top of changes in ocean carbonate chemistry, warming will have a strong impact on the oceans: according to the IPCC report (Collins *et al.*, 2013), sea surface temperature will increase between 1 and 5°C within this century. This is predicted to cause a decrease in phytoplankton biomass and productivity (Boyce *et al.*, 2010; Hoegh-Guldberg and Bruno, 2010; Sommer *et al.*, 2012). Such a decline in phytoplankton biomass has been attributed to a strengthened top-down control on phytoplankton (Rose and Caron, 2007), because growth and grazing rates of heterotrophic protists as well as copepods show a stronger temperature dependence than autotrophic protists (Aberle *et al.*, 2007, 2012; Lewandowska *et al.*, 2014). As grazing of both MZP and copepods is species- or size-selective, certain species are preferably grazed upon thus leading to changes in the phytoplankton community structure (Riegman *et al.*, 1993; Lewandowska and Sommer, 2010).

While investigations of single factors are of importance, there is a strong need to consider interactive effects of multiple stressors in future analyses (Caron and Hutchins, 2012). In one of the few experiments on the joint effects of OA and warming, Rose *et al.* (2009) found significant differences in MZP abundance and community composition for a combination of factors in a North Atlantic spring bloom plankton community. Their study suggests that indirect effects due to changes in the phytoplankton community could be more important in changing MZP community structure than direct effects of OA or warming. In contrast, Calbet *et al.* (2014) performed a multiple-stressor mesocosm experiment in a Norwegian fjord and added eutrophication as a third stressor. Contrasting effects of warming and acidification for different plankton groups were observed, pointing at the importance of indirect effects due to changes in phytoplankton food quality leading to a lower ciliate biomass maximum and a shift of the plankton community in the combined treatment (Calbet *et al.*, 2014).

Generally, pCO_2 in highly productive estuarine areas such as the Kiel Fjord is much more variable than in the open ocean (Feely *et al.*, 2010; Melzner *et al.*, 2013). Thus, the responses of plankton communities to warming and OA highly depend on the community composition and the ecosystem characteristics. Currently, seawater pCO_2 in the Kiel Fjord is often as high as 700 ppm, with peaks in summer and autumn reaching values of up to 2300 ppm (Thomsen *et al.*, 2010). While the community in the Fjord is thus expected to be resilient to a high pCO_2 (Melzner *et al.*, 2013; Rossoll *et al.*, 2013), there is evidence that Baltic Sea plankton communities are strongly affected by warming (Sommer and Lewandowska, 2011; Aberle *et al.*, 2012, 2015; Winder *et al.*, 2012; Lewandowska *et al.*, 2014).

Here, we present an indoor mesocosm study on the combined effects of enhanced CO_2 and warming on natural autumn plankton communities from Kiel Fjord, characterized by a diatom-dominated phytoplankton bloom in autumn (Wasmund *et al.*, 2008). Our working hypotheses considering the combined effects of warming and CO_2 were as follows:

(i) Warming will enhance MZP growth (timing and biomass), thus leading to a strong top-down control of phytoplankton and a strong copepod predation on MZP.

(ii) Based on the high pH tolerance of coastal MZP communities, only indirect effects on MZP due to an altered phytoplankton quality and community composition are likely.

(iii) Due to positive effects on photosynthesis, high pCO_2 will cause an increase in phytoplankton biomass leading to a higher MZP biomass.

(iv) The combined effects of warming and pCO_2 will lead to a dampening of the effects of high pCO_2. The increase in MZP biomass and growth rate with warming is expected to compensate for indirect effects on MZP due to changes in phytoplankton community composition and quality.

Material and methods

Experimental design

Twelve mesocosms with a volume of 1400 l each and a depth of 1 m were installed in four temperature-controlled culture rooms at GEOMAR, Kiel, Germany, for an experiment in autumn 2012. The setup is described in more detail by Sommer *et al.* (2007); however, mesocosm lids were added for the CO_2 manipulation. Two temperatures (9 and 15°C, hereafter called "cold" and "warm") and two CO_2 levels (target levels 560 and 1400 ppm, hereafter called "low" and "high") were crossed in a full-factorial design with each treatment in triplicate. The temperatures reflect a difference of 3°C from the ambient temperature of ~12°C. The symmetric design was chosen to avoid confounding the effects of the direction of temperature change (warming or cooling) with the effects of temperature change as such. The low target CO_2 concentration of 560 ppm was chosen to represent actual values measured in Kiel Fjord the day before filling the mesocosms, which is well below the average concentration of 700 ppm expected for the Kiel Fjord in autumn (Thomsen *et al.*, 2010). The high CO_2 level of 1400 ppm represents the value predicted for the end of the century for surface waters of the Baltic Sea (Collins *et al.*, 2013).

Light was provided by computer-controlled light units (GHL Groß Hard- und Softwarelösungen, Lampunit HL3700 and ProfiluxII). The light units consisted of five HIBay-LED spotlights (purpose built units by Econlux, each 100 W), illuminating each mesocosm from above. Light supply and daylength were calculated after Brock (1981), resembling the solar irradiance of a cloudless 21st October in Kiel and reduced by 50% to account for under water light attenuation. The light : dark cycle was 11 h50 min : 12 h10 min. The daily maximum light intensity in the middle of the water column was 252.3 μmol m^{-2} s^{-1}.

The mesocosms were filled with unfiltered seawater from Kiel Fjord on 19 October 2012, containing a natural autumn community of phytoplankton, bacteria, and protozoa. To ensure the same starting conditions in all mesocosms, the water was pumped from ~2 m

depth in a collecting tank using a rotary pump before distributing it into the mesocosms with a 12-way valve. Mesozooplankton was added from net catches with a target copepod concentration of 10 individuals l^{-1} to resemble natural densities and species composition for that time of the year (Javidpour *et al.*, 2009). The mesocosms were gently stirred by a propeller to minimize sedimentation and to ensure a homogenous distribution of plankton throughout the water column. Previous experiments with the same design have shown that this treatment does not lead to an increase in mesozooplankton mortality (Sommer *et al.*, 2007).

