Adaptation of methane oxidising bacteria to environmental changes: implications for coastal methane dynamics

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Abstract. Global warming induces alterations in ocean temperature regimes as well as precipitation patterns. This leads to shifts in water column properties, which will increasingly impact coastal ecosystems. These changes may have profound implications for microbial communities such as methane-oxidising bacteria (MOBs), which play a critical role in regulating methane fluxes and ecosystem dynamics. In this study, we investigate the resilience and adaptability of aerobic MOBs in response to changing environmental conditions. Microcosm incubations with waters from the North Sea and the Wadden Sea collected across different seasons, and adjusted for methane availability, temperature, and salinity were used to assess how these factors shape MOB community structure and functional capacity. Our results reveal an increase in the relative abundance of MOBs to up to 57% in experiments with elevated methane concentrations, highlighting the primary role of methane availability for MOB community development. Temperature and salinity variations, on the other hand, exerted lesser effects on MOB composition and relative abundance. A strong effect on MOB community development was furthermore caused by the origin of the inoculum (location and season). Our results thus suggest a functional redundancy in the variable pools of microbial inocula enabling multiple MOB clades to cope with drastic changes in environmental parameters. The adaptability of MOB communities is key to understand their role in mitigating methane emissions from coastal regions in a future ocean with potentially elevated methane, temperature and variable salinity levels.

1 Introduction

The impacts of global warming are anticipated to manifest in rising water temperatures and shifts in precipitation patterns, leading to fluctuations in salinity levels in the coastal ocean (Masson-Delmotte et al., 2021). Marine heatwaves are projected to increase in frequency, potentially exacerbated by factors such as ocean acidification and hypoxia (Collins et al., 2019). Elevated temperatures can accelerate methanogenesis in sediments, consequently increasing methane (CH_4) availability in both, oxic surface sediments and the water column. Once methane escapes to the atmosphere, it acts as a potent greenhouse gas (Etminan et al., 2016). While the majority of atmospheric methane originates from anthropogenic and natural, terrestrial sources (including inland aquatic systems), the coastal ocean is an important source of methane to the atmosphere, too (Weber et al., 2019; Saunois et al., 2024). Yet, methane serves as the primary carbon source for aerobic methane oxidising bacteria (MOBs; Hanson and Hanson, 1996), which constitute the final sink for methane before its release from the ocean to the atmosphere (Reeburgh, 2007; Steinle et al., 2015; Mao et al., 2022; de Groot et al., 2024). Alterations in methane availability thus substantially influence MOB community dynamics and their ability to retain methane in sediments and the water column in future ocean scenarios (Reeburgh, 2007; Crespo-Medina et al., 2014; Knief, 2015; de Groot et al., 2024).

Canonical aerobic methane oxidation (MOx), involves two main steps: methane is firstly converted into methanol by methane monooxygenases and then further oxidised to formaldehyde and finally to CO_2 either via the ribulose monophosphate (RuMP) or the Serine pathway (Hanson and Hanson, 1996; Reeburgh, 2007;):

$$CH_4 + 2\,O_2 \rightarrow CO_2 + 2\,H_2O$$

Two primary groups of MOBs were classified: the first are Type I and Type X MOB and consist of taxa from the phylum *Gammaproteobacteria* (Hanson and Hanson, 1996; Dedysh and Knief, 2018; ; Kalyuzhnaya et al., 2019; Kalyuzhnaya et al., 2019). The second group, Type II MOB, is composed of *Alphaproteobacteria* (Hanson and Hanson, 1996; Dedysh and Knief, 2018; ; Kalyuzhnaya et al., 2019; Haque et al., 2020). In addition, some members of the phylum *Verrucomicrobiota* (also referred to as Type III MOB; Dedysh and Knief, 2018) can mediate MOx (Pol et al., 2007). A distinct group, *Candidatus Methylomirabilis*, expresses the canonical MOx pathway but uniquely converts nitric oxide into oxygen and nitrogen before oxidising methane (Ettwig et al., 2009, 2010).

In marine ecosystems, the most important MOB are Type I MOBs, represented by taxa such as *Methylococcus* and *Methylomonas* are generally more dominant than Type II MOBs such as *Methylocystis* and *Methylosinus* (Hanson and Hanson, 1996; He et al., 2012; Knief, 2015; Steinle et al., 2016). The specific mechanisms driving the selection between Type I and Type II MOBs remains unclear. Nevertheless, it has been suggested that Type I MOB are better adapted to low methane levels, while Type II MOB may thrive in environments with high methane concentrations (Hanson and Hanson, 1996). This hypothesis is supported by recent environmental observations (Henckel et al., 2000; Macalady et al., 2002; Bodelier and Laanbroek, 2004; Kessler et al., 2011; He et al., 2012). However, this scenario likely applies primarily to environments already characterized by high methane concentrations. Knief and Dunfield (2005) found that Type II MOBs (belonging to the *Methylocystaceae*) demonstrated a higher potential for remaining active at low methane levels.

In addition to methane concentrations, the availability of copper and iron, essential components in the reaction centres of the soluble and particulate methane monooxygenase (sMMO and pMMO) respectively, may also influence enzyme expression, activity, and consequently the community structure of MOBs. For instance, under copper limitation, pMMO expression has been found to decrease compared to sMMO (Murrell, 2010).

Other bacterial strains have been classified as potential MOBs due to their close genetic resemblance to canonical MOBs and/or apparent MOx activity. However, their status as obligate or facultative MOB, or potential syntrophic associations with MOBs often remains ambiguous. For instance, a *Methyloceanibacter* strain express sMMO and was found to mediate MOx while other strains exhibit alternative metabolic behaviours (Vekeman et al., 2016). Also, some MOBs belonging to the family *Beijerinckiaceae* were found to metabolise C-compounds other than methane (Knief, 2015; Kox et al., 2019, Haque et al., 2020).

The detection and quantification of MOBs in marine environments are challenging due to their typically low abundance and the absence of reliable, MOB-specific molecular markers (Tavormina et al., 2011; Knief, 2015). For example, pMMO-primers were found to miss a substantial fraction of methanotrophs (Tavormina et al., 2011; Ghashghavi et al., 2017). Incubation-based methods, on the other hand, offer means for enriching MOBs, which

allows to identify potentially novel MOBs and to determine their ecological roles and metabolic capabilities (Ho et al., 2014; Ho et al., 2018; Li et al., 2021). However, inherent 'bottle effects' (Zobell, 1943, Herlemann et al., 2019) may result in the preferential selection of MOBs that are not representative of the original MOB population. Bottle effects arise from (slight) differences between incubation conditions and environmental parameters in situ, e.g. differences in macro (i.e., methane) and micronutrient levels (e.g. trace metals) as well as physicochemical parameters such as temperature and salinity (Ho et al., 2018; de Groot et al., 2023; Zhang et al., 2023). Also, (small) differences in the microbial community of the inoculate caused by stochastic processes during sampling and aliquoting can affect the abundance/presence/absence of rare organisms disproportionally adding to bottle effects. Nevertheless, such approaches hold great promises in identifying MOBs across ecosystems.

The future ocean will undoubtedly be impacted by climate change, but the potential of the ocean's microbiome for adaptation to changing methane dynamics remains unconstrained. In this study, we conducted in vitro microcosm experiments with coastal waters to investigate how MOB communities respond to variations in methane availability, temperature and salinity levels. We also aimed to assess whether these environmental factors or the origin of the microbial inoculum select for distinct communities, or whether environmental MOB communities exhibit functional redundancy.

2 Materials and methods

The composition of methanotrophic microbes was investigated in microcosms, which were inoculated with concentrated microbial biomass obtained from the water column. The North Sea inocula originated from two water depth and two seasons, while the Wadden Sea inocula originated from one water depth and 4 seasons. We adjusted physicochemical parameters (CH_4 availability and temperature in incubation setups with both, North Sea and Wadden Sea inocula, and salinity in Wadden Sea incubations only) and the microbial communities in the different incubations were compared to each other as well as to the microbial communities in the original environmental samples.

