

Effects of ocean acidification and warming on microzooplankton communities

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Summary

Among the environmental stressors affecting the oceans, warming and acidification have been identified as two of the most important ones. According to IPCC predictions, an increase in sea surface temperature of up to 3 °C and a further decrease in pH by up to 0.5 units can be expected towards the end of the 21st century. Currently, the understanding of the effects of the two stressors on plankton communities forming the base of the food web is still incomplete. Different direct and indirect effects of warming or acidification on some plankton components are known, mostly from small-scale laboratory or microcosm experiments. However, data availability for long-term effects, interactions effects of the two stressors and effects on a community level is limited. This is even more the case for microzooplankton, an important yet understudied intermediary between phytoplankton and higher trophic levels. The aim of this work was to bridge this gap by investigating the effects of warming and high CO₂ on microzooplankton on a community level.

The KOSMOS 2013 experiment in the Skagerrak was the first long-term mesocosm study investigating the effects of end-of-century acidification on a natural plankton community (Chapter I). During the transition from a spring bloom to a mid-summer situation, we did not observe any effects of high CO₂ on the community composition or diversity of microzooplankton. In addition, biomass and growth rates of total microzooplankton and ciliates were not significantly different between the ambient and the high CO₂ treatments. However, dinoflagellates were positively affected in terms of a higher biomass and growth rate. This was best visible under low nutrient concentrations towards the end of the experiment. Moreover, the observed effects on microzooplankton were most likely indirectly caused by changes in phytoplankton standing stocks. This was also suggested by the accompanying grazing experiments revealing positive effects of CO₂ on flagellate growth.

In the experiment presented in Chapter II, the effects of both warming and high CO₂ on a Baltic Sea plankton community were investigated in a multiple-stressor indoor mesocosm experiment during an autumn bloom. While warming led to an earlier bloom peak of microzooplankton, indicating a tighter coupling with phytoplankton, microzooplankton biomass or the magnitude of the peak were not affected. Furthermore, warming resulted in

higher growth rates and a higher diversity of microzooplankton. CO₂ or the interactions of warming and CO₂ did not have any significant effects on microzooplankton biomass, growth rate or species composition. However, unlike microzooplankton, copepods were positively affected by a high CO₂ level. It is thus likely that intense copepod grazing masked any positive effects of the stressors on microzooplankton that might have occurred via changes in the phytoplankton community.

A second multiple-stressor study with the same mesocosm setup was conducted using a nutrient-deplete late summer plankton community to allow for comparisons on the effects of warming and high CO₂ between seasons (Chapter III). While we observed no effects of CO₂ or temperature on diversity, growth and grazing rates of microzooplankton, the interaction of the stressors led to a higher biomass. More precisely, with increasing CO₂ levels, the negative effects of high temperatures on the biomass seemed to be less severe. The combination of the two stressors led to an earlier bloom peak of ciliates while warming as a single stressor resulted in a lower biomass and a delayed bloom peak. Grazing experiments additionally revealed positive and negative effects of CO₂ and temperature on growth rates of different phytoplankton taxa. Overall, the results suggest that the lack of a positive effect of warming was based on the increased top-down control by copepods in combination with overall low phytoplankton densities due to nutrient-depletion.

Apart from direct effects on micro- and mesozooplankton, high CO₂ levels have the potential to lead to a decrease in phytoplankton food quality in terms of carbon-to-nutrient ratios which is expected to indirectly affect primary consumers. As described in Chapter IV, we conducted a short-term laboratory study using model organisms to disentangle the direct and indirect effects of ocean acidification on zooplankton growth. By crossing a heterotrophic dinoflagellate and a copepod cultured at different CO₂ concentrations with different phytoplankton food qualities, direct and indirect effects could be distinguished. As a result, we observed that growth rates of zooplankton were significantly negatively affected by low food qualities while the direct effects of high CO₂ exposure seemed to be less important for both dinoflagellates and copepods. In addition, low food qualities, but not high CO₂ seawater, led to higher copepod respiration rates indicating a physiological response in order to excrete excess carbon that had been obtained by the low quality phytoplankton.

In conclusion, the results showed that a stronger effect of warming than of elevated CO₂ can be expected for coastal microzooplankton communities with respect to realistic IPCC end-of-century scenarios. As previously suggested, indirect effects of high CO₂ due to changes in the phytoplankton community composition and food quality were found to be more important than direct ones. Most likely, this can be attributed to the high tolerance of estuarine plankton communities to fluctuations in *p*CO₂ occurring already today. In contrast, warming can be expected to directly affect microzooplankton in terms of higher growth and grazing rates, thus strengthening the coupling with phytoplankton. However, the interactions of warming and acidification were shown to be additionally affected by nutrient concentrations. During periods of nutrient-repletion, no interactions were found, while during nutrient-depletion, a dampening effect of high CO₂ levels on the negative effects of warming on microzooplankton caused by an enhanced top-down control by mesozooplankton was observed. Overall, the results highlight the significance of mesocosm studies at the community level under close-to-natural conditions including trophic interactions when assessing the effects of climate change. Moreover, they emphasize the importance of multiple-stressor experiments conducted during different seasons.

Zusammenfassung

Innerhalb der Umweltfaktoren, von denen Auswirkungen auf die Meere zu erwarten sind, gehören Erwärmung und Versauerung zu den beiden wichtigsten. Nach Angaben des IPCC werden bis zum Ende des 21. Jahrhunderts eine Erwärmung des Oberflächenwassers von bis zu 3°C erwartet, sowie ein Sinken des pH-Wertes um bis zu 0,5 Einheiten. Zur Zeit ist das Verständnis der Effekte dieser beiden Stressfaktoren auf die Planktongemeinschaften an der Basis des Nahrungsnetzes jedoch noch immer lückenhaft. Für einige Planktongruppen sind bereits verschiedene direkte und indirekte Folgen von Erwärmung und Versauerung bekannt, zum überwiegenden Teil auf kleinformatischen Laborexperimenten oder Mikrokosmen basierend. Allerdings ist die Datenverfügbarkeit in Bezug auf Langzeitwirkungen, Interaktionen der beiden Stressfaktoren, sowie Auswirkungen auf der Ebene von Gemeinschaften sehr begrenzt. Das gilt um so mehr für das Mikrozooplankton, einem wichtigen Bindeglied zwischen Phytoplankton und höheren trophischen Ebenen. Das Ziel dieser Arbeit war es, dazu beizutragen, diese Lücke durch die Untersuchung der Effekte von Erwärmung und erhöhten CO₂-Konzentrationen auf Gemeinschaftsebene zu schließen.

Das KOSMOS 2013-Experiment im Skagerrak war die erste Studie, die erfolgreich die Langzeitauswirkungen der Versauerung, wie sie für das Ende des Jahrhunderts erwartet wird, auf eine natürliche Planktongemeinschaft untersucht hat (Kapitel I). Während des Übergangs von der Frühjahrsblüte zur Sommergemeinschaft konnte kein Effekt erhöhter CO₂-Konzentrationen auf die Zusammensetzung oder die Diversität der Mikrozooplanktongemeinschaft beobachtet werden. Auch die Biomassen und Wachstumsraten des Mikrozooplanktons unterschieden sich nicht signifikant zwischen den Ansätzen mit niedrigen und hohen CO₂-Konzentrationen. Für Dinoflagellaten wurden allerdings höhere Biomassen und Wachstumsraten bei erhöhten CO₂-Konzentrationen gemessen, insbesondere bei den gegen Ende des Experiments auftretenden niedrigen Nährstoffkonzentrationen. Die beobachteten Effekte auf Mikrozooplankton wurden höchstwahrscheinlich durch Veränderungen in den Phytoplanktonbeständen verursacht. Die Annahme wurde durch die während des Experiments durchgeführten Fraßversuche bestärkt, bei denen positive Folgen erhöhter CO₂-Konzentrationen auf die Wachstumsraten von Flagellaten festgestellt wurden.

In dem in Kapitel II vorgestellten Mesokosmos-Experiment wurde die Kombination der Effekte von Erwärmung und hohen CO₂-Konzentrationen auf eine Ostsee-Planktongemeinschaft während einer Herbstblüte getestet. Während Erwärmung zu einer früheren Mikrozooplanktonblüte führte, die eine engere Kopplung an das Phytoplankton vermuten ließ, wurden Biomasse und Maximum der Blüte nicht beeinflusst. Weitere Folgen der Erwärmung waren höhere Wachstumsraten und eine höhere Diversität des Mikrozooplanktons. Erhöhte CO₂-Konzentrationen sowie die Interaktionen von Erwärmung und erhöhten CO₂-Konzentrationen beeinflussten weder Biomassen noch Wachstumsraten oder Artenspektrum des Mikrozooplanktons. Im Gegensatz dazu hatten erhöhte CO₂-Konzentrationen positive Effekte auf Copepoden zur Folge. Die naheliegende Schlussfolgerung ist, dass der stärkere Copepoden-Fraßdruck sämtliche Auswirkungen der Stressfaktoren auf Mikrozooplankton verdeckt hat, die aufgrund der veränderten Phytoplanktongemeinschaft hätten auftreten können.

Mit dem gleichen Mesokosmos-Aufbau wurde eine zweite Studie kombinierter Stressfaktoren durchgeführt, diesmal mit einer nährstofflimitierten Spätsommer-Planktongemeinschaft, um die Auswirkungen von Erwärmung und erhöhten CO₂-Konzentrationen innerhalb verschiedener Jahreszeiten vergleichen zu können (Kapitel III). Während keine Auswirkungen von Erwärmung oder erhöhten CO₂-Konzentrationen auf die Diversität sowie die Wachstums- und Fraßraten von Mikrozooplankton beobachtet wurden, führte die Interaktion der beiden Stressfaktoren zu einer höheren Biomasse. Dies beruhte auf einer Abschwächung der negativen Effekte der wärmeren Temperatur, die im Zuge der erhöhten CO₂-Werte auftrat. Die Kombination der beiden Stressfaktoren führte zu einer früheren Ciliatenblüte, während Erwärmung als einzelner Stressor zu einer geringeren Biomasse und einem späteren Blütezeitpunkt führte. Anhand von Fraßversuchen wurden zudem sowohl positive als auch negative Folgen von Erwärmung und erhöhten CO₂-Werten auf Phytoplankton-Wachstumsraten festgestellt, abhängig vom jeweiligen Taxon. Die Ergebnisse deuten an, dass das Fehlen positiver Effekte der Erwärmung vermutlich an einer Kombination von verstärktem Copepoden-Fraßdruck (top-down control) und insgesamt niedriger Phytoplanktondichten aufgrund von Nährstofflimitierung lag.

Abgesehen von direkten Auswirkungen auf Mikro- und Mesozooplankton können erhöhte CO₂-Konzentrationen auch zu einer geringeren Phytoplankton-Futterqualität führen, indem sie die Verhältnisse zwischen Kohlenstoff und anderen Nährstoffen ändern, was sich wiederum indirekt auf die Konsumenten auswirkt. Bei dem in Kapitel IV beschriebenen

Experiment wurde ein Kurzzeit-Laborversuch durchgeführt, der mit Hilfe von Modellorganismen die direkten und indirekten Auswirkungen von Versauerung auf das Wachstum von Zooplankton klären sollte. Durch Hälterung eines heterotrophen Dinoflagellaten und eines Copepoden bei verschiedenen CO₂-Konzentrationen sowie der Fütterung mit zwei verschiedenen Phytoplankton-Qualitäten konnte zwischen direkten und indirekten Auswirkungen unterschieden werden. Die Ergebnisse zeigten, dass die Wachstumsraten des Zooplanktons von der geringeren Futterqualität des Phytoplanktons negativ beeinflusst wurden. Die direkten Folgen der Hälterung bei erhöhten CO₂-Konzentrationen schienen sowohl für Dinoflagellaten als auch für Copepoden weniger wichtig zu sein. Zudem führte eine geringere Futterqualität, im Gegensatz zur Hälterung bei hohen CO₂-Konzentrationen, zu einer erhöhten Respirationsrate der Copepoden. Dieser Effekt deutet auf eine physiologische Reaktion hin, die der Exkretion überschüssigen Kohlenstoffs dient, der mit der Nahrung aufgenommen wurde.

Als Schlußfolgerung kann festgehalten werden, dass die Ergebnisse auf stärkere Auswirkungen von Erwärmung im Vergleich zur Versauerung hinweisen, wenn man im Rahmen realistischer IPCC-Szenarien für das Ende des 21. Jahrhunderts bleibt. Wie bereits von anderen Studien angedeutet, waren indirekte Folgen der Versauerung aufgrund der Änderung der Artenzusammensetzung der Phytoplanktongemeinschaft und der Nahrungsqualität wichtiger als direkte Folgen. Dieser Effekt beruht höchstwahrscheinlich auf der hohen Toleranz ästuariner Planktongemeinschaften gegenüber den heutzutage bereits auftretenden CO₂-Fluktuationen. Im Gegensatz dazu werden vornehmlich direkte Auswirkungen der Erwärmung auf Mikrozooplankton erwartet, die zu höheren Wachstums- und Fraßraten führen können und damit zu einer engeren Kopplung an das Phytoplankton. Allerdings wurden die Interaktionen von Erwärmung und Versauerung zusätzlich von der Nährstoffkonzentration beeinflusst. Waren ausreichend Nährstoffe vorhanden, wurden keine Interaktionen festgestellt. Bei Nährstofflimitation wurde dagegen ein abschwächender Effekt erhöhter CO₂-Konzentrationen auf die negativen Folgen der Erwärmung auf Mikrozooplankton beobachtet, die auf verstärktem Fraßdruck durch Mesozooplankton beruhten (top-down control). Insgesamt unterstreichen die Ergebnisse die Wichtigkeit von Mesokosmos-Experimenten auf der Ebene von Gemeinschaften, wenn es um die Einschätzung der Auswirkungen des Klimawandels geht, da sie naturnahe Bedingungen bieten und trophische Interaktionen miteinbeziehen. Zudem zeigt sich deutlich, wie wichtig bei Experimenten die Kombination mehrerer Stressfaktoren und die Durchführung während verschiedener Jahreszeiten ist.

Introduction

The pelagic food web

The oceans cover 70 % of the earth's surface. Yet, for a long time, knowledge about trophic interactions within the oceans was surprisingly limited in comparison to what was known about terrestrial ecosystems. Up to the 1970s, the pelagic food web was usually depicted as a simple linear food chain (Figure 1). With phytoplankton as primary producer, mesozooplankton as primary consumers, followed by planktivorous and piscivorous fish as top-predator, the flow of energy was illustrated as a one-way flux. Detritus, dead material and dissolved organic carbon were thought to be remineralized by bacteria, providing new nutrients for the phytoplankton. This linear model turned out to be an oversimplification of the actual food web with its complicated interactions of different trophic levels. However, omnivory or mixotrophy were not included, and neither were effects like protozoa preying on higher trophic levels during certain life stages, e.g. dinoflagellates feeding on copepod eggs (Jeong, 1999).

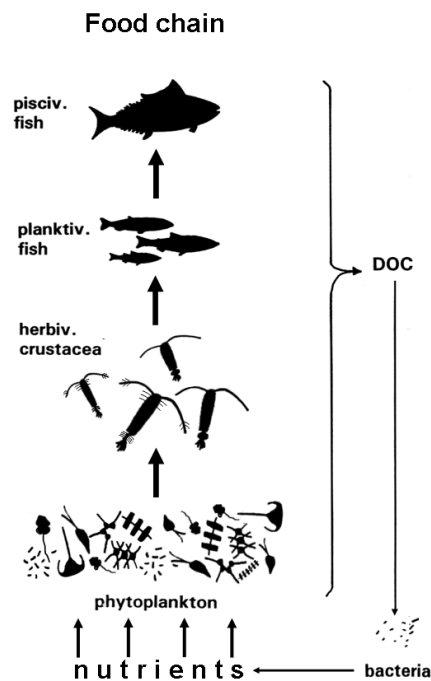


Figure 1: Traditional pelagic food chain (after Sommer, 2005).

But some effects described from the field could not be explained by this model. One of the main problems of the linear food chain was that mesozooplankton and especially copepods were assumed to be the main grazer of phytoplankton, expected to graze up to 90 % of the phytoplankton standing stocks (Steele, 1976). This was, however, in contradiction to carbon budget calculations suggesting that the consumption of herbivorous mesozooplankton could not account for more than about 50 % of the primary production (Walsh et al., 1981). Still, it was not until Azam et al. (1983) introduced the “microbial loop” that the importance of microzooplankton (MZP) grazing received more attention. This loop included bacteria being consumed by heterotrophic nanoflagellates which in turn were being preyed upon by MZP. With MZP being an important food source for mesozooplankton, the connection with the “classical” food web was established. The microbial loop concept indicated that it was in fact MZP contributing to large proportions to the remineralization of nutrients within the microbial loop.

Overall, the complexity of trophic interactions in the pelagic has increased considerably with the inclusion of the microbial loop and further predator-prey relationships between groups previously not considered, leading to a web-like structure (Pace et al., 1984, Figure 2). However, some connections are still not fully understood, like e.g. fish larvae feeding on MZP, proposed as the protozooplankton-ichthyoplankton link (Montagnes et al., 2010).

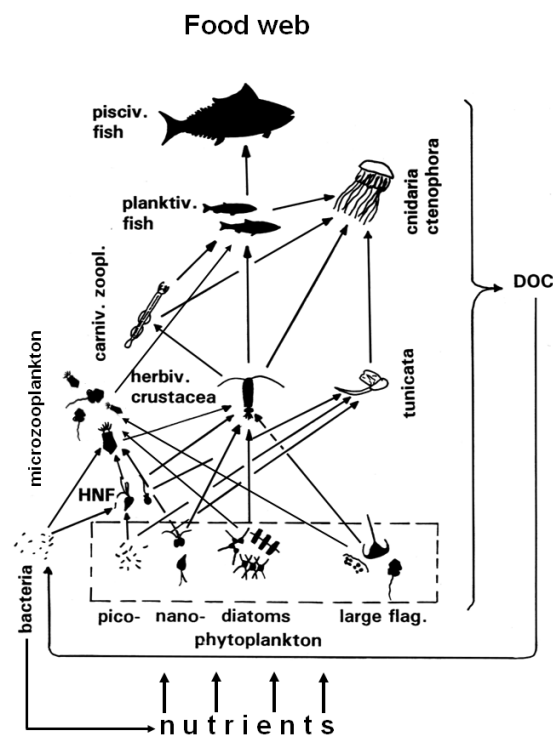


Figure 2: Simplified pelagic food web including heterotrophic nanoflagellates (HNF) and microzooplankton (after Sommer, 2005).

Microzooplankton: an integral part of the food web

MZP is a heterogeneous group of plankton defined by its size of 20-200 μm (Sieburth et al., 1978). It is composed of protozoa, among which ciliates and heterotrophic dinoflagellates are the most important ones, and metazoa like rotifers, copepod nauplii and other planktonic larvae. On average, MZP is considered to graze up to 75 % of the daily primary production, a proportion that varies between environments. The contribution of MZP to total grazing is considered to be lowest in coastal and polar environments (~60 %) and highest in the open ocean and tropical regions (70-75 %) (Calbet and Landry, 2004). As visualized by Figure 3, mesozooplankton usually grazes a much smaller percentage of the primary production, not more than about 30 % at maximum (Calbet et al., 2008). Due to its high growth and grazing rates, MZP is able to instantaneously follow changes in the phytoplankton concentration within days, which happens e.g. during the spring bloom (Johansson, 2004). In contrast, mesozooplankton reacts much slower to increases in phytoplankton or MZP due to its longer developmental times of weeks to months (Calbet et al., 2008).

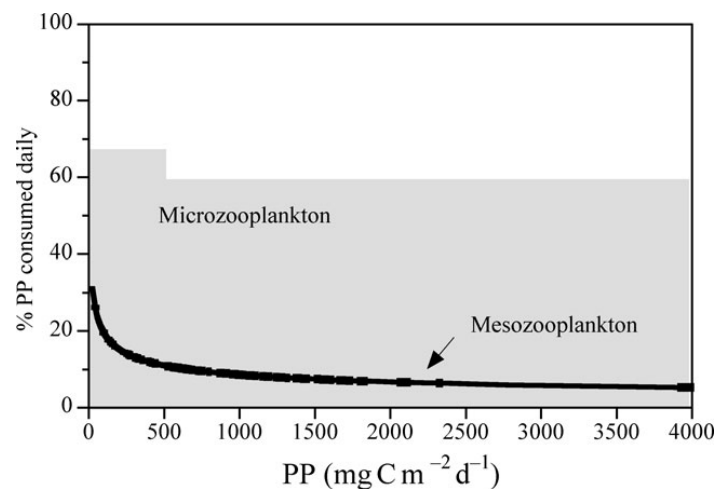


Figure 3: Approximation of the mean global grazing impact on phytoplankton. The percentage of primary production (PP in $\text{mg C m}^{-2} \text{ day}^{-1}$) consumed by MZP is shown in grey, with the percentage consumed by mesozooplankton shown as a black line (after Calbet, 2008).

As MZP shows species-specific phytoplankton grazing preferences, it has a strong influence on both phytoplankton biomass and community composition (Riegman et al., 1993; Löder et al., 2011). Species-specific grazing can promote the growth of unpalatable phytoplankton which has found a form of defence, e.g. due to the production of toxins (Rose et al., 2009). Nevertheless, the suppression of phytoplankton blooms by intense MZP grazing is also possible at times when mesozooplankton grazing is low (Aberle et al., 2007). In fact,

according to the “loophole” theory by Irigoien et al. (2005), phytoplankton blooms can only occur when it manages to successfully escape from MZP grazing pressure, due to defence mechanisms or inedibility. Given the wide array of phytoplankton suitable as food item for MZP, the overlap of the food spectrum with mesozooplankton can lead to competition between the two groups (Calbet and Landry, 2004; Fonda Umani, 2005). However, many MZP taxa are not strictly heterotrophic but mixotrophic, some even considered as primarily autotrophic with the capability of heterotrophic feeding modes (Sherr and Sherr, 2002).

Apart from its significance as grazer, MZP also represents an important food source for mesozooplankton, probably even a critical one for certain life stages (Paffenhofer, 1988; Stoecker and Capuzzo, 1990). For copepods it has been estimated that MZP can contribute 30-50 % of the daily diet, with the contribution being highest at low phytoplankton concentrations (Calbet and Saiz, 2005). The ability of MZP to buffer nutritional imbalances of phytoplankton makes it a preferred food source for mesozooplankton such as copepods, especially at times when phytoplankton food quality is low (Malzahn et al., 2010; Löder et al., 2011). This could be for example a change in carbon-to-nutrient ratio and biochemical composition in disfavour of the grazers due to environmental stressors directly affecting phytoplankton (Burkhardt et al., 1999; Schoo et al., 2013). Indeed, it has been hypothesized that ‘trophic upgrading’ by MZP will become more important for copepods with stressors like e.g. warming and acidification influencing phytoplankton characteristics (Malzahn et al., 2010)

Characteristics of ciliates

To date, about 7500 species of ciliates have been described. While most of them fall within the MZP range of 20-200 μm , there are exceptions of up to 2 mm length. Ciliates are motile and capable of very fast movements, with the arrangement of cilia on their cell surface forming a species-specific ciliature, even though for some taxa, cilia are only present during a certain life stage (Montagnes et al., 2001). Some taxa such as *Suctorina* sp. can also attach to surfaces. In general, ciliates are divided into two distinct groups. Loricated genera are surrounded by a cup-shaped shell made of organic materials like *Favella*, or with agglomerates of particles on the surface like *Tintinnopsis*, in contrast to naked or aloricated ones such as *Strombidium* or *Lohmaniella* (Montagnes et al., 2001, Figure 4).

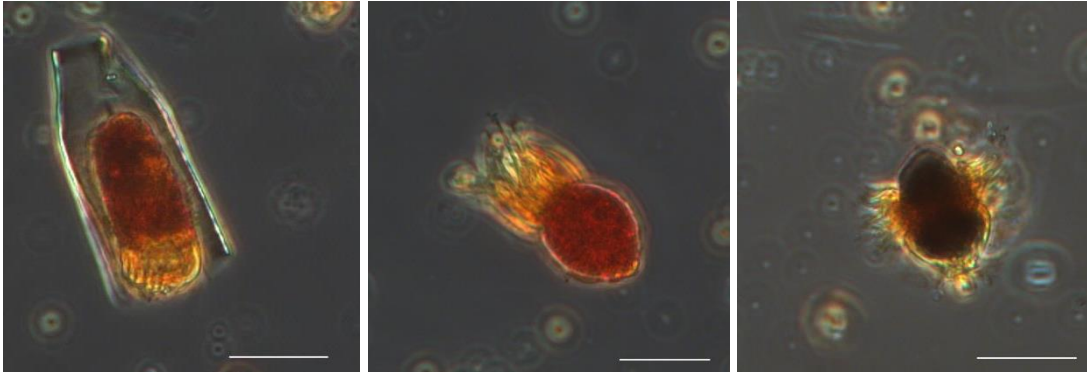


Figure 4: Loricated ciliate and aloricate *Lohmaniella oviformis* and *Myrionecta rubra*. Pictures taken from Lugol samples. Scale bars: 20 μm .

Ciliates can be filter feeders, suspension feeders or predators, also preying on other ciliates, and some obligate heterotrophic taxa are known (Montagnes et al., 2008). During a bloom, ciliates usually increase almost simultaneously with phytoplankton, but after the bloom peak, they decline relatively fast, either due to starvation or due to predation if mesozooplankton abundances are high (Montagnes and Lessard, 1999). In general, the ideal size of ciliate food items is estimated to be within the range of 2-40 μm , depending on ciliate size (Johansson, 2004). For loricated ciliates, the width of the lorica opening restricts the size of prey items. However, for small-sized and bacterivorous ciliates it has been shown that also the size class of 0.5-2 μm can be ingested as food item (Jonsson, 1986; Christaki et al., 1998). In addition, many ciliate species such as *Laboea strobila* or *Tontonia gracillima* are mixotrophic, and some like *Myrionecta rubra* even characterized as phototrophic over extended periods of time (Johnson and Stoecker, 2005). While ciliates do not necessarily contain chloroplasts, some taxa they are able to sequester chloroplasts from phytoplankton cells, referred to as kleptochloroplasts (Stoecker et al., 1987).

Characteristics of heterotrophic dinoflagellates

Currently, about 2500 species of dinoflagellates are known, with sizes ranging from to 2 μm to 2 mm in the case of *Noctiluca scintillans*. In the following, only the size classes from 20-200 μm will be considered as they comply with the definition of microzooplankton by Sieburth et al. (1978). Two groups of dinoflagellates are distinguished; thecate or armoured ones are encased in cellulose plates in a species-specific arrangement while athecate or naked ones lack this kind of armour (Kraberg et al., 2010). Examples for the former group are e.g. the common genera *Protoperidinium* and *Dinophysis* while the latter includes *Gymnodinium* and *Gyrodinium* (Figure 5). The majority of the species is motile by the means of two flagella.

While about half of the species are regarded as photosynthetic but in most cases capable of ingesting prey, the other half is obligatory heterotrophic and includes both free-living and parasitic forms (Hansen and Calado, 1999). Kleptochloroplasts can also be found in dinoflagellates, for example in the genus *Dinophysis* (Carvalho et al., 2008).

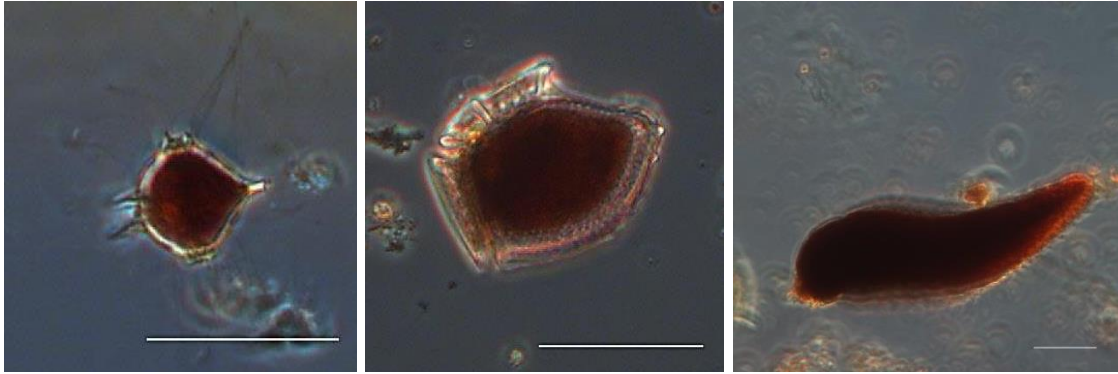


Figure 5: Thecate dinoflagellate of the genera *Protoperidinium* and *Dinophysis* as well as athecate *Gyrodinium* sp.. Scale bars: 50 μ m.

Heterotrophic dinoflagellates exhibit a variety of feeding modes, from filter feeding to raptorial. The engulfment of whole cells has been observed for many athecate dinoflagellate genera such as *Gymnodinium* or *Gyrodinium* (Hansen and Calado, 1999). Another feeding mode observed for e.g. the genus *Dinophysis* is the deployment of a tube to suck out the cytoplasm of a prey item (Tillmann, 2004). In addition, especially thecate dinoflagellates like *Protoperidinium* spp. have the ability to digest food items outside of their theca using a pallium reaching out of their flagellar pore (Hansen and Calado, 1999). Overall, dinoflagellates can feed on a much wider range of prey sizes than ciliates, from bacteria to particles which are more than their own body size, including injured metazoans (Jeong, 1999). Interestingly, heterotrophic feeding modes have also been observed for some dinoflagellate taxa traditionally considered as purely autotroph, e.g. *Ceratium* spp. (Hansen and Calado, 1999).

While it was initially assumed that ciliates were the most important grazers within the MZP community, there is now evidence that in fact dinoflagellates exert the strongest grazing impact on phytoplankton communities, especially during diatom blooms (Sherr and Sherr, 2007). In general, although dinoflagellates react somewhat slower to phytoplankton blooms due to lower growth rates in comparison to ciliates, it seems that their broad food spectrum and higher starvation potential enables them to form long-lasting bloom phases (Löder et al., 2011).

Environmental stressors

Marine ecosystems face many threats besides invasive species, overfishing or habitat destruction by human activities. Environmental stressors like ocean warming or acidification are expected to have pronounced effects on the physical and biological compartments of the oceans. As summed up in a series of reports of the Intergovernmental Panel on Climate Change (IPCC) starting in 1990, anthropogenic greenhouse gas emissions are the main driver behind these changes. Next to methane (CH₄) and nitrous oxide (N₂O), carbon dioxide (CO₂) was identified as one of the most important greenhouse gases. Since the industrial revolution in the mid-eighteenth century, CO₂ concentrations have increased at a rate exceeding all fluctuations in the last 800.000 years, which were between 180 and 300 ppm (Collins et al., 2013). This is also clearly illustrated by the long-time measurements at Mauna Loa, Hawaii. From ~320 ppm in 1959, average atmospheric CO₂ concentrations have increased to more than 380 ppm in 2009, with the pH value decreasing alongside with it (Feely et al., 2009, Figure 6). Depending on the IPCC scenario selected, a further increase of CO₂ to 1000 ppm in the worst case can be expected until the end of this century, and even if the anthropogenic CO₂ input is reduced, 700 ppm are still likely to be reached (Collins et al., 2013).

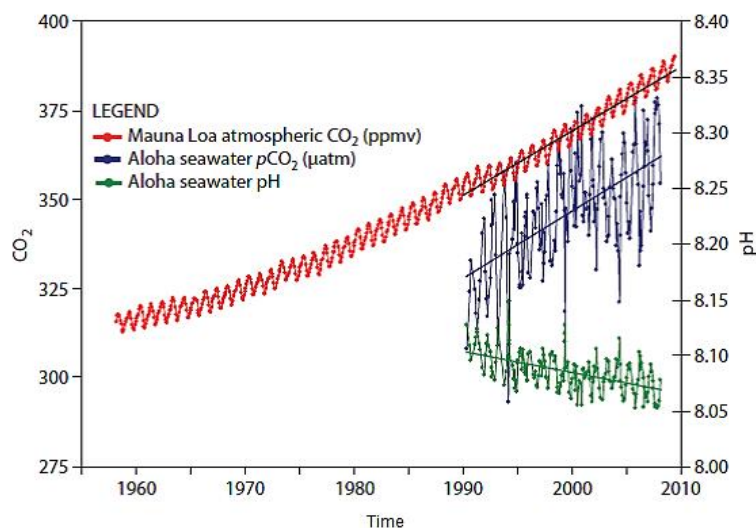


Figure 6: Mauna Loa time series showing the development of atmospheric CO₂ (red), seawater pCO₂ (blue) and seawater pH (green) since 1959 (after Feely et al., 2009).

To document how much has already changed in aquatic ecosystems due to these environmental stressors, long-term data series such as the Helgoland Roads Series are most valuable (Franke et al., 2004). It is not only the abiotic parameters that are measured, such as

water temperature or salinity, but also the biotic ones like phyto- or zooplankton abundance and taxonomic composition. As a result it was shown that the mean temperature in the North Sea has increased by 1.1 °C since 1962, one of the effects on the organisms being a shift in phytoplankton succession and a delayed diatom bloom (Wiltshire and Manly, 2004). However, it is difficult to assess from such data series how the change in temperature affects biotic interactions within the food web. The impacts visible in parameters such as zooplankton abundance do not necessarily allow conclusions on how exactly these outcomes happened to be – via direct effects on the physiology, or indirect effects due to changes in e.g. phytoplankton availability or community composition. This usually remains unanswered.

On the other end of the spectrum of experiments investigating the effects of global change are small-scale laboratory studies, where the physiology, stoichiometry and biochemistry of plankton can be analysed. They provide a detailed background on what to expect for single species or simplified food webs, but are quite artificial and thus limited in predicting what might be expected under future ocean scenarios. To be able to include both direct and indirect effects of stressors in a more natural environment, mesocosms are very useful tools. They allow enclosing a whole plankton community, thus interactions between different species and trophic levels in response to the stressors applied can be observed. While they are somewhat limited in reproducing the natural conditions and the time they can be sustained, sufficient volumes allow for experimental runtimes of weeks to months (Sommer et al., 2007; Riebesell et al., 2013a). For short-lived plankton, this means several generations living under future ocean conditions. As summarized by Riebesell and Gattuso (2015), it is not only the focus from single species that needs to be expanded to communities and ecosystems, but also from single- to multiple-stressor approaches. Likewise, long-term studies are necessary to further explore acclimatization or adaptations.

Global warming and ocean acidification are among the most important environmental stressors to investigate. Direct and indirect effects of these two factors on marine organisms have been studied in single-stressor and more recently also in multiple-stressor approaches on different scales (see for example overviews by Hoegh-Guldberg and Bruno, 2010; Cross et al., 2015; Riebesell and Gattuso, 2015). Nevertheless, for MZP, there is a scarcity of data both on single-species and on community level, especially with regard to realistic end-of-century scenarios. This is a gap that needs to be addressed as any model of future oceans lacking the MZP component will be inaccurate.

Global warming

As summarized by Collins et al. (2013) in the IPCC report (2014), average global surface temperature has increased by 0.85 °C since 1880, and this trend is expected to continue to up to 2-4 °C until the end of the century (Figure 7). The oceans have absorbed most of the additional energy, resulting in a sea surface temperature increase of 0.6°C within the last century and a predicted further increase by 1-3 °C until the year 2100 (Hoegh-Guldberg and Bruno, 2010). Physical impacts of warming are e.g. the decrease in sea ice cover in the Arctic or changes in the ocean currents due to an unequal distribution of warming, but also an intensified vertical stratification of the oceans (Collins et al., 2013).