A PVC lid covered each mesocosm with only a small sampling port being opened for daily samplings. CO_2 levels were achieved by a flow-through of CO_2-enriched air with 560 and 1400 ppm CO_2 through the headspace between the water surface and the mesocosm lid with a rate of 30–60 l h^{-1}. The headspace was used to simulate a more natural CO_2 addition compared with the addition of CO_2-saturated water. However, the biological drawdown of CO_2 due to photosynthetic CO_2 consumption in combination with an incomplete equilibration between headspace and mesocosm water led to CO_2 concentrations below the target level. This was compensated by the addition of sterile-filtered, CO_2-saturated mesocosm water three times during the experiment; the necessary volumes were calculated based on dissolved inorganic carbon and alkalinity.

Target temperatures and divergence of CO_2 levels were reached 3 d after filling in all treatments on 22 October (hereafter called day 0) and the experiment ran until 12 November 2012 (day 21) with constant light and temperature conditions.

Sampling and measurements

Daily measurements included water temperature, salinity, and pH. Three times per week, samples for *in situ* fluorescence, heterotrophic nanoflagellates, phytoplankton, and MZP were taken by siphoning seawater from the middle of the water column using a silicone tube. Similarly, particulate organic carbon, phosphorus, and nitrogen as well as inorganic nutrients were sampled three times per week. Mesozooplankton was sampled once per week by three vertical hauls with a plankton net (64 μm mesh size), resulting in a sampled volume of 5.1 l.

In situ fluorescence was measured directly after sampling with a 10 AU fluorometer (Turner Design). For the MZP samples, 250 ml of mesocosm water was transferred into brown glass bottles, fixed with acid Lugol's solution, and stored dark. Counting and taxonomic identification of MZP was carried out using the Utermöhl method (Utermöhl, 1958). Depending on the plankton density, either 50 or 100 ml of each sample was transferred to a sedimentation cylinder and allowed to settle for 24 h before counting with an inverted microscope (Zeiss Axiovert 135). To reduce the counting bias against rare species and to assure comparability of the counts both at high and low MZP abundances, the whole surface of the sedimentation chamber was counted at 200-fold magnification.

MZP was identified to the lowest possible taxonomic level (species or genus level) according to Carey (1992), Montagnes *et al.* (2001), and Kraberg *et al.* (2010) and otherwise grouped into size classes (small: <30 μm, medium: 30–55 μm, and large: >55 μm). Biovolumes of ciliates were calculated according to geometric proxies by Hillebrand *et al.* (1999). For each group, the dimensions of 20 random cells were measured digitally (AxioVision 4.9 and AxioCam, Carl Zeiss Microscopy GmbH). Ciliate carbon biomass was estimated from the biovolumes using the conversion factors provided by Putt and Stoecker (1989).

Details on phytoplankton, nutrients, and carbonate chemistry and copepod sampling and analysis are given by Paul *et al.* (2015) and Garzke *et al.* (2015), respectively. Copepod biomass was calculated from abundances of adults and copepodites using standard conversion factors (Lengfellner, 2008).

Data analysis

First, we identified the day (D_{max}) when biomass reached its peak in each mesocosm. Growth rates μ (d^{-1}) of total ciliates and single species of ciliates were calculated as the slope of a regression of biomass over time (ln transformed) from day 0 until D_{max}. This day was defined as the bloom timing (D_{max}) for the respective mesocosm. The biomass maximum was the highest measured value from each single mesocosm, independent of the experimental day. The species diversity index (H', $\log_e$) was calculated after Shannon and Weaver (1963) on a sample day basis.

For the statistical analysis, all data were tested for normality and homogeneity of variance and transformed (ln) if necessary. To investigate the interactions between the factors temperature, CO_2 level, and time, repeated-measures ANOVAs were calculated with ciliate biomass, total copepod biomass, ciliate diversity, or chlorophyll fluorescence as a dependent variable. Two-way ANOVAs were performed to test for significant effects of temperature and CO_2 level (independent variables) as well as the interactions of these two factors on biomass maximum, D_{max}, and growth rates for total ciliates and single species of ciliates (dependent variables). Likewise, chlorophyll fluorescence maximum and bloom timing were tested with two-way ANOVAs.

Statistica 12 (StatSoft, Inc.) was used for ANOVAs and SigmaPlot 12.5 (Systat Software, Inc.) for regressions and graphs.

Results

Owing to a technical problem with the light control units of mesocosm 9 at the beginning of the experiment, the plankton community of this specific mesocosm showed a strongly reduced plankton development and was thus excluded from further analysis (thus, the cold low CO_2 treatment only had two replicates instead of three).

Temperatures in the mesocosms were 9.44°C ($\pm$0.39) and 14.78°C ($\pm$0.31) and remained stable over the course of the experiment (Figure 1a). The pCO_2 values decreased over time, but this was compensated by the addition of CO_2-enriched water on days 7, 12, and 19 (Figure 1b). Overall, the average value was 439 ppm ($\pm$180) for the low and 1040 ppm ($\pm$228) for the high CO_2 treatments.

