

# Methanotrophic potential of Dutch canal wall biofilms is driven by *Methylomonadaceae*

Koen A.J. Pelsma<sup>1</sup>, Daniël A.M. Verhagen<sup>1</sup>, Joshua F. Dean<sup>2</sup>, Mike S.M. Jetten<sup>1</sup>, Cornelia U. Welte<sup>1,\*</sup>

<sup>1</sup>Department of Microbiology, Radboud Institute for Biological and Environmental Sciences, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

<sup>2</sup>School of Geographical Sciences, University of Bristol, Bristol BS8 1SS, United Kingdom

\*Corresponding author. Department of Microbiology, Radboud Institute for Biological and Environmental Sciences, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands. E-mail: [c.welte@science.ru.nl](mailto:c.welte@science.ru.nl)

Editor: [Tillmann Lueders]

## Abstract

Global urbanization of waterways over the past millennium has influenced microbial communities in these aquatic ecosystems. Increased nutrient inputs have turned most urban waters into net sources of the greenhouse gases carbon dioxide (CO<sub>2</sub>) and methane (CH<sub>4</sub>). Here, canal walls of five Dutch cities were studied for their biofilm CH<sub>4</sub> oxidation potential, alongside field observations of water chemistry, and CO<sub>2</sub> and CH<sub>4</sub> emissions. Three cities showed canal wall biofilms with relatively high biological CH<sub>4</sub> oxidation potential up to 0.48 mmol g<sub>DW</sub><sup>-1</sup> d<sup>-1</sup>, whereas the other two cities showed no oxidation potential. Salinity was identified as the main driver of biofilm bacterial community composition. *Crenothrix* and *Methyloglobulus* methanotrophs were observed in CH<sub>4</sub>-oxidizing biofilms. We show that microbial oxidation in canal biofilms is widespread and is likely driven by the same taxa found across cities with distinctly different canal water chemistry. The oxidation potential of the biofilms was not correlated with the amount of CH<sub>4</sub> emitted but was related to the presence or absence of methanotrophs in the biofilms. This was controlled by whether there was enough CH<sub>4</sub> present to sustain a methanotrophic community. These results demonstrate that canal wall biofilms can directly contribute to the mitigation of greenhouse gases from urban canals.

**Keywords:** climate change microbiology, eutrophication, greenhouse gases, *Methyloglobulus*, microbial community, urban waterways

## Introduction

Urban waters are increasingly recognized as important ecosystems that contribute significantly to global greenhouse gas emissions. As even low methane (CH<sub>4</sub>) emissions can have a great impact on climate warming (Myhre et al. 2013), CH<sub>4</sub> with its high global warming potential has been the focus of several studies of urban ponds (van Bergen et al. 2019, Peacock et al. 2021) and even whole cities (Martinez-Cruz et al. 2017, Herrero Ortega et al. 2019, Wang et al. 2021). While streams, rivers, and lakes are well studied (Bastviken et al. 2011, Stanley et al. 2016, Saunio et al. 2020), urban canals and ditches are poorly represented in recent datasets (Peacock et al. 2021).

Greenhouse gas emissions from aquatic systems are the result of microbial respiration and anaerobic degradation of organic matter (Conrad 2009, Dean et al. 2018). Urban canals are susceptible to many factors that could increase anoxia, and, consequently, higher CH<sub>4</sub> fluxes, such as slow, laminar water flow and high levels of nutrients (Needelman et al. 2007, Peacock et al. 2021). Lower availability of oxygen causes a larger methanogenic zone and increases CH<sub>4</sub> emissions. In the Netherlands, many urban waters are considered to have low water quality due to phosphate and ammonia loading, yearly algal blooms, and excessive human activity like boating and recreation (Teurlincx et al. 2019, Armstrong et al. 2022). Therefore, urban canals have the potential to be a substantial source of CH<sub>4</sub> in the Netherlands (Stanley et al. 2016, Peacock et al. 2021). Due to the extensive use of canals in many Dutch

cities, the environmental impact might be considerable but has been poorly constrained.

Methanotrophic bacteria (MOB) and archaea in the sediment or water column can consume CH<sub>4</sub>, acting as a biological filter. In the sediment, anaerobic CH<sub>4</sub> oxidation (AOM) can occur in freshwaters using a variety of electron acceptors such as NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, iron, and organic matter (Ettwig et al. 2010, Haroon et al. 2013, Ettwig et al. 2016, Valenzuela et al. 2019). For several lakes and streams, this AOM process has been observed to be ecologically relevant (Martinez-Cruz et al. 2018, Shen et al. 2019). The aerobic MOB *Bacillus methanica* was in fact first isolated from an urban canal in Delft by Söhngen (1906), and aerobic methanotrophs are considered to be an important CH<sub>4</sub> sink at the global scale due to their high CH<sub>4</sub> conversion rates (Frenzel et al. 1990, Hanson and Hanson 1996, Knief 2015). In lakes and rivers, MOB thrive at the sediment–water interface or in the water column. Several studies have determined the methanotrophic potential of the water column in lakes (e.g., Carini et al. 2005, Guérin and Abril 2007, Thottathil et al. 2019, Reis et al. 2020), but for flowing waters, this has been performed only for the rivers Saar and Elbe, Germany (Zaiss et al. 1982, Matoušů et al. 2017), the Yellow River, China (Hao et al. 2020), and the Condamine River, Australia (Burrows et al. 2021).

Recently, a study by Pelsma et al. (2022) reported a novel urban habitat for MOB in the form of the canal wall biofilm. Exposed to both air and water, these biofilms were hypothesized to be an excellent habitat for methanotrophs as they experience little

Received 31 May 2023; revised 18 August 2023; accepted 11 September 2023

© The Author(s) 2023. Published by Oxford University Press on behalf of FEMS. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

water turbulence and are exposed to high CH<sub>4</sub> concentrations. The biofilm methanotroph present was *Methyloglobulus morosus*, previously isolated from lake sediment (Deutzmann et al. 2014). However, little is known about methanotrophic biofilms in the built aquatic environment. We hypothesized that MOB are present and metabolically active in a broader range of urban aquatic systems. To test this hypothesis, we sampled canal wall biofilms in five cities in the Netherlands with canals ranging from saline to freshwater. In addition, we investigated the impact of surface material on the microbial community and methanotrophic activity. Lastly, we synthesized field observations and flux data to better identify potential urban hotspots of microbial CH<sub>4</sub> cycling.

## Materials and methods

### City selection and sample sites

The Netherlands has many cities with urban canals as part of the cityscape. We chose five representative cities along a salinity gradient with different environmental parameters in order to capture several types of biofilms in this cross-sectional study. The city of Middelburg was chosen as it is known to be saline due to its proximity to the Scheldt estuary and North Sea. Den Helder and Zwolle were chosen based on their location in the northern and eastern extremities of the Netherlands. Zaandam, like Amsterdam, is influenced by the saline Noordzeekanaal and has brackish canals. Leiden was chosen to represent a freshwater canal system in the central Netherlands. All cities were sampled between March and July 2022, with a minimum of four sample sites per city (Table 1, Fig. 1; [https://methanecanals.shinyapps.io/data\\_maps/](https://methanecanals.shinyapps.io/data_maps/)). Sample sites within each city were chosen based on the accessibility to the air–water interface of the canal wall from the street level, whilst also allowing for a dispersed spread of locations. Sampling was undertaken on a single day for each city and samples were processed to a stable state within 24 h after sampling. Maps with sampling sites and photographs of the locations are presented in the [Supplementary Materials](#).

