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Abundance, activity and diversity of methanotrophic bacteria in the Elbe Estuary and southern North Sea

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ABSTRACT: Shelf sea areas are the primary oceanic source for methane release, the most abundant hydrocarbon in the atmosphere. As such, the southern North Sea's methane concentration is mainly determined by river runoff and tidal marshes. Within such a highly variable temperate estuary, this study is the first to reveal detailed information on the *in situ* activity, abundance and community structure of methane oxidizing bacteria along a transect from the marine environment near Helgoland island to the riverine harbor of Hamburg, Germany. The *in situ* methane oxidation rate was determined with a radio tracer, and methane concentration with the head-space method. Abundance and diversity of the methanotrophic bacterial community in the water column was assessed with quantitative polymerase chain reaction for the particulate methane monooxygenase and monooxygenase intergenic spacer analysis. Median abundances ranged from 2.8×10^4 cells l^{-1} in the marine environment to 7.5×10^5 cells l^{-1} in the riverine environment. Except for salinity, no conclusive linear correlation between any environmental parameter and the abundance of methanotrophs could be determined. Relating activity with abundance of methanotrophs showed that about 70% of the population accounted for is inactive, especially in the coastal and marine environment. This study found distinct operational taxonomic unit (OTU) community compositions among the 3 environmental categories (river, coast, marine). Several identified OTUs have been reported previously and imply a wide geographic occurrence. Overall, we propose that salinity is the most important driver in shaping differing methanotrophic communities in the riverine, coastal and marine environment.

KEY WORDS: Methane oxidizing bacteria · Methane oxidation · Fingerprinting method · pMMO · qPCR · MISA-OTU · Estuarine environment

1. INTRODUCTION

Atmospheric methane (CH_4) is the most abundant hydrocarbon and the second most influential greenhouse gas in the atmosphere. Its concentration has been increasing since the 1980s and reached approx. 1900 ppb in 2018 (https://www.esrl.noaa.gov/gmd/dv/iadv/). However, it is not easy to assess the individual CH_4 sources and their role in the overall CH_4 budget. This is mainly because CH_4 is emitted by a variety of processes that need to be understood and quantified separately (Saunois et al. 2016).

Shelf sea areas comprise only 7% of the total ocean area, but contribute 75% of the total oceanic CH_4 flux (Bange et al. 1994). Dissolved CH_4 is near equilibrium concentration (2–3 nM) in open ocean upper layers, while in shelf sea areas saturation can be orders of magnitude higher (Middelburg et al. 2002). In the southern North Sea, the main sources influencing the CH_4 budget are the estuaries (Upstill-God-

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dard & Barnes 2016) and tidal marshes of the Wadden Sea environment (Grunwald et al. 2009, Osudar et al. 2015, Matousu et al. 2017). Important processes for removing CH_4 from the water column are diffusion into the atmosphere, biological CH_4 oxidation and dilution with methane-poor Atlantic water (Grunwald et al. 2009). Aerobic oxidation in the water column is a very efficient sink, which allows very little CH_4 to reach the atmosphere (Saunois et al. 2016).

Aerobic methane oxidation (MOX) is mostly carried out by methanotrophic *Proteobacteria*, which only use CH_4 as a carbon and energy source, as well as *Verrucomicrobia*, which perform MOX (van Teeseling et al. 2014). Based on phylogenetic analyses of 16S rRNA gene sequences, the methanotrophs were initially divided into 3 subgroups: type I and type X methanotrophs correspond to the *Gammaproteobacteria*, and type II to the *Alphaproteobacteria* (Knief 2015). However, there are several exceptions which sometimes impede classification (Op den Camp et al. 2009, Semrau et al. 2010). The key reaction of MOX is the initial oxidation from CH_4 to methanol. This conversion is mainly conducted by the membrane-associated particulate methane monooxygenase (pMMO). It is found in most known methanotrophs and is located in the cytoplasmic membrane. The gene encoding for this enzyme — the *pmoA* gene — is widely used as functional gene marker for the detection of methane oxidizing bacteria (MOB) (Op den Camp et al. 2009, Semrau et al. 2010). MOB have been mostly isolated from non-saline environments (soils, sediments and lakes) (Hanson & Hanson 1996, Khmelenina et al. 1999). However, there are also a few examples of MOB isolated from marine environments (Hirayama et al. 2013, Tavormina et al. 2015, Vekeman et al. 2016).

Estuaries can be seen as dynamic links between the freshwater/terrestrial and marine environments. Tidal processes lead to variations in salinity and mixing of organic matter derived from industrial and agricultural activities to the oligotrophic marine environment (Sherry et al. 2016, Upstill-Goddard & Barnes 2016). Little is known about the capability of methanotrophs to cope with this highly dynamic environment, with respect to abundance and community composition. Sherry et al. (2016) revealed the presence of likely inactive methanotrophs in the Tyne Estuary, which only become active under extreme environmental conditions. In addition, frequency of disturbance and site history are important factors in assessing the resilience of the methanotrophic community to changes in salinity (Ho et al. 2016). A recent study (Osudar et al. 2017) investigated the tolerance of single methanotrophic strains and the natural methanotrophic community towards salinity, determining that community composition is crucial for the overall response to salinity changes.

Using the monooxygenase intergenic spacer analysis method, the aim of our study was to identify relevant representatives of the methanotrophic community within the Elbe Estuary. Additionally, we linked the community composition of MOB to environmental parameters in this highly dynamic system. In previous studies in the southern North Sea and Elbe Estuary, we demonstrated that MOX rates were influenced by CH_4 concentration, temperature and salinity (Osudar et al. 2015, Matoušů et al. 2017). The aim of this study was to assess abundance and diversity of methanotrophic bacteria and to relate these data to their activity.

2. MATERIALS AND METHODS

2.1. Study site

We sampled the Elbe River estuary along a transect from Helgoland Island, in the German Bight, southeastern North Sea to Hamburg Harbor, which displays a strong salinity gradient (Fig. 1). Samples were taken almost every month from November 2013 until November 2014 (Table S1 in the Supplement at www.int-res.com/articles/suppl/a083p035_supp.pdf). Stns Elbe VI–VIII were sampled either on the western or eastern side of the waterway. Water masses were classified as 'riverine water' (salinity ≤ 5), 'coastal water' (5 < salinity < 20) and 'marine water' (salinity ≥ 20), modified from (Caspers 1959).

