Diversity of dimethylsulfide-degrading methanogens and sulfate-reducing bacteria in anoxic sediments along the Medway Estuary, UK

Stephania L. Tsola, Yizhu Zhu, Oshin Ghurnee, Chloe K. Economou, Mark Trimmer and Özge Eyice
School of Biological and Chemical Sciences, Queen Mary University of London, London, UK.

Summary
Methane is a powerful greenhouse gas but the microbial diversity mediating methylotrophic methanogenesis is not well-characterized. One overlooked route to methane is via the degradation of dimethylsulfide (DMS), an abundant organosulfur compound in the environment. Methanogens and sulfate-reducing bacteria (SRB) can degrade DMS in anoxic sediments depending on sulfate availability. However, we know little about the underlying microbial community and how sulfate availability affects DMS degradation in anoxic sediments. We studied DMS-dependent methane production along the salinity gradient of the Medway Estuary (UK) and characterized, for the first time, the DMS-degrading methanogens and SRB using cultivation-independent tools. DMS metabolism resulted in high methane yield (39%–42% of the theoretical methane yield) in anoxic sediments regardless of their sulfate content. *Methanomethylovorans*, *Methanolobus* and *Methanococcoides* were dominant methanogens in freshwater, brackish and marine incubations respectively, suggesting niche-partitioning of the methanogens likely driven by DMS amendment and sulfate concentrations. Adding DMS also led to significant changes in SRB composition and abundance in the sediments. Increases in the abundance of *Sulfurimonas* and SRB suggest cryptic sulfur cycling coupled to DMS degradation. Our study highlights a potentially important pathway to methane production in sediments with contrasting sulfate content and sheds light on the diversity of DMS degraders.

Introduction
Methane (CH₄) is a potent greenhouse gas with ~28 times the global warming potential of carbon dioxide (CO₂) over 100 years (Myhre et al., 2013). Total global methane emissions are estimated to range between 550 and 594 Tg per year (Saunois et al., 2020), around 60% of which is produced by methanogenic archaea via one of three described pathways, namely, hydrogenotrophic, acetoclastic and methylotrophic methanogenesis (Conrad, 2020). Research efforts have mostly focused on the acetoclastic (acetate) and hydrogenotrophic (CO₂ and hydrogen) methanogenesis pathways, although it has been acknowledged that methylotrophic methanogenesis is the dominant methanogenic pathway in saline ecosystems, e.g. marine and estuarine sediments (Lazar et al., 2011; Zhuang et al., 2016).

Dimethylsulfide (DMS) is an abundant methylated sulfur compound with an annual atmospheric flux of 24.5 ± 5.3 Tg and with significant roles in both the global carbon and sulfur cycles as well as in atmospheric chemistry (Charlson et al., 1987; Watts, 2000). The degradation of DMS and the related compound methanethiol (MT) via aerobic metabolism has been studied extensively in marine and terrestrial ecosystems, where high DMS and MT concentrations were measured (De Bont et al., 1981; Kiene and Bates, 1990; Eyice et al., 2015; Eyice and Schäfer, 2016). The majority of DMS is thought to be produced in the Earth’s upper ocean layers, however, DMS is also produced in anoxic freshwater lake sediments, saltmarsh sediments and peat bogs (Kiene and Visscher, 1987; Kiene, 1988; Kiene and Hines, 1995; Lomans et al., 1997).

Early studies reported both methane production and sulfate reduction coupled to the degradation of DMS and its metabolic intermediate MT in anoxic sediments, suggesting these compounds to be competitive substrates for methanogens and sulfate-reducing bacteria (SRB) in sulfate-containing sediments (Kiene et al., 1986; Kiene and Capone, 1988). Still, we know little about the pathways of DMS biotransformation in anoxic environments. Pioneering research was also undertaken to isolate DMS-degrading methanogens of the genera *Methanomethylovorans*, *Methanolobus*, *Methanosarcina*.
and Methanohalophilus from freshwater and saline sediments (Mathrani et al., 1988; Liu et al., 1990; Ni and Boone, 1991; Finster et al., 1992; Kadom et al., 1994; Oremland and Boone, 1994; Lomans et al., 1999). In addition to methanogens, DMS-degrading SRB of the genera Desulfotomaculum (Tanimoto and Bak, 1994) and Desulfosarcina (Lyimo et al., 2009) were also obtained from a thermophilic fermenter and mangrove sediment respectively. However, it is not known if these isolated species represent the actual diversity of the DMS-degrading microbial populations in anoxic sediments.