Biomass and growth rate

There was an immediate numerical response in terms of ciliate biomass to the increasing phytoplankton biomass in the warm mesocosms in contrast to a delayed response in the cold ones (Figure 2a and b). Ciliate biomass was significantly different between the temperature treatments, although it depended on the time of the experiment (significant interaction time × temperature, $p < 0.001$; Table 1). Neither CO_2 nor the interaction of CO_2 and temperature had a significant effect on ciliate biomass. The peak of ciliate biomass was reached on day 11 in the warm treatments, followed by a sharp decline to initial levels (Table 2 and Figure 2b). In the cold treatments, peak densities of ciliates were observed on day 18 or 21 (Table 2). The ciliate biomass maximum was not affected by warming or CO_2 or interactions of these factors (Table 3). However, the timing of the biomass maximum was significantly affected by the temperature ($p < 0.001$), and this was also the case

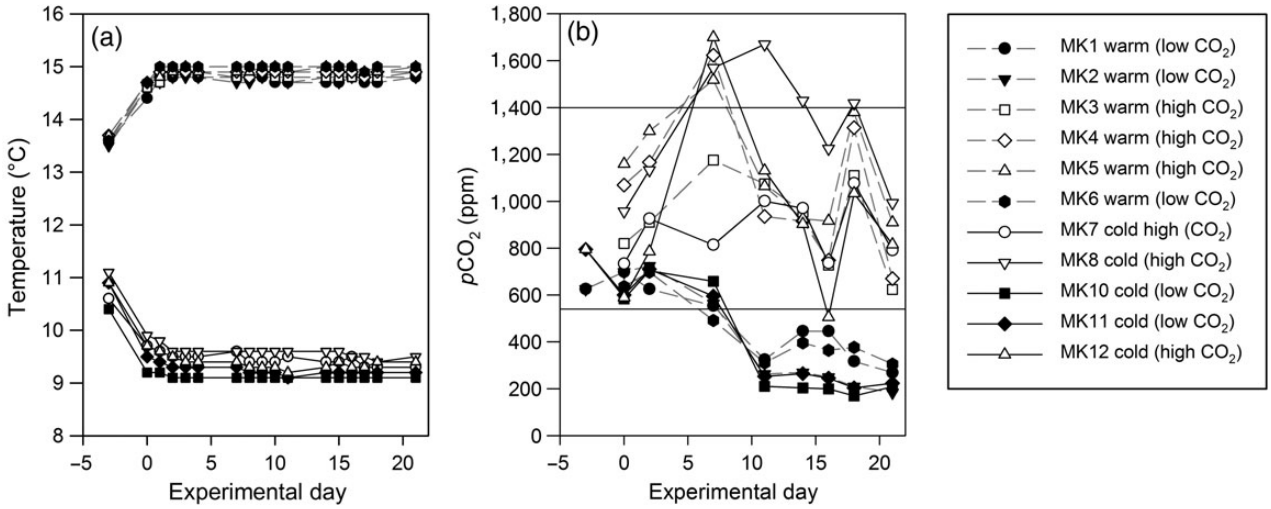

Figure 1. Actual temperatures and pCO_2 levels in the 12 mesocosms with the treatments of 9°C (black lines) and 15°C (dashed grey lines) at low (540 ppm; filled symbols) and high (1400 ppm; open symbols) CO_2 levels. Horizontal black lines denote target pCO_2 levels.

