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

Responses to comments from Reviewer 1

Comment 1

Line 30: "PIC" for the first appearance, should be marked it's the abbreviation of "particulate inorganic carbon". Also for "POC".

Response:

As suggested, all abbreviations are spelled out once in full upon their first appearance in Abstract and the main manuscript text.

Comment 2

Line 31-32: "10:1, 24:1 and 63:1" are the ratios of N:P, the unite "mol mol-1" , not necessarily shown.

Response:

As suggested, we removed the unit 'mol mol^{-1}' and state 'molar ratios 10:1, 24:1 and 63:1'. (See Page 2, Line 31; Page 8, Line 155)

Comment 3

Line 87-92: "E.huxleyi is expanding its range poleward", why then gave an example of the subtropical area.

Response:

The reference to a study in the subtropical area was removed. A study in the Bering Sea is added, in which Harada et al. (2012) found that warming and freshening have promoted *Emiliania huxleyi* blooms since the late 1970s. (See Page 5, Lines 92-94)

Comment 4

L149-151: "The target values were chosen to reflect a present and future regime of each factor", however, the pCO2 concentrations 560 and 2400 µatm they used, can hardly be considered reasonable. An explanation why a gap in the CO2 concentrations was so big.

Response:

In plankton-rich waters, respiration plus atmospheric CO_2-enrichment can drive regional pCO_2 up to 900 μatm at times even today. Considerable seasonal, depth and regional variations of pCO_2 have been observed in the present-day ocean (Joint et al. 2011). For example, up to 900 μatm of pCO_2 was observed in August in the Southern Bight of the North Sea, with a lower pCO_2 (192 μatm) in April (Schiettecatte et al. 2007). A natural pCO_2 gradient of 292 to 8828 μatm was reported off Culcano Island, Italy (Ziveri et al. 2014). In the future oceans, pCO_2 will increase with rising atmospheric CO_2, being 851-1370 μatm by 2100 and 1371-2900 μatm by 2150 (RCP8.5 scenario of the IPCC report 2014) (IPCC 2014).

In the present study, the chosen values of pCO_2 cover the range of typical levels of pCO_2 in the present-day ocean and future ocean projections. Such a big gap in the value of pCO_2 was used to test the response of *E. huxleyi* to a considerable, yet realistic variation of pCO_2.

To clarify the reason of pCO_2 set-up in our study, a detailed explanation is added in the revised manuscript. (See Page 7, Lines 134-141)

Comment 5

Line 172: Can they write in detail about how "the specific growth rate of 20% of µmax was applied". I'm curious and puzzled about the reason and methods of how the 20% of µmax (µ) was realized. Usually, specific growth rate is not expressed by %.

Response:

Using % of μ_{max} guarantees that the strength on nutrient deficiency is equal through all temperature and pCO_2 treatments. A fixed value of µ would mean weak deficiency when μ_{max} is low, and strong deficiency when it is high. Based on the gross growth rate (μ= 20% of μ_{max} (day^{-1})), the equivalent daily renewal rate (D, day^{-1}) can

be estimated according to the equation $D = 1- e^{-\mu t}$, where t is renewal interval (day) (here $t = 1$ day). Thus, the volume of the daily renewal incubation water can be calculated by multiplying D with the total volume of incubation water.

In the revised manuscript, we use the term 'gross growth rate' instead of specific growth rate. The term 'the gross growth rate' is explained on Page 9, Lines 184-185, which results from the process of reproduction alone. We also provide the detail about how 20% of μ_{max} was realized and applied and the reason of using % of μ_{max} in the revised manuscript. (See Page 9, Lines 185-191)

Comment 6

Line175-176: They said that the incubation water was exchanged with fresh seawater, since the culture medium was partially renewed according to the renewal rate D, the N:P ratios might deviate the target supply ratios in the remained medium due to differential consumption of N and P, can they give some information to show that the N:P supply ratios are stable after several rounds of renewal.

Response:

'fresh' seawater here implies freshly made seawater medium with the target N:P supply ratio, not only fresh seawater. Indeed, nutrient concentrations in semi-continuous culture may deviate from the target values due to consumption. Semi-continuous cultures, as a practical surrogate for fully continuous culture, have been successfully used to study the effect of nutrients on phytoplankton stoichiometry and fatty acid composition (Terry et al. 1985; Lynn et al. 2000; Piepho et al. 2012; Feng et al. 2017). While we did not measure the N and P in the media daily, semi-continuous cultures can be applied to study the effect of N:P supply ratio on *E. huxleyi* stoichiometry and fatty acid composition.

In the revised manuscript, we explain the successful usage of semi-continuous cultures in studies of phytoplankton stoichiometric and biochemical composition (See Pages 8, Lines 158-161). We also clarify that the incubation water was exchanged with freshly made seawater medium. (See Page 9, Line 192)

Comment 7

Line 178: It seems that the cell concentration was extremely high, the cell concentration range should be provided.

Response:

In our study, the final cell density at the steady state ranged between 1.50×10^5 – 17.8×10^5 cells mL^{-1}, with the average value of 7.95×10^5 cells mL^{-1}. High cell density ($> 1 \times 10^6$ cells mL^{-1}) was observed in six out of 18 treatments. The average value of cell density in our study was consistent with the range of those in previous studies. For example, De Bodt et al. (2010) reported the maximum cell density of 6.84×10^5 cells mL^{-1} when testing the effects of pCO_2 and temperature on *E. huxleyi* calcification.

As suggested, the range of cell densities is shown in the revised manuscript. (See Page 10, Lines 203-204)

Comment 8

Line 180: What do the authors mean by "the net growth rate (r)", what's the difference between r and μ? Confusing wordings or mis-understood definations?

Response:

To clarify the difference between μ and r, we now use the term 'gross growth rate' for μ (resulting from the process of reproduction alone), while r (net growth rate) is the difference between the gross growth rate and the loss rate ($r = \mu - D$).

The difference between gross growth rate and net growth rate is clarified on Page 9, Lines 184-185 and 197-198.

Comment 9

Line 203: Here "was" should be "were".

Response:

As suggested, the word 'was' was replaced by 'were'. (See Page 11, Line 222)

Comment 10

Line 241: Is this theory applicable in all species and in any conditions.

Response:

This hypothesis was proposed by Cherif and Loreau (2010), suggesting that realized maximum growth rates (i.e., the observed maximum growth rate in the present study, μ_{max}) should be equal for essential, non-substantial resources for phytoplankton species. This assumption was supported by both theoretical and empirical evidence, 1) lab experiments showed little or no luxury uptake of resources at the highest growth rate; 2) the maximum capacity of the uptake machinery should not be oversized for a given resource based on economical design (Cherif and Loreau 2010). Similar μ_{max} in different nutrient conditions has been observed for different phytoplankton species in empirical experiments (e.g., Ahlgren 1985; Baek et al. 2008; Bi et al. 2012). The model presented in Cherif and Loreau (2010) has also been successfully used to study how nutrient gradients influence stoichiometry of autotrophs in natural chemostats (Nifong et al. 2014).

In the present study, we had only one value of μ_{max} for each nutrient treatment under different temperature and pCO_2 conditions, thus the effect of N:P supply ratio cannot be tested with ANOVA efficiently. In the revised manuscript, we tested the response of μ_{max} to temperature, N:P supply ratio and pCO_2 using GLMMs. The results of GLMMs were consistent with those of ANOVA, showing a highly significant effect of temperature on μ_{max}. As the chosen best model contained only first order effects, no significant interactions between the three environmental factors were detected. The non-significant response of μ_{max} to N:P supply ratio in *E. huxleyi* is consistent with the assumption of Cherif and Loreau (2010).

In the revised manuscript, methods and results of ANOVA were removed. We also revised the Results and Discussion sections according to the new results of GLMMs on μ_{max}. (See Page 14, Lines 294-303; Page 19, Lines 399-403)

Comment 11

Line1103: Why there is no panel for the pCO2 effect in Fig.2.

Response:

The effect of pCO_2 on stoichiometric C:N:P is added in Fig. 3 in the revised manuscript.

Comment 12

Line 1112: As I read from the "experimental setup" part, this study investigates the combined effects of temperature, pCO2 and N:P supply ratios on E.huxleyi. Why in Fig 3. the combined effects of N:P supply ratio and pCO2 are not considered, i.e. pCO2 is not considered in panel (a), (b), (c), and N:P supply ratio is not considered in panel (d), (e) and (f). The same question for Fig. 4, 5 and 6.?

Response:

In the present study, we tested the effects of temperature, N:P supply ratio and pCO_2 on *E. huxleyi* using GLMMs. The selected best models contain only first order effects, or first order effects and second order interactions of the three factors, while models containing third order interactions of the three factors were not selected for any response variables (Table S2). Furthermore, significant interactions between temperature and N:P supply ratio, and between temperature and pCO_2 were detected for cellular PIC and POC contents, and the proportion of DHA. However, the significant interaction between N:P supply ratio and pCO_2 was only found for the proportion of SFAs. Please see Table 2 for a systematic summary. Thus, the interactions between temperature and N:P supply ratio, and between temperature and pCO_2 are shown in figures 1-4, while that between N:P supply ratio and pCO_2 is only shown for SFAs in the supporting information Fig. S2.

To clarify the information above, we took the following actions in the revised manuscript:

1) In the Methods, we explain more about the best models selected for different response variables. (See Page 13, Lines 272-278)

2) The selected best models and significant interactions are also briefly stated in figure legends in Fig. 1-4. (See Page 45, Lines 1059-1083)

Responses to comments from Reviewer 2

General comment I

My main concerns with the manuscript in its current form are the framing of the experimental manipulations to global patterns and the length of the discussion. There seems to be a mismatch between projected temperature and CO2 conditions in future oceans and the ones you manipulated. I would like to know how you would translate your results to a future ocean scenario as the CO2 concentrations in your lowest treatment are higher than currently measured in global oceans (max 440 ppm; Bakker et al. (2016)). On a similar note, how do the three temperature treatments with a difference of 12C relate to future ocean projections?

Response:

The chosen levels of pCO_2 and temperature in this study were set based on the reasons below:

1) pCO_2. Please see our reply to Reviewer 1, Comment 4. In plankton rich waters respiration plus atmospheric CO_2-enrichment can drive regional pCO_2 up to 900 μatm at times today. For example, up to 900 μatm of pCO_2 was observed in August in the Southern Bight of the North Sea (Schiettecatte et al. 2007). A much higher pCO_2 (a natural pCO_2 gradient of 292 to 8828 μatm) was observed off Culcano Island, Italy (Ziveri et al. 2014). In the future oceans, pCO_2 will increase with rising atmospheric CO_2, being 851-1370 μatm by 2100 and 1371-2900 μatm by 2150 (RCP8.5 scenario of the IPCC report 2014) (IPCC 2014). Therefore, the chosen values of pCO_2 in the present study cover the range of typical levels of pCO_2 in the present-day ocean and future ocean projections.

2) Temperature. Water surface temperatures at the Azores vary between ~12 to 29 ℃ (Lafon et al. 2004), with the inter-annual average temperature between 16 to 22 ℃ and peaks usually reaching a maximum of 24 to 25 ℃ (http://dive.visitazores.com/en/when-dive; last accessed date: 22.08.2017). Our temperature range setup was based on the study of Lewandowska et al. (2014), who chose a temperature increment of 6 ℃, according to the ocean general circulation model under the IPCC SRES A1F1 scenario. Annual mean sea surface temperature

across the North Atlantic (0–60 °N) is projected to reach 29.8 ℃ in 2100 according to the ocean general circulation model (Lewandowska et al. 2014). We also chose this setup to compare with our previous results (Bi et al. 2017).

3) The ranges of pCO_2 and temperature in our study are identical in design with our previous work (Bi et al. 2017), which makes the comparison easier between results for different species.

The reasons for pCO_2 and temperature set-up are pointed out in the revised manuscript. (See Pages 6-7, Lines 128-141; Page 8, Lines 161-164)

General comment II

The discussion is quite lengthy and would benefit in my opinion to focus more on the interaction effects observed in the study as these are the core strength of the work and could advance the field. Perhaps you could reduce the amount of wording if you first discuss the solo effects and then go into all interaction effects in one paragraph (for C:N:P stoichiometry, PIC:POC separately). It seems that there is currently a lot of overlap in the things discussed in separate paragraphs.

Response:

As suggested, we first discuss the single effects and then continue to discuss interactive effects on C:N:P stoichiometry and PIC:POC. (See Pages 19-25, Lines 414-533)

General comment III

In addition, I'm missing the inclusion of the PON and POP contents underlying the responses in C:N:P stoichiometry, the results of N:P supply ratio on maximal growth rate and the (though non-significant) results of C:N:P stoichiometry in the different CO2 treatments (Fig. 2).

Response:

As suggested, the results of PON and POP contents, the N:P effect on μ_{max} in Fig. 1, and the pCO_2 effect on C:N:P stoichiometry in Fig. 2 and Fig. 3 are now shown in the revised manuscript.

General comment IV

From the introduction it is not clear that PIC and POC production will be discussed. In my opinion, the focus on C:N:P stoichiometry and underlying biochemical composition is the core of your work and introduced very well in the manuscript. I understand the importance of PIC:POC for calcifiers specifically, but I would advise to focus less on the PIC and POC contents, production rates and population yields and more on the C:N:P and fatty acids.

Response:

As suggested, we removed the results of PIC and POC production rates and population yields in the section of Results, and moved the corresponding figures to the supporting information.

General comment V

A discussion on how changes in stoichiometry and fatty acids relate to each other would be a great addition to the discussion section.

