

Interactive comment on “Methane production by three widespread marine phytoplankton species: release rates, precursor compounds, and relevance for the environment” by Thomas Klintzsch et al.

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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: 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

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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 (CH₄) production within the oceanic mixed layer is a widespread phenomenon, while the underlying CH₄ producing mechanism is still topic of scientific debate” We further added two sentences to the introduction: “The world's oceans are considered to be a minor source of methane (CH₄) to the atmosphere (1-3 %, Saunois et al., 2016). However, in recent years the widespread occurrence of in situ CH₄ production in the ocean mixed layer has received much attention, since CH₄ formation in the oxygenated ocean mixed layer challenge the paradigm that biological methanogenesis is a strictly anaerobic process.” We fur-

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ther deleted the sentence “However, partitioning source categories to reduce uncertainties in the global CH₄ budget is a major challenge (Saunio 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 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 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 CH₄ production depends on algal growth. It is therefore highly unlikely that bacteria are solely responsible for CH₄ production in our cultures. However, bacteria might be involved in the CH₄ production process. One scenario which we cannot rule out would be a production of CH₄ precursors by algae and a usage of these precursors by bacteria to produce CH₄. While we think that this is less likely than CH₄ production by algae alone, it would, if true, show that bacteria need algae-produced precursors to produce CH₄. 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 headspace, this may be small but should be mentioned. Were samples equilibrated with headspace before methane measured? The

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authors mention that oxygen was sometimes supersaturated, but was this relative to headspace or equilibration with ambient air?

Authors: The amount of dissolved CH₄ was not included. As requested we have calculated the dissolved CH₄ concentration by using the equation of Wiesenburg and Guinasso (1979). The dissolved fraction of CH₄ has now been included in our calculations and added to the total amount of CH₄ produced. As correctly stated by the referee the addition of the dissolved CH₄ fraction has only a marginal effect on the overall CH₄ production. Calculation of dissolved CH₄ is mentioned in the method section (2.6) and a new reference for calculating dissolved CH₄ was added (Wiesenburg and Guinasso, 1979) to the revised manuscript. Cultures were turned 30 seconds overhead prior to analysis to ensure equilibration between dissolved and headspace CH₄. In the preliminary equilibration experiments, we found that further shaking does not affect the CH₄ 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 (S.2).”

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 ¹³C-hydrogen carbonate (NaH¹³CO₃) was 48.7 μmol L⁻¹ and 10 μM for ¹³C₂-DMS, ¹³C₂-DMSO and ¹³C-MSO. Concentrations (final) of added substrates are given in the manuscript in line 133 for NaH¹³CO₃ and at line 173 for ¹³C₂-DMS, ¹³C₂-DMSO and ¹³C-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 NaH¹³CO₃ corresponds to 2% of the DIC of the North Sea seawater. This information was added to the revised manuscript: “The DIC value falls within the range of typical DIC concentrations of North

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Sea seawater. "We added two sentence to the section were we explain labeling experiments: "For stable carbon isotope experiments $48,7 \mu\text{mol L}^{-1}$ ^{13}C -hydrogen carbonate ($\text{NaH}^{13}\text{CO}_3$) in final concentration was added to the F/2 medium. The added amount of $\text{NaH}^{13}\text{CO}_3$ corresponds to 2% of the DIC of the North Sea seawater ($2152 \pm 6 \mu\text{mol L}^{-1}$), resulting in a theoretical calculated ^{13}C value of DIC of $+2014 \pm 331\text{‰}$."

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, DMSO and DMSO concentrations were ca. 1 nM, 4 nM, and 16 nM for DMS, DMSO and DMSO respectively. Thus we conclude that the initial substrate concentration in the seawater is insignificant in comparison to the added amount ($10 \mu\text{M}$), 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).

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 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 un-

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ambiguously 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 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. The paragraph reads now: "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 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}C CH_4 quantity from conversion of added ^{13}C labelled substance contributed 0.03% (^{13}C -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 $\leq 1\%$ 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 concentra-

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tion can reach 1 mM (Sunda et al., 2002) in cells of *E. huxleyi*, while the concentration of added $^{13}\text{C}_2$ -DMS was 0.01 mM in medium (final concentration). If intracellular $^{13}\text{C}_2$ -DMS was in equilibrium with bulk seawater $^{13}\text{C}_2$ -DMS and all CH_4 would be produced from intracellular DMS, then the contribution of the ^{13}C labeled compound would be about 1%. However, even if the biggest fraction of CH_4 in algae cultures was not released by the ^{13}C labeled substances, the significant increase in delta notation in $^{13}\text{C}_2$ -DMS, $^{13}\text{C}_2$ -DMSO and ^{13}C -MSO treated cultures above the $\delta^{13}\text{C}_{\text{CH}_4}$ values of the control groups demonstrate that ^{13}C labeled 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 were ca. nine ($^{13}\text{C}_2$ -DMS), three ($^{13}\text{C}_2$ -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 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

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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 1.4×10^{-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 where no correlation was observed (e.g. Lamontagne et al., 1975; Foster et al., 2006; Watanabe et al., 1995) or at least 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 where elevated CH_4 concentrations were 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

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potential relevance of algal CH₄ production.”

We followed the reviewer suggestion and added an additional comparison of our CH₄ production rates by using field study data of Schmale et al., (2018). The respective section reads: “Schmale et al., (2018) reported CH₄ 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 CH₄ source in the oxygenated upper waters. While the authors ruled out a major contribution of algae to the observed sub-thermocline CH₄ enrichment because of the low sub-thermocline phytoplankton biomass, they considered a primary production associated CH₄ formation as one likely source in the phytoplankton-rich mixed layer. The average phytoplankton carbon biomass of the mixed layer was approximately 600 $\mu\text{g L}^{-1}$ (averaged from Fig. 9 in Schmale et al., 2018). For the reported average net CH₄ production rate in the mixed layer (95 pmol CH₄ L⁻¹ d⁻¹), we calculated that a production rate of 2.5 $\mu\text{g g}^{-1}$ POC d⁻¹ is required if the CH₄ is produced by the algal biomass. This rate would be within the range of CH₄ production rates observed in our study. These calculations should be considered as a first rough estimate to assess whether CH₄ production rates of laboratory grown cultures can significantly contribute to CH₄ supersaturation associated with phytoplankton. We did not distinguish between species and did not take into account environmental factors or the complexity of microbiological 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 recommended terms for expression of stable isotope-ratio and gas-ratio measurement”).

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

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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 CH₄ L⁻¹).

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.

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