Stoichiometric calculations and growth experiments using DMS-degrading isolates demonstrated that DMS degradation via sulfate reduction (Eq. 1) is energetically more favourable (−94.5 kJ mol⁻¹ DMS; Tanimoto and Bak, 1994; Scholten et al., 2003) than methanogenesis (Eq. 2; −69.6 kJ mol⁻¹ DMS; Finster et al., 1992; Scholten et al., 2003).

\[
\text{(CH}_3\text{)}_2\text{S} + 1.5\text{SO}_4^{2-} \rightarrow 2\text{HCO}_3^- + 2.5\text{HS}^- + 1.5\text{H}^+ \quad (1)
\]

\[
\Delta G^\circ = -94.5 \text{ kJ mol}^{-1} \text{ DMS}
\]

\[
\text{(CH}_3\text{)}_2\text{S} + 1.5\text{H}_2\text{O} \rightarrow 1.5\text{CH}_4 + 0.5\text{HCO}_3^- + \text{HS}^- + 1.5\text{H}^+ \quad (2)
\]

\[
\Delta G^\circ = -69.6 \text{ kJ mol}^{-1} \text{ DMS}.
\]

A few studies also tested for competition between methanogens and SRB for DMS and showed that SRB outcompeted methanogens in sulfate-rich anoxic saltmarsh and mangrove sediments, where DMS concentrations were low, e.g. 1–10 μM (Kiene, 1988; Lyimo et al., 2009). This was potentially due to SRB exhibiting high affinities for DMS, as is the case for Desulfosarcina strain SD1 (Kᵣ < 0.5 μM; Lyimo et al., 2009). However, this strain grows extremely slowly on DMS (0.01 h⁻¹) compared to DMS-degrading methanogen Methanosarcina bensensis (0.07 h⁻¹), which implies that slow-growing DMS-degrading SRB may be outcompeted by DMS-degrading methanogens at high DMS concentrations (Lyimo et al., 2009). The outcome of this competition has significant consequences for the global methane and carbon cycles as it directly affects how much of the DMS carbon is converted into methane and CO₂ (Eq. 2). Yet, the effect of sulfate on the diversity of microbial populations underlying DMS degradation in anoxic sediments remains largely unexplored.

Estuarine sediments are ideal ecosystems to study anaerobic DMS degradation and how sulfate availability affects the fate of its carbon (i.e. produced as methane or CO₂). They sustain high methane production (Middelburg et al., 2002; Borges and Abril, 2012) and contain comparable DMS concentrations (>50 nM) to marine surface waters (Sciare et al., 2002; Hu et al., 2005; Lana et al., 2011). Furthermore, tidal estuaries have a natural salinity (and sulfate as the major ion) gradient along the full extent of the estuary, where river water with low sulfate concentrations (0.01–0.2 mM) mixes with marine water, where sulfate concentration is usually high at ~28 mM (Canfield et al., 2005; Dürr et al., 2011). We, therefore, incubated freshwater, brackish and marine sediments collected from along the Medway Estuary (UK; Fig. 1) under anoxia with DMS as the only carbon and energy source under near in situ sulfate concentrations. Then, we quantified the DMS-dependent methane production and analysed the methanogen and SRB populations mediating this process in sediment incubations. This study represents the first in-depth analysis of the diversity and abundance of DMS-degrading microorganisms in anoxic sediments and provides new insights into DMS degradation in estuarine sediments with contrasting sulfate content.

