**Authors' response to referees: comments of the referees are in black, and responses are in blue.**

## Responses to comments from Reviewer 1

### General Comment 1

The authors of this paper investigate experimentally the effects of changes in multiple environmental parameters on the production of phytoplanktonic biomarkers including, PUFAs, sterols, and alkenones in 3 individual species of diatom, cryptophyte, and haptyophyte. These parameters included different temperatures, molar ratios of N:P, and pCO2 concentrations.

This manuscript provides valuable insights for the impacts of lipid remodeling on food web dynamics and biogeochemical cycling, particularly in sterol production. The discussion however is lacking in the specific potential effects of increased FA production and alkenoates on modern biogeochemical dynamics and could be expanded on.

Response:

Fatty acid (FA) composition and contents in phytoplankton have shown significant correlations with zooplankton production and trophic carbon transfer from phytoplankton to zooplankton (Jónasdóttir et al., 2009; Amin et al., 2011; Rossoll et al., 2012; Arndt and Sommer, 2014; Bi and Sommer, 2020). These impacts of FA production remodeling have been discussed in detail in our previous studies (Bi et al., 2014; Bi et al., 2018; Bi and Sommer, 2020). As suggested, we now add some discussion on the potential effects of FA production changes in the revised manuscript. (See Pages 14-15, Lines 439-448)

We think the reviewer referred to the potential effects of "alkenones", instead of "alkenoates" which were not measured in our study. *E. huxleyi* is mainly grazed by heterotrophic protists in the context of pelagic food webs (Braeckman et al. 2018; Nejstgaard et al. 1997). Moreover, previous studies on copepod feeding clearly showed excretion of alkenones and indicated faecal pellet transport of these

compound to sediments. In the present study, we observed non-significant changes in organic carbon-normalized contents of total alkenones in *E. huxleyi* under variable temperature, N:P ratios and $pCO_2$, demonstrating quantitative applicability of alkenones as proxies for *E. huxleyi* biomass in biogeochemical cycles. We expand the discussion on the potential effects of alkenones in the revised text. (See Page 15, Lines 456-462)

**General Comment 2**

The authors have a sound experimental design to investigate the effects of multiple environmental drivers however much of the discussion focuses on the impacts of individual variables on the production of each respective biomarker. Greater exploration of the potentially compounding effects of multiple environmental drivers may increase the impact of the manuscript. The interpretations from the PCA (Fig. 3) are hardly mentioned in the discussion and would be worth expanding on as well.

Response:

We accept the reviewer's comment on expanding the interactive effects of multiple environmental drivers. Because non-significant interactive effects were observed on the contents of sterols and alkenones in this study, we now add some discussion on the potential interactions between temperature and N:P supply ratios on carbon-normalized contents of brassicasterol/epi-brassicasterol in *E. huxleyi*. (See Pages 11-12, Lines 345-352)

We now interpret the PCA results in more detail and highlight specific physiological functions and biosynthetic pathways of three lipid biomarkers. For example, the PCA results were interpreted that the responses of TFA carbon-normalized contents to N:P supply ratio were opposite to that of brassicasterol/epi-brassicasterol and alkenones (Fig. 3; Table S5), e.g., strong positive correlations of TFAs with N:P ratios in *P. tricornutum* and *Rhodomonas* sp., but negative ones of brassicasterol/epi-brassicasterol in *E. huxleyi*. (See Pages 9-10, Lines 278-288)

**General Comment 3**

Greater connection to the interpretation of these the sterols in sediments could be provided by the authors as well. How might these results specifically influence the interpretation of biomarker analysis in the reconstruction of phytoplankton productivity and community composition?

Response:

As suggested, we now provide some interpretations of sterol changes in sediments. Briefly, our study revealed an overall 20% decrease in carbon-normalized contents of brassicasterol/epi-brassicasterol in *Rhodomonas* sp. and *E. huxleyi* in ocean-related global change scenarios (warming, N and P deficiency and enhanced $pCO_2$), but not in the diatom *P. tricornutum*. Because smaller ranges are expected in temperature, N:P supply ratio and $pCO_2$ in individual locations over time, our results provide additional support for the applicability of using sterols in paleoproductivity reconstruction, especially in diatom-dominated areas. (See Pages 15-16, Lines 474-478)

**Specific Comment 1**

How comparable are carbon or cell normalized concentrations when sterols are often interpreted as biomarkers for productivity and normalized to dry sediment weight? Would this affect paleo interpretations of community structure or productivity?

Response:

In paleoceanography, lipid biomarker data have been reported on the basis of both dry sediment weight and total organic carbon (TOC) content (Zimmerman and Canuel, 2002; Zhao et al., 2006; Xing et al., 2016). Dry sediments contain variable organic and inorganic components, thus biomarker contents normalized to TOC can partially eliminate the influence of sedimentation rates, and between-site or temporal changes in the amount of organic carbon deposition or preservation (Zimmerman and Canuel, 2002). The application of sediment biomarker contents for paleoproductivity reconstruction is based on two key assumptions: (1) relatively constant ratios of biomarker per cell or per particulate organic carbon (POC) in a specific phytoplankton

group, and (2) non-significant changes in biomarker/POC ratios during post production and deposition degradation.

In laboratory culture studies, carbon-normalized and cell-normalized lipid contents provide broadly similar responses to environmental parameter changes in most cases, but there are exceptions (Ding et al., 2019; this study). However, cell-normalization is difficult for sediment reconstruction, as phytoplankton cell counting is often not quantitative (Piepho et al., 2010; Ahmed and Schenk, 2017). In the present study, we have discussed the implications of our findings based on carbon-normalized contents of lipid biomarkers. Because the variations of temperature, N:P ratios and $pCO_2$ in certain locations over time are smaller than that in this study, carbon-normalized contents of lipid biomarkers can to some extent reflect paleoproductivity changes.

We accept the reviewer's comment and expand our discussion based on the above interpretation. (See Pages 15-16, Lines 463-478)

**Specific Comment 2**

In Fig.3, is pCO2 contributing to the distribution of fatty acids in P. triconutum?

Response:

PCA results show that both Dim1 and Dim 2 loadings for $pCO_2$ were very low (0.185 and 0.151, respectively). Thus, the effects of $pCO_2$ on lipid biomarkers were weaker than temperature and N:P supply ratios. Generally, $pCO_2$ showed a stronger correlation with TFAs in *E. huxleyi* along Dim2, while the contribution of $pCO_2$ to TFAs in *P. tricornutum* was weak.

We now add more interpretation on the PCA results to clarify the differential roles of temperature, N:P supply ratios and $pCO_2$ on lipid biomarkers in the Results (See Page 9, Lines 270-273) and Discussion (See Pages 9-10, Lines 278-288).

**Specific Comment 3**

How does the DIC and pH compare amongst cultures? Are they consistent across the temperature and nutrient trials?

Response:

pH values were monitored daily during the experiments. DIC and pH did not differ substantially between different temperature and N:P ratio treatments in each experimental run in our study. We now clarify this information in the Methods (See Page 6, Lines 170-171), and add pH values in Table S1 in the revised manuscript.

**Specific Comment 4**

The prediction of an overall decrease in carbon-normalized contents of sterols and PUFA is based on future open ocean or coastal conditions? The authors suggest that the open ocean may experience more thermal stratification and thus depleted nutrients while the coastal ocean may receive more external inputs of nutrients.

Response:

Surface ocean inorganic N and P concentrations are highly depleted throughout much of the low-latitude oceans (Moore et al., 2013). Nutrient availability in future will be altered by increasing external nutrient inputs, changes in surface ocean chemistry driven by anthropogenic increases in atmospheric $CO_2$, and changes in ocean circulation (Moore et al., 2013). Therefore, inorganic N:P ratios show a strong spatial variation in the oceans, e.g., a low ratio of 6:1 mol $mol^{-1}$ in the center of the South Pacific Gyre *versus* a global scenario of an increase driven by the high N:P atmospheric deposition of ~ 370:1 mol $mol^{-1}$ (Bonnet et al., 2008; Peñuelas et al., 2012).

In our study, N:P supply ratios cover large-scale spatial patterns of nutrient status, and thus our prediction of lipid biomarker production changes is based on both future open ocean and coastal conditions. This information is now clarified in the revised manuscript. (See Page 5, Lines 133-141)

**Technical Corrections 1**

Missing clear information on the preparation of the fatty acids and their measurement. Please provide details of methodology including quantification. Are only polyunsaturated fatty acids identified and measured or total fatty acids? Please clarify or include citation to previous work in methods section.

Response:

FA samples (15 – 50 ml) were taken on pre-combusted and pre-washed (5-10% HCl) GF/F filters (Whatman GmbH, Dassel, Germany). After filtration, samples were stored at -80℃. FAs were measured as FA methyl esters (FAMEs) using a gas chromatograph (Trace GC-Ultra; Thermo Fisher Scientific, Schwerte, Germany). Analytical procedure of FAs was modified after Christie (1989). Briefly, FAs were extracted with a solvent mixture of chloroform/dichloromethane/methanol (1:1:1 volume ratios). The FAME 19:0 was added as an internal standard and 21:0 as a esterification control. The extracted FAMEs were dissolved in 100 μL *n*-hexane for analysis. Sample aliquots (1 μL) were injected onto the GC with hydrogen as the carrier gas. Individual FAs were identified with reference to the standards (Supelco 37 component FAME mixture and Supelco Menhaden fish oil), and the peaks were integrated using Chromcard software (Thermo Fisher Scientific, Schwerte, Germany), which included saturated, monounsaturated and polyunsaturated FAs (Bi et al., 2017, 2018).

The methods of FA analysis are clarified in the method section. (See Page 6, Lines 172-184)

**Technical Corrections 2**

What N:P was used for the pCO2 experiments, 24:1? Please clarify.

Response:

The experiments were designed as shown in the following figure (Fig. S1). It can clearly show that three N:P supply ratios and three temperatures were set for each $pCO_2$ level. We now add this figure as a supplementary material (Fig. S1) in the revised manuscript. (See Page 4, Line 127)

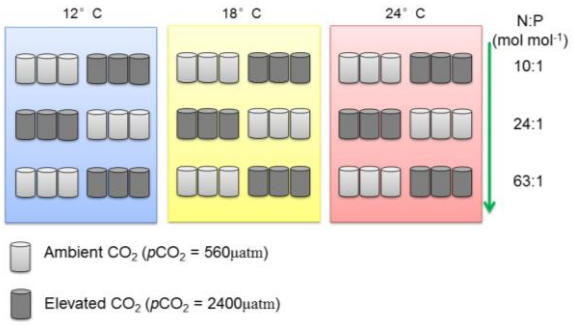


Fig. S1. Experimental setup. The three phytoplankton species were grown semi-continuously under a full-factorial combination of three temperatures (12, 18 and 24 °C), three N:P supply ratios (molar ratios 10:1, 24:1 and 63:1) and two $pCO_2$ levels (560 and 2400 µatm). Triplicates were set for each treatment.

**Technical Corrections 3**

Consider switching the order of sections 4.2 and 4.3.

Response:

In the manuscript, we consistently present the findings of sterols followed by alkenones in all sections including abstract, introduction, methods, results, discussion and conclusion.

We thank the reviewer for this comment. While the order of sections 4.2 and 4.3 could be switched, we prefer to keep the consistent order in all sections. We hope the reviewer will agree with this.

# Responses to comments from Reviewer 2

## General comment

The manuscript by Bi et al. describes the response of three species of phytoplankton to a combination of three parameters relevant for the adaptation of algae to future climate change. The highlights of the manuscript are the multifactorial nature of the experiments and their meticulous execution, including a full account of carbonate system parameters. That being said, the manuscript is a little lean on the methods section such as details of the cultivation. I have only minor comments on the manuscript and believe that all conclusions are sound, but I have the feeling that major opportunities to derive novel and far-reaching insights were missed. For instance, the discussion could be more specific on the geological implications of changing alkenone ratios and sterol compositions and abundance. Similarly, the impacts on the food web (past and present) could be discussed in more detail. I was also expecting an analysis of the caloric content or of energy storage molecules (e.g. triacylglycerols), which could be a great addition to the study.

Response:

We thank the reviewer for the positive comments on our paper and suggestions for improvements.

1) Previous studies showed that $C_{37}/C_{38}$ alkenone ratios not only varied with temperature and physiological stages (such as growth stage), but also differed between alkenone-producing species (Conte et al., 1998; Pan and Sun, 2011; Nakamura et al., 2014). We observed that $C_{37}/C_{38}$ alkenone ratios varied significantly with the changes in temperature, N:P supply ratios and $pCO_2$, indicating that besides temperature, other environmental factors such as nutrient availability and $pCO_2$ can also significantly influence $C_{37}/C_{38}$ alkenone ratios. In contrast, $C_{38}$ Et/Me ratios in our study responded significantly only to temperature changes. Our results denote the importance to consider the effects of multiple environmental factors on $C_{37}/C_{38}$ alkenone ratios, and further underline the importance of temperature in regulating $C_{38}$ Et/Me ratios in geological application of alkenone ratios.

Considering the implications of sterol contents, we now add discussion based on the differential responses of diatoms with other two species, i.e., non-significant changes in carbon-normalized contents of brassicasterol/epi-brassicasterol in the

diatom *P. tricornutum*, but significant decreases in brassicasterol/epi-brassicasterol in *Rhodomonas* sp. and *E. huxleyi* under ocean-related global change scenarios (warming, N and P deficiency and enhanced $pCO_2$). Because smaller ranges are expected in temperature, N:P supply ratio and $pCO_2$ in individual locations over time, our results imply applicability of sterols in paleoproductivity reconstruction, especially in diatom-dominated areas.

Based on these important applications of alkenone ratios and sterols, we expand the discussion on geological implications of these lipid biomarkers in the revised manuscript. (See Pages 15-16, Lines 463-483)

2) We accept the review's comment and add discussion on the impacts of changing lipid biomarker contents, particularly FA contents, on food webs. For example, an increase in PUFA contents in the diatoms in cold periods may have positive effects on zooplankton production; in contrast, a decrease in PUFA contents in *E. huxleyi* at enhanced $pCO_2$ may reduce trophic transfer efficiency at phytoplankton-zooplankton interface. (See Page 15, Lines 444-448)

3) We agree with the reviewer's suggestion that analysis of caloric content or of energy storage molecules would be a great addition for our study, but such analysis is beyond the scope of the present work and the methods. We now revise the conclusion and suggest future studies to consider the influence of these and other environmental changes on other energy storage molecules such as triacylglycerols. (See Page 16, Lines 493-494)

**Line comment 1:**

Line 65-70: It would be useful to describe the directionalities of these changes. Were these changes significant so that they prompted the present study?

Response:

Yes, some of previous studies presented significant changes in phytoplankton sterol contents. We now describe the directionalities of these changes. (See Page 3, Lines 67-73)

**Line comment 2:**

Line 102-139: Were the culture axenic? If not, how was bacterial growth controlled?

Response:

The algal cultures were non-axenic. We did not control bacterial growth. Because the biomass of bacteria was very low, the bacterial influence on the chemical composition of phytoplankton was negligible. This information is now clarified on Page 4, Lines 113-114.

**Line comment 3:**

Line 103: Could more information be supplied for the identity of the Rhodomonas strain such as a strain number and/or statements on deposition in a culture collection?

Response:

This strain was isolated from the Kiel Bight and identified by the working group at GEOMAR. But it has not been deposited in a culture collection, thus no strain number. However, it is maintained and still available from GEOMAR for laboratory culture. More information is supplied for the *Rhodomonas* strain. (See Page 4, Lines 108-109)

**Line comment 4:**

Line 117-132: It is not clear how target pCO2 was maintained in the semi-continuous cultures.

Response:

As suggested, detail information on the maintenance of target $pCO_2$ in semi-continuous cultures is added. Briefly, renewal of the cultures was carried out at the same hour every day using fresh filtered seawater pre-acclimated to target $pCO_2$, and $CO_2$-enriched water. (See Page 5, Lines 152-153)

**Line comment 5:**

Line 134: From the description it is not quite clear why these cultures are considered semi-continuous. Was a large volume of sample withdrawn directly from the reactor? How much volume was taken and what was the sampling interval? After what interval (turnovers?) was the culture considered to be back in steady state?

Response:

The renewal volumes were about 10% of the total culture volume (100 – 200 mL out of 920 mL). Renewal interval was 1 d. Steady state was reached at ca. 10 d with slight differences between the individual cultures.

More details are added to clarify the setup of semi-continuous cultures. (See Page 5, Lines 150-152)

**Line comment 6:**

Line 141: How were cells counted/fixed

Response:

Algal cells were counted using an improved Neubauer hemacytometer under a microscope. We clarify this information in the revised manuscript. (See Page 5, Lines 159-160)

**Line comment 7:**

Line 153-154: What was the volume of the samples?

Response:

The filtration volume of sterol and alkenone samples was 100 – 200 mL. Only 10% of the extractions were used for GC quantification, with the rest stored for later use. This information is now clarified on Page 6, Lines 186-187.

