Effect of climate warming on phytoplankton size structure and species composition: an experimental approach

Dissertation

in fulfilment of the requirements for the degree “Dr. rer.nat” of the Faculty of Mathematics and Natural Science at University of Kiel

Submitted by

Kalista Higini Peter

Kiel, 08.07. 2014

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Date of Examination: 08.07.2014

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Summary

Shrinking of body size has been proposed as one of the universal responses of organisms to global climate warming. Using phytoplankton as an experimental model system has supported the negative effect of warming on body size. However, there is no consensus about the underlying mechanisms. Explanation under the roof of Temperature Size Rule (TSR), clearly refer to size shift within species while community shift are often explained by intensified resource competition at higher temperatures and competitive advantages for smaller species. As an alternative explanation, intensified predation on larger prey items at higher temperatures has been suggested. This would apply only under specific food web configurations, e.g. if phytoplankton grazing is dominated by copepods which tend to feed on larger food particles while releasing small phytoplankton by predation from heterotrophic protists.

The current study aimed to clarify the underlying mechanisms which induce size reduction of organisms in response to warming. In this study, marine phytoplankton is used as a model system.

The first experiments were designed to test the mediating role of predation in the size response to temperature (TSR).Temperature was combined factorial with 3 types of grazing pressure, i.e. grazing by copepods, by microzooplankton and by nanozooplankton (Chapter 1). The predicted decrease in cell size with warming was confirmed for the majority of the phytoplankton species. Similarly, community mean cell size decreased with increasing temperatures. The results further showed that larger phytoplankton shrink more strongly than small ones, an effect which had not yet been reported in the literature before. Both, the interspecific and the community level size effects of warming were stronger under copepod gazing than under protists grazing. However, there was no reversal of sign under protist grazing, as would have been predicted from the feeding preference of protist grazers for smaller phytoplankton. This indicates that size selective predation has an influence on temperature-size relationship but that predation cannot be the dominant factor. Further factors are needed to explain the shrinking effect of temperature under protists grazing, which alone should be a selective advantage for bigger cell sizes. These results motivated the hypothesis that increasing nutrient stress at higher temperatures could be an important factor. Therefore, I performed a further experiment with a factorial combination of temperature and nutrient (nitrogen) stress (Chapter 2). Nutrient stress was manipulated by the rate of dilution according to the semi-continuous culture principle. However, a nutrient–independent role of temperature could not be assessed from a direct comparison of different treatments, because temperature itself influenced the strength of nutrient stress. Therefore C:N ratios of the biomass were taken as an indicator of the intensity of nutrient stress and the effects of temperature and

nutrient stress assessed by multiple regression with temperature and C:N ratios as independent variables. The results indicate that the direct temperature effect is much weaker than nutrient effect.

A further analysis of this experiment concentrated on the taxonomic response of phytoplankton to the impact of warming and of nutrient stress (Chapter 3). It confirmed the frequently reported replacement of large by small species under increasing temperature and nutrient stress. It was further asked, whether the response of the different species to the experimental treatment could also be explained by their phylogenetic position (diatoms vs. dinoflagellates). Compared to the effect of cell size, the effect of phylogenetic position turned out to be minor.

As demonstrated in Chapter 2 & 3 the nutrient (nitrogen) effect on phytoplankton cell size is dominant over direct temperature effects. However, the question remained, whether the nutrient effect would be similar for other potentially limiting nutrients. Therefore, a further experiment (Chapter 4) was carried out where temperature, nitrogen limitation and phosphorus limitation were employed as independent variables. The results indicated that the effect of N –limitation effect is stronger than the effect of P-limitation but both nutrient effects dominated over direct temperature effects.

In conclusion, direct temperature, nutrient, and grazing effects as explanations for temperature dependent size trends are not mutually exclusive, while general results indicate strongly nutrient effect dominance over direct temperature effect. However, the effect of grazing is expected to be less consistent, because phytoplankton groups of different size are grazed by different groups of grazers.

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Zusammenfassung

Die Reduktion der Körpergröße wird als eine universelle Reaktion von Organismen auf globale Klimaerwärmung gesehen. Bisherige experimentelle Untersuchungen mit Phytoplankton Modellsystem haben die negativen Effekte der Erwärmung auf Körpergröße überwiegende bestätigt, meist jedoch ohne die innerartliche Größenveränderungen und Verschiebungen zwischen großen und kleinen Arten getrennt zu betrachten. Es besteht jedoch kein Konsens über die zugrundeliegenden Mechanismen. Während die Temperatur-Größe-Regel (TSR) eine rein physiologische Erklärung bietet, wird für das Phytoplankton häufig eine Intensivierung der Ressourcenkonkurrenz (in erster Linie um limitierende Nährstoffe) und ein Konkurrenzvorteil für kleiner Zellen angenommen. Dominiert Grazing durch Copepoden, kommt auch ein intensivierter Fraßdruck durch Copepoden als Erklärung in Frage, da Copepoden bevorzugt größere Phytoplankter fressen und kleinere vom Fraßdruck durch heterotrophe Protisten entlasten..

Die aktuelle Studie zielte darauf ab, die zugrundeliegenden Mechanismen, die eine Größenreduzierung von Organismen bei Erwärmung hervorrufen, abzuklären. In dieser Studie wird marines Phytoplankton als Modellsystem verwendet.

Um die Rolle der Prädation zu testen, wurden Experimente mit einer faktoriellen Kombination von Erwärmung und verschiedneen Typen von Konsumenten (Copepoden, Mikrozooplankton, Nanozooplankton) durchgeführt (Kapitel 1). Die Abnahme der Zellgröße mit Erwärmung wurde für die Mehrheit der Phytoplanktonarten bestätigt. Ebenso nahm die mittlere Zellgröße der gesamten Phytoplanktongemeinschaft ab. Die Ergebnisse zeigten zum ersten Mal, dass bei erhöhten Temperaturen größere Phytoplanktonarten stärker schrumpfen als kleine Arten. Sowohl der innerartliche als auch zwischenartliche Effekt waren unter Copepoden-Grazing stärker als unter Protisten-Grazing, obwohl die erwarte Umkehrung des Effekts unter Protisten-Grazing ausblieb. Dies zeigt, dass größenselektiven Prädation einen Teil der Temperatur-Größe Beziehung erklären kann, nicht jedoch der alleine dominierende Faktor ist. Deshalb wurde in einem weiteren Experiment die Hypothese untersucht, dass Nährstoffstress ein weiterer Parameter sein könnte, der die Beziehung von Temperatur und Größe beeinflusst. Temperatur wurde mit Nährstofflimitation faktoriell kombiniert (Kapitel 2). Der Naährstoffstress (in disem Fall N-Stress) wurde durch unterschiedliche Verdünnungsraten nach dme Prinzip der semikontinuierlichen Verdünnung manipuliert. Allerdings zeigte sich, dass auch die Termperatur die Intensität der Nährstofflimitation beeinflusste, weshalb für eine vergleichende Auswertung von direkten, nährstoffunabhängigen Temperatureffekten und Nährstoffeffekten nicht die Verdünnungsrate nicht als unabhängige Variable eingesetzt werden konnte. Deshalb wurde das C:N-Verhältnis in dere Biomasse als

Indikator für die Intensität des Nährstoffstress herangezogen und dadurch konnte ein nährstoffunabhängiger Effekt mittels multipler Regression ermittelt werden, mit Temperatur und C:N-Verhältnisse als unabhängigen Variablen. Die Ergebnisse zeigten, dass der Temperatureffekt wesentlich schwächer ist als der Nährstoffeffekt.

In einer weitergehenden Analyse des selben Experiments wurde die Reaktion der verschiedenen Phytoplanktonarten auf die Faktoren Erwärmung und Stickstoffstress untersucht. Untersucht (Kapitel 3), In Übereinstimmung mit weit verbreiteten Annahmen, zeigte sich, dass kleinere Arten sowohl von höheren Temperaturen als auch von stärkerem Nährstoffstress begünstigt wurden. Außerdem wurde thematisiert, inwieweit die Reaktion der Arten auf Temperatur und Nährstoffe nicht nur von deren Größe sondern auch vom phylogenetischem Status abhängt (Diatomeen vs. Dinoflagellaten). Dabei erwies sich der Einfluss der Zellgröße als wichtiger als der phylogentische Status.

Während Kapitel 2 und 3 zeigten, dass der Nährstoffeffekt einen größeren Einfluss auf die Phytoplanktonzellgröße als der direkte Temperatureffekt hatte, war nicht klar, ob sich diese Ergebnis auch auf andere, portentiell limitierende Nährelemente übertragen ließe. . Deswegen wurde weiteres Experiment (Kapitel 4) durchgeführt um zu testen, ob die durch Stickstofflimitierung oder durch Phosphatlimitierung hervorgerufenen Temperatureffekte auf die Zellgröße gleich oder unterschiedlich in ihrer Richtung oder Intensität sind. Das Experiment wurde in einer faktoriellen Kombination von Temperatur, Art der Nährstofflimitierung (N oder P) und Intensität der Nährstofflimitierung durchgeführt. Die Ergebnisse zeigten, dass die N-Limitierung einen größeren Effekt hat als die P-Limitierung. Beide Nährstoffeffekte dominierten jedoch gegenüber dem direkten Temperatureffekt.

Abschließend lässt sich sagen, dass die direkte Temperatureffekte, Nährstoffeffekte und Fraßdruck als Erklärung für die temperaturabhängigen Größentrends einander nicht gegenseitig ausschließen. Allgemein zeigte sich jedoch eine Dominanz des Nährstoffeffektes gegenüber dem direkten Temperatureffekt. Der Effekt des Fraßdrucks ist weniger konsistent und kontextabhängig, da Phytoplanktongruppen unterschiedlicher Größe von unterschiedlichen Gruppen von Grazern gefressen werden.

General introduction

Phytoplankton responses to sea surface warming

Marine phytoplankton contributes to the biological regulation of the climate and provides half of the world's primary production (Baumert & Petzoldt, 2008, Boyce *et al.*, 2010). Due to importance of phytoplankton detecting the impact of global climate change on phytoplankton size structure or species composition is an essential task. Global climate change is predicted to alter the ocean's biological productivity. According to different climate scenarios (IPCC, 2007), the temperatures of the ocean surface waters are predicted to increase by 1 to 6 °C within the 21st century (IPCC, 2007). Furthermore, oceanographic studies have shown a decline of marine phytoplankton biomass and primary productivity in response to climate warming from the beginning of oceanographic observations until now and the trend will continue over the coming century (Behrenfeld *et al.*, 2006, Boyce *et al.*, 2010, Henson *et al.*, 2010, Hofmann *et al.*, 2011, Sommer *et al.*, 2012, Steinacher *et al.*, 2010). Similarly, the study of 9-years' time series suggested the expansion of oligotrophic portions of subtropical gyres of the world oceans (Polovina *et al.*, 2008) resulting in a reduction of chlorophyll and productivity in the large sub-tropical gyres. As a further consequence of rising temperature of ocean surface waters and associated hydrophysical changes (stratification, vertical mixing) changes in the distribution of phytoplankton species are expected. Some phytoplankton species are adapted to warm temperature and low nutrient levels (e,g small picophytoplankton) while other species prefer turbulent cool and nutrient-rich water e.g large phytoplankton.(Henson *et al.*, 2010).

Phytoplankton size structure and nutrient supply

Biogeographic ddifferences in present-day phytoplankton size structure in the ocean depend mainly on nutrient supply and not on direct effects of temperature (Maranon 2012). Phytoplankton nutrient uptake and growth are described as function of internal and external nutrient concentration (Droop, 1974) and differ strongly between species with small one being better adapted to take up nutrients at low nutrient concentrations (Litchman & Klausmeier, 2008). Furthermore, the rate of cell division for large cell sizes require greater nutrients uptake fluxes compared with small cells (Furnas, 1978). Therefore conditions which induced cell division such as temperature in a particular range of cell size will eventually favour the dominance of those size classes in terms of biomass. For example, when nutrients are low, phytoplankton community mainly are dominated by small cells. The advantage of smaller phytoplankton is usually explained by to their large-surface-area-to-volume ratio which helps to avoid nutrient diffusion limitation (Chisholm, 1992, Kiørboe, 1993).

Additionally, under nutrient limited conditions, small sized cells are able to use nutrient more effectively than larger ones (Harris 1992, Kormas et al 2002).

Biological temperature effect on phytoplankton species and size classes

The concern about climate change has re-vitalized the interest in the classic biogeographic rules relating body-size of organisms to latitude and temperature, e.g. Bergmann's rule (Bergmann, 1848) and Jame's rule (James, 1970). More recently, they were supplemented by the Temperature Size Rule (TSR) which predicts a smaller body size at maturity under higher temperatures. This is due to the fact that maturation is accelerated more strongly by warming than somatic growth (Atkinson 1994, Foster 2011). Furthermore, experimental studies on potential climate change effects have shown that elevated temperature lead to shifts in phytoplankton species composition and a decline in cell size even in the absence of temperature driven changes in stratification and mixing patterns (Hilligsøe *et al.*, 2011, Morán *et al.*, 2009, Sommer & Lengfellner, 2008, Yvon-Durocher *et al.*, 2010). Moreover, temperature directly alters photosynthesis and respiration rates but this direct effect can be outweighing by other factors e.g grazing (Gaedke *et al.,* 2010).

Importance of phytoplankton size in the marine ecosystem

Phytoplankton size structure plays a crucial role in controlling the trophic and biogeochemical functioning of pelagic ecosystem. Large phytoplankton cells tend to be grazed by larger zooplankton, resulting in shorter, simpler food webs and a more efficient matter and energy transfer to grazers and carnivores (Ryther, 1969) thus leading to a higher ration of fish production to primary production (Kiørboe, 1993). Furthermore, phytoplankton size structure not only determines the trophic organization of pelagic ecosystems and thus the efficiency with which organic matter produced by photosynthesis in channeled towards upper trophic level but also the export of organic matter to the ocean's interior (Falkowski & Oliver, 2007, Finkel *et al.*, 2010, Legendre & Rassoulzadegan, 1996). Large and dense phytoplankton cells are responsible for the majority of exported production (Tremblay *et al.*, 1997). Therefore, the dominance by large species allows a more efficient transfer of organic matter through short food chains towards upper trophic levels, as well as enhanced downward export fluxes and biological CO_2 drawdown than dominance by small cells (Maranón *et al.*, 2012). In contrast, small phytoplankton is associated with low sedimentation rates and intense recycling of matter through the microbial food web in the surface zone which results in little potential for carbon export.

Mechanisms inducing phytoplankton size shift

In summary the following hypothetic pathways of causation could operate:

1) Indirect temperature effects mediated via hydrography and nutrients: warming will intensify vertical stratification of the water column and thereby reduce the vertical upward flux of nutrients. The resulting reduced availability of nutrients will select for smaller cell sizes.

2) Indirect temperature effects mediated via hydrography and sedimentation: Intensified stratification and reduced turbulence will potentially increase the role of sinking as selective factor and favor small, slowly sinking phytoplankton cells.

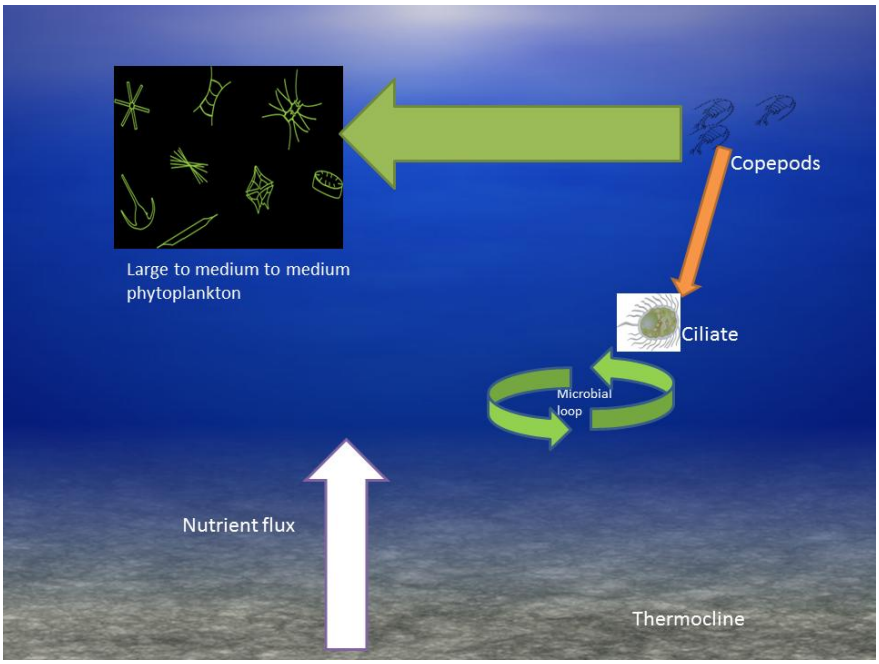
3) Indirect temperature effects mediated via grazing: Warming will increase grazing rates and thereby increase the selective advantage of more grazing resistant phytoplankton, which in the case of copepod grazing release the smaller ones.

4) Indirect temperature effects mediated via nutrient demand: In order to balance higher losses at higher temperatures phytoplankton will need higher growth rates which increase nutrients demands. This should lead to higher nutrient stress, if supply does not increase at the same time.

5) Direct temperature effects: This includes all physiological effects which happen in the absence of hydrographic change and changes in biotic interactions and includes different responses of ontogenic development and somatic growth, as hypothesized by the TSR.

   In principle, shifts in phytoplankton cell size can be brought about by shifts between different sized species and size shifts within species. So far, most studies have concentrated on only one of the two aspects

   (i) Well mixed sea in absence of surface warming



(ii) Climate warming, inhibits mixing and reduce upward nutrient transport

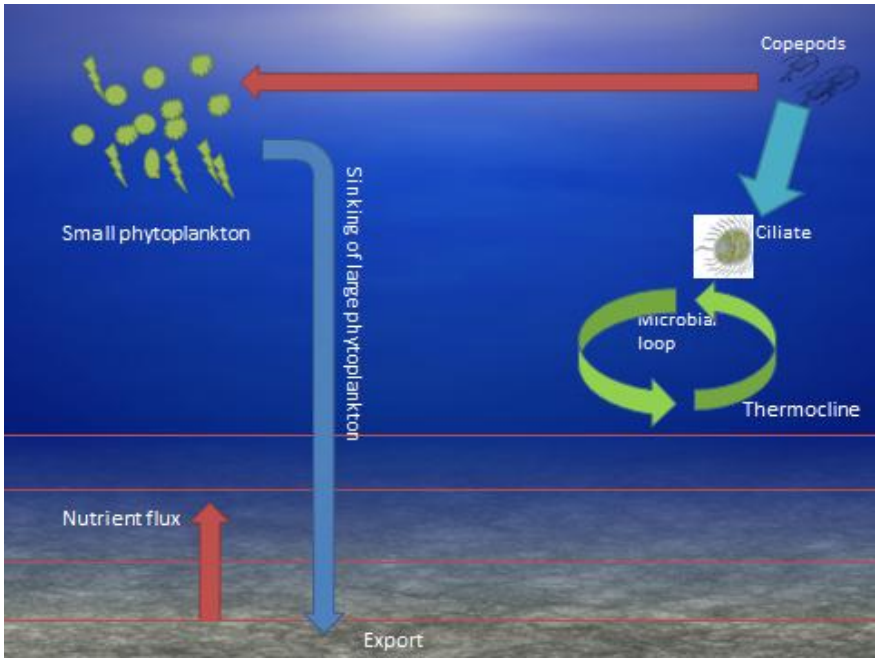


Fig 1: Response of marine pelagic ecosystems to sea surface warming. (i) In absence of surface warming, there is vertical mixing and high nutrient transport to the euphotic zone and large phytoplankton is dominant. These are grazed by copepods while ciliates feed on small phytoplankton. (ii) Under climate warming, surface temperatures' inhibit vertical mixing, increase the density gradient between the upper and lower water layers (thermocline) which supresses the upward flux of nutrients and reduces nutrient availability of nutrient for large phytoplankton. This condition favours small phytoplankton mainly flagellates than large phytoplankton. In this case, copepods feed more on ciliates and the importance of the microbial loop for energy flows will increase. Warming as indicated by gradient arrows elevates the thermocline and decrease vertical movement of nutrient and increase grazing pressure and eventually leads to shift towards small phytoplankton.Furthermore, as indicated (ii) warming induced faster sinking of large phytoplankton due to increasing potential for building aggregates

Objectives of the study

The current study aimed to elucidate several of the mechanism which may induce phytoplankton size reduction in response to warming which approached through different experiments. Due to logistic reasons, differences in stratification and vertical mixing could not be micked. Thus, the factors studied were direct temperature effects, gazing and nutrients

In **chapter 1**, it was aimed to test the role of predation in temperature-size relationship by performing phytoplankton experiments with factorial combination of warming and consumer type. It was hypothesized that, individual and community mean cell size decrease with warming and the effect is modified by grazers. Furthermore, it was expected the reversal of sign in the temperature-size relationship, i.e. negative under copepods and positive under protozoan grazing.

The **second chapter** assessed the extent of direct temperature effect on phytoplankton size mediated via nutrient limitation. The experiment was designed as a full factorial combination of temperature and levels of nutrient (nitrogen) stress.

Three level of temperature were used while the strength of nutrient limitation was manipulated by semi-continuous dilution.

In **Chapter 3,** a further statistical analysis from chapter 2 was carried out to assess the effect of warming on phytoplankton species composition. It was predicted that, nutrient stress and warming influence phytoplankton composition and the temperature effect should be stronger at more intense nutrient stress. It was also hypothesize that inter-specific difference in the response of phytoplankton species to temperature and nutrient limitation are primarily controlled by their size rather than phylogenetic status.

Lastly, it was aimed to compare the temperature effect on phytoplankton structure mediated under nitrogen and phosphorus limitation (**Chapter 4**). The experiment was performed in a factorial combined of temperature, type of nutrient limitation (N vs. P), and strength of nutrient limitation

CHAPTER 1

Phytoplankton cell size: intra- and interspecific effects of warming and grazing

ABSTRACT

Decreasing body size has been suggested as the third universal biological response to global warming after latitudinal/altitudinal range shifts and shifts in phenology. Size shifts in a community can be the composite result of intraspecific size shifts and of shifts between differently sized species. While metabolic explanations for the size shifts dominate in the literature top down effects, i.e. intensified size-selective consumption at higher temperatures, have been proposed as alternative explanation. Therefore, the phytoplankton experiment was performed with a factorial combination of warming and consumer type (protist feeding mainly on small algae vs. copepods mainly feeding on large algae). Natural phytoplankton was exposed to 3 (1^{st} experiment) or 4 (2^{nd} experiment) temperature levels and 3 (1^{st} experiment, nano-, microzooplankton, copepods) or 2 (2^{nd} experiment (microzooplankton, copepods) types of consumers. Size shifts of individual phytoplankton species and community mean size were analysed. Both, mean cell size of most of the individual species and mean community cell size decreased with temperature under all grazing regimes. Grazing by copepods caused an additional reduction in cell size. These results reject the hypothesis, that intensified size selective consumption at higher temperature would be the dominant explanation of decreasing body size. In this case, the size reduction would have taken place only in the copepod treatments but not in the treatments with protist grazing (nano- and microzooplankton).

1.1 INTRODUCTION

Changed biogeographic distributions and seasonal patterns are the two most general and most often reported biological responses to global climate warming (Parmesan & Yohe, 2003, Root *et al.*, 2003, Walther *et al.*, 2002). Recently, a debate emerged whether a reduction in body size can be considered the third universal response to warming (Gardner *et al.*, 2011, Yvon-durocher *et al.*, 2011). Such a trend would conform to classic biogeographic rules (Bergmann, 1848, James, 1970b) which predict smaller body sizes in warmer climates. While those rules were initially coined for endotherms and explained by easier thermoregulation at lower surface: volume ratios, they were later extended to ectotherms. A physiological explanation was provided by the Temperature Size Rule (TSR) which predicts a smaller final body size at maturity because maturation is accelerated more strongly by warming than somatic growth (Atkinson, 1994, Forster *et al.*, 2011) Changed body size distributions in a community or a trophic level consist of three different components: species replacements, changes in age structure of individual populations and size changes at a defined age or developmental stage within species (Daufresne *et al.*, 2009).

While size reduction in response to warming seems to become an accepted rule in spite of counter-examples (Table 1 in (Gardner *et al.*, 2011) for vertebrates; (Rüger & Sommer, 2012) for phytoplankton) there is no consensus about the underlying causality, given that the prevailing explanations are not being mutually exclusive. Explanations under the roof of the TSR (Atkinson, 1994) explicitly refer to size shifts within species. Community or trophic level wide shifts brought about by dominance shifts between species are often explained by intensified resource competition under higher temperatures and competitive advantages for smaller species (Finkel *et al.*, 2010, Finkel *et al.*, 2005, Finkel *et al.*, 2007, Irwin *et al.*, 2006, Winder *et al.*, 2009, Yvon-Durocher *et al.*, 2010). As an alternative explanation, intensified predation at higher temperatures has been suggested, particularly for primary producers, because heterotrophic metabolic rates grow faster with temperature than photosynthesis (Allen *et al.*, 2005, López-Urrutia *et al.*, 2006, Sommer & Lengfellner, 2008, Yvon-Durocher *et al.*, 2010). The predation effect should be particularly strong when predators prefer larger prey, such as copepods as predators on phytoplankton (Sommer & Lewandowska, 2011, Sommer & Sommer, 2006). However, the predation effect should be reversed or partially reversed, if the prevailing predators prefer small prey. In this case, stronger predation at higher temperature would lead to a stronger removal of small prey.

In order to test the role of predation in temperature-size relationships, marine phytoplankton used as a model system because of (a) their importance as primary producers by contributing ca.

50% of global primary production, (b) their short generation time and ease of experimental handling, and (c) because the size effects of their main predators are well known. Copepods tend to suppress medium to moderately large sized phytoplankton (lower limit 10^2 to 10^3 µm3, upper limit 10^4 or 10^5 µm3 cell volume, (Sommer & Sommer, 2006) but also microzooplankton (mainly ciliates and heterotrophic dinoflagellates). Thereby, they release smaller phytoplankton from grazing pressure, because most microzooplankton feed on phytoplankton <500 to 1000 µm3 (Sommer et al., 2005). Overall, interspecific grazer effects should have a stronger impact on community mean body size than intraspecific ones, because intraspecific size differences are usually much smaller than interspecific ones.