2.2. Water sampling

For North Sea stations, water was sampled with a water sampling rosette mounted with a sea-bird CTD sensor. In the Elbe River, water samples were taken with a Uwitec water sampler (Uwitec). Sampling depth was 1 m below the surface and 1 m above the sediment–water interface. For Stns #619–#699, only surface water was sampled, as previous cruises had shown no significant differences between bottom and surface water. During cruises along the Elbe River, temperature, salinity and oxygen in the water were measured immediately after sampling, using a universal pocket meter (Multi 340i). The amount of

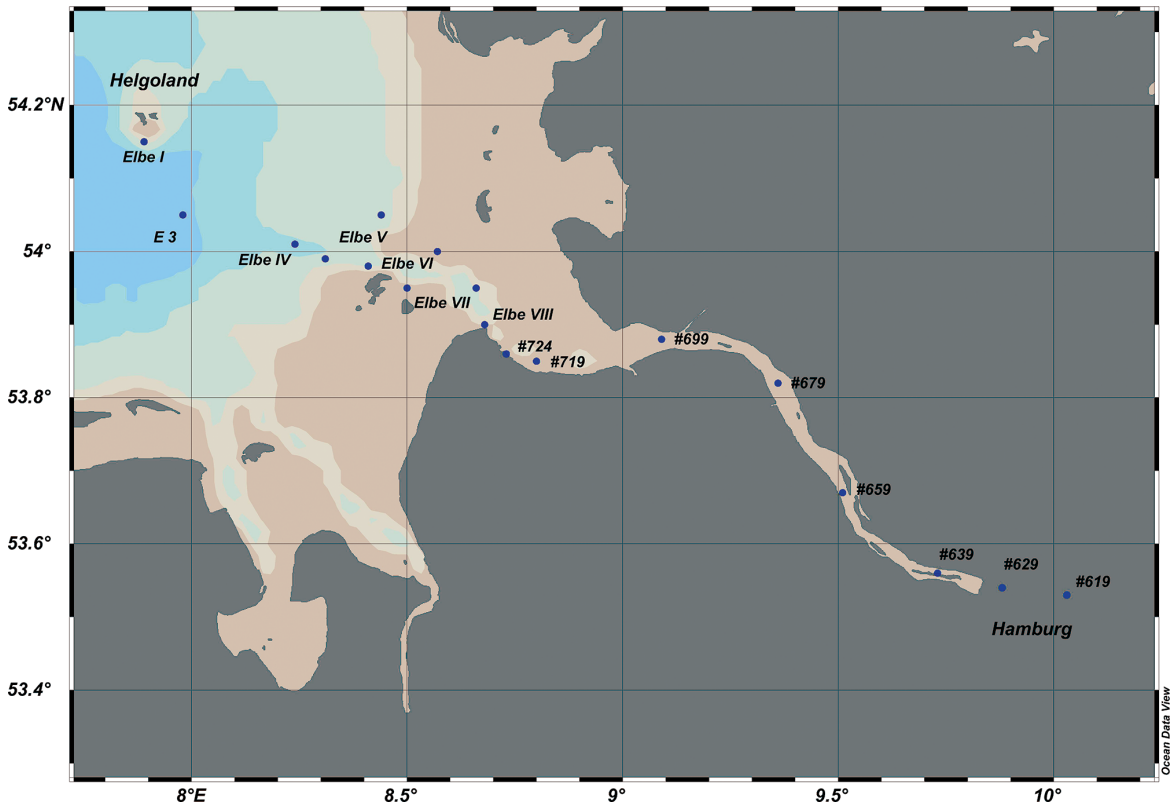

Fig. 1. Sampling area with sampling stations indicated along the transect from Hamburg to Helgoland. Stns Elbe VI–VIII were sampled either on the western or eastern side of the waterway

suspended particulate matter (SPM) and nutrients were analyzed as described previously (Text S1 in the Supplement) (Wiltshire et al. 2010).

2.3. Determination of CH_4 concentration and MOX rates

A total of 6 serum bottles (120 ml) were filled directly from the water sampler with thin silicon tubing. The bottles were flushed extensively with sample water (to prevent contact with the atmosphere) and were closed afterwards with butyl rubber stoppers. Excess water could escape via a needle in the stopper.

CH_4 concentrations were determined via the head-space method, as described previously. Samples were preserved by adding 0.2 ml of 25% H_2SO_4. After adding 20 ml of N_2 as head-space, samples were analyzed using gas chromatography. Calculations of CH_4 concentrations were performed according to Magen et al. (2014), considering different CH_4 solubilities across the wide range of salinities (1–33).

The MOX rate was determined by adding radioactive, tritiated CH_4 to triplicate samples. The principle of MOX rate determination is based on the ratio of produced tritiated hydrogen from the added tritiated CH_4 (for more details see Text S1). This ratio, corrected for incubation time, gives the fractional turnover rate (k' [d^{-1}]). To obtain final MOX, k' is multiplied by the *in situ* CH_4 concentration. For MOX, the limit of detection was calculated as described in Bussmann et al. (2015) and was determined to be ≤0.021 nmol l^{-1} d^{-1} for this data set.

2.4. Polymerase chain reaction and amplification of methane monooxygenase genes

Samples (250–400 ml) from surface and bottom water were filtered through 0.2 µm cellulose acetate filters (Sartorius) and stored frozen until further processing. No pre-filtration step was applied because methanotrophs are commonly found attached to particulate matter (Schut et al. 1997). High molecular weight DNA was extracted following the protocol of the PowerWater DNA Isolation Kit (MoBio). DNA concentrations were determined with UV absorbance (260:280 nm ratio).

The environmental DNA samples were checked for the presence of methanotrophic DNA with the water column-specific primers wcpmoA189f and wcpmoA661r (Tavormina et al. 2008) according to Bussmann et al. (2017); more details on the primers can be found in Text S1.

2.5. Quantitative polymerase chain reaction of methane monooxygenase genes

Extracted DNA from each sample was amplified by quantitative polymerase chain reaction (qPCR) using a LightCycler R 480 (Roche) and SYBR Green assay (Roche) as described by Bussmann et al. (2017). Briefly, *Methylobacter luteus* (NCIMB 11914) was used as standard for total *pmoA* gene.

The qPCR reaction mix was run with the primer set wcpmoA189f/wcpmoA661r; more details on the PCR conditions can be found in Text S1. The relative abundance of MOB was calculated as the percentage of MOB-DNA in the total extracted DNA of each sample. Relative abundances of ≤2% were assumed to be realistic (Bornemann et al. 2016, Samad & Bertilsson 2017). All other data were excluded from further analysis.

2.6. Methane monooxygenase intergenic spacer analysis

Methane monooxygenase intergenic spacer analysis (MISA) is a way of analyzing taxon-related length polymorphism in methane monooxygenase genes (Tavormina et al. 2010). MISA was used in this study to analyze MOB community composition in regards to their geographical distribution. Hence, all environmental samples including *pmoA* genes were analyzed with MISA to differentiate the methanotrophic populations and to estimate their diversity. MISA fingerprints were determined as described by Bussmann et al. (2017). PCR fragments from bulk environmental DNA were amplified using the primers spacer_pmoC599f and spacer_pmoA192r. A nested amplification was performed with the primer spacer_pmoC626_IRD and spacer_pmoA189r, using purified PCR product from the first PCR as a template. Amplified samples were separated on polyacrylamid gels using a DNA Analyzer 4300 (Licor). Details on the PCR conditions and subsequent analyses can be found in Text S1.

