*We would like to thank both reviewers and the editor for the time, effort and helpful suggestions. Below, we provide a point-by-point reply (in italic font and highlighted in green) to the reviewers' concerns (in normal font):*

Reviewer 1
The manuscript "Adaptations of methane oxidising bacteria to environmental changes" describes the diversity of methane oxidizing community under natural conditions and after experimental modifications. Overall, the study is well written

*Answer: Thank you very much for the appraisal.*

However there are some major concerns:
There is a mix between environmental observations and the experimental modifications. For the environmental observations the authors describe 2 locations at 4 seasons and 2 water depths. These should be described in a single way, so the readers can easily see if there is for example an influence of water depth or season on the MOB community.
However, as these environmental set-ups are quickly combined with the experiments, these basic and natural description of the MOB community remains unclear.

*Answer: We have clarified the setup of the experimental and observational datasets in the Materials and Methods section of the revised version of the MS (the environmental observational North Sea dataset comprised two water depth and two seasons; the Wadden Sea setup was only one water depth and 4 seasons). The presentation of results (in section 3.1) with respect to segregating environmental versus experimental data was streamlined.*

The experimental set ups involve modifications of the methane concentrations (3 levels), salinity (4 levels) and temperature (3 levels). First it should be explained why these levels were chosen in comparison to the natural range of these factors. The temperature range seems rather high, does the North Sea ever has water temperatures > 25°? What about lower temperatures < 15°? For the methane concentrations it should be clarified how much 5% CH4 in the headspace relates to nmol/L of dissolved methane; and how these concentrations relate to the natural concentrations at the study site. The same holds for the chosen levels of salinity.

*Answer: The reviewer is correct in the assessment of the different environmental parameters. We have chosen temperatures that are typical or higher for the seasons spring, summer and autumn and in all cases higher than in winter. This was chosen as the aim of the MS is to investigate MOB communities in a future, likely warmer coastal ocean. The same holds true for CH4 concentrations, these were deliberately chosen to represent extreme CH4 concentrations (5% HS CH4 is equivalent to ~55 µM CH4 at 25°C and 35 psu salinity). We added details in the revised MS (see new tables 1 and 2 in the supplementary information) and expand on the motivation in the revised version the MS (see first paragraph of the Discussion).*

The incubation time of 20 – 30 days seems to be rather long. Can the authors verify that other parameters such as oxygen concentrations, depletion of nutrients or biofilm on the glass bottle were not changing the system off from the "normal" situation?

*Answer: The reviewer is right that some authors chose a shorter incubation time period, however, others even longer ones. Our incubation times are similar in length to previous publications, e.g. Li et al., JGR Ocean, 20205. We'd like to stress that there is no consensus incubation time period for investigating methanotrophic community development.*

*We monitored O2 levels, which never dropped to less than 50% of the starting value (100% air saturation). This information has been added to table 1 and 2 in the supplements; biofilms were not apparent on the glass walls (though we did not specifically investigate this by eg microscopy). We did not measure nutrient levels (it seems however unlikely that the incubations run out of (micro)nutrients considering that the abundance of MOB increased to up 60% of all reads). We can, however, not exclude that the increase in MOB abundance was in expense of other microorganisms (ie., starvation and subsequent lysis of microbes other than MOBs may have supplied nutrients for MOB growth). We will add this information in the revised MS.*

As the authors have chosen to use 3 experimental factors, a full factorial analysis would include a full combination of all three factors, i.e. 3 x 4 x 3 = 36 combinations. A subsequent ANOVA could then state if these factors do have a significant influence on the MOB diversity. Therefore, the experimental part of the Ms should formulate clear hypothesis, which than can be accepter or not, such as: methane does have an influence on the diversity.

*Answer: We thank the reviewer for this suggestion and agree that testing whether experimental parameters have significant effects on microbial community composition is important. In our study, we used DESeq2, which is specifically designed for count-based community data and allows robust modelling of differential abundance across multifactorial experimental designs. DESeq2 tests the null hypothesis that changes of a given parameter (e.g., methane availability) do not lead to changes in microbial community structure. We have added explanations regarding this to the revised MS (section 2.4.3). The significant results (ie when a change in an experimental parameter led to a change in the community) were reported in the results in the original MS already. In contrast, a classical ANOVA would not be appropriate for our data, as it assumes normally distributed and homoscedastic residuals, which are not valid for microbial count data. Furthermore, ANOVA tests overall shifts and would not detect taxon-specific abundance changes that DESeq2 identifies. Therefore, our approach using DESeq2 directly addresses the reviewer's concern and provides a suitable and sensitive framework for analysing the influence of the experimental factors on MOB diversity.*

In addition, it could be shown (maybe with a heat map) at which combination a specific MOB group has a preference for which experimental combination.