2.1 Site description and sampling

In the North Sea, particulate organic matter (POM) suspended in the water column was collected at depths of 10 m and 35 m during two research cruises with R/V Pelagia, conducted in summer and late autumn, respectively (de Groot et al, 2024). Samples were obtained every 4 hours using McLane in-situ pumps (WTS-LV) equipped with glass fibre filters (142 mm, 0.3 µm nominal mesh size, Advantec MFS), with approximately 100 L of seawater continuously filtered during each sampling event. A total of 24 environmental samples (12 from each depth) were collected in summer, and 22 samples (11 from each depth) were collected in autumn.

Similarly, POM suspended in the water column of the Wadden Sea was collected during cruises with R/V Navicula across different seasons: winter, spring, summer, and autumn (de Groot et al., 2023). Sampling was conducted every 4 hours at 1 m and 3 m depths, except during summer when POM was only collected from 1 m depth. Glass fibre filters (142 mm, 0.3 µm nominal mesh size, Advantec MFS) or polycarbonate filters (142 mm, 0.22 µm nominal pore size, Sterlitech) were used for filtration of 3 L sea water per sampling event. A total of 25 water column samples were collected in winter, spring, and autumn, while 12 samples were collected in summer.

Following filtration, filters were cut in halves. One half was stored at -80 ˚C for molecular analysis of the original microbial community (de Groot et al., 2023, 2024), while the other half was combined with local seawater in a 5 L canister to concentrate microbial biomass (referred to as 'filter soup'). The filter soups thus contained an enrichment of water column microbes: the concentration was roughly 1:100 for the North Sea and 1:10 for the Wadden Sea. The 'filter soup' was maintained at 4 °C without additional methane amendment and used for setting up lab incubations (see section 2.2).

During each Wadden Sea cruise, we also collected surface sediments with a box corer at the same site where we collected water column samples (de Groot et al., 2023). Four subsamples of the sediment surface were scraped off with sterile spatulas and frozen at -20 °C until DNA extraction (sediment subsamples were processed individually). Similarly, sediment samples were recovered from the North Sea (de Groot et al., 2024).

2.2 Lab incubations

The impact of methane levels, temperature, and salinity on the structure of MOB communities was investigated in microcosms with North Sea or Wadden Sea inoculae, which were established in triplicate for each parameter. For this, we removed the filter halves from the 'filter soup' canister and placed them on aluminium foil. Immediately, the filter surface (with remaining microbes) was scraped off with a flamed razor blade and the scraped off material was returned to the canister and the scraped off filters were discarded. After all filters were scraped off, the 'filter soup' was homogenized by stirring and shaking. Then, 60 mL of 'filter soup' was dispensed into 160 mL borosilicate glass bottles. These bottles were sealed with black butyl stoppers and crimp-top sealed before adjusting the headspace methane concentrations, salinity levels, and temperature. All bottles were kept standing during incubations and manipulations to avoid contact of the aqueous phase with the stoppers as this can have adverse effects on methanotrophs (Niemann et al., 2015).

For both, North Sea and Wadden Sea incubations, standard conditions consisted of 5 % methane in the headspace gas phase, a temperature of 25 °C, and in situ salinity levels for the North Sea (35 psu) or either 15 or 35 psu (according to season, see below) for the Wadden Sea incubations (Table 1). Variations from these standard conditions included incubations with methane concentrations of 0.1 % and 1 % in the headspace at 25 °C, and incubations at different temperatures (15 °C and 30 °C) with 5% headspace methane. In total, the number of incubation set-ups with 'filter soup' from the North Sea was 31 (summer) and 36 (autumn). For setups using Wadden Sea water, the total number of incubations were 24 each for winter, spring and summer and autumn.

The higher number of incubations for the Wadden Sea is due to the additional treatments applied, which were designed to explore the effects of different salinity levels (15 psu and 35 psu) at 25 °C. The Wadden Sea salinity can change rapidly due to evaporation, precipitation, and runoff (de Groot et al., 2023). Initially, the salinity of the Wadden Sea inoculum was measured and adjusted with Milli-Q water to establish baseline salinities of 30 psu for summer and spring samples, and 20 psu for winter and autumn samples, closely reflecting in situ salinity conditions (de Groot et al., 2023). Separately, filtered Wadden Sea water (0.3 µm nominal mesh size, Advantec MFS) was subjected to controlled evaporation by boiling on a heater plate until a salinity of 100 psu was reached. To achieve the desired final salinity levels of 15 and 35 psu in a total volume of 60 mL, we mixed specific volumes of brine, Milli-Q water, and inoculum as follows: For a final salinity of 35 psu in spring and summer incubations, 16.5 mL of 100 psu brine were firstly mixed with 28.5 mL of Milli-Q water, and then with 15 mL of the 30 psu inoculum. Similarly, for autumn and winter incubations, 18 mL of 100 psu brine, 27 mL of MilliQ water and 15 ml of the 20 psu inoculumn were mixed. For a final salinity of 15 psu in spring and summer incubations, 4.5 mL of 100 psu brine, 40.5 mL of milliQ and 15 ml of the 30 psu inoculumn were mixed, while for autun and winter, 6 mL of 100 psu brine, 39 mL of Milli-Q water, and 15 mL of 20 psu inoculum were mixed.

After all incubations were set up, triplicates of microcosms were amended with 5% methane and immediately terminated (t0) to serve as reference points for comparing the community composition at final time points (tn). Note that only single incubations were set up for North Sea summer t0. Headspace methane concentrations were monitored weekly within each incubation using gas chromatography with flame ionization detection as described previously (de Groot et al. 2023). Oxygen concentrations were monitored in one replicate of selected treatments using a PyroScience PICO-O2 meter and OXSP5-SUB sensor spots. O_2 concentrations never dropped below 53% saturation (supplementary Tables 1 & 2). Concentrations of other electron acceptors as well as nutrients were not measured. Once headspace methane concentrations were at least 10% lower than the initial methane level (typically after 25 to 84 days of incubations, supp. Tables 1 & 2), incubations were terminated by opening the vials and filtering the liquid phase over a glass fibre filter (25 mm, 0.3 µm nominal mesh size, Advantec MFS). The filter was then stored at -80 ˚C for microbial community analysis.

Table 1. Setup of microcosm incubations from North Sea (NS) and Wadden Sea (WS) samples. All microcosms were setup in triplicates. Note: standard conditions are defined as 25°C, 5 % headspace methane and salinity levels of 35 psu (NS). In the WS, baseline salinity levels were 20 psu for autumn (A) and Winter (W), and 30 psu for spring (Sp) and summer (Su). For NS incubations, salinity was not a variable parameter.

Microcosm	Location	Variable	T (˚C)	sal (psu)	CH_4 (%)
Standard	NS and WS	N/A	25	NS: 35; WS: 20 (A, W), 30 (Sp, Su)	5
1	NS and WS	T	15	NS: 35; WS: 20 (A, W), 30 (Sp, Su)	5
2	NS and WS	T	30	NS: 35; WS: 20 (A, W), 30 (Sp, Su)	5
3	NS and WS	CH_4	25	NS: 35; WS: 20 (A, W), 30 (Sp, Su)	1

4	NS and WS	CH_4	25	NS: 35; WS: 20 (A, W), 30 (Sp, Su)	0.1
5	WS	sal	25	15	5
6	WS	sal	25	35	5

2.2 DNA extraction and 16S rRNA gene amplicon library preparation

DNA was extracted from particulate organic matter collected on filters and sediment core tops using the DNeasy PowerSoil Pro Kit (Qiagen). DNA extracts were stored at -20 ˚C until further analysis. SSU V4-V5 region amplification was performed in triplicate in a mix of Phusion polymerase (0.25µL), 5X HF PCR buffer (5µL), BSA (20 mg mL^{-1}: 1 µL), the universal primer pair 515F (CCGYCAATTYMTTTRAGTTT; Parada et al., 2016) and 926R GTGYCAGCMGCCGCGGTAA; Quince et al., 2011) targeting the V4 and V5 hypervariable regions (concentrations of either primer was 10 µM, 1.5 µL added), dNTPs (2.5 µM; 2µL), DNA extract (1 µL) and PCR water (12.75µL). The PCR program consisted of 5 minutes initial DNA denaturation at 98 ˚C, followed by 26 cycles of 98 ˚C for 1 minute, 58 ˚C for 1 minute, 72˚C for 2 minutes, with a final elongation of 72 ˚C for 10 min; after completion samples were stored at 5 ˚C. The 16S rRNA products (~400 bp including unique Golay barcodes on forward and reverse primers) were gel purified and further library preparation, pooling and Illumina MiSeq 2 × 300 sequencing was done as described previously (Vaksmaa et al., 2021).