A set of reference strains was used to assign the MISA operational taxonomic units (OTUs) in the environmental samples to previously described methanotrophs (see Table 2). Reference strains were grown on agar plates with nitrate mineral salts (NMS) medium and incubated in desiccators with a 50% CH_4 atmosphere. The respective MISA fragments were amplified and used as internal markers on the MISA gels.

2.7. Statistical analysis

Data on methanotrophic abundance and environmental parameters were log transformed (zero values excluded) and analyzed using R-Studio ('vegan' package). Multiple linear regression and Wilcoxon tests were used to find the independent variables that predicted the dependent variables (MOX, *k'*, abundance/relative abundance of MOB and OTU distribution; Table S2).

We also aimed to relate the occurrence of different OTUs to environmental parameters. To account for uneven frequencies of sampling (n_{river} = 33, n_{coast} = 32, n_{marine} = 103), we first calculated the sum of occurrences for each OTU in each category (location, season, MOX). For example, the OTUs 363, 407, 419 up to OTU-677 were detected 6, 2, 32 and 0 times in 'river'. These occurrences were then rank-transformed by relating them to the occurrences of all OTUs in 'river'. In this example, this resulted in the ranks 9, 11, 1 and 13, meaning OTU-419 was most frequent in 'river' (rank 1) while OTU-677 was least frequent (rank 13). This transformation was performed for 'coast' and 'marine' as well. When comparing the ranks for 'river', 'coast' and 'marine' for OTU-486, the ranks 5, 2, 1 were obtained, suggesting a marine preference.

For multivariate statistical analyses of the MISA, the software package PRIMER v.6 with PERMANOVA+ (PRIMER-E) was used. The analyses were performed on Jaccard matrices, generated from the presence or absence of MISA band class data for each sample. To test for the H_0 of no community assemblage differences on a temporal or spatial scale, either an analysis of similarity or a permutational multivariate analysis of variance (PERMANOVA, 999 permutations) was applied following Anderson (2001). The results were visualized by principal coordinate analysis. Distance-based multivariate multiple regression was used to calculate correlations of community composition with environmental factors, and distance-based redundancy analysis was used to visualize these correlations (Legendre & Gallagher 2001, Lucas et al. 2015).

Table 1. Oceanographic parameters (median and range) of the different water bodies; each category was tested against the other 2 categories (Wilcoxon rank sum test, significance level $\alpha < 0.05$; categories with the same index [a–c] do not differ significantly). SPM: suspended particulate matter; MOX: methane oxidation; *k′*: fractional turnover rate; nd: not detected

	Riverine	Coastal	Marine
Salinity (PSU)	0.5 (0.1–5.0) a	15.2 (5.2–20.0) b	28.9 (20.1–33.5) c
Temp. (°C)	10.6 (3.4–19.8) a	9.5 (1.4–19.3) a	11.7 (1.7–18.1) a
CH_4 (nmol l^{-1})	47 (12–1635) a	51 (23–155) a	30 (5–155) b
PO_4 (µmol l^{-1})	1.81 (0.64–3.11) a	2.07 (1.06–3.30) a	0.89 (0.06–3.17) b
NO_3 (µmol l^{-1})	164.3 (75.7–680.0) a	98.1 (23.9–379.0) b	18.7 (0.4–264.0) c
NO_2 (µmol l^{-1})	0.99 (0.01–4.34) a	0.90 (0.21–2.37) a	0.64 (0.00–5.29) a
NH_4 (µmol l^{-1})	4.7 (1.4–18.0) a	5.0 (2.1–12.0) a	3.2 (0.0–17.0) b
SPM (mg l^{-1})	58.5 (8–525) a	90.0 (59 –200) b	97.0 (47–157) b
O_2 (mg l^{-1})	9.2 (5.9–10.7) a	9.2 (6.8–11.0) a	8.9 (6.8–12.5) a
MOX (nmol l^{-1} d^{-1})	32.2 (2.7–2558.2) a	3.1 (0.2–132.5) b	0.4 (0.0–27.7) c
k′ (–)	0.771 (0.069–9.404) a	0.067 (0.006–1.657) b	0.020 (0.002–0.412) c
Abund. (cells l^{-1})	7.50×10^5 (nd–8.03×10^6) a	4.85×10^5 (nd–3.94×10^6) a	2.75×10^4 (nd–4.78×10^6) b
Rel. abund. (%)	0.25 (nd–1.74) a	0.32 (nd–1.66) a	0.02 (nd–1.77) b

3. RESULTS

3.1. Oceanographic parameters

Data were split according to salinity into riverine (salinity ≤ 5, n = 34), coastal (5 < salinity < 20, n = 30) and marine waters (salinity ≥ 20, n = 99). Riverine water was characterized by a median salinity of 0.5; coastal and marine waters had median salinities of 15.2 and 28.9, respectively. There was no difference in water temperature among the 3 water masses (median: 10.6, 9.5 and 11.7°C, respectively). The concentrations of phosphate and nitrate differed significantly among the water masses (Table 1). The highest concentration of phosphate was observed in the coastal water (median: 2.07 µmol l^{-1}) and lowest in marine water (median: 0.89 µmol l^{-1}). Nitrate concentration was highest in riverine water (median: 164.3 µmol l^{-1}) and lowest in marine water (median: 18.7 µmol l^{-1}). Nitrite and ammonium were distributed uniformly across the water masses (Table 1).

We also investigated the influence of different seasons on our parameters. However, no correlation or influence was found; thus, no further analyses are shown.

3.2. CH_4 concentrations and oxidation rates

CH_4 concentrations, oxidation rates and *k′* were all significantly different in the different water masses (Table 1). CH_4 concentrations ranged from a median of 47 nmol l^{-1} in river water to 51 nmol l^{-1} in coastal water and 30 nmol l^{-1} in marine water. MOX rates differed by one order of magnitude with a median of 32.2 nmol l^{-1} d^{-1} in river water, 3.1 nmol l^{-1} d^{-1} in coastal water and only 0.4 nmol l^{-1} d^{-1} in marine water (Fig. 2b). The detection limit for determining MOX was ≤0.021 nmol l^{-1} d^{-1}, and 3.6% of the data were below the detection limit. The *k′* showed the same pattern as MOX, with a median of 0.77 d^{-1} in river water, 0.07 d^{-1} in coastal water and only 0.02 d^{-1} in marine water.