In this study, the working hypotheses are:

1. Cell size of individual phytoplankton species decreases with temperature.
2. Temperature effects on cell sizes of species will be modified by grazers.
3. Warming leads to a decrease of community mean cell size of phytoplankton.
4. Temperature effects on community mean cell sizes will be modified by grazers.
   4a. (strong version): There will be a reversal of sign in the temperature – size relationship (negative under copepod grazing, positive under protozoan grazing)
   4b (weak version): Different grazer guilds will only modify the response, but not reverse it.

1.2 MATERIALS AND METHODS

1.2.1 Experiment Design

The first experiment was conducted from 1st to 28th April 2011. The experiment was performed in Erlenmeyer flasks of 700 mL incubated in temperature and light controlled climate cabinets. Twenty seven Erlenmeyer flasks of 700mls were filled with natural seawater from 1 to 3 m depth from Kiel Fjord (Western Baltic Sea) which contained the natural spring plankton community. No specific permits were required for the described field studies. They were placed in 3 climate cabinets with temperatures of 4.5, 6.5, and 10.5°C, respectively. Three grazing treatments were used, N: nanozooplankton only (natural seawater sieved through a 20 µm gauze), M: micro- and nanozooplankton (natural seawater sieved through a 200 µm gauze), and C: nano-, microzooplankton and copepod (natural seawater sieved through a 200 µm gauze and supplemented with the copepod *Acartia tonsa* nauplii at an initial density of 10 ind. L^{-1} after one week). Thus, the treatments N, M and C represented a gradient in grazer size. The three temperature levels (4.5, 6.5 and 10.5°C) were combined with the three grazing regimes in a full factorial design, resulting in 9 treatment combinations; each treatment replicated 3x.The coldest temperature (4.5 °C)

corresponded to the ambient water temperature in the Kiel Fjord at the time of sampling. The light intensity was 293 µmol m^{-2} s^{-1} and the light:dark cycle 13:11 hrs, in accordance with the season of the experiment. Erlenmeyer flasks were mixed by shaking twice per day. The salinity was 15.6 PSU. The water received no nutrient addition. Initial concentrations were 7.34 µmol l^{-1} nitrates, 2.6 µmol l^{-1} ammonium, 0.13 µmol l^{-1} dissolved phosphate and 16 µmol l^{-1} dissolved silicate.

The second experiment was conducted from 5th to 28th July 2012.Twenty four indoor mesocosms of 300 L were used, filled with natural summer plankton communities direct pumped from Kiel Fjord, western Baltic Sea. Copepods were excluded by sieving. The two grazing treatments consisted of absence of copepods (M) and of the addition of freshly caught copepods (C) at an initial density of 15 ind L^{-1}. Copepods were caught with 200 µm plankton net with a cod end and evenly distributed to the C-flasks. The natural community was strongly dominated (>95%) by *Acartia tonsa* which made it easy to offer the same species composition to all mesocosms. The four temperature levels (8, 12.5, 15.5 & 18ºC) were combined with the two grazing regimes in a fully factorial design , resulting into 8 treatment combination each replicated 3x. The coldest temperature corresponded to the ambient water temperature in the Kiel Fjord at the time of sampling. The light intensity was 249 µmol m^{-2} s^{-1} and the light: dark cycle 14:10 hrs. Due to low nutrients concentration in situ, all treatments were supplemented with moderate additions of nitrate and phosphate, leading to starting concentrations of 10.6 µmol l^{-1}, 0.6 µmol l^{-1} NH_4, 0.8 µmol l^{-1} PO_4 and 7.0 µmol l^{-1} dissolved Si. Mixing was by done manually by using standard boat paddle three times per day at 7.30 am, 2pm & 8pm.

1.2.2 Sampling and analysis

Samples for phytoplankton counts were taken once per week and immediately fixed with Lugol's iodine. Mixing was done before sampling to insure homogeneity. Water temperature, fluorescence, salinity and pH were measured every day to monitor the system. Phytoplankton smaller than 5µm were measured and sized by flow cytometry (FACScalibur, Becton Dickinson). Flow cytometry samples were sampled and immediately fixed with formeldehyde at 2% final concentration in vials. The vials were sealed and stored at -80ºC until analysis. In addition, these algae were identified by using a scanning electron microscope (SEM). SEM samples were taken and immediately filtered by using Nuclepore Track-Etch Membrane (Whatman) and dried at 0°C for 15 minutes. Only the diatom *Chaetoceros gracilis* could be identified, while the preparation method permitted no identification of picoplankton. Cell volumes of picoplankton were calculated as volumes of sphere.

Phytoplankton bigger than 5µm were counted using the inverted microscope method (Utermöhl, 1958) with settling cylinders of 50 ml and composite chamber with a bottom area of 500 mm2. Cells were allowed to settle for 24h before counting. It was attempted to count at least 100 cells of each taxon to achieve 95% confidence limits of ±20%. Cell size measurements were performed with the samples from the end of the experiments in order to get maximum time for the treatment to take effect. This was a period of slowly declining biomass after an interim peak in all treatments of both experiments. Linear cell dimensions were measured with the AxioVision programme (Zeiss) and the cell volumes were calculated after approximation to geometric models (Hillebrand *et al.*, 1999b). Twenty randomly selected cells from each species per sample were measured. Species biomass (B_i) was calculated form specific abundances (N_i) and cell volumes (V_i): $B_i = N_i * V_i$. Community mean cell size (V_c) was calculated by dividing the total biomass by the total cell number: $V_c = B_{tot}/N_{tot}$

Dissolved nutrients were measured according to oceanographic standard methods. At the end of experiment 2 also particulate matter C and N content were measured with CHN analyzer (Fisons, 1500 N, Fisons Instruments, MA, USA).

1.2.3 Statistical analysis

The significance of temperature and grazing effects and their interaction was tested by ANOVA (STATISTICA 7). The quantitative relationship between size and temperature was analyzed by regressions of cell sizes and biomass on temperature conducted separately for each grazing treatment. The best fits were obtained after logarithmic transformation of both the dependent and the independent variable.

1.3 RESULTS

1.3.1 Cell volumes of individual species

A total of 11 microsocpically counted species was abundant enough to perform size measurements, four species from the experiment 1, the silicoflagellate *Dictyocha speculum*, the dinoflagellate *Scrippsiella trochoidea*, the cryptophyte *Teleaulax amphioxeia*, and the diatoms *Chaeotoceros similis*, *and* seven species from experiment 2, the dinoflagellate *Amphidinium* sp., the diatoms *Guinardia delicatula*, *Chaetoceros brevis*, *Chaetoceros gracilis, Ditylum brightwellii, Skeletonema* cf. *costatum* , the cryptophyte *Teleaulax amphioxeia* and the raphidophyte *Chattonella* sp. (Table 1). Picophytoplankton counted by flow cytometry was treated as a collective category without

species distinction. Three species disappeared in the warmer treatments, *C. similis* at 10.5°C in experiment 1, *C. brevis* and *D. brightwelii* at 15.5 and 18.5°C in experiment 2.

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Table 1: Higher taxon and mean cell volume (V_m; μm^3; grand mean across all treatments) of phytoplankton species, arranged in descending order of size.

Species	**Taxon**	V_m
Experiment 1		
Scrippsiella trochoidea	Dinophyta	1046
Dictyocha speculum	Dictyochophyceae	235
Teleaulax amphioxeia	Cryptophyta	191
Chaetoceros similis	Bacillariophyceae	88.7
Picophytoplankton	*diverse higher taxa*	*5.55*
Experiment 2		
Ditylum brightwellii	Bacillariophyceae	12627
Guinardia delicatula	Bacillariophyceae	2207
Amphidinium sp.	Dinophyta	987
Chattonella sp.	Raphidophyceae	968
Chaetoceros brevis	Bacillariophyceae	960
Teleaulax amphioxeia	Cryptophyta	144
Skeletonema cf. *costatum*	Bacillariophyceae	93.7
Chaetoceros gracilis	Bacillariophyceae	51.8
Picophytoplankton	diverse higher taxa	4.62

The majority of species species (*D. speculum, S. trochoidea, T. amphioxeia, C. similis*, and picophytoplankton in experiment 1; *G. delicatula, A.* sp.*, T. amphioxeia, C. brevis, D. brightwelii*, and *S.* cf. *costatum* in experiment 2) decreased in cell size with increasing temperature (Fig 1 & 2; Table 2) while there was no significant temperature effect for *Chattonella sp* (experiment 2), *C. gracilis* (experiment 2) and for picophytoplankton in experiment 2.

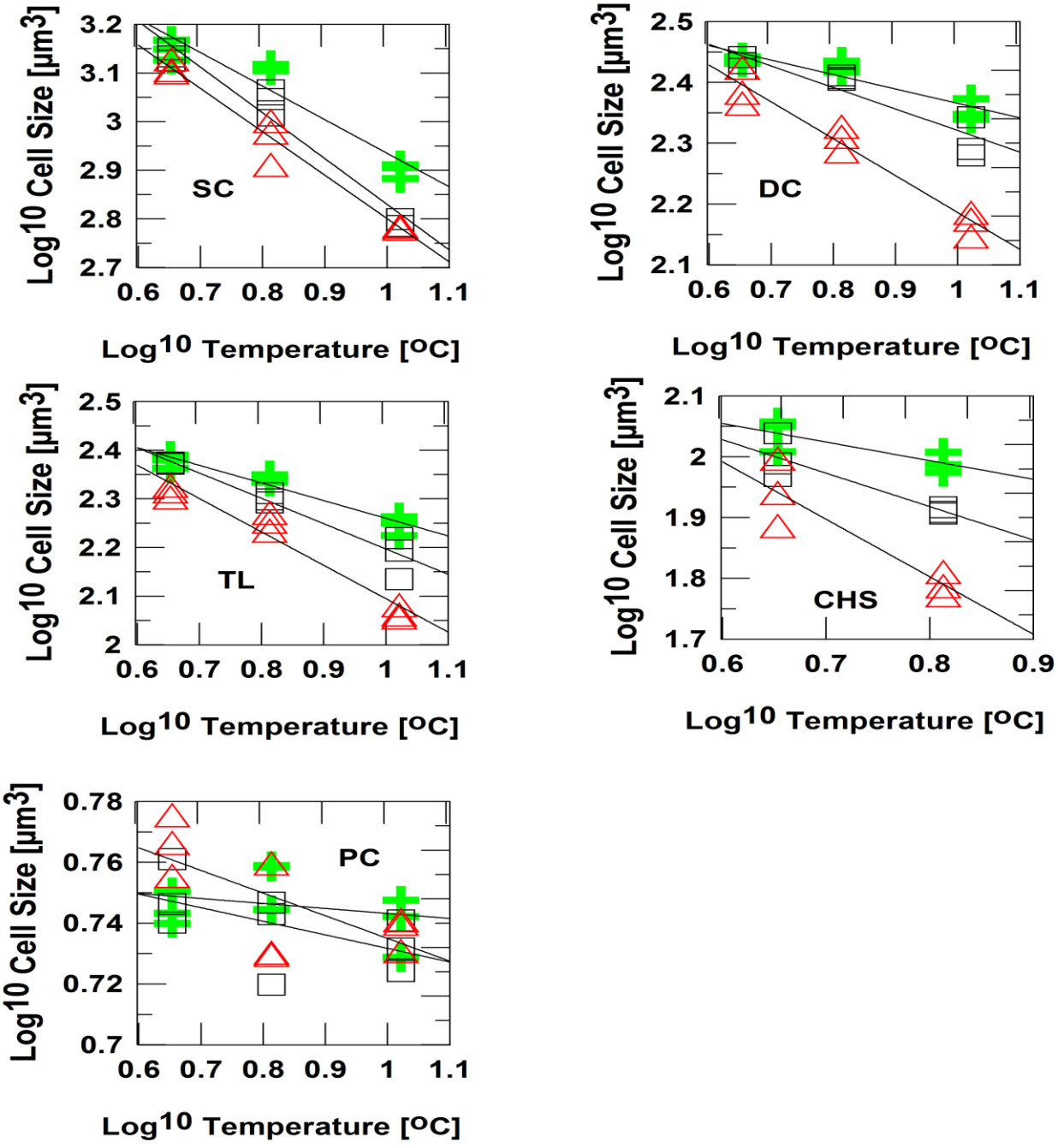


Fig 1. Temperature and grazing effects on the size of individual phytoplankton species, experiment 1: Regressions of mean cell sizes of individual species (log^{10} transformed, μm^3) on temperature (log^{10} transformed, °C) for the different grazing regimes (nanozooplankton-N: Crosses; microzooplankton-M: squares; copepods-C: triangles. Species codes: SC: *Scrippsiella trochiodea*, DC: *Dictyocha speculum*, TL: *Teleaulax amphioxeia*, CHS: *Chaetoceros similis*, PC: picophytoplankton

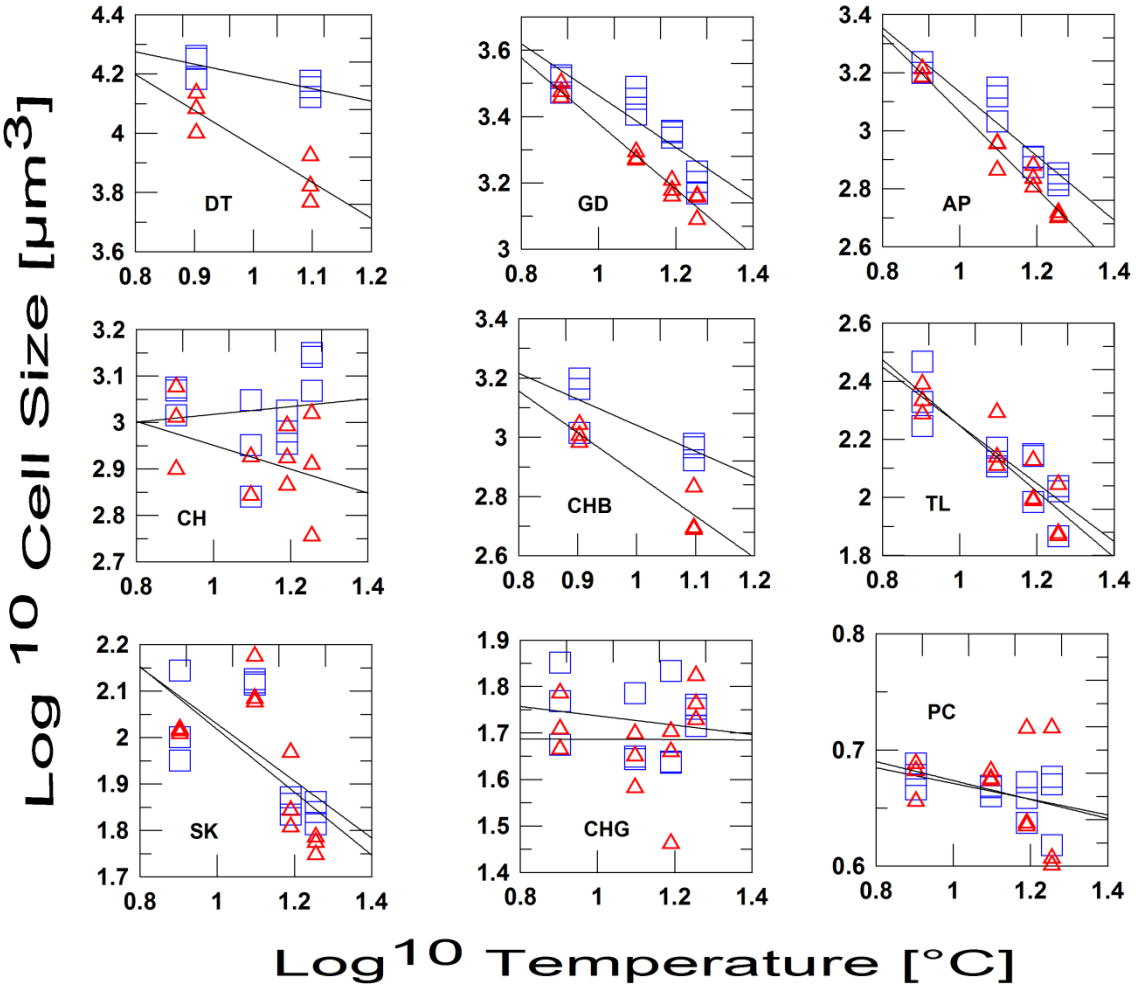


Fig 2. Temperature and grazing effects on the size of individual phytoplankton species, experiment 2: Regressions of mean cell sizes of individual species (log^{10} transformed, μm^3) on temperature (log^{10} transformed, °C) for the different grazing regimes (microzooplankton-M: Squares; copepods-C: Triangles. Species codes; DT:*Ditylum brightwellii*, GD: *Guinaridia delicatula*, AP: *Amphidinium* sp., CH: *Chattonella* sp., CHB: *Chaetoceros brevis*, TL: *Teleaulax amphioxeia*, SK: *Skeletonema* cf. *costatum*, CHG: *Chaetoceros gracilis*; PC: picophytoplankton

The grazing effect was significant in all cases except for *C. gracilis* (experiment 2), *S.* cf. *costatum* (experiment 2) *T.amphioxeia* (experiment 2) and picophytoplankton (experiments 1 and 2). Significant temperature – grazing interaction were found in most species during experiment 1 (*D. speculum, S. trochoidea, T. amphioxeia, C. similis*) and 4 species during experiment 2 (*G. delicatula, A.* sp., *C. brevis*, *D. brightwellii*). The mean cell sizes of all species showing a significant response to grazing declined with grazer size, i.e. at a given temperature cell sizes were smallest in the C-treatments. The grazing influence on the slopes of the size-temperature regressions showed interspecific differences. The slope was either most strongly negative in the C-treatments or

there were no differences in the slope (*C. gracilis, S.* cf. *costatum, S. trochoidea* and picophytoplankton (Table 3, Fig. 1 & 2).

Table 2: Two-factor ANOVA of temperature and grazing effects on cell sizes

Species	p-temperature	p-grazing	p-interaction	R^2
Experiment 1				
S. trochoidea	< 0.001	< 0.001	0.06	0.86
D. speculum	< 0.001	< 0.001	0.002	0.77
T. amphioxeia	<0.001	< 0.001	0.0001	0.83
C. similis	< 0.001	< 0.001	0.04	0.77
Picophytoplankton	0.01	0.23	0.10	0.37
Experiment 2				
D. brightwellii	<0.001	<0.001	0.007	0.89
G. delicatula	<0.001	<0.001	<0.001	0.85
A. sp.	<0.001	<0.001	0.04	0.82
Chattonella sp.	0.07	0.04	0.21	0.34
C. brevis	<0.001	0.005	0.003	0.83
T. amphioxeia	<0.001	0.65	0.73	0.72
S. cf. *costatum*	<0.001	0.39	0.5	0.81
C. gracilis	0.9	0.25	0.63	0.12
Picophytoplankton	0.47	0.94	0.91	0.16

Total phytoplankton biomass and mean cell size. Total phytoplankton biomass and community mean cell size declined with temperature and in the direction of N – M - C. The temperature and grazing effects and their interaction on total biomass were significant in both experiments (Table 4).

Table 3: Regressions (model: y = ax + b) of $\log^{10}$ cell volume (μm^3) on $\log^{10}$ temperature (°C) for the different species and grazing regimes.

Experiment 1

Species	Grazing	A	b	p	R^2
S. trochoidea	N	- 0.69	3.6251	0.0001	0.79
	M	- 0.9399	3.7702	0.0004	0.85
	C	- 0.9655	3.7452	0.00007	0.87
D. speculum	N	-0.2378	2.6034	0.0004	0.78
	M	- 0.3447	2.6685	2.6685	0.82
	C	- 0.5951	2.7888	2.7888	0.85
T. amphioxeia	N	- 0.3631	2.6234	0.0004	0.83
	M	- 0.4627	2.6552	0.002	0.76
	C	- 0.6863	2.7812	0.0001	0.86
C. similis	N	- 0.3087	2.2362	0.05	0.64
	M	- 0.5565	2.3580	0.014	0.79
	C	- 0.9590	2.5629	0.011	0.82
Picophytoplankton	N	-0.0164	0.7596	0.46	0.07
	M	-0.0446	0.7764	0.11	0.32
	C	-0.0558	0.7962	0.15	0.26

Table 3: continued

Experiment 2					
D. brightwellii	M	- 0.5876	4.7962	0.0036	0.87
	C	- 1.5577	5.4801	0.0035	0.85
G. delicatula	M	- 0.7800	4.2430	<0.001	0.77
	C	- 0.9885	4.3677	<0.001	0.87
A. sp.	M	- 1.1708	4.3032	<0.001	0.78
	C	- 1.4190	4.5027	<0.001	0.87
Chattonella sp.	M	+ 0.0830	2.9345	0.6805	0.0177
	C	- 0.2566	3.2076	0.2068	0.1541
C. brevis	M	- 0.8787	3.9191	0.0441	0.67
	C	- 1.4088	4.2833	0.0055	0.88
T. amphioxeia	M	- 1.0012	3.2500	0.00020	0.75
	C	- 1.1354	3.3832	0.00007	0.80
S. cf. *costatum*	M	-0.612	2.641	0.0244	o.41
	C	-0.6757	2.6933	0.0214	0.43
C. gracilis	M	-0.1004	1.8374	0.5758	0.0324
	C	- 0.0036	1.6902	0.9871	0.0024
Picophytoplankton	M	-0.0972	0.7742	0.0429	0.34
	C	-0.051	0.7244	0.4640	0.06

Table 4: Two-factor ANOVA of temperature and grazing effects on total Biomass (B_{tot}) and community cell size (B_{tot}/N_{tot})

	p-temperature	**p-grazing**	**p-interaction**	**R^2**
Experiment 1				
B_{tot}	<0.001	<0.001	0.004	0.86
B_{tot}/N_{tot}	<0.001	<0.001	0.02	0.75
Experiment 2				
B_{tot}	<0.001	<0.001	<0.001	0.86
B_{tot}/N_{tot}	<0.001	<0.001	0.003	0.87

Phytoplankton biomass declined with temperature and grazer size (Fig. 3).

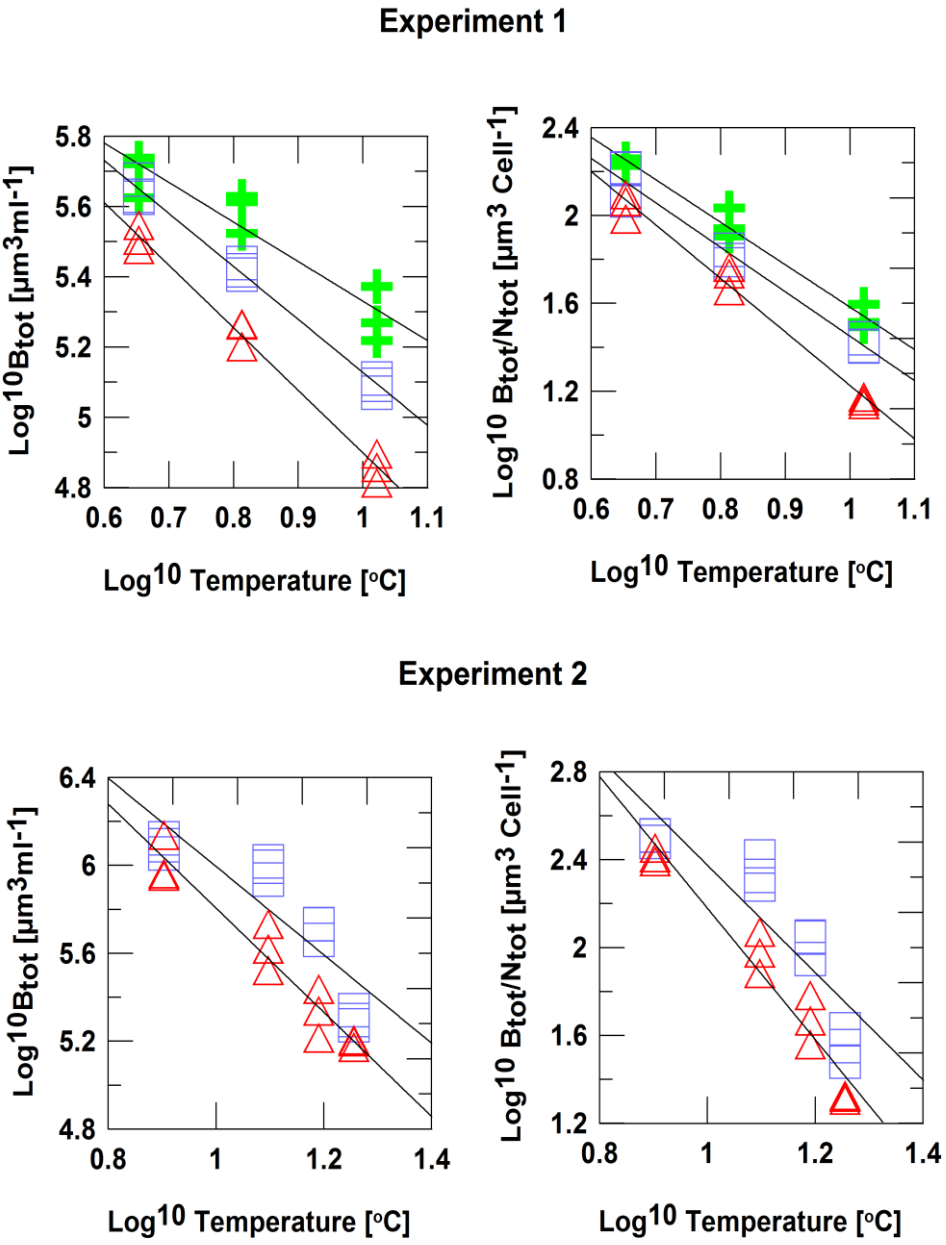


Fig 3. Temperature and grazing effects on biomass and mean size of the phytoplankton community: Regressions of total biomass (B_{tot}) and community cell sizes (B_{tot}/N_{tot}) to temperature (log^{10} transformed, °C) for the different grazing regimes (nanozooplankton-N: Crosses; microzooplankton-M: Squares; copepods-C: Triangles).

The slope of the biomass-temperature regressions became more negative with increasing grazer size (Table 5). Phytoplankton community cells size also decreased with temperature and grazer size (Fig 3), and there was significant interaction between temperature and grazing (Table 4). The slope of the size-temperature regressions became more negative with increasing grazer size (Table 5).

