Point-by-point response to the issues raised by referee#1 (Mary Scranton)

We thank the referee for the constructive comments and suggestions which have helped to improve the manuscript.

Referee #1 *(referee's comments are in italics)*

This paper presents an interesting discussion about the importance of methane production by several species of algae under aerobic conditions in the ocean. The authors' experiments are original and convincing but I think they overstate (or ignore) the extent to which this process can result in methane excess concentrations in open ocean surface water. In turn the minor role of that excess production to the atmospheric methane budget is not clearly explained. Below are some substantive criticisms and some minor corrections.

Authors: We appreciate the positive evaluation of our manuscript. The criticisms are addressed and corrections are made below.

Referee #1 ***Line 17:*** *The abstract indicates that the importance of oceanic methane production to the global methane budget is unknown but this is not discussed further in article and is misleading in any case since the ocean is known to be a very small contributor to the atmosphere. I am tired of proposals and papers that use the atmospheric methane budget to justify all studies of basic methane geochemistry. Surely it is enough to note a widespread and unexplained phenomenon which one is trying to explain mechanistically. I suggest adding a sentence or two to the introduction indicating why you are bothering to do this study and de-emphasizing how it might affect global methane budget. You are better off being straightforward and admitting that the real question is that methane is known to be produced in the oxic oceanic mixed layer and after more than 40 years no one really understands why. Give some idea of what actual flux of methane to atmosphere from ocean is thought to be. This HAS been calculated a number of times.*

Authors: We agree with the referee and thus have modified the Abstract and Introduction. The first sentence of the abstract now reads:

"Methane (CH_4) production within the oceanic mixed layer is a widespread phenomenon, but the underlying mechanisms are still under debate."

We further added two sentences to the introduction:

"The world's oceans are considered to be a minor source of CH4 to the atmosphere (1-3 %, Saunois et al., 2016). However, in recent years the widespread occurrence of in situ CH_4 production in the ocean mixed layer has received much attention, since CH_4 formation in the oxygenated ocean mixed layer challenge the paradigm that biological methanogenesis is a strictly anaerobic process."

We further deleted the sentence "However, partitioning source categories to reduce uncertainties in the global CH_4 budget is a major challenge (Saunois et al., 2016)."

Referee #1 ***Line 98:*** *(Were cultures axenic? How was this determined? Sterile technique is not enough if bacteria are intrinsic to algal cultures. Bob Guillard told me this when I was using his culture collection. I personally don't think that there are*
35 *anaerobic bacteria producing methane in rapidly photosynthesizing cultures, but one should be accurate.*

Authors: We can't consider our approach as fully axenic and the reviewer is right that it is extremely difficult to grow algal cultures without bacteria. However, the algal cultures were diluted many times, resulting in exponential algal growth while minimizing bacterial cell density. This is a common practice to keep non-axenic algae cultures largely free of bacteria and it was applied in many other physiological algal studies before, which used non-axenic cultures. Please see also answers
40 regarding comments by reviewer 2 (manuscript line 98ff and line 381), where we discuss a potential contribution of heterotrophs and/or methanogenic archaea. Briefly, the correlations we describe clearly show that CH_4 production depends on algal growth. It is therefore highly unlikely that bacteria are solely responsible for CH_4 production in our cultures. However, bacteria might be involved in the CH_4 production process. One scenario which we cannot rule out would be a production of CH_4 precursors by algae and a usage of these precursors by bacteria to produce CH_4. While we think that this is less likely
45 than CH_4 production by algae alone, it would, if true, show that bacteria need algae-produced precursors to produce CH_4. The latter scenario would be relevant in the field because algae co-exist with bacteria in the oceans. We have modified the Discussion and Abstract to make this clear. For more details see reply to reviewer #2 (manuscript line 98ff and line 381).

Referee #1 Line 115: *When calculating the amount of methane produced, was fraction dissolved included? With a large*
50 *headspace, this may be small but should be mentioned. Were samples equilibrated with headspace before methane measured? The authors mention that oxygen was sometimes supersaturated, but was this relative to headspace or equilibration with ambient air?*

Authors: The amount of dissolved CH_4 was not included. As requested we have calculated the dissolved CH_4 concentration
55 by using the equation of Wiesenburg and Guinasso (1979). The dissolved fraction of CH_4 has now been included in our calculations and added to the total amount of CH_4 produced. As correctly stated by the referee the addition of the dissolved CH_4 fraction has only a marginal effect on the overall CH_4 production. Calculation of dissolved CH_4 is mentioned in the method section (2.6) and a new reference for calculating dissolved CH_4 was added (Wiesenburg and Guinasso, 1979) to the revised manuscript.
60 Cultures were turned 30 seconds overhead prior to analysis to ensure equilibration between dissolved and headspace CH_4. In the preliminary equilibration experiments, we found that further shaking does not affect the CH_4 measurement and therefore all samples can be considered as equilibrated.
We modified the sentence line 260: "The measured oxygen concentrations were always saturated or supersaturated relative to equilibration with ambient air (Fig. S2)."
65 ***Referee #1 Line 133:*** *Concentrations (final) of added substrates should be given for comparison with natural concentrations. If possible give concentrations of these substrates in medium at start of incubation with and without addition of substrate.*

Authors: The final concentration of ^{13}C-hydrogen carbonate ($NaH^{13}CO_3$) was 48.7 µmol L^{-1} and 10 µM for $^{13}C_2$-DMS, $^{13}C_2$-DMSO and ^{13}C-MSO. Concentrations (final) of added substrates are given in the manuscript in line 133 for $NaH^{13}CO_3$ and at
70 line 173 for $^{13}C_2$-DMS, $^{13}C_2$-DMSO and ^{13}C-MSO.
Cultures were grown in sterile filtered (0.2 µm Ø pore size) natural North Sea seawater (sampled off Helgoland, Germany) enriched in nutrients according to F/2 medium. The dissolved inorganic carbon (DIC) was 2152 ± 6 µmol L^{-1} (line 104). This value falls within the range of typical DIC concentrations of North Sea seawater. The added amount of $NaH^{13}CO_3$ corresponds to 2% of the DIC of the North Sea seawater. This information was added to the revised manuscript: "The DIC value falls
75 within the range of typical DIC concentrations of North Sea seawater. "We added two sentence to the section were we explain labeling experiments: "For stable carbon isotope experiments 48,7 µmol L^{-1} ^{13}C-hydrogen carbonate ($NaH^{13}CO_3$) in final concentration was added to the F/2 medium. The added amount of $NaH^{13}CO_3$ corresponds to 2% of the DIC of the North Sea seawater (2152 ± 6 µmol L^{-1}), resulting in a theoretically calculated ^{13}C value of DIC of +2014 ± 331‰."

80 Unfortunately the natural DMS, DMSO and MSO concentrations in our seawater were not determined. However, the global DMS mean concentration has been reported to be ca. 2 nM (Galí et al., 2018). A rough estimation can also be made for DMSO concentrations in the ocean as DMSO is generally present in concentrations 1–2 orders of magnitude greater than DMS (Lee et al., 1999). These estimates are also in line with data reported from a cruise of the western Pacific Ocean that were reported by Zindler et al. (2013). The average (total) DMS ,DMSP and DMSO concentrations were ca. 1 nM, 4 nM, and 16 nM for
85 DMS, DMSP and DMSO respectively. Thus we conclude that the initial substrate concentration in the seawater is insignificant in comparison to the added amount (10µM), the latter being roughly two orders of magnitude higher than typically reported for oceanic concentrations (please see also reply to referee#2: line 172). Moreover, intracellular concentrations of methyl-sulfur compounds also play a significant role. We will discuss this issue below (see answer to next comment).

90 ***Referee #1 Line 327:*** *If the labelled methyl groups yield only a small percentage (less than 1%) of total methane produced where is the other methane coming from? Is this result consistent with field observations that show only a weak link if any between DMS or DMSO and excess methane in surface water? This point needs more elaboration since the question of the source of excess methane in seawater has been plagued by studies that show methane can be produced by a process but that rates are far lower than are needed to explain natural surface water values. Here is where a link to ambient DMS, DMSO or*
95 *MSO concentrations should be made. I think this point is a key issue.*
Authors: Please note that the main reason for the isotope experiments was to unambiguously show that the tested compounds might be able to form CH_4 under oxic conditions. The ^{13}C-labeling experiment showed that DMS, DMSO, and MSO are potentially important methyl-precursors for CH_4 but the contribution of these compounds to the overall CH_4 production in cultures of *E. huxleyi* could not be determined in our experiments due to the complexity of the formation of these compounds
100 in the algal cells. Hence, the stable isotope labeling approach should be considered as a proof of concept, showing that methyl

groups of all tested substance serve as precursor compounds of CH_4. Althoff et al. (2014) and Benzing et al. (2017) suggested a chemical reaction of DMSO, DMS and MSO that leads to CH_4 formation in eukaryotes, especially, in marine algae containing elevated concentration of these compounds. We have therefore tested whether the methyl groups of these substances can actually be converted to CH_4 in marine algae cultures. We made this point clearer in the discussion of the revised manuscript.
105 The paragraph reads now:

"The ^{13}C-labelling experiment showed that DMS, DMSO, and MSO are potentially important methyl-precursors for CH_4 but the contribution of these compounds to the overall CH_4 production in cultures of *E. huxleyi* could not be determined in our experiments due to the complexity of the formation of these compounds in the algal cells. This can be illustrated by the following. The contribution of a substance to the total CH_4 released is the product of both the added ^{13}C-labeled fraction (added
110 to the waters sample and uptake by the cells) and the internally formed fraction of these compounds (DMS, DMSO, and MSO) which will roughly show natural ^{13}C abundance. Therefore the stable isotope value of CH_4 will be diluted by the fraction of naturally formed methyl sulfur compounds in the algal cells and thus the contribution of DMS, DMSO, and MSO to CH_4 formation can therefore not be estimated on the basis of their added amount alone. The $^{13}CH_4$ quantity from conversion of added ^{13}C labelled substance contributed 0.03% ($^{13}C_2$-DMSO) up to 0.84% (^{13}C-MSO) to overall released CH_4. However, even
115 if the added ^{13}C labelled compounds might only explain $\leq$ 1% of CH_4 formed by the algae their overall contribution (including non-labelled sulfur compounds which we are not able to measure) might provide a much larger fraction of the released CH_4. The intracellular DMS concentration can reach 1 mM (Sunda et al., 2002) in cells of *E. huxleyi*, while the concentration of added $^{13}C_2$ -DMS was 0.01 mM in medium (final concentration). If intracellular $^{13}C_2$ -DMS was in equilibrium with bulk seawater $^{13}C_2$ -DMS and all CH_4 would be produced from intracellular DMS, then the contribution of the ^{13}C labeled compound
120 would be about 1%. However, even if the biggest fraction of CH_4 in algae cultures was not released by the ^{13}C labelled substances, the significant increase in delta notation in $^{13}C_2$-DMS, $^{13}C_2$-DMSO and ^{13}C-MSO treated cultures above the $\delta^{13}CH_4$ values of the control groups demonstrate that ^{13}C labelled precursor substances were converted to CH_4 by algal cultures (Fig.4 a-c).

This is also indicated, when the absolute conversion quantities of ^{13}C-labelled substance in algal cultures are considered: these
125 were ca. nine ($^{13}C_2$-DMS), three ($^{13}C_2$-DMSO) and thirty (^{13}C-MSO) times higher than in seawater control groups. Hence, the stable isotope labeling approach should be considered as a proof of concept, that methyl groups of all tested substance serve as precursor compounds of CH_4."

We furthermore deleted the paragraph (line 341-354), since the main points regarding the CH_4 conversion rates of ^{13}C labeled
130 compounds were discussed in the section above.

Referee #1 Line 400: *Weller et al may have found a correlation between chlorophyll a and methane concentrations but there were many studies in the older literature (1970s and 80s) where no such correlation was observed. I recommend authors go back and read over some of these earlier papers and confirm that measured production rates from this study can support other*

previously observed methane fluxes. Also see thesis by Scranton (1977) where methane production was examined in cultures by several species including Emiliani huxleyi (called Coccolithus huxleyi in my thesis) and T. pseudonana. I observed methane production in a much less sophisticated experimental setup and concluded that natural populations of the algae I studied might be adequate to support the widespread supersaturations of methane seen in the open ocean (including in places where no dense algal blooms were observed). Perhaps your results can be compared to mine or to other studies that report cell abundances and air-sea fluxes. A citation to a downloadable copy of my thesis is below. Scranton MI (1977) The marine geochemistry of methane. Citable URI https://hdl.handle.net/1912/1616. DOI10.1575/1912/1616.

Authors: We followed the recommendation of the reviewer (Mary Scranton) and compared the CH_4 production rates of *E. huxleyi* reported by Scranton (1977) with those of our study.

In line 392 we added: "We also compared the cellular CH_4 production rates of *E. huxleyi* reported by Scranton (1977) with those of our study. Scranton (1977) reported a production rate of 2×10^{-10} nmol CH_4 cell^{-1} hr^{-1}. This value is close to the production rate of 1.6×10^{-10} nmol CH_4 cell^{-1} hr^{-1} in our study. Scranton (1977) concluded from observed CH_4 production rates in laboratory experiments that natural populations might be adequate to support the widespread supersaturations of CH_4 seen in the open ocean."

As the referee stated correctly the distribution of chlorophyll has not shown a consistent correlation with CH_4 distributions in field studies.

The following section was added in Chapter 4.3: "In general, the distribution of chlorophyll has not shown a consistent correlation with CH_4 distributions in field studies. There are studies in which no correlation was observed (e.g. Lamontagne et al., 1975; Foster et al., 2006; Watanabe et al., 1995) or a correlation was found within a few depth profiles (Burke et al., 1983; Brooks et al., 1981). Many field measurements in oxygenated surface waters in marine and limnic environments have shown examples of elevated CH_4 concentrations spatially related to phytoplankton occurrence (e.g. Conrad and Seifer, 1988; Owens et al., 1991; Oudot et al., 2002; Damm et al., 2008; Grossart, et al., 2011; Weller et al., 2013; Zindler et al., 2013; Tang et al., 2014; Bogard et al., 2014; Rakowski et al., 2015). Taken together these studies suggest that phytoplankton is not the sole source of CH_4 in oxygenated surface waters, but importantly they also suggest that phytoplankton is one of the sources of CH_4. We therefore compared the CH_4 production rates of our cultures with two field studies for the Pacific Ocean (Weller et al., 2013) and the Baltic Sea (Schmale et al., 2018) to evaluate the potential relevance of algal CH_4 production. "

We followed the reviewer suggestion and added an additional comparison of our CH_4 production rates by using field study data of Schmale et al., (2018).