CH_4 concentration and MOX increased significantly with longitude, i.e. from Helgoland to Hamburg (Fig. 2a, with log CH_4: $r^2 = 0.33$, $p < 0.001$; Fig. 2b, with log MOX + 1: $r^2 = 0.72$, $p < 0.001$). To determine which environmental parameters were hidden behind 'longitude', a multiple linear regression (MLR) analysis was performed (Table S2). This analysis was able to explain parameters influencing MOX to a high extent (multiple $R^2 = 0.96$, n = 164). The most important environmental parameters were salinity ($p < 0.001$), oxygen concentration ($p < 0.001$) and PO_4 ($p = 0.001$), while the influence of water temperature was not significant ($p = 0.105$). The factors explaining *k′* values were the same as for MOX with additional nitrite and nitrate concentrations ($p = 0.004$ and 0.002) and SPM ($p = 0.002$) (Table S2). In addition, *k′* was also strongly influenced by the CH_4 concentration. All together, the MLR explained 91% of variability of *k′* ($R^2 = 0.89$).

3.3. Abundance of methanotrophs

The abundance of MOB ranged from not detectable to a maximum of 8.03×10^6 cells l^{-1} with a median of 5.17×10^4 cells l^{-1}. The detection limit was given by

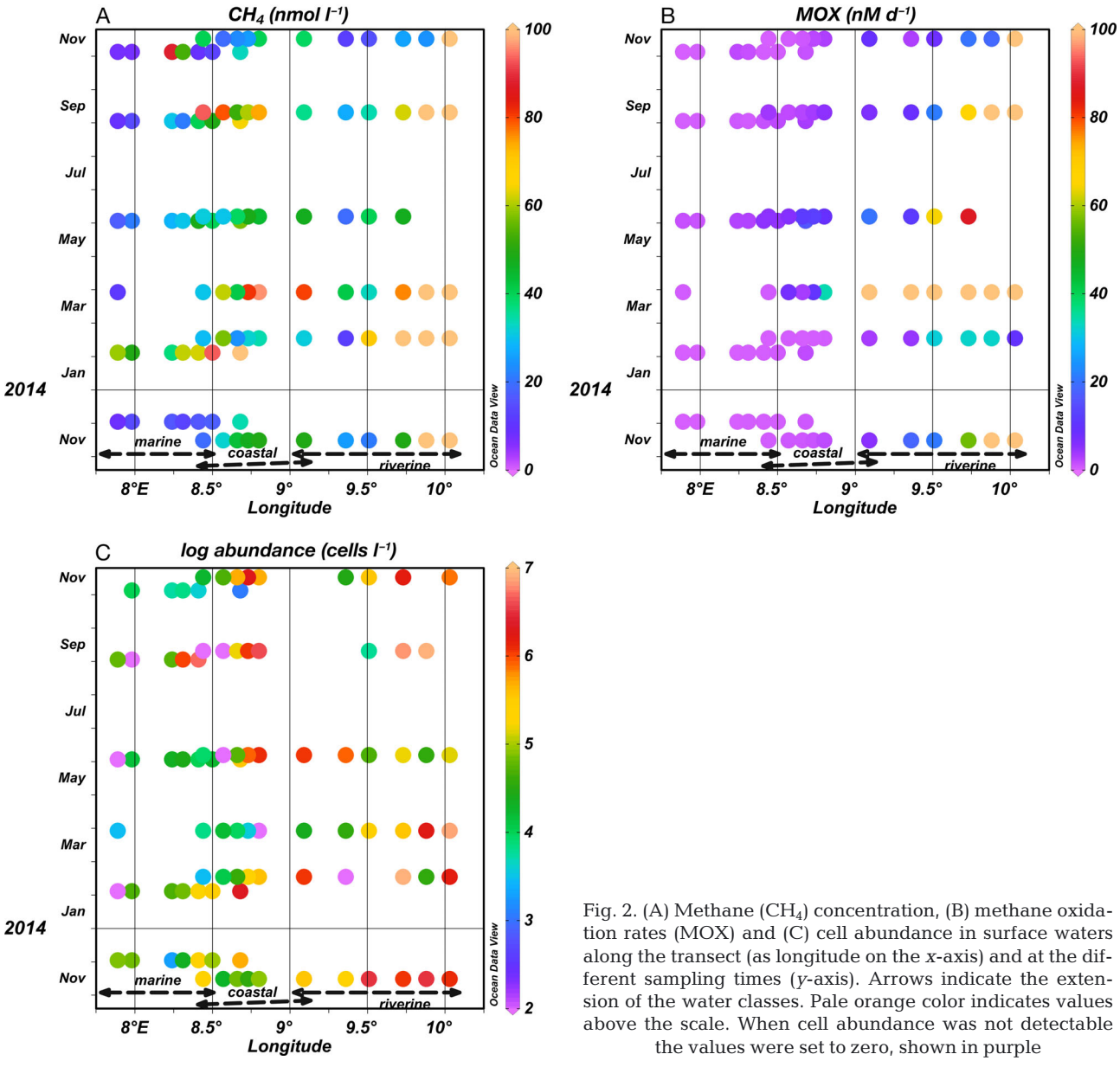

Fig. 2. (A) Methane (CH_4) concentration, (B) methane oxidation rates (MOX) and (C) cell abundance in surface waters along the transect (as longitude on the *x*-axis) and at the different sampling times (*y*-axis). Arrows indicate the extension of the water classes. Pale orange color indicates values above the scale. When cell abundance was not detectable the values were set to zero, shown in purple

the accuracy of reproducible standards in the qPCR, equivalent to 2.5×10^3 cells l^{-1}. In total, 18 out of 159 samples (11%) were below the detection limit.

Abundance and relative abundance of MOB were significantly influenced by the parameter 'water mass' (ANOVA with log-transformed data, $p < 0.001$, df = 140 and 144). The abundance of MOB differed by one order of magnitude, from a median of 7.50×10^5 cells l^{-1} in riverine water, over 4.85×10^5 cells l^{-1} in coastal water to 2.75×10^4 cells l^{-1} in marine water (Fig. 3A). The relative abundance of MOB was similar in riverine and coastal waters (median: 0.25 and 0.32%, respectively) versus only 0.02% in marine waters (Fig. 3B).