Table 5: Regressions (model: y = ax + b) of log^{10} total biomass (B_{tot}) and Community cell size (B_{tot}/Nt_{ot}) on log^{10} temperature (°C) for the different species and grazing regimes

Experiment 1					
	Grazing	A	b	p	R^2
Total Biomass	N	- 1.1296	6.4594	0.002	0.77
	M	- 1.4114	6.5359	0.0001	0.84
	C	- 1.7091	6.629	0.00003	0.86
Community mean cell size	N	- 1.9371	3.5187	0.00004	0.80
	M	- 2.1193	3.5417	0.00001	0.86
	C	- 2.4582	3.678	0.000002;	0.82
Experiment 2					
Total Biomass	M	-2.066	8.0606	0.0002	0.75
	C	-2.4534	8.2585	<0.0001	0.84
Community mean cell size	M	-2.2879	4.6646	0.0007	0.70
	C	-2.9993	5.1787	0.00002	0.84

Taxonomic composition. In experiment 1, the biomass of *D. speculum, S. trochoidea, T. amphioxeia,* and *C.similis* showed a significant negative response to temperature, while picophytoplankton showed a positive response (Table 6 and 7, Fig. 4).

Table 6: Two-factor ANOVA of temperature and grazing effects on biomass of species

Species	**p-temperature**	**p-grazing**	**p-interaction**	**R^2**
Experiment 1				
S. trochoidea	< 0.001	< 0.001	0.07	0.79
D. speculum	< 0.001	< 0.001	0.003	0.83
T. amphioxeia	<0.001	< 0.001	0.003	0.83
C. similis	< 0.001	< 0.001	0.004	0.77
Picophytoplankton	<0.001	0.53	0.06	0.81
Experiment 2				
D. brightwellii	<0.001	<0.001	0.004	0.89
G. delicatula	<0.001	<0.001	0.005	0.85
A. sp.	<0.001	<0.001	0.04	0.82
Chattonella sp.	0.13	0.11	0.18	0.18
C. brevis	<0.001	<0.001	0.19	0.79
T. amphioxeia	<0.001	0.42	0.55	0.83
S. cf. *costatum*	<0.001	0.56	0.08	0.81
C. gracilis	<0.001	0.59	0.27	0.58
Picophytoplankton	<0.001	0.56	0.08	0.81

Table 7: Regressions (model: y = ax + b) of log^{10} species biomass ($\mu m^3 ml^{-1}$) on log^{10} temperature (°C) for the different species and grazing regimes.

Species	Grazing	A	B	p	R^2
Experiment 1					
S. trochoidea	N	- 1.7394	6.4283	0.0003	0.76
	M	- 2.5319	6.8926	0.0002	0.84
	C	-2.6512	6.8767	0.0001	0.73
D. speculum	N	- 0.8629	5.9224	0.027	0.52
	M	- 1.1799	6.0683	0.0001	0.80
	C	- 1.8966	6.3697	0.00005	0.87
T. amphioxeia	N	- 0.5537	4.6458	0.006;	0.69
	M	- 1.0141	4.841	0.00009;	0.82
	C	- 1.7437	5.252	0.0003	0.78
C. similis	N	- 0.6796	5.2569	0.01	0.73
	M	-1.0356	5.4372	0.03	0.61
	C	- 2.0538	5.9669	0.001	0.84
Picophytoplankton	N	+1.6856	2.7581	0.00005	0.81
	M	+1.2065	3.1659	0.001	0.71
	C	+1.2262	3.1846	0.00002	0.86
Experiment 2					
D. brightwellii	M	- 1.2890	6.2320	0.0157	0.80
	C	- 2.3933	6.8632	0.0020	0.88
G. delicatula	M	- 2.4671	8.3421	0.0014	0.65
	C	- 3.2014	8.8194	0.00004;	0.89
A. sp.	M	- 1.1123	5.1461	0.0006	0.70
	C	- 2.0581	5.9788	0.0017	0.64
Chattonella sp.	M	- 0.1064	4.2246	0.7582;	0.0099
	C	- 0.4929	4.5723	0.0562	0.32
C. brevis	M	- 1.6340	5.8205	0.0217	0.76
	C	- 2.7162	6.5278	0.0119	0.82
T. amphioxeia	M	- 0.9858	5.8770	0.0004	0.72
	C	- 1.1083	5.9999	0.0004	0.62
S. cf. *costatum*	M	-3.3654	7.1868	0.0017	0.64
	C	-2.7763	6.5633	0.0030	0.60
C. gracilis	M	+ 0.7815	3.3717	0.0032	0.59
	C	+ 1.0952	2.9985	0.0075	0.52
Picophytoplankton	M	+0.6751	+3.4856	0.0017	0.64
	C	+0.8364	+3.3181	0.0008	0.69

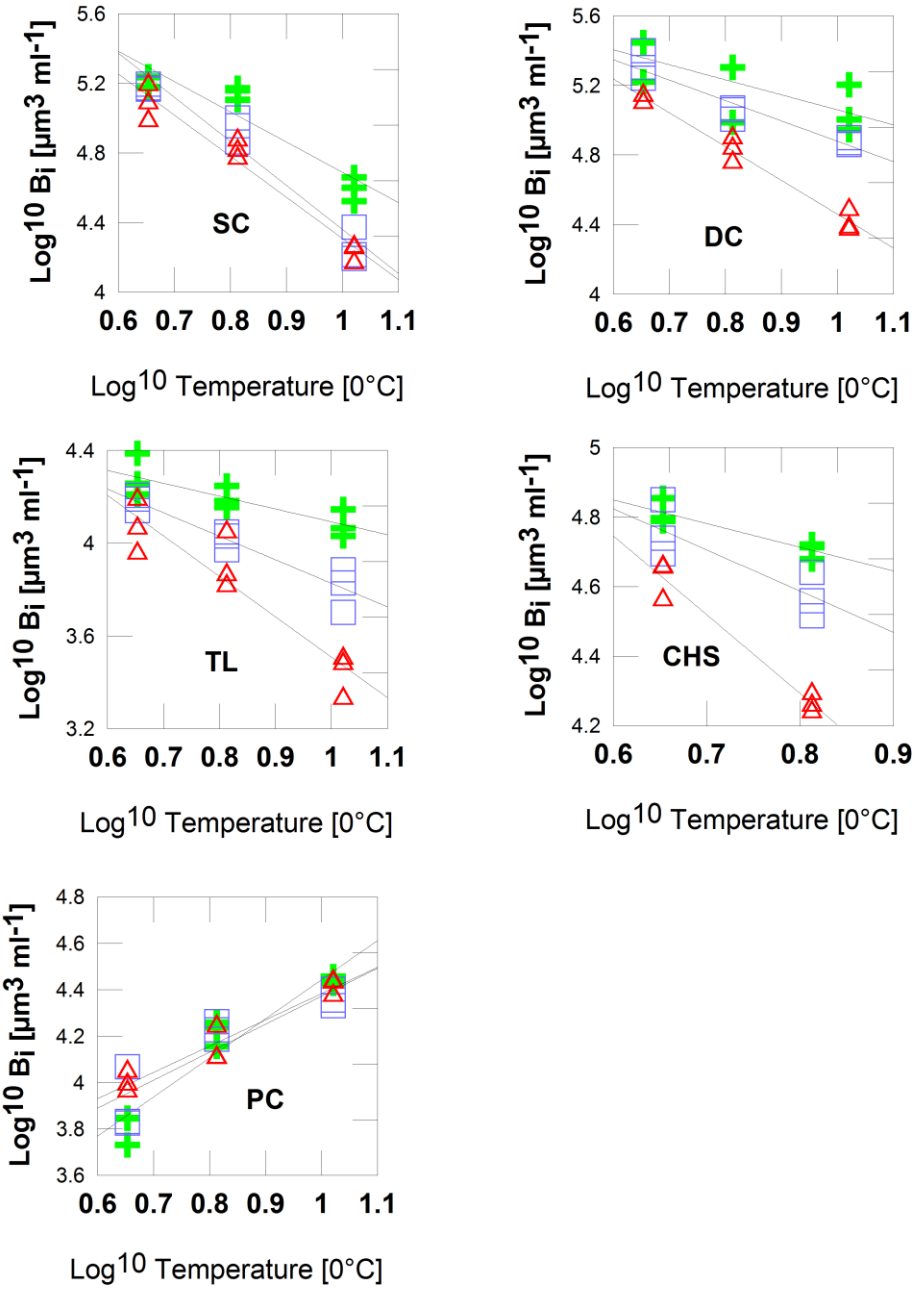


Fig 4. **Temperature and grazing effects on the biomass of individual phytoplankton species, experiment 1:** Regressions species specific biomass (log^{10} transformed, $\mu m^3 ml^{-1}$) on temperature (log^{10} transformed, °C) and grazing regimes (nanozooplankton-N: Croses; microzooplankton-M: squares; copepods-C: triangles); SC: *Scrippsiella trochiodea*, DC: *Dictyocha speculum*, TL: *Teleaulax amphioxeia*, CHS: *Chaetoceros similis*, PC: picophytoplankton

In experiment 2, a significant negative response to temperature was found in *G. delicatula, A.* sp., *T. amphioxeia, C. brevis*, *D. brightwelii*, and *S.* cf. *costatum*. No significant temperature effect was found in *Chattonella sp.* The biomass of of *C.gracilis* and picophytoplankton increased with temperature (Table 6 and 7, Fig. 5).

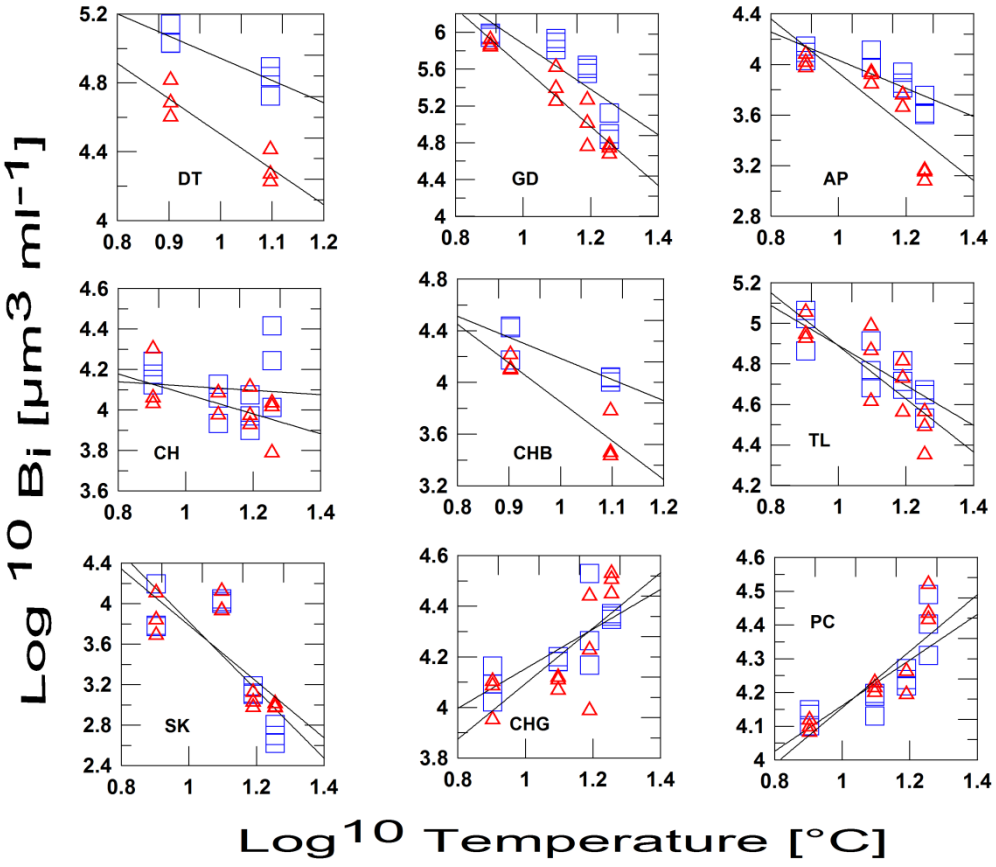


Fig 5: **Temperature and grazing effects on the biomass of individual phytoplankton species, experiment 2:** Regressions species specific biomass (log^{10} transformed, $\mu m^3 ml^{-1}$) on temperature (log^{10} transformed, °C) and grazing regimes (microzooplankton-M: Squares; copepods-C: Triangles); DT: *Ditylum brightwellii*, GD: *Guinaridia delicatula*, AP: *Amphidinium* sp., CH: *Chattonella* sp., CHB: *Chaetoceros brevis*, TL: *Teleaulax amphioxeia*, SK: *Skeletonema* cf. *costatum*, CHG: *Chaetoceros gracilis*; PC: picophytoplankton

Grazing treatments had a significant effect on all species in experiment 1, except for picophytoplankton. In all significant cases, biomass decreased with increasing grazer size. The interaction term between temperature and grazer treatment was significant in all cases. In experiment 2, the biomass of *G. delicatula*, *Chattonella* sp., *A.* sp., *C. brevis*, and *D. brightwelii* was significantly lower in the C-treatments than in the M-treatments. The biomass of *C. gracilis, T. amphioxeia, S.* cf. *costatum,* and picophytoplankton showed no response to grazing treatment. A significant interaction term between temperature and grazing was only found in *G. delicatula, A.* sp and *D. brightwellii*.

1.4 DISCUSSION

Hypothesis 1: For the majority of species, the predicted decrease in cell size with warming was confirmed. Exceptions where the small diatom *C. gracilis* (experiment 2), the raphidophyte *Chattonella* sp. (experiment 2) and picophytoplankton (both experiments). However, the latter case is not as clear cut, because picophytoplankton is an aggregate category comprising an unknown number of species. Therefore, any size change of this category can also be a consequence of species shifts. The slopes of the size – temperature regression had a mean value of -0.60 ($\pm$0.46 SD) which corresponds to a ca. 4-fold decrease at a one order of magnitude increase in temperature. This is a much stronger effect than the average 2.5% shrinkage per °C reported from meta-analysis of experiments with clonal cultures from a wide array of auto- and heterotrophic protists (Atkinson *et al.*, 2003). At present, we can only offer a tentative explanation for this discrepancy. Contrary to the experiments reported by Atkinson *et al* (2003), clonal was not used in this study i.e. genetically uniform cultures but a natural assemblage which also includes genetic variability within species. Therefore, there was also a selection effect in the experiments, while in clonal cultures size shifts can only resulted from phenotypic plasticity.

There is a potential caveat for diatoms, because one of the two daughter cells of many diatom species becomes smaller during division. If cell division rates increase with temperature this should lead to an automatic shrinkage of mean size with warming irrespective of other mechanisms. However, faster cell divisions should also lead to a higher biomass accumulation, unless the increased production of cells is removed by increasing losses. While we cannot exclude diatom grazing by copepods, we can exclude grazing by micro- and nanozooplankton for the large celled *D. brightwelii*, *G. delicatula*, *C. brevis* and the chain forming *S.* cf. *costatum* (Sommer *et al.*, 2005, Sommer & Sommer, 2006).Protist grazing on the small *C. similis* is possible. *C.gracilis* is only diatom species whose biomass increased with warming, while the biomass of all other decreased. We conclude that the diatom division effect did not contribute substantially to the temperature effects on cell size.

The temperature sensitivity of cell size was clearly size dependent. A regression of the slopes *a* from Table 3 on the grand mean of cell sizes of each species (V_{im}) yielded the following regression (pooled data for both experiments):

$a = 0.14 - 0.32\ (\pm 0.07\ \text{S.E.})\ \log^{10} V_{m;}\ \text{d.f.} = 31;\ R^2 = 0.41;\ p = 0.0001$

This means, that larger phytoplankton shrink more strongly under warming conditions, an effect which has not yet been reported to the best of our knowledge.

Hypothesis 2: There was a significant temperature*grazing interaction term in 7 of 14 cases (Table 1). However, these interactions consisted of a change of the negative slope of the size – temperature relationships, but not in a reversal between a negative and a positive dependence. In general, cell sizes were smaller when phytoplankton was subject to larger grazers, a difference which is particularly obvious when comparing the M- and the C-treatments. However, there were some notable exceptions: Picophytoplankton in both experiments, *T. amphioxeia, S.* cf. *costatum* and *C. gracilis* in experiment 2.

Hypothesis 3: Community mean cell size strongly declined with warming. The slopes for this tendency ranged from -1.94 (N-treatments in experiment 1) to ca. -3 (C-treatment in experiment 2), i.e. from a ca. 90-fold to a 1000-fold decrease of community mean cell size at a temperature increase of one order of magnitude. Thus, the interspecific size effect by far exceeds the intraspecific one. While only three species disappeared from the warmer treatments (*C. similis* at 10.5°C in experiment 1, *C. brevis* and *D. brightwellii* at 15.5 and 18.5°C in experiment 2) the relative composition changed to the disadvantage of the large species, which can be seen by a regression nalysis of the slopes of the biomass – temperature relationships in Table 7 on cell siz

$a = 1.32 - 0.41(\pm 0.09) \log^{10} V_m$; d.f. = 31; $R^2 = 0.49$; $p < 0.0001$

Hypothesis 4: Community mean cell volume was significantly influenced by grazing and the interaction term temperature*grazing was significant in both experiments. However, while grazing influenced the slope of the temperature response, it did not influence the sign of the relationship. Thus only the weak version of the hypothesis (4b) was supported while the strong version (4a) was rejected. S switch in sign would have been expected if grazing were the dominant source of size shifts. A higher activity of copepods at higher temperature would have selectively reduced the larger phytoplankton and thereby reduced community mean cell size, while in the absence of copepods a higher activity of protozoans (nano- and microzooplankton) would have selectively removed smaller phytoplankton and thereby increased mean cell volume (Sommer *et al.*, 2001). It seems that a grazing-independent temperature effect on size effect was strong enough to prevent this reversal of sign. However, as expected, the slope of the community mean cell size – temperature regressions was more negative in the copepod than in the microzooplankton treatments and also more negative in the microzooplankton than in the nanozooplankton treatments of experiment 1.

The shifts in mean cells size are in agreement with the biomass response of the individual species. There was a significant grazer effect on the biomass of phytoplankton species in 8 of 14 cases and significant grazing*temperature interactions in 6 cases. The grazer effect was absent in picophytoplankton in both experiments, and in *T. amphioxeia, S.* cf. *costatum* and *C. gracilis* in experiment 2. These were the same species, where also no grazing intraspecific size effect of grazing could be found. Since these were the smallest (experiment 1) or the 4 smallest (experiment 2) species, it seems probable that they were spared from copepod grazing.

The difference between the slopes of the size – temperature regression of the microzooplankton treatments (a_m) and the copepod treatments (a_c) became more negative with cell size:

$a_c - a_m = 0.26 - 0.21(\pm 0.06 \text{ S.E.}) \log^{10} V_m$; d.f. = 12; $R^2 = 0.51$; p = 0.004

This means that, the increased activity of copepods at higher temperature select more strongly against larger individual. This is in agreement with the known preference of copepods for relatively large phytoplankton (Sommer & Sommer, 2006). Phytoplankton species exceeding the food niche of copepods in size were lacking in our species pool, but one of the larger species (*S. trochiodea*) showed no copepod effect. *S. trochiodea* is a heavily armored dinoflagellate which is protected from copepod grazing by its cellulose plates (Sommer *et al.*, 2005)

Alternative explanations and outlook. While our experiments demonstrated an influence of size selective predation on temperature – size relationships, predation cannot be the dominant factor driving temperature- size relationships. Other mechanisms must have been stronger; otherwise the negative temperature size-relationship under protist grazing would not have been possible. Maturation (in our case: cell division) at smaller size as postulated by the TSR (Daufresne *et al.*, 2009, Forster *et al.*, 2011) can only explain a part of the observed trends. Already the intraspecific effect of most species studied was much stronger than the 2.5% shrinkage per °C found in a meta-analysis of experiments with clonal cultures (Sommer *et al.*, 2005) and shifts between differently sized species had a stronger effect on community mean cell size than size shifts within species.

The experiments do not support the hypothesis that decreased phytoplankton cell sizes can be explained by intensified nutrient competition at higher temperatures (Finkel *et al.*, 2010, Finkel *et al.*, 2005, Finkel *et al.*, 2007, Irwin *et al.*, 2006, Winder *et al.*, 2009).In stratified oceans and lakes, the increased nutrient stress is caused by increased strength of the vertical stratification and, therefore, decreased upward nutrient supply to the illuminated surface layer. Bottle and mesocosm experiments do not account for the stratification effect on nutrient supply but only for direct

temperature effects on nutrient demand. In our experiments, availability of nutrients was identical across all treatments and, in agreement with other studies (Müren *et al.*, 2005, O'Connor, 2009, O'Connor *et al.*, 2009, Sommer *et al.*, 2007, Sommer *et al.*, 2012b, Yvon-Durocher *et al.*, 2010), biomass accumulation decreased with warming. This means, that less biomass was built per unit of the limiting nutrient, i.e. biomass specific N-and P-quotas (Droop, 1973, Droop, 1983, Goldman *et al.*, 1979, Sommer, 1991) must have been higher under warmer conditions. This conclusion is supported by the N:C ratios in the particulate matter at the end of experiment 2, which we take as a proxy for the biomass specific nitrogen quota and as an indicator of nutrient stress, because initial and final dissolved nutrient concentration indicate a shortage of N relative to P. A two-factor ANOVA shows no significant influence of the grazing regime ($p = 0.53$) but a significant effect of temperature ($p = 0.0033$). A multiple range test (Fisher's LSD) shows two homogenous groups; 8.5 and 12°C with N:C ratios of 0.119+0.010 (S.D.) and 15.5 and 18°C with a N:C ratios of 0.143+0.014 (S.D.). This can either imply less nutrient stress under warmer conditions or intra- and interspecific shifts towards algae with higher minimal cell quotas under warmer conditions. The latter explanation has been proposed by (Yvon-durocher *et al.*, 2011) who also found a shift towards smaller algae under warmer conditions and identical nutrient supply in a long-term mesocosm experiment.

This study do not deny the frequently reported effect on nutrient supply on phytoplankton cell sizes which was demonstrated by a recent meta-analysis of size fractionated chlorophyll data from the global ocean (Maranón *et al.*, 2012) but the results require an explanation different from nutrient supply, grazing and the TSR. Daufresne *et al* (2009) invoked the metabolic theory of ecology (Allen *et al.*, 2002, Brown *et al.*, 2004) which predicts that at a constant supply rate of the limiting resource biomass should decline with increasing temperature ("energy equivalence rule") because of increasing metabolic demands per unit biomass. As presented by Daufresne *et al* (2009) this explanation is not complete, because there is no logical necessity that the reduction of biomass should be achieved by a reduction of the mean body size instead of a reduction of abundance. However, if warming increases resource demand then it increases resource stress and competition even under constant resource supply. This could lead to a shift towards smaller cells if they compete better (Yvon-durocher *et al.*, 2011).

CHAPTER 2

Phytoplankton cell size reduction in response to warming mediated by nutrient limitation

ABSTRACT

Shrinking of body size has been proposed as one of the universal responses of organisms to global climate warming. Using phytoplankton as an experimental model system has supported the negative effect of warming on body-size, but it remains controversial whether the size reduction under increasing temperatures is a direct temperature effect or an indirect effect mediated over changes in size selective grazing or enhanced nutrient limitation which should favour smaller cell-sizes. An experiment with a factorial combination of temperature and nutrient stress shows that most of the temperature effects on phytoplankton cell size are mediated via nutrient stress. This was found both for community mean cell size and for the cell sizes of most species analysed. At the highest level of nutrient stress, community mean cell size decreased by 46% per °C, while it decreased only by 4.7% at the lowest level of nutrient stress. Individual species showed qualitatively the same trend, but shrinkage per °C was smaller. Overall results support the hypothesis that, temperature effects on phytoplankton cell size are to a great extent mediated by nutrient limitation. This effect is expected to be exacerbated under field conditions, where higher temperatures of the surface waters reduce the vertical nutrient transport.

1.1 INTRODUCTION

Shrinking of body size has been proposed as one of the universal responses of organisms to global climate warming (Daufresne *et al.*, 2009, Gardner *et al.*, 2011) and related to classic biogeographic rules (Bergmann, 1848, James, 1970) and to the temperature-size rule (Atkinson *et al.*, 2003b). Smaller body sizes in warmer climates have been the domain of biogeographic rules since more than 1½ centuries (Bergmann, 1848, James, 1970).More recently, interest in the temperature response to size has been revised by Global Change research and by the "metabolic theory of ecology" (Allen *et al.*, 2002, Brown *et al.*, 2004) and phytoplankton has become one of the model systems to study the size effect of warming. While most phytoplankton studies support the general trend (Sommer *et al.*, 2012, Sommer & Lengfellner, 2008, Sommer & Lewandowska, 2010, Yvon-durocher *et al.*, 2011), the mechanism remain still unresolved. A meta-analysis of monoculture studies with protist found on average a 2.5% shrinkage per °C (Atkinson *et al.*, 2003), which is far less than the size trends observed in-situ and in experiments with naturally mixed plankton communities. Besides direct temperature effects, also enhanced size-selective grazing under warmer conditions (O'Connor, 2009, O'Connor *et al.*, 2009, Peter & Sommer, 2012, Sommer *et al.*, 2012, Sommer & Lengfellner, 2008, Sommer & Lewandowska, 2010) has been suggested as proximate cause, but it is general knowledge in biological oceanography that small phytoplankton tend to dominate in warm, nutrient poor waters while large ones tend to dominate in cold, nutrient rich waters (Chisholm, 1992a, Kiørboe, 1993, Maranón *et al.*, 2012, Raven, 1998). However, identification of the causal mechanism is difficult in form of field data because of the global anti-correlation between temperature and nutrients in the ocean (Kamykowski & Zentara, 1986). Warming of the surface waters intensifies vertical density stratification and, thereby, reduces vertical nutrient transport through the thermocline into the well it surface zone.

In order to disentangle nutrient from temperature effects on phytoplankton cell size, the experiment was performed with a factorial combination of temperature and nutrient stress. In this study, mixed plankton assemblages from Kiel Bight, western Baltic Sea was subjected to three temperature levels and three levels of nutrient limitation in a fully factorial design. The levels of nutrient limitation were manipulated by semi-continuous dilution of the cultures three times per week with fresh media and assessed by measuring the particulate matter C:N ratio, which is the inverse of the carbon-normalized N-cell quota (Droop, 1973) and shows a linear relationship to the extent of nutrient limitation (Goldman *et al.*, 1979).

