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# Activity and occurrence of methane oxidizing bacteria in the water column along the River Elbe

Ph.D. Thesis

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**Annotation**

During this PhD. thesis, the importance of methane-oxidizing bacteria (methanotrophs) and their ecological demands were studied on the longitudinal transect along an important European river - the River Elbe. However, it was necessary to adjust methodologies for precise measurements of methane oxidation in such a variable aquatic environment. Based on laboratory experiments and field measurements, several key methodological recommendations for future planning of methane oxidation rate estimations in an unknown environment have been identified or specified. In line with the variability of the river habitats, considerable heterogeneity was also found in the obtained data on methane concentration and methanotrophical activity. Probably, some of the most important information gathered during many field sampling campaigns is that sites with the highest methane concentration usually showed a very low activity of methanotrophic bacteria (resulting in higher methane emissions). These sites are predominantly human modified sections of the river, such as locks, weirs, harbors and canals. On the contrary, the free-flowing parts of the river, modified only by groynes, showed low level of methane concentration. And so groynes could represent a more effective solution and "natural-close" habitats of navigability of rivers.

**Declaration [in Czech]**

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Anna Matoušů

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**List of papers and author's contribution**

The thesis is based on the following papers (listed chronologically):

1. Bussmann I., **Matoušů A.**, Osudar R., Mau S. (2015) Assessment of the radio $^{3}H$-$CH_4$ tracer technique to measure aerobic methane oxidation in the water column. Limnology and Oceanography: Methods 13: 312–327. doi: 10.1002/lom3.10027

*AM was responsible for parts of the experiments, analyses and data evaluation, she contributed to the improvement of the method; participated on the manuscript revision.*

2. Osudar R., **Matoušů A.**, Alawi M., Wagner D., Bussmann I. (2015) Environmental factors affecting methane distribution and bacterial methane oxidation in the German Bight (North Sea). Estuarine, Coastal and Shelf Science 160: 10–21. doi: 10.1016/j.ecss.2015.03.028

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3. **Matoušů A.**, Osudar R., Šimek K., Bussmann I. (2016) Methane distribution and methane oxidation in the water column of the Elbe estuary, Germany. Aquatic Sciences, in press. doi: 10.1007/s00027-016-0509-9

*AM was responsible for part of the sampling campaigns, analyses and evaluation of the data, she wrote the manuscript.*

4. **Matoušů A.**, Rulík M., Tušer M., Bednařík A., Šimek K., Bussmann I. Methane dynamics in a large river – a case study of the River Elbe. Manuscript in prep.

*AM organized all sampling campaigns, analyzed a major part of the samples, evaluated the data and wrote the manuscript*

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## 1. Background rationale of the thesis

### 1.1. Importance of methane in the environment

Due to systematic measurements of the global tropospheric methane ($CH_4$) mixing ratios since 1978, it has become obvious that $CH_4$ acts as the most important anthropogenic greenhouse gas (GHG), after water vapor and carbon dioxide ($CO_2$) (e.g., Steele et al. 1987). This is mainly due to its significant contribution to the global warming with the ability to absorb infrared radiation at a relatively higher efficiency per molecule than $CO_2$ (Lelieveld et al. 1993). Analyses of the air trapped in ice cores taken from the Antarctic and Arctic regions showed that $CH_4$ atmospheric concentration has increased by 150% since pre-industrial times (Etheridge et al. 1998), with some projections indicating a further doubling by the year 2100 (www.esrl.noaa.gov/gmd/ccgg/iadv/). Besides, the analyses revealed increased emissions in the Northern Hemisphere (Fung et al. 1991) where major anthropogenic sources exist - landfills, biomass burning, gas drilling, coal mining, waste treatments, rice agriculture or intensive livestock farming due to $CH_4$ production in stomachs of cud-chewing animals. However, $CH_4$ is emitted also from natural sources, including wetlands (i.e. swamps, fens, or tundra), oceans, termites or freshwaters (i.e. lakes, reservoirs, rivers, and streams). The major individual microbial source of $CH_4$ is represented by wetlands and flooded rice fields (which could be actually seen as a sort of anthropogenic wetlands).

Long time has been thought that $CH_4$ production in freshwater habitats, like lakes and rivers, is an insignificant source of atmospheric $CH_4$. However, estimates on the contribution of $CH_4$ emissions to the global atmospheric budget from freshwater habitats have been refined in the light of new scientific discoveries. And so studies performed during the last four decades have confirmed that $CH_4$ production is considerably more important in freshwater habitats than predicted (Hamdan and Wickland 2016). Still, owing to their large morphological, ecological and geographical heterogeneity, as well as diverse anthropogenic impact on these ecosystems, we still undergo to large uncertainties about their position in the global $CH_4$ budget.

### 1.2 The microbial methane cycle

Biogenic methane is produced by methanogenic Archea (Garcia et al. 2000) during the final stages of organic matter degradation. This process is stricktly anaerobic and so occurs only in the absence of oxygen or other oxidants, i.e.

nitrate, sulphate, or ferric iron. There are two pathways of biogenic $CH_4$ production - either via acetate fermentation (i.e. acetotrophic methanogens convert acetic acid to $CH_4$ and $CO_2$) or via $CO_2$ reduction (i.e. hydrogentrophic methanogens convert $CO_2$ with hydrogen to $CH_4$), (Conrad 2007). In aquatic ecosystems $CH_4$ may be transported from the sediments into the water, through the water column into the atmosphere by several pathways: Via a simple diffusive flux, sediment resuspension, plant-mediated flux, or in case of high $CH_4$ concentrations by ebullition flux (e.g. Goodrich et al. 2011; Bussmann 2005; Bastviken et al. 2004; Van der Nat and Middelburg 2000; Whiting and Chanton 1996).

As counterpart to $CH_4$ production, the microbial consumption and oxidation of $CH_4$ (MOX) may provide a so called natural "biofilter" reducing the effective flux of $CH_4$ from the sediments to the water column and, to a lower extent, from the water column to the atmosphere (Frenzel et al. 1990, Krüger et al. 2005). Methane could be oxidized at different levels. Already inside the anoxic sediments, $CH_4$ can be oxidized by specialized microbes via so called microbial anaerobic oxidation of $CH_4$ (AOM). In this process, the $CH_4$ oxidation is coupled with sulphate reduction:

(1) $CH_4 + SO_4^{2-} \rightarrow HS^- + HCO_3^- + H_2O$

Anaerobic $CH_4$ oxidation occurs in freshwater wetlands, but mainly in the deep marine sediments, where it is responsible for reducing up to 50-80% of possible $CH_4$ emissions (Reebough 2007; Segarra et al. 2015).

In oxic freshwater sediments, $CH_4$ is mostly oxidized by aerobic methane oxidizing bacteria (MOB; or so called methanotrophs) at the oxycline - either at the top sediment layer or in the water column. Methanotrophs are a subset of bacteria (called Methylotrophs) capable of utilizing one-carbon compounds (besides $CH_4$ also methanol, methylamine, formate, carbon monooxide, and dimethylsulfide; Bowman 2006). Thus, they do not have the ability to grow on organic compounds with carbon-carbon bonds (Bowman 2006) but they use $CH_4$ as their sole source of energy and carbon. Methane can be either completely oxidized to $CO_2$ (Equation 2) or partially oxidized and partially assimilated into the microbial biomass ("x"; Equation 3):

(2) $CH_4 + 2O_2 \rightarrow CO_2 + H_2O$

(3) $CH_4 + (2-x)O_2 \rightarrow (1-x)CO_2 + CH_2O + (2-x)H_2O$

Traditionally, MOB have been divided into two major groups: Type I with the subgroup type X, and type II, belonging to the γ- and α-*Proteobacteria*, respectively (Hanson and Hanson 1996). The biology of type I and type II differs besides their phylogeny, also in chemotaxonomy, internal membrane structure, carbon assimilation pathways or type of enzymes (summary in Tab. 1; Fig. 1).

The type I belongs into the family *Methylococcaceae*, which has six genera: *Methylococcus* (the type genus), *Methylocaldum*, *Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylosphaera*. The first two genera are also referred to as type X, and this group is distinguished by certain/specific physiological, biochemical and phylogenetic characteristics. The type II is grouped in a family called *Methylocystaceae* with *Methylocystis* (type genus) and *Methylosinus* as the member genera (Bowman 2006).

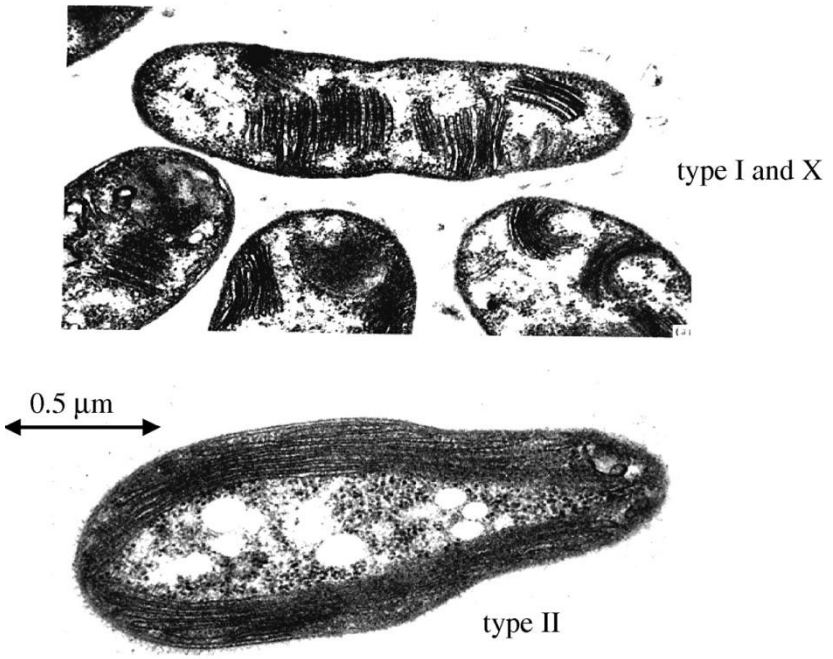


**Figure 1**
Transmission electron micrographs of sections of type I and X and type II methane-oxidizing bacteria (Dalton 2005).

Only small percentage of MOB can be grown in a pure culture, because of their low abundance, slow growth, eventual metabolic dependence on co-occurring bacteria, and contamination of non-methanotrophs on agar isolation

plates. Most-probable-number counting based on liquid media under specific $O_2$:$CH_4$ atmosphere gives better results (Bender and Conrad 1994; Bowman et al. 1997; Bussmann et al. 2006; Escoffier et al. 1997) with the added advantage that MOB can be isolated more easily from the highest dilutions. Hence, cultivation independent molecular methods and field measurements have been frequently used to assess the diversity and activity of methanotrophs present in a natural environment.

Methanotrophs are seen nearly as "heroes" not only by reducing the contribution of $CH_4$ emissions to global warming, but they also play important role in applied microbiology and biochemical engineering, including bioremediation of pollutants. This ability is provided by an enzyme called methane-monooxygenase (MMO). This enzyme catalyzes the first step of their metabolism - oxidation of methane to methanol (Bowman 2006). In addition MMO can oxidize also alkanes, alkenes, alicyclics, aromatics, ethers, heterocyclics and ammonia (Jiang et al. 2010). There are two forms of MMO: a cytoplasmic soluble MMO (sMMO), and a membrane-bound or particulate MMO (pMMO) (Murrell et al. 2000). All methanotrophs contain the pMMO, but some are also capable of producing the sMMO which determines their position in the environment (see below).

### 1.3 Methods for studying aerobic methane-oxidizing bacteria

With the least effort, activity of aerobic MOB in the water column can be determined by an "old fashioned" yet useful method measuring $CH_4$ dynamics in a created headspace using gas-chromatograph analysis (e.g., Abril and Iversen 2002; Abril et al. 2007; Carini et al. 2003; Dzyuban 2002, 2010; Pasche et al. 2001; Zais et al. 1982). Methane oxidation rate is then calculated by quantifying the decrease of $CH_4$ in headspace over time. Unfortunately, the disadvantage of this method is its very low sensitivity at those sites where low rates are expected. In these cases the use of a radiotracer method is not only a useful but probably mandatory tool.

Radiotracer method is based on transformation of the added radioactively labeled $CH_4$ (either $^{14}C$-$CH_4$ or $^{3}H$-$CH_4$) in the oxidation products over an incubation period at near in situ conditions. Both radiotracers have been applied in a variety of studies, in terrestrial and marine environments (e.g., Bastviken et al. 2002; Carini et al. 2005; Griffiths et al. 1982; Reeburgh et al. 1991; Schubert et al. 2010; Valentine et al. 2001). However, there are some

difficulties and differences in using both tracers. For example, $^{3}H$-labeled $CH_4$ is more sensitive as a tracer, because it is commercially available at considerably higher activities. Thus the $CH_4$ concentration added to the enclosed samples is relatively low, which minimizes the possible bias related to sample amendments and keeps its concentration fairly close to those at in situ conditions. On the other hand, $^{14}C$-methane is often preferred in sulphidic environments, as $CH_4$-bound tritium may undergo isotope exchange reactions with sulphide-bound $^{1}H$ (Blees 2011).

There are studies that use a stable carbon isotope ($\delta^{13}C$) ratios analyses as it is well known, that the isotope signature of $CH_4$ is highly depleted due to carbon fractionation associated with methanogenesis (Sugimoto and Wada 1995) and even more due to $CH_4$ oxidation (Summons et al. 1994). In consequence, organisms that assimilate carbon originating from $CH_4$ oxidation are characterized by further depletion in stable carbon isotopes (Coleman et al. 1981). Therefore, stable isotopes have become the key tracer infollowing the fate of $CH_4$-derived carbon originating in anaerobic sediments during methanogenesis, subsequently through its consumption via MOB up to higher trophic levels via grazing (Grey 2016). In some cases these observations were coupled with other supporting evidence using MOB-specific fatty acids analysis (e.g. Sanseverino et al. 2012).

**Table 1**
Some characteristics of methanotrophs. Adapted from Amaral and Knowles (1995), Bowman (2006), Börjesson and others (2004), and Dedysh et al. (2000, 2002).

| | type I | type X | type II |
|---|---|---|---|
| Phylogenetic affiliation | γ-Proteobacteria | γ-Proteobacteria | α-Proteobacteria |
| Family | Methylococcaceae | Methylococcaceae | Methylocystaceae |
| Genera | Methylosphaera | Methylococcus | Methylosinus |
| | Methylobacter | Methylocaldum | Methylocystis |
| | Methylomicrobium | | |
| | Methylomonas | | |
| Resting stages | Azetobacter-type cysts or none | Azetobacter-type cysts | Exospores or lipoidal cysts |
| Cell morphology | Short rods, usually occur singly; some cocci or ellipsoids | Cocci, often found as pairs | Crescent-shaped rods, rods, pear-shaped cells, sometimes occur in rosettes |
| Intracytoplasmic membranes | Flat disc-shaped vesicles distributed throughout the cell | Flat disc-shaped vesicles distributed throughout the cell | Vesicles arranged along the periphery of the cell |
| Carbon assimilation pathway | Ribulose monophosphate (RuMP) | RuMP, sometimes serine | Serine |
| Major PLFA | 16:01 | 16:01 | 18:01 |
| G+C content of DNA (mol %) | 49 – 60 | 59 – 65 | 62 – 67 |
| Nitrogen fixation | - | + | + |
| Soluble methane monooxygenase | - | - | + |
| Relationship to $CH_4$, $O_2$ and T | able to utilize $CH_4$ at high concentrations, low $O_2$, T >20°C | able to utilize $CH_4$ at high concentrations, low $O_2$, T >20°C | able to utilize $CH_4$ at low concentrations, high $O_2$, T: 5°-10°C |

Nevertheless, these techniques still need to be complemented with other methods and analyses to get more detailed information about the MOB community structure and function. In the previous research, different techniques were used for culture-independent detection of aerobic MOB (e.g., Lin et al. 2005; Sundh et al. 2005; McDonald et al. 2008; Taipale et al. 2011). Among these, fluorescence in situ hybridization (FISH) combined with catalyzed reporter deposition (CARD) turned out to be a promising tool, allowing the detection and counting of MOB cells directly in the environmental samples.

### 1.4 Ecology of aerobic methane-oxidizing bacteria

Despite all the well-known methods and diverse investigations, available data about ecological demands and adaptability of MOB in aquatic environments are still scarce. Moreover, only few publications investigated both $CH_4$ concentration and $CH_4$ oxidation rates in lotic systems. One study reports on $CH_4$ dynamics in a non-tidal estuary (Abril and Iversen 2002), another in diverse small tributaries of a large Russian reservoir (Dzyuban 2011), or on a short stretch of a river in Oregon (Anthony et al. 2012). So far only Zaiss and coworkers (1982) in their study investigated a 240 km long riverine transect. Even when the recent advances in this field are taken into account, our knowledge is still insufficient in respect to the large variability of different habitats and diverse ecological aspects of lotic ecosystems. However, these are important insights into the topic because of the crucial role of MOB in the global carbon cycle. Especially due to changing climatic conditions, reflected in the significant increase in $CH_4$ dynamics, it is fundamental to detect the main factors shaping the methanotrophic activity, which may significantly enhance the amount of GHG emissions.

Microbial $CH_4$ oxidation in water column of rivers and estuaries is considered to be much less efficient (Zaiss et al. 1982; Anthony et al. 2012) than in lakes or other freshwater habitats, where high $CH_4$ concentrations occur. The contribution of $CH_4$ consumption by MOB to $CH_4$ losses can range from 5%, in turbulent rivers (Lilley et al. 1996), up to 70% in some estuaries (De Angelis and Scranton 1993; Abril and Iversen 2002). As an equivalent to primary production, the net carbon fixation via methanotrophy can represent up to 6-46% (Trimmer et al. 2010; Shelley et al. 2014) in oxygenated riverbeds.

One of the key issues is the understanding of temperature influence on MOX and MOB community structure, while temperature may affects enzymatic

activity (Börjesson et al. 2004; Mohanty et al. 2007). Studies conducted on samples from soil and landfills showed that low temperature favores the type I methanotrophs over type II methanotrophs. Temperatures around 20°C or higher are preferred by both groups, but mainly type II (Gebert et al. 2003; Börjesson et al. 2004).

Dissolved nutrients like copper, nitrate, ammonium or oxygen content can strongly affect the relative distributions of each MOB type and their activity (Graham et al. 1993; Hanson and Hanson 1996), but the key factor is probably the availability of $CH_4$ as a sole source of carbon for MOB. As noted in the Tab. 1, each type of MOB may exhibit different affinity to a $CH_4$ concentration, but also to temperature and $O_2$ (Amaral and Knowles 1995). This could actually help to predict what MOB type would be present at any given habitat. For example, in soils the type II is found more frequently than the type I, while it can utilize $CH_4$ even at atmospheric concentrations (~ 2 ppm; Ricke et al. 2005). In contrast, the type I is usually dominant in aquatic environments, where generally higher $CH_4$ concentrations occur (Costello et al. 2002). However, it was shown that MOB types tend to co-occur with other bacteria in mixed communities (Lin et al. 2005; Sundh et al. 2005; He et al. 2012; Osudar et al. 2016). Some genera within one type (e.g., *Methylocystis* sp.) have shown different affinities for $CH_4$ and their occurrence in a variety of environments (Baani and Liesack 2008). This probably relates to the synthesis of different types of $CH_4$-monooxygenase. It has been demonstrated that the cells containing a particulate $CH_4$-monooxygenase display higher growth capabilities and higher affinity for $CH_4$ than another type, so called soluble $CH_4$-monooxygenase (Hanson and Hanson 1996).

It has been found that copper ions may play a key role in regulation and catalysis of the $CH_4$-monooxygenase, thus limiting the appearance of the particulate $CH_4$-monooxygenase cells to more copper-rich environments than the soluble $CH_4$-monooxygenase producing cells (Lieberman and Rosenzweig 2004). It seems that representatives of the MOB type I are able to adapt and profit from changing environmental conditions and rapidly reproduce (r-strategists), whereas the type II MOB maintain relatively stable abundances (K-strategists), particularly in habitats with high $CH_4$ production (Vecherskaya et al. 1993; Henckel et al. 2000).

In aquatic environments, microbial $CH_4$ oxidation could be to a large extent controlled by physical processes, i.e. gas-phase diffusion and transport of

$CH_4$ to the cells (Rudd et al. 1976; Bender and Conrad 1994; Bodelier et al. 2000), rather than by the enzymatic reactions (Urman et al. 2009). Another important factor could be also light. For instance King (1990) showed that light markedly contributes to the control of $CH_4$ oxidation rates via photosynthesis by the extension of the region of MOB activity under favorable oxic conditions. On the other hand, investigations made by other scientists suggest that $CH_4$ oxidation can be inhibited by visible light (Dumestre et al. 1999; Murase and Sugimoto 2005).

In estuaries and marine environments, salinity appears to be the crucial factor controlling the $CH_4$ oxidation (De Angelis and Scranton, 1993; Conrad et al. 1995). Estuaries are also typical for their high loads of suspended particles, which could have a positive effect on $CH_4$ oxidation in such highly dynamic ecosystems where freshwater meets marine water and diverse biological, physical and chemical parameters constantly change. According to one study, $CH_4$ oxidation can be particularly enhanced in the estuarine turbidity maximum, where surface suspended particles exceed 100 mg $L^{-1}$ (Abril et al. 2007). Thus, areas of mixing waters with different suspended particular matter content and $CH_4$ concentrations could probably represent the ‘‘hot-spots’’ for $CH_4$ oxidation, while MOB transported with particles can encounter high dissolved $CH_4$ levels. Similarly, short events like floods or sediment resuspensions could be likely the ‘‘generator’’ for $CH_4$ oxidation in aquatic systems (McClain et al. 2003).

Different factor shaping the MOB abundance and activity could be represented by the so called “top-down control” represented by predation on MOB. Many studies show that MOB may provide a food source for flagellates, zooplankton and even for insect larvae (e.g., Bastviken et al. 2003; Deines et al. 2007; Jones and Lennon 2009; Ravinet et al. 2010; Jones and Grey 2011; Sanseverino et al. 2012). In terms of the effect of predation on the MOB activity (i.e. $CH_4$ oxidation), grazing can result in reduced $CH_4$ oxidizing rates and so higher $CH_4$ emissions (Bunn and Boon 1993; Kankaala et al. 2007). On the other hand, due to assimilation of $CH_4$ via MOB, $CH_4$-derived carbon may become available as carbon source for aquatic food web via bacterial consumers (Kohzu et al. 2004). This would represent an alternative pathway of energy production supporting the river food webs and communities (Trimmer et al. 2012), which could change the traditional view that aquatic food webs are fuelled only by phototrophic carbon production.

From the current observations we can assume that MOB could represent even a "good-quality food source", which is supported by findings that MOB possess significant, and within prokaryotes even quite unique, amounts of sterols and sterol-like compounds (e.g. Bird et al. 1971; Schouten et al. 2000). These compounds act as a crucial factor determining food quality for zooplankton, especially for daphnids (e.g., Martin-Creuzburg et al. 2011). Nevertheless, some authors (Kankaala et al. 2007; Agasild et al. 2014) outlined an important member of the aquatic food web - heterotrophic nanoflagellates, as an intermediate step in transferring $CH_4$-derived carbon from MOB to crustacean zooplankton or other organism not capable of direct consumption of bacteria. Heterotrophic nanoflagellates are considered as the highly abundant key group of bacterivores in a planktonic food web of marine and freshwater lentic ecosystems (lakes, reservoirs; e.g. Boenigk and Arndt 2002; Sherr and Sherr 2002; Šimek et al. 2013). However, much less attention has been paid to their role in rivers, especially if the riverine plankton community, which is controlled by different drivers than in lentic ecosystems, e.g. often by limited contribution of metazooplankton (Phillips 1995).

To give a better knowledge of the complex and varying $CH_4$ and $CH_4$-derived carbon cycles, it is important to understand the ecology of MOB, which is still an incomplete story.

## 2. Hypotheses and Objectives

1) The use of a sensitive method is essential for accurate methane oxidation rate assessments in aquatic ecosystem with wide range of methanotrophic activities.
**Methodology adjustment will be performed using experimental as well as field measurements for an effective investigation of the ecology of methane-oxidizing bacteria in diverse habitats along a large river continuum.**

2) Methane concentration will vary along a large river system in relation to the changing riverine habitats. Canalized parts with weirs and heavily polluted sites are expected to be "hot-spots" generating large amounts of methane in the water column.
**During several sampling campaigns methane dynamics will be examined along the Elbe River, representing a broad diversity of habitat-specific methane variations in a large temperate river system.**

3) Methane-oxidizing bacteria are important sink of methane in temperate riverine ecosystems. The efficiency of this "methane-biofilter" is determined by diverse water parameters and compositions of the methanotrophic communities.
**Methane assimilation processes by methane-oxidizing bacteria and "hot-spots" of their activity will be studied along the River Elbe. Important factors influencing the activity and adaptations of MOB in different "Elbe-environments" will be examined. The efficiency of this microbial "biofilter" will be quantified. Presence and abundance of the target bacteria along the river continuum will be determined using the CARD-FISH approach.**

## 3. Results and Discussion

### 3.1 Method adjustment (Paper I)

Measurements of methane oxidation rates in aquatic environments are sparse. However, using a radiotracer method may provide an easy, fast and invaluable way of MOX data acquisition in water, especially were low $CH_4$ concentrations are expected. Although this method is widely used and thus rather well established, a comprehensive review of each procedure and assumption has been absent. In addition, some important details and uncertainties should be considered before planning any experiment or a field campaign. Together with my colleagues, we evaluate this method by implementing several measurements from diverse aquatic habitats. We highly recommend, among others, to always use extra bottles for $CH_4$ concentration estimation. Although, I was able to adapt the method also for a case, when there is no other possibility than using the radioactively marked samples also for this purpose. The key prerequisite is to work very precisely - always incubate the samples at *in situ* temperatures; in case of unknown activity in some habitat better to apply a time series for an estimate of proper incubation time; not leave killed samples standing more than 4 days for later analyses; measure the total radioactivity right after opening the samples; and last but not least work gently but quickly with the scintillation mixture. The methodological upgrade described in paper I will hopefully encourage more scientists and so enable to obtain complex data on MOX rates across environments, which will be comparable among other data sets, whereas widespread MOX rate estimations are needed in order to refine the global $CH_4$ budget.

### 3.2 Methane dynamics and related methanotrophic activities in a large river (Paper II, III and IV)

The firstly suggested goal of my thesis, i.e. to cover and describe $CH_4$ dynamics, its related processes (i.e. $CH_4$ production and -oxidation), and changing bacterial communities along the Elbe River sounded quite attractive, being however, almost impossible. Although with several compromises and improvised solutions I was able to gain very interesting and new information and in the final also comprehend almost the entire transect of the River Elbe. Despite some expectations, I found inconsiderable amounts of $CH_4$ dissolved in the Elbe water and, moreover, extraordinary microbial activities that I was not able to explain through analysis of other measured parameters. The key factor seemed to be the

availability of $CH_4$, however, I found that even in $CH_4$-rich environments the methanotrophic potential was very limited, or practically undetectable (e.g. at Střekov weir)! This opens an important question of so far overlooked importance of artificial water structures, distracting river continuum, that severely limit our possibility to reasonably estimate the global GHG budget.

During the Elbe-cruise in October 2013 we were able even to outline the coupling of the $CH_4$-related processes between sediment and water column (Rulík et al., unpublished data). Our investigations will complement the existing paucity of studies revealing the $CH_4$ pathway from the sediments through the water column into the atmosphere.

## 4. Conclusions and further perspectives

Almost no quantitative assessment of the role of MOB in a river continuum of temperate zones has been currently available and there is still very limited knowledge on the environmental factors shaping these processes in rivers. The conducted sampling campaigns have brought some detailed information and new insights into the major $CH_4$ pathways and general $CH_4$ trends along a large European river. Such a longitudinally highly heterogeneous ecosystem consists of more or less natural parts, turning downstream to moderately polluted zones up to highly disturbed regions. Thus the current situation offers diverse combinations of various physical as well as chemical parameters. Regardless the fact that the key factor seemed to be the availability of $CH_4$, I found that even in methane-rich environments the methanotrophic potential was very limited, or practically undetectable. As it appears that the main drivers are rather specific local combinations of different environmental conditions co-acting at each location. It seems that near natural habitats with active biofilms and functional interactions with their surrounding (e.g. hyporheal, riparian zones) they do function better in terms of self-purification processes. This is in contrary to large-scale human altered habitats, which may lead to greater eutrophication and GHG emissions. I was also able to confirm the assumption that methanotrophy indeed serves as an important "alternative way" of carbon flow to higher trophic levels.

This topic is for me still highly challenging - each sampling cruise or experiment it has brought new questions and ideas, and so opened up a potential new understudied area for more advanced research. I hope, that information and knowledge gathered during my PhD-study have an inconsiderable potential to contribute in the further research of $CH_4$ cycle in the global GHG context. In my future work and already upcoming manuscripts, a quantitative assessment of a grazing impact and contribution on $CH_4$-derived carbon shift to higher trophic levels will bring other missing piece of the "$CH_4$ story" in aquatic environments. This could be beneficial especially in river ecosystems where a negligible importance of heterotrophic nanoflagellates is predicted. Moreover, taking into account the fact that predation on MOB can cause reduced $CH_4$ consumption and so higher emissions from the river ecosystem, such information may be crucial for understanding the global carbon cycling and climate change.

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## 6. Attached publications

## 6.1 Assessment of the radio 3H-CH4 tracer technique to measure aerobic methane oxidation in the water column

LIMNOLOGY
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# Assessment of the radio $^3$H-$CH_4$ tracer technique to measure aerobic methane oxidation in the water column

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***Abstract***

Microbial methane oxidation rates in ocean and freshwater systems reveal how much of emitted methane from the sediments is oxidized to $CO_2$ and how much can reach the atmosphere directly. The tracer-method using $^3$H-$CH_4$ provides a way to measure methane oxidation rates even in water with low methane concentrations. We assessed this method by implementing several experiments, collecting data from various environments, and including recent literature concerning the method to identify any uncertainties that should be considered. Our assessment reveals some difficulties of the method but also reassures previous assumptions to be correct. Some of the difficulties are hardly to be avoided, such as incubating all samples at the right in situ temperature or limiting the variability of methane oxidation rate measurements in water of low methanotrophic activity. Other details, for example, quickly measuring the total radioactivity after stopping the incubation, are easy to adapt in each laboratory. And yet other details as shaking during incubation and bottle size seem to be irrelevant. With our study, we hope to improve and to encourage future measurements of methane oxidation rates in different environments and to provide a standard procedure of methane oxidation rate measurements to make the data better comparable.

### *Introduction*

Measurements of methane oxidation (MOX) rates are essential to understand why the large input of methane into oceans, lakes, rivers is in opposition to the relatively low flux of the gas to the atmosphere (Reeburgh 2007). Methane ($CH_4$) is after water vapor and $CO_2$, the most important greenhouse gas with a global warming potential that exceeds carbon dioxide ($CO_2$) 34-fold over a 100 yr timescale (IPCC, 2013). Methane is produced in aquatic sediments as well as in the water itself. In sediments, methane is generated by microbial breakdown as the last step of anaerobic degradation of organic matter (biogenic methane) and by thermocatalytic processes by increased temperature and pressure in deeply buried sediments [thermogenic methane (Tissot and Welte 1984)]. In the water column of the ocean, methane production has been linked to methylphosphonic acid (Karl et al. 2008; Metcalf et al. 2012) and dimethylsulfoniopropionate (Damm et al. 2010) as substrates for methanogenesis, as well as to anaerobic microenvironments (Reeburgh 2007). Also in the water column of lakes an until now unknown process of methane production in oxygenated water has been suggested based on incubation experiments and isotope analysis (Tang et al. 2014).

Despite of all these methane sources only little of the gas actually escapes to the atmosphere. About 11 Tg $CH_4$ $yr^{-1}$ is emitted to the atmosphere from the ocean (Bange et al. 1994) contributing 2% to the ~ 550 Tg $CH_4$ $yr^{-1}$ from all natural and anthropogenic sources (IPCC, 2013). A similar quantity of 40 Tg $yr^{-1}$ (range 8–73 Tg $yr^{-1}$) originates from freshwater sources like rivers, lakes, and reservoirs (Bastviken et al. 2011; IPCC, 2013). These limited quantities are thought to be due to microbial oxidation of the gas in the water, which maintains the bulk of the ocean at low nanomolar concentrations (Reeburgh 2007).

Aerobic MOX is realized by methanotrophs, who oxidize methane with oxygen to $CO_2$ and water. Methane oxidizing bacteria are found in oxic sediments and in the water column of most marine and freshwater settings, where oxygen is available (Jensen et al. 1992; Ding and Valentine 2008;

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# Assessment of the radio $^{3}H$-$CH_4$ tracer technique to measure aerobic methane oxidation in the water column

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## Abstract

Microbial methane oxidation rates in ocean and freshwater systems reveal how much of emitted methane from the sediments is oxidized to $CO_2$ and how much can reach the atmosphere directly. The tracer-method using $^{3}H$-$CH_4$ provides a way to measure methane oxidation rates even in water with low methane concentrations. We assessed this method by implementing several experiments, collecting data from various environments, and including recent literature concerning the method to identify any uncertainties that should be considered. Our assessment reveals some difficulties of the method but also reassures previous assumptions to be correct. Some of the difficulties are hardly to be avoided, such as incubating all samples at the right in situ temperature or limiting the variability of methane oxidation rate measurements in water of low methanotrophic activity. Other details, for example, quickly measuring the total radioactivity after stopping the incubation, are easy to adapt in each laboratory. And yet other details as shaking during incubation and bottle size seem to be irrelevant. With our study, we hope to improve and to encourage future measurements of methane oxidation rates in different environments and to provide a standard procedure of methane oxidation rate measurements to make the data better comparable.

## Introduction

Measurements of methane oxidation (MOX) rates are essential to understand why the large input of methane into oceans, lakes, rivers is in opposition to the relatively low flux of the gas to the atmosphere (Reeburgh 2007). Methane ($CH_4$) is after water vapor and $CO_2$, the most important greenhouse gas with a global warming potential that exceeds carbon dioxide ($CO_2$) 34-fold over a 100 yr timescale (IPCC 2013). Methane is produced in aquatic sediments as well as in the water itself. In sediments, methane is generated by microbial breakdown as the last step of anaerobic degradation of organic matter (biogenic methane) and by thermocatalytic processes by increased temperature and pressure in deeply buried sediments (thermogenic methane; Tissot and Welte 1984). In the water column of the ocean, methane production has been linked to methylphosphonic acid (Karl et al. 2008; Metcalf et al. 2012) and dimethylsulfoniopropionate (Damm et al. 2010) as substrates for methanogenesis, as well as to anaerobic microenvironments (Reeburgh 2007). Also in the water column of lakes an until now unknown process of methane production in oxygenated water has been suggested based on incubation experiments and isotope analysis (Tang et al. 2014).

Despite of all these methane sources only little of the gas actually escapes to the atmosphere. About 11 Tg $CH_4$ $yr^{-1}$ is emitted to the atmosphere from the ocean (Bange et al. 1994) contributing 2% to the 550 Tg CH4 $yr^{-1}$ from all natural and anthropogenic sources (IPCC, 2013). A similar quantity of 40 Tg $yr^{-1}$ (range 8–73 Tg $yr^{-1}$) originates from freshwater sources like rivers, lakes, and reservoirs (Bastviken et al. 2011; IPCC, 2013). These limited quantities are thought to be due to microbial oxidation of the gas in the water, which maintains the bulk of the ocean at low nanomolar concentrations (Reeburgh 2007).

Aerobic MOX is realized by methanotrophs, who oxidize methane with oxygen to $CO_2$ and water. Methane oxidizing bacteria are found in oxic sediments and in the water column of most marine and freshwater settings, where oxygen is available (Jensen et al. 1992; Ding and Valentine 2008; Rahalkar et al. 2009; Tsutsumi et al. 2012). Aerobic methanotrophic bacteria have been classified as type I and type II methanotrophs based on their phylogenetic position, carbon assimilation pathways, and the arrangement of intracellular membranes, and they belong to the classes Gammaproteobacteria and Alphaproteobacteria, respectively (Bowman 2006). Reported turnover times of methane range from 100s of years in the open ocean (Jones 1991; Angelis et

al. 1993) to several years in the coastal seas (Heintz et al. 2012) and to days in freshwater environments (Abril and Iversen 2002). Besides the aerobic oxidation of methane, it can also be oxidized in the absence of oxygen, that is, in sediments and in anoxic waters. Especially the oxidation in sediments by mostly archaea but also some bacteria plays an important role in reducing the methane flux from the sediment into the water column (Krüger et al. 2005).

Several techniques have been implemented to qualify and quantify the microbial methane consumption. MOX can be measured by the decrease of methane concentration over time (Scranton and Brewer 1978; Abril et al. 2007), the change in isotopic composition (Bastviken et al. 2002) or by a combination of stable isotope and conservative tracer measurements (Rehder et al. 1999; Heeschen et al. 2004). However, adding a radioactive tracer such as $^{3}H\text{-}CH_4$ (Valentine et al. 2001; Mau et al. 2013) or $^{14}C\text{-}CH_4$ (Reeburgh et al. 1992; Pack et al. 2011) makes quantification more sensitive.

Especially the method using $^{3}H\text{-}CH_4$ has become more and more established over the last decade as sample processing requires only a few steps, which can be carried out on board a research vessel, the tracer has become commercially available, and it has several advantages compared to $^{14}C\text{-}CH_4$ (Table 1). $^{3}H\text{-}CH_4$ has a higher specific activity ($3.7 – 7.4 \times 10^{11}$ Bq mmol$^{-1}$) than $^{14}C\text{-}CH_4$ with $3.7\text{-}18 \times 10^{7}$ Bq mmol$^{-1}$. Thus, the methane concentration of a water sample is changed by <3 nmol l$^{-1}$ $^{3}H\text{-}CH_4$ whereas the addition of $^{14}C\text{-}CH_4$ increases methane concentration of a sample by up to 500 nmol L$^{-1}$. This significantly alters the concentration of the gas especially in samples collected from environments with low in situ methane concentrations. However, recently also a low-level $^{14}C\text{-}CH_4$ method has been proposed, where $^{14}C$ is measured with accelerator mass spectrometry (Pack et al. 2011). Furthermore, the $^{3}H\text{-}CH_4$ has the advantage of a higher permitted radioactivity ($10^{9}$ Bq) compared to $^{14}C$ with $10^{7}$ Bq, which can be transported without special license (German Radiation Protection Ordinance, 2001, and ADR 2.2.7.1.1, Tab. 2.2.7.2.2.1, U.S. DoT, CFR Part 173.436). The working limits seem to differ in each country, thus, we will not give further information here. For transportation with ships, documents according to the IMO are recommended (IMO class 7, UN number 2910). For transportation with airplanes, documents according to the IATA are recommended (IATA DGR 10.3.1, Tab 10.3.A).