For the marine biota, warming results in a variety of effects on different levels of organisation. On an organisms' level, growth and respiration rates as well as body size and stoichiometry have been shown to be affected (Cross et al., 2015). Nevertheless, strong deviations from the optimum temperature result in physiological stress, and even within the tolerance range this means additional energetic costs (Pörtner, 2008). On species and community level, range and distribution shifts or local extinction can occur in response to unfavourable temperature conditions, as observed e.g. in the poleward migration of cold water fish or, in the case of sessile organisms, coral bleaching and mortality (Hoegh-Guldberg and Bruno, 2010).

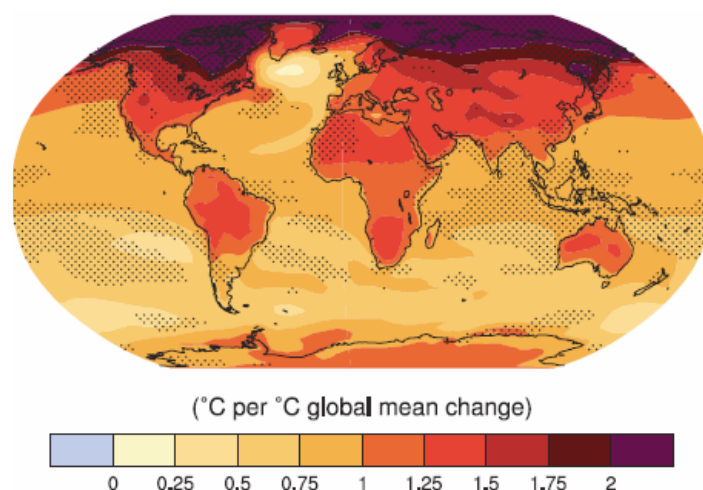


Figure 7: Average global surface temperature change simulated for the years 2081-2100 with respect to the time period from 1985-1999 (after Collins et al., 2013).

In the pelagic, effects on the plankton community at the base of the food web can have a strong influence on higher levels of the food web. Both autotrophic and heterotrophic plankton can benefit from a moderate temperature increase as it increases their metabolic

rates. However, heterotrophic processes are more sensitive to temperature than autotrophic ones (Bernacchi et al., 2001; Brown et al., 2004; O'Connor et al., 2009). There is indication that higher grazing rates of zooplankton have already led to an overall decrease of phytoplankton with warming during the last century (Boyce et al., 2010), a pattern which has also been shown experimentally (O'Connor et al., 2009; Sommer and Lewandowska, 2011). Nonetheless, the nutrient status of the system needs to be considered as an additional factor. As described in the model by Lewandowska et al. (2014), phytoplankton is usually dominated by large-sized cells at nutrient-replete conditions and, with an increased top-down control by copepods, this leads to a decrease in phytoplankton biomass. In contrast, nutrient-deplete conditions favour small-sized phytoplankton inedible for copepods but grazed by MZP, resulting in a strong top-down control by copepods and a decrease in MZP biomass thus fuelling the microbial loop.

Furthermore, phenological mismatches are predicted to occur with warming. As predators and their main prey are unlikely to react to warming in the same manner, the pattern of synchrony established between e.g. fish larvae and zooplankton could become uncoupled (Durant et al., 2007). It has been estimated that spring time events in the northern hemisphere occur between 4.4 and 5.5 days earlier per decade, and meta-analyses indicate that there is a significant trend towards earlier biological events as well (Thackeray et al., 2010). This is in line with experiments showing that warming can lead to an earlier onset of the phytoplankton spring bloom (Sommer et al., 2012). In addition, a reduced time-lag between phytoplankton and MZP bloom peaks was observed, with the enhanced MZP grazing potentially changing the cycling of energy and matter through the food web and affecting also higher trophic levels (Aberle et al., 2012).

Ocean acidification

High CO₂ concentrations in the atmosphere also affect the surface oceans by interfering with the carbonate system and resulting in ocean acidification (OA). Overall, the pH value has already decreased by ~0.1 units since pre-industrial times (Figure 8), and a further decrease by up 0.3-0.5 units until the end of this century is expected (Caldeira and Wickett, 2003). Due to the higher solubility of CO₂ in cold waters, the cold regions will suffer more from acidification. Upon dissolving, CO₂ reacts with water and forms carbonic acid (H₂CO₃) which further reacts to bicarbonate (HCO₃⁻) and hydrogen ions (H⁺) thus decreasing the pH. In a second step, CO₂, water and carbonate ions (CO₃²⁻) react and produce bicarbonate ions (HCO₃⁻) thus reducing the concentration of carbonate ions in the water. Both biological and

biogeochemical processes in the oceans are predicted to be affected by OA, and this could potentially result in adverse effects both on species and on community and ecosystem level (Riebesell et al., 2007). Consequently within the last ten years, “the other CO₂ problem” has become one of the three top global ocean research priorities (Riebesell and Gattuso, 2015).

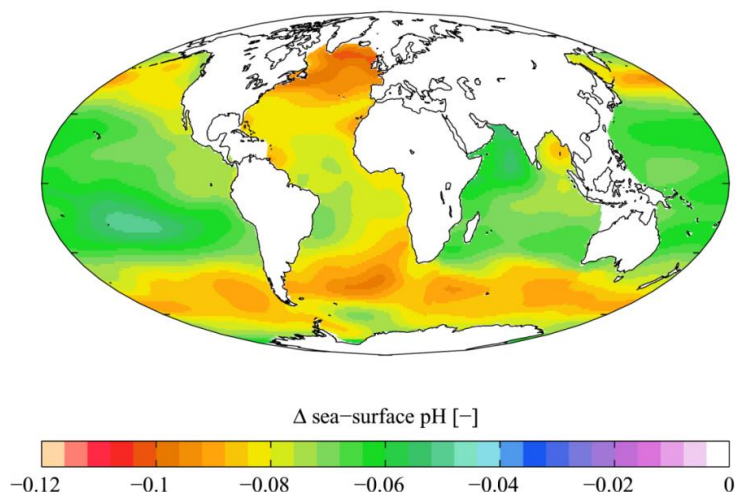


Figure 8: Average decrease in sea surface pH from pre-industrial times until the 1990s from the Global Ocean Data Analysis Project (Key et al., 2004).

Apart from the lowered pH, the reduction of carbonate ion concentration is especially problematic for calcifying organisms as it interferes with the build-up of calcareous structures (Fabry et al., 2008). The dissolution of calcium carbonate structures as a direct effect of acidification has already been observed in molluscs, corals, crustaceans, and calcifying algae (Kroeker et al., 2013). Other effects range from e.g. hypercapnia, the acidification of body fluids (Melzner et al., 2013) to changes in predator avoidance behaviour in fish (Munday et al., 2009). In addition, some phytoplankton taxa are negatively affected (Orr et al., 2005; Rost et al., 2008). Nevertheless, elevated CO₂ levels can also have positive effects. In cases where carbon was the limiting nutrient, the increased inorganic carbon content in the water can have a fertilizing effect on phytoplankton (Low-Decarie et al., 2014). However, OA can also affect phytoplankton stoichiometry, resulting in a higher C:N and C:P ratio (Riebesell et al., 2007; Schulz et al., 2013; Eggers et al., 2014). This turns phytoplankton into low-quality food for herbivores and can result in an indirect negative effect which could travel up the food chain (Schoo et al., 2013).

Already today, estuaries are often subject to fluctuations of CO₂ that are stronger than what is expected for the open oceans at the end of the century (Feely et al., 2008; Feely et al., 2010). This is mainly based on the low buffering capabilities of brackish estuarine waters, but also

upwelling events of corrosive waters from deeper layers (Feely et al., 2008; Melzner et al., 2013). Organisms from these regions are thus expected to be somewhat robust towards OA. However, for e.g. blue mussels in the Kiel Fjord, even though they are used to high CO₂ fluctuations and currently maintaining populations in the Fjord, there is evidence that they are already negatively affected by high CO₂ values in terms of external shell dissolution (Thomsen et al., 2010). Considering that fluctuations are predicted to get more extreme with increasing anthropogenic CO₂ input, they will eventually become harmful (Caldeira and Wickett, 2003).

For MZP, negative effects of high CO₂ on abundances have been shown in laboratory experiments using scaled-down food webs at extreme concentrations (Nielsen et al., 2010), but not for more realistic concentrations (chapter IV of this thesis). Moreover, mesocosm studies at future ocean conditions have revealed a low CO₂ sensitivity of MZP communities from Norwegian Fjords (Suffrian et al., 2008; Calbet et al., 2014), the Baltic Sea (Lischka et al., 2015) and the Arctic (Aberle et al., 2013).

Furthermore, environmental stressors do not occur separately, so factors like warming, eutrophication or hypoxia have to be taken into account as they might lead to an intensification or dampening of the effects of OA (Pörtner, 2008; Rost et al., 2008). Hence, there is an urgent need to use multiple-stressor approaches, if possible on a community level, to be able to predict what to expect in the future.

Thesis outline

It was only recently that MZP has received more attention in its function as one of the main grazers of phytoplankton in the oceans while simultaneously being an important food source for higher trophic levels. In previous studies investigating the impacts of environmental stressors on single species or on plankton community level, MZP was often not included. The aim of this thesis was to investigate the effects of ocean acidification and global warming on MZP communities, in terms of biomass, taxonomic composition, growth and grazing.

In Chapter I, the results from the KOSMOS 2013 large-scale mesocosm study in the Gullmar Fjord, Skagerrak, are presented. The experiment with ten mesocosms ran for 113 days, spanning the time from a spring-bloom to a late summer situation. This was the first time a seagoing mesocosm system had been successfully sustained over such a long duration, giving us the unique opportunity to study the long-term effects of end-of-century CO₂ conditions. The large size of the mesocosm allowed not only assessing how CO₂ affected the enclosed plankton community, but also to include the effects of fish larvae added as top predators to increase the complexity of the food web. It was possible to investigate the succession of the plankton community from nutrient-rich bloom to nutrient-deplete post-bloom conditions that occurred towards the end of the experiment. In addition, two different kinds of grazing experiments were conducted to further explore the effects of CO₂ on phytoplankton growth and MZP grazing rates when released from grazing pressure by higher trophic levels.

Chapter II describes the indoor mesocosm experiment with an autumn plankton community from the Kiel Fjord in October 2012. The autumn phytoplankton bloom taking place at that time of the year was dominated by large diatoms. It has been hypothesized that the interaction of two stressors can result in different effects than observed for single-stressor treatments as synergistic or antagonistic effects can occur. We thus crossed two temperatures with two CO₂ levels using twelve mesocosms in a full-factorial design in order to investigate possible interaction effects. While direct effects of an elevated CO₂ level on MZP were not expected due to the low CO₂ sensitivity of MZP from the Kiel Fjord, indirect effects due to changes in the phytoplankton community and thus prey availability were likely to be observed. Warming, on the other hand, was expected to have pronounced effects on MZP, leading to a tighter coupling between phytoplankton and MZP and an earlier timing of MZP blooms.

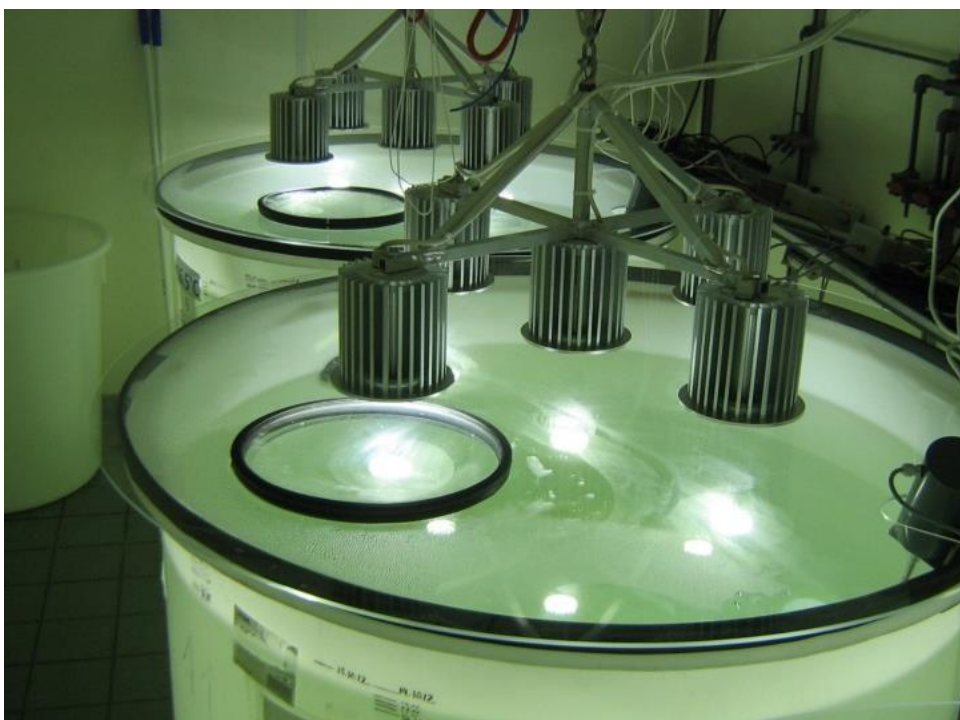
Chapter III investigates the combined effects of warming and increased CO₂ but focusses on a nutrient-deplete late summer plankton community from the Kiel Fjord in August 2013. The summer phytoplankton composition was characterized by high contributions of dinoflagellates and small-sized flagellates, with blooms of cyanobacteria frequently occurring. Using a regression design, two temperatures were crossed with six target CO₂ levels from 500 to 3000 µatm, from present day levels to an end-of-century scenario. Additional dilution experiments gave detailed insight into the effects of CO₂ and warming on growth rates of different phytoplankton groups and on MZP grazing rates. Based on the results from previous indoor mesocosm experiments, strong effects of warming on MZP communities were expected. Indirect positive effects of CO₂ on MZP were also likely to occur based on the fertilizing effect at high CO₂ levels on small-sized phytoplankton which made up the main part of the phytoplankton community.

In Chapter IV, effects of high CO₂ on zooplankton growth were examined in more detail using a laboratory setup with model organisms that allowed discrimination between the direct and indirect effects. Direct effects of short-term exposure were assessed by incubating the heterotrophic dinoflagellate *Oxyrrhis marina* and nauplii of the copepod *Acartia tonsa* at low and high CO₂ levels. In addition, two phytoplankton food qualities were obtained by culturing the cryptophyte *Rhodomonas salina* at the different CO₂ levels. A full-factorial design was used by crossing the two incubation treatments with the two food qualities. This way, direct effects of the incubation could be disentangled from indirect effects caused by food quality. For both phyto- and zooplankton, analysis of the stoichiometry gave insights into changes of the carbon-to-nutrient ratio. Further, concentrations of dissolved organic carbon and respiration were measured for both zooplankton species, with the developmental stages of the nauplii and copepodites being analysed as well at the end of the experiment.

In the general conclusions following Chapter IV, the results from the different experiments will be set into context to each other and to studies from other marine provinces. While there are some striking similarities between the mesocosm experiments from the Skagerrak and the Baltic Sea, encompassing different seasons and nutrient regimes (spring, autumn, and summer situations), some major differences in the outcome of the experiments need to be addressed. Finally, the results of warming and acidification experiments using natural pelagic communities are compared to studies on benthic communities within BIOACID as there are some interesting connections between them, pointing at the importance of further stressors that should be considered when assessing future impacts on high CO₂ oceans.



KOSMOS outdoor mesocosms in the Gullmar Fjord, Sweden, and schematic drawing including the underwater part (Photo: J. Bellworthy).



BIOACID indoor mesocosms, GEOMAR, Kiel

CHAPTER I – Low CO₂ sensitivity of microzooplankton communities in the Gullmar Fjord, Skagerrak: evidence from a long-term mesocosm study

Abstract

Ocean acidification is considered as a crucial stressor for marine communities. In this study, we tested the effects of the IPCC RPC6.0 end-of-century acidification scenario on a natural plankton community in the Gullmar Fjord, Sweden, during a long-term mesocosm experiment from a spring bloom to a mid-summer situation. The focus of this study was on microzooplankton and its interactions with phytoplankton and mesozooplankton. The microzooplankton community was dominated by ciliates, especially small *Strombidium* sp., with the exception of the last days when heterotrophic dinoflagellates increased in density. We did not observe any effects of high CO₂ on the community composition and diversity of microzooplankton. While ciliate abundance, biomass and growth rate were not affected by elevated CO₂, we observed a positive effect of elevated CO₂ on dinoflagellate abundances. Additionally, growth rates of dinoflagellates were significantly higher in the high CO₂ treatments. Given the higher Chlorophyll *a* content measured under high CO₂, our results point at mainly indirect effects of CO₂ on microzooplankton caused by changes in phytoplankton standing stocks, in this case most likely an increase in small-sized phytoplankton of <8 μm. Overall, the results from the present study covering the most important part of the growing season indicate that coastal microzooplankton communities are rather robust towards realistic acidification scenarios.

Introduction

Atmospheric CO₂ concentrations have increased considerably from 280 μatm at pre-industrial times to currently about 400 μatm (Le Quéré et al., 2013) and are predicted to reach up to 1000 μatm by the end of this century (IPCC scenario RPC6.0) (Collins et al., 2013). The oceans act as a major CO₂ sink and have absorbed about 30% of the anthropogenic CO₂ since the beginning of the industrial revolution (Sabine et al., 2004). This obviously has affected the ocean's carbonate system, leading to increased CO₂ and bicarbonate (HCO₃⁻) concentrations as well as a decrease in carbonate ion concentrations (CO₃²⁻) and pH. This drop in pH is referred to as ocean acidification (OA). While there are differences in CO₂ uptake depending on the region (Sabine et al., 2004), an overall pH decrease of about 0.3 units is expected until the end of the 21st century (Ciais et al., 2013).

Microzooplankton (MZP), plankton within the size class from 20 to 200 μm, is a heterogeneous group consisting of heterotrophic protozoa and metazoa. Often grazing on an average 60-75 % of the daily primary production, it is a dietary competitor for larger mesozooplankton (Calbet and Landry, 2004; Löder et al., 2011). Accordingly, MZP can have a strong impact on biomass and species composition of the phytoplankton community and even play an important role in suppressing phytoplankton blooms, especially at times when mesozooplankton grazing is low (Aberle et al., 2007). Moreover, MZP can also be strongly top-down controlled itself, as it is a preferred food source for mesozooplankton such as copepods (Calbet and Saiz, 2005; Löder et al., 2011).

OA is predicted to affect different marine biological and biogeochemical processes, potentially resulting in adverse effects not only on the species level but also on the community and ecosystem level (Riebesell et al., 2007). Potential direct effects of OA on MZP have been identified as e.g. changes in intracellular pH or enzyme activities (Nielsen et al., 2010), and indeed negative effects such as a decrease in biomass or the inhibition of growth have been reported for some species of MZP (Hinga, 1992; Pedersen and Hansen, 2003). Others, however, were not directly affected by a change in pH (Meunier et al., 2016). Nonetheless, CO₂ effects can also be transmitted indirectly, via changes in phytoplankton availability, community composition, or food quality (Rose et al., 2009; Calbet et al., 2014). Based on the enhanced growth of especially small-sized phytoplankton species which benefit from the higher carbon availability under OA (Rost et al., 2008; Brussaard et al., 2013; Low-Decarie et al., 2014; Sala et al., 2016), OA has the potential to lead to an increase in MZP productivity as well.

Despite of the pivotal role of MZP, small-scale laboratory experiments providing information about the impacts of OA on single species or simplified food webs are comparatively rare in contrast to the number of studies available for phytoplankton or mesozooplankton (e.g. Caron and Hutchins (2012); Kroeker et al. (2013)). Additionally, there is a lack of information about OA impacts on community level. Mesocosm studies are useful to fill this gap as they allow us to gain insight into the effects of OA on the plankton community and whether biotic interactions could dampen or amplify known responses in MZP (Rossoll et al., 2013; Calbet et al., 2014). The studies available so far indicate that MZP communities, especially in coastal areas, are rather tolerant to OA at incubation times up to weeks (Suffrian et al., 2008; Aberle et al., 2013; Rossoll et al., 2013; Horn et al., 2016). Yet, to study evolutionary adaptations to OA which are likely to occur due to the short generation times of planktonic organisms, long-term experiments would be required (Lohbeck et al., 2012). Experiments with longer durations are also necessary to allow observing possible numerical responses of mesozooplankton as it reacts time-delayed to CO₂-induced changes on phytoplankton or MZP level. Consequently, an in-situ mesocosm approach using a natural plankton assemblage and a sufficiently large incubation volume to allow for a long self-sustained runtime is a step towards the understanding of effects on ecosystem level (Riebesell et al., 2013a).

We investigated the impacts of high CO₂ levels on natural plankton communities during a long-term mesocosm study in the Gullmar Fjord, Skagerrak. Starting before the onset of the spring bloom in March, the long runtime until end of June made it possible to follow the natural succession of a plankton community during the transition from spring to summer. In the following, we will present the analysis of MZP succession patterns focusing on ciliates and heterotrophic dinoflagellates and their interactions with phytoplankton, while results on the dynamics with the microbial food web will be presented by Brussaard et al. (in prep.).

Moreover, grazing experiments should provide additional information both regarding the grazing impact of MZP and indirect effects of a high CO₂ level which are more likely to be detected when MZP is released from grazing pressure. Our hypotheses considering the effects of high CO₂ on MZP were as follows:

- 1) Elevated CO₂ will not directly affect MZP communities due to their high CO₂ tolerance.
- 2) An increase in phytoplankton biomass at high CO₂ conditions (due to positive effects on photosynthesis) will lead to enhanced MZP biomass and grazing rates.
- 3) A high CO₂ level will enhance trophic pathways through MZP due to CO₂-induced alterations in phytoplankton size classes and taxonomic composition.

Material and methods

Experimental design

The setup is described in detail by Bach et al. (in review), including mesocosm design, CO₂ addition, and maintenance work during the experiment, to which we refer the reader for further information. In short, ten "Kiel offshore mesocosms for future ocean simulations" (KOSMOS) (Riebesell et al., 2013a) with a volume of 55 m³ each were moored in the Gullmar Fjord on the Swedish west coast at 58°15'9 N, 11°28'7 E in January 2013. The experiment ran from 7 March till 26 June 2013. Upon closing of the mesocosms, a net with 3 mm mesh size was passed through the enclosed pre-bloom seawater to remove large organisms. Five mesocosms served as control with ambient CO₂ levels while CO₂-enriched seawater was added to the other five.

The chosen CO₂ level of 760 µatm corresponds to the conditions expected for the end of the 21st century according to IPCC scenario RPC6.0 (Collins et al., 2013). In order to compensate for outgassing, CO₂-enriched water was added to the high CO₂ treatments at five time points (days 17, 46, 48, 68 and 88). Herring larvae (*Clupea harengus*) and sea urchin larvae (*Strongylocentrotus droebrachiensis*) were added to the mesocosms on day 48 and day 56 of the experiment, respectively. Regular sampling every 2nd day included CTD casts, water column sampling, and sediment sampling. Plankton net hauls were performed every eight days. For the water samples, two 10 L carboys per mesocosm were filled using an integrating water sampler (IWS III, Hydro-Bios) over a depth of 0-17 m. Plankton samples were taken from the carboys as soon as they were back on shore.

Sampling and identification of MZP

MZP samples were taken once per week. 250 mL of mesocosm water was transferred into brown glass bottles and fixed with acid Lugol's solution (1% final concentration). MZP was counted and identified with an inverted microscope (Zeiss Axiovert 135) using the Utermöhl method (Utermöhl, 1958). Depending on the plankton density, 50 or 100 mL of each sample were transferred into a sedimentation chamber. After 24 h of sedimentation, the whole surface of the chamber was counted at 200-fold magnification to reduce the counting bias against rare species and to assure comparability of the counts both at high and low abundances.

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. Most dinoflagellates are capable of heterotrophic feeding modes and can be considered as mixotrophic MZP. Only few taxa such as *Ceratium* sp. were considered as predominantly autotrophic and thus included in the phytoplankton. Based on the digitally measured dimensions of 20 random cells per group (AxioVision 4.9 and AxioCam, Carl Zeiss Microscopy GmbH), biovolumes of MZP were calculated using geometric proxies by Hillebrand et al. (1999). MZP carbon biomass was estimated from the biovolumes using the conversion factors provided by Putt and Stoecker (1989) and Menden-Deuer and Lessard (2000) for ciliates and dinoflagellates, respectively.

Sampling and identification of phytoplankton

Samples of small-sized phytoplankton (<8 μm) were measured every 2nd day with an Accuri C6 flow cytometer (BD Biosciences) within three hours after mesocosm sampling was completed. Another 250 mL of mesocosm water was fixed with acidic Lugol's iodine (1% final concentration). Large phytoplankton cells (>8 μm) were counted on ten sampling days around the start of the experiment, the phytoplankton bloom peaks and the end. Counts were done on 50 mL concentrated sample water with an inverted microscope (Zeiss Axiovert 100) after Utermöhl (1958). The cells were counted either on half or total area of the chamber at 100-fold magnification or on 2 to 4 stripes at 200 or 400-fold magnification. Plankton were identified following Tomas et al. (1997), Hoppenrath et al. (2009), and Kraberg et al. (2010).

Data analysis

Diversity (H' , \log_e) was calculated after Shannon and Weaver (1963) on a sample day basis. The bloom timing (D_{max}) was defined as the experimental day when phytoplankton abundance or MZP biomass reached its peak in each mesocosm (max.). Net growth rates μ were calculated using an exponential growth model (Landry and Hassett, 1982):

$$\mu = \frac{1}{t} \ln \frac{P_{24}}{P_0} \quad (1)$$

where P_0 and P_{24} are the plankton concentrations on the first day of finding and on D_{max} and t is the time in between these days.

D_{max} , maximum and net growth rate data were first tested for normality and homogeneity using a Shapiro-Wilk test and Levene's Test for Homogeneity of Variance and log transformed if necessary. For analysing the effects of $p\text{CO}_2$ on these variables, ANOVAs

were performed. Tukey's HSD was used as post-hoc test. To test for significant effects of $p\text{CO}_2$ on MZP and phytoplankton biomass, abundance and species diversity over time, a Generalized Additive Mixed Model (GAMM) was applied. "Mesocosm" was added as random effect to test if there was an effect of the position of the mesocosms on the parameters. Biomass, abundance and diversity data were log transformed if it improved the outcome of the GAMM as indicated by the R^2 value. R Studio was used for all analyses with the additional packages *mgcv*, *vegan* and *car* (Version 0.95.265, RStudio, Inc.).

Dilution experiment

Setup

In order to further investigate the impact of microzooplankton grazing on phytoplankton, a dilution experiment after Landry and Hassett (1982) was conducted. The experiment took place on day 34 during the 1st phytoplankton bloom peak to ensure high phytoplankton densities in the samples. By releasing the MZP from copepod grazing pressure, indirect effects of CO_2 on MZP based on changes in phytoplankton density or composition are more likely to become visible. Additionally, this grazing setup allows the determination of the natural taxon-specific phytoplankton growth rates despite of a separation of phytoplankton and micrograzers not being possible due to their similar size.

15 L mesocosm water was sampled with an integrating water sampler from 6 out of 10 mesocosms selected for the experiment. To exclude mesozooplankton, the mesocosm water was pre-screened with a 200 μm mesh. For setting up the dilutions, filtered mesocosm water was obtained by using 0.45/0.2 μm sterile inline membrane filters (Sartobran® P 300, Sartorius AG). Three dilutions of 10, 25 and 50% as well as a setup with 100% undiluted mesocosm water were prepared in carboys. To prevent a bias due to nutrient limitation, sterile filtered nutrient solutions (Guillard and Ryther, 1962) were added to the carboys.

In triplicates, the dilutions were gently transferred into 0.5 L polycarbonate incubation bottles to avoid damaging the plankton. Three additional incubation bottles of 100% undiluted water were set up per mesocosm with the addition of five copepods (*Pseudocalanus* sp.) collected from a net tow the day before to analyze mesozooplankton grazing. Another three incubation bottles per mesocosm were set up without the addition of nutrients to serve as control. Initial samples for MZP and phytoplankton starting densities were obtained from the carboys, transferred into brown glass bottles, and fixed with acid Lugol's solution. Incubation bottles were placed on a plankton wheel turning at low speed (~1.1 rpm) at ambient temperature (5°C

during this experiment) and light conditions in a climate controlled room. After 24 h, samples were taken from every incubation bottle.

MZP was analyzed as described in the previous section. For phytoplankton counts, in principle the same method was used as previously described but with a sample volume between 10 and 50 mL depending on the phytoplankton density. At least 400 cells per abundant taxon were counted in tracks of the sedimentation slide using a Zeiss Axiovert 135 inverted microscope. Phytoplankton was identified after Tomas et al. (1997) and Kraberg et al. (2010) and otherwise assigned to a size class. For the estimation of phytoplankton biovolume 20 pictures per group were taken (AxioVision 4.9 and AxioCam, Carl Zeiss Microscopy GmbH) and digitally measured with ImageJ (Version 1.49). Phytoplankton biovolumes were calculated from the measurements according to Hillebrand et al. (1999) and converted to carbon biomass (Putt and Stoecker, 1989; Menden-Deuer and Lessard, 2000).

From the values obtained from the initial 100% sample, starting values for the diluted samples were calculated according to their dilution factor. Phytoplankton net growth per day μ was calculated using an exponential growth model as described in equation (1). The actual phytoplankton growth rate k and phytoplankton grazing mortality m were obtained from a linear regression of the dilution factor α against the phytoplankton growth μ_α where k is the intercept with the y-axis and m is the slope of the regression (Landry and Hassett, 1982; Landry et al., 1995):

$$\mu_\alpha = k + m \alpha \quad (2)$$

The MZP grazing rate g is the negative phytoplankton mortality. All negative grazing rates were set to zero. To calculate the instantaneous (natural) phytoplankton growth rate μ_0 , grazing mortality m was added to the net growth rate μ obtained from the controls grown without the addition of nutrients. All data were first tested for normality and homogeneity with a Shapiro-Wilk test and a Levene's Test for Homogeneity of Variance. Effects of CO₂ and copepod addition on phytoplankton growth rate k , phytoplankton grazing mortality m and instantaneous phytoplankton growth rate μ_0 were tested using ANOVAs. Tukey's HSD test was used as post hoc test.

Community grazing experiment

Setup

In addition to the dilution experiment, community grazing experiments were performed twice during the mesocosm study. While they do not allow for determination of natural phytoplankton growth rates, phytoplankton net growth rates μ including the MZP grazing impact can be obtained. Moreover, they allow the calculation of MZP growth rates. The time points chosen were day 37 and 53, after the 1st and the 2nd phytoplankton bloom peaks.

From each mesocosm, 5 L of seawater was sampled with an integrating water sampler. Two incubation bottles were set up per mesocosm, one containing unfiltered mesocosm water including mesozooplankton grazers (+G treatment) while the other was filled with pre-screened water (100 μm mesh size) to exclude them (-G treatment). Nutrients were not added to the incubation bottles. 250 mL sample of unfiltered and of pre-screened mesocosm water was transferred to brown glass bottles and fixed with acid Lugol's solution at the beginning of each experiment. Samples from every incubation bottle were taken after 24 h of incubation at ambient conditions using a plankton wheel. MZP and phytoplankton were counted microscopically as described for the dilution experiment.

Phytoplankton and MZP growth rates were calculated with equation (1) as previously described. Data were tested for normality and homogeneity and transformed ($\log x+1$) if necessary prior to analyzing the effects of CO_2 and grazer presence using a Two-Way ANOVA.

Results

The experiment ran from 7 March (day -2) until 26 June (day 111). MZP sampling took place from 10 March until 20 June. Within this time, temperature increased from 1.5°C (± 0.06) to 15.4°C (± 0) (Figure 1). Average $p\text{CO}_2$ was 383 μatm (± 100.46) in the low and 739 μatm (± 167.11) in the high CO_2 treatments. Despite of CO_2 fluctuations in the mesocosms due to outgassing and subsequent addition of CO_2 -enriched water, the treatments did not overlap at any time point.

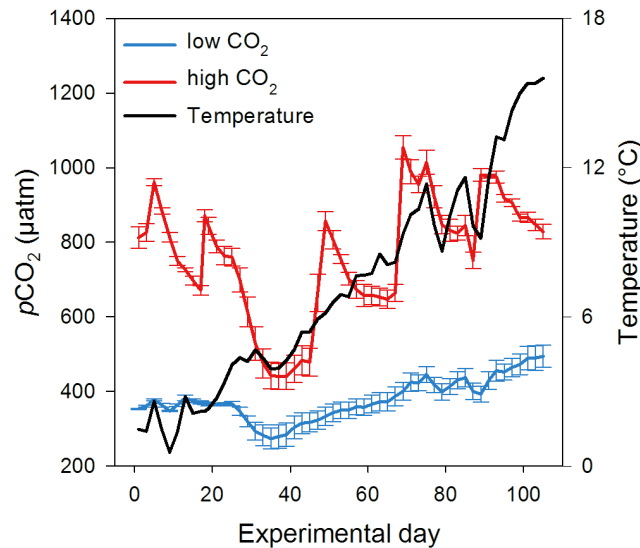


Figure 1: Mean CO₂ concentrations in the low (blue line) and high CO₂ treatments (red line) are shown from day 1 to day 105 as well as mean temperature (black line). Error bars indicate the standard deviation.

Phytoplankton succession and community composition

Starting conditions on 10 March 2013 were Chlorophyll *a* (in the following: Chl *a*) concentrations of 0.363 (± 0.014) and 0.357 (± 0.013) $\mu\text{g L}^{-1}$ in the low and high CO₂ mesocosms (Figure 2A). Based on the Chl *a* development, the experiment was divided in four phases: pre-bloom (until day 16), 1st phytoplankton bloom (day 17-40), 2nd phytoplankton bloom (day 41-79) and post-bloom phase (from day 80 on).

Total phytoplankton abundances at the beginning of the experiment (day -1) were at 1.89×10^7 ($\pm 5.06 \times 10^5$) and 2.06×10^7 ($\pm 5.33 \times 10^5$) cells L^{-1} in the low and high CO₂ treatments (Figure 2B). During the 1st phytoplankton bloom, the large phytoplankton size class ($>8 \mu\text{m}$) reached up to 7.91×10^4 and 9.43×10^4 cells L^{-1} in the low and high CO₂ treatments. Afterwards it decreased to $\sim 4.43 \times 10^3$ cells L^{-1} and did not form another bloom. In contrast, small-sized phytoplankton ($<8 \mu\text{m}$) had abundances of up to 1.48×10^8 cells L^{-1} during the 1st and 2nd bloom phase under high CO₂. In the low CO₂ treatments, this size class reached lower abundances (p -value = 0.010, Table 1) and peaked at 1.10×10^8 cells L^{-1} and 8.07×10^7 cells L^{-1} during the 1st and 2nd bloom phase, respectively. On day 105 at the end of the experiment, total phytoplankton abundance was reduced to $\sim 7.70 \times 10^6$ cells L^{-1} with high deviations between mesocosms.