Response:

We add a graph (Fig. 5) to show how changes in stoichiometry and fatty acids relate to each other, i.e., the responses of PON:PUFAs and POP:PUFAs to temperature, N:P supply ratios and pCO_2. A section 'PON:PUFAs and POP:PUFAs' is added in Results (See Page 17, Lines 357-364). Accordingly, we discuss the implications of our results for ecology based on the relative changes in stoichiometry and fatty acids (See Pages 29-30, Lines 629-642).

General comment VI

What would be a great addition to the introduction are hypotheses on how temperature, CO2 and nutrient supply affect the C:N:P stoichiometry and fatty acid composition. Something similar to Figure 7, but then hypothetical. This would then furthermore help shape the discussion as you could refer back to these hypotheses.

Response:

In the last paragraph of the introduction, we add hypotheses on how temperature, pCO_2 and nutrient supply affect elemental stoichiometry and fatty acid composition. (See Page 7, Lines 141-149)

General comment VII

A smaller comment, but the use of N:C and P:C ratios instead of C:N and C:P is not very commonly used in literature. The readability and comparison of these ratios to other studies would benefit greatly if they are expressed in C:N and C:P.

Response:

We now use POC:PON and POC:POP, instead of N:C and P:C biomass ratios, in the revised manuscript.

General comment VIII

Furthermore, the reasoning behind the statistical methods used are not entirely clear for me. Some information in the method section on why this type of statistics are used and what the associated parameters mean would aid the reader in the understanding of the manuscript.

Response:

Generalized linear mixed models (GLMMs) are appropriate for non-normal data such as counts or proportions, while classical statistical procedures such as ANOVA rely on normally distributed data (Bolker et al. 2009). GLMMs combine the properties of two statistical models (linear mixed models and generalized linear models) (Bolker et al. 2009) and have been widely used in ecology (e.g., Fr ère et al. 2010; Jamil et al. 2014; Bracewell et al. 2017), in which data sets are often non-normally distributed.

We explain the reason why to choose GLMMs and what the associated parameter (link function) means in the revised manuscript. (See Page 12, Lines 248-254 and 259-264)

Specific comment 1

73 Do you mean community structure of phytoplankton?

Response:

According to Doney et al. (2012), climate change may alter the physiological functioning, behavior, and demographic traits of organisms. These changes cascade from primary producers to upper trophic levels such as fish, seabirds and marine

mammals. Therefore, community structure in the sentence in our manuscript means not only for phytoplankton but also for other trophic levels.

We hence clarify this as '---- community structure of different trophic levels ---'. (See Page 4, Line 73)

Specific comment 2

80-82 'via releasing CO2' is not really clear for me what this means and why coccolithophores are important components of the carbon cycle.

Response:

This sentence was revised to clarify that coccolithophores are not only important photosynthetic producers of organic matter (causing a draw-down of CO_2 in the surface layer), but also play predominant roles in the production and export of calcium carbonate to deeper layers (causing a net release of CO_2 to the atmosphere). (See Page 4, Lines 79-85)

Specific comment 3

112 What do you mean by a core feature? 'Element' –> 'elemental'

Response:

This sentence was revised to ' --- variability in *Emiliania huxleyi* C:N:P stoichiometry (cellular quotas and ratios of C, N and P) can also be important in ocean biogeochemistry.' (See Page 6, Lines 111-113)

'element' was changed to 'elemental'. (See Page 6, Line 113)

Specific comment 4

117 Food for which organism? Phytoplankton or zooplankton?

Response:

According to Rosenblatt and Schmitz (2016), shifts in resource nutrient content generally occur with shifts in consumer physiology and behavior, and they are often overlooked in studies of the responses of food web dynamics to climate change.

We thus clarify this sentence as ‘ --- shifts in resource nutrient content for consumers are often overlooked in climate change ecology --.’. (See Page 6, Line 118)

Specific comment 5

129-136 This comes as a surprise for me here and seems to fit better in the methodological section than in the introduction.

Response:

As suggested, these two sentences were moved to the methods section. (See Page 11, Lines 227-231; Pages 11-12, Lines 242-246)

Specific comment 6

138 PIC:POC is already a ratio

Response:

Wording was revised as PIC:POC throughout the text.

Specific comment 7

147 The manipulated CO2 levels came as a surprise to me in the framework of current and future projections. Do you have specific reasons to choose these levels as I would have expected a lower ‘ambient’ CO2 level (around 400 ppm)?

Response:

Please see our reply to General comment I.

Specific comment 8

148 Does the strain have a specific reference number (to make possible comparisons with other studies easier)?

Response:

As suggested, the specific reference number (internal culture collection reference code: A8) is now added. (See Page 8, Line 156)

Specific comment 9

175 does ‘fresh’ seawater imply that is was taken from sea at that day?

Response:

'fresh' seawater implies that freshly made seawater medium, but the seawater was not taken from the sea on that day. To clarify this, the sentence was revised as 'The incubation water was exchanged with freshly made seawater medium -----'. (See Page 9, Line 192)

Specific comment 10

222 Is there a specific reason why you choose GLMM's instead of the more classic ANOVA's?

Response:

Please see our reply to General comment VIII.

Specific comment 11

228 What are link functions?

Response:

The link function is a transformation of the target that allows estimation of the model (https://www.ibm.com/support/knowledgecenter/SSLVMB_21.0.0/com.ibm.spss.statistics.help/idh_glmm_target.htm; last accessed date: 14.08.2017). For example, identity link function is appropriate with any distribution except for multinomial, while logit can be used only with the binomial or multinomial distribution. We explain in the text what the link function is. (See Page 12, Lines 259-264)

Specific comment 12

222-239 This part of the statistics is quite difficult for me to follow. Could you explain a bit more about the different procedures and what they do?

Response:

In the revised manuscript, we took the following actions to explain more about GLMMs:

1) The reason why to choose GLMMs instead of classical statistical procedures is explained on Page 12, Lines 249-254.

2) We explain what link function is. (See Page 12, Lines 259-264)

3) According to differences in AICc values, the best model was selected for each response variable, which is now explained more on Page 13, Lines 272-278.

Specific comment 13

242 I would not assume mumax to be the same between nutrient treatments as that was the case in another study. Did you test this and was this the case in your study?

Response:

We tested the changes of μ_{max} between different nutrient treatments. Because there was only one value of μ_{max} in each nutrient treatment under different temperature and pCO_2 conditions, the effect of N:P supply ratio cannot be tested with ANOVA efficiently. In the revised manuscript, we show the results of GLMMs on the response of μ_{max} to temperature, N:P supply ratio and pCO_2 using GLMMs, which are consistent with those of ANOVA, showing a highly significant effect of temperature and non-significant effect of N:P supply ratio and pCO_2. As the chosen best model contained only first order effects, no significant interactions between the three environmental factors were detected.

In the literature, there are limited data on the response of μ_{max} in *E. huxleyi* to nutrient availability, while several studies reported the response of specific growth rate. According to Cherif and Loreau (2010), realized maximum growth rates (i.e., the observed maximum growth rate in the present study, μ_{max}) should be equal for essential, non-substantial resources for phytoplankton species. This assumption was supported by both theoretical and empirical evidence, 1) lab experiments showed little or no luxury uptake of resources at the highest growth rate; 2) the maximum capacity of the uptake machinery should not be oversized for a given resource based on economical design (Cherif and Loreau 2010). For *E. huxleyi*, luxury consumptions for phosphate and nitrate are lower than other phytoplankton taxa (Rost and Riebesell

2004). Thus, the non-significant response of μ_{max} to N:P supply ratio in *E. huxleyi* in our study is consistent with the assumption of Cherif and Loreau (2010). Future work is suggested to study the response of μ_{max} in *E. huxleyi* under a wider range of nutrient conditions.

We revised the Results and Discussion sections according to the new results of GLMMs on μ_{max}. (See Page 14, Lines 294-303; Page 19, Lines 399-403)

Specific comment 14

244 Is there a specific reason why you only used w2 for the mumax results and not for other results as for instance figure 7?

Response:

Error mean square cannot be obtained from GLMMs and thus w^2 cannot be calculated for response variables tested with GLMMs. In the revised manuscript, we show the response of μ_{max} using GLMMs. Thus, w^2 for μ_{max} was also removed.

Specific comment 15

248 Why would you use nested models when you have a full factorial design? In other words, what is the added value of these statistical tests? Can you relate your chosen temperatures to acclimatization of E.hux in your lab or the original population that was sampled? How are average annual water temperatures at the Azoren?

Response:

It is possible to use a nested model in a full-factorial design setting. The question a nested model addresses is that, whether one factor plays a role under one (or several) configuration(s) of another factor, but not under all configurations of that factor equally. The difference to e.g. a test including straight-forward interaction effects is that interaction terms describe systematic variation of one factor's effects over a gradient of the other, whereas a nested model can highlight if for example pCO_2 plays a role for fatty acid content only at intermediate temperature.

The added value of a nested model is explained on Page 13, Lines 281-283. Please see our reply to General comment I regarding average water surface temperatures.

The chosen temperature setup in our study is within the range of sea surface temperature at the Azores.

Specific comment 16

274 Did you determine the CO2 effects by post-hoc tests? As there was no overall effect of CO2 on maximum growth rate while you have a significant interaction effect, wouldn't that mean that the effect of temperature is dependent on the CO2 level, but not vice versa?

Response:

In our study, a post hoc test was applied only if there were significant effects in ANOVA. We thus did not determine the effect of pCO_2 by the post hoc test, as the effect of pCO_2 was not significant according to ANOVA.

We agree with the reviewer that the effect of temperature is dependent on the CO_2 level. In the revised manuscript, we used GLMMs to test the response of μ_{max} (Please see our response to Specific comment 13). The results showed no significant interactions between temperature and pCO_2, while there was still a different trend of μ_{max} to increase with increasing temperature between the two pCO_2 treatments.

We revised the results of μ_{max} responses on Page 14, Lines 294-303. The discussion on μ_{max} responses was also revised accordingly (See Page 19, Lines 399-403).

Specific comment 17

280 any particular reason to use N:C ratios as opposed to C:N ratios? The latter is used more often in literature and makes the comparison with the Redfield Ratio easier. For instance, a hump-shaped curve to temperature (or Ushaped curve, line 286) is also observed for a marine cyanobacterium (Fu et al. 2014). By having the ratios in N:C instead of C:N, comparison with other studies like these can get confusing. Furthermore, you did not report interaction effects of temperature and N:P supply ratio on N:P ratios. So how does the difference in temperature response under N and P deficiency (lines 287-288) relate to that?

Response:

We present the results of POC:PON and POC:POP in the revised manuscript to make the comparison with the Redfield Ratio and the results in the literature easier.

We found that, similar to the results in Fu et al. (2014), a hump-shaped curve to temperature was also observed for POC:PON in response to increasing temperature under N deficiency in our study.

Indeed, there was non-significant interaction between temperature and N:P supply ratio on PON:POP according to GLMMs. However, POC:PON responded significantly to temperature, showing a different trend of changes to increasing temperature under different N:P supply ratio. We thus present this nutrient-dependent response, as it need not be universal to constitute 'significant discovery'.

Specific comment 18

283 instead of biomass ratios, would it make sense to use PON:POP or POC:PON as that would already imply that it is biomass related. Related to that question, is the C:N ratio composed of TPC:PON or POC:PON? Furthermore, what is underlying the changes in stoichiometry? You have the results for POC content in Figure 4, but how do PON and POP change?

Response:

As suggested, we use POC:PON, POC:POP and PON:POP in the revised manuscript. The C:N biomass ratio is composed of POC:PON.

To explore what is underlying the changes in C:N:P stoichiometry, we analyzed the responses of cellular PON and POP contents. For example, a U-shaped curve was observed for the responses of cellular POC and PON contents to increasing temperature under N deficiency, which can explain the observed hump-shaped curve for the response of POC:PON. The detail results are shown on Pages 14-15, Lines 305-317, and discussed on Pages 20-21, Lines 429-435, 449-452 in the revised manuscript.

Specific comment 19

292 What is a PIC population yield?

Response:

A population yield of PIC is the PIC content per ml (μg ml^{-1}). This is now clarified in the revised manuscript. (See Page 25, Lines 540-541)

Specific comment 20

347 Technically, C:N:P is not a ratio but is composed of C:N and C:P ratios. Additionally, why did you chose to only highlight the N:P results?

Response:

As suggested, C:N:P biomass ratio was changed to C:N:P stoichiometry throughout the text.

The response of N:P biomass ratio is highlighted here because it had the highest percent changes among the three stoichiometric ratios. To clarify this, this sentence was revised as '----, showing a maximum of 62% changes under nutrient deficiency'. (See Page 17, Lines 369-370)

Specific comment 21

356 These interactions effects don't become clear from table 2, as there you only report the effects of the individual stressors.

Response:

Indeed, we did not observe significant effects of the three stressors on all response parameters, with significant interactive effects only observed for cellular POC content, and SFA and DHA proportions.

We clarify this information as '----, we also detected significant interactions between temperature, N:P supply ratios and pCO_2 on certain response variables (e.g., cellular POC content and DHA proportion) (Table 1),----'. (See Page 18, Lines 375-377)

Specific comment 22

369 'strains' instead of 'strain'

Response:

As suggested, the word was corrected to 'strains'. (See Page 18, Line 390)

Specific comment 23

370 It would be interesting to link this result with the origin of your strain. Does it fall in excepted patterns?

Response:

In our study, μ_{max} of *E. huxleyi* (from the Azores, ~ 38° N) was two to three times higher at the highest temperature than that at the lowest temperature, showing a similar change pattern with that in *E. huxleyi* (1.6 times higher at the higher temperature) from the Sargasso Sea (~20-35° N).