**Table 1**
Comparison of the main characteristics of $^{3}H$ labeled methane vs. $^{14}C$-labeled methane.

| | $^{3}H$-methane | $^{14}C$-methane |
|---|---|---|
| Reaction | $^{3}H\text{-}CH_4 + 2O_2 \Rightarrow CO_2 + 2\ ^{3}H\text{-}H_2O$ | $^{14}C\text{-}CH_4 + 2O_2 \Rightarrow\ ^{14}C\text{-}CO_2 + 2H_2O + {}^{14}C$ - biomass |
| Specific activity | High (0.37–0.74 TBq mmol$^{-1}$), thus, < 3 nmol L$^{-1}$ methane are added to a water sample | Low (37–185 MBq mmol$^{-1}$), thus, < 500 nmol L$^{-1}$ methane are added to a water sample |
| Tracer storage | Limited tracer storage due to decomposition of the tracer over time | No problems with tracer storage known to date |
| Lab-equipment | GC for methane concentrations<br>Liquid scintillation counter for radioactivities. | GC for methane concentrations<br>Liquid scintillation counter for radioactivities.<br>Tube furnace and shaker for quantification of $^{14}C\text{-}CH_4$ and $^{14}C\text{-}CO_2$<br>Filtering devices for biomass determination. |
| Environment | Applicable to aerobic environments only | Applicable to aerobic and anaerobic environments |
| Exemption limit | Higher exemption limit | Lower exemption limit |
| Ionization energy | Low ionization energy (19 keV), thus, possible interference with chemo luminescence | Higher ionization energy (156 keV) less interference with chemo luminescence |

Regardless of the radiotracer, more and more often tracer incubations are conducted on board of research vessels, where similar to any laboratory country specific radiation regulations apply. The transport of the radioactive tracer, radioactive samples, and radioactive waste has to be organized according to these safety regulations. Ideally, the handling is done in an isotope van, especially if other parties of a cruise are interested in the natural abundance of $^{3}H$ or $^{14}C$. Areas on deck, where incubations are carried out, should be monitored for potential spills on a regular basis and these areas should be regularly hosed. This is particularly important on small vessels with too little space for an isotope van. Furthermore, sufficient ventilation should be assured by an open window, open door, or working outside if no ventilation hood is provided. Of principle importance are regular wipe tests to screen for any potential contaminations. With sufficient care the radiotracer technique provides a great tool to investigate and quantify microbial methane oxidation.

Although we describe here a rather established method, we felt the need to publish an extensive testing of the method to get the best results for current and future users. The idea arose during the Pergamon workshop in Kiel in 2011 where several parties met to discuss MOX rate measurements. Every user tested the method by implementing different experiments. Here, we summarize the tests to make the method of measuring MOX by adding $^{3}H\text{-}CH_4$ more comparable between laboratories, to facilitate it for newcomers and also to report on the little tricks and pitfalls hidden in the detail of every method.

**Material and methods**

To test parts of the MOX rate measurements using $^{3}H\text{-}CH_4$ as tracer, we followed the general method described below. Deviations and verifications of this protocol are indicated in the assessment part.

**Methane oxidation rate**

Water is sampled directly from Niskin bottles attached to a CTD/rosette. A 10–20 cm long tubing, which fits to the stop-cock of the Niskin bottle and reaches to the bottom of the sampling bottle, is used to collect water from the water sampling device. Water samples are collected in 12–160 ml glass bottles by filling the bottle from the bottom to the top and flushing the bottle twice to minimize contact of the sampled water with the surrounding air avoiding changes of the in situ methane and oxygen concentrations. The glass bottles are then closed avoiding air bubbles with rubber stoppers and are crimp sealed. Several water samples have to be collected: two to three bottles for determination of MOX rates, one bottle as killed control, and one to two bottles for analysis of in situ methane concentrations.

After crimp sealing of the water sample, the radioactive tracer is added to the sample and poisoned control bottles. $^{3}H\text{-}CH_4$ (as gas), commercially available from Biotrend (Köln, Germany) or from American Labeled Chemicals (St. Louis) is added by syringe using a second needle to allow for displacement of water. The amount of added $^{3}H\text{-}CH_4$-tracer has to be adapted to the environment, it should be high enough to produce a measurable quantity of $^{3}H\text{-}H_2O$, and as low as possible, to minimize any significant methane concentration changes in the water sample.

The addition of the $^{3}H\text{-}CH_4$ is >1000 Bq resulting in methane addition in the pico molar range ($10^{-12}$ mol). After injection of the $^{3}H\text{-}CH_4$, all bottles are vigorously shaken for at least 30 s to equilibrate the gaseous tracer with the liquid phase.

The samples are then incubated in the dark at near in situ temperature. After incubation for hours until days, microbial activity is stopped by poisoning or the samples are directly processed.

Then the total radioactivity ($^{3}H\text{-}CH_4$ and $^{3}H\text{-}H_2O$) added to the sample and the product of the oxidation, $^{3}H\text{-}H_2O$, have to be measured in the sample and control bottles (Fig. 1). To determine the total radioactivity of the sample, the sample bottll (or control bottle) is opened and 1-2 ml subsample is pipetted into

a seven milliliter scintillation vial. Five milliliter scintillation cocktail is added to the subsample. The scintillation cocktail should be specific for $^{3}H$-samples and aqueous solutions (e.g., Ultima Gold LLT from Perkin Elmer). Scintillation cocktail and sample are mixed by shaking the scintillation vial and counted in a liquid scintillation counter. This can be a laboratory-based counter (e.g., from Perkin Elmer) or a small, transportable one (e.g., Triathler from Hidex, Finland). Decays per minute (dpm) are calculated by the instruments based on the efficiency determined from the internal quench correction and calibration.

After counting the total radioactivity ($^{3}H$-$CH_4$ and $^{3}H$-$H_2O$) of the sample, the $^{3}H$-$CH_4$ is removed from the sample and the remaining $^{3}H$-$H_2O$ counted. For the removal of the $^{3}H$-$CH_4$, part of the sample is discarded to prevent overflow when sparging the sample with nitrogen or air. A synthetic air or a nitrogen gas bottle is connected to a sparging device that consists of several long needles or tubes to process more than one sample at a time. During expeditions, we also used an aquarium pump. The needles or tubes should reach nearly to the bottom of the bottle (i.e., be ~ 10-mm long) and the tubes should have a small diameter (~ 1 mm) to leave sufficient space for ventilation at the neck of the bottle. After sparging the sample for at least 30 min, a 1–2 ml subsample is mixed with five milliliter scintillation cocktail in a seven milliliter scintillation vial and counted in a liquid scintillation counter.

**Methane concentration**

To calculate the MOX rate, the methane concentration of the respective sample has to be known. Commonly, an additional water sample is collected from the same Niskin bottle and poisoned to stop microbial MOX. The sample is then stored cold until methane concentration analysis. However, if the samples were manipulated by adding different amounts of $^{3}H$-$CH_4$ or plain $CH_4$, it is necessary to measure the methane concentration in each sample before measuring its radioactivity (Fig. 2). Therefore, a 10 ml syringe without piston is inserted into the killed sample (120-160 ml sample). With a second syringe 10 ml of nitrogen are injected into the sample bottle while water flows into the syringe without piston assuring atmospheric pressure conditions in the sample bottle. Part of this water is used for measurement of the total radioactivity ($^{3}H$-$CH_4$ and $^{3}H$-$H_2O$; see above). The sample bottle with headspace is shaken and left standing for at least one day to equilibrate. An aliquot of the headspace is then analyzed with a

gas chromatograph (GC) equipped with a flame ionization detector to determine the methane concentration.

**Calculation**

After measuring methane concentration, the total radioactivity ($^3$H-$CH_4$ and $^3$H-$H_2O$) and the radioactivity of the produced water ($^3$H-$H_2O$), turnover time (s in d) and MOX rates (nmol $L^{-1}$ $d^{-1}$) can be calculated assuming first-order kinetics (Valentine et al. 2001):

(1) $$k' = \frac{\left(\frac{^3H-H_2O}{^3H-CH_4 + {}^3H-H_2O}\right)}{t}$$

(2) $$MOX = k' \times [CH_4]_{in\ situ}$$

(3) $$\tau - \frac{1}{k'} = \frac{[CH_4]}{MOX}$$

where k´ is the first-order rate constant calculated as the fraction of $^3$H-$CH_4$ oxidized per unit time (t) and $[CH_4]_{in\ situ}$ is the ambient methane concentration in nmol $L^{-1}$. The methane concentration should be measured in separate bottles, without tracer addition. k´ was termed the pseudo first-order rate constant for the methane transport into the cells at constant cell population by Button (1991). The turnover time can also been seen as an indication of the relative activity of various water samples as described by Koschel (1980).

The radioactivity of the $^3$H-$H_2O$ fraction in the killed controls is used to check for the amount of not biologically produced water. In case this background value is >1% the radioactivity of the total fraction, it should be subtracted from the sample value (Jørgensen 1978). In marine waters about 0.1% of the injected tracer was found to be “abiotic water.” In freshwaters the percentage can increase to about 5%.

**Statistics**

Wilcoxon Rank Sign Tests for nonparametric data were performed with Kaleidagraph 4.1.

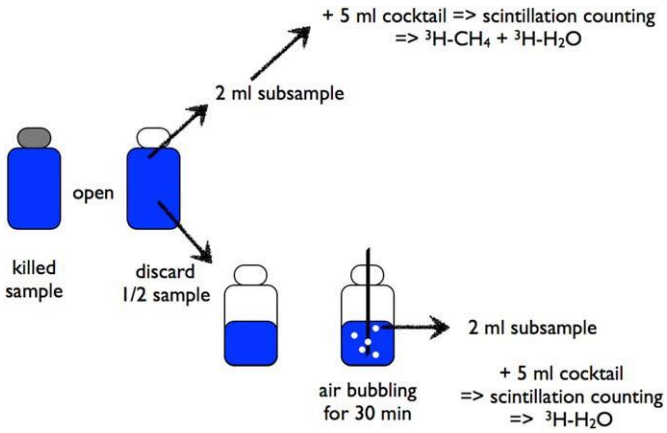


**Figure 1**
Scheme for analysis of the $^3$H-radioactivity in the total fraction and in the water fraction of a sample (or control bottle).

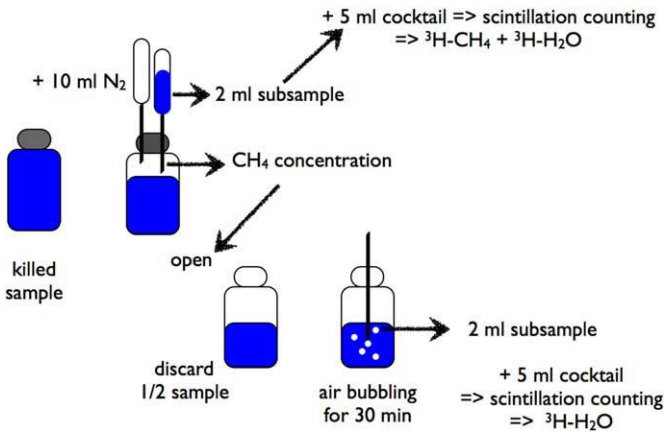


**Figure 2**
Modified scheme for measuring the methane concentration in the sample bottle, before assessing the radioactive fractions.

## Assessment

### Number of replicates

Replicates are essential to obtain a good estimate of MOX rates. Commonly, 2–3 replicates are used to quantify MOX rates (Schubert et al. 2010; Mau et al. 2013). To test the quality of data obtained from triplicate measurements, the coefficient of variation (cv=standard deviation X 100/mean) was calculated for data collected during eight cruises in the North Sea and Elbe in 2013. Our results show that the coefficient of variations is rather high (23%±11%, n=58) at low activities (< 10 nmol $L^{-1}$ $d^{-1}$) and lower (7%±5%, n=17) at higher activities (> 10 nmol $L^{-1}$ $d^{-1}$; Fig. 3). For comparison, when measuring the bacterial production in the water column using the leucine incorporation method, the coefficient of variation ranges from 6% to 10%, with 3–4 replicates (Ducklow et al. 2012; Simon et al. 2012). Hence, if a better precision and discriminatory power is needed, for example, when comparing different experimental setups, more than three replicates are necessary. The precision of replicates describes the total error of MOX rates. This total error consists of the error of each step of the method and the heterogeneity of the methanotrophic population in the water sample. All the tested error causes evaluated below, thus, most likely affect low rate measurements more than high rate measurements.

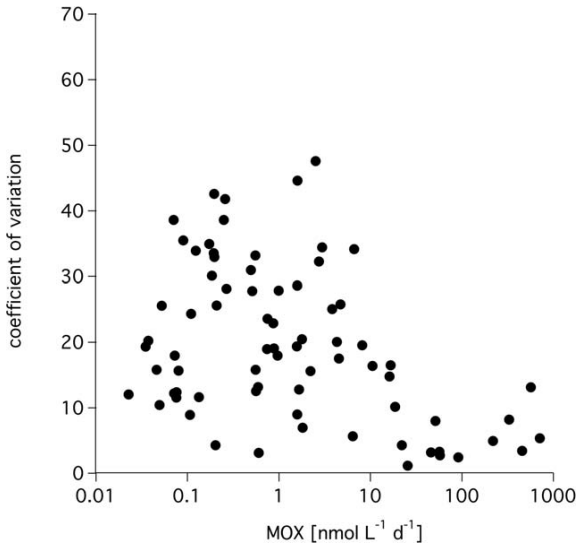


**Figure 3**
Coefficients of variation of triplicate samples in relation to MOX rates collected during eight research cruises in the North Sea and in the Elbe in 2013.

**Bottle size**

Glass bottles of different sizes (25-160 ml) are used to measure aerobic MOX rates. While larger samples contain a higher absolute number of bacteria, and thus, might be more representative, small sample vials have laboratory advantages. Less tracer has to be added to smaller samples, less space is needed during incubation, and less radioactive waste is produced. To test if small sample volumes are representative or if the coefficient of variation increases due to the small sample volume, a batch of North Sea water was filled in 12-160 ml glass bottles and incubated as described above. The comparison of incubation of different sample volumes showed no significant differences in turnover times (Wilcoxon Rank Sum Test, n=55). Therefore, small water samples are adequate, at least in waters with a turnover time of less than 20 d.

**Stoppers**

There are several different rubber stoppers available to firmly close the sample bottles. The stoppers should be gas tight to prevent any losses of methane and at the same time should be "soft" enough to allow easy handling with needles. However, most of the commercially available rubber stoppers leach organic and inorganic contaminants, which can inhibit or limit MOX. An extensive study was conducted by Niemann et al. (2015) recommending halogenated butyl rubber stoppers.

**Adding the $^{3}H$-$CH_4$ tracer**

The tracer can be added to the sample as a small bubble of concentrated $^{3}H$-$CH_4$ (10 µl) or as a bigger bubble (100 µl) diluted with nitrogen. According to the ratio of surface to volume of a sphere, more gas is diluted from small bubbles than from large ones. Furthermore, large bubbles may result in stripping of methane from the sample into the bubble in case of methane rich waters. However, handling of a 10 µl volume in contrast to a 100 µl volume is rather tricky especially on a moving boat. Furthermore, using diluted tracer has the advantage that the rate of decomposition is decreased. To test the effect of the bubble size, North Sea water was incubated with a 10 µl and a 100 µl bubble containing 131±40 Bq and 190±23 Bq, respectively (n=10 each, 24 h, at ambient methane concentration of 15 nmol $L^{-1}$ and at 18°C). The turnover time of the 10 µl samples (5.1±0.4 d) was slightly higher than the turnover time of the 100 µl

samples (4.5±0.4 d). The difference is in the range of the error of replicates indicating that the enhanced solubility for the 10 µl sample was negligible.

**Incubation time**

The incubation time has to be adapted to each environment. The time period should be long enough to produce a measurable amount of $^{3}H$-$H_2O$ and as short as possible to minimize incubation artifacts such as decomposition of the $^{3}H$-$CH_4$ or isotopic exchange reactions. To test the appropriate incubation time, one or more time series should be conducted. A time series is implemented by taking water samples from one location and incubating duplicate or triplicate samples for 3 h, 6 h, 12 h, 1 d, 2 d, etc. However, during field campaigns such time series may be difficult to perform due to the lack of time, space, or counting instruments. To provide a general idea of appropriate incubation times, we compiled data of time series of freshwater and marine environments. Our compilation suggests incubation times of <24 h in freshwater and 1–3 d in marine systems (Fig. 4). The time series indicate that $^{3}H$-$CH_4$ uptake per time is linearly related as long as the substrate (methane) is not limited. In case of the freshwater sample, the linear relation (first-order kinetics) is given during the first day of incubation and in case of the seawater samples over up to 5 d.

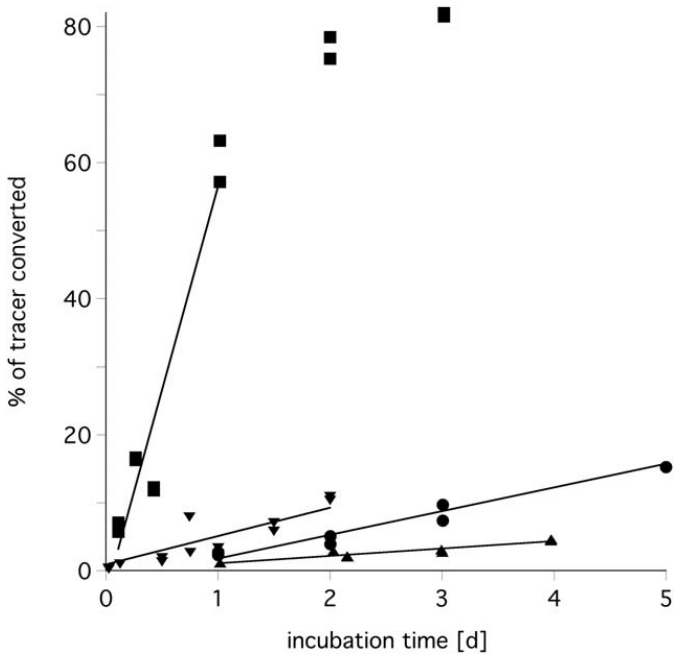


**Figure 4**

Time series conducted by incubating samples from different environments for 0.1–5 d. Freshwater from the pond of the MPI-Bremen (squares) and seawater from Storfjorden, Svalbard (circles), the North Sea (upward triangle), and the Santa Barbara Basin (downward triangle).

**During the incubation**

Incubation conditions for rate measurements should mimic the natural environment to determine a rate that resembles as close as possible the in situ rate. Typically, water samples are incubated at near in situ temperature, in the dark, and without motion.

In a set of experiments, we assessed the influence of temperature on the MOX rate. Elbe and North Sea water samples that were incubated at temperatures from 2°C to 25°C show the temperature curve of the MOX reaction (Fig. 5). We determined the $Q_{10}$-factor, which indicates the temperature dependence of a process: according to Raven and Geider (1988):

(4) $Q_{10} = \exp(-10 \times m \div T_{is}^2)$

where $T_{is}$ is the in situ temperature and m the slope of the regression line of the Arrhenius plot (the inverse of the absolute temperature vs. the natural logarithm of the MOX rate). We calculated $Q_{10}$ values to range between 1.52 and 1.75, for Elbe and North Sea water, respectively. These values are in accordance with $Q_{10}$ values between 1.4 and 2.1 determined for MOX rates in northern peatlands by (Dunfield et al. 1993). Also, $Q_{10}$ values of 1–1.84 were reported for ammonia oxidation in a marine setting (Horak et al. 2013). $Q_{10}>1$ indicates that the reaction rate increases with increasing temperature. Therefore, incubation temperature should be as close as possible to the in situ temperature or should be corrected if one wants to determine the in situ MOX rate.

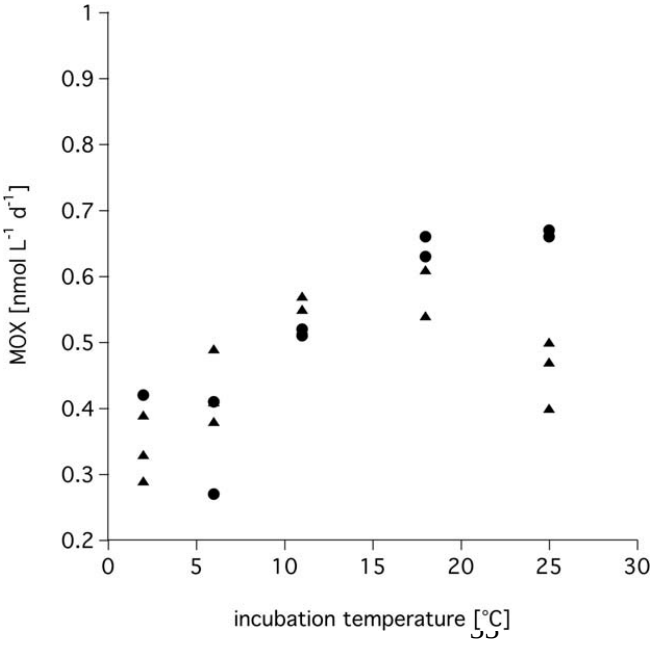


**Figure 5**

Influence of the incubation temperature on the MOX rate. North Sea water (triangles) and Elbe water (circles) with in situ methane concentrations of 20 nmol $L^{-1}$ and 32 nmol $L^{-1}$, respectively, were incubated for 24 h at different temperatures. Water samples were collected in January 2011 and had in situ temperatures of 3°C.

Samples are generally incubated in the dark; even though the influence of light on microbial MOX is unknown. Some studies suggest an inhibitory effect of light on methanotrophic growth and activity (Dumestre et al. 1999). Other studies suggest that the inhibitory effect depends on type of methanotrophs (Osudar et al. unpublished data). Unless more knowledge is available, we recommend to incubate the samples in the dark and also to minimize samples processing under high laboratory light.

The influence of motion/shaking of a sample during the incubation was tested. The motion not only increases the solubility of the tracer bubble but also imitates the motion of a research vessel at sea. The 120 ml sample bottles were put on a rocker during incubation to move the tracer bubble along the side of the bottle. No significant difference was found between samples from the rocker and samples incubated without motion (n=5 each). Also, the coefficient of variation was not different. Shaking of the sample is, thus, not necessary during incubation.

**Stopping the incubation**

If immediate analysis of samples after incubation is impracticable, a "killing" substance to stop microbial activity has to be added to the sample. This can be a toxic substance or a substance that shifts the pH. Strong toxic substances are mercury chloride ($HgCl_2$) and sodium azide ($NaN_3$), which both require environmental safe disposal. Sulfuric acid ($H_2SO_4$) or sodium hydroxide solution (NaOH) can be used to shift the pH and to stop microbial activity. We compiled data of the different "killing" substances in Table 2, which shows the amount of 3H-H2O in the killed control as percent of the total radioacitivity ($^3H$-$CH_4$ and $^3H$-$H_2O$). In seawater samples, we observed 2–15 times as high counts in control samples using NaN3 in contrast to $HgCl_2$ (Table 2).

For $HgCl_2$, it should be noted that some methanotrophs may be able to reduce $HgCl_2$ to elemental Hg, but they need to use most of the energy that they gain from methane metabolism to fuel mercury (II) reduction (Boden et al. 2011). Therefore, especially methanotrophs might not be stopped by adding $HgCl_2$. In seawater both NaOH and $H_2SO_4$ can be used to poison the samples. The $^3H$-$H_2O$ radioactivity of the controls using 10 mol $L^{-1}$ NaOH was 1.0%±0.3% (n=35) in North Sea water, while 25% $H_2SO_4$ had a slightly better performance (Table 2). In freshwater in most cases 5 mol $L^{-1}$ NaOH was superior to 25% $H_2SO_4$ in stopping the samples (Experiments I and II in Table

2). Field data revealed a low residual activity when stopping with 5 mol $L^{-1}$ NaOH (Elbe River near Hamburg; Lake Constance). However, in some cases (Czech part of the Elbe) NaOH was not sufficient and best results were obtained with concentrated $H_2SO_4$ (96%; Table 2). Overall $H_2SO_4$ was the best killing reagent, with a better performance than NaOH, which in turn is advantageous compared to $HgCl_2$ and $NaN_3$ that are both environmentally hazardous. In the marine environment diluted (25%) $H_2SO_4$ was sufficient, whereas in freshwater concentrated $H_2SO_4$ appears to be superior.

**Table 2**
Field and experimental data on the influence of the stopping reagent on the amount of $^3H$-$H_2O$ in the killed control as percent of the total radioactivity ($^3H$-$CH_4$ and $^3H$-$H_2O$). Given are the averages ± standard deviation and the number of samples in brackets. The experiments were performed with water from the Elbe near Hamburg and North Sea water near Helgoland. We added 0.2-0.3 mL of base or acid to 120 mL sample bottles, resulting in a pH of <1.5 or >10.

| | Location/ experiment | 5M NaOH | 10 mol $L^{-1}$ NaOH | 25% $H_2SO_4$ | 95% $H_2SO_4$ | $HgCl_2$ | $NaN_3$ |
|---|---|---|---|---|---|---|---|
| Seawater | Santa Barbara | | | | | 0.1±0.0 (6) | 0.5±0.2 (6) |
| Seawater | North Sea | | 1.0±0.3 (35) | 0.1±0.2 (39) | | | |
| Freshwater | Elbe near Hamburg | 3.8±4.0 (37) | | | | | |
| Freshwater | Elbe near Prague | 8.5±2.6 (12) | | 9.5±4.5 (12) | 0.8±1.7 (31) | | |
| Freshwater | Lake Constance | 5.2±7.1 (50) | | | | | |
| Freshwater | Exp. I | 5.4±1.0 (4) | | 37.9±0.3 (4) | | | |
| Seawater | | 0.2±0.1 (4) | | 0.0±0.0 (4) | | | |
| Freshwater | Exp. II | 0.4±0.1 (3) | | 28.0±12.6 (3) | | | |

After stopping the incubation, the samples should be analyzed as soon as possible. Experiments with storage time of $^3H$-$CH_4$-labeled samples show that within the first week there is a significant loss of $^3H$-$CH_4$, while the $^3H$-$H_2O$ fraction remains stable (Fig. 6a). Presumably the $^3H$-$CH_4$ is lost through diffusion through the stoppers. This loss results in an increase of the ratio ($^3H$-$H_2O$/$^3H$-$CH_4$+$^3H$-$H_2O$; Fig. 6b). This increase of the ratio will result in an overestimation of the MOX rate by 20–40%. Thus, samples should be analyzed within 3–4 d, or if this is not possible an overestimation of the MOX rate of approx. 20% has to be accepted.

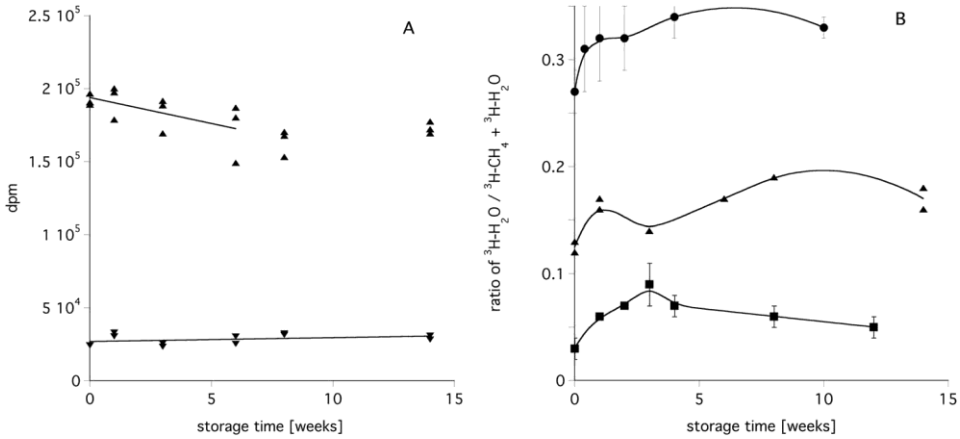


**Figure 6**

The influence of the storage time on the radioactivity in the different fractions and on their ratio. Incubations of North Sea water were stopped after 24 h with 25% $H_2SO_4$ and two to four bottles were analyzed immediately. The other samples were stored at 4°C and measured after the indicated time. (A) The radioactivity in dpm in the fraction ($^3$H-$CH_4$ + $^3$H-$H_2O$) with upward triangles and the radioactivity in the $^3$H-$H_2O$ fraction with downward triangles. (B) The ratio ($^3$H-$H_2O$/$^3$H-$CH_4$ + $^3$H-$H_2O$) of three experiments, the triangles are from the same experiment shown in figure A.

## Total radioactivity ($^3$H-$CH_4$ and $^3$H-$H_2O$) of the sample

After incubation, the total radioactivity ($^3$H-$CH_4$ and $^3$H-$H_2O$) that was added to the water sample has to be determined. We determined the total radioactivity in all bottles, samples, and controls. This allowed for a better precision, as we found a high variability (> 10%) between different bottles.

However, as methane has a low solubility, it rapidly equilibrates with the headspace in the scintillation vial and can leak from the scintillation vial. $^3$H-$CH_4$ in the headspace cannot be counted in a liquid scintillation counter. To measure the total radioactivity ($^3$H-$CH_4$ and $^3$H-$H_2O$) most accurately, we tested vigorous vs. gently mixing of a sample with scintillation cocktail, how long the mixed sample can be left standing before analysis, and the use of polyethylene vs. glass vials.

Vigorous shaking vs. gentle mixing was tested by adding one milliliter sample to five milliliter scintillation cocktail in each of two scintillation vials. One of the vials was gently turned upside down 3–4 times whereas the other was vigorously shaken by hand. The results show on average 7% less radioactivity in the vigorously shaken samples than in the gentle mixed samples (Supporting Information 1). Vigorous shaking of the samples with the cocktail, thus, leads to

a faster equilibration and higher leakage; therefore, mixing should be accomplished gently.

Furthermore, we observed that the total radioactivity rapidly decreases over time after addition of the scintillation cocktail and mixing (Fig. 7). Glass vials were found to have a slightly better performance than polyethylene vials. In glass vials, the total radioactivity decreased by ~ 15% within 60 h, whereas in polyethylene vials radioactivity was reduced by 25% in 60 h. Already in the first hour, 4% of the total radioactivity is lost using polyethylene vials.

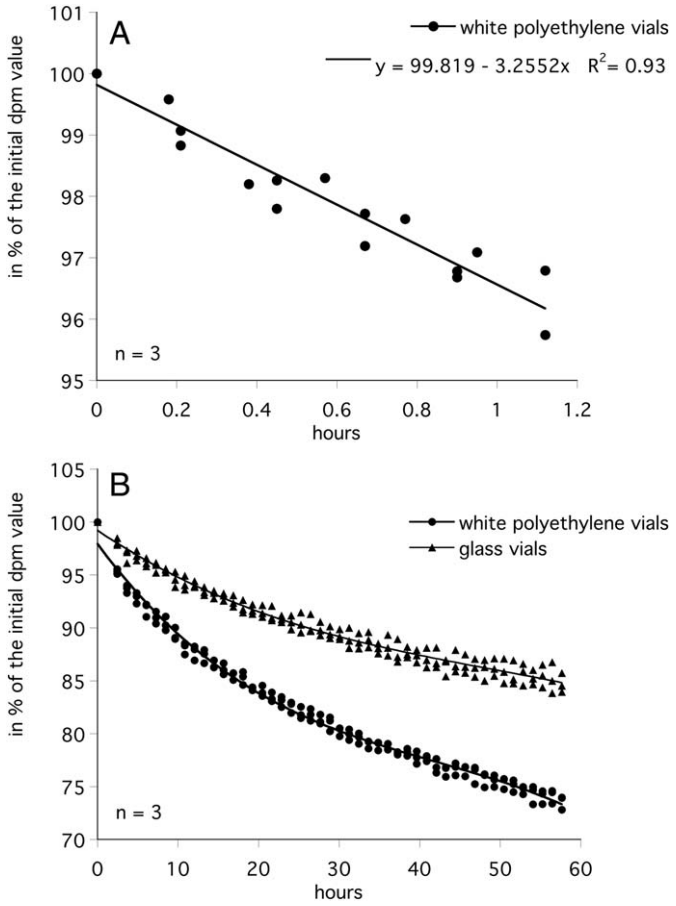


**Figure 7**
Loss of $^{3}H\text{-}CH_4$ from the total radioactivity ($^{3}H\text{-}CH_4$ and $^{3}H\text{-}H_2O$) in a scintillation vial with time. The scintillation vials contained a two milliliter subsample and five milliliter scintillation cocktail. The same vials were counted at different times.

As the loss of radioactivity is due to equilibration between the fluid and the gas phase, the loss depends on the methane concentration in the vial, and might be lower at low methane concentrations. However, the experiments show that samples should be immediately counted after mixing. Further, we recommend opening and processing less than 10 samples at once and set the

counting time to 1–3 min as otherwise the total radioactivity of the last sample analyzed is biased due to $^3H$-$CH_4$ leakage in the headspace.

**Radioactivity of the water fraction ($^3H$-$H_2O$)**

To determine the amount of water that has been produced by the methanotrophic bacteria through the oxidation of methane, the radioactivity of the $^3H$-$H_2O$ has to be quantified (Table 1). Therefore, the remaining $^3H$-$CH_4$ has to be removed from the sample, which can be done by sparging the sample with nitrogen or air. We moistened the air by directing it through a washing flask to prevent evaporation of sample water. However, this effect appears negligible and accounts for <0.2% of the $^3H$-$H_2O$ if one assumes an even distribution of the $^3H$-$CH_4$ in the sample, an evaporation rate of 200 g water $m^{-2}$ $h^{-1}$ and a sparging time of 30 min.

To test for the appropriate time to remove all of the $^3H$-$CH_4$, we sparged samples with a high and low activity for 5–30 min, that is, Elbe water and North Sea water, respectively. In the active samples about 28% of the added $^3H$-$CH_4$ was found in the water fraction. In the less active samples, only 2% of the $^3H$-$CH_4$ was found in the water fraction. In both cases, a stable counting was reached after 20–30 min of sparging (Fig. 8).

In contrast to the total radioactivity ($^3H$-$CH_4$ and $^3H$-$H_2O$), the radioactivity of the $^3H$-$H_2O$ in the scintillation vial was stable over time (70 h).

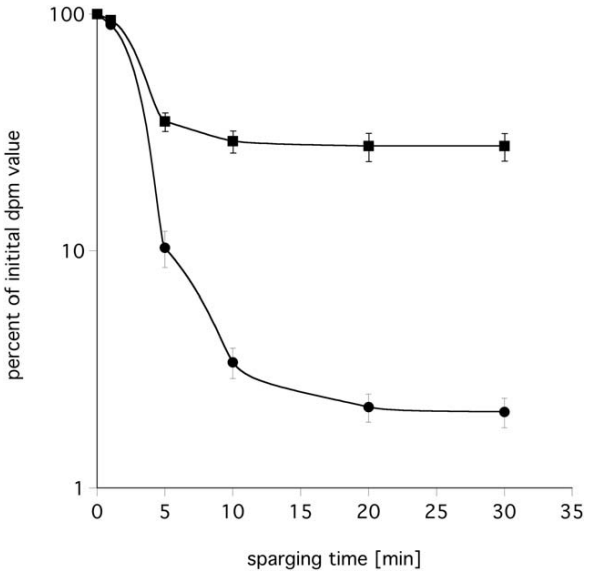


**Figure 8**

Identification of the necessary sparging time to remove not microbially oxidized $^3H$-$CH_4$. Elbe water (squares, n=3) and North Sea water (circles, n=3) samples were incubated for 24 h and stopped by adding a poison. Radioactivity was measured after different times of sparging. Note the logarithmic scale of the y-axis.

Therefore, the counting time can be extended to 10 min, to get a better statistical quality of the counts. The 2% sigma value as measured by the liquid scintillation counter for a given counting time decreased from 2% at 1 min to 0.6% at 10 min counting time.

**Storage of the $^{3}H$-$CH_4$ tracer**

Because $^{3}H$-compounds generally have a high specific activity and, thus, a high radioactive decomposition rate, this section provides advice on the optimal conditions for storing the $^{3}H$-$CH_4$ tracer. Decomposition is the interaction of emitted particles with the immediate surroundings and/or with the molecules of the labeled compound causing destruction of the labeled substance. To lessen the decomposition, it is necessary to keep the number of interactions as low as possible. This can be achieved by storing the tracer at low temperatures and by dilution of the tracer. For the $^{3}H$-$CH_4$ tracer dilution from the original ampoule, we recommend to use nitrogen, instead of air. In this way, reactions with OH and other compounds of the air will not occur. If the $^{3}H$-$CH_4$ tracer is to be used over months, it is best to have subsamples in a number of vials. This way, vials to be used later can be kept in the refrigerator to avoid reopening and warming/cooling cycles. Furthermore, $^{3}H$-$CH_4$ tracer is commonly stored on saturated salt solution, which decreases the likelihood of formation of reactive species. The water molecules surround the highly charged $Na^+$ and $Cl^-$ ions, increasing the structure of water and reducing the number of "free" water molecules, which can form reactive species such as $OH^-$ radicals (Emerson and Hedges 2009).

**Background $^{3}H$-$H_2O$**

Some of the $^{3}H$-$H_2O$ is often suggested not to result from microbial MOX but from isotopic exchange reactions or decomposition of the tracer. Salinity and reactive species (e.g., $OH^-$, $H^+$) are assumed to influence these processes (see storage of the tracer). That is, background $^{3}H$-$H_2O$ is supposed to be higher in less saline water because more "free" water molecules are around, which are broken by radiation to, for example, $OH^-$ and $H^+$ ions, and react with the tracer forming $^{3}H$-$H_2O$.

Similarly, pH modifies water to contain more reactive species. To test the influence of (i) salinity and (ii) pH on the concentration and stability of the background $^{3}H$-$H_2O$, we autoclaved water (deionized water, freshwater,

seawater, a saturated salt solution, and freshwaters and seawaters with different pH) to assure that all microbial activity is stopped. Then, we added the same amount of tracer to all samples. The samples were subsequently incubated in the dark at 10°C for up to 11 d. The results show no significant differences between the different setups, nor a significant change of the background value over time (Fig. 9). Apparently neither the availability of "free" water molecules in low salinity water, nor the increase in $H^+$ (acidic water) or $OH^-$ ions (basic water) led to elevated concentrations of $^3H$-$H_2O$ over the 11-d time period. Therefore, the isotopic exchange reaction and decomposition of the $^3H$-$CH_4$ tracer is negligible over typical time periods of incubations (hours to 3 d).

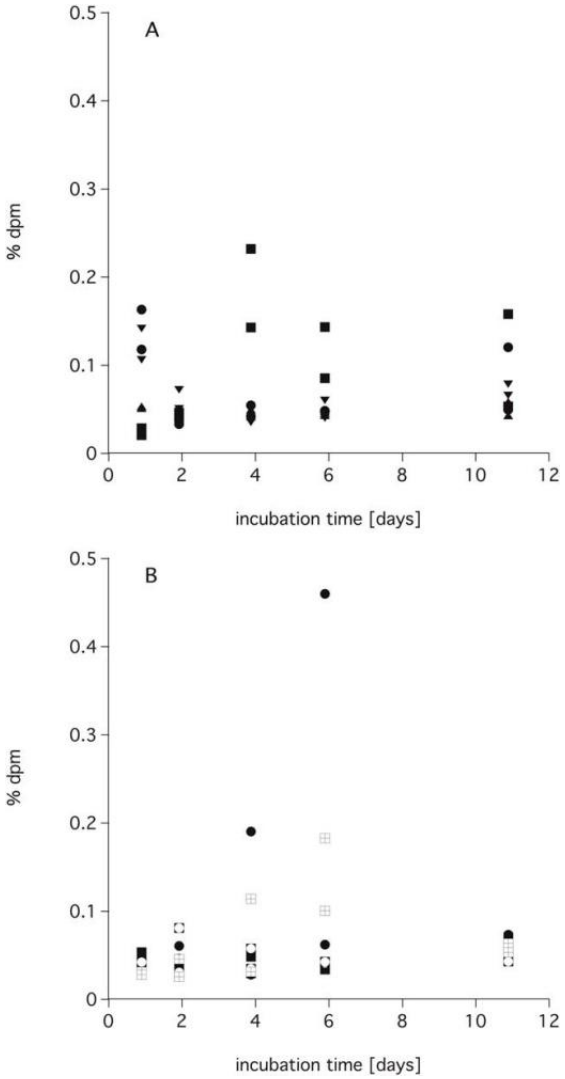


**Figure 9**
Percent of $^3H$-tracer remaining in autoclaved water after incuba- tion and sparging for 30 min to remove $^3H$-$CH_4$. Samples were stored at 10°C for up to 11 d. (A) Incubations with different salt concentrations: Milli Q (downward triangles), tap water (squares), seawater (circles), and brine (upward triangles). (B) Incubations with different pH: tap water with pH 5 (filled circles) and 9 (engulfed circles), seawater with pH 5 (filled squares) and 9 (crossed squares).