The respective section reads: "Schmale et al., (2018) reported CH_4 enrichments that were observed during summer in the upper water column of the Gotland Basin, central Baltic Sea. They furthermore found that zooplankton is one but not the only CH_4 source in the oxygenated upper waters. While the authors ruled out a major contribution of algae to the observed sub-thermocline CH_4 enrichment because of the low sub-thermocline phytoplankton biomass, they considered a primary production associated CH_4 formation as one likely source in the phytoplankton-rich mixed layer. The average phytoplankton carbon

biomass of the mixed layer was approximately 600 µg L^{-1} (averaged from Fig. 9 in Schmale et al., 2018). For the reported
170 average net CH_4 production rate in the mixed layer (95 pmol CH_4 L^{-1} d^{-1}), we calculated that a production rate of 2.5 µg g^{-1} POC d^{-1} is required if the CH_4 is produced by the algal biomass. This rate would be within the range of CH_4 production rates observed in our study. These calculations should be considered as a first rough estimate to assess whether CH_4 production rates of laboratory grown cultures can significantly contribute to CH_4 supersaturation associated with phytoplankton. We did not distinguish between species and did not take into account environmental factors or the complexity of microbiological
175 communities."

Minor issues

***Referee #1**: **Equation 7:** There should be a factor of 1000 to convert ratios to per mille values*

Authors: We would like to keep equation 7 as is as it follows the recommendations by Coplen (2011) ("Guidelines and
180 recommended terms for expression of stable isotope-ratio and gas-ratio measurement").

***Referee #1 Figure 1:** Plot control values here too.*

Authors: Control groups in Figure 1 were plotted in the revised manuscript. In order to add control groups the unit was changed in concentration (ng CH_4 L^{-1}).

185 ***Referee #1 Line 268:** Should it be "were applied" not "where applied"?*

Authors: Yes. Corrected.

***Referee #1 Line 308:** Inoculation OF cells?*

Authors: Yes. "of" was added.

190

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Point-by-point response to the issues raised by referee #2 (Anonymous)

We thank the reviewer for efforts in reviewing our manuscript and for the helpful comments which have improved the manuscript.

5

Referee #2 *(referee's comments are in italics)*

The present paper presents an interesting study about methane production under oxic conditions in marine environments. This so called "methane paradox" is a very important research field to understand methane emissions from oceans (and lakes) and has recently received strong interest by a number of investigators from different scientific disciplines. The author presents
10 *data from incubation experiments conducted with three different algal species. Methane production rates were determined with different methanogenic substrates (^{13}C-labled) using a stable isotope approach. A similar kind of studies was previously conducted for Emiliania huxleyi by Lenhart et al. (2016) and the isotope approach was successfully used in diverse investigations by Frank Keppler before to examine terrestrial methane production. The novel outcome in the present study is (1) that also other widespread haptophytes have the potential to produce methane under anoxic conditions; and (2) methylated*
15 *sulphur compounds (e.g. DMS), that are known to be enriched in the investigated algae species, present potential substrates. In addition, the authors present an attempt to transfer their results to an algal bloom in the Pacific Ocean to discuss the potential relevance of algal methane production.*
The experiments are well thought out and the results present an additional piece in the complex puzzle. There are lots of little corrections needed and from my point of view some sentences need another structure to make the content more accessible for
20 *readers that are not familiar with the topic in detail (especially in the method section, e.g. PP, exponential growth rate). I will give a few examples below.*
Some minor and major points need to be addressed and I therefor recommend a publication after major revision.

Authors: We thank the referee for the positive evaluation of our manuscript and for the helpful comments. Requested changes were taken into account, as detailed in the following.

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Referee #2: Line 95ff. *The experimental design is very complex. A flowchart for the method section would be helpful for the reader.*

Authors: We added a graphic/flowchart to the method section of the revised manuscript.

30 ***Referee #2: Line 98ff.*** *How clean are the algal culture samples (purity)? Small differences in the_degrees of contamination with archaea/bacteria (nitrogen limited bacteria, Line 69,Damm) between the cultures may have an impact on CH_4 production rate. Does the web link give information about the purity of the culture?*

Authors: Unfortunately, the weblink gives no detailed information about the purity of culture. We cannot consider our approach as axenic because it is extremely difficult to grow algal cultures without any bacteria. However, the algal cultures were diluted
35 regularly, resulting in exponential algal growth and minimal bacterial growth. This is a common practice to keep non-axenic algae cultures largely free of bacteria (please see also answer to comment of reviewer #1 concerning manuscript line 98).
We now mention that this cultures were non- axenic. We added the following sentence: “In order to keep non-axenic algae cultures largely free of bacteria, the cultures were diluted regularly, resulting in quasi constant exponential algal growth while minimizing bacterial cell density.”
40 However, we are aware that bacteria might play a role in CH_4 production, but even if they did they still would depend on algal growth in our cultures as demonstrated by the following points.

1) The CH_4 production rates decreased with decreasing algal growth rates:

In batch cultures, the algae cultures undergo various stages of growth (see section 3.1, Fig. 1 a-c). Bacterial density increases tremendously when algae culture reach stationary growth phase and excretion of organic products from senescent alga cells
45 together with the decomposition of cells is providing substratum for heterotrophs. This was described in literature (Salvesen et al., 2000) and is in line with our own experience with growing alga cultures in batch mode. In section 3.1 cultures have undergone transitionary growth phase leading up to the stationary phase. We calculated daily incremental CH_4 production rates (not shown in the manuscript). The CH_4 production rates of each species decreased with decreasing growth rates and decreased drastically when approaching stationary phase. This observation is the opposite of what we would have expected, if CH_4 were
50 mainly produced by bacteria. It would however be compatible with the idea that algae produce precursors which are subsequently used by bacteria to produce CH_4.

2) Light is a prerequisite for CH_4 formation in algae cultures:

Cultures of *E. huxleyi,* and *P. globose* were incubated under a day-night-cycle and continuous darkness. Methane concentrations did not increase when cultures were incubated in darkness while concentrations increased in cultures growing
55 under a day-night-cycle. This is a strong indication that CH_4 formation is dependent on the light-dependent metabolism of the algae, since the metabolism of heterotrophs or archaea is independent of light. While the latter conclusion does not rule out the “algae precursor scenario”, our experimental setup makes it rather unlikely. In these experiments we inoculated high cell densities ($\approx 10^5$ cells mL^{-1}) because they were designed to be short term which requires a high start cell density to yield measurable production. Therefore the start conditions will have included a seawater replete with precursors. It is unlikely that
60 the relatively few bacteria present should have become precursor-limited over a single dark phase. It is rather more likely that the pool of precursors was sufficient to sustain bacterial CH_4 production over the dark phase. In this scenario an extra precursor production by cultures exposed to light would have been without effect on CH_4 production.

3) It is highly unlikely that methanogenic ***archaea*** are the source of CH_4 in cultures where
CH_4 is produced alongside oxygen (incubation under day-night-cycle).
65 If archaea were the CH_4 source we would have expected a higher CH_4 production in the dark.

4) Selectively inhibition of algal growth reduced CH_4 production rates:

We compared emission rates of *E. huxleyi* that have been treated with and without 3-(3,4 dichlorophenyl)-1,1-dimethyl-urea (DCMU). DCMU acts as an inhibitor of photosynthesis (Wessels and Van Der Veen, 1956). Selectively inhibition of algal photosynthesis reduced both algal growth rate and CH_4 production rates. In the inhibition experiments, the growth rate was
70 only 29% of the uninhibited culture and the CH_4 production rate dropped to 18% of the uninhibited culture. Since the inhibition effect of DCMU is very selective for algae (Francoeur et al., 2007) the result may indicate direct CH_4 production from algae. Although we regard it as unlikely, we cannot strictly rule out the "precursor-scenario": Bacteria use algae-derived precursors to produce CH_4, and these bacteria require constant production of these precursors by algae. In other words, the precursor-production by algae is the rate limiting step of CH_4 production by bacteria (as evident from points 1, 2, and 4 above). If true
75 the CH_4 production observed in our experiments would be the result of a "collaborative effort" which needs both partners, algae and bacteria. This would be a significant finding and prompt further research. Questions to be addressed would include: what are the precursors? Which algae can produce the precursors? Which bacteria can produce CH_4 using these precursors? Is it possible to grow the respective algae without the bacteria (not all algal cultures can survive in an axenic state). Can the same CH_4 production be achieved by growing the bacteria without algae and adding the precursors? This selection of questions
80 would suffice for an entire research project. Meanwhile we are content with describing CH_4 production that depends on algae, whether solely or in cooperation with bacteria.

To sum up, our ***main finding*** is that CH_4 production in ***mixed algae/bacteria cultures*** depends on ***algal growth*** and is not supported when algae become senescent. Future research will clarify whether algae alone produce CH_4 or whether they produce precursors which in turn are used by bacteria to produce CH_4. We have made this important point clear in the revised
85 manuscript.

We added this information in supplementary material. We now discuss the possible contribution of bacteria and archaea in the main text and refer to further discussion in the supplementary material (see discussion above). The revised the paragraph (Chapter 4.1) now reads:" The algal metabolites DMSP, DMS and DMSO are ubiquitous in marine surface layers and nanomolar concentrations were found in blooms of *Chrysochromulina sp., P. globosa* and *E. huxleyi.* Several field studies
90 showed that these compounds are linked to CH_4 formation in seawater (Damm et al., 2008; Zindler et al., 2013; Florez-Leiva et al., 2013). The authors proposed that DMSP and their degradation products DMSO and DMS are used by methylotrophic methanogenic archaea, inhabiting anoxic microsites, as substrates for methanogenesis. In addition it was reported that, if nitrogen is limited but phosphorus is replete, marine bacteria might also use DMSP as a carbon source, thereby releasing CH_4 (Damm et al., 2010).
95 One scenario which we cannot rule out would be a production of CH_4 precursors by algae and a usage of these precursors by bacteria to produce CH_4. While we think that this is less likely than CH_4 production by algae alone, it would, if true, show that bacteria need algae-produced precursors to produce CH_4. The latter scenario would be relevant in the field because algae co-exist with bacteria in the oceans. Therefore bacteria might be involved in the CH_4 production process in our cultures, but even if they were they still would depend on algal growth. For further discussion of a potential contribution of heterotrophs and/or
100 methanogenic archaea see supplementary material. The correlations we describe in the supplementary material clearly show

that CH_4 production depends on algal growth. It is therefore highly unlikely that bacteria are solely responsible for CH_4 production in our cultures."

Referee #2: Line 133*. What is the difference in concentration of $NaHCO_3$ between natural and inoculated water sample? Why*
105 *did the authors added this amount of tracer? Should be mentioned.*

Authors: Natural North Sea surface seawater contains ca 2000 µmol L^{-1} bicarbonate. We added 48,7 µmol L^{-1} ^{13}C-bicabonate , i.e. about 2 % of the natural concentration. This bicarbonate concentration was chosen for two reasons, one analytical, the other physiological. The physiological reason is that phytoplankton is sensitive to changes in seawater carbonate chemistry (see reviews on "ocean acidification"). We aimed at a negligible physiological effect of the added bicarbonate. The chosen
110 bicarbonate concentration fulfills this criterion. The analytical reason is this: On the basis of the amount of added ^{13}C-bicarbonate we calculated the theoretical $\delta^{13}C$ -DIC value (see also manuscript line 134). Based on the theoretical $\delta^{13}C$ -DIC value and from the previously determined CH_4 increases in the cultures, the $\delta^{13}CH_4$ values can be estimated. The amount of ^{13}C- bicarbonate was chosen on the basis of expected changes of $\delta^{13}CH_4$ values which were measured using GC-IRMS. A change of tenth to few hundred per mil is ideal regarding statistical issues (applying keeling plots for source identification) but
115 also concerning linearity issues of the isotope ratio mass spectrometer.

Referee #2: Line 141. *Here, you should explain in more detail why an exponential growth rate is important to best compare CH_4 formation between the experiments. From this sentence one could assume that Langer performed already methane production rate experiments with algae that indicated that exponential growth rates are important. From my point of view the*
120 *activity of the cell is important for the turnover of these substrates and not their reproduction. It should be mentioned in the method or result section that microbial methane turnover takes place in the incubations and the production rates presented are minimum rates > because methane oxidation is not considered in the calculations (e.g. see methods in de Angelis and Lee).*

Authors: We agree with the reviewer that from a physiological point of view the activity of the cell is the relevant parameter
125 here. But as detailed below our point is purely methodological, not physiological.

We have clarified this in the revised manuscript:

"Exponential growth is a prerequisite for calculating production on the basis of growth rate and quota (here CH_4 quota). The point is a general, technical one, and is not confined to CH_4 production. The studies by Langer et al. (2012, 2013) discuss this point in the context of batch culture experiments. Briefly, production on this account is the product of growth rate and quota
130 (e.g. CH_4, calcite, organic carbon). Production here is an integrated value, typically over many cell divisions. For this calculation of production to be meaningful a constant growth rate is required. The exponential growth phase fulfills this criterion whereas the transition phase and the stationary phase do not. Therefore production cannot be calculated meaningfully in the non-exponential phases. The problem can, however, be minimized by using small increments (one day) because growth rate can be regarded as quasi-constant (see also Lenhart et al., 2016). "

135

We agree with the reviewer that our calculated rates should be regarded as minimum rates because of *microbial methane turnover*. Please see also answer to comment on manuscript line 98ff.

Referee #2: Line 137. *Can you explain if aggregates or sediment was visible in the incubation?*

140 Authors: Cells were counted under bright field microscopy and we did not observe any aggregates.

Referee #2: Line 172. *Why was exactly this amount of substrate (DMS...) injected and is this comparable with natural environments (concentrations). Substrate concentrations definitely affect the turnover and the addition of tracers/substrates should not impact the sample too drastically. Why did the authors did not applied MET (and DMSP) as a precursor that was*

145 *tested before successfully by Lenhart et al.?*

Authors: The amount added was chosen based on the practical experience from previous experiments with *E. huxleyi* and methionine (Lenhart et al., 2016), so that the expected $\delta^{13}CH_4$ fall within a measurable range with statistical significance. The growth of algae was not effected and changes of the overall CH_4 production did not change by the addition of substrate (within error of measurement).

150 The added amount (10µM) of methylated sulfur compounds ($^{13}C_2$-DMS, $^{13}C_2$-DMSO or ^{13}C-MSO) was higher than those expected in ocean water samples (please see also answer to reviewer #1 comment on manuscript line 133). However, the intracellular concentrations of these compounds can reach mM levels (Keller, 1989; Rafel et al.,1998; Keller et al., 1999; Sunda et al., 2002), which is two orders of magnitude higher than the added concentration of 10 µM (final) in our experiments. For this reason, it can be expected that the amount of ^{13}C labelled substance taken up by the algal cells is low in relation to the

155 amount of methylated sulfur compounds what they synthesize during metabolism.

The turnover of DMS, DMSO or MSO (including non-labeled compounds) to CH_4 could not be determined on the basis of their added amount of ^{13}C-labelled substance in cultures of *E. huxleyi*. Please see answers regarding comments of reviewer#1 (manuscript 327), where we discuss this issue in detail.

Neither was this the goal of the experiment. However, it can be determined exactly how the ratio of ^{13}C in CH_4 increases, when

160 ^{13}C labeled methylated sulfur compounds were added (Fig. 4 a-c). It has therefore been shown that methyl groups of these compounds can be converted into CH_4 in algal cultures.