Abundance significantly increased with longitude from Helgoland towards Hamburg ($r^2 = 0.27$, $p < 0.001$; Fig. 2c). No differences among season, temperature or day of the year could be detected. Abundances at the surface seemed to be slightly higher than in bottom water, although not significantly (Wilcoxon test, $p = 0.02$; bottom data shown in Fig. S1). To determine which environmental parameters were hidden behind 'longitude', a multiple linear regression analysis was performed (Table S2). This analysis of environmental factors and abundance of MOB explained an overall multiple R^2 of 0.51. The abundance of MOB was significantly influenced by oxygen concentrations and

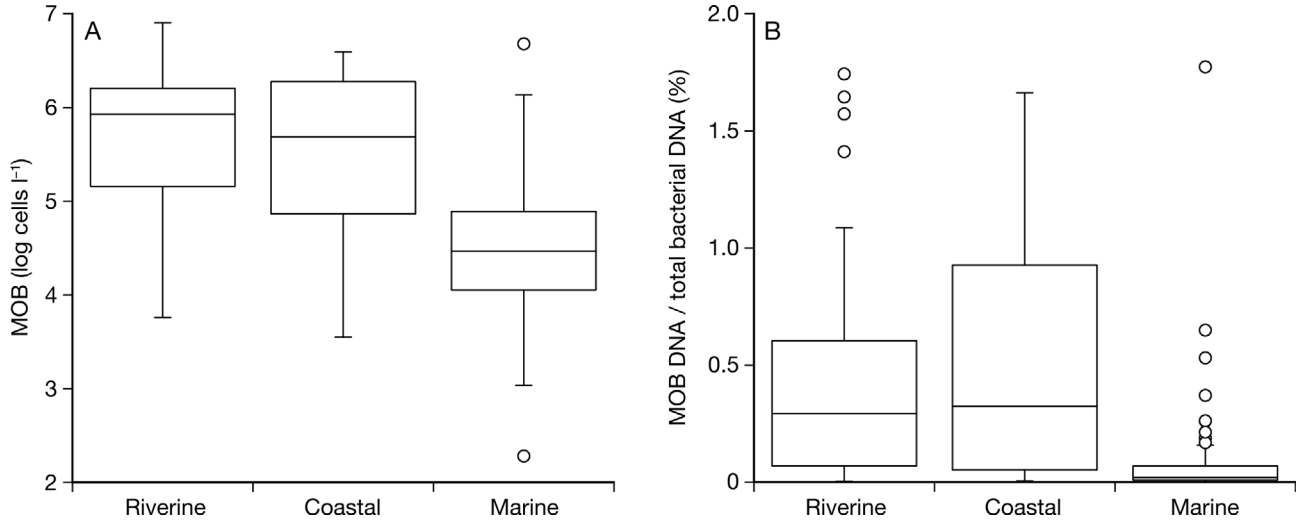

Fig. 3. (A) Total methane oxidizing bacteria (MOB) cell numbers and (B) relative abundance of MOB in the different water classes (n = 123). Each box encloses 50% of the data with the median value of the variable displayed as a line. The top and bottom of the box mark the limits of ± 25% of the variable population. The lines extending from the top and bottom of each box mark the minimum and maximum values within the data set that fall within an acceptable range. Any value outside of this range, called an outlier, is displayed as an individual point

temperature, as well as by phosphate and nitrite concentrations (Table S2). No parameter was found to significantly influence the relative abundance.

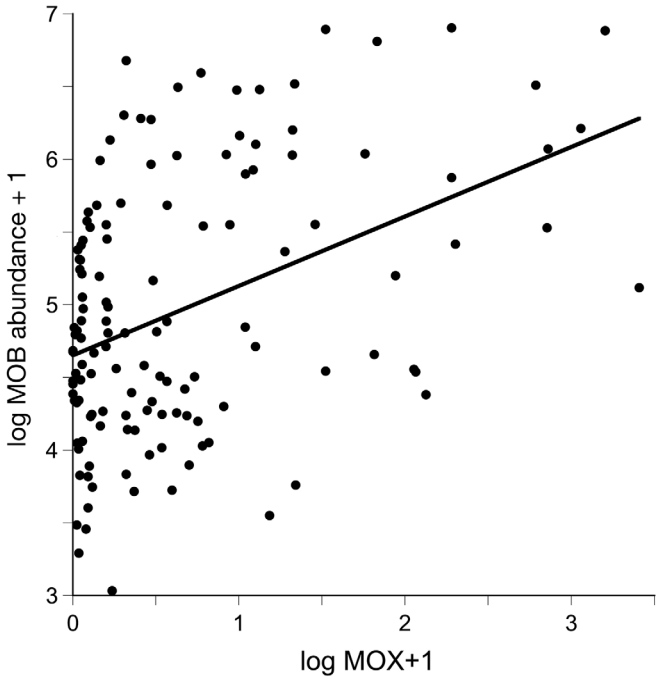

Fig. 4. Correlation between methane oxidizing bacteria (MOB) abundances and methane oxidation (MOX) rates from all samples ($r^2 = 0.17$, slope = 0.48, $p < 0.0001$). To avoid negative values, '1' was added to all values

To relate the activity of MOB with their abundance, log-transformed data of MOX were compared to the abundance and relative abundance of MOB. There was a weak but significant correlation between these parameters ($r^2 = 0.17$, slope = 0.48, $p < 0.0001$ and $r^2 = 0.13$, slope = 0.05, $p < 0.0001$, respectively). Thus, with no detectable activity (≤0.021 nmol l^{-1} d^{-1}) we still detected MOB, at 4.50×10^4 cells l^{-1} or 0.08%, respectively (Fig. 4). The median cell-specific MOX rates in marine and coastal waters were similar (0.55 and 0.23 fmol $cell^{-1}$ h^{-1}), and significantly lower than the riverine median (7.85 fmol $cell^{-1}$ h^{-1}; Wilcoxon test, $p < 0.001$).

3.4. Methanotrophic diversity

The MISA of the 168 environmental samples (15 stations on 11 sampling dates) along the Hamburg–Helgoland transect resulted in 13 different band classes or MISA-OTUs. The associated fragment sizes ranged from 677 to 363 bp. Subsequently, the different band classes were named according to their specific length (bp); e.g. MISA-OTU-677. All data are reported as presence/absence data (1/0).

MISA of the reference strains (Table 2) revealed single-characteristic OTUs for most of the strains. Only the type II methanotrophs (*Methylosinus trichosporium* and *M. sporium*) showed 2 distinct OTUs (470/592 and 475/580).

Table 2. Comparison of environmental methane monooxygenase intergenic spacer analysis operational taxonomic units (MISA-OTUs) with OTUs from pure cultures and other environmental studies (Tavormina et al. 2010). The frequency of the different OTUs in the study is also indicated

Environmental OTU	Frequency (%)	OTUs from cultures
MISA-OTU-363	17.9	
MISA-OTU-407	2.4	*Methylomonas methanica* (408 bp)
MISA-OTU-419	89.9	*Methylobacter luteus* (416 bp)
		Methylomonas albis (416 bp)[a]
		Methylomonas fluvii (416 bp)[a]
		Methylobacter marina (416 bp)[b]
		Methylosacrcina fibrate (417 bp)[b]
		Methylomicrobium agile (421 bp)[b]
MISA-OTU-430	33.9	*Methylovulum psychrotolerans* Eb1 (428 bp)[a]
		OPU-3 (433 bp)[b]
MISA-OTU-445	74.4	OPU-1 (442)[b]
MISA-OTU-471	6.5	*Methylosinus trichosporium* (471/592 bp)
		Methylosinus sporium (472/580 bp)
MISA-OTU-486	89.3	
MISA-OTU-513	64.9	
MISA-OTU-536	79.2	Group Z (535 bp)
MISA-OTU-560	65.5	
MISA-OTU-570	31.0	
MISA-OTU-635	7.7	
MISA-OTU-677	1.8	