### In situ flux measurements and canal water chemical analyses

Diffusive fluxes of CH<sub>4</sub> and carbon dioxide (CO<sub>2</sub>) were measured using the floating chamber method (Lorke et al. 2015) and a portable greenhouse gas analyser (LI-7810 CH<sub>4</sub>/CO<sub>2</sub>/H<sub>2</sub>O Trace Gas Analyser; LI-COR Inc., USA). Triplicate measurements were done at each site and measurements influenced by gas bubbles were discarded from the analysis. A bubble event was recognized either by eye or when a very sudden CH<sub>4</sub> spike was detected that went upwards of 5000 ppb. Fluxes were calculated through linear regression of the CH<sub>4</sub> or CO<sub>2</sub> concentration over the measurement time (Van Bergen et al. 2019):

$$F_{\text{CH}_4} = \frac{\Delta \text{ppb}}{\Delta t} \frac{P M_{\text{W}} V_{\text{Chamber}}}{R T \text{Area}_{\text{Chamber}} 1000}. \quad (1)$$

In situ pH, conductivity, temperature, and dissolved oxygen were measured using a Hach HQ4300 multi-parameter probe (Hach, The Netherlands). Canal water concentrations of NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, and PO<sub>4</sub><sup>3-</sup> were measured using colorimetric assays on an AutoAnalyzer3 (Bran+Luebbe, Germany) after filtering 40 ml water using a nylon 0.22 µm syringe filter into a sterile 50 ml centrifuge tube. Samples were stored at –20°C if they were not measured within 1 week. Total organic carbon (TOC) and total nitrogen were measured on a TOC-L CPH/CPN analyser (Shimadzu Benelux, The Netherlands) using unfiltered canal water. Metal

concentrations of Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Cd, Ba, La, Ce, Nd, Hg, and Pb were measured by inductively coupled plasma mass spectrometry on an Xseries-I (Thermo-Fisher Scientific, Germany). For metals, 10 ml of canal water was acidified with 1% nitric acid (Suprapur, Merck, Germany) to pH ~2 prior to analysis.

### Biofilm sampling

At each site, three biofilms were scraped from the surroundings and collected in sterile 50 ml centrifuge tubes. Scraping was done using a disposable metal razor that was disinfected using 70% ethanol and air-dried before scraping. For each biofilm subsample, a new razor was used to prevent cross-contamination. Accessibility to the canal wall prevented sampling in some locations, so another static object with visible biofilm growth was sampled instead like a wooden pole or floating pontoons in a harbour. For comparison to the water column, water samples were taken in autoclaved glass bottles by rinsing them with canal water three times and sealing the bottles under water.

### Microcosm CH<sub>4</sub> oxidation incubations

Oxidation rates of CH<sub>4</sub> were determined in triplicate per biofilm subsample and were started within 24 h after sampling. Biofilms were distributed equally in autoclaved 120 ml glass serum bottles. Forty millilitre AMS medium (0.5 g l<sup>-1</sup> NH<sub>4</sub>Cl, 1 g l<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g l<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.272 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, and 0.717 g l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O; made in Milli-Q water and autoclaved before adding the phosphate buffer; Whittenbury et al. 1970) supplemented with SL-10 trace elements (German Collection of Microorganisms and Cell Cultures) was used for all biofilm incubations. Additionally, to supply XoxF-type methanol dehydrogenases with lanthanide metals (Keltjens et al. 2014), LaCl<sub>3</sub> was added to a final concentration of 2 µM. Bottles were capped with grey rubber stoppers and sealed with aluminium crimp caps. While these grey rubber stoppers are less thick than red butyl rubber stoppers, they do not have the potential to inhibit pmoA-based methanotrophy (Niemann et al. 2015). For the canal water incubations, 40 ml of the canal water was used per replicate without any amendments and capped in the same way as the biofilm incubations. Abiotic controls of each biofilm subsample were measured in duplicate by autoclaving the sealed bottles.

A volume of 5 ml of CH<sub>4</sub> was added to each bottle, after which the bottles were brought up to an overpressure of 0.25 bar by injecting lab air through a 0.22 µm cellulose acetate membrane filters, amounting to a final headspace CH<sub>4</sub> concentration of 5%. Pressures were checked using a digital pressure meter (GMH 3111, GHM Messtechnik GmbH, Germany). Prior to the first measurement, bottles were left to equilibrate for 3 h at room temperature (21°C). Headspace CH<sub>4</sub> concentrations were measured daily for at least 2 weeks using 50 µl injections on an HP 5890 Series II (Agilent Technologies, USA) gas chromatograph equipped with a Porapak Q column (80/100 mesh) and a flame ionization detector. Headspace pressures were measured after each measurement day. Using a calibration curve, headspace CH<sub>4</sub> concentrations were calculated and adjusted using the measured pressures. Oxidation rates were calculated as the slope of a linear regression fitted to data points of the first eight days of incubation. Within this timeframe, biofilm incubations could be considered linear (median R<sup>2</sup> of 0.9). For comparison, biofilms were normalized to gram dry weight (g<sub>DW</sub>) by drying out 10 ml of well-mixed biofilm incubation medium in an 80°C stove.

To determine whether a CH<sub>4</sub> oxidation rate was due to biological activity or due to other physical effects, all rates were

