Quantification of methanogenic Archaea within Baltic Sea copepod faecal pellets

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Abstract

Methane enrichments are frequently observed in the oxic upper water column of the central Baltic Sea during summer months. However, methane sources as well as the fate of methane produced in surface near waters still remain unclear. In the present study, we conducted ship-based grazing experiments to examine the presence of methanogenic archaea in copepod faecal pellets. We quantified bacterial and archaeal 16S rRNA and the *mcrA* gene and transcripts within copepod faecal pellets by using droplet digital polymerase chain reaction. We showed that the pellets (< 150-µm) harbour a small number of methanogenic archaea; however, *mcrA* transcripts indicating methanogenic activity were not detected. This suggests that copepod faecal pellets from the central Baltic Sea, similar to analogous data on copepod guts, harbour the potential but are an unlikely hotspot for methane production by methanogenic archaea.

Introduction

The total global emissions of methane (CH₄) are known reasonably well; however, individual sources are poorly understood (Dlugokencky et al. 2011). In the ocean, in addition to methanogenesis in anoxic marine sediments, there is another, less characterised CH₄ source in the shallow, oxygenated water column that may add significant amounts of this potent greenhouse gas to the atmosphere. This still unexplained source of CH₄ has been examined for over 40 years (Scranton and Brewer 1977), but the pathways are still not fully clear. The relevance of individual CH₄ sources, oxic versus anoxic, and their according fluxes especially in the surface mixed layer are still not sufficiently distinguished in total CH₄ budgets as recently discussed for lakes (Günthel et al. 2019). However, a growing number

of studies identified numerous pathways that could explain CH₄ enrichments in shallow oxygenated waters. These suggested CH₄ production pathways include the following: (1) algal metabolites such as methylated sulphur compounds as a possible CH₄ precursor, potentially catalysed by nonheme iron-oxo (IV) forming methyl radicals from homolytically broken sulphur methyl bounds (Althoff et al. 2014; Benzing et al. 2017; Klintzsch et al. 2019); (2) bacterial break-down of methylphosphonate (MPn) under phosphate-stressed conditions (Karl et al. 2008; Repeta et al. 2016; Teikari et al. 2018; Ye et al. 2020); (3) the microbial degradation of dimethylsulphoniopropionate (DMSP) (Damm et al. 2010; Zindler et al. 2013; Stawiariski et al. 2019); (4) the microbial demethylation of trimethylamine (TMA) (Bižić-Ionescu et al. 2018a); (5) the conversion of readily fixed CO₂ to CH₄ under light conditions with a potential close link to the photosynthesis process in cyanobacteria as well as in diatoms, green algae and cryptophytes (Bižić et al. 2020; Günthel et al. 2020); (6) activity of methanogenic archaea in anoxic microniches within the gastrointestinal tract of zooplankton, decaying organic aggregates or zooplankton faecal pellets (Oremland 1979; Traganza et al. 1979; Karl and Tilbrook 1994; Holmes et al. 2000).

Regarding the latter, different zooplankton grazing experiments showed copepod species-specific CH₄ production (de Angelis and Lee 1994; Stawiariski et al. 2019). Strong support to the copepod-gut flora theory was given by microsensor profiles that measured highly reduced

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conditions in copepod guts (Tang et al. 2011). Moreover, 16S rRNA gene-based analyses identified putative hydrognotrophic (*Methanogenium* and *Methanobacterium*) and methylotrophic (*Methanlobus*) methanogens in faecal pellets of copepods (Cynar and Yayanos 1991; Ditchfield et al. 2012). In 1992 Bianchi et al. observed CH₄ production associated with copepods and egested faecal pellets. The authors suggested that methanogens within the faecal pellets originated from the digestive tract of the zooplankton. Furthermore, they proposed the symbiotic relationship between zooplankton and methanogens for the production of CH₄ in the upper ocean. The accumulation of methane below the thermocline has been observed repeatedly in the Eastern Gotland Basin of the central Baltic Sea. Stable carbon isotope ratios of CH₄ ($\delta^{13}\text{C-CH}_4$) indicate a biogenic origin of the CH₄ (Jakobs et al. 2014). Furthermore, Schmale et al. (2018) found a positive correlation of *T. longicornis*-dominated seston and CH₄ production rates, as well as *Methanomicrobiaceae* in the water depth of high CH₄ concentrations. Methanogens were also detected in copepod guts, but the overall relative abundance was low (Wäge et al. 2019). However, none of these studies quantified methanogenic archaea associated with faecal pellets to assess their contribution to the CH₄ accumulation in the shallow oxygenated water column.