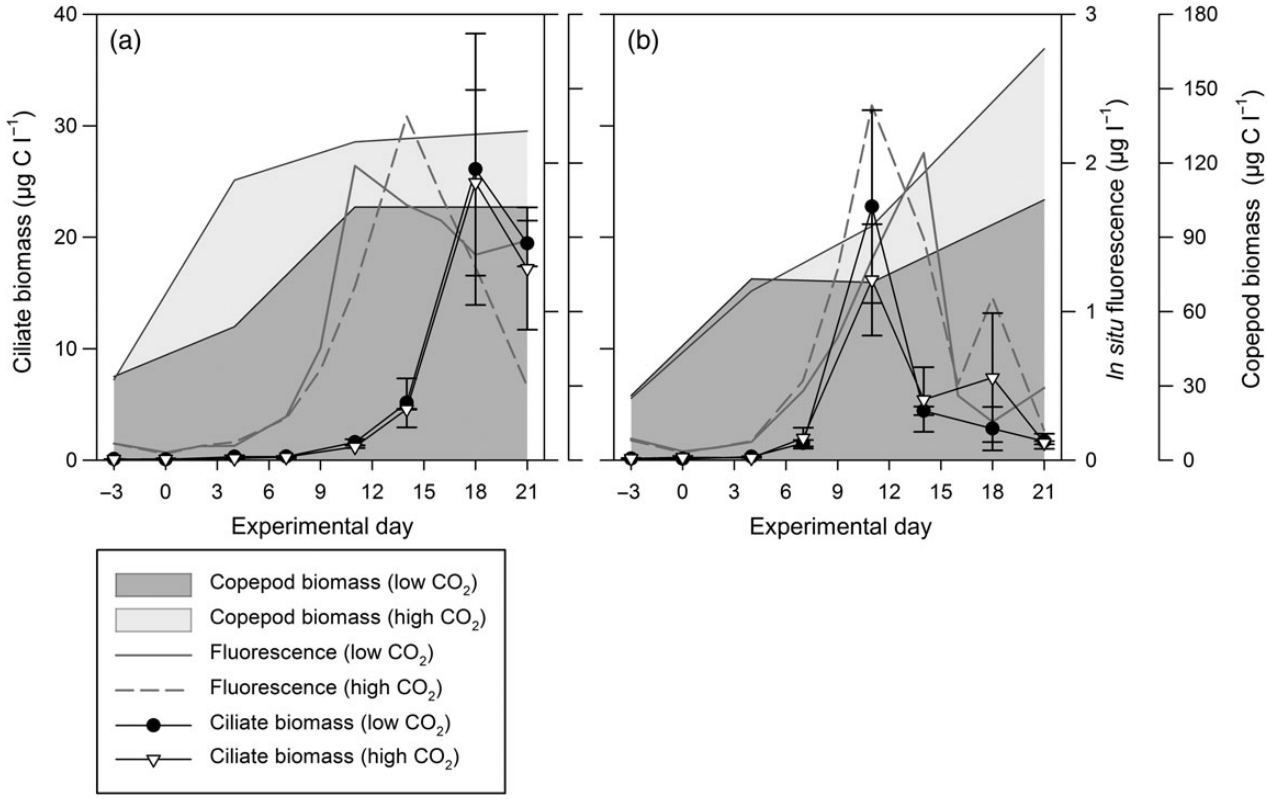

Figure 2. Ciliate biomass (mean ± SD) in µg C l^{-1} at low (filled symbols) and high CO_2 levels (open symbols) and total copepod biomass (adult copepods and copepodites) in µg C l^{-1} at low (dark grey fields) and high CO_2 levels (light grey fields) as well as *in situ* fluorescence (µg l^{-1}) at low (grey lines) and high CO_2 levels (dashed grey lines) in the (a) cold and (b) warm treatments over the course of the experiment.

for the ciliate growth rate µ ($p < 0.017$). It was higher in the warm treatments (mean 0.45 ± 0.08 d^{-1}) than in the cold ones (mean 0.31 ± 0.03 d^{-1}). An effect of the interactions of temperature and CO_2 could not be found.

Chlorophyll fluorescence was significantly affected by temperature and the interactions of temperature and time (Table 1). Maximum fluorescence was not different between any of the treatments; however, its timing was marginally affected by temperature ($p < 0.097$), leading to a slightly earlier bloom in the warm mesocosms (Table 3). Total copepod biomass was significantly higher in the high CO_2 treatments, but not affected by temperature or the interaction of both factors (Table 1).

Table 1. Results of the repeated-measures ANOVA for the effects of time, CO_2, temperature, and their interactions on ciliate biomass, total copepod biomass, chlorophyll fluorescence, and ciliate species diversity H' over the duration of the experiment.

Variable	Effect	d.f.	MS	*F*	*p*-values
ln total ciliate biomass ($\mu g\ C\ l^{-1}$)	CO_2	1	0.000	0.000	0.994
	Temperature	1	0.402	1.707	0.233
	CO_2 × temperature	1	0.441	1.870	0.214
	Time	6	33.873	123.448	<0.001***
	Time × CO_2	6	0.307	1.120	0.367
	Time × temperature	6	8.755	31.905	<0.001***
	Time × CO_2 × temperature	6	0.067	0.243	0.960
ln total copepod biomass ($\mu g\ C\ l^{-1}$)	CO_2	1	1.023	5.683	0.044*
	Temperature	1	0.034	0.187	0.677
	CO_2 × temperature	1	0.186	1.031	0.340
	Time	2	0.658	7.038	0.006**
	Time × CO_2	2	0.051	0.544	0.591
	Time × temperature	2	0.274	2.931	0.082
	Time × CO_2 × temperature	2	0.155	1.660	0.221
ln fluorescence ($\mu g\ l^{-1}$)	CO_2	1	0.116	0.278	0.614
	Temperature	1	2.764	6.653	0.037*
	CO_2 × temperature	1	1.167	2.808	0.138
	Time	9	15.569	58.539	<0.001***
	Time × CO_2	9	0.190	0.716	0.692
	Time × temperature	9	0.893	3.359	0.002**
	Time × CO_2 × temperature	9	0.242	0.909	0.523
Species diversity H'	CO_2	1	0.010	0.425	0.535
	Temperature	1	0.799	34.436	<0.001***
	CO_2 × temperature	1	0.024	1.055	0.339
	Time	6	0.319	5.240	<0.001***
	Time × CO_2	6	0.082	1.352	0.256
	Time × temperature	6	0.190	3.113	0.013*
	Time × CO_2 × temperature	6	0.092	1.516	0.197

Significant results are marked by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Table 2. Ciliate biomass maximum values (max.), bloom timing D_{max}, and ciliate growth rate μ for all treatments.

Temperature (°C)	CO_2 (ppm)	Biomass max. ($\mu g\ C\ l^{-1}$)	D_{max} (d)	μ (d^{-1})
15°C	439	34.52	11	0.55
	439	19.78	11	0.44
	439	13.95	11	0.46
	1040	20.93	11	0.53
	1040	9.27	11	0.30
	1040	18.33	11	0.41
9°C	439	21.49	21	0.29
	439	38.27	18	0.29
	1040	28.47	18	0.29
	1040	23.82	21	0.30
	1040	32.82	18	0.38

Species composition

Ciliate species diversity H' was significantly higher in the warm treatments, but not affected by CO_2 or the interactions of both factors (Table 1). The taxonomic composition of ciliates over the course of the experiment is given in Figure 3. Although present at very low initial densities, the small oligotrich *Strobilidium* sp. rapidly increased in density in all treatments and contributed up to 80% of the total ciliate community at D_{max} (day 11 for the warm mesocosms and day 18 for the cold ones) irrespective of the temperature or CO_2 level. While the growth rate of *Strobilidium* sp. was not significantly different in any of the treatments, the timing of the peak was significantly earlier in the warm treatments independent of the CO_2 level (Table 3). The biomass maximum of *Strobilidium* sp. was marginally affected by temperature ($p < 0.075$). In the warm treatments, they declined after the peak to almost initial levels which was not the case in the cold ones where they still made up >50% of the biomass on day 21.