**Line comment 8:**

Line 278: POC is mentioned here for the first time but it was not described in the methods how it was determined.

Response:

POC was measured using an elemental analyzer. We add this information in the methods. (See Page 6, Lines 172-176)

**Line comment 9:**

Line 310-313: Would this comparison change if the maximum growth rates of the batch cultures would be used for comparison? Can a stationary batch culture be reasonably compared to a continuous culture in terms of energy availability for remodeling?

Response:

Because growth rate strongly affects sterol contents according to previous studies, the comparison on lines 310-313 would change if the maximum growth rates of batch cultures were used for comparison. In a continuous culture, phytoplankton has a constant growth rate under a certain dilution rate, while the growth rate at the stationary phase of batch culture is zero. Thus, energy availability for sterol remodeling differ between the two culture systems. We now revise the paper to address this suggestion. (See Page 12, Lines 367-371)

**Line comment 10:**

Line 410-412: Could this be expanded on by considering expression of lipid biosynthesis genes and/or energy storage?

Response:

As suggested, we now expand the discussion here by considering expression of lipid biosynthesis genes and its links to transcriptomic and metabolomic responses. (See Page 16, Lines 491-492)

**Line comment 11:**

Line 417: Unclear if the “strong increase” in PUFAs is significant.

Response:

The increase in PUFA contents were significant ($p$ = 0.003). We clarify this response in the revised manuscript. (See Page 16, Line 498)

**Line comment 12:**

Line 810: Spell out Dims.

Response:

The word “Dims” refers to “dimensions”. We clarify this in the legend of Fig. 3. (See Page 28, Line 915), and the caption of Table S5.

**Line comment 13:**

Figures 1-3: It would be great to add information on error bars (standard error? triplicates?) to all figure and table captions.

Response:

Error bars in figures refer to standard errors, and triplicates were set for each treatment. As suggested, we make the information clearly in all figure and table captions (Figs. 1 and 2; Table S1, S3, S4 and Fig. S1).

**The list of some minor changes to the original manuscript**

All changes to the original manuscript within the document are highlighted in blue.

1. Table 2:

Mean percent changes in per-cell contents of POC in *P. tricornutum* are corrected (from +17% to +23% under N deficiency, and from -6% to -0.3% under P deficiency).

2. Table S3:

The SE value is corrected (from 0.002 to 0.003) for per-cell contents of brassicasterol/epi-brassicasterol in *P. tricornutum* at 12 ℃, high $pCO_2$ and N:P = 24:1.

3. Table S4:

The mean value is corrected (from 0.30 to 0.29) for per-cell contents of $C_{37:3}$ Me in *E. huxleyi* at 18 ℃, low $pCO_2$ and N:P = 10:1. The SE value is corrected (from 0.003 to 0.004) for $U_{37}^{K'}$ in *E. huxleyi* at 12 ℃, high $pCO_2$ and N:P = 24:1.

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# Ocean-related global change alters lipid biomarker production in common marine phytoplankton

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**Abstract.** Global change concurrently alters multiple environmental factors, with uncertain consequences for marine ecosystems. Lipids, in their function as trophic markers in food webs and organic matter source indicators in water column and sediments, provide a tool for reconstructing the complexity of global change effects. It remains unclear how ongoing changes in multiple environmental drivers affect the production of key lipid biomarkers in marine phytoplankton. Here, we tested the responses of sterols, alkenones and fatty acids (FAs) in the diatom *Phaeodactylum tricornutum*, the cryptophyte *Rhodomonas* sp. and the haptophyte *Emiliania huxleyi* under a full-factorial combination of three temperatures (12, 18 and 24°C), three N:P supply ratios (molar ratios 10:1, 24:1 and 63:1) and two $pCO_2$ levels (560 and 2400 µatm) in semi-continuous culturing experiments. Overall, N and P deficiency had a stronger effect on per-cell contents of sterols, alkenones and FAs than warming and enhanced $pCO_2$. Specifically, P deficiency caused an overall increase in biomarker production in most cases, while N deficiency, warming and high $pCO_2$ caused non-systematic changes. Under future ocean scenarios, we predict an overall decrease in carbon-normalized contents of sterols and polyunsaturated fatty acids (PUFAs) in *E. huxleyi* and *P. tricornutum*, and a decrease in sterols but an increase in PUFAs in *Rhodomonas* sp. Variable contents of lipid biomarkers indicate a diverse carbon allocation between marine phytoplankton species in response to changing environments. Thus, it is necessary to consider the changes in key lipids and their consequences for food web dynamics and biogeochemical cycles, when predicting the influence of global change on marine ecosystems.

## 1 Introduction

Ocean phytoplankton has profoundly responded to and driven natural climatic variability throughout Earth's history (Riding, 1992; Falkowski and Oliver, 2007; Falkowski, 2015). In the contemporary ocean, human-induced physical and chemical modifications are complex and concurrent, including warming, acidification, deoxygenation, and changes in nutrient availability (Doney et al., 2012; Moore et al., 2013; DeVries et al., 2017). The ocean-related global change fundamentally affects marine ecosystems (Hoegh-Guldberg and Bruno, 2010). These include especially global

phytoplankton biomass decreases (Boyce et al., 2010; Moore et al., 2018; Lotze et al., 2019) and plankton communities changes (Richardson and Schoeman, 2004; Jonkers et al., 2019), which consequently alters food-web dynamics (Kortsch et al., 2015; du Pontavice et al., 2020) and biogeochemical cycles (Hofmann and Schellnhuber, 2009; Gruber, 2011; Doney et al., 2012). A major challenge is the lack of a better understanding of the complexity of biological impacts of global change, which has hindered the prediction of potential feedbacks between marine ecosystems and projected environmental changes. Some of phytoplankton-produced biomolecules (biomarkers), functioning as indicators of nutritional food quality (Müller-Navarra, 2008) and tracers of organic matter sources (Volkman et al., 1998), have provided crucial insight into the trajectory of ecological responses to changing environment along food webs in the present-day ocean (Ruess and Müller-Navarra, 2019), and over geological time (Brocks et al., 2017).

Lipids are amongst the most important and widely used biomarkers, because they have far-reaching biochemical and physiological roles in cells and are sensitive to environmental changes (Arts et al., 2009), but also because of their dominance in the geological record as fossil molecules to reveal life's signatures on Earth (Falkowski and Freeman, 2014). There are also growing applications of lipids as proxies for global climate and marine ecosystem change. Of all biomarkers, fatty acids (FAs), the basic constituents of most algal lipids, have received the most intense attention. Polyunsaturated fatty acids (PUFAs) are essential for many animals and have been applied as nutritional components to study trophic interactions (Brett and Müller-Navarra, 1997; Müller-Navarra et al., 2000; Dalsgaard et al., 2003; Kelly and Scheibling, 2012; Ruess and Müller-Navarra, 2019). The impact of environmental changes on phytoplankton FAs has been well studied, mostly with a focus on the effects of temperature and nutrient changes (reviewed by Guschina and Harwood, 2009; Galloway and Winder, 2015; Hixson and Arts, 2016), while the interplay between different environmental drivers has been recently tested (Bermúdez et al., 2015; Bi et al., 2017, 2018). However, determining how phytoplankton lipids respond to global change still faces substantial challenges, partly because data on other important lipid classes such as sterols and alkenones are scarce. Understanding the impact of environmental change on these lipid classes is critical to achieve a better application of lipid biomarkers to contemporary issues and to the past record of marine ecosystems.

Sterols are tetracyclic triterpenoids present in all eukaryotes (Volkman, 2003, 2016). Sterols function primarily as structural components of plasma membranes, but also play key roles in cellular defense against toxic compounds and signal transduction, and serve as precursors to several important compounds (e.g., steroid hormones in animals) involved in cellular and developmental processes (Hartmann, 1998; Guschina and Harwood, 2009; Fabris et al., 2012). In ecology, sterols have been used as indicators of dietary nutritional quality, because some invertebrates are incapable of synthesizing sterols *de novo* and thus must obtain sterols from their diets (reviewed by Martin-Creuzburg and von Elert, 2009b). In geochemistry, sterols such as 24-methylcholesta-5,22E-dien-3β-ol (brassicasterol/epi-brassicasterol) and 4α,23,24-trimethylcholest-22E-en-3β-ol (dinosterol) have been applied to reconstruct diatom and dinoflagellate production and community structure on historical and geological timescales (Volkman, 1986; Schubert et al., 1998; Zimmerman and Canuel, 2002; Xing et al., 2016). Given the multiple biochemical roles and source specificity of sterols, their composition and biosynthetic pathways in

phytoplankton have been identified in different phyla (Fabris et al., 2014; Villanueva et al., 2014; Volkman, 2016). It has been observed that sterol contents (per carbon or dry weight or percentage of total lipids) in phytoplankton vary with environmental conditions. Véron et al. (1996) found a dramatic decrease in total sterol contents (per dry weight) in *Phaeodactylum tricornutum* as temperature increased. In contrast, a significant increase in carbon-normalized sterol contents with increasing temperature was found in other algal species (Piepho et al., 2012; Ding et al., 2019). Enhanced $pCO_2$ caused an increase in the percentage of sterols (% of total lipids) in *Dunaliella viridis* (Gordillo et al., 1998), but no clear change in per-cell contents of sterols in *Emiliania huxleyi* (Riebesell et al., 2000). Although significant interactions between two environmental factors have been observed on carbon-normalized or per-cell sterol contents in certain phytoplankton species (Piepho et al., 2010, 2012; Ding et al., 2019), the impacts of multiple environmental drivers on phytoplankton sterol contents have not been thoroughly investigated.

Long-chain alkenones are major lipids produced only by certain species of haptophytes, e.g., oceanic species *E. huxleyi* and *Gephyrocapsa oceanica* (Volkman et al., 1980c; Conte et al., 1995) and coastal species *Isochrysis galbana* (reviewed by Conte et al., 1994). Alkenones in *E. huxleyi* are believed to be used for energy storage (Epstein et al., 2001; Eltgroth et al., 2005), while little is known about the entire biosynthetic pathway of alkenones and their evolutionary and ecological functions (Rontani et al., 2006; Kitamura et al., 2018). Alkenones may have fitness and trophic benefit for their producers, because these unusual lipids are not only more photostable than other neutral lipids such as triacylglycerols, but also resistant to digestion, perhaps making alkenone producers less suitable for grazers (Eltgroth et al., 2005). Moreover, alkenones are well preserved in sediments over millions of years and thus their unsaturation ratios [e.g., the $U_{37}^{K'}$ index (= $C_{37:2}/(C_{37:2} + C_{37:3})$) (Brassell et al., 1986; Prahl and Wakeham, 1987)] are widely applied for reconstructing sea surface temperatures (Rosell-Melé and Prahl, 2013; Herbert et al., 2016). A long-standing issue for the use of alkenones to infer paleo-ocean surface temperature is how the production of these compounds is influenced by other environmental factors such as nutrients. Thus, culture studies have been conducted to test alkenone contents (mostly per-cell contents) in several species of haptophytes such as *E. huxleyi* under different growth phases (Wolhowe et al., 2009; Pan and Sun, 2011; Wolhowe et al., 2015), salinity (Sachs et al., 2016), temperature (Ding et al., 2019), and nutrient concentrations (Rokitta et al., 2014; Wördenweber et al., 2018). Conflicting results have been observed in different studies, e.g., independence of $C_{37}$ - $C_{39}$ alkenone contents on temperature (Prahl et al., 1988) *versus* significant responses of $C_{37}$ alkenone contents to temperature changes in *E. huxleyi* (Ding et al., 2019). More empirical evidence appears necessary to determine how the total contents and the ratios of specific alkenone isomers respond to multiple environmental drivers, which would allow us to better understand their roles in ecology and biogeochemistry.

Here, we present data from semi-continuous culture experiments to tackle the question of how important lipid biomarkers (FAs, sterols and alkenones) respond to the changes in multiple environmental drivers (temperature, N:P supply ratios and $pCO_2$) in three phytoplankton species (the diatom *P. tricornutum*, the cryptophyte *Rhodomonas* sp. and the haptophyte *E. huxleyi*). Specifically, we analyze the changes of particulate organic carbon (POC)-normalized (carbon-normalized hereafter)

and per-cell contents of major sterols and alkenones in the three species, and compare these responses with those of published FA data from the same experiments. Our aims are to determine (i) how sterols and alkenones respond to the changes of multiple environmental drivers, and (ii) how the responses of sterols, alkenones and FAs differ between each other. The goal of this study is to generate a better understanding of the impact of ocean-related global change on lipid biomarker productions in marine phytoplankton, which will help to quantitatively apply lipid biomarkers as proxies for ecosystem change, and to finally scale up from specific physiological roles of lipids to their effects on energy flow in food webs in the changing ocean.

## 2 Materials and Methods

### 2.1 Experimental design

The three phytoplankton species used in the experiments were the diatom *P. tricornutum* [SAG, 1090-1b; isolated from Plymouth, UK (De Martino et al., 2007)], the cryptophyte *Rhodomonas* sp. (isolated from the Kiel Bight, Baltic Sea and identified by the working group at GEOMAR; maintained and still available from GEOMAR for laboratory culture), and the haptophyte *E. huxleyi* (internal culture collection reference code: A8; isolated from waters off Terceira Island, Azores). *P. tricornutum* and *Rhodomonas* sp. are model species widely used in studies of diatom genomes, cryptophyte photosynthesis and planktonic trophic dynamics (Bi et al., 2017), and *E. huxleyi* is one of the major calcifying organisms in the pelagic ocean (Winter et al., 2014). The algal cultures were non-axenic. Because the biomass of bacteria was very low, the bacterial influence on the chemical composition of phytoplankton was negligible. Over the course of the experiments, the cultures of all species were exposed to a salinity of 37 psu and a light intensity of 100 $\mu$mol photons $m^{-2}$ $s^{-1}$ following a light:dark cycle of 16:8 h in temperature-controlled rooms of 12, 18 and 24°C. The culture medium was prepared according to the modified Provasoli's medium (Provasoli, 1963; Ismar et al., 2008), with enrichment nutrient solutions added to sterile filtered (0.2 $\mu$m pore size, Sartobran® P 300; Sartorius, Goettingen, Germany) North Sea water. Sodium nitrate and potassium dihydrogen phosphate were added to achieve the molar ratios of 10:1 (35.2 $\mu$mol $L^{-1}$ N and 3.6 $\mu$mol $L^{-1}$ P), 24:1 (88 $\mu$mol $L^{-1}$ N and 3.6 $\mu$mol $L^{-1}$ P) and 63:1 (88 $\mu$mol $L^{-1}$ N and 1.4 $\mu$mol $L^{-1}$ P). Sodium silicate pentahydrate was also added to diatom cultures at a concentration of 88 $\mu$mol $L^{-1}$. Initial $pCO_2$ was manipulated by bubbling with $CO_2$-enriched air (560 and 2400 $\mu$atm). Subsequently, the culture medium was transferred into sealed cell culture flasks with a 920-mL culture volume. Each treatment was replicated three times. All culture flasks were carefully agitated twice per day at a set time to minimize sedimentation.

At the onset of the experiments, each species was grown in batch cultures across a fully factorial combination of three temperatures (12, 18 and 24°C), three N:P supply ratios (molar ratios 10:1, 24:1 and 63:1) and two $pCO_2$ levels (560 and 2400 $\mu$atm) (Fig. S1). The chosen levels of temperature, N:P supply ratio and $pCO_2$ cover the ranges of typical changes of the three factors in natural environments, and they are in general agreements with projections. The temperature regimes

broadly conform to sea surface temperatures in the source regions for the three taxa studied: Plymouth, UK for *P. tricornutum* (~9 – 17°C) (Highfield et al., 2010), the Kiel Bight for *Rhodomonas* sp. (~3 – 18°C) (Hiebenthal et al., 2013), and the Azores for *E. huxleyi* (16 – 22°C; http://dive.visitazores.com/en/when-dive). The 6°C elevation also mimicks the largest projected warming under climate change scenarios (Sommer and Lengfellner, 2008). N:P molar ratio of 24:1 was selected as the balanced ratio under which phytoplankton cultures are typically maintained (Guillard, 1975). Surface ocean inorganic N and P concentrations are highly depleted throughout much of the low-latitude oceans (Moore et al., 2013). Nutrient availability in the future will be altered by increasing external nutrient inputs especially in coastal oceans, changes in surface ocean chemistry driven by anthropogenic increases in atmospheric $CO_2$, and changes in ocean circulation (Moore et al., 2013). Therefore, inorganic N:P ratios show a strong spatial variation in the oceans, e.g., a low ratio of 6:1 mol $mol^{-1}$ in the center of the South Pacific Gyre *versus* a global scenario of an increase driven by the high N:P atmospheric deposition of ~ 370:1 mol $mol^{-1}$ (Bonnet et al., 2008; Peñuelas et al., 2012). N:P ratios of 10:1 and 63:1 selected in this study cover large-scale spatial patterns of nutrient status, and thus our prediction of lipid biomarker production changes is based on both future open ocean and coastal conditions. Partial $CO_2$ pressure of 560 $\mu$atm is double the pre-industrial value and is a standard level for determining climate model sensitivity to $pCO_2$ forcing (e.g., IPCC, 2014). The value of 2400 $\mu$atm is at the mid-range of the projected values (1371 – 2900 $\mu$atm) by 2150 (RCP8.5 scenario; IPCC, 2014). Also, a high $pCO_2$ has been observed in the areas where one of the studied algae was isolated, e.g., 375 – 2309 $\mu$atm in the Kiel Bight (Thomsen et al., 2010).