Results

**DMS degradation leads to methane production along the sulfate gradient of the Medway Estuary**

DMS was consumed and methane produced concurrently in sediments incubated with DMS, while no transient MT was observed. The DMS-amended incubations produced between 29 and 40 μmol methane g⁻¹ wet sediment, whilst all the negative control incubations, which lacked DMS, produced significantly lower amounts of methane (0.15–0.35 μmol methane g⁻¹ wet sediment; p < 0.001). Another set of negative control incubations was prepared using autoclaved sediment samples and amended with DMS to quantify DMS adsorption and whether methane would be produced. These controls showed no methane production, but ~4 μmol of DMS was removed in all samples due to sediment complexation (Kiene et al., 1986). Incubations were terminated when the methanogens entered the stationary phase at 37, 22 and 62 days in the freshwater, brackish and marine sediment incubations respectively (Fig. 2A).

The freshwater sediment incubations consumed a total of 56 ± 0.1 μmol DMS g⁻¹ wet sediment and produced 34 ± 0.8 μmol methane g⁻¹ wet sediment by the end of their 37-day incubation (Fig. 2B). Assuming that 1 mol of DMS yields 1.5 mol of methane (Eq. 2; Finster et al., 1992), then the average amount of methane produced corresponds to ~41% of the theoretical methane yield (84 μmol g⁻¹). As CO₂ is an end product of both methanogenic and sulfidogenic DMS-degradation pathways (Eqs. 1 and 2), CO₂ concentrations were measured at the end of the incubation period. The total amount of CO₂ in the
freshwater incubations was 4.8 ± 0.6 μmol g⁻¹ wet sediment (Fig. 2C), which was significantly lower than the stoichiometric value for the methanogenic DMS degradation (28 μmol; p < 0.001).

The brackish sediment incubations, despite being amended with sulfate, required the shortest incubation time (22 days) to reach stationary phase in comparison to the freshwater and marine sediment incubations. A total of 49 ± 7 μmol DMS g⁻¹ wet sediment was degraded, while 29 ± 2 μmol methane g⁻¹ wet sediment was produced, which is equivalent to ~39% of the theoretical methane production (73.5 ± 10.5 μmol g⁻¹; Fig. 2B). The total amount of CO₂ was similar to the freshwater incubations at 4.1 ± 0.5 μmol g⁻¹ wet sediment (Fig. 2C), which was greatly lower than the theoretical value for the methanogenic route (24.5 μmol g⁻¹). Sulfate reduction was also observed in the brackish incubations, where ~60% of the sulfate amended to the incubations (114 μmol g⁻¹ wet sediment) was consumed, decreasing the sulfate concentration to 45 μmol g⁻¹ wet sediment (Supplementary Fig. 1).

DMS degradation, methane production and sulfate reduction were also detected in the marine sediment incubations; however, a prolonged lag phase for methane production was observed in these samples (35 days). After the lag phase, a total of 64 ± 0.6 μmol DMS g⁻¹ wet sediment was consumed and 40 ± 1.1 μmol methane g⁻¹ wet sediment was produced within 27 days (Fig. 2B). This amount of methane corresponds to ~42% of the theoretical methane yield (96 μmol g⁻¹). The total amount of CO₂ was 3 ± 0.6 μmol g⁻¹ wet sediment when the marine incubations were terminated, which was significantly lower than the 32 μmol g⁻¹ theoretical yield assuming the methanogenic route (p < 0.001; Fig. 2C). Sulfate added to the incubations (230 μmol g⁻¹ wet sediment) was also consumed in the marine sediments; however, as in the brackish sediments, it was not totally depleted and only reduced to 70 μmol g⁻¹ wet sediment at the end of the incubation. This indicates sulfate availability was not the limiting factor for the SRB to degrade DMS in the brackish and marine sediments (Supplementary Fig. 1).