We add now the comparison between our results and the results in the literature in the revised manuscript. (See Page 18, Lines 394-396)

Specific comment 24

375 I could also argue it the other way, that the biogeographic origin of an E. huxleyi strain is important for their response to temperature. Like mentioned before, could you elaborate on this more?

Response:

We agree that the results show the importance of the biogeographic origin of an *E. huxleyi* strain for their response to temperature. We revised this sentence as 'The results above suggest that the biogeographic origin of an *E. huxleyi* strain is important for their response to temperature'. (See Pages 18-19, Lines 396-398)

Specific comment 25

378 Seems to contrast Table 1 and lines, were you show no effect of CO2 on maximal growth rate. Or is this based on post-hoc comparisons?

Response:

In the revised manuscript, GLMMs were used to test the response of μ_{max} to temperature, N:P supply ratio and pCO_2, showing non-significant interactions between the three factors. Thus, the discussion on the significant interactions between temperature and pCO_2 was removed in the section 'Responses of maximal growth rate' in the Discussion.

Please also see our reply to Specific comment 13 and 16.

Specific comment 26

387-389 Can you quantify these slopes as they come a bit as a surprise at this point in the manuscript.

Response:

These slopes were quantified. At the low pCO_2, the slopes were 0.13 and 0.026 at lower (12 and 18 ℃) and higher temperatures (18 and 24 ℃), respectively; at the high pCO_2, the slopes (0.04 – 0.06) were relatively constant. (See Page 19, Lines 399-403)

Specific comment 27

393 remove 'and'

Response:

'and' was removed. (See Page 19, Line 406)

Specific comment 28

394 If it is a conceptual graph you're referring to, I would be interested in the conceptual reasoning behind this response.

Response:

The conceptual reasoning behind is still unclear. The authors who proposed the conceptual graph suggested that one possible explanation is that increasing temperature may modulate the balance between a fertilizing effect of ocean carbonation and a metabolic repression by ocean acidification (Sett et al. 2014). This possible explanation is stated at the end of this paragraph. (See Page 19, Lines 408-412)

Specific comment 29

403 I would opt for 'C:N:P stoichiometry' instead of biomass ratios.

Response:

'C:N:P biomass ratios' was revised to 'C:N:P stoichiometry'. (See Page 19, Line 414)

Specific comment 30

409 What do you mean by 'prevailed the governing effect'?

Response:

Skau (2015) tested the effects of temperature and phosphorus on stoichiometry in three haptophytes, showing that phosphorus treatments had a stronger effect on C:P ratios in *E. huxleyi* compared to temperature.

We revised this sentence as '--- nutrient availability played a more important role than temperature (and pCO_2) for elemental stoichiometry ----'. (See Pages 19-20, Lines 418-419)

Specific comment 31

415-417 I really like Figure 7 as it gives a nice overview about your results. But what I'm missing there is the change in cellular N and P content. These results could help you in making conclusions about the changes in PON:POP, whether that is mainly due to N or P deficiency. Furthermore, should it be a table instead of a figure?

Response:

We add now the responses of cellular N and P contents in the revised manuscript. Please check our response to General comment III and Specific comment 18.

Fig. 7 is now shown as a table (Table 2) in the revised manuscript.

Specific comment 32

445-447 But given your result that the changes in N:C (or C:N) are stronger than those of P:C, what would be the mechanism behind that? Is there any current literature on that respect? Furthermore, if you bring in the argument of less P rich ribosomes with warming, wouldn't you have expected an decrease in P:C instead of the increase you observed?

Response:

In the literature, variable changes of POC:PON and POC:POP to warming were observed in *E. huxleyi*, showing positive (Borchard and Engel 2012), negative (Feng et al. 2008; Matson et al. 2016) and U-shaped responses (Rosas-Navarro et al. 2016). Similar to our study, Borchard and Engel (2012) also found a stronger change of

POC:PON than of POC:POP at higher P condition, while both biomass ratios increased with increasing temperature. The mechanism behind the stronger changes in POC:PON compared to POC:POP may be explained by the temperature-dependent physiology hypothesis, which shows that organisms in warmer conditions require fewer P-rich ribosomes, relative to N-rich proteins (Toseland et al. 2013). In our study, both POC:PON and POC:POP decreased with increasing temperature, while the change in POC:PON (8%) was larger than that in POC:POP (5%). Thus, the relative changes in POC:PON and POC:POP, as well as the increase in PON:POP, in response to increasing temperature in our study are consistent with the temperature-dependent physiology hypothesis (Toseland et al. 2013).

We revised this part to compare our results with current literature and to clarify the mechanisms (temperature-dependent physiology hypothesis) behind the changes of C:N:P stoichiometry in response to warming. (See Pages 21-22, Lines 452-465)

Specific comment 33

461 I'm missing here a coupling to your own experimental set-up, did you not find effects of CO2 on stoichiometry due to light conditions or nutrient loads? Can you compare your set-up with those from the studies you mentioned?

Response:

We add the comparison of experimental set-up between our study and previous work. For example, Feng et al. (2008) reported that rising pCO_2 caused the increase in POC:PON only at the high light condition (400 μmol photons · m^{-2} · s^{-1}). The light intensity in our study (100 μmol photons · m^{-2} · s^{-1}) was lower than that in Feng et al. (2008). In our study, we used relatively low light intensity (100 μmol photons · m^{-2} · s^{-1}), did not investigate irradiance effects. Additional research is required to assess the effects of other important environmental factors such as irradiance and their interactions on C:N:P stoichiometry in our *E. huxleyi* strain. (See Page 22, Lines 474-481)

Specific comment 34

462 This is a rather fast transition for me from stoichiometry to cellular biomass. Perhaps this part fits better with the discussion paragraph on growth rates.

Response:

In this sentence, we discuss the responses of C:N:P stoichiometry and not cellular biomass. We revised this sentence as 'Taken together, our results indicate that C:N:P stoichiometry in *E. huxleyi* largely reflected the changes in N:P supply ratios, across different temperatures and pCO_2 levels.'. (See Page 22, Lines 482-484)

Specific comment 35

472 But you haven't looked at taxonomic composition as you study one species. As there is already such variability between strains and experiments with E.hux, I would shorten this paragraph and focus more on the drivers of variation in responses.

Response:

As suggested, the discussion in this paragraph was revised to focus more on the drivers of variation in stoichiometric responses: 'Taken together, our results indicate that C:N:P stoichiometry in *E. huxleyi* largely reflected the changes in N:P supply ratios, across different temperatures and pCO_2 levels. However, for two algal species from non-calcifying classes (the diatom *P. tricornutum* and the cryptophyte *Rhodomonas* sp.) temperature had the most consistent significant effect on stoichiometric ratios in our previous work (Bi et al. 2017). The results above are consistent with the ranking of environmental control factors in Boyd et al. (2010), which showed that temperature, nitrogen and phosphorus were ranked as important factors for major phytoplankton groups.'. (See Pages 22-23, Lines 482-489)

Specific comment 36

492 Refrain from starting a sentence with 'and'

Response:

'and' was removed from the beginning of the sentence. (See Page 24, Line 508)

Specific comment 37

498 This is vague for me, what other environmental drivers do you mean specifically?

Response:

According to previous studies, the interaction of pCO_2 with other environmental factors such as irradiance and temperature may be potential drivers on the changes in PIC:POC (Feng et al. 2008; De Bodt et al. 2010).

This sentence was removed as the whole paragraph was deleted.

Specific comment 38

507 'and the present study' should be within the brackets?

Response:

'the present study' is added within brackets. (See Page 24, Line 513)

Specific comment 39

519 CO2 would not be related to future oceans as the lowest treatment is already elevated.

Response:

Please see our reply to General comment I.

Specific comment 40

524 This argument is not clear to me and does not follow logically from your work. Yes, you have changes in PIC and POC yields with environmental changes, but why would that not scale up to carbon export?

Response:

We revised this sentence as 'It is worth noting that cellular PIC and POC contents are a measure for physiological response and cannot be directly used to infer population response, as different responses between cellular and population yields of PIC (and POC) (as μg ml^{-1}) to environmental changes were evident in previous work (Matthiessen et al. 2012) and the present study (Table S5, S6; Fig. S3, S4). Thus,

scaling our results up to coccolithophores carbon export should consider these uncertainties.'. (See Page 25, Lines 538-543)

Specific comment 41

529 'dynamic' –> 'dynamics'

Response:

'dynamic' was revised to 'dynamics', while this sentence was removed in the revised manuscript.

Specific comment 42

595 'low trophic levels consumers': do you mean first order consumers?

Response:

Here we would prefer 'low trophic levels consumers', which includes not only first order consumers but also second order consumers. Dietary preferences of zooplankton may change with environmental conditions such as temperature (Boersma et al. 2016). For example, the copepod *Temora longicornis* preferred the cryptophyte *Rhodomonas salina* at higher temperatures, while it preferred the heterotrophic dinoflagellate *Oxyrrhis marina* at lower temperatures (Boersma et al. 2016). In the studies we cited (Garzke et al. 2016; Garzke et al. 2017), the influences of warming and ocean acidification were studied in a community of calanoid copepods, which showed feeding preferences between phytoplankton and microzooplankton. Thus, it is more precise to use the term 'low trophic levels consumers' here.

Specific comment 43

606 'relationship' –> 'relationships'

Response:

'relationship' was revised to 'relationships'. (See Page 28, Line 612)

Specific comment 44

612 How does the temperature and CO2 relate to future ocean scenarios? That would be good to add to the introduction.

Response:

Please see our reply to General comment I.

Specific comment 45

614 Wouldn't that contradict the argument you made in line 523-524 that these results cannot be scaled up to carbon export?

Response:

The argument about carbon export was revised. Please see our reply to Specific comment 40.

Specific comment 46

Table S2: the meaning of the column effect builder is not clear to me. What does main, two way and three way mean and how do these model outputs relate to the ones in table 2?

Response:

In Table S2, 'main', 'two way' and 'three way' mean models containing first order effects of the three factors, second order interactions of all factors, and third order interactions of all factors, respectively. The selected models in Table 1 are shown in bold in Table S2.

We clarify the meaning of the column effect builder and the relationship between Table S2 and Table 1 in the revised manuscript.

Specific comment 47

Table 2: It is not clear to me what a significant intercept in these models mean? Furthermore, I'm missing interaction terms for some of the variables. I would change PIC (ug/ml) to PIC population yield (ug/ml) to make it easier to connect with the text.

Response:

A significant intercept means that the regression curve (or in case of linear correlations: regression line) does not pass through the origin.

Table 1 only shows the results of selected models. For some variables such as POC:PON, the model with only first order effects of the three factors was selected,

because it can best predict targets. Thus, there were no interaction terms for the variable POC:PON.

PIC (and POC) population yield is used in the revised manuscript. (See Table S5)

Specific comment 48

Figure 1: I'm missing the results for N:P supply in this figure.

Response:

The results for N:P supply ratio are shown in Fig. 1.

Specific comment 49

Figure 2: I'm missing the results for CO2 in this figure.

Response:

The results for pCO_2 are shown in Fig. 2 and Fig. 3.

Specific comment 50

Table S4 seems to be the only results in which standard deviations instead of standard errors are reported. For consistency reasons I would opt for standard errors here.

Response:

We show now standard errors in Table S4.

Specific comment 51

Fig S2 is missing the (mean +/- SE) from the legend. Or is standard deviation expressed here?

Response:

Data in Fig. S2 are expressed as mean ± SE. As suggested, this information is clarified in the revised manuscript.

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Simultaneous shifts in elemental stoichiometry and fatty acids of *Emiliania huxleyi* in response to environmental changes

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Abstract

Climate-driven changes in environmental conditions have significant and complex effects on marine ecosystems. Variability in phytoplankton elements and biochemicals can be important for global ocean biogeochemistry and ecological functions, while there is currently limited understanding on how elements and biochemicals respond to the changing environments in key coccolithophore species such as *Emiliania huxleyi*. We investigated responses of elemental stoichiometry and fatty acids (FAs) in a strain of *E. huxleyi* under three temperatures (12, 18 and 24 ℃), three N:P supply ratios (molar ratios 10:1, 24:1 and 63:1) and two pCO_2 levels (560 and 2400 μatm). Overall, C:N:P stoichiometry showed the most pronounced response to N:P supply ratios, with high ratios of particulate organic carbon vs. particulate organic nitrogen (POC:PON) and low ratios of PON vs. particulate organic phosphorus (PON:POP) in low N-media, and high POC:POP and PON:POP in low P-media. The ratio of particulate inorganic carbon vs. POC (PIC:POC) and polyunsaturated fatty acid proportions strongly responded to temperature and pCO_2, both being lower under high pCO_2 and higher with warming. We observed synergistic interactions between warming and nutrient deficiency (and high pCO_2) on elemental cellular contents and docosahexaenoic acid (DHA) proportion in most cases, indicating the enhanced effect of warming under nutrient deficiency (and high pCO_2). Our results suggest differential sensitivity of elements and FAs to the changes in temperature, nutrient availability and pCO_2 in *E. huxleyi*, which is to some extent unique compared with non-calcifying algal classes. Thus, simultaneous changes of elements and FAs should be considered when

predicting future roles of *E. huxleyi* in the biotic-mediated connection between biogeochemical cycles, ecological functions and climate change.

Key words: Coccolithophores; elements; biochemicals; warming; nutrients; CO_2

1 Introduction

Climate change and intensive anthropogenic pressures have pronounced and diverse effects on marine ecosystems. Physical and chemical properties in marine ecosystems are changing simultaneously such as the concurrent shifts in temperature, CO_2 and oxygen concentrations, and nutrient availability (Boyd et al., 2015). These changes have altered trophic interactions in both bottom-up and top-down directions and thus result in changes in community structure of different trophic levels and ecosystem functions (Doney et al., 2012). Phytoplankton are the base of marine food webs and major drivers of ocean biogeochemical cycling, and thus quantifying their responses to changing oceanic conditions is a major challenge in studies of food web structure and ocean biogeochemistry.