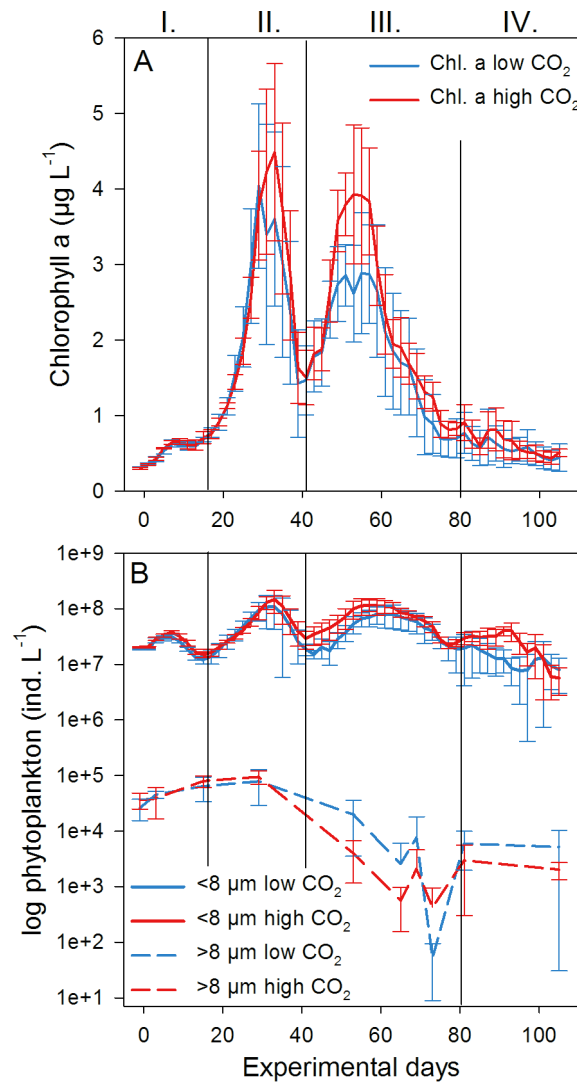


Figure 2: (A) Chlorophyll *a* concentrations from HPLC analysis in $\mu\text{g L}^{-1}$ at low (blue) and high CO_2 (red). Error bars represent the standard deviation. Vertical black lines and Latin numbers indicate the experimental phases (I-IV). (B) Phytoplankton abundances in log ind. L^{-1} in the low and high CO_2 treatments for pico- and nanophytoplankton (<8 μm , solid lines) as well as large phytoplankton (>8 μm , dashed lines).

In terms of abundance, pico- and nanophytoplankton of <8 μm contributed up to 99 % of the total community. For the size class <8 μm , abundance data from flow cytometry was used as it is more reliable than microscopy for small-sized taxa. Still, microscopic analysis of this size class revealed a dominance of the diatom *Arcocellulus* sp. as well as high abundances of the cryptophyte *Hemiselmis* sp.. Considering phytoplankton of >8 μm which was analyzed by microscopic counts, *Teleaulax* sp. and *Thalassiosira* sp. contributed the main part from the start of the experiment until the 1st bloom (Figure 3). The large phytoplankton community changed considerably after day 29 when both taxa disappeared and the remaining taxa

reached very low abundances only, with the exception of *Fragilaria* sp.. Also *Coscinodiscus* sp. increased in numbers after day 29, especially in the low CO₂ treatments. However, the increase of this large-sized diatom was mostly visible with regard to phytoplankton biomass. Nevertheless, while there was a positive CO₂ effect on phytoplankton abundance for the size class <8 μm (Figure 2), abundance and species diversity for taxa >8 μm were not affected by the CO₂ level in the data set we analyzed (Table 1). There was also no effect on phytoplankton abundance maximum or growth rates (Table 2).

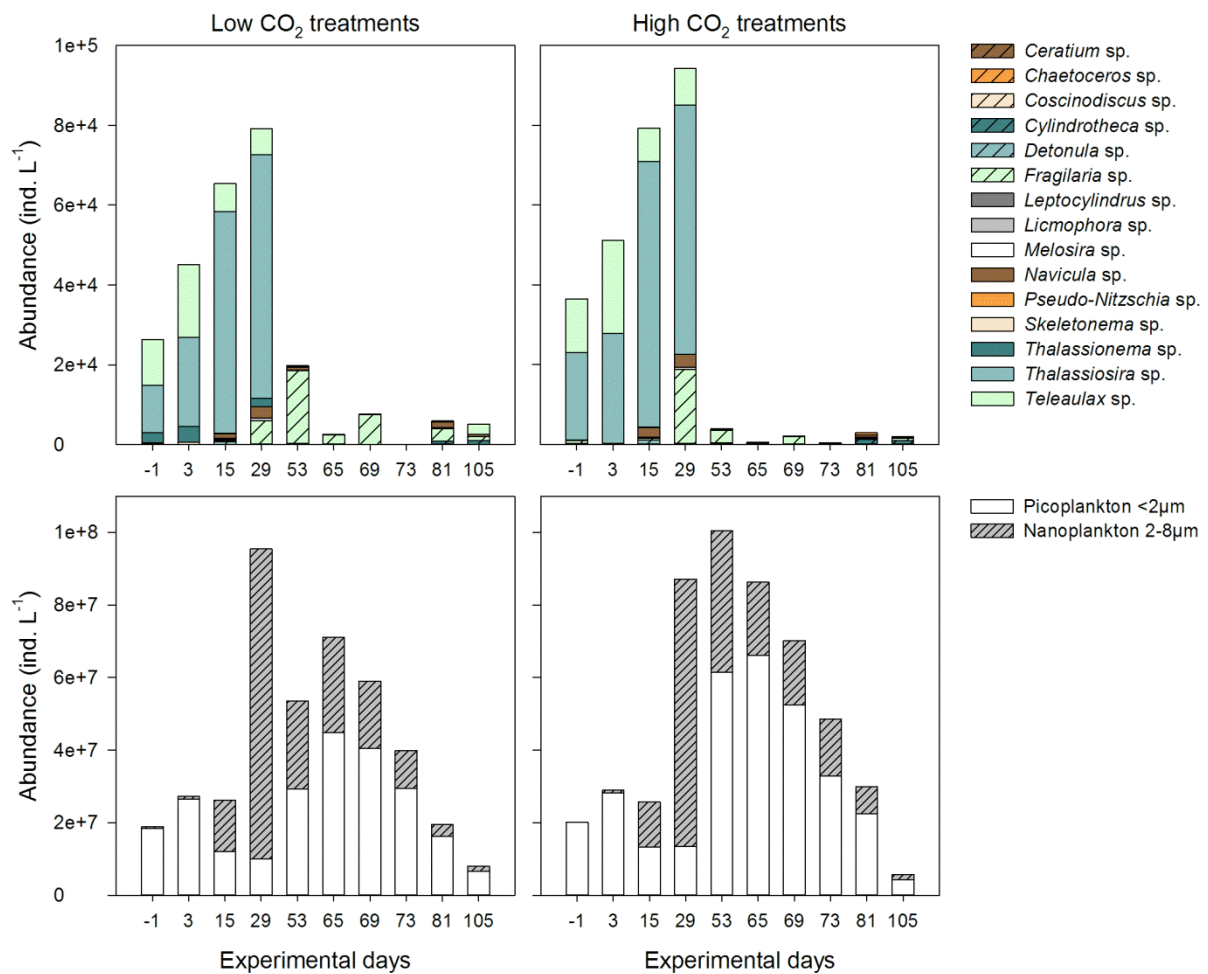


Figure 3: Taxonomic composition of phytoplankton based on mean abundances in the low and high CO₂ treatments in ind. L⁻¹. Abundances of the phytoplankton size classes >8 μm are from microscopic counts while abundances from the size classes <8 μm are from flow cytometry.

Table 1: Results from the GAMM analysis of the effects of high CO₂ on phytoplankton and MZP abundance, biomass, and diversity index. Significances are indicated by asterisks.

Variable		t	p-value
Abundance	log total phytoplankton	-2.626	0.010 *
	log phytoplankton <8 µm	-2.628	0.010 *
	log phytoplankton >8 µm	0.937	0.351
	log total ciliates	0.542	0.589
	log ciliates <30 µm	0.704	0.483
	log ciliates 30-55 µm	-0.020	0.984
	log ciliates >55 µm	1.128	0.263
	Total dinoflagellates	-26.490	<0.001 ***
	log dinoflagellates <30 µm	-1.353	0.179
	Dinoflagellates 30-55 µm	-4.009	<0.001 ***
	Dinoflagellates >55 µm	0.315	0.754
Biomass	log total ciliates	0.945	0.347
	log ciliates <30 µm	0.519	0.605
	log ciliates 30-55 µm	-0.154	0.878
	log ciliates >55 µm	1.302	0.197
	Total dinoflagellates	-1.872	0.064
	Dinoflagellates <30 µm	-1.840	0.068
	Dinoflagellates 30-55 µm	-1.473	0.144
	Dinoflagellates >55 µm	-0.365	0.716
Diversity index	Phytoplankton >8 µm	-0.234	0.816
	Ciliates	-0.264	0.792
	Dinoflagellates	-0.857	0.393

MZP succession

Initial ciliate biomass was 4.23 (± 0.82) for the low and 4.05 (± 0.55) $\mu\text{g C L}^{-1}$ for the high CO₂ mesocosms (Figure 4A). Ciliate biomasses did not react to the 1st bloom of the phytoplankton; only at the onset of the 2nd bloom on day 40 we observed a first increase in biomass for both treatments. After a decline around day 73, ciliate biomass increased again until day 103, especially in the high CO₂ treatments. The growth was most pronounced for the high CO₂ mesocosms MK7 and MK8, reaching 73.18 and 46.74 mg C L^{-1} . There was no CO₂ effect on ciliate biomass, abundance or diversity throughout the experiment (Table 1).

Total dinoflagellate biomass stayed below 1.27 (± 1.54) in the low and 1.56 (± 2.05) $\mu\text{g C L}^{-1}$ in the high CO₂ treatments until day 81 (Figure 4B). Afterwards, an increase in athecate dinoflagellates was observed until day 103 where values peaked at 8.92 (± 3.38) at low and 23.25 (± 12.01) $\mu\text{g C L}^{-1}$ at high CO₂. Thecate dinoflagellates contributed only ~ 2 $\mu\text{g C L}^{-1}$ on the last day. While biomass and diversity of dinoflagellates were not affected by the CO₂

treatment, a positive effect of elevated CO_2 on the total dinoflagellate abundance was found (p -value < 0.001 , Table 1). When size classes were regarded separately, the effect was visible for dinoflagellate abundances from 30-55 μm only (p -value < 0.001).

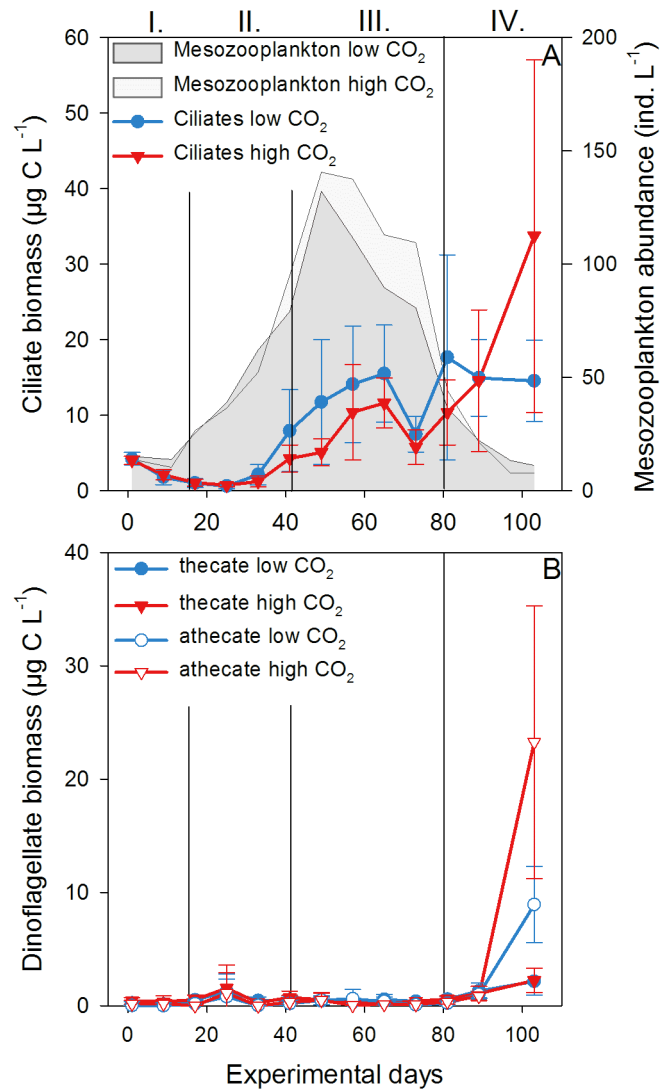


Figure 4: (A) Ciliate biomass in $\mu\text{g C L}^{-1}$ in the low (blue) and high CO_2 (red) treatments. Grey fields show total mesozooplankton abundance in ind. L^{-1} at low (dark grey) and high CO_2 (light grey). Error bars represent the standard deviation and vertical black lines the four experimental phases denoted by the Latin numbers. (B) Biomass of thecate (filled symbols) and athecate dinoflagellates (open symbols) in $\mu\text{g C L}^{-1}$.

Table 2: Results from the ANOVAs of the effects of CO₂ on abundance or biomass maximum (max.), growth rates, and timing of the maximum (D_{max}) for phytoplankton, ciliates and dinoflagellates. Significances are indicated by asterisks.

Variable	Df	Mean Sq.	F value	p-value	
Abundance max.	log total phytoplankton	1	0.094	1.086	0.328
	log phytoplankton <8 μm	1	0.094	1.090	0.327
	log phytoplankton >8 μm	1	0.205	0.562	0.475
Biomass max.	Total ciliates	1	457.300	1.539	0.250
	<i>Strombidium</i> sp. <40μm	1	0.112	0.324	0.585
	<i>Strombidium</i> sp. >40μm	1	3.999	3.131	0.115
	<i>Strobilidium</i> sp.	1	0.774	0.882	0.375
	<i>Tontonia gracillima</i>	1	3.565	1.779	0.219
	<i>Laboea strobila</i>	1	0.720	0.340	0.578
	<i>Lohmaniella oviformis</i>	1	0.644	0.980	0.351
	<i>Myrionecta rubra</i>	1	0.018	0.238	0.639
	<i>Suctorina</i> sp.	1	0.332	0.440	0.528
	<i>Euplotes</i> sp.	1	0.015	2.180	0.178
	Total dinoflagellates	1	520.250	5.024	0.055
	Thecate dinoflagellates <30μm	1	0.042	0.411	0.539
	Thecate dinoflagellates 30-55μm	1	0.298	0.170	0.691
	Thecate dinoflagellates >55μm	1	0.589	2.564	0.148
	Athecate dinoflagellates <30μm	1	1.663	7.137	0.028 *
Athecate dinoflagellates 30-55μm	1	3.339	4.439	0.068	
Athecate dinoflagellates >55μm	1	0.256	0.318	0.588	
Growth rate	Total phytoplankton	1	0.304	3.260	0.109
	Phytoplankton > 8μm	1	0.176	0.427	0.532
	Total ciliates	1	0.345	1.448	0.263
	<i>Strombidium</i> sp. <40μm	1	0.126	0.359	0.566
	<i>Strombidium</i> sp. >40μm	1	1.727	1.627	0.243
	<i>Tontonia gracillima</i>	1	0.170	0.137	0.724
	<i>Laboea strobila</i>	1	0.041	0.028	0.873
	<i>Lohmaniella oviformis</i>	1	0.226	0.882	0.379
	Total dinoflagellates	1	1.586	5.807	0.043 *
	Thecate dinoflagellates	1	0.013	0.023	0.883
	Athecate dinoflagellates <30μm	1	3.127	12.821	0.007 **
	Athecate dinoflagellates 30-55μm	1	2.400	4.478	0.072
D _{max}	Total ciliates	1	2624.400	21.337	0.002 **
	<i>Strombidium</i> sp. <40μm	1	3459.600	15.797	0.004 **
	<i>Strombidium</i> sp. >40μm	1	144.400	0.224	0.649
	<i>Strobilidium</i> sp.	1	0.008	0.629	0.451
	<i>Tontonia gracillima</i>	1	0.075	1.084	0.328
	<i>Myrionecta rubra</i>	1	25.600	0.118	0.740
	Thecate dinoflagellates <30μm	1	3027.600	3.125	0.115
	Thecate dinoflagellates >55μm	1	48.400	0.074	0.793

Analysis of the biomass maxima and growth rates revealed no effect of CO₂ on total ciliate biomass or ciliate taxa (Table 2). In contrast, the timing of the biomass maximum was significantly later in the high CO₂ mesocosms for total ciliates (p-value = 0.002) and *Strombidium* sp. <40 µm (p-value = 0.004).

For athecate dinoflagellates <30 µm, a positive effect of CO₂ on the biomass maximum was observed (p-value = 0.028) while thecate dinoflagellates and total dinoflagellate biomass were not affected. We also found a positive effect of high CO₂ on the growth rates of total dinoflagellates (p-value = 0.043) and athecate dinoflagellates <30 µm (p-value = 0.007). There was no effect of CO₂ on the timing of the biomass maxima D_{\max} .

MZP community composition

The ciliate community was dominated by small *Strombidium* sp. <40 µm in both CO₂ treatments, contributing up to 90 % of the total biomass (Figure 5). On day 81 and 89, *Strombidium* sp. >40 µm increased in biomass, providing about half of the total *Strombidium* sp. group. The cyclotrich *Myrionecta rubra* increased in density until day 25 where it contributed 18 % in the low and 16 % in high CO₂ treatments. It was virtually absent from all mesocosms after day 49. In contrast, the choreotrich *Lohmaniella oviformis* formed a bloom between days 65 and 89 but was not found at other time points. The oligotrich group of *Strobilidium* sp. were present throughout the experiment in small numbers and formed a bloom around day 73, including large *Rimostrombidium* sp.. Large species such as *Tontonia gracillima* and *Laboea strobila* (size class 55-100 µm) were present throughout the experiment. The latter one reached high densities on the last day of the experiment, contributing 36 % and 19 % in the low and high CO₂ treatments, respectively. *Suctorina* sp. was found almost exclusively on day 81 in both treatments, in similar densities. The group of rare species included *Balanion comatum*, *Mesodinium pulex*, *Leegardiella* sp., *Tiarina fusus*, *Favella* sp. and *Acineta* sp. in changing proportions at overall low concentrations.

Ciliate diversity was low at the start, increasing until day 33. After a sharp decrease around day 41, caused by the peak of *Strombidium* sp. <40 µm, it increased again afterwards. The diversity of the treatments was not significantly different (Table 1). Although the taxonomic composition of ciliates was very similar in both treatments; standard deviations between single mesocosms were high, especially in the high CO₂ treatments at the end of the experiment.

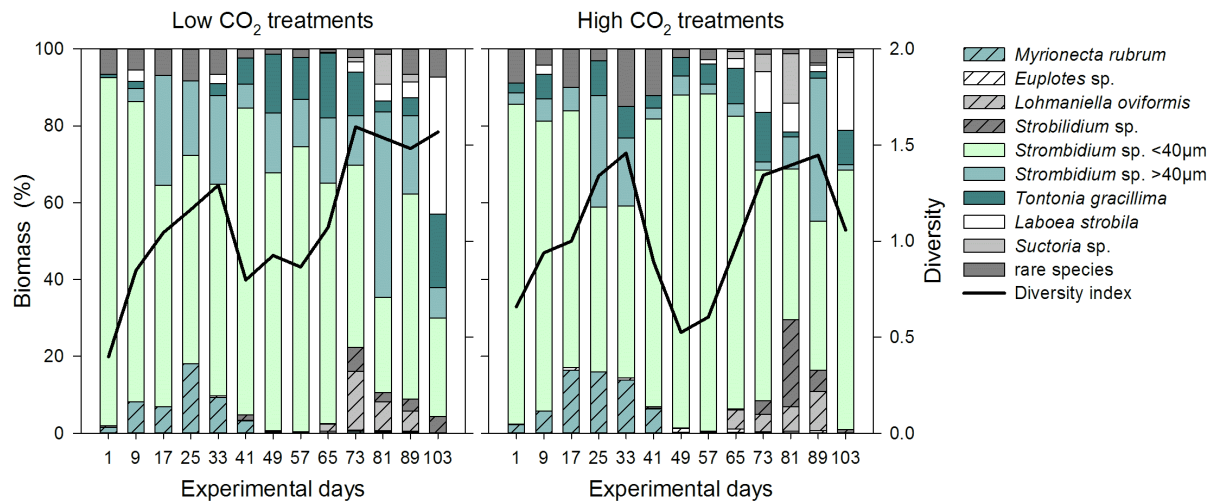


Figure 5: Taxonomic composition of the ciliate community over the course of the experiment based on mean biomass of the low and high CO₂ treatments. The black line indicates the species diversity H'.

During the first half of the experiment, the dinoflagellate community was dominated by thecate dinoflagellates, contributing up to 100 % in both CO₂ treatments (Figure 6). While the contribution of the different size classes varied over time, the main part of the size classes >30 µm was made up by different *Protoperidinium* sp. and *Dinophysis* sp., a mixotrophic taxon. Diversity was around 0.8 at the start and decreased in both treatments until day 49. It increased during the second half of the experiment, starting on day 57 in the low CO₂ and day 65 in the high CO₂ treatments. This was due to an increase in athecate dinoflagellates, mainly large *Gyrodinium* sp. (size class >55 µm). After a sharp decrease (low CO₂: day 65, high CO₂: day 73), the contribution of athecate dinoflagellates increased again to almost 90 % on day 103. In this case, athecate taxa of the size classes <30 µm and 30-55 µm each contributed about half of the community while taxa >55 µm occurred in low numbers only. While this pattern was similar for both treatments, differences in biomass between single mesocosms increased towards the end of the experiment, most notably in the high CO₂ treatments.

Overall, ciliates were the main player of the MZP community in terms of abundance and biomass, showing a similar succession pattern in the two CO₂ treatments. They contributed 67-98 % to the total MZP biomass with the exceptions of day 25 and 103 (Figure 7). On these two occasions, dinoflagellates made up ~66 % and ~44 %, respectively.

In summary, the MZP community composition or diversity was not affected by CO₂. MZP was dominated by small *Strombidium* sp., with especially athecate dinoflagellates substantially contributing to the community only during the last days.

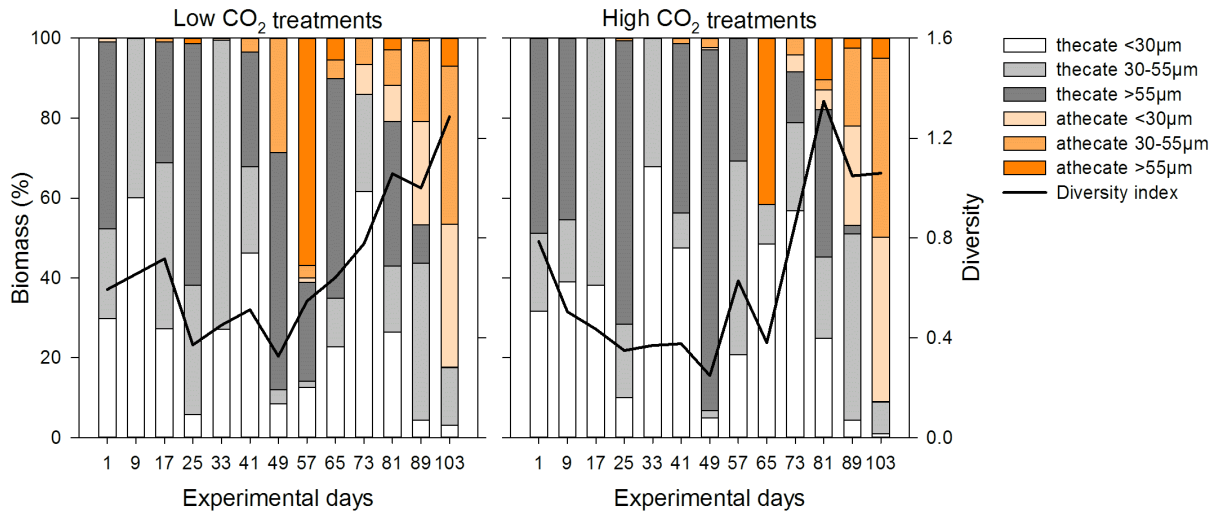


Figure 6: Size class composition of the thecate (grey bars) and athecate (orange bars) dinoflagellates for the size classes $<30\ \mu\text{m}$, $30\text{-}55\ \mu\text{m}$ and $>55\ \mu\text{m}$ based on mean biomass of the low and high CO_2 treatments. Dinoflagellate diversity H' is indicated by the black line.

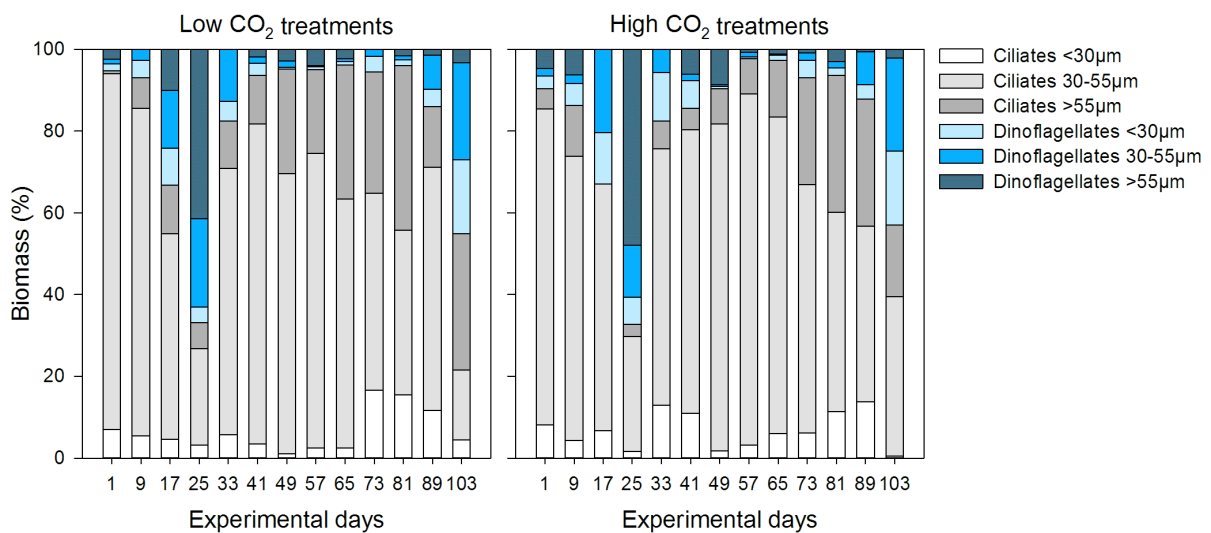


Figure 7: Composition of ciliates (grey bars) and dinoflagellates (blue bars) for the size classes $<30\ \mu\text{m}$, $30\text{-}55\ \mu\text{m}$ and $>55\ \mu\text{m}$ based on mean biomass of the low and high CO_2 treatments.

Grazing experiments

For the dilution experiment conducted during the 1st phytoplankton bloom peak, growth rates and natural growth rates could be calculated for the four most common phytoplankton groups as well as for total phytoplankton biomass. Phytoplankton growth rates k were entirely negative with k of total phytoplankton, flagellates $<5\ \mu\text{m}$ and flagellates $>5\ \mu\text{m}$ being significantly lower in the high CO_2 treatments ($p\text{-value} < 0.05$, Table 3). Likewise, all instantaneous phytoplankton growth rates μ_0 without nutrient addition were negative.

However, they were higher under high CO₂ conditions for total phytoplankton and flagellates <5 µm (p-value < 0.01). Additionally, the actual phytoplankton grazing mortality m was higher under high CO₂ conditions for total phytoplankton and flagellates <5 µm (p-value = 0.002) and close to significant for flagellates >5 µm (p-value = 0.052). We found no effect of CO₂ on the chlorophyte *Dunaliella* sp. or the diatom *Arcocellulus* sp. for k , m and μ_0 . Furthermore, the presence or absence of copepods had no effect on k and μ_0 (data not shown). MZP grazing rates g were zero for almost all treatments and could not be further evaluated.

Table 3: Results from the ANOVAs of the effects of CO₂ on phytoplankton growth rate k , phytoplankton grazing mortality m and instantaneous phytoplankton growth rate μ_0 without nutrient addition from the dilution experiment. Significances are indicated by asterisks.

Variable		Df	Mean Sq	F value	p-value	
k	Total phytoplankton	1	0.392	15.587	0.017	*
	Flagellates <5µm	1	0.351	9.854	0.035	*
	Flagellates >5µm	1	1.151	8.254	0.045	*
	<i>Dunaliella</i> sp.	1	2.211	6.718	0.061	
	<i>Arcocellulus</i> sp.	1	0.049	0.046	0.840	
m	Total phytoplankton	1	1.281	37.078	0.004	**
	Flagellates <5µm	1	1.494	57.024	0.002	**
	Flagellates >5µm	1	1.232	7.491	0.052	
	<i>Dunaliella</i> sp.	1	1.413	7.278	0.054	
	<i>Arcocellulus</i> sp.	1	0.550	0.562	0.495	
μ_0	Total phytoplankton	1	2.333	34.348	0.004	**
	Flagellates <5µm	1	3.091	42.526	0.003	**
	Flagellates >5µm	1	0.865	1.363	0.308	
	<i>Dunaliella</i> sp.	1	0.611	1.635	0.270	

Analysis of the two community grazing experiments revealed no effect of CO₂ concentration, grazer presence or an interaction of the two factors on net growth rates of total phytoplankton and ciliates (Table S1). There was also no effect on the most common phytoplankton taxa, *Teleaulax* sp., *Arcocellulus* sp., *Paralia sulcata*, flagellates <5 µm and flagellates >5 µm as well as the ciliates *M. rubra*, *Strombidium* sp. <40 µm and *Strobilidium* sp. <30 µm. Dinoflagellate growth rates were also not affected in the 1st experiment (Table 4). However, during the 2nd experiment, growth rates of thecate dinoflagellates <30 µm were significantly lower in the high CO₂ treatment (p-value = 0.012).

Table 4: Results from the ANOVAs of the effects of CO₂, grazer presence, and the interaction of the two factors on growth rate of total dinoflagellates and thecate dinoflagellates in the community grazing experiments. Significances are indicated by asterisks.

Experiment	Variable	Factor	Df	Mean Sq	F value	p-value
1	Total dinoflagellates	CO ₂	1	0.216	0.452	0.511
		Grazer	1	0.596	1.249	0.280
		CO ₂ x Grazer	1	0.064	0.134	0.719
	Thecate <30µm	CO ₂	1	0.530	0.558	0.466
		Grazer	1	0.014	0.015	0.904
		CO ₂ x Grazer	1	0.504	0.531	0.477
	Thecate >30µm	CO ₂	1	0.150	0.123	0.731
		Grazer	1	1.812	1.490	0.242
		CO ₂ x Grazer	1	0.038	0.031	0.863
2	Total dinoflagellates	CO ₂	1	0.001	0.000	0.984
		Grazer	1	0.738	0.324	0.577
		CO ₂ x Grazer	1	0.343	0.150	0.703
	Thecate <30µm	CO ₂	1	6.734	8.659	0.012 *
		Grazer	1	0.401	0.515	0.487
		CO ₂ x Grazer	1	0.713	0.917	0.357

Discussion

The mesocosms were sustained for more than 100 days by counteracting problems occurring during long-term studies with extensive maintenance work, such as regular cleaning of the in- and outside of the mesocosms to avoid wall growth. While there were fluctuations of $p\text{CO}_2$ for the two treatments over time due to photosynthetic carbon fixation and outgassing, there was no overlap between treatments at any time point.

The abundances of MZP in the mesocosms ranged from 4320 to 7489 ind. L⁻¹ at the start point which is within the expected range for the Gullmar Fjord area in the beginning of March (Calliari and Tiselius, 2009). MZP abundance stayed comparatively low during the experiment, with the exception of the post-bloom phase for both dinoflagellates and ciliates. Overall, the observed effects of elevated CO₂ concentrations on the measured MZP parameters were comparatively small and subtle.

Effects on phytoplankton

While some phytoplankton groups like calcifying algae are negatively affected by OA (Riebesell et al., 2000b; Kim et al., 2006), a fertilizing effect on other groups due to the

increased availability of carbon has been observed (Riebesell et al., 2007; Havenhand, 2012; Low-Decarie et al., 2014). For example for diatoms, a shift in species composition has been found in different field studies (Rost et al., 2008). Comparable to other studies using this mobile mesocosm system, the composition of phytoplankton and the development of abiotic factors such as light and temperature during a spring-bloom situation mimic the natural situation to a high degree. While a 1st bloom peak in the fjord was reached on day 27 with Chl *a* concentrations of 3.52 $\mu\text{g L}^{-1}$, the mesocosm bloom peaked on day 29 in the low and day 33 in the high CO₂ mesocosms at 4.04 and 4.49 $\mu\text{g L}^{-1}$, respectively.

According to the GAMM analysis, Chl *a* concentrations were significantly higher at high CO₂ even though the effect seemed to be most pronounced in phase III around the 2nd phytoplankton bloom peak (Figure 2A). We found no effect of high CO₂ on the abundance of phytoplankton >8 μm . Nonetheless, considering abundances, large sized phytoplankton played only a minor role during both phytoplankton bloom phases. Based on previous studies from other regions, a positive CO₂ effect on pico- and nanoeukaryotes is more likely to occur (Brussaard et al., 2013; Sala et al., 2016). In fact, a positive effect of a high CO₂ level on picophytoplankton was observed in this experiment (Bach et al., in review) which could explain the higher Chl *a* concentrations in these treatments to some extent.

Effects on MZP community composition

A direct effect of a lowered pH on MZP has been shown for areas such as the Baltic Sea or the North Atlantic (Hinga, 1992; Pedersen and Hansen, 2003), visible e.g. in the inhibition of growth (Nielsen et al., 2010). However, these effects were only shown for extreme pH values that are unlikely to occur in the near future (Nielsen et al., 2010). Results from a laboratory study applying more realistic OA scenarios on a single MZP species showed no direct effect (Meunier et al., 2016). In support of that, most mesocosm studies with a CO₂ level expected for the end of the 21st century also show no or only subtle effects on the MZP community composition and diversity (Suffrian et al., 2008; Aberle et al., 2013; Calbet et al., 2014). This can be partly attributed to the high tolerance of coastal communities to frequently occurring habitat pH fluctuations (Feely et al., 2010; Melzner et al., 2013). In general, open ocean communities are considered to be more susceptible to OA as they do not experience these fluctuations, still Rose et al. (2009) reported no direct effects of an elevated CO₂ level on the MZP community in a study in the open Atlantic Ocean.

The aforementioned mesocosm studies lasted 14 to 41 days. Nonetheless, even the longer runtime of 113 days in our study did not result in an effect of the applied CO₂ level on the

MZP community composition and diversity. Thus, hypothesis (1) stating that an elevated CO₂ level will not directly affect MZP communities due to their high CO₂ tolerance could not be rejected. In fact, there was an almost parallel development of the composition over time in the two treatments, both for ciliates and heterotrophic dinoflagellates.