The opposite trend was observed for the cyclotrich *Myrionecta rubrum* which made up the main proportion (40–80%) of the biomass in the cold treatments until day 7, followed by a rapid decline thereafter until day 21. In the warm mesocosms, density increased again after D_{max}. The hypotrich *Euplotes* sp. was present in all mesocosms but more abundant in the warm ones, especially towards the end of the experiment. A significant effect of the manipulated factors on the biomass maxima of *M. rubrum* and *Euplotes* sp. was not found (data not shown). There was also no clear trend for the succession of *Strombidium* sp. (Oligotrichids), *Balanion comatum* (Prorodontids), *Lohmaniella oviformis* (Choreotrichids), and thecate tintinnids which were found in small numbers only. However, *B. comatum* and *L. oviformis* were absent from the warm treatments after day 14. An increase in tintinnids was only observed in the cold treatments for the last day. Owing to a high variability between mesocosms, no significant effect on the biomass maxima of different taxa over time in response to warming or high CO_2 could be observed.

At the beginning of the experiment, some dinoflagellates were observed in the mesocosms: *Ceratium* sp. was present in all mesocosms until day 7 and *Prorocentrum micans* was present at very small numbers in some of the treatments. Since these species are considered as mainly autotroph (*Ceratium* sp.) or mixotroph (*P. micans*), they were not included in the analyses and are presented by Sommer *et al.* (2015) instead.

Table 3. Results of the two-way ANOVA for the effects of temperature, CO_2, and their interactions on total ciliate biomass, *Strobilidium* sp. biomass, and chlorophyll fluorescence regarding maximum (max.), bloom timing D_{max}, and growth rate μ.

Response variable	Factor	d.f.	MS	*F*	*p*-values
Biomass max. total ciliates (μg C l^{-1})	CO_2	1	43.568	0.634	0.452
	Temperature	1	248.998	3.623	0.099
	CO_2 × temperature	1	17.091	0.249	0.633
Biomass max. *Strobilidium* sp. (μg C l^{-1})	CO_2	1	0.146	1.145	0.320
	Temperature	1	0.553	4.350	0.075
	CO_2 × temperature	1	0.149	1.173	0.315
Fluorescence max. (μg l^{-1})	CO_2	1	0.079	0.214	0.658
	Temperature	1	0.173	0.472	0.514
	CO_2 × temperature	1	0.362	0.987	0.354
ln D_{max} total (d)	CO_2	1	0.001	0.110	0.749
	Temperature	1	0.826	208.680	<0.001**
	CO_2 × temperature	1	0.001	0.110	0.749
ln D_{max} *Strobilidium* sp. (d)	CO_2	1	0.001	0.110	0.075
	Temperature	1	0.826	208.680	<0.001**
	CO_2 × temperature	1	0.001	0.110	0.075
ln D_{max} fluorescence (d)	CO_2	1	0.001	0.015	0.907
	Temperature	1	0.141	3.663	0.097
	CO_2 × temperature	1	0.057	1.481	0.263
ln μ total ciliates (d^{-1})	CO_2	1	0.001	0.164	0.697
	Temperature	1	0.052	9.784	0.017*
	CO_2 × temperature	1	0.007	1.287	0.294
ln μ *Strobilidium* sp. (d^{-1})	CO_2	1	0.160	1.229	0.304
	Temperature	1	0.110	0.848	0.388
	CO_2 × temperature	1	0.126	0.965	0.359

Significant results are marked by *$p < 0.05$, and **$p < 0.001$.

Discussion

Although mesocosm approaches show some limitations when mimicking natural conditions such as diurnal variations in abiotic conditions (e.g. light and temperature) or vertical migration of zooplankton, mesocosms are a useful tool to simulate changes in abiotic conditions (e.g. warming and OA) and investigate their effects on plankton communities under near-natural conditions. While biases in species composition and foodweb complexity cannot be ruled out, the given experimental setup allowed the combined manipulation of temperature and CO_2, thus enabling an analysis on short-term reactions of a near-natural plankton community to future ocean conditions.

This indoor mesocosm facility has already been successfully used during a series of previous experiments investigating the effects of ocean warming on Baltic Sea plankton communities (Sommer *et al.*, 2007; Lewandowska and Sommer, 2010; Sommer and Lewandowska, 2011). As shown by Sommer *et al.* (2007), the mesocosms allowed the simulation of *in situ* species composition and plankton succession. Furthermore, the mechanical conditions did not have an adverse impact on the biota.

Effects of warming

Autotrophic protists are relatively temperature insensitive as long as their photosynthesis is light-limited (Tilzer *et al.*, 1986). In contrast, heterotrophic MZP responds to temperature, and a relationship between an increase in production and an increase in temperature has often been observed (Weisse and Montagnes, 1998; Montagnes and Lessard, 1999; Rose and Caron, 2007). The different reactions of heterotrophs and autotrophs to warming are based on the basic difference in the former being temperature-dependent due to the biochemical processes of their metabolism and the latter being in large parts light-dependent due to photosynthesis (Bernacchi *et al.*, 2001). The response of autotrophs and heterotrophs to warming is therefore unbalanced and thus will create shifts in interactions (McGowan *et al.*, 2003; Smol *et al.*, 2005).

In our study, we found a reduced time-lag between the phytoplankton bloom and the MZP biomass maximum. High temperatures resulted in a significantly higher MZP growth rate and an earlier bloom followed by a subsequent decline, an observation supporting hypothesis (i) which is in line with findings from previous studies (Aberle *et al.*, 2007, 2012; O'Connor *et al.*, 2009). Additionally, phytoplankton cell size decreased with warming thus providing better food for ciliates (Sommer *et al.*, 2015), an effect also indicated by previous studies (Aberle *et al.*, 2015). Overall, only a weak indication for taxonomic shifts in phytoplankton was found during the present mesocosms study (Sommer *et al.*, 2015) while we observed a higher diversity for MZP communities in the warm treatments.