Due to time constraints, we omitted the methionine treatment. Unfortunately, isotopically labelled $^{13}C_2$ DMSP was not commercially available. Moreover, this compound could not be synthesized in our laboratories.

165 ***Referee #2: Line 327ff.*** *Is it possible that a natural microbial community is needed for the turnover of these substances to methane? If the incubations are without contaminations (sterile filtered seawater, pure culture), the production rates might be low because of the missing community. The algae may only provide the precursors. Might be a point that could be discussed here.*

Authors: Please see reply to referee #2: line 98ff .

170

Referee #2: Line 381. *Argumentation is difficult. Only because Lenhart could prove a contamination-free incubations, this result cannot be transferred to all the incubation that will be performed by the working group afterwards. See comments/concerns to this topic above. Since the argumentation is difficult to follow, I suggest to discuss this topic less dominant and integrate this part somewhere else (not under a separate title). Also 50% of the text is nearly copied from the*

175 *introduction (doubling!).*

Authors: Agreed. This section has been modified according the referee's suggestions. Please see also reply to referee #2: line 98ff.

Referee #2: Line 335. *I have a different impression. Figure 4a: At day 2 the d13C values are very close to each other. In*

180 *Figure 4b all values from beginning to the end of the incubation time are very similar. Only Figure 4c shows a clear difference between culture and control over the course of the experiment. Add in the figure caption that also controls are plotted, not only results from cultures.*

Authors: $\delta^{13}CH_4$ values for $^{13}C_2$-DMS are presented in Figure 4 a. We found that DMS is also converted chemically in sterile filtered seawater. This is in line with observations of Zhang et al. (2015) (please see manuscript line 332-339). However, the

185 formation rates are very low and only become obvious when applying sensitive stable isotope labelling techniques. We agree with the reviewer that the $\delta^{13}CH_4$ values of the DMS spiked seawater group and the DMS spiked algae group are very close to each other up to day 2. A section regarding control values was added to the revised manuscript (Chapter 4.1) :" The $^{13}C_2$-DMS spiked seawater group and the $^{13}C_2$-DMS spiked algae group are very close to each other up to day 2 (see Fig.3a and Fig.4a). For this time period, it can be assumed that the chemical conversion has taken place in both samples to the same extent, since

190 the samples are relatively similar, because the algal cell density is only 5% (day 2) of the final cell density. However, the following days (day 3 to day 6), when algal cell density increased drastically, the $\delta^{13}CH_4$ values of the algae cultures also increased significantly compared with $\delta^{13}CH_4$ values of the seawater. This clearly indicates that conversion of $^{13}C_2$-DMS to CH_4 increases with increasing cell counts."

Referee #2: Chapter 4.3 *I would recommend to perform an additional calculation to show that algal CH_4 production is an*

195 *important mechanism that can explain air/sea methane fluxes and methane enrichments. For example Schmale et al. (2018) gives detailed data about phytoplankton biomass (e.g. Prymnesiales) and production rates needed to maintain air/sea fluxes and subthermocline methane enrichments. There are probably also other papers available that could be used for such calculation.*

Authors: We followed the recommendation and used the detailed data of Schmale et al. (2018) to estimate a possible

200 contribution of algal CH_4 production to the CH_4 production rate in the field. An additional calculation was added to Chapter 4.3 in the revised manuscript. See reply to referee #1: line 400.

Minor issues:

Referee #2: Title: *I recommend writing "potential relevance for the environment". A direct transfer*

205 *of laboratory studies/results into field observations is difficult.*

Authors: Change applied.

Referee #2: Line 24. *Please also give the productions rates per cell in the abstract. Temperature is not needed to be mentioned in the abstract.*

210 Authors: Changes applied.

Referee #2: Line 27ff. *It should be mentioned here that the conversion of methylated sulphur compounds to methane was only responsible for less than 1% of the observed methane production (line 327ff).*

Authors: We do not think that this information is important for the reader. The information might misleading here. Please see

215 answers regarding comments of reviewer#1 (manuscript line 327), where we discuss this issue in detail.

Referee #2: Line 26-29. *The word "clearly" is used to often.*

Authors: We have rephrased line 26-29.

220 ***Referee #2: Line 30.*** *"Relevance for the environment" is one major issue in the title but is reduced here to a little sentence. This part should be extended.*

Authors: We have emphasized this issue in the abstract as requested. We added the following sentence: "By comparing the algal CH_4 production rates from our laboratory experiments with results previously reported in two field studies of the Pacific Ocean and the Baltic Sea we might conclude that algae mediated CH_4 release is contributing to CH_4 oversaturation in oxic

225 waters. Therefore, we propose that haptophyte mediated CH_4 production could be a common and important process in marine surface waters."

Referee #2: Line 49. *How can "emissions from freshwater" explain the CH4 concentration in ocean surface water?*

230 Authors: We deleted "emissions from freshwater and" from line 49.

Referee #2: Line 50. *Shorten the sentence and delete "that has been often: : :". "Well-known" means "often reported"*

Authors: We have appropriately modified the sentence.

235

Referee #2: Line 55-58. *This paragraph should be moved to line 46. It might be better to start with this overall review before listing the recent specific studies to oxic methane production*
in lakes and ocean.

Authors: As requested, we have restructured this section.

240

Referee #2: Line 60. May *also mention Valle and Karl (2014) who used in situ MPn concentration in a 14C approach and showed that dissolved MPn in surface waters cannot account for methane oversaturation.*

Authors: The revised manuscript contains the results of del Valle and Karl (2014) as well as the results of Repeta et al. (2016), who, on the other hand, reported that the cycling of the organic matter phosphonate inventory could be sufficient to support

245 the total atmospheric CH_4 flux at their study site.

Referee #2: Line 98. *A bracket is missing (RC: : :). Is it clear for the reader for what the web link is*
good for?

Authors: The bracket was added. We have added the names of the culture collections to the front of the weblink to make it

250 clear that the links lead to the respective collections.

Referee #2: Line 102. *Delete "in" in front of "natural"*

Authors: "in was" deleted.

255 ***Referee #2: Line 110ff***. *Why did the authors used different volumes (medium and headspace)?*

Authors: For practical reasons. We have limited space in the climatic chamber and a limited supply of natural seawater, therefore the size of the vessels were adjusted.

Referee #2: Line 119. *What is meant with "main cultures"? Is this the investigated culture in the*

260 *incubation?*

Authors: Yes, it means the culture that was studied during the incubation. We rephrased this sentence to make it clear.

Referee #2: Line 119ff. *I would suggest to transfer the cell densities to the result section (3.1).*

Authors: Changes applied as suggested.

265 ***Referee #2: Line 122.*** *E. huxleyi was sampled daily! What do you mean with overall sampling*
interval: 9,11,6 days? Why this odd order? And why did you sample the cultures in different intervals? If there is a reason for that it should be explained.

Authors: Overall sampling interval means the incubation time (from inoculation to the end of the experiment/incubation) for
270 each species, that correspond to the sampling time. The incubation time varies from species to species and depends on the growth rate and the cell density in the stationary phase. The stationary phase for each species is dependent on a species specific cell density. As the species grow at different rates the sampling intervals differ. *E. huxleyi* has by far the highest growth rate and was sampled daily. *Chrysochromulina sp.* and *P. globosa* grow slower and were sampled at longer intervals. We revised this section in the manuscript and provided explanations.
275

Referee #2: Line 128. *Why only three data points for E. huxleyi. From Figure 1c it seems to be plausible to use four.*
Authors: The phase of exponential growth (from which μ was calculated) was defined by the cell densities which correspond to the best fit of the exponential regression. The fourth data point clearly deviates from exponential regression.

280 ***Referee #2: Line 131***. *It is always worth to have a repetition to support the previous results.*
Authors: Due to the time constraints, we decided not repeat the experiments with stable isotope measurements that were already done by Lenhart et al. (2016).

Referee #2: Line 134. *The delta is missing in d13C*
285 Authors: Corrected.

Referee #2: Line 135. *Suggest to write: ": : :values of the methane precursor: : :"*
Authors: The expression "source" is usually used in combination with Keeling plots. We would therefore like to keep the expression "source".
290

Referee #2: Line 144. *": : :measured at the end: : :"*
Authors: Corrected.

Referee #2: Line 145. *Suggest to write "For this additional experiment: : :"*
295 Authors: Agreed. Changes applied.

Referee #2: Line 146. *Suggest putting the cell densities in the result section (see above).*
Authors: Changes applied as suggested.

300 ***Referee #2: Line 153***. *"ag" is the abbreviation for what?*
Authors: Please see bracket in line 153. The unit "ag" means 10^{-18} g. The SI prefix "a" stand for atto (10^{-18}).

Referee #2: Line 158. *The program "Image J" is produced by which company?*

Authors: "Image J" is an open source software. A reference was added.

305

Referee #2: Line 174. *I still think that cell densities should be implemented in the result section (see above).*

Authors: Changes applied as suggested.

Referee #2: Line 176. *The target/design of the experiment in section 3.2 is still unknown!*

310 Authors: We have revised section 3.2 to make the experimental target and design clearer. Please see also the answer to the comment concerning manuscript line 95ff.

Referee #2: Line 189. *Analyzed. See line 180*

Authors: Corrected.

315

Referee #2: Line 193. *Write: ": : : (based on three: : :)".*

Authors: We changed line 193 as requested.

Referee #2: Line 204. *Delete: ": : :at a temperature: : :". Here and in some other parts of the result section you mention*

320 *details that were mentioned before in the method section. I would start with a sentence that makes clear that you are talking about the incubation with ^{13}C-labelled hydrogen carbon (2.3).*

Authors: Line 204 "at a temperature" was deleted. The result section was revised to avoid repetition of details from the method section. An introductory sentence was added in section 2.3 as suggested.

325 ***Referee #2: Line 208.*** *Also here delete the repeated information: "These rates were obtained: : :". Check the entire result section to avoid redundancy.*

Authors: The result section was revised to avoid repetition of details from the method section.

Referee #2: Line 211. *The cell density should only be mentioned here and not in the method*

330 *section!*

Authors: All final cell densities were removed from the method section.

Referee #2: Line 214. *Where is the control group plotted? Figure 2. Black and blue dots are difficult to distinguish. Even if it is "only" the control sample – make the visibility easier. The x-axis should be 1/CH4. Right?*

335 Authors: Control groups in Figure 1 were plotted in the revised manuscript. Please also look at the answer to referee#1's comment regarding figure 1. Figure 2 was revised: by changing colors and correcting labeling of x-axis. Yes, "1/CH_4" is correct.

Referee #2: Line 249*. See above. Not clear why the exponential growth phase is important and not the cell activity.*

340 Authors: Please see the answer above to the comment concerning manuscript line 141.

Referee #2: Line 251. *The equation is already described above (2 and 4). Avoid doubling. See comment above.*

Authors: We revised this section in order to avoid doubling. The sentence "By doing so the CH_4 production rate is the product

345 of exponential growth rate μ and cellular or POC quota." was removed from line 251.

Referee #2: Line 254. *The sentence should end with (Tab. 1).*

Authors: Corrected.

350 ***Referee #2: Line 256.*** *"community level" sounds odd in this context. May you can find a better description.*

Authors: This term was used in the context of the production potential which was established by Gafar et al. (2018). The phrase "community level" is also used in this context by Gafa et al. (2018). We would therefore like to keep this phrase in order to avoid misunderstandings.

355

Referee #2: Line 266. *It starts again with information that was mentioned before in the methods.*

Authors: We removed this information from line 266.

Referee #2: Line 271*. The sentence should end with "(Fig. 4)".Figure 4b. Change the x-axis to 13C2 (add 2).Figure 4c.*

360 *Change the x-axis to MSO (not MES, see caption).*

Authors: Line 271 and Figure 4c were corrected.

Referee #2: Line 279. *Add the control sample in the Figure.*

Authors: Control groups were added.

365

Referee #2: Line 302*. In the present study only the turnover of ^{13}C-hydrogen carbonate by two algal species was investigate. Lenhart applied the isotope technique for E. huxleyi.*

Authors: We have corrected this sentence.

370 ***Referee #2: Line 307.*** *(with highest cell numbers) is out of context. Please rephrase.*
Authors: The phrase "(with highest cell numbers)" was replaced by "(where the POC content is highest)".

Referee #2: Line 333. *In future investigations I would suggest a dark incubation to exclude methane production by UV or visible light (line 70ff).*
375 Authors: This topic is currently being investigated by us. Please see also answer to the comment of referee #2 concerning manuscript line 98. There we discuss a dark incubation experiment. In addition we discuss there a experiment with and without inhibition of algae by DCMU.

Referee #2: Line 352ff. *Did Althoff really proved that the "reactivity" is the driving force in her experiments? Or are point 1*
380 *(label concentration) and 2 (penetration) also possible explanations*
for her observations?
Authors: Althoff et al. (2014) used a defined chemical system to study the conversion of methylated sulfur compounds to CH_4. It turned out that the yield was not the same for all substances under otherwise identical reaction conditions. The CH_4 yield was therefore also dependent on the substance and thus its reaction behavior.
385

Referee #2: Line 360ff. *Sentence too complex. Devide in two parts.*
Authors: As requested, we have reformulated and restructured this sentence.

Referee #2: Line 363 *and 365. Too often "furthermore". Rephrase.*
390 Authors: Changes applied.

Referee #2: Line 391. *Interesting. But it needs be explained in more detail why the growth rate impacts the methane production. See above.*
Authors: A detailed explanation was added to the method section. Please see reply referee #2: line 141.
395

Referee #2: Line 411. *Include/explain why PP is meaningful parameter.*
Authors: Further explanation regarding the meaning of PP was added to the manuscript. Line 410 now reads: " However, several recent studies have emphasized that the production potential (PP), as opposed to cellular production, is a biogeochemically meaningful parameter (Gafar et al., 2018, Marra 2002, Schlüter et al., 2014, Kottmeier et al., 2016). The
400 concept of the production potential goes back at least to the first half of the 20th century (Clarke et al., 1946). Briefly, the production potential of substance X is the amount of X which a phytoplankton community or culture produces in a given time.

For details see Material and Methods and references above. The cellular production by contrast is the rate of production of X of a single cell, and therefore the cellular production is ill qualified to express community-level production.“

405

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List of substantial changes

In **chapter 4.1** we discuss the possible contribution of bacteria and archaea in the main text and refer to further discussion in
5 the supplementary material.
We added a new paragraph **(chapter 4.1)** where we discuss/explain that the turnover of DMS, DMSO or MSO (including non-labeled compounds) to CH_4 could not be determined on the basis of their added amount of ^{13}C-labelled substance in cultures of *E. huxleyi*. We also point out, that the stable isotope labelling approach should be considered as a proof of concept, that methyl groups of all tested substance serve as precursor compounds of CH_4.
10 In **chapter 4.3** we added a new section where we compared the CH_4 production rates of our cultures with field studies for Baltic Sea (Schmale et al., 2018) to evaluate the potential relevance of algal CH_4 production.