Taxonomic and functional diversities in the sediment incubations

A total of 1.9 × 10⁶, 6.4 × 10⁵ and 4.8 × 10⁵ quality-filtered, chimera-free sequences were obtained for the 16S rRNA, mcrA and dsrB genes respectively. A total of 15 111 distinct bacterial and archaeal operational taxonomic units (OTUs) were assigned at 99% identity, whilst 523 and 1702 OTUs were assigned for mcrA and dsrB genes at 85% identity.
Methanogen diversity

The mcrA gene sequences (Fig. 3A) and the Shannon diversity indices (Supplementary Table 1) showed that each sediment type had a distinct methanogen diversity before and after being incubated with DMS \( (p < 0.001) \). There was a statistically significant difference between the original sediments and the DMS-amended sediments (pairwise PERMANOVA; \( p = 0.0001 \)), while the control incubations with no additional DMS were not significantly different to the original sediments (Fig. 3A; pairwise PERMANOVA, \( p > 0.05 \)). This was also supported by the PCA of the mcrA sequences (Fig. 3B), which showed a clear separation of the DMS-amended sediments from the original sediments and the controls. Correlation analysis (Table 1) revealed that the DMS consumption \( (p = 0.007) \), methane production \( (p = 0.005) \), the initial sulfate concentrations \( (p = 0.03) \) and the concentrations of the total sulfate consumed in the incubations \( (p = 0.02) \) affected the methanogen diversity in the samples.

In both the original and DMS-amended freshwater sediments, the most dominant family was *Methanosarcinaceae*. Within this, the genera *Methanomethylovorans* (27% ± 4%) and *Methanosalum* (23% ± 1%), which were shown to degrade DMS previously were the most abundant in the original samples (Liu et al., 1990; Lomans et al., 1999; Fig. 3A). After incubating with DMS, *Methanomethylovorans* dominated the freshwater sediment at 66% ± 8% of the methanogen community \( (p = 0.04) \), whereas *Methanosalum* relative abundance decreased to 1% ± 2% \( (p < 0.001; \text{Fig. 3A}) \). *Methanococcoides* relative abundance also increased in the freshwater DMS incubations, where they comprised 29% ± 7% of the sequences \( (p = 0.008; \text{Fig. 3A}) \) despite being undetectable in the original samples. These results are consistent with the archaeal 16S rRNA sequencing data, which showed that the methanogens in

![Fig. 2.](image_url)

A. Average DMS amounts degraded (primary axis) and average cumulative methane amounts produced (secondary axis) in the DMS-amended incubations. Top - freshwater, middle - brackish and bottom - marine sediments.

B. Total amounts of DMS degraded and methane produced at the end of the incubation in sediments incubated with DMS.

C. Total amount of CO₂ produced at the end of the incubation. Error bars represent standard error above and below the average of five replicates.
the DMS-amended samples were predominantly affiliated with *Methanomethylovorans* (52% ± 18%; Supplementary Fig. 2). Quantification of the *mcrA* gene showed that the methanogen abundance increased 47-fold from 1.4 × 10^6 ± 3.9 × 10^5 copies g⁻¹ to 6.6 × 10^7 ± 1.5 × 10^7 copies g⁻¹ in the freshwater sediments, showing growth of methanogens in DMS-amended samples (Supplementary Fig. 3).

In the original brackish sediment samples, unclassified *Methanosarcinales* (26% ± 3%) and unclassified *Methanomicrobiales* (25% ± 1%) were the most abundant taxa (Fig. 3A). Their relative abundances decreased to 2% ± 1% and 7% ± 2% (p < 0.001) respectively, when the sediment was incubated with DMS, whilst *Methanolobus* became the most prevalent genus with a dramatic increase to 73% ± 11% from 3% (p < 0.001). This was also supported by the archaeal 16S rRNA sequencing data, which demonstrated the dominance of *Methanolobus* (87% ± 2%) in the DMS-amended incubations (Supplementary Fig. 2). Moreover, the abundance of methanogens increased from 4.9 × 10^6 ± 8.5 × 10^5 copies g⁻¹ to 1.3 × 10^7 ± 1.8 × 10^6 copies g⁻¹ in the DMS-amended brackish sediments (Supplementary Fig. 3).