**Methane concentrations**

The methane concentration is crucial for the calculation of the MOX rate. However, the concentrations can be determined either in separate bottles, in the control bottles or in the sample itself. The first possibility represents the in situ concentrations, while in the other ones, methane concentrations are altered due to the addition of $^{3}H\text{-}CH_4$. The addition of $^{3}H\text{-}CH_4$ should not increase the in situ methane concentrations significantly.

In several sets of samples, we determined the methane concentration (i) in separate bottles, (ii) in the killed controls, and (iii) in the sample bottles. Adding a diluted $^{3}H\text{-}CH_4$ ($1 \times 10^{11}$ Bq $mol^{-1}$) increased the methane concentration in the samples and controls by 3 nmol $L^{-1}$ or to 103% and 135% of the in situ concentration (Fig. 10). Subsequently, the MOX rate calculated with the different methane concentrations also were very similar. However, on a second cruise the $^{3}H\text{-}CH_4$ was more concentrated ($5 \times 10^{12}$ Bq $mol^{-1}$) and the methane concentrations in the samples and controls increased 1.5–5 times (to 138% and 469% of the in situ concentration). Thus, the MOX rates calculated with the methane concentrations of the control or the sample increased also by 1.5–5 times and were 1.5–5 times faster than the MOX rate calculated with the in situ methane concentration (Fig. 10). The experiment shows that (1) an additional sample for analysis of the in situ concentration is the easiest and most accurate way to calculate MOX rates and (2) the methane concentration of the $^{3}H\text{-}CH_4$-tracer should be adjusted for low methane concentration environments.

Using the same dataset, MOX rates were calculated based on the difference in methane concentration between the sample and control bottles. As methane consumption only takes place in the sample but not in the control bottle. In samples with high methanotrophic activity, calculation of the MOX rate with the 3H-CH4-tracer measurement vs. calculation of the difference in concentration were comparable (158±4 nmol $L^{-1}$ $d^{-1}$ vs. 144±31 nmol $L^{-1}$ $d^{-1}$), however, at low activities the difference in methane concentrations were within the measurement error of the GC (0.8±2.4 nmol $L^{-1}$ $d^{-1}$) and, thus, not comparable with the radiotracer measurements (0.05±0.01 nmol $L^{-1}$ $d^{-1}$). The latter comparison illustrates, why tracer experiments are essential in determining aerobic MOX in waters with low methane waters where slow MOX rates are expected. In these waters, the change in methane concentration over time cannot be measured by GC.

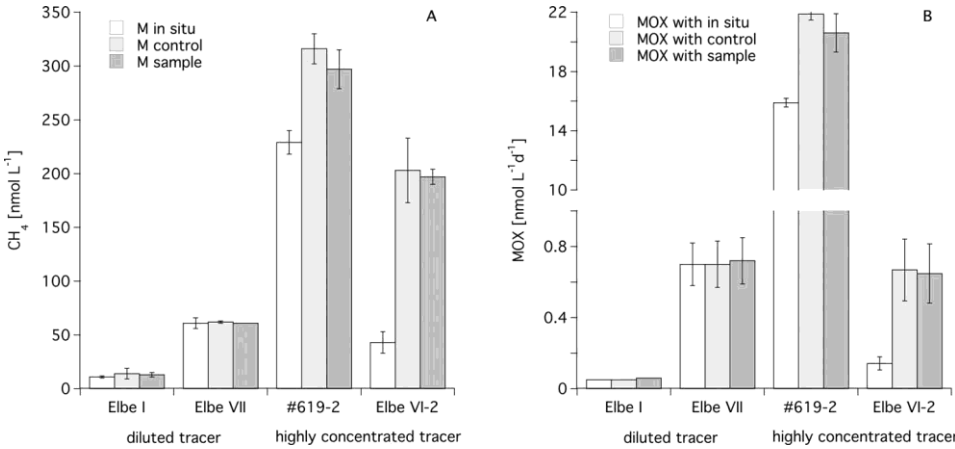


**Figure 10**

Methane concentrations measured in a separate water samples, in control bottles, and samples bottles, the latter two contain tracer (A). MOX rates calculated with the corresponding in situ methane concentrations, the methane concentrations in the control and sample bottles (B). North Sea and Elbe water was collected for this experiment. Note the break in the y-axis.

## MOX rate calculation with time series or single end point measurement

The MOX rate of a specific water sample can be calculated from a time series or as most often is the case from a single end-point measurement. During a time series, consumption of $^{3}H\text{-}CH_4$ is measured after an incubation time for example 0.5 d, 1 d, 2 d, 3 d, the slope of a linear regression of the fraction of the $^{3}H\text{-}CH_4$ oxidized vs. time is used to calculate k´ and then the MOX rate. The rate constant, k´, is, thus, determined from a dataset ($n \geq 8$). In contrast, single end-point measurements derive k´ from replicate samples ($n \geq 2$). Commonly single end-point measurements are made assuming first-order kinetics, that is, the reaction depends solely on the availability of one substrate, which is methane in this case. Further, it is assumed that the cell population is not growing. To test the reliability of these assumptions, we compared k´ derived from (i) a linear regression of time series data, (ii) from the average of the time points of a time series, and (iii) the commonly used 24 h incubation. We used data from marine environments (North Sea and Svalbard), as well as freshwater environments (MPI-pond and Lake Constance), in total 10 datasets. Incubation times ranged from 2 h to 24 h for the freshwater samples and from one day to five days for the marine samples.

As a first step for the time series data, we tested if a linear regression sufficiently describes the data. For all datasets, the average of the residuals was

equal to zero (t-test for "0"). Also the residuals were normally distributed (Shapiro–Wilk Normality Test with p=0.01, except one dataset). This indicates that the difference from the measured data to the calculated line fit was randomly distributed and showed no systematic deviation. Comparing k´ as calculated by (i) a linear regression or by (ii) average of single end points, revealed no significant difference (Wilcoxon Rank Sign Test for paired data, n=10, p=0.34), as also shown in Fig. 11.

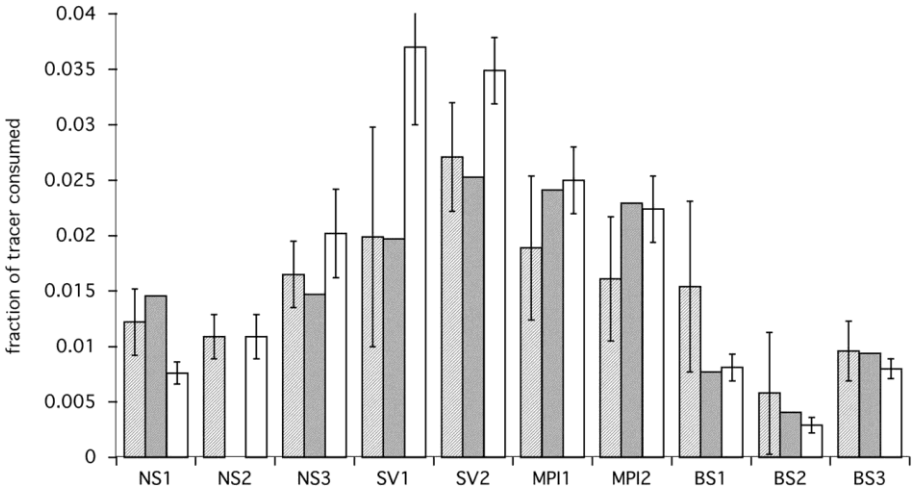


**Figure 11**
Comparison of the fraction of consumed tracer as calculated either from a linear regression of a time series (white columns), from the average of 3–4 single time points (light shade columns) and from one single end point at 24 h (dark shaded column). Details of the calculation are described in the text. The calculations were applied for three samples from the North Sea (NS1, 2, 3), two samples from off Svalbard (SV1, 2) and for methane rich freshwater settings (MPI1, 2) and Bodensee (BS1, 2, 3).

In general only one single time point (i.e., 24 h) is used for MOX rate calculations. Thus, this one single time point calculation is included among the single time point calculations (see also Fig. 4). As shown in Fig. 11 these values range within the values for the linear regression and the single end point calculation and their standard deviations. On average the one single end point calculation was 5% different from the linear regression calculation. Therefore, we assume that the above conclusions are also valid for single time point calculation, as long as this time point lies within the linear range (Fig. 4).

**Detection limit**

Control samples are frequently taken and are poisoned immediately after the addition of the $^{3}H\text{-}CH_4$ and the "initial ratio" ($^{3}H\text{-}H_2O/^{3}H\text{-}CH_4+^{3}H\text{-}H_2O$) is determined. The mean (x) and the standard deviation (s) of all controls sampled during different cruises in different areas were calculated and the limit of detection (LOD) was set as:

(5) $LOD = x + 3 \times s$

All samples with the "initial ratio" below this LOD-value were considered as below the detection limit and had to be set as zero. We applied this strict rule to different datasets of MOX rates (Table 3) with methane concentrations ranging from background concentrations of 1 nmol $L^{-1}$ to high seep concentrations of 1456 nmol $L^{-1}$. In some cases, 70% of the data were below the detection limit and had to be set to zero. The lowest detected value was 0.001 nmol $L^{-1}$ $d^{-1}$ based on the datasets of Table 3.

The use of laboratory based vs. portable LSD may also influence the LOD. Especially at low activities the counting efficiency of the liquid scintillation counter may be critical. For example, the same samples which were counted with a laboratory-based machine with 760 dpm and 815 dpm, retrieved only 189 dpm and 181 dpm with a portable liquid scintillation counter. While samples with higher counts (47,000 dpm) did not indicate any differences between the two liquid scintillation counters.

**Table 3**
LOD calculated for datasets of different areas. The * indicates samples which were measured with a portable Liquid scintillation counter.

| Cruise, Year | Area | $CH_4$ (nmol $L^{-1}$) | LOD MOX (nmol $L^{-1}$ $d^{-1}$) | Data below LOD (%) |
|---|---|---|---|---|
| *HE333*, 2010 | Svalbard | 5–86 | 0.02 | 4 |
| *PO419*, 2011 | Svalbard | 1–546 | 0.02* | 68 |
| *HE406*, 2013 | North Sea | 4–1456 | 0.02* | 70 |
| *PS-ANTXXIX*, 2013 | South Georgia | 1–56 | 0.001 | 38 |
| *HE413*, 2014 | North Sea | 9–686 | 0.1* | 11 |

## Discussion

Although it is known that methane is microbially oxidized in lakes and oceans and that this process reduces the gas flux into the atmosphere, only a small number of MOX data are available. $^{3}H\text{-}CH_4$ being relatively new commercially available provides a convenient tracer to determine the MOX rate in natural waters. Compared to the $^{14}C\text{-}CH_4$ method, the $^{3}H\text{-}CH_4$ method requires minimal sample processing and few specialized equipment. However, as known from the common saying "the devil is in the details," we checked the method to produce a best practice guide hoping to encourage people to take up the method and indicating the important parameters that can cause large errors. Below, we first discuss the errors found during the method assessment before evaluating existing data.

## Error discussion

We compared the different parameters causing uncertainties of the results as outlined in the assessment part by calculating for each tested modification the deviation from the common method in percent. For example, the deviation of the result caused by applying a 10 µl tracer bubble instead of the commonly used 100 µl tracer injection or the deviation caused by storing killed samples for 60 h in contrast to processing samples right after incubation as commonly done (Fig. 12). We also applied different methane concentrations to calculate the MOX rate, but recommend using the real in situ methane concentration as measured in separate bottles. Otherwise the MOX rates will increase by the same factor as the methane concentrations are increased. However, we did not include this comparison in Fig. 12 as we think this is rather a calculation error than a methodological error.

The largest uncertainty is due to the precision of the MOX rate measurements in waters with low methanotrophic activity (i.e., MOX-rates <10 mol $L^{-1}$ $d^{-1}$). By increasing the number of replicates, the precision of the data can be improved, however, at high costs of work effort and material.

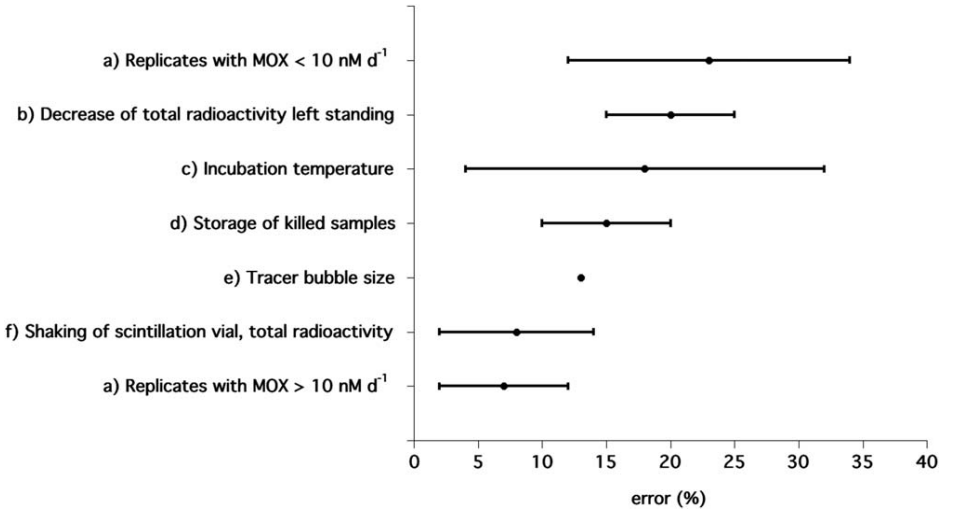


**Figure 12**
Mean and standard deviation of error associated with different parts of the method: (A) error of replicates indicates the difference from the mean value, (B) decrease of total activity left standing shows the difference between measuring the total activity right after stopping the incubation and measuring after 60 h left standing, (C) incubation temperature illustrates the error of MOX rates if incubation temperatures differ by 1–5°C and $Q_{10}$ ranges between 1.52 and 1.75, (D) storage of killed samples illustrates the error associated with storing a sample instead of rapid post-processing, (E) bubble size indicates the measured difference between a 100 µl bubble and a 10 µl bubble, (F) shaking of scintillation vials indicates the error associated with vigorous shaking of the total activity sample in a scintillation vial.

This large uncertainty can be viewed as the total error of MOX rates that consists of the error of each step of the method and the heterogeneity of the methanotrophic population in a water sample. This total error is influenced by the following uncertainties which apparently impact low rate measurements more than high rate measurements. The largest impact on the total error is caused by measuring of the total radioactivity ($^3H$-$CH_4$ and $^3H$-$H_2O$) not right after opening a sample. The second largest influence is due to incorrect incubation temperature. By incubating samples at temperatures as close as possible to the in situ temperature over- or underestimation of MOX rate can be avoided. The error associated with temperature can also be corrected using published $Q_{10}$ factors. However, as the data base for methanotrophic $Q_{10}$ is very small, we recommend to incubate the samples as close as possible to the in situ temperature or to determine a $Q_{10}$ for the respective environment. A similarly high error results from the storage of killed samples in comparison to post processing the samples right after incubation. The fourth largest error results

from the size of the injected $^3H$-$CH_4$ bubble and minor errors are due to vigorous shaking of the scintillation vial and subsequent counting of the total activity.

Some of these errors are easily avoidable while others can only be limited with more effort. Errors that can be avoided are: (1) measuring the total radioactivity ($^3H$-$CH_4$ and $^3H$-$H_2O$) right after opening the sample, (2) not leaving killed samples standing for later analysis, (3) using an appropriate $^3H$-$CH_4$ bubble volume, and (4) shaking the total radioactivity ($^3H$-$CH_4$ and $^3H$-$H_2O$) scintillation mixture gently. The other errors: (1) precision of low activity samples, (2) incubation at in situ temperature or derivation of $Q_{10}$, and (3) derivation of a more accurate k´ by implementing a time series, can also be minimized, but only by processing more samples. It depends on the scientific question and the according precision of the analysis needed, if the additional work and also the additional radioactive waste justify the higher effort.

In contrast to the parameters discussed above, bottle size, background, and produced $^3H$-$H_2O$, as well as shaking of the samples during incubation cause insignificant MOX rate errors in natural waters. Bottle size insignificantly altered MOX rate measurements; thus, bottle size can be reduced to lessen radioactive waste. Measurements of background $^3H$-$H_2O$ in microbial inactive waters remained low over 11 d.

Therefore, the chemical reaction that is self-decomposition and ionic exchange are negligible in the time frame of a typical incubation (< 3 d). If killed controls show a high $^3H$-$H_2O$ content, microbial metabolism was apparently not efficiently stopped as was shown by Boden and Murrell (2011), who investigated methanotrophic resistivity to $HgCl_2$. Not only the background, but also the produced $^3H$-$H_2O$ during microbial MOX was found stable over time, thus, these measurements can be delayed, for example, for on-shore analysis. Finally, shaking of the samples during incubation was not found to be relevant and is, thus, not necessary.

Other details of the method investigated resulted in suggestions for best practice to determine MOX rates. Stoppers should consist of halogenated butyl rubber. The tracer is best stored on saturated NaCl-solution, diluted in $N_2$ and at low temperatures (refrigerator). The incubation time is best determined by conducting a time series and the sparging time should be 30 min. We also recommend determining the in situ methane concentration used for MOX rate calculations in separate bottles. Thus, one does not need to correct for increased

methane concentrations through the addition of $^{3}H\text{-}CH_4$ nor for decreased methane concentrations due to methane consumption by methanotrophs.

**Existing data evaluation**

MOX rates have been measured using $^{14}C\text{-}CH_4$ and $^{3}H\text{-}CH_4$; data which cannot be readily compared. In the 20$^{th}$ century, water column MOX rates were determined using $^{14}C\text{-}CH_4$ (Scranton and Brewer 1978; Ward et al. 1987) while the $^{3}H\text{-}CH_4$ moved toward becoming commercial accessible in the 21$^{st}$ century. The data are not comparable as 100-fold more methane is added to a sample using $^{14}C\text{-}CH_4$ compared to $^{3}H\text{-}CH_4$ resulting in potential rates at elevated substrate concentration and near in situ rates, respectively. This difference was evaluated in water samples from Storfjorden and discussed in Mau et al. (2013).

For both tracers, the precision of the rate measurement in waters of low methane concentrations and activity appears to be the main uncertainty. In this study, we found the precision of MOX rates in waters of low methanotrophic activity to be the largest error, which might be due to the general higher error associated with low concentration experiments but could also be due to tracer-back-flux as reported for the anaerobic oxidation of methane (Holler et al. 2011). Also Blees et al. (2014) and Jakobs et al. (2013) indicate a great variability in low concentration-samples using $^{14}C\text{-}CH_4$.

However, existing publications all include measurements of replicate samples stating the precision. In most studies, duplicate or triplicate sampling is performed. When duplicate samples are used, both data points are shown (Gentz et al. 2013; Mau et al. 2013). Studies with triplicate sampling either show the error bars (Blees et al. 2014) or provide the standard deviation (Jakobs et al. 2013). Hence, the data is of good quality especially when considering rate measurements in the sediment where duplicate or triplicate measurements are not feasible.

In contrast to the precision of the measurements, the difference between in situ temperature and incubation temperature was hardly ever corrected, even though we found temperature to cause the second largest influence on error of MOX rates. Most often samples are incubated at a temperature that is close to the in situ temperature, but usually not exactly at in situ temperature. It is normally also not feasible to incubate all samples at in situ temperature, especially in summer when surface ocean temperatures are significantly elevated in comparison to deep water temperatures. Even if two or three incubators are

available and set to different temperatures, some water samples are still not incubated at in situ temperatures. Besides, all cooling devices have cooling cycles which might cause temperature variations of up to 5°C (refrigerator). It would be also helpful to monitor more exactly the incubation temperature. Using the $Q_{10}$ correction underestimation or overestimation of the MOX rates can be evaluated, although published $Q_{10}$ values are bulk values and might differ between regions due to the presence of different methanotrophic communities. Certainly, more $Q_{10}$ need to be derived to overcome this insufficient correction of MOX rates.

Another drawback of existing datasets is that no detection limit (LOD) was provided so far. Therefore, very low rates were published, which are most likely below the detection limit. We calculated LOD to be on the order of 0.001 nmol $L^{-1}$ $d^{-1}$ to 0.01 nmol $L^{-1}$ $d^{-1}$ and recommend to use Eq. 5 to derive the detection limit and use only the data that show significant MOX.

**Comments and recommendations**

Based on the experiments performed for this study, we recommend considering the following aspects when planning MOX rate measurements in an unknown environment. With a time series, the assumption of first-order kinetics can be checked and the appropriate incubation time can be defined. We encountered a rather high variability of the MOX rates, thus, at least three parallel samples are necessary to obtain a sufficient precision. Especially in methane poor environments with low activities the aspect of the detection limit has often been neglected. Therefore, a sufficient number of killed controls have to be setup to allow distinguishing between "real" methane consumption and background noise.

In our study, we investigated some of the important aspects of MOX measurements. However, even when writing the manuscript, we are well aware and realized that more aspects still would be interesting or important to look at.

Such aspects could be: Is there a difference when poisoning the controls before or after the tracer addition? What is the influence of different scintillation cocktails and different liquid scintillation counter on the rates? Are there differences between MOX measurements when using gaseous $^{3}H\text{-}CH_4$ compared to an aqueous tracer solution? The kinetics of MOX in natural waters is still not well-known, as most studies were done with pure cultures of methanotrophs or with soil samples. The priming effect (injection of additional methane and, thus,

increasing substrate concentrations) could be tested with nonlabeled methane as well as with labeled methane as was done only by Mau et al. (2013) so far. As with all/many methods an inter-laboratory comparison of MOX measurements would be very instructive.

Nevertheless, with our study we hope to improve and to encourage future measurements of MOX rates in different environments. We also hope to develop a standard procedure of MOX rate measurements to make data of MOX better comparable.

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## 6.2 Environmental factors affecting methane distribution and bacterial methane oxidation in the German Bight (North Sea)

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# Environmental factors affecting methane distribution and bacterial methane oxidation in the German Bight (North Sea)

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ABSTRACT

River estuaries are responsible for high rates of methane emissions to the atmosphere. The complexity and diversity of estuaries require detailed investigation of methane sources and sinks, as well as of their spatial and seasonal variations. The Elbe river estuary and the adjacent North Sea were chosen as the study site for this survey, which was conducted from October 2010 to June 2012. Using gas chromatography and radiotracer techniques, we measured methane concentrations and methane oxidation (MOX) rates along a 60 km long transect from Cuxhaven to Helgoland. Methane distribution was influenced by input from the methane-rich mouth of the Elbe and gradual dilution by methane-depleted sea water. Methane concentrations near the coast were on average 30 ± 13 nmol $L^{-1}$, while in the open sea, they were 14 ± 6 nmol $L^{-1}$. Interestingly, the highest methane concentrations were repeatedly detected near Cuxhaven, not in the Elbe River freshwater end-member as previously reported. Though, we did not find clear seasonality we observed temporal methane variations, which depended on temperature and presumably on water discharge from the Elbe River. The highest MOX rates generally coincided with the highest methane concentrations, and varied from 2.6 ± 2.7 near the coast to 0.417 ± 0.529 nmol $L^{-1}$ $d^{-1}$ in the open sea. Turnover times varied from 3 to >1000 days. MOX rates were strongly affected by methane concentration, temperature and salinity. We ruled out the supposition that MOX is not an important methane sink in most of the Elbe estuary and adjacent German Bight.



### 1. Introduction

Methane is the most abundant organic gas in the atmosphere. Being a potent greenhouse gas, it plays an important role in warming the Earth's atmosphere (Kirschke et al., 2013). Its contribution to global warming is attenuated by comparatively low concentrations and short lifetime in the atmosphere. Methane has the second-largest radiative forcing of the long-lived greenhouse gases, after $CO_2$ (Ramaswamy et al., 2001). The atmospheric concentration of methane has increased to a level unprecedented in at least the last 800,000 years (Stocker et al., 2013). Its average concentration nowadays is around 1.8 ppm (Kirschke et al., 2013). Changes in methane concentration, which are defined by the balance between sources and sinks of methane, have led to investigations on the microbial-driven methane cycle in various environments.

About 60% of the methane released to the atmosphere is from anthropogenic sources such as agriculture, waste treatment, biomass burning, and fossil fuels. The remaining 40% originates from natural sources, mainly wetlands (Kirschke et al., 2013). Among these sources, the world's oceans contribute <0.1–10% of the methane emissions (Scranton and McShane, 1991; Bates et al., 1996; Middelburg et al., 2002; Conrad, 2009). Digestive tracts of zooplankton (Bianchi et al., 1992; De Angelis and Lee, 1994), and

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**Environmental factors affecting methane distribution and bacterial methane oxidation in the German Bight (North Sea)**

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**Introduction**

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About 60% of the methane released to the atmosphere is from anthropogenic sources such as agriculture, waste treatment, biomass burning, and fossil fuels. The remaining 40% originates from natural sources, mainly wetlands (Kirschke et al. 2013). Among these sources, the world's oceans contribute <0.1e10% of the methane emissions (Scranton and McShane 1991; Bates et al. 1996; Middelburg et al. 2002; Conrad 2009). Digestive tracts of zooplankton (Bianchi et al. 1992; De Angelis and Lee 1994) and organic particulate matter (Karl and Tilbrook 1994) are the main methane sources in the open ocean. However, about 75% of global oceanic methane emission occurs in shelf areas and estuaries (Bange et al. 1994; Bange, 2006). The main sources of methane in these areas are sediments, adjacent rivers, tidal flats, and marshes (Bange et al. 1994; Bange, 2006; Abril et al. 2007; Grunwald et al. 2009; Bussmann, 2013). Sedimentary release of methane which follows methanogenesis (Koch et al. 2009; Bussmann, 2013) and abiotic methane production (Hovland et al. 1993) is supplemented with lateral methane transport. Water discharge from rivers, as well as tidal influence, are factors that greatly control methane distribution (Rehder et al. 1998; Grunwald et al. 2009). Thus, these areas represent diverse and complex hydro-dynamic systems. Besides, estuarine systems are subject to significant seasonal changes (Sansone et al. 1998; Middelburg et al. 2002; Abril and Borges 2005), a factor which is not

considered in many studies. Bacterial methane oxidation, along with degassing and dilution of methane rich water, are also important factors controlling methane distribution in the water column (Grunwald et al. 2009). Up to 80-90% of the available methane can be metabolized and thereby disposed of by bacterial methane oxidation in the freshwater (Reeburgh et al. 1993; Guerin and Abril 2007). In the marine environment, however, methane oxidation (MOX) rates are in general Loir (Valentine et al. 2001; Mau et al. 2013). Though, knowledge on bacterial methane oxidation in the water column rapidly improves, MOX rates measurements in estuaries are still scarce. Additionally, an improved knowledge of the environmental factors controlling bacterial methane oxidation and the physiological properties of these organisms is crucial and this topic needs further investigation (Valentine 2011; Mau et al. 2013).

The objectives of this study were to describe the spatial and seasonal variations of methane in the German Bight, near the Elbe estuary. The methane concentration, as well as several hydro-chemical parameters from the bottom and surface waters, were measured over a period of two years along a 60 km long transect in the German Bight. Along with temperature, the concentrations of inorganic nutrients (ammonium, nitrite, nitrate, phosphate, and silicate) and suspended particulate matter (SPM) were measured. Salinity was an especially important parameter, as it is a direct indicator of the extent of water discharge of the Elbe River. The aim of this study was to bring the importance of bacterial methane oxidation as a significant methane sink into focus. Therefore, we made highly sensitive radiotracer measurements to estimate MOX rates in the water column (Valentine et al. 2001). Only on the basis of these comprehensive examinations we were able to determine the main environmental factors that control methane distribution and MOX rates.


**Figure 1**
Map of the German Bight with sampling stations along the Cuxhaven - Helgoland transect.

## Materials and methods

### Study area

The North Sea (including its estuaries and fjords) has a surface area of about 750,000 $km^2$ and a volume of about 94,000 $km^3$ (Commission, 2000). The non-tidal circulation of the North Sea is dominated by a cyclonic residual current. Water from the Atlantic (Fair Isle Current, Shetland Flow) flows southward along the British coast and returns northward, together with influxes from the English Channel and various rivers along the coasts of the Netherlands, Germany, and Denmark (Rehder et al. 1998). The German Bight is the south-eastern bight of the North Sea, bounded by the Netherlands and Germany to the south, and Denmark and Germany to the east (the Jutland peninsula). To the north and west, it is bounded by the Dogger Bank. The depths in this area vary mainly from 20 to 40 m (Czitrom et al. 1988). The German Bight consists mainly of a mixture of the Central (Southern) North Sea water masses and continental coastal waters (Becker et al. 1992). The water column in the central (southern) North Sea can be stratified into two slightly different layers (Czitrom et al. 1988; Becker et al. 1992). Inshore water did not show any stratification in either summer or winter. Freshwater discharge from the Elbe and Weser rivers causes a large salinity contrast near the shore (Czitrom et al. 1988). Analysis of horizontal density gradients did not show a clear annual cycle either near the shore or offshore (Czitrom et al. 1988). Surface sediments affected by tidal or residual current, wave action, and heavy bottom trawling are very mobile (ICES 1988; Becker et al. 1992).

The Elbe is one of the major rivers of central Europe, with a total catchment area of about 150,000 $km^2$. It runs from the Czech Republic through Germany, and reaches the German Bight in its north-eastern region, near Cuxhaven. The Elbe's mean annual discharge at the river mouth is 860 $m^3 s^{-1}$. The discharge regime is mainly controlled by rainfall and snowmelt; therefore it peaks in April/May (Simon 2005).

### Water sampling

Samples were collected for 2 years from 2010 to 2012. Eleven one-day sampling cruises took place on the 7.10., 8.12. in 2010, 12.1., 2.3., 4.5., 6.7., 29.9. in 2011 and 11.1., 29.2., 28.3., 11.6. in 2012. All sampling procedures and some of the processing of the samples were done on board the research vessels ‘Uthörn’ and ‘Ludwig Prandtl’. Seven sampling stations were distributed along the

Helgoland-Cuxhaven transect (Fig. 1). The names of the stations were determined by their distance from the northernmost coastal point near Cuxhaven, so the most south-eastern station was denoted 'Sea kilometer (Sk) 1', and the most north-western, near Helgoland, Sk 59. We determined stations Sk 1, Sk 14, and Sk 20 to be "coastal stations", and stations Sk 27, Sk 32, Sk 49, and Sk 59 to be "marine stations". Water samples were collected with Niskin bottles from the water surface (1m below the surface) and from the bottom (1m above the bottom).

**Oceanographic parameters**

Water temperature was measured to monitor seasonality during the study period. Salinity was measured to account for the proportion of freshwater from the Elbe River in North Sea water. Oxygen concentration was measured to investigate its effect on MOX rate, and accordingly, on methane distribution. During the "Prandtl cruises", temperature, salinity, and oxygen in the water column were measured immediately after sampling on board using a Universal Pocket Meter (Multi 340i) with precisions of 1% for salinity, 0.1°C for temperature, and 0.5% for oxygen. Salinity was measured in mS $cm^{-1}$, and then converted according to the Practical Salinity Scale. For the "Uthörn cruises", a sea-bird CTD sensor was mounted to the water sampling rosette. In July 2011, temperature measurements were not made due to technical problems. Thus, we obtained temperature data from the database of the River Basin Community Elbe (RBC Elbe; in German, "Flussgebietsgemeinschaft (RBC) Elbe"; http://www.fgg-elbe.de/elbe-datenportal.html). These data were collected near Cuxhaven two days before our cruise. Comparisons of RBC measurements with ours for other months did not reveal any significant difference (±1°C). These data from July were excluded from the comparison of temperatures on the surface and on the bottom, but were used for the correlation analysis between temperature and other factors.

**Suspended particulate matter (SPM)**

Sampled water was filtered using pre-washed and pre-weighed GFC filters (Whatman TM), which were afterwards dried for 24 hours at 60°C and weighed again. Water volumes varied from 100 to 250 ml, depending on turbidity. Filtration was done on board right after collecting water samples. The other procedures were done in the laboratory.

**Inorganic nutrient analysis**

Samples for inorganic nutrients ($NO_2$, $NO_3$, $NH_4$, $PO_4$) and silicite concentrations were taken after sampling for methane and MOX rates. During the "Prandtl cruises", sampled water was filtered with GFC filters (Whatman TM), transferred into 50 ml Falcon tubes, and frozen until further analysis in the laboratory. Sampling during the "Uthörn cruises" and analysis of all the samples was performed by Karen Wiltshire's group (Wiltshire, 2011, 2012) following the technique described by Grasshoff et al. (1983). Nutrient analysis was performed only for surface waters.

**Methane concentration**

We measured methane concentrations along the transect in different months to assess seasonal and spatial variations. Right after sampling collected water was transferred, bubble-free, into 120 ml glass serum bottles. The bottles were rinsed with approximately two volumes of sample water, capped with black rubber stoppers, and sealed with an aluminum crimp.

To eliminate agents (such as soap) that inhibit methane oxidation, the glass bottles had been washed and soaked in 3% HCl for 12 hours, and then soaked in distilled water overnight. The stoppers had been autoclaved for 20 min at 120°C three times, and then rinsed with distilled water.

To determine methane concentrations, samples from each depth and each station were collected in single (March-September 2011) or duplicate bottles (October 2010 - January 2011, January - June 2012). Immediately after filling the bottles, samples were poisoned with 0.3 ml of 25% $H_2SO_4$ (samples from marine stations) or 0.3 ml of 5N NaOH (samples from coastal stations) to prevent methane oxidation. Poisoning agents were chosen according to the results of preliminary testing of the efficiency of both chemicals in marine and brackish water (Bussmann et al. 2015). Methane concentrations were measured using a headspace technique, by adding 10 ml of $N_2$ according to McAuliffe (1971). Three 0.1 ml headspace aliquots from each sample were analyzed with a gas chromatogram (GC 2014; Shimadzu) equipped with a flame ionization detector and a molecular sieve column (Hay Sep N, 80/100, Alltech). The temperatures of the oven, the injector, and detector were 40, 120, and 160°C, respectively. The carrier gas ($N_2$) flow was 20 ml $min^{-1}$. The gas flow of the FID was 40 ml $min^{-1}$ for $H_2$ and 400 ml $min^{-1}$ for synthetic air. Gas standards (Air Liquide) with concentrations of 10 and 100 ppm methane were used for calibration. The measurement error of the GC was less than 5%.

Equilibrium concentrations were calculated according the formula proposed by Wiesenburg and Guinasso (1979). Data on the methane concentration in the atmosphere were obtained from the Mace Head Atmospheric Research Station, located on the west coast of Ireland (http://agage.eas.gatech.edu). Saturation rates were calculated as the ratio between observed methane concentrations in the water column and equilibrium concentrations with respect to the ambient methane concentrations in the atmosphere, multiplied with 100%.

**Methane oxidation (MOX) rate**

In addition to the chemical parameters, we also measured the microbial methane oxidation, as a possible important methane sink. Samples for MOX rates were collected from each depth and each station in triplicate bottles. According to Bussmann et al. (2015) the coefficient of variation in this case is about 23 ± 11% at low activities (<10 nmol $L^{-1}$ $d^{-1}$) and 7 ± 5% at higher activities (>10 nmol $L^{-1}$ $d^{-1}$). MOX rates were measured following radiotracer techniques using tritiated methane (American Radiolabeled Chemicals, 20 Ci $mmol^{-1}$) according to a method modified from Valentine et al. (2001). A diluted tracer (0.1 ml) was added to the samples (2 kBq $ml^{-1}$). Samples were vigorously shaken and incubated for 24 h in the dark at near in situ temperatures. After incubation, methane oxidation was stopped by adding 0.3 ml of 25% $H_2SO_4$ (for marine station samples) or 5N NaOH (for coastal station samples). Controls were stopped before the addition of the tracer.

Under oxic conditions methane is oxidized according the following reaction:

1) $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$

The consumption of tritiated methane ($C^*H_4$) leads to the production of tritiated water ($^*H_2O$) and to the decrease of radioactive tracer in the gas phase:

2) $C^*H_4 + 2O_2 \rightarrow CO_2 + 2^*H_2O$

Then, the total radioactivity ($^3$H-$CH_4$ and $^3$H-$H_2O$) added to the sample and the fraction of the labeled gas oxidized or produced water ($^3$H-$H_2O$) have to be measured. To determine the total radioactivity of the sample, the sample bottle is opened, and 2ml of the subsample is mixed with 5 ml scintillation cocktail. To determine the radioactivity of the tritiated water, the rest of the sample was sparged with air to expel all methane. Bottles were then half

emptied, a long needle was inserted to the bottom of the bottle, and the sample was purged with air for 30 min. Two ml water aliquots after sparging were mixed with 5 ml of the scintillation cocktail (Ultima Gold LLT, Perkin Elmer) and analyzed on a liquid scintillation counter (Tri-Carb® 2910 TR, Perkin Elmer) using decays per minute (dpm) values. The MOX rate in nmol $L^{-1}$ $d^{-1}$ was calculated by taking the ratio of the produced radioactive water to the amount of added tracer (r = $^{*}H_2O/C^{*}H_4$, in dpm) and multiplying it with the ambient methane concentration ([$CH_4$] in nmol $L^{-1}$) and correcting for the incubation time (t in $d^{-1}$) (Valentine et al. 2001):

3) $MOX = r \times \frac{CH_4}{t}$

The turnover time (tt in days) is the time it would take to oxidize all the methane at given MOX rate. It was calculated as the inverse of the ratio (r), corrected for the incubation time (t). Thus, this parameter is independent of the ambient methane concentration, and gives a good measure of the methanotrophic potential (Heintz et al. 2012).

However, there might be some $^{*}H_2O$ which was not the result of methane oxidation but was a contamination of the tracer. The $^{*}H_2O$ from the killed controls reveals this value. In marine waters, about 0.06% of the injected tracer was found to be "abiotic water". In freshwater, the percentage increased to about 0.62%. The value of $^{*}H_2O$ were corrected for this "abiotic" water.

**Statistical analyses**

For comparison of surface and bottom values and for comparison of values at different stations (spatial variation), we used a paired t-test in the case of a normal distribution, and a nonparametric Wilcoxon signed- rank test when a normality test (Shapiro-Wilk) failed. Marine and coastal stations were analyzed separately. When no significant difference between data sets was found, the respective data were pooled. To investigate the dependence of methane concentration, turnover time, and MOX rate on hydrographical and chemical factors such as temperature, salinity, methane, phosphate, oxygen, and SPM, we performed simple linear regression analyses. If the linear correlation was not significant, we performed a Spearman rank order correlation analysis, which shows if variables are related monotonically, i.e. an increase of one variable causes an increase/decrease of the other variable. The Spearman correlation

coefficient ($r_s$) is defined as the Pearson correlation coefficient between the ranked variables. For a sample of size n, the n raw scores $X_i$, $Y_i$ are converted to ranks $x_i$, $y_i$, and $r_s$ is computed as:

4) $$r_s = \frac{\sum_i (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_i (x_i - \bar{x})^2 \sum_i (y_i - \bar{y})^2}}$$

Calculations were performed using SigmaPlot for Windows Version 11.0 software.