Effects on MZP biomass and growth rates

For autotrophic phytoplankton, it has already been shown that high CO₂ can have an either positive or negative direct impact, depending on the plankton group in focus (Kroeker et al., 2013; Low-Decarie et al., 2014). In contrast, indirect effects are considered to be more important for heterotrophic or mixotrophic zooplankton than direct ones, such as changes in phytoplankton availability or food quality (Iglesias-Rodriguez et al., 2008; Rose et al., 2009; Rossoll et al., 2012).

Generally, effects of CO₂ are likely to be more intense in a nutrient-deplete system than in a nutrient-replete one (Paul et al., 2015a; Sala et al., 2016; Bach et al., in review) even though this is not always the case, as e.g. in the Arctic Ocean (Riebesell et al., 2013b). Nutrient concentrations in the mesocosms were high in the beginning, caused by entrapping nutrient-rich deep water in the mesocosms which is distributed through the whole water column due to wind-induced mixing during wintertime (Lindahl et al., 1998). Nutrient depletion occurred already during the 1st phytoplankton bloom/phase II resulting in the 2nd phytoplankton bloom/phase III being nutrient-deplete and nutrients such as NO₃⁻/NO₂⁻, Si(OH)₄ and PO₄³⁻ being at concentrations close to or below detection limit (Bach et al., in review). In conclusion, nutrients must have been provided by remineralization to support the observed 2nd bloom, but were immediately used up and thus did not accumulate in the nutrient pool.

While community composition of large phytoplankton was not affected by CO₂ in the data set we analyzed, positive effects on picoeukaryotes were observed (Bach et al., in review). Additionally, we found a positive effect of high CO₂ on the abundances of heterotrophic dinoflagellates over time. Total biomass, however, was not affected. This is in agreement with biomass maximum and growth rates of small athecate dinoflagellates <30 μm being higher at elevated CO₂. The dominance of small athecate dinoflagellates in the community was most likely also the reason why a positive CO₂ effect on the total dinoflagellate growth rate was found despite of the other size classes not being affected by CO₂ (Figure 7). Thus, not only phytoplankton but also smaller size classes of microzooplankton seemed to benefit from an elevated CO₂ level.

A high contribution of athecate dinoflagellates to the total MZP community during the bloom or in the post-bloom phase as in our case has been described for several coastal areas (Sherr and Sherr, 2007). In contrast, total ciliate biomass was lower under high CO₂ conditions during the 2nd bloom, even though this effect was not significant. Overall, abundances of large phytoplankton and copepods (Algueró-Muñiz et al., in prep.) were not significantly affected by the CO₂ level in this phase. Still, abundance of pico- and nanophytoplankton was higher at high CO₂ so the negative effect on MZP was probably caused by additional factors.

The dilution experiment conducted right after the 1st bloom peak took place at a time when MZP densities were still low. Considering that the phytoplankton community composition was not different from the regular samples from the mesocosms, we can assume that there was no loss of any groups from handling the water samples. The overall negative phytoplankton growth rates were most probably caused by senescence as nutrient addition prevented limitation and the MZP grazing impact was low due to low MZP densities.

Nonetheless, there was an indication of a higher phytoplankton biomass at high CO₂ in the dilution experiment as we observed higher instantaneous phytoplankton growth rates μ_0 in the high CO₂ treatments. As μ_0 is calculated without the grazing impact, the results suggest that phytoplankton was indeed growing better under high CO₂, especially small flagellates. This would also fit to Chl *a* maximum of the 1st phytoplankton peak being higher at high CO₂ and has been described by other authors (Rost et al., 2008; Brussaard et al., 2013; Low-Decarie et al., 2014; Sala et al., 2016). We also observed an overall higher phytoplankton grazing mortality *m* in the high CO₂ treatments pointing at a higher MZP density at high CO₂ even though actual grazing rates could not be calculated. This is mirrored in the phytoplankton growth rates *k* being more negative for high CO₂ due to the MZP grazing impact.

Although the two community grazing experiments took place during the 1st and 2nd bloom peak, thus consisting of two somewhat different phytoplankton communities, MZP communities and MZP biomass, the result was the same for both phytoplankton and ciliate growth rates which were not affected by CO₂. Contrastingly, there was once more an effect on dinoflagellates during the 2nd bloom, but in this case a negative CO₂ effect on the growth rates of heterotrophic thecate dinoflagellates of the size class <30 μm . In addition, we once more found an indication for a higher phytoplankton density at high CO₂ in the community grazing experiments which could potentially lead to an increase in MZP biomass. An increase in MZP biomass was indeed what we observed in the mesocosms on day 103 when grazing pressure by copepods had all but disappeared.

We hypothesized that (2) an increase in phytoplankton biomass at high CO₂ conditions due to positive effects on photosynthesis will lead to enhanced MZP biomass and grazing rates. While grazing rates could not be calculated, hypothesis (2) was confirmed with regard to biomass of dinoflagellates of the size class 30-55 µm as we did observe effects of an elevated CO₂ level, even though most phytoplankton groups were not affected. However, the hypothesis was rejected for ciliates as this group showed no response except for a delayed bloom peak under high CO₂.

Food web effects

While an increase in phytoplankton has the potential to positively influence MZP, the effect might be masked by grazing pressure by mesozooplankton as a numerical response of copepods to increasing MZP densities has been described (Stoecker and Capuzzo, 1990). As mentioned before, copepods are known to be size-selective in their feeding behavior, and while ciliates have the ideal size, phytoplankton cells are often either too small or too large (Frost, 1972). In our experiment, phytoplankton <8 µm contributed almost 99 % of the phytoplankton community during the two blooms in terms of abundance. In general, this size class is considered inedible for most copepod species but represents ideal food items for MZP, especially ciliates (Jonsson, 1986). It has already been shown in other experiments that nanoflagellates are selectively grazed upon by ciliates even if other phytoplankton groups are present in sufficient densities (Löder et al., 2011). In contrast, dinoflagellates can consume phytoplankton cells larger than their own size and also cannibalistic feeding behavior has been reported (Hansen and Calado, 1999).

Noticeable was the appearance of large *Coscinodiscus* sp. (>200 µm) during the two phytoplankton blooms, reaching abundances of ~428 ind. L⁻¹ during the 2nd bloom and contributing to a large part of the total phytoplankton biomass. However, this large-sized diatom is usually not considered as a copepod food source, even though some copepod species have been reported to feed on them (Jansen, 2008). In conclusion, there was hardly any phytoplankton present in a size class edible for mesozooplankton despite of high phytoplankton abundances during the bloom phases. The low concentration of MZP was therefore most likely caused by intense top-down control by mesozooplankton, thus MZP functioned as a “trophic link” between different levels of the present food web (Calbet and Saiz, 2005).

In addition, grazing pressure could also explain the trend towards higher MZP biomass at high pCO₂ as observed during the post-bloom phase (IV), despite deviations between mesocosms

being high. The MZP succession pattern fits to the development of the mesozooplankton population in both CO₂ treatments (Figure 4A). Starting at low initial abundances, total mesozooplankton increased in numbers reaching on average 136 ind. L⁻¹ (±23) on day 49. The dominating mesozooplankton group was copepods, most notably *Pseudocalanus* sp.. By the end of the 2nd bloom mesozooplankton was reduced to ~40 ind. L⁻¹, and continued decreasing even more, thus releasing the MZP from grazing pressure (details on mesozooplankton presented by Algueró-Muniz et al., in prep.). This was the time point when ciliates and dinoflagellates started to increase in the high CO₂ treatments, despite the decline in phytoplankton densities. While MZP, and especially dinoflagellates, show a variety of feeding mechanisms (Sherr and Sherr, 2007), most taxa are considered as mixotroph and do not necessarily rely on high phytoplankton concentrations alone.

Finally, the occurrence of fish larvae as top-predators of the system had the potential to reduce both copepod and MZP densities. The herring larvae which were released into the mesocosms on day 63 could explain the drop in biomass observed for ciliates on day 73 (Figure 4A) as early stage larval fish are known to feed on MZP (De Figueiredo et al., 2007). Around day 71, the larvae should have been at an age when they switch from yolk-sack stage to feeding on nauplii and large MZP (>55 µm) (Schnack, 1972). Consequently, large ciliates increased in density again afterwards, at the point when the fish larvae started feeding on larger food items such as copepods thus releasing the MZP from grazing pressure. The effect was not visible for large dinoflagellates, probably due to their overall low numbers at this time point.

Apart from grazing pressure by copepods, an explanation for the low MZP biomass at the beginning of the experiment and the lack of response to the 1st phytoplankton bloom (Figure 4A) could have been the low temperatures. In contrast to phytoplankton, which is in large parts light-dependent due to photosynthesis, MZP shows a temperature-dependence due to the biochemical processes of its metabolism (Bernacchi et al., 2001). Therefore, a relationship between an increase in temperature and an increase in production has been observed (Montagnes and Lessard, 1999; Rose and Caron, 2007; Aberle et al., 2012). At the beginning of this experiment, sea surface temperatures were ~1°C and during the 1st phytoplankton bloom phase ~5°C (Figure 1). The low temperature seemed to prevent MZP from growth as no biomass increase was observed in response to increases in phytoplankton standing stock. Only during the 2nd phytoplankton bloom phase when temperatures reached up to 10°C, a biomass peak in MZP was observed.

Hypothesis (3) predicted that high CO₂ levels will enhance trophic pathways through MZP due to CO₂-induced alterations in phytoplankton size classes and taxonomic composition. Overall, it was accepted as an alteration of the phytoplankton community was observed in favor of small-sized phytoplankton. Moreover, as MZP most likely made use of the increase in small phytoplankton while simultaneously being a preferred food item for copepods at the same time, the hypothesized enhanced trophic pathways through MZP due to high CO₂ was observed.

Conclusion

Complex near-natural systems like the one used in this mesocosms study are associated with a higher buffering capacity towards the effects of OA in comparison with lab studies using simplified food webs (Suffrian et al., 2008). Nevertheless, while we found a high tolerance of most MZP groups to a realistic acidification scenario, we observed effects on both phytoplankton and MZP. While large phytoplankton species remained unaffected by high CO₂, abundances of small taxa and Chl *a* concentrations were positively affected. We observed no effects on ciliates apart from a delayed bloom peak under high CO₂. There was, however, a positive effect of CO₂ on heterotrophic dinoflagellate abundances as well as the biomass maximum and growth rate of athecate dinoflagellates (<30 µm).

This highlights the importance of long-term studies lasting for a complete succession period to follow e.g. an entire build-up and decline during bloom periods in spring. Previous mesocosm studies of comparable size from the Baltic Sea, the North Sea and the Arctic considered only shorter time spans. This might have masked effects of high CO₂ which are visible only under long-term exposure (Suffrian et al., 2008; Aberle et al., 2013; Calbet et al., 2014; Lischka et al., 2015). Based on the results, MZP communities from coastal regions comparable to the study site are not expected to be strongly affected by end-of-century CO₂ levels.

Supplement

Table S1: Complete results from the community grazing experiments. Results from the ANOVAs from the two community grazing experiments. Effects of CO₂, grazer presence, and the interaction of the two factors on growth rate of total phytoplankton and ciliates as well as the most common taxa of the two groups are shown. Transformations are indicated.

Experiment	Variable	Factor	Df	Mean Sq	F value	p-value	
1	Total phytoplankton	CO ₂	1	0.026	0.047	0.831	
		Grazer	1	0.035	0.064	0.804	
		CO ₂ x Grazer	1	0.129	0.233	0.636	
	Flagellates <5µm	CO ₂	1	0.000	0.000	0.992	
		Grazer	1	0.013	0.014	0.909	
		CO ₂ x Grazer	1	0.030	0.030	0.864	
	Flagellates >5µm	CO ₂	1	0.196	0.293	0.596	
		Grazer	1	0.773	1.157	0.298	
		CO ₂ x Grazer	1	0.194	0.290	0.598	
	<i>Teleaulax</i> sp.	CO ₂	1	0.023	0.021	0.890	
		Grazer	1	0.261	0.235	0.641	
		CO ₂ x Grazer	1	0.236	0.212	0.658	
	<i>Paralia sulcata</i>	CO ₂	1	0.006	0.004	0.953	
		Grazer	1	1.296	0.769	0.401	
		CO ₂ x Grazer	1	0.485	0.288	0.603	
1	Total ciliates log (x+1)	CO ₂	1	0.002	0.009	0.925	
		Grazer	1	0.124	0.669	0.425	
		CO ₂ x Grazer	1	0.604	3.260	0.090	
	<i>Myrionecta rubra</i>	CO ₂	1	0.589	0.870	0.365	
		Grazer	1	0.719	1.064	0.318	
		CO ₂ x Grazer	1	1.267	1.873	0.190	
	<i>Strobilidium</i> sp. <30µm	CO ₂	1	4.940	3.835	0.069	
		Grazer	1	0.078	0.060	0.809	
		CO ₂ x Grazer	1	0.036	0.028	0.870	
	<i>Strombidium</i> sp.<40µm log (x+1)	CO ₂	1	0.049	0.228	0.639	
		Grazer	1	0.410	1.906	0.186	
		CO ₂ x Grazer	1	0.448	2.081	0.168	
	2	Total phytoplankton	CO ₂	1	0.045	0.365	0.554
			Grazer	1	0.340	2.730	0.118
			CO ₂ x Grazer	1	0.005	0.042	0.841
Flagellates <5µm		CO ₂	1	0.134	1.913	0.186	
		Grazer	1	0.028	0.406	0.533	
		CO ₂ x Grazer	1	0.003	0.041	0.843	
Flagellates >5µm		CO ₂	1	0.036	0.046	0.834	
		Grazer	1	0.668	0.864	0.373	
		CO ₂ x Grazer	1	0.115	0.148	0.708	
<i>Arcocellulus</i> sp.		CO ₂	1	0.199	0.155	0.702	

		Grazer	1	0.001	0.001	0.978
		CO ₂ x Grazer	1	0.887	0.691	0.425
2	Total ciliates log (x+1)	CO ₂	1	0.225	2.256	0.153
		Grazer	1	0.205	2.060	0.170
		CO ₂ x Grazer	1	0.307	3.081	0.098
	<i>Myrionecta rubra</i>	CO ₂	1	0.124	0.180	0.678
		Grazer	1	0.000	0.000	0.999
		CO ₂ x Grazer	1	0.081	0.119	0.735
	<i>Strobilidium</i> sp. <30µm	CO ₂	1	0.067	0.055	0.818
		Grazer	1	0.546	0.451	0.515
		CO ₂ x Grazer	1	1.835	1.515	0.242
	<i>Strombidium</i> sp. <40µm	CO ₂	1	0.448	2.481	0.135
		Grazer	1	0.377	2.088	0.168
		CO ₂ x Grazer	1	0.542	3.000	0.103

CHAPTER II – Effects of high CO₂ and warming on a Baltic Sea microzooplankton community

Abstract

Global warming and ocean acidification (OA) 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 CO₂ 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 a 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 pCO₂ 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 CO₂ and temperature. We attribute this to the high tolerance of this estuarine plankton community to fluctuations in pCO₂, often resulting in CO₂ 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.

Introduction

The concentration of CO₂ in the atmosphere has increased considerably in the last decades, from 280 ppm in pre-industrial times to currently around 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 CO₂ has a direct effect on the surface oceans. Acting as major sinks for CO₂, the increase in dissolved CO₂ 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 CO₂ taken up by the oceans depending on the region (Sabine et al., 2004). Linked to the predicted increase in CO₂, 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., 2014b) 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 in order 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 20 to 200 µm is a major phytoplankton consumer in planktonic food webs where it plays a vital role as intermediary between the microbial loop and higher trophic levels (Calbet and Landry, 2004; Calbet et al., 2008). Due 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 micro- and mesozooplankton (Löder et al., 2011). At the same time, higher trophic levels use MZP as 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 to 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; Aberle et al., 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, pCO₂ in highly productive estuarine areas such as the Kiel Fjord are 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 ecosystems characteristics. Currently, seawater pCO₂ 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 pCO₂ (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; Winder et al., 2012; Lewandowska et al., 2014; Aberle et al., 2015).

Here we present an indoor mesocosm study on the combined effects of enhanced CO₂ and warming on natural autumn plankton communities from Kiel Fjord, characterised by a

diatom-dominated phytoplankton bloom in autumn (Wasmund et al., 2008). Our working hypotheses considering the combined effects of warming and CO₂ were as follows:

- (1) 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.
- (2) 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.
- (3) Due to positive effects on photosynthesis, high pCO₂ will cause an increase in phytoplankton biomass leading to a higher MZP biomass.
- (4) The combined effects of warming and pCO₂ will lead to a dampening of the effects of high pCO₂. 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 & 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 CO₂ manipulation. Two temperatures (9 and 15°C, hereafter called “cold” and “warm”) and two CO₂ 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 CO₂ 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 CO₂ 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 day length 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 11h50 min:12h10 min. The daily maximum light intensity in the middle of the water column was $252.3 \mu\text{mol m}^{-2} \text{s}^{-1}$. The mesocosms were filled with unfiltered seawater from Kiel Fjord on October 19th 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 about 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} in order 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 to 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 DIC and alkalinity.

Target temperatures and divergence of CO_2 levels were reached three days after filling in all treatments on October 22nd (hereafter called day 0) and the experiment ran until November 12th 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 10AU fluorometer (Turner Design). For the MZP samples, 250 mL of mesocosm water were transferred into brown glass bottles, fixed with acid Lugol's solution and stored dark. Counting and taxonomic identification of microzooplankton was carried out using the Utermöhl method (Utermöhl, 1958). Depending on the plankton density, either 50 or 100 mL of each sample were transferred to a sedimentation cylinder and allowed to settle for 24h prior to 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: $< 30\mu\text{m}$, medium: $30\text{-}55\mu\text{m}$, large: $> 55\mu\text{m}$). Biovolumes of ciliates were calculated according to geometric proxies by Hillebrand et al. (1999). For each group, the dimensions of twenty 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. (2015b) 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–Wiener (Shannon and Weaver, 1963) on a sample day basis.

For the statistical analysis, all data was 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 measure ANOVAs were calculated with ciliate biomass, total

copepod biomass, ciliate diversity or chlorophyll fluorescence as dependent variable. Two-way ANOVAs were performed to test for significant effects of temperature and CO₂ level (independent variables) as well as the interactions of these two factors on biomass maximum, D_{\max} and growth rate for total ciliates and single species of ciliates, respectively (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

Due 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 treatment cold low CO₂ only had two replicates instead of three).

Temperatures in the mesocosms were 9.44°C (± 0.39) and 14.78°C (± 0.31) and remained stable over the course of the experiment (Figure 1 A). The pCO₂ values decreased over time, but this was compensated by the addition of CO₂-enriched water on day 7, 12 and 19 (Figure 1 B). Overall, the average value was 439 ppm (± 180) for the low and 1040 ppm (± 228) for the high CO₂ treatments.

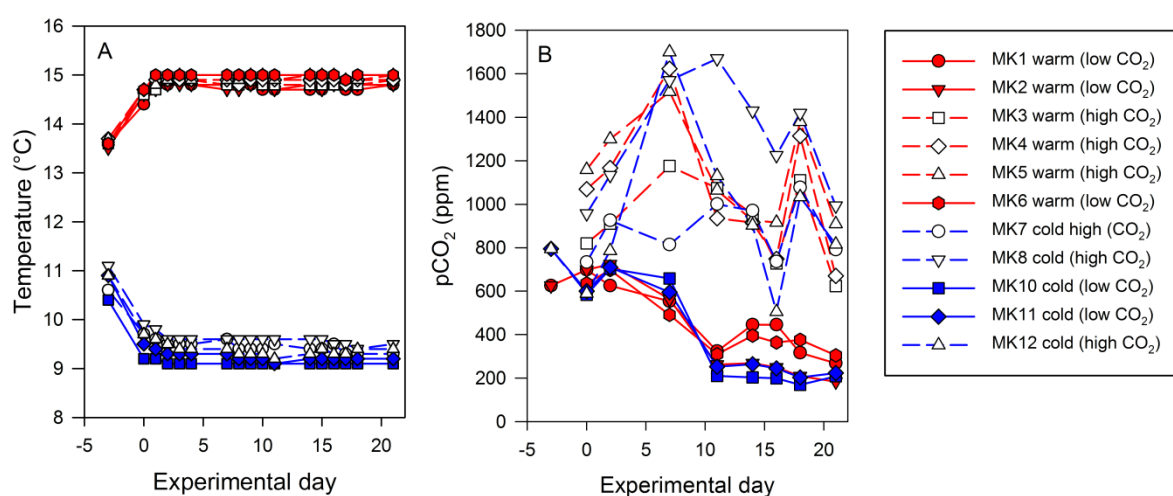


Figure 1: Actual temperatures and pCO₂ levels in the 12 mesocosms with the treatments of 9°C (blue) and 15°C (red) at low (540 ppm; full symbols) and high (1400 ppm; open symbols) CO₂ levels.

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 2 A, B). Ciliate biomass was significantly different between the temperature treatments although it depended on the time of the experiment (significant interaction time \times 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, Figure 2 B). 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 for the ciliate growth rate μ ($p < 0.017$). It was higher in the warm treatments (mean $0.45 \text{ d}^{-1} \pm 0.08$) than in the cold ones (mean $0.31 \text{ d}^{-1} \pm 0.03$). An effect of the interactions of temperature and CO_2 could not be found.

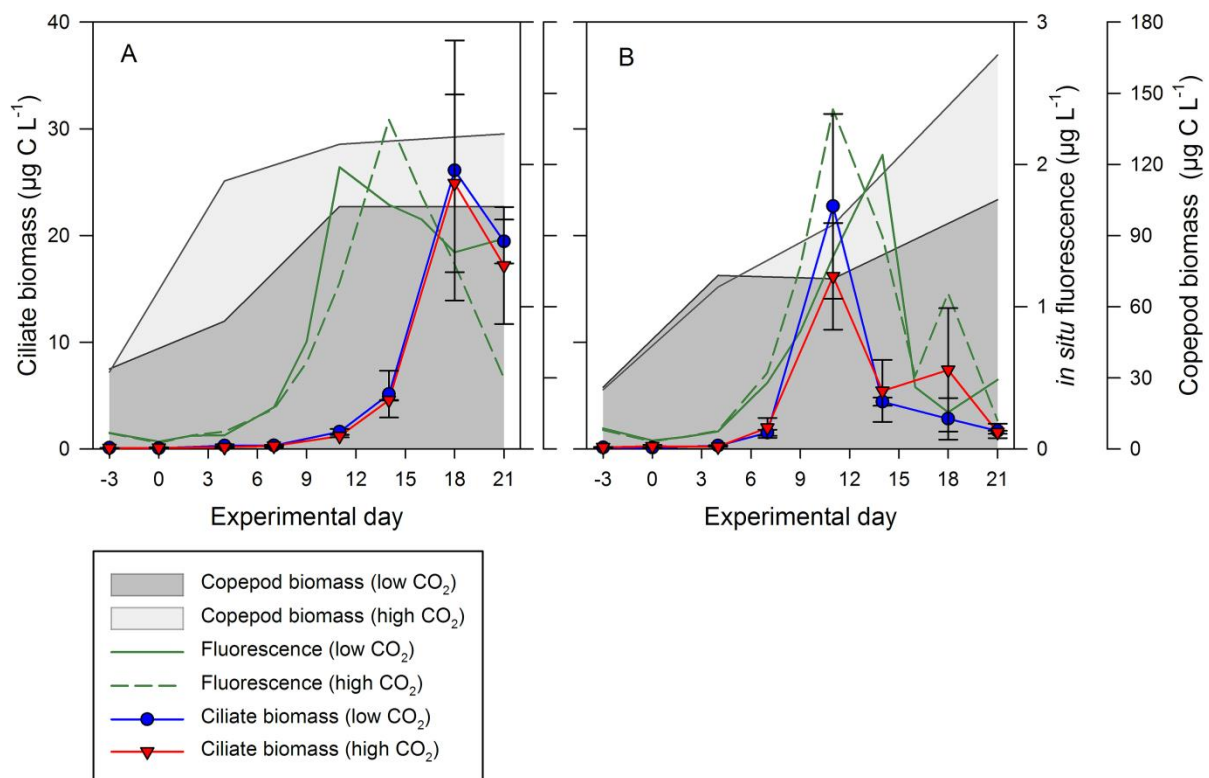


Figure 2: Ciliate biomass (mean \pm SD) in $\mu\text{g C L}^{-1}$ at low (blue) and high CO_2 levels (red) and total copepod biomass (adult copepods and copepodites) in $\mu\text{g C L}^{-1}$ at low (dark grey fields) and high CO_2 levels (light grey fields) as well as *in situ* fluorescence ($\mu\text{g L}^{-1}$) at low (green line) and high CO_2 levels (dashed green line) in the (A) cold and (B) warm treatments over the course of the experiment.

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 CO₂ treatments but not affected by temperature or the interaction of both factors (Table 1).

Table 1: Results of the repeated measure ANOVA for the effects of time, CO₂, temperature and their interactions on ciliate biomass, total copepod biomass, chlorophyll fluorescence and ciliate species diversity H' over the duration of the experiment. Significant results are marked by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Variable	Effect	dF	MS	F	p
ln total ciliate biomass ($\mu\text{g C L}^{-1}$)	CO ₂	1	0.000	0.000	0.994
	Temperature	1	0.402	1.707	0.233
	CO ₂ x temperature	1	0.441	1.870	0.214
	Time	6	33.87	123.45	<0.001 ***
	Time x CO ₂	6	0.307	1.120	0.367
	Time x temperature	6	8.755	31.91	<0.001 ***
	Time x CO ₂ x temperature	6	0.067	0.243	0.960
ln total copepod biomass ($\mu\text{g C L}^{-1}$)	CO ₂	1	1.023	5.683	0.044 *
	Temperature	1	0.034	0.187	0.677
	CO ₂ x temperature	1	0.186	1.031	0.340
	Time	2	0.658	7.038	0.006 **
	Time x CO ₂	2	0.051	0.544	0.591
	Time x temperature	2	0.274	2.931	0.082
	Time x CO ₂ x temperature	2	0.155	1.660	0.221
ln fluorescence ($\mu\text{g L}^{-1}$)	CO ₂	1	0.116	0.278	0.614
	Temperature	1	2.764	6.653	0.037 *
	CO ₂ x temperature	1	1.167	2.808	0.138
	Time	9	15.57	58.54	<0.001 ***
	Time x CO ₂	9	0.190	0.716	0.692
	Time x temperature	9	0.893	3.359	0.002 **
	Time x CO ₂ x temperature	9	0.242	0.909	0.523
Species diversity H'	CO ₂	1	0.010	0.425	0.535
	Temperature	1	0.799	34.44	<0.001 ***
	CO ₂ x temperature	1	0.024	1.055	0.339
	Time	6	0.319	5.240	<0.001 ***
	Time x CO ₂	6	0.082	1.352	0.256
	Time x temperature	6	0.190	3.113	0.013 *
	Time x CO ₂ x temperature	6	0.092	1.516	0.197

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. ($\mu\text{g C L}^{-1}$)	D_{\max} (d)	μ (d^{-1})
15°C	540	34.52	11	0.55
	540	19.78	11	0.44
	540	13.95	11	0.46
	1400	20.93	11	0.53
	1400	9.27	11	0.30
	1400	18.33	11	0.41
9°C	540	21.49	21	0.29
	540	38.27	18	0.29
	1400	28.47	18	0.29
	1400	23.82	21	0.30
	1400	32.82	18	0.38

Table 3: Results of the two-way ANOVA for the effects of temperature, CO₂ and their interactions on total ciliate biomass, *Strobilidium* sp. biomass and chlorophyll fluorescence regarding maximum (max.), bloom timing D_{\max} and growth rate μ . Results for ciliate species diversity H' are also given. Significant results are marked by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Response variable	Factor	dF	MS	F	p	
Biomass max. total ciliates ($\mu\text{g C L}^{-1}$)	CO ₂	1	43.57	0.634	0.452	
	Temperature	1	248.99	3.623	0.099	
	CO ₂ x temperature	1	17.09	0.249	0.633	
Biomass max. <i>Strobilidium</i> sp. ($\mu\text{g C L}^{-1}$)	CO ₂	1	0.146	1.145	0.320	
	Temperature	1	0.553	4.350	0.075	
	CO ₂ x temperature	1	0.149	1.173	0.315	
Fluorescence max. ($\mu\text{g L}^{-1}$)	CO ₂	1	0.079	0.214	0.658	
	Temperature	1	0.173	0.472	0.514	
	CO ₂ x 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	208.68	<0.001	***
	CO ₂ x temperature	1	0.001	0.110	0.749	
ln D_{\max} <i>Strobilidium</i> sp. (d)	CO ₂	1	0.001	0.110	0.075	
	Temperature	1	0.826	208.68	<0.001	***
	CO ₂ x 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 x 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 x temperature	1	0.007	1.287	0.294	
ln μ <i>Strobilidium</i> sp. (d^{-1})	CO ₂	1	0.160	1.229	0.304	
	Temperature	1	0.110	0.848	0.388	
	CO ₂ x temperature	1	0.126	0.965	0.359	

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 more than 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 afterwards 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 low 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. Due to a high variability between mesocosms, no significant effect for 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 low 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.

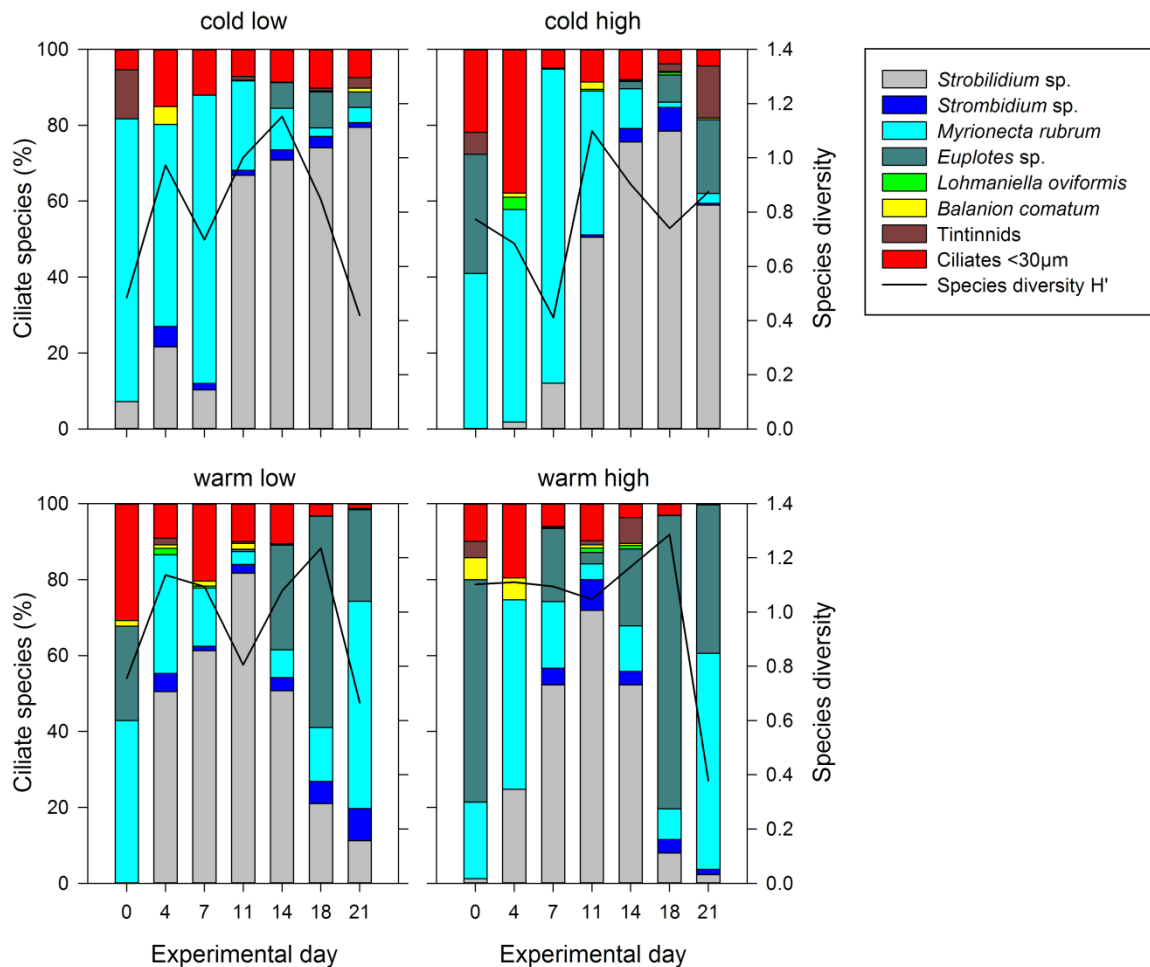


Figure 3: Relative ciliate species composition and species diversity index (H' ; black line) over the time of the experiment for the two temperature (warm and cold) and CO_2 treatments (low and high).

Discussion

Despite the fact that mesocosm approaches show some limitations when mimicking natural conditions such as diurnal variations in abiotic conditions (e.g. light, temperature) or vertical migration of zooplankton, mesocosms are a useful tool to simulate changes in abiotic conditions (e.g. warming, OA) and investigate their effects on plankton communities under near-natural conditions. While biases in species composition and food web complexity cannot be ruled out, the given experimental setup allowed the combined manipulation of the factors 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 to a very high degree. 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 fundamental difference of 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 imbalanced 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 (1) which is in line with findings from previous studies (Aberle et al., 2007; O'Connor et al., 2009; Aberle et al., 2012). Additionally, phytoplankton cell size decreased with warming thus rendering it into potentially 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 (1). 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 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 pCO₂

An increase in pCO₂ 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 pCO₂ 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 CO₂ elevation of effectively 1040 ppm, we showed that the present coastal MZP community was tolerant against the effects of CO₂. This might be related to a high pH tolerance of the Baltic Sea coastal plankton community in the Kiel Fjord to habitat pCO₂ 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 CO₂-rich water originating from the heterotrophic degradation of organic material (Melzner et al., 2013). Upwelling of CO₂-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 CO₂ input (Feely et al., 2008; Feely et al., 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 pCO₂ 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 (2) was not confirmed.

Further, we hypothesised that an elevated pCO₂ might result in a higher carrying capacity of phytoplankton thus leading to increases in MZP biomass (hypothesis 3). In the literature there is some evidence that such indirect effects are induced by an increase in pCO₂, 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 pCO₂ concentration can be e.g. an increased photosynthetic rate at high CO₂ levels due to an increased availability of CO₂ and HCO₃⁻ (Burkhardt et al., 2001; Rost et al., 2008), changes in stoichiometry affecting phytoplankton food quality (Burkhardt et al., 1999; Schoo et al., 2013) and inhibiting the development in case of calcifying algae (Riebesell et al., 2000b; Orr et al., 2005; Iglesias-Rodriguez et al., 2008). In our study, an increased phytoplankton biomass at high pCO₂ was observed at high temperatures only (Sommer et al., 2015). Consequently, a general increase in MZP biomass due to higher phytoplankton biomass at high pCO₂ could not be confirmed and hypothesis (3) was rejected. Nonetheless, copepod densities were higher in the high pCO₂ 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 pCO₂ were found; all of which can be attributed to the high tolerance of the phytoplankton community to a high pCO₂ (Paul et al., 2015b; Sommer et al., 2015). However, one effect reported from the present mesocosm study is an increased cell size of phytoplankton at high pCO₂, turning them into less preferred food items for ciliates (Sommer et al., 2015).