[a]Cultures isolated from the Elbe river (I. Bussmann et al. unpubl. data)
[b]From Tavormina et al. (2010)

Table 3. Rank distribution (1–13) of the sum of the presence/absence data (1/0) for all methane monooxygenase intergenic spacer analysis operational taxonomic units (MISA-OTUs) within each environment (river, coast, marine with their median salinities). MISA-OTUs with a clear preference for one environment are written in **bold**

	River (PSU = 0.5)	Coast (PSU = 15.2)	Marine (PSU = 28.9)	Distribution pattern
MISA-OTU-363	9	9	9	Rare and even
MISA-OTU-407	11	11	13	
MISA-OTU-635	11	10	10	
MISA-OTU-677	13	11	12	
MISA-OTU-419	1	3	2	Dominant and even
MISA-OTU-536	3	3	3	
MISA-OTU-513	4	6	5	None
MISA-OTU-445	**1**	**1**	**6**	**Riverine preference**
MISA-OTU-471	**8**	**13**	**11**	
MISA-OTU-570	**6**	**7**	**8**	
MISA-OTU-430	**10**	**8**	**7**	**Marine preference**
MISA-OTU-486	**5**	**2**	**1**	
MISA-OTU-560	**6**	**5**	**4**	

Several MISA-OTUs discovered in our environmental samples could have been assigned to corresponding methanotrophic strains (MISA-OTU-407, -419, -430 and -471). However, most of the discovered MISA-OTUs in this study did not match any known methanotrophic strain (MISA-OTU-363, -445, -486, -513, -536, -560, -570, -635 and -677; Table 2). The most prominent strain, MISA-OTU-419 (frequency of 90%), could not be assigned to a single strain but corresponded to several reference strains. Several MISA-OTUs detected in the environmental samples corresponded to the strains *M. albis*, *M. fluvii* and *M. psychrotolerans* Eb1, which had been isolated from the Elbe River (Stn #659, I. Bussmann et al. unpubl. data; Table 2).

Single MISA-OTUs could be related to the riverine, coastal or marine environment, respectively (Table 3). Several MISA-OTUs were evenly distributed across the environments, either with low or high frequency or no obvious pattern (MISA-OTU-677, -635, -407 -363 and OTU-536, -419 and -513). However, 3 MISA-OTUs (MISA-OTU-445, -471 and -570) preferred the riverine environment, i.e. they had the lowest ranks in the river (e.g. rank 6/7/8 for MISA-OTU-570 in riverine, coastal and marine environments, respectively). Low rank and a preference for the marine environment was shown by MISA-OTU-430, -486 and -560 (ranks 10/8/7 and 5/2/1 and 6/5/4 in riverine, coastal and marine environments, respectively).

The same analysis was applied to the different MOX levels (Table S3). We classified our MOX data into 3 levels: low activity (<10 nmol l^{-1} d^{-1}), medium activity (>10 and <100 nmol l^{-1} d^{-1}) and high activity (>100 nmol l^{-1} d^{-1}). Most MISA-OTUs were evenly distributed across different levels of MOX, either with low or high frequency or no pattern evident (MISA-OTU-677, -635, -570, -536, -470, -445, -407 and -363). Two MISA-OTUs occurred mostly at high levels of MOX (MISA-OTU-513, -430) and 2 (MISA-OTU-486, -560) occurred mostly at low levels of MOX.

When applying the same analysis for the different seasons with their characteristic temperatures, MISA-OTU-536 could be assigned to a warm preference and MISA-OTU-430 preferred a cold environment (Table S4).

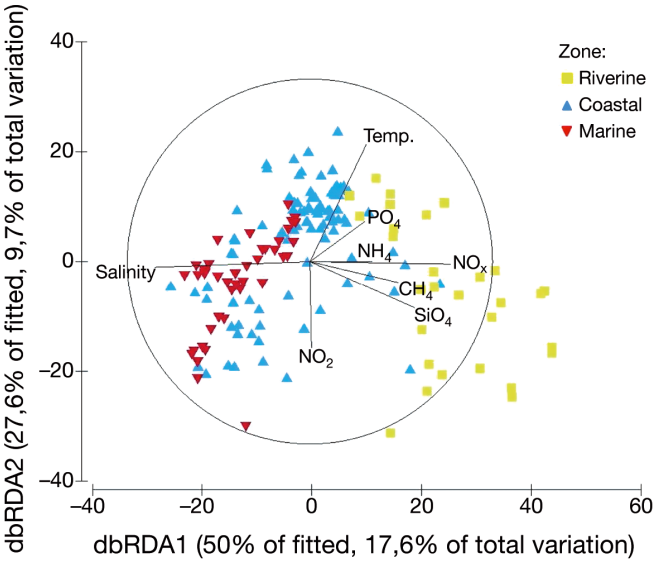

Fig. 5. Resemblance matrix based on the Jaccard index. Vector overlay of variation explaining variables (Temp: temperature; PO_4: phosphate; NH_4: ammonium; NO_x: nitrogen compounds; CH_4: methane; SiO_4: silicate; NO_2: nitrite)

A distance-based linear model of the MISA-OTUs was applied to detect potential drivers of the observed diversity patterns (Fig. 5). The model explained 35.2% of the total variation, wherein salinity accounted for the largest portion (14%), followed by temperature (5.5%), nitrate (3.8%) and CH_4 (2.5%).

Diversity (number of MISA-OTUs station^{-1}) was significantly different among riverine, coastal and marine waters (Kruskal-Wallis test, H = 7.23, p = 0.03) with the highest values in marine and coastal waters (median: 6 MISA-OTUs station^{-1}) versus only 4 MISA-OTUs station^{-1} in river water. In contrast, diversity was distributed evenly among seasons. No correlation was found between the diversity of methanotrophs and their activity (MOX or k') or their abundance by multiple linear regression analysis (Table S2). Salinity and SPM, as well as the concentrations of oxygen and phosphate revealed a significant correlation with diversity (Table S2).

4. DISCUSSION

4.1. Abundance of methanotrophs

In freshwater (river and lakes) and marine areas the minimum numbers of MOB have been reported to range from 10^1 to 10^4 cells l^{-1} (Steinle et al. 2015, Samad & Bertilsson 2017). In freshwater the maximum numbers are about 10^5 cells l^{-1}, while at marine seep sites up to 10^8 cells l^{-1} have been reported (Table 4). The absolute abundances of MOB detected in this study ranged from 5.75 × 10^3 to 8.03 × 10^6 cells l^{-1} in the river, and from not detected to 4.78 × 10^6 cells l^{-1} at the marine sites, which is well within the range reported in the literature. The relative abundance in our study ranged from 0.001–1.773% (median: 0.052%) and coincides with the ranges of 0.13–1.3% and 0.1–0.6% reported for freshwater lakes (Bornemann et al. 2016, Samad & Bertilsson 2017). However, at sites with high CH_4 release, much higher relative abundances have also been reported (7–9%; Bornemann et al. 2016, Steinle et al. 2015).