The observed maximal growth rate ($\mu_{max}$, $d^{-1}$) was calculated from the changes of cell numbers within the exponential growth phase in batch cultures (Bi et al., 2012). Once the early stationary phase was reached, semi-continuous cultures were started with the algae from batch cultures, and the gross growth rate ($\mu$, $d^{-1}$) was set as 20% of $\mu_{max}$. We calculated the volume of the daily renewal incubation water by multiplying daily renewal rate [$D$, $d^{-1}$; $D = 1- e^{-\mu \cdot t}$, where $t$ is renewal interval (here $t$ = 1 day)] with the incubation volume of 920 mL. According to the gross growth rate of 20% of $\mu_{max}$, the renewal volumes were about 10% of the total culture volume (100 – 200 mL out of 920 mL), and renewal interval was 1 d. Steady state was reached at about 10 d with slight differences between the individual cultures. Renewal of the cultures was carried out at the same hour every day using fresh filtered seawater pre-acclimated to target $pCO_2$, and $CO_2$-enriched water. The difference between the gross growth rate and the loss rate, i.e., the net growth rate [$r$ ($d^{-1}$); $r = \mu - D$] was used to assess the steady state, at which $r$ was zero, and $\mu$ was equivalent to $D$.

**2.2 Sampling and measurements**

At steady state in semi-continuous cultures, samples were collected for the measurements of cell density, dissolved inorganic carbon (DIC), total alkalinity, pH, POC, FAs, sterols and alkenones. Cell density was measured daily in batch and semi-continuous cultures using an improved Neubauer hemacytometer (Glaswarenfabrik Karl Hecht GmbH, Rhön Mountains, Germany) under a microscope (Hund, Wetzlar, Germany). Also, pH measurements were carried out daily in

semi-continuous cultures, and the electrode was calibrated using standard pH buffers (pH 4 and pH 7; WTW, Weilheim, Germany).

DIC samples were taken on sampling days with 10-mL glass vials (Resteck, Germany) filled using a peristaltic pump and an intake tube containing a single-use syringe filter (0.2 $\mu$m, Minisart RC25; Sartorius, Goettingen, Germany). Vials were immediately sealed and stored in the dark at 4°C. DIC was measured according to Hansen et al. (2013) using a gas chromatographic system (8610C; SRI-Instruments, California, USA). For total alkalinity analysis, samples were filtered (GF/F filters; Whatman GmbH, Dassel, Germany) and analyzed with the Tirino plus 848 (Metrohm, Filderstadt, Germany). The remaining carbonate parameter $pCO_2$ was calculated from DIC and total alkalinity using CO2SYS (Pierrot et al., 2006) and the constants of Hansson (1973) and Mehrbach et al. (1973) that were refitted by Dickson and Millero (1987) (Table S1; Bi et al., 2017, 2018). DIC and pH did not differ substantially between different temperature and N:P ratio treatments in our study (Table S1).

POC and FA samples (15 – 50 ml) were taken on pre-combusted and pre-washed (5-10% HCl) GF/F filters (Whatman GmbH, Dassel, Germany). After filtration, filters for POC analysis were immediately dried at 60°C and stored in a desiccator, and those for FA measurements were stored at -80°C. For POC analysis in *E. huxleyi*, particulate inorganic carbon was removed by exposing filters to fuming hydrochloric acid for 12h. POC was determined using an elemental analyzer (Thermo Flash 2000; Thermo Fisher Scientific, Schwerte, Germany) after Sharp (1974). FAs were measured as FA methyl esters (FAMEs) using a gas chromatograph (Trace GC-Ultra; Thermo Fisher Scientific, Schwerte, Germany). Analytical procedure of FAs was modified after Christie (1989). Briefly, FAs were extracted with a solvent mixture of chloroform/dichloromethane/methanol (1:1:1, volume ratios). The FAME 19:0 was added as an internal standard and 21:0 as a esterification control. The extracted FAMEs were dissolved in 100 µL *n*-hexane for analysis. Sample aliquots (1 µL) were injected onto the GC with hydrogen as the carrier gas. Individual FAs were identified with reference to the standards (Supelco 37 component FAME mixture and Supelco Menhaden fish oil), and the peaks were integrated using Chromcard software (Thermo Fisher Scientific, Schwerte, Germany), which included saturated, monounsaturated and polyunsaturated FAs (Bi et al., 2017, 2018).

Samples for sterol and alkenone analysis were filtered (GF/F filters; Whatman GmbH, Dassel, Germany) and measured according to the procedure of Zhao et al. (2006). The filtration volume of sterol and alkenone samples was 100 – 200 mL, while only 10% of the extractions were used for GC quantification and the rest was stored for later use. Lipids were extracted ultrasonically eight times from the freeze-dried filter samples utilizing dichloromethane and methanol (3:1, volume ratios) as extraction solvent, with $C_{19}$ *n*-alkanol added for quantification. After hydrolysis with 6% potassium hydroxide in dichloromethane, the lipids were separated into a polar fraction and a non-polar fraction using silica gel chromatography. The polar lipid fractions containing sterols from the extracts of all the three species, as well as $C_{37}$ - $C_{39}$ alkenones from the *E. huxleyi* extracts, were eluted with 22 mL dichloromethane and methanol (95:5, volume ratios). Subsequently, the polar lipid fractions were silylated with 80 $\mu$L *N,O*-bis(trimethylsilyl)-trifluoroacetamide at 70°C for 1h. The sterol and alkenone

fractions were analyzed and quantified using an Agilent 7890A GC with flame ionization detection (50 m HP-1 capillary column, 0.32 mm i.d., 0.17 $\mu$m film thickness) by comparing analyte peak area to known amount of the internal standard $C_{19}$ *n*-alkanol. The oven temperature held initially at 80°C for 1 min, increased to 200°C at 25°C $min^{-1}$ and then programmed to 250°C at 4°C $min^{-1}$ and to 300°C at 1.7°C $min^{-1}$ holding for 12 min, and finally to 315°C at 5°C $min^{-1}$ with an 8 min isothermal period.

The identification of brassicasterol/epi-brassicasterol, the major sterol in all three species, was conducted with reference to laboratory standards. In marine systems, the most likely epimer for 24-methylcholesta-5,22E-dien-3β-ol is epi-brassicasterol (24α) synthesized by diatoms (Gladu et al., 1990, 1991), cryptophytes (Goad et al., 1983) and haptophytes (Maxwell et al., 1980). In contrast, 24β-methylcholesta-5,22E-dien-3β-ol (brassicasterol) has been also found, e.g., in a strain of the haptophyte *Isochrysis* (Gladu et al., 1990). As the stereochemistry at C-24 could not be determined in our study, we use the trivial name brassicasterol/epi-brassicasterol for 24-methylcholesta-5,22E-dien-3β-ol.

Alkenones were identified using an Agilent GC 7890B (30 m HP-5MS column, 0.25 mm i.d., 0.25 $\mu$m film thickness) connected to an Agilent MSD 5977B mass selective detector (70 eV constant ionization potential, ion source temperature 230°C). The temperature program started with a 1 min hold time at 80 °C and then increased to 200°C at 25°C $min^{-1}$, followed by a 4°C $min^{-1}$ ramp to 250°C and 1.8°C $min^{-1}$ to 300°C holding for 10 min, and finally increased to 315°C at 5°C $min^{-1}$ holding for 5 min. Alkenone identifications were performed by comparing sample mass spectra generated by GC-MS to previous published EI mass spectra, based on the molecular ion and prominent ions of each alkenone component (de Leeuw et al., 1980; Volkman et al., 1980c; Lopez and Grimalt, 2006). Note that the molecular ions for $C_{39:3}$ ethyl ketone ($C_{39:3}$ Et) (at m/z 556) and $C_{39:2}$ ethyl ketone ($C_{39:2}$ Et) (at m/z 558) were below detection, only prominent ion fragments were detected. In addition, the comparisons of the GC retention time of alkenone molecules between our study and previous work (Volkman et al., 1980c) indicated the presence of $C_{39:3}$ Et and $C_{39:2}$ Et.

### 2.3 Statistics

Generalized linear mixed models (GLMMs; Bolker et al., 2009) were used to test the effects of temperature, N:P supply ratios and $pCO_2$ on carbon-normalized and per-cell contents of brassicasterol/epi-brassicasterol and $C_{37}$ - $C_{39}$ total alkenones (as $\mu$g mg $C^{-1}$ and pg $cell^{-1}$), per-cell contents of $C_{37}$ alkenones, $C_{38}$ alkenones, $C_{38}$ ethyl ketones ($C_{38}$ Et) and $C_{38}$ methyl ketones ($C_{38}$ Me), $C_{37}/C_{38}$ alkenone ratios and $C_{38}$ Et/$C_{38}$ Me ratios ($C_{38}$ Et/Me), with temperature, N:P supply ratios and $pCO_2$ as fixed effects. Target distributions were tested before GLMMs were taken. Subsequently, appropriate link functions were chosen, e.g., identity link function for any distribution except for multinomial, and logit link function for the binomial or multinomial distribution. To find the model that best predicted targets, we tested models containing first order effects, and second and third order interactions of temperature, N:P supply ratios and $pCO_2$. The Akaike Information Criterion corrected (AICc) was used to select the best model for each response variable, with a lower AICc value representing a better fit of the model. When the changes of AICc values were 10 units or more, it was considered as a reasonable improvement in the

fitting of GLMMs (Bolker et al., 2009). In case AICc values were comparable (< 10 units difference), the simpler model was chosen. Based on the differences in AICc values, models containing only first order effects of temperature, N:P supply ratios and $pCO_2$ were chosen as the best models for all response variables (bold letters in Table S2), while those containing second or third order interactions were not selected.

Principal component analysis (PCA) was conducted to visualize the responses of carbon-normalized contents of brassicasterol/epi-brassicasterol and total fatty acids (TFAs) in the three species, and total alkenones in *E. huxleyi* to the changes of temperature, N:P supply ratios and $pCO_2$. Data for TFAs were from previous studies (Bi et al., 2017, 2018).

GLMMs were performed using SPSS 19.0 (IBM Corporation, New York, USA). PCA were conducted using R package factoextra (Kassambara and Mundt, 2017) and FactoMineR (Le et al., 2008) in R version 3.5.1 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). All statistical tests were conducted at a significance threshold of $p = 0.05$.

## 3 Results

### 3.1 Sterol and alkenone composition

Brassicasterol/epi-brassicasterol was the major sterol observed in all the three species, while alkenones were only detected in *E. huxleyi*. The alkenones in *E. huxleyi* consisted of four pairs of homologues, i.e., $C_{37}$ methyl ketones ($C_{37}$ Me including $C_{37:4}$ Me, $C_{37:3}$ Me and $C_{37:2}$ Me), $C_{38}$ Me ($C_{38:3}$ Me and $C_{38:2}$ Me), $C_{38}$ Et ($C_{38:3}$ Et and $C_{38:2}$ Et) and $C_{39}$ ethyl ketones ($C_{39}$ Et including $C_{39:3}$ Et and $C_{39:2}$ Et). $C_{37}$ Me were the most abundant, followed by $C_{38}$ Et, $C_{38}$ Me and $C_{39}$ Et.

### 3.2 Responses of brassicasterol/epi-brassicasterol to environmental changes

GLMM results showed that per-cell contents of brassicasterol/epi-brassicasterol responded significantly to changes in N:P supply ratios in the three species (bold letters in Table 1). Moreover, per-cell contents of brassicasterol/epi-brassicasterol in *P. tricornutum* also showed significant responses to temperature changes, while non-significant effects of $pCO_2$ were observed in all species. Specifically, higher per-cell contents of brassicasterol/epi-brassicasterol were observed at higher temperatures and higher N:P supply ratios in *P. tricornutum* (Fig. 1a; Table S3), under the lowest and highest N:P supply ratios in *Rhodomonas* sp. (Fig. 1c), and under higher N:P supply ratios in *E. huxleyi* (Fig. 1e).

Carbon-normalized contents of brassicasterol/epi-brassicasterol responded significantly to $pCO_2$ in *Rhodomonas* sp., and to temperature and N:P supply ratios in *E. huxleyi* (bold letters in Table 1), but with non-significant responses in *P. tricornutum*. Carbon-normalized contents of brassicasterol/epi-brassicasterol in *Rhodomonas* sp. decreased as $pCO_2$ increased (Fig. 1d), while those in *E. huxleyi* were generally higher at higher temperatures and under the balanced N:P supply ratio (N:P = 24:1 mol $mol^{-1}$; Fig. 1e).

### 3.3 Responses of alkenones to environmental changes

Total alkenone content per cell in *E. huxleyi* increased with increasing N:P supply ratios (Fig. 2 a; bold letters in Table 1; Table S4). However, carbon-normalized contents of total alkenones showed non-significant responses to changes in temperature, N:P supply ratios and $pCO_2$.

$C_{37}/C_{38}$ alkenone ratios responded significantly to all three environmental factors (bold letters in Table 1), showing a clear increase with increasing temperature, a higher value under the lowest and highest N:P supply ratios, and a slight decrease at high $pCO_2$ (Fig. 2 c and d). $C_{38}$ Et/Me ratios had significant responses only to temperature changes, clearly higher at the highest temperature (Fig. 2 e and f).

### 3.4 Comparisons of sterol, alkenone and fatty acid responses

PCA extracted four axes with eigenvalues > 1, and the first two axes in PCA explained 44.1% of total variance (Table S5). PC axis 1 explains 26.4% of the total variance and largely differentiates between the highest (N:P = 63:1 mol $mol^{-1}$) and lower N:P treatments (N:P = 10:1 and 24:1 mol $mol^{-1}$). Along PC axis 1, N:P supply ratios correlated positively with carbon-normalized contents of TFAs in *P. tricornutum* and *Rhodomonas* sp., but negatively with brassicasterol/epi-brassicasterol in *E. huxleyi* (Fig. 3). PC axis 2 (17.7% of the total variance) differentiates between the highest and both colder temperature treatments, along which temperature showed positive correlations with brassicasterol/epi-brassicasterol in *Rhodomonas* sp. and alkenones in *E. huxleyi*, but a negative correlation with TFAs in *E. huxleyi*. Both Dim1 and Dim 2 loadings for $pCO_2$ were very low (0.185 and 0.151, respectively), showing a weaker effect of $pCO_2$ on lipid biomarkers compared to temperature and N:P supply ratios. Along PC axis 2, $pCO_2$ plays a negative role particularly on TFAs in *E. huxleyi*, while the contribution of $pCO_2$ to other lipid biomarkers was weak.

## 4 Discussion

To our knowledge, this is the first study to disentangle the effects of multiple environmental drivers on sterol and alkenone productions, and to compare the responses of sterols, alkenones and FAs in marine phytoplankton. The mean percent changes of the three lipid biomarkers were elucidated (Table 2), particularly showing obvious changes in per-cell lipid contents which underly specific modes of biosynthesis. Furthermore, the PCA results highlight that the responses of TFA carbon-normalized contents to N:P supply ratios were opposite to that of brassicasterol/epi-brassicasterol and alkenones (Fig. 3; Table S5), e.g., strong positive correlations of TFAs with N:P ratios in *P. tricornutum* and *Rhodomonas* sp., but negative ones of brassicasterol/epi-brassicasterol with N:P ratios in *E. huxleyi*. Also, carbon-normalized alkenone contents in *E. huxleyi* correlated positively with temperature, but TFAs contents showed a negative correlation with temperature. Such variable responses of the three classes of lipid biomarkers can be attributed to their specific physiological functions and biosynthetic pathways (Riebesell et al., 2000). Carbon-normalized contents of FAs were particularly sensitive to

environmental changes, because FAs can be incorporated into different types of lipids, and thus play multiple roles within the cells such as energy storage, membrane components and metabolic regulations (Guschina and Harwood, 2009; Van Mooy et al., 2009). Conversely, less pronounced changes in carbon-normalized contents of sterols and alkenones may reflect their major roles in membrane functions and energy storage, respectively. The varying production of lipid biomarkers indicate potential changes of energy flow in marine food webs in response to ocean-related global change.