**Table 1.** Sample locations within the cities.

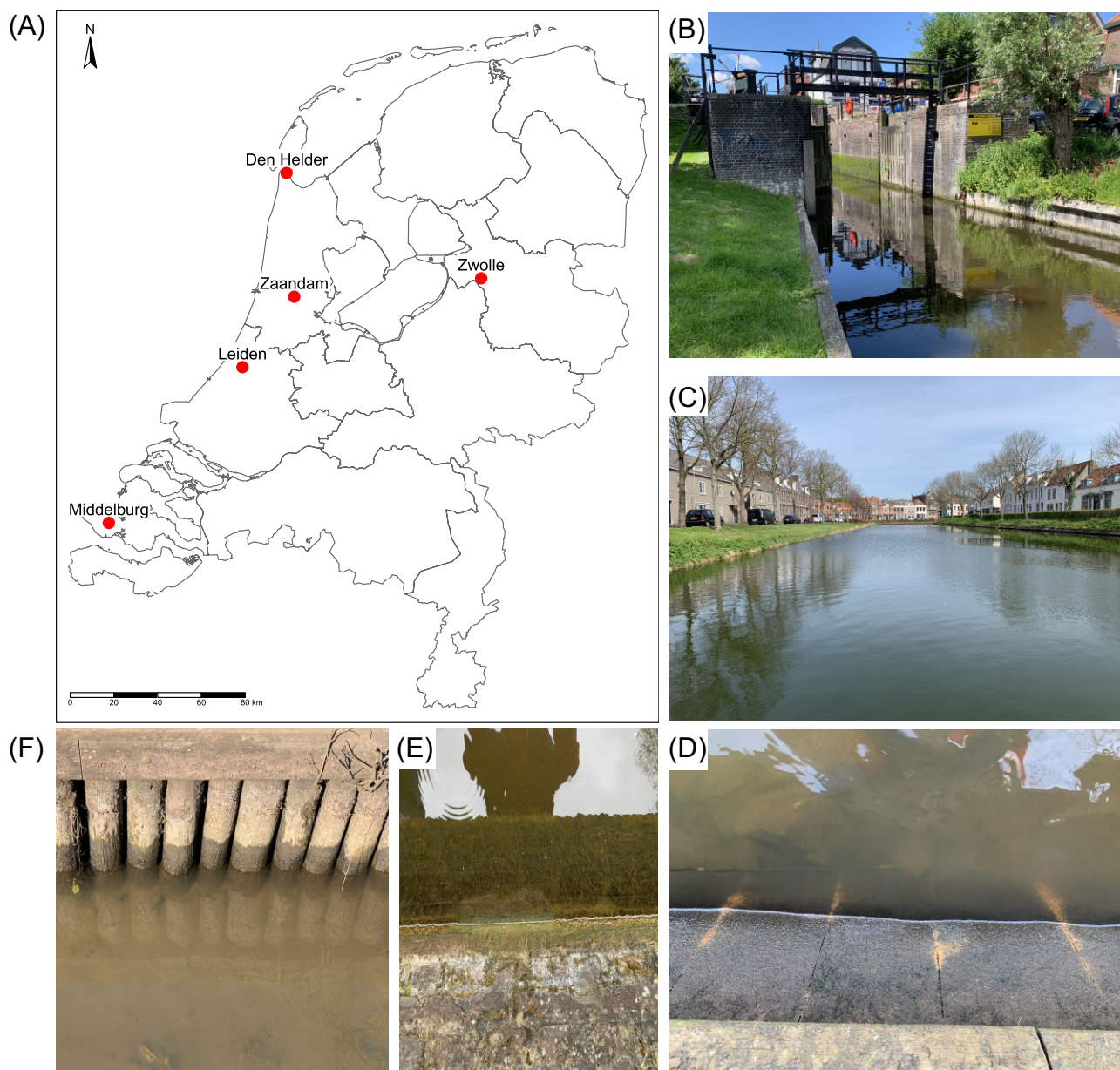
City	Site	Date	Coordinates	Water temperature [°C]	Salinity [µS/cm]	pH	Site description
Zwolle	1	08/03/2022	52°30'57.2' 6°05'04.9"E	6.3	684	7.36	Shallow broad canal next to a main motorway (A28)
	2	08/03/2022	52°30'51.7'N 6°05'26.4"E	6.8	443	7.93	Main urban canal in the city (Thorbeckegracht)
	3	08/03/2022	52°30'57.6'N 6°05'34.0"E	6.1	437	7.81	A northern canal that used to be part of a defensive moat, now used as a small harbour
	4	08/03/2022	52°30'34.1'N 6°05'39.8"E	7.1	440	7.62	Southern part of the defensive moat, flanked by a busy road to the south and a park
Middelburg	1	11/04/2022	51°29'44.7'N 3°36'33.9"E	11	20 640	8.64	A southern stretch of the Binnengracht, the city's main canal
	2	11/04/2022	51°30'01.0'N 3°36'30.8"E	11.1	21 170	8.68	A western site of the Binnengracht
	3	11/04/2022	51°30'09.9'N 3°36'38.4"E	12.7	21 460	8.89	The most northern part of the Binnengracht. Here the canal ends
	4	11/04/2022	51°30'03.5'N 3°37'19.7"E	11.5	21 550	8.77	The main harbour of Middelburg, northern part
	5	11/04/2022	51°29'58.9'N 3°37'20.7"E	10.6	22 490	8.59	The main harbour of Middelburg, southern part
	6	11/04/2022	51°30'11.7'N 3°37'34.0"E	12.3	8500	8.54	A suburban ditch that enters the city from the northeast
Leiden	1	25/04/2022	52°09'23.3'N 4°29'10.4"E	12.8	780	8.02	One of the main canals (Rapenburg)
	2	25/04/2022	52°09'45.6'N 4°29'17.4"E	14	770	7.99	A northern central canal (Oude Vest)
	3	25/04/2022	52°09'36.5'N 4°29'45.0"E	12.5	776	7.85	A central canal that used to be the main river supply (Oude Rijn)
	4	25/04/2022	52°09'27.9'N 4°29'31.6"E	14.1	793	7.91	A central canal, next to the city hall (Nieuwe Rijn)
	5	25/04/2022	52°09'21.7'N 4°30'17.8"E	13.2	742	7.76	A canal just east of the main city centre (Rijnkade)
Den Helder	1	31/05/2022	52°57'31.4'N 4°44'52.5"E	18	2960	7.89	A suburban canal, flanked by a grassy shoreline
	2	31/05/2022	52°57'44.7'N 4°45'12.8"E	16.9	3020	8.01	A northern section of the main canal (Kerkgracht)
	3	31/05/2022	52°57'04.7'N 4°46'46.5"E	18	2310	7.94	A southwestern canal now used for houseboats and small quays (Bassingracht)
	4	31/05/2022	52°57'40.1'N 4°46'05.0"E	17.2	2610	7.74	A canal in front of the Dutch maritime museum
Zaandam	1	04/07/2022	52°25'58.4'N 4°50'01.7"E	20.6	9910	7.54	A canal that connects to Zijnkanaal G, in front of the sluice gates (Hanenpadsluis)
	2	04/07/2022	52°25'59.5'N 4°50'04.6"E	20.1	3760	7.51	A canal that connects to Zijnkanaal G, behind the sluice gates (Hanenpadsluis). Here the sluice gates opened and an ebullition event occurred
	3	04/07/2022	52°26'11.3'N 4°49'02.3"E	21.8	1889	7.8	A canal in the west of the city centre in a residential area
	4	04/07/2022	52°26'20.7'N 4°49'10.4"E	21.8	2260	7.57	A central canal in the main shopping area of the city (Gedempte Gracht)

statistically compared to the abiotic control using a Brunner-Munzel test. Only when the median oxidation rate of the biofilms was statistically significant from the abiotic controls ( $P < .05$ ) did we report that a canal wall biofilm can oxidize  $\text{CH}_4$ .

