Author comments on “Organic matter exudation by *Emiliania huxleyi* under simulated future ocean conditions” by C. Borchard and A. Engel

Answers to the referees by C. Borchard and A. Engel

We greatly appreciate the time and effort both referees spent on the critical reading of our manuscript to provide valuable comments improving our text and presentation - but also acknowledging good presentation, careful conduction and valuable data.
Some general comments made by referee #1 and #2 (*in italics*) addressed similar issues and are therefore answered together.

R#1
In situ the carbonate system does not remain constant during growth of phytoplankton, as photosynthesis drastically changes the carbonate chemistry. Thus while these experiments are very useful to investigate certain behavior they certainly do not mimic in situ conditions (conclusion, P1203 line 17pp).
Moreover, oligotrophic conditions are also not mimicked in this experimental design with fairly high nitrate concentrations (p1219 line 15).
R#2:
The authors should, however, be careful to say that this experiment mimics an oligotrophic situation (conclusion), as they use nutrient concentrations which are high above what you find in Atlantic water during a winter situation.

Response to referee #1 and #2:
We agree with the referees and will therefore use the terms “in situ”, “mimic natural conditions”, and “oligotrophic” more carefully. We will shift the focus of the manuscript to the investigation effect of the applied conditions in order to achieve a mechanistic understanding of organic matter production by *Emiliania huxleyi*.
Data and description of the carbonate system are presented and discussed in the companion paper Borchard et al. 2011.

R#1
Phosphor limited growth rates are implied. What were the actual nutrient concentrations during the experiment? Can nutrient limitation be inferred e.g. from phosphate concentrations or POP production? E. huxleyi are “low nutrient opportunists” and able to utilize organic phosphor.
R#2
In these experiment the authors want to mimic a situation of future ocean by testing the effect of phosphorus limitation, and a N:P ratio of 26 was used. They do not say why phosphorus and not nitrogen was chosen as the limiting nutrient. N:P = 26 is not a very high ratio, and E. huxleyi is known to grow perfectly well at much higher N:P ratios. One of the reasons is that E. huxleyi produce a lot of alkaline phosphatase when phosphate concentration is low and by that perfectly well can utilize organic phosphorus compounds. The enzyme activity that could have shown P-limitation was not measured here. The nutrient concentration in the chemostats which is not reported could also give an indication of nutrient limitation.

Response to referee #1 and #2:
The referee´s comments clearly show that our manuscript lacks clearly referring to the companion paper (Borchard et al. 2011) where these issues, data and discussion

are thoroughly presented. We agree with the referees, that this some of this information is needed to be given for the present manuscript as well.
We are aware, that *E. huxleyi*, grows well on a very wide range of N:P ratios. However, an even higher N:P- ratio would not necessarily indicate a limitation, since the ratio itself provides no information of the actual nutrient availability. The supplied nutrient media in the present experiment was enriched to yield 29 µmol L^{-1} NO_3^- and 1.1 µmol L^{-1} PO_4^{3-}. In all cultures, PO_4^{3-} and organic phosphorous were below the detection limit during steady state (while residual NO_3^- concentration in a range of 0.42-1.08 µmol L^{-1} were determined). Since POP-concentration equaled Pi-concentration in the nutrient media it can be assumed, that organic phosphorous did not influence the present experiment. We will provide a more detailed summary of experimental conditions and implement nutrient concentrations in table 1.

R#1
Growth rate is discussed as a parameter in itself, however, in this experiment growth rate reflected phosphorus limitation. If growth rate were limited by light climate for example the "impact of growth rate" on carbon partitioning may be very different. Discussing this explicitly would clarify this issue. The measured growth rate is a combined result of the environmental conditions, not a determining factor in itself.
R#2
In a chemostat, growth rate of the culture is determined by the by the dilution rate, or more precise the available nutrients as these are proposal with the dilution rate. The effect of different growth rate on organic production is a main focus in this paper, but as these are given by available nutrients it will be more exact to change the focus to "effects of different nutrient stress or limitation".

Response to referee #1 and #2:
PO_4^{3-} and dissolved organic phosphorous were below the detection limit during both growth rates. At the lowered growth rate (where nutrient media of identical concentration is supplied at a lowered rate), cell densities were the same as for the higher growth rate. Thus, cells responded by growing more slowly on identical nutrient concentrations. Since identical nutrient concentrations were supplied throughout the experiment and only the supply-rate was varied influencing the growth rate, focusing on the varied growth rate describes the experimental assessment accurately. In order to clarify this highlight/advance of the use of chemostats (and reason for it´s application for the present study) we will provide additional information on this issue. Nevertheless, relative to the point of view, we partly agree with the referee: we investigated 2 levels of P-limitation, since the lowered growth rate can also be interpreted as enhanced nutrient stress. Highlighting the importance of the growth rate as a parameter itself, not only the nutrient supply or the N:P ratio however, in our opinion is crucial in order to gain a mechanistic understanding of growth and production processes because this assessment provides the possibility to identify the coupling – or decoupling - of physiological processes.