*Answer: The DESeq2 analyses revealed that mostly CH4 availability led to changes in community composition. We have shown this in a heatmap already (supplementary figure 3).*

As it is now in the Ms there are certainly a lot of information, but the reader (or at leas I) remains confused about the presented results. Thus I recommend a separation of environmental and experimental results. For the experimental results, either each parameter or better combination of parameters should be described separately.

*Answer: We have already separated environmental and experimental results presenting firstly the results from microcosm incubations with North Sea and then the Wadden Sea inocula. We have further motivated this approach in the beginning of the results (section 3.1) and highlighted that section 3.2.1. and 3.3.1 deal with the diversity of MOB in microcosms with North Sea and Wadden Sea inocula. '3.4 Comparison with environmental data' encompasses the environmental data for the North and Wadden Sea – we think that the title was already clear. We would like to refrain, however, from changing the presentation of results and combining eg MOB community shifts in relation to CH4 modifications from the different environments as this would make representation of results rather confusing and lengthy.*

Reviewer 2

De Groot et al. have performed incubation experiments with samples they collected from several marine sources. They have sequenced samples of the endpoints of all of these incubations, and present that sequencing data in this paper. The incubations were provided with methane, and incubated under different temperature and salinity conditions.

Although it is nice that the authors put effort into incubations and sequencing, this paper in its current form unfortunately does not provide valuable insights to the scientific community. It reads more like a data collection description than a scientific paper. In this form, I think it would be highly useful in a repository, so that others can include this data into their research. This feeling is enhanced by the very short discussion section of the paper.

*Answer: We thank Reviewer 2 for the time and effort spent on providing feedback. While we agree that the manuscript contains a substantial amount of sequencing data and statistical analysis, leading to a longer Methods and Results section, we respectfully disagree with the assessment that the manuscript does not provide valuable insights to the scientific community. Firstly, it is currently not well understood how MOB communities in coastal marine systems respond to environmental changes such as variations in methane concentration, temperature, and salinity. Our results show that coastal ocean environments harbour a high diversity of MOBs, including types that typically remain below detection thresholds in classical NGS surveys, but proliferate under elevated methane conditions. Secondly, our findings strongly suggest functional redundancy within the MOB community, as no specific clade consistently increased or decreased in relative abundance in response to changes in the tested environmental parameters. Thirdly, our experiment allowed us to successfully show that the origin (environment, season) of the incubation inoculum shaped the MOB community (we made this clearer in the discussion and conclusion of the revised version of the MS). While we recognise that microcosm incubation experiments are inherently subjected to bottle effects, as discussed in the original manuscript, we believe that our results contribute important new insights into the ecology and resilience of marine MOBs under future ocean change scenarios.*

The setup of the experiments is also flawed for the conclusions drawn here. As stated in the methods, the incubations were stopped when methane levels were below 10% of initial concentration, and as a result the duration of the incubations varied. The communities are clearly not stable, but in a process of change from their initial composition to a community more adapted to the conditions they were placed in. However, if the incubations differ in duration, than one cannot simply compare them and then attribute the changes to the treatments. It is likely that certain community members are fast responders, but deminish later, or vice versa. Therefore, to compare communities, they need to be from incubations of the same duration.

*Answer: We thank the reviewer for this thoughtful comment. However, we respectfully disagree with the criticism. As the reviewer correctly points out, microbial communities require time to adapt to new conditions. However, this adaptation is driven by substrate consumption (in our case, methane), not simply by elapsed time. To meaningfully compare community responses, it is essential that the communities experience similar levels of carbon substrate depletion, rather than identical incubation durations. If we had used a uniform incubation time across all treatments, the incubations would have differed in the extent of methane consumption -*

*resulting in communities exposed to very different levels of carbon-substrate availability, which potentially introduces additional bias (ie some incubations might have run out of methane while others could have been methane replete). By monitoring methane concentrations and terminating incubations once methane dropped by 10% of the initial concentration (not to 10% of the initial concentration, this was explained wrongly in the original MS and has been corrected now, our apologies for the confusion), we ensured that each community experienced comparable degrees of methane-driven selective pressure without becoming carbon-limited. We have more specifically informed on the incubation times and CH4 concentration levels (supplementary tables 1 and 2).*

The figures are hardly refered to in the discussion. The NMDS figures are not mentioned at all in the discussion. They are also hardly mentioned in the results. Therefore, those figure do not add anything at the moment.