In the close-to-natural high pCO₂ 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 $p\text{CO}_2$ today, it could happen that the values in terms of acidification will be even higher than what is currently predicted as 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 $p\text{CO}_2$

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 only few multiple-stressor studies dealing with the effects of warming and high CO_2 in combination with e.g. 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; Kim et al., 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 food web 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., 2015). 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 $p\text{CO}_2$ is close to the atmospheric values. Generally, the aforementioned studies point towards the importance of indirect effects of elevated $p\text{CO}_2$ 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 pCO₂ and warming as far as MZP growth rate, total biomass and D_{max} are concerned thus supporting hypothesis (4). We observed no effects of high pCO₂ on MZP biomass or growth, not even in the cold treatments, where a masking of possible CO₂ 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 pCO₂ 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 CO₂ and warming between trophic levels (Riebesell et al., 2008; Maugendre et al., 2015).

Implications for the food web

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 CO₂ level did not affect any of the measured parameters. Phytoplankton stoichiometry was also not affected by CO₂ while biomass decreased and growth rates increased with warming (Paul et al., 2015b). Additionally, phytoplankton cell size increased at high pCO₂ (Sommer et al., 2015).

However, our study also included copepods as mesograzers. Ciliates are ideal food items for copepods due their ideal size compared to 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 µg C L⁻¹ initially and increasing in all treatments during the experiment, most notably in the high temperature/high CO₂ treatment. In fact, copepod biomass was significantly higher in the high CO₂ mesocosms which is in contrast to previous studies where no such effect was found (Rossoll et al., 2012; Cripps et al., 2014b). 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 CO₂ 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 copepods on MZP could be one of the factors explaining the comparatively low MZP biomass during the experiment. As pointed out by other studies, a high CO₂ 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 CO₂ 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 bottom-up control of plankton communities while a high CO₂ seems to promote top-down 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 pCO₂ in the future. This is most likely related to a high tolerance of MZP species to average pCO₂ levels of 700 ppm (Thomsen et al., 2010). In fact, most ecologically important groups in the Baltic Sea food web 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 CO₂ (see Paul et al. (2015b) 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).

CHAPTER III – Dynamics in the lower food web of a Baltic Sea summer plankton community in a changing ocean: a microzooplankton perspective

Abstract

Aquatic ecosystems face a multitude of environmental stressors among which warming and acidification might be the most important ones. While warming is expected to have a pronounced effect on plankton communities, many components of the plankton seem robust towards realistic end-of-century acidification conditions. However, interactions of the two stressors and the inclusion of further factors such as nutrient concentration and trophic interactions are expected to change this outcome. We investigated the effects of warming and high CO₂ on a nutrient-deplete late summer plankton community from the Kiel Fjord, Baltic Sea, using a mesocosm setup crossing two temperatures with a gradient of *p*CO₂ levels. Biomass, taxonomic composition, growth and grazing rates of microzooplankton (MZP) as well as growth rates of phytoplankton were analysed. Depending on the taxon in focus, we observed positive or negative effects of CO₂, temperature and their interactions on phytoplankton growth rates. Positive effects of the interaction of warming and CO₂ on MZP biomass were found while diversity, growth and grazing rates of MZP were not affected. In addition, ciliates were positively affected by the interactions of warming and CO₂ in terms of a higher biomass and earlier bloom peak while warming as a single stressor led to a lower biomass and a delayed bloom peak. Our results indicate that increased top-down control by copepods can mask any effects of warming and high CO₂ on MZP communities especially at times when phytoplankton biomass is low and mainly inedible phytoplankton (<2 µm) is present. This highlights the importance of considering trophic interactions when assessing climate change effects on marine plankton communities.

Introduction

Currently, CO₂ concentrations in the atmosphere have reached about 400 µatm (Le Quéré et al., 2013). This considerable increase from 280 µatm in pre-industrial times is predicted to continue, and according to the IPCC worst-case-scenario (RCP8.5), concentrations of up to 1000 µatm could occur by the end of this century (Collins et al., 2013). The oceans have absorbed about one third of the anthropogenic CO₂ emissions (Sabine et al., 2004) which has led to a change in carbonate chemistry and ocean acidification (OA). The decrease in pH with OA is most critical for organisms such as molluscs, corals, or calcifying phytoplankton, as their carbonate structures face higher incidences of dissolution (Orr et al., 2005; Kroeker et al., 2013). In contrast, positive effects have been shown for some phytoplankton groups which benefit from the fertilizing effect of an increased carbon concentration in the surface waters, including picophytoplankton (Rost et al., 2008; Brussaard et al., 2013; Low-Decarie et al., 2014; Sala et al., 2016). The dissimilar response of different phytoplankton groups is predicted to lead to changes in phytoplankton composition, stoichiometry, and biomass which then have the potential to affect higher trophic levels (Rose et al., 2009; Calbet et al., 2014).

In this respect, it is necessary to study the impact of OA not only on species but also on whole communities where biotic interactions and a possible dampening of the effects can be taken into account (Riebesell et al., 2013a; Rossoll et al., 2013). Mesocosm experiments are useful tools to investigate the impact of OA on natural plankton communities and have already been applied in different marine regions, e.g. the Baltic Sea, North Sea, North Atlantic, or the Arctic (Suffrian et al., 2008; Rose et al., 2009; Aberle et al., 2013; Calbet et al., 2014; Lischka et al., 2015). However, most of these studies were conducted during the spring bloom or with the addition of nutrients while the nutrient-deplete post bloom situation e.g. during late spring and summer has rarely been investigated so far. Yet, it seems that effects of OA are more likely to be visible under nutrient depletion which has been indicated by recent outdoor mesocosm experiments (Paul et al., 2015a; Sala et al., 2016; Bach et al., in review), even though it is not always the case (Riebesell et al., 2013b).

Apart from investigating the effects of an elevated CO₂ level under different nutrient concentrations, it is important to consider additional factors of environmental change. In multiple-stressor approaches, possible amplifications or dampening of the effects of single stressors can be observed (Pörtner, 2008; Rost et al., 2008). One important factor to add in a two-stressor system is warming as an increase of sea surface temperature of 1 to 5°C is predicted within this century (Collins et al., 2013). With warming, an overall decline in

phytoplankton biomass and productivity is expected (Boyce et al., 2010; Hoegh-Guldberg and Bruno, 2010). Even though small-sized phytoplankton has been shown to benefit from warming in experiments, the positive effects will likely be overcompensated by more intense zooplankton grazing based on the stronger temperature dependence of heterotrophs in comparison to autotrophs (Rose and Caron, 2007; Lewandowska et al., 2014). As a result from the unequal effects of warming on different trophic levels, a temporal mismatch between predators and their prey may occur (Durant et al., 2007; Thackeray et al., 2010).

Microzooplankton (MZP) of the size class 20-200 μm , with its high growth and grazing rates allowing an immediate response to changes in the phytoplankton community, is an important trophic intermediary between phytoplankton and higher trophic levels (Calbet and Landry, 2004). With a grazing rate of up to 75 % of the daily primary production, it can have a strong impact on the phytoplankton community leading to strong competition with mesozooplankton (Calbet and Landry, 2004; Aberle et al., 2007). At the same time, it functions a preferred food source for mesozooplankton such as copepods, especially at times when phytoplankton food quality is low (Malzahn et al., 2010; Löder et al., 2011).

In this study, we examined the interaction effects of temperature and elevated $p\text{CO}_2$ on MZP in a mesocosm experiment during a nutrient-deplete summer situation in the Kiel Fjord, Baltic Sea. In contrast to the nutrient-replete spring bloom dominated by diatoms, late summer phytoplankton communities are characterized by a high contribution of dinoflagellates, nanoflagellates and cyanobacteria (Smetacek, 1985; Sellner, 1997). CO_2 fluctuations in the Kiel Fjord are generally stronger than in the open ocean which is typical for highly productive coastal areas (Feely et al., 2010; Melzner et al., 2013). Today, average concentrations are about 700 μatm , with peak values of more than 2300 μatm occurring over short times in summer (Thomsen et al., 2010). While there is evidence for direct negative effects of high CO_2 levels on MZP (Hinga, 1992; Nielsen et al., 2010), previous Baltic Sea mesocosm studies have revealed no or only subtle effects of end-of-century CO_2 levels on MZP (Rossoll et al., 2013; Lischka et al., 2015; Horn et al., 2016). In contrast, a pronounced effect of warming on MZP can be expected (Aberle et al., 2007; Sommer et al., 2007). However, trophic interactions such as grazing pressure by copepods are likely to obscure potential effects on MZP biomass or taxonomic composition (Lewandowska et al., 2014). To unmask such predation effects, grazing experiments can provide further insights into MZP grazing and specific phytoplankton growth rates.

We hypothesize that (i) warming will enhance MZP growth, leading to an earlier bloom. Furthermore, dissimilar effects of CO_2 on different phytoplankton groups are expected to lead

to changes in the phytoplankton community composition, with picophytoplankton benefitting from the increase in CO₂. Hence (ii) an increase in small-sized phytoplankton with increasing CO₂ should indirectly affect MZP in terms of a higher biomass while direct effects of a high CO₂ level on MZP are unlikely to occur due to a high degree of tolerance of coastal MZP communities to elevated CO₂ conditions. For the combination of high CO₂ and warming, we expect that (iii) MZP biomass will be overall positively affected even though changes in the community composition might be observed in relation to CO₂-induced changes in prey availability.

Methods

Experimental design

The mesocosm experiment took place at GEOMAR, Kiel, Germany, where twelve mesocosms were set up in temperature-controlled rooms in summer 2013. On August 13th 2013, the mesocosms were filled with unfiltered water from the Kiel Fjord containing a natural assemblage of bacteria, phytoplankton and protozoa from about 2 m depth. By using a rotary pump with a twelve-way tube distribution system, equal starting conditions in all mesocosms were obtained. During the whole experiment, the mesocosms were gently stirred by slowly rotating propellers to ensure mixing and avoid plankton sedimentation. Previous experiments using the same setup have shown that this treatment does not damage plankton community (Sommer et al., 2007).

Two temperatures of 16.5 and 22.5°C (“cold” and “warm” in the following) were crossed with a gradient of *p*CO₂ values ranging from 500 to 3000 µatm. The temperatures represent a delta of 3°C from ambient conditions of 19.5°C on the filling day. This symmetrical temperature treatment avoids confounding the effects of the direction of temperature change with the effects of temperature change as such. The lowest target CO₂ concentration is based on ambient values of about 540 µatm measured on the filling day while the high target concentration represents the worst case summer scenario. Already today, peak values of more than 2300 µatm can occur in the Kiel Fjord over short time spans (Thomsen et al., 2010).

The mesocosms were closed with transparent PVC lids to reduce outgassing. A sampling port inserted in the lids was opened during measurements only. The target CO₂ levels of 500, 1000, 1500, 2000, 2500 and 3000 µatm were obtained by stepwise addition of CO₂-saturated

seawater. Additionally, CO₂-saturated seawater was added three times per week during the runtime of the experiment to compensate for outgassing and the natural drawdown of CO₂ caused by photosynthetic activity. The seawater for the addition was sampled from the Kiel Fjord on the mesocosm filling day, sterile filtered (0.2 µm) and stored dark and cool until use. Prior to addition, 20 L were aerated with CO₂ for at least 6 h to obtain saturation. The required amount of CO₂-saturated seawater was calculated with the CO2SYS program (version 2.1) by Lewis and Wallace (1998) based on the measurements of total alkalinity and dissolved inorganic carbon (DIC).

Purpose built light units equipped with HIBay-LED spotlights by Econlux (100 W each, lamp units HL3700 and ProfiluxII) were used for the illumination of the mesocosms. Day length and light intensity were calculated after Brock (1981) according to the season to resemble a cloudless day with a reduction of intensity by 40 % to account for underwater attenuation. The light:dark cycle of 14:10 h including dawn and dusk phases was computer controlled (GHL Advanced Technology GmbH & Co. KG, Kaiserslautern, Germany).

To simulate a high density of cyanobacteria as it is common for the Kiel Fjord during summer (Sellner, 1997), the filamentous cyanobacterium *Nodularia spumigena* was added to the mesocosms. *N. spumigena* was pre-cultured at 18°C and introduced into the mesocosms on August 14th 2013 to a final density of about 5160 cells L⁻¹. As the way the mesocosms were filled typically reduces the catchability for larger zooplankters, these were added separately. This addition of mesozooplankton from net catches from the Kiel Fjord took place on August 15th (day -1 in the following). The mesozooplankton, mainly copepods, had been caught the day before and was allowed to acclimatize in the temperature controlled rooms for 24 h prior to addition to the mesocosms. The density of 20 ind. L⁻¹ corresponded to summer copepod abundances measured in the Kiel Fjord (Javidpour et al., 2009). Full manipulation of the mesocosms was reached on August 16th 2013 (day 0). The experiment ran for 28 days until September 13th 2013.

Sampling and measurements

Temperature, salinity, pH and fluorescence were measured daily. Samples for total alkalinity and DIC for the calculation of the carbonate chemistry system were taken three times per week directly from the mesocosms. At the same time, 20 L of mesocosm water were sampled from the middle of the water column using a flexible silicone tube and transferred into carboys. Samples for nutrients, heterotrophic nanoflagellates, phyto- and microzooplankton were taken from the carboys. Details on measurements and analysis of carbonate chemistry,

abiotic parameters, nanoflagellates, and phytoplankton are presented by Paul et al. (2016). All data sets from this experiment are available at PANGAEA (www.pangaea.de).

Microzooplankton sampling and measurements

MZP samples of 250 mL were taken from the carboys once per week, transferred into brown glass bottles and fixed with acid Lugol's solution (final concentration: 1 %). These were stored cool and dark until counting. MZP was counted after Utermöhl (1958) with a Zeiss Axiovert 135 microscope. Sedimentation cylinders of 50 or 100 mL were used depending on plankton density and allowed to settle for 24 h prior to counting. To assure comparability of the counts both at high and low abundances and to reduce the counting bias against rare species, the whole surface of the sedimentation chamber was counted at 200-fold magnification. MZP was identified on species or genus level and otherwise grouped into size classes, using Carey (1992), (Montagnes et al., 2001) and Kraberg et al. (2010). The most frequently found dinoflagellates were *Prorocentrum micans*, *Prorocentrum minimum* and *Dinophysis* sp.. As these three taxa are capable of heterotrophic feeding modes, they were included in the MZP (Stoecker et al., 1997; Kraberg et al., 2010). Biovolumes were calculated using geometric proxies by after Hillebrand et al. (1999) based on the dimensions of 20 digitally measured random cells per group (AxioVision 4.9 and AxioCam, Carl Zeiss Microscopy GmbH). Using conversion factors provided by Putt and Stoecker (1989) for ciliates and Menden-Deuer and Lessard (2000) for dinoflagellates, MZP carbon biomass was calculated from the biovolumes.

Diversity of ciliates and dinoflagellates (H' , \log_e) was calculated on a sample day basis following Shannon and Weaver (1963). The day of the MZP biomass maximum in each mesocosm was defined as the bloom peak (D_{\max}). The time frame in consideration was from day 3 to 28 while day 0 had to be excluded due to the pronounced decrease in biomass in all mesocosms between day 0 and 3. MZP net growth rates μ were calculated based on an exponential growth model from day 3 to D_{\max} for each mesocosm.

Mesozooplankton sampling and measurements

Zooplankton was sampled once a week by three vertical net hauls, starting on day 0 of the experiment, with a hand-held plankton net (64 μm mesh size, 12 cm diameter, and from 150 cm depth). Each net haul sampled a volume of 5.1 L. All samples were fixed with Lugol's iodine. The zooplankton samples were divided with a sample splitter (HydroBios), so that $\frac{1}{4}$

of the sample volume was counted and identified. Copepods of the copepodite stage 1 to adult were identified to genus and developmental stage level. Prosome lengths (length of carapax) of each genus and developmental stage was measured digitally via photographs and digital software (AxioVision 4.8 and AxioCamMRc, Carl Zeiss Microscopy GmbH) with a precision to the nearest μm . Temporal biomass estimations were calculated from taxon and stage average sizes from day 0 samples. Average prosome lengths were used for taxon and stage abundances of each sampling date and treatment. Copepod biomass was calculated from length to carbon using conversions factors (Table 1).

Table 1: Length (L) to carbon (mg C) conversion factors used for copepodites (C1 to adult).

Taxon	a	b	Reference	Remarks
<i>Acartia</i> spp. ¹	1.11×10^{-11}	2.92	Berggreen et al. (1988)	Modified by Thor et al. (2005), L in μm
<i>Centropages</i> spp. ²	2.4492	-6.0984	Klein Breteler et al. (1982)	L in μm
<i>Oithona</i> spp. ¹	9.47×10^{-10}	2.16	Sabatini and Kiørboe (1994)	L in μm
<i>Pseudocalanus</i> spp. ¹	6.12×10^{-11}	2.7302	Klein Breteler et al. (1982)	Modified by Thor et al. (2005), L in μm

¹ Calculated from the following equation $a \cdot L^b$

² Calculated from the following equation $a \cdot \log(L) + b$, multiplied with 0.45 to convert into carbon.

Statistical analysis

A Generalized Least Squares (GLS) model was applied to analyse the effects of CO_2 , temperature, time and the interactions of these factors on MZP biomass and diversity. Autocorrelation of the data was tested by computing an Auto- and Cross-Covariance and -Correlation Function Estimation (ACF) and included in the model if required. Residuals were tested for normality using a Shapiro-Wilk test. If necessary, the data was log or $\log(x+1)$ transformed prior to re-fitting the model. Heteroscedasticity of the residuals was checked using conditioning plots and accounted for by including a variance component term. The full model was then optimized by stepwise removal of insignificant interactions. For selection of the best-fit model, Akaike's Information Criterion (AIC) was used as it takes into account both the complexity of the model and the goodness of fit.

To test for correlations between total copepod (adults and copepodites) and phytoplankton biomass with MZP biomass, a GLS analysis was conducted in the same way while excluding the impacts of CO_2 and temperature. The phytoplankton biomass fractions tested were phytoplankton $>2 \mu\text{m}$ and picophytoplankton $<2 \mu\text{m}$. The large phytoplankton fraction was

not further divided in edible or inedible groups as food size class preferences of MZP are generally difficult to assess. Especially heterotrophic dinoflagellates are able to consume phytoplankton from a wide range of cell sizes up to their own size (Hansen and Calado, 1999). Picophytoplankton was considered separately because only few small MZP species are known to feed on it while the majority prefers cells sizes $> 2 \mu\text{m}$ (Jonsson, 1986; Christaki et al., 1998; Johansson, 2004).

The effects of CO_2 and temperature on MZP, ciliate and dinoflagellate biomass maximum, D_{max} and net growth rate μ were analyzed using linear regressions. Residuals were tested for normality and heteroscedasticity with a Shapiro-Wilk normality test and a studentized Breusch-Pagan test. To optimize the model, insignificant interactions terms were removed.

R Studio was used for all analyses (R Development Core Team, 2011; RStudio Team, 2011) with the additional packages *lmtest* (Hothorn et al., 2011), *nlme* (Pinheiro et al., 2011), and *vegan* (Oksanen et al., 2011). Graphs were created with SigmaPlot 12.5 (Systat Software, Inc.).

Dilution Experiments

Setup

Dilution experiments after Landry and Hassett (1982) were conducted twice during the mesocosms study, on day 13 and 20. They allow both calculating the phytoplankton net growth rate μ and the instantaneous growth rate μ_0 excluding the grazing impact of MZP. Six of the twelve mesocosms were chosen for the experiment; low (500 μatm), medium (1500 μatm) and high CO_2 concentrations (3000 μatm) at both cold and warm temperature. For the dilutions we used the water that had been collected on the mesocosm filling day. This water was filtered with a 0.45/0.2 μm sterile inline filter (Sartobran P 300, Sartorius AG) using a peristaltic pump and stored dark and cool until use. Prior to addition to the dilutions, it was filtered once more with the same kind of filter cartridge.

Dilutions of 10, 25, 50 and 100% undiluted mesocosm water were set up in separate carboys by adding mesocosm water to the sterile filtered water. Additionally, one undiluted sample was set up to serve as control. With exception of the control, nutrient solutions after Guillard and Ryther (1962) reduced by half were added to carboys to prevent nutrient limitation.

Fluorescence of phytoplankton start samples from each carboy were measured using a BBE Algae Analyzer (Moldaenke, Kiel, Germany). Incubation bottles were set up in triplicates and fixed on plankton wheels placed in the respective climate rooms to ensure the same temperature and light conditions as in the mesocosms. After 24 h, each incubation bottle was

measured with the Algae Analyzer. Due to the handling time necessary for each mesocosm, the dilutions were started time-delayed. One set of three mesocosms was started the first day, the other set on the following day.

Data analysis

Growth rates were determined based on total fluorescence as proxy for the total phytoplankton and for the algae groups distinguished by the BBE Algae Analyzer, namely green algae, bluegreen algae, cryptophytes, and diatoms. Phytoplankton net growth rate μ was calculated using an exponential growth model (Landry and Hassett, 1982; Landry et al., 1995):

$$\mu = \frac{1}{t} \ln \frac{P_{24}}{P_0} \quad (1)$$

where t is the incubation time in days and P_0 and P_{24} are the phytoplankton concentrations at the start time and after 24 h. In the dilutions, P_0 was below detection limit of the Algae Analyzer in some cases (resolution: $0.01 \mu\text{g Chl } a \text{ L}^{-1}$), resulting in a measured concentration of zero for the start time while the measurements from P_{24} showed that phytoplankton growth had taken place. Thus all measured concentrations were transformed by +1. From a linear regression of the dilution factor α against phytoplankton net growth μ , the phytoplankton growth rate k and phytoplankton mortality m were obtained as the intercept with the y-axis and the slope of the regression, respectively:

$$\mu = k + m \alpha \quad (2)$$

The MZP grazing rate g is the negative phytoplankton mortality. All negative grazing rates were set to zero. The instantaneous (natural) phytoplankton growth rate μ_0 was calculated by adding phytoplankton mortality m to the net growth rate μ obtained from the controls grown without the addition of nutrients. Effects of CO_2 , temperature and the interactions of these factors on μ , μ_0 , m and g were tested with linear regressions for the algae classes distinguished by the Algae Analyzer. Distributions of the residuals were tested with a Shapiro-Wilk normality test and a studentized Breusch-Pagan test. Insignificant interactions terms were removed to optimize the model.

Results

There was a certain degree of $p\text{CO}_2$ fluctuation in the mesocosms during the experiment, due to outgassing, photosynthetic drawdown of CO_2 and subsequent CO_2 addition. Nevertheless,

distinct treatments were sustained over the duration of the experiment even though the actual CO₂ values stayed below the target concentrations (Figure 1). This was especially the case for the 2500 and 3000 µatm target levels.

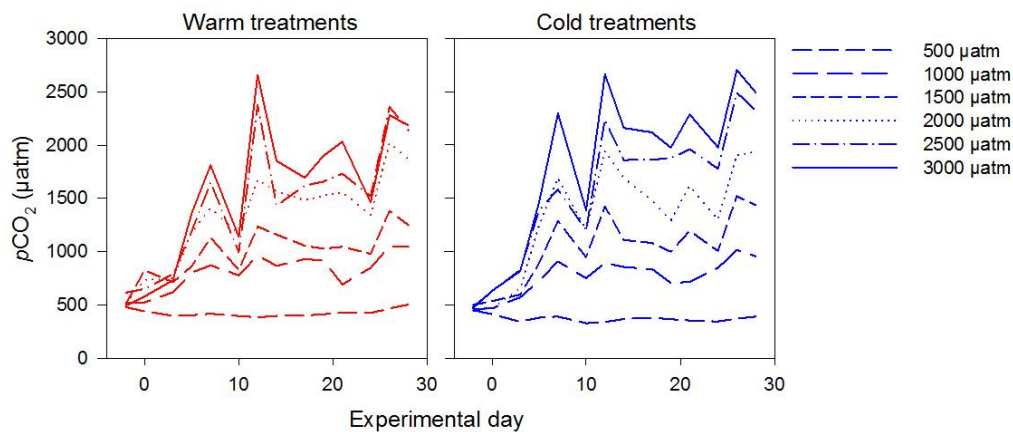


Figure 1: CO₂ development in the warm (red) and cold (blue) mesocosms from day -2 to day 28.

Phytoplankton succession

Initial total phytoplankton biomass of 184.12 (± 55.91) $\mu\text{g C L}^{-1}$ on day 0 decreased in all treatments over the course of the experiment to an average concentration of 33.67 (± 12.21) in the cold and 24.69 (± 8.58) $\mu\text{g C L}^{-1}$ in the warm treatments on day 28 (Figure 2). When regarding the different size classes separately, picophytoplankton $< 2 \mu\text{m}$ made up the main part of the total phytoplankton biomass during the experiment with an average concentration of 143.68 (± 54.18) $\mu\text{g C L}^{-1}$ on day 0. Until day 12, it decreased in all treatments, forming bloom peaks around day 5 in the warm mesocosms and day 10 in the cold ones, with the exception of the warm 3000 µatm treatments where the peak was on day 10 as well. After day 12 in the post-bloom phase, picophytoplankton biomass decreased to concentrations of 23.02 (± 8.29) and 30.81 (± 13.22) $\mu\text{g C L}^{-1}$ on day 28 in the warm and the cold treatments, respectively.

Biomass of phytoplankton $> 2 \mu\text{m}$ started at ~ 40.44 (± 8.03) $\mu\text{g C L}^{-1}$ on day 0. However, this size class did form blooms around day 7 in the cold mesocosms during which biomasses between 42.33 and 100.22 $\mu\text{g C L}^{-1}$ were measured. This increase in phytoplankton $> 2 \mu\text{m}$ was not observed in the warm treatments. Biomass concentrations in the cold and warm treatments were reduced to 2.86 (± 1.84) and 1.67 (± 0.74) $\mu\text{g C L}^{-1}$ on day 28.

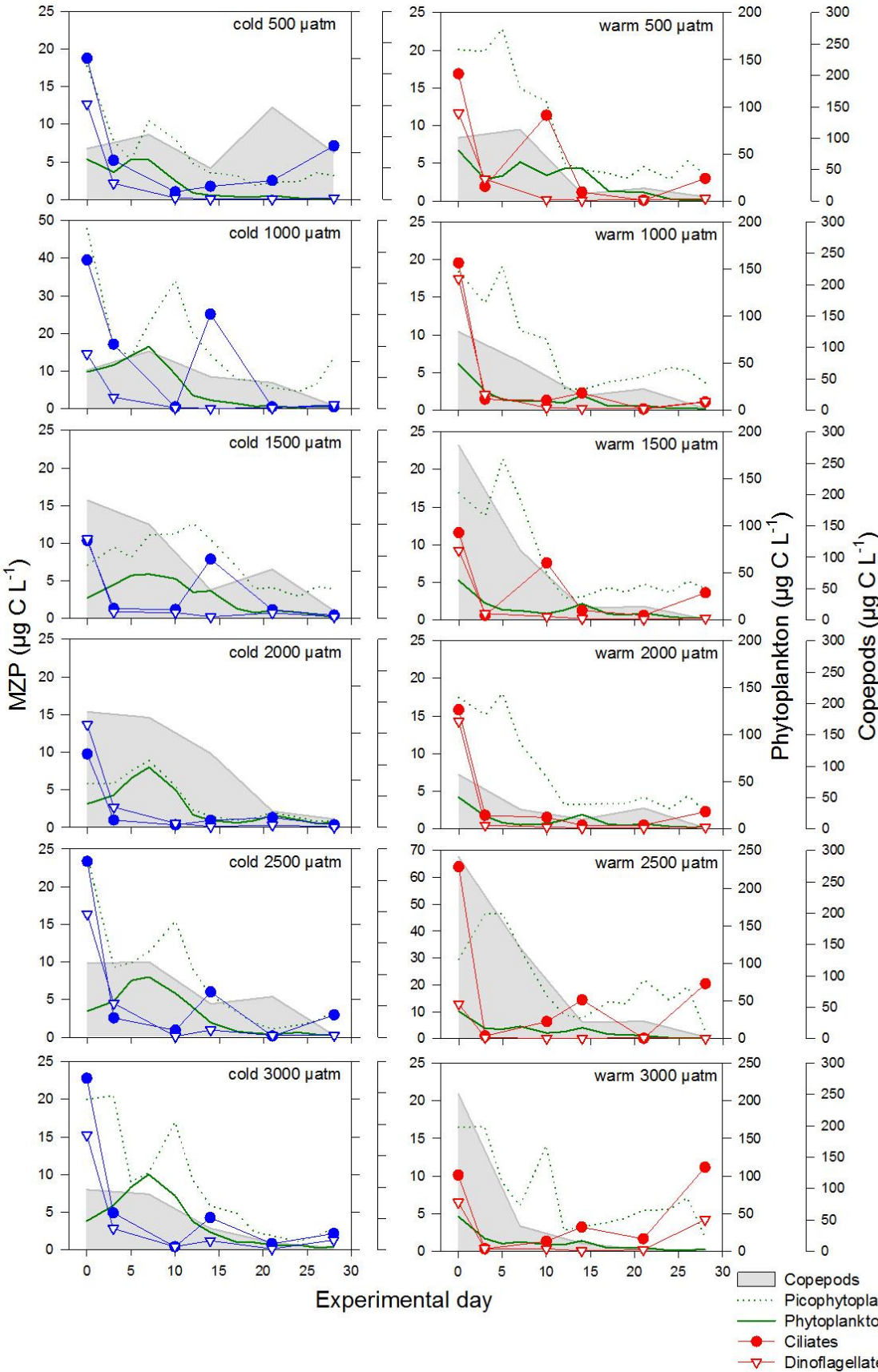


Figure 2: Ciliate (filled symbols) and dinoflagellate biomass (open symbols) in the cold (blue) and warm (red) treatments. Green lines show phytoplankton biomass (dotted: picophytoplankton $<2\ \mu\text{m}$; solid: phytoplankton $>2\ \mu\text{m}$) and grey areas the total copepod biomass (adults and copepodites). All parameters in $\mu\text{g C L}^{-1}$.

Mesozooplankton succession

On day 0, total copepod biomass in the cold and warm mesocosms was at 121.52 (± 48.91) and 188.57 (± 85.89) $\mu\text{g C L}^{-1}$, respectively (Figure 2). It decreased in all treatments during the runtime of the experiment, most notably in the warm ones, where concentrations of ~ 17.81 (± 5.29) $\mu\text{g C L}^{-1}$ were reached on day 14. In comparison, mean biomass of copepods in the cold treatments was at ~ 58.73 (± 27.27) $\mu\text{g C L}^{-1}$ on day 14. In the cold mesocosms at 500, 1500, and 2500 μatm , a moderate increase in copepod biomass was observed around day 21. Final copepod densities on day 28 were 18.57 (± 25.34) in the cold and 2.95 (± 1.88) $\mu\text{g C L}^{-1}$ in the warm treatments.

Microzooplankton succession

Total MZP had an average initial biomass of 34.81 (± 16.06) $\mu\text{g C L}^{-1}$ on day 0. We observed a significant positive effect of the interactions of CO_2 , temperature and time ($p = 0.010$, Table 2) as indicated by the t-value. If size classes were regarded separately, MZP $< 30 \mu\text{m}$ was also significantly positively affected by the 3-way interaction ($p = 0.007$), while MZP $> 30 \mu\text{m}$ was negatively affected by time or temperature as a single factors ($p = 0.044$). Total MZP biomass maximum and MZP net growth rate μ were not affected by CO_2 or temperature (Figure 3), but we observed a significant positive effect of the interaction of CO_2 and temperature leading to an earlier timing of the maximum ($p = 0.017$, Table S1 in the Supplement).

Ciliate biomass (Figure 2) in the cold treatments decreased from 20.75 (± 9.96) to 5.35 (± 5.51) $\mu\text{g C L}^{-1}$ on day 3. In the warm treatments, this decline was more pronounced, from 22.98 (± 18.57) to 1.19 (± 0.59) $\mu\text{g C L}^{-1}$. Between day 10 and 14 in the warm treatments, an increase in biomass could be observed for the 500, 1500, 2500 and 3000 μatm mesocosms. In addition, ciliates increased in biomass in the warm 2500 and 3000 μatm mesocosms towards day 28. The increase was observed later on day 14 in the cold treatments.

Overall, ciliate biomass was significantly negatively affected by temperature ($p = 0.037$, Table 2) while CO_2 or the interaction of these factors had no effect. There was also no effect of CO_2 or temperature on the ciliate biomass maximum or net growth rate μ (Figure 3, Table S1). The timing of the biomass maximum, D_{max} , was negatively affected by CO_2 ($p = 0.037$) and temperature ($p = 0.016$), while the interactions of temperature and time resulted in an earlier timing ($p = 0.004$).

Dinoflagellates were reduced from an initial biomass of 13.34 (± 1.61) to 2.38 (± 0.91) $\mu\text{g L}^{-1}$ on day 3 in the cold treatments (Figure 2). In the warm treatments, they decreased from 11.91

(± 1.61) to $1.18 (\pm 0.96) \mu\text{g L}^{-1}$ until day 3. A moderate increase in athecate dinoflagellates was observed in the cold treatments at $3000 \mu\text{atm}$ on day 28, leading to a final dinoflagellate biomass of $1.24 \mu\text{g L}^{-1}$ (Figure 2). In the warm treatments, a similar trend was observed for the $3000 \mu\text{atm}$ mesocosm resulting in a final concentration of $3.71 \mu\text{g L}^{-1}$, respectively.

While total dinoflagellate biomass was not affected by CO_2 or temperature, there was a significantly negative effect of the interactions of CO_2 and temperature ($p = 0.001$, Table 2). In contrast, the interaction of CO_2 and time affected dinoflagellate biomass positively ($p = 0.005$). For dinoflagellates, growth rates could be calculated for two high CO_2 treatments at cold temperatures. D_{max} was on day 3 for all other treatments, thus the timing of the biomass peak could not be evaluated and no additional growth rates could be determined.

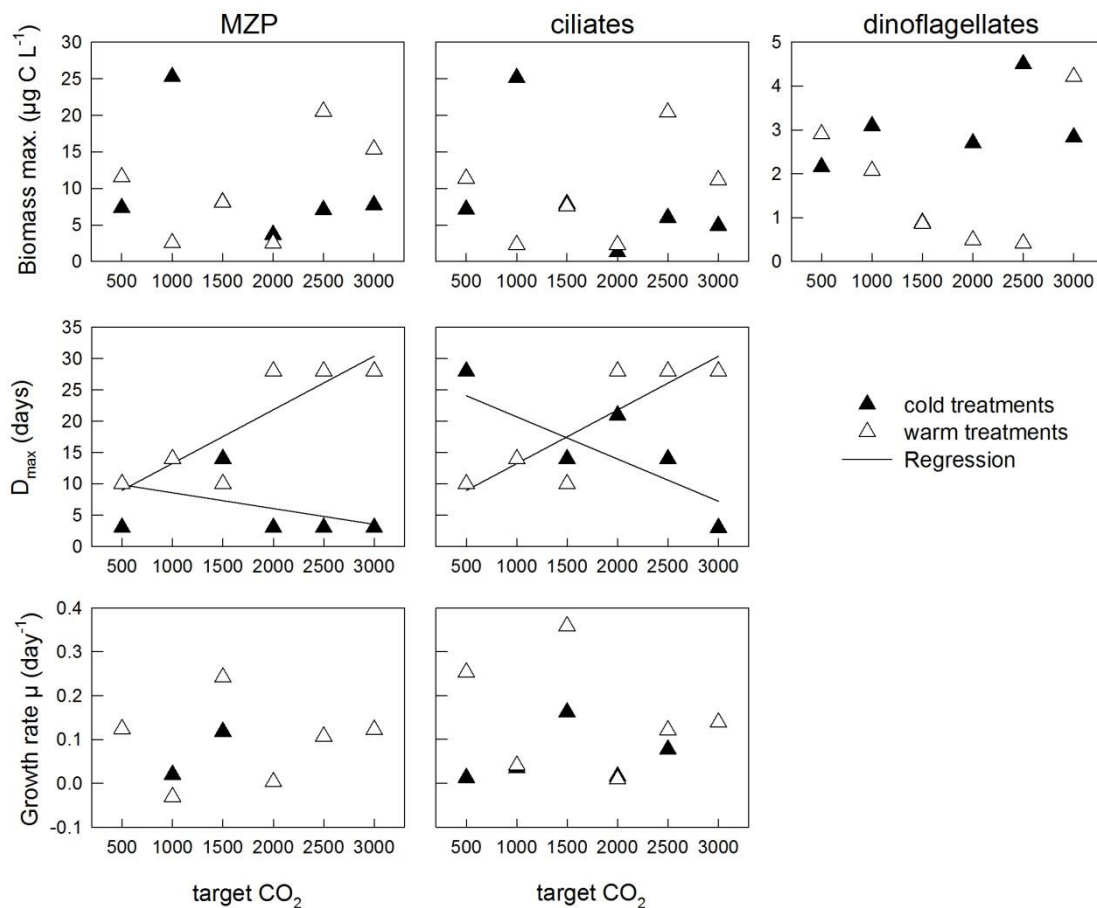


Figure 3: Biomass maximum, timing of the maximum D_{max} and net growth rate μ plotted for total MZP, ciliates and dinoflagellates for the different CO_2 levels at cold (black symbols) and warm (white symbols) temperatures. Significant effects and interactions are indicated by regression lines.