## Results

### Oceanographic parameters

No significant difference between surface and bottom temperature was observed (paired t-test, n=39, p=0.917), so the data were pooled. The lowest temperatures were measured in January 2011, with an average of 2.6±1.2°C. The highest temperature (19.0±1.4°C) was observed in September 2011.

Surface water generally had a lower salinity than bottom water (paired t-test, n=49, p<0.001). The stratification of the water column was most pronounced from June to September. Due to the input of freshwater from the Elbe River, salinity increased with distance from the shore. This is well illustrated by two independent sampling campaigns in the summer (July 2011) and winter (February 2012) (Fig. 2). For all sampling dates, at marine station Sk 59, the average salinity was 32.2±1.0, while coastal station Sk 1 had an average salinity of 16.3±5.7. The lowest salinity at this station (7.8) was detected in September 2011, and the highest salinity at this station (24.7) was detected in June 2012.

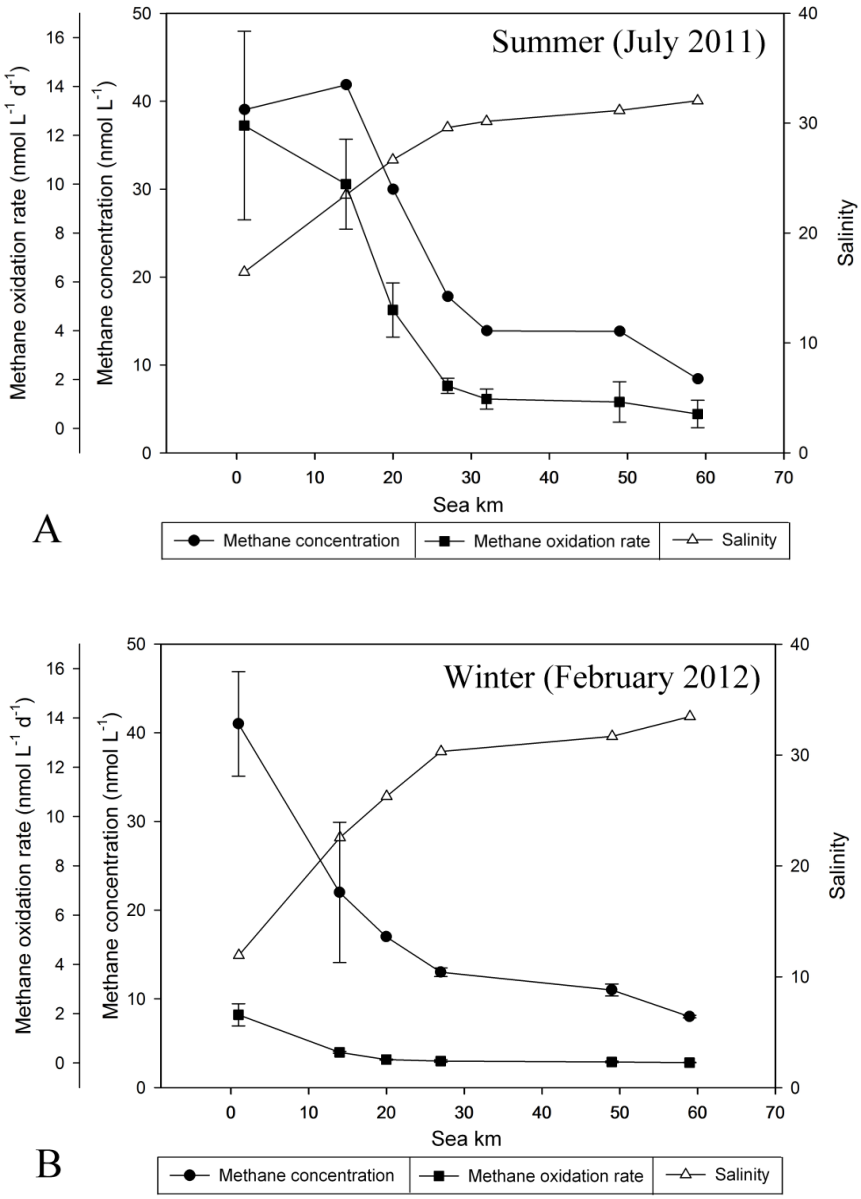
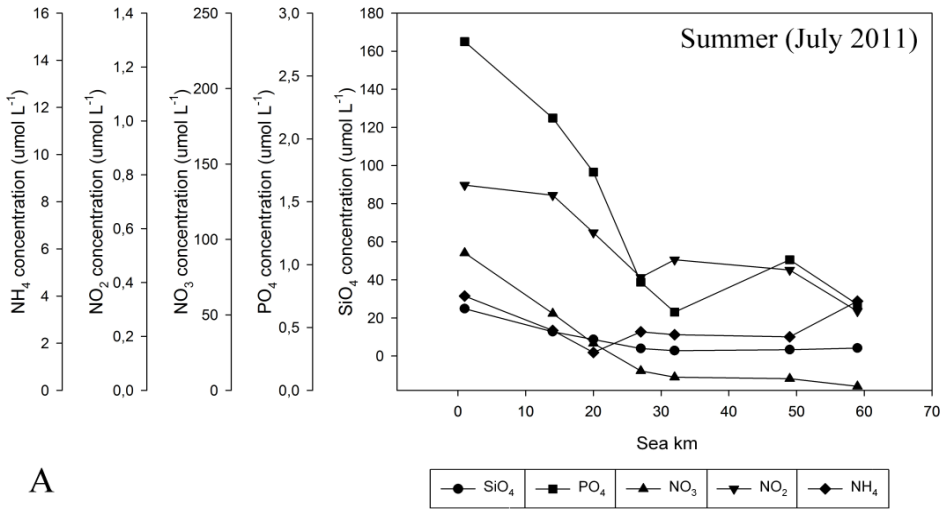



**Figure 2**
Distribution of methane concentration, MOX rate, and salinity along the transect in A) summer (July 2011) and B) winter (February 2012). Shown is the average of surface and bottom water.



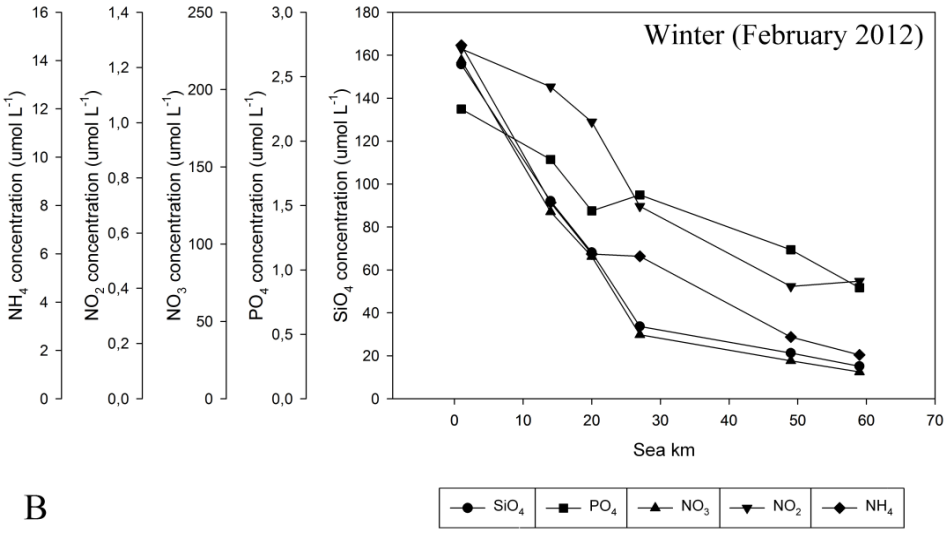




**Figure 3**
Distribution of inorganic nutrients in A) summer (July 2011) and B) winter (February 2012). Shown are only data from surface water.

**Suspended particulate matter, oxygen and inorganic nutrients**

Values for SPM ranged from 8 to 167 mg $L^{-1}$, but neither seasonal nor spatial (along the transect) differences could be detected. Bottom water was always more turbid than corresponding surface water (paired t-test, n=49, p<0.001, data not shown).

Oxygen concentrations did not show spatial differentiation along the transect, nor seasonal trend. The lowest concentration of oxygen (6.1 mg $L^{-1}$) was detected in October 2010 at station Sk 59, while the highest concentration (11.4 mg $L^{-1}$) was detected in January 2012 near station Sk 14. Values near the surface (average value of 8.7 mg $L^{-1}$) were generally slightly higher than near the bottom (average value of 8.5 mg $L^{-1}$) (paired t-test, n=42, p=0.021).

Overall concentrations of $NO_2$, $NO_3$, $NH_4$, and $PO_4$ decreased towards the sea and were nearly depleted at a distance of 30 km from the coast. Fig. 3 shows representative results from two individual sampling campaigns. Phosphate concentrations ranged from 0.3 mmol $L^{-1}$ (May 2011) to 1.8 mmol $L^{-1}$ (December 2010) at station Sk 59, and from 1.2 mmol $L^{-1}$ (May 2011) to 3.3 mmol $L^{-1}$ (January 2012) at station Sk 1. Nitrite concentrations ranged from 0.1 mmol $L^{-1}$ (October 2010) to 1.2 mmol $L^{-1}$ (March 2011) at station Sk 59, and from 0.7 mmol $L^{-1}$ (May 2011) to 1.5 mmol $L^{-1}$ (January 2012) at station Sk 1. Nitrate concentrations ranged from 0.1 mmol $L^{-1}$ (October 2010) to 25.0 mmol $L^{-1}$ (May 2011) at station Sk 59, and from 81.6 mmol $L^{-1}$ (May 2011) to 219.0 mmol $L^{-1}$ (February 2012) at station Sk 1. Ammonium concentrations ranged from 0.1 mmol $L^{-1}$ (May 2011) to 3.8 mmol $L^{-1}$ (July 2011) at station Sk 59, and from 0.5 mmol $L^{-1}$ (May 2011) to 14.6 mmol $L^{-1}$ (February 2012) at station Sk 1. In the summer months, e.g. July 2011, the ammonium concentrations varied significantly over the entire transect. From Sk 1 to Sk 49, the concentration decreased, but suddenly rapidly increased again, until it reached a maximum at Sk 59, which is similar to Sk 1. Silicate concentrations ranged from 1.6 to 15.1 mmol $L^{-1}$ at station Sk 59, and from 21.8 to 155.7 mmol $L^{-1}$ at station Sk 1 in May 2011 and February 2012, respectively.

**Methane concentrations**

The results of all the measurements are summarized in Fig. 4 and are published in the data library PANGAEA (Bussmann et al. 2014a). No significant difference between surface and bottom values was observed either for the marine (paired t-test, n=28, p=0.412), or coastal stations (paired t-test, n=20, p=0.522),

so the data were pooled. The dissolved equilibrium concentrations varied from 2.9 to 3.1 nmol $L^{-1}$. Methane concentrations in the water column for both marine and coastal stations were supersaturated. At the coastal stations saturation rate varied from 340 to 1880%, at the marine stations it varied from 170 to 1110%. At the coastal stations, methane concentrations were, in general, higher than at the marine stations, and decreased offshore from station Sk 1 to station Sk 20. The only exception was detected in September 2011, when values were significantly higher than during the rest of the season. These data were the only ones that did not follow the trend, and stood apart from the rest of the observations, so we omitted it from further processing. The three coastal stations had an average methane concentration of 30±13 nmol $L^{-1}$. The average methane concentration at station Sk 1 was 40.9±9.4 nmol $L^{-1}$, ranging from 24.0 nmol $L^{-1}$ in May 2011 to 58.3 nmol $L^{-1}$ in March 2012. The average methane concentration at station Sk 20 was 19.8±8.9 nmol $L^{-1}$, ranging from 10.0 nmol $L^{-1}$ in May 2011 to 36.0 nmol $L^{-1}$ in June 2012. For the marine stations, no spatial variation of methane concentrations was observed, so the data were pooled. The average methane concentration for the marine stations was 14.0±4.8 nmol $L^{-1}$ (n=56), with a minimum of 5 nmol $L^{-1}$ in March 2011 and a maximum of 32 nmol $L^{-1}$ in December 2010.

To highlight the strong effect of the riverine water and the seasonal trends, two individual sampling campaigns (summer/winter) are shown in Fig. 2. At the coastal stations, methane concentrations were higher in summer (July 2011) than in winter (February 2012). At the marine stations, methane concentrations were approximately the same. Methane concentration decreased offshore, clearly coinciding with the salinity increase in both summer and winter. Regression analysis showed that about 57% of methane variability could be explained by salinity (for all data, n=93, Fig. 5). On four occasions, we were able to sample all stations on one day. For these dates (May 2011, July 2011, January 2012, and February 2012), the correlation was much stronger and the salinity explained 70-98% of the methane variability. When the regression line (Fig. 5) was extrapolated to a salinity of zero, we obtained a prospective methane concentration of 72 nmol $L^{-1}$ for Elbe waters. The corresponding methane concentrations from the single cruise analysis ranged from 56 to 80 nmol $L^{-1}$.

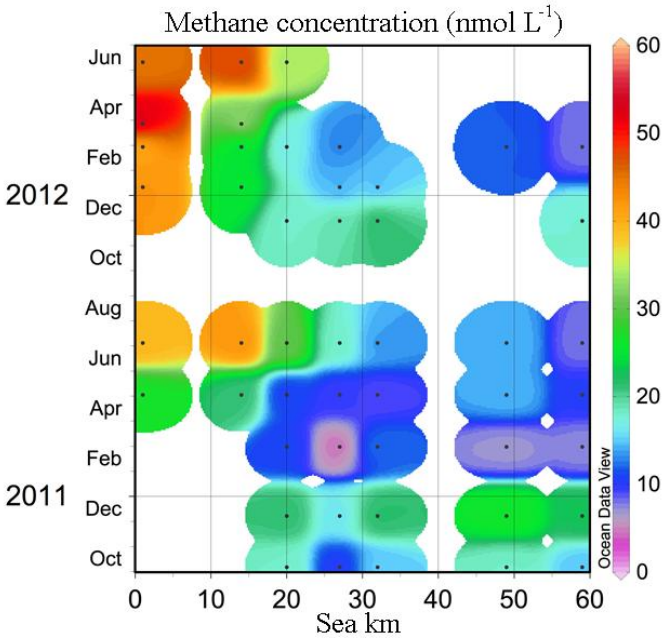



**Figure 4**
Methane concentration along the transect during the whole period of investigation (surface and bottom).

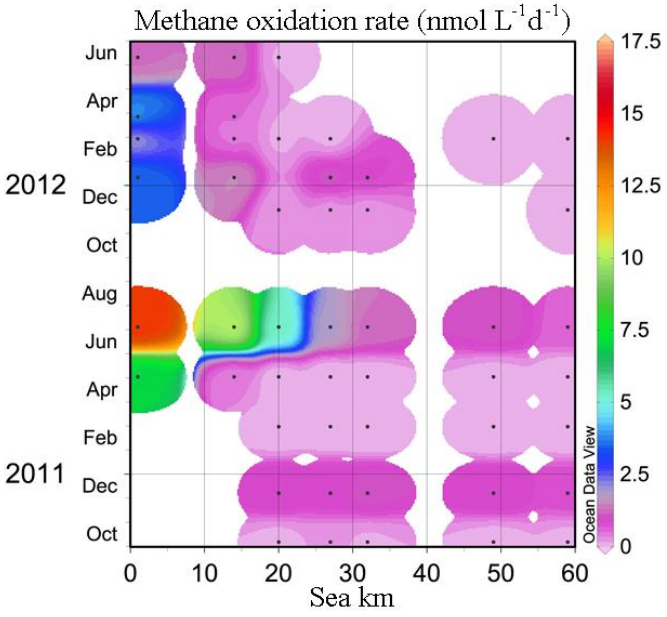



**Figure 7**
MOX rates along the transect during the whole period of investigation (surface and bottom).

No linear correlation between methane concentration and temperature was observed, however we detected temperature dependence after rank transformation of the data. At the coastal stations, the highest methane concentrations were found during periods of highest temperatures ($r_s$=0.49, n=36). Since two independent parameters correlated with methane concentration at the coastal stations, we applied multiple linear regressions between methane concentration, salinity, and temperature, and obtained a model with an even higher correlation coefficient ($r^2$=0.74, n=30) (Fig. 6). No linear correlation, but a weak positive dependence of methane concentration on oxygen, was found along the entire transect ($r_s$=0.37, n=80). We also found a strong correlation between concentrations of methane and inorganic nutrients, except $NO_2$ ($r_s$ varied from 0.43 to 0.66 depending on the inorganic nutrient). However, we assumed that this was mainly due to the input of nutrient-rich waters from the mouth of the Elbe, as salinity and inorganic nutrient concentrations were also strongly correlated ($r^2$ for different mineral nutrients varied from 0.46 to 0.87).

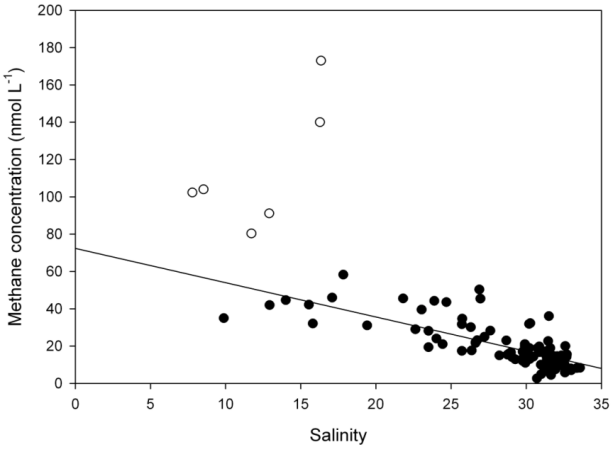



**Figure 5**
Linear regression between salinity and methane concentration. Empty circles indicate outliers, which were excluded from regression analysis (measurements from September 2011).

No linear correlation between SPM and methane concentrations was found. Moderate negative dependence was indicated after rank transformation of the marine data. Thus, we can state that at the marine stations, the highest methane concentrations were found at the lowest SPM values ($r_s$=-0.45, n=54).

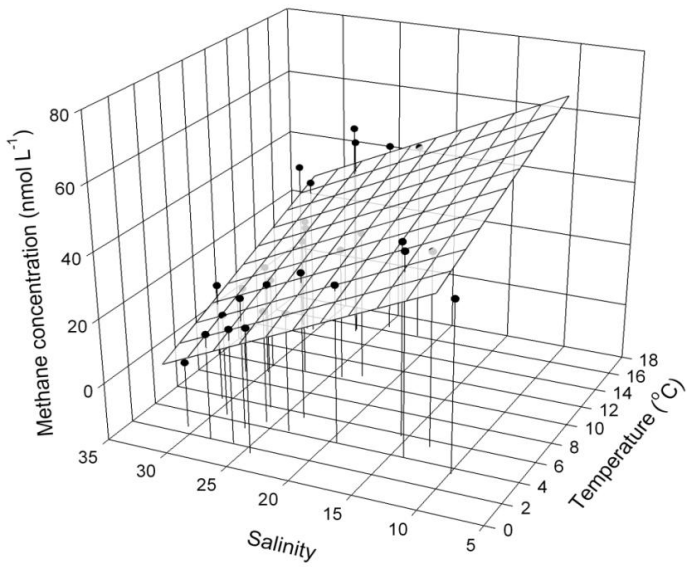



**Figure 6**
Methane distribution at the coastal stations according to salinity and temperature. Multiple linear regression: $CH_4$ = 55.798 + (1.697*Temperature) - (1.709*Salinity); $r^2$ = 0.737.

**Methane oxidation rate**

The results of all the measurements are summarized in Fig. 7 and are published in the data library PANGAEA (Bussmann et al. 2014a). No significant differences between surface and bottom values were observed either for the coastal stations (paired t-test, n=18, p=0.154), or for the marine stations (paired t-test, n=28, p=0.290), so the data were pooled. At the coastal stations, MOX rates were in general higher than at the marine stations, and decreased offshore from station Sk 1 to station Sk 20. The coastal stations had an average MOX rate of 2.6±2.7 nmol $L^{-1}$ $d^{-1}$. The average MOX rate at station Sk 1 (nearest the coast) was 5.26±4.72 nmol $L^{-1}$ $d^{-1}$, ranging from 0.78 nmol $L^{-1}$ $d^{-1}$ in June 2012 to 16.5 nmol $L^{-1}$ $d^{-1}$ in July 2011. The average MOX rate at station Sk 20 was 0.97±1.85 nmol $L^{-1}$ $d^{-1}$, ranging from 0.04 nmol $L^{-1}$ $d^{-1}$ in May 2011 to 5.81 nmol $L^{-1}d^{-1}$ in July 2011. The average MOX rate for the marine stations was 0.417±0.529 nmol $L^{-1}$ $d^{-1}$, with a minimum of 0.009 nmol $L^{-1}d^{-1}$ in February 2012 and a maximum of 1.96 nmol $L^{-1}$ $d^{-1}$ in July 2011.

For the turnover time no significant differences between surface and bottom values were observed either for coastal stations (paired t-test, n=18, p=0.182), or marine stations (paired t-test, n=28, p=0.136). Thus, the data were pooled. The coastal stations had an average turnover time of 64±75 days. The average turnover time at station Sk 1 was 16±15 days, with a minimum of 3 days in July 2011 and a maximum of 56 days in June 2012. The average turnover

time at station Sk 20 was 120±92 days, with a minimum of 5 days in July 2011 and a maximum of 293 days in October 2010. The average turnover time for the marine stations was 196 days, with a minimum of 9 days in July 2011 and a maximum of 1049 days in February 2012. To highlight the strong effect of the riverine water and the seasonal trends, two individual sampling campaigns (summer/winter) are shown in Fig. 2. MOX rates along the transect generally followed a trend similar to the methane concentrations and salinity. However, MOX rates were significantly lower in winter (February 2012) than in summer (July 2011), especially at the coastal stations.

Because of great variability in the turnover times and MOX rates, linear regression analysis did not reveal clear results. Therefore, we rank transformed the data and performed Spearman rank order correlation tests (Table 1). The tests showed that the variability of the turnover time was influenced by methane concentration ($r_s$=-0.60, n=94) in a negative way, i.e. highest turnover times (the slowest rates) were found at lowest methane concentrations. Salinity influenced the variability of the turnover time in a positive way ($r_s$=0.56, n=91), especially in the coastal area ($r_s$=0.64, n=37), i.e. highest turnover times were found at highest salinities. Turnover time was moderately correlated in a negative way with temperature ($r_s$=-0.46, n=86), i.e. highest turnover times were found at lowest temperatures. Thus, we found that turnover time was correlated to a greater or lesser extent with three factors: methane concentration, salinity, and temperature.

Because of the dilution of the methane-rich freshwater, we mostly found low methane concentrations at high salinities. To exclude this interaction, and to check for the 'real' influence of salinity on MOX rate and turnover time, we grouped the methane concentrations, according to their frequency distribution, to low methane concentrations (<15 nmol $L^{-1}$, n=37), medium methane concentrations (15-30 nmol $L^{-1}$, n=41), and high methane concentrations (>30 nmol $L^{-1}$, n=21, Table 1). Only when methane concentrations were high (>30 nmol $L^{-1}$), MOX rate and the turnover time (rank transformed data) were correlated with salinity ($r_s$=0.39 and 0.34, respectively). At lower methane concentrations, these parameters were independent of salinity. Thus at 'high' methane concentrations, salinity had a negative effect on the turnover time and MOX rate. At lower methane concentrations, the most important factor for the turnover time was simply the methane concentration.

The same tendency was observed for MOX rates (Table 1), which were negatively correlated with salinity ($r_s$=0.66, n=92), but positively correlated with methane concentration ($r_s$=0.78, n=94) and temperature ($r_s$=0.43, n=87), i.e. highest MOX rates were detected at lowest salinities and highest methane concentrations and temperatures. However, the correlation between MOX rate and methane concentration should be viewed with caution, because MOX rate calculations are dependent on methane concentrations.

Methane oxidation rates were correlated with phosphate concentrations ($r_s$=0.54, n=42), but phosphate was strongly correlated with salinity. Therefore, to circumvent the co-correlation of phosphate and salinity, we grouped the salinity data into meso-, poly-, and euhaline (5-18, 18-30, and >30; (Caspers 1959). For each of these salinity groups, no influence of phosphate on turnover time or MOX rate was observed (rank transformed data). No influence of the other inorganic nutrients, SPM, or oxygen on MOX rates or turnover times could be detected by statistical analyses.

**Table 1**
Spearman rank correlation of the turnover time and MOX rates with hydrographic parameters.

| Dependent parameter | Independent parameter | Spearman rank order correlation | Comments |
|---|---|---|---|
| Turnover time | Methane concentration | −0.60 | |
| | Salinity | +0.56 | Due to co-correlation of salinity with methane, methane data were grouped, then only with methane >30 nmol $L^{-1}$, salinity influences turnover time with $r_s = 0.39$ |
| | Temperature | −0.46 | |
| MOX rate | Methane Concentration | n. d. | |
| | Salinity | −0.66 | Due to co-correlation of salinity with methane, methane data were grouped, than only with methane >30 nmol $L^{-1}$, salinity influences MOX with $r_s = -0.34$ |
| | Temperature | +0.43 | |

n.d. – not determined.

**Table 2**
Methane concentrations and MOX rates, measured in different estuaries and in the open ocean.

| Location | Methane concentrations (nmol $L^{-1}$) | MOX rates (nmol $L^{-1}d^{-1}$) | Reference |
|---|---|---|---|
| Estuaries | | | |
| Elbe (German Bight) | 3–60 | 0.01–17 | This study |
| Elbe | 4–111 | n.m. | (Middelburg et al., 2002) |
| North Sea around Helgoland | 200 | n.m. | (Grunwald et al., 2009) |
| Southern Bight (North Sea) | 3–22 | <<1.3 | (Scranton and McShane, 1991) |
| Ems | 91–51 | n.m. | (Middelburg et al., 2002) |
| Thames | 5–273 | n.m. | (Middelburg et al., 2002) |
| Rhine | 4.1–1437 | n.m. | (Middelburg et al., 2002) |
| Scheldt | 20–485 | n.m. | (Middelburg et al., 2002) |
| Loire | 16–671 | n.m. | (Middelburg et al., 2002) |
| Gironde | 4–559 | n.m. | (Middelburg et al., 2002) |
| Douro | 15–128 | n.m. | (Middelburg et al., 2002) |
| Sado | 37–40 | n.m. | (Middelburg et al., 2002) |
| Hudson river estuary | 48–858 | 0.1–68 | (de Angelis and Scranton, 1993) |
| Randers Fjord, Denmark | 28–420 | <0.2–15.2 | (Abril and Iversen, 2002) |
| Bay of Temmesjoki River (Baltic Sea) | 62–588 | n.m. | (Silvennoinen et al., 2008) |
| Estuary of the Yangtze River | 3–89 | n.m. | (Zhang et al., 2008) |
| Open ocean | | | |
| Gulf of Mexico | 10–343 | 0–57 | (Kelley, 2003) |
| Cape Lookout Bight | 18–246 | 0 | (Kelley, 2003) |
| Arctic fjord Storfjorden | 5–80 | 0–3 | (Mau et al., 2013) |

n.m. – not measured.

**Discussion**

Methane distribution is defined as the balance between methane sources and sinks. Estuaries are one of the main sources of methane in the North Sea. They, in turn, are supplied with methane by riverine input, tidal flats, and marshes (Rehder et al. 1998; Middelburg et al. 2002; Abril and Borges 2005; Grunwald et al. 2009). The main methane sinks are represented by the dilution of methane-rich waters with methane-depleted waters, outgassing, and bacterial methane oxidation (Scranton and McShane 1991).

Methane concentrations were monitored over two years along a transect from the Elbe estuary towards the German Bight. The highest methane concentrations were detected near the coast (the first coastal station, Sk 1). Methane concentrations for the next 20 km offshore, decreased until they reached a plateau, and did not significantly vary any further (20e60 km). Therefore, we can conclude that in terms of methane distribution, the direct impact zone of the Elbe riverine waters is in the range of 20 km from the river mouth in Cuxhaven. The coastal stations (1-20 km from the coast) had an average methane concentration of 30±13 nmol $L^{-1}$, comparable with the measurements of Rehder et al. (1998), which were in the range of 10-40 nmol $L^{-1}$. Methane concentrations at the marine stations were on average 14±6 nmol $L^{-1}$. These concentrations are slightly higher than those reported by Rehder et al. (1998), but remarkably lower than values reported by Grunwald et al. (2009), which were around 200 nmol $L^{-1}$ near Helgoland. In general, methane concentrations in the Elbe estuary and in the German Bight are comparable with those measured in other estuaries (de Angelis and Scranton 1993; Abril and Iversen 2002; Middelburg et al. 2002; Silvennoinen et al. 2008; Zhang et al. 2008; Grunwald et al. 2009) and open ocean locations (Kelley 2003; Mau et al. 2013) (Table 2).

For all stations and at all times the water was supersaturated with methane (170-1880%), thus the German Bight acts as a methane source to the atmosphere both at the coastal and at the open sea part.

As was done in previous studies, we extrapolated methane concentration at zero salinity using a linear correlation model. In March and June 2012, we had the opportunity to also sample water from the Elbe River itself. These data are published in the PANGAEA data base (Bussmann et al. 2014b). Thus, we were able to examine the actual riverine input more closely (Fig. 8). At salinities of 10-15, methane concentrations were 65±5 nmol $L^{-1}$ (Bussmann et al. 2014b), which is very close to our interpolated value of 72 nmol $L^{-1}$. The corresponding stations EC-719 and EC-724 are close to the port of Cuxhaven. In contrast, the real riverine methane concentrations with salinities <5 (river stations EC-679, 699 and 709) were much lower (26±8 nmol $L^{-1}$). This shows that the Elbe is not the main methane source in the estuary, and that the mixing model can only be applied at salinities 10-15. Thus, application of extrapolating approaches as proposed by Rehder et al. (1998) is not possible.

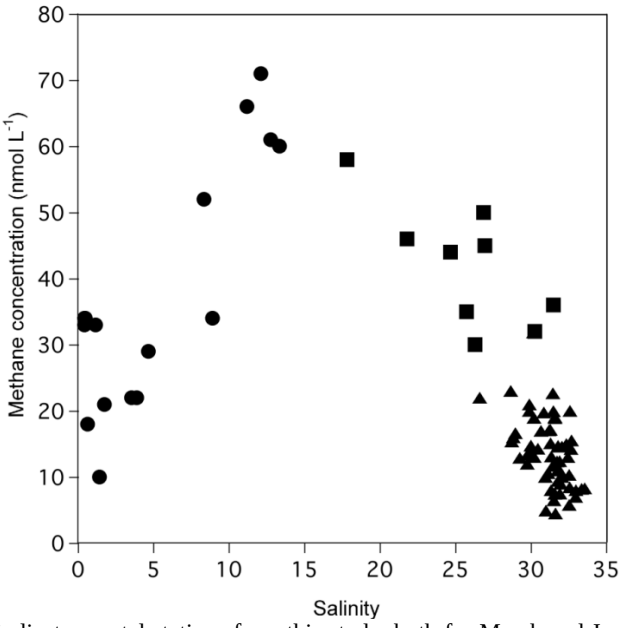



**Figure 8**
Methane concentrations with corresponding salinities. Circles indicate river stations (Bussmann et al. 2014b), and squares indicate coastal stations from this study, both for March and June 2012. The triangles represent the values from all marine stations from this study.

Middelburg et al. (2002) measured a significantly higher methane concentration of 111 nmol $L^{-1}$ at zero salinity in 1997. However, as the water quality in the Elbe has improved over the last 20 years (Amann et al. 2012) methane concentration may have decreased. Besides, Middelburg et al. (2002) made measurements during only one sampling campaign in April, and as we have shown, methane concentrations are subject to significant temporal variations (this will be discussed later).

Explanations for the methane increase near Cuxhaven are: a) increased methane production in the underlying sediment, b) lateral input. Additionally, we would expect decreased methane oxidation in the estuary in comparison with the freshwater end-member (discussed later). Methane is mostly produced in anoxic sediment zones, from where it can diffuse into the overlying water column. Our comparison of bottom and surface waters gave no indication of strong methane production in the sediments. The sediment in this area is supposed to be very coarse due to the strong currents, and even though methanogenesis also

occurs in oxygenated, organic-rich sediments just several cm under the sediments surface (Deborde et al. 2010), we did not observe a strong direct sedimentary input. This is in agreement with the observation made by Scranton and McShane (1991), who stated that sandy sediments of the North Sea are not a significant methane source.

Tidal flats in the North Sea are known to be active sites of mineralization of organic material, which eventually leads to methane accumulation. Due to advective flow in the tidal areas, which is of special importance in permeable sandy sediments, pore waters enriched in re-mineralized nutrients and methane are actively released from sediments into the overlying water column (Beck and Brumsack 2012; Segarra et al. 2013). As Cuxhaven is surrounded by tidal flats, we assume a strong lateral input of methane from these tidal flats.

Other potential methane sources are inputs from other rivers draining into the estuary, and sewage discharge. The Oste, a small river which flows into the North Sea near Cuxhaven, has low water velocities and therefore could have much higher methane concentrations. However, no data were available for this river. A wastewater treatment plant in Cuxhaven, as well as industrial activity, could lead to additional input of organics which might trigger in situ methane production both in sediments and in the water column. Input of inorganic nutrients was also detectable in the hydrochemical data; the phosphate and nitrate concentrations were especially high at the nearby station. We can thus conclude that the Elbe River gets enriched with methane at its mouth due to additional methane sources, whereby methanogenesis might also play an important role. Furthermore, water in the estuary gets diluted with methane-depleted water from the North Sea. The last section of the transect represents North Sea water almost exclusively, and methane concentrations there do not vary significantly.

We expected seasonality for our methane concentration data, with higher methane concentrations in summer and low ones in winter. However, we could not find any clear seasonal pattern, which may be also due to the too large sampling intervals. But as seasonality is also reflected in changing temperatures, we could find a correlation with water temperature. The effect of temperature on methane production has been shown for many aquatic systems (Pulliam 1993; Bange et al. 1994; Duc et al. 2010; Lofton et al. 2014), including river and estuary systems (Fedorov et al. 2003). Enhanced methane production in the warmer months is known for various river and estuary systems, such as the Don River, with the Taganrog Bay in the Sea of Azov, Russia (Fedorov et al. 2003), and the Baltic Sea (Bange et al. 1994). Clear seasonality with increased methane in the summer and autumn was also shown for the Rhine estuary (Middelburg et al. 2002). However, in the Scheldt and Gironde estuaries, seasonality was not pronounced (Middelburg et al. 2002), nor in Humber estuary, where methane concentrations were lowest in the summer (Upstill-Goddard et al. 2000). In our investigation, moderate correlation of methane concentration with temperature was shown, but only for the coastal stations ($r_s$=0.49). Absence of the correlation at the

marine stations can be explained by insignificant methane production and rather stable temperature regimes in the open sea and sediments.

Further on, we have to consider that a seasonality of methane concentrations could also originate from variation in the Elbe water discharge. As discussed before, methane distribution along the transect was correlated with salinity. Finally, a multiple linear regression combining salinity and temperature as parameters affecting the methane concentration explained 74% of the methane distribution. Water discharge measured at Neu Darchau from September 2010 to June 2012 ranged from 300 to 3500 $m^3 s^{-1}$ (River Basin Community Elbe, http://www.fgg-elbe.de). Indeed, during the entire period of our sampling campaign at station Sk 1, the salinity varied widely from 8 to 25. However, we did not manage to find a strong correlation between water discharge and methane variations. This can be partly explained by the remoteness between Neu Darchau and station Sk1. Additionally; tides are likely to also be a significant factor affecting short term methane distribution, as shown in previous studies (Grunwald et al. 2009). However, in our investigation, we did not have enough information to correlate methane concentration with the tidal surge.

MOX rates, as well as turnover times, were calculated to assess the role of methane oxidation as a methane sink, and to define its role in methane distribution. The highest MOX rates were found at the coastal stations (2.6±2.7 nmol $L^{-1} d^{-1}$), and the lowest MOX rates were found at the marine stations (0.4±0.5 nmol $L^{-1} d^{-1}$). Despite the importance of methane oxidation processes in the water column, very few measurements have been made in estuaries.

MOX rates in the Hudson estuary (in the salt intrusion area) varied from 0.1 to 68 nmol $L^{-1}d^{-1}$ (de Angelis and Scranton 1993). In the Randers fjord MOX rates reached 15 nmol $L^{-1} d^{-1}$ (Abril and Iversen 2002). Summarized data on aerobic methane oxidation in ocean waters from different locations show that MOX rates are generally in the range of 0.001-10 nmol $L^{-1} d^{-1}$ (Kelley 2003; Mau et al. 2013). MOX rates measured at different estuaries and in the open ocean are summarized in Table 2. Thus, our measurements along the entire transect are in the same range of MOX rates in other aquatic environments.

For the southeast coast of the North Sea a flushing time of 11 days and for the central North Sea of 40 days is given by Ilyina et al. (2006). Our average turnover times for the coastal stations and marine stations were 64 days and 196 days, respectively. Thus, the water masses are faster exported than MOX could consume the methane. Scranton and McShane (1991), who studied methane distribution in the Southern Bight (North Sea) also came to a conclusion that bacterial methane oxidation was not a significant methane sink. However, a more detailed modelling would be necessary to adequately address the question if methane oxidation is a significant methane sink in the Elbe estuary and adjacent German Bight.

Microbial activities are often controlled by substrate concentration, following the enzyme kinetics as described by Michaelis Menten. In our case this means, that with increasing methane concentration the MOX rate also increases, until (enzyme) saturation is reached and no further increase of MOX can be observed.

To describe this phenomenon and make it comparable between different studies, the parameter $V_{max}$ and $K_m$ are used to describe the maximal velocity and the half saturation concentration (Lehninger 1985). Unfortunately, to our knowledge there are no kinetic studies on marine methanotrophs, which usually endure at low methane concentrations. Recent studies from arctic lakes reveal Km from 4 to 10 mM (Lofton et al. 2014) and the review from Hanson and Hanson (1996) gives a Km of 1 mM. In a study on the kinetics of cultured methanotrophic strains under low methane concentration (10-100 ppm), it was shown that these strains, which have a Km within the above described range, are able to grow under these limiting conditions (Knief and Dunfield 2005). In our study, natural methane concentrations ranged from 3 to 58 nmol $L^{-1}$, which are concentrations well below half saturation.

Thus we assume that the methanotrophic population of the North Sea is strongly limited by methane concentration and an increase in methane concentration would lead to increased activity. With our data we did not calculate such a relation between methane concentration and MOX rate due to methodological restraints (the MOX rate is calculated by multiplying the ratio of consumed tracer with the methane concentration). But even when setting these restraints apart only a weak correlation was observed.

