Microbial megacities fueled by methane oxidation in a mineral spring cave

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Supplementary Materials and Methods

Water sample analysis

Water for analysis of dissolved organic carbon (DOC) and ion chromatography was filtered through 0.45 μm syringe driven filter units (Merck Millipore, Germany) and stored at 4° C in sterile, pretreated (soaked overnight in bidest water) glassware containing no residual carbon, or collected in polystyrene vials for ion chromatography (IC).

DOC samples that were not analyzed immediately were acidified using 1M HCl (< pH 2). DOC was analyzed using high temperature combustion with infrared detection of CO_2 on a TOC-V (Shimadzu, Japan) with an ASI-V autosampler having a detection limit of 0.1 mg L^{-1}. Major cations (calcium, magnesium, potassium, ammonium, sodium) and anions (nitrite, nitrate, chloride, bromide, sulfate) were measured on a DX-100 (Dionex, USA) ion chromatograph equipped with a CS12 and AS14A Ion Pac columns (Faye *et al.,* 2005, Stoewer *et al.,* 2015) equipped with an AS40 auto-sampler.

Gas sample analysis

Methane concentrations were determined on a gas chromatograph (SRI Instruments, USA) equipped with a helium ionization detector and a thermal conductivity detector. A 250 µl gas sample was manually injected into a HayeSep D column (80-100 mesh, 6 m 1/8", SRI Instruments, USA) which was kept at 50 °C. Measuring time was 4 min, which allowed the recording of nitrogen, methane and carbon dioxide. For quantification, a 5-point standard curve (100000 – 1000 ppm) was generated (R^2 = 0.98, n = 3) using mixtures of N_2, CH_4, and CO_2 (all gases Linde, Germany).

Compound specific isotope ratios were measured using a TRACE GC Ultra (Thermo Fisher Scientific; Italy), coupled to a Finnigan MAT 253 IRMS (Thermo Fisher Scientific, Germany) connected by a Finnigan GC Combustion III Interface as previously described (Bergmann *et al.,*

2011). The combustion oven temperature was 980 °C for carbon isotope analysis, while for hydrogen isotopes a pyrolytic interface was used (1390 °C). The GC was equipped with a programmable temperature vaporizer (PTV) injector (Optic3, ATAS GL International B.V.; Netherlands) heated to 100 °C. For isotope analysis of methane, the GC was equipped with a RT –QPLOT capillary column (30m x 0.32, Restek, USA) connected to fused-silica pre- and postcolumns (FS-Methyl-Sil, 2 m x 0.32 mm and 1 m x 0.32 mm, respectively; CS Chromatographie Service GmbH, Germany). For carbon analysis, carrier gas flow was set to 1.4 mL min^{-1} and a 100 µl sample was injected for each measurement making three measurements per run (800 s). Gas samples were injected by hand into the heated (100 °C) injection port. Oxidation of carbon was achieved at 980 °C. For hydrogen isotope analysis, the same settings and columns were used but carrier gas flow was set to 1.2 mL min^{-1} and 500 µl was injected for each measurement. The GC-oven was programmed to permanently hold at 40 °C.

Biofilm stable isotopes

Isotope analysis of biofilm samples was carried out on an elemental analyzer (EA; Euro Vector SPA, Italy) coupled with a combustion unit (Hekatech, Germany) connected to a Finnegan Mat 253 isotope-ratio mass spectrometer (IRMS; Thermo Fisher Scientific, Germany) to assess the carbon and nitrogen isotopic signature. Samples were lyophilized by means of freeze-drying and then ground to powder, which was weighted into tin capsules for analysis. The temperature of the oxidation tube in the EA was 1000 °C and the temperature of the reduction tube was 600 °C. The temperature of the combustion tube was 1480 °C. For calibration, three different standards were analyzed in triplicate before and after the analysis of the samples. Standards used for $\delta^{13}C$ and $\delta^{15}N$ calibration were caffeine (IAEA-600) and l- glutamic acid (USGS-40 and USGS-41). In addition, a caffeine standard was analyzed after every seventh sample to calibrate shifts in the measurements.