*Answer: We respectfully disagree with the assessment that the NMDS figures are not mentioned and do not contribute meaningfully to the manuscript. Two subsections focus on NMDS analysis in the results: In Section 3.4.1, we present the NMDS analysis for the entire bacterial community, and in Section 3.4.2, we present the NMDS analysis that focused specifically on the MOB community. Both analyses demonstrate the effect of sample origin on community structure. This is also discussed in Section 4.3 of the Discussion where we elaborate on the observed segregation between North Sea and Wadden Sea water column and sediment communities, and how initial community composition influences subsequent adaptation under experimental conditions. We have added more reference to the results (and figs/tabs) in the discussion to guide the reader. Specifically, we have clarified in the discussion (first paragraph of section 4.3), that the community changed in the incubations when compared to the original sample but that a clustering both on the MOB as well as whole-community level was visible in the NMDS.*

This paper feels to me as wrangling the maximum out of data. And although I encourage the reuse and the public availability of data, I do not think this paper is of interest to the readers of BG.

*Answer: We believe that every study should accept the challenge to extract the maximum scientific insight from its available data. While the bioinformatic analyses conducted here required detailed analyses and reporting, we did not perform unnecessary or speculative analyses. The combination of relative abundance profiling, DESeq2-based differential abundance testing, and NMDS ordination revealed statistically significant patterns in the structure and adaptation of MOBs, which would not have been detectable using a single method alone. Considering the paramount role of MOBs in mediating fluxes of the greenhouse gas CH4 across the geo-hydro-atmosphere continuum, we believe that the insights provided by this study are of relevance to the readership of BG, particularly in the context of environmental change and microbial ecosystem functioning. Our study clearly relies on a set of hypothesis/aims that we have made clearer in the revised version of the MS (end of introduction).*

Specific comments:

The non-continuous line numbering is unpractical.
*Answer: Changed*

Abstract

1. The first sentence of the abstract is hard to follow. I'd recommend changing it to make sure the readers are on board.

*Answer: We thank the reviewer for this comment. We have split the sentence and changed the phrasing to improve clarity.*

The result that methanotrophs increase in abundance when more methane is added, is presented as the key result in the abstract. This is very obvious, as it's the only microbe that is provided with improvement of their living conditions, whereas the other members of the community are not. Therefore I don't think this is really the result to highlight.

*Answer: We think it is important to firstly point out that the CH4 amendment led to a strong increase in the relative abundance of MOBs. The body of literature on effects of different CH4 concentrations on natural MOB communities is rather limited and we show that different genera increase in abundance at different concentrations. This highlights that the effect of CH4 availability on MOB community structure is not so predictable. We also observed similar effects of temperature on community composition in the number of genera affected and the size of the fold changes, but we did not see such an increase in the total of the relative abundances of the MOBs. Furthermore, in the literature, salinity has been suggested as a key parameter affecting MOB community composition, but our experiment could not underscore this. Finally, the abstract highlights additional key findings, emphasising the functional redundancy of MOB communities across seasons and locations, rather than focusing solely on $CH_4$ availability as an isolated driver.*

**Figures**

Using HS for headspace in a paper about marine settings is somewhat confusing, as it makes me think of hydrogen sulfide.

*Answer: we have changed HS to head space throughout the MS to avoid this confusion.*

Fig. 1

I'd choose to put % instead of relative abundance on the y axis. I am also not a big fan of this scaling. Abundances below 0.5% should not be reported in my opinion as 16S sequencing and PCR based methods are just not reliable enough for that. Therefore, the lower regions of the scale are not necessary anyway.

*Answer: We have revised the y-axis of Figure 1 and 2 to show percentages instead of relative abundances for clarity. We agree that sequence abundances in the sub-percent range should be interpreted with caution, due to stochastic variation in 16S rRNA gene amplicon sequencing and PCR amplification. However, though non-detection of a taxon may indeed reflect abundance below the detection limit, we carefully removed spurious counts from the samples based on the (few) counts observed in the negative controls such that we are confident that a positive detection - even at low relative abundance - provides meaningful ecological information. This is also commonly done in papers dealing with rare communities. We would thus like to refrain from introducing a 0.5% RA cutoff.*

I have not tried it but I think these colors don't print well in gray scale (probably cant be recognized anymore). It would also look better if the bars had a line between them so they don't

become a big blob. It is hard to understand what I need to take home from this figure. The bars are small, there is a lot of info, the labels don't tell me anything so I need to go back and forth to the legend.. I would recommend making this figure more attractive and perhaps move some information to the supplemental info that is not key information. Or split the figure into several figures to make it easier to highlight key findings.