Referee #1: page 1208 line 14: steady state between days 10 and 12 – how is that possible if each dilution regime was only enforced for 12 days? Also see line 10: lowering dilution rate . . .day 17 – the counting of days seems inconsistent throughout the MS. The development of these cultures in each treatment must be described more accurately […] Later sampling is referred to as 1,2 and 3? Please clarify what that signifies.

Referee #2: P1205 L4. It is a bit confusing how many days the experiments ran. The cultures were grown as batch cultures for 3 days, then 12 days at dilution rate 0.3d-1 and finally 12 days at dilution rate 0.1d-1. This adds up to 27 days, while in the Methods sampling days 10, 14, 17 and 22, 25, 28 are given (?). These sampling days are later named "sampling 1, 2, 3 and 4, 5, 6". Whys not use the sampling days instead of introduce a new sampling identification which is confusing?

Response to referee #1 and #2:
We missed to introduce the initial equilibration phase in this manuscript, which clarifies the consistency of "experimental" and "sampling days". After an initial equilibration of CO_2 in the chemostats, cells of *E. huxleyi* were inoculated on day 3 of the experiment and grown as batch culture without flow through. From day 5 onwards, the nutrient media was supplied at a rate of D=0.3d^{-1} and reduced to D=0.1 d^{-1} on day 17, directly after the last sampling of the higher dilution rate and kept until the last sampling on day 28 of the experiment. Therefore, each dilution rate was kept and observed for 12 days.
We will describe the development of the cultures and the time-schedule of the experiment more clearly.

Answers to specific comments *(in italics)* made by Referee #1:

R#1
TEP correlated with particulate combined carbohydrates – but it may be assumed that both also correlated well with cell concentration (not mentioned or shown). A large fraction of the particulate carbohydrates may be assumed to be cell associated (both internal and external) rather than representing TEP. A correlation between TEP and particulate carbohydrates thus gives very little reason to assume that the former represent the latter.

TEP-C concentrations were corrected for the cell abundance as written in the methods section (p. 1207, l. 25). Moreover, cell densities within each treatment remained constant throughout the experiment (no matter what growth rate), while TEP and carbohydrate concentrations differed significantly between D=0.3 d^{-1} and D=0.1 d^{-1}. A correlation of TEP and carbohydrates therefore provides a strong indication that they represent each other. We will provide cell densities for each treatment and growth rate in table 1 for clarification.

R#1
Low HMW-dCCHO: DO14C in combination with high TEP formation were interpreted to reflect fast partitioning (? transfer) from DOC to POC. This argument needs to be developed more carefully. Bacterial activity (uptake of HMW-dCCHO) may also convert dissolved carbon to particulate carbon, for example, but bacteria are not mentioned at all.
We agree with the referee, that we should discuss bacterial activity while presenting data of freshly produced organic matter. We checked bacterial cell number by flow cytometry during the experiment and estimated the carbon demand from the growth rate of bacteria with an assumed biomass of 20 fg C $cell^{-1}$ (Lee & Fuhrman 1987), implementing growth efficiency (30%) and respiration (70%) into estimations. Relative to the huge concentrations of organic carbon produced by *E. huxleyi*, the bacterial carbon demand was negligible for the present study.

R#1
Also the composition of released DOC varies with physiological stage of phytoplankton, thus a low HMW-dCCHO: DO14C ratio may reflect changes in phytoplankton exudation as well. When discussing TEP formation from precursors under different CO2 scenarios, it may be worthwhile to look at a recently published paper on the topic by Passow 2012 in Marine Chemistry.

Passow 2012 showed that the abiotic formation of TEP is not impacted by ocean acidification. Here we report on the biological production of dissolved pre-cursors and therefore the biological production of TEP which is significantly affected by global change. Our study furthermore showed that the composition of dissolved carbohydrates was not significantly affected by varied CO_2 concentrations. A higher TEP formation at future ocean conditions is therefor rather a result of higher concentrations of pre-cursos due to enhanced biological activity. We will use the opportunity to implement conclusions made by Passow 2012 (which was published shortly after we submitted our manuscript) and highlight the differences and connections between abiotic and biotic factors influencing TEP-formation in the future ocean.