Elemental composition of biofilms

Freeze-dried sample material was extracted by means of ashing under pressure at 170 °C in a Seif device with HNO_3 (Schramel 2012) respectively with tetramethyl ammonium hydroxide (TMAH) (Schramel and Hasse 1994) for the determination of iodine. Elements in the extracts were determined using inductively coupled plasma optical emission spectrometry (ICP-OES) (Michalke and Witte 2015). Iodine content in the water samples was measured equally by adding TMAH and using ICP-OES or ICP-MS according to ISO 17294.

DNA Extraction

Biofilms were disrupted (Cury and Koo 2007) and DNA was extracted from replicate samples following the routine of Pilloni *et al.,* (2012). Briefly, the frozen biofilms were suspended in 1x PBS buffer and pretreated by repetitive (3x) sonication (35 kHz, Sonorex RK102; Bandelin Electronic GmbH & Co, Germany), shaking and spinning (5500 g for 10 min at 4 °C) to interrupt the extracellular matrix. The cell slurry and filters from the water samples were suspended in 650 µl PTN buffer (120 mM Na_2HPO_4, 125 mM Tris, 0.25 mM NaCl, pH 8) and incubated at 37°C for 15 min with 40 µl lysozyme (50 mg ml^{-1} in TE) and 10 µl proteinase K (10 mg ml^{-1} in TE). After the addition of 150 µl SDS (20% (wt/v) sodium dodecyl sulfate, 1.6 M NaCl), the incubation was continued for 15 min at 65°C with shaking at 500 rpm. The sediments were bead beaten (45 s at 6 ms^{-1} in a FastPrep-24 (MP Biomedicals, USA)) with, 0.2 ml of zirconia-silica beads (1:1 mix of 0.1- and 0.7-mm diameter; Roth, Germany) and 100 µl of phenol- chloroform-isoamyl alcohol (25:24:1) in 2-ml screw-cap vials. This was followed by an additional bead beating step (20 sec 6.5 ms^{-1}). Afterwards nucleic acids were sequentially purified by extraction with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and 1 volume of chloroform-isoamyl alcohol (24:1). The purified nucleic acid was then precipitated with 2 volumes of 30% polyethylene glycol by incubation at 4°C for 12 h and subsequently centrifuged at 20,000 g and 20°C for 30 min. All used chemicals were from Sigma-

Aldrich (USA), if not otherwise stated. For each biological sample, two parallel extractions were pooled and stored at -20°C until further analysis.

Molecular analyses

PCR for 16S rRNA and functional gene amplification was performed in a Mastercycler ep gradient (Eppendorf, Germany). The PCR reactions for 16S rRNA amplification were set up with the following cycling conditions: initial denaturation (94°C, 5 min), followed by 28 cycles of denaturation (94°C, 30 s), annealing (52°C, 30 s) and elongation (70°C, 60 s). Each 50 µl PCR reaction contained 1x PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 1.25 U recombinant Taq polymerase (Fermentas, Germany), 0.2 mg ml^{-1} bovine serum albumin, 0.3 mM of each specific forward and reverse primer (Biomers, Germany) and 1 µl of template DNA. Amplification reactions for the functional marker genes *mxaF* (α subunit of the methanol dehydrogenase), *mmoX* (α subunit of the hydroxylase component of the soluble methane monooxygenase) and *pmoA* (α subunit of the particulate methane monooxygenase) differed in using higher annealing and elongation temperatures (55 and 72 °C , respectively) as well as longer annealing times (*mxaF* and *mmoX*: 60 sec, *pmoA*: 90 sec) as originally recommended (Costello and Lidstrom 1999, Dumont and Murrell 2005, Horz *et al.,* 2001). Products were verified on an agarose gel, and all samples were purified using PCRExcract mini kit columns according to the manufacturers protocol (5prime, Germany).