*Answer: We thank the reviewer for the constructive feedback regarding figure clarity. Stacked bar plots, as used in Figures 1 and 2, are a widely accepted method to represent microbial community compositions. In these figures, each bar shows the overall MOB community abundance, while the stacked colours represent the relative abundances of different MOB genera. We agree that with 15 groups, interpretation in grayscale would be challenging, regardless of the specific colour or pattern scheme. We have therefore selected a colour palette with high contrast to maximize readability. We have also indicated in the figure caption that the figure is intended for colour viewing. We prefer not to add lines between the bars, as this would require additional vertical space and/or obscure smaller groups. Currently, biological triplicates are grouped closely together, and gaps between treatments help distinguish experimental conditions.*

Table 2.
Is the abundance in the autumn higher? Or the abudance after incubation of a sample taken in autumn? That should be more clear from either the table or the caption. What is base mean?

*Answer: The table caption already states that the changes refer to incubations started from inocula taken in autumn or summer. The base mean refers to the mean normalized count as provided by DESeq2. We have added this information to the legend of table 2-8 for easier reference.*

Table 2 + 3 + 4. I think this data could presented in a much more attractive way. The dry numbers are useful but don't need to be a table, they can be in the text or as a supplemental table.

*Answer: We thank the reviewer for this comment. We agree that the numerical results are important. However, presenting these data (Tab 2-8) within the main text would substantially complicate readability and increase text volume unnecessarily. Moreover, transferring these tables to the Supplementary Information would reduce the visibility of key findings, particularly regarding which MOB groups exhibited statistically significant differential abundance changes. We believe that retaining these tables in the main manuscript ensures optimal accessibility and transparency of the results.*

Figure 2. Same comments as to figure 1: hard to interpret, unattractive to look at.

*Answer: see above*

**Methods:**
P4L29: So the incubations were all different in duration? How can you then say that the differences in community composition were due to the conditions? It can also have been due to the duration of incubation, as the communities are clearly in a non-stable state. The exact duration should also be reported somewhere for each incubation.

*Answer: see above. We have added information on the duration of the incubations.*

**Discussion**

P16l9 I do not agree to this statement. It is presented as the main finding of the paper, but I think it is incorrect to say that you can deduct from your experiments that methane is the primary factor shaping microbial community composition. Because methane is the only redox-active element that you changed. The experiments with different temperatures and salinities change completely different factors. Therefore, making such statements based on these incubations is not possible.

*Answer: We thank the reviewer for the critical feedback and respectfully disagree with the assertion that it is incorrect to conclude that methane availability is the primary driver shaping MOB community composition under our experimental conditions, at least when considering the parameters tested here. The MOBs investigated in this study are (mostly) obligate methanotrophs, and thus methane is the relevant and central electron donor for their metabolism. Altering other electron donors would not have been biologically meaningful for this group. Temperature and salinity were tested under comparable methane availability conditions (5% headspace $CH_4$), while methane variation experiments were conducted under standardized temperature (25 °C) and salinity (30 PSU). Thus, the experimental setup allows direct comparison of the relative influence of these environmental parameters. Our DESeq2 differential abundance analysis revealed that temperature had similar effects on MOB community composition as CH4, while salinity induced changes were much less profound (see above, we clarified that further in the revised version of the MS). This finding is important as previous studies have suggested niche differentiation of MOBs along salinity gradients. Our conclusion is based on a controlled experimental design and statistically supported results, and provides new insights into the resilience and adaptability of coastal MOB communities.*

P16l11 This seems very obvious and not that surprising.

*Answer: See above; the effect of CH4 availability on MOB community structure is not so predictable.*

P16L18 Can play, not a given that they do.

*Answer: We don't fully understand what the reviewer means here; MOBs are the only methane consuming organisms in the oxic part of the ocean.*

P16l29 it would be interesting if you placed this into context of your incubations, whether you seem similar trends in the origin material versus the results of your incubations

*Answer: We are not sure what the reviewer's intentions are here. Seasonal effects are most strongly pronounced in the environmental dataset, but they are still present in the incubations showing the origin effect with respect to season. We have clarified this in the revised version of the MS (see comment above).*

P16L44 it would be useful to have your figures show these findings more clearly

*Answer: We think that this is very obvious from Fig 1 and 2. The different colours of the bars, representing the different MOB genera, show that different groups of MOB dominate different environmental conditions.*