### DNA extraction and 16S rRNA gene amplicon sequencing

Biofilm DNA was extracted using the DNeasy PowerSoil DNA extraction kit (Qiagen, The Netherlands) by weighing ~300 mg in a

PowerBead tube. The manufacturer's instructions were followed except for the homogenization step as this was done using a TissueLyser LT (Qiagen) at 50 Hz for 10 min. DNA was eluted in diethyl pyrocarbonate-treated water (Invitrogen, USA). Eluted DNA was stored at  $-20^\circ\text{C}$  until sequencing. Due to difficulties during DNA extraction, biofilms from Leiden were not sequenced, and 16S rRNA gene amplicon sequencing was done by MacroGen (MacroGen, Inc., Korea) using the Illumina MiSeq, next-generation sequencing platform. Paired-end libraries were constructed using



**Figure 1.** Location of the five sampled cities within the Netherlands (A). Examples of canals in Zaandam (B) and Middelburg (C). Sampled biofilms in Zwolle (F and D) and Leiden (E) showed that there is great diversity in the types of canals and biofilm growth surfaces. An interactive version of a sample site map is available at [https://methanecanals.shinyapps.io/data\\_maps/](https://methanecanals.shinyapps.io/data_maps/).

the Illumina Hercules II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina, The Netherlands). Primers used for bacterial amplification were Bac341F (5'-CCTACGGGNGGCWGCAG-3'; Herlemann et al. 2011) and Bac806R (5'-GGACTACHVGGGTWTCTAAT-3'; Caporaso et al. 2012). Raw sequencing reads were deposited in the European Nucleotide Archive at the European Bioinformatics Institute under project number PRJEB62150 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB62150>).

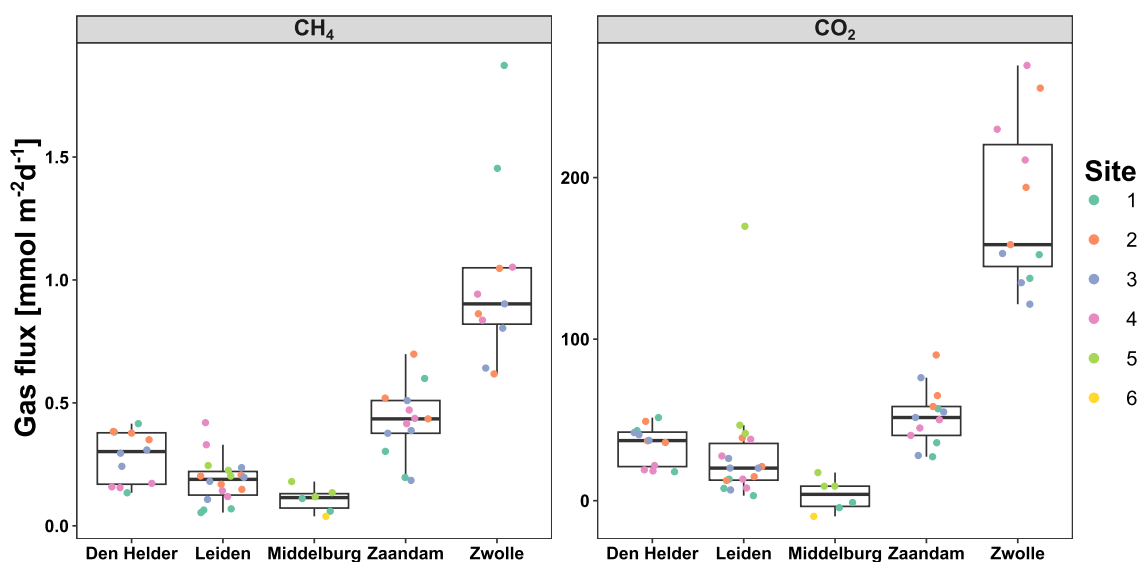
### Sequencing data analysis

Raw sequencing reads were filtered and called to amplicon sequence variants (ASVs) using the package *dada2* (Callahan et al. 2016; v1.26) in R4.1.2 (R Core Team 2022). ASVs were assigned taxonomy based on the SILVA SSU 138.1 database (Quast et al. 2012). After trimming, denoising, dereplication, and chimera removal, each sequencing sample had a minimum 24 103 merged reads

with a maximum of 67 592. Biofilm raw reads from the Amsterdam 2019 dataset (Pelsma et al. 2022; PRJEB40426) were re-analysed with the updated packages and added to the analysis. Using the *phyloseq* package (McMurdie and Holmes 2013, v1.42.0), sequence abundance tables were converted to R dataframes. Graphs were constructed using packages available in the package library *tidyverse* (Wickham et al. 2019).

### Statistical analyses

Comparison of  $\text{CH}_4$  oxidation rates to the abiotic control was done using the permuted non-parametric Brunner–Munzel test available in the R package *brunnermunzel* (Neubert and Brunner 2007, Ara 2022). The test was single tailed with a significance of 5% ( $\alpha = 0.05$ ). Comparison of the biofilm surface type and oxidation potential was done using the Mann–Whitney *U* test through the function *wilcox\_test* available in the R package *rstatix*



**Figure 2.** Diffusive flux measurements for CH<sub>4</sub> (left panel) and CO<sub>2</sub> (right panel) for the five investigated cities. All individual data points are shown and coloured by sample sites within the city. Note the different y-axes for each gas.

(Kassambara 2023). Correlation analyses on environmental variables were done using Kendall's  $\tau$  coefficient. Ordination using non-metric multidimensional scaling (NMDS) was done using the function *metaMDS* from the package *vegan* (Oksanen et al. 2022, v2.6.4). Environmental parameters were tested for significance and fitted to the ordination using *envfit*. All environmental parameters shown were statistically significant after testing with 999 permutations ( $p < .001$ ).

## Results

### Physico-chemical properties of several Dutch urban canals

Five cities with urban canals were sampled to determine the potential of the canal wall biofilm to consume CH<sub>4</sub>. The canals showed large differences in salinity, with Middelburg canals being highly saline, while Leiden and Zwolle had freshwater canals. Zaandam and Den Helder were in between freshwater and brackish canals (Table S1). The canal water temperature increased from Zwolle (6°C) to Zaandam (20°C) because of the time of year during sampling.

Nutrient concentrations differed between cities, with freshwater canals in Zwolle, Den Helder, and Leiden containing increased levels of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> up to 90 and 25  $\mu\text{mol l}^{-1}$ , respectively. In contrast, the saline canals of Middelburg contained little NO<sub>3</sub><sup>-</sup> (0.13–3.53  $\mu\text{mol l}^{-1}$ ) and NH<sub>4</sub><sup>+</sup> (~3.5  $\mu\text{mol l}^{-1}$ ). The highest concentrations of PO<sub>4</sub><sup>3-</sup> were measured for Den Helder and Zaandam (5.4–29.7  $\mu\text{mol l}^{-1}$ ), whereas in the other cities, concentrations were below 1  $\mu\text{mol l}^{-1}$ . Total organic carbon values ranged from 5.7 to 15.5 mg l<sup>-1</sup> (Table S1), indicating increased organic carbon loading in the canals, especially in the city of Leiden. The highest iron and cerium concentrations were measured in Zwolle, specifically at site 1 with the lowest pH of 7.36, at 2.98 mg l<sup>-1</sup>. Copper concentrations were highest in Middelburg (saline) with 6.93  $\mu\text{g l}^{-1}$  and Leiden (freshwater) with 9.37  $\mu\text{g l}^{-1}$ . Dissolved oxygen was always above 50% air saturation, indicating that anoxia was not occurring in the water columns at the sampling sites.