For detailed information about changes made to the manuscript please see point by point response to the referee and track changes version of the manuscript.

Methane production by three widespread marine phytoplankton species: release rates, precursor compounds, and potential relevance for the environment

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Abstract. ~~The world's oceans are considered to be a minor source of methane (CH_4) to the atmosphere although the magnitude of total net emissions is highly uncertain. In recent years the origin of the frequently observed in situ CH_4 production in the ocean mixed layer has received much attention.~~ Methane (CH_4) production within the oceanic mixed layer is a widespread phenomenon, but the underlying mechanisms are still under debate. Marine algae might contribute to the observed CH_4 oversaturation in oxic waters, but so far direct evidence for CH_4 production by marine algae has only been provided for the coccolithophore *Emiliania huxleyi*.

In the present study we investigated, next to *Emiliania huxleyi*, other widespread haptophytes, i.e. *Phaeocystis globosa* and *Chrysochromulina sp*. ~~for CH_4 formation~~. ~~Our results of~~We performed CH_4 production and stable carbon isotope measurements and provide unambiguous evidence that all three investigated marine algae are involved in the ~~produce~~ production of CH_4 ~~per se~~ under oxic conditions. ~~and at r~~Rates ~~ranging~~ ranged from 1.~~6~~ 9 ± 0.~~5~~ 6 to ~~2~~3.~~7~~ 1 ± 0.~~7~~ 4 µg CH_4 per g POC (particulate organic carbon) d^{-1} ~~at a temperature of 20°C~~ with *Chrysochromulina sp.* and *Emiliania huxleyi* showing the lowest and highest rates, respectively. Cellular CH_4 production rates ranged from 16.8 ± 6.5 (*Phaeocystis globosa*) to 62.3 ± 6.4 ag CH_4 $cell^{-1}$ d^{-1} (*Emiliania huxleyi*; ag = 10^{-18} g). In cultures that were treated with ^{13}C-labelled hydrogen carbonate $\delta^{13}CH_4$ values increased with incubation time, ~~clearly~~ resulting from the conversion of ^{13}C-hydrogen carbonate to $^{13}CH_4$. The addition of ^{13}C labelled dimethyl sulfide, dimethyl sulfoxide and methionine sulfoxide – known algal metabolites that are ubiquitous in marine surface layers ~~–~~ ~~enabled us to clearly monitor~~resulted in the occurrence of ^{13}C-enriched CH_4 in cultures of *Emiliania huxleyi* clearly indicating that methylated sulphur compounds are also precursors of CH_4. By comparing the algal~~e~~ CH_4 production rates from

our laboratory studiesexperiments with results previously reported fromin two field studies ofform the Pacific Ocean and the Baltic Sea we might concludeconcluded that algae mediated CH_4 releaseproduction from algae is could likely contributing to CH_4 oversaturation in oxic waters. Therefore, wWe propose that haptophyte mediated CH_4 production could be a common and important process in marine surface waters. likely contributing to CH_4 oversaturation in oxic wateBy comparing the algae CH_4 production rates with two field studies form the Pacific Ocean and the Baltic Sea we concluded that CH_4 production could likely contributing to CH_4 oversaturation in oxic waters.

1. Introduction

Methane (CH_4), the second most important anthropogenic greenhouse gas after CO_2, is the most abundant reduced organic compound in the atmosphere and plays a central role in atmospheric chemistry (Denman et al., 2007; Kirschke et al., 2013; Lelieveld et al., 1998). The mixing ratio of CH_4 in the atmosphere has been increasing dramatically from pre-industrial values of about 715 parts per billion by volume (ppbv) to about 1868 ppbv (October 2018, NOAA). The global atmospheric CH_4 budget is determined by the total emission (540-568 Tg CH_4 yr^{-1}) of various sources from terrestrial and aquatic surface areas, that are balanced primarily by one major sink (hydroxyl radicals) in the atmosphere. However, partitioning source categories to reduce uncertainties in the global CH_4 budget is a major challenge (Saunois et al., 2016). The world's oceans are considered to be a minor source of CH_4 to the atmosphere (1-3 %, Saunois et al., 2016). However, in recent years the widespread occurrence of in situ CH_4 production in the ocean mixed layer has received much attention, since CH_4 formation in the oxygenated ocean mixed layer challenge the paradigm that biological methanogenesis is a strictly anaerobic process.

Methane is primarily formed by degradation of buried organic matter under heat and pressure (thermogenic) inside the earth crust or produced by the incomplete combustion of biomass (pyrogenic). On the other hand, CH_4 resulting from microbial processes, carried out by methanogenic archaea under anoxic conditions in soils and sediments or the digestion system of ruminants are categorized as biogenic or microbial (Kirschke et al., 2013). In contrast to these well-known sources, recent studies have confirmed direct CH_4 release from eukaryotes, including plants, animals, fungi, lichens, and the marine alga *E. huxleyi* even in the absence of methanogenic archaea and in the presence of oxygen or other oxidants (Keppler et al., 2006; Ghyczy et al., 2008; Lenhart et al., 2012; Lenhart et al., 2016; Lenhart et al., 2015b). A very recent study also confirmed *cyanobacteria*, as CH_4 producers suggesting that CH_4 production occurs in all three domains of life (Bizic-Ionescu et al., 2018). These novel sources, from the domains *eucarya* and *bacteria,* might be classified as biotic non-archaeal CH_4 (Boros and Keppler, 2018).

In situ CH_4 production in oxygenated surface waters in the marine environment was first reported by Scranton and Farrington (1977) and Scranton and Brewer (1977) and some decades later also for lakes (Grossart et al., 2011). Significant quantities of CH_4, produced in upper oxic waters, near the air-water interface, might overcome oxidation, and thus significantly contributing

to CH_4 fluxes from aquatic environments to the atmosphere (Bogard et al., 2014). It turned out that in situ CH_4 production in the upper oxic waters is a common feature of both oceans and lakes (Forster et al., 2009; Reeburgh, 2007; Tang et al., 2014;Donis et al., 2017; Bižić-Ionescu et al., 2018; Bange et al., 1994). These results have stimulated the scientific community to study in more detail the phenomenon of CH_4 occurrence in oxygenated surface waters.

In this context, emissions from ~~freshwater and marine~~ cyanobacteria or algae might help to explain the ~~well-known~~ phenomenon of dissolved CH_4 oversaturation. ~~in the upper oxic waters of both oceans and lakes (Forster et al., 2009;Reeburgh, 2007;Tang et al., 2014;Donis et al., 2017; Bižić-Ionescu et al., 2018;Bange et al., 1994Significant quantities of CH_4, produced in upper oxic waters, near the air-water interface, might overcome oxidation, and thus significantly contributing to CH_4 fluxes from aquatic environments to the atmosphere (Bogard et al., 2014)In situ CH_4 production in oxygenated surface waters in the marine environment was first reported by Scranton and Farrington (1977) and Scranton and Brewer (1977) and some decades later also for lakes (Grossart et al., 2011). These results have stimulated the scientific community to study in more detail the phenomenon of CH_4 occurrence in oxygenated surface waters.~~

In addition, ~~For example,~~ it has been suggested that CH_4 might be produced by the bacterial cleavage of methylphosphonate (MPn) in oligotrophic marine P~~p~~acific waters during phosphorus limitation~~.~~ (Karl et al., 2008; Metcalf et al., 2012 ;Repeta et al., 2016). While dissolved MPn in surface waters cannot account for the CH_4 oversaturation observed in the oligotrophic waters of the North Pacific (Valle and Karl, 2014), the cycling of the organic matter phosphonate inventory might be sufficient to support the total atmospheric CH_4 flux (Repeta et al., 2016).

In contrast to this apparently non-oxygen sensitive pathway, many other studies have identified the "traditionally" archaeal methanogenesis in anoxic microenvironments as a CH_4 source. Floating particles (Karl and Tilbrook, 1994), the digestive tracts of zooplankton (de Angelis and Lee, 1994; Stawiarski et al., 2019; Schmale et al., 2018) or fishes (Oremland, 1979) have been found as anoxic micro niches for methanogens. It has been suggested that some methanogens might be active under oxic conditions by being equipped with enzymes to counteract the effects of molecular oxygen during methanogenesis (Angel et al., 2011). Potential substrates for methylotrophic methanogens in such micro niches are the algae metabolites dimethylsulfoniopropionate (DMSP) and their degradation products dimethyl sulfide (DMS) or dimethyl sulfoxide (DMSO) (Zindler et al., 2013; Damm et al., 2008; Florez-Leiva et al., 2013). Furthermore, DMSP might also be converted to CH_4 by nitrogen limited bacteria (Damm et al., 2010;Damm et al., 2015). However, in coastal waters where DMS and DMSP production is enhanced, CH_4 was found to mainly related to sedimentary sources (Borges et al., 2018).

In contrast to microbial processes, which are considered to be driven by enzymes, CH_4 might also be derived by the chemical reaction of chromophoric dissolved organic matter (CDOM) and DMS induced by UV or visible light under both oxic and anoxic conditions (Zhang et al., 2015). A similar photochemical CH_4 formation was earlier described for acetone by Bange and Uher (2005) but the production of CH_4 from acetone was considered negligible under oxic conditions.

Another chemical reaction that readily forms CH_4 from the methyl thioethers and their sulphoxides under highly oxidative conditions and catalyzed by nonheme iron-oxo (IV) species was presented by Althoff et al. (2014) and Benzing et al. (2017). Iron-oxo species have been identified as active intermediates in the catalytic cycles of a number of biological enzymatic

100 systems (Hohenberger et al., 2012). Thus, marine algae containing elevated concentrations of methyl thiolethers and their
sulfoxides such as DMSP, DMSO, methionine (MET) or methionine sulphoxide (MSO), might be biochemical reactors for
non-archaeal CH_4 production as it was already proposed by Lenhart et al. (2016) and Keppler et al. (2009).
Marine phytoplankton plays a central role in the global carbon cycle: Approximately a half of earth's primary production is
carried out by marine phytoplankton (Field et al., 1998). In this context it is important to mention that almost 40 years ago
105 researchers (Scranton and Brewer, 1977; Scranton and Farrington, 1977; Scranton 1977) already mentioned the possibility of
in-situ formation of CH_4 by marine algae, since CH_4 production was examined in cultures of *E. huxleyi* and *T. pseudonana.*
Furthermore, a ~~So far,~~ direct isotopic evidence for CH_4 production by marine algae in the absence of methanogenic archaea
has only been provided for *E. huxleyi* (Lenhart et al., 2016). Based on the application of stable carbon isotope techniques, it
could be clearly shown that both hydrogen carbonate and a position-specific ^{13}C-labelled MET were carbon precursors of the
110 observed CH_4 production. However, it remains unclear whether CH_4 production also occurs among other marine algae and if
there are also other carbon precursors, involved in the formation process.
In the present study we investigated, next to the coccolithophore *E. huxleyi*, two other marine, non-calcifying *haptophy*tes,
namely *P. globosa* and *Chrysochromulina sp.* for CH_4 formation. The investigated species are all bloom-forming and often
found as dominant members in marine phytoplankton community worldwide (Schoemann et al., 2005; Thomsen, 1994; Brown
115 and Yoder, 1994). Furthermore, they are well-known for their high DMSP, DMS and DMSO productivity (Liss et al., 1994;
Keller, 1989; Holligan et al., 1993; Stefels et al., 2007; Matrai and Keller, 1993). We therefore conducted stable isotope
experiments using ^{13}C labelled DMS, MSO and DMSO to identify potential methyl group precursor compounds that eventually
lead to CH_4 production. Finally, we discuss the laboratory CH_4 production rates in relation to its potential significance in
marine environments.

120

2. Material & Methods

2.1 Cultures and culture conditions

Three algal species, *Emiliania huxleyi* RCC1216 obtained from the Roscoff Culture Collection (http://roscoff-culture-
collection.org/) *Phaeocystis globosa* PLY 575 and *Chrysochromulina sp.* PLY 307 obtained from the Marine Biological
125 Association of the United Kingdom (https://www.mba.ac.uk/facilities/culture-collection) were studied. In order to keep non-
axenic algae cultures largely free of bacteria, the cultures were diluted regularly, resulting in quasi constant exponential algal
growth while minimizing bacterial cell density.
All incubation experiments were carried out in controlled and sterile laboratory conditions under a 16/8 hour light/dark cycle
at a light intensity of 350 µmol photons m^{-2} s^{-1} and a temperature of 20°C. All samples were taken at the end of the light cycle.

130 Monoclonal cultures were grown in full-batch mode (Langer et al., 2013) in sterile filtered (0.2 µm Ø pore size) ~~in~~ natural North Sea seawater (sampled off Helgoland, Germany) enriched in nutrients according to F/2 medium (Guillard and Ryther, 1962). The initial dissolved inorganic carbon (DIC) of the F/2 medium was 2152 ± 6 µmol L^{-1} (measured by Shimadzu TOC-V CPH). The DIC value falls within the range of typical DIC concentrations of North Sea seawater.

2.2 Determination of cell densities

135 Cell densities were determined from four aliquots of each culture sample, using either a Fuschs-Rosenthal or Neubauer counting chamber, depending on cell density.

2.3 Incubation with ^{13}C- labelled hydrogen carbonate

To investigate CH_4 production by algal cultures borosilicate glass bottles (Schott, Germany) filled with 2.0 L 0.2 µm filtered F/2 medium and with 0.35 L headspace volume were used in our investigations of C*hrysochromulina sp.* and *P. globosa*. For 140 the investigations of *E. huxleyi* 0.85 L medium and 0.4 L headspace volume were used (Schott, Germany). The ~~flasks~~ vails were sealed airtight with lids (GL 45, PP, 2 port, Duran Group) equipped with one three-way port for liquid and a second port fitted with a septum for gas sampling. For measurements of the mixing ratio and stable carbon isotope value of ~~methane~~ CH_4 ($\delta^{13}C$-CH_4) samples of headspace (20 mL) were taken from each vial. Afterwards, samples (2 mL) for determining cell densities were taken. In order to maintain atmospheric pressure within the vial, the surrounding air was allowed to enter via 145 the three-way port and trough a sterile filter to avoid biological contamination. The inflow of surrounding air was taken into consideration when CH_4 production was calculated.

~~Main cultures~~Cultures that were studied during the incubation were inoculated from a pre-culture grown in dilute-batch mode (Langer et al., 2009). ~~The initial cell densities were 26.9 ± 4.0 × 10^3 cells mL^{-1} for C*hrysochromulina sp.*, 25.6 ± 1.2 × 10^3~~ 150 ~~cells mL^{-1} for *P. globosa* and 17.5 ± 2.0 × 10^3 cells mL^{-1} for *E. huxleyi*.~~ To investigate algal derived CH_4 formation six vials were inoculated with algae and another six ~~jars~~vials contained medium only.