### 4.1 Sterol and alkenone composition

Brassicasterol/epi-brassicasterol was the only major sterol in the three algal species under wide ranges of temperature, N:P supply ratios and $pCO_2$. It is well established that the diversity of sterols is low in most phytoplankton species (Martin-Creuzburg and Merkel, 2016; Volkman, 2016). The predominance of brassicasterol/epi-brassicasterol has been reported for *P. tricornutum* (Orcutt and Patterson, 1975; Ballantine et al., 1979; Rampen et al., 2010), *Rhodomonas* (Dunstan et al., 2005; Chen et al., 2011), and *E. huxleyi* (Volkman et al., 1981; Riebesell et al., 2000). A few other minor sterols have been reported but they were not detected or below detection in our study, such as cholesterol, 24-methylcholest-5-en-3β-ol, and cholesta-5,22-dienol in *P. tricornutum* (Ballantine et al., 1979; Véron et al., 1996; Cvejić and Rohmer, 2000), and cholesterol and 24-methylcholesta-5,7,22-trien-3β-ol in *Rhodomonas/Proteomonas* sp. (Dunstan et al., 2005) and *E. huxleyi* (Yamamoto et al., 2000; Sawada and Shiraiwa, 2004; Mausz and Pohnert, 2015).

Alkenones were only observed in *E. huxleyi* in our study, consistent with previous results showing that these compounds were only synthesized by a few haptophytes including *E. huxleyi* (Volkman et al., 1980b; Volkman et al., 1998). The alkenone composition of *E. huxleyi* was characterized by the presence of four pairs of isomers, including eight alkenone compounds (Table S4) (Marlowe et al., 1984; Riebesell et al., 2000; Sachs et al., 2016). Moreover, higher abundance of several alkenone components have been also observed in some *E. huxleyi* strains under certain culture conditions, e.g., $C_{37:4}$ Me in the strain 1742 (Eltgroth et al., 2005) or at low temperatures (our study; Prahl and Wakeham, 1987). In addition, two other compounds $C_{38:4}$ Et and $C_{38:4}$ Me were also found in one *E. huxleyi* strain (Marlowe et al., 1984).

Collectively, the results above highlight the similarity of sterol and alkenone composition in algal species in our study with those in conspecifics or congeneric phytoplankters in previous studies. Sterol and alkenone composition can vary between algal strains and can be affected by environmental changes (Conte et al., 1994; Volkman, 2003), which may explain the differences between our findings and previous results. In the following section, specific response patterns of brassicasterol/epi-brassicasterol and alkenones are evaluated and quantified, which are further compared with FA responses under changing temperature, N:P supply ratios and $pCO_2$.

### 4.2 Responses of brassicasterol/epi-brassicasterol contents

Increasing temperature caused an overall 12% increase in carbon-normalized contents of brassicasterol/epi-brassicasterol in *E. huxleyi*, but non-significant changes in *P. tricornutum* and *Rhodomonas* sp. (Table 1; Table 2). Consistent with our

findings, positive correlations between increasing temperature and sterol carbon-normalized contents have been observed in the dinoflagellates *Karenia mikimotoi* and *Prorocentrum minimum* (Ding et al., 2019), and the green alga *Scenedesmus quadricauda* (Piepho et al., 2012). High sterol contents at high temperature could be predicted based on its biochemical function, because increasing levels of sterols can reduce membrane fluidity to enable an organism's functional activity as temperature increases (Ford and Barber, 1983).

Enhanced partial $CO_2$ pressure caused a 21% decrease in carbon-normalized brassicasterol/epi-brassicasterol contents in *Rhodomonas* sp., and non-significant responses in *P. tricornutum* and *E. huxleyi*; however, per-cell contents of brassicasterol/epi-brassicasterol and POC showed non-significant changes in all three species (Table 1; Table 2). Minor effects of $CO_2$ concentration on per-cell contents of sterols have been observed in another strain of *E. huxleyi* (PML B92/11) (Riebesell et al., 2000) and the Chlorophyceae *D. viridis* (Gordillo et al., 1998). While the mechanism underlying sterol responses to $pCO_2$ is still unclear, our results indicate that enhanced $pCO_2$ did not induce substantial changes in per-cell contents of sterols in phytoplankton due to the role of sterols in membrane composition and functions (Riebesell et al., 2000). Nevertheless, enhanced $pCO_2$ might change carbon metabolism in phytoplankton (Gordillo et al., 2001), as revealed by variable carbon-normalized contents of brassicasterol/epi-brassicasterol in *Rhodomonas* sp. in our study.

N and P deficiency caused overall 8% and 37% decreases in carbon-normalized brassicasterol/epi-brassicasterol contents, respectively, in *E. huxleyi*, but non-significant changes in other two species (Table 1; Table 2). Carbon-normalized or dry-weight contents of sterols in phytoplankton generally reduced in response to N or P deficiency (Breteler et al., 2005; Piepho et al., 2010; Ding et al., 2019). Furthermore, the relatively higher per-cell contents of sterols in response to P deficiency than N deficiency have been also found in the three species in this study and in the freshwater diatom *Stephanodiscus minutulus* (Lynn et al., 2000). Lipid modifications triggered by nutrient deficiency have been well studied in the plant *Arabidopsis* and more recently elucidated in typical phytoplankters (Van Mooy et al., 2009; Abida et al., 2015; Shemi et al., 2016). In *P. tricornutum*, N deficiency exerted more severe stress on membrane glycerolipids than P deficiency which caused a stepwise adaptive response, resulting in undetectable phospholipids and instead the increase in the synthesis of non-phosphorus lipids (Abida et al., 2015). Also in plants, P deficiency resulted in the replacement of phospholipids by non-phosphorous glycolipids such as glucosylceramide, sterol glucoside and acylated sterol glucoside (Siebers et al., 2015). Consequently, sterols are synthesized and accumulate in the plasma membrane in response to P deficiency. Thus, N deficiency may inhibit the capacity of the cells to synthesize sterols, while upon P deficiency membrane glycerolipid remodeling with the accumulation of non-phosphorous lipids may explain the relatively higher per-cell contents of sterols in response to P deficiency in our study.

In summary, our study shows that temperature, N:P supply ratios and $pCO_2$ had significant separate effects on per-cell and per-carbon contents of brassicasterol/epi-brassicasterol in certain algal species. Previous studies have shown significant interactions of two environmental factors on carbon-normalized contents of sterols in phytoplankton (Piepho et al., 2012; Chen et al., 2019; Ding et al., 2019). We here found potentially confounding effects of multiple environmental drivers on

phytoplankton sterol contents. For example, the responses of *E. huxleyi* brassicasterol/epi-brassicasterol carbon-normalized contents to increasing temperature varied with N:P supply ratios, i.e., an increase under lower N:P supply ratios, but no clear changes under the highest N:P ratio (Fig. 1e), suggesting that nutrient availability may potentially alter the effects of warming on sterol contents in *E. huxleyi*.

### 4.3 Responses of alkenone contents and ratios

Carbon-normalized contents of total alkenones showed non-significant responses to the changes in temperature, N:P supply ratios or $pCO_2$ in *E. huxleyi* (Table 1), which can be attributed to similar response patterns of per-cell contents of alkenones and POC (Table 2). We observed that per-cell contents of alkenones changed significantly in response to N and P deficiency, but showed non-significant responses to warming or enhanced $pCO_2$. In the following, the responses of per-cell contents of alkenones and the ratios of certain alkenone isomers are discussed.

N deficiency in semi-continuous *E. huxleyi* cultures grown at 20% of $\mu_{max}$ led to a 35% decrease in per-cell contents of alkenones in our study (Table 2). However, all published culture studies we could find in which *E. huxleyi* was grown under N deficiency were performed with batch cultures and reported either an increase in per-cell alkenone contents or a non-significant change (Epstein et al., 1998; Prahl et al., 2003; Bakku et al., 2018; Wördenweber et al., 2018). Several possible explanations exist for these contradictory findings. An increase in growth rate has been shown to reduce alkenone concentrations (ng $mL^{-1}$) in continuous cultures of *E. huxleyi* (Sachs and Kawka, 2015). Thus, the higher growth rate of *E. huxleyi* (20% of $\mu_{max}$) in semi-continuous cultures in our study is a possible cause of lower alkenone contents compared to the batch culture studies where cells were harvested at or near the stationary phase of growth (i.e., growth rate approaching 0) (Epstein et al., 1998; Prahl et al., 2003; Bakku et al., 2018; Wördenweber et al., 2018). Because growth rate and growth phase strongly affect sterol contents (Sachs and Kawka, 2015; Chen et al., 2019), the conjunctions above-described may change if the maximum growth rates of batch cultures were used for comparison. In addition, phytoplankton in a continuous culture has a constant growth rate under a given dilution rate, while the growth rate at the stationary phase of a batch culture is zero. Thus, energy-availability for sterol remodeling differs between the two culture systems. Contradictory results obtained in batch and semi-continuous cultures could indicate different alkenone contents produced by *E. huxleyi* during the bloom period and summertime growth of this species, respectively (Lampert and Sommer, 2007). Another possibility is that gene complements within the species of *E. huxleyi* vary considerably, which may explain different phenotypic variations, including differences in N and P uptake in this species (Read et al., 2013). N deficiency severely impairs the synthesis of nucleotides, amino acids and ultimately all enzymatic machinery, consequently resulting in a decrease of most central metabolites (Wördenweber et al., 2018). Intense lipid turnover with the reduction of most central metabolites have been reported in *E. huxleyi* under N deficiency based on transcriptomic and metabolomic studies (Rokitta et al., 2014; Wördenweber et al., 2018), which may also result in lower per-cell alkenone contents in our study.

In contrast, P deficiency caused an increase (49%) in per-cell contents of alkenones in *E. huxleyi* (Table 2), as well as in other strains and life-cycle stages of *E. huxleyi* (Wördenweber et al., 2018). Experimental data presented here agree with a metabolic model predicted from transcriptomic data (Rokitta et al., 2016), and the findings in a comprehensive metabolome study showing a significant accumulation of several key metabolites, especially neutral lipids such as triacylglycerols, alkenones and alkenes in response to P deficiency (Wördenweber et al., 2018). *E. huxleyi* contains only very small amounts of triacylglycerols and hence alkenones have been suggested to have a storage role (Volkman et al., 1980c; Bell and Pond, 1996; Eltgroth et al., 2005). Our results support this view and suggest that P deficiency can induce the accumulation of alkenones which can serve as storage molecules in *E. huxleyi* cells. The increased abundance of metabolites in response to P deficiency is likely derived from the arrest of cell-cycling due to decreased nucleic acid synthesis, and the reduction equivalents are preserved by lipogenesis as enzymatic functionality (Wördenweber et al., 2018).

The carbon chain-length distribution of alkenones ($C_{37}/C_{38}$ alkenone ratios) showed a 13 – 21% increase from the cold to warm treatments and in response to N and P deficiency, and a slight decrease (6%) with enhanced $pCO_2$ (Table 1; Table 2). Previous studies have shown that $C_{37}/C_{38}$ alkenone ratios not only varied with temperature and physiological stages (such as growth stage), but also differed between alkenone-producing species (Conte et al., 1998; Pan and Sun, 2011; Nakamura et al., 2014). In agreement with our findings, a slight increase in $C_{37}/C_{38}$ alkenone ratios at higher temperatures has been also found in four *E. huxleyi* strains in exponential phase cultures (Conte et al., 1998). In contrast to our results, it has been reported lower $C_{37}/C_{38}$ alkenone ratios occurred under nutrient deficiency at the stationary phase of *E. huxleyi* in comparison to those at the exponential phase (Conte et al., 1998; Pan and Sun, 2011). As discussed above, different culturing approaches may cause conflicting results in different studies, as the effects of nutrient deficiency and growth rate cannot be well distinguished in the batch approach. The proposed biosynthetic pathways of classical $C_{37}$ - $C_{40}$ alkenones show that biosynthesis of $C_{37}$ Me involve chain elongation with malonyl-CoA, while $C_{38}$ Et are formed by the condensation of methylmalonyl-CoA and $C_{38}$ Me are produced after the involvement of an additional α-oxidation (Rontani et al., 2006). Our results suggest that warming, N and P deficiency and enhanced $pCO_2$ may have independent effects on the synthesis of $C_{37}$ Me and $C_{38}$ alkenones, that ultimately result in changes in $C_{37}/C_{38}$ ratios.

The relative abundance of $C_{38}$ homologs ($C_{38}$ Et/Me ratios) showed an 82% increase from the cold to warm treatments (Table 1; Table 2). The prominent increase in $C_{38}$ Et/Me ratios resulted from non-significant changes in per-cell contents of $C_{38}$ Et and the decrease in $C_{38}$ Me. An increase in $C_{38}$ Et/Me ratios with increasing temperatures has been found in four *E. huxleyi* strains in mid-exponential phase of batch cultures (Conte et al., 1998). More importantly, our experimental results agree well with the findings in the sedimentary records back to ~ 120.5 Ma (Brassell et al., 2004), showing the absence of all $C_{38}$ Me but the occurrence of $C_{38}$ Et in Cretaceous sediments (warm climate, indicating a high $C_{38}$ Et/Me ratio) but the presence of $C_{38}$ Me from Cretaceous to Quaternary ages (warm to cold climate, suggesting a potentially declined $C_{38}$ Et/Me ratio). The strong increases in $C_{38}$ Et/Me ratios with warming in our study may reflect the differences in biosynthetic

pathways between $C_{38}$ Et and $C_{38}$ Me (Rontani et al., 2006). Therefore, the distribution of alkenones in sediments over time can be linked to evolutionary adaption of alkenone biosynthesis in response to global climate change (Brassell, 2014).

The results discussed above show significant changes in per-cell contents of alkenones in response to N and P deficiency, indicating the important role of alkenones as storage molecules. Another type of biomolecules, alkenoates, has been identified in *E. huxleyi* and may biochemically link with alkenones (Marlowe et al., 1984; Conte et al., 1994). However, alkenoates were converted to FAs by saponification in our sample preparation steps and thus not evaluated. There might be interesting variations in alkenone/alkenoate ratios with changing multiple environmental conditions, which can be assessed in future studies.

### 4.4 Implications for ecology and biogeochemistry

There has been evidence that carbon allocation in algal cells is highly responsive to environmental changes (Palmucci et al., 2011; Halsey and Jones, 2015). Our new study demonstrated that, under future ocean scenarios (warming, N and P deficiency and enhanced $pCO_2$), carbon-normalized contents of brassicasterol/epi-brassicasterol, alkenones and FAs have differential responses, i.e., significant but non-systematic changes in sterols and FAs, and non-significant changes in alkenones (Table 1). Our results further suggest rearrangements of cellular carbon pools under future ocean scenarios, and such variations would have important impacts on marine ecological functions and biogeochemical cycles.

Our study revealed an overall decrease (~ 20%) in carbon-normalized contents of brassicasterol/epi-brassicasterol in *Rhodomonas* sp. and *E. huxleyi* under ocean-related global change scenarios (Table 2). The low availability or absence of dietary sterols has been shown to constrain growth, reproduction and survival in *Daphnia* (Martin-Creuzburg et al., 2005; Martin-Creuzburg and von Elert, 2009a), and development and egg production in copepods (Hassett, 2004; Klein-Breteler et al., 2005). The potential influence of sterol deficiency on ecosystem functioning is the reduction of carbon transfer efficiency across autotroph-herbivore interface, leading to a low production of higher trophic levels (von Elert et al., 2003; Martin-Creuzburg and von Elert, 2009b). Brassicasterol/epi-brassicasterol are cholesterol precursors and can be converted to cholesterol by most crustaceans (Martin-Creuzburg and von Elert, 2009b; Kumar et al., 2018), and thus can efficiently support somatic growth of crustacean zooplankton such as *Daphnia magna* (Martin-Creuzburg et al., 2014). It is therefore possible that reduced brassicasterol/epi-brassicasterol under projected future ocean conditions may have deleterious ecological consequences in plankton communities, particularly where *Rhodomonas* or *E. huxleyi* is dominant.

Carbon-normalized contents of PUFAs showed an overall increase (~ 65%) in *Rhodomonas* sp., and an overall decrease (~ 10 – 20%) in *P. tricornutum* and *E. huxleyi* (Table 2). FA composition and contents in phytoplankton have shown significant effects on zooplankton production and trophic carbon transfer from phytoplankton to zooplankton (Müller-Navarra et al., 2000; Jónasdóttir et al., 2009; Rossoll et al., 2012; Arndt and Sommer, 2014). An example of this is the positive effect of increased docosahexaenoic acid (22:6n-3; DHA) content in the diatom *Skeletonema marinoi* on egg production rates of the calanoid copepod *Acartia tonsa* (Amin et al., 2011). These impacts of FA production remodelling

have been discussed in detail in our previous studies (Bi et al., 2014; Bi et al., 2018; Bi and Sommer, 2020). The varying PUFA contents observed in the present study may thus potentially influence zooplankton nutrition. For example, an increase in PUFA contents in the diatoms in cold periods may have positive effects on zooplankton production; in contrast, a decrease in PUFA contents in *E. huxleyi* at enhanced $pCO_2$ may reduce trophic transfer efficiency at phytoplankton-zooplankton interface. Moreover, we found that the overall responses of brassicasterol/epi-brassicasterol were opposite to PUFAs in *Rhodomonas* sp., while both showed an overall decrease in *E. huxleyi* and only brassicasterol/epi-brassicasterol decreased in *P. tricornutum* under future ocean scenarios (Table 2). Co-limitation of sterols and PUFAs has been well studied in a freshwater herbivore *D. magna*, showing a negative effect on the growth of this herbivore (Martin-Creuzburg et al., 2009; Sperfeld et al., 2012; Marzetz et al., 2017). Less is known about how sterols and PUFAs regulate the performance of marine herbivorous zooplankton. The differential responses of sterols and PUFAs we observed may have tremendous implications for the study of marine food webs, especially in habitats where phytoplankton succession is highly dynamic with environmental changes.