The newly obtained 16S rRNA gene amplicon sequences from incubation experiments and environmental samples, along with previously published environmental sequences from the North Sea and Wadden Sea cruises (ENA project PRJEB76184; de Groot et al., 2024), were analysed collectively using the NIOZ in-house pipeline Cascabel (Abdala Asbun et al., 2020). In short, prior to ASV identification, reads were truncated to 260bp and 200bp for forward and reverse reads, respectively. ASV designation was done using DADA2 v.1.19.1 (Callahan et al., 2016). Identification by consensus across samples was used to identify chimeras, and sequence variants identified as chimeric were removed. Taxonomies were assigned using DADA2's native implementation of the naïve Bayesian classifier method RDP using the Silva v138.1 release as reference database and a minimum bootstrap value of 50. MOBs were identified based on the taxonomic classifications as reviewed previously (Dedysh and Knief, 2018; Kalyuzhnaya et al., 2019; Haque et al., 2020; see Supplementary Table 1). That is, we considered all genera of the families *Methylomonadacea*, *Methylococcaceae* and *Methylohalobiaceae* and selected genera of the families *Beijerinckiaceae*, *Methyloligellaceae*, *Methylacidiphilaceae* and *Methylomirabilaceae* as MOBs.

2.4 Bioinformatics and statistical analysis

2.4.1 Preprocessing of microbial abundance data

ASVs that were taxonomically annotated to organelles, i.e., chloroplast or mitochondrial DNA were removed from the ASV table. ASVs without annotation at the Domain level, and ASVs annotated as Eukaryotic sequences, were also removed. After inspecting negative control samples, we subtracted two counts from each ASV in each sample to account for spurious counts caused by contamination. Singletons, that is ASVs with an abundance lower than 2, were excluded from further analyses. We further removed ASVs annotated as common skin microbiome genera: *Enhydrobacter*, *Cutibacterium*, *Staphylococcus*, *Pseudomonas*, *Acinetobacter*, *Corynebacterium*, *Listeria*, *Staphylococcus*, *Escherichia-Shigella*, and members of the Family *Neisseriaceae*. The final dataset consists of 27012 ASVs in 318 samples with a sequencing depth of 58690 (mean) or 41805 (median) counts per sample.

2.4.2 Nonparametric multidimensional scaling (NMDS)

ASVs read counts from incubation and environmental samples were first square root transformed and then standardized with double Wisconsin transformation. To reduce clustering artifacts caused by ASVs with excessive zero values, we included only ASVs that were present (non-zero values) in ≥ 5% of the samples, thereby minimizing the influence of rare or sporadically observed ASVs. A Bray-Curtis dissimilarity matrix was then calculated based on the transformed and filtered abundance data, quantifying the dissimilarities in microbial

community composition between samples ('vegdist' function from R package 'vegan'). NMDS analysis was performed on the Bray-Curtis dissimilarity matrix using the metaMDS function from the 'vegan' package version 2.6-6.1 (Dixon, 2003). The resulting plot (Figure 3) represents the samples as points in a two-dimensiona; coordinate space where the distances between points reflect the extent of dissimilarity in terms of bacterial community composition resembling true dissimilarities as closely as possible. A second NMDS was performed on a subset of ASVs classified as MOBs (Figure 4). For this analysis, we included only ASVs annotated at the genus level. We kept the relative abundances (Wisconsin-standardized counts) of MOB ASVs that were computed based on the whole community, so that the dissimilarity also takes into account the differences in the variability of the total relative abundance of MOBs in the microbial community across samples. The stress values of these two NMDS ranged between 0.09 and 0.16, suggesting that most of the Bray-Curtis dissimilarity could be represented in a two-dimensional ordination plot.

2.4.3 Differential abundance analysis

ASV counts were summed-up at the genus level for differential abundance analysis. ASVs that could not be classified at the genus level were clustered at the most specific taxonomic level available. Differential abundance analysis was then conducted using the 'DESeq2 ' package version 1.38.1 (Love et al., 2014) within R. After adding a pseudocount of 1, dispersions were estimated using the 'local' fit type. The generalised linear model (glm) included methane (0.1 %, 1 %, 5 %), season (WS: spring, summer, autumn, winter; NS: summer, autumn), temperature (15 ˚C, 25 ˚C, 30 ˚C) and salinity (WS only: 15, 20, 30 and 35 psu). Generalised linear models were computed and analysed separately for the Wadden Sea and North Sea to account for differences in the experimental design. Differential abundance across incubation conditions was tested with the Wald significance test and using the t-distribution as null model. 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 tested the different incubation settings against 0.1% as reference level for methane, 15 ºC for temperature, and summer for the seasons. Methane levels in t0 samples were set to 0.1% as this approximately reflects the in situ conditions. Log2 fold changes were moderated with the 'ashr' method (Stephens, 2016), and the False Discovery Rate (FDR) was used to adjust p-values for multiple testing. We then selected MOB genera and the family *Methylomonadaceae* (*Methylomonadaceae* ASVs without genus taxonomy assignment, see Supplementary Table 1) for downstream analyses. The Base Mean indicates the average abundance of a genus across all incubation samples including t0 samples. Further analyses focused on MOB with a base mean value of at least 10. Genera with an FDR (adjusted p-value) of less than 0.05 were considered to be significantly different in their abundance across conditions.

3. Results

3.1 General patterns in MOB diversity and abundance

The diversity and abundance of MOBs in incubations subjected to varying abiotic conditions (Figs. 1 & 2) were compared both among treatments and against the original environmental samples (Supplementary Figs. 1 & 2). MOBs were consistently detected in environmental seawater samples, except in the North Sea surface waters during summer (de Groot et al., 2024) and several time points during spring in the Wadden Sea. In total, we identified 8 MOB genera and additional members of the *Methylomonadaceae* and *Methylacidiphilaceaeae* that we could not assign to the genus level (Supplementary Figures 1 and 2). MOBs were also consistently observed at the beginning and end of all incubation experiments regardless of the origin of the inocula (i.e., water depth, season or sampling area) and incubation conditions (Figure 1 and 2). In total, we identified 12 different MOB genera in incubation experiments (all present in Wadden Sea and 8 present in the North Sea incubations) and additional members of the *Methylomonadaceae* that we could not assign to the genus level. However, substantial differences were observed in community composition and abundance of MOB genera. While this was in some cases consistent for distinct conditions, we also found notable variation between replicates in other instances (Figure 1, 2), as described below. Furthermore, we frequently detected high abundances of MOB groups at the end of the incubations that were below detection limit at t0.

3.2 North Sea

3.2.1 Diversity and abundance of methanotrophs in microcosms

Summer – Methanotrophs in the inoculum from the North Sea sampling (t0 incubations) from summer and 35m water depth were predominantly composed of Type I MOB of the *Methylomonas* genus with minor admixtures of *Marine Methylotrophic Group 2* (*MMG2*) (Figure 1). In these incubations, *MMG2* was the most dominant group independently of incubation conditions. Nevertheless, we also detected elevated levels of *Methyloprofundus* in incubations with higher methane levels (5% at 25 °C, 15 °C and 30 °C). MOB diversity was generally higher in incubations with a surface inoculum. In contrast to deep waters, *MMG2* was dominant in incubations carried out at higher methane levels (5%) but only at 15 °C and 30 °C. Surface incubations carried out at low methane levels (0.1%) were heterogenous and either dominated by *Methylomicrobium* or *Methylomarinum*, while incubations at 1% headspace methane levels where either dominated by *Methylomonas* or members of the *pltb-vmat-59* group. At higher methane levels (5%) and at 25 °C, samples were either dominated by *Methyloprofundus* or *Methylomonas*. *Methyloceanibacter* was the only (potential) type II MOB detected, more frequently in deep water samples, but generally at very low relative abundances of typically $<10^{-4}$, though this group was dominating the original water column samples (Supplementary Figure 1).