*Methanococcoides* (*Methanosarcinaceae* family) was the most abundant methanogenic genus in the original marine sediment samples at 23% ± 5% of the sequences, followed by *Methanothermococcus* (from the *Methanococcaceae* family) at 18% ± 5% (Fig. 3A). After incubations with DMS, *Methanococcoides* remained the most abundant genus with a sharp increase in the relative abundance to 98% ± 1% (p < 0.001; Fig. 3A), suggesting this genus to be the only detectable DMS-degrading methanogenic taxon in the marine sediment. 16S rRNA sequencing data showed 97% ± 2% of the
sequences in the DMS-amended marine sediment were closely related to unclassified Methanosarcinaea (Supplementary Fig. 2), which are likely from the Methanococcoides in the samples as was determined by mcrA sequencing. The original marine sediment had 6.1 \( \times 10^5 \) ± 1.9 \( \times 10^5 \) copies g\(^{-1}\) methanogen abundance, which increased to 1.8 \( \times 10^7 \) ± 3.9 \( \times 10^5 \) copies g\(^{-1}\) after growth on DMS.

**SRB diversity**

Analyses of the dsrB sequences (Fig. 3A) and Shannon diversity indices (Supplementary Table 1) showed each original sediment sample had a significantly different SRB diversity (\( p < 0.001 \)). Pairwise PERMANOVA tests with the brackish sediment samples showed a statistically significant difference in SRB diversity between the original sediment, DMS-amended incubations and control incubations (Fig. 4A; \( p = 0.0001 \)). The PCA of the dsrB sequences explained 89% of the total variability between the samples from the different sites (Fig. 4B). The second principal component of the dsrB analysis correlated significantly with the total concentrations of sulfate consumed by the end of the incubations (\( p < 0.001 \); Table 1). This indicates the role that sulfate concentration plays on the SRB diversity while the effect on the DMS degradation was not significant.

A remarkable increase in the relative abundance of unclassified Deltaproteobacteria from 29% ± 6% to 63% ± 16% was observed in the freshwater sediments incubated with DMS (\( p = 0.02 \)), making it the most abundant recognized SRB taxon in these samples (Fig. 4A). The addition of DMS resulted in a dramatic ~50-fold increase in the dsrB abundance to 3.7 \( \times 10^7 \) ± 3.3 \( \times 10^5 \) copies g\(^{-1}\) from 3.4 \( \times 10^5 \) ± 1.4 \( \times 10^4 \) copies g\(^{-1}\) (\( p = 0.04 \)).

The dsrB sequences from the original brackish sediments were mainly composed of unclassified Deltaproteobacteria supercluster (27% ± 6%) and uncultured DsrAB lineage 9 (56% ± 24%) whilst unclassified Deltaproteobacteria supercluster dominated the DMS-amended sediments (71% ± 7%; Fig. 4A). Concurrently, the relative abundance of uncultured DsrAB lineage 9 reduced to 16% ± 5%. Despite the change in the composition of SRB, there was not a marked difference in the average dsrB abundances between the original and DMS-amended brackish samples (3.3 \( \times 10^6 \) ± 2.7 \( \times 10^5 \) and 1.6 \( \times 10^6 \) ± 1.6 \( \times 10^5 \) respectively).

In the original marine sediment, the majority of the dsrB sequences were affiliated with Desulfobacteraceae and uncultured dsrAB lineage 9, which represented 48% ± 5% and 38% ± 5% of the SRB community respectively (Fig. 4A). After DMS addition, Desulfobacteraceae accounted for 20% ± 4% (\( p < 0.001 \)), whilst uncultured dsrAB lineage 9 had a sharp increase to

![Fig. 4. A. Family-level taxonomy of the dsrB sequences in the original and DMS incubated freshwater, brackish and marine sediments from the Medway Estuary. Percentages show the average relative abundances of the taxa calculated using five replicates. Ori: Original sediment samples. Con: Control incubations with no added DMS. DMS: Sediment samples incubated with DMS. B. Principal components analysis of the dsrB sequences (PC1–48%, PC2–40%). F: freshwater sediment DMS incubations (black, dot), FO: freshwater original sediment (black, square), FC: freshwater sediment control incubation (black, square), B: brackish sediment DMS incubations (blue, dot), BO: brackish original sediment (blue, square), BC: brackish sediment control incubation (blue, square), M: marine sediment DMS incubations (red, dot), MO: marine original sediment (red, square) and MC: marine sediment control incubation (red, square). Note that OTUs with relative abundances <2% were not shown in the figure. [Color figure can be viewed at wileyonlinelibrary.com] © 2021 The Authors. Environmental Microbiology published by Society for Applied Microbiology and John Wiley & Sons Ltd., Environmental Microbiology.
62% ± 3% and became the most dominant SRB in the samples (p < 0.001). A significant decline was observed in the dsrB abundance, which reduced from $1.3 \times 10^8 \pm 3.8 \times 10^7$ copies g$^{-1}$ to $2.1 \times 10^6 \pm 2.5 \times 10^5$ copies g$^{-1}$ after DMS amendment (Supplementary Fig. 3).