The reduced carrying capacity of phytoplankton (Table 1), in relation to warming observed in our experiment as well as in other Baltic Sea experiments (Lewandowska and Sommer, 2010; Sommer *et al.*, 2012; Suikkanen *et al.*, 2013) and in the North Atlantic (Rose *et al.*, 2009), led to an overall decrease in MZP biomass in the warm treatments, confirming hypothesis (i). In fact, Lewandowska *et al.* (2014) pointed out that a potential positive reaction of phytoplankton to warming is likely to occur, but would be masked by grazing pressure from MZP. This might have happened in our experiment as MZP followed the phytoplankton increase in the warm treatments almost instantaneously, pointing towards a strengthened coupling between phytoplankton and MZP based on warming, a finding which is in line with observations by Aberle *et al.* (2015).

A stronger top-down effect caused by warming has been reported in previous studies, which was explained by the temperature insensitivity of photosynthesis in combination with the temperature-stimulated

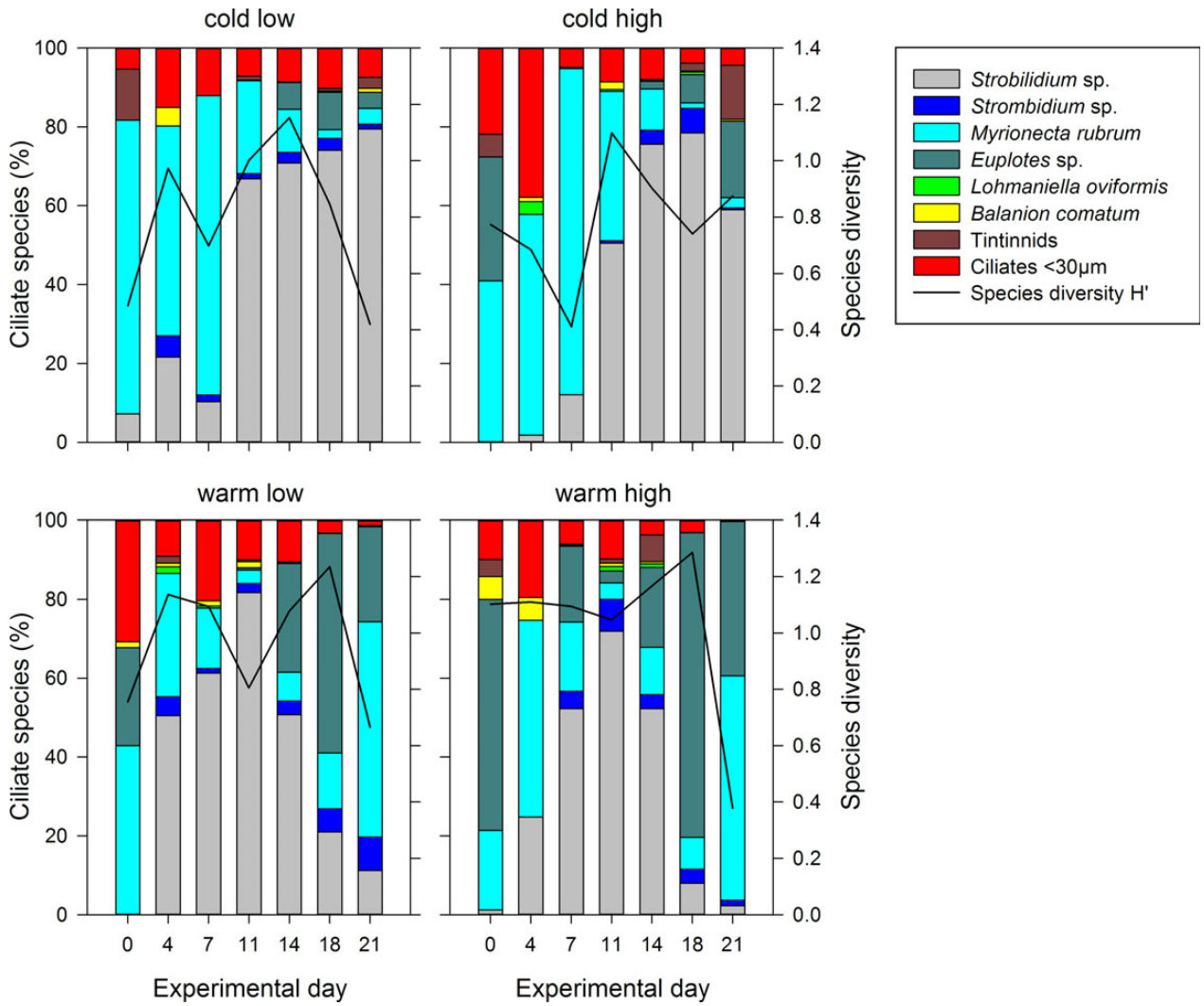

Figure 3. Relative ciliate species composition and species diversity index (H'; black lines) over the time of the experiment for the two temperature (warm and cold) and CO_2 treatments (low and high).

MZP biomass increase (O'Connor *et al.*, 2009; Sommer and Lewandowska, 2011). Similarly, it has been shown for North Sea plankton communities that intense grazing by zooplankton caused by warm autumn or winter temperatures could lead to a depression and delay of the spring bloom in the subsequent year (Wiltshire and Manly, 2004). The more intense grazing seems to be primarily caused by warming, not zooplankton densities (Wiltshire *et al.*, 2008). An intensified grazing by copepods on MZP caused by warming could be an explanation for the overall low MZP densities we found in our study. It seems likely that a high copepod predation in our mesocosms resulted in a strong suppression of ciliates as MZP is considered as a preferred food item for copepods (Löder *et al.*, 2011).