In a first step, the whole data set spanning the gradient from riverine water to mostly marine water was analyzed with a multiple linear regression. However, as no conclusive pattern was evident, the data were then split into 3 categories of water masses, and more simplified, but consistent patterns became evident.

The abundance of methanotrophs was only partly explained by all recorded environmental parameters in this study (Table 1). The multiple linear regression analysis (with the complete data set) revealed a significant positive influence of dissolved oxygen and temperature on MOB abundance, and to a lesser extent, nutrient concentrations. Other studies that quantified MOB with qPCR revealed oxygen and

Table 4. Compilation of methanotrophic abundance in different aquatic environments. qPCR: quantitative polymerase chain reaction; FISH: fluorescence *in situ* hybridization

Source	Area	Min. (cells l^{-1})	Max. (cells l^{-1})	Method
This study	Elbe River	6 × 10^3	1 × 10^8	qPCR
	North Sea	2 × 10^2	8 × 10^8	
Bussmann et al. (2017)	Lena Delta	4 × 10^4	3 × 10^6	qPCR
Samad & Bertilsson (2017)	Swedish lakes	5 × 10^4	5 × 10^5	qPCR
Osudar et al. (2016)	Siberian lakes	7 × 10^2	1 × 10^5	qPCR
	Lena River			
Crespo-Medina et al. (2014)	Gulf of Mexico	2 × 10^4	2 × 10^8	qPCR
Tavormina et al. (2010)	Pacific, non-seep sites,	2 × 10^3	2 × 10^5	qPCR
OPU-3, assuming 2 copies DNA cell^{-1}	Seep site	2 × 10^3	2 × 10^6	
Steinle et al. (2016)	North Sea	1 × 10^4	5 × 10^7	FISH
Steinle et al. (2015)	North Atlantic	1.9 × 10^1	3.0 × 10^7	FISH

phosphate as positive influences. In Samad & Bertilsson (2017), however, these parameters were observed in stratified lakes and their concentrations were much lower (and thus probably also limiting) than in our study area. However, the same parameters (oxygen and nutrients) that had a positive effect on abundance had a negative influence on methanotrophic activity (MOX and *k'*). This would indicate that the same environmental parameters should have contrasting effects on activity and abundance. For the relative abundance of MOB, none of the recorded environmental parameters seem to be important. In addition, abundance and relative abundance were not significantly different in our environmental or seasonal classes (Table 1). Therefore, in our study, we can not reveal any plausible environmental parameter that influences methanotrophic abundance.

4.2. Activity and abundance

The microbial activity of a sample is the combination of its cell number (population size) and the activity of each single cell (log-MOX versus log-MOB abundance; Röling 2007). Thus, higher activity is based either on more cells with constant activity or a constant number of cells that are more active. In our study, the slope of this correlation was different from zero (slope = 0.48; Fig. 4). Thus, the MOX activity was controlled both by changes in population size and by a changed activity per cell. Other water column studies of MOB have confirmed this relationship between MOX and MOB abundance (Sundh et al. 2005, Siljanen et al. 2011), but mostly without being quantitative. However, in our study, the relationship between activity and abundance was rather weak ($r^2 = 0.17$; Fig. 4), indicating that higher activities were related only to a minor extent to higher abundance.

This weak relationship may also be due to methodological constraints. With the radio tracer method we measured *in situ* activity with a much lower detection limit, in contrast to other studies with 'artificial' potential methanotrophic activity (Sundh et al. 2005, Siljanen et al. 2011). In addition, the intercept of the *y*-axis revealed the number of MOB that showed no activity ($4.5 \pm 1.2 \times 10^4$ cells l^{-1}; Fig. 4). As qPCR quantifies the total amount of *pmoA* genes present in a sample, irrespective of whether the cells are actively growing, inactive members of a population are also detected. When we relate this inactive part of the population to the median of all observed numbers (6.07×10^4 cells l^{-1}), about 70% of the population accounted for is inactive. For the marine population, this implies that with a median abundance of only 2.75×10^4 cells l^{-1} on average the total population was inactive. However, at times with higher abundances the marine population would still contain active MOB.

In our study, higher activities were related only to a minor extent to higher abundances; thus, cell-specific activity (i.e. moles of oxidized CH_4 per cell and time) was also considered: the riverine MOB were more active on the cellular level than the MOB in coastal and marine waters (1.8 vs. 0.3 and 0.7 fmol $cell^{-1}$ h^{-1}, respectively). When corrected for the inactive part of the population, the cell-specific activities were 1.9, 0.3 and 0 fmol $cell^{-1}$ h^{-1}, respectively. This is in contrast to the study of Steinle et al. (2015), where a low but constant cell-specific activity was observed. Higher or lower MOB abundance resulted in switches between high and low activities. Especially in the coastal and marine environments, we observed a high percentage of inactive cells due to low cell-specific activities. This fact may be one reason that no distinct environmental parameter influenced MOX and MOB abundance. The reason for the low cell-specific activities in the marine environment could be the dilution of riverine MOB into the marine environment where higher salinities inhibit their activity. There are several reports on inactive or dormant methanotrophs in the environment, but there is little information on their percentage of the whole population (Pester et al. 2004, Schubert et al. 2006). However, especially in a dynamic environment, a microbial seed bank is an important prerequisite to maintain functioning (Krause et al. 2012).

4.3. Methanotrophic diversity

The MISA method used in this study generated the first successful fingerprinting of the methanotrophic population in a temperate estuary. Until now, only 2 studies had applied MISA to environmental samples: Tavormina et al. (2010) and Bussmann et al. (2017). We were able to identify a diverse number of MISA-OTUs (up to 13 distinct MISA-fragments) for which methanotrophic strains or cultures with single OTUs were characteristic. As already shown by Tavormina et al. (2010), we revealed one prominent MISA-OTU, MISA-OTU-419, which was related to several known methanotrophic strains. MISA-OTU-419 also occurred in most of our environmental samples and no environmental preference could be assigned to this MISA-OTU. With MISA it is not possible to connect the MISA-fragment to a specific species identity,

therefore it might be assumed that several bacterial strains, likely with different environmental preferences, contain a MISA-OTU of the same length. Hence, several identities might hide behind MISA-OTU-419, and a reliable ecological interpretation for this MISA-OTU is thus not possible.