In addition, three vials of each group were treated with ^{13}C-hydrogen carbonate ($H^{13}CO_3^-$) to investigate CH_4 formation by measuring stable carbon isotope values of CH_4. ~~Four~~ Four different treatments were ~~used~~used: medium either ~~either~~ with $H^{13}CO_3^-$ (medium + $H^{13}CO_3^-$) or without (medium, data not available) ~~or without (medium) a treatment of $H^{13}CO_3^-$~~ and cultures 155 supplemented either with $H^{13}CO_3^-$ (medium + culture + $H^{13}CO_3^-$) or without (medium + culture). ~~$H^{13}CO_3^-$ (n=3)~~ The different treatments and the number of replicates for the experiments with C*hrysochromulina sp.* and *P. globosa* are provided in Fig. 1. ~~Please note that~~ Please note that stable isotope measurements using $H^{13}CO_3^-$ were not performed for *E. huxleyi* as evidence for isotope labelling of CH_4 ~~formation~~ was already provided by Lenhart et al. (2016). To study CH_4 formation of *E. huxleyi* by measuring headspace concentration three replicates (culture and medium group, n=3) were used.

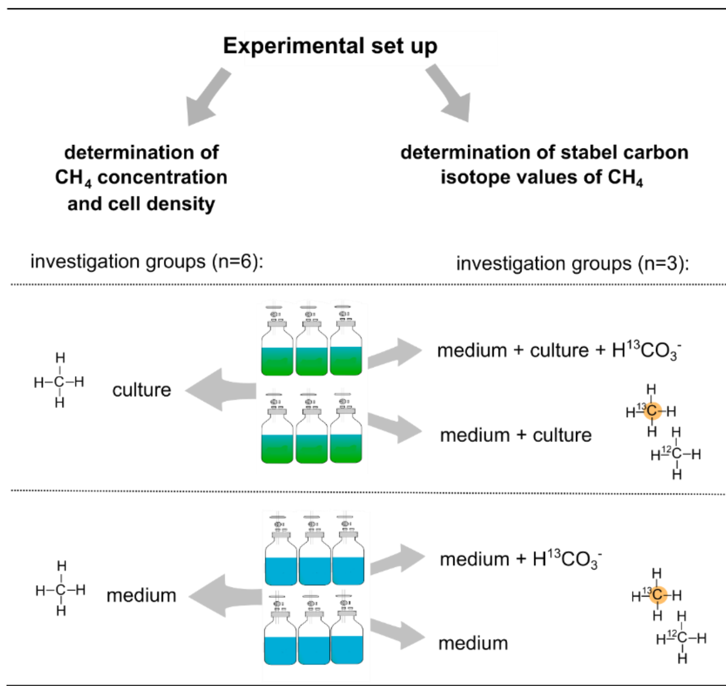


Fig. 2 Experimental setup for measuring CH_4 formation by *Chrysochromulina sp.* and *P. globosa*. Methane formation was investigated by concentration measurements within six vials containing either algae or medium only (left column). For stable isotope measurements of CH_4 ^{13}C labelled hydrogen carbonate ($H^{13}CO_3^-$) was added to three vials of both groups (right column).

The overall incubation time –was 9, 11 and 6 days for *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi* respectively. H~~h~~eadspace and liquid samples were collected on a daily basis for *E. huxleyi* and in 2-3 days intervals from cultures of *Chrysochromulina sp.* and *P. globosa*. The incubation time and sampling intervals varied between species because of variations in the growth rate and the cell density in the stationary phase. ~~The overall sampling time was 9, 11 and 6 days with final cell densities of $0.18 \pm 0.01 \times 10^6$ cells mL^{-1}, $1.77 \pm 0.15 \times 10^6$ cells mL^{-1} and $1.70 \pm 0.09 \times 10^6$ cells mL^{-1} for *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi* respectively.~~

Cell densities were plotted versus time and the exponential growth rate (μ) was calculated from exponential regression using the natural logarithm (Langer et al., 2013). The ~~phase of~~ exponential growth phase (from which μ was calculated) was defined by the cell densities which corresponded to the best fit ($r^2 > 0.99$) of the exponential regression. This was done by using the first three (*Chrysochromulina sp.* and *E. huxleyi*) or four data points (*P. globosa*) of the growth curve.

~~Four different treatments were used: medium either with (medium + HCO_3^-) or without (medium) a treatment of $H^{13}CO_3^-$ and cultures supplemented either with (culture + HCO_3^-) or without (culture) $H^{13}CO_3^-$ (n=3). Please note that stable isotope measurements using $H^{13}CO_3^-$ were not performed for *E. huxleyi* as evidence for isotope labelling of CH_4 formation was already provided by Lenhart et al. (2016).~~

For stable carbon isotope experiments 48,7 µmol L^{-1} ~~^{13}C hydrogen carbonate (~~$NaH^{13}CO_3$~~)~~ in final concentration was added to the F/2 medium. The added amount of $NaH^{13}CO_3$ corresponds to 2% of the DIC of the North Sea seawater (2152 ± 6 µmol L^{-1}), resulting in a theoretically calculated $\delta^{13}C$ value of DIC of +2014 ± 331‰. To determine the $\delta^{13}C$-CH_4 values of the source, the Keeling-plot method was applied (Keeling, 1958). For a detailed discussion of the Keeling plot method for determination of the isotope ratio of CH_4 in environmental applications, please refer to (Keppler et al., 2016). Oxygen concentration was monitored daily (using inline oxygen sensor probes, PreSens, Regensburg) at the end of the light cycle (Fig. S1).

2.4 Determination of CH_4 production rates

Since the experiment in the section ~~3.2~~2.3 was not designed to obtain POC quotas (POC = particulate organic carbon), we conducted an additional experiment. To best compare CH_4 formation rates of the three algae species it is necessary to obtain exponential growth to ensure constant growth rates and constant (at a given time of day) cellular POC quotas over the course of the experiment. ~~(Langer et al., 2012, 2013).~~ Exponential growth is a prerequisite for calculating production on the basis of growth rate and quota (here CH_4 quota). The point is a general, technical one, and is not confined to CH_4 production. The studies~~papers~~ by Langer et al. (2012, 2013) discuss this point in the context of batch culture experiments. Briefly, production on this account is the product of growth rate and quota (e.g. CH_4, calcite, organic carbon). Production here is an integrated value, typically over many cell divisions. For this calculation of production to be meaningful a constant growth rate is required. The exponential growth phase fulfills this criterion whereas the transition phase and the stationary phase do not. Therefore production cannot be calculated meaningfully in the non-exponential phases. The problem can, however, be minimized by using small increments (one day) because growth rate can be regarded as quasi-constant (see also Lenhart et al., 2016). ~~.~~The CH_4 production rates can be calculated by multiplying the growth rate µ with the corresponding cellular or POC-CH_4 quota, that was measured ~~on~~ at the end of the experiment.

For this additional experiment t~~T~~he cultures were grown in 160 ~~ml~~ mL crimped serum bottles filled with 140 ~~ml~~ mL medium and 20 ~~ml~~ mL headspace (n=4). ~~The initial cell density of 22.5 ± 3.1 × 10^3 cells mL^{-1}, 80.9 ± 11.5 × 10^3 cells mL^{-1} and 29.0 ± 5.5× 10^3 cells mL^{-1} for *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi*, respectively were inoculated.~~ ~~Cultures were gown up to 37.0 ± 9.2 × 10^3 cells mL^{-1} (*Chrysochromulina sp.*), 219 ± 24.1 × 10^3 cells mL^{-1} (*P. globosa*) and 283 ± 15.6 × 10^3 cells mL^{-1} (*E. huxleyi*). These cell densities corresponded to the cell densities of exponential growth phase obtained from the experiment in section 3.2.~~ Oxygen concentration was monitored (using inline oxygen sensor probes, PreSens, Regensburg) at the end of each light and dark cycle (Fig. S2).

The growth rate (µ) was calculated from cell densities of the beginning and end of the experiment according to Eq.1:

$$\mu = \frac{Ln(N_1) - Ln(N_0)}{(t_1 - t_0)} \quad (1)$$

where N_0 and N_1 are the cell densities at the beginning (t_0) and end of the experiment (t_1). The daily cellular CH_4 production rates (CH_4P_{cell}, ag CH_4 $cell^{-1}$ d^{-1}, ag = 10^{-18} g~~-~~) were calculated according to Eq.2:

$$CH_4P_{cell} = \mu \times \frac{m(CH_4)}{cell} \quad (2)$$

where $m(CH_4)$ is the amount of CH_4 that was produced at the end of the experiment.

To calculate POC based CH_4 production rates the cellular organic carbon content (POC_{cell}) was derived from cell volume (V_{cell}) by using the Eq. 3 according to (Menden-Deuer and Lessard, 2000):

$$POC_{cell} = 0.216 \times V_{Cell}{}^{0.939} \quad (3)$$

The cell volume was determined measuring the cell diameter in light micrographs using the program Image~~-~~J (Schindelin et al., 2012).

According to (Olenina, 2006) a ball shape can be assumed for calculating the cell volume for the three species investigated here. The daily cellular CH_4 production rates (CH_4P_{POC}, µg CH_4 g^{-1} POC d^{-1}) were calculated from growth rate and CH_4-POC quotas at the end of the experiment according to Eq. 4.

$$CH_4P_{POC} = \mu \times \frac{m(CH_4)}{POC} \quad (4)$$

The CH_4 production potential (CH_4-PP) was used to translate differences in cellular production rates to community level. According to ~~(~~Gafar et al.~~,~~ (2018), the CH_4-PP can be calculated for different periods of growth, by calculating a cellular standing stock for each time period from a known starting cell density (N_0) (whereby constant exponential growth is assumed). The corresponding amount of produced CH_4 (CH_4PP) for each period of growth and standing stock is the product of the cellular standing stock and CH_4 quota (Eq. 5).

$$CH_4PP = N_0 \times e^{\mu \times t} \times \frac{m(CH_4)}{cell} \quad (5)$$

In the present study the CH_4-PP was calculated for a standing stock that is obtained after 7 days of growth ~~and~~ starting ~~by a cell count of one~~with a single cell.

2.5 Incubation with ^{13}C labelled DMS, DMSO and MSO

The sulphur bonded methyl group(s) in DMS, DMSO and MSO were investigated as precursors for algal-derived CH_4 in an incubation experiment with *E. huxleyi*. For all tested compounds only the C atom of the sulphur bonded methyl group(s) was labelled with ^{13}C (R-S-$^{13}CH_3$, 99%). A final concentration of 10 µM were used for each compound. ~~The treatments were initiated in parallel from batch culture by inoculating 17.5 ± 2.0 × 10^3 cells mL^{-1} and cultures were grown to final cell densities of 1.77 ± 0.08 × 10^6 cells mL^{-1}. For every single compound ($^{13}C_2$-DMS, $^{13}C_2$-DMSO, ^{13}C-MSO)~~ The different treatments to investigate potential CH_4 formation by $^{13}C_2$-DMS, $^{13}C_2$-DMSO, ^{13}C-MSO are provided in Fig. 2.~~four treatment groups with~~ T~~t~~hree independent replicates and repeated measurements over time were used. Headspace and vail size were analogous to the experiment~~s~~ described in section ~~3~~2.~~2~~ 3 for *E. huxleyi*~~. With reference to section 3.2 the experiment~~

differs in sampling frequencies and overall incubation time.: Ssamples were taken daily during an overall incubation time of
235 6 days.

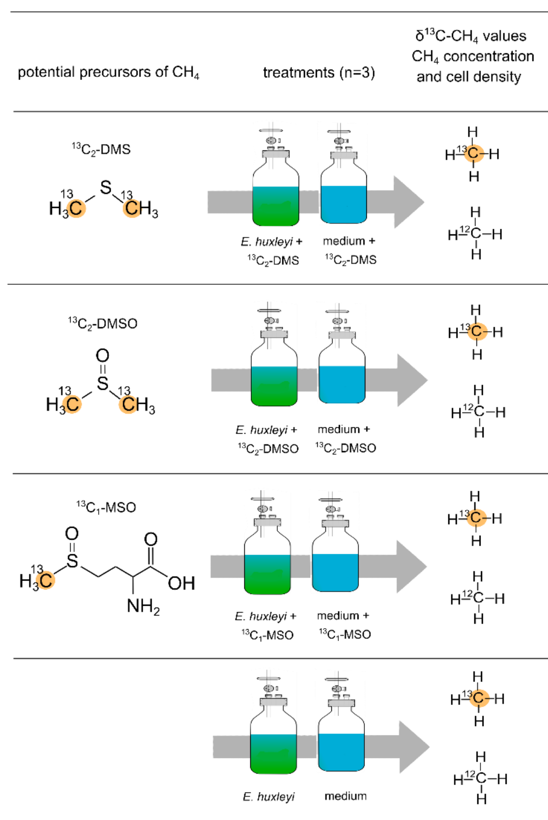


Fig. 3 Experimental setup to investigate potential precursor compounds of CH_4. Dimethyl sulfide ($^{13}C_2$-DMS), dimethyl sulfoxide ($^{13}C_2$-DMSO) and methionine sulfoxide (^{13}C-MSO) were added to the jarsvials containing either a culture of *E. huxleyi* or medium
240 **only. For all tested compounds only the carbonC atom of the sulphur bonded methyl group(s) was labelled with ^{13}C.**

2.6 Determination of CH_4 mass

Five mL of a gas sample was collected from the head space of the vials using a gas tight Hamilton gas syringe. The sample
245 was analyzed by gas chromatography (GC-14B, Shimadzu, Japan; column: 2 m, Ø = 3.175 mm inner diameter, high-grade steel tube packed with Molecular Sieve 5A 60/80 mesh from Supelco) equipped with a flame ionization detector (FID). Quantification of CH_4 was carried out by comparison of the integrals of the peaks eluting at the same retention time as that of the CH_4 authentic standard, using two reference standards containing 9837 and 2192 parts per billion by volume (p.p.b.v).

Mixing ratios were corrected for head space pressure that was monitored using a pressure measuring device (GMSD 1,3 BA,
250 Greisinger).

The CH_4 mass (m_{CH_4}) was determined by its mixing ratio (x_{CH_4}) and the ideal gas law (Eq. 6),

$$m_{CH_4} = M_{CH_4} \times x_{CH_4} \frac{p \times V}{R \times T} \quad (6)$$

where M_{CH_4}= molar mass, p = pressure, T = temperature, R = ideal gas constant, V = volume.

The dissolved CH_4 concentration was calculated by using the equation of Wiesenburg and Guinasso (1979).