Effects of pCO_2

An increase in pCO_2 resulting in a decrease in pH could directly affect the physiology of both autotrophic and heterotrophic protists and lead to e.g. changes in intracellular pH, membrane potentials, and enzyme activities (Nielsen *et al.*, 2010). There are indications for a pH sensitivity of MZP from a variety of ecosystems like the Baltic Sea (Pedersen and Hansen, 2003) and the North Atlantic, Rhode Island, USA (Hinga, 1992). However, as Hinga (2002) pointed out, it largely depends on the inherent pH tolerance of a plankton species if it can grow at a broad or a narrow range of pH values. Unusually, high or low pH values often occurring in coastal systems can favour the selection towards the growth of species adapted to a wide range of pH values.

While there is evidence from other experimental studies showing that high pCO_2 negatively affected heterotrophic ciliates in terms of biomass and growth (Calbet *et al.*, 2014, western Norway) or even inhibited growth (Nielsen *et al.* 2010, Baltic Sea), we observed the opposite. In our experiment with its comparatively moderate CO_2 elevation of effectively 1040 ppm, we showed that the present coastal MZP community was tolerant against the effects of CO_2. This might be related to a high pH tolerance of the Baltic Sea coastal plankton community in the Kiel Fjord to habitat pCO_2 fluctuations (Melzner *et al.*, 2013). Generally, the Kiel Fjord is characterized by a low buffering capacity due to its low salinity (Rossoll *et al.*, 2013) and a stratification with a bottom layer of CO_2-rich water originating in the heterotrophic degradation of organic material (Melzner *et al.*, 2013). Upwelling of CO_2-rich deep water masses, especially during summer and autumn, leads to acidification of

the surface waters (Hansen *et al.*, 1999), an effect which is also found on the west coast of the United States and which is predicted to worsen in the future with additional anthropogenic CO_2 input (Feely *et al.*, 2008, 2010).

A similarly high tolerance was found in mesocosm studies using coastal plankton communities in the Arctic, Svalbard (Aberle *et al.*, 2013), off Bergen, Norway (Suffrian *et al.*, 2008) and another study from Kiel Fjord (Rossoll *et al.*, 2013). While food availability and phytoplankton composition were affected by the different pCO_2 treatments, the authors observed no or only very subtle indirect effects of OA on the MZP community composition and biomass maxima. While, in our study, indeed no direct effects on MZP species composition were found, there were also no indirect effects despite there being a changed phytoplankton community. Thus, hypothesis (ii) was not confirmed.

Furthermore, we hypothesized that an elevated pCO_2 might result in a higher carrying capacity of phytoplankton, thus leading to increases in MZP biomass (hypothesis iii). In the literature, there is some evidence that such indirect effects are induced by an increase in pCO_2, mainly due to changes in phytoplankton availability (Suffrian *et al.*, 2008; Rose *et al.*, 2009; Calbet *et al.*, 2014). Concerning phytoplankton, direct effects from an elevated pCO_2 concentration include an increased photosynthetic rate at high CO_2 levels due to an increased availability of CO_2 and HCO_3^- (Burkhardt *et al.*, 2001; Rost *et al.*, 2008), changes in stoichiometry affecting phytoplankton food quality (Burkhardt *et al.*, 1999; Schoo *et al.*, 2013), and inhibition of the development for calcifying algae (Riebesell *et al.*, 2000b; Orr *et al.*, 2005; Iglesias-Rodriguez *et al.*, 2008). In our study, an increased phytoplankton biomass at high pCO_2 was observed at high temperatures only (Sommer *et al.*, 2015). Consequently, a general increase in MZP biomass due to higher phytoplankton biomass at high pCO_2 could not be confirmed and hypothesis (iii) was rejected. Nonetheless, copepod densities were higher in the high pCO_2 treatment, thus the increased grazing pressure on MZP and, to a smaller part, on phytoplankton could have masked changes in the carrying capacity resulting from enhanced copepod predation.

Additionally, no change in elemental ratios of phytoplankton and only weak changes in species composition due to a high pCO_2 were found; all of which can be attributed to the high tolerance of the phytoplankton community to a high pCO_2 (Paul *et al.*, 2015; Sommer *et al.*, 2015). However, one effect reported from the present mesocosm study is an increased cell size of phytoplankton at high pCO_2, turning them into less preferred food items for ciliates (Sommer *et al.*, 2015).

In the close-to-natural high pCO_2 scenario, we chose for the experiment with a value of 1040 ppm we observed a strong tolerance of the Kiel Fjord MZP community. Nevertheless, with the already strong fluctuations of pCO_2 today, it could happen that the values in terms of acidification will be even higher than what is currently predicted as a worst case scenario (Caldeira and Wickett, 2003). In this case, a direct effect on MZP could be expected as some species do react to extreme pH values as shown by Pedersen and Hansen (2003). Furthermore, additional factors, such as light regime, hypoxia, and eutrophication, have been identified to affect plankton communities (Lewandowska and Sommer, 2010; Melzner *et al.*, 2013; Suikkanen *et al.*, 2013). However, whether these factors act antagonistically or synergistically remains still unclear. Also, long-term adaptations of organisms are a factor that needs further investigation as they can result in adaptation of previously OA-sensitive plankton species (Lohbeck *et al.*, 2012).

Combination of the effects of warming and pCO_2

While there is an increasing number of studies available addressing the impacts of either ocean warming or OA, multiple-stressor approaches are rare, despite the importance of finding synergistic or antagonistic effects of these two stressors (Pörtner, 2008; Rost *et al.*, 2008; Calbet *et al.*, 2014). So far, there are few multiple-stressor studies dealing with the effects of warming and high CO_2 in combination with, for example, a focus on copepods (Mayor *et al.*, 2012), bacterioplankton (Lindh *et al.*, 2013), phytoplankton (Hare *et al.*, 2007; Feng *et al.*, 2009; Kim *et al.*, 2011, 2013), and MZP (Rose *et al.*, 2009; Calbet *et al.*, 2014).