Diffusive flux measurements of CH<sub>4</sub> and CO<sub>2</sub> showed that every city except Middelburg could be considered a consistent source of CH<sub>4</sub>. For Zaandam and Zwolle, the observed CH<sub>4</sub> flux

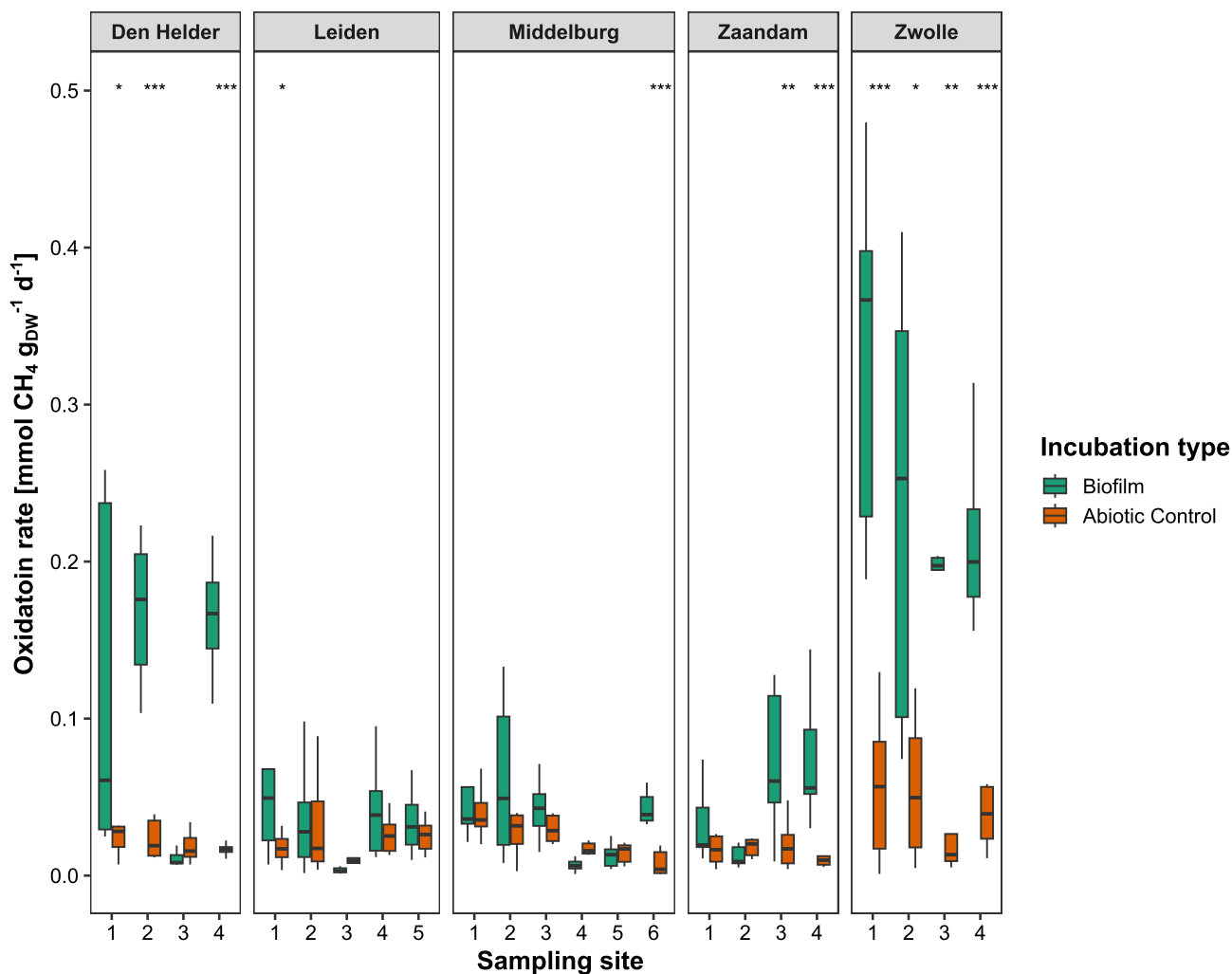
was highest at 1.8 and 0.69 mmol m<sup>-2</sup> d<sup>-1</sup>, respectively (Fig. 2). Both Zwolle's and Zaandam's canals were a source of CO<sub>2</sub> as well at median flux of 158 and 51 mmol m<sup>-2</sup> d<sup>-1</sup>, respectively. For the saline canals of Middelburg, CH<sub>4</sub> emissions were only detectable for site 5 (0.14 mmol m<sup>-2</sup> d<sup>-1</sup>, three replicate measurements), whereas the CO<sub>2</sub> flux was mostly negative except for site 5 (average of 11.79 mmol m<sup>-2</sup> d<sup>-1</sup>). Despite high nitrate and TOC, the canals in Leiden did not emit CO<sub>2</sub> and median CH<sub>4</sub> emissions were only 0.18 mmol m<sup>-2</sup> d<sup>-1</sup>. Canals in Den Helder, like Zaandam, were a net source of both CH<sub>4</sub> and CO<sub>2</sub> but with some spatial variability within the city.

### CH<sub>4</sub> oxidation on the canal wall is driven by *Methylomonadaceae*

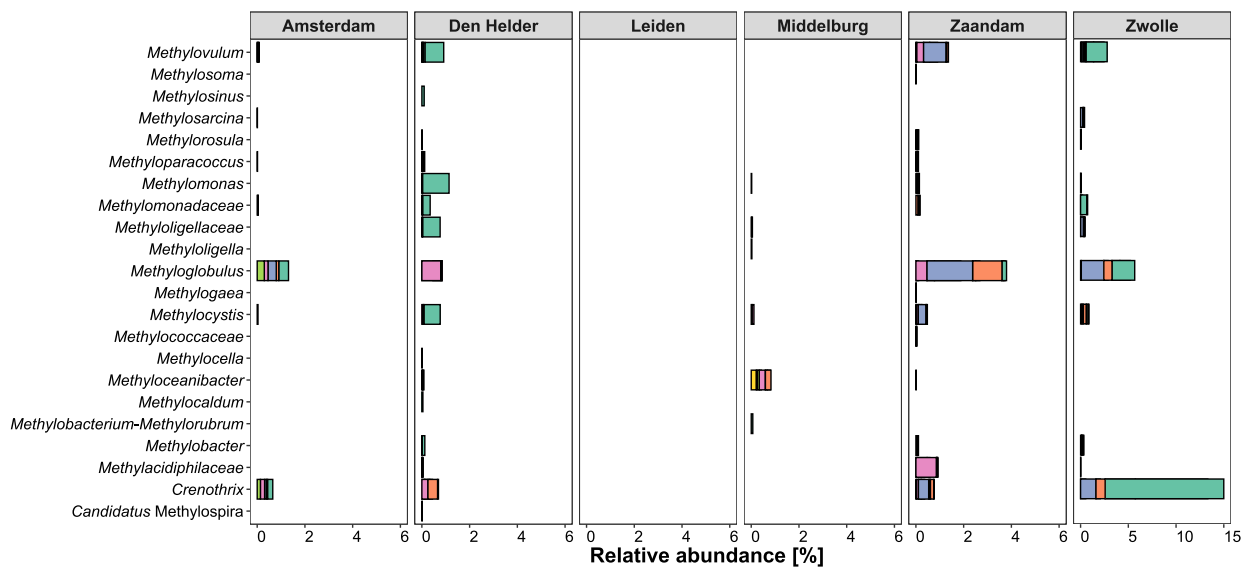
Den Helder, Zaandam, and Zwolle hosted canal wall biofilms with clear biological CH<sub>4</sub> removal (Fig. 3). However, several biofilms, for example, sites 1–5 in Middelburg and sites 2–5 in Leiden, showed CH<sub>4</sub> oxidation rates of <0.1 mmol g<sub>DW</sub><sup>-1</sup> d<sup>-1</sup>, which were not significantly different from the control incubations. The highest observed CH<sub>4</sub> oxidation rates were 0.48 mmol g<sub>DW</sub><sup>-1</sup> d<sup>-1</sup> in Zwolle (March), 0.31 mmol g<sub>DW</sub><sup>-1</sup> d<sup>-1</sup> in Den Helder (May), and 0.12 mmol g<sub>DW</sub><sup>-1</sup> d<sup>-1</sup> in Zaandam (July). The variability between biofilm subsamples was high as indicated by the large interquartile range (0.01–0.35 mmol g<sub>DW</sub><sup>-1</sup> d<sup>-1</sup> in Zwolle) for the biofilm incubations (Fig. 3). Water column methanotrophy was observed in all cities except Zwolle but with low statistical significance (Fig. S1).