2.7 GC-C-IRMS measurements

255 Stable carbon isotope values of CH_4 of headspace samples were analyzsed by gas chromatography stable isotope ratio mass spectrometry (GC-C-IRMS, Deltaplus XL, Thermo Finnigan, Bremen, Germany). All $\delta^{13}C\text{-}CH_4$ values were corrected using two CH_4 working standards (isometric instruments, Victoria, Canada) with values of -23.9 ± 0.2‰ and -54.5 ± 0.2‰. The results were normalized by two-scale anchor calibration according to (Paul et al., 2007). The average standard deviation of the analytical measurements was in the range of 0.1 ‰ to 0.3 ‰ (based on three repeated measurements of CH_4 working
260 standards). All $\delta^{13}C\text{-}CH_4$ values are expressed in the conventional δ notation, in per mille (‰) vs. Vienna Pee Dee Belemnite (VPDB), using Eq.7.

$$\delta^{13}C = \frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{standard}} - 1 \quad (7)$$

For a detailed description of the $\delta^{13}C\text{-}CH_4$ measurements by GC-IRMS and technical details of the pre-concentration system we would like to refer to previous studies by (Comba et al., 2018) and (Laukenmann et al., 2010)

2.8 Statistics

265 To test for significant differences in cell density, CH_4 formation, and CH_4 content between the treatments, two-way analysis of variance (ANOVA) (considering repeated measurements) and a post hoc test [Fisher least significant difference (LSD) test; alpha 5 %] were used.

3. Results

3.1 Algal growth and CH_4 formation

270 To investigate CH_4 production by algal cultures incubations with ^{13}C-labelled hydrogen carbon were applied as described in section 2.3. The growth curves during incubation of the three algal species ~~at a temperature of 20°C and a day-night-cycle of 16 h day 8 h night~~ are presented in Fig. ~~1~~ 3 (upper panel a, b, c). The initial cell densities were 26.9 ± 4.0 × 10^3 cells mL^{-1} for C*hrysochromulina sp.*, 25.6 ± 1.2 × 10^3 cells mL^{-1} for *P. globosa* and 17.5 ± 2.0 × 10^3 cells mL^{-1} for *E. huxleyi*. The exponential growth rate μ was highest for *E. huxleyi* (1.71 ± 0.04 d^{-1}) i.e. three or five times higher than for *P. globosa* and

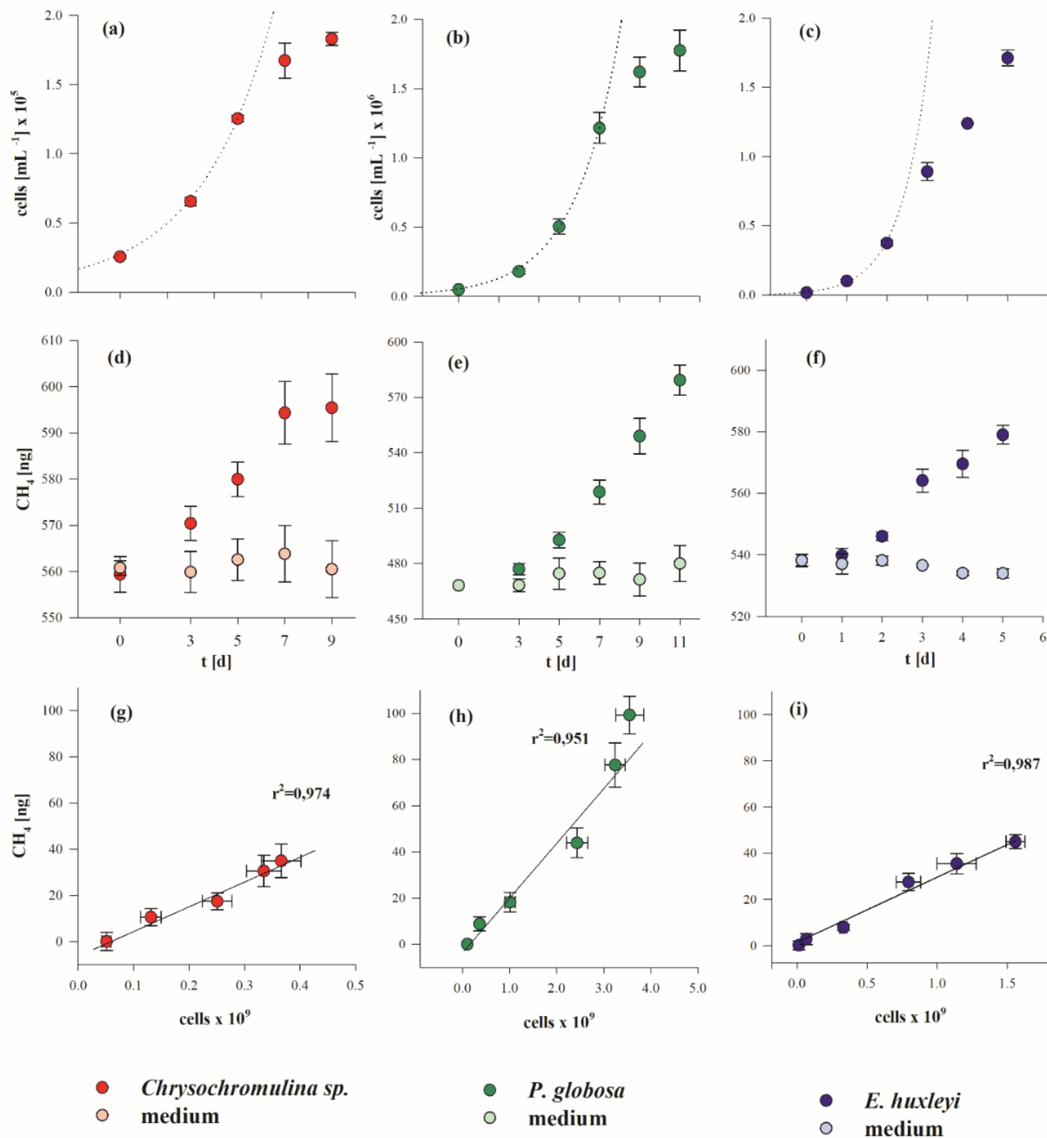
275 C*hrysochromulina sp.* (with 0.33 ± 0.08 d^{-1} and 0.52 ± 0.07 d^{-1}, respectively). The dotted lines in Fig. 1 a, b, c marks the time points of exponential growth.

~~These rates were obtained by exponential regression from the first three (*Chrysochromulina sp.* and *E. huxleyi*) or four time points (*P. globosa*) of the growth curve, while cell densities on the following timepoints departs from the one expected from exponential growth (dotted line, Fig. 1 a, b, c,).~~ Maximum cell densities were lowest for C*hrysochromulina sp.* with 0.18 ±

280 0.01 × 10^6 cells mL^{-1} followed by *E. huxleyi* with 1.70 ± 0.09 × 10^6 cells mL^{-1} and highest for *P. globosa* with 1.77 ± 0.15 × 10^6 cells mL^{-1}.

Significant CH_4 formation was observed in all three cultures over the whole incubation period of 5 to 11 days (Fig. ~~1~~ 3 d, e, f) whereas no increase in CH_4 over time was observed in the control groups. For all species the increase in headspace CH_4 was significant ($p \leq 0.05$) at second time point of measurement and at all following time points ($p \leq 0.001$). At the end of the

285 incubation period the amounts of produced CH_4 were ~~30.0~~34.9 ± ~~6.2~~7.3 ng, ~~85.2~~99.3 ± ~~10~~8.~~0~~ 2 ng and 45.0 ~~43.5~~ ± ~~4.3~~3.1 ng for *Chrysohromulina. sp.*, *P. globosa* and *E. huxleyi*, respectively. A linear correlation was found between the absolute number of cells and the amount of produced CH_4 of *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi* (Fig. ~~1~~ 3 lower panel, g, h, i).



290 **Fig. 4: Cell growths (first panel), CH_4 production (middle panel) in course of time and correlation between the total number of cells and produced CH_4 (lower panel) from three algae species. *Chrysochromulina sp.* (left column a, d, g,), *P. globosa* (middle column b, e, h) and from *E. huxleyi* (right column c, f ,i). Please note that the cell numbers of *Chrysochromulina sp.* are presented in 10^5 and P. *globosa*, *E. huxleyi* in 10^6. Mean values of six (*Chrysochromulina sp., P. globosa*) and three (*E. huxleyi*) replicated culture experiments are shown and error bars mark the SD.**

295

3.2 Stable carbon isotope values of CH_4 during incubation with ^{13}C-hydrogen carbonate

Stable carbon isotope values of CH_4 ($\delta^{13}CH_4$ values) for *Chrysochromulina sp.* and *P. globosa* are presented in Fig. 2 4 (a, c). We observed conversion of ^{13}C carbon (provided by ^{13}C-hydrogen carbonate) to $^{13}CH_4$ in cultures of both species, indicated by increasing $\delta^{13}CH_4$ values over time. Stable isotope values increased from initial atmospheric (laboratory air) levels of - -48.7
300 ± 0.3 ‰ and -48.4 ± 0.10 ‰ up to +30.1 ± 10.2 ‰ and +245 ± 16 ‰ for *Chrysochromulina sp.* and *P. globosa,* respectively, whilst the $\delta^{13}CH_4$ values of the control groups (algae without ^{13}C-hydrogen carbonate or ^{13}C-hydrogen carbonate in medium without culture) did not change over time. The increase of $\delta^{13}CH_4$ values in the headspace-CH_4 depended on the amount of released CH_4 that was added to the initial (atmospheric) background level. To calculate the $\delta^{13}CH_4$ values of the CH_4 source which has raised CH_4 quantity above background level the Keeling-plot method (Keeling, 1958; Pataki et al., 2003) was used
305 (Fig. 2 4 b, d).

The calculated $\delta^{13}CH_4$ values of the CH_4 source were +1300 ± 245 ‰ (*Chrysochromulina sp*.) and +1511 ± 35 ‰ (*P. globosa*) and thus close to the theoretical calculated ^{13}C value of the DIC (2014 ± 331‰) resulting from the addition of ^{13}C-hydrogen carbonate. Please note that ^{13}C-hydrogen carbonate stable isotope labelling experiment with *E. huxleyi* were already performed by Lenhart et al. (2016) and were not repeated in this study. This is why $\delta^{13}CH_4$ values and the respective Keeling plot of *E.*
310 *huxleyi* are not shown in Fig. 24.

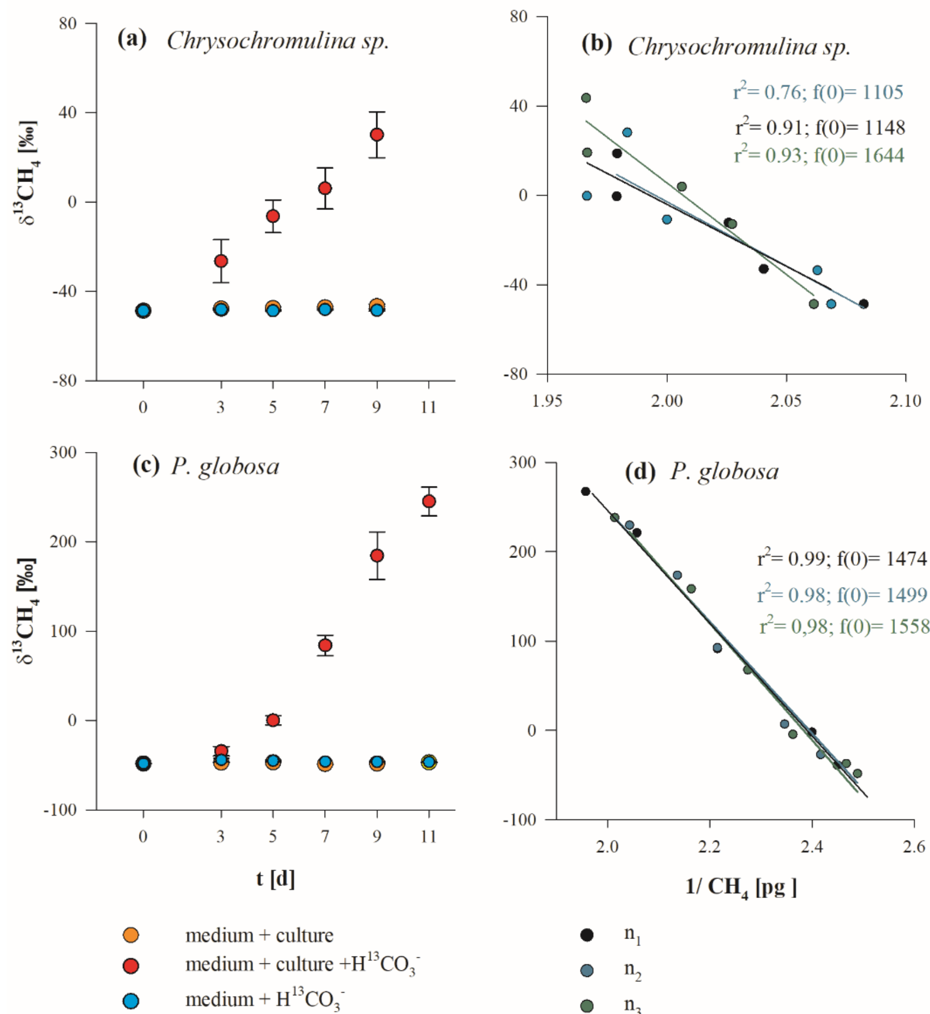


Fig. 5: δ^{13}CH₄ values (left column) and respective Keeling plots (right column) from *Chrysochromulina sp.* (a,b) and *P. globosa* (c,d) after the addition of $H^{13}CO_3^-$. The left column (a, c) shows the δ^{13}CH₄ values of three investigation groups ("culture + $H^{13}CO_3^-$ ", "culture" and "$H^{13}CO_3^-$"), whereas each data point presented is the mean value of three replicated culture experiments with error bars showing SD. The right column shows the Keeling plots for the treatments "culture + $H^{13}CO_3^-$" from each replicated culture experiments (n_1, n_2, n_1) where f (0) refers to the ^{13}C value of the CH_4 source.

315

3.3 CH_4 production and production potential

~~To estimate CH_4 production rates of the three algal species it is necessary to ensure exponential growth. We normalized CH_4~~
320 ~~production rates to cell and to particulate organic carbon (POC) content. By doing so the CH_4 production rate is the product of~~

exponential growth rate μ and cellular or POC quota. Since the experiment in the section above (isotope measurements) was not designed to obtain POC quotas, we conducted an additional experiment to estimate CH_4 production rates of the three algal species.

From initial cell density of 22.5 ± 3.1 × 10^3 cells mL^{-1}, 80.9 ± 11.5 × 10^3 cells mL^{-1} and 29.0 ± 5.5× 10^3 cells mL^{-1} Cultures cultures were gown up to 37.0 ± 9.2 × 10^3 cells mL^{-1} (*Chrysochromulina sp.*), 219 ± 24.1 × 10^3 cells mL^{-1} (*P. globosa*) and 283 ± 15.6 × 10^3 cells mL^{-1} (*E. huxleyi*).for *Chrysochromulina sp.*, *P. globosa* and *E. huxleyi*, respectively. These cell densities corresponded to the cell densities of exponential growth phase obtained from the experiment in section 3.21.