To improve our understanding of microorganisms associated with copepod faecal pellets and their potential for CH₄ production in the oxygenated water column of the central Baltic Sea, we quantified bacterial and archaeal 16S rRNA genes and the methyl coenzyme M reductase (*mcrA*) gene as well as transcripts from copepod faecal pellets originating from the central Baltic Sea, by using droplet digital polymerase chain reaction (ddPCR).

Materials and methods

Sampling

Sampling and ship-based experiments were conducted during the expedition Alkor 483 between 6 and 24 August 2016 in the central Baltic Sea. Zooplankton was sampled with a WP2 net (100- μm mesh size) equipped with a non-filtering cod-end, which was pulled with a speed of 0.1 m/s in depth ranges of 45–25 m. This layer is commonly dominated by the copepod species *Temora longicornis*, which was confirmed by zooplankton sampling during the same research expedition (Stawiarski et al. 2019). The collected copepods were transferred into a bucket with 15 L of water, collected using 10 L free-flow bottles (Hydrobios, Germany) attached to a rosette water sampler, from the same station and same depth range.

Faecal pellet experiment

To separate the dead and unfit copepods from the healthy ones, the copepods were kept in 15 L of water in a cold room at 10 °C for 90 min. Subsequently, 50 mL of water including vital copepods was transferred to each 500 mL incubation crystallising glass dish ($n=7$), avoiding the bottom layer of the water, as the dead and unfit copepods sank to the bottom of the bucket. In three control glass dishes, 50 mL of the same water without the copepods was added. To all glass dishes ($n=10$), 50 mL *Rhodomonas salina* (provided by the Technical University of Denmark; particulate organic carbon (POC) concentration of the culture: 1.46 mg/L) was added as additional food source and topped up with 300 mL 0.22- μm filtered sea water collected from the same depth as the copepods. All glass dishes were incubated at 10 °C in darkness for 3 h, to allow the copepods to feed on the algae *R. salina* and defecate their gut content. After the 3 h incubation the copepods were separated with a 200- μm gauze and the water containing the faecal pellets was filtered using a 3- μm polycarbonate filter (Millipore GVWP, 47 mm diameter, Darmstadt, Germany), frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

Nucleic acid extraction

Genomic DNA and total RNA was extracted from the 3- μm filters using the AllPrep DNA/ RNA Mini Kit (Qiagen, Hilden, Germany). The filters were cut into pieces and vortexed twice with silanised glass beads (2 mm and 3 mm) and 600 μL RLT-Buffer for 5 min. For all other steps the manufacturer's procedures were followed. The integrity of total RNA was tested on a denaturing tris-acetate-EDTA agarose gel stained with ethidium bromide (Life Technologies, Paisley, UK). cDNA was synthesised using SuperScript™ VILO™ cDNA Synthesis reagents (Life Technologies, Darmstadt, Germany), with approximately 10 ng of total RNA and following the manufacturer's protocol.

Quantification of methanogens, bacteria and archaea

Molecular quantification of the bacterial 16S rRNA gene, the archaeal 16S rRNA gene and the *mcrA* gene, as well as their transcripts, were conducted with the QX200™ Droplet Digital PCR System (Bio-Rad, München, Germany). For quantification, the bacterial primer pair Com1f (CAGCAG CCGCGGTAATAC) and Com2r (CCGTCAATTCCTTTG AGTTT) targeting the V-4 and V-5 regions described in Schwieger and Tebbe (1998), the archaeal 16S rRNA primer pair A519F (CAGCMGCCGCGGTAA) and A906R (CCC