Biofilm bacterial community profiling showed a dominance of the gammaproteobacterial methanotrophic species *Crenothrix* and *Methyloglobulus* (Fig. 4). Most sampled canal walls were made of either wood or brick. Depending on the city, methanotrophs were more abundant on brick than on wood, but no clear pattern was observed between abundance and canal wall material. In Middelburg, no methanotrophy was observed and the sequencing results indeed showed no reads classified as either *Crenothrix* or *Methyloglobulus*. In Zaandam, these species were observed but only at a relative abundance of 2%, which corresponds quite well to the lower CH<sub>4</sub> oxidation rates (Fig. 3).

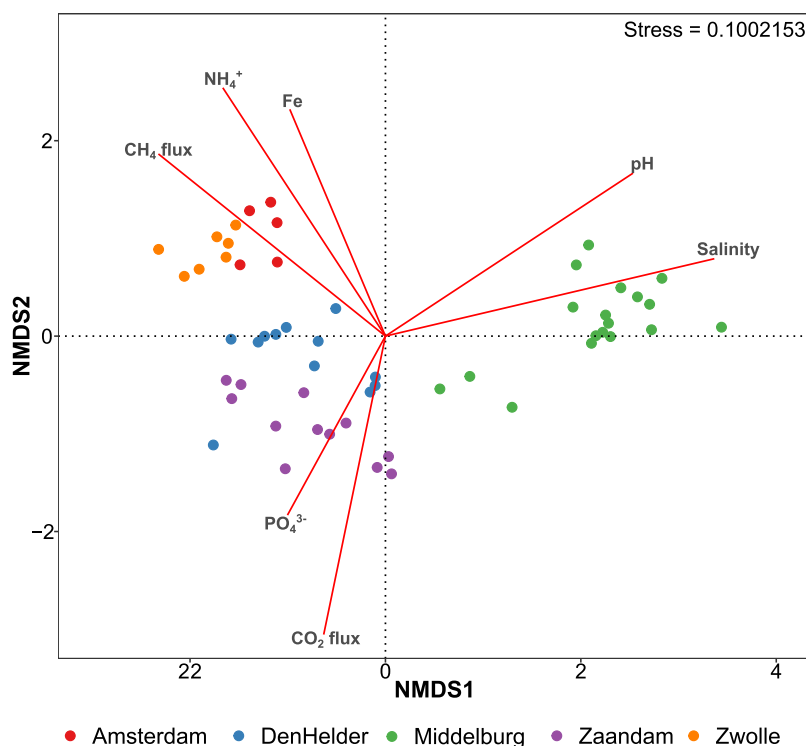
Analysis of the total bacterial community revealed a strong pattern based on salinity and nutrient levels (Fig. 5). High NH<sub>4</sub><sup>+</sup> and Fe concentrations in Zwolle clustered it together with



**Figure 3.** Biofilm methanotrophic rates for each sampling site, ordered by city. Comparison against the abiotic control was done using a Brunner–Munzel test. Per site, nine biofilm incubations were compared against six control incubations. Biofilm incubations with statistically significant differences from the control are marked by an asterisk. \* $p < .05$ , \*\* $p < .01$ , and \*\*\* $p < .001$ .



**Figure 4.** Relative abundances of total bacterial 16S rRNA gene reads of known methanotrophic genera for six sequenced cities. Bars are coloured by sampling site within each city. Leiden biofilms were not sequenced due to DNA extraction difficulties, which is indicated by a blank column. The Amsterdam dataset was obtained from a previous study (Pelsma et al. 2022). Note the different scales for the Zwolle samples.



**Figure 5.** Ordination of the total bacterial community based on 16S rRNA gene amplicon sequencing with Bray–Curtis dissimilarity as distance metric. Environmental variables are scaled based on their effect.

Amsterdam (data from Pelsma et al. 2022), while Den Helder and Zaandam also grouped together, separately. Despite differences in  $\text{NO}_3^-$  concentrations, it did not explain the differences in biofilm community composition as significantly as  $\text{CH}_4$  emissions and  $\text{PO}_4^{3-}$ . The single brackish site in Middelburg formed a grouping closer to Den Helder and Zaandam, suggesting a stronger influence of salinity than nutrient levels on the biofilm microbial community.

## Discussion

### Regulators of $\text{CH}_4$ emissions

The potential of urban canals to emit significant amounts of  $\text{CH}_4$  has been well documented in recent years (Stanley et al. 2016, Peacock et al. 2021, Rosentreter et al. 2021, Wang et al. 2021) and the results obtained here support this. Out of the five sampled cities, only the saline canals in Middelburg were not a source of  $\text{CH}_4$ . As the canals of Middelburg contained the least amount of dissolved nutrients and were fully oxygenated, they can be considered a marine system. However, during the fieldwork, we observed turbid water with a lot of algal growth in these canals. Due to the time of year we sampled in Middelburg (early April), algal death in late summer could contribute to  $\text{CH}_4$  production and emission in other seasons.

There was some spatial separation of  $\text{CH}_4$  fluxes within the Zwolle city centre, with site 1 having the highest  $\text{CH}_4$  emissions (Fig. 2). This site was characterized by a shallow waterway, so the increase in emissions could be explained by a more turbulent water column that resuspends sediment, likely also causing high dissolved Fe concentrations at this site. However, the other sites were subject to boating, leading to similar resuspension of canal sediment. The  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations measured in these canals could contribute to eutrophication of the canal sys-

tem and subsequent increases in sediment methanogenesis. With our sampling date for Zwolle in early spring, these canals could have higher  $\text{CH}_4$  emissions in late summer not captured by our study. The other freshwater city, Leiden, had substantially lower  $\text{NH}_4^+$  concentrations and  $\text{CH}_4$  emissions (Fig. 2, Table S1), but higher  $\text{PO}_4^{3-}$  concentrations. The sediment floor of these canals was dominated by plants, which could help in aerating the sediment and thereby decreasing methanogenesis in the sediment (Bastviken et al. 2023).