The POC normalized daily CH_4 production rate was highest in *E. huxleyi*, followed by *P. globosa*, and *Chrysochromulina sp.*. However, the cellular or POC normalized daily production rates of the three algal species were in the same order of magnitude (Table. 21). We calculated the CH_4 production potential (CH_4PP), that is the amount of CH_4 produced within a week of growth (Gafar et al., 2018), to translate the cellular production rates (μ × CH_4 $cell^{-1}$) of each species to community level. The CH_4PP was two order of magnitude higher for *E. huxleyi* than the other two species. This is a consequence of the higher growth rate of *E. huxleyi*.

We furthermore observed the oxygen concentrations during the light and dark periods to ensure oxic conditions. The measured oxygen concentrations were always saturated or supersaturated relative to equilibration with ambient air (Fig. S2).

Table 1: Growth rate, cellular POC, CH_4 production rates and $CH_4PP_{(7days)}$ of *Chrysochromulina sp*. (n=4), *P. globosa* (n=4) and *E. huxleyi* (n=4). Values are the mean of four replicated culture experiments with SD.

	growth rate (μ)	cellular POC	CH_4 production rate		$CH_4PP_{(7days)}$
	d^{-1}	pg $cell^{-1}$	ag CH_4 $cell^{-1}$ d^{-1}	μg CH_4 g^{-1} POC d^{-1}	fg CH_4
Chrysochromulina sp.	0.21 ± 0.04	25.4 ± 4.0	4438.59 ± 130.96	1.96 ± 0.65	10.08 ± 0.3
P. globosa	0.50 ± 0.06	7.0 ± 0.4	164.84 ± 65.56	2.41 ± 0.98	1.10 ± 0.43
E. huxleyi	1.09 ± 0.02	20.1 ± 0.7	62.353.3 ± 65.45	32.17 ± 0.47	12104 ± 98.0

3.4 CH_4 formation from ^{13}C labelled methyl thiol ethers

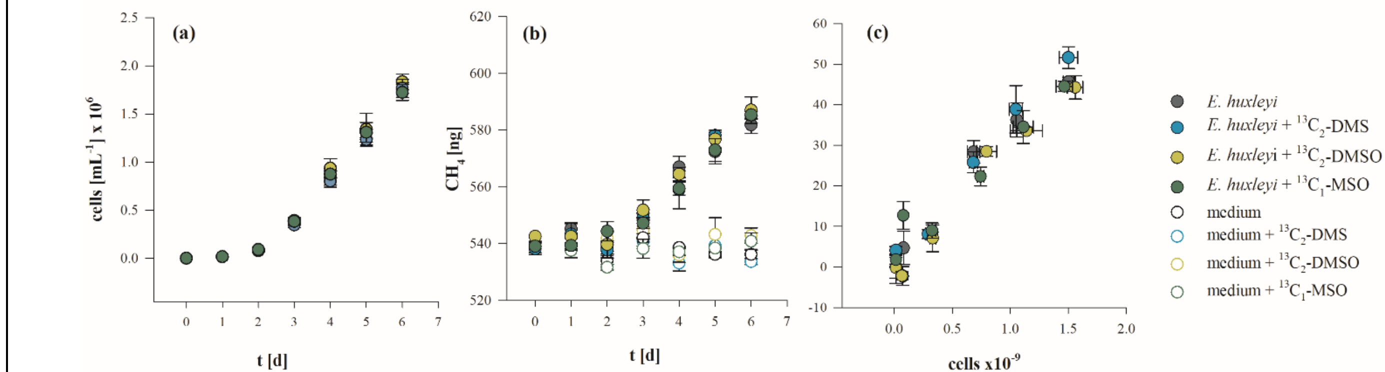
The three methylated sulphur compounds MSO, DMSO and DMS were tested for potential CH_4 formation in incubation experiments with *E. huxleyi*. For all tested compounds ^{13}C isotope labelling technics where applied where only the methyl bonded sulphur group(s) (R-S-$^{13}CH_3$) was fully labelled with ^{13}C atoms.The treatments were initiated in parallel from batch culture by inoculating 17.5 ± 2.0 × 10^3 cells mL^{-1} and cultures were grown to final cell densities of 1.77 ± 0.08 × 10^6 cells mL-

345 1 ,(Fig. 5 a). Cell densities and CH_4 formation correlated in all treatments, while no difference in cell growth pattern or CH_4 formation was observed when isotope labelled methyl thioether and sulfoxides were added to the culture (Fig. 3 5 a, b, c). Differences between treatments were found in $\delta^{13}CH_4$ values of headspace CH_4. The initial $\delta^{13}CH_4$ value of headspace (-47.9 ± 0.1 ‰, laboratory air) increased slightly over time in untreated cultures (without isotope treatment) to -46.8 ± 0.3 ‰ (Fig 46.b).

350 In contrast, experiments where $^{13}C_2$-DMSO, $^{13}C_2$-DMSO and ^{13}C-MSO was applied to cultures of *E. huxleyi* $\delta^{13}CH_4$ values increased to -31.0 ± 1.1 ‰, -45.7 ± 0.1 ‰ and +18.3 ± 7.7 ‰, respectively over a time period of 6 days (Fig. 4 6 a, b, c) and differed significantly from control groups ($p<0.05$).

The results unambiguously show that a fraction of the ^{13}C-labelled methyl groups of the added substances was converted to $^{13}C-CH_4$ in cultures of *E. huxleyi*. Much smaller changes in $\delta^{13}CH_4$ values were observed for controls of sterile filtered media

355 where only $^{13}C_2$-DMS and ^{13}C-MSO was added (-42.8 ± 1.7 ‰ and -43.9 ± 0.2 ‰ respectively, Fig. 4 6 a, c, day 6), whereas $\delta^{13}CH_4$ values did not change over time in the seawater controls (no addition of isotopic labelled compounds) and in the seawater controls treated with $^{13}C_2$-DMSO (Fig. 4 6 b). Based on the initial amount of ^{13}C label substance that were added to the cultures and the total amount of $^{13}CH_4$ at the end of the incubation period, 9.5 ± 0.2 pmol ($^{13}C_2$-DMS), 3,0 ± 3,2 pmol ($^{13}C_2$-DMSO) and 30,1 ± 3,6 pmol (^{13}C-MSO) of 8.5 µmol were converted to CH_4.



360

Fig. 6 Cell growths (a), CH_4 production (b) and relation between the total number of cells and produced CH_4 (c) from *E. huxleyi* treated with $^{13}C_2$-DMS, $^{13}C_2$-DMSO and ^{13}C-MSO or without any treatment. Mean values of three replicated culture experiments are shown and error bars mark the SD.

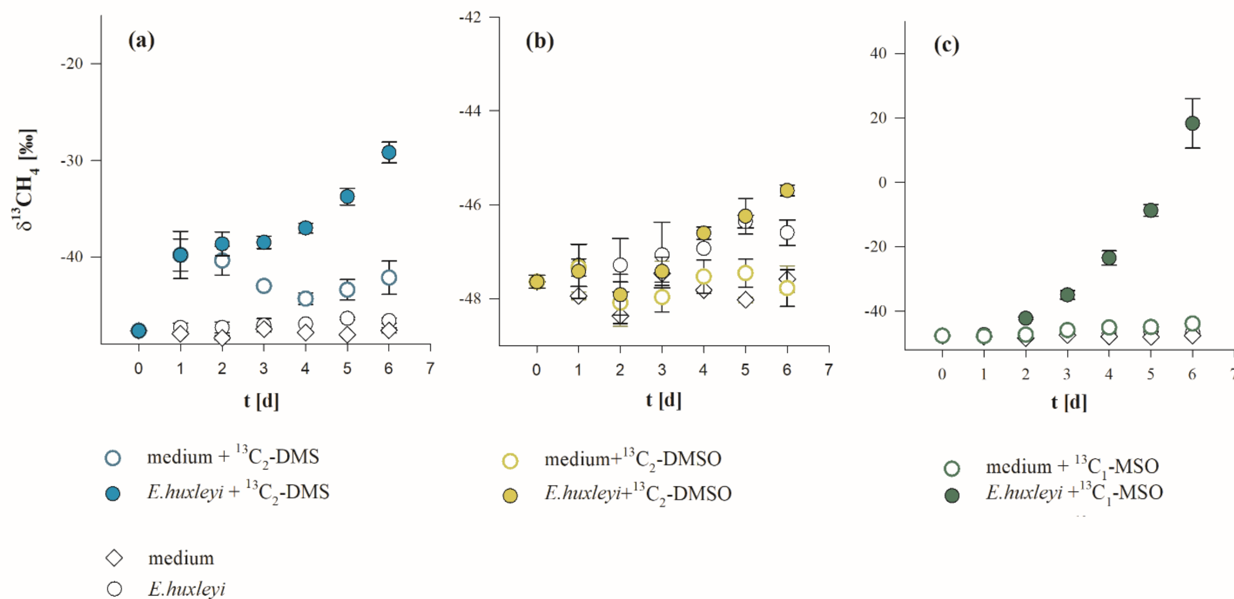


Fig.~~.~~ 7. $^{13}CH_4$ values of headspace CH_4 in cultures of *E. huxleyi* supplemented with (a) $^{13}C_2$-DMS, (b) $^{13}C_2$-DMSO and (c) ^{13}C-MSO. Mean values of three replicated culture experiments are shown and error bars mark the SD.

4. Discussion

Our results of CH_4 production and stable carbon isotope measurements provide unambiguous evidence that next to *E. huxleyi* (Lenhart et al., 2016) other widespread marine algal species namely *Chrysochromulina sp.* and *P. globosa* are involved in the production of ~~produce~~ CH_4 ~~per se~~ under oxic conditions at rates of 1.~~6~~ 9 ± 0.~~5~~ 6 to ~~2~~3.~~7~~ 1 ± 0.~~7~~ 4 µg CH_4 g^{-1} POC d^{-1}. The three investigated genera of marine phytoplankton have a world-wide distribution and they are representatives of the most widespread marine haptophytes (Schoemann et al., 2005; Thomsen, 1994; Brown and Yoder, 1994). The results indicate that CH_4 production could be a common process across marine haptophytes. We first discuss the stable isotopic evidence of CH_4 formation, the role of precursor compounds and likely mechanisms involved. Finally, we discuss the laboratory CH_4 production rates in relation to its potential significance in marine environments and provide a first rough estimation how these production rates might contribute to CH_4 concentration in oxic surface waters previously reported in open ocean algal blooms.

In cultures of *Chrysochromulina sp*. and *P. globosa*, that were treated with ^{13}C-labelled hydrogen carbonate, $\delta^{13}CH_4$ values increased with incubation time, clearly resulting from the conversion of ^{13}C-hydrogen carbonate to $^{13}CH_4$. These results demonstrate that all three investigated algal species are instrumental in the production ~~produce~~ of CH_4 ~~per se~~ under oxic conditions (Fig. S~~.~~1) and that hydrogen carbonate serves as a carbon source for $^{13}CH_4$. Our findings are in agreement with the stable isotope evidence of CH_4 production by *E. huxleyi* (Lenhart et al., 2016). However, we do not consider hydrogen

carbonate as the direct carbon precursor of CH_4. In a first step hydrogen carbonate and its isotope label is converted to CO_2 and subsequently fixed by algal primary production forming POC. Therefore, we would expect a large fraction of the ^{13}C label of the hydrogen carbonate (+2014 ± 331‰) to be transferred to the POC towards the end of the experiment (where the volume 385 normalized POC content is highest~~with highest cell numbers~~). The experiments were started by inoculation of cells from pre-cultures, that were grown on DIC with natural $^{13}C/^{12}C$ abundance ($\delta^{13}C$ values ~0 ‰). This means that during ongoing incubation the $\delta^{13}C$-POC value should get close to $\delta^{13}C$-DIC values, resulting from the addition of ^{13}C-hydrogen carbonate, when cultures grow in the new ^{13}C enriched medium. Consequently, the $\delta^{13}C$-POC values are considered to be somewhat lower than the theoretically calculated $\delta^{13}C$-DIC values (+2014 ± 331‰) of the medium. Our assumptions are in line with the $\delta^{13}CH_4$ 390 source signature values (averaged over 9 or 11 days respectively), obtained via Keeling plot method, which were +1300 ± 245 ‰ and +1511 ± 35 ‰ for *Chrysochromulina. sp*. and *P. globosa*, respectively and thus were somewhat lower than for the theoretical calculated ^{13}C value of the DIC (+2014 ± 331‰) resulting from the addition of ^{13}C-hydrogen carbonate. Unfortunately, $\delta^{13}C$-DIC and $\delta^{13}C$-POC values could not be determined in our set of experiments to allow more detailed calculations. However, our results clearly indicate that hydrogen carbonate is the principle inorganic carbon precursor of $^{13}CH_4$ 395 produced in algae, but intermediate metabolites are likely to be formed from which CH_4 is released, possibly by cleavage of sulphur-bonded methyl groups of methyl thioethers and sulfoxides (Althoff et al., 2014, Lenhart et al., 2016, Benzing et al., 2017).

4.1 CH_4 formation from ^{13}C labelled methyl thioethers

400 *Methyl thioethers are precursors of CH_4*

Methyl thioethers and their sulphoxides are ubiquitous in marine environments as they are often produced by algae at substantial rates. It is also known that these compounds are metabolized in the three investigated algal species (Liss et al., 1994; Keller, 1989). Based on the addition of $^{13}C_2$-DMSO, $^{13}C_2$-DMS and ^{13}C-MSO, where only the sulphur-bonded methyl 405 groups (–S-CH_3) were 99% labelled with ^{13}C, it was possible to clearly monitor $^{13}CH_4$ formation by stable carbon isotope measurements in cultures of *E. huxleyi*. The $\delta^{13}CH_4$ values, increased over time significantly in $^{13}C_2$-DMS, $^{13}C_2$-DMSO and ^{13}C-MSO treated cultures, above the $\delta^{13}CH_4$ values of the control groups~~.~~ (Fig. ~~4~~ 6 a-c). The ^{13}C-labelling experiment showed that DMS, DMSO, and MSO are potentially important methyl-precursors for CH_4 but the contribution of these compounds to the overall CH_4 production in cultures of *E. huxleyi* could not be determined in our experiments due to the complexity of the 410 formation of these compounds in the algal cells. This can be illustrated by the following. The contribution of a substance to the total CH_4 released is the product of both the added ^{13}C-labeled fraction (added to the waters sample and uptake by the cells) and the internally formed fraction of these compounds (DMS, DMSO and MSO) which will roughly show natural ^{13}C abundance. Therefore the stable isotope value of CH_4 will be diluted by the fraction of naturally formed methyl sulfur

compounds in the algal cells and thus the contribution of DMS, DMSO, and MSO to CH_4 formation can therefore not be estimated on the basis of their added amount alone. The $^{13}CH_4$ quantity from conversion of added ^{13}C labelled substance contributed 0.03% ($^{13}C_2$-DMSO) up to 0.84% (^{13}C-MSO) to overall released CH_4. However, even if the added ^{13}C labelled compounds might only explain ≤ 1% of CH_4 formed by the algae their overall contribution (including non-labelled sulfur compounds which we are not able to measure) might provide a much larger fraction of the released CH_4. The intracellular DMS concentration can reach 1 mM (Sunda et al., 2002) in cells of *E. huxleyi*, while the concentration of added $^{13}C_2$-DMS was 0.01 mM in medium (final concentration). If intracellular $^{13}C_2$-DMS was in equilibrium with bulk seawater $^{13}C_2$-DMS and all CH_4 would be produced from intracellular DMS, then the contribution of the ^{13}C labelled compound would be about 1%. However, even if the biggest fraction of CH_4 in algae cultures was not released by the ^{13}C labelled substances, the significant increase in delta notation in $^{13}C_2$-DMS, $^{13}C_2$-DMSO and ^{13}C-MSO treated cultures above the $\delta^{13}CH_4$ values of the control groups demonstrate that ^{13}C labelled precursor substances were converted to CH_4 by algal cultures (Fig. 6 a-c). This is also indicated, when the absolute conversion quantities of ^{13}C-labelled substance in algal cultures are considered: these were ca. nine ($^{13}C_2$-DMS), three ($^{13}C_2$-DMSO) and thirty (^{13}C-MSO) times higher than in seawater control groups. Hence, the stable isotope labelling approach should be considered as a proof of concept, that methyl groups of all tested substance serve as precursor compounds of CH_4.