The freshwater, brackish and marine sediment control incubations with no DMS had increased relative abundances of unclassified Deltaproteobacteria (49% ± 18%), unclassified Deltaproteobacteria supercluster (58% ± 12%) and uncultured dsrAB lineage 9 (56% ± 12%) respectively (Fig. 4A). These taxa likely utilized organic carbon in the sediments and sulfate provided in the growth medium.

**Total bacterial diversity**

The 16S rRNA sequence analysis and the Shannon diversity indices (Supplementary Table 1) showed that in the brackish and marine sediments, incubated with DMS, bacterial diversity changed significantly ($p = 0.0013$ and $p = 0.02$ respectively, Fig. 5A); however, the freshwater samples exhibited no statistically significant change; although unclassified Methylophilales increased from 1% to 5% in the freshwater DMS incubations. Pairwise PERMANOVA tests revealed significant differences between the original sediments, control incubations and DMS-amended incubations ($p = 0.0001$) except for the brackish control and DMS-amended incubations (Fig. 5B). The first PCA component significantly correlated with the amount of DMS consumed ($p = 0.002$), the methane yield ($p = 0.001$), and both the initial sulfate concentrations and total concentrations of sulfate consumed in the incubations ($p < 0.001$; Fig. 5B; Table 1), indicating an effect of both sulfate and DMS on the bacterial community structure in the sediments.

The most striking result of the 16S rRNA sequence analysis was the significant increase in the relative abundance of the chemoautotrophic, sulfur-oxidizing bacterial genus *Sulfurimonas* in all sediment samples after incubation with DMS ($p < 0.001$ for freshwater and brackish, $p = 0.04$ for marine sediments). *Sulfurimonas* comprised a higher relative abundance in the DMS-amended, sulfate-containing brackish and marine sediments at 30% ± 7% and 18% ± 12 respectively, compared to the
DMS-amended freshwater sediment (4.0% ± 0.1%; Fig. 5A). It should be noted that the relative abundance of Sulfurimonas also increased in the control incubations but to a smaller extent than in the DMS-amended incubations (3% ± 0.1%, 26% ± 5% and 8% ± 3%, for freshwater, brackish and marine incubations respectively). Furthermore, the relative abundance of sequences closely related to Methylophilales and Piscirickettsiaceae increased significantly to 5% ± 2% and 7% ± 2%, in freshwater and marine DMS incubations respectively. These taxa have methylotrophic members that can degrade DMS, however, their relative abundances increased also in freshwater and marine control incubations (8% ± 1% and 6% ± 2% respectively; de Zwart et al., 1996; Eyice et al., 2015). This either suggests that Methylophilales and Piscirickettsiaceae were not directly involved in DMS degradation in our sediments or that they carried out different functions in the control and DMS-amended incubations.