The effect of salt as a chemical agent inhibiting methane oxidation in freshwater has been shown before (de Angelis and Scranton 1993). The changing salinity likely causes osmotic stress for freshwater methanotrophic bacteria (Hanson and Hanson 1996). At the same time, most studies report that microbial methane oxidation is already significantly reduced at salinities <10 (Abril and Borges 2005), whereas in our investigation, high MOX rates (17 nmol $L^{-1}$ $d^{-1}$) were detected even at a salinity of 17. Our results show that salinity, as such, only has a (negative) effect when methane concentrations are >30 nmol $L^{-1}$. Thus, we conclude that, at the coastal stations, the negative effect of osmotic stress is counteracted by the positive effect of high methane concentrations in the estuary. An explanation might be that microorganisms which are frequently exposed to changing salinity (like the Elbe estuary, due to the significant influence of tides) are adapted to changing salinities. However, it remains unclear as to what extent freshwater or marine methanotrophic bacteria participate in methane oxidation.

Our results show that temperature also has a moderate effect on methane oxidation. Thus, the lowest turnover times and highest MOX rates were detected during the highest temperatures. This is in contrast with observations made by Lofton et al. (2014), who states that temperature can influence methane oxidation only in substrate-saturated environment.

Another factor which can stimulate methane oxidation is turbidity or SPM. It was mentioned before that SPM can serve as a vehicle for methanogens, and the same principle can be applied to methane-oxidizing microorganisms (Middelburg et al. 2002; Abril et al. 2007). In vitro experiments showed that removal of particles smaller than 11 mm can decrease the MOX rate by 50% (de Angelis and Scranton 1993). In our study, we did not detect any correlation of turnover times/MOX rates with SPM. The easiest explanations would be that stronger factors (such as methane concentration and temperature) simply suppressed the influence of SPM.

Oxygen is another important factor for methane oxidation. Summarized data on oxygen half-saturation constants for aerobic methane oxidation (the concentration at which the growth rate of bacteria reaches the half-maximum) show a wide range, from 0.14 to 58 mM (Guérin and Abril 2007). Oxygen concentrations measured along the transect in our study were within this range, thus oxygen was never a limiting factor, and no correlation with methane oxidation was detected.

Methanotrophs are regarded as slow growing bacteria, and hence tend to lose out on nutrients to faster growing heterotrophs. Besides, some mineral nutrients, like ammonium, are known to inhibit methane oxidation (Alam and Jia 2012) while others, like phosphate, increase MOX rates (Boiesen et al. 1993). Also, the availability of nitrogen can become a limiting factor for the growth of methanotrophs in nitrogen-limited environments (Bodelier et al. 2000). In our study, large quantities of inorganic nutrients were brought to the German Bight from the mouth of the Elbe, thus providing all the inorganic nutrients needed for intensive methane oxidation. However, we did not detect any correlation between MOX rates and any mineral nutrients at the marine stations. Due to low MOX rates at the marine stations, the supply of inorganic nutrients was still sufficient, and thus did not influence the methanotrophy.

**Conclusion**

We observed a wide variation of methane concentration in the water column along a 60 km transect from Cuxhaven to Helgoland. Highest concentrations were detected near the coast, where the water is enriched with methane by river water and lateral sources. Over the next 20 km, in the direction of Helgoland, we observed decrease in methane concentration due to dilution with methane depleted sea water from the German Bight. The last 40 km of the transect represents seawater almost exclusively, with a consistently low methane concentration. We also discovered that most of the methane in the Elbe estuary does not come from the Elbe River itself, as was thought before, but from an area near Cuxhaven. Thus, the conservative mixing model, which describes methane distribution as a simple dilution of methane-rich freshwater from the river with marine water, is only applicable at

salinities >10. Possible methane sources near Cuxhaven are input from small rivers, and methane rich tidal flats.

Though we did not find any clear seasonal pattern, sampling through the year also enabled us to discover that the methane distribution in the Elbe estuary was subject to significant temporal changes. We assume that these changes were controlled by the interaction of Elbe water discharge and methane concentrations in the mouth of the Elbe, which were higher in the warmer seasons. More information on the tidal surge, as well as the current regime would be useful for a better interpretation of the methane variations. However in the present study we could show that salinity explained about 57% of the methane variability.

Methane oxidation measurements were made in this area for the first time during at least the last 10 years. We discovered that methane oxidation was likely not significant methane sink in most of the Elbe estuary. However, more data on water residence time is needed to make definite conclusions. The main factors affecting methanotrophic activity were methane concentration, salinity, and temperature. However, further kinetic studies would be useful to gain more insight into the influence of methane concentrations on MOX.

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## 6.3 Methane distribution and methane oxidation in the water column of the Elbe estuary, Germany

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Aquatic Sciences

RESEARCH ARTICLE

# Methane distribution and methane oxidation in the water column of the Elbe estuary, Germany

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**Abstract** The River Elbe, as one of the major waterways of central Europe, is a potential source of high amounts of methane into the North Sea. Twelve sampling cruises from October 2010 until June 2013 were conducted from Hamburg towards the mouth of the Elbe at Cuxhaven. The dynamic of methane concentrations in the water column and its consumption via methane oxidizing bacteria was measured. In addition, physico-chemical parameters were used to estimate their influence on the methanotrophic activity. We observed high methane concentrations at the stations in the area of Hamburg harbour ("upper estuary") and about 10 times lower concentrations in the lower estuary (median of 416 versus 40 nmol $L^{-1}$, respectively). The methane oxidation rate mirrored the methane distribution with high values in the upper estuary and low values in the lower estuary (median of 161 versus 10 nmol $L^{-1}$ $day^{-1}$, respectively). Methane concentrations were significantly influenced by the river hydrology (falling water level) and the biological oxygen demand while interestingly, no clear relation to the amount of suspended particulate matter (SPM) was found. Methane oxidation rates were significantly influenced by methane concentration and to a lesser extent by temperature. Methane oxidation accounted for 41 ± 12 % of the total loss of methane in summer/fall periods, but for only 5 ± 3 % of the total loss in the winter/spring periods (total loss = methane oxidation + diffusion into the atmosphere). The average sea-air flux of methane was 33 ± 8 g $CH_4$ $m^{-2}$ $y^{-1}$. We applied a box model taking into account the residence times of each water parcel depending on discharge and tidal impact. We observed almost stable methane concentrations in the lower estuary, despite a strong loss of methane through diffusion and oxidation. Thus we postulate that losses in the lower Elbe estuary were balanced by additional inputs of methane, possibly from extensive salt marshes near the river mouth.

**Keywords** Estuary · Methane · Methane budget · Methane oxidation · River Elbe

## Introduction

Estuaries are highly dynamic ecosystems where freshwater meets marine water and diverse biological, physical and chemical parameters constantly change, which makes estuaries to hot-spots of biological production and biodiversity (Bianchi 2007). Due to the physical and chemical mechanisms present in brackish water (e.g. long residence time, low salinity) turbidity zones are produced with high concentrations of suspended particulate matter (SPM). In such zones riverine phytoplankton becomes limited by light and dies, which results in a considerable enhancement of the easily degradable organic matter available to microbes (Cole et al. 1992). This material is subsequently

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# Methane distribution and methane oxidation in the water column of the Elbe estuary, Germany

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**Introduction**

Estuaries are highly dynamic ecosystems where freshwater meets marine water and diverse biological, physical and chemical parameters constantly change, which makes estuaries to hot-spots of biological production and biodiversity (Bianchi 2007). Due to the physical and chemical mechanisms present in brackish water (e.g. long residence time, low salinity) turbidity zones are produced with high concentrations of suspended particulate matter (SPM). In such zones riverine phytoplankton becomes limited by light and dies, which results in a considerable enhancement of the easily degradable organic matter available to microbes (Cole et al. 1992). This material is subsequently decomposed and re-mineralized by heterotrophic bacteria resulting in elevated levels of carbon dioxide ($CO_2$) and methane ($CH_4$) emissions (Frankignoulle et al. 1998; Borges and Abril 2011), being the two most abundant carbon-containing greenhouse gasses in the atmosphere with a considerable impact on global warming. Estuaries present an interface between rivers and oceans, where rivers act as major sources of $CH_4$ to the atmosphere (Borges et al. 2015a). The role of the ocean as atmospheric methane source (including geological sources) is estimated to be 16% of natural sources, which is the same range as freshwater (including lakes and rivers) with 12% of the natural sources (IPCC 2013).

On the contrary to methane production, microbial $CH_4$ oxidation via specialized microbes, so called methanotrophs (MOB, methane-oxidizing bacteria) represents a biological sink of methane in aquatic environments. Methanotrophy can significantly reduce the amount of $CH_4$ escaping from the sediments and subsequently from the water column into the atmosphere (Kankaala et al. 2006; Conrad 2009; Bastviken 2009). Methanotrophs are physiologically specialized groups of methylotrophic prokaryotes capable of utilizing single carbon compounds (methane, methanol and few strains can utilize methylamine and a narrow selection of other C1 compounds; Bowman 2006) as an electron donor and source of cell carbon. Aerobic methanotrophs are widely distributed in nature, typically at anoxic–oxic interfaces (Bowman 2006). They can make an important contribution to the biomass entering the food web and can consume up to 50% of $CH_4$ diffusing from the sediments in some estuaries (de Angelis and Scranton 1993; Abril and Iversen 2002). However, the conditions influencing the methanotrophic activity in the water column are still largely unknown, making it even more difficult to uncover the major driving forces of the activity of MOB in such a variable ecosystem as an estuary. For instance, temperature may affect enzymatic activity and temperature variations may also lead to changes in the structure of the methanotrophic community (Sundh et al. 2005; Mohanty et al. 2007). It seems likely that methane oxidation (MOX) is mainly controlled by physical processes, i.e. gas-phase

diffusion and transport of $CH_4$ to the cells, rather than by enzymatic reactions (Urman et al. 2009). Besides $CH_4$, the limitation of other simple organic substrates and nutrients may also affect methanotrophic activity (Rudd et al. 1976; Bender and Conrad 1994; Bodelier et al. 2000). King (1990) showed that light strongly controls MOX via photosynthesis by the extension of the oxic zone suitable for methanotrophic activity. On the contrary, the investigations made by Dumestre et al. (1999) in Petit Saut Reservoir and later by Murase and Sugimoto at mesotrophic Lake Biwa (2005) suggested that methane oxidation can be inhibited by visible light. Especially in estuarine habitats, two parameters may strongly affect the MOX - salinity and suspended particulate matter (SPM) content. Whereas salinity has an inhibitory effect on MOX (de Angelis and Scranton 1993; Conrad et al. 1995), SPM shows a positive influence on MOX rate. The MOB may settle on organically rich particles (Zimmermann 1997, 2002) and thus exploit the micro-patchiness with locally enhanced $CH_4$ concentrations to become available for MOB (Abril et al. 2007).

In an effort to clarify the relationship between MOB and their environment, the aims of this study were: (i) to examine the distribution of $CH_4$ in the water column of the River Elbe estuary, (ii) to assess the activity of MOB as well as major physical and chemical parameters influencing their activities and (iii) to attempt to budget the different methane related processes along a large estuarine ecosystem.

## Material and methods

### Study site

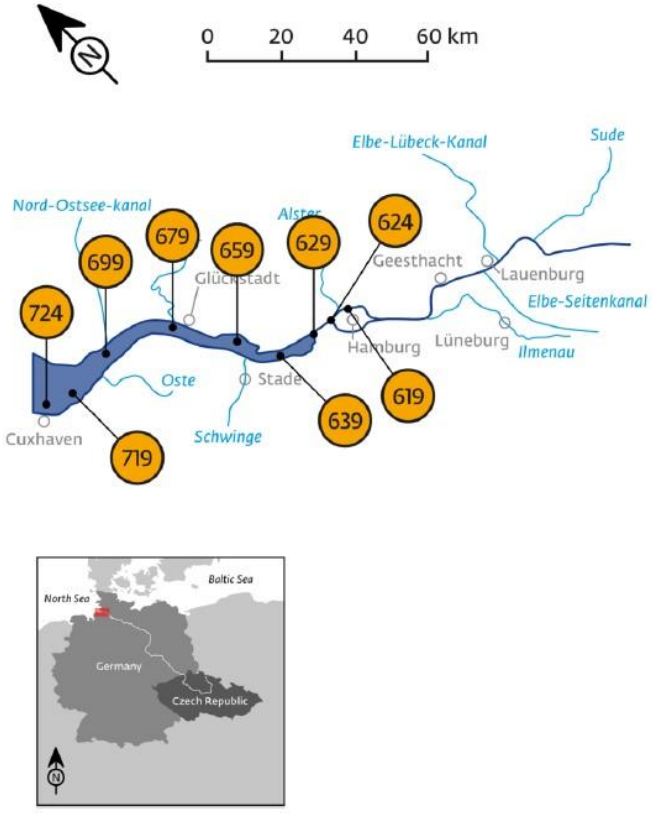
The River Elbe rises at an elevation of 1386 m above sea level in the Krkonoše (Giant Mountains) in the northeast of the Czech Republic, flows through the central part of the Czech Republic and central and northern Germany before discharging into the North Sea at Cuxhaven (Fig. 1). The Elbe estuary is a eutrophic ecosystem receiving urban, agricultural and industrial waste. It is an intermediate-turbid, well-mixed estuary with a pronounced estuarine turbidity maximum (ETM; approximately from #659 to #719, see Fig. 1) in the brackish zone of the estuary; note that these station codes are derived from the Elbe-km, which refer to the distance from the Czech-German border (= 0 river-km; IKSE 1995). The range of semi-diurnal tides rise from 2 m (at the Geesthacht weir) towards the port of Hamburg where it attains its maximum of 3.5 m. High tidal current velocities (up to 1.8 m $s^{-1}$; Bergemann and Gaumert 2010) cause a steep horizontal salinity gradient, particularly near Brunsbüttel (#690). The residence time of water varies from 15 to 30 days (Frankignoulle and Middelburg 2002).

Based on the different biogeochemical processes and oxygen demands the Elbe estuary can be divided into two sections (according to Amann et al. 2012): 1) the pre-oxygen minimum zone (pre-OMZ; approximately from #585 to #624), and 2) the oxygen minimum zone (OMZ; approximately from #624 to #659), both fall into the tidal freshwater area. The

Elbe estuary is a unique, well monitored system with data on physical and chemical parameter available from www.portal-tideelbe.de and www.fgg-elbe.de. Our sampling stations started in the port of Hamburg at station #619 and were spaced at intervals of 10 km and 20 km, terminated at Cuxhaven with #724 (Fig. 1). For the purpose of this study, the sampling sites were divided "upper estuary" and the "lower estuary": the upper estuary includes sampling sites #619 and #629; the lower estuary part covers the sampling sites from #659 till #724; site #639 was designed as the "transition zone".

**Sampling**

Twelve sampling campaigns were performed from October 2010 till June 2013. All data are available in the Pangaea data base (www.pangaea.de; Bussmann et al. 2014, Matousu et al. 2015). The water samples were collected in the main channel of the Elbe from the research vessel "Ludwig Prandtl" (Helmholtz-Zentrum Geesthacht, Germany) and were taken from two different depths of the water column: approximately 1 m above the river bed ("bottom") and approximately 1 m below the water surface ("surface"). Samples for $CH_4$ concentration and $CH_4$ oxidation rate were transferred directly from a water sampler (Uwitec, Austria) into serum bottles (120 ml). Bottles were overfilled with approximately two volumes of water, capped with black butyl stoppers (Ochs, Germany), and sealed with an aluminum crimp. Care was taken to exclude air bubbles from capped samples. An additional water sample was taken for analyses of other water properties.




(A)

| Station code | Latitude | Longitude |
|---|---|---|
| #619 (n=13) | 53°31,92 N | 10°1,53 E |
| #624 (n=7) | 53°32,59 N | 9°57,25 E |
| #629 (n=10) | 53°32,56 N | 9°52,77 E |
| #639 (n=16) | 53°33,72 N | 9°43,93 E |
| #659 (n=18) | 53°39,99 N | 9°30,76 E |
| #679 (n=18) | 53°49,07 N | 9°21,50 E |
| #699 (n=18) | 53°52,51 N | 9°5,54 E |
| #719 (n=24) | 53°51,10 N | 8°47,76 E |
| #724 (n=20) | 53°52,55 N | 8°43,94 E |

(B)

| | Range | Length [km] | Depth [m] | Area [$km^2$] | Volume [$km^3$] | Ratio (A / V) |
|---|---|---|---|---|---|---|
| Box 1 | #619 - #629 | 10 | 8 - 10 | 3 | 0.04 | 97 |
| Box 2 | #629 - #639 | 10 | 10 - 15 | 5 | 0.05 | 100 |
| Box 3 | #639 - #659 | 20 | 12 - 15 | 16 | 0.13 | 125 |
| Box 4 | #659 - #679 | 20 | 12 - 15 | 25 | 0.19 | 134 |
| Box 5 | #679 - #699 | 20 | 11 - 16 | 35 | 0.27 | 131 |
| Box 6 | #699 - #719 | 20 | 9 - 16 | 43 | 0.34 | 128 |
| Box 7 | #719 - #724 | 5 | 9 - 23 | 23 | 0.11 | 204 |

**Figure 1**

Overview of sampling sites in the Elbe River estuary. The station codes are derived from the river-kilometres, which refer to the distance from the Czech-German border (=0 river-km; IKSE 1995). Station coordinates are given in table A. Parameters of used box-model are given in table B Original map source: https://commons.wikimedia.org/wiki/File:Lauf_der_Elbe.png, modified

**Analytical methods**

The duplicate methane concentration samples were killed immediately, by injecting 0.3 ml of 5M NaOH through the septum into the serum bottle filled with water to stop microbial activity. The samples were analyzed within 1 week in the laboratory by using a headspace technique according to McAuliffe (1971): a 20 ml headspace was created by adding $N_2$ through a syringe causing displacement of 20 ml of water through another needle in the stopper. Afterwards the samples were vigorously shaken and then stored for 24 hour at room temperature to allow equilibration of the gases in the headspace. Subsequently the samples were analyzed with a gas chromatograph equipped with a flame ionization detector (GC 2014 Shimadzu Corp). Calibration was performed with 10 ppm and 100 ppm methane standards (Air Liquide). Precision of the analyses was 2%; the $r^2$ of the calibration curve > 97%. Dissolved $CH_4$ concentrations were calculated with the solubility coefficients of Yamamoto et al. (1976).

The $CH_4$ oxidation rates were determined as outlined in Bussmann et al. (2015). Water samples were processed in triplicates with one "killed control". Immediately after collecting the samples, 100 µl (10 µCi) of $^3$H-$CH_4$ (American Radiolabeled Chemicals, Inc.) was injected into each sample through the septum. Control samples were killed by injecting 200 µl of 5 M NaOH before the tracer was added. The samples were vigorously shaken for 60 s and incubated in the dark at near in situ temperature. Activities in "live" samples were stopped in the same way as "killed controls", but after approximately 24 hours of incubation. Samples were stored in the dark at 4°C, prior to being analyzed (within 1 week). In the laboratory, the samples were opened, 2 ml aliquot of each sample was mixed with 5 ml of scintillation cocktail (Ultima Gold™ LLT) and analyzed with a liquid scintillation counter (Tri-Carb® 2910 TR, Perkin Elmer) for estimation of the total radioactivity of the sample, i.e. the labeled $CH_4$ and labeled produced $H_2O$ (see Eq. 1). Subsequently the samples were sparged with air for 30 min to expel all remaining labeled $CH_4$. Afterwards, again 2 ml aliquot of each sample was mixed with 5 ml of scintillation cocktail and analyzed with a liquid scintillation counter for estimation of the microbially produced radioactive water.

The principle of the calculation of the microbial mediated MOX is based on the transformation of added radioactively labeled tracer ($^3$H-$CH_4$) into the oxidation product ($^3$H-$H_2O$) during incubation:

(1) $^*CH_4 + 2O_2 \rightarrow CO_2 + 2\ ^*H_2O$

In order to calculate the methane oxidation rate ($RO_x$), first the fraction of methane that was turned over is calculated:

(2) $F_{[CH_4]} = \frac{[H_2O]_{produced}}{[CH_4]_{injected}}$

The fractional turnover rate constant (k) is then determined by dividing it by the incubation time (t):

(3) $k = \frac{F_{[CH_4]}}{t}$

Finally the oxidation rate (MOX) is obtained by multiplication with the in situ methane concentration:

(4) $MO_x = k \times [CH_4]$

The inverse of the fractional turnover rate (k) is the turnover time (τ):

(5) $\tau = \frac{1}{\mathrm{k}}$

The turnover time is the time which it would take to oxidize the ambient amount of $CH_4$ dissolved in water with a given methane oxidation rate. The turnover time rate is an effective measure of the methanotrophic potential (Heintz et al. 2012).
The "killed controls" served for estimation of abiotically formed radioactive $H_2O$, which could lead to an overestimation and these (on average about 3.5 % of total transformation) were subtracted.

During the sampling campaigns along the Elbe River temperature, salinity, and oxygen in the water were measured right after sampling using a Universal Pocket Meter (Multi 340i) with a precision of 0.01 for salinity, 0.1˚C for temperature, and 0.5 % for oxygen.

Depending on the load of suspended particulate matter (SPM) 250 ml of water were filtered through prewashed (with MQ water) and pre-weighed Whatman GF/C filters and dried for 24 hour at 60°C, and weighed for determination of the total SPM content.

For nutrient analysis, water samples were filtered through GF/C filters (Whatman™) immediately after collection. Afterwards the filtrates were transferred into 50 ml Falcon tubes and frozen until further analysis in the laboratory. Samples were analyzed for phosphate, silicate, ammonia, nitrate, and nitrite with an autoanalyzer (AA3 from Seal-Analytical, Germany) and using standardized techniques as detailed in Wiltshire et al. (2010), following techniques described by Grasshoff et al. (1983). The error is estimated to be 10%.

Additional data for the water level (Ganglinie) were obtained from the "Wasser- und Schifffahrtsverwaltung des Bundes", given through the Bundesanstalt für Gewässerkunde

(BfG). We assigned the data from our stations to their locations according to the following: #619 with #623 (St. Pauli), #659 with #660 (Grauerort), #679 with #674 (Glückstadt) and #724 with #724 (Cuxhaven Steubenhöft). We took the original water level of the sampling time and calculated the difference from – 10 to + 10 minutes from sampling time and defined this parameter as "delta water level".

Data for the biological oxygen demand in 7 days (BOD-7, in mg $O_2$ $L^{-1}$) were obtained from the Elbe data portal (http://www.elbe-datenportal.de). The stations were allocated within 6 km distance to our stations and within 3 days range of our sampling date. Data ranged from 1 – 5.7 mg $L^{-1}$, n = 24.

Data for the discharge ($m^3$ $s^{-1}$) were also obtained from the Elbe data portal (http://www.elbe-datenportal.de). These data were measured at "Pegel Neu Darchau". Data were only allocated to the stations of the upper estuary (#619 and #624), because for the following stations we assume an increasing tidal input. As the water takes about one day from Neu Darchau to reach Hamburg, we also took the data from one day earlier than the sampling date.

**Statistical analysis**

To determine the possible dependence of $CH_4$ concentration and $CH_4$ oxidation rate on measured physical (water depth, water temperature) and chemical factors (salinity, $NH_4^+$, $NO_3^-$, $NO_2^-$, $PO_4^{3-}$, $SiO_4$, $O_2$, SPM) Spearman rank order correlation analyses were performed. Calculations and graphical representation were performed using SigmaPlot for Windows Version 11.0 software. One way ANOVA, linear correlation after log transformation and Wilcoxon test were performed with Kaleidagraph Version 4.1.3. Redundancy analyses (RDA) were performed using CANOCO program Version 4.5 (Ter Braak and Šmilauer 2002). Environmental parameters were selected using forward selection with 999 Monte Carlo permutations. The results of the RDA were visualized by CanoDraw for Windows. The box plots (Fig. 2) show the median (middle line), the lower and upper quartile (bottom and top of the box) with 25 and 75% of the data, the minimum and maximum of the data, being the lines extending from the bottom and top of the box. The dots are outliers, whose values are either greater / smaller than 1.5 * upper or lower quartile (Kaleidagraph Version 4.1.3).

**Calculations of the sea-air fluxes and the methane budget**

To reduce interferences with tidal currents we selected 4 cruises when the water level was falling during sampling time, i.e. the overall water flow and the ship movement was towards the sea. These cruises were 3. 8. 2011, 12. 4. 2013 and 12. 6. 2013 for stations #619 - #679; and 1. 8. 2012 for stations #659 - #724. For flux calculation and budgeting we defined boxes between the different stations. With the maps provided by "Wasser- und

Schifffahrtsverwaltung des Bundes", given through www.portal-tideelbe.de, we estimated the length and width of the river at each station. The depth of the "navigational channel" was set as 15 m and for the "deep water zone" ranging from 10 to 2 m, an average depth of 6 m was assumed. The boxes were split into different triangles and squares and the respective areas and volumes were calculated (Fig. 1; Supplementary material 1).

Gas exchange across an air–water interface can be described in general by the following function (Wanninkhof et al. 2009):

(6) $F = k \times (c_m - c_e)$

where F is the rate of gas flux per unit area, $c_m$ is the methane concentration measured in surface water and $c_e$ is the atmospheric gas equilibrium concentration based on Wiesenburg and Guinasso (1979).

The gas exchange coefficient (k) is a function of water surface agitation. However, in oceans and estuaries k is more determined by wind speed, while in rivers water velocity dominates (Alin et al. 2011). The determination of k is very important for the calculation of the sea-air flux. We decided to calculate $k_{600}$ in the Elbe according to the empirical equation 2 from Raymond et al. (2012):

(7) $k_{600} = 4725 \times (V \times S)^{0.86} \times Q^{-0.14} \times D^{0.66}$

with V as stream velocity (m $s^{-1}$), S as slope, Q as discharge ($m^3$ $s^{-1}$), and depth (m). Data on stream discharge were obtained from "Pegel Neu Darchau", data on stream velocity were provided by www.portal-tideelbe.de, for the respective stations and dates. The slope of the Elbe estuary at its delta was 0.00005 (tidal range of 5cm/km for the upper estuary; Vandenbruwaene 2013). For the two lower stations (#719 and #724) we took the empirical model from Borges et al. (2004), which is only wind driven. Wind data were obtained for Cuxhaven from the German Weather Service. Further details are given in Supplementary material 2. The calculated $k_{600}$ (value for $CO_2$ at 20°C) was converted to $k_{CH4}$ according to (Striegl et al. 2012):

(8) $\frac{k_{CH_4}}{k_{600}} = \left(\frac{Sc_{CH_4}}{Sc_{CO_2}}\right)^{0.69}$

where Schmidt numbers (Sc) are determined by water temperature and salinity, according to (Wanninkhof 1992).

For each station with its "navigational channel"and its "deep water zone", $k_{CH4}$ was determined as described above. The atmospheric equilibrium concentration and difference to the measured methane concentrations were calculated. With eq. 6 we calculated the respective sea-air fluxes (mol $m^2$ $d^{-1}$). The average of this flux between two neighboring stations was then multiplied with the respective area of the box. Then the sea-air flux for the

"navigational channel" and the flux for the "deep water zone" were added, resulting in the total sea-air flux for each box in mol $d^{-1}$.

For calculation of the total MOX rate, we took the average MOX between two neighboring stations and multiplied it by the total volume of the respective box ($V_{navigational\ channel} + V_{deep\ water}$). This resulted in the total MOX rate of each box in mol $d^{-1}$.

## Results

### Physico-chemical parameters of water

The physical parameters of the water samples are shown in Tab. 1. The values of the chemical parameters fluctuated considerably (Tab. 1). Phosphate had a clear seasonal trend with the highest values being in winter at #699 and #719. Oxygen also varied seasonally, with the lowest values being in summer. Other nutrients showed no clear seasonal or spatial pattern, in contrast to SPM with the highest values being in spring at #679 and #699 (i.e., in the estuarine turbidity maximum zone). Salinity increased towards the river mouth with 2.2 ± 1.6 at #699 up to 12.4 ± 6.2 at #724, no clear seasonal pattern was, however, evident.

### Distribution of methane in the water column

We observed rather high methane concentrations in the upper estuary (#619 – #629; median 416 nmol $L^{-1}$). At stations #659 – #724 $CH_4$ concentrations were about 10 times lower with a median of 40 nmol $L^{-1}$ (Fig. 2). According to this pattern, we grouped our stations into the "upper" and "lower" estuary (one way ANOVA with df = 81, $p < 0.001$, excluding station #639 as a transition zone). This grouping was also valid for most of the nutrients monitored. The site with the highest $CH_4$ concentration for each cruise moved up- or downstream between #619 and #639. Linear regression analysis (Tab. 2) and RDA (Fig. 3) revealed no seasonal trend for methane (no $CH_4$ - temperature correlation), for either all stations or for the upper or lower estuary. In the lower estuary, where salinity increases from 5 to 25 PSU towards to the North Sea, $CH_4$ concentrations remain stable and only a slightly negative yet insignificant influence of salinity (dilution) was detected (Fig. 3D), but with no significant correlation.

We found a clear negative correlation between SPM and $CH_4$ for all stations ($r^2$ = 0.36, for log transformed data), indicating high methane concentrations at low SPM, i.e. clear water. (Tab. 2, Fig. 3A). However, when splitting the data into upper and lower estuary, no significant correlation could be found. Using RDA analyses we found a relevant negative correlation only in the case of the transition zone (Fig. 4C), but a slight indication for all stations and even a positive in the upper estuary (Fig. 3B).

**Table 1**
Overview of collected data on physico-chemical parameters in the water column of the River Elbe estuary during all sampling campaigns from October 2010 to June 2013: median and the range of the data (in brackets) are given.

| Station code | Salinity [PSU] | Oxygen [mg $L^{-1}$] | Temperature [°C] | $SiO_4$ [µmol $L^{-1}$] | $PO_4^{3-}$ [µmol $L^{-1}$] | $NO_2^-$ [µmol $L^{-1}$] | $NO_3^-$ [µmol $L^{-1}$] | $NH_4^+$ [µmol $L^{-1}$] |
|---|---|---|---|---|---|---|---|---|
| #619 | 0.4 ± 0.2 | 8.1 ± 1.5 | 14.6 ± 5.2 | 133.6 (0.0–177.0) | 1.5 (0.1–4.8) | 1.4 (0.6–2.4) | 278.2 (196.5–1451.2) | 4.9 (3.6–20.3) |
| #624 | 0.3 ± 0.2 | 9. ± 1.4 | 15.3 ± 5.7 | 135.2 (129.5–213.3) | 1.3 (1.0–5.9) | 2.5 (2.0–2.8) | 230.2 (216.4–289.9) | 9.6 (8.3–11.4) |
| #629 | 0.3 ± 0.2 | 6.9 ± 2.4 | 12.7 ± 4.7 | 133.8 (0.0–135.2) | 1.5 (0.3–3.7) | 1.3 (0.6–2.6) | 290.0 (119.3–1386.7) | 5.7 (4.2–12.6) |
| #639 | 0. ± 0.2 | 7.6 ± 2.2 | 13.9 ± 5.4 | 130.1 (0.0–175.9) | 1.5 (1.2–6.2) | 1.5 (0.2–2.9) | 273.0 (64.7–1490.1) | 4.6 (1.3–12.9) |
| #659 | 0.5 ± 0.2 | 7.7 ± 2.0 | 14.1 ± 4.8 | 113.1 (1.7–172.5) | 1.4 (0.6–6.5) | 1.3 (0.1–2.5) | 253.1 (136.1–1523.7) | 3.2 (1.5–15.1) |
| #679 | 0.7 ± 0.5 | 8.0 ± 1.7 | 14.2 ± 4.9 | 118.3 (12.0–169.8) | 1.6 (0.8–8.4) | 0.3 (0.0–2.7) | 253.0 (116.4–1510.0) | 2.9 (0.5–15.7) |
| #699 | 2.6 ± 2.1 | 8.3 ± 1.6 | 14.2 ± 4.7 | 116.3 (20.8–184.9) | 2.5 (1.6–11.9) | 0.4 (0.1–1.6) | 237.8 (104.4–1618.6) | 3.7 (0.9–9.5) |
| #719 | 9.9 ± 6.0 | 8.2 ± 1.5 | 13.8 ± 4.8 | 86.2 (17.1–168.5) | 2.3 (1.4–14.1) | 0.9 (0.4–2.6) | 268.2 (40.2–1659.2) | 5.5 (2.8–10.7) |
| #724 | 12.2 ± 6.5 | 8. ± 1.8 | 12.8 ± 4.8 | 81.0 (26.4–116.4) | 1.7 (1.2–2.6) | 1.1 (0.5–2.6) | 371.0 (60.1–1659.0) | 6.8 (2.8–9.6) |

**Table 2**
Linear correlations between methane concentration/methane oxidation rate/methane turnover time and environmental factors, with log transformed data

| | Methane concentration | | Methane oxidation rate | | Methane turnover time | |
|---|---|---|---|---|---|---|
| | Upper estuary $n = 30$ | Lower estuary $n = 98$ | Upper estuary $n = 30$ | Lower estuary $n = 95$ | Upper estuary $n = 30$ | Lower estuary $n = 95$ |
| Temperature | | | 0.18/0.06 | 0.13/0.001 | −0.47*/<0.001 | −0.11/0.002 |
| Salinity | | | | −0.10/0.001 | | 0.16/<0.001 |
| Oxygen | | | | | 0.28/0.009 | |
| SPM (all stations) | all stations: −0.36/<0.001 | | | | | |
| Delta water level | −0.86**/<0.001 (n = 10) | | | | | |
| BOD7 | 0.80/0.02 (n = 6) | | all stations (n = 24): 0.31/0.005 | | | |
| | all stations (n = 24): 0.29/0.007 | | | | | |
| $PO_4^{3-}$ | | | | | | |
| $NO_2^-$ | −0.27/0.01 | | | | | |
| $NO_3^-$ | | | | | | |
| $NH_4^+$ | 0.15/0.08 | | | | −0.18/0.07 | |
| $CH_4$ | | | 0.55/<0.001 | 0.49/<0.001 | | |

Shown are the regression coefficients ($r^2$), positive or negative signs indicate a positive or negative correlation, followed by the respective $p$ value ($r^2$/p). Notes: * three very high values were omitted; ** two very high values were omitted; when not indicated otherwise the number of sampling occasions (n) ranged from 19 to 24

The RDA analyses indicated a slight influence of $NO_3^-$ and $SiO_4$ on the methane dynamics in the upper estuary (Fig. 3B). In the lower estuary, the RDA analysis showed only a minor influence of $NO_2^-$ and water temperature (Fig. 3D). A similar pattern was also evident for the $NH_4^+$ concentration and we even found a positive correlation between $CH_4$ and $NH_4^+$ for the upper estuary (log transformed data, $r^2 = 0.15$), but no correlation for the lower estuary. In the upper estuary, methane concentrations were negatively correlated with nitrite concentrations ($r^2 = 0.27$), but not in the lower estuary.

The water discharge of a river is an important factor in its chemistry. The obtained discharges (see Material and Methods) can be considered as the total freshwater discharge of the Elbe since the tributary discharges are negligible compared to the discharge of the main channel (factor 100) (Vandenbruwaene 2013). However, no direct correlation between $CH_4$ concentration and water discharge was detected in the upper estuary.

From the Elbe Daten Portal we obtained data on the BOD-7 and the water level for the respective stations and sampling times. However, these data were not available for all our sampling occasions. When the overall biodegradable material (i.e. BOD-7) in the water was high, we also observed high methane concentrations in the whole estuary ($r^2 = 0.29$, $n = 24$) and particularly pronounced in the upper estuary ($r^2 = 0.80$, $n = 6$) but not in the lower estuary. We calculated the difference between the water level 10 min before and 10 min after sampling to get an estimate of tidal influence. We found that when the water level was dropping the higher $CH_4$ concentrations were observed ($r^2 = 0.86$, $n = 10$) in the upper estuary, but no correlation was found in the lower estuary.

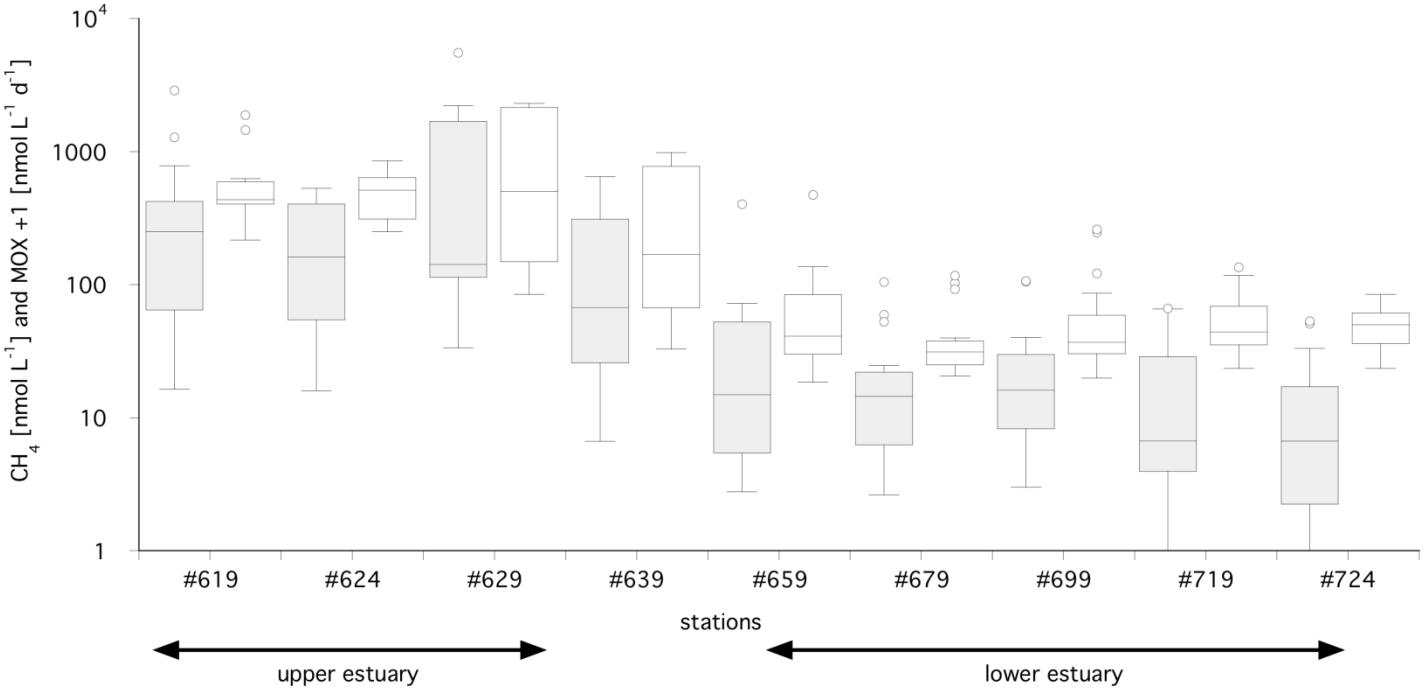



**Figure 2**
Box plots of the methane concentrations (grey boxes) and oxidation rates (white boxes) at the river stations in the Elbe estuary during the sampling campaigns from October 2010 to June 2013. We grouped the stations into upper (#619 - #629) and lower estuary (#659 - #724), with the station #639 as a transition zone. The number of samples ranged from n = 7 at station #624 to n = 24 at station #724. For definition of the box plot see Material & Methods.

**Methane oxidation and turnover time in the water column**

Methane oxidation rates were rather high in the upper estuary (#619 - #629) with a median of 161 nmol $L^{-1}$ $d^{-1}$. In the lower estuary (#659 – #724) methane oxidation rates were more than 10 times lower with a median of 10 nmol $L^{-1}$ $d^{-1}$ (Fig. 2). As described for methane concentrations, a one way ANOVA showed that methane oxidation rates and turnover times were significantly different (df = 124, $p < 0.001$) between the upper (#619 - #629) and lower estuary (#659 - #724).