1.2 MATERIAL AND METHODS

1.2.1 Experimental design

The experiment was conducted over three weeks from 9th to 30th August 2012. Twenty seven Erlenmeyer flasks of 700 mL incubated in temperature and light controlled climate cabinets served as experimental units. They were filled with Baltic Sea water (Kiel Fjord) from 1 to 3 m depth containing the natural plankton community and were sieved through plankton gauze of 200µm mesh size to keep out large zooplankton. Microscopic inspection of the initial plankton community indicated that micro zooplankton were extremely rare. Before the start of the experiment, the initial nutrient concentrations were assessed: 16.7 µmolL^{-1} nitrate (NO_3^-), 3.47 µmolL^{-1} phosphate (PO_4^+) and 19 µmoll^{-1} silicate. All treatments were supplemented with a moderate addition of 16 µmolL^{-1} NO_3, 10 µmolL^{-1} Si and 1 PO_4 µmolL^{-1} yielding initial concentrations of 32.7 µmolL^{-1} NO3, 4.47 µmolL^{-1} PO_4 and 29 Si µmolL^{-1}, respectively. The flasks were placed in 3 climate cabinets with temperatures of 13.5, 16.5 and 19.5ºC, respectively, 16.5 ºC being the ambient temperature at the start of the experiment. The strength of nutrient limitation was manipulated by semicontinuous dilution three times per week on Monday, Wednesday and Friday in which 0%, 25%, and 50% of the culture volume were replaced by fresh medium. The medium was sterile filtered (0.2 µm pore size) Baltic Sea water enriched by 16 µmol$L^{-1}$$NO_3$, 1 µmol$L^{-1}$$PO_4$, 10 µmol L^{-1} SiO_4 and stored at low temperature (2ºC) in darkness. Nutrient regimes are denoted by N1 (50% replacement, weak nutrient stress), N2 (25%, medium nutrient stress), and N3 (0%, strong nutrient stress), respectively. Each nutrient regime was combined with three temperature levels in a fully factorial design, leading to 9 treatments, each replicated 3 times.

1.2.2 Sampling and analysis

Samples were taken at the end of the experiment in order to get maximum time for the treatment to take effect. Water temperature, salinity, and pH were measured every day to monitor the experiments. Samples for dissolved nutrients were filtered by cellulose acetate filters of 0.8 µm pore size and kept at -20ºC until analysis. Dissolved nutrients were measured according to oceanographic standard methods (Grasshoff *et al.*, 1983). For the determination of particulate organic carbon (POC), nitrogen (PON) and phosphorus (POP), samples were filtered onto precombusted Whatman GF/F filters (Whatman GmbH, Dassel, Germany). After filtration, the samples were immediately dried and stored in desiccators. Analysis of POC and PON were carried out after Sharp (1974) by gas chromatography in the elemental analyzer (Thermo Flash 2001) (Thermo Fisher Scientific Inc., Schwerte, Germany), while POP was determined

colorimentrically after converting organic phosphorus compounds into orthophosphate (Hansen & Koroleff, 2007).

Samples for microscopic phytoplankton counts and size measurements were immediately fixed with Lugol's iodine. Phytoplankton smaller than 5µm were analyzed by flow cytometry (FACScalibur, Becton Dickinson). Flow cytometry samples were fixed with formaldehyde at 2% final concentration. The vials were sealed and stored in the -80ºC freezer until analysis. Cell volumes were calculated after approximation to geometric standard models (Hillebrand *et al.*, 1999) In total it was possible to distinguish and count 15 phytoplankton species but other protists including heterotrophic ones were rare to be counted.Diatoms smaller than 5 µm (only *Cylindrotheca closterium*) were identified and sized by using a Scanning Electron Microscope (SEM). 10 ml of samples were taken and immediately filtered by using Nucleopore Track-Etch Membrane (Whatman) and kept in oven for 15 minutes. Phytoplankton bigger than 5µm were counted using the inverted microscope method (Utermöhl, 1958) with settling cylinders of 50 ml volume and a bottom area of 500 mm^2. Cells were allowed to settle for 24 h and counted under an inverted light microscope. It was attempted to count at least 100 cells of each taxon to achieve 95% confidence limits of ~20% but this was not applicable in some of the treatments where the biggest species (e.g. *Ceratium tripos* and *Ceratium tripos)* were rare, like. Twenty randomly selected cells from each species per sample were for size measurements. Species biomass was calculated from specific abundances (N_i) and cell volumes (V_i): $B_i = N_i * V_i$. The community mean cell size (V_c) was calculated by dividing the total biomass by the total cell number.

1.2.3 Statistical analysis:

Statistical analysis: The temperature and nutrient level effects and their interaction effect on individual cell volume were tested by a two factor ANOVA (STATISTICA 7). The quantitative relationships between C:N ratio with Temperature at different nutrient levels were analysed by linear regression. The quantitative relationship between individual cell sizes and mean cell size with temperature at different nutrient levels was also analysed by linear regression.

1.3 RESULTS

The C: N ratios of particulate matter increased in the direction N1 to N3 and with temperature (Fig. 1).A two-factor ANOVA with log-transformed C:N data showed a significant main effect of

nutrient treatment and of temperature, but no interaction effect (P_{nutr} <0.0001; P_{temp} = 0.0031; N = 27). The molar C:N ratios ranged from 8.5 to 31, thus indicating weak to severe nitrogen limitation (Goldman *et al.*, 1979) while P-limitation could be excluded because of N:P ratios <16 in all experimental units.

The community mean cell volume (Fig. 2) and the cell volumes of the majority of the individual species (shown for the all spp. in Fig. 3) showed a significant negative effect of nutrient stress (14 of 15 spp.), a negative temperature effect (11 of 15 spp.) and an interaction effect (9 of 15 spp.) (Table 1).

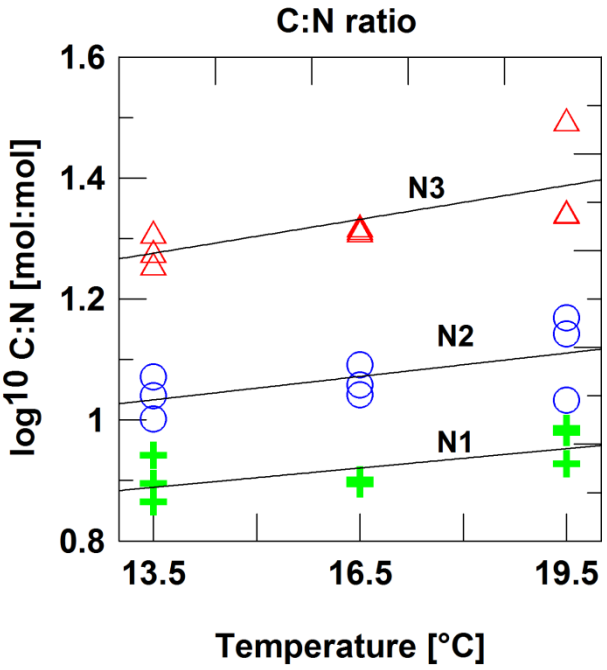


Figure 1. Response of C:N ratios. Molar C:N ratios of particulate, organic matter in response (log^{10}-scale) to temperature and nutrient regime; N1: 50% dilution three times per week; N2: 25% dilution three times per week; N3: no dilution

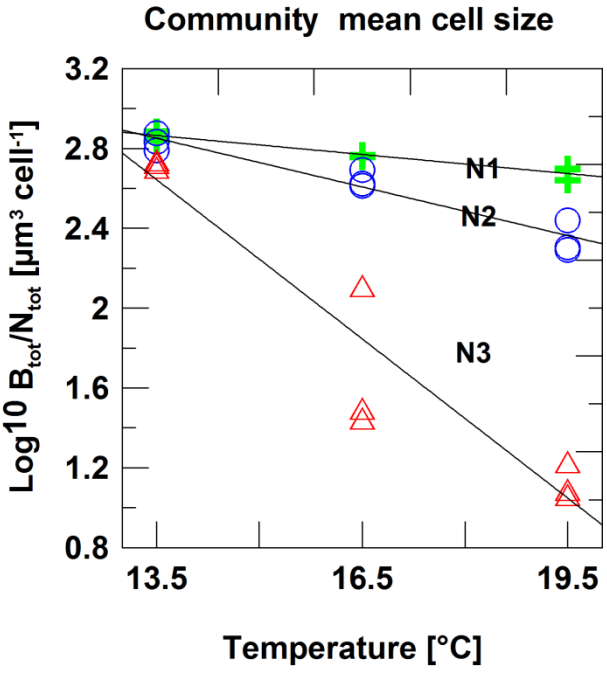
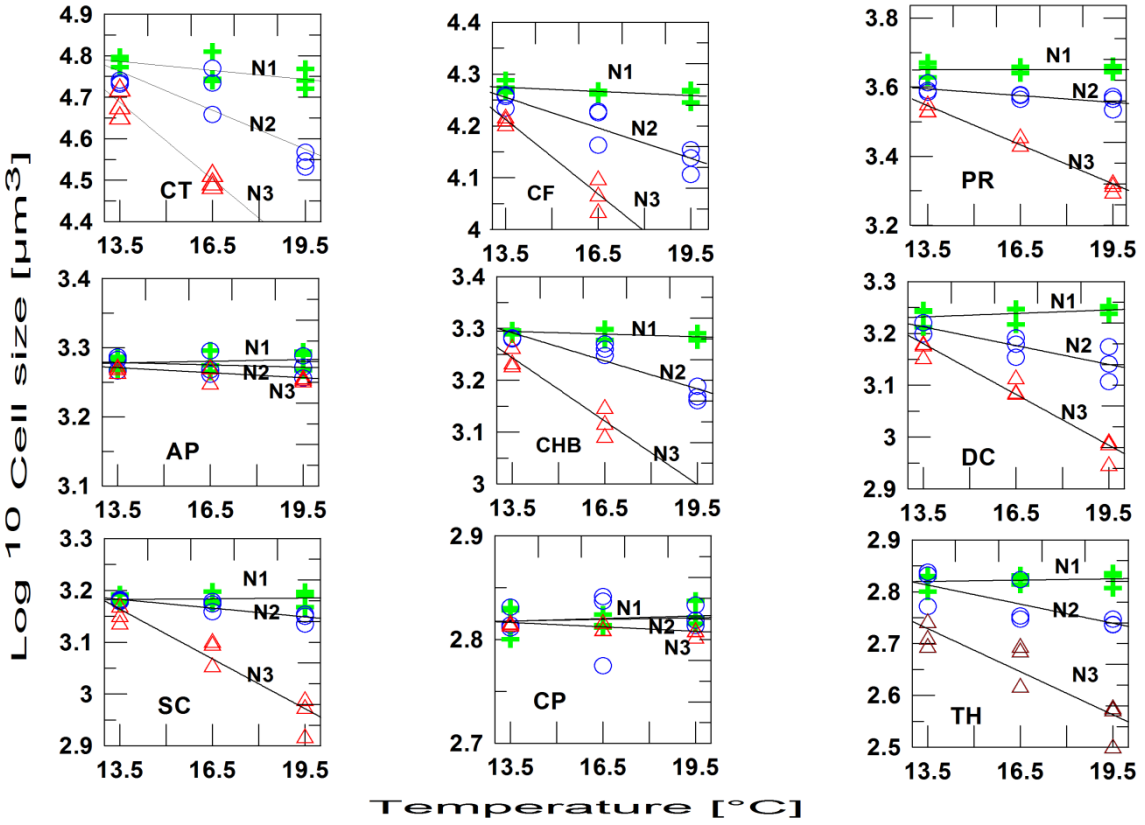


Figure 2. Response of community mean cell size. Community mean ell volume in μm^3 (log^{10}-scale) in response to temperature and nutrient regime; N1: 50% dilution three times per week; N2: 25% dilution three times per week; N3: no dilution.



Continuous

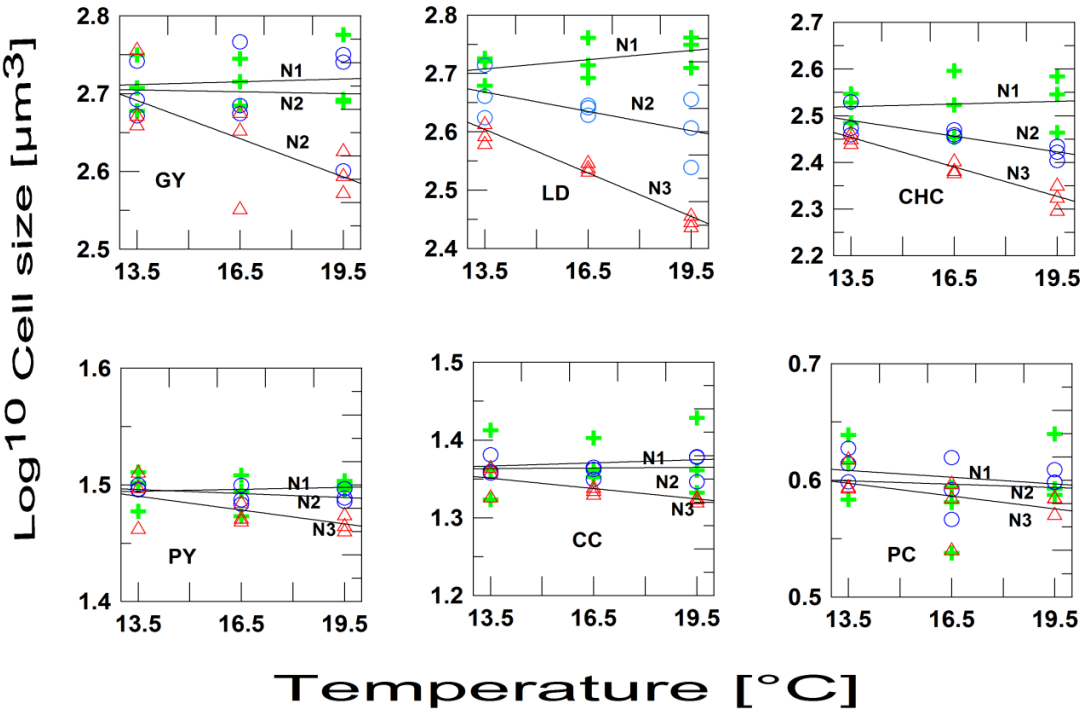


Figure 3. Response species cell sizes. Cell volume in µm^3 (log^{10}-scale) in response to temperature and nutrient regime; N1: 50% dilution three times per week; N2: 25% dilution three times per week; N3: no dilution.Species codes;CT: *Ceratium tripos*; CF: *Ceratium fusus*; PR: *Prorocentrum micans*; AP: *Amphidinium* sp.; CHB: *Chaetoceros brevis*; DC: *Dictyocha speculum*; SC: *Scrippsiella trochoidea*; CP: *Cerataulina pelagica*; TH: *Thalassionema nitzschioides*; GY: *Gymnodinium* sp.; LD: Leptocylindrus *danicus*; CHC: *Chaetoceros curvisetus*; PY: *Pyramimonas* sp.; CC: *Cylindrotheca closterium*; PC: picophytoplankton.

Table 1: ANOVA of temperature and nutrient effects: Two-factor ANOVA of temperature and nutrient level effects on $\log^{10}$ cell volume (μm^3) of the entire phytoplankton community and of the individual species arranged by size from the largest to the smallest; *N*=27, except for *Ceratium tripos, Ceratium fusus,* and *Chaetoceros brevis* which disappeared from the N3 – 19.5°C treatment combination (*N*=24).

Species	**Higher taxon**	P_{temp}	P_{nutr}	P_{int}	R^2
Community mean cell size		<0.001	<0.001	<0.001	0.92
Ceratium tripos	Dinophyta	< 0.001	< 0.001	< 0.001	0.89
Ceratium fusus	Dinophyta	< 0.001	< 0.001	< 0.001	0.88
Prorocentrum micans	Dinophyta	< 0.001	< 0.001	< 0.001	0.92
Amphidinium sp.	Dinophyta	0.65	0.03	0.74	0.37
Chaetoceros brevis	Bacillariophyceae	< 0.001	< 0.001	< 0.001	0.94
Dictyocha sp.	Dictychophyceae	< 0.001	< 0.001	< 0.001	0.89
Scrippsiella trochoidea	Dinophyta	< 0.001	< 0.001	0.002	0.79
Thalassionema nitzschioides	Bacillariophyceae	< 0.001	< 0.001	0.001	0.85
Cerataulina pelagica	Bacillariophyceae	0.93	0.49	0.92	0.12
Gymnodinium sp.	Dinophyta	0.42	0.01	0.4	0.24
Leptocylindrus danicus	Dinophyta	0.002	<0.001	0.002	0.79
Chaetoceros curvisetus	Bacillariophyceae	0.01	<0.001	0.07	0.73
Pyramimonas sp.	Prasinophycea	0.24	0.02	0.5	0.48
Cylindrotheca closterium	Bacillariophycea	0.8	0.03	0.8	0.35
Picophytoplankton	diverse taxa	0.04	0.3	0.7	0.15

Individual regression analyses for different levels of nutrient stress showed, that the slopes of the temperature-size relationship became more negative at more stringent nutrient stress (Table 2).

At the lowest nutrient stress level, no species showed a significant response to temperature. Comparing the response of V_c to the responses of V_i, it is also obvious that compositional changes, .i.e. dominance shifts between differently sized species, by far outweigh intraspecific size shifts. The slopes of the *Vc*-temperature regression roughly conform to ca. 4.7% size reduction per °C at N1, 17.2% at N2, and 46% at N3, respectively. The most responsive individual species, the dinoflagellate *Ceratium tripos*, decreased by 1.7% per °C at N1 (insignificant), 6.8% at N2, and 13.3% at N3, respectively.

Table 2: Regression analysis of temperature-size relationships. Regression (Model: y = ax+b) of log^{10} cell volume (μm^3) on temperature (°C) for the different species and nutrient levels; *N*=9, except for *Ceratium tripos, Ceratium fusus,* and *Chaetoceros brevis* which disappeared from the N3 – 19.5°C treatment combination (*N*=6).

Species	**Nutrient level**	***A***	***B***	***P***	***R2***
Community mean cell size	N1	-0.0207	3.0903	0.0009	0.88
	N2	-0.0820	3.9612	0.0003	0.90
	N3	-0.2661	6.2376	0.00008	0.92
Ceratium tripos	N1	-0.0073	4.8846	0.075	0.38
	N2	-0.0306	5.184	00038	0.72
	N3	-0.0619	5.5233	0.001	0.94
Ceratium fusus	N1	-0.0026	4.3100	0.08	0.35
	N2	-0.0196	4.5197	0.0008	0.81
	N3	-0.0479	4.8597	0.0016	0.93
Prorocentrum micans	N1	+0.0001	3.6486	0.950	0.0006
	N2	-0.0065	3.6831	0.012	0.61
	N3	-0.0377	4.0556	0.00003	0.96
Amphidinium sp.	N1	+0.0008	3.2672	0.6478	0.03
	N2	-0.0011	3.2941	0.5736	0.05
	N3	-0.1124	3.3042	0.04561	0.46
Chaetoceros brevis	N1	+0.0017	3.3174	0.1539	0.26
	N2	-0.0180	3.5354	0.0003	0.86
	N3	-0.0408	3.7946	0.0033	0.90
Dicytocha sp	N1	+0.0022	3.218	0.2799	0.16
	N2	-0.0121	3.3756	0.00470	0.70
	N3	-0.0326	3.6202	0.00001	0.93
Scrippsiella sp	N1	+0.0004	3.1765	0.78550	0.01
	N2	-0.0056	3.2590	0.0019	0.76
	N3	-0.0320	3.5964	0.0001	0.90
Thalassionema nitzschioides	N1	+0.0008	2.809	0.6558	0.03
	N2	-0.0123	2.979	0.0195	0.56
	N3	-0.0277	3.103	0.0012	0.80
Cerataulina pelagica	N1	+0.0008	2.8065	0.6178	0.04
	N2	+0.0005	2.8110	0.8644	0.005
	N3	-0.0014	2.8358	0.0113	0.62
Gymnodinium sp	N1	+0.0012	2.6954	0.8142	0.09
	N2	-0.0008	2.7155	0.9204	0.04
	N3	-0.0164	2.9123	0.00439	0.68
Leptocylindrus danicus	N1	+0.0053	2.6367	0.20	0.22
	N2	-0.0111	2.8173	0.08	0.37
	N3	-0.0249	2.9397	0.00006	0.94
Chaetoceros curvisetus	N1	+0.0019	2.4940	0.8022	0.009
	N2	-0.0112	2.6416	0.0077	0.66
	N3	-0.0211	2.7378	0.0004	0.92
Pyramimons sp	N1	+0.005	1.4871	0.779	0.009
	N2	-0.0011	1.5115	0.2010	0.23
	N3	-0.0040	1.5436	0.05015	0.45
Cylindrotheca closterium	N1	+0.0013	1.3485	0.8052	0.009
	N2	+0.0003	1.3598	0.8929	0.003
	N3	-0.0044	1.4106	0.0239	0.54
Picophytoplankton	N1	-0.0010	0.6125	0.8400	0.006
	N2	-0.0019	0.6344	0.4637	0.008
	N3	-0.0037	0.6467	0.2290	0.19

Table 3: Multiple Regression of cell volume on temperature and C:N ratios: Regression according to the model $\log^{10} V = a+b.t+c.\log^{10}(C:N)$, where t is expressed in °C and V in μm^{3C}; partial correlation coefficients (R_t, R_{CN}), partial probabilities of error (P_t, P_{CN}), R^2 for the full model, and probability of error for the full model (P_{model}); N=9, except for *Ceratium tripos*, *Ceratium fusus*, and *Chaetoceros brevis* which disappeared from the N3 – 19.5°C treatment combination (N=6). The temperature effect is also shown as % volume reduction per °C.

Species	***a***	***B***	**%°C^{-1}**	***c***	P_t	R_t	P_{CN}	R_{CN}	P_{model}	R^2
Community mean cell size	6.55	-0.093	-19.3	-2.22	0.0011	-0.40	<0.0001	-0.69	<0.0001	0.71
Ceratium tripos	5.55	-0.018	-4.1	-0.49	0.0019	-0.35	<0.0001	-0.55	<0.0001	0.66
Ceratium fusus	4.8	-0.011	-2.5	-0.36	0.005	-0.34	<0.0001	-0.54	<0.001	0.66
Prorocentrum micans	4.28	-0.008	-1.8	-0.51	0.045	-0.18	<0.0001	-0.86	<0.001	0.81
Amphidinium sp.	3.33	-0.001	-0.23	-0.04	0.67	-0.07	0.0046	-0.54	0.012	0.66
Chaetoceros brevis	3.76	-0.01	-2.3	-0.31	0.0038	-0.35	<0.0001	-0.54	<0.0001	0.68
Dictyocha sp.	3.75	-0.009	-2.1	-0.36	0.011	-0.28	<0.0001	-0.78	<0.0001	0.75
Scrippsiella trochoidea	3.62	-0.009	-2.1	-0.29	0.017	-0.30	<0.0001	-0.72	<0.0001	0.66
Thassionema nitzschioides	3.38	-0.007	-1.6	-0.44	0.024	-0.20	<0.0001	-0.86	<0.0001	0.83
Cerataulina pelagica	2.83	0.0002	+0.05	-0.017	0.85	+0.38	0.24	-0.23	0.54	0
Gymnodinium sp.	2.97	-0.003	-0.7	-0.20	0.49	-0.11	0.0006	-0.64	0.0014	0.37
Leptocylindrus danicus	3.23	-0.004	-0.92	-0.45	0.23	-0.11	<0.0001	-0.87	<0.0001	0.79
Chaetoceros curvisetus	2.93	-0.006	-1.4	-0.32	0.071	-0.20	<0.0001	-0.80	<0.0001	0.71
Pyramimonas sp.	1.56	-0.001	-0.23	-0.047	0.26	-0.17	0.0029	-0.50	0.0038	0.32
Cylindrotheca closterium	1.45	0.0002	+0.05	-0.085	0.92	+0.02	0.003	-0.57	0.0102	0.26
Pico-phytoplankton	0.65	-0.002	-0.46	-0.017	0.33	-0.20	0.52	-0.13	0.43	0

1.4 DISCUSSION

While the dominance of the nutrient effect in mediating the temperature-size effect is obvious, a remaining nutrient-independent role of temperature cannot be assessed from a direct comparison of the different treatments, because temperature itself influenced the strength of nutrient stress, as can be seen from the response of the N:C ratios to temperature. However, if the N:C ratio is taken as indicative of the intensity of nutrient stress (Goldman *et al.*, 1979) a nutrient-independent effect can be assessed by a multiple regression with temperature and C:N-ratios as independent variables (Table 3). The dominance of the nutrient effect is obvious, both from the number of significant cases and from the partial correlation coefficients. The mean nutrient-independent temperature regression slope was -0.0059±0.0028 (95% CL). The slope for the community level effect indicates a 19.3% size reduction, while the most temperature sensitive species, *Ceratium tripos,* showed a 4.1% size reduction per °C. The mean value of species specific size reduction was 1.36% (SD = 1.16; Shapiro-Wilks test for normality: $p = 0.24$), while several species did not show any nutrient-independent temperature response. Overall, the range of variation overlaps with the results obtained from clonal cultures of a wide array of protists (Atkinson *et al.*, 2003).

An alternative explanation for the observed temperature effect could lie in the dilution effect on protistan grazers (microzooplankton) which are more strongly diluted at higher dilution rates. Since microzooplankton in general prefer smaller prey and thereby benefit the larger prey both inter- and intraspecifically. Therefore, the grazing and the nutrient effect on cell sized should have the same sign, i.e. smaller sized at lower dilution rates. However, there are good reasons to consider the contribution of the microzooplankton effect as relatively unimportant:

1) Microzooplankton densities were too low to count them in the phytoplankton samples, as opposed to at least 100 phytoplankton cells counted per species and sample. Therefore, grazing rates must have has little influence on the outcome of our experiment.
2) In this experiment, the nutrient effect on the cell size was generally stronger for the larger species which are outside the feeding spectrum of most microzooplankton species.
3) If the effect of microzooplankton grazing dominates the size response of phytoplankton, higher grazing rates at warmer temperature should lead to a positive temperature effect on cell size. This hypothesis was tested in chapter one and rejected. Even under protist grazing, warming led to a shrinkage of cell size, although not as strongly as under copepod grazing.

Direct temperature effects, nutrient effects, and grazing effects as explanations for temperature dependent size trends are not mutually exclusive. However, the results strongly indicate that the direct temperature effect is much weaker than the nutrient effect. This was found both at the intra- and the interspecific level. The community effect was much stronger than the intraspecific effect,

but this is no surprise, because the scope for interspecific size difference by far exceeds the scope for intraspecific ones: Size differences between species span about 9 orders of magnitude while intraspecific size changes are almost always <1 order of magnitude on a volumetric base (Reynolds, 1984). Additional mechanisms such as enhanced grazing under higher temperatures cannot be excluded. However, the effect of grazing would be less consistent, because different guild of grazer affect different parts of the phytoplankton size spectrum (Sommer *et al.*, 2005) , e.g. protozoan grazer would rather suppress phytoplankton <5 or 10 µm, while copepods would rather suppress larger ones, i.e. temperature effects mediated by grazing should depend on the dominance of different grazer guilds. , the expected stronger negative temperature effect on phytoplankton under copepod grazing was found, but there was no reversal of sign under protist grazing. This means, that a grazing independent negative temperature effect on phytoplankton must have outweighed the supposed positive effect of protist grazers. Then, in chapter one (Peter & Sommer 2013) it was only speculated about the possible importance of nutrient limitation, while in this chapter provide direct evidence for it.