Discussion

DMS-dependent methanogenesis in Medway Estuary sediments

The formation of methane to stoichiometrically relevant concentrations in freshwater, brackish and marine sediment samples after DMS amendment strongly suggests that the production of methane was a direct result of DMS degradation as has been shown previously (Zinder and Brock, 1978; Kiene et al., 1986; Kiene, 1988; Van der Maarel and Hansen, 1997; Lomans et al., 2001). No MT was observed in incubations, indicating that MT, produced as a by-product of DMS degradation in the samples, was also degraded by the microbial communities. Between 39% and 42% of the theoretical methane yield was observed in sediments incubated with DMS, despite their contrasting sulfate content, thus indicating that sulfate availability has little or no effect on the methane conversion efficiencies along the Medway Estuary sediments. The methane yields of our DMS-amended samples are comparable to those observed by Kiene et al. (1986), who obtained 52%–63% yield in various anoxic sediments including from saltmarshes, freshwater, hypersaline and alkaline lakes. The difference between the methane yields is potentially due to the different characteristics of the sediments used in these studies. For instance, Kiene et al. (1986) obtained the highest methane yield from a saltmarsh, where high DMS production was shown to occur (Kiene, 1988). Other studies that used lake and saltmarsh sediments found approximately 28% methane conversion efficiency (Zinder and Brock, 1978; Kiene, 1988), which was likely a result of short incubation times (<2 days), prohibiting the DMS-degrading methanogens from reaching the exponential phase of growth.

A delay in methanogenesis was observed in our incubations (14, 12 and 35 days in freshwater, brackish and marine sediments respectively), which might be due to multiple reasons. First, methylotrophic methanogens in the sediments we studied were a small fraction of the total microbial diversity and might preferentially be mineralising methylamines, abundant methylated compounds in marine sediments (Sun et al., 2019). Hence, they likely required time to acclimate to the laboratory conditions and become more abundant to produce detectable levels of methane in the incubations. Second, DMS concentrations are lower in natural estuarine sediments than those used in our experiments, therefore, in the brackish and marine sediments, SRB might be the active DMS degraders due to their higher DMS affinity (Lyimo et al., 2009). Interestingly, the brackish sediment we studied had a shorter lag phase for measurable methanogenesis to begin than the freshwater and marine sediments, although the sulfate concentration in the brackish sediment was sufficient for high rates of sulfate reduction. Furthermore, there was remaining sulfate in both the brackish and marine sediments at the end of the incubation period, implying that the sulfate reduction likely depended on the availability of DMS and the initiation of methanogenesis was not directly related to sulfate concentration (Nedwell and Abram, 1979; Trimmer et al., 1997). The effect of sulfate concentration on methanogenesis was studied in the Yarqon Estuary, where incubations with acetate and lactate under a sulfate gradient from 1 to 10 mM (Sela-Adler et al., 2017) showed that the sulfate concentration did not affect the rates of methanogenesis or sulfate reduction. Overall, our study confirms the high methane production potential via DMS degradation in estuarine sediments, thus indicating DMS to be potentially an important methanogenic substrate in ecosystems even with high sulfate concentrations and emphasizing the requirement to better understand the environmental control over the metabolism of DMS-degrading methanogens.

Niche partitioning of DMS-degrading methanogens along the estuarine sediments

Using cultivation-independent methods, we detected the dominance of distinct methylotrophic methanogens in different parts of the sediments from the Medway Estuary. The genera Methanomethylovorans, Methanolobus and Methanococcoides dominated in the DMS-incubated freshwater, brackish and marine sediments respectively. The clear grouping of putative DMS-degrading methanogens that correlated with the sulfate concentration in the incubations strongly suggests sulfate-driven niche
partitioning of DMS-degrading methanogens along the salinity gradient of the Medway Estuary sediments. Although several studies have focused on niche partitioning among methanogenic archaea in estuaries and shales (Webster et al., 2014; Carbonero et al., 2014; Youngblut et al., 2015; Borton et al., 2018), only a few have assessed the effect of sulfate on this process. Oakley et al. (2012) studied the niche occupancy of methanogen genus *Methanoseta* and sulfate-reducing genus *Desulfohalobus* in Colne Estuary (UK) sediments and found particular genotypes from both taxa at distinct points along the salinity gradient, which is consistent with niche partitioning. In another study on the Colne Estuary, Webster et al. (2014) reported that *Methanoseta* dominated the brackish sediment, whilst *Methanococcosoides*, *Methanolobus* and *Methanosarcina* dominated the marine sediment, suggesting that salinity may be a significant factor controlling the diversity and distribution of methanogens. Furthermore, Youngblut et al. (2015) tested the diversity among 56 *Methanosarcina mazei* isolates from the salinity gradient along the Columbia River Estuary and demonstrated that *M. mazei* strains had clade-level differences when they were grown on methylamine, indicating niche partitioning by substrate utilization. A similar mechanism could have affected the distribution of DMS-degrading methanogens, particularly *Methanococcosoides* spp., which were detected in all our samples at different relative abundances.

