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RESEARCH ARTICLE

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Versatile methanotrophs form an active methane biofilter in the oxycline of a seasonally stratified coastal basin

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Abstract

The potential and drivers of microbial methane removal in the water column of seasonally stratified coastal ecosystems and the importance of the methanotrophic community composition for ecosystem functioning are not well explored. Here, we combined depth profiles of oxygen and methane with 16S rRNA gene amplicon sequencing, metagenomics and methane oxidation rates at discrete depths in a stratified coastal marine system (Lake Grevelingen, The Netherlands). Three amplicon sequence variants (ASVs) belonging to different genera of aerobic *Methylomonadaceae* and the corresponding three methanotrophic metagenome-assembled genomes (MOB-MAGs) were retrieved by 16S rRNA sequencing and metagenomic analysis, respectively. The abundances of the different methanotrophic ASVs and MOB-MAGs peaked at different depths along the methane oxygen counter-gradient and the MOB-MAGs show a quite diverse genomic potential regarding oxygen metabolism, partial denitrification and sulphur metabolism. Moreover, potential aerobic methane oxidation rates indicated high methanotrophic activity throughout the methane oxygen counter-gradient, even at depths with low in situ methane or oxygen concentration. This suggests that niche-partitioning with high genomic versatility of the present *Methylomonadaceae* might contribute to the functional resilience of the methanotrophic community and ultimately the efficiency of methane removal in the stratified water column of a marine basin.

INTRODUCTION

Coastal and estuarine-shelf ecosystems contribute up to 75% of marine methane fluxes to the atmosphere (Dean et al., 2018; Rosentreter et al., 2021; Upstill-Goddard & Barnes, 2016). Currently, coastal ecosystems are facing warming, enhanced eutrophication, increased stratification and expansion of hypoxic or anoxic zones (Breitburg et al., 2018; Seidel et al., 2022). This will likely alter methane cycling and possibly result in larger methane emissions to the atmosphere. The quantification of methane emissions from these ecosystems remains challenging due to the dynamic nature of the occurring biogeochemical processes (Rosentreter et al., 2021; Weber et al., 2019). Consequently, there is a large uncertainty in estimates of global marine methane emissions (4–27 Tg of CH_4 per year) (Rosentreter et al., 2021; Saunois et al., 2020; Weber et al., 2019).

Environmental methane emissions are a result of an imbalance between methane production and

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methane removal. In coastal ecosystems, methane is mostly produced in the anoxic sediment by methanogenic archaea. Methane can also be produced in the oxic zone, for example, from methyl-phosphonate by a variety of microorganisms, but their contribution is not yet fully quantified (Liu et al., 2022). Up to 90% of this methane is estimated to be removed by archaeal methanotrophs in a syntrophic relationship with sulphate-reducing bacteria in the sulphate–methane transition zone or coupled to various other electron acceptors (Knittel & Boetius, 2009; Wallenius et al., 2021). The efficiency of this so-called methane-oxidation filter in the sediment can vary over the season and may lead to high benthic CH_4 fluxes (Egger et al., 2016) and elevated methane concentrations in the water column of hypoxic, stratified coastal waters (Dalcin Martins et al., 2022; Myllykangas et al., 2020; Steinle et al., 2015). If methane is diffusing from the sediment to the bottom water, an active microbial biofilter in the water column can further mitigate methane emissions to the atmosphere, as demonstrated for other coastal ecosystems (Matoušů et al., 2017; Pack et al., 2015; Steinsdóttir et al., 2022).

To improve environmental policy-making and predictions of future methane emissions, the understanding of the microbial methane dynamics in the water column is crucial. The activity and distribution of methanotrophs in the water column are influenced by factors such as oxygen availability, methane concentrations, salinity, temperature and hydrodynamics (Madigan et al., 2006; Nijman et al., 2021; Steinle et al., 2015). The efficiency and drivers of this methane filter, however, are less well-understood in the water column of coastal systems.

Aerobic methanotrophic bacteria (MOB) are phylogenetically distributed among *Alphaproteobacteria*, *Gammaproteobacteria*, *Verrucomicrobia* and the NC10 phylum (Ettwig et al., 2010; Hanson & Hanson, 1996; Op den Camp et al., 2009; Semrau et al., 2010). As a result of the phylogenetic and metabolic diversity (Kits et al., 2015; Hirayama et al., 2022; Schmitz et al., 2022), different groups of methanotrophs inhabit distinctive niches in the environment. While *Verrucomicrobia* MOB seem to inhabit extreme environments such as low pH ecosystems (Dunfield et al., 2007; Pol et al., 2007), alpha-MOB are mostly found under oligotrophic conditions (Ho et al., 2013; Kaupper et al., 2020). Gamma-MOB seem to be less resilient to disturbances and are found where methane and other nutrients are not limiting (Ho et al., 2013). When methane, oxygen and nutrient availability in the water column varies with depth during summer stratification, this niche specification within the methanotrophic community may foster efficient methane removal in the water column (Mayr, Zimmermann, Guggenheim, et al., 2020). However, we have little insight into the structure of the methanotrophic community in the water column of coastal waters during summer stratification and hypoxia. By unravelling the biogeochemical niches of the present methanotrophic community, we can improve our understanding of what drives methanotrophic activity in situ and evaluate the efficiency and stability of the water column microbial methane filter.

Here, we study the role of aerobic methane oxidation in the water column of Marine Lake Grevelingen (NL) during water column stratification in late summer. We present water column profiles of methane and oxygen paired with methane oxidation assays and test the effect of oxygen concentration on methane oxidation potential using batch incubations. In addition, we use 16S rRNA gene sequencing and metagenomics to explore both microbial community composition and the metabolic potential of the methanotrophic communities, respectively.