GCCAATTCCTTTAAGTTTC) targeting the V-4 and V-5 regions (Teske and Sørensen 2008) and the *mcrA* primer pair (forward: GGTGGTGTGCGATTACACARTAYGCWACAGC and reverse: TTCATTGCRTAGTTWGGRTAGTT) (Luton et al. 2002) were used. For the ddPCR reaction 11 µL QX200™ ddPCR™ EvaGreen® Supermix was mixed with 100 nM forward/reverse primer (final concentration in total reaction), 10 ng gDNA or cDNA, and filled up with DEPC H₂O to prepare a final volume of 22 µL. The positive reaction for the archaea and methanogens included template DNA from *Methanogenium organophilum* (DSMZ No. 3596) and *Vibrio vulnificus* (DSMZ No. 10143) for the bacteria. For details on PCR programme and primer test see Wäge et al. (2019). A standard curve showing the amplification range of the *mcrA* primer pair was generated, which showed a detection limit at 0.1 pg template DNA (Fig. S1A). To test the detection limit of the *mcrA* primer pair using RNA as template, a positive cDNA control was generated using the in vitro MAXIscript™ T7 Transcriptionkit (ThermoFisher, Darmstadt, Germany) following the manufactures procedures. cDNA was synthesised using MultiScribe™ Reverse Transcriptase (ThermoFisher, Darmstadt, Germany) and the reverse *mcrA* primer (Luton et al. 2002). A serial dilution revealed a detection limit at 3 pg template cDNA (Fig. S1B). The archaeal 16S rRNA primer pair A519F and A906R was previously used for amplicon sequencing of free-living microorganisms, particle-associated microorganisms and copepod guts (Wäge et al. 2019). Since the sequencing results showed low numbers of archaeal reads, we present the ddPCR results with the original measurements as well as with a primer correction of 2.5% being actual archaeal reads, which is the average read number obtained from the previous sequencing analysis of three different sample types. Bacterial genomes display up to 15 operons, whereas about 40% of the organisms have only one or two. Instead, Archaea typically have a single operon, but some show up to five (Acinas et al. 2004). Because of these diverse numbers of operons, we decided not to correct our ddPCR quantifications. Hence, the reader should be aware that the presented ddPCR copies are not equal to the exact cell number. The extract of one incubation dish showed amplification problems and was therefore removed from further analyses.

Statistical analysis

Statistical analysis for the absolute copy number in 10 ng DNA/cDNA of 16S rRNA genes/transcripts from *Bacteria* and *Archaea* and the *mcrA* gene/transcripts was conducted using IBM® SPSS Statistics Version 25 (IBM Corp, Armonk, NY, USA). All data were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov test and Levene's test. Significant gene and transcript expressions were tested using an unpaired *t* test. For all analyses,

statistical significance was accepted at $p < 0.05$. All graphs were designed with XACT 8.03 (SciLab, Germany).

Results and discussion

The aim of this study was to determine the number of methanogenic archaea and their activity within copepod faecal pellets, in order to understand their contribution to CH₄ production in the oxygenated water column in the central Baltic Sea. To achieve this, archaeal and bacterial 16S rRNA genes as well as the functional gene *mcrA*, indicative of methanogenesis, were quantified by ddPCR. The quantification revealed the presence of a small number of methanogens associated with the faecal pellets; however, no *mcrA* transcriptional activity was detected (Figs. 1, 2).

Although previous studies identified methanogens (*Methanogenium*, *Methanobacterium* and *Methanobolus*) by 16S amplicon sequencing in copepod faecal pellets (Ditchfield et al. 2012) and described copepod faecal pellets and sedimenting particles as hotspots for microbial activity (e.g. Simon et al. 2002; Tang 2005), no study quantified methanogenic archaea. To the best of our knowledge, this study represents the first quantification of methanogens in copepod faecal pellets using a culture-independent method. The archaeal and bacterial 16S rRNA gene primer systems revealed that the number of 16S rRNA genes determined varied between individual faecal pellet incubations and the natural control particle incubations (Fig. 1a, b). We speculate that a variety of microorganisms, including methanogens, are incorporated from the gut during the faecal pellet production, as suggested by de Angelis and Lee (1994). Our previous study showed that the copy number of methanogens is positively correlated with the copy number of archaea for the particle-associated water fraction and negatively correlated for the free-living water column (Wäge et al. 2019). The reason for different numbers of 16S rRNA gene copies in the parallel incubation dishes might be due to some copepods having full guts from the beginning of the incubation experiment, and the excretion of faecal pellets was possibly faster. Potentially, this also allowed more additional microorganisms to accumulate on the particles. It is known that particle-attached bacteria are motile and can actively seek particles. Therefore, the colonisation of particles occurs in a matter of minutes and the bacteria have an average of 3 h residential time. Hence, a high exchange of bacteria between aggregates and the surrounding water can take place (Kiørboe et al. 2002). Likewise, the small number of natural particles in the control dishes was not all identical and some might have harboured more microorganisms, explaining the different copy numbers for both bacteria and archaea (Fig. 1a, b). This is supported by the study of Bižić-Ionescu et al. (2018b), which showed that the active microbial communities are highly