Table 2: Results from the GLS analysis of the effects of CO₂, temperature, time and the interactions of these factors on MZP biomass and diversity. Significances are indicated by asterisks.

Variable		Factor	t	p
Biomass	Total MZP	CO ₂	-0.240	0.811
		Temperature	-0.386	0.701
		Time	-1.945	0.056
		CO ₂ x temperature	-1.553	0.125
		CO ₂ x time	-0.678	0.501
		Temperature x time	-0.003	0.998
		CO ₂ x temperature x time	2.669	0.010 *
	MZP <30 µm	CO ₂	0.418	0.677
		Temperature	-1.070	0.289
		Time	1.188	0.239
		CO ₂ x temperature	-1.488	0.142
		CO ₂ x time	0.859	0.393
		Temperature x time	1.618	0.111
		CO ₂ x temperature x time	2.765	0.007 **
	MZP >30 µm	CO ₂	-0.662	0.510
		Temperature	-2.056	0.044 *
		Time	-4.282	<0.001 ***
	Ciliates	CO ₂	-0.918	0.362
		Temperature	-2.123	0.037 *
		Time	0.698	0.488
<i>Strombidium</i> sp.	CO ₂	-1.515	0.135	
	Temperature	-3.166	0.002 **	
	Time	-2.743	0.008 **	
Dinoflagellates	CO ₂	0.486	0.629	
	Temperature	0.890	0.377	
	Time	-2.876	0.005 **	
	CO ₂ x temperature	-3.336	0.001 **	
	CO ₂ x time	2.885	0.005 **	
Diversity	Ciliates	CO ₂	1.193	0.237
		Temperature	-0.640	0.524
		Time	-1.734	0.088
		CO ₂ x temperature	-2.110	0.039 *
	Dinoflagellates	CO ₂	0.581	0.563
		Temperature	-1.791	0.078
		Time	2.005	0.049 *

Ciliate community composition

Initial taxonomic composition on day 0 was similar between all treatments (Figure 4) with the exception of the warm 500 and 2500 µatm mesocosm, where higher proportions of *Strombidium* sp. occurred. The dominating taxa in terms of biomass were *Strombidium* sp., *Strombidium* sp. and *Balanion comatum*; also loricate Tintinnids and *Myrionecta rubra* were

present. However, while biomass was generally low from day 3 on (Figure 2), the composition of ciliate taxa was different for the cold and warm treatments.

The cold mesocosms were dominated by *Strombidium* sp. and *B. comatum*. Indeed GLS analysis showed significantly negative effects of time and of temperature on *Strombidium* sp. (p-value = 0.002, Table 2). The *B. comatum* dominance (up to 90%) in the cold treatments between day 10 and 14 only appeared in the three cold mesocosm with the highest CO₂ levels. At 500 and 1500 µatm CO₂, *Strombidium* sp. was the most common taxon while at 1000 µatm a high contribution of *Euplotes* sp. and *Suctorina* sp. was found. Diversity decreased from day 0 to day 10 but increased again afterwards until day 28. At high CO₂, diversity was lowest on the last day. In the warm treatments, diversity showed a similar trend, decreasing towards day 10 when small *Strombidium* sp. contributed 84-97 % to the biomass irrespective of the CO₂ level. Subsequently, diversity increased again in all treatments when *Strombidium* sp., *Euplotes* sp. and *Suctorina* sp. reached higher proportions. On day 28 choreotrich *Lohmaniella oviformis* appeared at varying percentages in the warm treatments only while diversity was very low due to a domination of *Strombidium* sp..

Overall, no significant effect of CO₂ or temperature on ciliate diversity was found while the interaction of these factors had a negative effect (p-value = 0.039, Table 2) which could be seen in the higher diversity at low CO₂ in the cold mesocosms.

Dinoflagellate community composition

We observed a dominance of thecate dinoflagellates in both the cold and warm treatments (Figure 5). In terms of biomass the size class >30 µm was represented mostly by mixotrophic *P. micans* but its contribution decreased towards day 28 when it was almost completely absent. Large *Dinophysis* sp. were found in low concentrations throughout the experiment. This was the same development for both temperature treatments. Likewise, the size class <30 µm was dominated by mixotrophic *P. minimum* in all mesocosms.

The contribution of athecate dinoflagellates increased in all treatments between day 21 and 28 except for the warm 1500 and 2500 µatm mesocosms. The highest proportion of athecate dinoflagellates was reached in the 3000 µatm mesocosms in the cold and warm treatments with 88 % and 95 %, respectively. We found no effect of CO₂ or temperature or the interactions of these factors on dinoflagellate diversity, only time had a significantly positive effect (Table 2).

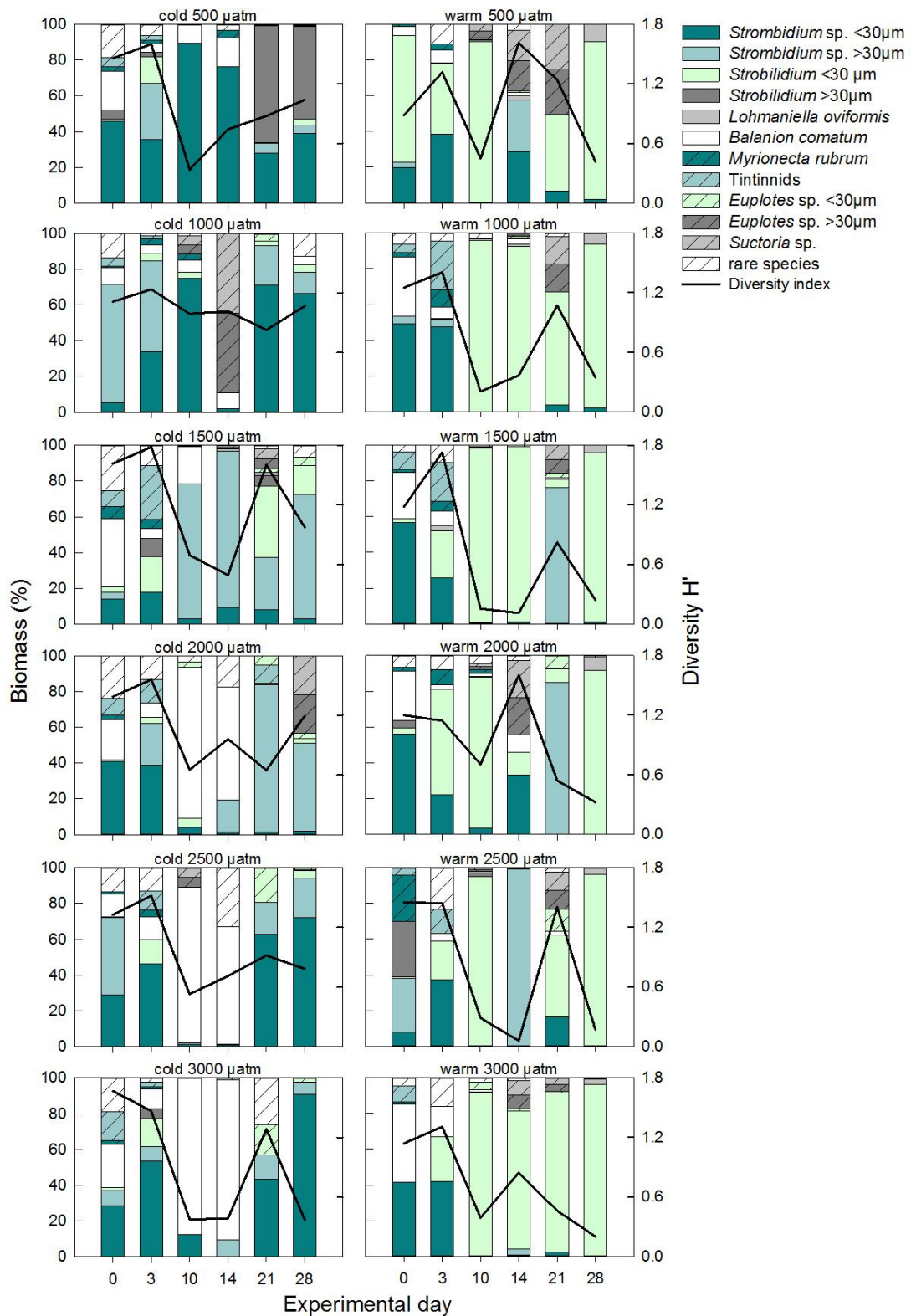


Figure 4: Taxonomic composition of the ciliate community in the cold and warm treatments based on biomass. The black line indicates species diversity H' .

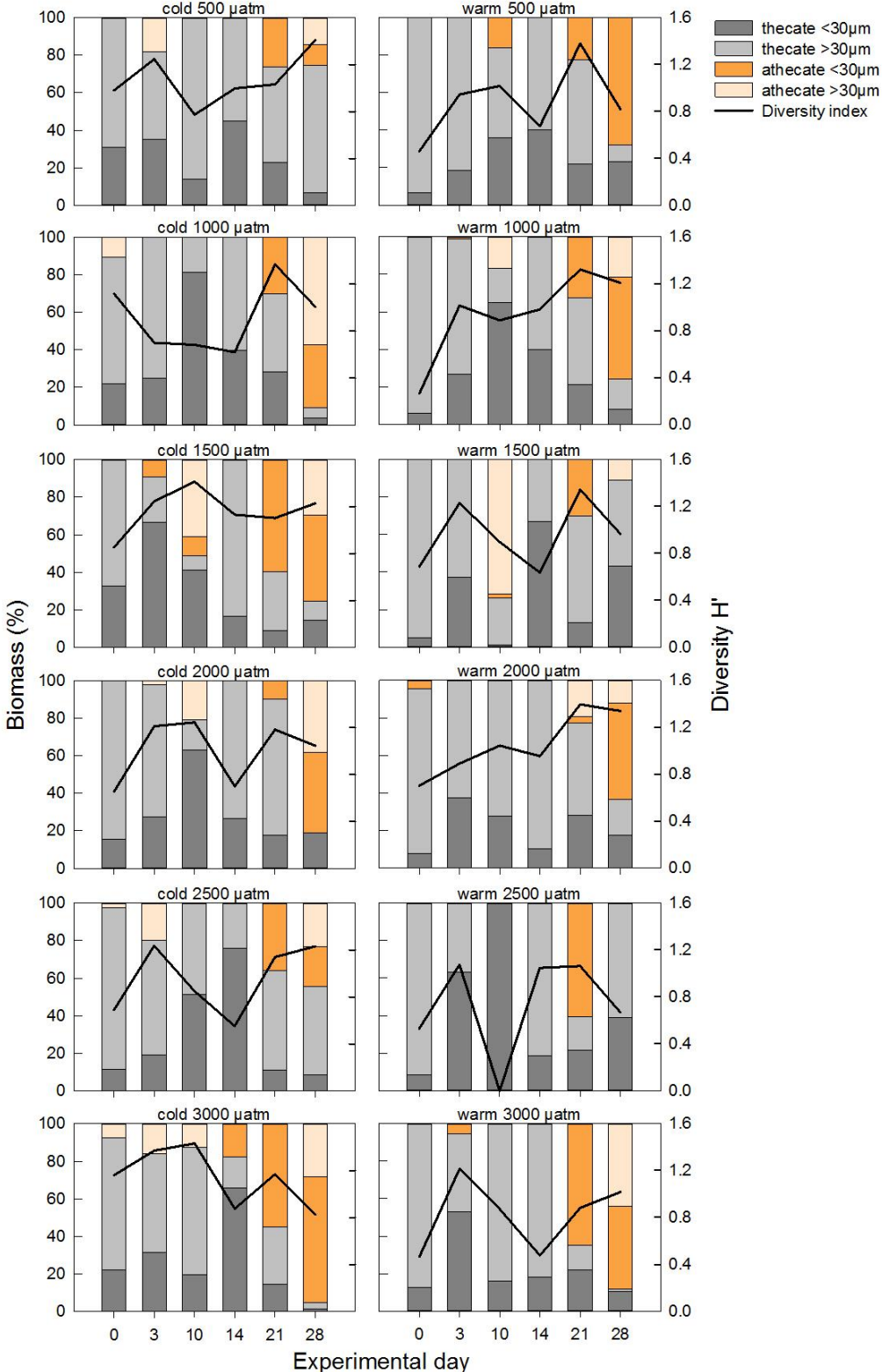


Figure 5: Size-class distribution of thecate and athecate dinoflagellates in the cold and warm treatments based on biomass. The black line indicates the species diversity H['].

Dilution experiments

In the 1st dilution experiment which took place on day 13, we observed no effect of CO₂ on the net growth rate μ and instantaneous growth rate μ_0 of total phytoplankton and diatoms. However, a positive effect of temperature ($p < 0.001$, Table 3) and a negative effect of the interaction of CO₂ and temperature ($p < 0.001$) were found. μ of green algae was not affected by CO₂ or temperature. Cryptophytes were only present in the warm mesocosms during experiment 1 where a significantly positive effect of CO₂ ($p = 0.004$) and a negative effect of temperature ($p = 0.002$) on the net growth rate μ was observed. Bluegreen algae were the only group where a positive effect of CO₂ on μ_0 ($p = 0.020$) and a negative temperature effect ($p < 0.001$) were found.

Table 3: Results from the dilution experiments. Effects of CO₂, temperature and the interactions of these factors on phytoplankton net growth rates μ and instantaneous growth rates μ_0 for total fluorescence (total f) and the different phytoplankton groups are shown. Significances are indicated by asterisks.

Experiment	Variable	Factor	t	p		
1	μ	Total f	CO ₂	1.614	0.129	
			Temperature	12.122	<0.001	***
			CO ₂ x temperature	-8.880	<0.001	***
		Green algae	CO ₂	0.666	0.515	
			Temperature	0.192	0.850	
		Diatoms	CO ₂	1.712	0.109	
			Temperature	10.998	<0.001	***
			CO ₂ x temperature	-8.470	<0.001	***
		Cryptophytes	CO ₂	3.393	0.004	**
	Temperature		-3.813	0.002	**	
	μ_0	Total f	CO ₂	-0.621	0.545	
			Temperature	12.508	<0.001	***
			CO ₂ x temperature	-5.677	<0.001	***
		Diatoms	CO ₂	1.346	0.200	
			Temperature	17.037	<0.001	***
			CO ₂ x temperature	-9.947	<0.001	***
		Bluegreen algae	CO ₂	2.606	0.020	*
			Temperature	-12.604	<0.001	***
2		μ	Green algae	CO ₂	-3.179	0.006
	Temperature			3.767	0.002	**
	Bluegreen algae		CO ₂	-0.170	0.867	
			Temperature	-9.611	<0.001	***
			CO ₂ x temperature	6.605	<0.001	***
	μ_0	Green algae	CO ₂	-4.943	<0.001	***
			Temperature	3.720	0.002	**
		Bluegreen algae	CO ₂	3.274	0.005	**
			Temperature	-4.101	0.001	**

During the 2nd dilution experiment on day 20 (post-bloom phase), the phytoplankton community had changed. The only groups in high densities for which growth rates could be calculated were green and bluegreen algae. For green algae, both growth μ and μ_0 were positively affected by temperature ($p = 0.002$) but negatively by CO_2 ($p = 0.006$ and $p < 0.001$). There was also no effect of CO_2 on net growth rate μ of bluegreen algae, but a negative effect of temperature ($p < 0.001$) while the interaction of the two factors had an overall positive effect ($p < 0.001$). μ_0 of bluegreen algae was positively affected by CO_2 ($p = 0.005$) and negatively by temperature ($p = 0.001$).

Neither in the 1st nor in the 2nd experiment effects of CO_2 , temperature or the interactions of these factors on phytoplankton mortality m or MZP grazing rate g were observed (Table S2).

Discussion

Temperature effects

Warming is predicted to affect autotrophic and heterotrophic components of the plankton unequally. For heterotrophic MZP, a positive relationship between an increase in temperature and an increase in productivity has been described (Weisse and Montagnes, 1998; Montagnes and Lessard, 1999; Rose and Caron, 2007). The temperature-stimulated increase in MZP can result in a strong top-down effect on phytoplankton which is less affected by warming due to the temperature insensitivity of the photosynthesis (O'Connor et al., 2009; Sommer and Lewandowska, 2011). Thus an overall decline in phytoplankton biomass and productivity is expected with warming (Boyce et al., 2010; Hoegh-Guldberg and Bruno, 2010), and indeed a lower phytoplankton biomass with elevated temperatures has been observed in Baltic Sea plankton communities (Sommer et al., 2007; Sommer and Lewandowska, 2011; Winder et al., 2012).

In this study, changes in MZP community composition with warming were observed even though the diversity was not affected by temperature as a single stressor. In addition, total phytoplankton biomass decreased with warming (Paul et al., 2016). However, we observed no effect of warming on biomass of total MZP, MZP $< 30 \mu\text{m}$, and dinoflagellates while warming led to a lower biomass of ciliates and MZP $> 30 \mu\text{m}$ (Table 2). Additionally, warmer temperatures led to a delayed timing of the MZP biomass maximum of ciliates. This is in contrast to our expectations predicting an enhanced MZP growth and an earlier bloom peak with warmer temperatures, thus hypothesis (i) was rejected. Furthermore, this is also in

contrast to previous studies where the group of ciliates was positively affected by temperature in terms of higher growth rates and an earlier bloom peak (Aberle et al., 2007; Lewandowska et al., 2014; Horn et al., 2016). Considering that copepod biomass was higher in the warm treatments during the first half of the experiment but decreased during the second half, the delayed bloom peak of ciliates might have been caused by intense grazing pressure as warming is expected to enhance top-down control by copepods as well.

Especially the genus *Strombidium* sp. is known to strongly respond to temperature, usually showing a positive response to warming (Montagnes, 1996). This finding was not supported by the present study, but as Aberle et al. (2007) pointed out it is not only the effect of warming which leads to changes in ciliate species composition, but rather a combination with other biotic factors, e.g. phytoplankton density and composition, which need to be considered in detail. We observed that both phytoplankton density and community composition were affected by warming (Paul et al., 2016), suggesting that such changes in prey availability and community composition directly translated into changes in MZP composition and diversity as MZP is regarded being highly controlled by the food supply (Irigoien et al., 2004).

It is unlikely that the increase in temperature to up to 22.5 °C might have had a direct negative impact on the MZP as temperatures up to 25 °C usually lead to an increase in growth rate in comparison to a decrease at temperatures <18 °C (Rose and Caron, 2007). Furthermore, we found an overall low MZP biomass in this study, not just in the warm treatments. As mesozooplankton is also positively affected by warmer temperatures in terms of increased grazing activities (Lewandowska et al., 2014), the low MZP biomass was most likely caused by intense copepod grazing. The most common copepod taxon in the mesocosms was *Acartia* sp. (data not shown) which is known to have a high predation rate on ciliates (Jonsson and Tiselius, 1990). In addition, the density of phytoplankton of the size range edible for copepods (>2 µm) was very low during the experiment, especially during the second half and in the warm treatments, so it is most likely that the copepods grazed substantially on MZP instead.

The nutrient-deplete status of the late summer community used in this experiment can also explain the negative effect of warming observed (O'Connor et al., 2009; Lewandowska et al., 2014). It has already been suggested by Lewandowska et al. (2014) that effects of warming on marine plankton depend on the nutrient regime. Under nutrient-rich conditions as e.g. during a spring-bloom situation, large phytoplankton mainly consisting of diatoms presents a food source not only for MZP but also for copepods, resulting in a moderate grazing pressure on MPZ. In nutrient-limited waters, phytoplankton mainly consists of small-sized groups

inedible for copepods, thus the grazing pressure on MZP increases considerably and so does the importance of the microbial loop. Indeed, the pronounced top-down effect leading to a low MZP biomass in our experiment seems to have been caused by picophytoplankton being by far the most abundant phytoplankton size class. For MZP, which is also known to prey on heterotrophic nanoflagellates and bacteria, picophytoplankton is within the range of edible phytoplankton (Christaki et al., 1998).

Interestingly, the negative effect of warming on MPZ concentrations in a nutrient-limited situation we observed for the Baltic Sea was also found in a study by Rose et al. (2009) conducted in the North Atlantic. Obtaining similar results from these two different provinces, the eutrophic Baltic Sea and the oligotrophic North Atlantic, stresses the relevance of considering the nutrient status of a system in order to understand the complex responses of plankton communities to warming in different regions and during different seasons.

In short, the bottom-up regulation caused by intensified phytoplankton growth at warmer temperature was most likely overcompensated by intense grazing which turned the system from a bottom-up into a top-down controlled one (Lewandowska et al., 2014).

CO₂ effects

There is evidence that high CO₂ concentrations can have direct negative effects on MZP, resulting e.g. in inhibition of growth or decrease in biomass, but this only occurred when unrealistically high values were applied (Hinga, 1992; Nielsen et al., 2010). However, it seems that indirect effects caused by changes in the phytoplankton community composition are more important for MZP than direct CO₂ effects, a pattern which was observed in previous laboratory and mesocosm studies at end-of-century acidification conditions (Rose et al., 2009; Calbet et al., 2014; Meunier et al., 2016).

Effects on autotrophic phytoplankton are in fact more often observed than for heterotrophs, e.g. changes in phytoplankton community composition, cell size, or stoichiometry (Schoo et al., 2013; Low-Decarie et al., 2014; Sommer et al., 2015). In general, an increase in CO₂ leading to a higher availability of carbon in the water has a fertilizing effect on the phytoplankton species for which carbon had been the limiting nutrient, while especially calcifying algae are affected negatively by a lowered pH (Schulz et al., 2013; Suikkanen et al., 2013; Low-Decarie et al., 2014). In contrast, pico- and nanophytoplankton seem to be positively affected by an elevated CO₂ level (Feng et al., 2009; Brussaard et al., 2013; Sala et al., 2016). As a result, the unequal responses of the different phytoplankton taxa lead to

changes in the community composition which in turn has the potential to affect their respective grazers (Suffrian et al., 2008; Aberle et al., 2013; Calbet et al., 2014).

In our study, total phytoplankton biomass was positively affected by high CO₂, even though the effect seemed to be caused mainly by the size fraction from 2-70 µm (Paul et al., 2016). Yet, this did not translate to the next trophic level. Biomass, biomass maximum, diversity and net growth rate of MZP were not affected by CO₂ as a single stressor, thus hypothesis (ii) predicting positive indirect effects on MZP due to changes in the phytoplankton community was rejected. Only dinoflagellate biomass was positively affected by an increase in CO₂ in interaction with time. This was visible at the highest CO₂ concentration at warm temperature on day 28 (Figure 2) while in all other treatments, the highest dinoflagellate concentration was on day 0. A similar result was found in a mesocosm study at the Gullmar Fjord, Skagerrak, where under nutrient-replete post-bloom conditions a significantly positive effect of CO₂ on dinoflagellate, but not on ciliate biomass was found (Horn et al., in review). In addition, we observed a delayed timing of the ciliate biomass maximum at high CO₂ (Figure 3) which was also the case in the Gullmar Fjord study. Only at 2500 and 3000 µatm target concentrations, an increase in ciliate biomass on day 28 was observed, at the same time when copepod biomass had gone down (Figure 2). At warm temperatures, this increase was more pronounced than the moderate peak around day 14.

In general, a dampening of the effects of CO₂ found in single-species experiments can be expected on community level where species richness and trophic interactions are able to partly compensate for effects (Rossoll et al., 2013; Calbet et al., 2014). This is even more the case for communities from highly productive coastal and estuarine areas where CO₂ concentrations fluctuate more than in the open ocean and thus it is likely that species from coastal and estuarine environments are adapted to grow under a wide range of pH values (Hinga, 2002). The plankton community of the Kiel Fjord is considered to be accustomed to the frequently occurring periods of high CO₂ concentrations (Melzner et al., 2013; Rossoll et al., 2013). While our ambient treatment of ~500 µatm was well below the average *p*CO₂ concentration of 700 µatm in the Kiel Fjord, the chosen target level of 3000 µatm represents the worst case scenario of more than 2300 µatm which can be reached over short time intervals during summer and autumn (Thomsen et al., 2010). The Kiel Fjord is characterized by a low buffering capacity and a bottom layer of CO₂-rich water caused by heterotrophic degradation of organic material (Melzner et al., 2013; Rossoll et al., 2013). Under prevailing south-westerly winds, upwelling events lead to acidification of the surface waters during

summer (Hansen et al., 1999), and these CO₂ fluctuations are expected to get worse with additional anthropogenic CO₂ input (Feely et al., 2008; Feely et al., 2010).

In conclusion, MZP from this coastal plankton community seems to be rather robust towards realistic acidification conditions expected for the end of this century (Collins et al., 2013). The results of our experiment are in line with other mesocosm studies from the Baltic Sea, North Sea, North Atlantic and the Arctic showing no or only subtle effects of an elevated CO₂ level on MZP communities despite the fact that effects on phytoplankton can be visible (Suffrian et al., 2008; Rose et al., 2009; Aberle et al., 2013; Rossoll et al., 2013; Lischka et al., 2015).

Combined effects of temperature and CO₂

In reality, environmental stressors will not occur separately but in concert, and it is of vital importance to also consider possible synergistic and antagonistic effects in multiple-stressor experiments (Pörtner, 2008; Rost et al., 2008). There has been an increasing number of studies on community level taking into account the effects of both temperature and CO₂, e.g. with a focus on bacterioplankton (Lindh et al., 2013), phytoplankton (Hare et al., 2007; Feng et al., 2009; Kim et al., 2013) or copepods (Mayor et al., 2012). Studies with a focus on MZP, however, are still comparatively rare.

In mesocosm studies with plankton communities from Kiel Fjord, a Norwegian Fjord and the Mediterranean, no effects of the combination of warming and acidification were observed in comparison to the ambient treatments (Calbet et al., 2014; Maugendre et al., 2015; Horn et al., 2016). Nevertheless, all of the aforementioned studies were conducted under nutrient-replete or enriched conditions while this study used a nutrient-deplete community, and there is growing evidence that the nutrient level is an influential factor for the impacts of ocean acidification (Sala et al., 2016; Bach et al., in review). In a series of short-term mesocosm acidification studies in an oligotrophic region of the Mediterranean, Sala et al. (2016) observed that at nutrient-rich conditions, no or negative effects on plankton abundances were observed while nutrient-limited conditions resulted in higher abundances of different planktonic groups. The effects on abundance were most distinct during summer situations when compared to winter communities thus showing that the combination of low nutrients, warm temperatures and acidification led to positive effects on prokaryotes (Sala et al., 2016). These results are in line with observations from the present Baltic Sea mesocosm experiment conducted at nutrient-deplete summer conditions, pointing towards the importance of the

nutrient status when considering effects of ocean acidification and warming on plankton communities.

Furthermore, in a multiple-stressor experiment in the oligotrophic North Atlantic, Rose et al. (2009) observed that the combination of CO₂ and temperature (“greenhouse treatment”) resulted in a lower total MZP abundance than in single factor treatments. Overall, the MZP community in our study seemed to respond rather positively to the interaction of both factors by showing a higher biomass for total MZP, MZP <30 µm, and dinoflagellates (Table 2). In addition, the timing of the bloom peak was earlier for both total MZP and ciliates when warming and CO₂ were combined in comparison to the single-stressor response (Figure 5), which is in line with the greenhouse treatment in the experiment in the North Atlantic (Rose et al., 2009). Therefore, hypothesis (iii) predicting an overall positive effect of the greenhouse treatment on MZP biomass was confirmed. However, it must be pointed out that the combined effects of CO₂ and temperature in turn resulted in a lowered ciliate diversity.

Trophic interactions

Apart from the effects of CO₂ or temperature, we also analysed effects of copepods and phytoplankton on MZP as both grazing and food supply can influence the effects of abiotic factors (Rossoll et al., 2013; Lewandowska et al., 2014). Copepods are known to feed on MZP and especially ciliates (Frost, 1972) and have been shown to be positively affected by temperature (Lewandowska et al., 2014) which was also the case for copepod abundances in our experiment (Paul et al., 2016). Considering that warming and CO₂ could only explain a small part of the variability in MZP biomass, we additionally analysed the effects of copepod and phytoplankton biomass on the data set using a GLS. However, as this analysis excluded any impacts of warming and CO₂ that occurred, it did not allow assessing causality of the observed effects, but instead showed correlations between the different plankton groups.

We observed a negative correlation between copepod biomass and dinoflagellates (Table S3); yet, none of the other MZP groups were correlated with this factor. Even though ciliate biomass decreased with warming, this was obviously not due to a higher grazing pressure at warm temperatures. Furthermore, if the low MZP biomass was only based on top-down control, there should have been a recovery of MZP as soon as the copepod biomass had been reduced in the second half of the experiment. However, such a release from grazing pressure was observed only in the high CO₂ mesocosms at warm temperatures, where the decrease in copepods was strongest. This might not be surprising considering the higher biomass of

phytoplankton edible for copepods at cold temperatures and high CO₂ concentrations (Paul et al., 2016).

Taking direct and indirect effects of the stressors on MZP community composition into account, changes in the phytoplankton community are expected to affect MZP as well. Analysis of the edible phytoplankton (>2 µm) showed an evenly distributed species composition with low biomass in the warm treatments, high biomass in the cold ones, and increasing phytoplankton biomass with increasing CO₂ (Paul et al., 2016). It is questionable if both of these effects led to negative impacts on MZP, indicating that there were more factors involved, most likely the nutrient concentration. Still, different effects of the single- and multiple-stressor treatments on phytoplankton and MZP community were also observed in other mesocosm studies (Rose et al., 2009; Calbet et al., 2014). In this study, phytoplankton (>2 µm) was indeed positively correlated with total MZP biomass, MZP >30 µm and <30 µm as well as ciliates and dinoflagellates (Table S3). For picophytoplankton (<2 µm), a positive correlation was only observed with MZP >30 µm and dinoflagellates while the correlation with ciliates was negative (Table S3). The interaction of phytoplankton with time had a negative effect for all MZP groups given the decline in phytoplankton over time.

The dilution experiments conducted on day 13 and day 20 allowed a more detailed look into effects of the stressors on the phytoplankton community. As MZP grazing pressure can have a strong influence on the composition of the phytoplankton up to the suppression of blooms (Calbet and Landry, 2004; Irigoien et al., 2005), effects of CO₂ or temperature obscured by grazing pressure are likely to become visible in the instantaneous growth rate. In general, picophytoplankton is one of the groups positively affected by high CO₂ concentrations in acidification studies (e.g. Brussaard et al., 2013; Sala et al., 2016) which is in line with our experiment.

However, effects of CO₂ on phytoplankton depend on the group in focus. Apart from picophytoplankton, a positive effect of high CO₂ has been described for green algae and autotrophic dinoflagellates while other taxa are negatively affected, especially calcifying ones such as coccolithophores (Riebesell et al., 2000b; Kim et al., 2006; Schulz et al., 2013; Suikkanen et al., 2013; Low-Decarie et al., 2014). For diatoms and bluegreen algae, both positive and negative effects have been reported, which among other factors seems to be controlled by the nutrient concentration (Feng et al., 2009; Kroeker et al., 2013; Schulz et al., 2013; Suikkanen et al., 2013).

Contrary to expectations for a cyanobacteria bloom during late summer situations in the Baltic Sea (Sellner, 1997; Suikkanen et al., 2013), large-sized filamentous cyanobacteria like

Nodularia sp. and *Anabaena* sp. were only present in very low abundance throughout the experiment and no cyanobacteria blooms developed in the mesocosms. Nevertheless, based on the results from the dilution experiment, there was a positive effect of CO₂ on the instantaneous growth rate μ_0 of bluegreen algae in both experiments. The dominant taxa of bluegreen algae were picophytoplanktonic *Synechocystis* and *Cyanodictyon* (Paul et al., 2016), both of which are considered as inedible for copepods. Still, they are within the food size range for MZP such as *B. comatum* (Johansson, 2004) which contributed to large parts to the MZP community during both dilution experiments. It seems thus likely that the missing CO₂ effect on the net growth rate μ was thus caused by MZP grazing pressure. Temperature as a single stressor had a negative effect on μ and μ_0 of bluegreen algae; a pattern which has been observed in other studies as well (O'Connor et al., 2009). Contrastingly, the interaction of CO₂ and temperature on μ of bluegreen algae was positive in the 2nd experiment, indicating that at this point of low MZP concentrations the positive effects of CO₂ probably outweighed the negative ones of increased grazing at warm temperatures.

Cryptophytes were another group which showed a positive effect of CO₂ on μ during the 1st dilution experiment. They were missing in the 2nd experiment which fits to the phytoplankton counts showing that the cryptophyte *Plagioselmis* sp. (<2 μm) was quite abundant during the first part of the experiment and disappeared afterwards (Paul et al., 2016). The positive CO₂ effect measured for cryptophytes was most probably solely based on *Plagioselmis* sp. as the other cryptophyte present in higher densities, *Teleaulax* sp., had been reduced to almost zero in all treatments when the 1st dilution experiment took place (on day 13) as shown by Paul et al. (2016). With a size >2 μm , *Teleaulax* sp. is considered a suitable copepod food source even though not an optimal one (Stoecker and Capuzzo, 1990) and was thus grazed upon while *Plagioselmis* sp. is considered as inedible for copepods (Sommer et al., 2005). Cryptophytes were negatively affected by temperature, which has been observed for this group in other studies (Suikkanen et al., 2013; Calbet et al., 2014).

In contrast, there was no effect of CO₂ or temperature on μ of green algae in the 1st dilution experiment. During the 2nd dilution experiment, μ and μ_0 of green algae were negatively affected by CO₂ although they are generally expected to be positively by affected an increase in CO₂ if C is the limiting nutrient (Low-Decarie et al., 2014). Thus, the nutrient-deplete conditions in this study were probably responsible for the observed negative effect. In addition, green algae were positively affected by temperature. Picoplanktonic *Bathycoccus* sp. was one of the main contributors to this group and it was still present during the 2nd

experiment (Paul et al., 2016). For this taxon a positive effect of temperature has been observed before (Sommer et al., 2015).