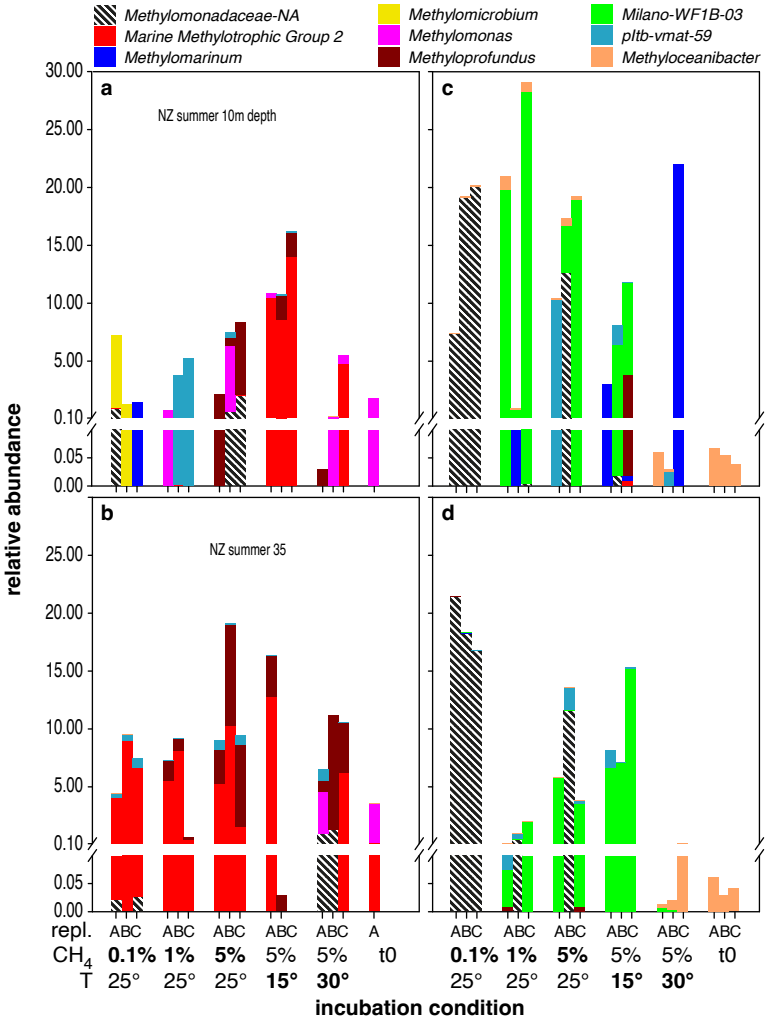

Figure 1. Distribution of MOB genera in microcosm incubations with North Sea inocula sampled during su mmer at depth of 10 m (a) and 35 m water depth (b) as well as during autumn at 10 m (c) and 35 m water depth (d). Each set of bars represents microcosm incubations conducted in triplicate (labelled A, B, C) under varying conditions as indicated on the x-axis: starting conditions (t0), methane head space concentrations (%) and temperature (˚C). Baseline conditions were set to methane

headspace concentrations at 5 % and temperature at 25 ˚C. Salinity was 35 psu in all incubations. All deviations in methane headspace concentrations or temperature are indicate in bold face type. Note that sequence abundances in the sub-percent range should be interpreted with caution and considered only in terms of presence/absence, due to stochastic variation inherent in 16S rRNA gene amplicon sequencing and PCR amplification. The figure is intended for colour viewing.

Autumn – The composition of the MOB community in microcosms with inocula from the autumn sampling campaign was notably different when compared to summer (Figure 1, Table 2). All t0 incubations only contained *Methyloceanibacter* as the sole potential MOB though its abundance was low ranging between 0.05 and 0.1%. Furthermore, we found high abundances of unassigned *Methylomonadaceae* that predominated incubations form both water depth with 0.1% methane headspace levels. Unassigned *Methylomonadaceae* also dominated single replicates in incubations with 5% methane headspace levels at 15 °C. However, the other replicates at these conditions were dominated by members of the *Milano-WF1B-03* group (35 m water depth) or *Milano-WF1B-03* together with *pltb-vmat-59* (10 m water depth). In incubations with deep water inocula, *Milano-WF1B-03* was also predominant in incubations with 5% methane headspace concentrations; at the surface, only two replicates of this incubation conditions were dominated by *Milano-WF1B-03*, while *Methylomarinum* and *Methyloprofundus* were important genera, too. *Methylomarinum* also dominated one replicate of incubations at 30 °C and 5% methane with surface water inocula, while the remaining two replicates were dominated by members of the *pltb-vmat-59* group or *Methyloceanibacter*. *Methyloceanibacter* was also dominant in incubations at 30 °C and 5% methane with a deep water inoculum. In general, abundances of unidentified *Methylomonadaceae*, *Methyloceanibacter* and *Milano-WF1B-03* were higher in autumn when compared to summer, while *MMG2*, *Methylomonas*, *Methyloprofundus* and *ptlb-vmat-59* were less abundant (Table 2)

Table 2. Differential abundance of MOB genera in incubations with North Sea inocula from autumn and summer samples. 'Standard Error (lfcSE)' denotes the standard error of the log2 fold change estimate. 'Adjusted p-values' indicate the False Discovery Rate. Log2 fold changes indicate abundance differences between incubations with inocula from autumn and summer. The base mean refers to the mean normalised count as provided by DESeq2.

Taxa	Base Mean	Log2 Fold Change	Standard Error (lfcSE)	Adjusted p-value
Abundance in autumn higher than in summer				
Methylomonadaceae-NA	794.3	2.68	0.74	< 0.001
Methyloceanibacter	32.1	3.1	0.47	< 0.001
Milano-WF1B-03	870.2	6.24	0.59	< 0.001
Abundance in autumn lower than in summer				
Marine Methylotrophic Group 2	692.6	-9.75	0.59	< 0.001
Methylomonas	109.1	-6.93	0.66	< 0.001
Methyloprofundus	375.2	-5.67	0.66	< 0.001
pItb-vmat-59	213.7	-2.94	0.67	< 0.001

3.2.2 Effects of methane concentrations and temperature on methanotroph communities in microcosms.

Methane – MOB variability within treatments was high across incubations with different methane levels. MOB abundances were typically higher at higher methane concentrations and incubations temperatures <30 °C (Figure 1, Table 3, Supplementary Figure 3). The lower MOB abundance in incubations at 30 °C was especially pronounced in incubations with an inoculum from 35 m water depth recovered in autumn (Figure 1). However, no distinct MOB group consistently dominated across all water depths and seasons at a specific methane headspace level.

Analysis of differential MOB abundance revealed significant changes in methanotroph populations in response to varying methane concentrations (Table 3). For instance, abundances of *pltb-vmat-59* and *Milano-WF1B-03* were substantially higher at both 1% and 5% when compared to 0.1% headspace methane levels. In contrast, the abundance of unassigned *Methylomonadaceae* was lower at 1% when compared to 0.1% headspace methane levels.

Table 3. Differential abundance of methanotrophs in incubations with North Sea inocula, comparing the effects of different headspace methane concentrations independent of the season. Log2 fold changes indicate abundance differences between incubations with varying methane levels. The base mean refers to the mean normalised count as provided by DESeq2.

Taxa Name	Base Mean	Log2 Fold Change	Standard Error (lfcSE)	Adjusted p-value
Abundance in 5% higher than 0.1% CH_4				
Methyloprofundus	375.2	3.86	0.94	< 0.001
pItb-vmat-59	213.7	6.02	0.96	< 0.001
Milano-WF1B-03	870.2	6.68	0.8	< 0.001
Abundance in 5% lower than 0.1% CH_4				
none				
Abundance in 1% higher than 0.1% CH_4				
Milano-WF1B-03	870.2	5.88	0.82	< 0.001
pItb-vmat-59	213.7	2.54	1.23	0.008
Abundance in 1% lower than 0.1% CH_4				
Methylomonadaceae-NA	794.3	-5.3	1.09	< 0.001

Temperature – Differential abundance analysis revealed that 4 distinct MOB genera were significantly affected by temperature (Table 4), however, the effect of temperature on MOBs was highly variable. MOBs generally exhibited lower abundances in incubations at 30 ˚C, particularly in samples collected in autumn, contrasting with the high abundances of MOBs in incubations at 15 ˚C and 25 ˚C using the same inoculum (Figure 1). For instance, *Methyloprofundus* was higher in abundance at 15 °C when compared to 25°C and 30°C (Table 4). In contrast, potential MOBs belonging to the (potential) Type II genus *Methyloceanibacter* showed generally higher abundances at higher incubation temperatures. However, no distinct MOB group consistently dominated across all water depths and seasons at a specific incubation temperature.