EXPERIMENTAL PROCEDURES

Fieldwork location and sampling methodologies

Lake Grevelingen is a former estuary in the southwest Netherlands. The lake has a surface area of 115 km^2 with an average water depth of 5.1 m. Here, we sampled Scharendijke basin, which is part of a former tidal channel and has a depth of 45 m. A more detailed description of the system and the study site can be found elsewhere (Egger et al., 2016; Żygadłowska et al., 2023). Lake Grevelingen was chosen to investigate microbial methane cycling as previous studies indicated that methanogenesis exceeded methane oxidation in the sediment during summer stratification, resulting in a periodic release of methane into the water column.

We took water column samples at the deepest point in Scharendijke basin (51.742°N; 3.849°E, 45 mbs; Figure 1), in September 2020 during a two-day sampling campaign with RV Navicula.

To determine the extent of stratification of the water column in September 2020, we measured salinity, temperature and depth with a CTD unit (SBE 911 plus, Sea-Bird Electronics Bellevue [WA], USA). The oxygen distribution was simultaneously recorded by a seabird sensor. Water samples were taken at 14 depths with an 8 L Niskin bottle. The distribution of the sampling depths is depicted in (Table S1).

For DNA samples, we filled two sterile 1 L plastic bottles with unfiltered water from each depth and stored them at 4°C until further processing (within 24 h).

Samples for methane concentrations were taken by filling 120 mL borosilicate serum bottles (duplicates) directly via gas-tight tubing. To prevent air contamination,

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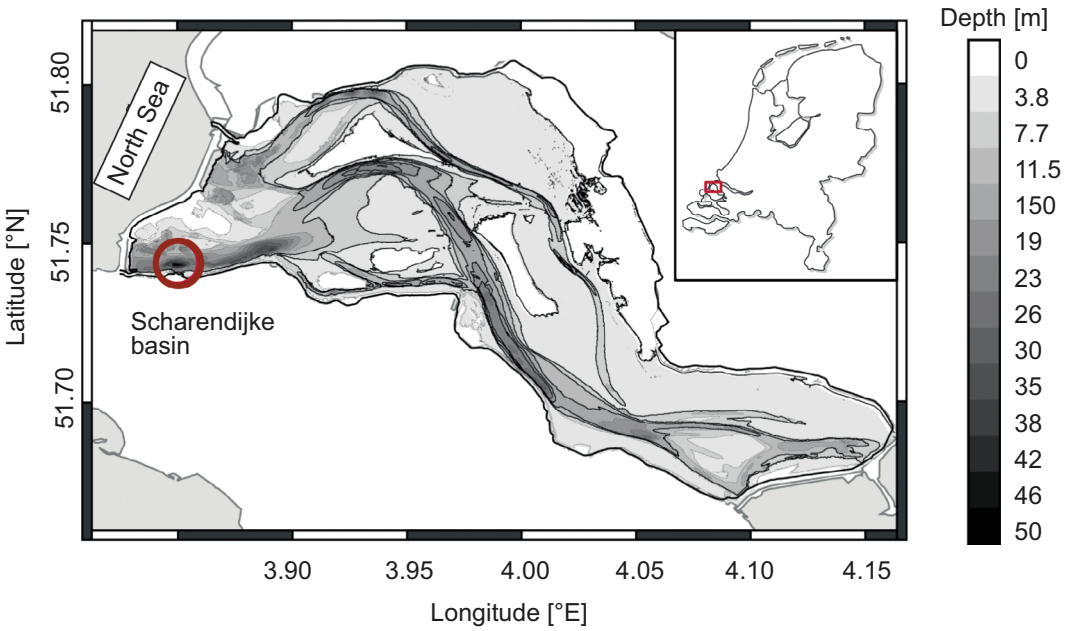

FIGURE 1 Map of marine Lake Grevelingen modified from Egger et al. (2016). Sample location Scharendijke (circle) (51.742 N; 3.849 E).

the bottle was filled up from the bottom while letting the water overflow three times. Then the bottle was crimp-capped with a butyl stopper and aluminium cap and stored upside down to avoid air contamination during storage. Subsequently, 1 mL of anoxic 50% $ZnCl_2$ solution (w:v) was added to stop microbial activity, and samples were stored at room temperature until further processing.

To test the activity and the response to different oxygen concentrations of the MOB community, we took four additional samples along the oxycline at 20, 32, 35 and 42 m depth. Samples were filled into sterile 1 L borosilicate Schott bottles and stored at 4°C in the dark.

Methane concentration measurements

To determine methane concentrations in the water column, 5 mL of nitrogen gas was added to all the methane samples simultaneously removing the same volume of the sample. After equilibrating for at least 2 h methane concentrations were measured with a Thermo Finnigan Trace™ gas chromatograph (Flame Ionisation Detector).

DNA extraction, 16S rRNA gene sequencing and data analysis

DNA from the water column samples was obtained by filtering between 0.5 and 1 L of water on Supor® PES 0.22 μm filters using a vacuum pump, within a day after sampling. The filters were then stored at −80°C until further use. DNA was extracted following the protocol of the DNeasy Power water DNA Isolation kit (Qiagen, Venlo, the Netherlands).

The composition of the microbial community was analysed, by sequencing the V3–V4 region of the 16S rRNA gene (Illumina MiSeq platform, Macrogen, Amsterdam, the Netherlands), using the primer pairs Bac341F (CCTACGGGNGGCWGCAG; Herlemann et al., 2011) with Bac806R (GGACTACHVGGGTWTCTAAT; Caporaso et al., 2012) for bacteria, and Arch349F (GYGCASCAGKCGMGAAW) with Arch806R (GGACTACVSGGGTATCTAAT; Ken & Koki, 2000) for archaea (Martin & Rahmann, 2012). RStudio was used to process 16S rRNA sequencing data. First, primers were removed with cutadapt (Martin & Rahmann, 2012) using the options −g, −G and –discard-untrimmed. Reads were truncated to a length of nt 270 forward nt 260 reverse by following the DADA2 pipeline (Callahan et al., 2017) and low-quality reads were removed. Sequences were dereplicated after error 252 models were built. Amplicon sequence variants (ASVs) were then inferred and forward and reverse reads were merged, and chimaeras were removed. The 254 Silva non-redundant train set v138 downloaded from https://zenodo.org/record/3731176#.XoV8D4gzZaQ was used for taxonomic assignment. Phyloseq was then used for further clustering and calculation of relative abundance. Plots were made with ggplot2. Raw reads of the 16S amplicon sequencing data can be accessed on the European Nucleotide Archive under the accession number PRJEB57287 (https://www.ebi.ac.uk/ena/browser/view/PRJEB57287).