CHAPTER 3

Interactive effect of warming and nutrient limitation on phytoplankton composition

ABSTRACT

Biogeographic patterns in marine phytoplankton composition and changes driven by climate change are commonly thought to be driven by oceanographic patterns and their consequences for nutrient supply. However, recent experiments motivated by climate impact research, also suggest a direct effect of temperature on phytoplankton composition and size structure. In situ temperature and nutrient effects cannot be separated easily because of the global anti-correlation of surface temperatures and nutrient availability. Therefore, an experiment was performed with a full factorial combination of temperature and nutrient stress. Nutrient limitation was manipulated by semi-continuous dilution with fresh medium at three levels. Temperature was also offered at three levels. Within each level of dilution rate, increasing temperatures increased the extent of nitrogen stress as indicated by increasing C:N ratios in the biomass and decreasing concentrations of dissolved nitrogen. When using biomass C:N-ratios as index of nitrogen stress, a multiple regression analysis showed that both increasing temperature and increasing C:N ratios favoured dominance shift from large to small phytoplankton species. A separate analysis of two higher taxa, diatoms and dinoflagellates, showed that the advantage of smaller species under higher temperatures and stronger nutrient limitation was more pronounced among the dinoflagellates, the signs of the trends were equal between both higher taxa.

1.1 INTRODUCTION

Nutrient supply, absolute nutrient levels and nutrient ratios in the upper layers of the ocean are often considered to exert the primary control on the size and taxonomic structure of phytoplankton communities (Chisholm, 1992, Nightingale *et al.,* 1996). Eutrophication in coastal areas increases N:Si and P:Si nutrient ratios and changes phytoplankton composition to the disadvantage of diatoms (Egge & Aksnes, 1992, Officer & Ryther, 1980) while nutrient input from upwelling or deep mixing favours dominance by large size diatoms because of high Si:N and Si:P ratios (Del Amo *et al.*, 1997). Climate warming is expected to enhance thermal stratification, reduce nutrient supply from deep water and thereby shift the competitive advantage to smaller algae under oligotrophic conditions (Falkowski & Oliver, 2007) or to algae which are able to regulate their vertical position in euphotic zone under nutrient rich conditions (Huisman *et al.*, 2004) , e.g. dinoflagellates. In summary, low nutrient conditions favor pico- and nanophytoplankton, mixing and stratified, nutrient rich conditions favors diatoms (Agawin *et al.*, 2000, Bopp *et al.*, 2005, Chisholm, 1992, Reynolds, 2006, Sellner *et al.*, 2001, Sommer, 1996). Apart from stratification, warming influences the change in phytoplankton composition also through direct effects and through grazing. Stratification independent temperature effects on phytoplankton composition have been shown in microcosms and mesocosms experiments (Hilligsøe *et al.*, 2011, Morán *et al.*, 2009, Sommer & Lengfellner, 2008, Yvon-Durocher *et al.*, 2010) to increase the importance of small-sized species in response to warming. Apart from direct effect, temperature enhanced size-selective grazing under warm condition contributed to this effect (Lewandowska & Sommer, 2010, O'Connor, 2009, O'Connor *et al.*, 2009, Sommer *et al.*, 2012\, Sommer & Lengfellner, 2008, Sommer & Lewandowska, 2011, Yvon-durocher *et al.*, 2011). Moreover, a meta-analysis of size structure data from cold, temperature and warm oceans (Maranón *et al.*, 2012) which include different combination of nutrient richness and temperature found that, the partitioning between phytoplankton size classes could be explained to 62% by primary productivity as a proxy for a nutrient supply and 2% by temperature.

However, under field conditions, the role of nutrients supply and temperature cannot be disentangled easily because of the global negative correlation between nutrient concentrations and temperature (Kamykowski & Zentara, 1986). A balanced contribution of both factors was assumed by (Agawin *et al.*, 2000) except for coastal ecosystems (Seitzinger *et al.*, 2002). In a recent study, we have published an experimental analysis on the combined effect of temperature and nutrient stress on the inter- and intraspecific size variation of phytoplankton (Peter & Sommer, 2013). Based on the same experiment, it is the aim of the current study to examine the effect of temperature and nutrient limitation on the phytoplankton community structure, i.e. species replacements and

dominance shifts, in a fully factorial design. Nutrient stress was manipulated by the dilution with fresh medium at three levels and temperature was manipulated at three levels as well. Therefore, the working hypotheses are:

1. Nutrient stress and warming influence phytoplankton composition and temperature effects should be stronger at more intense nutrient stress.

2. Inter-specific differences in the response of the phytoplankton species to temperature and nutrient stress are primarily explained by their size rather than phylogenetic position.

1.2 MATERIAL AND METHODS

1.2.1 Experimental design,

The experiment was conducted over three weeks from 9th to 30th August 2012. Twenty seven Erlenmeyer flasks of 700 mL incubated in temperature and light controlled climate cabinets served as experimental units. They were filled with Baltic Sea water (Kiel Fjord) from 1 to 3 m depth containing the natural plankton community and were sieved through plankton gauze of 200µm mesh size to keep out large zooplankton. Microscopic inspection of the initial plankton community indicated that micro zooplankton were extremely rare. Before the start of the experiment, the initial nutrient concentrations were assessed: 16.7 µmolL^{-1} nitrate (NO_3^-), 3.47 µmolL^{-1} phosphate (PO_4^+) and 19 µmoll^{-1} silicate. All treatments were supplemented with a moderate addition of 16 µmolL^{-1} NO_3, 10 µmolL^{-1} Si and 1 PO_4 µmolL^{-1} yielding initial concentrations of 32.7 µmolL^{-1} NO3, 4.47 µmolL^{-1} PO_4 and 29 Si µmolL^{-1}, respectively. The flasks were placed in 3 climate cabinets with temperatures of 13.5, 16.5 and 19.5ºC, respectively, 16.5 ºC being the ambient temperature at the start of the experiment. The strength of nutrient limitation was manipulated by semicontinuous dilution three times per week on Monday, Wednesday and Friday in which 0%, 25%, and 50% of the culture volume were replaced by fresh medium. The medium was sterile filtered (0.2 µm pore size) Baltic Sea water enriched by 16 µmol$L^{-1}NO_3$, 1 µmol$L^{-1}PO_4$, 10 µmol L^{-1} SiO_4 and stored at low temperature (2ºC) in darkness. Nutrient regimes are denoted by N1 (50% replacement, weak nutrient stress), N2 (25%, medium nutrient stress), and N3 (0%, strong nutrient stress), respectively. Each nutrient regime was combined with three temperature levels in a fully factorial design, leading to 9 treatments, each replicated 3 times.

Sampling and analysis

Samples were taken at the end of the experiment in order to get maximum time for the treatment to take effect. Water temperature, salinity, and pH were measured every day to monitor the experiments. Samples for dissolved nutrients were filtered by cellulose acetate filters of 0.8 µm pore size and kept at -20ºC until analysis. Dissolved nutrients were measured according to oceanographic standard methods (Grasshoff *et al.*, 1983). For the determination of particulate organic carbon (POC), nitrogen (PON) and phosphorus (POP), samples were filtered onto precombusted Whatman GF/F filters (Whatman GmbH, Dassel, Germany). After filtration, the samples were immediately dried and stored in desiccators. Analysis of POC and PON were carried out after Sharp (1974) by gas chromatography in the elemental analyser (Thermo Flash 2001) (Thermo Fisher Scientific Inc., Schwerte, Germany), while POP was determined colorimentrically after converting organic phosphorus compounds into orthophosphate (Hansen & Koroleff, 2007).

Samples for microscopic phytoplankton counts and size measurements were immediately fixed with Lugol's iodine. Phytoplankton smaller than 5µm were analysed by flow cytometry (FACScalibur, Becton Dickinson).Flow cytometry samples were fixed with formaldehyde at 2% final concentration. The vials were sealed and stored in the -80ºC freezer until analysis. Cell volumes were calculated after approximation to geometric standard models (Hillebrand *et al.*, 1999a) In total it was possible to distinguish and count 15 phytoplankton species but other protists including heterotrophic ones were rare to be counted. Diatoms smaller than 5 µm (only *Cylindrotheca closterium*) were identified and sized by using a Scanning Electron Microscope (SEM). 10 ml of samples were taken and immediately filtered by using Nucleopore Track-Etch Membrane (Whatman) and kept in oven for 15 minutes. Phytoplankton bigger than 5µm were counted using the inverted microscope method (Utermöhl, 1958) with settling cylinders of 50 ml volume and a bottom area of 500 mm^2. Cells were allowed to settle for 24 h and counted under an inverted light microscope. It was attempted to count at least 100 cells of each taxon to achieve 95% confidence limits of ~20% but this was not applicable in some of the treatments where the biggest species (e.g. *Ceratium tripos* and *Ceratium tripos)* were rare, like. Twenty randomly selected cells from each species per sample were for size measurements. Species biomass was calculated from specific abundances (N_i) and cell volumes (V_i): $B_i = N_i * V_i$ and relative biomass (P_i) was calculated by diving species biomass (B_i) to total biomass (B_{tot}) $P_i = B_i / B_{tot}$

1.2.2 Statistical analysis

The correlations of dissolved nutrients with temperature and dilution rate were analysed through linear regression while correlations of relative biomass ($P_i = B_i / B_{tot}$) with temperature, dilution rate and C:N ratio were analysed through multiple regressions after arcsine-square root transformation of p_i. The correlation of C: N ratio with temperature and dilution rate was analysed also through multiple regressions model.

1.3 RESULTS

1.3.1 Taxonomic composition

A total of 16 species was counted and sized. Initial species composition was mainly dominated by the dinoflagellates *Ceratium tripos* (25% of biomass), *Ceratium fusus* (10%), *Scripssiella trochoidea* (6%), *Prorocentrum micans* (10%), *Gymnodium* sp. (4%), *Amphidinium* sp (3%) and by the diatoms species *Chaetoceros brevis* (16%), *Chaetoceros curvisetus* (3%), *Cerataulina pelagica* (3%), *Chaetoceros curvisetus* (3%), *Leptocylindrus danicus* (3%) and *Thalassionema nitzschioides* (2%). The silicoflagellate *Dictyocha speculum* contributed 10% to the total biomass while the smallest species i.e. picophytoplankton, the flagellate *Pyramimonas* sp, the cyanobacterium *Anabaena* sp. and the diatom *Cylindrotheca closterium* contributed only 2% of the total phytoplankton biomass.

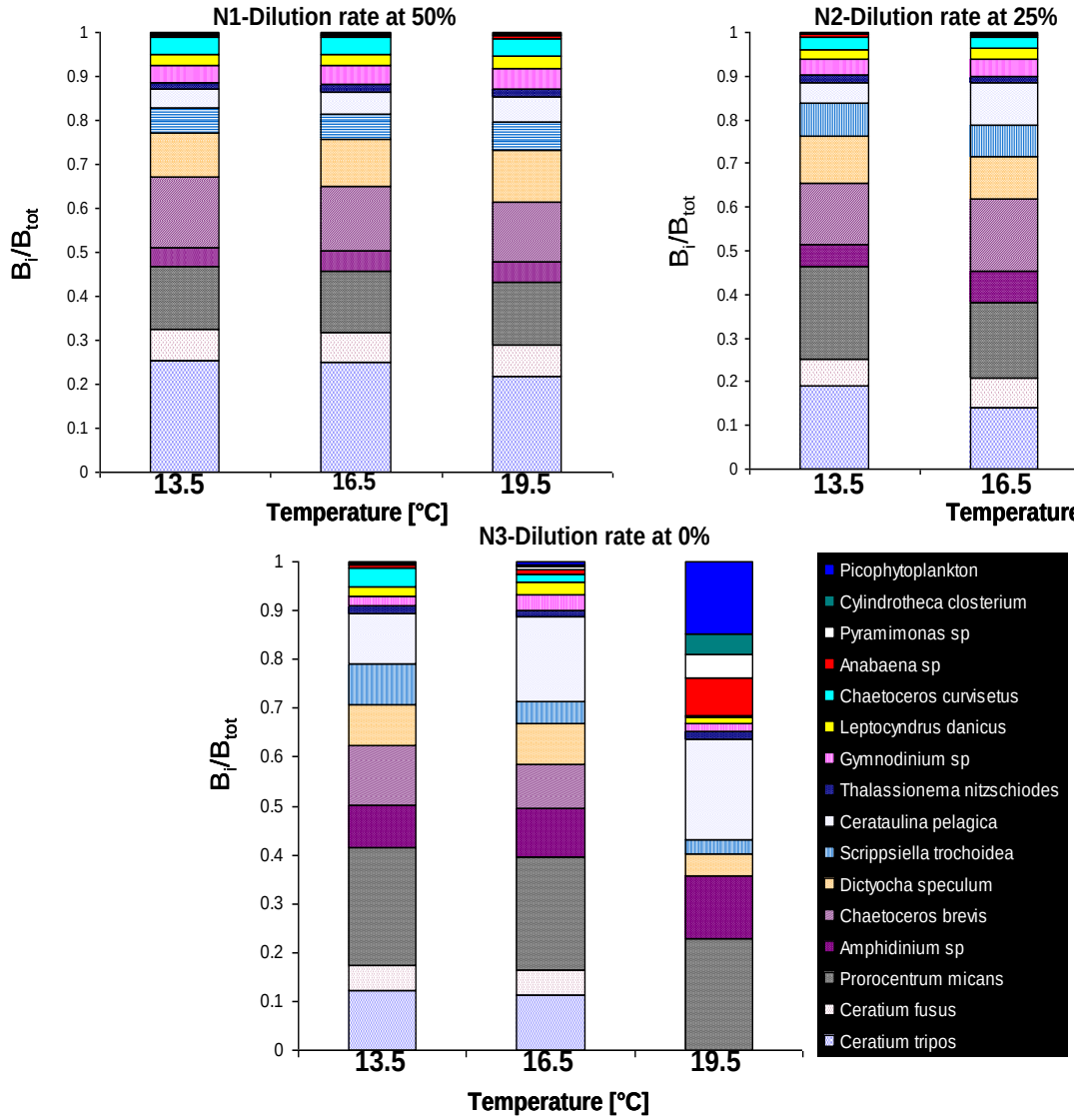


Figure 2: Change in relative biomass (arcsine-square root-transformed- B_i/B_{tot}) of different species in respond to temperature and dilution rate

At all levels of temperature combined with high and medium dilution rates, final phytoplankton communities were mostly dominated by *C. tripos, micans, C. fusus,. Ch. brevis, D, speculum, S. trochoidea, C. pelagica and curvisetus* (Fig 1). However, at the highest temperature, *C. tripos, C. fusus* and *Ch. brevis* disappeared in the cultures without dilution and were replaced by an increase of small species (*Pyramimonas* sp., *C., closterium, Anabaena* sp. and picophytoplankton).

1.3.2 Nutrient conditions

Concentrations of all dissolved nutrients increased with dilution rates and decreased with temperature. Temperature effects were significant for NO_3 (including also NO_2), NH_4 and SiO_4, while the temperature effect on PO_4 was not significant (Fig 2, Table 1).

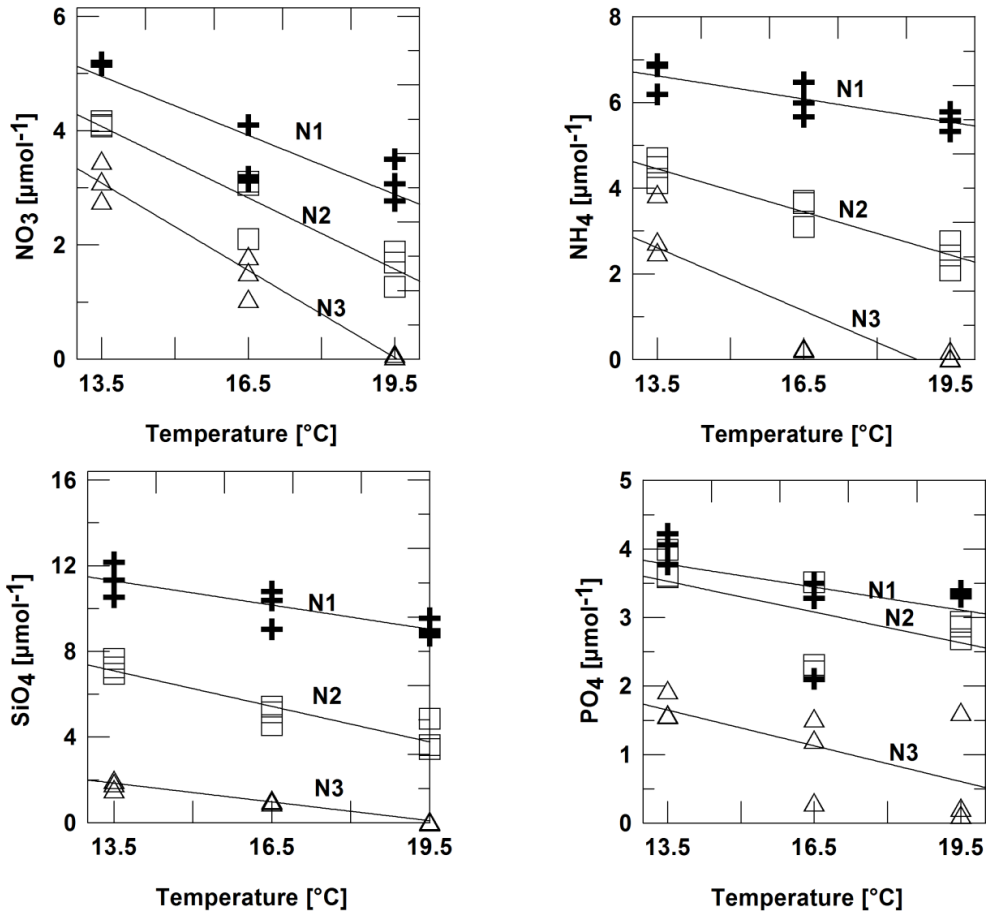


Figure 2: Change in dissolved inorganic nutrient $\log^{10}$ (µmolL-1) in respond to temperature and different dilution rate. 50% dilution rate-N1: Crosses; 25% dilution rate-N2: open squares and 0% dilution rate-N3: open triangles

Table 1: Regression (Model: y = ax +b) of dissolved inorganic nutrients (Log ^{10} µmol l^{-1}) on temperature (°C) at different dilution rates.

		A	B	P	R^2
NO_3	N1	-0.0297	1.1735	0.001	0.77
	N2	-0.0489	1.3719	<0.001	0.89
	N3	-0.0975	1.9525	<0.001	0.91
NH_4	N1	-0.011	1.0309	0.005	0.7
	N2	-0.0326	1.1944	<0.001	0.88
	N3	-0.0937	1.7947	<0.005	0.81
SiO_4	N1	-0.017	1.3207	0.001	0.77
	N2	-0.0238	1.2254	0.007	0.82
	N3	-0.0877	1.7343	<0.001	0.87
PO_4	N1	-0.0103	0.8141	0.26	0.17
	N2	-0.0152	0.857	0.09	0.34
	N3	-0.0407	0.974	0.006	0.41

DIN (NO_3+NO_2+NH_4) to PO_4 ratios were always clearly <16:1, indicating that N rather than P would be the potentially limiting nutrient, except for Si-limited diatoms. Therefore, we used biomass C:N ratios, i.e. the inverse of the carbon normalized cell quota of N (Droop, 1973, Droop, 1983, Goldman *et al.*, 1979) as indicator of cellular nutrient stress. C:N ratios increased with temperature but decreased with dilution rate as shown by multiple regression analysis (Table 2; for graphical representation see Figure 1 in chapter 2).

Table 2: Multiple regression of $\log^{10}$ C:N (dependent variable) on the temperature and dilution rate both as independent variables

C:N ratio	a	B	c	R-temp	R-dilution	p_{-temp}	$p_{-dilution}$	r^2	p-model
	1.092	0.013	-0.008	0.28	-0.84	0.0026	<0.001	0.92	<0.0001

1.3.3 Temperature, dilution and C:N ratio effects on phytoplankton species.

The multiple regression model (Table 3) was significant for all species. The temperature effect was significant for 13 species while the dilution effect was significant for 14 species.

Linear regressions of the parameters b (temperature response) and c (dilution response) from the multiple regressions (Table 3) on $\log^{10}$ cell volume demonstrated a clear size dependence of the temperature effect (Fig 3a) as indicated by the regression.

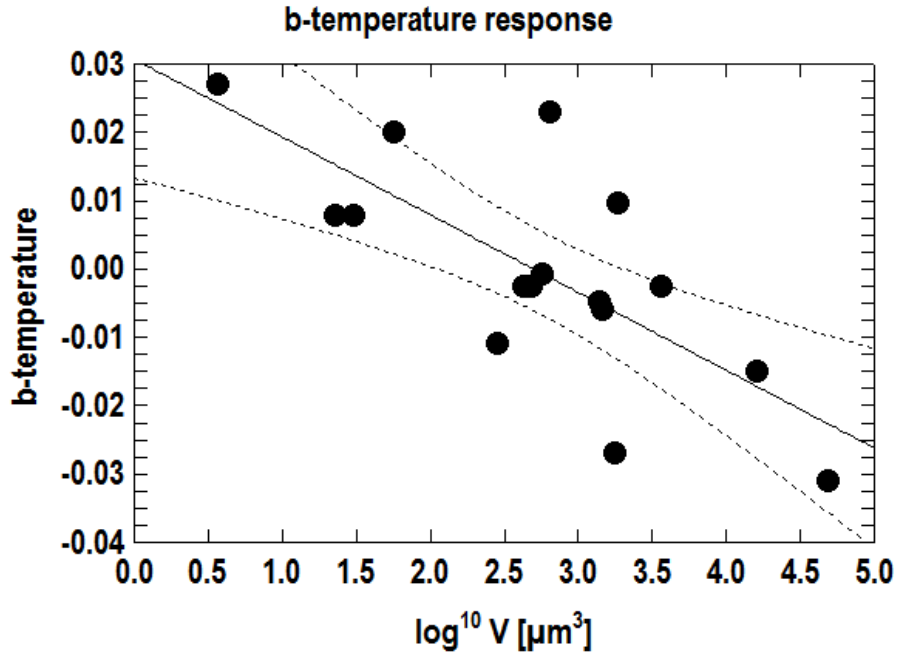


Fig .3a: Linear regression of b (temperature response) on $\log^{10}$ cell volume

Table 3. Multiple Regression of arcsine-square root-transformed relative biomass ($p_i = B_i/B_{tot}$) on temperature and dilution rate both as explanatory variables. Regression according to the model $a\sin\sqrt{p_i} = a + b.t + c.d$. Where t expressed as °C , d expressed in % : dilution rate at 50%, 25% ,and 0% .Probability of error for temperature ($P\text{-}_{temp}$),probability of error for dilution ($P\text{-}_{dilution}$), R^2 for full model, and probability of error for the full model (P_{model}), N= 9 except for *Ceratium tripos*, *Ceratium fusus*, and *Chaetoceros brevis* which disappeared from the N3-19.5°C, treatment combination (N=6).

Species	a	b	C	$p_{\text{-temp}}$	$p_{\text{-dilution}}$	r^2	p-model
Ceratium tripos	0.73	-0.031	0.0064	<0,0001	<0,0001	0.77	<0,0001
Ceratium fusus	0.41	-0.015	0.0024	0.0012	0.0001	0.57	<0,0001
Prorocentrum micans	0.41	-0.0025	0.00006	0.0076	0.56	0.21	0.0232
Amphidinium sp.	0.089	0.0097	-0.0004	0.0007	0.14	0.37	0.0015
Chaetoceros brevis	0.67	-0.027	0.0039	0.0012	0.0002	0.54	<0,0001
Dictyocha speculum	0.38	-0.0061	0.001	0.0205	0.0017	0.4	0.001
Scrippsiella trochoidea	0.29	-0.0048	0.00081	0.054	0.0068	0.3	0.0058
Cerataulina pelagica	-0.04	0.023	-0.022	<0,0001	<0,0001	0.74	<0,0001
Thalassionema nitzschioides	0.13	-0.0009	0.00034	0.35	0.0046	0.25	0.0123
Gymnodinium sp.	0.21	-0.0025	0.00049	0.021	0.0002	0.47	0.0002
Leptocylindrus danicus	0.18	-0.0025	0.0005	0.093	0.0082	0.26	0.0095
Chaetoceros curvisetus	0.3	-0.011	0.0017	0.0002	<0,0001	0.65	<0,0001
Anabaena sp	-0.07	0.02	-0.0042	0.0006	<0,0001	0.7	<0,0001
Pyramimonas sp.	-0.04	0.0078	-0.0012	0.0012	0.0001	0.57	<0,0001
Cyliyndrotheca closterium	-0.06	0.0078	0.0011	0.001	0.0002	0.54	<0,0001
Picophytoplankton	-0.2	0.027	-0.0041	0.0015	0.0001	0.53	<0,0001

The regression coefficient for temperature effect showed a negative correlation to the mean cell volume of the species (V_m)

$b = 0.0114\text{-}0.037(\pm 0.004) \log^{10} V_m$, $r^2=0.55$, $P=0.0011$

This means that smaller species profited from higher temperatures while larger ones profited from lower ones. Conversely, no size dependence could be found for the regression coefficient for the dilution dilution effect:

$c = 0.0017+ 0.0056 (\pm 0.001) \log^{10} V_m$, $r^2 =0.09$, $p =0.26$.(Fig 3b).