Table 4. Differential abundance of genera associated with methanotrophs due to different incubation temperatures. Incubations were started with microbial biomass from the North Sea seawater inoculum. Log2 fold changes indicate abundance differences between incubations at different temperatures. The base mean refers to the mean normalised count as provided by DESeq2.

Taxa Name	Base Mean	Log2 Fold Change	Standard Error (lfcSE)	Adjusted p-value
Abundance higher at 30 ˚C than at 15 ˚C				
Methyloceanibacter	32.1	2.29	0.89	0.009
Methylomonadaceae-NA	794.3	5.11	1.24	< 0.001
Abundance lower at 30 ˚C than at 15 ˚C				
Milano-WF1B-03	870.2	-4.99	0.93	< 0.001
pItb-vmat-59	213.7	-3.56	1.09	0.002
Methyloprofundus	375.2	-3.12	1.02	0.003
Abundance higher at 25 ˚C than at 15 ˚C				
Methyloceanibacter	32.1	3.55	0.77	< 0.001
Methylomonadaceae-NA	794.3	5.48	1.1	< 0.001
Abundance lower at 25 ˚C than at 15 ˚C				
Methyloprofundus	375.2	-2.23	1.11	0.02

3.3 Wadden Sea

3.3.1 Diversity and abundance of methanotrophs in microcosms

Spring – the MOB community in the spring inoculum was predominantly composed of the potential MOB genus *Methyloceanibacter* at the beginning of the incubation (t0). Independent of season, this pattern was found in all inocula from the Wadden Sea and *Methyloceanibacter* was also a dominant MOB group in the original water column and in sediment samples (Supplementary Figure 2). In contrast to the North Sea incubations, Wadden Sea spring incubations were more variable among replicates and contained a higher degree of unassigned *Methylomonadaceae*. Only incubations with 35 psu salinity (and 5% methane in the headspace, incubated at 25 °C) showed a high abundance of *Methylomarinum*. *Methylomarinum* was also dominant in one replicates of all other incubations with 5% methane in the headspace. Other dominant groups in at least one replicate were *IheB2-23*, *Methyloprofundus*, *Methylomicrobium*, *pltb-vmat-59*, *MMG2* and unassigned *Methylomonadaceae*.

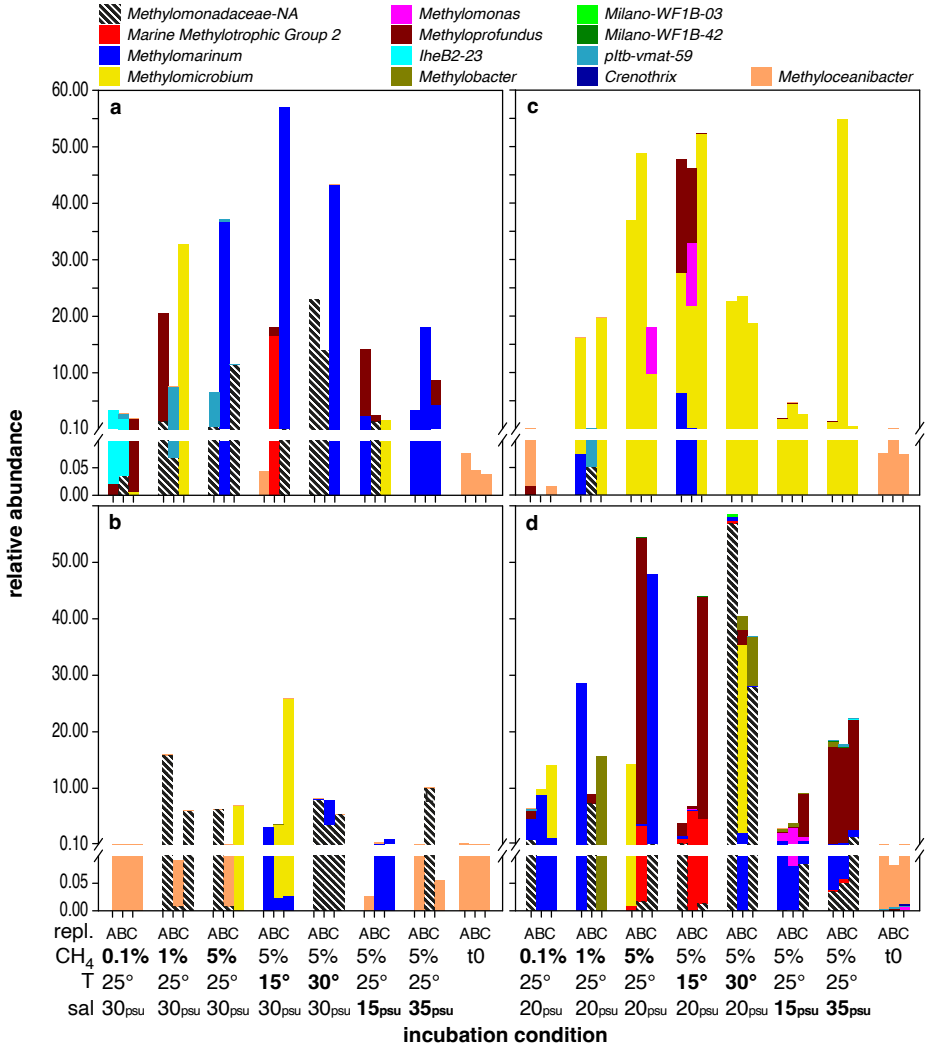

Figure 2. Distribution of MOB genera in microcosm incubations with Wadden Sea inocula sampled in spring (a), summer (b), autumn (c) and winter (d). Each set of bars represents microcosm incubations conducted in triplicate (labelled A, B, C) under varying conditions as indicated on the x-axis: starting conditions (t0), methane head space concentrations (%), temperature (˚C) and salinity (psu). Baseline conditions were 5% methane headspace concentrations, 25 ˚C and 30 psu (spring, summer) or 20 psu salinity (autumn, winter). All deviations in methane headspace concentrations, temperature or salinity are indicated in bold face type. Note that sequence abundances in the sub-percent range should be interpreted with caution and considered only in

terms of presence/absence, due to stochastic variation inherent in 16S rRNA gene amplicon sequencing and PCR amplification. The figure is intended for colour viewing.

Summer – in contrast to spring, summer incubations showed a higher degree of consistency between replicates. *Methyloceanibacter* did, in addition to t0, also dominate incubations with 0.1% methane in the headspace. *Methylomicrobium* dominated two replicates in incubations with 15 psu salinity and one in incubations with 5% methane in the headspace. *Methylomarinum* was dominant in 2 replicates in incubations with 15 psu salinity. Unassigned *Methylomonadaceae* comprised the majority of MOB in all replicates of incubations conducted at 30 °C, and dominated 1 or 2 replicates of incubations with 1% or 5 % methane in the headspace and those conducted at salinity levels of 35 psu.

Autumn – incubations from autumn were often dominated by *Methylomicrobium* and *Methylomarinum* or unassigned *Methylomonadaceae*. Similar to spring, incubations with 0.1% methane in the headspace were also dominated by *Methyloceanibacter* (though overall abundance was low with <0.001).

Winter – similar to spring, winter incubations were more variable among replicates and contained a higher degree of unassigned *Methylomonadaceae*. Only incubations with 35 psu salinity (and 5% methane in the headspace, incubated at 25°C) showed a dominance of *Methyloprofundus*. Other important MOB groups dominating 1 or 2 replicates were *Methylomarinum*, *Methylomicrobium*, *Methylobacter*, *MMG2* or *Methylomonas*.