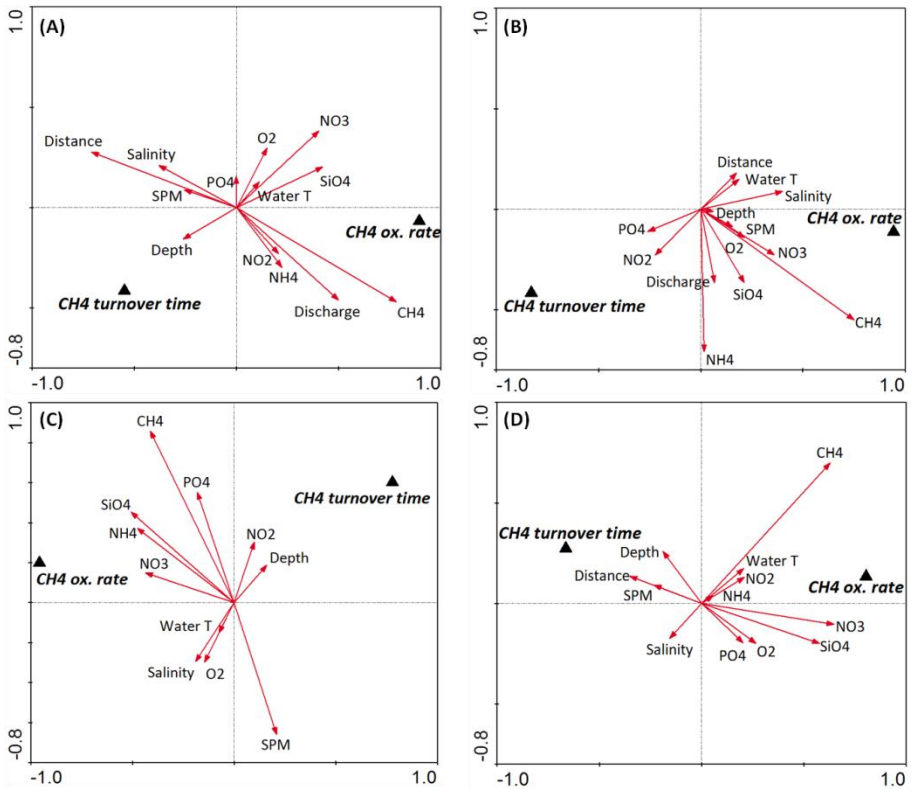



**Figure 3**

The ordination diagrams of the first and second axis of the redundancy analysis (RDA): (A) whole estuary; (B) upper estuary; (C) transition zone; (D) lower estuary. Note: $CH_4$ oxidation rate and $CH_4$ turnover time (triangle symbols) are explained variables; environmental parameters (arrows) are explanatory variables. The angle size between variables indicates their interrelationship, while arrows pointing in the same direction indicate positive correlations, and arrows pointing in opposite directions indicate negative correlations. The length of the arrows shows the strength of the environmental variable.

We found a positive correlation between MOX and temperature in the upper estuary ($r^2 = 0.18$) and a weaker correlation in the lower estuary ($r^2 = 0.13$, $p = 0.06$), as can also be seen in the RDA diagrams (Fig. 3 A-D). The MOX rate in the upper and lower estuary were both tightly correlated with $CH_4$ concentrations ($r^2 = 0.54$ and 0.66, respectively; both with $p < 0.001$). However, as the MOX is calculated by multiplying the fractional turnover rate constant (k) with the $CH_4$ concentration, this correlation has to be regarded with some caution. For the whole estuary MOX also was correlated with the BOD-7 ($r^2 = 0.31$, $n = 24$, $p = 0.005$). The analysis revealed no further statistically significant correlation. Only a slight indication of a positive correlation between MOX and some nutrients ($NH_4^+$, $NO_3^-$) was found in the transition zone (Fig. 3C).

Methane turnover times ($\tau$) were rather short at the upper estuary (#619 – #629) with a median of 1.6 days and a range of 1 - 25 days. In the lower estuary, (#659 – #724) $\tau$ were about twice as long with a median of 3.7 days, ranging from 1 - 66 days (Supplementary Material 6) being, moreover, significantly different (one way ANOVA, df = 124, $p = 0.01$) from the upper estuary.

In the upper estuary, RDA analyses (Fig. 3B) indicated a slightly negative relationship between $\tau$ and water temperature ($r^2 = 0.47$, $p < 0.001$), i.e. a slow turnover at lower temperature, and oxygen ($r^2 = 0.28$, $p = 0.009$), i.e. a fast turnover at higher oxygen concentrations. In the transition zone we found the same correlations between $\tau$ and water temperature and additionally a weak negative correlation also with salinity (Fig. 3C). For the lower estuary, we found only a less tight correlation with temperature ($r^2 = 0.11$, $p = 0.002$) and salinity ($r^2 = 0.16$, $p < 0.001$), however, the RDA analyses (Fig. 3D) revealed an influence of the changing nutrient concentrations ($NO_3^-$, $PO_4^{3-}$, $O_2$ and $SiO_4$).

**Calculation of the methane sea-air fluxes and budget**

The sea-air flux ranged from 6 up to 115 kmol $d^{-1}$ (Tab. 3). The higher numbers (> 60 kmol $d^{-1}$) were mainly due to high current velocities (100 - 130 $cm^3$ $s^{-1}$) at the respective stations and dates. The total methane oxidation rate ranged from 2 to 49 kmol $d^{-1}$. For all cruises the total MOX seemed to be most important in box 1 (#619-#629, Hamburg port) with 57% and 61% of the total loss assigned to MOX. Generally low MOX rates were observed in April 2013 with rather cold water (6.6°C) compared to the other dates (18 - 21°C), thus obviously the low

temperature slowing MOX. However, on average 41 ± 12% (without the April data) of the total loss can be attributed to microbial methane consumption.

**Table 3**
The loss of methane (in kmol $day^{-1}$) via diffusive flux (Diff) into the atmosphere and the microbial methane oxidation (MOX) at the different sampling dates and different boxes (the definition of the boxes is given in Fig. 1). The contribution of MOX to the total loss (diffusion + methane oxidation) is also given

| | 03. 08. 2011 | | | 01. 08. 2012 | | | 12. 04. 2013 | | | 12. 06. 2013 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diff | MOx | % MOx | Diff | MOx | % MOx | Diff | MOx | % MOx | Diff | MOx | % MOx |
| Box 1 | 13 | 17 | 57 | | | | 33 | 3 | 9 | 13 | 20 | 61 |
| Box 2 | 10 | 8 | 44 | | | | 67 | 4 | 5 | 23 | 24 | 50 |
| Box 3 | 7 | 3 | 27 | | | | 115 | 2 | 2 | 68 | 49 | 42 |
| Box 4 | 10 | 2 | 15 | 6 | 4 | 44 | 35 | 1 | 2 | 76 | 47 | 38 |
| Box 5 | | | | 13 | 8 | 36 | | | | | | |
| Box 6 | | | | 13 | 11 | 47 | | | | | | |
| Box 7 | | | | 6 | 4 | 37 | | | | | | |

In a second step we tried to calculate a budget for the boxes, i.e. to estimate the importance of the input and output of methane by the river flow, the methane reducing processes (evasion into the atmosphere and microbial oxidation), and finally – when assuming an equilibrium state and a closed budget – an extra input of methane. The amount of methane transported into or out of a box ($A_{in}$ and $A_{out}$ in mol) was calculated by multiplying the $CH_4$ concentration at the inflowing and outflowing station of the box with the volume of water entering/leaving this box. The residence time of a water parcel in the respective box was estimated from the data given in Bergemann (1996) at low, medium, and high discharge (see Supplementary Material 7). No information on the error of his estimate is given, thus we assume an error of at least 10%. The residence time is determined by the discharge of the river as well as the tidal influence, resulting in short residence times near the port of Hamburg. However, with the stronger tidal effects near the coast, the residence time increases. Also the duration of the $CH_4$ consuming process within one box is determined by the residence time. Thus we multiplied the rate of the removal process, which results in the amount of $CH_4$ lost through diffusion ($A_{diff}$) and the amount lost through $CH_4$ oxidation ($A_{MOX}$) in this box. In accordance with Anthony (2012) and Bergemann (1996) we then assume that

(9) $A_{in} - A_{diff} - A_{MOX} = A_{calc-out}$

When comparing the calculated amount of $CH_4$ ($A_{calc\ out}$) with the measured amount of $CH_4$ ($A_{meas\ out}$) finally gives us the additional input ($A_{additional}$) required to balance the budget for the respective box:

(10) $A_{calc-out} - A_{meas-out} = A_{additional}$

In August 2011, the Elbe discharge was moderate (716 $m^3\ s^{-1}$) and the residence times were estimated to range from 0.8 d in box 1 to 5.3 d in box 4. This contrasts to June 2013 with a very high discharge (4041 $m^3\ s^{-1}$) and a residence time ranging from 0.4 d in box 1 to only 2.9 d in box 4. In August 2012 water discharge was low (409 $m^3\ s^{-1}$) and for the boxes in the lower estuary we estimated residence times of 14 d in box 4 and 25 d in box 6. In figure 4 we visualize the different processes for the cruise in August 2011. All processes for all cruises are shown in the Supplementary material 7.

In August 2011, in box 1 the amount of methane leaving the box was only slightly lower than the incoming amount (Fig. 4). With a residence time of 0.8 day, the $CH_4$ consuming process was about half the amount of the input. The measured output was slightly lower than the input, and "adding" the $CH_4$ reducing process, we estimated an additional input of the same size as the measured input. As the residence times increases downstream, correspondingly we estimated a residence time of 5.3 days in box 4. Thus the $CH_4$ reducing processes strongly increased, and whereas the measured input and output were almost the same, the $CH_4$ reducing processes can only be balanced with additional input about 6 times as high as the input. A similar pattern was observed for the cruises in April and June 2013 (Supplementary material 7).

In August 2012 the discharge was low and the boxes were nearer to the coast, thus the residence time increased to 14 – 25 d (Supplementary material 7) and consequently the amount of $CH_4$ reduction also increased. Even though the measured input and output of these boxes was about the same, the strong loss of $CH_4$ can only be accounted for by a very strong additional input (22 – 33 times higher than the input).

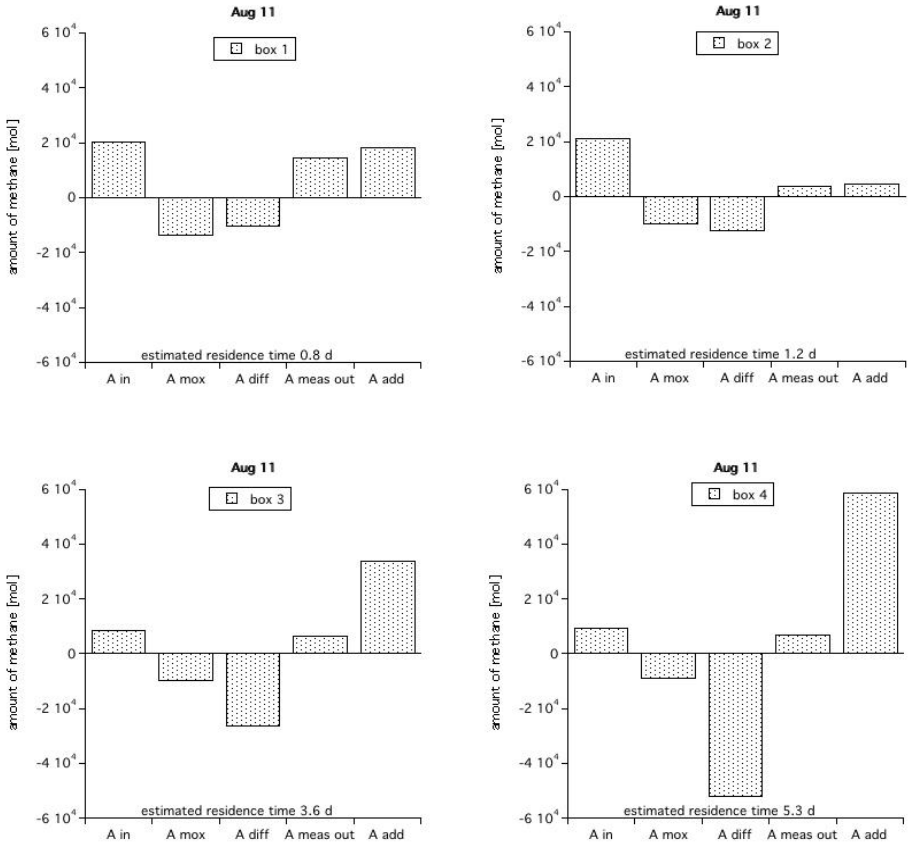



**Figure 4**
The amount of methane entering a box ($A_{in}$) with the methane reducing processes (oxidation $A_{MOX}$ and diffusion into the atmosphere $A_{diff}$) versus the measured amount of methane "leaving" the box ($A_{meas\ out}$). To obtain equilibrium an additional input ($A_{add}$) is required. The estimated residence time for each box is also given. Data are from 3.8.2011. For details of the calculation see results.

## Discussion

### Methane distribution in the water column of the Elbe estuary

Our exceedingly high values of methane concentration correspond more or less to tropical/warm water systems and large rivers (Zhang et al. 2008; Borges et al. 2015a; b). Middelburg et al. (2002) measured the freshwater end-member for the Elbe River at a concentration of 111 nmol $L^{-1}$ (salinity of approx. 0.4) and the marine end-member at a concentration of 4 – 6 nmol $L^{-1}$. This fits our data well, as at salinities between 0.3 - 0.5 we found a median methane concentration of 127 nmol $L^{-1}$. Wernecke et al. (1994) determined the $CH_4$ concentrations to be

between 60 and 120 nmol $L^{-1}$ in the main stream of the River Elbe, and up to 1200 nmol $L^{-1}$ in the stagnant water (the upper end of the harbor basin), which is in line with our data. Rehder et al. (1998) estimated the average $CH_4$ input into the North Sea to be 70 nmol $L^{-1}$ compared to our lower estimates with a median of 50 nmol $L^{-1}$ (for our outmost station #724). In contrast to previous studies (Middelburg et al. 2002; Rehder et al. 1998, Osudar et al. 2015) we observed no correlation between $CH_4$ and salinity (including for the two outmost stations). This indicates that there is no simple two-compartments based mixing of riverine and marine $CH_4$.

We found significantly higher $CH_4$ concentrations at falling water levels in the upper estuary. Grunwald et al. (2009) suggested that $CH_4$ concentrations usually peaked during low tide, probably due to the $CH_4$-rich freshwater input and that, conversely, low values may be caused by dilution with $CH_4$-poor marine water and degassing processes. This was revealed and postulated also by others (Koné et al. 2010; Upstill-Goddard and Barnes 2016). However, we did not find a correlation to water gauge/level, like Grunwald, but with the "falling water level". We assume that our sampling strategy from a moving boat versus fixed stations biased this effect. For the North Sea the tidal flat have been shown to be a methane source (Wu 2015), and we assume that the strong surge at falling water levels results in the release of methane into the river/estuary.

Methane in the whole estuary but more specifically in its upper part is positively correlated with the BOD-7, which represents a measure of the bioavailability of degraded organic matter. The high $CH_4$ concentrations in the upper part of the Elbe estuary likely result from the high heterotrophic activity related to remineralization processes of high loads of labile organic matter. The organic material originates mainly from phytoplankton biomass (Kerner 2000), which is subject to enhanced mortality in the upper zone of the Elbe Estuary (area of Hamburg Port) due to higher runoff, deeper water column mixing processes, and high water turbidity (Wolfstein and Kies 1995; Muylaert and Sabbe 1999). No data on waste water input and its influence on the $CH_4$ concentration in the Elbe were found, however, improvements in wastewater and in industrial inputs led to significant decrease of nitrogen and phosphorus loads since the 1990´s (Schlarbaum et al. 2010) and so minor effects are assumed.

Elevated $CH_4$ concentrations as well as elevated $CH_4$ oxidation rates are often associated with a high content of SPM or turbidity (Upstill-Goddard 2000; Abril et al. 2007). As most $CH_4$ is produced in anoxic zones within the sediment,

significant $CH_4$ amounts are also released during particle resuspension (Bussmann 2005). Particle attached bacteria normally show higher activity rates and diversity (Ortega-Retuerta et al. 2013). Additionally, a high variability (from 0 up to 98 %) of the contribution of particle-attached bacteria to total bacterial production is also reported (Garneau et al. 2009). Thus we suggest that MOB may also attach to particles and lead to an elevated particle-attached $CH_4$ oxidation rate (Abril et al. 2007). However, the described direct relationship does not hold for all rivers and estuaries, as our data do not support these hypotheses, for either $CH_4$ concentration or MOX. We even observed a negative relationship between $CH_4$ and SPM for the upper estuary (Fig. 3B). There are also examples in literature where no direct relationship between SPM and methane concentration was found (Grunwald et al. 2009). One possible reason for this inconsistency is that the chemical composition of SPM changes spatially within an estuary (Savoye et al. 2012) and probably also with season. In our data set we observed a great variability of the SPM data, which makes it even more difficult to interpret them. Further, experimental studies would shed light on the relation between SPM and methane, i.e. the composition of the SPM and the status of the particle-attached or free-living MOB being in an active or dormant physiological state (Ho et al. 2013).

**Methane oxidation and turnover time in the water column of the Elbe estuary**

In our study along the salinity gradient of the Elbe estuary we observed $CH_4$ oxidation rates from 0.8 nmol $L^{-1}$ $d^{-1}$ to very high about 5542 nmol $L^{-1}$ $d^{-1}$ (at the Hamburg harbour area; Fig. 2). This variability is in line with the literature data from diverse estuaries (Griffiths et al. 1982; Scranton and McShane 1991; deAngelis and Scranton 1993; Abril and Iversen 2002). The turnover time varied between 0 to 66 days. Very few investigations included $CH_4$ turnover time measurements, e.g. a study from the Ogeechee River estuary with very fast turnover time between 2 hours to 1 day (Pulliam 1993).

One important factor in both estuary parts was temperature, i.e. higher MOX rates and a fast turnover rate were observed at higher temperatures. This corroborates the observations made by Osudar et al. (2014) and Lofton et al. (2014) but contradicts the observations of Zaiss et al. (1982) and Utsumi et al. (1998), who revealed only a minor influence of temperature on MOX in situ. Additionally Lofton et al. (2014) suggested a specific substrate-temperature

interaction, i.e. only methane saturated MOX was influenced by temperature, at limiting methane concentrations temperature had no influence on MOX. These findings are supported by our data on the turnover time; the influence of temperature was much higher in the upper estuary with its higher $CH_4$ concentrations. As the oxidation of $CH_4$ also needs oxygen, the process of $CH_4$ oxidation is also a part of the BOD-7, as is shown by its correlation; however, this fact does not give any further ecological information.

The availability of $CH_4$, as a sole source of energy for MOB seems to be the key factor influencing their activity. Similar findings are reported for MOX kinetics in the marine water column (Mau et al. 2015). For riverine, but sediment borne $CH_4$ concentrations of 2000 – 4000 nmol $L^{-1}$ were still limiting levels (Shelley et al. 2015). Our observed $CH_4$ concentrations ranged from 18 – 2306 nmol $L^{-1}$ and support the fact that MOX was mostly limited by $CH_4$ concentration. Type I MOB responds rapidly to substrate availability and is the predominantly active community in many environments (Ho et al. 2013). In the Elbe Estuary a dominance of type I MOB was also observed, with no type II MOB being detected (Hackbush 2014).

The influence of nutrients showed no clear pattern, suggesting limited effects of nutrients for MOX as most parts of this in this system have typically high trophic status (Tab. 2). Other authors also report a complex influence of nutrients (N and P) on MOX (Veraart et al. 2015). Oxygen also had mostly no influence on the microbial parameters. Thus we assume that the minimal oxygen concentrations of 3.4 mg $L^{-1}$ were still sufficient to permit $CH_4$ oxidation. This is not surprising as aerobic methanotrophs can be active in microoxic conditions (Deutzmann et al. 2014).

**The sea-air fluxes of methane and budget calculations**

We calculated the diffusive methane flux along the Elbe estuary for four selected cruises when the overall water movement and the sampling scheme were downstream. For these calculations the values of the gas exchange coefficient ($k_{600}$) were crucial. They have previously been determined directly via chamber or eddy covariance measurements, or can be alternatively assessed via different models taking into account the stream hydrology. We applied the model from Raymond et al. (2012) and obtained an average $k_{600}$ value of 8.4 m $d^{-1}$ or 35 cm $h^{-1}$, which is fairly close to the range of 20 and 31 cm $h^{-1}$ previously reported for the Elbe (Abril and Borges 2004; Amann et al. 2015). Thus, we think that our

approach is reasonable and our slightly higher values can be related to the rather high water velocities (> 100 cm $s^{-1}$) recorded on some specific occasions.

A direct comparison with flux data from the literature is hindered by the usage of many different units. We calculated an average sea-air flux of $CH_4$ (for all boxes and all 4 cruises) of 33 ± 8 g $CH_4$ $m^{-2}$ per year and with a calculated total area of 1.5 x $10^8$ $m^{-2}$ and thus come up with an average $CH_4$ emission of 5.0 ± 1.2 x $10^9$ g per year for the whole Elbe estuary. For the Scottish Tay Estuary 1.02 g C $m^{-2}$ $y^{-1}$ and 5 x $10^7$ g C per year has been calculated (Harley 2015). For both units the flux in the Tay estuary is 30 times and one order of magnitudes lower than in the Elbe, respectively. However, other authors estimated 1.8 - 3 x $10^{12}$ g $CH_4$ per year for temperate estuaries, which is much higher than our estimate for the Elbe (Middelburg 2002). For a Baltic estuary $CH_4$ fluxes were generally below 15 g $CH_4$ $m^2$ $y^{-1}$ in the bay (Silvennoinen 2008). For a subtropical estuary $CH_4$ flux of 0.1 to 10 g $CH_4$ $m^{-2}$ $y^{-1}$ is reported (Musenze 2014), which is roughly in line with our estimates. In tropical, African estuaries the flux ranged from 0.1 – 14 g $CH_4$ $m^{-2}$ $y^{-1}$, which is less than half of our values (Konné 2010). In a worldwide compilation of $CH_4$ flux data, the $CH_4$ flux from estuaries is described as being 539 ± 602 g $CH_4$ $m^{-2}$ $y^{-1}$ (Ortiz-Llorente 2012), while Borges and Abril estimate the global estuarine $CH_4$ flux with 6.2 g $CH_4$ $m^{-2}$ $y^{-1}$ (Borges and Abril 2011). Our data are within the lower range of this data compilation. These examples clearly demonstrate the difficulties of such comparisons. One reason for the variability of the data may be the methods used in calculating the flux, especially the determination of $k_{600}$ (Musenze 2014). Other reasons are certainly the variability of environmental parameters across locations, such as water velocity, tides, river discharge and of course methane contents itself. Also the degree and stability of the stratification, the influence of tidal areas and urban pollution have to be taken into account (Koné 2010; Middelburg 2002; Marwick 2014). Thus the variability of $CH_4$ emissions is greatest for oceans and estuaries, with no clear seasonal pattern of $CH_4$ emission found for estuaries (Ortiz-Llorente 2012).

Another process which eliminates $CH_4$ from estuaries is microbial MOX. For all cruises the total MOX seemed to be most important in box 1 (Hamburg port). This can be attributed to the morphometry of the boxes, as in box 1 the river is deep and narrow i.e. with a low volume ratio: volume ratio, while the river becomes wider and shallower towards the river mouth i.e. high area: volume ratio. Rather low MOX rates were observed in April 2013 at a

water temperature of 6.6°C. The impeding effect of temperature on MOX also becomes obvious through the negative correlation between temperature and MOX (this study) and a $Q_{10}$ of 1.5 for Elbe water (Bussmann 2015). Thus in warm water i.e. in the summer/autumn on average 41 ± 12% of the total loss can be attributed to microbial $CH_4$ consumption and this portion of $CH_4$ is not lost to the atmosphere. In colder water (winter and spring) we estimate that only 5 ± 3% of the total $CH_4$ loss is due to MOX.

In previous studies box models were used to estimate the influence of the different $CH_4$ related processes in a river (de Angelis and Scranton 1993; Anthony 2012). However, in our study area we also have a strong tidal regime, thus the water is not simply moving downstream. Each day a hypothetic water parcel moves four times up- and downstream. Thus, it takes 12 weeks for a water parcel to get from Geesthacht to Cuxhaven (140 km, with a water discharge of 250 $m^3$ $s^{-1}$) (Bergemann 1996). Therefore any $CH_4$ reducing or producing processes have ample time to act. So we chose a modified box model taking into account the different residence times, depending on the water discharge of the river and the intensity of the tidal regime (Bergemann 1996). Certainly, numerical modeling would give us a better insight into this dynamic estuarine system (Schroeder 1997; Schöl 2014); however, though this approach may be realized in the future, it was beyond the scope of this study.

When the residence time for a box is low, the importance of the $CH_4$ consuming processes is also correspondingly low. In several cases we observed almost no difference between the amounts of $CH_4$ entering a box ($A_{in}$) and the amount leaving a box ($A_{out}$; Fig. 4, box 1). As the $CH_4$ consuming processes are active at a low level, a low amount of additional $CH_4$ is required. Near the coast or at times of low discharge the residence time increases and therefore the amount of consumed $CH_4$ also increases. To balance out this strong loss of $CH_4$, the additional input also has to increase (Fig. 4, box 4). So even when the $CH_4$ concentrations and the amount of $CH_4$ entering and leaving a box seem to be stable or equal, $CH_4$ reducing processes are active, and to counterbalance them an additional input of $CH_4$ is required. In box 1, the ratio of $A_{in}$ to $A_{add}$ was 1 - 2. In contrast to boxes 4 - 6 where the ratio was 22 - 44, indicating a tremendous additional input of methane. As the width of the Elbe increases (from 0.5 km at #629 to almost 7 km at station #724) the bordering tidal marshes could be the source of this additional $CH_4$ input. For dissolved inorganic carbon (DIC) in the Elbe it is known that marshes contribute up to one third of the annual DIC

excess (Amann et al. 2015). Tidal flats are known to emit $CH_4$ into the surface water and atmosphere in substantial amounts (Røy 2008; Weston 2014). Due to advective flow, which is of special importance in permeable sandy sediments, pore waters enriched in remineralized nutrients and $CH_4$ are actively released from sediments into the overlying water column (Beck 2012). Additionally, groundwater also releases $CH_4$ into estuarine waters (Porubsky 2014).

As the Elbe estuary is surrounded by the tidal flats from the Waden Sea, which are one of the largest sand- and mudflats worldwide (Marencic 2009), we suggest that the influence of these tidal flats is much stronger than the riverine input into the North Sea. Even though the $CH_4$ concentrations in the upper estuary are very high, this $CH_4$ will not reach the North Sea (due to oxidation and diffusion on its way). But it is the lower estuary with its lower $CH_4$ content which is flowing into the North Sea. This is supported by the fact that we did not see any effect of dilution (correlation between salinity and $CH_4$) in our data. Only at a greater distance from the coast, the $CH_4$ of the coastal water, being a mixture of riverine and tidal-flat-originated $CH_4$, becomes diluted with $CH_4$ - poor marine water (Osudar 2015). Detailed isotopic studies could help to clarify this hypothesis.

**Conclusions and outlook**

Estuaries represent a mosaic of habitats changing from freshwater to marine environment, which can be seen also in relation to methane related processes. High methane concentrations were observed in the upper estuary (Hamburg port), which decreased by one order of magnitude towards the lower estuary and the river mouth. Despite active methane oxidation, the microbial filter was estimated to be responsible for 5 – 41% of the methane total loss. The other part was attributed to methane diffusion into the atmosphere. We did not observe any dilution of methane-rich river water with methane poor marine water. On the contrary, we assume that marshes bordering the Elbe estuary release a substantial amount of methane into the river and further into the North Sea.

Our study is based on ship-borne measurements, mostly going downstream. However, it would be interesting to relate this approach and our conclusions with methane data from fixed stations along the river, along which the water and its processes are moving by. The other possibility would be measurements within a “water parcel” as it is moving by river discharge and tidal currents. However, in the Elbe estuary, with its high level of ship-traffic,

this would be rather difficult for ship-borne measurements. Permanent recording sensors may help to overcome these logistic restrictions.

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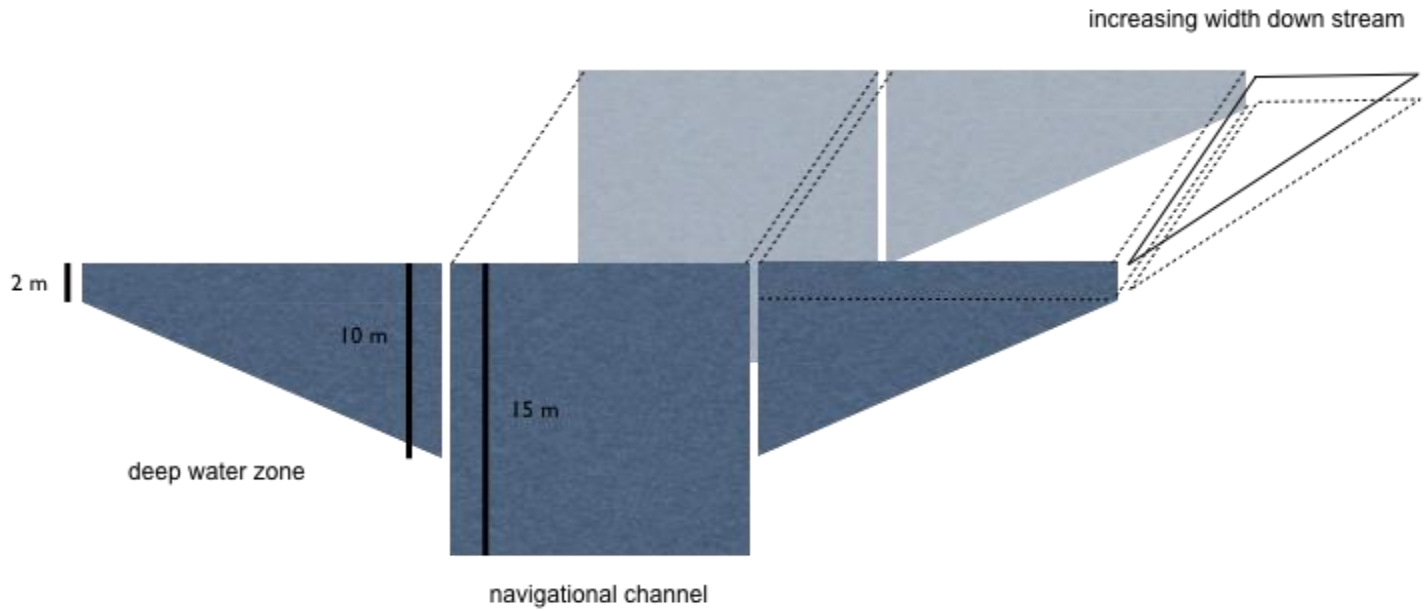
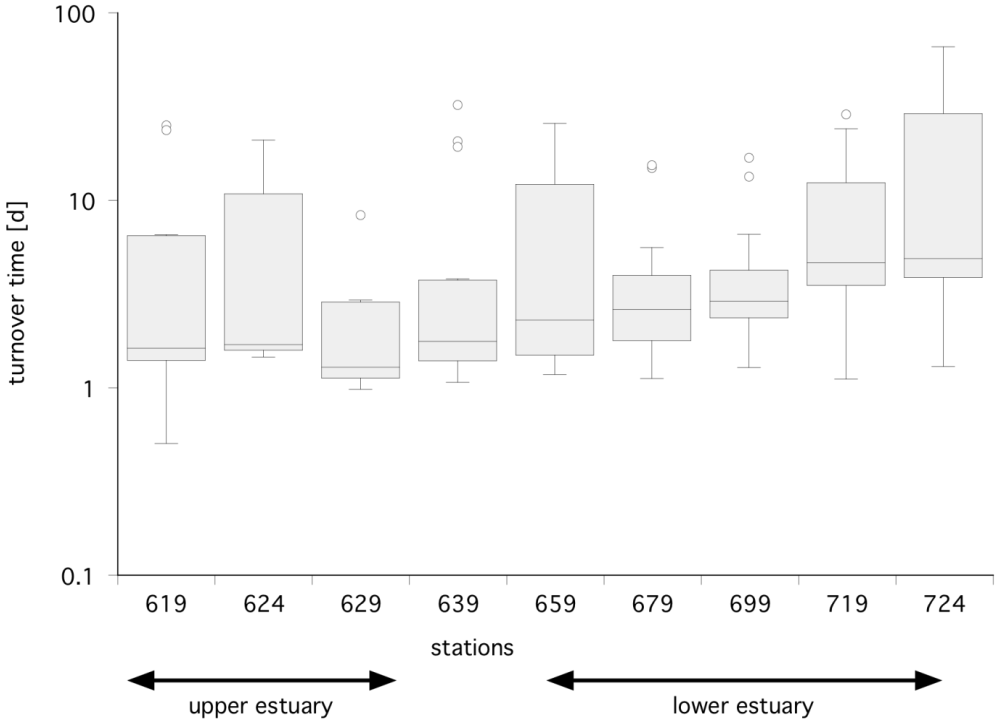
## Supplementary material



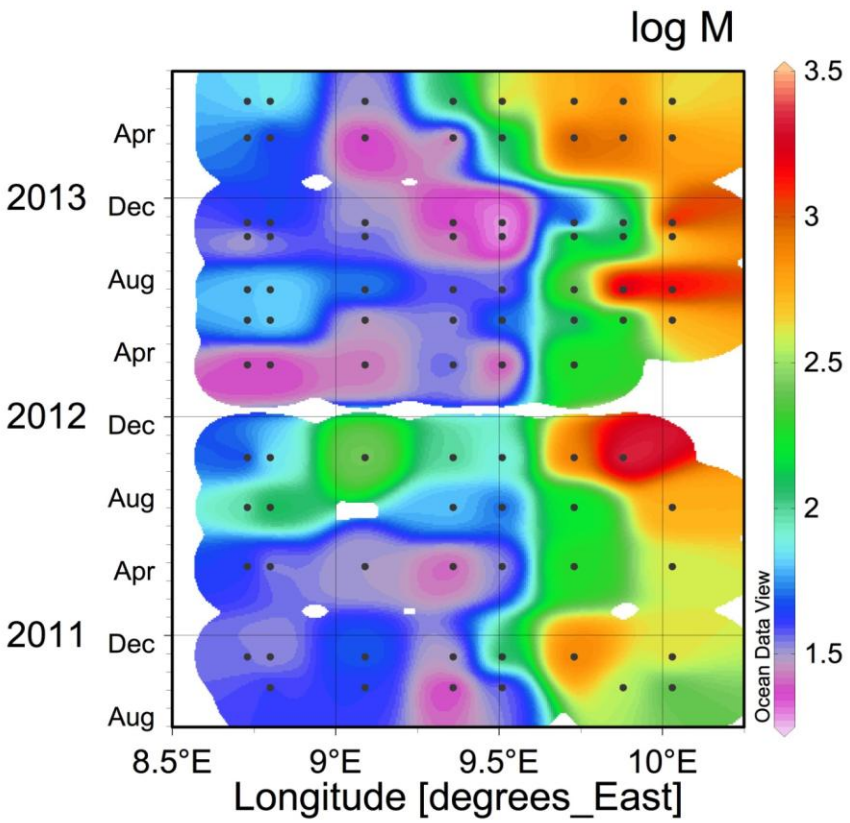

**Supplement 1**
Morphometric scheme for calculating the areas and volume of the navigational channel and the deep-water zone with increasing width downstream.

| Station | Date | k determined with |
|---|---|---|
| | 3.8.2011 | monthly mean of the current velocity in 2008 from #630, Teufelsbrück |
| #619 | 12.4.2013 | |
| | 12.6.2013 | |
| | 3.8.2011 | monthly mean of the current velocity in 2008 from #630, Teufelsbrück |
| #629 | 12.4.2013 | |
| | 12.6.2013 | |
| | 3.8.2011 | exact current velocity from #643 Hanskalb |
| #639 | 12.4.2013 | |
| | 12.6.2013 | |
| | 3.8.2011 | exact current velocity from #665 Pagensand |
| #659 | 1.8.2012 | |
| | 12.4.2013 | |
| | 12.6.2013 | |
| | 3.8.2011 | exact current velocity from #676 Rhiplatte |
| #679 | 1.8.2012 | |
| | 12.4.2013 | |
| | 12.6.2013 | |
| #699 | 1.8.2012 | exact current velocity from #697 Krumdeich |
| #719 | 1.8.2012 | wind speed in Cuxhaven |
| #724 | 1.8.2012 | wind speed in Cuxhaven |

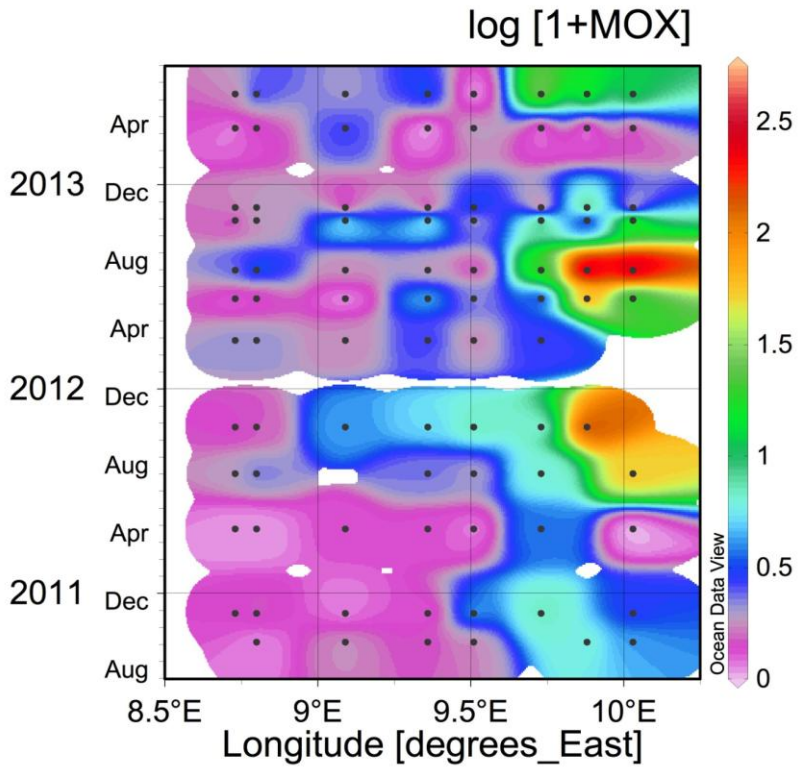
**Supplement 2**
Water velocities provided by www.portal-tideelbe.de, for k calculation.