Coccolithophores are a key phytoplankton group in the ocean because of their production of calcified scales called coccoliths. They are not only important photosynthetic producers of organic matter (causing a draw-down of CO_2 in the surface layer), but also play predominant roles in the production and export of calcium carbonate to deeper layers (causing a net release of CO_2 into the atmosphere) (Rost and Riebesell, 2004). Owning to the determination of these two processes on ocean-atmosphere exchange of CO_2, coccolithophores exhibit a complex and significant influence on global carbon cycle (Rost and Riebesell, 2004). Of all coccolithophores, *Emiliania huxleyi* is the most widely distributed and the most abundant species (Winter et al., 2014), with the capacity to form spatially extensive blooms in mid- to high-latitudes (Raitsos et al., 2006; Tyrrell and Merico, 2004).

Evidence from *in situ* and satellite observations indicates that *E. huxleyi* is increasingly expanding its range poleward in both hemispheres over the last two decades, and contributing factors to this poleward expansion may differ between regions and hemispheres (Winter et al., 2014). For example, warming and freshening have promoted *E. huxleyi* blooms in the Bering Sea since the late 1970s (Harada et al., 2012), while temperature and irradiance were best able to explain variability in *E. huxleyi*-dominated coccolithophore community composition and abundance across the Drake Passage (Southern Ocean) (Charalampopoulou et al., 2016). Hence, empirical data on the responses of *E. huxleyi* to different environmental drivers would be critical for fully understanding the roles of this prominent coccolithophore species in marine ecosystems.

Extensive experimental studies have shown highly variable responses of *E. huxleyi* to rising atmospheric CO_2 (reviewed by Feng et al., 2017a; Meyer and Riebesell, 2015), while other studies focused on the influence of other environmental factors such as temperature (Rosas-Navarro et al., 2016; Sett et al., 2014; Sorrosa et al., 2005), light intensity (Nanninga and Tyrrell, 1996; Xing et al., 2015) and nutrient availability (Oviedo et al., 2014; Paasche, 1998). Responses of *E. huxleyi* to the interactions between these different factors have recently received more attention (De Bodt et al., 2010; Feng et al., 2008; Milner et al., 2016; Perrin et al., 2016; Rokitta and Rost, 2012). Many of these studies above focused on the physiological, calcification and photosynthetic responses of *E. huxleyi* due to its considerable role in global carbon cycle. However, biogeochemical cycles of the major nutrient elements (nitrogen and

111 phosphorus) and carbon are tightly linked (Hutchins et al., 2009), and thus variability
112 in *E. huxleyi* C:N:P stoichiometry (cellular quotas and ratios of C, N and P) can also
113 be important in ocean biogeochemistry. Moreover, elemental budgets in organisms are
114 primarily determined by the physiology and biochemistry of biochemicals such as
115 proteins and fatty acids (FAs) (Anderson et al., 2004; Sterner and Elser, 2002). Thus,
116 studying simultaneous changes of elements and biochemicals enables the connection
117 between climate change and ecosystem functions such as elemental cycles; however,
118 shifts in resource nutrient content for consumers are often overlooked in climate
119 change ecology (Rosenblatt and Schmitz, 2016). Recently, Bi et al. (2017)
120 investigated responses of C:N:P stoichiometry and FAs to the interactions of three
121 environmental factors in the diatom *Phaeodactylum tricornutum* and the cryptophyte
122 *Rhodomonas* sp., showing dramatic effects of warming and nutrient deficiency, and
123 modest effects of increased pCO_2. However, for the key coccolithophore species *E.*
124 *huxleyi* much less is known about the simultaneous changes in elemental
125 stoichiometry and biochemicals in response to multiple environmental factor changes.
126 In the present study, we conducted semi-continuous cultures of *E. huxleyi* to
127 disentangle potential effects of temperature, N:P supply ratios and pCO_2 on *E. huxleyi*
128 elemental stoichiometry and FAs. The elevated levels of temperature and pCO_2 in our
129 study are within the predicted ranges of future ocean scenarios. The inter-annual
130 average temperature varied between 16 to 22 ℃ at the Azores
131 (http://dive.visitazores.com/en/when-dive; last accessed date: 22.08.2017), the source
132 region of our *E. huxleyi* strain, while annual mean sea surface temperature across the

North Atlantic (0–60 °N) is projected to reach 29.8 ℃ in 2100 according to the ocean general circulation model (Lewandowska et al., 2014). Considerable seasonal, depth and regional variations of pCO_2 have been observed in the present-day ocean (Joint et al., 2011). In plankton-rich waters, respiration plus atmospheric CO_2-enrichment can drive high regional pCO_2 at times today, e.g, up to 900 μatm in August, with the minimum value of 192 μatm in April, in the Southern Bight of the North Sea (Schiettecatte et al., 2007). In the future oceans, pCO_2 will increase with rising atmospheric CO_2, being 851-1370 μatm by 2100 and 1371-2900 μatm by 2150 (RCP8.5 scenario of the IPCC report 2014) (IPCC, 2014). We tested the following hypotheses in the present study: (i) elemental stoichiometry and FAs in *E. huxleyi* show different sensitivity to considerable variations in temperature, N:P supply ratios and pCO_2; (ii) the ratios of particulate organic carbon vs. particulate organic nitrogen (POC:PON), POC vs. particulate organic phosphorus (POC:POP), and particulate inorganic carbon vs. POC (PIC:POC) in *E. huxleyi* will reduce and the proportions of unsaturated fatty acids will increase under projected future ocean scenarios; and (iii) there are synergetic interactions between warming, nutrient deficiency and rising pCO_2 on *E. huxleyi* elemental stoichiometry and FA composition.

2 Material and methods

2.1 Experimental setup

To address our questions on how multiple environmental drivers influence elemental and FA composition in *E. huxleyi*, we performed a semi-continuous culture experiment crossing three temperatures (12, 18 and 24 ℃), three N:P supply ratios

(molar ratios 10:1, 24:1 and 63:1) and two pCO_2 levels (560 and 2400 µatm). The strain of *E. huxleyi* (Internal culture collection reference code: A8) was isolated from waters off Terceira Island, Azores, North Atlantic (38°39′22″ N 27°14′08″ W). Semi-continuous cultures, as a practical surrogate for fully continuous culture, have been successfully used to study the responses of phytoplankton stoichiometric and biochemical composition to environmental changes such as nutrient availability (Feng et al., 2017a; Lynn et al., 2000; Terry et al., 1985). Our temperature range setup was based on the study of Lewandowska et al. (2014), who chose a temperature increment of 6 ℃, according to the ocean general circulation model under the IPCC SRES A1F1 scenario.

All cultures were exposed to a light intensity of 100 µmol photons · m^{-2} · s^{-1} at a 16:8 h light:dark cycle in temperature-controlled rooms. The culture medium was prepared with sterile filtered (0.2 µm pore size, Sartobran® P 300; Sartorius, Goettingen, Germany) North Sea water with a salinity of 37 psu. Macronutrients were added as sodium nitrate ($NaNO_3$) and potassium dihydrogen phosphate (KH_2PO_4) to achieve three N:P supply ratios, i.e., 35.2 µmol ·L^{-1} N and 3.6 µmol ·L^{-1} P (10:1 mol mol^{-1}), 88 µmol ·L^{-1} N and 3.6 µmol ·L^{-1} P (24:1 mol mol^{-1}) and 88 µmol ·L^{-1} N and 1.4 µmol ·L^{-1} P (63:1 mol mol^{-1}). Vitamins and trace metals were added based on the modified Provasoli's culture medium (Ismar et al., 2008; Provasoli, 1963). Initial pCO_2 of the culture medium was manipulated by bubbling with air containing the target pCO_2. Three replicates were set up for each treatment, resulting in 54 experimental units. Each culture was kept in a sealed cell culture flask with 920 mL

177 culture volume. Culture flasks were carefully rotated twice per day at a set time to
178 minimize sedimentation.

179 First, batch culture experiments were performed to obtain an estimate of the
180 observed maximal growth rate (μ_{max}, day^{-1}) under three temperatures, three N:P supply
181 ratios and two pCO_2 levels. μ_{max} was calculated based on the changes of population
182 cell density within exponential phase (Bi et al., 2012). Once batch cultures reached
183 the early stationary phase, semi-continuous cultures were started with the algae from
184 batch cultures. The gross growth rate (μ, resulting from the process of reproduction
185 alone) was applied as 20% of μ_{max} (day^{-1}). Using % of μ_{max} guarantees that the
186 strength on nutrient deficiency is equal through all temperature and pCO_2 treatments.
187 A fixed value of μ would mean weak deficiency when μ_{max} is low, and strong
188 deficiency when it is high. Based on μ, the equivalent daily renewal rate (D, day^{-1})
189 can be calculated according to the equation $D = 1- e^{-\mu t}$, where t is renewal interval
190 (day) (here t = 1 day). The volume of the daily renewal incubation water can be
191 calculated by multiplying D with the total volume of incubation water (920 mL). The
192 incubation water was exchanged with freshly made seawater medium with the target
193 N:P supply ratios, as well as pre-acclimated to the desired pCO_2 level. To
194 counterbalance the biological CO_2-drawdown, the required amount of CO_2-saturated
195 seawater was also added. Renewal of the cultures was carried out at the same hour
196 every day. The steady state in semi-continuous cultures was assessed based on the net
197 growth rate [r, the difference between the gross growth rate and the loss rate ($r = \mu - D$)].
198 When r was zero (at steady state), μ was equivalent to D.

199 **2.2 Sample analysis**

200 Sampling took place at steady state for the following parameters: cell density,
201 dissolved inorganic carbon (DIC), total alkalinity (TA), pH, total particulate carbon
202 (TPC), POC, PON, POP and FAs. Cell density was counted daily in batch and
203 semi-continuous cultures (final cell density at steady state ranging between 1.50×10^5
204 - 17.8×10^5 cells mL^{-1}, with the average value of 7.95×10^5 cells mL^{-1}). pH
205 measurements were conducted daily in semi-continuous cultures (Fig. S1), and the
206 electrode was calibrated using standard pH buffers (pH 4 and pH 7; WTW, Weilheim,
207 Germany).

208 DIC water samples were gently filtered using a single-use syringe filter (0.2 µm,
209 Minisart RC25; Sartorius, Goettingen, Germany) which was connected to the intake
210 tube of a peristaltic pump. Samples were collected into 10 ml glass vials, and all vials
211 were immediately sealed after filling. DIC was analyzed following Hansen et al.
212 (2013) using a gas chromatographic system (8610C; SRI-Instruments, California,
213 USA). Samples for TA analysis were filtered through GF/F filters (Whatman GmbH,
214 Dassel, Germany) and analyzed with the Tirino plus 848 (Metrohm, Filderstadt,
215 Germany). The remaining carbonate parameter pCO_2 was calculated using CO2SYS
216 (Pierrot et al., 2006) and the constants supplied by Hansson (1973) and Mehrbach et
217 al. (1973) that were refitted by Dickson and Millero (1987) (Table S1).

218 TPC, POC, PON and POP samples were filtered onto pre-combusted and
219 pre-washed (5% ~ 10% HCl) GF/F filters (Whatman GmbH, Dassel, Germany). For
220 POC samples, PIC was removed by exposing filters containing TPC to fuming

hydrochloric acid for 12h. Before analysis, filters were dried at 60 ℃ and stored in a desiccator. POC and PON were simultaneously determined by gas chromatography in an organic elemental analyzer (Thermo Flash 2000; Thermo Fisher Scientific Inc., Schwerte, Germany) after Sharp (1974). POP was analyzed colorimetrically by converting organic phosphorus compounds to orthophosphate (Hansen and Koroleff, 1999). PIC was determined by subtracting POC from TPC. PIC and POC production were estimated by multiplying μ with cellular PIC and POC content, respectively. As the physiological (i.e., cellular) PIC and POC variations cannot directly be up scaled to total population response (Matthiessen et al., 2012), PIC and POC contents in our study were shown both on the cellular (as pg $cell^{-1}$) and the population (as µg ml^{-1}) levels.

Fatty acid samples were taken on pre-combusted and hydrochloric acid-treated GF/F filters (Whatman GmbH, Dassel, Germany), stored at -80 ℃ before measurement. FAs were measured as fatty acid methyl esters (FAMEs) using a gas chromatograph (Trace GC-Ultra; Thermo Fisher Scientific Inc., Schwerte, Germany) according to the procedure described in detail in Arndt and Sommer (2014). The FAME 19:0 was added as internal standard and 21:0 as esterification control. The extracted FAs were dissolved with n-hexane to a final volume of 100 µL. Sample aliquots (1 µL) were given into the GC by splitless injection with hydrogen as the carrier gas. Individual FAs were integrated using Chromcard software (Thermo Fisher Scientific Inc., Schwerte, Germany) and identified with reference to the standards Supelco 37 component FAME mixture and Supelco Menhaden fish oil. FA data were

243 expressed as a percentage of total fatty acids (TFAs) (FA proportion, % of TFAs) to
244 better compare our results with those in previous studies. FAs were also quantified on
245 a per unit biomass (μg mg C^{-1}), which is an ideal approach when considering
246 nutritional quality of phytoplankton for herbivores (Piepho et al., 2012).