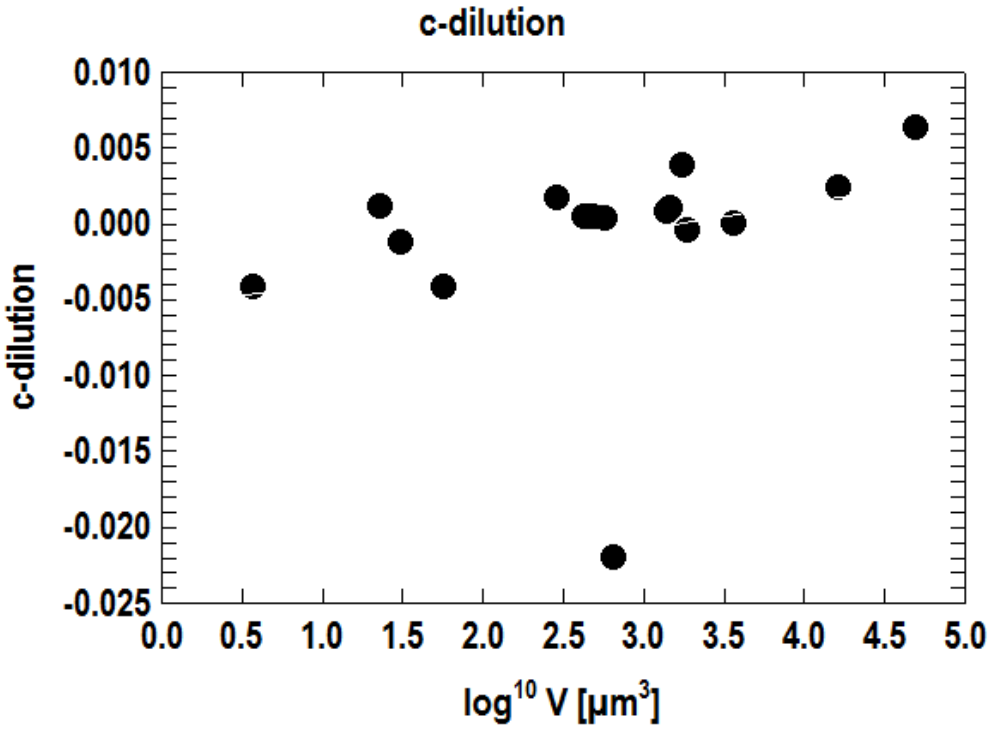


Fig. 3b: Linear regression of c (dilution) on log^{10} cell volume

However, when leaving out *Cerataulina pelagica* as an outlier, a clear size effect dependence on dilution was found: c = 0.0019 +0.0045 (±0.006) log^{10} V_m, r^2 =0.58, P=0.00009. The regression equation indicates that larger size species profit from dilution, i.e, from a less intense nutrient limitation. However, the coefficient b does not indicate a pure temperature effect, because of the temperature dependence of nutrient stress as expressed by the C:N-ratio. Therefore, a multiple regression analysis was also performed of the arcsine-square root transformed relative biomasses on temperature and C:N-ratios (log- transformed). The full regression model was significant for all species. Linear regression analysis of the parameter b (response to temperature) and c (response to C:N ratios) from the multiple regression in Table 4 on log^{10} cell volume showed a size dependence of both the temperature (Fig 4a) and the C:N-effect (Fig 4b) as indicated by the regression equations:

b-temp, b= 0.0084 -0.0219 (±0.006 S.E) log ^{10} V_m, r^2= 0.48, P = 0.0061

c-C:N , c=0.2282-0.6181 (± 0.04 S.E) log^{10} V_m, r^2 =0.58, P=0.0021

Table 4. Multiple Regression of arcsine-square root-transformed relative biomass ($p_i = B_i/B_{tot}$) on temperature and C:N ratio both as explanatory variables. Regression according to the model $a\sin\sqrt{p_i} = a + b.t + c.\log^{10}$ (C:N), where t expressed as °C , C:N ratio expressed as mol:mol. Probability of error for temperature ($P\text{-}_{temp}$),probability of error for C:N ratio ($P\text{-}_{CN}$). R^2 for full model, and probability of error for the full model (P_{model}), N= 9 except for *Ceratium tripos*, *Ceratium fusus*, and *Chaetoceros brevis* which disappeared from the N3-19.5°C, treatment combination (N=6)

Species	a	b	c	$p_{\text{-temp}}$	$P_{\text{-CN}}$	r^2	p-model
Ceratium tripos	1.424	-0.023	-0.631	0.011	<0.0001	0.76	<0.0001
Ceratium fusus	0.734	-0.011	-0.302	0.0089	<0.0001	0.62	<0.0001
Prorocentrum micans	0.420	-0.002	0.007	0.0109	0.5824	0.21	0.0236
Amphidinium sp.	0.046	0.009	0.036	0.0017	0.3266	0.34	0.0027
Chaetoceros brevis	0.201	-0.021	-0.491	0.075	<0.0001	0.60	<0.0001
Dictyocha speculum	-0.531	-0.004	0.1362	0.0784	0.003	0.47	0.0002
Scrippsiella trochoidea	0.414	-0.003	0.1155	0.1553	0.0005	0.42	0.0005
Cerataulina pelagica	-0.294	0.020	0.221	<0.0001	0.0004	0.67	<0.0001
Thalassionema nitzschioides	0.180	-0.005	-0.0115	0.7689	0.0009	0.33	0.0029
Gymnodinium sp.	0.277	-0.002	-0.057	0.1151	0.0002	0.47	0.0002
Leptocylindrus danicus	0.234	-0.005	-0.081	0.239	0.0214	0.21	0.023
Chaetoceros curvisetus	0.512	-0.004	-0.074	0.0028	<0.0001	0.64	<0.0001
Anabaena sp	-0.640	0.013	0.521	0.0091	<0.0001	0.77	<0.0001
Pyramimonas sp.	-0.208	0.006	0.156	0.0082	<0.0001	0.64	<0.0001
Cyliyndrotheca closterium	-0.205	0.006	0.138	0.0054	<0.0001	0.61	<0.0001
Picophytoplankton	-0.769	0.019	0.529	0.0086	<0.0001	0.63	<0.0001

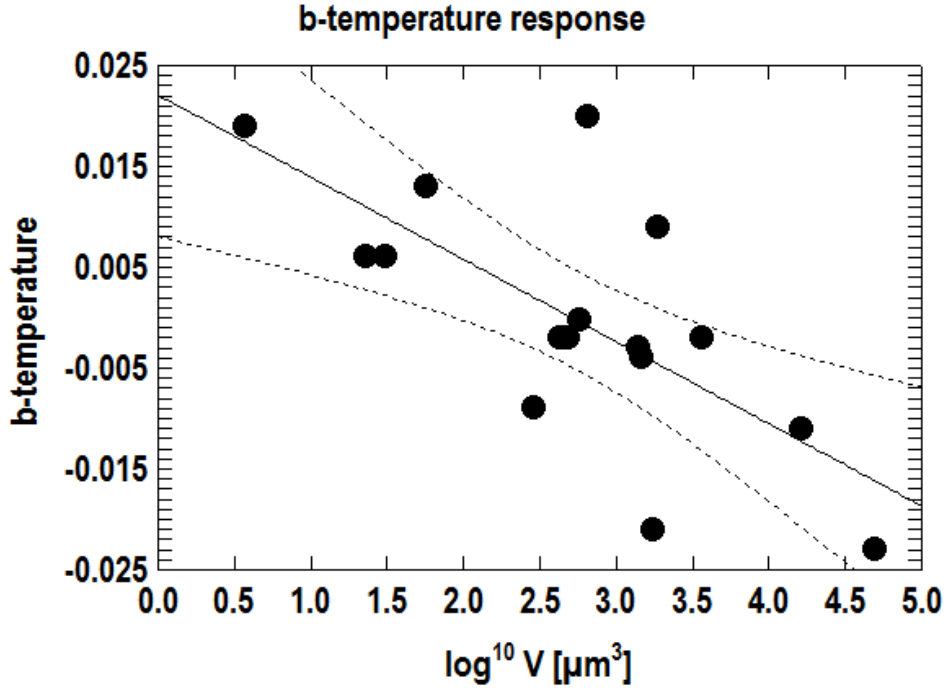


Fig .4a: Linear regression of b (temperature response) on $\log^{10}$ cell volume

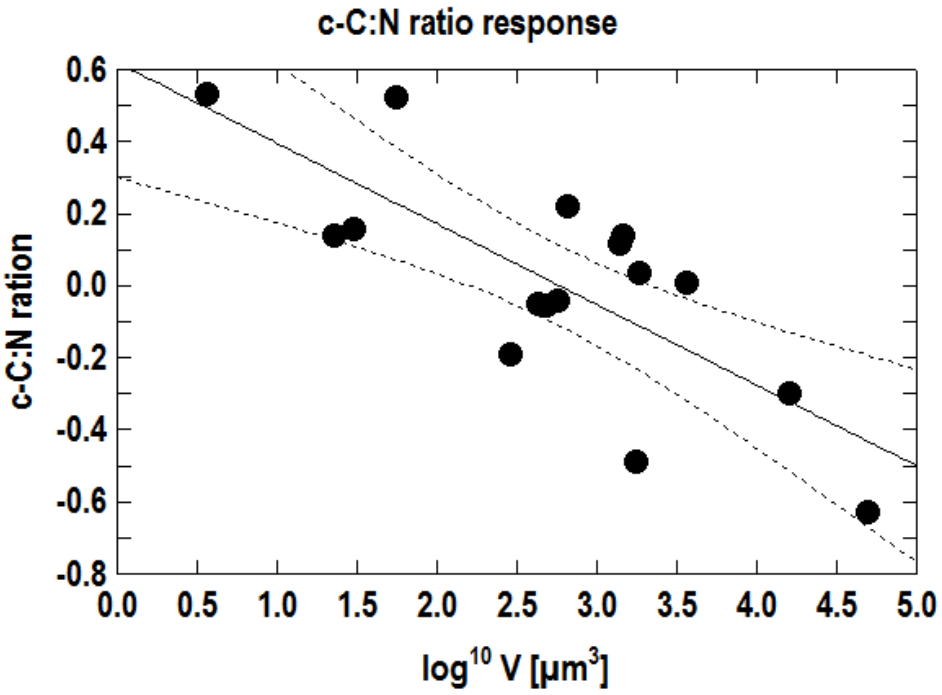


Fig 4b: Linear regression of c (C:N ratio response) on $\log^{10}$ cell volume

The relative abundance of small species increased with temperature (positive b), while the relative abundance of large species decreased (negative b). Similarly, small species profited from high C:N ratios (positive c), indicating strong nutrient limitation, while larger species profited from smaller ratios (negative c), indicating weak nutrient limitation. In total, we analyzed the response of 15 species, 1: a-taxonomically defined size category (picophytoplankton) and 2: higher taxa (diatoms, dinoflagellates). The remaining higher taxa (Dictyochophyceae, Prasinophyceae, Cyanobacteria) were only represented as single species (Table 5).

Table 5. Higher taxon and mean cell Volume (V_m, μm^3); grand mean) of phytoplankton species arranged in descending order of size.

Species	Taxon	V_m
Ceratium tripos	Dinoflagellates (Dinophyta)	49772.82
Ceratium fusus	Dinoflagellates (Dinophyta	16373.26
Prorocentrum micans	Dinoflagellates (Dinophyta	3671.92
Amphidinium sp.	Dinoflagellates (Dinophyta	1876.92
Chaetoceros brevis	Dinoflagellates (Dinophyta	1766.69
Dictyocha speculum	Dictyochophyce	1490.01
Scrippsiella trochoidea	Dinoflagellates (Dinophyta	1394.33
Cerataulina pelagica	Dinoflagellates (Dinophyta	657.14
Thalassionema nitzschioides	Diatom (Bacillariophyceae)	571.29
Gymnodinium sp.	Dinoflagellates (Dinophyta)	490.24
Leptocylindrus danicus	Diatom (Bacillariophyceae)	435.19
Chaetoceros curvisetus	Diatom (Bacillariophyceae)	290.39
Anabaena sp.	Cynobacteria	56.77
Pyramimonas sp.	Prasinophyceae	30.82
Cyliyndrotheca closterium	Bacillariophyceae	22.82
Picophytoplankton	Higher taxa diverse	3.72

The same linear regression analysis was performed of the parameter b (temperature response) and c (C:N ratio response) from the multiple regression in Table 4 on $\log^{10}$ cell volume separately for the two major higher taxa, i.e. diatoms and dinoflagellates. Once more, this showed a size dependence of the responses to temperature and C:N ratio (Fig 5) for dinoflagellates: b-temp, b = 0.00117-0.0366(±0.002 S.E) $\log^{10} V_m$, r^2 = 0.64, p=0.005, c-C:N ratio: c =0.3278 – 1.0405 (±0.003 S.E) $\log^{10} V_m$ r^2 =0.75, p=0.0026; After deleting *Cerataulina pelagica* as an outlier also diatoms showed a significant size dependence of the temperature and the C:N-effect b-temp: b= 0.0126-0.0257 (± 0.005 S.E) $\log^{10} V_m$, r^2 = 0.80, p=0.0322, c-C:N ratio : c = 0.291-0.6009 (+ 0.002) $\log^{10} V_m$, r^2= 0.79,p=0.0174), though both size effects appeared to be smaller than in the case of dinoflagellates.

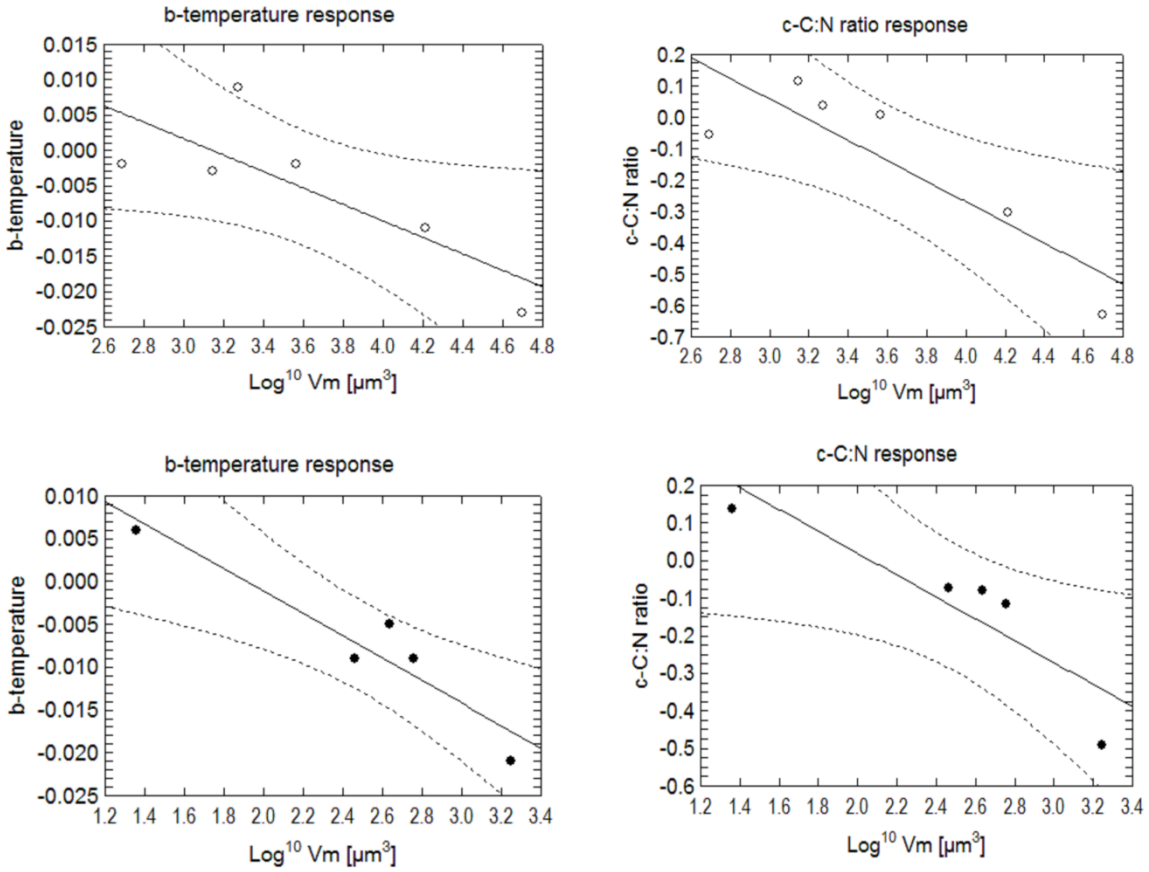


Fig 5: Linear regression of b (temperature response) and c (C: N ratio respond) on log^{10} cell volume (V_m, μm^3). Filled circles for Dinoflagellates and open circles: Diatoms

3.4 DISCUSSION

Hypothesis 1: The aim was to assess the effect of nutrient and warming on phytoplankton composition and also to determine whether temperature effects would intensify under nutrient stress. The idea of the study was based on previous studies reporting replacements of large by smaller phytoplankton species under increasing temperatures (Daufresne *et al.*, 2009, Hilligsøe *et al.*, 2011, Morán *et al.*, 2010, Sommer & Lengfellner, 2008, Yvon-durocher *et al.*, 2011) but there is lack clarity whether temperature effects are direct or are mediated via nutrient limitation. Therefore, the analysis of factorial design combining temperature and nutrient stress was performed. However, the results showed that, the extent of nutrient limitation, as indicated by the concentrations of dissolved, available nitrogen species (nitrate, ammonium) (Table 1) and, more importantly, by the C:N ratio in the particulate matter (Table 2) was also influenced by the temperature treatment. The C:N ratio in the biomass is the inverse of the nitrogen cell quota sensu Droop (1973) when normalized to biomass. A linear, negative relationship between the quotient realized growth rate/maximal growth rate and the ratio of C to the limiting nutrient in

phytoplankton biomass has been demonstrated by Goldman et al. (1979) and confirmed by later studies, (e.g. (Sommer, 1991). We identified N as the limiting nutrient and excluded P because DIN:PO_4 and PON:POP ratios in all cultures were below 15:1, which is considered indicative of a balanced N:P supply for average phytoplankton (Goldman et al. 1979). Biomass C:N ratios near the Redfield-ratio (ca. 6.6:1) indicate nitrogen replete conditions, while increasing ratios indicate increasingly stronger nitrogen stress. C:N-ratios were only slightly above the Redfield-ratio in the treatment combination of low temperature - high dilution rate but increased up to 31.4 in the combination high temperature – no dilution. Taking C:N ratios instead of dilution rates as indicator of nutrient stress permitted us to use test by multiple regression analysis whether there is still a nutrient-independent temperature effect. The relationship of the regression coefficients b and c further shows, that both warming and increasing nitrogen stress favour the replacement of larger by smaller species.

In situ, both effects will enforce each other in a warming ocean because surface warming will strengthen vertical temperature stratification and reduce vertical nutrient transport to the euphotic zone (Behrenfeld *et al.*, 2006, Doney, 2006, Polovina *et al.*, 2008).

Hypothesis 2: The aim was to investigate whether temperature and nutrient responses of the different species also depended on their phylogenetic status. Since several of the higher taxa (Dictyochophyceae, Prasinophyceae, and Cyanobacteria) were represented only by single species, we can only conclude that they did not stand out from the general size related trend, i.e. they responded to warming and CO_2-enrichment according to their cell size. Therefore, a separate analysis for dinoflagellates and diatoms was performed. Their temperature- and CO_2-responses depended on size qualitatively in the same way, i.e. higher temperatures and stronger nutrient limitation selected for smaller cell size. However, the different slopes of the equation b-temp and c-C:N indicated a stronger sensitivity of dinoflagellates. Overall, this chapter conclude that there is some phylogenetic effect, but that the size effect is dominant.

1.3.4 The Global Change perspective

Nutrient and temperature effects on phytoplankton cell size will most probably reinforce each other in a future, warmer ocean. Surface sea water warming will lead to an enhanced thermal stratification (Doney, 2006) and, therefore, to a decreased nutrient import from deeper waters on the global scale, though local and short term exceptions because of increasing storm events might

happen. In this experiment, a single Si:N:P ratio was used in the medium. Therefore, our prediction relates only to cell size, not to taxonomic composition. However, there is a rich body of literature on the influence of nutrient ratios on the outcome of taxonomic composition (Karl & Lukas, 1996, Sommer, 1996, Tyrrell, 1999). For instance increase N:Si and P:Si nutrient ratios change the phytoplankton species to non-diatomic species (Egge & Aksnes, 1992, Officer & Ryther, 1980) while high Si:N and Si:N ratio favours large diatoms (Babin *et al.*, 2004, Del Amo *et al.*, 1997).Since Si is mainly imported into the surface layer via vertical transport, diatoms will suffer a disadvantage from enhanced stratification.

The consequences of size shift are in two ways: First, the dominance by small, non-siliceous algae will reduce the export of organic matter to the ocean interior because of low sedimentation rates and a shift towards a more intense recycling of matter through microbial food web (Wohlers *et al.*, 2009). Second, a dominance of primary production by small algae will lead to a less efficient food web transfer of matter and energy because of the addition of intermediate trophic levels (Sommer *et al.*, 2002). While copepods, the premier food source of zooplankton feeding fish, directly feed on phytoplankton >5 to 10 µm (Sommer & Sommer, 2006), smaller phytoplankton are primarily consumed by heterotrophic protists.

CHAPTER 4

Interactive effect of warming, Nitrogen and Phosphorus limitation on phytoplankton cell size

ABSTRACT

Cell size is one of the ecologically most important traits of phytoplankton. The cell size variation is frequently related to temperature and nutrient limitation. In order to disentangle the role of both factors an experiment was conducted to determine the possible interactions of these factors. Baltic Sea water containing the natural plankton community was used. We performed a factorial combined experiment of temperature, type of nutrient limitation (N vs. P), and strength of nutrient limitation. The type of nutrient limitation was manipulated by altering the N: P ratio of the medium (balanced, N- and P-limitation) and strength by the dilution rate (0 and 50%) of the semi-continuous cultures. The negative effect of temperature on cell size was strongest under N-limitation, intermediate under P-limitation and weakest when N and P were supplied at balanced ratios. However, temperature also influenced the intensity of nutrient imitation, because at higher temperature there was a tendency for the identical dilution rates and medium composition dissolved nutrient concentrations to be lower while the C:N or C:P ratio being higher. Analysing the response of cell size to C:N ratios (as index of N-limitation) and C:P ratios (as index of P-limitation) indicated a clear dominance of the nutrient effect over the direct temperature effect, though the temperature effect was also significant

I.1 INTRODUCTION

The relationship between body size and temperature has experienced a recent revival due to the concerns about anthropogenic climate change and because several studies have confirmed a tendency towards smaller body size at higher temperatures for phytoplankton (Atkinson *et al.*, 2003, Daufresne *et al.*, 2009, Morán *et al.*, 2010, Yvon-durocher *et al.*, 2011). With the increased evidence for the size decline, interest in the relative importance of direct and indirect temperature effects has emerged. The mechanism driving intraspecific and community level size reductions differs between systems and may be associated with higher grazing (Ryther & Sanders, 1980), nutrient limitation which promotes small size algae (Finkel *et al.*, 2010, Winder *et al.*, 2009) and higher sedimentation of large phytoplankton (Piontek *et al.*, 2009). Moreover, temperature directly alters photosynthesis and respiration rates but this direct effect can be outweighed by other factors e.g grazing (Gaedke *et al.,* 2010). Even in experimental systems, where indirect effects of temperature via stratification and nutrient supply to the surface layer can be excluded, temperature effects were often mediated by biotic factors e.g. grazing (Gaedke *et al.*, 2010). Recently several studies have supported a role of increased size in selective grazing at higher temperatures, which leads to a disadvantage for larger phytoplankton if grazing is dominated by copepods (Lewandowska & Sommer, 2010, Peter & Sommer, 2012, Sommer & Lengfellner, 2008, Sommer & Lewandowska, 2011). A widespread alternative explanation for the well-known biographic shift from large phytoplankton in cold to small phytoplankton in warm ocean regions (Maranón *et al.*, 2012) is provided by the coupling between temperature, vertical stratification and nutrient supply from deeper waters and the resulting negative correlation between sea surface temperature and nutrient availability (Kamykowski & Zentara, 1986).

Small phytoplankton cells, due to a higher surface-area-to-volume ratio and smaller thickness of the diffusion boundary layer, have a competitive advantage over larger cells in nutrient-poor environments (Chisholm, 1992, Kiørboe, 1993, Raven, 1998). On the other hand, large phytoplankton are able to sustain higher rates of biomass-specific production rates in nutrient-rich waters (Cermeno *et al.*, 2005, Maranón *et al.*, 2007). Furthermore, the rate of cell division for large cell sizes require greater nutrients uptake fluxes compared with small cell size (Furnas, 1978). Moreover, the reduction picophytoplankton in nutrient-rich waters has been explained by loss rates (Agawin *et al.*, 2000) while decreased productivity is well related to increase in sea-surface temperatures and vertical temperature gradients in the upper-ocean (Doney, 2006) which intensifies vertical nutrients density stratification and thereby reduces vertical nutrient transport leading to nutrient limitation at the well illuminated surface zone. Thus stratified, oligotrophic environment are dominated by small-sized phytoplankton while weakly stratified or mixed, turbulent

environments are dominated by large-sized phytoplankton (Cushing, 1989, Kiørboe & Nielsen, 1990).

Interestingly, the identity of the limiting nutrient has not yet been related to phytoplankton cell size, while there are numerous examples relating taxonomic composition to nutrient ratios (Karl & Lukas, 1996, Sommer, 1996, Tyrrell, 1999) following Tilman (1982) seminal resource ration hypothesis. In a precursor of this study, the intensity of nitrogen limitation was manipulated by semi-continuous dilution at different rates (Peter & Sommer, 2013). These experiments showed that the effect of nitrogen limitation was dominant over a direct temperature effect. In the current research a further statistical analysis was necessary to determine whether the effect phosphorus and nitrogen limitation on cell size are the same or differ from each other , either in direction or intensity. The question is plausible, because the bulk of biomass nitrogen is contained in proteins, while the bulk of phosphorus is contained in nucleic acids, in particular in ribosomal RNA. Therefore, the synthesis of different biomass components may be affected by N- or P-limitation.

I.2 MATERIAL AND METHODS

I.2.1 Experimental design

The experiment was conducted for three weeks from 6th to 28th April 2013. Thirty six Erlenmeyer flasks of 700 mL were incubated in temperature and light controlled climate cabinets. The flasks were filled with Baltic Sea water (Kiel Fjord) from 1 to 3 m depth containing the natural plankton community and sieved through plankton gauze of 200µm mesh size in order to keep out large zooplankton. The flasks were placed in 2 climate cabinets with temperatures of 3ºC above and below in-situ conditions, respectively (1 and 7°C). The strength of nutrient limitation was manipulated by semi-continuous dilution three times per week on Monday, Wednesday and Friday by replacing 0% (strong limitation) and 50% (weak limitation) of the culture volume by 3 types of fresh medium. All media were sterile filtered with 0.2 µm pore size of Baltic Sea water and thereafter enriched. Medium 1 (P-limited) was enriched with 20 µmolL^{-1} NO_3, 14 µmolL^{-1}Si, and 0.5 µmolL^{-1} PO_4; medium 2 (balanced) enriched with 20 µmol$L^{-1}$$NO_3$, 14 µmol$L^{-1}$Si and 1.25 µmol$L^{-1}$$PO_4$; medium 3 (N-limited) enriched with 5 µmolL^{-1} NO_3, 14 µmolL^{-1}Si and 1.25 µmolL^{-1} PO_4. The media were stored at low temperature (1ºC) in darkness. In the following, the nutrient regimes are described by the following abbreviations: Plim1 (50% dilution rate, P-limited medium), Plim2 (0% dilution, P-limited medium), Bal1 (50% dilution, balanced medium) and Bal2 (0% dilution, balanced medium), Nlim1 (50% dilution, N-limited medium), and Nlim2 (0% dilution, N-limited medium). Each nutrient regime was combined with each temperature level in a fully

factorial design, leading to 12 treatments, each replicated 3 times. The light intensity was 249 µmol m^{-2} s^{-1} and the light: dark cycle 14:10 hrs for all treatments.