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Metagenome sequencing, genome binning and sequence analysis

For deeper insight into the metabolic potential of MOB across the oxycline in the water column of Lake Grevelingen, samples from 25, 32, 35 and 42 m (congruent with the samples used for the incubations) were sent for full metagenome sequencing (TruSeq Nano DNA [350] kit, Novaseq platform, Macrogen, Amsterdam, the Netherlands). The metagenomic data were analysed with an in-house automated pipeline 'BinMate'. FASTQC was used to assess the quality of the reads and trimming of adapters and low-quality bases were performed with BBTools. Reads were assembled into contigs with megahit. The coverage of each contig was obtained by mapping the reads back to contigs with bbmap. For optimised binning results, BinMate used CONCOCT, MaxBin2 and MetaBAT to perform metagenomic binning. Bins were then dereplicated with DASTool. Final quality checks were performed with CheckM. Medium quality was defined as >50% completeness and <10% contamination. Bins were classified taxonomically with gtdb-tk and annotated with DRAM. Raw reads of the metagenome sequencing data can be accessed on the European Nucleotide Archive under the accession number PRJEB57287 (https://www.ebi.ac.uk/ena/browser/view/PRJEB57287).

Potential methane oxidation rates and oxygen manipulation experiments

To investigate the potential activity of methanotrophs and the effect of oxygen on the efficiency of methane removal in the water column of Lake Grevelingen, we conducted methane oxidation experiments in batch incubations. Within 24 h after sampling, 20 mL of each water sample was transferred into autoclaved 120 mL borosilicate serum bottles, closed with Bromo-butyl stoppers, and crimped with an aluminium cap. To remove the ambient gases, the bottles were purged with argon gas for 15 min.

Each of the four depths was provided with four different oxygen saturations of 50%, 25%, 10% and 5% resulting in a dissolved oxygen concentration of 100, 50, 20 and 10 μM. The total amount of oxygen available 20 mL water incubated was 350, 175, 70 and 35 μmols. To track methane oxidation, 1% of headspace volume ^{13}C-CH_4 (99% ^{13}C-CH_4 and 1% ^{12}C-CH_4 gas mix) was provided to give a methane concentration of 12 μM dissolved and a total of 40 μmols of CH_4 available.

To follow the oxidation of labelled methane, the increase of produced ^{13}C-CO_2 was measured directly from the headspace with a GC–MS (Agilent 5975C inert MSD). In addition, we measured methane headspace concentrations with a GC-FID (see Figure S2). The total concentrations of ^{13}C-CO_2 and ^{13}C-CH_4 in the incubation bottles were calculated using Henry's law (Methods S1). The rates were then calculated using linear regression of the first linear increase in total ^{13}C-CO_2 in the incubation bottle after the lag phase (Table S5). The rates were then calculated using linear regression of the first linear increase in total ^{13}C-CO_2 after the lag phase (Table S5). Rates were calculated from total μmol ^{13}C-CO_2 in the incubation bottles (CO_2 headspace + CO^*_2liquid). CO^*_2 (total DIC) was calculated accounting for dissociation of CO_2 into HCO_3- and H_2CO_3 at a pH of 8, salinity of 31 and temperature of 21°C (Methods S1, equation 4a and b, see Data S1 for all equations and assumptions).

Statistical analysis

Statistical analyses to test the effect of sampling depth and initial headspace oxygen concentrations on methane oxidation rates were performed in Rstudio with the following packages tidyverse, ggpubr and rstatix.

We used independent two-way ANOVA. The assumption of normal distribution was tested with the Shapiro–Wilk normality test after log transformation. All data points were normally distributed ($p > 0.05$) except for 35 m 1% ($p = 0.016$) and 25 m 2% ($p = 0.083$). However, we did not find any extreme outliers using the identify_outliers() function. Therefore, we decided to not exclude any rates from the biological replicates and considered them as true variations. Moreover, there was homogenous variance in different groups (Levene's-Test $p = 0.728$).

After a significant two-way effect (anova.test(), $p < 0.05$) was found and a simple pairwise comparison was performed as post hoc test (emmeans_test()).

RESULTS

Water column chemistry

The methane and oxygen concentrations in the water column of Lake Grevelingen were measured in September 2020. The methane and oxygen profiles showed a clear counter gradient (Figure 2A). As the oxygen concentrations remained at around 7 μM below 35 m, we assume that this is rather a common background signal problem of the Seabird oxygen sensor (SBE43) than a real signal. Therefore, oxygen concentrations below 7 μM were considered anoxic. In the anoxic bottom water methane concentrations were high (73 μM). A narrow oxycline was observed between 32 and 35 m with oxygen concentrations from 37.6 μM at 35 m to 115.4 μM at 32 m. Here, the concentration of methane decreased rapidly from 6 μM at 35 m to 0.2 μM at 32 m and remained this high in the surface water.