Community fingerprinting of the 16S rRNA gene and the *pmoA* gene was done via terminal restriction fragment length polymorphism (TRFLP). The respective primers were fluorescently labeled with FAM (Carboxyfluorescein) and purified PCR products (80 ng µl-1) by adding 0.3 µL restriction enzymes (*Msp*I 10 units μL^{-1}) and 1 µL buffer (all Thermo Fisher, USA) and incubating the mixture for 2h at 37 °C. Fragments were subsequently desalted with DyeEx 2.0 Spin Kit columns (QIAGEN, Germany). Aliquots of 1 and 3 µL were combined with a mixture of high definition formamide and 6-carboxy-X-rhodamine-labeled MapMarker 1000 ladder (BioVentures, USA) in a

1:400 dilution. Fragments were denatured at 95 °C for 5 min and stored in the fridge (4 °C) until fragment analysis. Automated fragment electrophoresis was performed at the genome analysis center (Helmholtz Zentrum München), where fragments were separated by capillary electrophoresis on an ABI 3730 DNA analyzer (Applied Biosystems, Germany). Electrophoresis was executed with POP-7 polymer in a 50 cm capillary array under the following conditions: 10 s injection time, 2 kV injection voltage, 7 kV run voltage, 66 °C run temperature and 63 min analysis time.

The electropherograms were then evaluated with the Gene Mapper 5.1 software (Applied Biosystems, Germany) and T-RFLP data was analyzed with the online T-RF analysis software T-REX (Culman *et al.,* 2009). Background noise filtering (Abdo *et al.,* 2006) selected peaks with a height >1 standard deviation of all peaks and the clustering threshold for aligning peaks across the samples was set to 2, binning all peaks within two bp. Relative T-RF abundance was inferred from peak heights. For reduction of data complexity, T-RFs that occurred in less than 5 % of the samples were excluded from further analysis.

Barcoded amplicons were generated for forward and reverse reads. Primers were fused with A or B adapters (for forward and reverse discrimination) and multiplex barcode identifiers (MID). PCR products were purified with the Agencourt AMPure magnetic beads (Beckman Coulter, Germany) as specified by the manufacturers. After the first purification a second purification step was necessary to guarantee the removal of all short fragments. Furthermore, each amplicon was checked for primer dimer contamination and correct fragment size using the Bioanalyzer2100 (Agilent, USA) by loading High Sensitivity DNA assay chips (Agilent, USA), as described by the manufacturer. Amplicons generated with barcoded primers (pyrotag PCR) were quantified by the Quant-iT PicoGreen dsDNA quantification kit (Life Technologies, USA) and pooled in an equimolar ratio of 10^9 molecules ml^{-1}. Emulsion PCR, emulsion breaking and sequencing were performed applying the GS FLX Titanium chemistry following supplier protocols (Roche 2013).

Quantitative PCR was applied for 16S rRNA genes on a Stratagene MX3000P qPCR cycler (Agilent, USA). Gene copy numbers per DNA extract were measured for three dilutions in triplicate. Dilution factors were $1x10^{0}$, $1x10^{-1}$ and $1x10^{-2}$. Quantitative PCR was performed using the PCR settings described above while also adding the fluorescent dyes SybrGreen (0.25 µl 1/500) for DNA detection and ROX (0.75 µl 1/500) (both Life Technologies, USA) for equilibration and adding 2 µl sample instead of 1 µl. Quantitative PCR was performed at an initial denaturation temperature of 94 °C (3 min) followed by 40 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and elongation (70 °C 30 s), and subsequent denaturation (95 °C, 1 min), reassociation (55 °C, 30 s) and a dissociation ramp (55 °C to 95 °C, 30 min). The specificity of the PCR products was verified by melting curve analysis. A full length 16S rRNA of Azoarcus sp. strain T, with a known concentration, was used in a dilution series between $1x10^{7}$ and $1x10^{1}$ molecules per µl as a standard curve to convert measured threshold cycles to rRNA gene copy numbers (Kunapuli *et al.,* 2007).

Supplementary References

Abdo Z, Schuette UME, Bent SJ, Williams CJ, Forney LJ, Joyce P (2006). Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. *Environ Microbiol* **8:** 929-938.

Amann R, Stromley J, Devereux R, Key R, Stahl D (1992). Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl Environ Microbiol* **58:** 614-623.

Bergmann FD, Abu Laban NM, Meyer AH, Elsner M, Meckenstock RU (2011). Dual (C, H) isotope fractionation in anaerobic low molecular weight (poly) aromatic hydrocarbon (PAH) degradation: potential for field studies and mechanistic implications. *Environ Sci Technol* **45:** 6947-6953.

Costello AM, Lidstrom ME (1999). Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl Environ Microbiol* **65:** 5066-5074.

Culman S, Bukowski R, Gauch H, Cadillo-Quiroz H, Buckley D (2009). T-REX: software for the processing and analysis of T-RFLP data. *BMC Bioinformatics* **10:** 171.