247 **2.3 Statistical analysis**

248 Generalized linear mixed models (GLMMs) were applied to test the best model
249 explaining the variations in μ_{max}, elemental stoichiometry and FA composition, as this
250 method is more appropriate for non-normal data than classical statistical procedures
251 (Bolker et al., 2009). GLMMs combine the properties of two statistical models (linear
252 mixed models and generalized linear models) (Bolker et al., 2009) and have been
253 widely used in ecology (e.g., Bracewell et al., 2017; Frère et al., 2010; Jamil et al.,
254 2014), in which data sets are often non-normally distributed. In our study, response
255 variables included μ_{max}, elemental stoichiometry [elemental cellular contents (as pg
256 $cell^{-1}$) and their molar ratios], PIC and POC population yield (as μg ml^{-1}) and
257 production (as pg $cell^{-1}$ d^{-1}), FA proportion (as % of TFAs) and contents (as μg mg C^{-1}),
258 with temperature, N:P supply ratios and pCO_2 as fixed effects. Target distributions
259 were tested and link functions were consequently chosen. The link function is a
260 transformation of the target that allows estimation of the model
261 (https://www.ibm.com/support/knowledgecenter/SSLVMB_21.0.0/com.ibm.spss.statis
262 tics.help/idh_glmm_target.htm; last accessed date: 14.08.2017). For example, identity
263 link function is appropriate with any distribution except for multinomial, while logit
264 can be used only with the binomial or multinomial distribution. For all response

265 variables, we tested models containing first order effects, and second and third order
266 interactions of the three factors. The model that best predicted targets was selected
267 based on the Akaike Information Criterion corrected (AICc), i.e., a lower AICc value
268 representing a better fit of the model. Changes of 10 units or more in AICc values
269 were considered as a reasonable improvement in the fitting of GLMMs (Bolker et al.,
270 2009). In case AICc values were comparable (< 10 units difference), the simpler
271 model was thus chosen, unless there were significant second or third order
272 interactions detected. According to differences in AICc values, models containing
273 only first order effects of the three factors were selected as the best models for most
274 response variables, while those also containing second order interactions were chosen
275 for cellular POC, PON, POP and PIC contents, and the proportions of saturated fatty
276 acid (SFA) and docosahexaenoic acid (22:6n-3; DHA) (bold letters in Table S2).
277 Models containing third order interactions were not selected for any response
278 variable.

279 Nested models were applied to test whether the response pattern to one factor (a
280 nested factor) was significant within another factor, in case significant second order
281 interactions were detected in GLMMs. The question a nested model addresses is that,
282 whether one factor plays a role under one (or several) configuration(s) of another
283 factor, but not under all configurations of that factor equally. Also, the nature
284 (antagonistic, additive, or synergistic) of significant second order interactions was
285 analysed according to Christensen et al. (2006). The observed combined effect of two
286 factors was compared with their expected net additive effect [e.g., (factor$_1$ - control) +

287 (factor$_2$ - control)], which was based on the sum of their individual effects. If the
288 observed combined effect exceeded their expected additive effect, the interaction was
289 defined as synergism. In contrast, if the observed combined effect was less than the
290 additive effect, the interaction was defined as antagonism.

291 All statistical analyses were conducted using SPSS 19.0 (IBM Corporation, New
292 York, USA). Significance level was set to $p < 0.05$ in all statistical tests.

293 **3 Results**

294 **3.1 Maximal growth rate (μ_{max})**

295 We observed a highly significant effect of temperature (bold letters in Table 1) and
296 non-significant effect of N:P supply ratios and pCO_2 on μ_{max} in *E. huxleyi*. Increasing
297 temperature stimulated μ_{max}, causing μ_{max} to be two to three times higher at the highest
298 temperature than those at the lowest temperature (Fig. 1). Although non-significant
299 interactions between the three factors were detected, the effect of temperature was
300 dependent on the pCO_2 level (Fig. 1b). At the low pCO_2, the slope of μ_{max} response to
301 increasing temperature was higher from 12 to 18 ℃ and it became lower from 18 to
302 24 ℃, while at the high pCO_2 the slope of μ_{max} response showed no clear difference
303 between three temperatures.

304 **3.2 Elemental stoichiometry**

305 GLMMs results showed that cellular contents of POC, PON, POP and PIC
306 responded significantly to temperature and the interaction between temperature and
307 N:P supply ratios (bold letters in Table 1). Moreover, there were significant effects of
308 pCO_2 on cellular PIC content, and significant interactions between temperature and

309 pCO_2 on cellular PIC and POP contents. For cellular contents of POC, PON and POP,
310 increasing temperature and nutrient deficiency showed synergistic interactions (Table
311 S3), resulting in lower values at higher temperatures under N deficiency (N:P supply
312 ratio = 10:1 mol mol^{-1}) and an increasing trend with increasing temperature under P
313 deficiency (N:P supply ratio = 63:1 mol mol^{-1}) (Fig. 2 a-c; Nested model, $p < 0.001$).
314 Synergistic interactions were also observed between increasing temperature and
315 enhanced pCO_2 on cellular POP content (Table S3), showing the lowest value at low
316 pCO_2 level and the highest one at enhanced pCO_2 in response to increasing
317 temperature (Fig. 2g; Nested model, $p = 0.003$). For cellular PIC content, increasing
318 temperature and N deficiency had antagonistic interactions, while increasing
319 temperature and P deficiency showed synergistic interactions (Table S3). As a result,
320 cellular PIC content showed a slight decreasing trend with increasing temperature
321 under N deficiency and an increasing trend under higher N:P supply ratios (Fig. 2d;
322 Nested model, $p = 0.030$). Increasing temperature and enhanced pCO_2 affected
323 cellular PIC contents synergistically (Table S3), with the negative response of cellular
324 PIC contents to enhanced pCO_2 being significantly weaker as temperature increased
325 (Fig. 2h; Nested model, $p < 0.001$).

326 POC:PON, POC:POP and PON:POP responded significantly to N:P supply ratios
327 (bold letters in Table 1), while only POC:PON showed significant responses to
328 temperature, with non-significant effect of pCO_2 detected. Increasing N:P supply
329 ratios caused a decreased trend in POC:PON (Fig. 3a) and an increase in POC:POP
330 (Fig. 3b), resulting in a positive relationship between PON:POP and N:P supply ratios

(Fig. 3c). The response of POC:PON to increasing temperature was complex, showing a hump-shaped response under N deficiency and negative responses under higher N:P supply ratios (Fig. 3a). PIC:POC responded significantly to temperature and pCO_2, with non-significant effect of N:P supply ratios detected (Table 1). PIC:POC increased with increasing temperature and decreased with enhanced pCO_2 (Fig. 3 d and h).

3.3 Fatty acids

The most abundant FA group was polyunsaturated fatty acids (PUFAs) (33%-54% of TFAs), followed by SFAs (22%-46%) and monounsaturated fatty acids (MUFAs) (13%-27%), across the entire tested gradients of temperature, N:P supply ratios and pCO_2 (Table S4). The high proportion of PUFAs was predominantly caused by high amounts of DHA (12%-31%) and 18:4n-3 (3%-13%), and SFAs was mainly represented by 14:0 (13%-23%) and 16:0 (5%-11%). The major individual MUFA was 18:1n-9 (8%-21%).

GLMMs results showed significant effects of temperature and pCO_2 on the proportions of both MUFAs and PUFAs (bold letters in Table 1). Increasing temperature caused a decrease in the proportion of MUFAs and an increase in PUFAs (Fig. 4 a). In contrast, enhanced pCO_2 resulted in an increase in MUFAs and a decrease in PUFAs at higher temperatures (Fig. 4 c).

The proportion of major individual PUFAs (DHA) showed significant responses to temperature and N:P supply ratios, and the interactions between temperature and N:P supply ratios (and pCO_2) (bold letters in Table 1). Increasing temperature and nutrient deficiency caused an overall increase in DHA (Fig. 4 b). The interactions between

353 increasing temperature and nutrient deficiency (and enhanced pCO_2) affected DHA
354 synergistically (Table S3), and the positive effect of temperature became more
355 pronounced at lower N:P supply ratios (Nested model, $p < 0.001$) and at the low pCO_2
356 (Nested model, $p < 0.001$) (Fig. 4 b and d).

357 **3.4 PON:PUFAs and POP:PUFAs**

358 Both PON:PUFAs and POP:PUFAs varied with the changes in temperature, N:P
359 supply ratios and pCO_2, showing high values under the balanced nutrient condition
360 (N:P supply ratio = 24:1 mol mol^{-1}) at the highest temperature (24 ℃) and high pCO_2
361 level (2400 μatm) (Fig. 5). The lowest value of PON:PUFAs was observed under N
362 deficiency at the intermediate temperature (18 ℃) and high pCO_2 level (Fig. 5 a and
363 c), while that of POP:PUFAs was under P deficiency at the intermediate temperature
364 and low pCO_2 level (560 μatm) (Fig. 5 b and d).

365 **4 Discussion**

366 Our study scales the impacts of temperature, N:P supply ratios and pCO_2 on
367 elemental stoichiometry and FA composition of the ubiquitously important calcifier *E.*
368 *huxleyi*, while accounting for their interactive effects. Overall, C:N:P stoichiometry
369 changed markedly in response to N:P supply ratios, showing a maximum of 62%
370 changes under nutrient deficiency (Table 2). Both PIC:POC and PUFA proportion
371 increased with warming and decreased under high pCO_2, indicating a partial
372 compensation by pCO_2 of a predominantly temperature-driven response. The overall
373 response patterns of C:N:P stoichiometry and PUFAs in our study are consistent with
374 those on a global scale (Martiny et al., 2013), and conform with the meta-analysis

results on haptophytes (Hixson and Arts, 2016). In line with these studies, we also detected significant interactions between temperature, N:P supply ratios and pCO_2 on certain response variables (e.g., elemental cellular content and DHA proportion) (Table 1), indicating variable response patterns of elemental stoichiometry and FA composition in *E. huxleyi* under any given constellation of environmental factors. Our results thus underscore the importance of simultaneous consideration of multiple environmental drivers, demonstrating differential effects of the three environmental factors on elemental stoichiometry and FA composition of *E. huxleyi*.

4.1 Responses of maximal growth rate

Increasing temperature significantly accelerated μ_{max} of *E. huxleyi* in our study (Fig. 1; Table 1). This positive correlation between increasing temperature and growth rate is typical for many *E. huxleyi* strains within the range of temperature 12 to 24 ℃ used in our study (Feng et al., 2008; Rosas-Navarro et al., 2016; Sett et al., 2014; van Bleijswijk et al., 1994). However, the extent to which growth rate of *E. huxleyi* increases with increasing temperature varies between *E. huxleyi* strains, which may contribute to specific biogeographic distribution of different strains (Paasche, 2002). For example, growth rate of *E. huxleyi* from the Gulf of Maine (~42 °N) was 1.2 times higher at 26 ℃ than that at 16 ℃, while growth rate of *E. huxleyi* from the Sargasso Sea (~20-35 °N) was 1.6 times higher at the higher temperature (Paasche, 2002). In our study, μ_{max} of *E. huxleyi* (from the Azores, ~ 38 °N) was two to three times higher at the highest temperature than that at the lowest temperature, showing a similar change pattern with that in the *E. huxleyi* strain from the Sargasso Sea. The

results above suggest that the biogeographic origin of an *E. huxleyi* strain is important for their response to temperature.

Moreover, the response of μ_{max} to temperature was dependent on the pCO_2 level in our study, showing a pronounced decrease in the slope of μ_{max} in response to increasing temperature (0.13 at lower temperatures and 0.026 at higher temperatures) at the low pCO_2 and a relatively constant slope (0.04 – 0.06) at the high pCO_2 (Fig. 1b). This result is consistent with a conceptual graph proposed by Sett et al. (2014). The graph showed a clear increase in metabolic rates from low to intermediate temperature and a slight increase from intermediate to high temperature at the low pCO_2 (~560 µatm), while the changes of metabolic rates are similar from low to intermediate temperature and from intermediate to high temperature at the high pCO_2 (~2400 µatm) (Sett et al., 2014). The conceptual reasoning behind conceptual graph proposed by Sett et al. (2014) is still unclear. One possible explanation is that increasing temperature may modulate the balance between a fertilizing effect of ocean carbonation and a metabolic repression by ocean acidification (Bach et al., 2011; Sett et al., 2014).

4.2 Responses of C:N:P stoichiometry

N:P supply ratios showed highly significant effects on C:N:P stoichiometry (up to 62% changes in response to nutrient deficiency) in *E. huxleyi* in our study, with a weaker effect of warming (-6% to 5% changes) and non-significant effect of pCO_2 observed (Table 1; Table 2). Similarly, previous lab experiments also reported that nutrient availability played a more important role than temperature (and pCO_2) for

elemental stoichiometry in different strains of *E. huxleyi* such as those from outer Oslofjord (Skau, 2015) and from the Chatham Rise, east of New Zealand (Feng et al., 2017b). Also, for marine phytoplankton community biomass on a global scale nitrate concentration as a proxy of nutrient availability explained 36% and 42% of variation in N:P and C:P, respectively, with the less variation explained by temperature (33% and 38% of the variation in N:P and C:P, respectively) (Martiny et al., 2013).