Fig. 1 Absolute numbers of **a** bacterial 16S rRNA genes, **b** archaeal 16S rRNA genes (dark grey bars represent absolute number as measured; light grey bars show corrected number (2.5%) as determined by the primer specificity; note the log scale) and **c** *mcrA* genes in faecal pellets of copepods as well as in controls, as determined by ddPCR. Analysis was performed by unpaired *t* test. *Significant differences ($p < 0.05$) of DNA copies between faecal pellets and control

heterogeneous even with an identical particle source. This suggested random initial colonisation most likely explains also the differences in our incubation dishes. With regard to the number of archaeal or bacterial 16S rRNA genes, as well as transcripts, no significant differences were found between the faecal pellets and the controls (Figs. 1a, b, 2a, b). In contrast, and despite the fact that these were only present in low numbers, significantly higher numbers of *mcrA* gene copies were determined for the faecal pellets ($t_8 = 2.835$, $p = 0.022$; Fig. 1c). Previous studies suggested that faecal pellets can temporarily act as anoxic microniches for methanogenic archaea (Oremland 1979; Bianchi et al. 1992; Marty 1993; Karl and Tilbrook 1994; Ditchfield et al. 2012). However, copepod faecal pellets have a large surface-to-volume ratio, and due to their small size anoxia was argued to unlikely occur inside the pellets (Alldredge and Cohen 1987). Also Ploug et al. (2008) doubted that anoxic conditions persist

within faecal pellets outside the anoxic digestive tract of copepods. Faecal pellet production experiments with the copepod *Temora longicornis* feeding on the nanoflagellate *Rhodomonas* sp. showed that the diffusive boundary layer of the faecal pellet, which is important for the gas exchange, was particularly thin and no indication of anoxic conditions was found (Ploug et al. 2008). An earlier study revealed that anoxia in marine aggregates has a short persistence and that stable anoxic conditions require a high and continuous carbon supply to support the oxygen consumption rates (Ploug et al. 1997). Ditchfield et al. (2012) discussed that anoxia within pelagic particulate material is limited to larger macroaggregates (> 600- μ m). The faecal pellet size from the surface and subthermocline measured during our cruise was with < 150- μ m rather small in comparison to larger crustaceans (Alldredge and Cohen 1987; Stawiariski et al. 2019). Consequently, the quantification of the *mcrA* transcripts in

Fig. 2 Absolute numbers of transcripts of **a** bacterial 16S rRNA genes, **b** archaeal 16S rRNA genes and **c** *mcrA* genes in faecal pellets of copepods as well as in controls, as determined by ddPCR

our incubations, as an indicator for active CH_4 production, showed that these were undetectable in all samples investigated (Fig. 2c). Hence, our results support the assumption that anaerobic methanogenesis within small faecal pellets is rather unlikely to occur. This suggests that CH_4 production by methanogens within faecal pellets plays only a minor or no role in maintaining the CH_4 accumulation in the oxygenated water column in the central Baltic Sea. This is in line with our previous study which showed that the guts of *Temora* sp. and *Acartia* sp. have the potential to produce CH_4 by methanogenesis, but are unlikely a hotspot (Wäge et al. 2019). Therefore, there must be other major sources responsible for the CH_4 production. For instance, bloom-forming *Nodularia spumigena* were discovered to be able to degrade methylphosphonate and release CH_4 in the Baltic Sea (Teikari et al. 2018). Undiscovered in the Baltic Sea yet are other postulated CH_4 sources as the microbial degradation of dimethylsulphoniopropionate (DMSP) or the decomposition of methylphosphonate (MPn) (Damm et al.

2008; Karl et al. 2008). An alternative, yet not in the field investigated metabolic pathway could be the conversion of readily fixed inorganic carbon into CH_4 by cyanobacteria under light and dark conditions (Bižić et al. 2020).

The present study showed that faecal pellets of copepods from the central Baltic Sea harbour small numbers of methanogenic archaea; however, active *mcrA* transcripts remained undetected. Similarly to our previous study on copepod guts, the faecal pellets are also an unlikely hotspot for CH_4 production by methanogenic archaea. In order to find a suitable explanation for the CH_4 accumulation in the oxygenated water column, further research is necessary, which should consider the indirect contribution of zooplankton by the release of CH_4 precursor substrates in the surrounding water.

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Compliance with ethical standards

Conflict of interest None declared.

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