These isotope labelling results are also in good agreement with recent results from laboratory experiments where ^{13}C-MET was added to cultures of *E. huxleyi* (Lenhart et al., 2016). In addition, we also found an indication for a purely chemical CH_4 formation pathway from control samples using sterile seawater and addition of either $^{13}C_2$-DMS and ^{13}C-MSO. The $^{13}C_2$-DMS spiked seawater group and the $^{13}C_2$-DMS spiked algae group are very close to each other up to day 2 (see Fig. 5a and Fig. 6a). For this time period, it can be assumed that the chemical conversion has taken place in both samples to the same extent, since the samples are relatively similar, because the algal cell density is only 5% (day 2) of the final cell density. However, the following days (day 3 to day 6), when algal cell density increased drastically, the $\delta^{13}CH_4$ values of the algae cultures also increased significantly compared with $\delta^{13}CH_4$ values of the seawater. This clearly indicates that conversion of $^{13}C_2$-DMS to CH_4 increases with increasing cell counts.

However, the relatively slight increase in $\delta^{13}CH_4$ values in the control samples (Fig.4 6 a, c) implicates that this is only a minor pathway. The CH_4 conversion from ^{13}C-DMS and ^{13}C-MSO in seawater was approximately 3- and 30-fold lower than in the corresponding treatments with algae and becomes only obvious when applying very sensitive stable isotope labelling experiments. A similar observation was already made by Lenhart et al. (2016) when applying ^{13}C-MET in seawater. However, this observation might be in agreement with previously findings by Zhang et al. (2015), who described a photochemically and CDOM related conversion of DMS to CH_4 in oxygenated natural seawater.

~~*Reasons for variable conversion of methyl thioethers to CH_4*~~

~~The conversion of the initial added amount of ^{13}C labelled substance (8,5 µmol) into $^{13}CH_4$ varied drastically between supplemented compounds in algal cultures ranging from 3.0 pmol ($^{13}C_2$-DMSO), 9.5 pmol ($^{13}C_2$-DMS), to 30.1 pmol (^{13}C-~~

MSO). However, these conversion quantities of ^{13}C labelled substance in algal cultures were about nine ($^{13}C_2$-DMS), three ($^{13}C_2$-DMSO) and thirty (^{13}C-MSO) times higher than in control groups. Several explanations for variable conversion of methyl

450 thioethers to CH_4 can be given: firstly newly synthesized, non-labelled molecules of the applied ^{13}C substances are produced by the cells to a different extent, which in turn leads to different cell concentrations of ^{13}C labelled molecules (due to the relative depletion of ^{13}C labelled substances). Secondly, the applied compounds penetrate the cells to a different extent leading to different final concentrations inside the cells. For instance, amino acids like MET and MSO are taken up by transporter systems (Cho and Komor, 1985) while DMS is much more hydrophobic than DMSO and is highly permeable to cell

455 membranes (Sunda et al., 2002;Spiese et al., 2015) . Finally, the reactivity of the methyl bonded sulphur group of the methyl thioethers and their sulfoxides differs between the chemical compounds. For instance, the CH_4 conversion were variable between methylated sulphur compounds, when Althoff et al. (2014) used different methylated sulphur compounds to mimic CH_4 formation in eukaryotes via chemical reaction.

460

Potential mMechanism of CH_4 formation from thioethers

The CH_4 formation from thioethers (MET, DMS) and their corresponding sulphoxides (MSO, DMSO) might be catalysed by nonheme oxo iron (IV), thus forming methyl radicals ($\cdot CH_3$) from homolytically broken sulphur methyl bounds ($R-CH_3$) leading to CH_4 under oxidative conditions (Althoff et al., 2014; Benzing et al., 2017). Since TThe tested compounds are found

465 in high cellular concentrations in *E. huxleyi*, *Chrysochromulina sp.* and *P. globosa* and non heme oxo iron (IV) have been identified as active intermediates in the catalytic cycles of a number of biological enzymatic systems (Hohenberger et al., 2012),. Therefore, the postulated reaction might be a likely pathway for CH_4 production in investigated alga species. Furthermore, DMS and DMSO were described to be part of an antioxidant system as these compounds can readily scavenge hydroxyl radicals in cells of *E. huxleyi* (Sunda et al., 2002). Furthermore, CH_4 is released via a methyl radical, that is subtracted

470 from DMSO when hydroxyl radicals being scavenged – and accordingly DMS after its sulphoxidation (Herscu-Kluska et al., 2008). Since MET and MSO have similar functional groups to DMS and DMSO respectively, it was proposed that the reaction described above is taking place analogously for these compounds (Bruhn et al., 2012; Lenhart et al., 2015a). Consequently, the CH_4 formation in investigated algal species might be a response of oxidative stress, that forms hydroxyl radicals or other reactive oxygen species (ROS), which in turn might react with the applied methylated sulphur compounds generating methyl

475 radicals and eventually CH_4.

Methyl thioethers are not converted by methanogenic archaea in our cultures

The algal metabolites DMSP, DMS and DMSO are ubiquitous in marine surface layers and nanomolar concentrations were found in blooms of *Chrysochromulina sp*., *P. globosa* and *E. huxleyi*. Several field studies showed that these compounds are

480 linked to CH_4 formation in seawater (Zindler et al., 2013; Damm et al., 2008; Florez-Leiva et al., 2013). The authors proposed that DMSP and their degradation products DMSO and DMS are used by methylotrophic methanogenic archaea, inhabiting in anoxic microsites, as substrates for methanogenesis. In addition it was reported that, if nitrogen is limited but phosphorus is replete, marine bacteria might also use DMSP as a carbon source, thereby releasing CH_4 (Damm et al., 2010).

One scenario which we cannot rule out would be a production of CH_4 precursors by algae and a usage of these precursors by

485 bacteria to produce CH_4. While we think that this is less likely than CH_4 production by algae alone, it would, if true, show that bacteria need algae-produced precursors to produce CH_4. The latter scenario would be relevant in the field because algae co-exist with bacteria in the oceans. Therefore bacteria might be involved in the CH_4 production process in our cultures, but even if they were they still would depend on algal growth. For further discussion of a potential contribution of heterotrophs and/or methanogenic archaea see supplementary material (S3). The correlations we describe in the supplementary material clearly

490 show that CH_4 production depends on algal growth. It is therefore highly unlikely that bacteria are solely responsible for CH_4 production in our cultures. While methanogenesis in anoxic microsites is a proposed source in the field, an involvement of methanogenic archaea is highly unlikely in our culture experiments because experiments were performed under sterile and oxic conditions. High oxygen accumulation due to algal photosynthesis was observed in all experiments (S1, S2). Furthermore,

~~the absence of methanogenic archaea in culture experiments of *E. huxleyi* strain RCC1216 using several molecular tools was clearly shown in a previous study (Lenhart et al., 2016).~~

4.2 POC normalized production

For all three algal species significant correlations between CH_4 mass and cell density was found ($r^2 > 0.95$ for all species, Fig ~~1~~ 3 g, h, i), suggesting that CH_4 formation occurred over the entire growth curve. However, since CH_4 production can only be determined in the exponential phase (Langer et al., 2013) we additionally ran dilute batch cultures to determine CH_4 production. All three species displayed similar CH_4 production ranging from 1.9~~6~~ ± 0.~~5~~6 to ~~2~~3.~~7~~1 ± 0.~~7~~4 µg CH_4 g^{-1} POC d^{-1} with *Chrysochromulina sp.* and *E. huxleyi* showing the lowest and highest rates, respectively. The CH_4 production for *E. huxleyi* was found to be twofold higher than rates reported for the same strain and comparable culture conditions by Lenhart et al. (2016) (0.7 µg CH_4 g^{-1} POC d^{-1}). The lower production reported by Lenhart et al. (2016) may be explained by the fact that CH_4 production was not obtained from exponentially growing cultures. We also compared the cellular CH_4 production rates of *E. huxleyi* reported by Scranton (1977) with those of our study. Scranton (1977) reported a production rate of 2×10^{-10} nmol CH_4 $cell^{-1}$ hr^{-1}. This value is close to the production rate of 1.6×10^{-10} nmol CH_4 $cell^{-1}$ hr^{-1} in our study. Scranton (1977) concluded from observed CH_4 production rates in laboratory experiments that natural populations might be adequate to support the widespread supersaturations of CH_4 observed in the open ocean. However, we do suggest that CH_4 production of various algae might differ substantially under changing environmental conditions, as already shown for terrestrial plants (Abdulmajeed and Qaderi, 2017; Martel and Qaderi, 2017). Moreover, the cellular concentrations of potential precursor compounds such as methylated sulphur compounds might vary greatly between species and cultures. The investigated algal species can reach millimolar intracellular concentrations of DMS and DMSP (Sunda et al., 2002; Liss et al., 1994; Keller, 1989) and even if the conversion rate of methylated sulphur compounds to CH_4 in algal cells might be low, they could be sufficient to explain a substantial fraction of the CH_4 production rates by marine algae.

4.3 Implication for the marine environment and algal blooms

In general, the distribution of chlorophyll has not shown a consistent correlation with CH_4 distributions in field studies. There are studies in which no correlation was observed (e.g. Lamontagne et al., 1975; Foster et al., 2006; Watanabe et al., 1995) or a correlation was found within a few depth profiles (Burke et al., 1983; Brooks et al., 1981). Many field measurements in oxygenated surface waters in marine and limnic environments have shown examples of elevated CH_4 concentrations spatially related to phytoplankton occurrence (e.g. Conrad and Seifer, 1988; Owens et al., 1991; Oudot et al., 2002; Damm et al., 2008; Grossart, et al., 2011; Weller et al., 2013; Zindler et al., 2013; Tang et al., 2014; Bogard et al., 2014; Rakowski et al., 2015).

Taken together these studies suggest that phytoplankton is not the sole source of CH_4 in oxygenated surface waters, but importantly they also suggest that phytoplankton is one of the sources of CH_4. We therefore compared the CH_4 production rates of our cultures with two field studies for the Pacific Ocean (Weller et al., 2013) and the Baltic Sea (Schmale et al., 2018) to evaluate the potential relevance of algal CH_4 production.

It was estimated that the gross CH_4 production in a southwest Pacific Ocean mesoscale eddy is 40 - 58 pmol CH_4 L^{-1} d^{-1} (Weller et al., 2013). Using reported phytoplankton cell densities (1.7×10^8 to 2.9×10^8 cells L^{-1}, Weller et al., 2013), we calculated a maximal cellular production of 5.5 ag CH_4 $cell^{-1}$ d^{-1} for this eddy. The species investigated in this study showed ca. 3-~~9~~ 11 times higher cellular production (Table 1). Hence each of the three haptophyte algae studied here could account for the CH_4 production reported by Weller et al. (2013).

Schmale et al., (2018) reported CH_4 enrichments that were observed during summer in the upper water column of the Gotland Basin, central Baltic Sea. Furthermore they found that zooplankton is one but not the only CH_4 source in the oxygenated upper waters. While the authors ruled out a major contribution of algae to the observed sub-thermocline CH_4 enrichment because of the low sub-thermocline phytoplankton biomass, they considered a primary production associated CH_4 formation as one likely source in the phytoplankton-rich mixed layer. The average phytoplankton carbon biomass of the mixed layer was approximately 600 µg L^{-1} (averaged from Fig. 9 in Schmale et al., 2018). For the reported average net CH_4 production rate in the mixed layer (95 pmol CH_4 L^{-1} d^{-1}), we calculated that a production rate of 2.5 µg g^{-1} POC d^{-1} is required if the CH_4 is produced by the algal biomass. This rate would be within the range of CH_4 production rates observed in our study. These calculations should be considered as a first rough estimate to assess whether CH_4 production rates of laboratory grown cultures can significantly contribute to CH_4 supersaturation associated with phytoplankton. We did not distinguish between species and did not take into account environmental factors or the complexity of microbiological communities.

Judging from cellular production, the species studied here are of similar importance for oceanic CH_4 production in biogeochemical terms. Regarding the highest cellular production, that of *E. huxleyi* as 100%, *P. globosa* produces 27% and *Chrysochromulina sp.* ~~8~~71% (Table 1). However, several recent studies have emphasized that the ~~Gafar et al. (2018suggested the~~ production potential (PP), as opposed to cellular production, ~~as~~ is a biogeochemically meaningful parameter (Gafar et al., 2018, Marra 2002, Schlüter et al., 2014, Kottmeier et al., 2016). The concept of the production potential goes back at least to the first half of the 20th century (Clarke et al., 1946). Briefly, the production potential of substance X is the amount of X which a phytoplankton community or culture produces in a given time. For details see Material and Methods and references above. The cellular production by contrast is the rate of production of X of a single cell, and therefore the cellular production is ill qualified to express community-level production.

~~because the PP includes the impact of growth rates on cell densities in an exponentially growing community whereas cellular production rates do notHowever, while cellular production is an interesting parameter in physiological terms, it is not useful in biogeochemical terms. Gafar et al. (2018) suggested the production potential (PP), as opposed to cellular production, as a biogeochemically meaningful parameter.~~ We calculated the CH_4-PP (Material and Methods) for our three species, and when the one of *E. huxleyi* is considered 100%, *P. globosa* has a CH_4-PP of ~~1~~0.9%, and *Chrysochromulina sp.* 0.8% (~~Table~~ Table

1). In terms of CH_4 production in the field, therefore, *E. huxleyi* out-performs the other two haptophytes by two orders of magnitude. It can be concluded that the CH_4-PP under given environmental conditions is species-specific and therefore
560 community composition will have an influence on algal sea surface water CH_4 production.

It can be hypothesized that changing environmental conditions might drastically affect algal CH_4 production, which has to be taken into account when calculating annual averages. The effect of dominant environmental parameters such as light intensity and temperature on algal CH_4 production will therefore be the subject matter of future studies.

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