Diatoms which were present in the 1st experiment showed no effects of μ and μ_0 in response to CO₂ conditions but a positive effect of temperature. They consisted mainly of *Chaetoceros* sp. and *Ditylum brightwellii* which were still present during the 1st experiment but heavily grazed upon. While CO₂ alone had no effects on diatoms in our study, the combined effects of CO₂ and temperature were negative thus confirming the negative effects of CO₂ on diatoms and especially *Chaetoceros* sp. reported from previous experiments (Riebesell et al., 2000b; Sommer et al., 2015).

Overall, the present results point at the pivotal role of top-down control mechanisms on the summer phytoplankton community in our mesocosms. This indicates that grazing was probably the most important factor for the development of phytoplankton which fits to the model by Lewandowska et al. (2014) developed for nutrient-deplete conditions. As a result, the effects on phytoplankton visible in the dilution experiment did not translate to the MZP level as far as growth or grazing rates were concerned. In addition, there was no effect of the stressors on phytoplankton grazing mortality m or MZP grazing rate g in the 1st and 2nd experiment, and neither on the net growth rate of MZP.

In this study, we observed effects of CO₂, temperature and their interactions on growth rates of different specific phytoplankton groups and total phytoplankton biomass. However, on the MZP level, effects were not so clear and at times even controversial. This indicates that indirect effects on MZP e.g. via changes in the phytoplankton prey spectrum might have played a substantial role in shaping this summer plankton community and, in turn, that such effects might have additionally been masked by a strong top-down control by mesozooplankton due to warming. This emphasizes once more the dampening effect of natural communities comprising of multiple trophic levels towards environmental stressors (Rossoll et al., 2013; Calbet et al., 2014).

Conclusions

Overall, the effects of CO₂ and temperature on MZP from this experiment had a closer resemblance to multiple-stressor studies from oligotrophic regions than to eutrophic ones like the Kiel Fjord under nutrient-replete conditions. Most MZP communities, including those from the Kiel Fjord, have shown a low sensitivity towards realistic future CO₂ concentrations and were positively affected by warming. However, in a nutrient-deplete late summer situation, a negative effect of warming and positive effects of the interactions of CO₂ and

warming on MZP were observed. This indicates that under high CO₂, negative effects of warming were less severe. These effects point at the importance of a top-down control by mesozooplankton controlling MZP communities, especially at elevated temperature conditions and at times when phytoplankton comprises mainly of inedible species and biomass is low. Our results suggest that under nutrient depletion and low phytoplankton densities, trophic interactions can turn a system from bottom-up to top-down control thus stressing the relevance of considering trophic interactions when estimating climate change effects on marine plankton communities. Furthermore, the results emphasize the importance of the nutrient status when estimating the effect of high CO₂ concentrations and warming on plankton communities.

Supplement

Table S1: Results from the linear regression analysis of effects of CO₂ and temperature on biomass maximum (max.), timing of the maximum D_{max} and net growth rate μ of total MZP, ciliates and dinoflagellates. Significances are indicated by asterisks.

Variable		Factor	t	p	
Biomass max.	Total MZP	CO ₂	-0.917	0.386	
		Temperature	-1.249	0.247	
		CO ₂ x Temperature	1.415	0.195	
	Ciliates	CO ₂	-1.173	0.275	
		Temperature	-1.177	0.273	
		CO ₂ x Temperature	1.359	0.211	
	Dinoflagellates	CO ₂	0.766	0.466	
		Temperature	-0.024	0.982	
		CO ₂ x Temperature	-0.473	0.649	
D _{max}	Total MZP	CO ₂	-0.961	0.365	
		Temperature	-0.888	0.400	
		CO ₂ x Temperature	2.996	0.017	*
	Ciliates	CO ₂	-2.492	0.037	*
		Temperature	-3.059	0.016	*
		CO ₂ x Temperature	4.001	0.004	**
	μ	Total MZP	CO ₂	0.640	0.557
Temperature			0.630	0.563	
CO ₂ x Temperature			-0.600	0.581	
Ciliates		CO ₂	0.301	0.772	
		Temperature	1.192	0.272	
		CO ₂ x Temperature	-0.661	0.530	

Table S2: Results from the analysis of the effects of CO₂, temperature and the interactions of the two factors on phytoplankton mortality m and MZP grazing rate g from the dilution experiments.

Experiment	Variable	Factor	t	p	
1	m	Total f	CO ₂	-0.465	0.674
			Temperature	-2.483	0.089
		Green algae	CO ₂	0.618	0.580
			Temperature	-0.714	0.527
		Bluegreen algae	CO ₂	-0.205	0.871
			Temperature	1.357	0.404
	Diatoms	CO ₂	-0.422	0.701	
		Temperature	-2.940	0.061	
	g	Total f	CO ₂	1.303	0.284
			Temperature	1.345	0.271
		Green algae	CO ₂	-0.921	0.425
			Temperature	0.096	0.930
		Bluegreen algae	CO ₂	0.205	0.871
			Temperature	-1.357	0.404
Cryptophytes		CO ₂	-1.036	0.489	
		Temperature	-0.656	0.630	
2	m	Total f	CO ₂	-3.092	0.054
			Temperature	-2.548	0.084
		Green algae	CO ₂	-3.165	0.051
			Temperature	-1.668	0.194
		Bluegreen algae	CO ₂	1.378	0.262
			Temperature	1.848	0.162
	Diatoms	CO ₂	-1.372	0.264	
		Temperature	-2.562	0.083	
	g	Total f	CO ₂	1.477	0.236
			Temperature	1.138	0.338
		Bluegreen algae	CO ₂	-1.689	0.190
			Temperature	-1.889	0.155

Table S3: Results from the GLS analysis of the effects of total copepod, phytoplankton (>2 µm) and picophytoplankton (<2 µm) biomass on MZP biomass. Significances are indicated by asterisks.

Variable	Factor	t	p	
Total MZP	Copepods	-0.566	0.573	
	Time	-2.371	0.021	*
MZP <30 µm	Copepods	0.249	0.805	
	Time	-2.382	0.021	*
MZP >30 µm	Copepods	0.214	0.831	
	Time	-3.154	0.003	**
Ciliates	Copepods	-0.386	0.701	
	Time	-2.428	0.018	*
Dinoflagellates	Copepods	0.345	0.731	
	Time	-3.287	0.002	**
	Copepods x time	-2.583	0.012	*
Total MZP	Phytoplankton	3.244	0.002	**
	Time	-0.017	0.986	
	Phytoplankton x time	-3.755	<0.001	***
MZP <30 µm	Phytoplankton	2.904	0.005	**
	Time	0.074	0.941	
	Phytoplankton x time	-3.681	0.001	**
MZP >30 µm	Phytoplankton	4.333	<0.001	***
	Time	0.109	0.914	
	Phytoplankton x time	-4.702	<0.001	***
Ciliates	Phytoplankton	2.093	0.040	*
	Time	-0.202	0.841	
	Phytoplankton x time	-2.861	0.006	**
Dinoflagellates	Phytoplankton	7.115	<0.001	***
	Time	0.633	0.529	
	Phytoplankton x time	-7.866	<0.001	***
Total MZP	Picophytoplankton	-0.425	0.672	
	Time	-2.328	0.023	*
MZP <30 µm	Picophytoplankton	-0.588	0.559	
	Time	-2.077	0.042	*
MZP >30 µm	Picophytoplankton	2.275	0.026	*
	Time	-0.548	0.585	
	Picophytoplankton x time	-3.764	<0.001	***
Ciliates	Picophytoplankton	1.079	0.285	
	Time	-0.349	0.728	
	Picophytoplankton x time	-2.321	0.023	*
Dinoflagellates	Picophytoplankton	-1.798	0.077	
	Time	-3.787	<0.001	***
	Picophytoplankton x time	2.302	0.024	*

CHAPTER IV – Direct and indirect impact of near-future $p\text{CO}_2$ levels on zooplankton dynamics

Abstract

Ocean acidification has direct physiological effects on organisms by, for example, dissolving the calcium carbonate structures of calcifying species. However, non-calcifiers may also be affected by changes in seawater chemistry. To disentangle the direct and indirect effects of ocean acidification on zooplankton growth, we carried out a study with two model organisms. We investigated the individual effect of short term exposure to (1) high and low seawater $p\text{CO}_2$ and (2) different phytoplankton qualities as a result of different CO_2 incubations on the growth of a heterotrophic dinoflagellate and a copepod species. It has been previously observed that higher CO_2 concentrations can decrease phytoplankton food quality in terms of carbon:nutrient ratios. We therefore expected both seawater $p\text{CO}_2$ (pH) and phytoplankton quality to result in a decrease of zooplankton growth. Although we expected lowest growth rates for all zooplankters under high seawater $p\text{CO}_2$ and low algal quality, we found that direct pH effects on consumers seem to be of lesser importance than the associated decrease in algal quality. The decrease of primary producers' quality under high $p\text{CO}_2$ conditions negatively affected zooplankton growth, which may lead to lower availability of food for the next trophic level and thus potentially affect the recruitment of higher trophic levels.

Introduction

Human industrial activities have increased atmospheric carbon dioxide (CO₂) concentrations which have now reached values of over 400 ppm on average (Tans and Keeling, 2013), the highest level for millions of years (Royer, 2006; Pagani et al., 2011). About 25% of the CO₂ enters the oceans which can act as carbon (C) sinks (Canadell et al., 2007). As a result, the carbonate chemistry of oceans has changed, especially in the upper 100 meters where ocean acidification has a major influence (Doney et al., 2009).

Ocean acidification negatively affects a number of organisms directly by, for example, dissolving the calcium carbonate structures of calcifying species (Orr et al., 2005). However, marine organisms, such as micro- and mesozooplankton, may be subjected to other adverse effects. Lower seawater pH resulting from increasing *p*CO₂ could directly affect the physiology of both phyto- and zooplankton by changing intracellular pH, membrane potentials, and enzyme activities (Nielsen et al., 2010). This acidification of body fluids is known as hypercapnia (Fabry et al., 2008). When CO₂ levels increase in seawater, dissolved CO₂ more readily diffuses across body surfaces and equilibrates in both intra- and extracellular spaces. As in seawater, CO₂ reacts with internal body fluids causing H⁺ ions to increase and pH to decrease. Hypercapnia can cause the suppression of metabolic processes (Michaelidis et al., 2005; Pörtner, 2008) and disrupt acid-base homeostasis (Miles et al., 2007), thus decreasing growth rate and reproductive success and increasing mortality of marine organisms (Yamada and Ikeda, 1999). Smaller organisms are likely to be more affected by changes in seawater chemistry than larger ones as a result of the differences in volume to surface ratios and future conditions will increase variations in pH at the cell surface (Flynn et al., 2012). However, despite their ecological importance, only few studies have focused on the impact of ocean acidification on microzooplankton so far, and, to our knowledge, their sensitivity to hypercapnia has never been investigated. Microzooplankton are an essential component in planktonic ecosystems. Indeed, they often comprise the major predatory group in microbial food webs (Sherr and Sherr, 2002), and microzooplankters form a trophic link between pico-, nano- and microplankton on the one hand and higher trophic levels, such as copepods, on the other hand (Sommer et al., 2005). Although there is indication that microzooplankton are sensitive to elevated pH (Hinga, 2002; Pedersen and Hansen, 2003), to our knowledge no studies have directly tested the effects of CO₂-induced lowering of the pH on marine microzooplankton. The few existing experimental studies were carried out using natural plankton communities and observed no effects of a high *p*CO₂/low

pH on microzooplankton, independent on whether the systems were fixed pH, or whether pH was allowed to drift as it would in reality (Suffrian et al., 2008; Rose et al., 2009; Aberle et al., 2013). Ocean acidification does not only reduce the global base pH but also influences smaller-scale fluctuations. For example, ocean acidification may influence spatial and seasonal variations by modulating seawater alkalisation during intense C-fixation associated with phytoplankton blooms (Flynn et al., 2012). Having said this, as the knowledge on effects of ocean acidification on microzooplankton is so limited we decided to focus on effects of fixed changes in $p\text{CO}_2$ only. However, it is generally difficult to observe physiological effects in natural community experiments whereas smaller and more controlled microcosm studies are more helpful to understand physiological responses such as microzooplankton sensitivity to hypercapnia. Further, most studies measuring copepod physiological performance at lower seawater pH observed that copepods are relatively tolerant to hypercapnia (Mayor et al., 2012; McConville et al., 2013). Yet, recent studies observed strong negative effects of near-future ocean acidification levels on copepods (Lewis et al., 2013; Thor and Dupont, 2015), and others suggest that inappropriate experimental designs might have underestimated the actual direct effect of ocean acidification on copepods, and potentially microzooplankton (Cripps et al., 2014a; Cripps et al., 2014b). Since micro- and mesozooplankton play different roles in the pelagic ecosystem, it is important to determine whether those two groups are differently affected by high $p\text{CO}_2$.

Apart from direct acidification effects, the increasing C availability in the marine environment will likely change primary productivity and the quality of phytoplankton as food for higher trophic levels (Low-Decarie et al., 2014). As primary producers reflect the nutrient composition of their surrounding medium, they are expected to show higher C:nutrient ratios as CO_2 availability increases (Burkhardt et al., 1999; Urabe et al., 2003; van de Waal et al., 2010). Further, algae with high C:nutrient ratios are known to often be food of inferior quality for herbivorous consumers since there is a larger difference between resource chemical composition and consumer metabolic requirements (Sterner and Elser, 2002). Recent results indicate negative indirect effects of ocean acidification on copepods caused by a decline in prey quality when grown under high $p\text{CO}_2$ (Rossoll et al., 2012; Schoo et al., 2013). In the light of the predicted increase of $p\text{CO}_2$ and the observed trend of decreasing nitrogen (N) and phosphorus (P) inputs to coastal areas (Grizzetti et al., 2012), the potential for an excess of C and a concurrent nutrient limitation at the base of the food web is considerably heightened. Although evidence is increasing that the growth rate of zooplankters decreases with increasing CO_2 availability to the algae (Olson and Kawaguchi, 2011; Rossoll et al., 2012;

Schoo et al., 2013), it remains unclear whether direct or indirect effects on consumer fitness play the more prominent role. Indeed, only one study investigated the direct and indirect effects of acidification on zooplankton growth and reproduction (Rossoll et al., 2012). This study concluded that high CO₂ availability decreases copepods fitness mostly indirectly. Further, Melzner et al. (2011) showed that the effects of high CO₂ may be less pronounced when there is enough food available, as the energy needed to deal with the unhealthy environment is available. If energy is the limiting compound in the study of Melzner et al. (2011), at low food concentrations higher algal carbon:nutrient ratios might benefit herbivores since algae grown under high CO₂ conditions are more energy rich as they often contain more lipids (Rossoll et al., 2012). The generality of this result remains unclear, as trophic upgrading and differential algae sensitivity in terms of growth rate and stoichiometry to pCO₂ may compensate for low food quality. For instance, Isari et al. (2016) recently observed that increased pCO₂ does not affect the stoichiometric quality of the phytoplankton species *Heterocapsa sp.* and, logically, did not alter performances of copepods feeding on that prey. Further, Klein Breteler et al. (1999) showed that inadequate algal food could be biochemically upgraded by protozoans to high quality food for copepods. Hence, protozoan herbivores might dampen the negative effect of acidification on algal food quality through trophic upgrading. In fact, Caron and Hutchins (2012) identified lack of data on the effects of ocean acidification on microzooplankton as one of the major knowledge gaps.

To disentangle the direct and indirect effects of ocean acidification on both microzooplankton and mesozooplankton, we carried out a study with model organisms. We investigated the individual effect of short term exposure to (1) high and low seawater pCO₂ and (2) different qualities of the alga *Rhodomonas salina* on the growth and development of two model zooplankton species *Oxyrrhis marina* (Montagnes et al., 2011) and *Acartia tonsa* (Mauchline et al., 1998). The different algal qualities were obtained by growing *R. salina* in high and low seawater pCO₂ relative to current scenarios. We hypothesize that a significant interaction of seawater pCO₂ and phytoplankton quality should affect zooplankton growth and that lowest growth rates for both micro- and mesozooplankters should be observed under high pCO₂ seawater and low algal quality.

Material and methods

To test whether planktonic herbivores growth is affected by seawater pCO₂ or by algal quality differences caused by seawater pCO₂ (direct versus indirect effect), we conducted a laboratory

experiment with nauplius and copepodite stages of the copepod *A. tonsa* as well as with the heterotrophic dinoflagellate *O. marina*. The zooplankton species were cultured under high and low $p\text{CO}_2$ and were fed with two algal qualities in full factorial design, i.e. four treatments. The different algal qualities were obtained by growing the model organism *R. salina* in high and low seawater $p\text{CO}_2$ relative to current scenarios. Using model organisms such as *O. marina* and *R. salina* entails limitations regarding the extent to which experimental results can be interpreted. Nevertheless, due to its high growth rate and to the reproducibility of nutrient treatments, *R. salina* is a useful model organism when studying the importance of phytoplankton food quality for zooplankton. Further, Davidson et al. (2010) made a critical assessment of the advantages and disadvantages of using *O. marina* as a model organism. Their study supports the use of this dinoflagellate in experimental studies since its feeding mode and predator:prey size ratio are comparable to most protozoa. Both *O. marina* and *R. salina* are planktonic, not benthic, and in this sense, they are appropriate model organisms for planktonic processes.

Phytoplankton

R. salina (Wislouch) Hill et Wetherbee was kept in F/2 medium prepared with 0.2 μm filtered seawater. *R. salina* was grown in continuous chemostat cultures maintained at steady state. The phytoplankton cultures were constantly aerated with a mixture of air stripped of CO_2 by soda lime and pure CO_2 adjusted to 200 and 800 μatm (Rho 200 and Rho 800) to represent pre-industrial and predicted future scenarios. The pre-defined $p\text{CO}_2$ level was achieved following Schoo et al. (2013). A sensor (HTK Hamburg) continuously monitored the $p\text{CO}_2$ of the gas mixture distributed to the algal cultures and automatically adjusted the CO_2 content and flow rate. Preliminary experiments showed that the $p\text{CO}_2$ of the growth medium was in equilibrium with the target level of the gas $p\text{CO}_2$ within 1 h of aeration. The algal chemostat cultures (5 L) were stirred continuously and kept at 18 °C under a 16h:8 h light:dark regime (185 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Subsamples from the surplus culture collected in an overflow container were taken daily to measure the cell density of the cultures with a CASY cell counter (SCHÄRFE SYSTEMS, Reutlingen, Germany) as well as the algal stoichiometry (see procedure below).

To feed the zooplankton populations, two new *R. salina* batch cultures were created daily at concentrations of 0.5×10^6 cells L^{-1} to ensure that, within each treatment, zooplankters were feeding on the same algal quality for the duration of the experiment. We needed to prepare batch cultures since the volume of algae needed to feed the zooplankton populations was too

important to be taken from the chemostats. The phytoplankton cultures were constantly aerated with air at $p\text{CO}_2$ of 200 and 800 μatm (Rho 200 and Rho 800). It is important to note that this study was conducted using a controlled system with fixed pH which might induce different responses than in the field where pH drifts with phytoplankton growth (Flynn et al., 2015). The algae were cultivated in 1 L batch cultures in F/2 medium at 18 °C under a 16h:8 h light:dark regime ($185 \mu\text{mol m}^{-2} \text{s}^{-1}$). After three days of growth, an aliquot of each *R. salina* culture was filtered onto pre-combusted Whatman GF/F filters. The particulate C and N content of *R. salina* was measured with a Vario Micro Cube/CN-analyser (Elementar). Particulate P was analysed as orthophosphate after acidic oxidative hydrolysis with 5 % H_2SO_4 (Grasshoff et al., 1999).

Microzooplankton

O. marina Dujardin was obtained from the Göttingen culture collection (Strain B21.89) and fed *R. salina* at 18 °C under a dim continuous light regime ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$). Prior to the experiment, the *O. marina* culture was starved for 1 week in order to eradicate any effects of preculture conditions. This culture was then split into 28 separate cultures (four treatments, seven replicates) which were all diluted to a start concentration of 20,000 cells mL^{-1} with CO_2 preconditioned artificial, sterile and nutrient-free seawater (Aqua Marin) at a salinity of 32. Cell concentrations of the cultures were determined using a CASY particle counter (SCHÄRFE SYSTEMS, Reutlingen, Germany). The *O. marina* cultures were gently aerated with a mixture of air and pure CO_2 adjusted to 200 (Water200) and 800 μatm (Water800). As for phytoplankton cultures, a sensor continuously monitored the $p\text{CO}_2$ of the gas mixture distributed to the algal cultures and automatically adjusted the CO_2 content and flow rate. Pre-experiments indicated that gentle bubbling does not affect *O. marina* growth rate. The cultures had pH of 8.3 (± 0.06) and 7.8 (± 0.05) when aerated with 200 and 800 $p\text{CO}_2$, respectively. The total alkalinity of the CO_2 preconditioned artificial seawater was 3300 $\mu\text{mol L}^{-1}$ for the 200 μatm and 3197 $\mu\text{mol L}^{-1}$ for the 800 μatm $p\text{CO}_2$ treatment. The pH was measured with a ProLab 3000 pH meter with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A170MFDIN-N). TA was estimated from open-cell duplicate potentiometric titration and calculation with modified Gran plots (Bradshaw et al., 1981), using a TitroLine alpha plus titrator with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A120MF-DIN-N). The carbonate system was calculated from TA, pH, temperature and salinity using CO2Sys (Lewis and Wallace, 1998), the $p\text{CO}_2$ values obtained were 292 and 911 ppm for the 200 and 800 treatments, respectively. To

calculate the carbonate system, we used the equilibrium constants of (Mehrbach et al., 1973) refitted by Dickson and Millero (1987). Each of the two pCO₂ culture sets (Water200 and Water800) was fed *ad libitum* with 35 algal cells per *O. marina* daily during four days with either Rho 200 or Rho 800 (Rho 200-Water 200, Rho 200-Water 800, Rho 800-Water 200, Rho 800-Water 800). The quantity of food given daily was sufficient to prevent food quantity limitation and was adjusted at the last feeding day to minimize the amount of remaining algal cells after 24 h. After four days of growth, the cell concentration of each *O. marina* culture was determined by CASY counting and the relative growth rate of each culture was calculated.

Mesozooplankton

Eggs of the calanoid copepod *A. tonsa* were produced in 200-liter cylindrical tanks, where the animals were cultivated at 18 °C at a 16:8 h light:dark cycle. Copepods were fed with *R. salina*. Eggs were siphoned from the bottom of the tanks daily and stored in seawater at 4 °C for later use. The stored eggs were incubated in fresh seawater at 18 °C for hatching. Since hatching peaks between 24 h and 36 h of incubation, we collected the nauplii hatched during this period to minimize age differences between individuals. Nauplii were placed at 18 °C under a dim 16:8 h light:dark regime in the same four treatments above-described for microzooplankton and fed daily for 12 days. The cultures were put in 1 L glass containers at densities of 1,000 individuals L⁻¹ in seven replicates per treatment. Copepods were fed *ad libitum* with 20,000 algal cells per copepod and all experimental animals were washed daily over a sieve (75 µm mesh size) to separate them from any algae before being introduced to fresh CO₂ preconditioned artificial seawater prior to feeding in order to minimise changes in the nutrient composition of the algae by waste products of the animals. The developmental rate of the juvenile copepods was determined after 12 days. The developmental stages of the copepods were determined for at least 100 individual animals from subsamples at the end of the experiment. For the calculation of the developmental rates, all naupliar larvae were assigned to stage 6, the last naupliar stage before transition to the copepodite stages. The number of individuals per stage was divided by the number of days of growth, and the developmental rate per treatment was then calculated by dividing this sum by the number of individuals counted. We also tested the copepods reaction to the treatments by measuring the respiration rates of stage 4 nauplii and stage 3 copepodites. Copepods respiration rates were measured following the procedure described by Schoo et al. (2013). Respiration rates were determined with a microsensor oxygen metre (PreSens Precision Sensing, Germany) equipped

with oxygen microoptodes. Approximately 100 nauplii and 75 copepodites were sampled from the incubation containers at day 5 and 10 and were washed over a sieve (75 μm mesh size) to separate them from any algae before being introduced into the 5 mL incubation vessel. Oxygen air saturation values were below 80 % at the end of the 1 h measurements. Bacterial respiration rates were measured as a control treatment at the same time and the measured bacterial respiration rates were deduced from the total respiration rates of the copepod measurements. The animals were collected and counted after the incubation to determine the precise number of animals in each vessel, permitting an accurate calculation of respiration rates per individual animal. Respiration rates were calculated by linear regression of oxygen concentration over time. Technical issues unfortunately prevented us from measuring the respiration for *O. marina*. It is not possible to separate *O. marina* from *R. salina* due to small size differences between the two species and pre-experiments showed that using a control with algae only generates too large standard deviations.

Results

The population density and the stoichiometry of the algae grown in the continuous chemostat cultures were affected by the exposure to the different $p\text{CO}_2$ during growth (Figure 1). The phytoplankton cultures had significantly higher cell densities when grown under elevated $p\text{CO}_2$ (Figure 1A, Repeated Measures ANOVA, $F_{2,19} = 44.46$, $p < 0.01$). Both C:N (Figure 1B, Repeated Measures ANOVA, $F_{2,19} = 44.46$, $p < 0.01$) and C:P ratios (Figure 1C, Repeated Measures ANOVA, $F_{2,18} = 159.57$, $p < 0.01$) were significantly higher in *R. salina* cultures reared under elevated $p\text{CO}_2$.

We cultured zooplankton in low and high $p\text{CO}_2$ seawater and fed them algae grown in batch cultures under low and high $p\text{CO}_2$. The CO_2 treatments significantly affected algal C content; Rho 800 was 30 % richer in C than Rho 200 (Table 1, t-test $p < 0.05$). This difference in C content resulted in lower C:N (10.1 ± 3.1) and C:P ratios in Rho 200 (294 ± 24) than in Rho 800 (14.7 ± 0.9 and 396 ± 31 ; t-test $p < 0.05$). Further, the CO_2 treatments did not affect the N and P content and the N:P ratio of *R. salina*. Although one may argue that the change in algal C:N:P reflects a short-term response caused by the small duration of adaptation, our chemostat culture indicates that such changes persist over generations (Schoo et al., 2013).

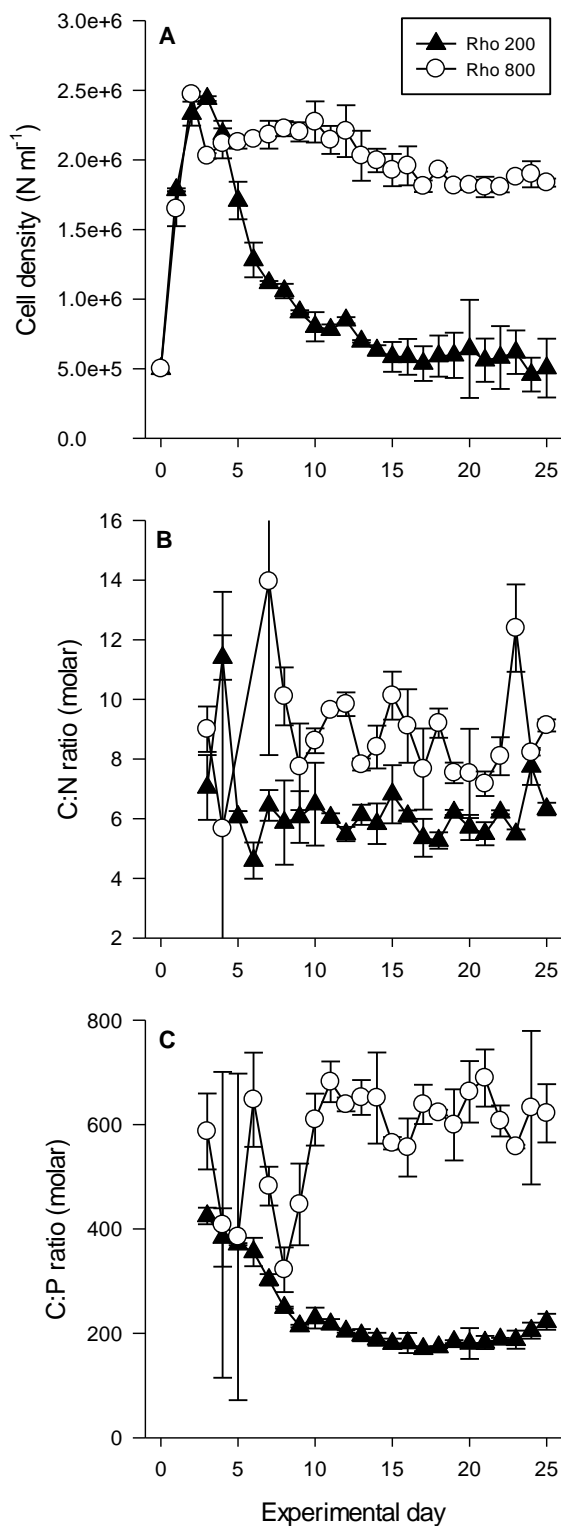


Figure 1: *R. salina* (A) cell densities, (B) C:N ratios, and (C) C:P ratios grown in chemostats under different $p\text{CO}_2$. Data presented are means and standard deviations of three replicates.

Table 1: Mean carbon, nitrogen, phosphorus cell content (pg cell^{-1}) and C:N:P of *R. salina* used to feed the zooplankton cultures. Numbers in brackets are standard deviations of five replicates and stars indicate significant differences ($n = 5$; $FG = 8$; $p < 0.05$).

	Rho 200	Rho 800
C (pg cell^{-1})	57.2 (1.1)*	79.5 (0.6)*
N (pg cell^{-1})	6.2 (1.1)	6.2 (0.4)
P (pg cell^{-1})	0.57 (0.01)	0.53 (0.03)
CN (molar)	10.1 (3.1)*	14.7 (0.9)*
CP (molar)	294 (24)*	396 (31)*
NP (molar)	22.6 (3.4)	26.3 (3.8)
Growth rate (d^{-1})	0.42 (0.04)	0.43 (0.03)
ESD (μm)	9.46 (0.08)	9.81 (0.12)

We observed that the growth rate of *O. marina* was significantly affected by the food quality treatment (Figure 2, two-way ANOVA, $p < 0.05$), while seawater $p\text{CO}_2$ did not alter the dinoflagellate growth. *O. marina* growth rate was 40 % lower when feeding on the C-rich Rho 800 (0.18 d^{-1}) than when feeding on Rho 200 (0.3 d^{-1}). Further, although we expected to observe the lowest growth rate under high $p\text{CO}_2$ seawater and low algal quality, the ANOVA interaction effects between seawater $p\text{CO}_2$ and algal quality did not significantly affect the growth of *O. marina* (two-way ANOVA $p > 0.05$). Similarly, only algal quality had an effect on the development of *A. tonsa* (Figure 3). While seawater $p\text{CO}_2$ did not affect *A. tonsa* development, copepod populations feeding on C-rich Rho 800 contained a lower percentage of the older C6 and C5 copepodite stages at the end of the 12 days growth experiment than those feeding on Rho 200 (Figure 3A). Consequently, *A. tonsa* developmental rates were influenced by algal quality and we observed a significant development decrease in the Rho 800 treatment compared to the Rho 200 treatment (Figure 3B, two-way ANOVA, $p < 0.05$); while seawater $p\text{CO}_2$ had no significant effect.

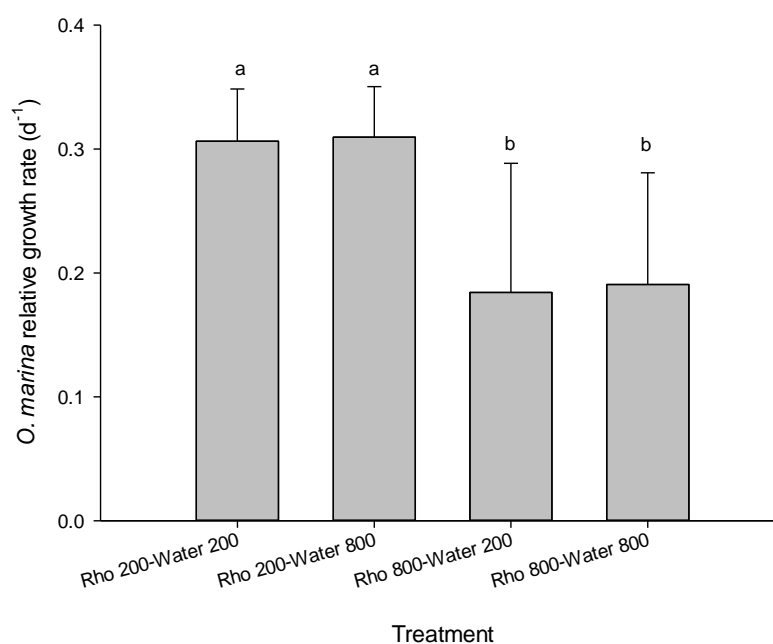


Figure 2: Growth rates of *O. marina* reared under different $p\text{CO}_2$ and fed with different $p\text{CO}_2$ *R. salina*. Data presented are means and standard deviations of seven replicates. Statistically significant differences ($p < 0.05$) are indicated by letters.

The different food qualities also led to differences in copepod respiration rates. Copepods feeding on C-rich Rho 800 had significantly higher respiration rates than those feeding on Rho 200 (Figure 4, two-way ANOVA, Tukey's honest significant difference posthoc test, $p < 0.01$); while no effect of seawater $p\text{CO}_2$ could be identified. Further, although we expected the combination of high $p\text{CO}_2$ seawater and low algal quality to have a strong effect, the ANOVA interaction effects between seawater $p\text{CO}_2$ and algal quality did not significantly affect the development and respiration of *A. tonsa* (two-way ANOVA $p > 0.05$).