N deficiency caused overall high POC:PON and low PON:POP, while P deficiency resulted in high POC:POP and PON:POP in *E. huxleyi* in this and most previous studies (Langer et al., 2013; Leonardos and Geider, 2005b; Perrin et al., 2016). An important biogeochemical question is the extent to which C:N:P stoichiometry changes in response to N and P deficiency. We found that the high percent change in PON:POP (a 62% increase) under P deficiency was mainly due to a 60% increase in POC:POP, associated with the higher percent change in cellular POC content (a 50% increase) and the lower percent change in cellular POP content (a 8% decrease) (Table 2). Under N deficiency, the 36% decrease in PON:POP was driven by a 33% increase in POC:PON and a 15% decrease in POC:POP, along with similar percent changes in cellular element contents (32% to 53% decrease). The more variable POC:POP under P deficiency and the less variable POC:PON under N deficiency in our study are consistent with the findings in global suspended particle measurements, which showed the high variability of P:C in response to changes in phosphate and the less variable N:C to changes in nitrate (Galbraith and Martiny, 2015). The consistence of C:N:P stoichiometric responses in our study with those on a global scale may reflect

441 the capacity of *E. huxleyi* to thrive under a wide range of environmental conditions.
442 This capacity was largely revealed by a pan-genome assessment, which distributed
443 genetic traits variably between strains and showed a suit of core genes for the uptake
444 of inorganic nitrogen and N-rich compounds such as urea (Read et al., 2013). In spite
445 of strain diversity within *E. huxleyi*, a recent study suggested that the global
446 physiological response of this species to nutrient environments is highly conserved
447 across strains and may underpin its success under a variety of marine environments
448 (Alexander, 2016).

449 Warming resulted in slight decreases in POC:PON (-6%) and POC:POP (-3%) and
450 an 5% increase in PON:POP, associated with a 8% decrease in cellular POC content
451 and 5% to 9% increases in cellular contents of PON and POP in *E. huxleyi* (Table 2).
452 In the literature, variable changes of POC:PON and POC:POP to warming were
453 observed in *E. huxleyi*, showing positive (Borchard and Engel, 2012), negative (Feng
454 et al., 2008; Matson et al., 2016), and U-shaped responses (Rosas-Navarro et al.,
455 2016). Similar to our study, Borchard and Engel (2012) also found a stronger change
456 of POC:PON than of POC:POP at higher P condition in the strain PML B92/11 from
457 Bergen, Norway. The mechanism behind the stronger changes in POC:PON compared
458 to POC:POP may be explained by the temperature-dependent physiology hypothesis,
459 which shows that organisms in warmer conditions require fewer P-rich ribosomes,
460 relative to N-rich proteins (Toseland et al., 2013). In our study, both POC:PON and
461 POC:POP decreased with increasing temperature, while the change in POC:PON
462 (-6%) was larger than that in POC:POP (-3%). Thus, the relative changes in

463 POC:PON and POC:POP, as well as the increase in PON:POP, in response to
464 increasing temperature in our study are consistent with the temperature-dependent
465 physiology hypothesis (Toseland et al., 2013).

466 The single effects of nutrient availability and temperature described above can be
467 modulated by their interactions. In our study, significant interactions were observed
468 between temperature and N:P supply ratios (and pCO_2), with warming and nutrient
469 deficiency synergistically affecting cellular element contents (Table 1; Table S3). An
470 overall synergistic effect was also observed across 171 studies on the responses of
471 marine and coastal systems to multiple stressors (Crain et al., 2008). Furthermore,
472 although 25% to 29% changes emerged in cellular PON and POP contents in response
473 to rising pCO_2, we found non-significant single effect of pCO_2 on *E. huxleyi* C:N:P
474 stoichiometry. Previous studies showed that rising pCO_2 seems to change
475 phytoplankton stoichiometry under specific conditions, e.g., at high light condition
476 (400 μmol photons · m^{-2} · s^{-1}) (Feng et al., 2008) and low nutrient loads (500 μmol
477 photons · m^{-2} · s^{-1} at N:P supply ratio ≤ 15 or N:P supply ratio ≥ 30) (Leonardos and
478 Geider, 2005a). In our study, we used relatively low light intensity (100 μmol photons
479 · m^{-2} · s^{-1}), did not investigate irradiance effects. Additional research is required to
480 assess the effects of other environmental factors such as irradiance and their
481 interactions on C:N:P stoichiometry in our *E. huxleyi* strain.

482 Taken together, our results indicate that C:N:P stoichiometry in *E. huxleyi* largely
483 reflected the changes in N:P supply ratios, across different temperatures and pCO_2
484 levels. However, for two algal species from non-calcifying classes (the diatom *P.*

485 *tricornutum* and the cryptophyte *Rhodomonas* sp.) temperature had the most
486 consistent significant effect on stoichiometric ratios in our previous work (Bi et al.,
487 2017). The results above are consistent with the ranking of environmental control
488 factors in Boyd et al. (2010), which showed that temperature, nitrogen and
489 phosphorus were ranked as important factors for major phytoplankton groups.

490 **4.3 Responses of PIC:POC**

491 Both pCO_2 and temperature had highly significant effects on PIC:POC in our study,
492 with enhanced pCO_2 and warming resulting in an overall 49% decrease and a 41%
493 increase in PIC:POC, respectively, while N:P supply ratios showed no significant
494 effect (Table 1; Table 2). This result is in agreement with rankings of the importance
495 of environmental drivers on PIC:POC in a Southern Hemisphere strain of *E. huxleyi*
496 (isolated from the Chatham Rise), showing the order of pCO_2 (negative effect) >
497 temperature (positive effect) and non-significant effect of nitrate or phosphate (Feng
498 et al., 2017b).

499 The negative effect of enhanced pCO_2 on PIC:POC has been widely observed for
500 different strains of *E. huxleyi* (Meyer and Riebesell, 2015 and references therein). The
501 negative response of PIC:POC to rising pCO_2 in our study was driven by the
502 significant decrease in cellular PIC content (calcification) and non-significant change
503 in cellular POC content (photosynthesis) (Table 1; Table 2). Previous studies also
504 showed a greater impact of ocean acidification on calcification than on photosynthesis
505 in coccolithophores (De Bodt et al., 2010; Feng et al., 2017a; Meyer and Riebesell,
506 2015). Feng et al. (2017a) suggested that the decreased calcification in *E. huxleyi* may

be caused by the increased requirement of energy to counteract intracellular acidification. The increased activity of carbonic anhydrase (CA) at low pCO_2 may explain the lack of a significant effect of pCO_2 on the photosynthetic or growth rate (Feng et al., 2017a), as up-regulation of CA at low DIC was previously observed (Bach et al., 2013).

Warming causes diverse responses of calcification and photosynthesis within *E. huxleyi* species (Rosas-Navarro et al., 2016 and references therein; the present study). Overall, our study showed that the increase in PIC:POC at high temperatures was driven by a marked increased cellular PIC content (28%) and a decreased cellular POC content (-8%) (Table 1; Table 2), consistent with the responses of PIC:POC to warming in other *E. huxleyi* strains such as the strain PML B92/11 (Sett et al., 2014) and the strain CCMP3266 from the Tasman Sea (Matson et al., 2016). The positive response of PIC:POC to increasing temperature may be explained by the allocation of carbon to calcification rather than photosynthesis at high temperatures (Sett et al., 2014).

Significant interactions between temperature and N:P supply ratios (and pCO_2) were observed on cellular particulate carbon contents in our study (Table 2). For example, the negative relationship between cellular PIC contents and enhanced pCO_2 became weaker at the highest temperature (Fig. 2h). This result is in agreement with the modulating effect of temperature on the CO_2 sensitivity of key metabolic rates in coccolithophores, due to the shift of the optimum CO_2 concentration for key metabolic processes towards higher CO_2 concentrations from intermediate to high

529 temperatures (Sett et al., 2014). Specifically, the interactions between warming and
530 nutrient deficiency (and high pCO_2) synergistically affected both PIC and POC
531 cellular contents in most cases in our study (Table S3), indicating that nutrient
532 deficiency and high pCO_2 are likely to enhance the effect of warming on *E. huxleyi*
533 calcification and photosynthesis efficiency.

534 In summary, our results showed an overall reduced PIC:POC in *E. huxleyi* under
535 future ocean scenarios of warming and higher pCO_2 (Fig. 3h and Table 2), consistent
536 with the reduced ratio of calcium carbon production to organic carbon during the *E.*
537 *huxleyi* bloom in previous mesocosm experiments (Delille et al., 2005; Engel et al.,
538 2005). It is worth noting that cellular PIC and POC contents are a measure for
539 physiological response and cannot be directly used to infer population response, as
540 different responses between cellular and population yields of PIC (and POC) (as μg
541 ml^{-1}) to environmental changes were evident in previous work (Matthiessen et al.,
542 2012) and the present study (Table S5, S6; Fig. S3, S4). Thus, scaling our results up to
543 coccolithophores carbon export should consider these uncertainties.

544 **4.4 Responses of fatty acids**

545 Our study provides the first experimental demonstration of the relative importance
546 of temperature, N:P supply ratios and pCO_2 on *E. huxleyi* FA composition. Both
547 temperature and pCO_2 had significant effects on the proportions of MUFAs and
548 PUFAs, with warming causing larger changes in MUAFs and PUFAs than rising
549 pCO_2, while significant effects of N:P supply ratios was only observed for DHA
550 proportion (Table 1; Table 2).

551 Increasing temperature caused a 20% decline in MUFA proportion and a 13%
552 increase in PUFA proportion in our study (Table 2). This result is consistent with the
553 negative response of MUFA proportion and positive response of PUFA proportion to
554 warming in other haptophytes based on a meta-analysis on 137 FA profiles (Hixson
555 and Arts, 2016), showing an opposite response to general patterns of phytoplankton
556 FAs to warming. Although warming is expected to have a negative effect on the
557 degree of fatty acid unsaturation to maintain cell membrane structural functions
558 (Fuschino et al., 2011; Guschina and Harwood, 2006; Sinensky, 1974), variable FA
559 responses to warming were widely observed in different phytoplankton groups (Bi et
560 al., 2017; Renaud et al., 2002; Thompson et al., 1992). Contradictory findings were
561 even reported in meta-analyses on large FA profiles such as the absence (Galloway
562 and Winder, 2015) or presence (Hixson and Arts, 2016) of the negative correlation
563 between temperature and the proportion of long-chain EFAs in freshwater and marine
564 phytoplankton. While the underling mechanisms of variable FA responses are still
565 unclear, it is known that both phylogeny and environmental conditions determine
566 phytoplankton FA composition (Bi et al., 2014; Dalsgaard et al., 2003; Galloway and
567 Winder, 2015). In our study, we found significant interactions between temperature
568 and pCO_2 (and N:P supply ratios) on the individual FA component DHA, showing that
569 pCO_2 and nutrient availability may alter the effect of warming on *E. huxleyi* FA
570 composition.

571 Enhanced pCO_2 led to an overall 7% increase in MUFAs and a 7% decrease in
572 PUFAs (Table 2), consistent with FA response patterns in the *E. huxleyi* strain PML

573 B92/11 (Riebesell et al., 2000) and the strain AC472 from Western New Zealand,
574 South Pacific (Fiorini et al., 2010). Also in a natural plankton community (Raunefjord,
575 southern Norway), PUFA proportion was reduced at high pCO_2 level in the nano-size
576 fraction, suggesting a reduced Haptophyta (dominated by *E. huxleyi*) biomass and a
577 negative effect of high pCO_2 on PUFA proportion (Bermúdez et al., 2016). To date,
578 several mechanisms have been suggested to explain the reduced PUFAs at high pCO_2
579 in green algae (Pronina et al., 1998; Sato et al., 2003; Thompson, 1996), with much
580 less work conducted in other phytoplankton groups. One possible mechanism was
581 demonstrated in the study on *Chlamydomonas reinhardtii*, showing that the repression
582 of the CO_2-concentrating mechanisms (CCMs) was associated with reduced FA
583 desaturation at high CO_2 concentration (Pronina et al., 1998). Our observed decrease
584 in the proportion and content of PUFAs at higher pCO_2 (Table S6) fits well with the
585 mechanism proposed by Pronina et al. (1998), which may be attributed to the
586 repression of CCMs at high pCO_2 in *E. huxleyi*.

587 N and P deficiency caused no clear changes in the proportions of MUFAs and
588 PUFAs, with 14% to 22% increase in DHA proportion observed (Table 2). While
589 nutrients often play a major role on phytoplankton lipid composition (Fields et al.,
590 2014; Hu et al., 2008), the less pronounced effects of nutrient deficiency in our study
591 indicate a unique lipid biosynthesis in *E. huxleyi*. Indeed, Van Mooy et al. (2009)
592 suggested that *E. huxleyi* used non-phosphorus betaine lipids as substitutes for
593 phospholipids in response to P scarcity. Genes are also present in the core genome of
594 *E. huxleyi* for the synthesis of betaine lipids and unusual lipids used as

595 nutritional/feedstock supplements (Read et al., 2013). Therefore, the lack of
596 significant nutrient effects on most FA groups in *E. huxleyi* in our study may be
597 caused by the functioning of certain lipid substitutions under nutrient deficiency.

598 In summary, our study showed stronger effects of pCO_2 and temperature, and a
599 weaker effect of N:P supply ratios on the proportions of unsaturated FAs in *E. huxleyi*.
600 It should be noted that using different units to quantify FA composition may cause
601 contradictory results, e.g., an increase in PUFA proportion (% of TFAs) but an overall
602 decline in PUFA contents per biomass (μg mg C^{-1}) with increasing temperature in our
603 study (Table S5, S6). Moreover, PUFA contents per biomass in two species of
604 non-calcifying classes (*P. tricornutum* and *Rhodomonas* sp.) showed a similar
605 response pattern with those in *E. huxleyi* in our study (Table S6), responded
606 negatively to warming and positively to N (and P) deficiency (Bi et al., 2017).
607 However, differential responses were also observed, e.g., a significant negative effect
608 of enhanced pCO_2 on PUFA contents in *E. huxleyi*, but a non-significant effect of
609 pCO_2 on PUFA contents in *P. tricornutum* and *Rhodomonas* sp. (Bi et al., 2017). This
610 different response between phytoplankton groups is in agreement with findings in
611 mesocosm studies (Bermúdez et al., 2016; Leu et al., 2013), suggesting that changes
612 in taxonomic composition can cause different relationships between PUFAs and pCO_2
613 in natural phytoplankton community.