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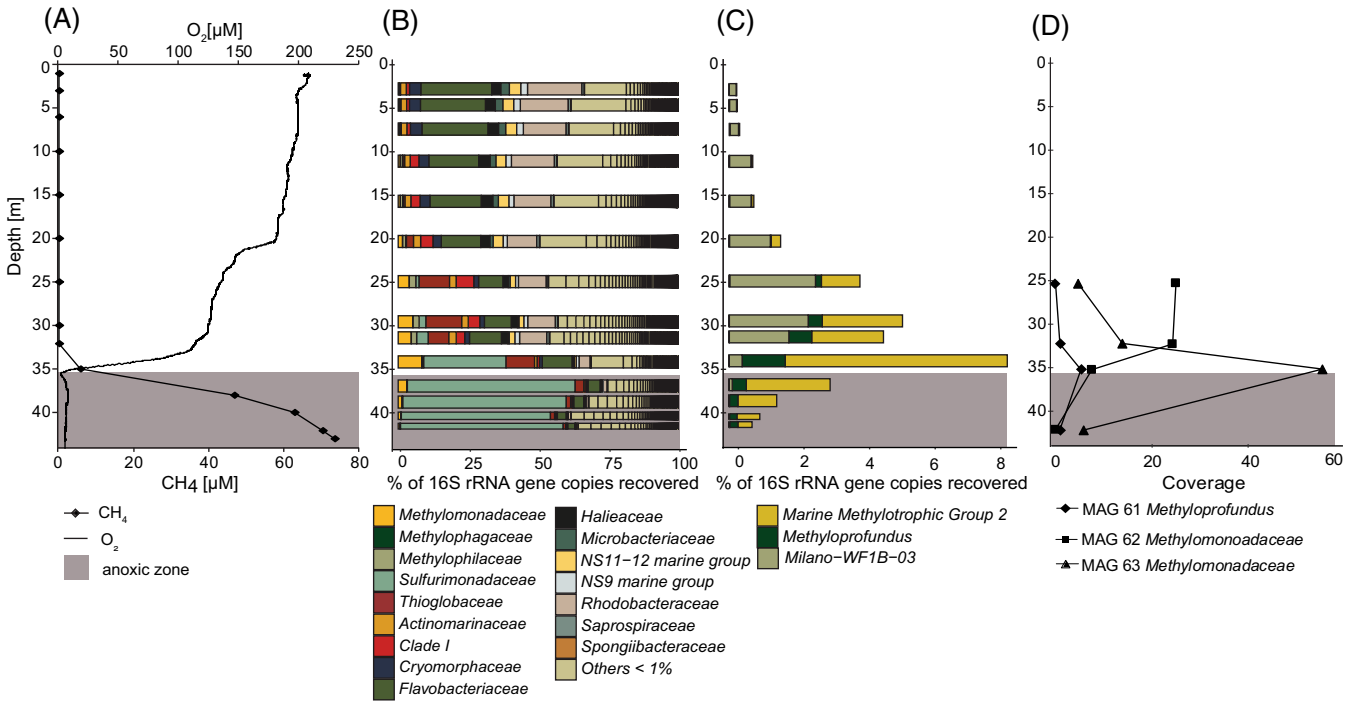

FIGURE 2 Water column depth profiles of methane and oxygen and the methanotrophic community structure during summer stratification in marine Lake Grevelingen. The grey area indicates anoxic water. (A) Methane (diamonds) and oxygen concentrations (black line) with depth in the lake. (B) Relative abundance of bacterial families obtained from 16S rRNA sequencing. (C) Vertical distribution of MOB-genera obtained by 16S rRNA sequencing. (D) Vertical distribution of MOB-MAGs obtained from metagenomic analysis. MOB, methanotrophic bacteria; MAG, metagenome-assembled genome.

Concurrent with the oxycline, the temperature decreased at 32 m from 19.9°C in the upper water, to 9.4°C in the bottom below 38 m (Table S1). Salinity showed little change with depth (range of 31 to 32; Table S1). A detailed discussion of the water column chemistry at the time of sampling can be found in (Żygadłowska et al., 2023).

Changes in microbial community structure and methanotroph abundance in the stratified water column

The high-resolution depth profile of 16S rRNA gene amplicon sequencing revealed a clear vertical pattern in the microbial community structure of bacteria and archaea in the stratified water column. The aerobic methanotrophic family of *Methylomonadaceae* was found at all water depths. However, the abundance increased from 20 m downwards and peaked at 35 m in the hypoxic zone, reaching an abundance of 8% of bacterial families (Figure 2B). There, also the anaerobic methane-oxidising archaeal family of *Methanoperedenaceae* with a relative abundance of 2% of archaeal families was observed, but they were not found at other depths (Figure S1). Although the relative abundance of *Methylomonadaceae* decreased below 35 m, the relative abundance was still considerably high in the anoxic waters with relative abundances of 3% and 1.5% at 38 and 40 m, respectively.

The genus-level vertical distribution of *Methylomonadaceae* revealed three genera, *Methyloprofundus*, *Marine_Methylotrophic_Group_2* and *Milano-WF1B-03* with a shift in abundance with depth (Figure 2C). *Milano-WF1B-03* was most abundant in the oxic part of the water column, reached a maximum at 25 m and decreased until it disappeared at and below 40 m. *Marine_Methylotrophic_Group_2* and *Methyloprofundus* showed an inverted pattern compared to *Milano-WF1B-03*. Although present in oxic waters, the abundance increased with decreasing oxygen concentrations, peaking at the lower oxycline at 35 m and they remained the dominant methanotrophs in the anoxic bottom water. We also observed sequences belonging to the aerobic Alphaproteobacterial methanotrophs (*Methyloligellaceae*) in the microbial community. Although present in the entire water column, the relative abundance never exceeded 0.1%. Therefore we focussed on the dominant *Methylomonadaceae* hereafter.