Carbon-normalized contents of total alkenones in *E. huxleyi* showed non-significant changes (Table 2). *E. huxleyi* is mainly grazed by heterotrophic protists in the context of pelagic food webs (Braeckman et al. 2018; Nejstgaard et al. 1997). Also, early studies on copepod feeding clearly showed ingestion of *E. huxleyi* (Harris, 1994; Nejstgaard et al., 1997; Vermont et al., 2016) and excretion of alkenones (Volkman et al., 1980a), indicating faecal pellet transport of these compounds to sediments (Volkman et al., 1980a). In the present study, we observed non-significant changes in carbon-normalized contents of total alkenones in *E. huxleyi* under variable temperature, N:P ratios and $pCO_2$, demonstrating quantitative applicability of alkenones as proxies for *E. huxleyi* biomass in biogeochemical cycles.

From a paleoceanographic perspective, lipid biomarker data have been reported on the basis of both dry sediment weight and total organic carbon (TOC) content (Zimmerman and Canuel, 2002; Zhao et al., 2006; Xing et al., 2016). Dry sediments contain variable organic and inorganic components, thus biomarker contents normalized to TOC can partially eliminate the influence of sedimentation rates, and between-site or temporal changes in the amount of organic carbon deposition or preservation (Zimmerman and Canuel, 2002). The application of sediment biomarker contents for paleoproductivity reconstruction is based on two key assumptions: (1) relatively constant ratios of biomarker per cell or per POC in a specific phytoplankton group, and (2) non-significant changes in biomarker/POC ratios during post production and deposition degradation. In laboratory culture studies, carbon-normalized and cell-normalized lipid contents provide broadly similar responses to environmental parameter changes in most cases, but there are exceptions (Ding et al., 2019; this study). However, cell-normalization is difficult for sediment reconstruction, as phytoplankton cell counting is often not quantitative (Piepho et al., 2010; Ahmed and Schenk, 2017). In the present study, we focus on the implications of our findings based on organic carbon (POC)-normalized contents of lipid biomarkers. For example, our study revealed an overall 20% decrease in carbon-normalized contents of brassicasterol/epi-brassicasterol in *Rhodomonas* sp. and *E. huxleyi* in ocean-related global change scenarios, but not in the diatom *P. tricornutum*. Because smaller ranges are expected in temperature, N:P supply ratio

and $pCO_2$ in individual locations over time, our results provide additional support for the applicability of using sterols in paleoproductivity reconstruction, especially in diatom-dominated areas. Furthermore, we observed that $C_{37}/C_{38}$ alkenone ratios varied significantly with the changes in temperature, N:P supply ratios and $pCO_2$, indicating that besides temperature, other environmental factors may also significantly influence $C_{37}/C_{38}$ alkenone ratios. In contrast, $C_{38}$ Et/Me ratios in our study responded significantly only to temperature changes. These results denote the importance to consider the effects of multiple environmental factors on $C_{37}/C_{38}$ alkenone ratios, and further underline the importance of temperature in geological application of alkenone ratios.

## 5 Conclusions

The responses of sterols, alkenones and FAs to projected future scenario changes in temperature, N:P supply ratios and $pCO_2$ were experimentally examined in three phytoplankton species. Our results reveal that N and P deficiency had a stronger effect on per-cell contents of the three lipid biomarkers, while the effects of warming and high $pCO_2$ were relatively moderate. We also show that P deficiency caused an increase but N deficiency led to a decrease in per-cell contents of lipids in most cases. Our results provide important new evidence to previous transcriptomic and metabolomic studies, which showed that key metabolites were up-regulated in response to P deficiency while most central metabolites were down-regulated in response to N deficiency. Such transcriptomic and metabolomic rearrangements are linked to the regulation of lipid biosynthesis-related genes (Read et al., 2013; Wördenweber et al., 2018). Future studies are suggested to consider the influence of these and other environmental changes on the composition of major and minor sterols, alkenoates and other energy storage molecules such as triacylglycerols, for example, whether varying environmental conditions influence C-24 alkylation in sterols.

Our study demonstrates that, under future ocean scenarios, the overall carbon-normalized contents of brassicasterol/epi-brassicasterol and PUFAs decreased in most cases in the three algal species; however, non-significant changes were also observed in brassicasterol/epi-brassicasterol and alkenones, and a significant increase was found in PUFAs in one of the three species (*Rhodomonas* sp.). This result highlights that a diverse allocation of carbon would potentially occur between lipid biomarkers and between phytoplankton taxa when they acclimate to large fluctuations in environmental conditions. Such variations in the contents of essential lipids (sterols and PUFAs) and in carbon allocation strategies may influence the structures and functions of food webs and the future ocean ecosystems.

**Data availability:** Data supporting the conclusions will be publicly available at PANGAEA at the time of publication of this paper.

**Author contribution**: Rong Bi (RB), Stefanie M. H. Ismar-Rebitz (SI), Ulrich Sommer (US) and Meixun Zhao (MZ) designed the experiments. RB carried the experiments out, with assistance of Hailong Zhang (HZ). RB prepared the manuscript with contributions from all co-authors.

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The authors declare that they have no conflict of interest.

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**Table 1**. Results of the selected GLMMs testing for the effects of temperature, N:P supply ratios and $pCO_2$ on carbon-normalized and per-cell contents of brassicasterol/epi-brassicasterol (brassi./epi-brassi./POC and brassi./epi-brassi./cell; $\mu$g mg $C^{-1}$ and pg $cell^{-1}$) in *Phaeodactylum tricornutum*, *Rhodomonas* sp. and *Emiliania huxleyi*, carbon-normalized and per-cell contents of $C_{37}$ - $C_{39}$ total alkenones (Alkenones/POC and Alkenones/cell), per-cell contents of $C_{37}$ alkenones ($C_{37}$/Cell), $C_{38}$ alkenones ($C_{38}$/Cell), $C_{38}$ ethyl ketones ($C_{38}$ Et/Cell) and $C_{38}$ methyl ketones ($C_{38}$ Me/Cell), and the ratios of $C_{37}/C_{38}$ alkenones and $C_{38}$ Et/$C_{38}$ Me ($C_{38}$ Et/Me) in *E. huxleyi*. Significant *p* values are shown in bold; T: temperature; N:P: N:P supply ratios.

| Species | Variable | Factor | Coefficient | *SE* | *t* | *p* | *n* |
|---|---|---|---|---|---|---|---|
| *P. tricornutum* | Brassi./epi-brassi./cell | Intercept | -1.482 | 0.077 | -19.187 | < 0.001 | 50 |
| | | T | 0.010 | 0.004 | 2.842 | **0.007** | |
| | | $pCO_2$ | < 0.001 | < 0.001 | 1.656 | 0.105 | |
| | | N:P | 0.003 | 0.001 | 4.199 | **< 0.001** | |
| | Brassi./epi-brassi./POC | Intercept | 7.043 | 0.820 | 8.589 | < 0.001 | 52 |
| | | T | 0.040 | 0.039 | 1.021 | 0.312 | |
| | | $pCO_2$ | < 0.001 | < 0.001 | 0.452 | 0.653 | |
| | | N:P | -0.012 | 0.008 | -1.464 | 0.150 | |
| *Rhodomonas* sp. | Brassi./epi-brassi./cell | Intercept | -0.388 | 0.103 | -3.750 | < 0.001 | 51 |
| | | T | -0.006 | 0.005 | -1.290 | 0.203 | |
| | | $pCO_2$ | < 0.001 | < 0.001 | 0.368 | 0.714 | |
| | | N:P | 0.002 | 0.001 | 2.092 | **0.042** | |
| | Brassi./epi-brassi./POC | Intercept | 1.484 | 0.186 | 7.987 | < 0.001 | 54 |
| | | T | 0.012 | 0.008 | 1.435 | 0.158 | |
| | | $pCO_2$ | < 0.001 | < 0.001 | -2.706 | **0.009** | |
| | | N:P | -0.002 | 0.002 | -0.868 | 0.389 | |
| *E. huxleyi* | Brassi./epi-brassi./cell | Intercept | -1.189 | 0.131 | -9.085 | < 0.001 | 54 |
| | | T | -0.003 | 0.006 | -0.450 | 0.655 | |
| | | $pCO_2$ | < 0.001 | < 0.001 | 1.485 | 0.144 | |
| | | N:P | 0.003 | 0.001 | 2.367 | **0.022** | |
| | Brassi./epi-brassi./POC | Intercept | 5.860 | 0.709 | 8.262 | < 0.001 | 54 |
| | | T | 0.081 | 0.033 | 2.447 | **0.018** | |
| | | $pCO_2$ | < 0.001 | < 0.001 | -0.203 | 0.840 | |
| | | N:P | -0.043 | 0.007 | -6.017 | **< 0.001** | |
| | Alkenones/Cell | Intercept | 0.143 | 0.126 | 1.134 | 0.263 | 52 |
| | | T | -0.009 | 0.006 | -1.452 | 0.153 | |
| | | $pCO_2$ | < 0.001 | < 0.001 | 1.663 | 0.103 | |
| | | N:P | 0.006 | 0.001 | 4.973 | **< 0.001** | |
| | Alkenones/POC | Intercept | 122.935 | 25.130 | 4.892 | < 0.001 | 52 |
| | | T | 0.468 | 1.183 | 0.396 | 0.694 | |
| | | $pCO_2$ | -0.001 | 0.006 | -0.090 | 0.929 | |
| | | N:P | -0.172 | 0.258 | -0.665 | 0.509 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| $C_{37}$/Cell | Intercept | -0.192 | 0.121 | -1.591 | 0.118 | 52 |
| | T | -0.004 | 0.006 | -0.772 | 0.444 | |
| | $pCO_2$ | < 0.001 | < 0.001 | 1.509 | 0.138 | |
| | N:P | 0.007 | 0.001 | 5.466 | **< 0.001** | |
| $C_{38}$/Cell | Intercept | -0.181 | 0.134 | -1.351 | 0.183 | 52 |
| | T | -0.012 | 0.006 | -1.922 | 0.061 | |
| | $pCO_2$ | < 0.001 | < 0.001 | 1.765 | 0.084 | |
| | N:P | 0.006 | 0.001 | 4.356 | **< 0.001** | |
| $C_{38}$ Et/Cell | Intercept | -0.509 | 0.150 | -3.396 | < 0.001 | 52 |
| | T | -0.004 | 0.007 | -0.569 | 0.572 | |
| | $pCO_2$ | < 0.001 | < 0.001 | 1.512 | 0.137 | |
| | N:P | 0.006 | 0.002 | 4.054 | **< 0.001** | |
| $C_{38}$ Me/Cell | Intercept | -0.305 | 0.113 | -2.702 | 0.010 | 52 |
| | T | -0.032 | 0.005 | -6.045 | **< 0.001** | |
| | $pCO_2$ | < 0.001 | < 0.001 | 2.181 | **0.034** | |
| | N:P | 0.005 | 0.001 | 4.648 | **< 0.001** | |
| $C_{37}/C_{38}$ | Intercept | 0.922 | 0.084 | 11.010 | < 0.001 | 54 |
| | T | 0.024 | 0.004 | 6.094 | **< 0.001** | |
| | $pCO_2$ | < 0.001 | < 0.001 | -2.179 | **0.034** | |
| | N:P | 0.003 | 0.001 | 3.348 | **0.002** | |
| $C_{38}$ Et/Me | Intercept | 0.895 | 0.091 | 9.872 | < 0.001 | 54 |
| | T | -0.028 | 0.004 | -6.524 | **< 0.001** | |
| | $pCO_2$ | < 0.001 | < 0.001 | 0.464 | 0.644 | |
| | N:P | -0.001 | 0.001 | -0.749 | 0.457 | |

**Table 2.** The mean percent differences in carbon-normalized and per-cell contents of brassicasterol/epi-brassicasterol (brassi./epi-brassi./POC and brassi./epi-brassi./cell; $\mu$g mg $C^{-1}$ and pg $cell^{-1}$), total fatty acids (TFAs/POC and TFAs/cell), polyunsaturated fatty acids (PUFAs/POC and PUFAs/cell), and per-cell contents of particulate organic carbon (POC/cell) in *Phaeodactylum tricornutum*, *Rhodomonas* sp. and *Emiliania huxleyi*; carbon-normalized and per-cell contents of total alkenones (Alkenones/POC and Alkenones/cell), and per-cell contents of $C_{37}$ alkenones ($C_{37}$/cell), $C_{38}$ alkenones ($C_{38}$/cell), $C_{38}$ ethyl ketones ($C_{38}$ Et/cell), $C_{38}$ methyl ketones ($C_{38}$ Me/cell) in *E. huxleyi*; $C_{37}/C_{38}$ alkenone ratios ($C_{37}/C_{38}$) and $C_{38}$ ethyl/$C_{38}$ methyl ratios ($C_{38}$ Et/Me) in *E. huxleyi* between cold and warm treatments, under N and P deficiency (-N and -P) and enhanced $pCO_2$ conditions. Only significant changes are shown according to GLMMs (Table 1).

| Lipids or POC | Species | Effect | | | | |
|---|---|---|---|---|---|---|
| | | Warming | -N | -P | Enhanced $pCO_2$ | Interaction |
| Brassi./epi-brassi./cell | *P. tricornutum* | +23% | -7% | +37% | | |
| | *Rhodomonas* sp. | | +21% | +51% | | |
| | *E. huxleyi* | | -46% | -3% | | |
| Brassi./epi-brassi./POC | *P. tricornutum* | | | | | |
| | *Rhodomonas* sp. | | | | -21% | |
| | *E. huxleyi* | +12% | -8% | -37% | | |
| Alkenones/cell | *E. huxleyi* | | -35% | +49% | | |
| Alkenones/POC | *E. huxleyi* | | | | | |
| $C_{37}$/cell | *E. huxleyi* | | -34% | +59% | | |
| $C_{38}$/cell | *E. huxleyi* | | -38% | +37% | | |
| $C_{38}$ Et/cell | *E. huxleyi* | | -47% | +27% | | |
| $C_{38}$ Me/cell | *E. huxleyi* | -39% | -16% | +62% | +25% | |
| $C_{37}/C_{38}$ | *E. huxleyi* | +15% | +13% | +21% | -6% | |
| $C_{38}$ Et/Me | *E. huxleyi* | +82% | | | | |
| TFAs/cell | *P. tricornutum*[a] | +19% | +1% | +77% | | |
| | *Rhodomonas* sp.[a] | | +124% | +218% | | |
| | *E. huxleyi*[b] | -29% | -11% | +72% | | |
| TFAs/POC | *P. tricornutum*[a] | | +2% | +12% | | |
| | *Rhodomonas* sp.[a] | | +78% | +108% | | |
| | *E. huxleyi*[b] | -22% | | | -20% | |
| PUFAs/cell | *P. tricornutum*[a] | | -4% | +71% | | |
| | *Rhodomonas* sp.[a] | | +86% | +165% | | |
| | *E. huxleyi*[b] | -19% | -9% | +93% | | |
| PUFAs/POC | *P. tricornutum*[a] | -20% | -2% | +9% | | T × N:P |
| | *Rhodomonas* sp.[a] | | +58% | +76% | | |
| | *E. huxleyi*[b] | | | | -24% | |
| POC/cell | *P. tricornutum*[a] | +23% | -0.3% | +64% | | |
| | *Rhodomonas* sp.[a] | -12% | +26% | +59% | | T × N:P |
| | *E. huxleyi*[b] | -8% | -39% | +50% | | T × N:P |

Decrease Increase

[a and b] Data are from Bi et al. (2017, 2018), respectively.