Seasonal variations were further investigated by means of differential abundance analysis (Table 5). These highlighted significant increases of certain MOB genera in incubations with inocula from colder conditions (winter and spring) compared to summer, namely *Methyloprofundus* and *pltb-vmat-59*, while members of the *MMG2* and *Methylobacter* genera where in abundance only in spring higher when compared to summer. MOB abundances in winter incubations showed the strongest differences compared to summer incubations, displaying the highest number of more abundant MOB genera, and the highest log2 fold changes. Although autumn incubations were dominated by *Methylomicrobium*, its abundance was not significantly higher in autumn than in summer incubations. Only *Methyloprofundus* and *pltb-vmat-59* were significantly more abundant in autumn than in summer, but with lower log2 fold changes than between winter and summer. With the exception of *MMG2*, autumn incubations exhibited high abundances of the dominant genera found in both summer and winter incubations. Similarly, spring incubations showed microbial abundance profiles that were intermediate between winter and summer.

Table 5. Differential abundance of methanotrophs in incubations of Wadden Sea seawater inoculum generated during summer, autumn winter and spring. Log2 fold changes indicate abundance differences between incubations with inocula from spring, summer, autumn and winter. The base mean refers to the mean normalised count as provided by DESeq2.

Taxa Name	Base Mean	Log2 Fold Change	Standard Error (lfcSE)	Adjusted p-value
Abundance in winter higher than in summer				
Marine Methylotrophic Group 2	30.29	3.64	0.68	< 0.001
pItb-vmat-59	26.8	3.78	0.68	< 0.001
Methylobacter	68.8	6.79	0.72	< 0.001
Methyloprofundus	1007	9.01	1.0	< 0.001
Abundance in winter lower than in summer				
none				
Abundance in spring higher than in summer				
pItb-vmat-59	26.75	4.32	0.60	< 0.001
Methyloprofundus	1007	6.18	0.81	< 0.001
Abundance in spring lower than in summer				
Methyloceanibacter	19.5	-3.28	0.48	< 0.001
Methylomicrobium	919.5	-3.46	0.92	0.002
Abundance in autumn higher than in summer				
Methyloprofundus	1007	1.5	1.11	0.044
pItb-vmat-59	26.75	2.12	0.79	0.005
Abundance in autumn lower than in summer				
Methyloceanibacter	19.5	-3.52	0.54	< 0.001
Methylomonadaceae-NA	529.5	-5.36	0.81	< 0.001

3.2.1 Effects of methane concentrations, temperature and salinity on methanotroph communities

Methane - The relative abundance of MOBs roughly reflected methane concentrations during incubations, independent of season, though overall MOB abundances were lowest in summer incubations. The impact of elevated headspace methane concentrations (5%) had a more pronounced effect on MOB community structure, resulting in a broader range of MOBs exhibiting higher abundance compared to lower methane levels of 0.1% (Supplementary Figure 3). This included *pltb-vmat-59*, *MMG2*, *Methyloprofundus*, *Methylomicrobium* and *Methylomonas* (Table 6, Supplementary Figure 3). However, just as for North Sea incubations, no single MOB genus dominated incubations at specific headspace methane levels across all seasons (Figure 2).

Table 6. Differential abundance of methanotrophs in varying headspace methane concentrations in incubations inoculated with microbial biomass from the Wadden Sea. Log2 fold changes indicate abundance differences between incubations with varying methane levels. The base mean refers to the mean normalised count as provided by DESeq2.

Taxa Name	Base Mean	Log2 Fold Change	Standard Error (lfcSE)	Adjusted p-value
Abundance higher in 1% vs. 0.1% CH_4				
Methylomicrobium	919.5	6.68	1.19	< 0.001
Methylomonadaceae-NA	529.5	5.28	1.03	< 0.001
Abundance lower in 1% vs. 0.1% CH_4				
none				
Abundance higher in 5% vs. 0.1% CH_4				
pItb-vmat-59	26.8	1.01	0.62	0.044
Marine Methylotrophic Group	30.3	1.1	0.65	0.04
Methyloprofundus	1007	2.72	0.73	< 0.001
Methylomicrobium	919.5	3.25	0.83	< 0.001
Methylomonadaceae-NA	529.5	3.38	0.64	< 0.001
Methylomonas	83.8	3.48	0.64	< 0.001
Abundance lower in 5% vs. 0.1% CH_4				
none				

Temperature – Similar to the North Sea incubations, no distinct MOB group consistently dominated across all seasons at a specific incubation temperature. Differential abundance analysis (Table 7) showed that at 30 ˚C, compared to 15 ˚C, several MOB genera were less abundant, including *Methyloprofundus*, *MMG2*, *Methylomicrobium*, and *Methylomonas* Conversely, unassigned *Methylomonadaceae* showed a higher abundance at 30 ˚C than at 15 °C. At 25 ˚C, compared to 15 ˚C, we observed a similar pattern. The MOB genera *MMG2*, *Methyloprofundus*, and *Methylomicrobium* were less abundant at higher temperature.

Table 7. Differential abundance of methanotrophs due to incubation temperature, in incubations of Wadden Sea inoculum taken in four different seasons. Log2 fold changes indicate abundance differences between incubations at different temperatures. The base mean refers to the mean normalised count as provided by DESeq2.

Taxa Name	Base Mean	Log2 Fold Change	Standard Error (lfcSE)	Adjusted p-value
Abundance higher in 30 vs 15 degree				
Methylomonadaceae-NA	529.5	6.87	1.12	< 0.001
Abundance lower in 30 vs 15 degree				
Methyloprofundus	1007	-6.84	1.23	< 0.001

Marine Methylotrophic Group 2	30.3	-5.38	0.85	< 0.001
Methylomicrobium	919.5	-3.3	1.37	0.006
Methylomonas	83.75	-2.89	1.04	0.005
Abundance higher in 25 vs 15 degree				
Methylomonadaceae-NA	529.5	2.93	0.85	< 0.001
pItb-vmat-59	26.75	1.8	0.84	0.015
Abundance lower in 25 vs 15 degree				
Marine Methylotrophic Group 2	30.3	-4.62	0.64	< 0.001
Methyloprofundus	1007	-3.26	0.86	< 0.001
Methylomicrobium	919.5	-2.4	1.11	0.009

Salinity – Just as for methane concentrations and temperature, we did not find a distinct MOB group consistently dominating across all seasons at a specific salinity. However, MOB abundance was generally higher at baseline incubation conditions, i.e. 5% methane, 25°C and salinity levels of 30 psu (spring/summer) or 25 psu (autumn/winter), but typically lower at either higher or lower salinity. Any of the groups (or combination of groups) dominating incubations at lower or higher salinity also contributed substantially to the MOB community in other incubations. For example, *Methylomarinum* was predominant in all replicates with 35psu from spring but the group was also found in incubations with elevated and decreased temperature (i.e., 15°C and 30°C). Also, differential abundance analysis (Table 8) only identified two genera namely *pltb-vmat-59* and *Methylomonas* that were, respectively, positively and negatively affected by salinity.

Table 8. Differential abundance of methanotrophs due to differences in salinity, in incubations of Wadden Sea inoculum taken in four different seasons. Log2 fold changes indicate abundance differences between incubations at different salinity levles. The base mean refers to the mean normalised count as provided by DESeq2.

Taxa Name	Base Mean	Log2 Fold Change	Standard Error (lfcSE)	Adjusted p-value
Abundance higher when salinity is higher				
pItb-vmat-59	26.8	1.50	0.26	< 0.001
Abundance lower when salinity is higher				
Methylomonas	83.8	-2	0.29	< 0.001

3.4 Comparison with Environmental data

Environmental methanotrophs in the North Sea water column were predominantly composed of *Methyloceanibacter*, with minor contributions from *pltb-vmat-59* (Supplementary Figure 1). However, the relative abundance of reads classified as MOB was low (<0.03%); in fact, MOBs were not detected in surface waters during summer. In contrast, sediments contained a higher relative abundance of MOB reads (up to 4.5%) and shwed a greater MOB diversity, primarily dominated by *Methyloceanibacter* and *Methyloprofundus*.

In the Wadden Sea, environmental MOBs in the water column were also dominated by *Methyloceanibacter*, again at low relative abundances. Sediments from the Wadden Sea showed higher MOB abundances as well, with *Methyloceanibacter* as the dominant genus, and minor contributions from *pltb-vmat-59*, *Methylomarinum*, and the Marine Methylotrophic Group 2.

Most environmental sediment-derived MOBs from both the North Sea and the Wadden Sea were also found in the microcosm incubations (Figs. 1 & 2), with exceptions. For example, *Methylomicrobium* was not detected in North Sea sediments but appeared in microcosms, while *plw-20* was found in sediments but not detected in incubations.