614 **4.5 Implications for marine biogeochemistry and ecology**

615 We observed that warming and nutrient deficiency caused an overall increase in
616 POC:PON and POC:POP (i.e., decreases in cellular PON and POP quotas), while

enhanced pCO_2 showed no clear effects. This result indicates that nitrogen and phosphorus requirements in *E. huxleyi* are likely to reduce under projected future changes in temperature and nutrient availability, and show minor changes in response to higher pCO_2. Likewise, Hutchins et al. (2009) suggested negligible or minor effects of projected future changes in pCO_2 on most phytoplankton phosphorus requirements. Moreover, the overall low PIC:POC under future ocean scenarios (warming and enhanced pCO_2) indicates that carbon production by the strain *E. huxleyi* in our study acts as a carbon sink. This argument is consistent with the findings of the decreased calcification with increasing pCO_2 in most coccolithophores (Beaufort et al., 2011; Hutchins and Fu, 2017), which may reduce vertical exported fluxes of sinking calcium carbonate and minimize calcification as a carbon source term, ultimately downsizing the ocean's biological carbon cycle (Hutchins and Fu, 2017).

The C:N and C:P stoichiometry and PUFAs have been used as indicators of nutritional quality of phytoplankton for consumers (Hessen, 2008; Müller-Navarra, 2008). We found that C:N:P stoichiometry and PUFAs co-varied in *E. huxleyi* in response to the changes in culture conditions, with the highest values of both PON:PUFAs and POP:PUFAs observed under the balanced nutrient condition at the highest temperature and high pCO_2 level (Fig. 5). The high PON:PUFAs and POP:PUFAs indicate a high probability of PUFA limitation relative to PON (and POP) for zooplankton feeding *E. huxleyi* based on the extended stoichiometric hypothesis (Anderson and Pond, 2000). Studies on plant-herbivore interactions reported that changes in elemental and biochemical composition in phytoplankton can translate to

higher trophic levels (Kamya et al., 2017; Rossoll et al., 2012) and refer to direct effects of environmental changes on low trophic level consumers, which can be modified by indirect bottom-up driven impacts through the primary producers (Garzke et al., 2016; Garzke et al., 2017).

5 Conclusions

Our study shows that N:P supply ratios had the strongest effect on C:N:P stoichiometry, while temperature and pCO_2 played more influential roles on PIC:POC and PUFA proportions in *E. huxleyi*. The specific response patterns of elemental ratios and FAs have important implications for understanding biogeochemical and ecological functioning of *E. huxleyi*. The observations presented here suggest differential responses of elements and FAs to rising temperature, enhanced pCO_2 and nutrient deficiency in *E. huxleyi*, being to some extent unique compared with algal species from non-calcifying classes. Thus, the role of multiple environmental drivers under the biodiversity context should be considered to truly estimate the future functioning of phytoplankton in the changing marine environments.

661 **Data availability**: data sets are available upon request by contacting Meixun Zhao
662 (maxzhao@ouc.edu.cn and maxzhao04@yahoo.com).
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664 experiments and R. Bi carried them out. R. Bi prepared the manuscript with
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681
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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 the observed maximal growth rate (μ_{max}), elemental stoichiometry and fatty acid proportions in *Emiliania huxleyi*. Significant *p* values are shown in bold; T: temperature; N:P: N:P supply ratios; TFA: total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; DHA: docosahexaenoic acid. Results of AICc are shown in Table S2.

Variable	Factor	Coefficienct ± SE	*t*	*p*
μ_{max} (d^{-1})	Intercept	-1.368 ± 0.225	-6.075	**<0.001**
	T	0.074 ± 0.010	7.082	**<0.001**
	pCO_2	<0.001 ± <0.001	-0.472	0.644
	N:P	<0.001 ± 0.002	-0.162	0.873
POC cellular content (pg $cell^{-1}$)	Intercept	3.683 ± 0.377	9.779	<0.001
	T	-0.089 ± 0.020	-4.577	**<0.001**
	pCO_2	<0.001 ± <0.001	-0.929	0.358
	N:P	-0.008 ± 0.008	-0.996	0.324
	T × pCO_2	<0.001 ± <0.001	1.886	0.066
	T × N:P	0.001 ± <0.001	3.477	**0.001**
	pCO_2 × N:P	<0.001 ± <0.001	-0.359	0.721
PON cellular content (pg $cell^{-1}$)	Intercept	1.208 ± 0.491	2.458	**0.018**
	T	-0.083 ± 0.026	-3.259	**0.002**
	pCO_2	<0.001 ± <0.001	-0.873	0.387
	N:P	-0.008 ± 0.011	-0.709	0.482
	T × pCO_2	<0.001 ± <0.001	1.549	0.128
	T × N:P	0.001 ± 0.001	2.802	**0.007**
	pCO_2 × N:P	<0.001 ± <0.001	0.165	0.870
POP cellular content (pg $cell^{-1}$)	Intercept	-0.564 ± 0.468	-1.206	0.234
	T	-0.091 ± 0.024	-3.751	**<0.001**
	pCO_2	<0.001 ± <0.001	-1.656	0.104
	N:P	-0.018 ± 0.010	-1.840	0.072
	T × pCO_2	<0.001 ± <0.001	2.396	**0.021**
	T × N:P	0.001 ± <0.001	2.410	**0.020**
	pCO_2 × N:P	<0.001 ± <0.001	0.572	0.570
PIC cellular content (pg $cell^{-1}$)	Intercept	3.293 ± 0.406	8.122	<0.001
	T	-0.067 ± 0.021	-3.193	**0.003**
	pCO_2	-0.001 ± <0.001	-5.519	**<0.001**
	N:P	-0.003 ± 0.009	-0.292	0.772
	T × pCO_2	<0.001 ± <0.001	4.584	**<0.001**
	T × N:P	0.001 ± <0.001	2.340	**0.024**

	pCO_2 × N:P	<0.001 ± <0.001	0.111	0.912
POC:PON (mol mol^{-1})	Intercept	2.741 ± 0.081	33.823	**<0.001**
	T	-0.008 ± 0.004	-2.169	**0.035**
	pCO_2	<0.001 ± <0.001	0.153	0.879
	N:P	-0.004 ± 0.001	-5.430	**<0.001**
POC:POP (mol mol^{-1})	Intercept	5.423 ± 0.128	42.300	**<0.001**
	T	-0.007 ± 0.006	-1.242	0.220
	pCO_2	<0.001 ± <0.001	0.069	0.945
	N:P	0.012 ± 0.001	9.617	**<0.001**
PON:POP (mol mol^{-1})	Intercept	2.702 ± 0.145	18.590	<0.001
	T	0.001 ± 0.007	0.157	0.876
	pCO_2	<0.001 ± <0.001	-0.169	0.866
	N:P	0.016 ± 0.001	11.200	**<0.001**
PIC:POC	Intercept	0.460 ± 0.066	7.010	<0.001
	T	0.025 ± 0.003	8.184	**<0.001**
	pCO_2	<0.001 ± <0.001	-12.837	**<0.001**
	N:P	<0.001 ± 0.001	-0.166	0.869
SFA proportion (% of TFAs)	Intercept	3.506 ± 0.145	24.178	<0.001
	T	-0.012 ± 0.008	-1.538	0.131
	pCO_2	<0.001 ± <0.001	-0.238	0.813
	N:P	-0.004 ± 0.003	-1.248	0.218
	T × pCO_2	<0.001 ± <0.001	1.816	0.076
	T × N:P	<0.001 ± <0.001	1.657	0.104
	pCO_2 × N:P	<0.001 ± <0.001	-2.487	**0.016**
MUFA proportion (% of TFAs)	Intercept	30.259 ± 1.344	22.518	<0.001
	T	-0.579 ± 0.063	-9.240	**<0.001**
	pCO_2	0.001 ± <0.001	2.269	**0.028**
	N:P	-0.014 ± 0.014	-1.050	0.299
PUFA proportion (% of TFAs)	Intercept	32.264 ± 2.300	14.028	<0.001
	T	0.638 ± 0.107	5.949	**<0.001**
	pCO_2	-0.002 ± 0.001	-2.769	**0.008**
	N:P	0.034 ± 0.023	1.453	0.152
DHA proportion (% of TFAs)	Intercept	2.204 ± 0.185	11.887	<0.001
	T	0.054 ± 0.010	5.611	**<0.001**
	pCO_2	<0.001 ± <0.001	1.874	0.067
	N:P	0.010 ± 0.004	2.735	**0.009**
	T × pCO_2	<0.001 ± <0.001	-2.946	**0.005**
	T × N:P	-0.001 ± <0.001	-2.898	**0.006**
	pCO_2 × N:P	<0.001 ± <0.001	1.249	0.218

Table 2. The changes in elemental cellular contents (as pg cell^{-1}), elemental molar ratios and the proportions of major fatty acid groups and docosahexaenoic acid (DHA) (as % of total fatty acids) in response to warming, N and P deficiency and enhanced pCO_2 in *Emiliania huxleyi*. Here, not only significant effects are depicted, but also non-significant and substantial effects on response variables. Significant interactions are presented based on GLMM results in Table 1. Red and blue arrows indicate a mean percent increase and decrease in a given response, respectively.

Response	Effect				
	Warming	-N	-P	Enhanced pCO_2	Interactions
POC cellular content	↓ -8%	↓ -39%	↑ 50%	–	T×N:P supply
PON cellular content	↑ 5%	↓ -53%	↑ 52%	↑ 25%	T×N:P supply
POP cellular content	↑ 9%	↓ -32%	↓ -8%	↑ 29%	T×N:P supply T×CO_2
PIC cellular content	↑ 28%	↓ -31%	↑ 65%	↓ -36%	T×N:P supply T×CO_2
POC:PON	↓ -6%	↑ 33%	–	–	
POC:POP	↓ -3%	↓ -15%	↑ 60%	–	
PON:POP	↑ 5%	↓ -36%	↑ 62%	–	
PIC:POC	↑ 41%	–	–	↓ -49%	
SFA proportion	↑ 5%	↓ -7%	↓ -15%	↑ 7%	N:P supply×CO_2
MUFA proportion	↓ -20%	–	–	↑ 7%	
PUFA proportion	↑ 13%	–	–	↓ -7%	
DHA proportion	↑ 16%	↑ 14%	↑ 22%	↓ -7%	T×N:P supply T×CO_2

↑↓ Changes ≥ 25% ↑↓ Changes < 25% – No clear changes

Fig. 1 Responses of the observed maximal growth rate (μ_{max}; mean ± SE) to temperature, N:P supply ratios and pCO_2 in *Emiliania huxleyi*. The selected model contains only the first order effects of the three environmental factors, with the results of AICc shown in Table S2.

Fig. 2 Responses of cellular contents of (a, e) particulate organic carbon (POC), (b, f) particulate organic nitrogen (PON), (c, g) particulate organic phosphorus (POP) and (d, h) particulate inorganic carbon (PIC) (mean ± SE) to temperature, N:P supply ratios and pCO_2 in *Emiliania huxleyi*. The selected models contain the first order effects, and second order interactions of the three environmental factors for the four response variables, with the results of AICc shown in Table S2.

Fig. 3 The ratios of (a, e) particulate organic carbon vs. particulate organic nitrogen (POC:PON), (b, f) POC vs. particulate organic phosphorus (POC:POP), (c, g) PON vs. POP (PON:POP) and (d, h) particulate inorganic carbon vs. POC (PIC:POC) (mean ± SE) in response to temperature, N:P supply ratios and pCO_2 in *Emiliania huxleyi*. The selected models contain only the first order effects of the three environmental factors for the four response variables, with the results of AICc shown in Table S2.

Fig. 4 Responses of the proportions of (a, c) monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), and (b, d) docosahexaenoic acid (DHA) (mean ± SE) to temperature, N:P supply ratios and pCO_2 in *Emiliania huxleyi*. For MUFA and PUFA proportions, the selected models contain only the first order effects of the three environmental factors, and that for DHA proportion contains also second order interactions, with the results of AICc shown in Table S2.

Fig. 5 The ratios of (a, c) particulate organic nitrogen vs. polyunsaturated fatty acids (PON:PUFAs) and (b, d) particulate organic phosphorus vs. PUFAs (POP:PUFAs) in response to temperature, N:P supply ratios and pCO_2 in *Emiliania huxleyi*.