Cury JA, Koo H (2007). Extraction and purification of total RNA from *Streptococcus mutans* biofilms. *Anal Biochem* **365:** 208-214.

Dumont MG, Murrell JC (2005). Community - level analysis: Key genes of aerobic methane oxidation. *Methods Enzymol* **397:** 413-427.

Faye S, Maloszewski P, Stichler W, Trimborn P, Cissé Faye S, Bécaye Gaye C (2005). Groundwater salinization in the Saloum (Senegal) delta aquifer: minor elements and isotopic indicators. *Science of The Total Environment* **343:** 243-259.

Holmes AJ, Costello A, Lidstrom ME, Murrell JC (1995). Evidence that participate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. *FEMS Microbiol Lett* **132:** 203-208.

Horz HP, Yimga MT, Liesack W (2001). Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxaF*, and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. *Appl Environ Microbiol* **67:** 4177-4185.

Kunapuli U, Lueders T, Meckenstock RU (2007). The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *ISME J* **1:** 643-653.

Lane D (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds). *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons: New York. pp 125-175.

McDonald I, Kenna E, Murrell J (1995). Detection of methanotrophic bacteria in environmental samples with the PCR. *Appl Environ Microbiol* **61:** 116-121.

Michalke B, Witte H (2015). Characterization of a rapid and reliable method for iodide biomonitoring in serum and urine based on ion chromatography–ICP-mass spectrometry. *J Trace Elem Med Biol* **29:** 63-68.

Pilloni G, Granitsiotis MS, Engel M, Lueders T (2012). Testing the limits of 454 pyrotag sequencing: reproducibility, quantitative assessment and comparison to T-RFLP fingerprinting of aquifer microbes. *PLoS One* **7:** e40467.

193 Roche (2013). Amplicon Library Preparation Manual 454 Life Sciences Corp.: Branford, CT 06405,
194 USA. p 12.

195 Schramel P, Hasse S (1994). Iodine determination in biological materials by ICP-MS. *Microchim Acta*
196 **116:** 205-209.

197 Schramel P (2012). Aufschlussverfahren zur Bestimmung von Metallen in biologischem Material
198 [Biomonitoring Methods in German language, 2002]. *The MAK Collection for Occupational Health and*
199 *Safety*. pp 280-320.

200 Stoewer MM, Knöller K, Stumpp C (2015). Tracing freshwater nitrate sources in pre-alpine
201 groundwater catchments using environmental tracers. *J Hydrol* **524:** 753-767.

202 Suzuki MT, Giovannoni SJ (1996). Bias caused by template annealing in the amplification of
203 mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* **62:** 625-630.
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Supplementary Tables

Table S1. Primers and Restriction enzymes

Primer name	Sequence 5'→3'	Target gene	Reference
27f *†	AGA GTT TGA TCM TGG CTC AG	16S rRNA	Suzuki and Giovannoni (1996)
519r†	TAT TAC CGC GGC KGC TG	16S rRNA	Lane (1991)
907r	CCG TCA ATT CCT TTG AGT TT	16S rRNA	Amann *et al.,* (1992)
A189f *	GGN GAC TGG GAC TTC TGG	*pmoA*	Holmes *et al.,* (1995)
mb661	CCG GMG CAA CGT CYT TAC C	*pmoA*	Costello and Lidstrom (1999)
mxaf1003	GCG GCA CCA ACT GGG GCT GGT	*mxaF*	McDonald *et al.,* (1995)
mxar1561	GGG CAG CAT GAA GGG CTC CC	*mxaF*	McDonald *et al.,* (1995)
mmoX534f	CCG CTG TGG AAG GGC ATG AA	*mmoX*	Horz *et al.,* (2001)
mmoX1393r	CCG CTG TGG AAG GGC ATG AA	*mmoX*	Horz *et al.,* (2001)

* these primers were FAM labeled for T-RF fingerprint analysis
† these primer were used for qPCR and sequencing, linked to a barcode for multiplexing for the latter
nucleotide wobbles: M = A+C, K = G+T, N = A+C+T+G, Y = C+T

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216 **Table S2.** Sequences and OTUs