I.2.2 Sampling and analysis

Phytoplankton and nutrient samples were taken at the end of the experiment while water temperature, salinity, and pH were measured every day to monitor the experiments. Samples for dissolved nutrients were filtered by cellulose acetate filters of 0.8 µm pore size and kept in the -20 ºC until analysis. Dissolved nutrients were measured according to oceanographic standard methods (Grasshoff *et al.*, 1983). For the determination of particulate organic carbon (POC), nitrogen (PON) and phosphorus (POP), samples were filtered onto precombusted Whatman GF/F filters (Whatman GmbH, Dassel, Germany). After filtration, the samples were dried immediately and stored in desiccators. Analysis of particulate matter (POC and PON) were carried out after Sharp (1974) by gas chromatography in the elemental analyzer (Thermo Flash 2001, Thermo Fisher Scientific Inc., Schwerte, Germany), while POP was determined calorimetrically by converting organic phosphorus compounds to orthophosphate (Hansen & Koroleff, 2007). Particulate matter C:N and C:P ratios were used as an index of nutrient limitation (Goldman et al. 1979).

Samples for microscopic phytoplankton counts and size measurements were immediately fixed with Lugol's iodine. Phytoplankton bigger than 5µm were counted using the inverted microscope method (Utermöhl, 1958) with settling cylinders of 50 ml volume and a bottom area of 500 mm^2. Cells were allowed to settle for 24 h and counted under an inverted light microscope. It was attempted to count at least 100 cells of each taxon to achieve 95% confidence limits of $\pm$20%. Cell size measurements were done by measuring linear dimension with the AxioVisoin programme (Zeiss) and the cell volumes were calculated after approximation to geometric models (Hillebrand *et al.*, 1999). Twenty randomly selected cells from each species per sample were measured. Species biomass was calculated from specific abundances (N_i) and Cell volumes (V_i): $B_i = N_i * V_i$. The relative biomass was calculated by dividing the individual species biomass by the total biomass ($p_i = B_i/B_{tot}$) while community mean cell size were calculated by total biomass dividing by total number of cells ($V_c = B_{tot}/N_{tot}$)

I.2.3 Statistical analysis

Factorial ANOVA (STATISTICA 8) was used to analyze the effect of temperature, nutrient level and dilution rate both as categorical factors and their interaction on cell volume and community

mean cell size and relative biomass (dependent variables). General Linear Models (Sigma-restricted, Type VI unique) were used to analyze the effect temperature (categorical factor), C:N and C:P ratio (both as continuous factors) on phytoplankton cell size and community mean cell size. The same models was used also to analyze separately the effect of C:N and C:P ratio on cell volume and community mean cell size. For normal distribution of data, cell volume, C:P and C:N ratios were log^{10} transformed while relative biomass was arcsine-square root-transformed.

I.3 RESULTS

I.3.1 Species composition

A total of 7 phytoplankton species were abundant enough to perform analysis. The phytoplankton community was manly dominated by diatoms: *Chaetoceros curvisetus*, *Thalassionema nitzschioides, Thalassiosir*a sp., *Chaetoceros similis* and *Skeletonema costatum*. The other taxa available for analysis were the dinoflagellate *Scrippsiella trochoidea* and the cryptophyte *Teleaulax amphioxeia.*

I.3.2 Dilution effects

(i) **Cell volume: Phytoplankton cell sizes responded to dilution rate.** The cell sizes of all species showed increased with increasing dilution rates, indicating a shift towards larger size at less stringent nutrient limitation. The same pattern applied also to community mean cell size (Fig1a).

(ii) Biomass. Total biomass declined with decreasing dilution rate (Fig 2). Particulate matter C:N and C:P ratios were maximal in the undiluted cultures (Fig 3). There were significant correlations between total biomass and particulate matter stoichiometry. C:N & C:P had significant effect on total biomass: $Log^{10} B_{tot} = 6.79\text{-}0.25(\pm 0.005) \log^{10}$ C:N, $r^2 = 0.53$, $p < 0.0001$ and $Log^{10} B_{tot} = 6.59\text{-}0.39 (\pm 0.004) \log^{10}$ C:P, $r^2 = 0.47$; $p < 0.0001$ (Fig 4)

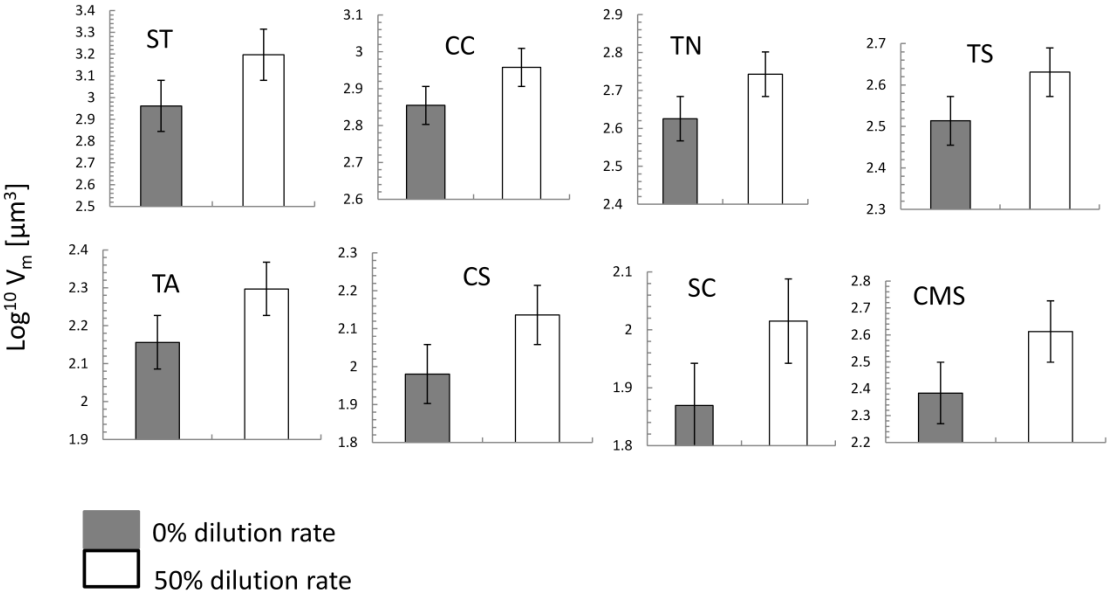


Figure1a: Decrease of individual cell sizes (**log**^{10} V_m[µm^3]) with decreasing dilution rate: **ST**-*Scrippsiella trochoidea* , **CC**-*Chaetoceros curvisetus,* **TN**-*Thalassionema nitzschioides*, **TS**-*Thassiosira* sp, **TA**-*Teleaulax amphioxeia*, **CS** -*Chaetoceros similis*, **SC**-*Skeletonema costatum,* **CMS**-Community mean cell size.

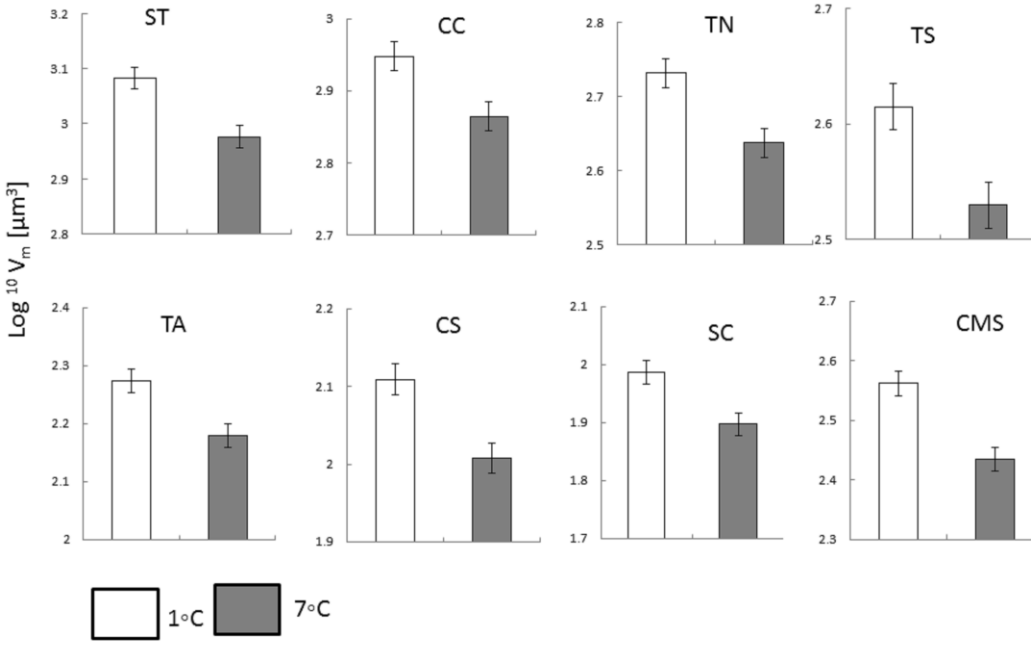


Figure1b: Decrease of individual cell sizes (log^{10} V_m[µm^3]) with increasing temperature: **ST**-*Scrippsiella trochoidea* , **CC**-*Chaetoceros curvisetus,* **TN**-*Thalassionema nitzschioides*, **TS**-*Thassiosira* sp, **TA**-*Teleaulax amphioxeia*, **CS** -*Chaetoceros similis*, **SC**-*Skeletonema costatum,* **CMS**-Community mean cell size.

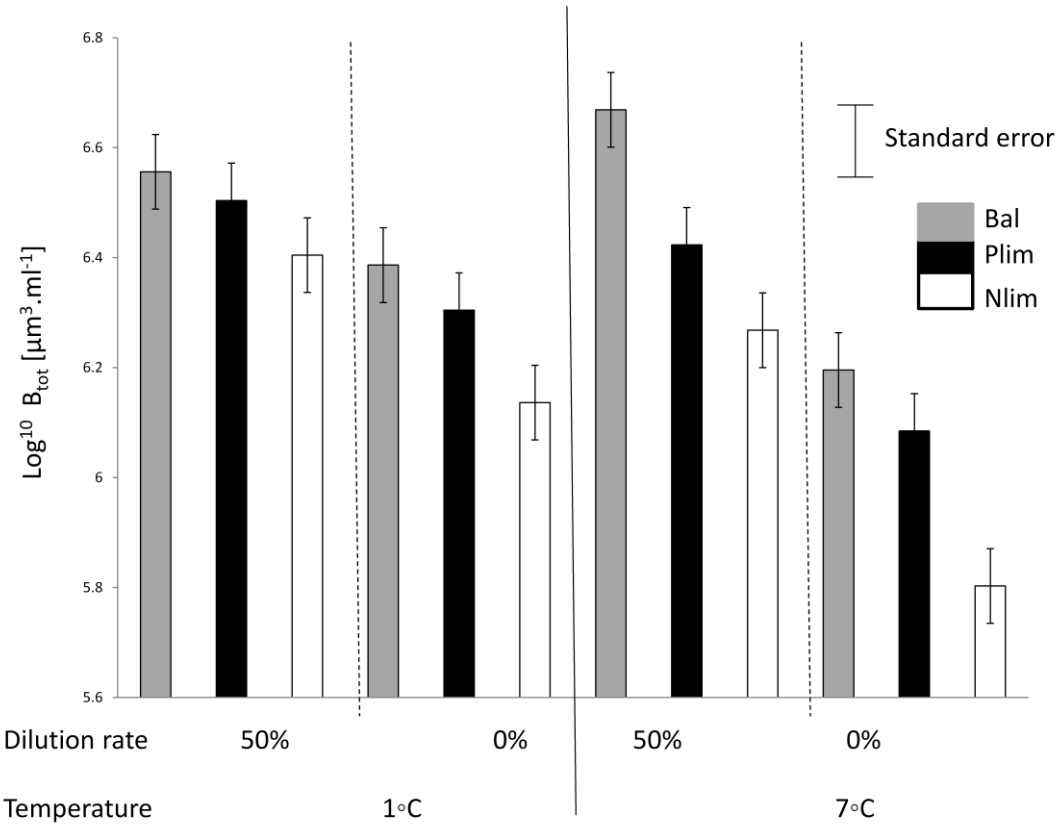


Figure 2: Variation of Total biomass (Log^{10} B_{tot} [μm^3 ml^{-1}]) with temperature (°C) and dilution rate and intensity of nutrient limitation (**Bal**-Balanced, **Plim**-P-limited and **Nlim**-N-limited and.

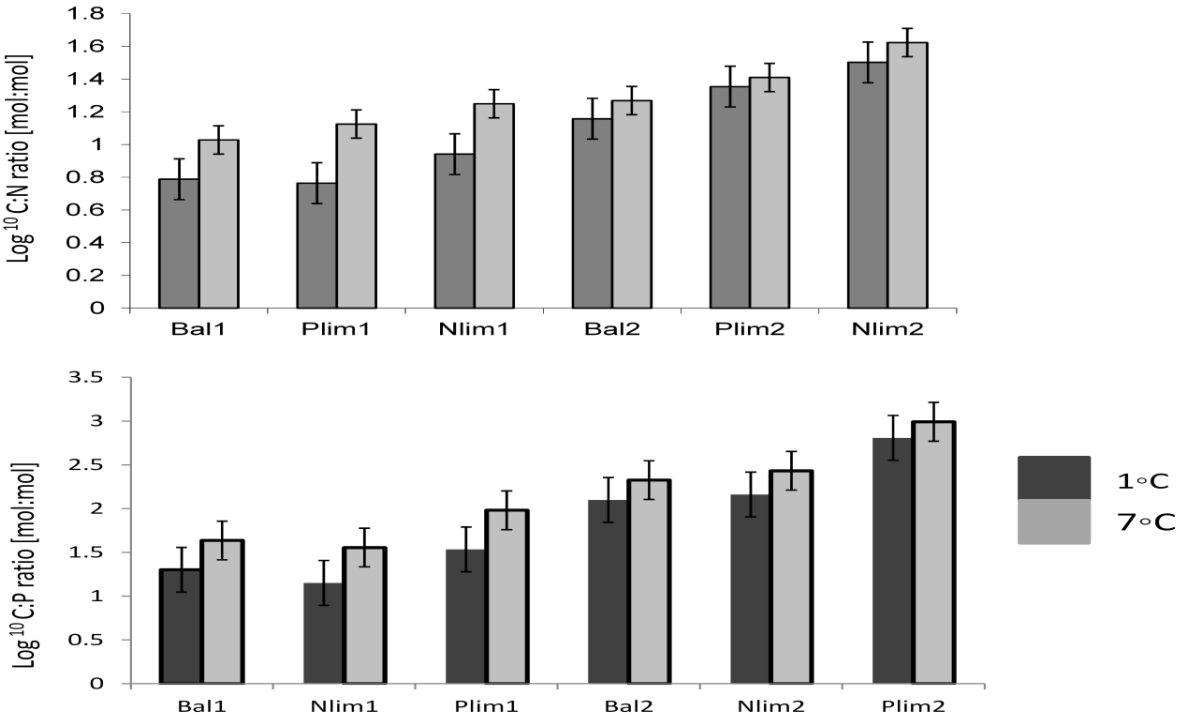


Figure 3: Variation of C:N and C:P ratios with dilution rate, intensity of nutrient limitation (**Bal**-Balanced, **Nlim**-N-limited and **Plim**-P-limited) and temperature.

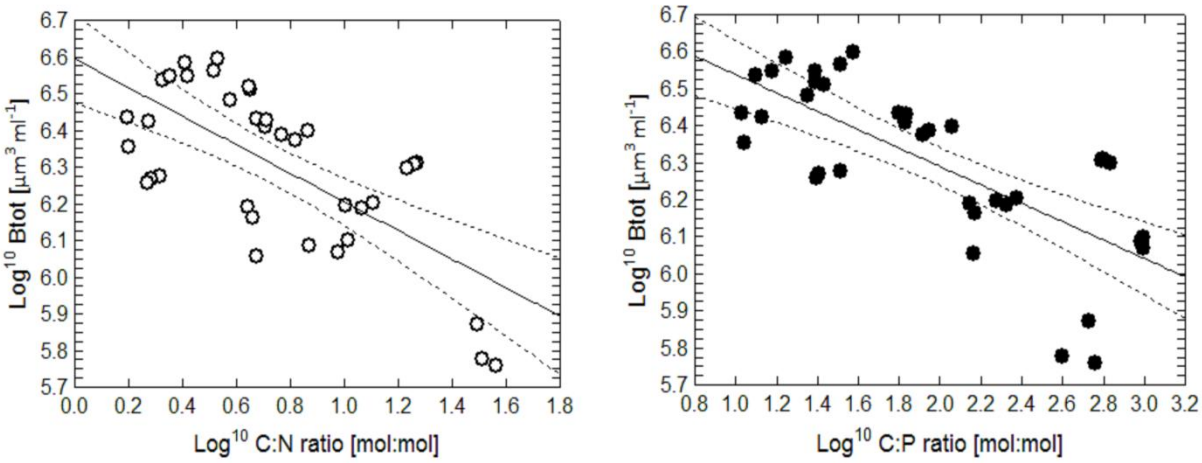


Figure 4: Decrease in total biomass (Log^{10} B_{tot} [μm^3 ml^{-1}]) with increasing C:P and C:N ratios [mol:mol].

I.3.3 Temperature effects

(i) **Cell size and community mean cell sizes:** Both cell sizes of individual species and the community mean cell size decreased with increasing temperature (Fig1b, 5 & 6,).

(ii) **C: N and C:P ratios:** Both C:N and C:P ratios increased with temperature. (Fig 3).

(iii) **Total biomass:** The response of total biomass (B_{tot}) to temperature depended on nutrient conditions (Fig. 2). While B_{tot} increased slightly with temperature in the Bal1 treatment and it decreased most strongly with temperature in the Nlim2 treatment.

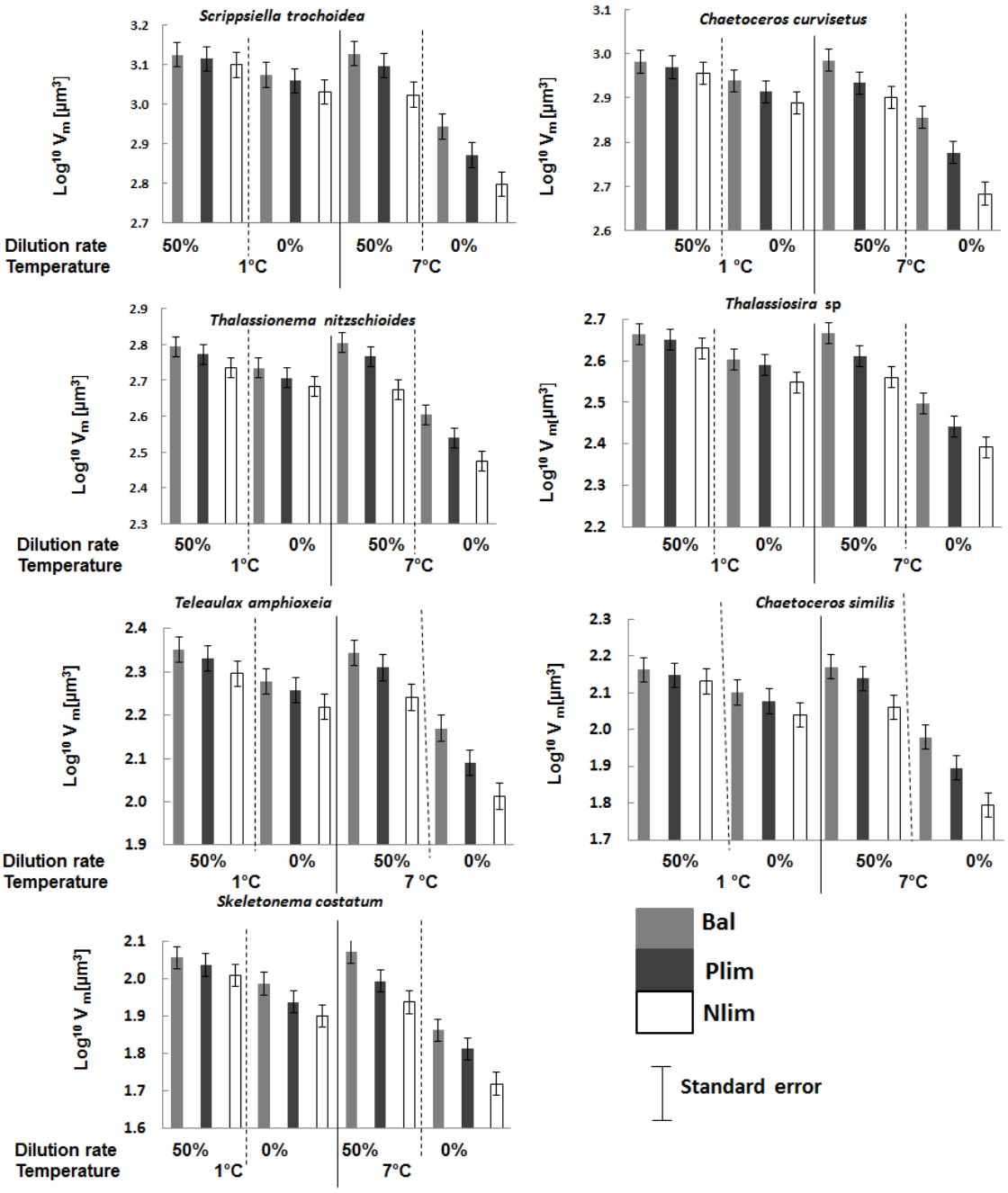


Figure 5: Change of species cell size (log^{10} $V_m[\mu m^3]$) with dilution rate, intensity of nutrient limitation (**Bal**-Balanced, **Nlim**-N-limited and **Plim**-P-limited) and temperature (°C)

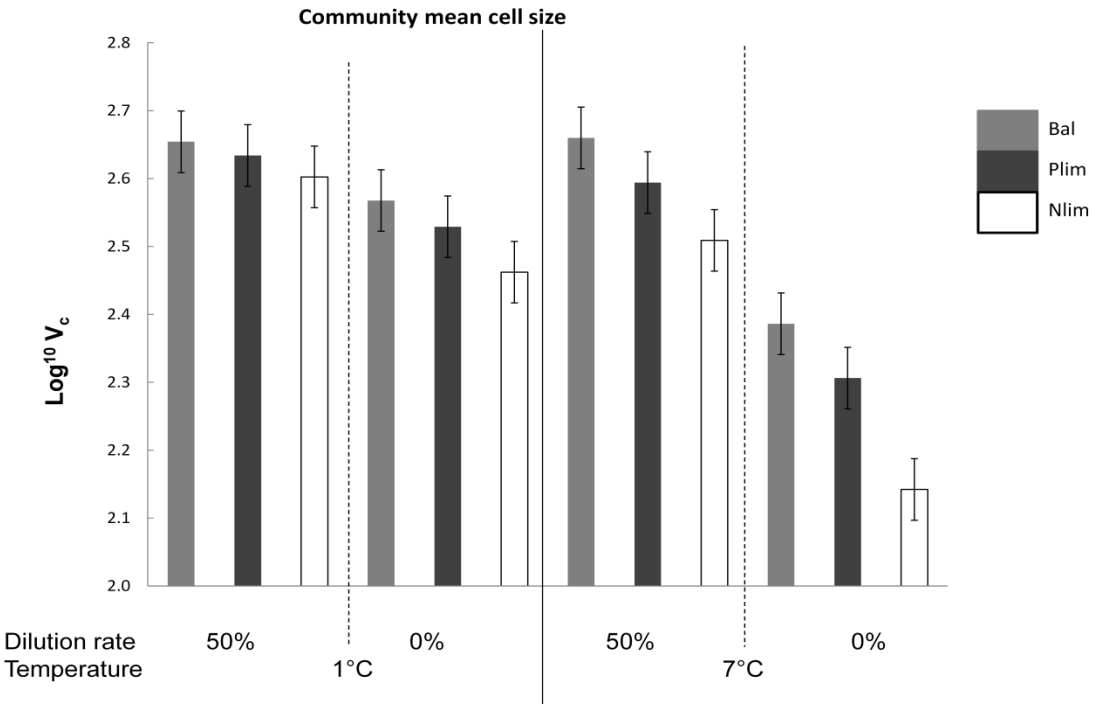


Figure 6: Change in Community mean cell size (log^{10} V_c) with dilution rate, intensity of nutrient limitation (**Bal**-Balanced, **Nlim**-N-limited and **Plim**-P-limited) and temperature (°C).

I.3.4 Effect of nutrient limitation type (balanced, N- and P-limitation) and temperature

i. **C: N and C:P ratios**. C: N ratios were maximal in the Nlim2 treatment under the higher temperature and minimal in the Bal1 and Plim1 treatments under the lower temperature. C: P ratios were maximal in the Plim2 treatment under the warmer temperature and minimal in the Nlim1 and Bal1 treatment under the lower temperature (Fig 3). This indicates maximally strong nutrient limitation at low dilution, warm temperature and extreme nutrient rations in the medium.

ii. **Cell volume:** The response patterns of the different species showed similar trends in the response to nutrient treatments and declined in the direction of intensity of nutrient limitation ie Bal1 > Plim1 > Nlim1 > Bal2 > Plim2 > Nlim2 while the temperature effect was strong only in the treatments without nutrient renewal (Bal2, Plim2 and Nlim2) (Fig 5). Temperature showed stronger effect on cell sizes in the Nlim2 than in Plim2 treatments.

iii. **Total biomass**: Total biomass influenced by nutrients limitation. The maximum value of total biomass was found in the treatment with balanced nutrient supply at high dilution rates in the warm treatments. Temperature showed a stronger negative effect on total biomass in N than

P-Limited treatment thereby minimum value was found in Nlim2. Total biomass decreased in the direction of Bal > Plim> Nlim (Fig 2).

iv. **Community mean cell size**: The community mean cell size declined with increasing temperature in the direction of Bal1 > Plim1 > Nlim1 > Bal2 > Plim2 > Nlim2 (Fig 6). However, the temperature effect was strong only in the treatments without dilution (Bal2, Plim2 and Nlim2).The minimum value of community mean cell size was found in the treatments with nitrogen limitation (N-lim2) at the higher temperature.

v. **Species composition:** The diatom *C, curvisetus* formed ca. half of total phytoplankton biomass (47-51%) in the treatments with weak nutrient limitation at both temperatures and about a third (26-36%) in the strongly nutrient limited treatments (Fig 7).