R#1
Bacterial enzymatic reactions are not always and generally accelerated at elevated CO2 (P 1216 line 13). Other papers show the opposite or no response to CO2 or different responses for different enzyme activities. A more balanced approach might prove wise.

The referee is right; we will provide a concise and more careful discussion of literature reporting on the response of bacterial enzyme activity to elevated CO_2.

R#1
As small differences in experimental conditions impact results, these need to be described in more detail; e.g. especially data on the carbonate chemistry and nutrient concentrations are needed. These may be summarized from the Borchard et al paper.

We agree. See comments above.

R#1
Throughout the MS the treatments are referred to as cultures, but as the same culture was used for all treatments, the word treatment may be more appropriate.

We agree with the referee and are grateful for already providing a suitable solution: We will change “culture” to “treatment” and furthermore add the respective growth rate for clarification of the 8 different treatments (4*2) investigated.

P1200 line 26 pp: suggesting a stronger partitioning of PP from DOC to POC by coagulation of exudates. - Please clarify what exactly is meant (see below).

We present and discuss how the partitioning of dissolved (exudation; $DO^{14}C$) and particulate ($PO^{14}C$) primary production (PP) is affected by CO_2, temperature and growth rates. A more general partitioning of dissolved (DOC) and particulate organic carbon (POC) has to take an abiotic formation of POC from dissolved precursors in

form of TEP into account. Our findings indicate that global change favors 1. exudation of DOC but also 2. the formation of POC via TEP, most likely due to the higher concentrations of biological produced dissolved pre-cursors provided by excess exudation. This affects the general partitioning between DOC and POC, potentially resulting in a generally higher proportion of particulate matter. Briefly, as written for the abstract: A stronger partitioning of PP from DOC to POC.
We will describe this hypothesis more careful and precise in the introduction and discussion. For the abstract however, we try to clarify, but keep the expression short and concise.

P1201 line 1: omit "processes":
"Processes" will be omitted.

P1201 line 17: ...for light and temperature – does this mean the light intensity or the fluctuation or day length was varied?

Both. We will clarify this sentence.

R#1
Experimental set-up: Some details need to be expanded on:
Where nutrients and carbonate chemistry measured regularly – It states that pH was measured, how about other carbonate parameters and what was the pH range experienced in incubators? Refer to Borchard 2011 when necessary, but give some overall results of ranges. What were nutrient concentrations in the incubators during the measuring phases and before? Again the method is described but no data given.

Nutrients and total alkalinity were determined regularly at every sampling day and the full data set is given in Borchard et al. 2011. As mentioned above we will give a more clearly description of the experimental design and provide nutrient data in table 1.

R#1
How did the carbonate system parameters change during 14C incubations? This could be estimated if it was not measured.

We did not measure parameters of the carbonate system for the ^{14}C incubations due to methodical constraints while working with radioactive samples. However, we do not see the advance/information from evaluation of this issue for this short term incubation. Of course we implemented the DIC value of each treatment (samples were taken before the spiking with ^{14}C) into rate calculations as it is common practice for this method.

R#1
Where treatments conducted as replicates or was there only 1 incubation per treatment? Do averages represent the 3 measuring dates of one incubation?
As described in the methods section, incubations (light and dark) were accomplished in triplicates for each treatment. Fig. 1 shows the average for each dilution rate.

R#1
Was the bubbling of the incubators with adjusted air continued during the experiment (after incubation), or was only the media bubbled after the initial equilibration phase?

If the latter, than information on the variability of the carbonate chemistry in incubators is even more essential.

The incubators were constantly aerated with gas with the target CO_2 concentrations. This experimental part will be described briefly and the reader will be referred to Borchard et al. 2011, where the method of the CO_2 perturbation is explained very detailed.

R#1
After inoculation a 3 d batch growth was followed by 12 days at D=0.3 d-1, followed by 12 days D=0.1 d-1. (p 1205 line 5 pp) – How long after the different dilution rates were established did it take until cell concentrations in the incubator remained constant?

The cell densities remained constant throughout the experiment, clearly showing an immediate response of the growth rate of the cells to the dilution rate. This is a central aspect of our experiment. This and several other comments of the referee urge us to explain and clarify the experimental set up. It is however, strongly recommended to read the companion paper (Borchard et al., 2011) since we cannot and should not, reproduce all data presented there.

R#1
To what conditions were cells acclimatized before inoculation?
Cells were acclimatized to light conditions as applied during the experiment. Stock-cultures of *Emiliania huxleyi* were grown on reduced f/2-media (100µmol L-1 NO_3^- and 10 µmol L-1 PO_4^{3-} at 15°C. As discussed in Borchard et al. 2011, in comparison with publications reporting on acclimatization (Zondervan et al. 2002, Müller et al. 2010, Barcelos e Ramos, 2010), cells were not long term acclimated to experimental conditions.