900

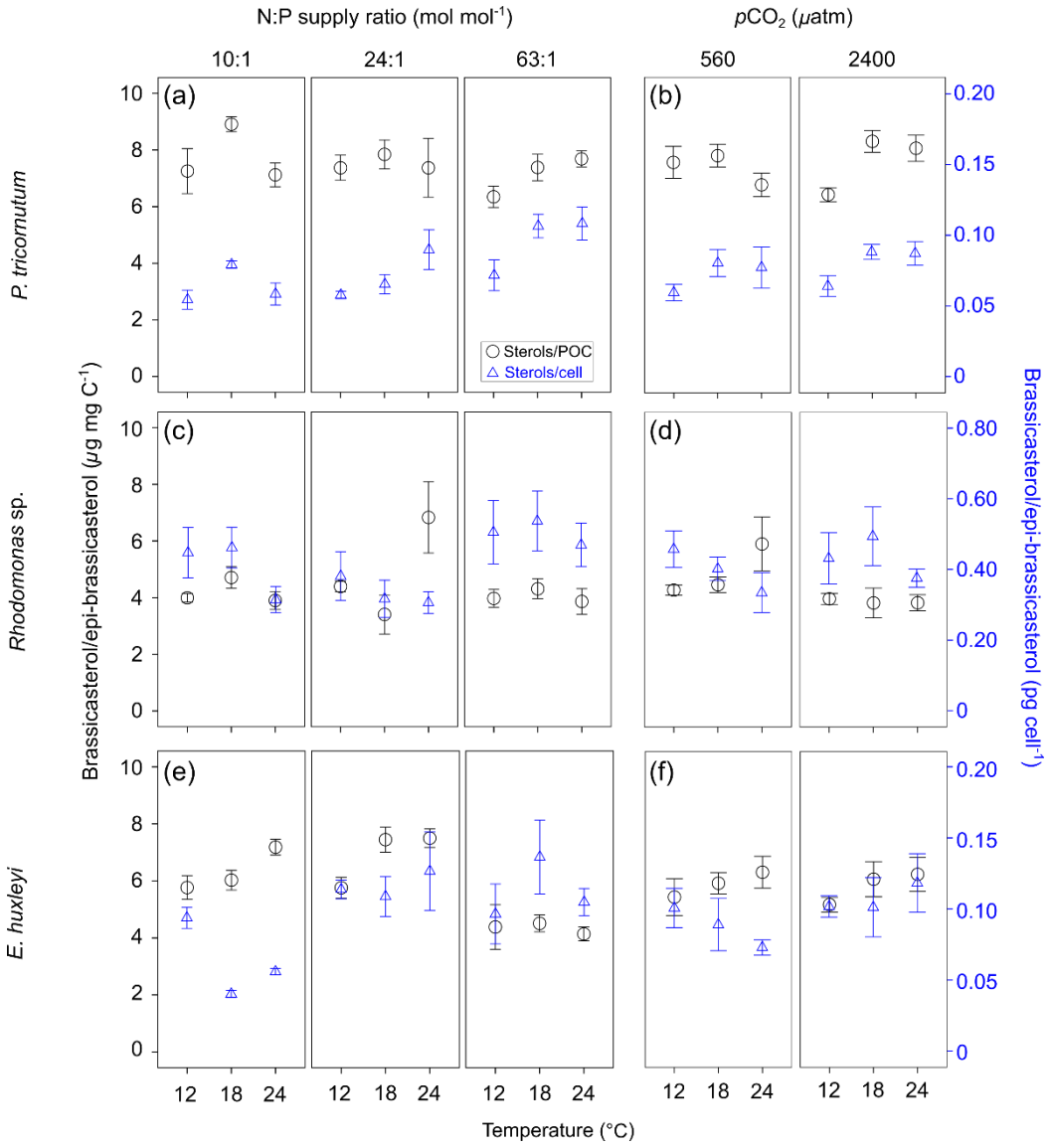
**Fig. 1** Carbon-normalized (open circles) and per-cell (open triangles) contents of brassicasterol/epi-brassicasterol (mean ± SE; $\mu$g mg $C^{-1}$ and pg $cell^{-1}$, respectively) in response to the changes in temperature, N:P supply ratios and $pCO_2$ in *Phaeodactylum tricornutum* (a and b), *Rhodomonas* sp. (c and d) and *Emiliania huxleyi* (e and f). Triplicates were set for each treatment.

**Fig. 2** Carbon-normalized (open circles) and per-cell (open triangles) contents of $C_{37}$ - $C_{39}$ total alkenones (mean ± SE; $\mu$g mg $C^{-1}$ and pg $cell^{-1}$, respectively) (a and b), $C_{37}/C_{38}$ alkenone ratios (c and d) and $C_{38}$ ethyl/$C_{38}$ methyl ratios ($C_{38}$ Et/Me alkenone ratios) (e and f) in *Emiliania huxleyi* in response to the changes in temperature, N:P supply ratios and $pCO_2$. Triplicates were set for each treatment.

**Fig. 3** PCA biplot based on the carbon-normalized contents of the major sterol (brassicasterol/epi-brassicasterol) and total fatty acids ($\mu$g $mgC^{-1}$) in *Phaeodactylum tricornutum*, *Rhodomonas* sp. and *Emiliania huxleyi*, and $C_{37}$ - $C_{39}$ total alkenones in *E. huxleyi* under different temperatures, N:P supply ratios and $pCO_2$. Blue, black and red symbols represent 12, 18 and 24°C, respectively. Open triangles, open circles and closed circles represent N:P molar ratios of 10:1, 24:1 and 63:1, respectively. The first two dimensions (Dims) account for 44.1% of the total variance. The length of each vector reflects the combined loading of each variable in the first two Dims (Table S5).

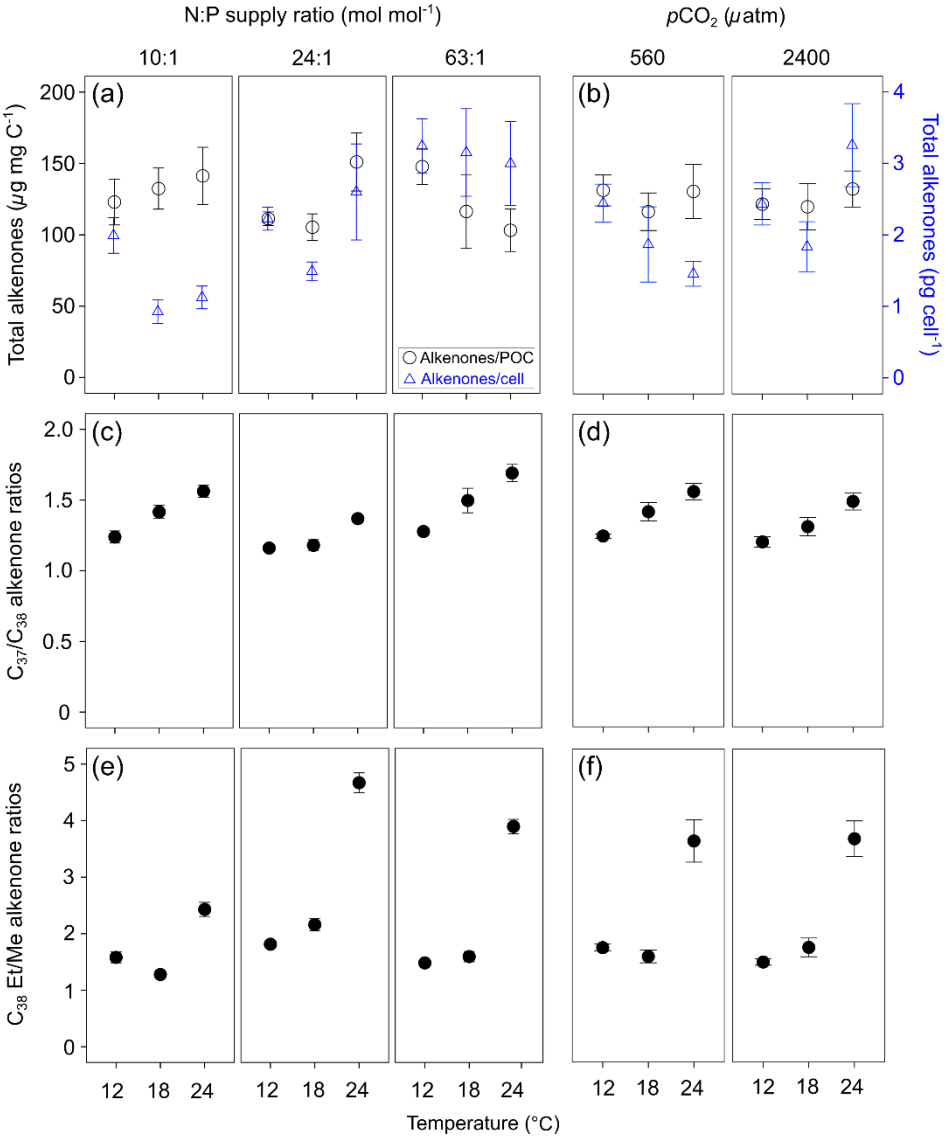
935

**Fig. 1**


N:P supply ratio (mol mol-1)
pCO2 (µatm)
10:1
24:1
63:1
560
2400
(a)
(b)
(c)
(d)
(e)
(f)
P. tricornutum
Rhodomonas sp.
E. huxleyi
Sterols/POC
Sterols/cell
Brassicasterol/epi-brassicasterol (µg mg C-1)
Brassicasterol/epi-brassicasterol (pg cell-1)
Temperature (°C)
12
18
24

940

**Fig. 2**


N:P supply ratio (mol mol-1)
pCO2 (μatm)
10:1
24:1
63:1
560
2400
(a)
(b)
(c)
(d)
(e)
(f)
Total alkenones (μg mg C-1)
Total alkenones (pg cell-1)
C37/C38 alkenone ratios
C38 Et/Me alkenone ratios
Alkenones/POC
Alkenones/cell
Temperature (°C)

945

950

**Fig. 3**

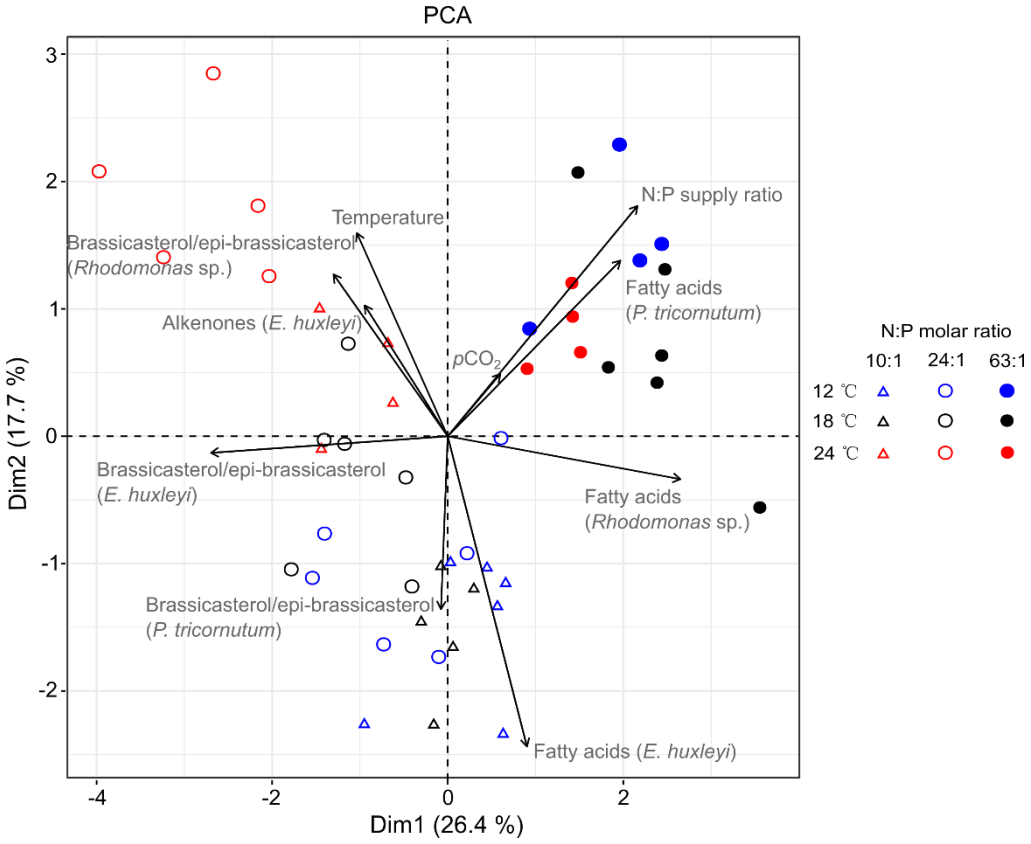

PCA
Dim2 (17.7 %)
Dim1 (26.4 %)
3
2
1
0
-1
-2
-4
-2
0
2
Temperature
N:P supply ratio
Brassicasterol/epi-brassicasterol
(Rhodomonas sp.)
Fatty acids
(P. tricornutum)
Alkenones (E. huxleyi)
pCO2
Brassicasterol/epi-brassicasterol
(E. huxleyi)
Fatty acids
(Rhodomonas sp.)
Brassicasterol/epi-brassicasterol
(P. tricornutum)
Fatty acids (E. huxleyi)
N:P molar ratio
10:1
24:1
63:1
12 ℃
18 ℃
24 ℃

**Table S1.** Measured dissolved inorganic carbon (DIC) and total alkalinity (TA), calculated $pCO_2$, and pH (mean ± SE) in the cultures of *Phaeodactylum tricornutum*, *Rhodomonas* sp. and *Emiliania huxleyi* on the sampling days at steady state. Triplicates were set for each treatment.