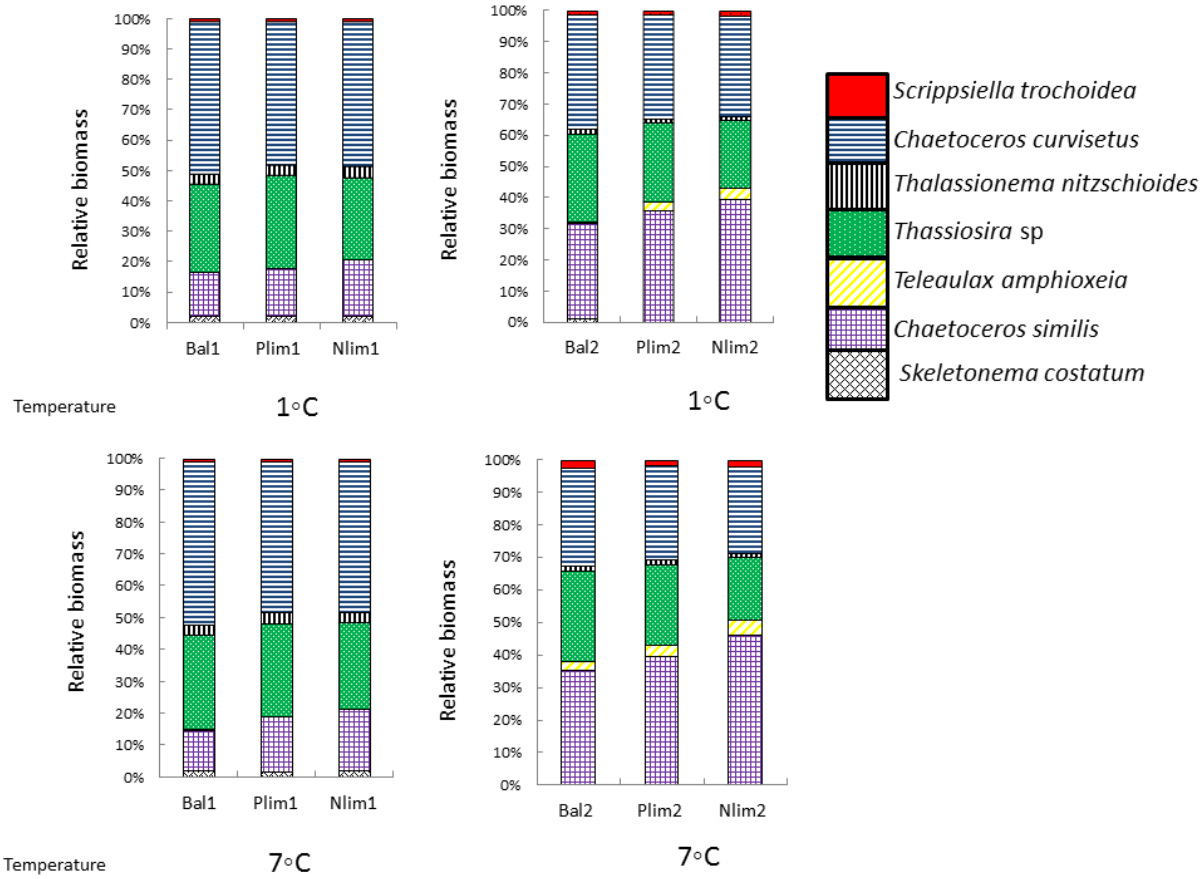


Fig 7: Change in phytoplankton structure with dilution rate, intensity of nutrient limitation (**Bal**-Balanced, **Nlim**-N-limited and **Plim**-P-limited) and temperature (°C)

The smaller congener *C. similis* was favored by nutrient limitation, forming ca. 20% (13-19%) in the treatments with weak nutrient limitation, but ca. one third (30-46%) in under strong nutrient limitation. *T. amphioxeia* contributed only 0.1-0.2% to total biomass in Bal1, P-lim1, N-lim1, and

Bal2, while it contributed 2-5% under strong and one-sided nutrient limitation (N-lim2, P-lim2). The relative biomass of other diatoms species decreased with increasing dilution rate (Fig 7).

I.3.5 Interactive effect of dilution rate, nutrient limitation and temperature;

(i) **Cell volume**: The multifactor ANOVA showed significant main effects of temperature, nutrient limitation, and dilution, and significant interaction effects temperature*nutrient and temperature*dilution on cell size for all species. The interaction effect of dilution*nutrient on cell size was significant for only 5 species while temperature*nutrient level*dilution interaction was significant for 4 species (Table 1).

(ii) **Community mean cell size**: Phytoplankton cell sizes responded both to temperature and nutrient treatment. There were significant main effects of temperature, nutrient, dilution and significant interaction effects of temperature*nutrient and temperature*dilution on community mean cell size. However, there was no significant interaction effect of temperature*dilution*nutrient level on community mean cell size (Table 1)

(iii) **Relative biomass (P_i):**The multifactorial ANOVA with arcsine-square root-transformed relative biomass ($P_i = B_i/B_{tot}$) of the different species (Table 2) showed significant temperature effect on relative biomass for 4 species, the nutrients and the dilution effects were significant for all species. A significant nutrient*temperature interaction was found for 4 species, and the interaction effect of temperature*dilution rate was significant for 5 species. The triple interaction temperature*nutrient*dilution rate was never significant.

Table 1: Factorial ANOVA of species size (Log^{10} V μm^3) as dependent factor on temperature (Temp-°C) , limiting nutrient level (Nutr) and dilution rate (Dil), P-values for main effects and interactions.

Species	Temp	Nutr	Dil	Temp*nutr	Temp*Dil	Nutr*dil	Temp*Nutr*dil
Scrippsiella trochoidea	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.0035
Chaetoceros curvisetus	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.0001
Thalassionema nitzschioides	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0008	0.022
Thalassiosira sp	<0.0001	<0.0001	<0.0001	0.020	0.0001	0.491	0.892
Teleaulax amphioxeia	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	0.068	0.943
Chaetoceros similis	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	0.0005
Skeletonema costatum	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	0.0722	0.2718
Community mean cell size	<0.0001	<0.0001	<0.0001	<0.0006	<0.0001	0.0005	0.521

Table 2: Factorial ANOVA of temperature, nutrient limitation, dilution rate effects on arcsine-square root-transformed biomass ($P_i=B_i/B_{tot}$) of different species

Species	P-temp	P-Nutrient	P-Dil	P-Tem*Nutr	P-Temp*dil	P-Nutr*dil	P-Temp*nutr*dil
Scrippsiella trochoidea	0.006	0.056	0.003	0.265	0.025	0.018	0.781
Chaetoceros curvisetus	0.051	0.002	<0.0001	0.051	0.06	0.031	0.917
Thalassionema nitzschioides	0.061	0.001	0.040	0.479	0.052	0.054	0.960
Thalassiosira sp	0.008	0.054	0.01	0.052	0.035	0.045	0.872
Telaulax amphioxeia	0.06	0.0006	<0.001	0.042	0.0035	0.023	0.444
Chaetoceros similis	0.0004	0.002	<0.001	0.014	0.051	0.026	0.871
Skeletonema costatum	0.071	0.002	0.003	0.057	0.197	0.004	0.119

Dissolved nutrients: The intensity of nutrient limitation was higher in the warm than cold treatments. The concentration of NO_3, PO_4 and SiO_4 were higher in the cold than in the warm treatments (Fig 8).

The final concentrations of dissolved nutrient $NO_{2+}NO_3$, NH_4, PO_4 and SiO_4 were also influenced by dilution rate. Maximal concentrations of NO_3+NO_2 and of NH_4 were found in the Bal1 and Plim1 treatments, minimal levels in the Nlim2 treatments. Maximal levels of PO_4 were found in the Bal1 and Nlim1 treatments and minimal ones in the Plim2 treatments. SiO_4 concentrations were high in the treatments with high dilutions rates and low in the undiluted ones. The intensity of nutrients limitations were lower in the treatments with high dilution rate and high in the treatments with low dilution rate. Nutrient limitation was also influence by temperature. The intensity of nutrient limitation was higher in the warm than cold treatments. The maximum values of $NO_{2+}NO_3$, NH_4, PO_4 and SiO_4 were found in the cold treatments (Fig 8).

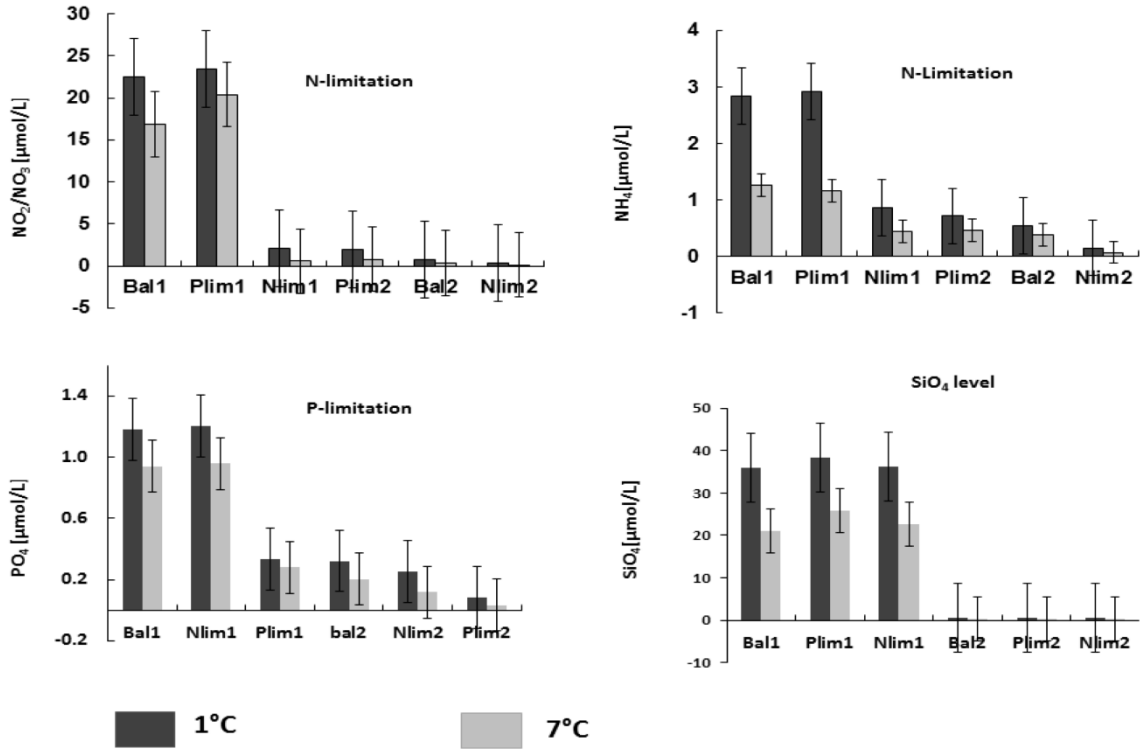


Figure 8: Decrease of dissolved nutrients with increasing temperature (°C)

I.3.6 Effects of particulate matter stoichiometry and temperature on cell sizes

Since both indicators of nutrient limitation (dissolved nutrients, cellular stoichiometry) were not only influenced by the nutrient treatment but also by temperature, it is not possible to derive direct, nutrient-independent temperature effects from the direct comparison of experimental

treatments. Therefore, C:N and C:P ratios was used as indicator for nutrient limitation (Goldman et al., 1979). GLM analyses using temperature as categorical independent variable and C:N and C:P ratios as continuous variable (Table 3). This analysis showed significant a significant effect of the particulate matter C:N ratio on cell sizes of all species and community mean cell size while the effect of C:P ratios was not significant. The temperature effect was significant only for 4 species and not significant for community mean cell size (Table 3). The full model was significant for all species and community mean cell size. In order to exclude the cases of P-limitation from the analysis of C:N-effects and the cases of N-limitation from the analysis of C:P-effects; the GLM analysis was also performed for the combination temperature with C:N ratio without the P-limited treatments and the combination temperature with C:P ratio without the N-limited treatments (Tables 4 & 5). In these separate analyses, particulate matter stoichiometry had significant effects in all cases while the effect of temperature was non-significant in most cases of N-limitation (Table 4). There were more cases of significant temperature effects (6 of 7 spp.; Table 5) in the P- than N-limited cultures.

Table 3: General Linear Model-GLM (Sigma-restricted, Type VI unique) of species size (Log^{10} V μm^3) as independent factor on temperature (Temp-°C) categorical factor, Log^{10} C:N ratio and Log^{10} C:P as continuous factors by including both N-and P limitation, P-values and R^2

Species	P-C:N ratio	P-C:P ratio	Temp	R^2	P-model
Scrippsiella trochoidea	0.001	0.934	0.0235	0.67	<0.0001
Chaetoceros curvisetus	0.0006	0.661	0.052	0.63	<0.0001
Thalassionema nitzschioides	0.0044	0.774	0.0362	0.62	<0.0001
Thalassiosira sp	0.0001	0.8103	0.0486	0.70	<0.0001
Teleaulax amphioxeia	0.0001	0.623	0.056	0.73	<0.0001
Chaetoceros similis	0.0009	0.6931	0.118	0.67	<0.0001
Skeletonema costatum	<0.0001	0.771	0.189	0.74	<0.0001
Community mean cell size	<0.0001	0.960	0.323	0.72	<0.0001

Table 4: General Linear Model (Sigma-restricted, Type VI unique) of species size (Log^{10} V μm^3) on temperature [°C] as categorical factor and log ^{10} C:N ratio [mol:mol] as continuous factor after excluding P-limitation treatments, P-values and R^2

Species	P-C:N ratio	P-Temperature	R^2	P model
Scrippsiella trochoidea	<0.0001	0.038	0.70	<0.0001
Chaetoceros curvisetus	<0.0001	0.116	0.63	<0.0001
Thalassionema nitzschioides	<0.0001	0.132	0.61	<0.0001
Thalassiosira sp	<0.0001	0.128	0.70	<0.0001
Teleaulax amphioxeia	<0.0001	0.173	0.74	<0.0001
Chaetoceros similis	<0.0001	0.323	0.67	<0.0001
Skeletonema costatum	<0.0001	0.323	0.68	<0.0001
Community mean cell size	<0.0001	0.398	0.73	<0.0001

Table 5: General Linear Model (Sigma-restricted, Type VI unique) of species cell sizes (Log^{10} V-μm^3) on temperature [°C] as categorical factor and log ^{10} C:P [mol:mol] as continuous factor after excluding N-limitation treatments, P-values and R^2.

Species	P-C:P ratio	P-Temperature	R^2	P model
Scrippsiella trochoidea	0.0001	0.031	0.68	<0.0001
Chaetoceros curvisetus	<0.0001	0.010	0.73	<0.0001
Thalassionema nitzschioides	0.0004	0.0263	0.69	<0.0001
Thalassiosira sp	<0.0001	0.0212	0.71	<0.001
Teleaulax amphioxeia	<0.0001	0.0127	0.72	<0.0001
Chaetoceros similis	<0.0001	0.0219	0.73	<0.0001
Skeletonema costatum	<0.0001	0.2119	0.73	<0.0001
Community mean cell size	<0.0001	0.085	0.78	<0.0001

I.4 DISCUSSION

While field (Hilligsøe *et al.*, 2011, Marañón *et al.*, 2001) and experimental (Morán *et al.*, 2010, Sommer & Lengfellner, 2008, Yvon-durocher *et al.*, 2011) evidence for a phytoplankton size decline at increasing temperatures is widespread, there was still a lack of clarity how much of the temperature influence is mediated via hydrographic factors (enhanced stratification with less nutrient supply and higher sedimentary losses) or biotic factors (shifts in biotic nutrient cycling and grazing). In two preceding experimental studies, it demonstrated a strong role of biotic shifts. A factorial combination of grazing and warming in chapter 1 (Peter & Sommer, 2012) showed, that the cell size decline with warming was strongest under copepod grazing, intermediate under microzooplankton grazing and minimal under nanozooplankton grazing.This supported the tentative explanation of experimental studies on the phytoplankton spring bloom (Sommer & Lengfellner, 2008, Sommer & Lewandowska, 2011) at stronger copepods grazing pressure under elevated temperature. This agrees with the known grazing selectivity of copepods which preferentially remove the larger phytoplankton while releasing the smaller ones from protist grazing (Sommer, 1986). However, the experiment also demonstrated a grazing-independent role of temperature, because community mean cell sizes and cell sizes of the majority of species decrease even under nanozooplankton grazing although it is highly improbable that heterotrophic nanoflagellates would selectively remove the larger algae.

Moreover, (chapter 2) Peter & Sommer (2013) analysed how nutrient limitation and temperature would interact to determine phytoplankton cell size. Nitrogen was used as limiting nutrient and the strength of nutrient limitation was manipulated by semi-continuous dilution. Similarly, the present study showed that nutrient limitation was not only influenced by the dilution rate but temperature also affected the limitation. According to (Droop, 1973), a direct nutrient-independent temperature effect could only be assessed by taking the biomass C:N ratio i.e. the inverse of the biomass specific nitrogen cell quota; as proxy for the strength of nutrient limitation. The subsequent analysis showed a dominant effect of nitrogen limitation. However, a direct temperature effects was only detected in some of the species and for community mean cell size.

While the study of Peter & Sommer (2013) was performed only with N as a limiting element; there was still an open question, whether the same effect would show up with other limiting nutrients. Therefore, in the current research an additional factor on the dimension quality of nutrient limitation (balanced, supply of N- and P-limitation) was necessary.

During the present study, biomass stoichiometry (C:N ratios for N-limitation, C:P-ratios for P-limitation) was used. The rationale for this choice was provided by (Goldman *et al.*, 1979) who

demonstrated a linear relationship between the "relative growth rate" (μ/μ_{max}) and the C: limiting nutrient ratio in biomass which was relatively uniform between species. This operation permitted to disentangle direct temperature effects on cell size from effects mediated via nutrient limitation (Tables 4 & 5). The GLM show highly significant effects of C:N and C:P ratios on the cell size of all species and on community mean cell size. In the nitrogen-limited cases the nutrient effect was so dominant that a direct temperature effect could only be seen in one species (*Scrippsiella trochoidea)* but vanished when applying a Bonferroni-correction to the threshold of significance. In the case of P-limitation, a temperature effects were seen in 6 of 7 species, but not in community mean cell size. N-limitation showed stronger effect on cell size than P-limitation, this could be associated with a reduction in light absorption under nitrogen limitation (Stramski *et al.*, 2002).

In conclusion, the effects of nitrogen limitation on phytoplankton cell size are stronger than the effects of P-limitation, and nutrient effects clearly dominate over direct temperature effects, which sometimes are detectable or undetectable.

Extrapolating to Global Change issue, I could predict a shift towards smaller cell sizes of phytoplankton. This prediction is particularly robust, because the hydrographic effects of warming and warming effects mediated via biotic interaction operate in the same direction. The consequences for ecosystem services are twofold: (1) Not only will intensified vertical stratification reduce nutrient supply and thereby low ocean productivity, but also smaller cell size will reduce the efficiency of energy transfer to fish, because copepods inefficient feeder of small phytoplankton and more of primary production will be channelled through the microbial loop. Thereby, the trophic level of fish will increase which inevitably decrease the ratio of fish production: primary production (Sommer *et al.*, 2002). (2) The shift towards the microbial food chain will lead to increase respiration of organic carbon and reduce production of sinking organic matter (Wohlers *et al.*, 2009). Large diatoms therefore are important for carbon export to the deep water because of high sinking velocity, their tendency to form even faster sinking aggregates after senescence and because they strongly contribute to the C-content of fast sinking faecal pellets when consumed by copepods (Dugdale *et al.*, 2002, Smayda, 1971, Smetacek, 1999). Thus, the efficiency of the biological carbon pump will be impaired by the shift towards smaller algae

General Conclusion

This study has demonstrated the temperature effect on phytoplankton cell sizes are mediated via nutrient and grazing to a great extent. However, the grazing effect cannot be a universal dominant factor driving temperature-size relationships. The grazing effect is context dependent to large extent phytoplankton groups of different size that are grazed by different groups of grazers. Therefore, the selective predation grazing effect of warming depends on the selectivity of the dominant grazers. Grazing effects should be stronger if predators prefer large phytoplankton such as copepods (Sommer & Lewandowska, 2010, Sommer & Sommer, 2006). In contrast, if the feeders/predators for small algae are dominant, the expected grazing effect is opposed to the nutrient effect and the direct, physiological temperature effect.

Generally, phytoplankton size structure determines the trophic organization of pelagic ecosystem and thus the efficiency with which organic matter produced by photosynthesis is channelled towards trophic level (Falkowski & Oliver, 2007, Finkel *et al.*, 2010). Through a series of experiments, the study demonstrated shift towards small phytoplankton with increasing temperature under laboratory conditions without stratification effects. In situ, climate warming is expected to inhibit mixing and to reduce the upward nutrient supply and even further increase the advantage for smaller cell sizes. The shift towards smaller phytoplankton will lead to low sedimentary losses and intense recycling of matter through the microbial food web resulting in little potential for carbon export. Furthermore, since copepods feed on medium to moderately large phytoplankton, the shift towards small algae will impair the transfer efficiency from primary production to copepods and eventually affect fish production. In conclusion, the shift towards small phytoplankton as result of sea surface warming will impair the efficiency of the biological pump.

Future research

Based on the findings of the current study, several questions cannot be answered. Therefore, I suggest 4 future researches which may improve the understanding of phytoplankton size shift in response to global warming:

I. The current study, investigate the effect of temperature on the primary producers (phytoplankton) mediated via grazing. However, for better understand the whole context effect on prey and predators relationship to global warming; future study is important on how predators (copepods, microzooplankton and nanozooplankton) respond to the elevated temperature.

II. As described in this study, the interspecific difference in responses of phytoplankton species to temperature and nutrients stress are both explained by size and taxonomic

composition. However, only two major taxonomic groups were involved. In future research, a wide range of taxonomic groups (probably culture phytoplankton species) should be included for better prediction of the consequences of climate change on phytoplankton taxonomic groups.

III. The study examined the effect of elevated temperature on marine phytoplankton. However, for better understanding the whole context of effect of climate warming to aquatic ecosystem, a further study is necessary to establish the extent of this effect in freshwater because of large differences in phytoplankton community structure between marine and freshwater phytoplankton (Stibor *et al.*, 2004).

IV. The study determined the effect of temperature on phytoplankton through series of mesocosm experiments. However, indoor experiments are sometimes criticized due to lack of their artificial nature and limitation in space and time. Further research is needed to determine the relationship between field information and indoor experiment. This can link environmental changes with ecological pattern for better prediction of phytoplankton changes in response to climate warming.

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Contribution of Authors

The work presented in this thesis was conducted within the FB3-Experimental ecology- food webs entitled “Effect of climate warming on phytoplankton size structure and species composition: an experimental approach” Coordinated by Prof. Dr. Ulrich Sommer.

Chapter 1: Phytoplankton cell size: intra- and interspecific effects of warming and grazing

Published in PLoS ONE: Peter KH & Sommer U (2012) PLoS ONE 7(11): e49632

Conceived and designed the experiments: KHP and US. Performed experiment: KHP. Analysed data: KHP with assistance of US. Writing: KHP with assistance of US

Chapter 2: Phytoplankton cell size Reduction in response to warming mediated by nutrient limitation.

Published in PLoS ONE: Peter KH & Sommer U (2013) PLoS ONE 8:e71528

Conceived and designed the experiment: KHP and US. Performed experiment: KHP. Analysed data: KHP with assistance of US. Writing: KHP with assistance of US

Chapter 3: Interactive effect of warming and nutrient limitation on phytoplankton composition.

Submitted to journal of Aquatic Botany

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Chapter 4: Interactive effect of warming, nitrogen and phosphorus limitation on phytoplankton cell size

Accepted for publication in journal of Ecology and Evolution

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Acknowledgements

First and foremost, I owe my deepest gratitude to my supervisor, Prof. Dr. Ulrich Sommer for his guidance and support throughout the program. This thesis would not have been possible without his valuable ideas and encouragement that made me to understand the subject of my research and remained motivated.

Thank you to Prof. Dr. Stefanie Ismar for being my second reviewer.

The financial support provided by DAAD (German Academic Exchange Service) and Tanzania Ministry of Education training and Vocation is greatly acknowledged.

Special thanks to University of Dodoma (UDOM) for granting a study leave.

Thank to Dr. Birte Matthiessen for being my second referee to DAAD. I thank technicians: Thomas Hansen, Cordula Meyer, and Bente Gardner for their technical support.

Special thanks to Dr. Carmen Arndt for the German version of the summary of this thesis

I thank Jessica Garzke for good cooperation during mesocosm experiment and Dr. Juan-Carlos Melinero for statistical technics assistance

My cordial thanks also go to the whole EÖ-N working group for the great working atmosphere and Gabriele Barth for her coordination on administrative issues.

Last but not least, I am deeply indebted to my husband Dr. George Bandoma Swella for his love, encouragement and more importantly for his patience during the entire period of my studies. I also thank my mother and relatives for their encouragement. I also thank all who in one way or another helped me academically or socially to make all this possible.

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October 1998-September 2001: BSc. Agronomy majoring in Plant &Soil & Management: Sokoine University of Agriculture (SUA) -Morogoro-Tanzania.

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Publications

Peter KH, Sommer U (2013) Phytoplankton Cell Size Reduction in Response to Warming Mediated by Nutrient Limitation. *PloS one,* 8, e71528

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Peter KH, Swella GB, Mushobozy DM (2009) Effect of plant populations on the incidence of bean stem maggot (Ophiomyia spp.) in common bean intercropped with maize. Plant Protection Science-UZEI (Czech Republic).

Scientific Conferences and trainings

16-19, October, 2012: International Symposium "Recent achievement and future directions in Aquatic mesocosm Research' Oral presentation with a title: Effect of temperature and grazing on phytoplankton's species.an experimental approach-Heraklion, Crete, Greece.

01-12 March 2010: Training on Climate change Adaptation in Natural resources Management organised by Wageningen University and Research Centre and Addis Ababa University, Horn of Africa Regional Environment Center and Network

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Declaration

I here declare that this work is my own work apart from supervisor's guidance and acknowledged assistances. This dissertation has not been submitted for the award of doctoral degree in other examining body and was prepared according to the Rules of Good Scientific Practice of the German Research Foundation.

Kiel,

Kalista Higini Peter