Fig. 1

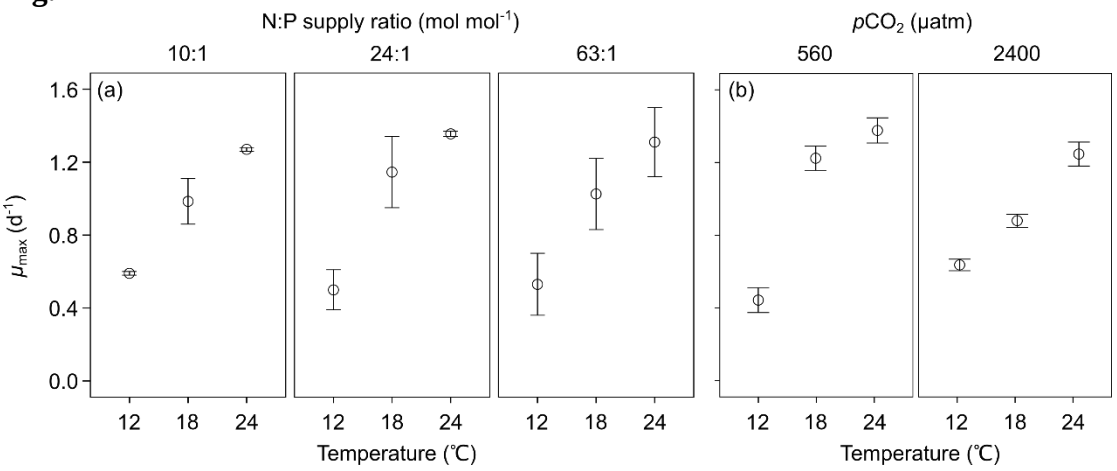

N:P supply ratio (mol mol-1)
10:1
24:1
63:1
pCO2 (μatm)
560
2400
(a)
(b)
μmax (d-1)
1.6
1.2
0.8
0.4
0.0
12
18
24
Temperature (℃)

Fig. 2

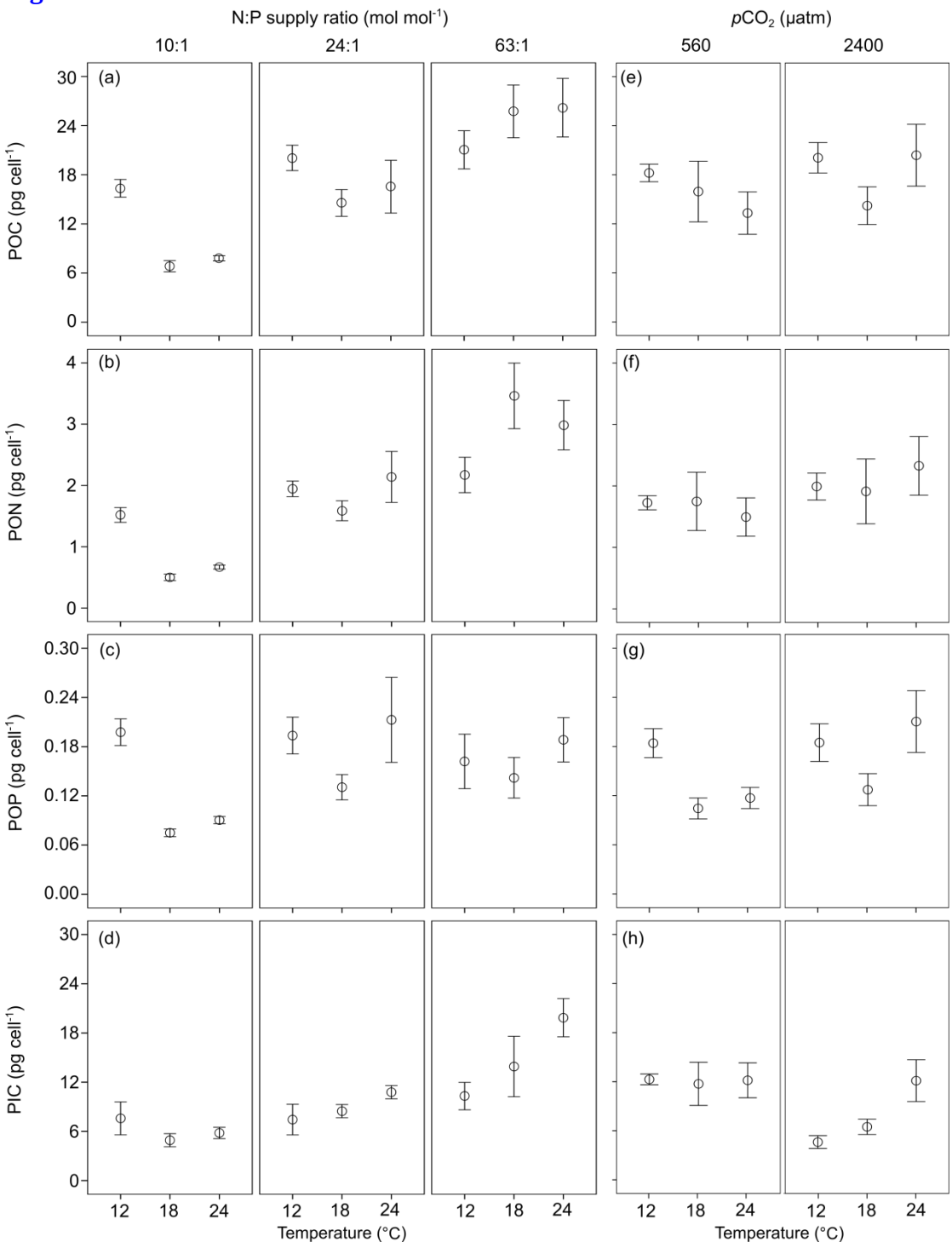
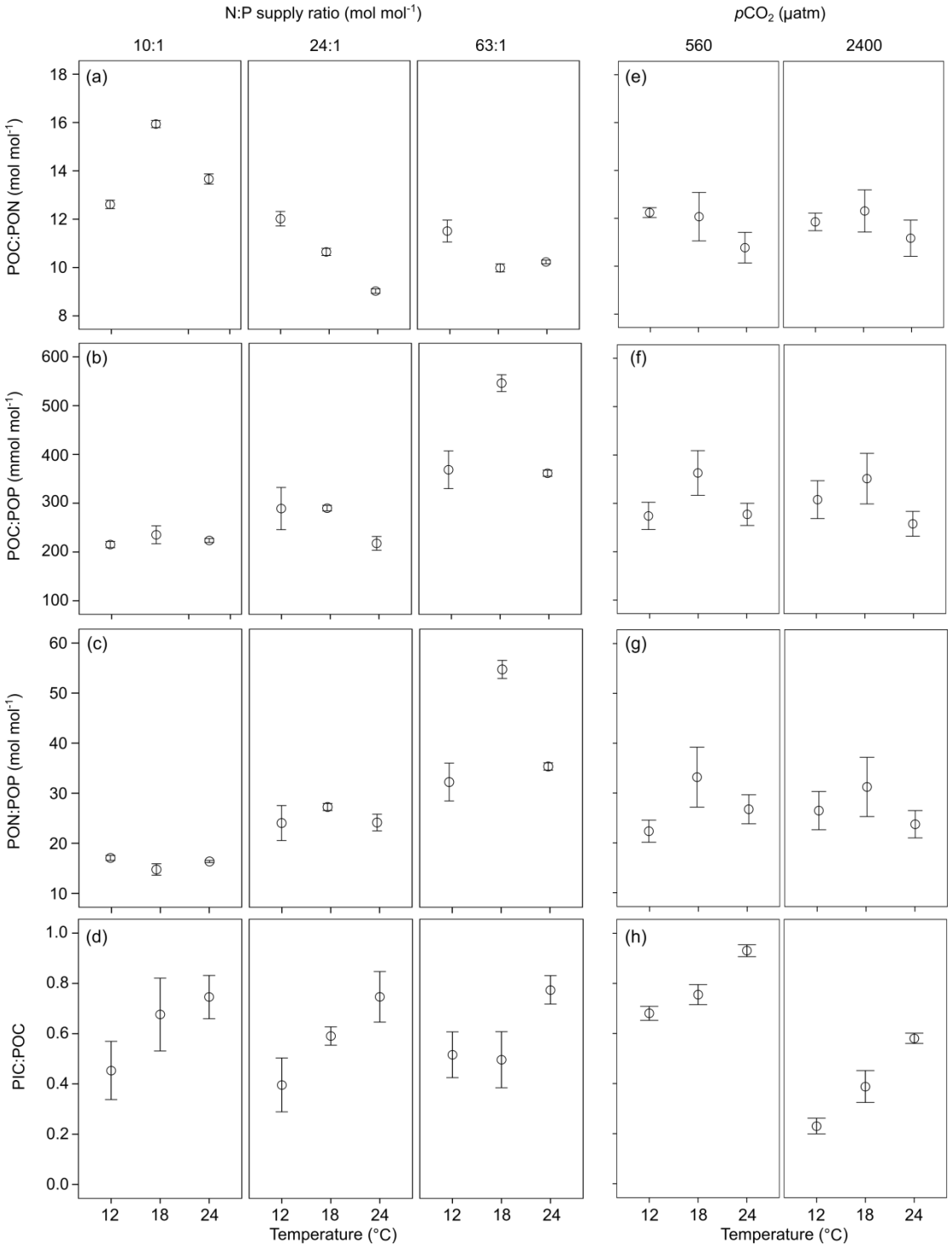

N:P supply ratio (mol mol-1)
10:1
24:1
63:1
pCO2 (µatm)
560
2400
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
POC (pg cell-1)
PON (pg cell-1)
POP (pg cell-1)
PIC (pg cell-1)
Temperature (°C)
Temperature (°C)

Fig. 3


N:P supply ratio (mol mol-1)
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24:1
63:1
pCO2 (µatm)
560
2400
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
POC:PON (mol mol-1)
POC:POP (mmol mol-1)
PON:POP (mol mol-1)
PIC:POC
Temperature (°C)
Temperature (°C)

Fig. 4

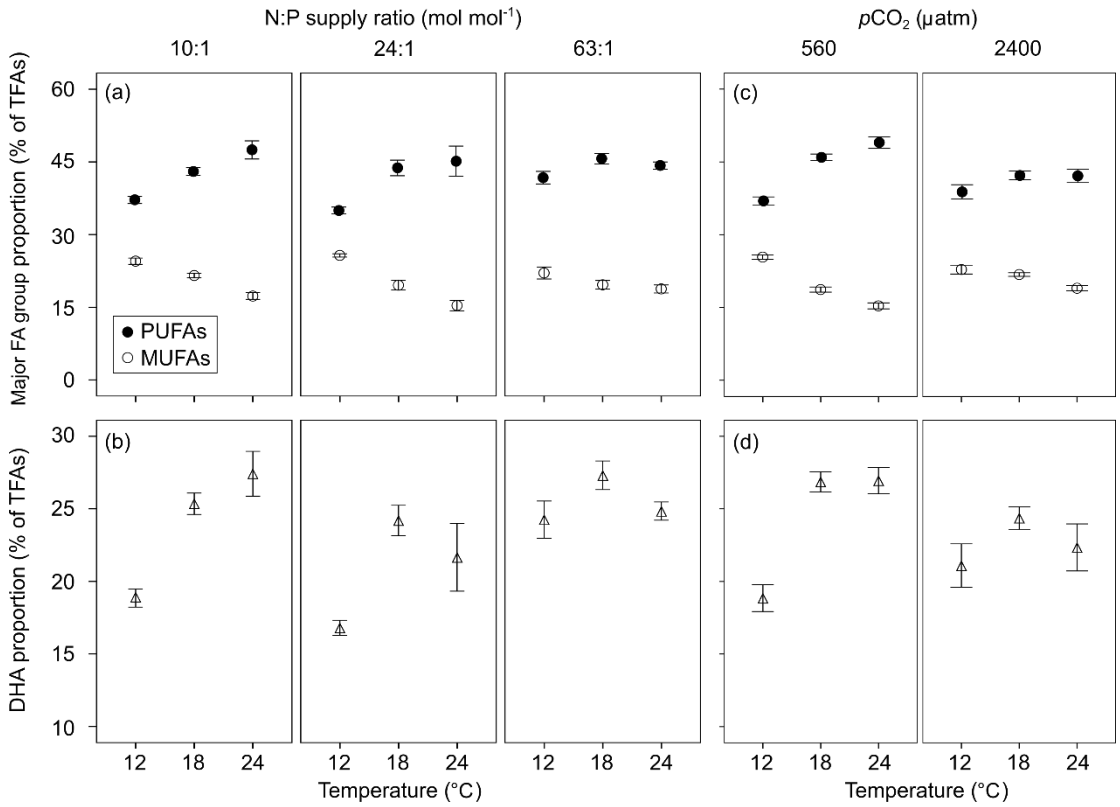
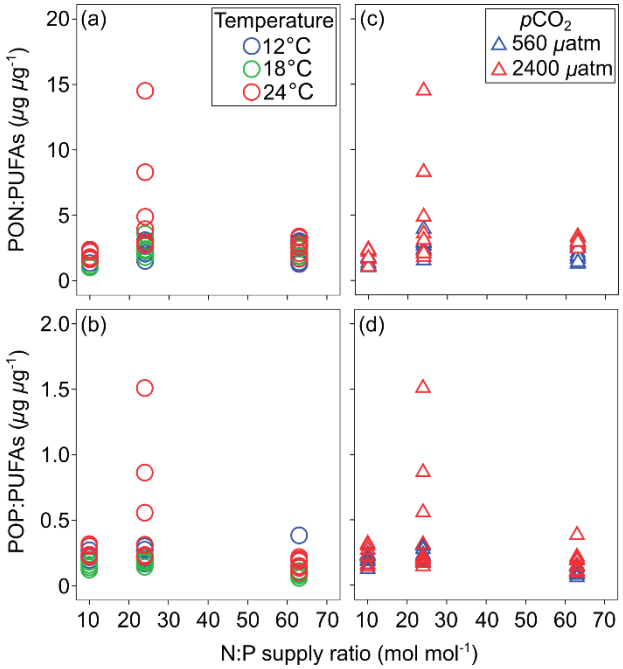

N:P supply ratio (mol mol-1)
10:1
24:1
63:1
pCO2 (µatm)
560
2400
(a)
(b)
(c)
(d)
Major FA group proportion (% of TFAs)
DHA proportion (% of TFAs)
PUFAs
MUFAs
Temperature (°C)
Temperature (°C)

Fig. 5


(a)
(b)
(c)
(d)
Temperature
12°C
18°C
24°C
pCO2
560 µatm
2400 µatm
PON:PUFAs (µg µg-1)
POP:PUFAs (µg µg-1)
N:P supply ratio (mol mol-1)