Genomic potential and distribution of methanotrophic MAGs in the oxycline

The genomic metabolic potential of the methanotrophs observed in the stratified water column was elucidated

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by high throughput shotgun-sequencing of four samples along the oxycline. In line with the 16S rRNA gene sequencing, we retrieved three metagenome-assembled genomes (MAGs) with more than 90% completeness, that were assigned to the family of *Methylomonadaceae*. As the 16S rRNA ASVs, the abundances of these three methanotrophic MAGs varied along the oxycline (Figure 2D). The abundance of MOB-MAG61 was the lowest with maximum coverage of 5.7 at 35 m. MOB-MAG62 was most abundant at 25 m with a coverage of 25, although at 35 m the coverage was also high with 24, and close to zero at 42 m. The most abundant MOB-MAG63 peaked at 32 m with a coverage of 54. In line with the suggested niche-partitioning of the methanotrophs, differences in the metabolic potential of each MAG were found (Figure 3). While all three MOB-MAGs had the particulate methane monooxygenase (pMMO), no soluble methane monooxygenase (sMMO) was found in any of the three MOB-MAGs. The nature of the gene encoding for methanol dehydrogenases (MDH) differed in each MAG. While MOB-MAG61 and MOB-MAG62 encoded for both, La-MDH (*xoxF*) and Ca-MDH (*mxaF*), MAG63 only possessed *xoxF* encoding La-MDH. Cytochrome c oxidase (*caa3*)—the low-affinity oxidase—was present in all MAGs. The high-affinity oxidase bd gene cluster was fully present in MOB-MAG61 and partially in MOB-MAG63. Interestingly, we found genes involved in denitrification in all MOB-MAGs. Moreover, in MOB-MAG63 we found all the genes involved in denitrification except for the nitrous oxide reductase (*nosZ*). MOB-MAG61 was the only one to possess *nifH*, a diagnostic gene for nitrogen fixation, despite the high ammonium concentration throughout the water column (Table S1). All MOB-MAGs showed potential for thiosulphate reduction and sulphide oxidation (*sox*). However, only MOB-MAG62 was equipped with the entire SOX gene cluster. MOB-MAG63 also showed potential for Fe, As and Hg-reduction.

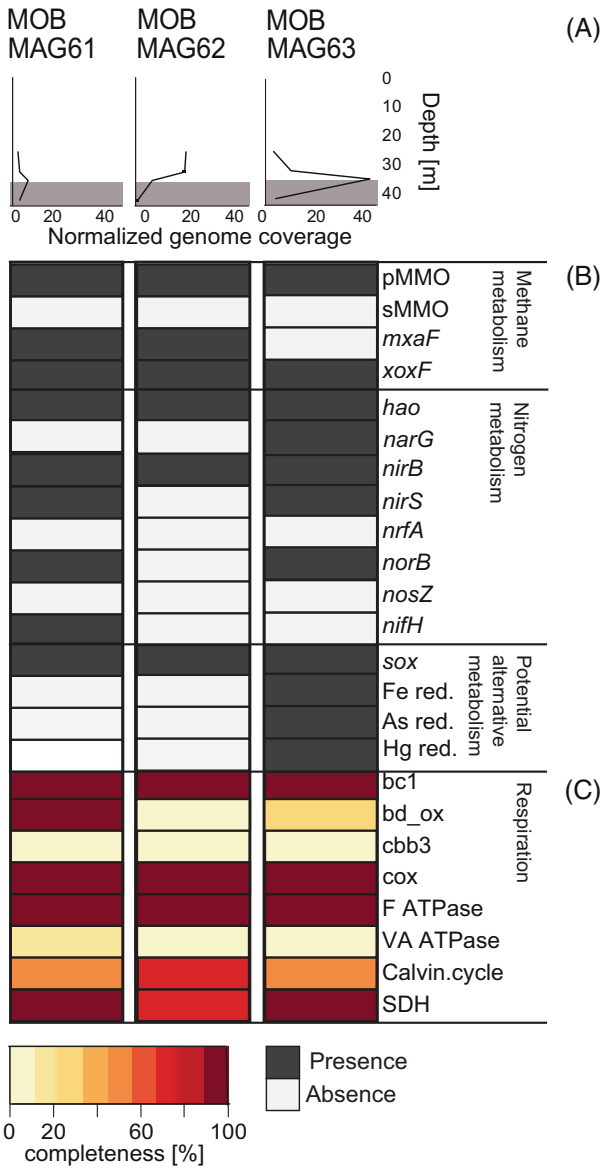

FIGURE 3 (A) Genome coverage of MOB-MAGs. Grey area indicates the anoxic zone. (B) Presence (black) and absence (white) of genes or gene complexes of MOB-MAGs. (C) Completeness of gene complexes of MOB-MAGs. MOB, methanotrophic bacteria; MAG, metagenome-assembled genome.

Potential aerobic methane oxidation rates along the methane oxygen counter-gradient

The potential methane oxidation rates were measured at four different depths, corresponding to four different oxygen concentrations (100, 50, 20 and 10 μM) and ranged from 3.6 to 282 μmol $L^{-1}d^{-1}$ (Figure 4; Table S2). Rates of methane oxidation were highest at 32 m with values ranging from 235 to 282 μmol L^{-1} d^{-1}. Although the two-way ANOVA showed an effect of oxygen concentrations and sampling depth, the multiple pairwise comparison (emmeans_test()) of the methane oxidation rates at different oxygen concentrations grouped by each sampling depth revealed only significant differences in methane oxidation rates at 35 m (Table S4). Thus, initial in situ conditions (e.g., methane and oxygen concentrations) and absolute abundance of MOB affected methane oxidation rates more strongly than oxygen concentrations in the incubations (Tables S3 and S4).