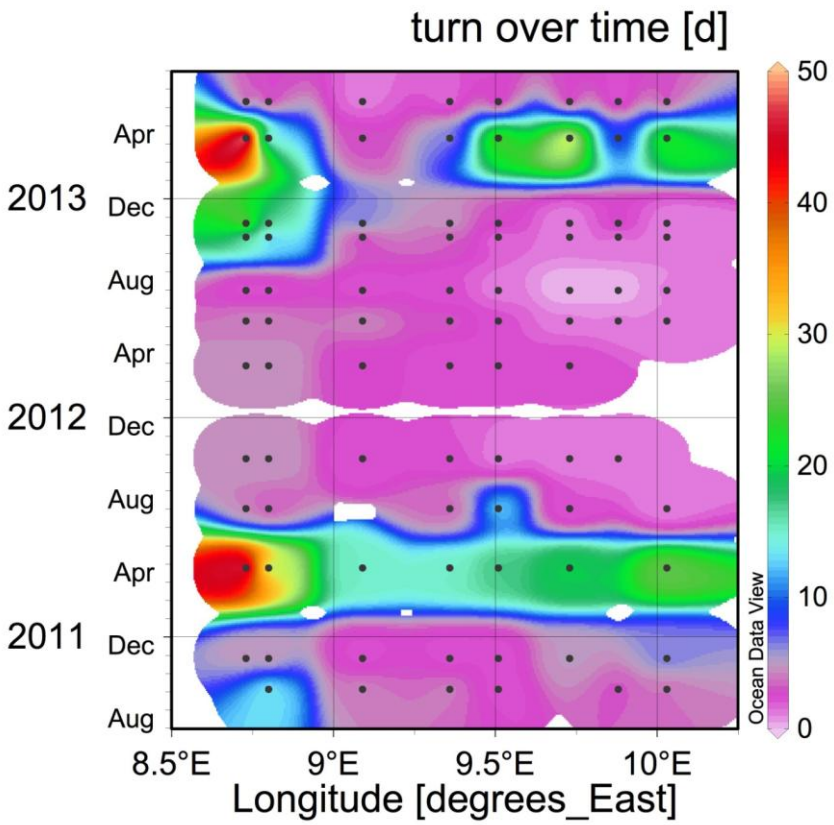

**Supplement 3**
Box plots of the methane turnover time at the river stations in the Elbe estuary during the sampling campaigns from October 2010 to June 2013. We grouped the stations into inner (#619 - #629) and outer estuary (#659 - #724), with the station #639 as a transition zone. The number of samples ranged from n=7 at station #624 to n=24 at station #724. For Definition of the box plot see Material & Methods.




**Supplement 4**
The methane distribution in the whole monitored stretch of the Elbe estuary during the sampling campaigns from October 2010 to June 2013. Note: To achieve a detailed resolution we used logarithmic transformation of the data.




**Supplement 5**
The methane oxidation in the whole monitored stretch of the Elbe estuary during the sampling campaigns from October 2010 to June 2013. Note: To achieve a detailed resolution we used logarithmic transformation of the data.




**Supplement 6**
The methane turnover time in the whole monitored stretch of the Elbe estuary during the sampling campaigns from October 2010 to June 2013.

| | | Box 1 | Box 2 | Box 3 | Box 4 | Box 5 | Box 6 |
|---|---|---|---|---|---|---|---|
| 3.8.2011 | residence time (d) | 0.8 | 1.2 | 3.6 | 5.3 | | |
| | Ain | 20 | 21 | 8 | 9 | | |
| | Amox | 14 | 10 | 10 | 9 | | |
| | Adiff | 10 | 12 | 26 | 52 | | |
| | Acalc out | -4 | -1 | -28 | -52 | | |
| | Ameas out | 15 | 4 | 6 | 7 | | |
| | Aadd | 18 | 4 | 34 | 59 | | |
| | Aadd / Ain | 1 | 0.2 | 4 | 6 | | |
| 12.4.2013 | residence time (d) | 0.8 | 1.2 | 3.6 | 5.3 | | |
| | Ain | 23 | 47 | 124 | 21 | | |
| | Amox | 3 | 4 | 8 | 4 | | |
| | Adiff | 26 | 80 | 415 | 185 | | |
| | Acalc out | -6 | -38 | -299 | -168 | | |
| | Ameas out | 32 | 52 | 14 | 4 | | |
| | Aadd | 38 | 90 | 314 | 172 | | |
| | Aadd / Ain | 2 | 2 | 3 | 8 | | |
| 12.6.2013 | residence time (d) | 0.4 | 0.7 | 2.2 | 2.9 | | |
| | Ain | 15 | 35 | 67 | 88 | | |
| | Amox | 8 | 17 | 107 | 136 | | |
| | Adiff | 5 | 16 | 150 | 221 | | |
| | Acalc out | 2 | 2 | -191 | -269 | | |
| | Ameas out | 24 | 28 | 60 | 22 | | |
| | Aadd | 22 | 26 | 251 | 291 | | |
| | Aadd / Ain | 1 | 1 | 4 | 3 | | |
| 1.8.2012 | residence time (d) | | | | 14 | 21 | 25 |
| | Ain | | | | 6 | 10 | 18 |
| | Amox | | | | 62 | 160 | 279 |
| | Adiff | | | | 80 | 281 | 319 |
| | Acalc out | | | | -135 | -431 | -580 |
| | Ameas out | | | | 7 | 14 | 22 |
| | Aadd | | | | 142 | 446 | 603 |
| | Aadd / Ain | | | | 22 | 44 | 33 |

**Supplement 7**

The amount of methane (Ain in kmol) entering a box with the amount of methane reduced in this box by methane oxidation and diffusion into the atmosphere (AMOX and Adiff). The difference between the calculated amount of methane leaving the box (Acalc out) and the measured amount of methane leaving the box (Ameas out) results in the additional amount of methane (Aadd). The Aadd is necessary to counterbalance for the methane consuming processes.

### 6.4 Methane dynamics in a large river – a case study of the River Elbe

(Manuscript in preparation)

**Methane dynamics in a large river – a case study of the River Elbe**

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**Keywords:** Aerobic methanotrophic bacteria; diffusive flux; ebullition; methane concentration; methane emissions; methane oxidation; methane turnover time

**Abstract**

We conducted multiple small sampling campaigns (2011 – 2012) and one large sampling campaign (2013) at selected profiles along the River Elbe. The obtained data have brought some important insights into the major methane ($CH_4$) pathways and general trends in the riverine ecosystem. The highest $CH_4$ concentrations were found in man-altered riverine habitats, i.e. in harbor and channel (median of 1075 nmol $L^{-1}$; 2013), and in the "impounded" river part (median 357 nmol $L^{-1}$; 2011-2013). The lowest $CH_4$ concentrations were found in the "lowland" part (median 67 nmol $L^{-1}$; 2011-2013). On the other hand, the $CH_4$ turnover time was nearly equal in the "impounded" part (with median 22 nmol $L^{-1}$ $d^{-1}$) and "lowland" part (with median 23 nmol $L^{-1}$ $d^{-1}$), but slightly faster at harbor (17 nmol $L^{-1}$ $d^{-1}$) and channel (13 nmol $L^{-1}$ $d^{-1}$); however, the fastest $CH_4$ turnover time was repeatedly found in the "natural" part with median of 12 nmol $L^{-1}$ $d^{-1}$. Methane emissions from the surface water into the atmosphere ranged from 0.4 to 11.9 mg $m^{-2}$ $d^{-1}$ (median of 2.1 ± 0.6 mg $m^{-2}$ $d^{-1}$), while the highest $CH_4$ emissions were found at a harbor - such man altered riverine habitats have not been taken into consideration in the $CH_4$ budget before; despite them being important parts of the modified river ecosystem since may be significant $CH_4$ hot-spots. Despite the $CH_4$ removal via emissions to the atmosphere and microbial oxidation in the water column, the amount of $CH_4$

leaving the Elber River from the "lowland" part to the estuary was 7 times higher than the incoming amount of $CH_4$ from the "impounded" river part. The value of the total $CH_4$ diffusive flux from the whole Elber River was estimated to be approximately 97 t $CH_4$ $y^{-1}$.

**Introduction**

Considering $CH_4$ as an efficient greenhouse gas, it is important to understand its sources and sinks in the global atmospheric budget. Among natural $CH_4$ sources, wetlands represent the largest (175 - 220 Tg $CH_4$ $yr^{-1}$; Kirschke et al. 2013), however, there is an increasing awareness of inland waters (i.e., lakes, reservoirs, rivers) being significant contributors to the global budget (103 Tg $CH_4$ $yr^{-1}$; Bastviken et al. 2011). Despite their small area, these freshwater systems can affect carbon balances, especially on a regional scale (Cole et al. 2007). While a considerable level of scientific interest has been given to studies of $CH_4$ emissions in lakes (e.g., Bastviken et al. 2004, 2008; Devlin et al. 2015; Sepulveda-Jauregui et al. 2015) and reservoirs (e.g., StLouis et al. 2000; Delsontro et al. 2011; Beaulieu et al. 2014), rivers remain in the background in terms of available data and their underestimated importance in the global greenhouse gas balance (Bastviken et al. 2011). Nevertheless, high $CH_4$ production documented in stream beds (Hlaváčová et al. 2005; Wilcock and Sorrell 2008) has also been identified as a considerable source of atmospheric $CH_4$ through ebullition and emissions (Baulch et al. 2011), especially in impounded rivers (Guérin et al. 2006; Maeck et al. 2013).

In contrast to $CH_4$ production, the $CH_4$ consumption by methanotrophic bacteria (methane-oxidizing bacteria, MOB) in the water column may provide a natural barrier reducing the flux of $CH_4$ from different ecosystems into the atmosphere. Methanotrophs are divided into two groups, type I (divided into type I and X) and type II, according to their internal membrane arrangement, carbon assimilation pathway, phylogenetic classification etc. (Bowman 2006). Methanotrophs inhabit anoxic–oxic interfaces, such as upper sediment layers and surfaces (Auman et al. 2000) or suspended particles in the water column (Abril et al. 2007). Notably, it has been documented that MOB can consume (i.e., oxidize) up to 90% of $CH_4$ diffusing from lake sediments (Utsumi et al. 1998; Kankaala et al. 2006). However, in the water columns of rivers, microbial $CH_4$ oxidation is believed to be much less efficient (Zaiss et al. 1982) – the

contribution of methanotrophic $CH_4$ consumption to $CH_4$ losses is about 5% in turbulent rivers (Lilley et al. 1996). However, we have primarily gained only snap shots of the processes shaping the methane budget of river ecosystems on a local basis and only seldom connected this knowledge to the environmental factors that shape methane related processes or monitored these processes for a whole river continuum.

In this study, we focused on processes closely related to the methane cycle, i.e. distribution of $CH_4$ in the water column ($CH_4$ concentration), methanotrophic potential ($CH_4$ turnover time) and $CH_4$ emissions (diffusive flux and ebullition) on the longitudinal transect of the River Elbe. The River Elbe rises at an elevation of 1386 m above sea level in the Krkonoše (Giant Mountains) in the northeast of the Czech Republic, flowing through the central part of the Czech Republic and through central and northern Germany before discharging into the North Sea at Cuxhaven (Fig. 1). The Elbe basin, comprising the main river and its tributaries, has a catchment area of 148268 $km^2$ and a total length of 1094 km. Prior to 1990, the Elbe was extremely polluted by industrial, communal, and agricultural wastes from the former Eastern bloc (Adams et al. 1996). Despite significant water quality improvement the Elbe is still largely affected by water transport and associated interventions into the river. Considering the different habitats, pollution, and velocity, we divided the River Elbe into four sections for purposes of this study: The first section of the river (upstream Pardubice) is characterized as a fast flowing natural stream with meanders and torrential zones (for the purpose of this study called "natural part"; $8^{th}$ - $116^{th}$ km; Fig. 1). The lower Czech reach of the Elbe from Hradec Králové to Střekov (for the purpose of this study called "impounded part"; $140^{th}$ - $326^{th}$ km; Fig. 1) has been recognized as one of the most polluted and modified stretches of the River Elbe in the Czech Republic (Prange et al. 2000). This section of the river contains 21 weirs and represents a channeled river serving mainly for boat navigation. Numerous agricultural, settlement, and industrialized areas are spread along the river and its tributaries. In the third part, downstream of the Střekov weir, the River Elbe flows freely over a distance of 600 km through low mountains, later becoming a lowland river (for the purpose of this study called "lowland part"; $366^{th}$ - $948^{th}$ km; Fig. 1). In this part the river is stabilized only by groynes (wing-dams) along most of its flow. This part of the river has a relatively natural morphology and large areas have

been preserved close to their natural state (IKSE 1997). The last section, the tidal part of the River Elbe, is separated from the non-tidal part by the Geesthacht weir; this section was the subject of another study (Matoušů et al., in press).

The aims of this study were (i) to achieve an understanding of the possible $CH_4$ sinks in river water, and to determine hotspots of methane dynamics along the River Elbe transect, (ii) to assess the metabolic activities of aerobic methanotrophic bacteria in the water column considering the different types of habitats along a river continuum, and (iii) to determine the possible physical and chemical parameters that could influence the activities and population dynamics of methanotrophic bacteria.

## Materials and Methods

### Sampling

We conducted four small sampling campaigns of the Czech part ($8^{th}$ till $366^{th}$ km) of the River Elbe on 24.-25. May 2011, 5.-6. October 2011, 13.-14., March 2012 and 1.-2. August 2012. The samples were collected from the river banks or pontoons, from approximately 0.5 m below the surface ("surface" sampling), or in case of higher water levels also from approximately 0.5 m above the bottom ("bottom" sampling). In these small campaigns, selected hydrological parameters (see further) as well as $CH_4$ concentration and turnover time were analyzed.

Additionally we conducted one large sampling campaign on 8.-26. October 2013. During this campaign a longitudinal transect of the River Elbe from Špindlerův Mlýn ($8^{th}$ km) to Geesthacht ($948^{th}$ km) and backwards upstream to Děčín ($356^{th}$ km) was sampled over 18 days. In the navigable part (from the $228^{th}$ km) the samples were collected from the main channel using the "Thor Heyerdahl" research vessel, from approximately 0.5 m below the water surface. At the $326^{th}$ km point (in the lock of the Střekov weir), we took additional samples at 10 m water depth. During this campaign water samples (and sediment samples; Rulík et al. in prep) were taken for hydrological, chemical and molecular analysis. In total we sampled 37 stations (22 during the downstream cruise, 15 upstream). These water samples were treated similarly to the small sampling campaigns.

**Table 1**
Overview of all sampling sites on the Elbe River. River km was approximately recalculated from the distance downstream the river (for the purposes of this study). Exact locations of sampling sites, riverine classification ("natural" / "impounded" / "lowland"; for the purposes of this study) and type of riverine habitat (R = river; C = conjunction; L = lock; H = harbor; G = groynefield; CH = channel) are given. Notes: n.a. = data not available; D = sampling downstream the course in October 2013, U = sampling upstream course in October 2013. Water velocity was measured only during the longitudinal sampling campaign in 2013.

| River km | Latitude | Longitude | River classification | River habitat | Water velocity [m $s^{-1}$] | Sampling campaign |
|---|---|---|---|---|---|---|
| 8 | 50.7360 | 15.6084 | natural | R | 0.3 | 2011, 2012, 2013 |
| 46 | 50.4959 | 15.7388 | natural | R | n.a. | 2012 |
| 56 | 50.4444 | 15.7932 | natural | R | 1.1 | 2011, 2012, 2013 |
| 64 | 50.4189 | 15.8529 | natural | R | n.a. | 2012 |
| 116 | 50.0969 | 15.8039 | impounded | R | 0.3 | 2011, 2012, 2013 |
| 140 | 50.0324 | 15.6175 | impounded | R | 0.1 | 2011, 2012, 2013 |
| 228 | 50.1861 | 14.6723 | impounded | R | 0.2 | 2013 |
| 250 | 50.2959 | 14.4820 | impounded | R | 0.1 | 2011, 2012, 2013 |
| 258 | 50.3528 | 14.4687 | impounded | C | 0.3 | 2011, 2012, 2013 |
| 302 | 50.5286 | 14.1284 | impounded | C | 0.3 | 2013 |
| 326 | 50.6396 | 14.0489 | impounded | L | 0.0 | 2011, 2012, 2013 |
| 366 | 50.8798 | 14.2372 | lowland | R | 1.1 | 2011, 2012, 2013 |
| 372 | 50.9108 | 14.1815 | lowland | R | 0.6 | 2013 |
| 411 | 51.0353 | 13.8315 | lowland | R | 1.6 | 2013 D + U |
| 447 | 51.1706 | 13.4744 | lowland | R + H | 1.4 / 0.0 | 2013 D + U |
| 489 | 51.4118 | 13.2088 | lowland | R | 1.0 | 2013 U |
| 563 | 51.8183 | 11.8358 | lowland | C | 1.4 | 2013 U |
| 578 | 51.8573 | 12.6410 | lowland | R | 0.4 | 2013 D + U |
| 632 | 51.8737 | 12.1481 | lowland | R | 1.8 | 2012; 2013 D + U |
| 655 | 51.9570 | 11.9113 | lowland | C | 1.3 | 2013 U |
| 703 | 52.2245 | 11.7032 | lowland | R | n.a. | 2012; 2013 D + U |
| 734 | 52.3990 | 11.9602 | lowland | R + G | 1.2 / n.a. | 2013 D + U |
| 767 | 52.6754 | 12.0139 | lowland | R + G | 1.1 / 1.3 | 2013 D + U |
| 792 | 52.8741 | 11.9984 | lowland | C | 1.1 | 2013 U |
| 802 | 52.9094 | 11.8709 | lowland | C | 1.3 | 2013 U |
| 871 | 53.1381 | 11.2472 | lowland | CH + G | n.a. | 2013 U |
| 886 | 53.1557 | 11.0503 | lowland | R | n.a. | 2013 D + U |
| 934 | 53.3694 | 10.5465 | lowland | C | n.a. | 2013 U |
| 948 | 53.4279 | 10.3671 | lowland | R | 1.2 | 2013 |

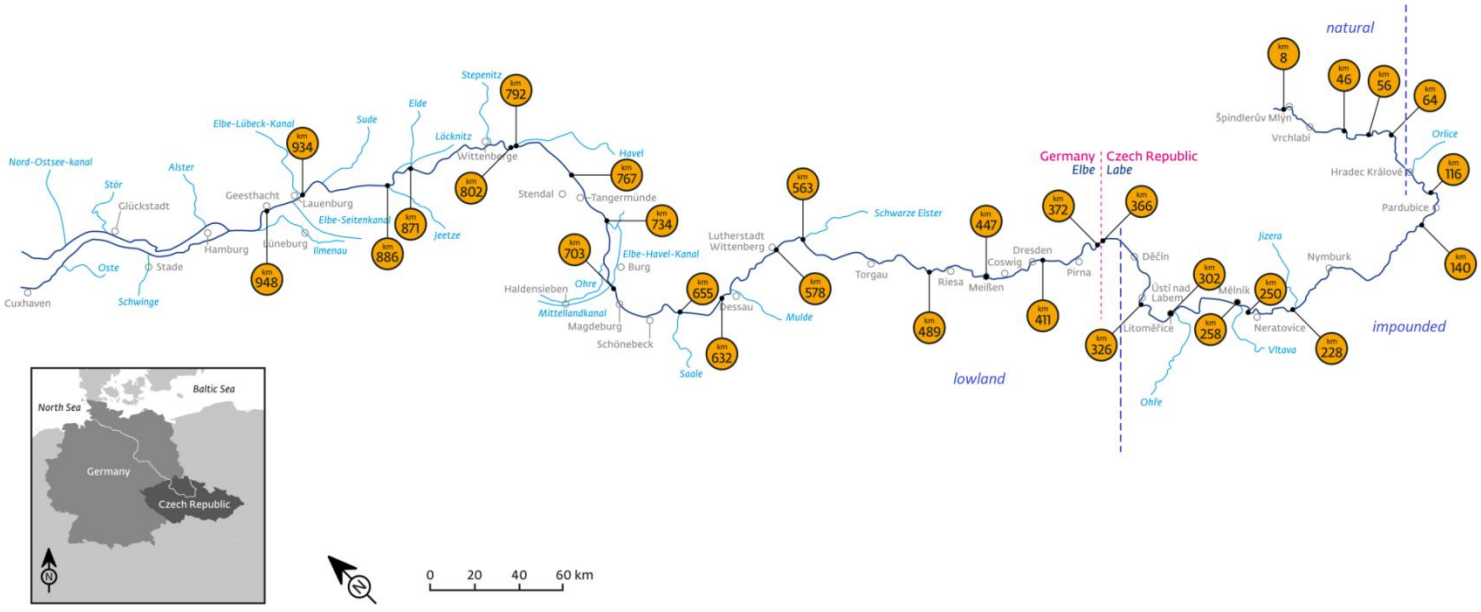


**Figure 1**
The sampling sites on the longitudinal profile of the River Elbe. The numbering is determined by the distance from the river source in km (see "River km" in Tab. 1). Original map: https://commons.wikimedia.org/wiki/File:Lauf_der_Elbe.png; modified.

**Hydrological parameters**

Water temperature, conductivity, pH and oxygen content in the water were measured during sampling using a Universal Pocket Meter (Multi 340i). The discharge velocities were determined with an electromagnetic water velocity meter (Marsh-McBirney FloMate 2000; Hach U.S.A.).

**Nutrient composition, suspended particulate matter**

Water samples from all sampling campaigns were collected from a vertical Van Dorn sampler (1 m length, 6.4 L volume) into the bottles.

During the small sampling campaigns (2011 - 2012) 200 – 500 ml (depending on the load) of water were filtered through pre-washed and pre-weighted Whatman GF/C filters (1.2 μm) for suspended particulate matter (SPM) estimation. Afterwards the filters were dried for 24 h at 60°C, and reweighed. The filtered water was transferred into 50 ml Falcon tubes and frozen for later analysis in the laboratory.

All samples for nutrient analyses were collected only from the surface water into plastic bottles. Total dissolved phosphorus (DP) was determined in the filtered (0.4 μm) samples with the molybdate method after perchloric acid digestion according to Kopáček and Hejzlar (1993). Water samples (only during the large sampling campaign in 2013) for the estimation of total dissolved nitrogen (TDN) were filtered through glass-fiber filters of 0.4-μm nominal pore size (GF-5, Macherey-Nagel, Düren, Germany) and analyzed with an Elementar vario TOC cube analyzer (Elementar Analysensysteme GmbH, Germany). The SPM was determined gravimetrically on GF-5 filters with drying at 105°C until a constant weight.

**Methane concentration and -turnover time**

To determine the $CH_4$ concentration and $CH_4$ turnover time the samples were filled directly from the sampler into serum bottles (100ml Crimp Neck Vials): water samples for $CH_4$ concentration determination in triplicates, water samples for $CH_4$ turnover time in triplicates plus two killed controls. The bottles were overfilled with approximately two volumes of water, capped with black butyl rubber stoppers (Ochs, Germany), sealed with aluminum crimps, and stored upside down. Care was taken to exclude air bubbles during the procedure.

Samples for $CH_4$ concentration determination in the water column were preserved immediately after sampling by injecting concentrated sulfuric acid (100 µl per 12 ml of sample) for terminating the microbial activity. The samples were analyzed within 1 month in the laboratory using a headspace technique according to McAuliffe (1971). A 5 ml headspace was created by adding $N_2$ through a syringe by displacement of an appropriate amount of water through another needle in the stopper. Afterwards, the samples were vigorously shaken and then stored for 24 h at room temperature to allow the equilibration of the gases in the headspace. Subsequently the samples were analyzed with an Agilent 6890N gas chromatograph (Agilent Technologies, USA) equipped with a flame ionization detector and a 0.53 x 30 m GS Alumina column at 45°C. Dissolved $CH_4$ concentrations were calculated with the solubility coefficient of 0.03469 given by Yamamoto (Yamamoto et al. 1976).

The $CH_4$ turnover time was determined as outlined in next text (detailed in Bussmann et al. 2015). Immediately after collecting the samples we injected 100 µl (10 µCi) of $^3H$-$CH_4$ (American Radiolabeled Chemicals, Inc.) through the rubber septa into the water samples. Subsequently the samples were vigorously shaken for 60 s and incubated in the dark at near in situ temperature. The two killed control samples were preserved by injecting 200 µl of concentrated sulphuric acid before the tracer was added. Activities in "live" samples were stopped in the same way, but after approximately 24 hours. Samples were stored in the dark at 4°C, prior to being analyzed (within 1 week). In the laboratory the samples were opened, and the total radioactivity of the sample including labeled $CH_4$ and labeled produced $H_2O$ were measured immediately by mixing a 1 ml aliquot of each sample with 5 ml of scintillation cocktail (Ultima Gold™ LLT) and analyzed with a liquid scintillation counter (Tri-Carb® 2910 TR, Perkin Elmer; or Tri-Carb® 2900 TR, Packard). Subsequently the samples were sparged with air for half an hour to expel all remaining $CH_4$. Afterwards the microbially produced radioactive water was analyzed as described above.

The principle of the calculation of the $CH_4$ turnover time is based on the transformation of added radioactively marked tracer ($^3H$-$CH_4$) into the oxidation products ($^3H$-$H_2O$) during the incubation:

(1) $C^*H_4 + 2O_2 \rightarrow CO_2 + 2^*H_2O$

The turnover time is the time it takes to oxidize the ambient amount of $CH_4$ dissolved in water with a given methane oxidation rate. In order to calculate the $CH_4$ turnover time, first the fraction of methane that was turned over is calculated:

(2) $F_{CH_4} = \frac{[H_2O]produced}{[CH_4]injected}$

The fractional turnover rate constant (k) is then determined by dividing by the incubation time (t):

(3) $k = \frac{F_{CH_4}}{t}$

The inverse of the fractional turnover rate (k) is the turnover time:

(4) $\tau = \frac{1}{k}$

The "killed controls" served for estimation of abiotically formed radioactive $H_2O$, which could lead to an overestimation and these (on average about 3.5 % of total transformation) were subtracted.

**Methane emissions**

Gaseous $CH_4$ emissions from the water column into the atmosphere were measured using a set of six floating chambers. The open-bottom floating PE chambers (5L domes with an area of 0.03 $m^2$) were maintained on the water surface by a floating body (Styrene) attached to the outside. The chambers were allowed to float on the water surface for 1-3 hours (see Rulík et al. 2013; Bednařík et al. 2015). Previous measurements based on the collection of gas samples every 30 min over a 3 h period confirmed a linear dependency between the methane concentration inside the chambers and time. The fluxes were calculated using linear regression based on the concentration change as a function of time (Duchemin et al. 1999; Silvennoinen et al. 2008).

At each sampling occasion, ambient air samples were also collected for determining initial background concentrations. Samples of headspace gas were collected through the rubber stopper inserted at the top of the chamber and stored in 12 ml Exetainers® (Labco Limited, UK). Emissions were calculated as the difference between initial background and final concentration in the chamber headspace, and expressed on the 1 $m^2$ area of the surface level per day according to the formula:

(5) $E = \frac{\left[\frac{(cI-cR)*24*V}{1000*t}\right]}{p}$

where F is the gas flux (mmol $m^{-2}$ $d^{-1}$), cI is the concentration of methane in the chamber headspace after 1-3 hours of collecting (µmol $L^{-1}$), cR is the concentration of methane in background air, V is the volume of the chamber in L, t is time of incubation (h), p is the area of chamber ($m^2$).

We also determined $CH_4$ diffusive fluxes to the atmosphere using calculations derived from recent studies (Striegl et al., 2012; McGinnis et al., 2014; Borges et al., 2015; Bodmer et al., 2016). The air-water $CH_4$ diffusion flux (F) was computed according to

(6) $F = k(c_w - c_a)$

where k is the gas transfer velocity for water-air gas exchange (m $d^{-1}$), cw − ca is the gas concentration gradient between the river and the atmosphere (mol $m^{-3}$). For the river flux calculations k is defined as (Fortescue and Pearson, 1967)

(7) $k = 1.46(\frac{DV}{h})^{-0.5}$

where D is the molecular diffusion coefficient of $CH_4$ in the water (Broecker and Peng, 1974), V is the water velocity (m $s^{-1}$) and h is the water depth (m).

In order to estimate the total $CH_4$ fluxes from the River Elbe we divided it into particular river sections according to sites of diffusive flux measurements (with the exception of emissions from harbors and groynefields, due to the difficulty of estimation their area) and calculated from the measured diffusive fluxes at individual sites along the River Elbe continuum according to the following equation:

(8) $E_T = \frac{(\Sigma P \times E_m \times 365 \times M_{CH_4})}{1000000}$

where $E_T$ is total $CH_4$ emission from the total river area in t $yr^{-1}$; P is the area of a particular river section in $m^2$; $E_m$ is the mean $CH_4$ diffusive flux from the particular river section expressed in mol $m^{-2}day^{-1}$; $M_{CH4}$ is the molar weight of $CH_4$ molecule.

The amount of $CH_4$ transported by the River Elbe out of the Czech Republic and into the estuary was calculated by multiplying the $CH_4$ concentration and current discharge. Data for the discharge ($m^3$ $s^{-1}$) were

obtained from the Elbe data portal (http://www.elbe-datenportal.de). We used discharge data measured 18th October 2013 at "Pegel Neu Darchau" (53.2284003N, 10.8858031E) and 26th October 2013 at "Pegel Schöna" (50.8752542N, 14.2350553E).

**Ebullition**

Floating subsurface gas collectors were deployed to measure ebullitive gas flux during the 2013 campaign at selected sites of various habitats (weir, lock, groynefield, harbor, free-flowing main stream of the river). Each gas collector consists of an inverted funnel with an opening of 0.3 $m^2$ attached to the lower end of an 80 cm long gas-storing tube, equipped with a septum-lined cap at the upper end. The collectors were submerged in the surface water, filled with water and enclosed with septum-lined caps. Consequently, they were moored with a loose rope that allowed them to hang at one place. The collectors were balanced so that only a small part of the upper capped end was visible above the surface facilitating gas sampling. Gas samples were taken by a syringe through the septum-lined cap and transported to Exetainers® for gas composition analysis via gas chromatography.

**Total bacterial abundance in water samples**

Water samples (50 ml) for total bacteria counting were preserved with formaldehyde (2% final concentration, vol/vol) immediately after collecting and stored refrigerated. In the laboratory subsamples (4.5 ml) were taken and filtered through white polycarbonate filters (25 mm, 0.2 μm; GE Healthcare UK Limited, UK), stained with 4, 6-diamidino-2-phenylindole (DAPI; final concentration 0.1 $g.mL^{-1}$ wt/vol) and enumerated using an epifluorescence microscope (Olympus AX 70).

**Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) with rRNA-targeted oligonucleotide probes for detecting Methane Oxidizing Bacteria**

We examined proportions of the probe-targeted MOB of total bacterial cells in the water column of each sampling site (in duplicate) during the large sampling campaign in 2013. The water samples were preserved with paraformaldehyde (4% final concentration, vol/vol) immediately after collecting for at least 2 h.

Subsequently, 4.5 ml of fixed water sample were filtered through white polycarbonate filters (25 mm, 0.2 μm; GE Healthcare UK Limited, UK), washed with phosphate buffered saline (PBS) and miliQ-water, and stored in liquid nitrogen. Further analyses were performed in the laboratory using group-specific 16S rRNA probes modified at 5'-end with horseradish peroxidase (biomers.net GmbH, Germany): Mα450 probe targeting type II methanotrophs and Mγ84 probe targeting type I methanotrophs (Eller et al. 2001). We applied the CARD-FISH protocol described previously (Sekar et al. 2003) modified for MOB by using 20% formamide as a hybridization buffer. The prepared filter sections (in triplicates per sample) were then stained with DAPI and the proportions of hybridized bacterial cells (in 500-1000 DAPI-stained cell inspected) were enumerated using the epifluorescence microscope (Olympus BX 53, 1000× magnification).

**Statistical analysis**

To determine the possible dependence of $CH_4$ concentration and $CH_4$ turnover time from measured hydrological and chemical factors a Spearman rank order correlation analyses were performed. For MOB abundance analyses the Spearman rank order correlation and Wilcoxon Signed Rank Test were used. Calculations and graphical representation were performed using SigmaPlot for Windows Version 11.0 software.

**Results**

**Hydrological parameters and nutrient composition of the water column**

The obtained data on chemical and hydrological parameters from the different sampling campaigns are summarized in Tab. 2. We did not observe any seasonal pattern for the measured parameters, except for the data on water temperature. The data obtained during the large campaign in 2013 from the Czech river ($8^{th}$ till $366^{th}$ km) section fit in the range of the previous small campaigns, with the exception of TDN which was rather high in contrast to the previous sampling campaigns (2011 – 2012). The oxygen content obtained during all sampling campaigns indicated sufficient oxygen supply for aquatic microorganisms.

**Table 2**
Overview of obtained data (median) on nutrients and physical parameters in the water column of the River Elbe during all sampling campaigns (2011 - 2013): oxygen content ($O_2$); Suspended particular matter (SPM); water temperature (Water T); pH; dissolved organic nitrogen (DN; i.e. sum of $NO_3^-$, $NO_2^-$ and $NH_4^+$); total dissolved nitrogen (TDN); total dissolved phosphorus (TDP). Note: n.a. = data not available.

| Sampling campaign | River section | $O_2$ [mg $L^{-1}$] | SPM [mg $L^{-1}$] | Water T [°C] | pH | Conductivity | DN [mg $L^{-1}$] | TDP [µg $L^{-1}$] |
|---|---|---|---|---|---|---|---|---|
| | natural (n=2) | 10.2 | 3.0 | 14.5 | n.a. | n.a. | 0.3 | 30.9 |
| May 2011 | impounded (n=8) | 8.9 | 10.7 | 21.1 | n.a. | n.a. | 0.9 | 66.3 |
| | lowland (n=2) | 9.7 | 23.0 | 16.2 | n.a. | n.a. | 0.7 | 35.9 |
| | natural (n=2) | 10.5 | 2.9 | 11.3 | 7.4 | n.a. | 0.2 | 26.8 |
| October 2011 | impounded (n=9) | 9.3 | 7.0 | 18.7 | 7.9 | n.a. | 0.8 | 88.5 |
| | lowland (n=2) | 9.6 | 9.0 | 19.1 | 8.7 | n.a. | 0.9 | 84.0 |
| | natural (n=2) | 12.2 | 25.6 | 5.6 | 7.0 | 163.7 | 1.2 | 28.0 |
| March 2012 | impounded (n=6) | 13.5 | 32.4 | 8.4 | 7.2 | 396.0 | 3.5 | 45.0 |
| | lowland (n=3) | 18.1 | 132.0 | 13.1 | 7.0 | 393.0 | 3.7 | 50.7 |
| | natural (n=4) | 8.1 | 10.3 | 16.5 | 7.9 | 204.5 | 1.4 | 71.9 |
| August 2012 | impounded (n=5) | 12.9 | 21.6 | 24.5 | 6.1 | 411.0 | 2.7 | 94.1 |
| | lowland (n=0) | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| | natural (n=2) | 11.4 | n.a. | 7.0 | 6.8 | 136.5 | n.a. | n.a. |
| October 2013 | impounded (n=9) | 10.7 | n.a. | 12.0 | 7.6 | 484.5 | n.a. | n.a. |
| | lowland (n=23) | 10.2 | 17.2 | 11.4 | 7.6 | 487.0 | $3.6^{TDN}$ | 68.7 |

## Methane concentration in the water column

The data on $CH_4$ concentrations obtained during the large sampling campaign in 2013 from the 8$^{th}$ till the 366$^{th}$ km (i.e., Czech part of the river) are consistent with the data-trend from the small sampling campaigns in 2011 and 2012 (Fig. 2). Therefore we describe the methane dynamics in the River Elbe complexly, i.e. including data from all the sampling campaigns together, divided into the above mentioned parts, i.e., "natural", "impounded" and "lowland". As we observed no difference between "surface" and "bottom" samples the data were pooled.

We observed the following trend for the $CH_4$ concentration: The spatial variation of $CH_4$ repeatedly showed very low concentrations at the pristine site at the 8$^{th}$ km point with a median of 11 nmol $L^{-1}$ (0.1 - 31 nmol $L^{-1}$). Further downstream $CH_4$ concentrations usually rapidly increased from the "natural" sampling site at the 56$^{th}$ km point with median of 259 nmol $L^{-1}$ (164 - 387 nmol $L^{-1}$) towards the "impounded" sampling site at the 140$^{th}$ km point with a median of 439 nmol $L^{-1}$ (133 - 825 nmol $L^{-1}$). The values for the additional sites at the 228$^{th}$ and 302$^{nd}$ km points sampled during the 2013 campaign did not differ from the data pattern obtained during the previous four sampling campaigns. Neither did we observe any unusually high $CH_4$ concentration after conjunctions

with other Czech main rivers - Vltava (258th km) and Ohře (302nd km) in the "impounded" river part.

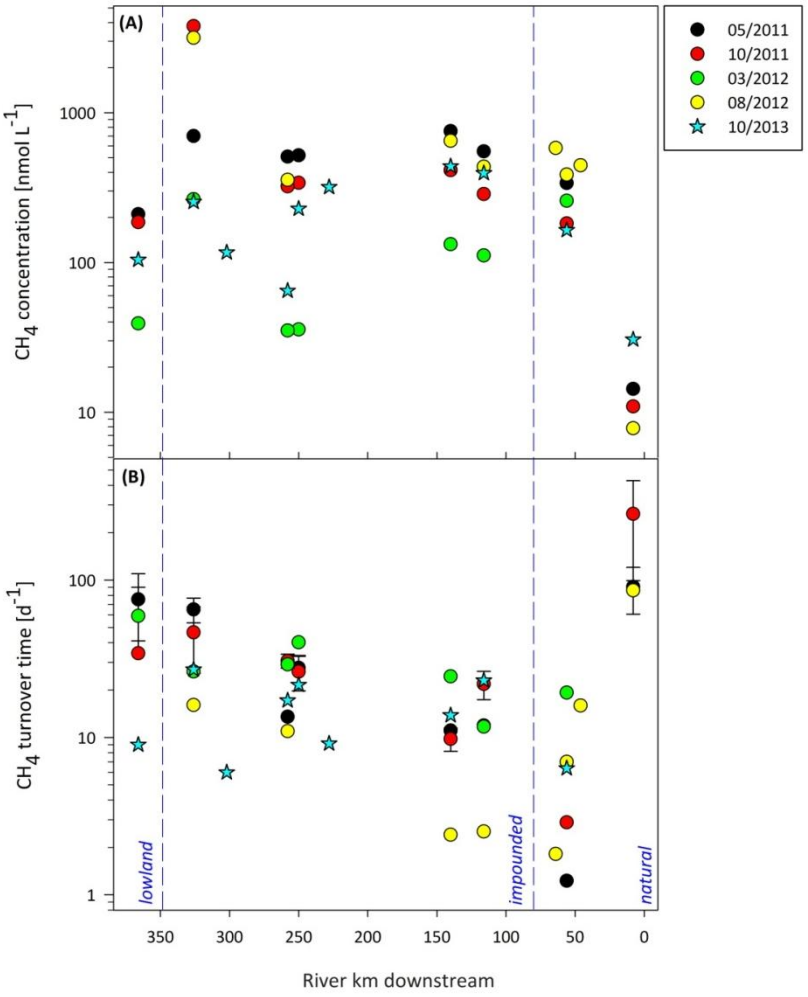


**Figure 2**
Methane distribution in the water column (A) and related methanotrophic potential (B) in 2011 - 2013 in the Czech part of the River Elbe (mean values ± SD; in case of overlapping within the symbol size error bars are not shown).