P1208 line 17: cell yield... – yield usually does not describe a concentration but total production/ harvest.
We will exchange “yield” by “cell density”.

P1208 line 24: ..sampling 1,2 and 3 – what does this mean? Are the three sampling days meant? Both in text and in figure legends.
We will assign “days of the experiment” and “sampling days” more clearly.

P1211 line 1: omit “now”
“now” will be omitted

P1211 line 14: While . . .higher . . .. – Sentence needs grammatical adjustment and is too long, “higher than what?”
Ratios of HMW-dCCHO:tCCHO were clearly higher for 300-14 and 500-14 at μ=0.1d^{-1} compared to μ=0.3d^{-1}. Sentence will be adjusted.

P1215 line 11: add “(diatom)” after T. pseudonana:
“diatom” will be added

R#1
Writing associated with figures is too small except for figure 4

The referee is right, the figures will be revised.

R#1
Fig 6: Do these values represent the ratio of rates (e.g. TEP production rate to POC production rate) or the ratio of standing stocks (TEP concentration vs POC concentration). Please clarify, in text and table also.

Values represent ratios of TEP concentration vs. POC concentration. Rates and concentrations were converted/ calculated from each other by the use of equation (1) and (2). This issue will be clarified in text, figures and tables.

Answers to specific comments *(in italics)* made by Referee #2

R#2
A culture in a chemostat it will for sure respond to the dilution rate or a change in dilution rate, but will the dilution rate have any influence on the culture when it is incubated in a bottle outside the chemostat? I am sceptical to that. When a sample is taken out and incubated in a bottle for 4h for primary production measurements, the algal cells will only respond on the present nutrient concentration, which is higher in the 0.3d-1 than in the 0.1d-1. This problem can easily be solved by focusing more on different degree of nutrient limitation/stress as mentioned above.

This aspect comes with the general methodical constraint of bottle incubations. However, since cell were not transferred to varied nutrient conditions, a continued growing of the cells at the adapted physiological state during the bottle incubation is most likely - and as the referee already pointed out, we also assume, that the degree of nutrient limitation between the 2 growth rates were sufficiently assessed during these rate measurements.

P1205 L. Methods for cell counting and nutrient analyses are described, but the results are not shown only referred in beginning of discussion (Borchard et al., 2011). It is better give this information in the Methods.
Cell densities and nutrient concentrations will be given in a revised version of the manuscript.

P1208 L. Cell normalized values of $PO^{14}C$ and $DO^{14}C$. Are there results reported?
The referee is right, we finally chose not to present the cell normalized concentrations since we decided to present cell normalized rates (table 1). We will adapt this information in the methods section. Cell normalized values of $PO^{14}C$ and $DO^{14}C$ (pmol $cell^{-1}$) can be easily derived from equation 2 and data (rate as pmol $cell^{-1}$ d^{-1}) given in table 1. To avoid this kind of a replicate presentation of data we decided to focus on the rates and omit showing the “per cell”- values. Furthermore, providing a cellular production rate for a dissolved component ($DO^{14}C$) describes the process, while a reporting on the concentration of a dissolved component per cell is very uncommon and does, at this point, not provide additional information.

P1206 L 8.” Activity in the samples was determined by removing a 100 µl aliquot . . .. . . and transferred to 6 ml liquid scintillation vial.” I hope the authors also added a strong base to the sample otherwise the 14C activity will change.

Yes! The referee is right. Actually, 200µl of 2N NaOH were pipetted into the vial first, then the aliquot from 14C-spiked bottle was added, before directly adding 4 ml of liquid scintillation cocktail (Three times for standardization on each sampling day). We will complete this part of the methods description.

R#2
One interesting thing I believe could have been done based on these data is to calculate the amount of organic carbon (particulate and dissolved) produced versus to the amount nitrogen and phosphorus consumed in the different treatment. This may give a more quantitative estimate of carbon overconsumption and exudation at low nutrient concentration.

Actually this is a nice idea and we will implement TOC:N and TOC:P ratios in the results section. The importance of growth/nutrient stress on exudation becomes very obvious by presenting data in the suggested way. DOC:TN ratios increase from ~1 in all treatments at the higher, to a range of ~3.7-7.2 at the lower growth rate. For DOC:TP the relative increase in exudation as a result of growth rate reduction and carbon overconsumption increased from 20-30 at μ=0.3 d^{-1} to 90-200 at μ=0.1 d^{-1}.

References:

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