DISCUSSION

Active microbial biofilter in the oxycline reduces methane emissions

The bottom water methane concentration in Lake Grevelingen was 73 μM, which is up to 100-fold higher than in comparable coastal ecosystems (Borges et al., 2016; Matoušů et al., 2017; Steinsdóttir et al., 2022). In coastal sediments, anaerobic oxidation of methane reduces

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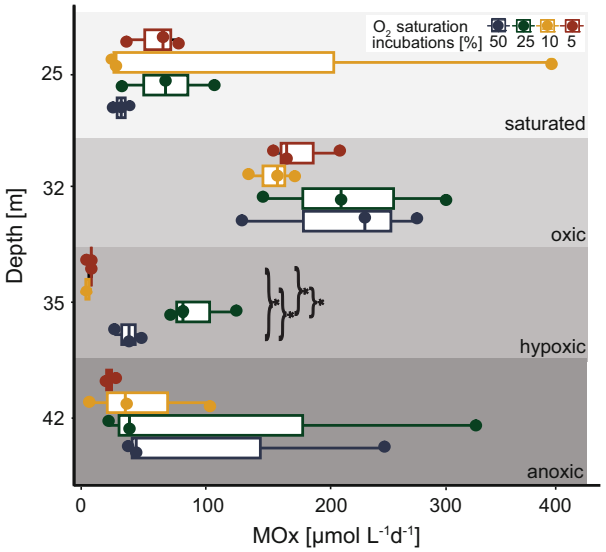

FIGURE 4 Potential methane oxidation rates (µmol L^{-1} d^{-1}) of four depths along the oxycline. Each depth was incubated in triplicate with different O_2 saturations (colours and shapes) and 1% of ^{13}C-CH_4 to follow ^{13}C-CO_2 production. Grey-scale backgrounds indicate oxygen saturation at the water depth at which the sample was taken for incubation. Boxes indicate the first and third quartiles, lines indicate the median, whiskers indicate outer data points if less than 1.5 interquartile range from quartiles. Individual data points are shown, $n = 48$. Asterisks indicate significant differences (emmeans_test(), $p < 0.05$).

methane flux to the overlying water column (Knittel & Boetius, 2009; Wallenius et al., 2021). In the Scharendijke basin sediment in Lake Grevelingen, however, methane production outpaces methane consumption, likely because of the high sedimentation rate (~13 cm yr^{-1}; Egger et al., 2016). This results in high benthic diffusive and ebullitive methane fluxes (Egger et al., 2016; Żygadłowska et al., 2023). Furthermore, in situ methane production in the anoxic bottom waters could add to these high methane concentrations, supported by the presence of methanogens, *Methanosarcinaceae*, *Methanomicrobiaceae* and *Methanocorpusculaceae*, as dominant archaeal families at these depths (Figure S1).

As methane is transported upwards to the oxycline through vertical mixing, the concentrations decrease moderately. From 35 m upwards, however, the methane concentration decreased strongly from 4 to 0.12 µM at 32 m, resulting in substantially lower methane concentrations above the oxycline. Such sharp methane oxygen counter-gradients in the stratified water column are typical for marine and freshwater ecosystems, where active microbial methane removal occurs (Blees et al., 2014; Mau et al., 2013; Reis et al., 2020).

Methanotrophic *Methylomonadaceae* ASVs were found in the entire water column. Strikingly, the relative abundance peaked concurrently with the strong decrease in methane concentrations within the oxycline. In addition, nitrate-dependent methanotrophic archaea (*Methanoperedens*) were also present in the hypoxic zone at 35 m, which implies a potential for anaerobic methane removal. However, we estimate the contribution to methane removal by *Methanoperedens* compared to MOB to be low, as the abundance of archaea in the water column is 100 times lower than the bacterial abundance (Steinsdóttir et al., 2022; Thamdrup et al., 2019). Overall, the methane-oxygen counter-gradient coinciding with the abundance peak of MOB strongly indicates that an active biofilter is removing a large proportion of the bottom water methane at the oxycline, thereby strongly lowering total methane emission to the atmosphere.

Niche partitioning of MOB along the methane-oxygen counter-gradient

In many aquatic ecosystems, the MOB community consists of both gamma- and alpha-MOB that have different adaptation strategies to different environmental conditions (Ho et al., 2013; Kaupper et al., 2020). The methanotrophic community in the water column of Lake Grevelingen mainly consisted of *Methylomonadaceae* belonging to the aerobic gamma-MOB. Although gamma-MOB were also the dominant MOB in the water column of other marine and freshwater ecosystems during water column stratification (Blees et al., 2014; Khanongnuch et al., 2022; Padilla et al., 2017; Rissanen et al., 2018), the small reservoir of alpha-MOB (<0.05% relative abundance), indicates that alpha-MOB might still be active at different stages of water column stratification as observed in freshwater lakes (Mayr, Zimmermann, Dey, et al., 2020).

Although the methanotrophic community in the water column of Lake Grevelingen only consisted of one family, these *Methylomonadaceae* showed clear niche-partitioning: different genera peaked at various depths, indicating adaptability to different biogeochemical niches. Such niche partitioning may especially be important for eutrophic, stratified systems, to efficiently counteract high benthic methane fluxes. The combination of water column stratification with distinct niches of varying oxygen and methane availability, together with the high metabolic versatility of the methanotrophic community, thus facilitates the formation of a multi-layered biofilter. This not only enables methane removal across the entire water column, but also potentially increases the functional resilience of the ambient methanotrophic community towards changes in oxygen and methane availability.

Versatile metabolic potential of the methanotrophic community

In line with our 16S rRNA gene amplicon sequencing results, three metagenome-assembled genomes (MAGs) belonging to the family of *Methylomonadaceae*

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were retrieved, with abundances peaking at four different depths along the oxycline. The diverse genomic potential and vertical distribution of these methanotrophic MAGs give further insight into the niche-specification of *Methylomonadaceae* along the methane-oxygen counter gradient in the water column of marine Lake Grevelingen. The genomic potential for denitrification implies the capability of the methanotrophic community to oxidise methane under anoxic or hypoxic conditions using nitrate as alternative electron acceptor. Additionally, the genomic potential to oxidise alternative electron donors (e.g., sulphur compounds or methanol) further implies potential coping mechanisms during methane or oxygen limitation (Gwak et al., 2022). Below, three aspects of the versatile metabolic potential of the three retrieved MOB-MAGs will be discussed: (1) the presence of high-affinity oxidases, (2) the use of alternative terminal electron acceptors and/or (3) alternative electron donors.