| Species | | Treatment | | DIC ($\mu$mol kg$^{-1}$) | TA ($\mu$mol kg$^{-1}$) | $pCO_2$ ($\mu$atm) | pH |
|---|---|---|---|---|---|---|---|
| *P. tricornutum* [a] | 12°C | Low $CO_2$ | N:P = 10:1 | 2476 ± 12 | 2505 ± 7.0 | 1479 ± 97 | 7.788 ± 0.008 |
| | | | N:P = 24:1 | 2425 ± 16 | 2551 ± 0.7 | 728 ± 78 | 8.057 ± 0.017 |
| | | | N:P = 63:1 | 2404 ± 18 | 2533 ± 5.6 | 714 ± 72 | 7.996 ± 0.004 |
| | | High $CO_2$ | N:P = 10:1 | 2634 ± 17 | 2502 ± 0.4 | 4032 ± 353 | 7.510 ± 0.016 |
| | | | N:P = 24:1 | 2628 ± 28 | 2534 ± 1.4 | 3300 ± 558 | 7.557 ± 0.044 |
| | | | N:P = 63:1 | 2629 ± 5.2 | 2520 ± 3.3 | 3542 ± 159 | 7.417 ± 0.010 |
| | 18°C | Low $CO_2$ | N:P = 10:1 | 2392 ± 11 | 2462 ± 0.5 | 1289 ± 103 | 7.856 ± 0.025 |
| | | | N:P = 24:1 | 2273 ± 9.7 | 2535 ± 7.4 | 435 ± 21 | 8.230 ± 0.029 |
| | | | N:P = 63:1 | 2421 ± 22 | 2512 ± 2.3 | 1167 ± 160 | 7.902 ± 0.019 |
| | | High $CO_2$ | N:P = 10:1 | 2564 ± 8.2 | 2466 ± 3.9 | 4045 ± 204 | 7.381 ± 0.019 |
| | | | N:P = 24:1 | 2594 ± 8.1 | 2545 ± 2.1 | 3059 ± 128 | 7.584 ± 0.025 |
| | | | N:P = 63:1 | 2551 ± 23 | 2540 ± 2.4 | 2401 ± 346 | 7.598 ± 0.013 |
| | 24°C | Low $CO_2$ | N:P = 10:1 | 2350 ± 15 | 2464 ± 1.8 | 1217 ± 110 | 8.033 ± 0.035 |
| | | | N:P = 24:1 | 2280 ± 32 | 2494 ± 1.1 | 704 ± 103 | 8.233 ± 0.043 |
| | | | N:P = 63:1 | 2330 ± 10 | 2470 ± 0.6 | 1015 ± 59 | 8.067 ± 0.009 |
| | | High $CO_2$ | N:P = 10:1 | 2521 ± 2.5 | 2480 ± 0.4 | 3473 ± 86 | 7.449 ± 0.025 |
| | | | N:P = 24:1 | 2514 ± 0.04 | 2503 ± 0.8 | 2865 ± 20 | 7.483 ± 0.054 |
| | | | N:P = 63:1 | 2557 ± 14 | 2497 ± 3.5 | 3928 ± 335 | 7.381 ± 0.018 |
| *Rhodomonas* sp. [a] | 12°C | Low $CO_2$ | N:P = 10:1 | 2181 ± 6.5 | 2289 ± 0.2 | 725 ± 20 | 8.016 ± 0.009 |
| | | | N:P = 24:1 | 2238 ± 16 | 2345 ± 2.6 | 745 ± 100 | 8.072 ± 0.017 |
| | | | N:P = 63:1 | 2255 ± 14 | 2317 ± 1.7 | 1012 ± 111 | 7.940 ± 0.005 |
| | | High $CO_2$ | N:P = 10:1 | 2368 ± 7.3 | 2298 ± 2.0 | 2726 ± 138 | 7.456 ± 0.003 |
| | | | N:P = 24:1 | 2418 ± 2.7 | 2349 ± 5.0 | 2724 ± 58 | 7.506 ± 0.017 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | N:P = 63:1 | 2420 ± 14 | 2313 ± 2.0 | 3420 ± 315 | 7.333 ± 0.005 |
| | 18°C | Low $CO_2$ | N:P = 10:1 | 2211 ± 15 | 2287 ± 0.3 | 1138 ± 136 | 7.958 ± 0.019 |
| | | | N:P = 24:1 | 2255 ± 8.0 | 2342 ± 2.1 | 1073 ± 70 | 7.979 ± 0.017 |
| | | | N:P = 63:1 | 2209 ± 7.7 | 2311 ± 1.5 | 945 ± 61 | 7.970 ± 0.021 |
| | | High $CO_2$ | N:P = 10:1 | 2387 | 2285 ± 2.3 | 4028 | 7.399 ± 0.013 |
| | | | N:P = 24:1 | 2423 ± 17 | 2347 ± 1.7 | 3489 ± 414 | 7.472 ± 0.014 |
| | | | N:P = 63:1 | 2388 ± 2.5 | 2310 ± 1.1 | 3486 ± 74 | 7.448 ± 0.012 |
| | 24°C | Low $CO_2$ | N:P = 10:1 | 2188 ± 7.4 | 2302 ± 0.5 | 1083 ± 56 | 7.939 ± 0.010 |
| | | | N:P = 24:1 | 2201 ± 17 | 2306 ± 3.2 | 1160 ± 109 | 7.927 ± 0.016 |
| | | | N:P = 63:1 | 2189 ± 12 | 2288 ± 4.6 | 1210 ± 126 | 7.920 ± 0.009 |
| | | High $CO_2$ | N:P = 10:1 | 2383 ± 15 | 2297 ± 1.3 | 4395 ± 347 | 7.372 ± 0.011 |
| | | | N:P = 24:1 | 2398 ± 8.4 | 2318 ± 5.1 | 4259 ± 95 | 7.372 ± 0.010 |
| | | | N:P = 63:1 | 2408 ± 3.4 | 2306 ± 2.2 | 4814 ± 138 | 7.789 ± 0.183 |
| *E. huxleyi* [b] | 12 °C | Low $pCO_2$ | N:P = 10:1 | 1302 ± 54 | 1269 ± 57 | 1509 ± 35 | 7.803 ± 0.007 |
| | | | N:P = 24:1 | 1328 ± 18 | 1292 ± 27 | 1564 ± 149 | 7.710 ± 0.039 |
| | | | N:P = 63:1 | 1374 ± 25 | 1349 ± 24 | 1412 ± 21 | 7.832 ± 0.004 |
| | | High $pCO_2$ | N:P = 10:1 | 1956 ± 46 | 1962 ± 50 | 1357 ± 14 | 7.504 ± 0.034 |
| | | | N:P = 24:1 | 2042 ± 17 | 2053 ± 17 | 1357 ± 76 | 7.466 ± 0.001 |
| | | | N:P = 63:1 | 1829 ± 22 | 1801 ± 49 | 1041 ± 191 | 7.494 ± 0.014 |
| | 18 °C | Low $pCO_2$ | N:P = 10:1 | 763 ± 15 | 793 ± 4 | 552 ± 118 | 8.032 ± 0.044 |
| | | | N:P = 24:1 | 885 ± 6 | 922 ± 12 | 567 ± 84 | 7.906 ± 0.015 |
| | | | N:P = 63:1 | 1065 ± 3 | 1108 ± 8 | 633 ± 44 | 8.072 ± 0.001 |
| | | High $pCO_2$ | N:P = 10:1 | 1415 ± 154 | 1454 ± 121 | 1113 ± 489 | 7.704 ± 0.077 |
| | | | N:P = 24:1 | 1278 ± 13 | 1196 ± 18 | 2944 ± 330 | 7.581 ± 0.079 |
| | | | N:P = 63:1 | 1613 ± 35 | 1620 ± 32 | 1507 ± 332 | 7.705 ± 0.010 |
| | 24 °C | Low $pCO_2$ | N:P = 10:1 | 785 ± 13 | 808 ± 10 | 845 ± 256 | 8.105 ± 0.007 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | N:P = 24:1 | 809 ± 10 | 682 ± 11 | - | 8.042 ± 0.017 |
| | | N:P = 63:1 | 1243 ± 16 | 1231 ± 10 | 1734 ± 163 | 7.980 ± 0.039 |
| | High $pCO_2$ | N:P = 10:1 | 1266 ± 22 | 1240 ± 20 | 2079 ± 406 | 7.625 ± 0.004 |
| | | N:P = 24:1 | 1596 ± 63 | 1691 ± 36 | 1163 ± 190 | 7.608 ± 0.007 |
| | | N:P = 63:1 | 1616 ± 27 | 1550 ± 34 | 3295 ± 171 | 7.553 ± 0.029 |

[a] Data for *Phaeodactylum tricornutum* and *Rhodomonas* sp. are modified after Bi et al. (2017). [b] Data for *Emiliania huxleyi* are from Bi et al. (2018).

**Table S2.** Results of Akaike information criterion corrected (AICc) in the generalized linear mixed models (GLMMs) testing for the effects of temperature, N:P supply ratios and $pCO_2$ on carbon-normalized and per-cell contents of brassicasterol/epi-brassicasterol (brassi./epi-brassi./POC and brassi./epi-brassi./cell; $\mu$g mg $C^{-1}$ and pg $cell^{-1}$) in *Phaeodactylum tricornutum*, *Rhodomonas* sp. and *Emiliania huxleyi*, and carbon-normalized and per-cell contents of $C_{37}$ - $C_{39}$ total alkenones (alkenones/POC and alkenones/cell), per-cell contents of $C_{37}$ alkenones ($C_{37}$/Cell), $C_{38}$ alkenones ($C_{38}$/Cell), $C_{38}$ ethyl ketones ($C_{38}$ Et/Cell) and $C_{38}$ methyl ketones ($C_{38}$ Me/Cell), $C_{37}/C_{38}$ alkenone ratios, and $C_{38}$ Et/$C_{38}$ Me ($C_{38}$ Et/Me) in *E. huxleyi*.

| Species | Response variable | Effect builder | AICc |
|---|---|---|---|
| *P. tricornutum* | Brassi./epi-brassi./POC | Main, two way and three way | 271 |
| | | Main, two way | 248 |
| | | **Main** | **207** |
| | Brassi./epi-brassi./cell | Main, two way and three way | 63 |
| | | Main, two way | 39 |
| | | **Main** | **-21** |
| *Rhodomonas* sp. | Brassi./epi-brassi./POC | Main, two way and three way | 197 |
| | | Main, two way | 194 |
| | | **Main** | **191** |
| | Brassi./epi-brassi./cell | Main, two way and three way | 94 |
| | | Main, two way | 66 |
| | | **Main** | **5** |
| *E. huxleyi* | Brassi./epi-brassi./POC | Main, two way and three way | 272 |
| | | Main, two way | 248 |
| | | **Main** | **200** |
| | Brassi./epi-brassi./cell | Main, two way and three way | 116 |
| | | Main, two way | 89 |
| | | **Main** | **31** |
| | Alkenones/POC | Main, two way and three way | 575 |

| | | |
|---|---|---|
| | Main, two way | 558 |
| | **Main** | **534** |
| Alkenones/Cell | Main, two way and three way | 109 |
| | Main, two way | 82 |
| | **Main** | **26** |
| $C_{37}$/Cell | Main, two way and three way | 105 |
| | Main, two way | 77 |
| | **Main** | **22** |
| $C_{38}$/Cell | Main, two way and three way | 115 |
| | Main, two way | 88 |
| | **Main** | **32** |
| $C_{38}$ Et/Cell | Main, two way and three way | 125 |
| | Main, two way | 98 |
| | **Main** | **42** |
| $C_{38}$ Me/Cell | Main, two way and three way | 100 |
| | Main, two way | 73 |
| | **Main** | **15** |
| $C_{37}/C_{38}$ alkenone ratios | Main, two way and three way | 79 |
| | Main, two way | 50 |
| | **Main** | **-13** |
| $C_{38}$ Et/Me | Main, two way and three way | 83 |
| | Main, two way | 55 |
| | **Main** | **-5** |

The selected models are shown in bold.

**Table S3.** Brassicasterol/epi-brassicasterol contents (each $\mu$g mg $C^{-1}$ and pg $cell^{-1}$; mean ± SE) in *Phaeodactylum tricornutum*, *Rhodomonas* sp. and *Emiliania huxleyi* as a function of temperature, N:P supply ratios and $pCO_2$. Triplicates were set for each treatment.

| Treatment | | | *P. tricornutum* | | *Rhodomonas* sp. | | *E. huxleyi* | |
|---|---|---|---|---|---|---|---|---|
| | | | $\mu$g mg $C^{-1}$ | pg $cell^{-1}$ | $\mu$g mg $C^{-1}$ | pg $cell^{-1}$ | $\mu$g mg $C^{-1}$ | pg $cell^{-1}$ |
| 12 ºC | Low $pCO_2$ | N:P = 10:1 | 8.00 ± 1.59 | 0.06 ± 0.01 | 4.02 ± 0.18 | 0.37 ± 0.02 | 6.08 ± 0.69 | 0.11 ± 0.01 |
| | | N:P = 24:1 | 8.34 ± 0.10 | 0.05 ± 0.002 | 4.49 ± 0.42 | 0.48 ± 0.11 | 6.24 ± 0.44 | 0.11 ± 0.01 |
| | | N:P = 63:1 | 6.33 ± 0.30 | 0.06 ± 0.01 | 4.31 ± 0.34 | 0.50 ± 0.10 | 3.99 ± 1.66 | 0.08 ± 0.04 |
| | High $pCO_2$ | N:P = 10:1 | 6.51 ± 0.29 | 0.05 ± 0.002 | 3.96 ± 0.28 | 0.50 ± 0.12 | 5.46 ± 0.55 | 0.08 ± 0.01 |
| | | N:P = 24:1 | 6.40 ± 0.18 | 0.06 ± 0.003 | 4.30 ± 0.04 | 0.29 ± 0.06 | 5.28 ± 0.51 | 0.11 ± 0.01 |
| | | N:P = 63:1 | 6.36 ± 0.78 | 0.08 ± 0.02 | 3.64 ± 0.52 | 0.51 ± 0.18 | 4.77 ± 0.38 | 0.11 ± 0.01 |
| 18 ºC | Low $pCO_2$ | N:P = 10:1 | 8.53 ± 0.11 | 0.08 ± 0.002 | 4.47 ± 0.80 | 0.41 ± 0.06 | 5.73 ± 0.16 | 0.04 ± 0.001 |
| | | N:P = 24:1 | 7.43 ± 0.94 | 0.05 ± 0.01 | 4.91 ± 0.13 | 0.32 ± 0.03 | 7.19 ± 0.21 | 0.08 ± 0.01 |
| | | N:P = 63:1 | 7.42 ± 0.82 | 0.11 ± 0.02 | 4.01 ± 0.23 | 0.48 ± 0.05 | 4.85 ± 0.44 | 0.15 ± 0.03 |
| | High $pCO_2$ | N:P = 10:1 | 9.29 ± 0.44 | 0.08 ± 0.003 | 4.95 ± 0.12 | 0.51 ± 0.10 | 6.33 ± 0.70 | 0.04 ± 0.01 |
| | | N:P = 24:1 | 8.26 ± 0.52 | 0.08 ± 0.01 | 1.91 ± 0.34 | 0.32 ± 0.16 | 7.70 ± 0.92 | 0.14 ± 0.01 |
| | | N:P = 63:1 | 7.35 ± 0.64 | 0.10 ± 0.01 | 4.61 ± 0.69 | 0.59 ± 0.17 | 4.17 ± 0.35 | 0.13 ± 0.05 |
| 24 ºC | Low $pCO_2$ | N:P = 10:1 | 6.31 ± 0.75 | 0.05 ± 0.01 | 3.36 ± 0.16 | 0.25 ± 0.04 | 7.54 ± 0.24 | 0.06 ± 0.003 |
| | | N:P = 24:1 | 6.36 ± 0.91 | 0.07 ± 0.02 | 9.56 ± 0.55 | 0.29 ± 0.03 | 7.15 ± 0.56 | 0.07 ± 0.002 |
| | | N:P = 63:1 | 7.47 ± 0.44 | 0.13 ± 0.01 | 4.79 ± 0.40 | 0.54 ± 0.16 | 4.25 ± 0.43 | 0.09 ± 0.01 |
| | High $pCO_2$ | N:P = 10:1 | 7.66 ± 0.17 | 0.07 ± 0.01 | 4.44 ± 0.44 | 0.38 ± 0.03 | 6.83 ± 0.46 | 0.05 ± 0.003 |
| | | N:P = 24:1 | 8.88 ± 2.10 | 0.11 ± 0.004 | 4.10 ± 0.39 | 0.32 ± 0.06 | 7.85 ± 0.31 | 0.19 ± 0.02 |
| | | N:P = 63:1 | 7.90 ± 0.40 | 0.09 ± 0.01 | 2.94 ± 0.08 | 0.42 ± 0.03 | 4.03 ± 0.30 | 0.12 ± 0.02 |

**Table S4.** Alkenone contents (each $\mu$g mg $C^{-1}$ and pg $cell^{-1}$; mean ± SE) in *Emiliania huxleyi* as a function of temperature, N:P supply ratios and $pCO_2$. Triplicates were set for each treatment.

| Treatment | | | $C_{37:4}$ Me | | $C_{37:3}$ Me | | $C_{37:2}$ Me | | $C_{38:3}$ Et | | $C_{38:3}$ Me | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $\mu$g mg $C^{-1}$ | pg $cell^{-1}$ | $\mu$g mg $C^{-1}$ | pg $cell^{-1}$ | $\mu$g mg $C^{-1}$ | pg $cell^{-1}$ | $\mu$g mg $C^{-1}$ | pg $cell^{-1}$ | $\mu$g mg $C^{-1}$ | pg $cell^{-1}$ |
| 12 ºC | Low $pCO_2$ | N:P = 10:1 | 5.10 ± 0.61 | 0.09 ± 0.01 | 52.6 ± 9.17 | 0.91 ± 0.12 | 13.3 ± 2.63 | 0.23 ± 0.03 | 22.5 ± 3.95 | 0.39 ± 0.05 | 16.2 ± 3.21 | 0.28 ± 0.04 |
| | | N:P = 24:1 | 4.90 ± 0.38 | 0.09 ± 0.01 | 43.3 ± 3.67 | 0.79 ± 0.09 | 10.5 ± 0.83 | 0.19 ± 0.02 | 21.1 ± 1.95 | 0.39 ± 0.05 | 13.6 ± 1.47 | 0.25 ± 0.03 |
| | | N:P = 63:1 | 7.17 ± 1.22 | 0.15 ± 0.05 | 56.9 ± 3.28 | 0.80 ± 0.42 | 18.7 ± 0.31 | 0.26 ± 0.13 | 24.3 ± 2.20 | 0.51 ± 0.15 | 19.3 ± 1.28 | 0.40 ± 0.11 |
| | High $pCO_2$ | N:P = 10:1 | 5.38 ± 0.25 | 0.08 ± 0.01 | 40.9 ± 7.59 | 0.61 ± 0.11 | 11.0 ± 3.32 | 0.16 ± 0.05 | 18.0 ± 3.28 | 0.27 ± 0.05 | 16.5 ± 3.34 | 0.25 ± 0.05 |
| | | N:P = 24:1 | 4.05 ± 0.19 | 0.09 ± 0.01 | 39.5 ± 1.67 | 0.86 ± 0.07 | 11.3 ± 0.46 | 0.24 ± 0.02 | 18.8 ± 1.04 | 0.41 ± 0.03 | 14.3 ± 0.68 | 0.31 ± 0.03 |
| | | N:P = 63:1 | 6.15 ± 0.63 | 0.14 ± 0.02 | 49.7 ± 6.17 | 1.13 ± 0.16 | 19.8 ± 4.47 | 0.45 ± 0.11 | 18.4 ± 1.79 | 0.42 ± 0.05 | 18.1 ± 2.35 | 0.41 ± 0.05 |
| 18 ºC | Low $pCO_2$ | N:P = 10:1 | 1.01 ± 0.06 | 0.01 ± 0.001 | 41.1 ± 8.74 | 0.29 ± 0.08 | 25.3 ± 6.89 | 0.18 ± 0.06 | 10.7 ± 2.25 | 0.08 ± 0.02 | 13.2 ± 3.02 | 0.09 ± 0.03 |
| | | N:P = 24:1 | 0.90 ± 0.07 | 0.01 ± 0.002 | 39.0 ± 3.39 | 0.45 ± 0.09 | 25.5 ± 2.14 | 0.30 ± 0.06 | 14.3 ± 1.07 | 0.17 ± 0.03 | 10.8 ± 0.88 | 0.13 ± 0.02 |
| | | N:P = 63:1 | 1.10 ± 0.15 | 0.03 ± 0.01 | 36.9 ± 8.36 | 1.15 ± 0.38 | 22.8 ± 9.16 | 0.72 ± 0.30 | 10.4 ± 2.95 | 0.33 ± 0.12 | 9.72 ± 2.41 | 0.31 ± 0.10 |
| | High $pCO_2$ | N:P = 10:1 | 1.58 ± 0.34 | 0.01 ± 0.00 | 51.8 ± 2.14 | 0.35 ± 0.07 | 27.3 ± 2.51 | 0.19 ± 0.06 | 14.1 ± 0.43 | 0.10 ± 0.02 | 16.4 ± 0.58 | 0.11 ± 0.03 |
| | | N:P = 24:1 | 0.72 ± 0.16 | 0.01 ± 0.002 | 28.7 ± 2.65 | 0.51 ± 0.04 | 14.7 ± 1.55 | 0.26 ± 0.01 | 14.4 ± 0.90 | 0.25 ± 0.01 | 8.06 ± 0.70 | 0.14 ± 0.01 |
| | | N:P = 63:1 | 0.97 ± 0.09 | 0.03 ± 0.01 | 38.0 ± 11.2 | 0.93 ± 0.10 | 30.8 ± 13.3 | 0.69 ± 0.21 | 11.4 ± 3.37 | 0.28 ± 0.03 | 10.8 ± 3.34 | 0.26 ± 0.03 |
| 24 ºC | Low $pCO_2$ | N:P = 10:1 | 0 ± 0 | 0 ± 0 | 18.7 ± 1.22 | 0.15 ± 0.01 | 78.3 ± 9.84 | 0.62 ± 0.09 | 3.90 ± 0.24 | 0.03 ± 0.003 | 3.91 ± 0.21 | 0.03 ± 0.003 |
| | | N:P = 24:1 | 0 ± 0 | 0 ± 0 | 17.4 ± 2.55 | 0.16 ± 0.01 | 60.6 ± 19.4 | 0.56 ± 0.15 | 5.50 ± 0.78 | 0.05 ± 0.004 | 2.51 ± 0.39 | 0.02 ± 0.002 |
| | | N:P = 63:1 | 0 ± 0 | 0 ± 0 | 12.8 ± 3.66 | 0.26 ± 0.05 | 41.7 ± 13.8 | 0.85 ± 0.17 | 3.05 ± 1.02 | 0.06 ± 0.02 | 1.69 ± 0.48 | 0.03 ± 0.01 |
| | High $pCO_2$ | N:P = 10:1 | 0 ± 0 | 0 ± 0 | 20.2 ± 5.41 | 0.15 ± 0.03 | 47.8 ± 13.0 | 0.69 ± 0.31 | 3.26 ± 0.50 | 0.04 ± 0.01 | 3.01 ± 0.39 | 0.03 ± 0.01 |
| | | N:P = 24:1 | 0 ± 0 | 0 ± 0 | 15.7 ± 1.41 | 0.37 ± 0.05 | 76.1 ± 9.40 | 1.82 ± 0.31 | 5.36 ± 0.38 | 0.13 ± 0.01 | 2.65 ± 0.31 | 0.06 ± 0.01 |
| | | N:P = 63:1 | 0 ± 0 | 0 ± 0 | 20.2 ± 3.18 | 0.56 ± 0.04 | 53.0 ± 1.03 | 1.96 ± 0.21 | 5.32 ± 0.90 | 0.15 ± 0.01 | 2.91 ± 0.46 | 0.08 ± 0.01 |