Similarly, *Methylomicrobium* was below the detection limit in Wadden Sea sediments but became dominant in the corresponding microcosms.

3.4.1 Total bacterial community

Non-metric multidimensional scaling (NMDS) analysis showed that microbial communities from Wadden Sea and North Sea water column incubations were remarkably distinct (Fig. 3) and clearly segregated from environmental water column and sediment microbiomes published previously (de Groot et al., 2024). Notably, t0 incubations more closely resembled their environmental counterparts in the NMDS plot, than the remaining incubation samples (t0 samples mostly cluster in-between environmental and incubation samples).

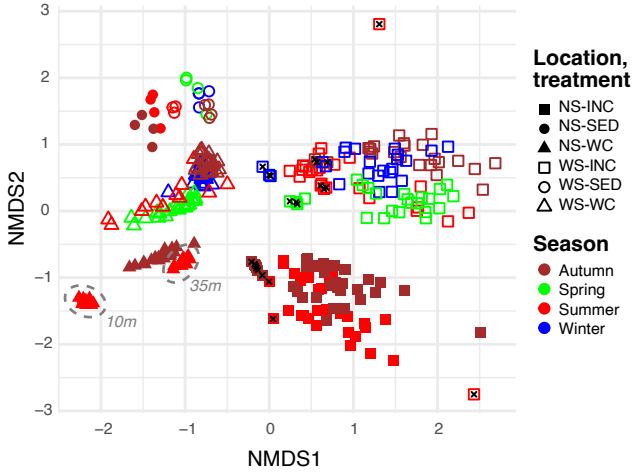

Figure 3. Non-metric multidimensional scaling (NMDS, stress = 0.16) plot of microbial taxa in environmental water column (WC) and sediment samples (SED), as well as incubated (INC) water column samples from the North Sea (NS) in summer and autumn, and Wadden Sea (WS) in winter, spring, summer, and autumn. *S*amples taken at the start of the incubations are indicated with a black cross and the water depth of environmental communities for summer is highlighted by a dashed grey line.

Sediments from both locations formed distinct clusters located on the side of the environmental water column samples considering the horizontal NMDS axis, and appearing more similar to the Wadden Sea than the North Sea samples on the vertical axis. The location of the sediment samples thus seems to have a smaller effect than the location of the water column samples. The environmental water column samples from the Wadden and North Sea furthermore clustered by season, and depth-related differences were apparent for the North Sea summer samples, too. While the NMDS analysis revealed slight seasonal differences for North Sea incubations, such differences were less apparent for Wadden Sea incubations.

3.4.2 MOB community variability

A second NMDS analysis focused solely on MOB genera, including only ASVs assigned to the genus level (Figure 4). Similar to the NMDS analysis of the total bacterial community, this MOB-focused NMDS revealed a segregation between North Sea and Wadden Sea incubations (horizontal axis), with seasonal differences particularly apparent for the North Sea and less pronounced for the Wadden Sea. The MOB community of all environmental samples (both water column and sediment) clustered tightly near the origin. The MOB community

in the t0 samples of our incubation experiments also clustered near the environmental samples at the origin of Figure 4, while incubation samples displayed variation from these by scattering further away from the origin.

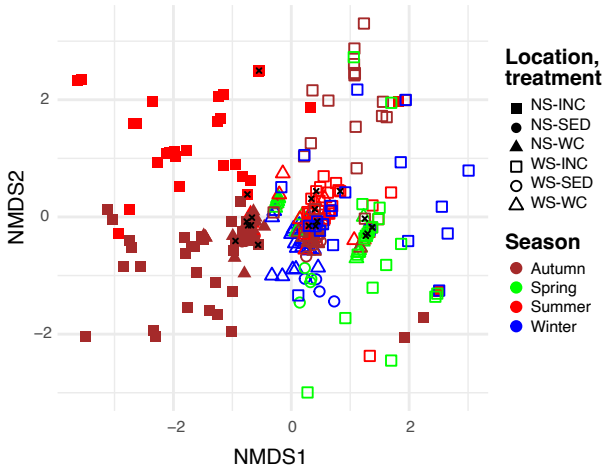

Figure 4. Non-metric multidimensional scaling (NMDS, stress = 0.09) plot of methane oxidising bacteria (MOB) in environmental water column (WC) and sediment samples (SED), as well as incubated (INC) water column samples from the North Sea (NS) in summer and autumn, and Wadden Sea (WS) in winter, spring, summer, and autumn. Samples taken at the start of the incubation are indicated with a black cross.

4 Discussion

Understanding how MOB communities adapt to environmental changes is crucial for predicting their role in coastal ecosystems amidst climate change. Our study explores the development of MOB communities in microcosms with inoculate from North Sea and Wadden Sea waters collected during different seasons and incubated at various methane concentrations, temperatures, and salinity levels. These factors are critical environmental variables influencing MOB community structure. We chose experimental conditions to investigate MOB communities in a future, likely warmer coastal ocean, ie., conditions reflecting rather drastic changes in water column temperature and CH_4 availability. In the area south of the Dogger Bank (North Sea), in situ temperature typically remain below 20°C in summer and around 5°C in winter (Greenwood et al., 2010; de Groot et al., 2024). Wadden Sea temperatures can exceed North Sea values, but generally stay below 25°C (de Groot et al., 2023). Methane headspace concentrations of 0.1%, 1%, and 5% (equivalent to ~1, 11, and 53 µM at 25°C and 30 psu) are on the high end compared to natural conditions, where in situ concentrations are typically <2 µM (North Sea) and <0.2 µM (Wadden Sea) at present (de Groot et al., 2023, 2024). Salinity was adjusted only in Wadden Sea incubations to reflect natural variability; for North Sea samples, salinity remained unchanged, as substantial shifts are unlikely in more offshore waters.

4.1 MOB community adaptation and incubation effects

Our investigations reveal a clear distinction between incubations from the North Sea and the Wadden Sea inoculates at both, the levels of the whole microbial community (Fig. 3) and within the MOB community (Fig. 4). This distinction is apparently independent of sampling time or how environmental parameters were experimentally changed during the incubation period. Additionally, we found a disparity in the apparent MOB community composition in environmental samples when compared to microcosms at t0. The original MOB community in environmental samples (water column and sediments) from the North Sea and Wadden Sea comprised predominantly *Methyloceanibacter*, with lesser contributions from *MMG2*, *Methyloprofundus*, *Crenothrix*, and *Methylomarinum* (Supplementary Figures 1 and 2). At t0, *Methyloceanibacter* was also present (except in the summertime North Sea inocula) but other canonical MOB genera such as *MMG2* and *Methylomonas* only contributed minimally to the MOB community (Figs. 1 & 2). Furthermore, *MMG2* and *Methylomonas* comprised

the majority of MOBs in incubations with North Sea waters collected during summertime, though *Methylomonas* was not detected in the original, environmental North Sea samples (Supplementary Figure 1). The apparent scarcity and observed diversity of canonical MOBs in the environmental samples and at the beginning of the incubation (t0) is likely related to the detection limit of the molecular methods used here, which was apparently not sufficient to capture rare microbial communities. Nevertheless, as the incubations progressed under manipulated environmental conditions, the initially undetectable MOB communities thrived and, in many cases, became the most abundant microbial groups (Supplementary figures 1, 2; Figs. 1 & 2). This successful enrichment of MOB communities from various ecosystems, including different seasons, depths, and locations, highlights the adaptability and resilience of these microorganisms to diverse environmental conditions. By subjecting natural environmental microbial communities to a range of different conditions such as variations in methane concentration, temperature, and salinity, we were able to observe how these parameters shape community composition, favouring microorganisms that are best suited to thrive under specific conditions.