High- and/or low-affinity oxidases enable the growth of gamma-MOB under varying oxygen concentrations (Oshkin et al., 2015). MOB-MAG61 had genes for both a low-affinity cytochrome c oxidase and a cytochrome bd oxidase with a presumed high affinity for oxygen. Considering the high relative abundance of MOB-MAG61 in the hypoxic zone at 35 m, it is very likely that the high-affinity oxidase enables these methanotrophs to oxidise methane with the low amounts of oxygen supplied through downward turbulent diffusion from the oxygenated water column. MOB-MAG62 was highly abundant in the oxic water layers and did not possess this high-affinity bd oxidase and is therefore likely outcompeted by MOB-MAG61 and heterotrophs such as *Flavobacteriaceae* at 35 m. Interestingly, although MOB-MAG63 was most abundant in the hypoxic and anoxic water, only one out of three oxidase-bd subunits were present. Therefore, other metabolic features must explain the high abundance of MOB-MAG63, such as the potential to use alternative electron acceptors or alternative metabolic pathways.

Gamma-MOB can oxidise methane under virtually anoxic conditions (<50 nM dissolved oxygen), using various strategies (Steinsdóttir et al., 2022; van Grinsven et al., 2020). Some aerobic methanotrophs can use nitrate as a terminal electron acceptor for the respiratory chain (Kits et al., 2015; van Grinsven et al., 2020). We found all key genes involved in denitrification in MOB-MAG63, except for nitrous oxide reductase. With nitrate as terminal electron acceptor, a non-oxygen-dependent respiratory mode could be sustained during anoxia (Chen & Strous, 2013), enabling methane oxidation in the hypoxic water column (Kits et al., 2015; Oswald et al., 2016; Rissanen et al., 2018). However, oxygen would still be required for the oxidation of methane to methanol (Liu et al., 1995; Wallar & Lipscomb, 2001). Despite the metabolic potential and indications for active methane oxidation by *Methylomonadaceae* under anoxic conditions in sediments and water columns of other aquatic ecosystems (Oswald et al., 2016; Steinsdóttir et al., 2022), the stable isotope signatures of the methane in Lake Grevelingen do not indicate high in situ activity of anaerobic methane removal in the anoxic bottom waters (Żygadłowska et al., 2023).

Under anoxic conditions, MOB can also use alternative electron donors such as methanol (Dam et al., 2013; Tavormina et al., 2015). Indications of methanol oxidation potential were present in all three MAGs. MOB-MAG61 and MOB-MAG62 had genes for both the lanthanide and calcium-dependent methanol dehydrogenases (*xoxF* and *maxF*). MOB-MAG63, the most abundant MOB-MAG in the anoxic water, only harboured the gene for lanthanide-dependent methanol dehydrogenase (*xoxF*) which has a higher methanol affinity and yields higher methanol oxidation rates than *mxaF* (Keltjens et al., 2014). This might enhance the competitiveness to use methanol as an electron donor during hypoxic conditions. At Scharendijke basin, the input of easily degradable organic matter to the anoxic water is high (Egger et al., 2016), which can provide a source of methanol (Mincer & Aicher, 2016). In addition, thiosulphate or polysulphides are potential electron donors for methanotrophs (Gwak et al., 2022; Kojima et al., 2014). We found the key gene *sox* in all MOB-MAGs. However, the only MOB containing all genes of the *sox* system (MOB-MAG62) peaked in the oxic water column. Lastly, under anoxic conditions, methanotrophs can switch to fermentation to maintain a mixture of respiring and fermenting metabolism (Gilman et al., 2017; Kalyuzhnaya et al., 2013; Roslev & King, 1994). The survival strategies of MOB during anoxia, their ability to re-activate methane oxidation, and the doubling time after anoxia are potentially important features to mitigate methane emissions during water column turnover (Blees et al., 2014; Mayr, Zimmermann, Dey, et al., 2020). Nonetheless, most importantly, the potential to remain metabolically active during oxygen limitation might ensure a fast re-activation of methane oxidation upon re-oxygenation, and therefore might contribute to the functional resilience of the methanotrophic community.

Potential aerobic methane oxidation at different depths and oxygen concentrations

Evidence of active aerobic methane oxidation in the water column of Lake Grevelingen was shown by our oxic incubation experiments. All incubated depths along the methane-oxygen counter gradient showed methane oxidation at relatively high rates (0.1–9.5 μmol L^{-1} d^{-1}). However, the rates significantly differed between depths. Despite the higher relative

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abundance of methanotrophic ASVs at the hypoxic depth (35 m), the 'sweet spot' for highest activity of methanotrophs was at 32 m, where oxygen and methane are readily available. This is in line with other studies that found the highest methane oxidation rates in the upper part of the methane oxygen counter-gradient (Cabrol et al., 2020). The methane oxidation rates in the anoxic zone at 42 m indicate that methane oxidation can be activated by oxygen addition, even after weeks to months of exposure to anoxia. Vice-versa, methane oxidation in the oxygenated water at 25 m could be activated by methane addition, although the methane profile did not indicate active methane removal at this depth. This suggests that methanotrophs throughout the Lake Grevelingen water column can quickly adapt to changes in methane and oxygen availability, in line with observations in other ecosystems (Blees et al., 2014; Mayr, Zimmermann, Dey, et al., 2020). Together with the genomic data and biogeochemical profiles, the incubations confirm the high potential of the microbial methane oxidation filter in the water column.