### Biofilm methanotrophy is present in diverse urban canals

The city of Zwolle harboured biofilms with up to 8% methanotrophs, and biological  $\text{CH}_4$  consumption was found at all sites. Compared to the Amsterdam data, we detected similar species but at higher relative abundances and oxidation rates. The absence of methanotrophs from all sampled biofilms in Middelburg in April is consistent with its very low  $\text{CH}_4$  oxidation potential. However, the incubation medium used in this study was not optimized for saline conditions and could have affected the observed methanotrophic rate. Similarly, it has been documented that in the nearby Lake Grevelingen, the relative abundance of methanotrophs and  $\text{CH}_4$  oxidation potential are also lowest around the end of March (Egger et al. 2016, Venetz et al. 2022, Żygadłowska et al. 2023). Only one sampled biofilm from Leiden consumed  $\text{CH}_4$ , suggesting it is unlikely that Leiden's biofilms host methanotrophs.

Biofilm incubations from Zaandam showed that both sites 1 and 2 did not consume  $\text{CH}_4$  and only site 2 harboured *Methylobionadaceae* methanotrophs. These sites are spatially separated from each other by one sluice gate (pictured in Fig. 1b). During the fieldwork, the sluice gate was opened causing a  $\text{CH}_4$  ebullition event. If most  $\text{CH}_4$  is emitted through bubbles, MOB do not have a

constant supply of substrate and could be outcompeted by other bacteria in the biofilm. For the other two sites in Zaandam, no ebullition was observed but biofilms did oxidize CH<sub>4</sub> and harboured methanotrophs, meaning a consistent, if lower, CH<sub>4</sub> supply is sufficient to sustain a biofilm community with methanotrophic potential. Den Helder was very similar to Zaandam in terms of water chemistry but had much higher oxidation rates. However, for Den Helder site 1, only one subsample had a relative abundance of 9% *Methylomonadaceae*, corresponding to the highest rate. For site 3, no CH<sub>4</sub> oxidation rate was measured and no MOB could be detected with sequencing (Fig. 4). This site was a small domestic harbour very similar to the other four sites. Therefore, it is evident that within a city canal network, there are local effects of which our study could only capture the heterogeneity to a certain extent. Future research is advised to take spatial heterogeneity into account during study design, as day-night cycles, point sources, and local infrastructure (like sluice gates) can have great effects on both the microbial community and CH<sub>4</sub> dynamics (Attermeyer et al. 2021, Stanley et al. 2022).

## Conclusions

Our data indicate the presence of biofilm methanotrophy in several distinct urban canal networks across the Netherlands. The two dominant methanotrophs were *Crenothrix* and *Methyloglobulus*, in line with what was previously found in canal biofilms in Amsterdam. The presence of these methanotrophs was associated with consistent diffusive CH<sub>4</sub> emissions. Their presence could be a way to identify canals with high CH<sub>4</sub>-cycling activity. CH<sub>4</sub> emissions were observed from most canals and could contribute substantially to total urban CH<sub>4</sub> emissions. NH<sub>4</sub><sup>+</sup> concentration was positively correlated with CH<sub>4</sub> emissions and CH<sub>4</sub> oxidation rate, while salinity correlated negatively. We showed that canal wall biofilms may assist in mitigating CH<sub>4</sub> emissions across a diverse range of Dutch cities. A widespread, year-round monitoring of CH<sub>4</sub> emissions (diffusive and ebullitive) from urban canals, in conjunction with microbial activity experiments, is required for a better understanding of these ubiquitous and unique Dutch landmarks.

## Author contributions

Koen A.J. Pelsma (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Writing – original draft, Writing – review & editing), Daniël Verhagen (Investigation), Joshua F. Dean (Conceptualization, Investigation, Methodology, Writing – review & editing), Mike S.M. Jetten (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing), and Cornelia U. Welte (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing)

## Acknowledgements

We would like to thank Sebastian Krosse and Paul van der Ven (Radboud University) for their help with the analysis of nutrients and metals. We thank Gerben Wulterkens (Radboud University) for the design and construction of the floating chamber.

## Supplementary data

Supplementary data are available at [FEMSEC Journal](#) online.

Conflict of interest: None declared

## Funding

This research was supported by the Dutch Organisation for Scientific Research and the Ministry of Education, Culture and Science through the gravitation programmes NESSC [024002001] and SIAM [024002002], and through the ERC Synergy grant MARIX [854088]. J.F.D. received additional support from a UKRI Future Leaders Fellowship MR/V025082/1.

## References

- Ara T. *Brunnermunzel*: (permuted) Brunner–Munzel Test. R package version 2.0. 2022.
- Armstrong M, Aksu Bahçeci H, van Donk E et al. Making waves: lessons learned from the COVID-19 anthropause in the Netherlands on urban aquatic ecosystem services provisioning and management. *Water Res* 2022;**223**:118934.
- Attermeyer K, Casas-Ruiz JP, Fuss T et al. Carbon dioxide fluxes increase from day to night across European streams. *Commun Earth Environ* 2021;**2**:118.
- Bastviken D, Tranvik LJ, Downing JA et al. Freshwater methane emissions offset the continental carbon sink. *Science* 2011;**331**:50.
- Bastviken D, Treat CC, Pangala SR et al. The importance of plants for methane emission at the ecosystem scale. *Aquat Bot* 2023;**184**:103596.
- Burrows RM, van de Kamp J, Bodrossy L et al. Methanotroph community structure and processes in an inland river affected by natural gas macro-seeps. *FEMS Microbiol Ecol* 2021;**97**:fiab130. <https://doi.org/10.1093/femsec/fiab130>
- Callahan BJ, McMurdie PJ, Rosen MJ et al. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;**13**:581–3.
- Caporaso JG, Lauber CL, Walters WA et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 2012;**6**:1621–4.
- Carini S, Bano N, LeCleir G et al. Aerobic methane oxidation and methanotroph community composition during seasonal stratification in Mono Lake, California (USA). *Environ Microbiol* 2005;**7**:1127–38.
- Conrad R. The global methane cycle: recent advances in understanding the microbial processes involved. *Environ Microbiol Rep* 2009;**1**:285–92.
- Dean JF, Middelburg JJ, Röckmann T et al. Methane feedbacks to the global climate system in a warmer world. *Rev Geophys* 2018;**56**:207–50.
- Deutzmann JS, Hoppert M, Schink B. Characterization and phylogeny of a novel methanotroph, *Methyloglobulus morosus* gen. nov., spec. nov. *Syst Appl Microbiol* 2014;**37**:165–9.
- Egger M, Lenstra W, Jong D et al. Rapid sediment accumulation results in high methane effluxes from coastal sediments. *PLoS One* 2016;**11**:1–22.
- Ettwig KF, Butler MK, Le Paslier D et al. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* 2010;**464**:543–8.
- Ettwig KF, Zhu B, Speth D et al. Archaea catalyze iron-dependent anaerobic oxidation of methane. *Proc Natl Acad Sci* 2016;**113**:12792–6.
- Frenzel P, Thebrath B, Conrad R. Oxidation of methane in the oxic surface layer of a deep lake sediment (Lake Constance). *FEMS Microbiol Ecol* 1990;**6**:149–58.
- Guérin F, Abril G. Significance of pelagic aerobic methane oxidation in the methane and carbon budget of a tropical reservoir. *J*