Differences observed in the community composition among individual replicates under the same incubation conditions can be partially attributed to what is commonly referred to as the 'bottle effect' (Zobell, 1943; Massana et al., 2001; Calvo-Díaz et al., 2011). This phenomenon describes the variation between replicates of the same inoculum, resulting in divergent community compositions over time. The 'bottle effect' arises from micro-variations in in-vitro conditions and stochastic processes, leading to slight disparities in cell numbers and composition among replicates of the same inoculum. These subtle variations can substantially influence the growth rates of individual microorganisms. Moreover, stochastic effects play a crucial role in driving community development within microcosms (Haro-Moreno et al., 2019). This is evidenced by instances where three replicates yield three distinct communities, as observed in the case of MOB communities incubated at 15 °C with Wadden Sea winter inoculum (Figure 2). The exact relationship between such stochastic effects and the initial microbial community composition in each individual inoculum added to the bottles, as well as the extent to which slight variations in growth and death rates contribute to the observed results, remains speculative (Hammes et al., 2010, Kalenitchenko et al., 2021: Debray et al., 2022). Despite these challenges, our incubations successfully enriched natural MOB communities from various ecosystems.

4.2 MOBs in the environment and influence of environmental parameters

The versatility of MOBs across ecosystems underscores the wide range of conditions in which MOBs may perform methane oxidation thereby reducing methane liberation from various ecosystems to the atmosphere (Knief, 2015; Bodelier et al., 2019). While type I MOBs seemingly dominate aquatic environments, both type I and type II MOBs inhabit terrestrial systems. In these terrestrial environments, MOBs can exhibit biogeographic patterns that are influenced by abiotic factors, such as temperature, pH, and moisture levels (Knief, 2015; Bodelier et al., 2019). It seems logical that abiotic factors such as methane levels shape marine MOB community composition, too. For instance, *Milano-WF1B-03* is prevalent in temperate and Arctic marine environments, thriving in methane-rich habitats like cold seeps (Mau et al., 2020; Gründger et al., 2021; Sert et al., 2023; Broman et al., 2024). Our study corroborates this, with *Milano-WF1B-03* flourishing in microcosms with high methane concentrations and a North Sea inoculum (Figure 1). Similarly, *Methyloprofundus*, common in marine sediments (Tavormina et al., 2015), dominates at high methane concentrations but also seems to become more dominant at elevated temperatures (Gründger et al., 2021; Hirayama et al., 2022; Sert et al., 2023). Type I MOB genera like *Methylomicrobium* and *Methylomarinum* thrive across various regions globally, with *Methylomicrobium* particularly successful in high-salinity environments, as observed in Wadden Sea incubations in autumn (Jensen et al., 2008; Yu et al., 2018; Bodelier et al., 2019; Gründger et al., 2021). Similar to the findings of Knief and Dunfield (2005), our results of high abundances of Type I MOB at high headspace methane levels does not corroborate results from other systems where a dominance of Type II MOB is typically associated with high methane levels (Henckel et al., 2000; Macalady et al., 2002; Bodelier and Laanbroek, 2004; Kessler et al., 2011; He et al., 2012, Steinle et al., 2016). However, we cannot rule out that our incubations might have become depleted in copper or other essential (micro) nutrients for Type II MOB (Murrell, 2010).

Our study underscores the influence of environmental parameters on MOB community dynamics, with methane availability emerging as a primary factor shaping microbial community composition (Hanson and Hanson, 1996; Sherry et al., 2016; Li et al., 2021; Yao et al., 2022). While temperature and salinity surprisingly exert less apparent

effects, higher methane concentrations led to more diverse and abundant MOB communities (Mau et al., 2013; James et al., 2016; Sherry et al., 2016; Li et al., 2019). Notably, no single MOB group dominated our incubations with high methane conditions, as evidenced by contrasting community compositions in different seasons and locations. For example, in incubations with 5% methane, the MOB community was dominated by *Methyloprofundus* and *MMG2* in the North Sea in summer, while *Milano-WF1B-03* dominated North Sea autumn incubations which is contrasted by a *Methylomicrobium* dominance in the Wadden Sea incubations in autumn. Despite being numerically rare when compared to other microbes, active methane oxidising strains within MOB communities play a crucial role in overall methane consumption (Bodelier et al., 2013). These findings underscore the importance of microbial diversity and traits in regulating global methane budgets and ecosystem functions, emphasizing the need for further research in this field.

4.3 The origin effect

MOB community composition in incubation samples were strongly dependent on the season in which they were taken. For example, the North Sea summer incubations were dominated by *Methyloprofundus* and *MMG2,* while the autumn incubations were dominated by *Milano-WF1B-03* and other unassigned *Methylomonadacea* (Fig. 1). All known genera of the *Methylomonadaceae* are MOB, hence it seems not unlikely that the unidentified *Methylomonadaceae* comprises (potentially novel) MOB, too. Though we found some variations when comparing surface and bottom waters, the seasonal effect on these incubations was more pronounced. Also, the Wadden Sea incubations showed a clustering of samples by season (rather than with respect to the manipulated conditions; Fig. 2); however, these samples were more variable and clusters of samples from different seasons overlapped, while they were more distinct and overlapped only slightly for the North Sea. While our results show that the microbial community in the incubations with adjusted abiotic factors (CH_4 availability, temperature, and salinity) changed during incubation or when compared to the original environmental sample (both for the entire microbial community and for MOBs alone), we still observed clustering of communities linked to the origin of the inoculum. This clustering was associated with geographic location, water column vs. sediment source, and season, and was visible in both the MOB fraction and the overall microbial community (Figs. 3 & 4).

The composition of the initial inoculum and the environmental conditions to which microbes in the inoculum were exposed apparently exert a profound influence on community development. This origin effect is seemingly influenced by factors such as geographic location and seasonal variations, which coincide with important gradients in environmental conditions (see previous section). Speculating on the true MOB community composition in the collected inocula is challenging due to the limitations of detecting rare community members with DNAsequence-based approaches resulting from e.g. sampling bias, stochastic effects during sample processing and sequencing depth (Leray and Knowlton, 2017; Shirazi et al., 2021). The prevalence of Methyloceanibacter as the dominant genus in our environmental samples and at t0 suggests their potential survival, even if undetected by sequencing (Takeuchi et al., 2019, 2021). Despite relatively uniform MOB community patterns observed across origins (environmental water column), the persistent differences within location and season indicate likely distinct initial MOB communities. Our incubations indicate functional redundancy within the variable pool of MOBs, enabling multiple members to cope with drastic changes in environmental parameters such as methane availability, temperature, and salinity. Stochastic effects and historical contingencies may, in addition to abiotic effect and the origin of a sample determine which individual member of a functionally redundant group dominates the community after a certain incubation period (Kalenitchenko et al., 2021; Ramond et al., 2024).

Summary and conclusions

In this study, we investigated shifts in the community composition of MOBs in controlled microcosm experiments using water column inocula collected from the North Sea and Wadden Sea during different seasons. Our findings reveal distinct differences primarily driven by the origin of the inoculant, particularly evident in experiments with methane amendments. Interestingly, variations in temperature and salinity exerted a lesser influence on MOB community structure compared to methane availability. These results highlight the remarkable functional redundancy within the MOB microbiome of the Coastal Ocean, contributing to its resilience and adaptability to

environmental changes. Our study suggests that multiple MOBs possess the capacity to occupy ecological niches characterized by high methane levels, elevated temperatures, and variable salinity levels, providing insights into potential future scenarios in ocean ecosystems.

*Data availability***.** All data and scripts as well as the parameter settings of the Cascabel pipeline will be archived and made publicly available in the DAS database (see supplementary information for details). Sequence data for this study have been deposited in the European Nucleotide Archive (ENA) under project numbers PRJEBxxx (incubation experiments, will be made available upon acceptance of the MS) and PRJEB76184 (environmental sequences from the North Sea and Wadden Sea expeditions).

Supplement. The supplementary information related to this article is available online at: https://doi.org/xxxx

Acknowledgments. We are grateful for the support provided by the captains and crews of R/V Pelagia and R/V Navicula. We extend further gratitude to the technical team of the molecular ecology laboratories at NIOZ – Maartje Brouwer, Sanne Vreugdenhil, Harry Witte, Wietse Reitsma, and Alejandro Abdala Asbun - for their instrumental support, which was vital to the success of this study.

Author Contributions. This study was conceptualized and designed by TRdG and HN. Incubation experiments were conducted by TRdG and JD. Bioinformatics analyses were performed by TRdG, PR, JCE, JvB, and HN. The research was supervised by JCE and HN. All authors contributed to writing and revising the manuscript.

Competing interests. Helge Niemann is a member of the editorial board of the journal Biogeosciences.

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