| | Mean per-cell content | | | | |
|---|---|---|---|---|---|
| | $C_{37:4}$ Me | $C_{37:3}$ Me | $C_{37:2}$ Me | $C_{38:3}$ Et | $C_{38:3}$ Me |
| All treatment | 0 – 0.15 | 0.15 – 1.15 | 0.16 – 1.96 | 0.03 – 0.51 | 0.02 – 0.41 |

**Table S4.** Continued.

| Treatment | | | $C_{38:2}$ Et | | $C_{38:2}$ Me | | $C_{39:3}$ Et* | | $C_{39:2}$ Et* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $\mu$g mg C$^{-1}$ | pg cell$^{-1}$ | $\mu$g mg C$^{-1}$ | pg cell$^{-1}$ | $\mu$g mg C$^{-1}$ | pg cell$^{-1}$ | $\mu$g mg C$^{-1}$ | pg cell$^{-1}$ |
| 12 °C | Low $pCO_2$ | N:P = 10:1 | 13.4 ± 2.85 | 0.23 ± 0.04 | 4.08 ± 1.01 | 0.07 ± 0.01 | 3.81 ± 0.82 | 0.07 ± 0.01 | 2.29 ± 0.53 | 0.04 ± 0.01 |
| | | N:P = 24:1 | 10.9 ± 0.63 | 0.20 ± 0.02 | 3.27 ± 0.50 | 0.06 ± 0.01 | 3.29 ± 0.33 | 0.06 ± 0.01 | 1.88 ± 0.19 | 0.03 ± 0.005 |
| | | N:P = 63:1 | 16.3 ± 0.43 | 0.33 ± 0.06 | 5.71 ± 0.01 | 0.12 ± 0.02 | 4.50 ± 0.21 | 0.09 ± 0.02 | 3.28 ± 0.05 | 0.07 ± 0.01 |
| | High $pCO_2$ | N:P = 10:1 | 10.6 ± 3.72 | 0.16 ± 0.05 | 4.10 ± 1.28 | 0.06 ± 0.02 | 3.84 ± 0.96 | 0.06 ± 0.01 | 2.37 ± 0.78 | 0.04 ± 0.01 |
| | | N:P = 24:1 | 12.1 ± 0.30 | 0.27 ± 0.03 | 3.81 ± 0.12 | 0.08 ± 0.01 | 3.60 ± 0.19 | 0.08 ± 0.01 | 2.33 ± 0.07 | 0.05 ± 0.01 |
| | | N:P = 63:1 | 16.4 ± 4.37 | 0.37 ± 0.10 | 6.42 ± 1.34 | 0.15 ± 0.03 | 3.77 ± 0.64 | 0.09 ± 0.01 | 3.37 ± 0.86 | 0.08 ± 0.02 |
| 18 °C | Low $pCO_2$ | N:P = 10:1 | 16.5 ± 4.73 | 0.12 ± 0.04 | 7.89 ± 2.21 | 0.06 ± 0.02 | 1.95 ± 0.49 | 0.01 ± 0.004 | 3.06 ± 0.93 | 0.02 ± 0.01 |
| | | N:P = 24:1 | 20.3 ± 1.70 | 0.23 ± 0.05 | 6.66 ± 0.47 | 0.08 ± 0.01 | 2.03 ± 0.19 | 0.02 ± 0.005 | 3.08 ± 0.27 | 0.04 ± 0.01 |
| | | N:P = 63:1 | 15.0 ± 6.30 | 0.48 ± 0.21 | 5.85 ± 2.29 | 0.19 ± 0.08 | 1.50 ± 0.42 | 0.05 ± 0.02 | 2.48 ± 1.01 | 0.08 ± 0.03 |
| | High $pCO_2$ | N:P = 10:1 | 18.1 ± 2.53 | 0.13 ± 0.05 | 8.85 ± 1.18 | 0.06 ± 0.02 | 2.54 ± 0.27 | 0.02 ± 0.01 | 3.49 ± 0.63 | 0.03 ± 0.01 |
| | | N:P = 24:1 | 13.5 ± 1.64 | 0.24 ± 0.02 | 3.94 ± 0.37 | 0.07 ± 0.003 | 1.99 ± 0.09 | 0.04 ± 0.001 | 2.07 ± 0.23 | 0.04 ± 0.003 |
| | | N:P = 63:1 | 21.5 ± 9.85 | 0.47 ± 0.16 | 8.24 ± 3.62 | 0.18 ± 0.06 | 1.75 ± 0.59 | 0.04 ± 0.01 | 3.68 ± 1.66 | 0.08 ± 0.03 |
| 24 °C | Low $pCO_2$ | N:P = 10:1 | 40.2 ± 5.94 | 0.32 ± 0.05 | 15.0 ± 1.88 | 0.12 ± 0.02 | 0.25 ± 0.13 | 0.002 ± 0.001 | 4.49 ± 0.63 | 0.04 ± 0.01 |
| | | N:P = 24:1 | 41.3 ± 14.1 | 0.38 ± 0.11 | 7.14 ± 2.07 | 0.07 ± 0.02 | 0.23 ± 0.11 | 0.002 ± 0.001 | 2.87 ± 0.83 | 0.03 ± 0.01 |
| | | N:P = 63:1 | 23.3 ± 8.87 | 0.46 ± 0.12 | 4.87 ± 1.71 | 0.10 ± 0.02 | 0 ± 0 | 0 ± 0 | 1.68 ± 0.68 | 0.03 ± 0.01 |
| | High $pCO_2$ | N:P = 10:1 | 25.3 ± 8.55 | 0.39 ± 0.19 | 9.00 ± 2.73 | 0.12 ± 0.05 | 0.17 ± 0.17 | 0.002 ± 0.001 | 2.75 ± 0.88 | 0.04 ± 0.02 |
| | | N:P = 24:1 | 51.0 ± 5.79 | 1.22 ± 0.19 | 9.59 ± 1.15 | 0.23 ± 0.04 | 0 ± 0 | 0 ± 0 | 4.21 ± 0.48 | 0.10 ± 0.02 |
| | | N:P = 63:1 | 41.2 ± 11.7 | 1.10 ± 0.12 | 8.84 ± 2.21 | 0.24 ± 0.03 | 0.11 ± 0.11 | 0.002 ± 0.002 | 3.24 ± 0.91 | 0.09 ± 0.01 |

| | Mean per-cell content | | | |
|---|---|---|---|---|
| | $C_{38:2}$ Et | $C_{38:2}$ Me | $C_{39:3}$ Et* | $C_{39:2}$ Et* |
| All treatment | 0.12 - 1.22 | 0.06 - 0.24 | 0 - 0.09 | 0.02 - 0.10 |

* Tentatively identified due to low molecular ion abundance.

**Table S4.** Continued.

| Treatment | | | Total alkenones | | $C_{37}/C_{38}$ | $C_{38}$ Et/Me | $U^{K'}_{37}$ |
|---|---|---|---|---|---|---|---|
| | | | $\mu$g mg $C^{-1}$ | pg $cell^{-1}$ | | | |
| 12 ºC | Low $pCO_2$ | N:P = 10:1 | 133.2 ± 24.8 | 2.29 ± 0.31 | 1.27 ± 0.02 | 1.79 ± 0.04 | 0.20 ± 0.003 |
| | | N:P = 24:1 | 112.7 ± 9.69 | 2.06 ± 0.24 | 1.20 ± 0.01 | 1.92 ± 0.08 | 0.20 ± 0.004 |
| | | N:P = 63:1 | 156.2 ± 7.51 | 3.24 ± 0.80 | 1.34 ± 0.08 | 1.55 ± 0.07 | 0.24 ± 0.01 |
| | High $pCO_2$ | N:P = 10:1 | 112.8 ± 24.0 | 1.68 ± 0.34 | 1.20 ± 0.09 | 1.37 ± 0.05 | 0.20 ± 0.03 |
| | | N:P = 24:1 | 109.9 ± 4.17 | 2.38 ± 0.22 | 1.12 ± 0.01 | 1.71 ± 0.03 | 0.22 ± 0.004 |
| | | N:P = 63:1 | 142.1 ± 21.4 | 3.24 ± 0.53 | 1.29 ± 0.04 | 1.41 ± 0.05 | 0.28 ± 0.03 |
| 18 ºC | Low $pCO_2$ | N:P = 10:1 | 120.7 ± 29.3 | 0.85 ± 0.25 | 1.41 ± 0.04 | 1.28 ± 0.02 | 0.37 ± 0.02 |
| | | N:P = 24:1 | 122.6 ± 10.1 | 1.42 ± 0.28 | 1.26 ± 0.01 | 1.98 ± 0.04 | 0.40 ± 0.005 |
| | | N:P = 63:1 | 105.7 ± 32.1 | 3.33 ± 1.22 | 1.58 ± 0.15 | 1.53 ± 0.17 | 0.34 ± 0.08 |
| | High $pCO_2$ | N:P = 10:1 | 144.1 ± 7.07 | 0.99 ± 0.27 | 1.42 ± 0.10 | 1.28 ± 0.03 | 0.35 ± 0.03 |
| | | N:P = 24:1 | 88.1 ± 6.21 | 1.55 ± 0.04 | 1.10 ± 0.05 | 2.34 ± 0.17 | 0.34 ± 0.02 |
| | | N:P = 63:1 | 127.0 ± 47.0 | 2.96 ± 0.60 | 1.41 ± 0.09 | 1.65 ± 0.10 | 0.41 ± 0.06 |
| 24 ºC | Low $pCO_2$ | N:P = 10:1 | 164.7 ± 20.0 | 1.30 ± 0.19 | 1.55 ± 0.03 | 2.31 ± 0.12 | 0.80 ± 0.01 |
| | | N:P = 24:1 | 137.6 ± 39.2 | 1.27 ± 0.30 | 1.40 ± 0.04 | 4.72 ± 0.37 | 0.76 ± 0.04 |
| | | N:P = 63:1 | 89.1 ± 29.9 | 1.80 ± 0.38 | 1.73 ± 0.10 | 3.90 ± 0.21 | 0.76 ± 0.02 |
| | High $pCO_2$ | N:P = 10:1 | 138.3 ± 35.9 | 0.85 ± 0.14 | 1.52 ± 0.09 | 2.54 ± 0.22 | 0.79 ± 0.04 |
| | | N:P = 24:1 | 164.6 ± 18.8 | 3.93 ± 0.63 | 1.34 ± 0.01 | 4.62 ± 0.09 | 0.83 ± 0.01 |
| | | N:P = 63:1 | 117.2 ± 3.17 | 4.19 ± 0.39 | 1.60 ± 0.03 | 3.89 ± 0.19 | 0.77 ± 0.02 |

| | % of total alkenones | | | |
|---|---|---|---|---|
| | $C_{37}$ Me | $C_{38}$ Et | $C_{38}$ Me | $C_{39}$ Et |
| All treatments | 48 – 65% | 18 – 35% | 6.6 – 19% | 0 – 5.9% |

**Table S5.** Dimension 1 (Dim1) and Dim 2 loadings for each variable in PCA (Fig. 3) testing the responses of carbon-normalized contents of the major sterol component (brassicasterol/epi-brassicasterol), $C_{37}$-$C_{39}$ total alkenones and total fatty acids (TFAs) ($\mu$g mg $C^{-1}$) in *Phaeodactylum tricornutum*, *Rhodomonas* sp. and *Emiliania huxleyi* to changes in temperature, N:P molar ratios and $pCO_2$.

| Variables | Dim 1 | Dim 2 |
|---|---|---|
| % of variance | 26.4 | 17.7 |
| Temperature | -0.320 | 0.493 |
| $pCO_2$ | 0.185 | 0.151 |
| N:P supply ratio | 0.668 | 0.559 |
| Brassicasterol/epi-brassicasterol (*P. tricornutum*) | -0.024 | -0.420 |
| Brassicasterol/epi-brassicasterol (*Rhodomonas* sp.) | -0.402 | 0.393 |
| Brassicasterol/epi-brassicasterol (*E. huxleyi*) | -0.833 | -0.040 |
| Alkenones (*E. huxleyi*) | -0.292 | 0.317 |
| TFAs (*P. tricornutum*) | 0.608 | 0.426 |
| TFAs (*Rhodomonas* sp.) | 0.819 | -0.104 |
| TFAs (*E. huxleyi*) | 0.279 | -0.753 |

**Fig. S1**

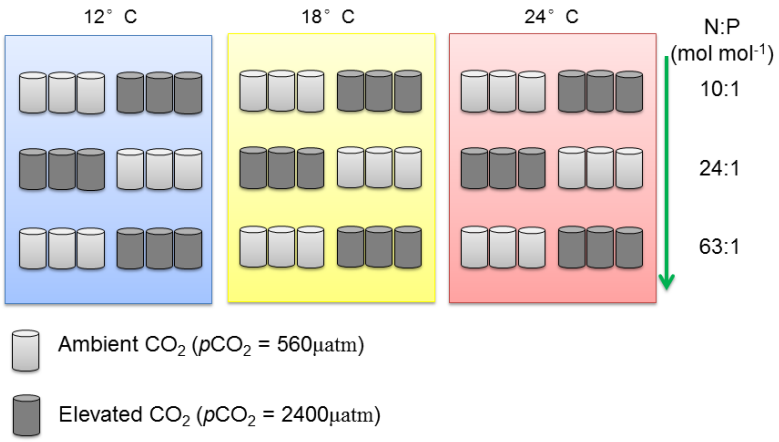



Fig. S1. Experimental setup. The three phytoplankton species were grown semi-continuously under a full-factorial combination of three temperatures (12, 18 and 24 °C), three N:P supply ratios (molar ratios 10:1, 24:1 and 63:1) and two $pCO_2$ levels (560 and 2400 µatm). Triplicates were set for each treatment.