- Geophys Res Biogeosci* 2007;**112**:G03006. <https://doi.org/10.1029/2006JG000393>
- Hanson RS, Hanson TE. Methanotrophic bacteria. *Microbiol Rev* 1996;**60**:439–71.
- Hao Q, Liu F, Zhang Y et al. Methylobacter accounts for strong aerobic methane oxidation in the yellow river delta with characteristics of a methane sink during the dry season. *Sci Total Environ* 2020;**704**:135383.
- Haroon MF, Hu S, Shi Y et al. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* 2013;**500**:567–70.
- Herlemann DP, Labrenz M, Jürgens K et al. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic sea. *ISME J* 2011;**5**:1571–9.
- Herrero Ortega S, González-Quijano CR, Casper P et al. Methane emissions from contrasting urban freshwaters: rates, drivers, and a whole-city footprint. *Global Change Biol* 2019;**25**:4234–43. <https://doi.org/10.1111/gcb.14799>
- Kassambara A. *rstatix: pipe-friendly framework for basic statistical tests*. R package version 0.7.2. 2023. <https://rpkgs.datanovia.com/rstatix/>
- Keltjens JT, Pol A, Reimann J et al. PQQ-dependent methanol dehydrogenases: rare-earth elements make a difference. *Appl Microbiol Biotechnol* 2014;**98**:6163–83.
- Knief C. Diversity and habitat preferences of cultivated and uncultivated aerobic methanotrophic bacteria evaluated based on pmoA as molecular marker. *Front Microbiol* 2015;**6**:1346.
- Lorke A, Bodmer P, Noss C et al. Technical note: drifting versus anchored flux chambers for measuring greenhouse gas emissions from running waters. *Biogeosciences* 2015;**12**:7013–24.
- McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013;**8**:1–11.
- Martinez-Cruz K, Gonzalez-Valencia R, Sepulveda-Jauregui A et al. Methane emission from aquatic ecosystems of Mexico City. *Aquat Sci* 2017;**79**:159–69.
- Martinez-Cruz K, Sepulveda-Jauregui A, Casper P et al. Ubiquitous and significant anaerobic oxidation of methane in freshwater lake sediments. *Water Res* 2018;**144**:332–40.
- Matoušů A, Osudar R, Šimek K et al. Methane distribution and methane oxidation in the water column of the Elbe estuary, Germany. *Aquat Sci* 2017;**79**:443–58.
- Myhre G, Shindell D, Bréon FM et al. Anthropogenic and natural radiative forcing. In Stocker T, Qin D, Plattner GK et al. (eds.), *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge: Cambridge University Press, 2013.
- Needelman BA, Kleinman PJA, Strock JS et al. Drainage ditches. *J Soil Water Conserv* 2007;**62**:171–8.
- Neubert K, Brunner E. A studentized permutation test for the non-parametric Behrens–Fisher problem. *Comput Stat Data Anal* 2007;**51**:5192–204.
- Niemann H, Steinle L, Bles J et al. Toxic effects of lab-grade butyl rubber stoppers on aerobic methane oxidation. *Limnol Oceanogr*: Methods 2015;**13**:40–52. <https://doi.org/10.1002/lom3.10005>
- Oksanen J, Simpson GL, Blanchet FG et al. *vegan: community ecology package*. R package version 2.6-4. 2022.
- Peacock M, Audet J, Bastviken D et al. Global importance of methane emissions from drainage ditches and canals. *Environ Res Lett* 2021;**16**:044010.
- Pelsma KAJ, in 't Zandt MH, Op den Camp HJM et al. Amsterdam urban canals contain novel niches for methane-cycling microorganisms. *Environ Microbiol* 2022;**24**:82–97.
- Quast C, Pruesse E, Yilmaz P et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2012;**41**:D590–6.
- R Core Team. *R: a Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing, 2022. <https://www.R-project.org/>
- Reis PCJ, Thottathil SD, Ruiz-González C et al. Niche separation within aerobic methanotrophic bacteria across lakes and its link to methane oxidation rates. *Environ Microbiol* 2020;**22**:738–51.
- Rosentreter JA, Borges AV, Deemer BR et al. Half of global methane emissions come from highly variable aquatic ecosystem sources. *Nat Geosci* 2021;**14**:225–30.
- Saunois M, Stavert AR, Poulter B et al. The global methane budget 2000–2017. *Earth Syst Sci Data* 2020;**12**:1561–623.
- Shen L, Ouyang L, Zhu Y et al. Active pathways of anaerobic methane oxidation across contrasting riverbeds. *ISME J* 2019;**13**:752.
- Söhngen N. *Het ontstaan van Waterstof onder den invloed van het organische leven*. Ph.D. Thesis, Technical University Delft, 1906.
- Stanley EH, Casson NJ, Christel ST et al. The ecology of methane in streams and rivers: patterns, controls, and global significance. *Ecol Monogr* 2016;**86**:146–71.
- Stanley EH, Loken LC, Casson NJ et al. Grimedb: the global river database of methane concentrations and fluxes. *Earth Syst Sci Data Discuss* 2022;**2022**:1–94.
- Teurlincx S, Kuiper JJ, Hoevenaer EC et al. Towards restoring urban waters: understanding the main pressures. *Curr Opin Environ Sustain* 2019;**36**:49–58.
- Thottathil SD, Reis PCJ, Prairie YT. Methane oxidation kinetics in northern freshwater lakes. *Biogeochemistry* 2019;**143**:105–16.
- Valenzuela EI, Avendaño KA, Balagurusamy N et al. Electron shuttling mediated by humic substances fuels anaerobic methane oxidation and carbon burial in wetland sediments. *Sci Total Environ* 2019;**650**:2674–84.
- van Bergen TJHM, Barros N, Mendonça R et al. Seasonal and diel variation in greenhouse gas emissions from an urban pond and its major drivers. *Limnol Oceanogr* 2019;**64**:2129–39. <https://doi.org/10.1002/lno.11173>
- Venez J, Żygadłowska OM, Lenstra WK et al. Versatile methanotrophs form an active methane biofilter in the oxycline of a seasonally stratified coastal basin. *Environ Microbiol* 2023;1–12. <https://doi.org/10.1111/1462-2920.16448>
- Wang G, Xia X, Liu S et al. Intense methane ebullition from urban inland waters and its significant contribution to greenhouse gas emissions. *Water Res* 2021;**189**:116654.
- Whittenbury R, Phillips KC, Wilkinson JF. Enrichment, isolation and some properties of methane-utilizing bacteria. *Microbiology* 1970;**61**:205–18.
- Wickham H, Averick M, Bryan J et al. Welcome to the tidyverse. *J Open Source Softw* 2019;**4**:1686.
- Zaiss U, Winter P, Kaltwasser H. Microbial methane oxidation in the River Saar. *Z Allg Mikrobiol* 1982;**22**:139–48.
- Żygadłowska OM, Venez J, Klomp R et al. Pathways of methane removal in the sediment and water column of a seasonally anoxic eutrophic marine basin. *Front Mar Sci* 2023;**10**:1085728.