	Snottites Ceiling	Subaerial Top Wall	Submersed Bottom Wall	Mineral spring water	Mixed Cave water
# Samples	4	2	3	3	2
# Sequences per sample	2158 ± 504	2940 ± 60	2036 ± 206	2220 ± 164	2112 ± 582
# OTUs per sample	116 ± 11	138 ± 20	121 ±7	147 ± 10	92 ± 4
Inverse Simpson	17.9 ± 7.4	22.4 ± 2.1	10.2 ± 1.9	12.9 ± 3.5	6.5 ± 2.1
Hill number (exp(H))	32.1 ± 8.9	39.5 ± 6.4	21.5 ± 4.8	27.3 ± 5	12.8 ± 2.6

217 Given are mean values and IQR as variation

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219

Supplementary Figure

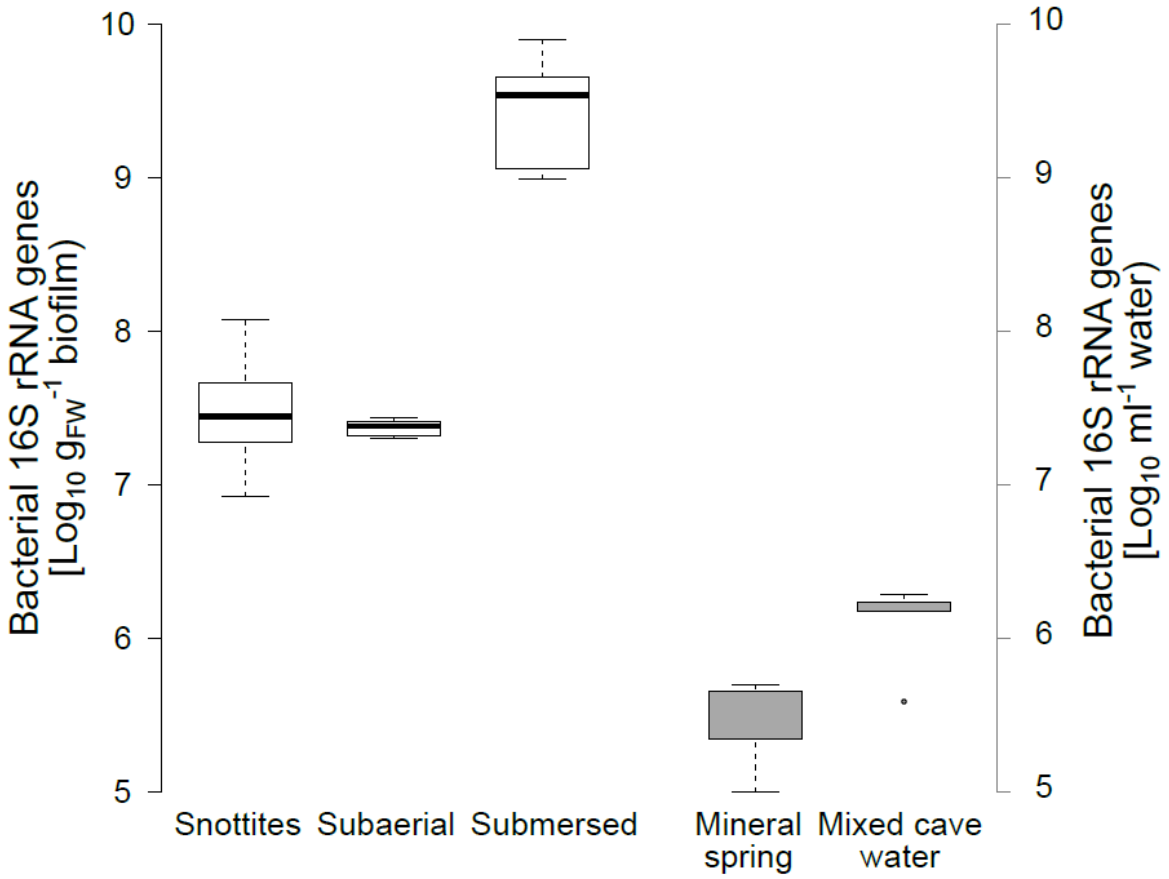

Figure S1. Quantitative PCR of biofilm samples reveal significant differences between submersed and subaerial biofilm compartments ($t=-13.58$, $df=29$, $p<0.05$).