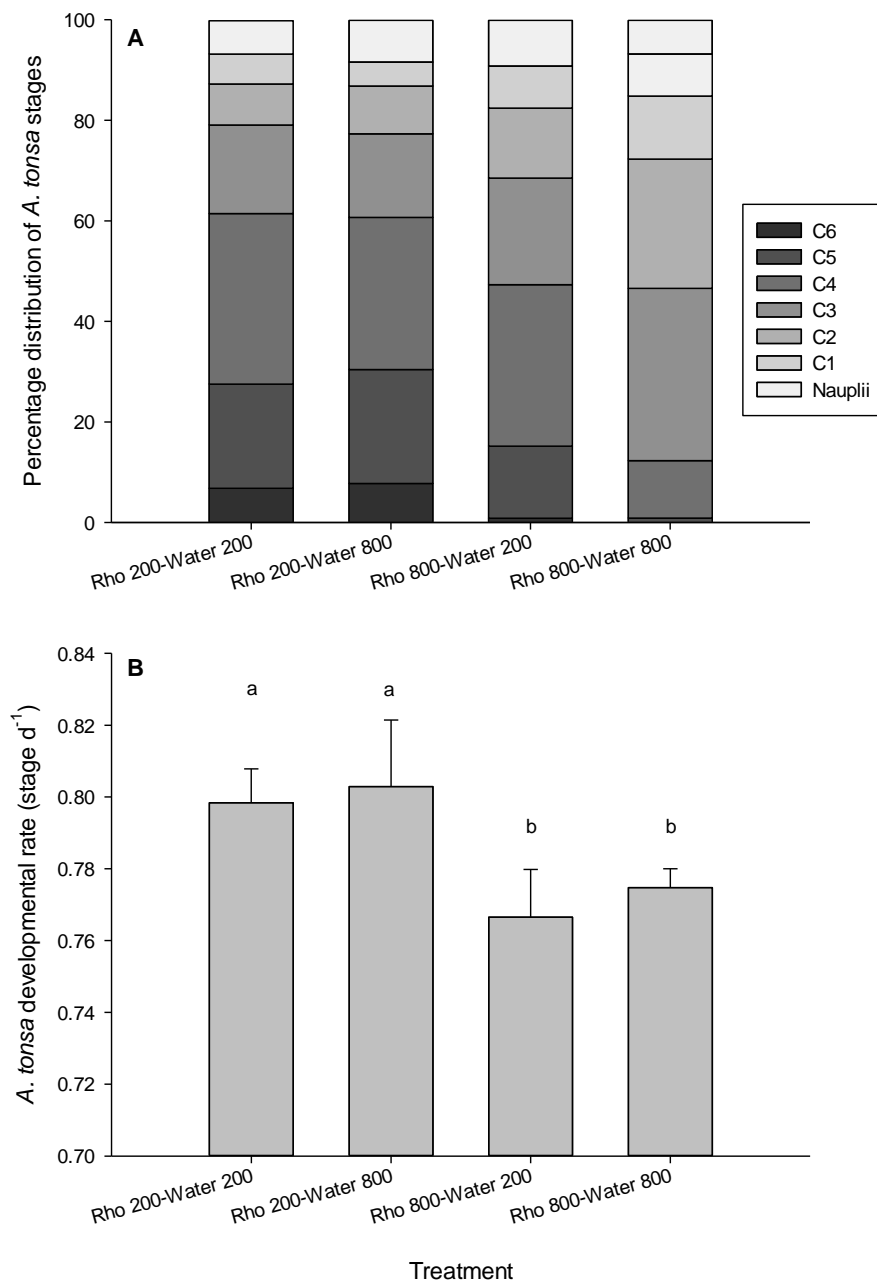


Figure 3: (A) Percentage distribution and (B) developmental rates of *A. tonsa* development stages reared under different $p\text{CO}_2$ and fed with different $p\text{CO}_2$ *R. salina*. Data presented are means and standard deviations of seven replicates. Statistically significant differences ($p < 0.05$) are indicated by letters.

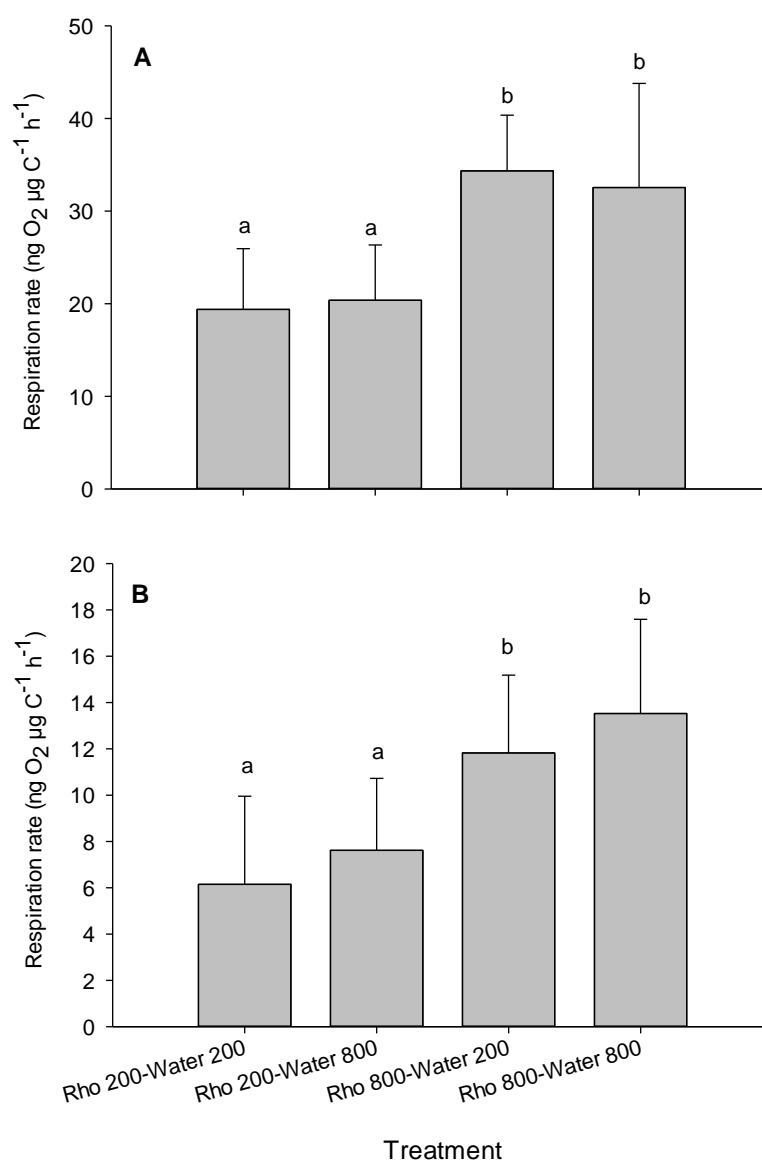


Figure 4: Respiration rates of (A) *A. tonsa* nauplii and (B) copepodites reared under different $p\text{CO}_2$ and fed with different $p\text{CO}_2$ *R. salina*. Data presented are means and standard deviations of seven replicates. Statistically significant differences ($p < 0.01$) are indicated by letters.

Discussion

Although it has previously been suggested that smaller organisms should be more affected by ocean acidification (Flynn et al., 2012), we found no direct effect of seawater $p\text{CO}_2$ on the dinoflagellate and copepod species we studied. This indicates that zooplankton might already be resistant to hypercapnia. Due to environmental variability (*e.g.*, upwelling, rock pools), diapause at depth, many zooplankton (including larval stages) already face pH levels much

lower than those predicted for surface waters in the coming century (Olson and Kawaguchi, 2011). Thus, predicted changes in surface seawater pH may be small relative to the range of pH zooplankton experience during their lifespan. These organisms could already be well adapted to seawater pH variations and potential effects of hypercapnia.

Our study, however, shows that the primary producer used in this study increased its cellular carbon content when cultured under elevated $p\text{CO}_2$. Both higher C fixation and increased growth rate under high $p\text{CO}_2$ could result in increased C:nutrient ratios. Culturing *R. salina* under different $p\text{CO}_2$ at identical dilution rates (i.e. growth rates) in chemostats yielded different C:nutrient ratios (Figure 1, see also Schoo et al., 2013). This indicates that elemental stoichiometric differences are caused by higher C fixation rather than by higher growth rates under high $p\text{CO}_2$. This change in algal biochemical composition, and therefore quality, decreased the growth of the dinoflagellate *O. marina* as well as the development of the copepod *A. tonsa*. Thus, not only copepodites (as shown by Schoo et al., 2013) but also nauplii and microzooplankton react with decreasing growth with increasing CO_2 availability to the algae. Altogether, the growth rate and development of microzooplankton and mesozooplankton decrease at higher $p\text{CO}_2$, coupled with the suppression of reproductive scope identified by other studies (Cripps et al., 2014a; Cripps et al., 2014b) have clear potential to damage population growth dynamics.

To our knowledge, none of the previous studies investigated the direct and indirect effects of near-future $p\text{CO}_2$ levels on zooplankton. Only Rossoll et al. (2012) conducted a full factorial experiment testing direct and indirect high $p\text{CO}_2$ effects on copepods. However, their work suffers from the fact that copepods did not contain any long chain fatty acids in acid treatments although they were provided by the algae (at decreased amounts). This points to an unexplained lack of feeding rather than an effect of food quality. Our results therefore bring important new knowledge on the impact of ocean acidification on copepods. Further, our study contradicts the conclusions of the few existing experimental studies on microzooplankton which found no effects of increased $p\text{CO}_2$ (Suffrian et al., 2008; Rose et al., 2009; Aberle et al., 2013). However, the major focus of those studies was on the direct effects of increased $p\text{CO}_2$, which, as we identified here, does not impact microzooplankton. Although we expected that the direct effect of high CO_2 would be most pronounced when the grazers were feeding on low algal quality, the interaction between these two treatments did not alter zooplankton performances. This could be the result of an elevated energy expenditure enabled by higher algal energy content and should result in higher grazers' respiration rates. However, nauplii and copepodites respiration was only increased by low

algal quality. As previously described by Schoo et al. (2013), we suggest that increased respiration rates represented a physiological response to excrete the excess C obtained from prey grown in high $p\text{CO}_2$ conditions, rather than a stress response to deal with low pH.

In this study, we found that direct $p\text{CO}_2$ effects on consumers seem to be of lesser importance than the associated decrease in algal quality. Several studies have investigated the direct effects of ocean acidification on zooplankton. While elevated $p\text{CO}_2$ does not seem to affect adult copepods, hatching rates are negatively affected by very high $p\text{CO}_2$ (Kurihara et al., 2004; Mayor et al., 2007; Cripps et al., 2014b). Further, the decrease of primary producers' quality under high $p\text{CO}_2$ conditions negatively affects zooplankton production and growth. However, the generality of this result remains uncertain, as community level dampening, such as species richness and complex trophic interactions, may compensate for low food quality (Rossoll et al., 2013). Indeed, the CO_2 effect in the one alga – one copepod species food chain in the study by Rossoll et al. (2012) vanished when the same zooplankton species fed on a semi-natural food mixture in mesocosms (Rossoll et al., 2013). Nevertheless, lower growth rates of zooplankton, as shown in this study, may lead to lower availability of food for the next trophic level and thus potentially affect the recruitment of higher trophic levels. Furthermore, quality effects have also been shown to travel up the food chains (Malzahn et al., 2007), and decreased algal quality may affect higher trophic levels as well.

General discussion

Overall, the results from the aforementioned studies suggest that warming can be expected to have a stronger effect on natural MZP communities than elevated CO₂ levels. Furthermore, they indicate that nutrient concentrations should be considered as an additional factor when assessing the effects of climate change on plankton communities. Our results support the hypothesis that indirect effects of CO₂ via changes in the phytoplankton community or stoichiometry are more important to MZP than direct effects under realistic end-of-century CO₂ concentrations. In contrast, warming can be expected to both directly affect MZP communities increasing their growth rates as well as indirectly affect them via increased mesozooplankton grazing pressure.

Single- and multiple-stressor experiments

Both the mesocosm studies and the laboratory experiment support the previously reported high tolerance of MZP communities towards realistic IPCC acidification scenarios. Nevertheless, we did find effects of CO₂ and effects of the interaction of CO₂ and warming on MZP, but only in a nutrient-deplete post-bloom situation. In the Skagerrak mesocosm study, dinoflagellate biomass was positively affected by the high CO₂ treatment towards the end of the experiment when nutrients had been used up. Likewise in the Baltic Sea mesocosm studies, we did not observe an effect of CO₂ on MZP during the autumn bloom while warming led to an earlier bloom peak, higher growth rates and a higher diversity of MZP. Interaction effects of CO₂ and warming on MZP were not found. Yet, MZP biomass was significantly positively affected by the combination of CO₂, warming, and time in the post-bloom summer situation. The negative slopes of the effects of CO₂ and warming as single stressors turning positive in interaction (Chapter III, Table 3) indicate that at a higher CO₂ level, the negative effect of warming was less severe. Exactly the same pattern was observed for the timing of the MZP and ciliate bloom peaks which were earlier under the combination of the stressors even though both CO₂ and warming led to a delay of the bloom when the factors were addressed separately. Furthermore, this was also the pattern observed for the phytoplankton biomass in this specific study where increasing CO₂ led to a higher biomass while higher temperatures led to a lower one (Paul et al., 2016).

The findings for MZP and phytoplankton are in line with one of the very few CO₂ mesocosm studies including protozoa conducted during both nutrient-deplete conditions in summer and nutrient-replete bloom conditions in winter. Sala et al. (2016) observed stronger effects of acidification in terms of higher abundances of phytoplankton and protozoa in the nutrient-deplete experiment while in the nutrient-replete one, only picophytoplankton was affected. For temperature, there is more information available about the influence of the nutrient concentration. The effects we observed in our studies indoor mesocosm studies match the model developed by Lewandowska et al. (2014, see also Figure 1), predicting a positive influence of warming on MZP in terms of a higher abundance when nutrient concentrations are high, but a negative effect at low nutrient concentrations, based on the intense grazing by copepods which is also increased by warming.

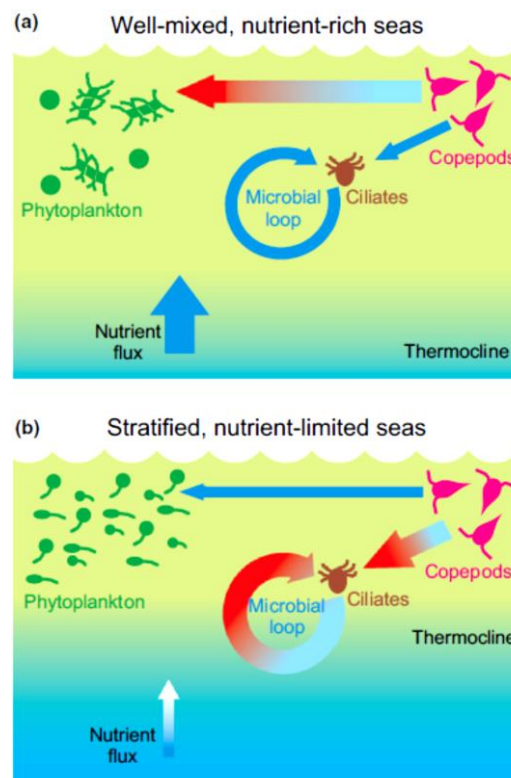


Figure 1: Models for the effects of warming on trophic interactions in plankton communities as indicated by the red arrows. In (a) nutrient-rich waters where grazer effects are dominant and (b) nutrient-limited waters where nutrient effects dominate (after Lewandowska et al., 2014).

The main difference here lies in the phytoplankton community mainly consisting of large diatoms providing a good food source for copepods at high nutrient concentrations. At low nutrient concentrations, small flagellates which are considered as inedible for most mesozooplankton (preferred food size $>5 \mu\text{m}$, Sommer et al., 2005) dominate the system.

However, they are within the feeding range of MZP (Christaki et al., 1998; Johansson, 2004; Aberle et al., 2007) thus boosting MZP standing stocks which are in turn grazed intensively by copepods. The dominance of small-sized phytoplankton taxa at nutrient-limited conditions was also found in our study (Chapter III). Similarly, in the experiment conducted at nutrient-rich conditions, a diatom bloom including medium- to large-sized taxa occurred (Chapter II). In short, warming can switch the system from bottom-up to top-down control, and provided high nutrient concentrations, MZP can profit from such a switching. At nutrient-limited conditions, CO₂ resulted in higher phytoplankton biomass, thus the hypothesized fertilizing effect of increased carbon content, especially on small-sized taxa (Low-Decarie et al., 2014) was indeed observed. The positive effect on MZP biomass was thus most likely indirectly caused by higher phytoplankton concentrations.

In the light of nutrient influence on the effects of CO₂, the analysis of the KOSMOS outdoor mesocosms experiment conducted off the coast of Gran Canary in autumn 2014 will be an interesting addition. The experiment took place over a time of 60 days using a regression layout with target CO₂ levels ranging from ambient to 2000 µatm in a total of 9 mesocosms. Unfortunately, the data analysis of this KOSMOS experiment is not completed so far due to analytical difficulties. But it will be a great supplement to the present studies since the North Atlantic is characterized by low nutrient and plankton concentrations, but a highly diverse plankton community where mesozooplankton grazing accounts for only a very small part of the total grazing impact on phytoplankton (Aristegui et al., 2001). Only during upwelling events in winter, nutrient-rich deep-water reaches the upper layers, resulting in a late winter bloom dominated by picophytoplankton (Schmoker et al., 2014). These upwelling events were simulated during the mesocosm experiment by adding deep-water which had been collected with a novel system. Our expectations are that increased CO₂ should enhance growth of picophytoplankton as observed for other regions (e.g. Rose et al., 2009; Aberle et al., 2013; Sala et al., 2016). Given the results of the positive effects of high CO₂ and warming on MZP biomass from the late summer experiment in the Baltic Sea (Chapter III), we expect an indirect positive effect of CO₂ on MZP during the oligotrophic phase prior to deep-water addition. However, given the generally low concentration of mesozooplankton in the Atlantic in comparison to the Baltic Sea, the result might yet differ from the expectations.

In conclusion, the additional effort to conduct multiple-stressor studies on a community level was rewarded with new insights about the interaction of stressors. Multiple-stressor experiments like the ones described in Chapter II and III are still rare despite the fact that many authors point out that single-stressor experiments are insufficient in investigating the

effects of global change on plankton communities (e.g. Caron and Hutchins, 2012; Calbet et al., 2014). It has been described before by Rose et al. (2009) and Calbet et al. (2014) that the combination of warming and CO₂ results in a different outcome than application of single stressors, which was also observed in our study under nutrient-depletion. To our knowledge, these are the only two plankton community studies available combining warming and CO₂ with a detailed analysis of the MZP community in terms of abundance/biomass and taxonomic composition.

With the continuing increase of CO₂ in the atmosphere promoting both temperature increase and acidification, it is obvious that the two stressors will occur in concert. Given the indication that as far as MZP is concerned both stressors might be acting as antagonists even though by different means, there is a clear need to continue with multiple-stressor approaches to further investigate this suggestion. The results presented in this work further emphasize that it is crucial to include seasonality patterns and differences in the nutrient regime as additional factors. Since eutrophication is another human-induced threat to ecosystems e.g. in the Baltic Sea, there is a strong need to study changes in nutrient influx in concert with climate change induced parameters (HELCOM, 2014). This is a factor that definitely needs more attention when assessing impacts of future climate change, as nutrient concentration might change the outcome of other stressors.

The need for multiple-stressor experiments does not invalidate single-stressor studies as conducted in the Skagerrak (Chapter I) and off Gran Canary (unpublished). These outdoor mesocosm studies followed a different aim, and the large volumes necessary to maintain a true long-term experiment at near-natural conditions cannot be realized indoors at the present. Nonetheless, while effects of CO₂ or nutrients can be tested with the KOSMOS mesocosms, light or temperature conditions are quite challenging to maintain at this scale. Overall, the KOSMOS experiments could be interpreted as multiple-stressor studies in the sense of combining the factors CO₂ and time.

Direct and indirect effects

As previously mentioned, MZP has been assumed to be mainly indirectly affected by CO₂ which is difficult to prove in a mesocosm study due to trophic interactions obscuring possible effects and also a dampening effect of the community (Rossoll et al., 2013). Possible indirect effects include most prominently changes phytoplankton species composition and stoichiometry (Aberle et al., 2013; Meunier et al., 2016). Even in studies reporting no or only subtle effects of high CO₂ levels on MZP, effects on one or more phytoplankton parameters

were observed (e.g. Suffrian et al., 2008; Rose et al., 2009). Yet, based on the dampening effect of the community (Rossoll et al., 2013), it is likely that any effects on MZP that could have been observed disappeared in the mesocosm studies due to factors such as grazing pressure by consumers or switching in prey preferences.

To disentangle effects of environmental stressors from trophic interactions, laboratory studies as described in detail in Chapter IV are important. We were able to show experimentally that indirect effects of CO₂ on MZP and mesozooplankton were indeed more important than direct ones, provided realistic CO₂ concentrations of up to 800 ppm. When mesozooplankton was fed with phytoplankton grown at high CO₂ conditions and thus lower-quality food items, they showed reduced growth and higher respiration rates. However, direct effects on mesozooplankton cultured in high CO₂ seawater could not be observed. Low quality in this case was defined as the higher C:N and C:P ratios measured for the phytoplankton, as the consumers metabolic requirements are usually favoured by lower C:nutrient ratios (Sterner and Elser, 2002). Our results are in line with findings of Schoo et al. (2013) who also reported higher carbon-to-nutrient ratios for the cryptophyte *R. salina* with increasing CO₂ levels as well as higher respiration rates to excrete excess carbon.

The importance of indirect effects also emphasizes the importance of grazing experiments conducted during mesocosm studies (Chapter I and III). Direct effects of environmental stressors on phytoplankton might otherwise remain obscured by grazing and also indirect effects on MZP based on these changes. With the release of MZP from grazing pressure, the interactions at the lower and upper food web level become more visible. Furthermore, the use of the standard dilution method introduced by Landry and Hassett (1982) allows the comparison of phytoplankton growth and zooplankton grazing rates from different marine regions with and without the impact of stressors.

Between-system comparisons

Marine experimental ecology usually addresses benthic and pelagic communities separately. However, during BIOACID II, both benthic and pelagic experiments were conducted using mesocosm approaches. This provides a unique data set thus enabling cross-system analyses on the effects of warming and CO₂ both in the benthos and the pelagial and to account for differences and similarities of these systems. The indoor mesocosm experiments described in Chapter II and III of this thesis covered the interactions in the pelagic in response to multiple stressors. The benthos part was covered using outdoor mesocosm facilities permanently installed at the Kiel Fjord in early summer, late summer, autumn/winter and winter/spring,

thus comparing different nutrient and temperature regimes. The so-called benthocosms allowed observing the impacts of end-of-century temperature and CO₂ levels under realistic light and nutrient conditions using a flow-through system of natural seawater (for detailed setup see Wahl et al., 2015). The benthocosms concentrated on the macroalgae *Fucus vesiculosus*, an important belt-forming brown algae in the Baltic Sea, with its epiphytes and the most important associated invertebrates, *Littorina littorea*, *Gammarus* sp. and *Idotea* sp. (Werner et al., 2016).

Positive effects of high CO₂ on macroalgae in terms of increased growth have been observed before (Kroeker et al., 2013), indicating a potential bottom-up effect with high CO₂ similar to the positive effect hypothesized and experimentally shown for an increase in phytoplankton abundances with high CO₂ (e.g. Paul et al., 2016). Nevertheless, while positive effects of CO₂ on *F. vesiculosus* were not observed in the benthocosm studies, the results point at the importance of the season with its specific nutrient concentrations in determining the effects of stressors (Werner et al., 2016). Nutrients thus seem to be a factor to include in assessing effects of climate change, both in pelagic and benthic systems. The effects of nutrient concentrations on phytoplankton and *F. vesiculosus*, however, are expected to differ. The increasing eutrophication in the Kiel Bight is expected to affect phytoplankton productivity positively, but it has also been shown to result in an overall negative effect on *F. vesiculosus* due to overgrowth by CO₂-stimulated epiphytes possibly affecting the community associated with the algae (Werner et al., 2016).

Effects of temperature differed with the season in both experimental approaches. In principle, a stronger positive effect of warming on herbivore grazing was observed in contrast to the effect on primary production in both benthic and pelagic studies, resulting in an indirect negative effect of higher temperatures on phytoplankton biomass (Paul et al., 2016; Werner et al., 2016, and Chapter II). However, in the pelagic experiments warming in combination with high CO₂ levels also led to an increase in grazing pressure on phytoplankton during summer (Chapter III). In the benthocosms, warming effectively disrupted the top-down grazing of epiphytes leading to overgrowth of *F. vesiculosus* during summer (Werner et al., 2016). This also led to an overall lower biomass of *F. vesiculosus* with warming, but due to different reasons.

The results obtained from mesocosms and benthocosms on the effects of environmental stressors on the community level emphasize once more the striking importance of multiple-stressor and cross-system approaches. While the inclusion of trophic interactions, CO₂ and warming as well as seasonality patterns complicate the interpretation of the results, it also

allows for a more comprehensive understanding on the impacts of global change on aquatic communities. Furthermore, the data collected within BIOACID II enables assessing effects of multiple environmental stressors, thus allowing for direct comparisons between different systems and future predictions.

Outlook

The detailed MZP data collected during the three mesocosm experiments in concert with the great a variety of additional parameters measured are at present the basis for extensive modelling approaches and meta-analyses during the final phase of BIOACID (BIOACID III). It is a valuable addition to the phyto- and mesozooplankton data sets available, addressing a plankton component which has only recently moved into focus despite the importance of MZP both as micrograzer and food source. With the inclusion of MZP, the cycling of matter and energy can be explained in more detail.

At present, it poses a challenge to include the sometimes contradicting results on the effects of environmental stressors on MZP abundance and biomass obtained from different experiments in a model simulating the effects of global change (M. Moreno, in prep.). Moreover, with the addition of another trophic level the system gets more complex. Furthermore, connecting the analyses on pelagic plankton communities with those on benthic communities will be a future challenge within BIOACID III.

In addition, not only different marine provinces but also different seasons might have to be considered separately given the importance of nutrient status and seasonality that was observed both in pelagic and benthic experiments during BIOACID II. This challenge is currently being addressed within BIOACID III relying on the data that was created during phase II, including those presented in this work.

With ongoing global change, it is of great interest to ecologists, politicians and the public to understand how marine communities will be affected. However, it is of utmost importance that actions are taken in order to reduce CO₂ emission and meet the goal of keeping warming beneath 2°C as implemented by the IPCC.

Contributions of multiple authors

Chapter I – Low CO₂ sensitivity of microzooplankton communities in the Gullmar Fjord, Skagerrak: evidence from a long-term mesocosm study

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Submitted to PLOS One

Conceived and designed the experiments: UR, LTB, MB, MGJL, HGH, NA, MAM

Conducted the experiments: HGH, MAM, LTB, UR, AS

Analyzed the data: HGH, NS, AS, MAM, LTB

Wrote the manuscript: HGH, NA, MB, MGJL, UR, LTB, MAM, AS, NS

Chapter II – Effects of high CO₂ and warming on a Baltic Sea microzooplankton community

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Analyzed the data: HGH, JG

Wrote the manuscript: HGH, NA, MB, US, MGJL, JG

Chapter III – Dynamics in the lower food web of a Baltic Sea summer plankton community in a changing ocean: a microzooplankton perspective

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About to be submitted

Conceived and designed the experiments: US, JG, HGH, NA, MB

Conducted the experiments: HGH, JG

Analyzed the data: HGH, JG

Wrote the manuscript: HGH, NA, MB, US, JG

Chapter IV – Direct and indirect impact of near-future $p\text{CO}_2$ levels on zooplankton dynamics

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Conducted the experiments: HGH, JALF, MAM, CLM

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Wrote the manuscript: CLM, MB, HGH, JALF, MAM

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Curriculum vitae

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 - At the GEOMAR Helmholtz Centre for Ocean Research Kiel, Marine Ecology, Marine Food Webs Group
- 2012-2016 Member of the Helmholtz Graduate School for Polar and Marine Research (POLMAR) at the AWI
- 2011 Diploma degree in Biology, University of Goettingen
Supervised by E.I. Meyer and M. Maraun
Thesis topic: Impacts of anthropogenic salinization on amphipods
- At the University of Muenster, Germany, Institute for Evolution and Biodiversity, Department of Limnology
 - In cooperation with the University of Lyon, France, Department of River System Ecology and EcoRing Company, Hardegsen, Germany
- 2010 Oral examinations for the Diploma degree, University of Goettingen
Subjects Ecology, Nature Conservation and Microbiology
- 2006 Intermediate Diploma Degree in Biology, University of Goettingen

International education

2006–2007 Erasmus exchange student, University of Lund, Sweden
Master courses: Limnology, Microbiology, Ecotoxicology, Animal Sensory Biology

International field work

- 2014 KOSMOS 2014 GC Project: large-scale mesocosm experiment, Plataforma Oceánica de Canarias (PLOCAN), Taliarte, Gran Canary, Spain
- 2013 KOSMOS 2013 Gullmar Fjord Project: large-scale mesocosm experiment, University of Gothenburg, Kristineberg Field Station, Sweden
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Work experience

- Since 2012 PhD candidate at the Biologische Anstalt Helgoland and GEOMAR, Kiel
- 2011-2012 Research assistant, University of Muenster, Institute of Evolution and Biodiversity, Department of Limnology
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Publications

- Horn, H.G.**, Boersma, M., Garzke, J., Löder, M.G.J., Sommer, U., Aberle, N. (2015): Effects of high CO₂ and warming on a Baltic Sea microzooplankton community. ICES Journal of Marine Science, doi: 10.1093/icesjms/fsv198
- Meunier, C.L., Algueró-Muñiz, M., **Horn, H.G.**, Lange, J.A.F., Boersma, M. (2016): Direct and indirect effects of near-future pCO₂ levels on zooplankton dynamics. Marine and Freshwater Research, doi: <http://dx.doi.org/10.1071/MF15296>

Publications in review or in preparation

- Bach, L.T., Taucher, J., Boxhammer, T., Ludwig, A., The Kristineberg KOSMOS Consortium, Achterberg E., Algueró-Muñiz, M., Anderson, L., Bellworthy, J., Büdenbender, J., Czerny, J., Ericson, Y., Esposito, M., Fischer, M., Haunost, M., Hellemann, D., **Horn, H.G.**, Hornick, T., Meyer, J., Sswat, M., Zark, M., Riebesell, U. (in review): Influence of ocean acidification on a natural winter-to-summer plankton succession: First insights from a long-term mesocosm study draw attention to periods of nutrient limitation. PLoS One
- Horn, H.G.**, Sander, N., Stuhr, A., Algueró-Muñiz, M., Bach, L.T., Löder, M.G.J., Boersma, M., Riebesell, U., Aberle, N. (submitted): Low CO₂ sensitivity of microzooplankton communities in the Gullmar Fjord, Skagerrak: evidence from a long-term mesocosm study. PLoS One
- Algueró-Muñiz, M., Álvarez, S., Bach, L.T., Esposito, M., **Horn, H.G.**, Ecker, U., Malzahn, A.M., Thor, P., Boersma, M. (in prep.): Ocean Acidification effects on mesozooplankton community development: results from a long-term near-natural conditions experiment. To be submitted to PLoS One
- Horn, H.G.**, Boersma, M., Sommer, U., Aberle, N. (in prep.): Effects of ocean acidification and warming on the succession of a summer plankton community in the Baltic Sea.
- Algueró-Muñiz, M., Álvarez, S., Bach, L.T., **Horn, H.G.**, Spisla, C., Boersma, M. (in prep.): Ocean acidification effects on an oligotrophic zooplankton community.
- Lange, J.A.F., **Horn, H.G.**, Schwenk, K., Boersma, M. et al. (in prep.) Ocean acidification: Long-term effects of low food quality on the copepod *Acartia tonsa*, a selection experiment.

Talks

- Horn, H.G.**, Sander, N., Algueró-Muñiz, M., Garzke, J., Löder, M.G.J., Ludwig, A., Boersma, M., Riebesell, U., Aberle, N.: Life in the future ocean: effects of end-of-century warming and acidification conditions on microzooplankton. AWI Science Meeting, Bremerhaven, Germany, 10/2015
- Horn, H.G.**, Sander, N., Algueró-Muñiz, M., Löder, M.G.J., Boersma, M., Riebesell, U., Aberle, N.: Does ocean acidification affect North Sea microzooplankton communities? 50. European Marine Biology Symposium (EMBS), Helgoland, Germany, 09/2015

Talks

- Meunier, C.L., Algueró-Muñiz, M., **Horn, H.G.**, Lange, J.A.F., Boersma, M.: Ocean acidification indirectly affects the growth of two zooplankters. 50. European Marine Biology Symposium (EMBS), Helgoland, Germany, 09/2015
- Horn, H.G.**, Sander, N., Algueró-Muniz, M., Löder, M.G.J., Boersma, M., Riebesell, U., Aberle, N.: Effects of ocean acidification on North Sea microzooplankton communities. AWI PhD Days, Sylt, Germany, 06/2015
- Horn, H.G.**, Sander, N.: Effects of ocean acidification on North Sea microzooplankton communities. KOSMOS 2013 Gullmar Fjord Data Workshop, Kiel, Germany, 04/2015
- Horn, H.G.**, Aberle, N., Löder, M.G.J, Boersma, M.: BIOACID summer experiment 2013: First microzooplankton results. BIOACID Phase II Data workshop, Kiel, Germany, 07/2014
- Horn, H.G.**, Aberle, N., Boersma, M. KOSMOS 2013 Kristineberg: First microzooplankton results. KOSMOS 2013 Data Workshop, Kiel, Germany, 12/2013
- Horn, H.G.**, Coring, E., Bäche, J., Riss, H.W., Meyer, E.I.: Die Auswirkungen anthropogener Versalzung. Workshop of the University of Muenster and the K+S Kali GmbH, Philippsthal, Germany, 10/2011
- Horn, H.G.**, Coring, E., Bäche, J., Riss, H.W., Meyer, E.I.: Die Auswirkungen anthropogener Versalzung auf heimische und invasive Gammariden. Annual Meeting of the DGL, Technical University of Munich, Freising, Germany, 09/2011

Poster

- Horn, H.G.**, Sander, N., Algueró-Muñiz, M., Löder, M.G.J., Boersma, M., Aberle, N.: Ocean acidification and global warming: can we expect effects on the microzooplankton communities? BIOACID Phase II Final Meeting, Kiel, Germany, 10/2015
- Sander, N., **Horn, H.G.**, Boersma, M., Aberle, N.: Microzooplankton as a trophic upgrader in a high CO₂ ocean: evidence from a small-scale experiment. 50. European Marine Biology Symposium (EMBS) Helgoland, Germany, 09/2015
- Horn, H.G.**, Sander, N., Algueró-Muñiz, M., Löder, M.G.J., Boersma, M., Riebesell, U., Aberle, N.: Effects of ocean acidification on North Sea microzooplankton communities. ASLO 2015 Aquatic Sciences Meeting, Granada, Spain, 02/2015
- Horn, H.G.**, Aberle, N., Löder, M.G.J., Boersma, M.: Effects of ocean acidification and warming on microzooplankton communities in North Sea and Baltic Sea. BIOACID Phase II Meeting, Kiel, Germany, 09/2014
- Horn, H.G.**, Aberle N., Löder, M.G.J., Boersma, M.: Small but important: Effects of ocean acidification and warming on microzooplankton communities. AWI PhD Days, Helgoland, Germany, 05/2014
- Algueró-Muñiz, M., Lesniewski, T.J, Meunier, C.L., Lange, J.A.F., **Horn, H.G.**, Ecker, U., Thor, P., Boersma, M.: Direct and indirect effects of CO₂ in mesozooplankton: a review. AWI PhD Days, Helgoland, Germany, 05/2014
- Horn, H.G.**, Aberle, N., Löder, M.G.J., Boersma, M.: The role of microzooplankton under future ocean acidification and warming scenarios. BIOACID Phase II Meeting, Warnemuende, Germany, 10/2013
- Horn, H.G.**, Aberle, N., Löder, M.G.J, Boersma, M.: The role of microzooplankton under future ocean acidification and warming scenarios. BIOACID Phase II Kick-off Meeting, Kiel, Germany, 11/2012

Eidesstattliche Erklärung

Hiermit erkläre ich, dass die vorliegende Dissertation, abgesehen von der Beratung durch meine Betreuer, selbständig von mir angefertigt wurde und nach Inhalt und Form meine eigene Arbeit ist. Ich habe diese Arbeit noch keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt oder zur Veröffentlichung eingereicht.

Die Promotion erfolgt im Fach Meeresbiologie. Des Weiteren erkläre ich mich damit einverstanden, dass Zuhörer bei der Disputation zugelassen werden.

Ort, Datum

Henriette Horn