Calbet *et al.* (2014) found negative effects of OA on the ciliate biomass maximum indirectly caused by stoichiometric changes in phytoplankton quality in a near-natural, large-scale mesocosm experiment in a Norwegian fjord. In contrast, the authors found that warming and acidification in concert did not affect the MZP biomass maximum, but led to a shift towards a more autotrophic foodweb based on the ratio of autotrophic to heterotrophic biomass. For an oligotrophic plankton community from the Mediterranean Sea, no effects of a multiple-stressor treatment on heterotrophic prokaryotes were reported (Maugendre *et al.*, 2014). In contrast, Rose *et al.* (2009) observed a significantly higher MZP abundance in a multiple-stressor treatment during a spring bloom experiment in the oligotrophic North Atlantic, although overall, the temperature effect was stronger. The study by Rose *et al.* (2009) was conducted in an open sea situation; however, where the seawater pCO_2 is close to the atmospheric values. Generally, the aforementioned studies point towards the importance of indirect effects of elevated pCO_2 on MZP and showed that the effects differ depending on the marine province.

Our experiment did not result in any significant interaction effects of high pCO_2 and warming as far as MZP growth rate, total biomass, and D_{max} are concerned thus supporting hypothesis (iv). We observed no effects of high pCO_2 on MZP biomass or growth, not even in the cold treatments, where a masking of possible CO_2 effects on MZP biomass and growth due to the pronounced reaction to warming could be excluded. This also supports previous findings that indirect effects of high pCO_2 observed for simple "one phytoplankton species—one consumer species" treatments can be compensated at the ecosystem level by species richness and trophic interactions (Rossoll *et al.*, 2013). Furthermore, it emphasizes the importance of using a near-natural plankton community instead of single-species systems that cannot provide enough information about indirect effects of high CO_2 and warming between trophic levels (Riebesell *et al.*, 2008; Maugendre *et al.*, 2014).

Implications for the foodweb

While warming was found to lower the biomass, increase the growth rates, lead to an earlier bloom and a higher diversity of MZP, an elevated CO_2 level did not affect any of the measured parameters. Phytoplankton stoichiometry was also not affected by CO_2 while biomass decreased and growth rates increased with warming (Paul *et al.*, 2015). Additionally, phytoplankton cell size increased at high pCO_2 (Sommer *et al.*, 2015).

However, our study also included copepods as mesograzers. Ciliates are ideal food items for copepods due to their ideal size compared with phytoplankton cells which are often either too small or too large (Frost, 1972). They make up 30–50% of the copepods daily diet depending on the phytoplankton concentration (Calbet and Saiz, 2005; Löder *et al.*, 2011). In our case, total copepod biomass was at 29 $\mu g\ C\ l^{-1}$ initially and increasing in all treatments

during the experiment, most notably in the high temperature/high CO_2 treatment. In fact, copepod biomass was significantly higher in the high CO_2 mesocosms, which is in contrast to previous studies where no such effect was found (Rossoll *et al.*, 2012; Cripps *et al.*, 2014). Considering that copepod, MZP, and phytoplankton starting conditions were the same for all mesocosms and no increase in MZP or phytoplankton biomass in the high CO_2 treatments was observed, the question arises: what caused the increase in copepod biomass?

As mentioned before, an increase in MZP usually supports an increase in copepods. This numerical response of copepods to increasing ciliate densities is an effect also described by other authors (Stoecker and Capuzzo, 1990). Generally, the strong top-down control of MZP by copepods could be one of the factors explaining the comparatively low MZP biomass during the experiment. As pointed out by other studies, a high CO_2 level can lead to an increase in phytoplankton biomass (Rost *et al.*, 2008; Havenhand, 2012; Low-Decarie *et al.*, 2014), which in turn has the potential to cause an increase in MZP biomass. It seems plausible that, in our experiment, a positive effect of high CO_2 on phytoplankton and subsequently on MZP was masked by a high copepod grazing pressure on both phytoplankton and MZP. This is in line with observations by Lewandowska *et al.* (2014) in a single-stressor mesocosm experiment, showing comparable impacts of copepod abundance and thus grazing being enhanced by warming.

Our results indicate that high temperatures favour a top-down control of plankton communities, whereas a high CO_2 seems to promote bottom-up controlled mechanisms. However, the near-natural mesocosms we used were complex systems and did not allow us to prove these conclusions. MZP grazing experiments would have been a valuable addition to disentangle the effects of the multiple stressors on the different community levels, but unfortunately we were unable to conduct additional grazing experiments.

Conclusions

Overall, the present study shows that productive coastal ecosystems like the Kiel Fjord and especially MZP communities are not expected to be directly affected by a high pCO_2 in the future. This is most likely related to a high tolerance of MZP species to average pCO_2 levels of 700 ppm (Thomsen *et al.*, 2010). In fact, most ecologically important groups in the Baltic Sea foodweb seem to be rather tolerant to acidification (Havenhand, 2012). Additionally, there was no indication of changes in phytoplankton food quality in terms of stoichiometry due to high CO_2 [see Paul *et al.* (2015) for details] that could indirectly affect MZP or higher trophic levels during our short-term experiment. Indirect positive effects resulting from increases in phytoplankton biomass can be expected. However, it seems that such effects might be masked by increased grazing pressure from mesozooplankton. Finally, our results indicate that global warming affects MZP plankton communities in terms of higher total biomass, increased growth rates, and earlier autumn bloom timing. This could, in turn, lead to changes in trophic dynamics due to a tighter coupling of phytoplankton and MZP, in particular, the phytoplankton-ciliate link, which is likely to enhance energy transfer efficiency to higher trophic levels (Aberle *et al.*, 2015).

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