Further downstream, at the 326th km point (the Střekov weir) in the "impounded" part always very high values were measured, ranging from 232 nmol $L^{-1}$ up to 3991 nmol $L^{-1}$ (median 700 nmol $L^{-1}$), with the highest $CH_4$ concentration obtained in October 2011 (Fig. 2). At this sampling site the lowest values were obtained during the large sampling campaign (2013) and in March 2012. Some kilometers downstream in the "lowland" part of the river at the

366th km point the $CH_4$ concentration in the water column decreased again and ranged between 39 and 229 nmol $L^{-1}$ (median of 186 nmol $L^{-1}$). During the large sampling campaign in 2013 (Fig. 3) we obtained a $CH_4$ concentration of 104 nmol $L^{-1}$ at this site and further downstream the concentration stayed at a comparable level ranging between 30 and 186 nmol $L^{-1}$ till the 886th km point (the Geesthacht weir), where we observed an elevated $CH_4$ concentration of 331 nmol $L^{-1}$ (Fig. 3). The $CH_4$ concentrations in the three sampled groynefields were equal to the values measured in the main stream of the river; only at the 871st km point did the samples show a slightly elevated $CH_4$ value. The conjunctions with important rivers – Schwarze Elster (563rd km), Saale (655th km), and Havel (792nd and 802nd km) in the "lowland" part did not result in elevated $CH_4$ concentrations. Very high $CH_4$ concentration in the water column at the German section of the river were observed at sites which have been considerably altered by man, e.g. the Meissen harbor (Winterhafen) or the harbor on the Elde Canal at Dömitz. Overall, the highest $CH_4$ concentration (3991 nmol $L^{-1}$) of the whole monitored river section (i.e. from 2011 - 2013) was found at the 326th km point (the Střekov weir) in the "impounded" river part.

On the way upstream the obtained $CH_4$ concentrations were significantly different at some of the "doubled" sites (i.e. 886th, 734th, 632nd, 578th, 447th and 411th km) than on the way downstream: at the 886th km point ($t = 18{,}594$, $P = <0{,}001$), at the 632nd km point ($t = 5{,}98$, $P = 0{,}002$), and at the 578th km point ($t = 4{,}475$, $P = 0{,}007$). The range of the obtained values varied from 55 nmol $L^{-1}$ to 170 nmol $L^{-1}$ (Fig. 3).

**Methane turnover time in the water column**

The data on the $CH_4$ turnover time obtained during the large sampling campaign in 2013 (8th - 366th river km, Fig. 3) were comparatively consistent with the data range from the four previous small sampling campaigns in 2011 and 2012 (Fig. 2). In the following text the pooled data ("surface" and "bottom") from the sampling campaigns 2011 - 2013 were used, unless otherwise noted.

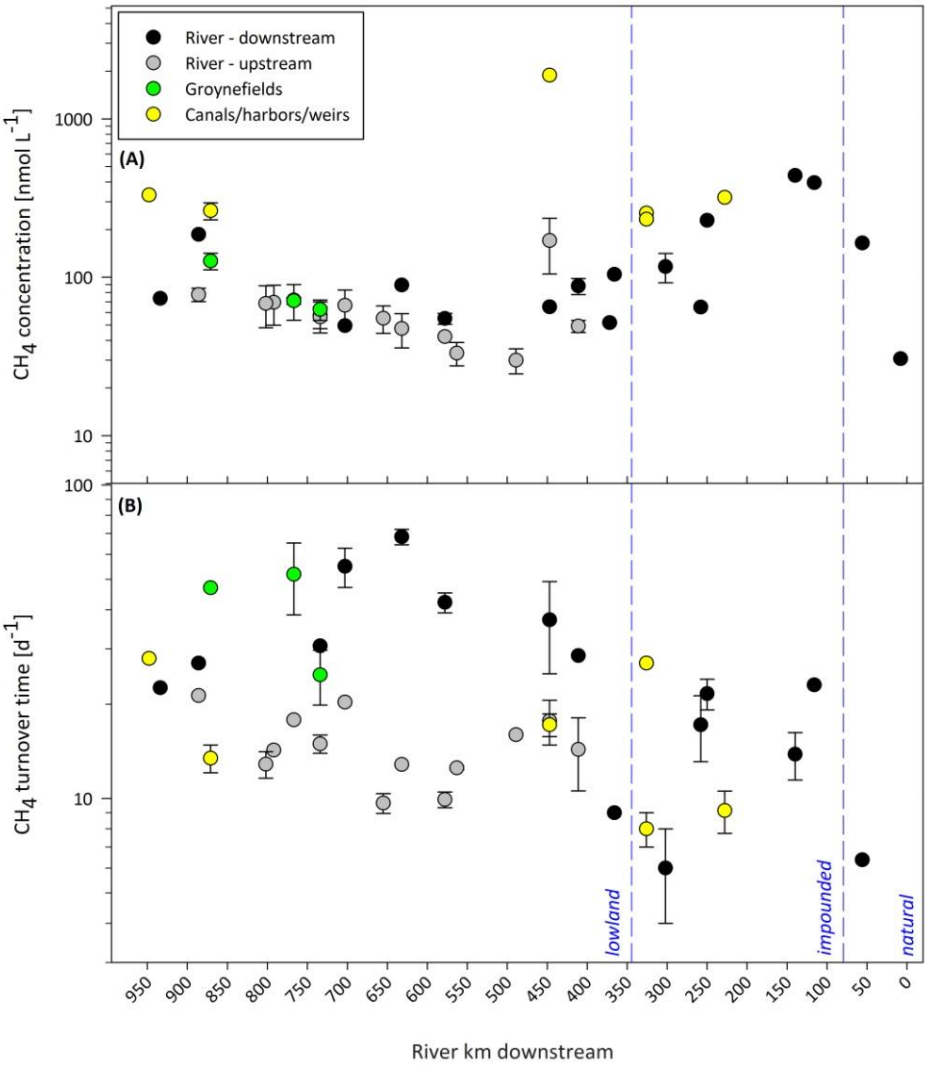


**Figure 3.** Methane distribution in the water column (A) and related methanotrophic potential (B) from the continuous sampling campaign in 2013 along the whole monitored stretch of the River Elbe (mean values ± SD; in case of overlapping within the symbol size error bars are not shown).

Compared to the $CH_4$ concentration, there is no such apparent trend in the data on the $CH_4$ turnover time. We always observed the longest $CH_4$ turnover time at the first sampling site (8$^{th}$ km), ranging from 88 to 1800 d (median of 128 d; 2011 - 2012), while the lowest activity of MOB at this site (most probably below the detection limit of the method) was measured in October 2013 (not shown in Fig. 2 and 3). Compared to this site, a rapid $CH_4$

turnover time was repeatedly observed at the 56$^{th}$ km point (still "natural" part), ranging from 1 to 19 d (median of 6 d; 2011 - 2013). In the "impounded" river part (from the 116$^{th}$ to the 326$^{th}$ km) the $CH_4$ turnover time fluctuated with slightly increasing tendency from 6 to 65 d (median 21 d; 2011 - 2013), only in August 2012 exceptionally rapid turnover time of 2 d were detected in the region between 116$^{th}$ - 140$^{th}$ km. From the first "lowland" site (366$^{th}$ km) the values on $CH_4$ turnover time showed increasing trend from 9 d to 68 d at 632$^{nd}$ km (Fig. 3), and decreasing again towards to the 948$^{th}$ km with 23 d. On the way upstream the obtained $CH_4$ turnover times were much shorter than on the way downstream, ranging from 10 to 20 d (Fig. 3).

Water samples collected at the three groynefields (734$^{th}$, 767$^{th}$ and 871$^{st}$ km) did not show any extraordinary $CH_4$ turnover time (25 - 52 d). Water samples collected after conjunctions of the River Elbe and some of its important tributaries (Schwarze Elster, Havel, and Saale) showed roughly the same $CH_4$ turnover time as samples from the main stream. Despite the great difference between the $CH_4$ concentration observed in the Meissen harbor and the mainstream at 447$^{th}$ km, both sites showed the same values of the $CH_4$ turnover time.

In general the shortest $CH_4$ turnover time (approximately 5 d) was observed repeatedly at the 56$^{th}$ km point ("natural" part). However, similarly rapid turnover time as at this site was reached by another Czech sampling site in October 2013 - at the 302$^{nd}$ km (Fig. 3 B) after conjunction with Ohře River.

**The abundance of aerobic methanotrophic bacteria in the water samples**

The total bacterial abundance ranged from approximately 0.1 x $10^6$ bacteria per ml (8$^{th}$ km point) to 4.6 x $10^6$ bacteria per ml (326$^{th}$ km point – the Střekov weir), with a median of 3 x $10^6$ bacteria per 1 ml in the riverine sampling sites (including samples from the groynefields). However, we found the highest bacterial numbers at the 447$^{th}$ km point (Meissen harbor) - up to 7.5 x $10^6$ bacteria per 1 ml.

The distribution of the aerobic MOB in the water along the monitored stretch of the River Elbe was remarkably variable in number and composition (Fig. 4). For instance, in some places such as the 632$^{nd}$ km point and the 871$^{st}$ km point (both "lowland" part), we did not observe single methanotrophic bacteria in our samples. The MOB type II (see examples in Supplement Fig. 2

A, B) was almost one order of magnitude more abundant than the MOB type I (examples in Supplement Fig. 2 C, D); with a median of approximately 8.37 x $10^3$ cells per 1 ml for type II in contrast to 1 x $10^3$ cells per 1 ml ($p < 0.001$; n = 42) for type I. On some sites we found only one type of MOB (Fig. 4). The proportion of MOB I to the total bacterial abundance was low with median of 0.03 %, the highest abundance (1%) was observed at the 250$^{th}$ km in the "impounded" river part. In contrast, the proportion of MOB II to the total bacterial abundance was slightly higher with median of 0.3%, the highest abundance (3%) was observed at the 703$^{rd}$ km in the "lowland" river part.

**Methane emissions and ebullition**

Methane emissions from the surface water into the atmosphere ranged from 0.4 to 11.9 mg $m^{-2}$ $d^{-1}$ (median of 2.1 ± 0.6 mg $m^{-2}$ $d^{-1}$), (Fig. 5). The highest values on $CH_4$ emissions were obtained at the 56$^{th}$ km in natural river part with methane concentration 164 nmol $L^{-1}$ and the lowest values were obtained at the 326$^{th}$ km (the lock at Střekov weir) with methane concentration 243 nmol $L^{-1}$ (mean value). In the lowland river we can see a decreasing trend in the $CH_4$ emissions along the whole river flow. The only exception was the Meissen harbor (447$^{th}$ km), with its extraordinarily high $CH_4$ emissions - up to 11.9 mg $m^{-2}$ $d^{-1}$. Only at this site we could also measure the ebullition, where $CH_4$ in the gas bubbles reached 488436 ppm (data not shown). Although we were able to take gas bubbles into the gas collectors on some sampling sites along the river, the gas content of the bubbles was dominated by carbon dioxide. No correlations with other measured parameters were found.

The $CH_4$ diffusive fluxes from Elbe River into the atmosphere calculated for the different river reaches along the Elbe River are showed in Table 3. The value of the total $CH_4$ diffusive flux from the whole Elbe River was estimated to be approximately 97 t $CH_4$ $y^{-1}$. The comparison of the measured $CH_4$ emissions versus calculated diffusive flux showed no statistically significant difference (Mann-Whitney U Statistic = 36; T = 126; n = 9; P = 0.142).

**Table 3.** The total $CH_4$ diffusive fluxes from Elbe River into the atmosphere calculated for the different river reaches along the Elbe River. Emissions from harbors and groynefields were not included.

| River reach [km] | Area [$km^2$] | Mean $CH_4$ emissions [mmol $m^{-2}$ $d^{-1}$] | Total emission [t $CH_4$ $y^{-1}$] |
|---|---|---|---|
| 0 - 142 | 3.12 | 0.55 | 10.1 |
| 142 - 300 | 11.6 | 0.38 | 24.5 |
| 300 - 410 | 12.65 | 0.35 | 25.9 |
| 410 - 468 | 6.32 | 0.21 | 7.7 |
| 468 - 534 | 6.47 | 0.22 | 8.4 |
| 534 - 641 | 15.9 | 0.03 | 2.4 |
| 641 - 787 | 23.21 | 0.07 | 10.0 |
| 787 - 948 | 31.72 | 0.05 | 8.5 |
| 0 - 948 | 109.6 | | 97.5 |

In October the 18$^{th}$ 2013, the Elbe discharge at the Neu Darchau measurement stand was 709 $m^3$ $s^{-1}$, while month discharges ranged from 442 to 736 $m^3$ $s^{-1}$. Assuming that $CH_4$ concentration in the water column of the Elber River near Lauenburg (886$^{th}$ km) (last measured site) was 331 nmol $L^{-1}$ at this time, river transports to its lower part above Hamburg annually 118.7 t of $CH_4$, which is consequently net input of $CH_4$ to Elbe River estuary. Methane concentration in surface water of Elbe River near the Czech-German border at the 366$^{th}$ km was found to be 104 nmol $L^{-1}$, while the water discharge was 324 $m^3$ $s^{-1}$. Based on these values, river water entering the German section transports annually approximately17.0 t of $CH_4$. Thus, despite the $CH_4$ removal via emissions to the atmosphere and microbial oxidation in water column, the amount of methane leaving the Elber River near the Lauenburg was 7 times higher than the incoming amount of methane from the Czech part of the Elbe River.

**Table 4**

Pearson Product Moment Correlation between $CH_4$ turnover time and hydrological parameters. Data from Dömitz canal and Meißen harbor were not included. Note: * = $p < 0.05$; ** = $p < 0.01$.

| | natural | impounded | lowland |
|---|---|---|---|
| $CH_4$ | -0.31 | 0.29 | 0.62** |
| $O_2$ | 0.08 | 0.15 | -0.10 |
| SPM | -0.16 | -0.09 | -0.10 |
| Water T | -0.37 | -0.13 | 0.74** |
| pH | -0.55 | 0.36 | 0.52* |
| Conductivity | -0.54 | -0.12 | -0.53 |
| (T)DN | -0.28 | -0.02 | -0.79** |
| TDP | -0.47 | **-0.50**** | 0.13 |

**Possible drivers of methanotrophic activity and abundance**

A significantly positive correlation (n = 29, r = 0.61, P < 0.01) between the $CH_4$ turnover time and $CH_4$ concentration was found only in the "lowland" river part (Tab. 4). In this part of the river we also found a significantly positive correlation (n = 26, r = 0.74, P < 0.01) between the $CH_4$ turnover time and temperature, which implies that higher water temperature causes longer turnover time (i.e. lower methanotrophic activities). We found no correlation between the $CH_4$ turnover time and oxygen content, conductivity, or SPM along the entire monitored stretch of the river (Tab. 4). A significantly negative relationship was found between $CH_4$ turnover time and pH in the "lowland" part of the Elbe. The obtained chemical parameters of the water (dissolved nitrogen and phosphorus) showed a strong correlation between total dissolved phosphorus content and $CH_4$ turnover time in the "impounded" river part (Tab. 4). We also took a closer look to the methanotrophic abundance (MOB type I versus MOB type II, see examples in Supplement Fig. 2). Unfortunately, we found no correlation between the occurrence of each MOB type and the $CH_4$ turnover time or $CH_4$ concentration; neither did we find correlation between MOB-abundance and the hydrological data (results not shown).

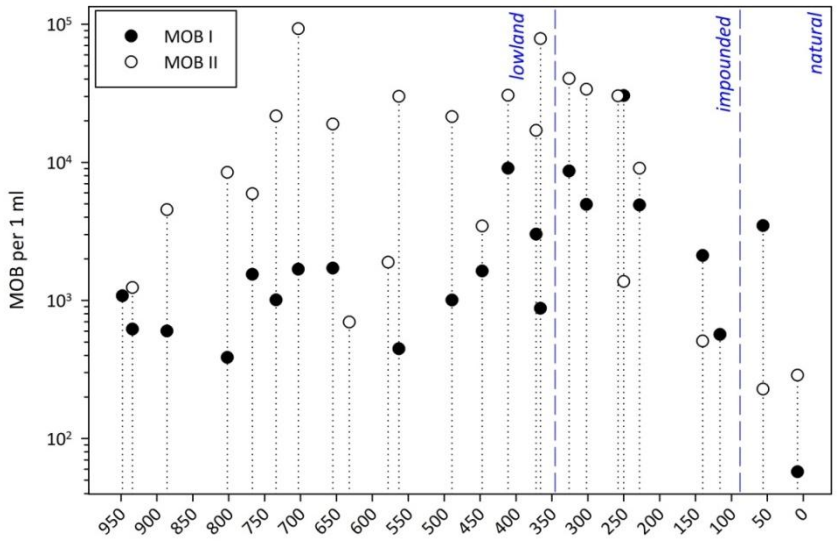


**Figure 4.** Displaying the total abundance of each type of methanotrophic bacteria (MOB I. and MOB II.) from the water samples from the continuous sampling campaign in October 2013.

## Discussion

### Methane distribution in the water column along the monitored stretch of the River Elbe

Our results on $CH_4$ concentration show that the water column of the River Elbe was supersaturated with respect to atmospheric equilibrium concentrations (2.5 - 4 nmol $L^{-1}$) along the entire monitored stretch of the river, with the exception of the most upstream oligotrophic sampling site at the 8$^{th}$ km point. In general, the range of detected $CH_4$ concentration in the water column of the River Elbe (Fig. 2 and 3) falls towards the higher end or even exceeds the range of $CH_4$ concentration reported from other rivers: for example up to 2000 nmol $L^{-1}$ in the mainstream of the River Saar (Zaiss et al. 1982); between 5 to 1730 nmol $L^{-1}$ in Oregon rivers (de Angelis and Lilley 1987); between 42 and 97 nmol $L^{-1}$ in a forest stream (Jones and Mulholland 1998); between 60 and 420 nmol $L^{-1}$ in the River Willamette (Anthony et al. 2012) or between 20 and 500 nmol $L^{-1}$ for various Amazonian rivers (Sawakuchi et al. 2014). In the Elbe River estuary the methane concentrations in the area of Hamburg harbour is about 416 nmol $L^{-1}$ but in the lower part of the estuary about 40 nmol $L^{-1}$ (Matoušů et al., in press). The range of reported $CH_4$ concentrations in other studies (for an overview see Zhang et al. 2008, and Middelburg et al. 2002) has a large variation and only some of them (Zaiss et al. 1982; de Angelis and Lilley 1987; Dzyuban 2011) took into account the differences between habitats along the river flow.

The $CH_4$ concentrations obtained during the longitudinal sampling campaign in October 2013 fit into the range of the data from the small sampling campaigns in 2011 and 2012 from the "natural" to the "lowland" river sections (Fig. 2). From this we can deduce that our data from the large sampling campaign in 2013 show no extraordinary values and might be seen as representative values on $CH_4$ dynamics of a large river ecosystem. The $CH_4$ concentration in the water column showed an increasing trend downstream of the first sampling site. However, the increase of $CH_4$ concentrations occurred until the 140$^{th}$ km site, thereafter the $CH_4$ concentrations were more or less at the same level or even decreased. The 140$^{th}$ km sampling point is located in the industrial zone typical for its higher load of pollution (Prange et al. 2000) which is in accordance with the hypothesis that $CH_4$ concentration increases with the increasing pollution of the river (Swinnerton et al. 1969; Dzyuban 2011). Unfortunately, we were not able to confirm this hypothesis because no correlation of $CH_4$ with our obtained chemical parameters of the water was

found ($r = 0.60$; $P = 0.20$; $n = 6$). However, we found a negative correlation between $CH_4$ and oxygen content ($r = -0.93$; $P < 0.01$; $n = 7$) and a positive correlation between $CH_4$ and water temperature ($r = 0.82$; $P = 0.02$; $n = 7$) at this sampling point. Both results imply possible effect of pollution, as warmer water lead to higher mineralization (Gudasz et al. 2010) which can cause anaerobic conditions in the water and especially at the sediment-water boundary, which favors the $CH_4$ production (Liikanen and Martikainen 2003). Some authors suggest that an increased concentration of $CH_4$ in rivers comes from the lateral diffusion of stream banks and the drainage of forest and agricultural soils or from the outflow of urbanized areas and sewage treatments (De Angelis and Lilley 1987; Lilley et al. 1996; Yang et al. 2012). Striegl et al. (2012) mentioned that river $CH_4$ could be derived from multiple sources, including groundwater, surface water runoff, and the benthic and water column microbial processing of organic carbon. This kind of further survey would definitely be of a great importance.

A special setting was found at the Střekov weir ($326^{th}$ km) with its extraordinary high $CH_4$ concentrations (3000 nmol $L^{-1}$ in August 2012 and up to 4000 nmol $L^{-1}$ in October 2011). Weirs (and locks) are typical anthropogenically modified river habitats, especially regarding the River Elbe with its 25 weirs along the whole flow. In this sense these data should be included to the total $CH_4$ riverine budget. According to Maeck and his colleagues (2013) dams and other small reservoirs in a river ecosystem (i.e. weirs, locks) represent hot-spots for $CH_4$ production and contribute significantly to freshwater emissions (Hertwich 2013). Weirs accumulate sediments and organic material from the upstream parts of the river. Since these sediments are anaerobic a massive microbial degradation occurs, which generates a high concentration of dissolved $CH_4$ (more than 1000 nmol $L^{-1}$), enhances $CH_4$ emissions (up to 20 mmol $m^2$ $d^{-1}$), and leads to gas bubble formation (Maeck et al. 2013). Notably, Zaiss et al. (1982) observed a high $CH_4$ concentration, up to 17000 nmol $L^{-1}$, at the Mettlach hydroelectric power plant on the German River Saar. However, we were not able to detect any $CH_4$ emissions at Střekov weir during the sampling campaign in 2013 (see further).

The Střekov weir is currently the last weir on the Czech part of the Elbe, and from this point the $CH_4$ concentration decreased and stayed below the values obtained at the last sampling site in the Czech Republic (i.e. the $366^{th}$ km point at Hřensko) and for the next 600 km till the weir at Geesthacht (i.e. the

"lowland" river part). This part of the river is stabilized with more than 3400 groynes (20–30 m long wing-dams) which support navigation for ships during low water levels. Due to the significant decrease of the flow velocity in comparison to the main stream, a counter flowing circulating water between the groynes (in so-called groynefields) leads to high sedimentation of sand and coarse organic material - the amount and location of sedimentation depend on hydraulic processes and type of groynes (Henning and Hentschel 2013). In a way groynefields represent an artificial transition area between river and floodplain, and so a higher $CH_4$ concentration in the water there, due to higher $CH_4$ production in contrast to the main stream, was expected. However, we did not record an increase of $CH_4$ concentration in groynefields. On the other hand, more sampling of different types of groynefields (with different level of sedimentation, size, etc.) through the season are needed before we generalize the statement; that in this way groynefields would represent an counterbalance to $CH_4$-producing weirs, while all these technologies are designed for navigation.

High amounts of SPM are reported to be positively correlated with high $CH_4$ concentrations (Upstill-Goddard 2000), but others (Abril et al. 2007; Grunwald et al. 2009) also showed a negative correlation (i.e. high $CH_4$ concentrations in clear-water systems) or no correlation (Matoušů et al. in press; Osudar et al. 2015). However, the idea behind a possible correlation between these two parameters is that the resuspension of sediment results in increased SPM (turbidity) content. As most of the $CH_4$ is produced in anoxic zones within the sediment, $CH_4$ is also released (Bussmann 2005). But this direct relationship does not seem to hold in rivers, since in rivers the SPM has its own set of dynamics i.e.: within the river continuum the characteristics of SPM quantity and composition alter in relation to changes in the mobilization of terrestrial versus in-stream sources as well as in-stream autotrophic processes (Dawson et al. 2012). We also observed that the filter used to analyze samples from the Czech section (small sampling campaigns in 2011-2012) often retained algae, which were not related to sediment resuspension. Abril et al. (2007) postulated that lower $CH_4$ concentrations at increased SPM content are due to an increased $CH_4$ oxidation associated with the particles. However, our data do not support their findings.

**Methane turnover time and its possible drivers in the water column**

Our data on $CH_4$ turnover time in the water column of the River Elbe present a very wide range, which might reflect the fact, that there are many drivers, and/or their specific combinations controlling the microbial $CH_4$ oxidation. With the given hydrological parameters we were able to reveal only some hints of possible effect on the methanotrophical potential.

The availability of $CH_4$ as a sole source of carbon for MOB was thought to be the key factor for their activity. We have found significant correlation between the $CH_4$ turnover time and $CH_4$ concentration only in the "lowland" part (Tab. 3), which, however, suggests the negative effect of $CH_4$ levels in the water column on the $CH_4$ turnover time. This fact means that a higher $CH_4$ concentration does not support equally higher methanotrophic activity, which contradicts our expectations. This can be exemplified on three sites with quite contrasting characteristics (during all sampling occasions) regarding the ambient $CH_4$ concentrations and related $CH_4$ turnover time: Verdek ($56^{th}$ km), Střekov ($326^{th}$ km), and Hřensko ($366^{th}$ km); data from sampling campaigns 2011-2012. At Verdek we found, at intermediate $CH_4$ concentrations (median of 299 nmol $L^{-1}$), a very active methanotrophic population (median of $CH_4$ turnover time approximately 5 days). At Střekov lock we found, at very high $CH_4$ concentrations (median of 1 226 nmol $L^{-1}$), an intermediate $CH_4$ turnover time (median of 30 days). This is in contrast to the site Hřensko, where we found a rather inactive methanotrophic population (median $CH_4$ turnover time of 93 days) at an intermediate high $CH_4$ concentration (median of 190 nmol $L^{-1}$). The explanation for these contradictory findings may lie in the habitat type. At the site Verdek the river stream is shallow (0.25 - 1 m) with high current velocity (1.05 m $s^{-1}$, measured during October 2013). The river bed is composed of rocks (approximately size 10 cm) covered with algae and visible biofilms. Notably, this part of the river still has a natural character, with connections between the river and its surrounding. High loads of red colored clay silts (Supplement Fig. 1) were observed during two sampling campaigns (08/2012 and 10/2013). The silt color comes from iron oxides (hematite, $Fe_2O_3$) which are present in the surroundings soils. Iron was found to increase the maximum methane utilization rate and strongly influence the yield coefficient in cultured MOB (Boiesen et al. 1993).

The Střekov lock has an opposite character: the water at the lock is stagnant with a deep water column (up to 13 m). There are no connections to the

surrounding terrestrial ecosystem. As mentioned before, many authors have proposed that weirs, locks, dams, and man-made reservoirs can act as important producers of $CH_4$ emissions (e.g. Guérin and Abril 2007; Hertwich 2013; Maeck et al. 2013) while others have found that the microbial filtering of this greenhouse gas may not always be effective in such habitats (Delsontro et al. 2010). Regarding the habitat type at Hřensko - the river at this site is a wide (approximately 110 m) and shallow (2.5 m water depth, October 2013), with a high current velocity (1.1 m $s^{-1}$, October 2013). This part of the river is under a high load of boat traffic and pollution.

On the other hand, we measured similarly high methanotrophic activity to that at Verdek at the 302$^{nd}$ km (Litoměřice; conjuction of the River Elbe with Vltava River) in October 2013. These two sites are completely different in character (water depth, velocity, temperature, etc.), the only common parameter was the $CH_4$ concentration (about 117 - 164 nmol $L^{-1}$), and still they had a similar, very fast $CH_4$ turnover time. Neither did the MOB abundance show us any common signs among the sampling sites and related $CH_4$ turnover time. This may be due to their different affinity to $CH_4$ concentrations (Amaral and Knowles 1995): So far it has been found that mainly MOB type I are capable of utilizing $CH_4$ at lower concentrations, higher oxygen content, and lower temperatures (5-10°C), while MOB type II are at an advantage at higher $CH_4$ concentrations, lower oxygen content, and higher temperatures (20°C) (Amaral and Knowles 1995; Börjesson et al. 2004). This might partially elucidate the similar methanotrophic activity at Verdek and Litoměřice, with similar $CH_4$ concentrations but a different MOB community: at Verdek we found a prevailing MOB type I, but at Litoměřice we found only MOB type II (Fig. 4).

In contrast to the Czech river section, with a significantly man-altered river flow by weirs and locks, the German river section is modified mainly via groynes. The groynefields build heterogeneous habitats consisting of a matrix of coarse gravel and also sand and fine organic components. The groynefields may offer a different environment in contrast to the rapid flow of the mainstream of the river in that they provide a longer water residence time, higher temperature due to the low water depth and higher particle sedimentation. We expected that this could lead to higher $CH_4$ production and also higher $CH_4$ consumption by methanotrophic bacteria. However, we discovered only one of three sampled groynefields as $CH_4$ productive - at the 871$^{st}$ km point (Dömitz). The $CH_4$ concentration as well as the $CH_4$ oxidation in the water column ranged between

values measured on nearby stretches of the main stream of the river (Fig. 3). Neither showed us the obtained $CH_4$ emissions from these habitats at any higher amounts (Fig. 5). In comparison to other man-altered sections of the river, such as weirs or channels, groynefields seem to be a more favorable way to regulate a river considering the $CH_4$ production. However, further sampling of groynefields is needed, with respect to their different material, location, and sediment type. An important role can also be played by a multiple sampling within one groynefield as the sedimentation and the movement of the water mass changes considerably with flow rate.

**Methane emissions and ebullition**

With the exception of the Meissen-harbor locality $CH_4$ emissions showed a decreasing trend along the River Elbe's longitudinal profile, indicating that both low $CH_4$ production (Rulík et al. in prep.) coupled with methanotrophy efficiently control $CH_4$ release into the atmosphere in the middle part of the Elbe River (Fig. 5). Generally, the values of methane emissions measured in this study correspond to the range of methane emissions published from other free-flowing US rivers (e.g. de Angelis and Scranton 1993; Jones and Mulholland 1998), Amazonian rivers (Sawakuchi et al. 2014) or rivers in Europe (Saarnio et al. 2009), regardless to used measuring methods. Data on the factors controlling methane emissions are commonly restricted to wetlands and lakes (Ortiz-Llorente and Alvarez-Cobelas 2012) and knowledge of these factors in rivers are more elusive. Nevertheless, our data show that both damming of the river as occurs in the impounded part of the Elbe River and the occurrence of emission hot spots may promote $CH_4$ emissions and also contribute to the poor estimation of $CH_4$ fluxes at the ecosystem scale. Unfortunately, despite very high $CH_4$ concentrations (and observed gas bubbles during previous sampling campaigns) measured in previous sampling occasions at the Střekov weir, we were not able to measure any $CH_4$ emissions in October 2013. This was due to the fact that shortly before the sampling in October 2013 a cargo ship passed through the lock and so the lock was completely drained and re-filled with new water. We assume that further measurements would indicate very high emission values at this site.

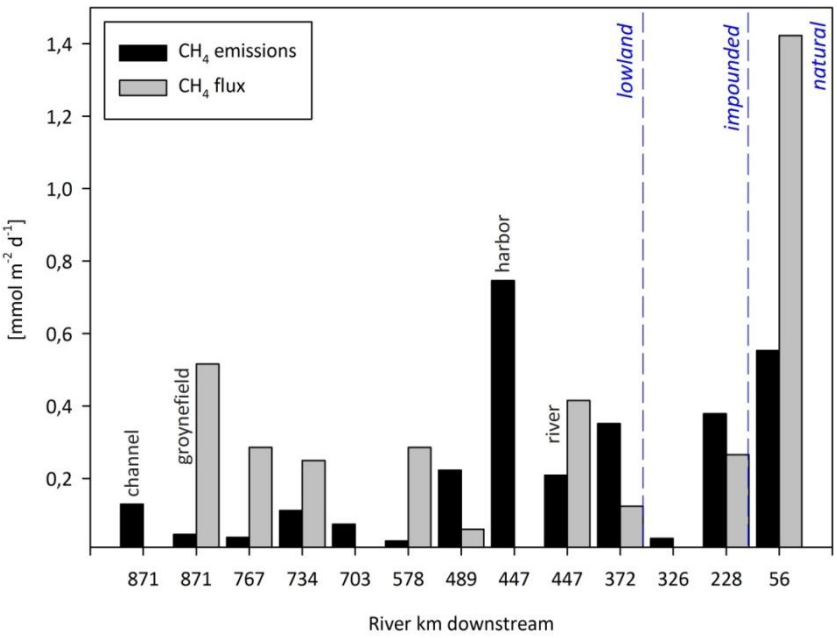


**Figure 5.** Measured $CH_4$ emissions versus calculated $CH_4$ flux at selected profiles along the River Elbe flow from the continuous sampling campaign in October 2013. Doubled sites are differentiated according to the habitat type. Note: In case of missing the $CH_4$ flux column (326; 447/harbor; 703; 871/channel) we were not able to calculate it because of lack of data.

Undetectable $CH_4$ content in bubbles captured during ebullition measurement in October 2013 supports our findings of very low $CH_4$ concentrations found within top sediment layers (Rulík et al. in prep.). For comparison, methane usually creates 20-70 % of the volume of bubbles released from river sediments (Wilcock and Sorrell 2008, Baulch et al. 2011). From this point of view, the source of $CH_4$ for emissions to the atmosphere is not likely the sediment, but more probably downstream transport of $CH_4$. One exception to this rule is the Meissen-harbor locality which represents a "hot-spot" for $CH_4$ production. In this case the high $CH_4$ content in the bubbles (~50 %) contributes substantially to the very high emissions of $CH_4$ into the atmosphere from this site.

Assuming that area of Elbe river main channel is approximately 109.6 $km^2$, the total $CH_4$ annual flux to the atmosphere might be calculated as 0.9 t $CH_4$ $km^{-2}$ $y^{-1}$ (see Table 3). This result is highly comparable with other studies estimating the total $CH_4$ fluxes from lotic ecosystems. For instance, Saarnio et al. (2009) estimated $CH_4$ efflux from European watercourses to be 2.6 t $CH_4$ $km^{-2}$ $y^{-1}$. Estimation of $CH_4$ emissions from rivers based on calculation of Bastviken

et al. (2011) is equal to 4.8 t $CH_4$ $km^{-2}$ $y^{-1}$. Mach et al. (2016) estimated total $CH_4$ efflux to be 3.5 t $CH_4$ $km^{-2}$ $y^{-1}$ for small temperate stream, while 0.9 t $CH_4$ $km^{-2}$ $y^{-1}$ has been estimated for mainstem of Amazon River (Richey et al. 1988). In context of studies mentioned above, an estimation published by Ortiz-Llorente and Alvarez-Cobelas (2012), 465 t $CH_4$ $km^{-2}$ $y^{-1}$, seems to be considerably overestimated.

**Conclusions**

Our study shows that the methane concentrations in the water column of the River Elbe exceed the data from previously reported river systems in the temperate zone. Along the flow of the Elbe $CH_4$ concentrations increased and culminated in the most polluted and man-altered parts of the river, while later in the free flowing part they stayed more or less constant. Overall the $CH_4$ concentration in the water column varied over two orders of magnitude. This implies that single measurement of river water will not reflect the heterogenic emissions along the river transect. The $CH_4$ turnover time of the water samples was similarly heterogenic, spanning over again one to two orders of magnitude. The $CH_4$ turnover time was, however, not correlated to the methane concentration of the water. We were not able to clarify the potential drivers of the methanotrophic activity; it is most probably a specific combination of factors and local features. However, the "$CH_4$ hot-spots" and concurrently low MOB activity spots were man-altered habitats, such as weirs, locks, harbors, and canals. As compared with the free flowing river modified only with groynes, where the $CH_4$ concentration stayed at a low level with only minor $CH_4$ emissions and so probably represent a more effective and nature-close solution for river navigability.

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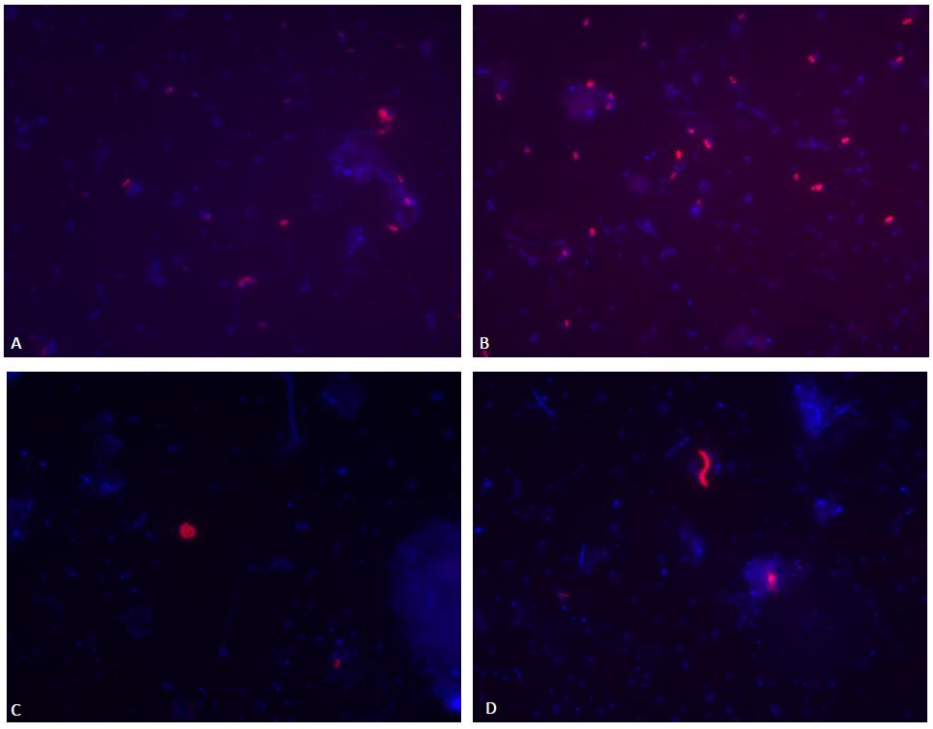
## Supplementary material

**Supplement 1**
Reddish-colored clay silts at the 46$^{th}$ and 56$^{th}$ km point in the natural part of the River Elbe.




**Supplement 2**
Photomicrographs of MOB type II (A and B; probe Mα450) and MOB type I (C and D; probe Mγ84) from the CARD-FISH analyses: MOB red-colored, other bacteria (DAPI stained) blue-colored.

## 7. Curriculum Vitae - Anna Matoušů

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### Date and place of Birth

10. 3. 1983 in Ostrov, Czech Republic

### Objective of research

Aerobic methanotrophic bacteria in the water column of diverse aquatic habitats; their activity and ecology. Methane dynamics in riverine systems, factors influencing methane emissions, hot-spots of methane emissions.

### Education

1998 - 2002 První české gymnázium in Karlovy Vary (high school)
2004 - 2007 University of South Bohemia in České Budějovice, Faculty of Science, bachelor degree in Biology
2007 - 2010 University of South Bohemia in České Budějovice, Faculty of Science, master degree in Conservation biology
2010 - 2017 University of South Bohemia in České Budějovice, Faculty of Science, School of Doctoral Studies in Biological Sciences, Hydrobiology

### Abroad stay

2010 - 2011 DAAD scholarship - Alfred-Wegener-Institut für Polar- und Meeresforschung, Meeresstation Helgoland, Germany

### International scientific conferences

2011: 12th Symposium on Aquatic Ecology, Germany (poster presentation)
2012: European Geosciences Union General Assembly, Austria (poster presentation)

2012: XVI. Conference of SLS and ČLS, Slovakia (oral presentation in Czech)
2013: 13th Symposium on Aquatic Ecology, Italy (poster presentation)
2015: 14th Symposium on Aquatic Ecology, Sweden (poster presentation)

**Publications**

Bussmann I., Matoušů A., Osudar R., Mau S. (2015) Assessment of the radio $^{3}H$-$CH_4$ tracer technique to measure aerobic methane oxidation in the water column. Limnology and Oceanography: Methods 13: 312-327. doi: 10.1002/lom3.10027

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