Comparing the occurrence of MISA-OTUs with another marine and a polar study, using the same methods, revealed a wide geographic distribution of several methanotrophic OTUs (Table 5) (Tavormina et al. 2010, Bussmann et al. 2017). Besides their detection in this study, 3 MISA-OTUs (MISA-OTU-363, -486 and -560) were also detected in the polar Lena Estuary. Another 3 (MISA-OTU-419, -430 and -472) were also detected in the marine Pacific and 2 (MISA-OTU-445 and -536) were found in all 3 regions. In contrast, 5 MISA-OTUs (MISA-OTU-407, 513, 570, 635 and -677) were only detected in the Elbe Estuary.

We also assigned ecological preferences to some of our MISA-OTUs (Table 5). MISA-OTU-430 was characterized by a preference for the marine environment, cold temperatures and high levels of activity. This OTU belongs to the clade OPU-3 and *Methylovulum psychrotolerans* strain Eb1 (Oshkin et al. 2016, I. Bussmann et al. unpubl. data). This strain is able to grow at low temperatures (4°C) in the laboratory. The *in situ* temperatures (5–7°C) at the sites where OPU-3 and MISA-OTU-430 occurred confirm the psychrophilic character of this OTU. Concerning the association of MISA-OTU-430 with high MOX, no information on OPU-3 or *M. psychrotolerans* are available.

The MISA-OTU-445 preference we defined as riverine is currently an assumption (Table 5). The corresponding OTU for the Lena Delta with the same fragment size has no riverine preference. This also holds for the respective phylotype identified in the Pacific samples (OPU-1), which was defined more as a coastal type. The other 'riverine' MISA-OTU-471 belongs to the type II methanotroph such as *Methylosinus* sp. (Table 2). These species have been described for freshwater and brackish environments (Bowman 2006), they can be cultivated up to a salinity of 10 (Krause et al. 2014) and are described as resistant towards salinity changes (Osudar et al. 2017). For MISA-OUT-570, no further information is available. For the riverine MISA-OTUs it is also possible that they were exported with the river into the marine environment, even though it is uncertain if they are active in the marine environment. Such a dilution into the marine environment has been described before for sulfate reducing bacteria; however, there was also an autochthonous and culturable marine population found offshore (Colin et al. 2017).

Examples for MISA-OTUs preferring the marine environment are MISA-OTU-560 and MISA-OTU-486. Both phylotypes also show preferences for low MOX (Table 5). MISA-OTU-560 was found in 2 estuaries (Elbe Estuary, Lena Delta), with similar environmental characteristics (median salinities: 29 and 27; MOX: 0.7 and 0.4 nmol l^{-1} d^{-1}). Unfortunately, there are few marine methanotrophic strains available in culture or culture collection (Tavormina et al. 2015, Vekeman et al. 2016) and no reference cultures with corresponding MISA-OTUs are known for either phylotype (MISA-OTU-560 or MISA-OUT-486).The ecological preference of MISA-OTU-536 is the Elbe Estuary with warm temperatures (median water temperature: 15°C; Table 5). This phylotype prefers the river/mixed environment in the Lena Estuary with a water temperature of 10°C (during the time of the study) and is assumed to relate to the group-Z phylotype (Tavormina et al. 2010). However, this is in contrast to the assigned preference of MISA-OTU-536, which is described here as having a wide marine distribution.

For most of our OTUs we were able to describe their environmental preference in accordance with the corresponding cultures or other OTUs.

Table 5. Distribution of methane monooxygenase intergenic spacer analysis operational taxonomic units (MISA-OTUs) at different locations and their environmental preferences. Dark/medium/light grey: detected at the Pacific, Lena estuary and Elbe estuary locations, respectively. Dark/light blue shading: marine/riverine preference; dark/light orange: high/low methane oxidation (MOX); dark/light green: cold/warm temperature preference; (–) not detected or no preference

MISA-OTU	Location			Salinity	Activity	Temperature
	Pacific	Lena	This study			
MISA-OTU-363	–			–	–	–
MISA-OTU-407	–	–		–	–	–
MISA-OTU-419		–		–	–	–
MISA-OTU-430		–				
MISA-OTU-445					–	–
MISA-OTU-471		–			–	–
MISA-OTU-486	–					–
MISA-OTU-513	–	–		–		–
MISA-OTU-536				–	–	
MISA-OTU-560	–					–
MISA-OTU-570	–	–			–	–
MISA-OTU-635	–	–		–	–	–
MISA-OTU-677	–	–		–	–	–

However, for several other phylotypes no further information is available. This implies a need to collect more environmental methanotrophic strains and to thoroughly describe these isolates. But the MISA method provides even more: a view on the occurrence and distribution but also the ecological preference of the OTUs in 3 different water masses displaying a salinity gradient. Some of the OTUs were identified to species level by comparing OTU length to previously described OTUs/species. This study also shows the presence of OTUs specifically detected in the Elbe Estuary, even though not identified in detail yet. It is a fact that the diversity of methanotrophs is still not covered both with respect to identity of the methanotrophs but also with respect to representative culturable isolates, e.g. for experimental studies. Additionally, further insights could be gained by next-generation sequencing, which provides an in-depth view of population structures. Metagenomic and metatranscriptomic analyses are needed to understand the distribution, diversity and activity of methanotrophic populations in the environment. Identification of functional genes and proof of activity with regards to MOX is highly relevant in order to describe the capability of methanotrophic populations (Dumont et al. 2013, Padilla et al. 2017, Rissanen et al. 2018).

The most prominent environmental parameter relating *in situ* activity, abundance and community structure of MOB was salinity, mostly in the form of 3 different water masses. This parameter was used to define the broader environmental categories riverine, coastal and marine. However, the question of a straightforward relationship between environmental parameters and the methanotrophic bacterial community remains unresolved.

The River Continuum Concept hypothesizes that microbial communities change along the aquatic continuum to adapt to the inefficiencies of upstream communities by forming new communities adapted to consume resources released from upstream environments (Vannote et al. 1980). However, this model does not apply to the methanotrophic community because the substrate — CH_4 — remains unaltered along the continuum.

A study based on microbial gene abundance and expression patterns of the whole microbial population suggests that river plumes host transitional mixtures of organisms that grow into more stable communities under elevated growth conditions, and that the composition of these communities depends not on salinity but instead on factors that drive those high growth conditions (Fortunato & Crump 2015). Referring to the methanotrophs, it seems to be the other way around, as activity and abundance decrease towards marine areas.

Experiments with methanotrophic cultures and natural populations suggest that marine methanotrophs are more sensitive to changes in salinity than the freshwater populations (Osudar et al. 2017). With regard to salinity, 10 PSU seems to be a threshold as the lower limit for growth of marine methanotrophs (Hirayama et al. 2013, Tavormina et al. 2015). Thus, the 3 different environments are characterized by different regimes of CH_4 concentration and nutrients; however, none of those parameters seems to be as critical as salinity in shaping the methanotrophic community.

Data archive. The data used in the publication can be found at www.pangaea.de (https://doi.org/10.1594/PANGAEA.897351).

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