Differences in oxygen availability can explain the differences in methane oxidation rates between depths. However, the drivers of in situ methane oxidation, and seasonal variability of methanotrophic community structure and function still require further investigation. However, varying initial oxygen saturations of 5%, 10%, 25% and 50% in our incubations (corresponding to 10, 20, 50 and 100 μM dissolved O_2), did not influence methane oxidation rates significantly at most depths. Although this does not exclude oxygen availability as a driver for methane oxidation in situ, it improves our understanding of the adaptability of methanotrophs to changes in in situ oxygen supply. Moreover, it sheds light on the competitive ability of methanotrophs when competing for oxygen with heterotrophs under oxygen-limited conditions. At 35 m we observed significant effects of oxygen availability on methane oxidation rates. There, incubations with 5% and 10% oxygen saturation (10 and 20 μM dissolved O_2.), resulted in lower methane oxidation rates compared to incubations with 25% and 50% (50 and 100 μM dissolved O_2.). The upwards flux of methane to 35 m likely exceeded oxygen downward flux, which results in oxygen limitation in situ. The dominant methanotroph at 35 m (MOB-MAG63), showed genomic potential for methane oxidation with nitrate. The in situ oxygen concentrations at this depth (37 μM) might be high enough for methane oxidation to methanol, and methanol could further be metabolised with nitrate. Yet, lower oxygen concentrations in our incubations (10 and 20 μM dissolved O_2), resulted in significantly lower methane oxidation rates than with 50 and 100 μM dissolved O_2. The overarching regulator for this could be the competition for oxygen with heterotrophs, which may be more efficient in scavenging available oxygen compared to MOB-MAG63 which did not harbour the high-affinity oxidase. Under high competitive pressure, it may be energetically favourable to remain in anaerobic mode until a threshold oxygen concentration is reached. In the incubations with 50% and 25% oxygen saturation (100 and 50 μM dissolved O_2), total oxygen concentrations could have remained high enough to reach such threshold, even with co-occurring heterotrophic oxygen consumption (e.g., with *Flavobacteriaceae*; Figure 1B), and resulted in higher methane oxidation rates. Intriguingly, methane oxidation rates with 50% initial oxygen saturation were lower than at 25%. This non-linear correlation between oxygen concentrations and methane oxidation rates has been shown in other ecosystems (Thottathil et al., 2019), which may point to other forms of nutrient limitation or inhibition. At 42 m, where methanotrophs had been exposed to anoxia for months, higher oxygen concentrations resulted in slightly higher methane oxidation rates, but the differences were not significant. As heterotrophs were less abundant in the anoxic water than at the oxic-anoxic interface, methanotrophs in the anoxic water were less likely to be outcompeted by active heterotrophs upon re-oxygenation. There, the ability of the methanotrophic community to enter a resting state and re-activate methane oxidation upon re-oxygenation, might be a more crucial factor for efficient methane removal than the potential use of alternative electron acceptors. Furthermore, interactions with other element cycles through the microbial network could also influence methane oxidation rates or the metabolic state during oxygen limitation (Li et al., 2021). Overall, the effect of oxygen on methane oxidation rates is influenced by both competitive effects and the active metabolic state of the methanotrophs in situ. This highlights the importance of measuring methane oxidation rates at high vertical resolution in stratified water columns and taking the entire microbial community into account, to improve estimations of methane removal and its drivers.

CONCLUSION

We showed that an active microbial methane filter in the water column of marine Lake Grevelingen counteracts high benthic methane fluxes during summer stratification and hypoxia. Metagenomic and 16S rRNA gene sequencing analysis showed that *Methylomonadaceae* were abundant in the entire water column, with a striking distinct abundance peak coinciding with a sharp methane oxygen counter-gradient. Furthermore, three MOB-MAGs belonging to *Methylomonadaceae*, with versatile metabolic potentials and distinct vertical distribution were retrieved. Potential methane oxidation rates were high at all incubated depths and did not show a strong influence of oxygen availability on methane oxidation at most depths.

We therefore, conclude that the versatile metabolic potential of members of *Methylomonadaceae* in the

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stratified water column of Lake Grevelingen (1) enables the population of different biogeochemical niches within the water column, (2) broadens the vertical range of the active methane oxidation filter, (3) improves competitiveness with other microorganisms and (4) increases the resilience of the in situ methanotrophic community towards changes in oxygen availability. Thus, the metabolic versatility of the ambient methanotrophic community is a crucial factor for the mitigation of methane emissions from seasonally stratified coastal ecosystems. Investigation of seasonal dynamics of methane oxidation, including the metabolic potential of the methanotrophic community, will further improve our understanding of the functioning and drivers of the microbial methane filter in the water column of these highly dynamic coastal ecosystems.

AUTHOR CONTRIBUTIONS

Jessica Venetz: Data curation (equal); formal analysis (lead); investigation (equal); methodology (equal); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Olga M Żygadłowska:** Data curation (supporting); formal analysis (supporting); investigation (equal); methodology (equal); writing – review and editing (equal). **Wytze K.L. Lenstra:** Data curation (supporting); formal analysis (supporting); investigation (equal); methodology (equal); writing – review and editing (equal). **Niels A.G.M. Van Helmond:** Data curation (supporting); formal analysis (supporting); investigation (equal); methodology (equal); writing – review and editing (equal). **Paula Dalcin Martins:** Data curation (supporting); formal analysis (supporting); investigation (supporting); visualization (supporting); writing – review and editing (supporting). **Anna J. Wallenius:** Data curation (supporting); formal analysis (supporting); methodology (supporting); writing – review and editing (supporting). **Guylaine H.L. Nuijten:** Methodology (supporting); writing – review and editing (supporting). **Caroline P. Slomp:** Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); project administration (equal); supervision (supporting); writing – review and editing (equal). **Mike S.M. Jetten:** Conceptualization (equal); data curation (supporting); formal analysis (supporting); funding acquisition (equal); investigation (equal); project administration (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (equal). **Annelies J. Veraart:** Conceptualization (equal); data curation (equal); formal analysis (supporting); investigation (equal); methodology (supporting); project administration (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Raw reads of the metagenome sequencing data can be accessed on the European Nucleotide Archive under the accession number PRJEB57287 (https://www.ebi.ac.uk/ena/browser/view/PRJEB57287). Experimental data and field data are available through DANS-EASY (tbd).

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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