*Methanomethylovorans*, which were found in the freshwater DMS incubations, are known methylotrophic methanogens with *M. hollandica* being the first DMS-degrading methanogen isolated from a freshwater sediment (Lomans et al., 1999). Now, it is well known that *Methanomethylovorans* spp. are typical freshwater methylotrophic isolates (Lomans et al., 1999; Jiang et al., 2005; Cha et al., 2013). Hence, the high abundance of *Methanomethylovorans* in low-salinity sediments such as our freshwater sediment was not surprising. We also demonstrated that *Methanolobus* and *Methanococcosoides* dominated the DMS-amended brackish and marine incubations respectively, suggesting that these genera degraded DMS in these sediment samples. *Methanolobus* and *Methanococcosoides* are prevalent methylotrophic methanogens in saline environments, like brackish lakes, tidal flats and estuaries (Munson et al., 1997; Purdy et al., 2002; Wilms et al., 2006; Watanabe et al., 2009). Furthermore, species of the genus *Methanolobus*, which were isolated from estuarine and sea sediments, were shown to degrade DMS (Kadam et al., 1994; Orem and Boone, 1994). Likewise, *Methanococcosoides* are obligate methylotrophic methanogens that can degrade methylated compounds such as methylamines, betaine, choline and methanol (L’Haridon et al., 2014; Jameson et al., 2019). None of the members of this genus have been shown to degrade DMS previously, yet our data suggest that known or novel *Methanococcosoides* species use DMS as the carbon source in the Medway Estuary sediments.

In contrast to previous studies, we observed the growth of *Methanococcosoides* also in DMS-amended freshwater and brackish sediments, using *mcrA* sequencing. This may be due to the members of this genus being transported from the marine site to the brackish and freshwater sites by tides where they then proliferated when the conditions became optimum for their growth. It is worth noting that archaeal 16S rRNA sequencing did not detect *Methanococcosoides* in the freshwater DMS incubations but found sequences affiliated with *Methanolobus*, which is likely because of a higher number of representative sequences from *Methanolobus* in the 16S rRNA database. Overall, our findings show that *Methanococcosoides* were the only detectable methanogens in the marine sediment incubations.

**DMS degradation by SRB and cryptic sulfur cycling in the sediments**

Incubating with DMS led to significant sulfur in the SRB composition and the total SRB abundance in all three sediments, despite the growth medium of the freshwater sediments having a low concentration of sulfate. Currently, there are around 240 pure cultures of sulfate-reducing microorganisms available, which greatly limit our ability to taxonomically define the SRB in our samples. *Deltaproteobacteria* were the most abundant SRB in the freshwater and brackish DMS incubations, whereas uncultured dsrAB lineage 9 dominated the marine DMS incubations. An increase in the relative abundances of *Deltaproteobacteria* and uncultured dsrAB lineage 9 was also observed in the control incubations with no DMS, however, this increase was greater in the DMS-amended incubations. It is likely that the SRB in the control incubations used available carbon in the sediments, while, in the DMS-amended incubations, they were involved in the cycling of DMS or its degradation products. The significant correlation between the sulfate concentration and SRB diversity indicates the effect of sulfate on the SRB community composition along the estuarine sediments, which is consistent with previous studies (Kondo et al., 2007; Oakley et al., 2010).

It is interesting that we detected a 50-fold increase in SRB abundance and a change in the relative abundances of distinct SRB in the freshwater sediments incubated with DMS, which were provided with low concentrations of sulfate representing estuarine conditions. These results suggest that SRB played a role in the cycling of DMS in these freshwater sediments. The notable changes in the SRB abundance in these
incubations were likely due to a cryptic sulfur cycle, where sulfide, produced as an end product of DMS degradation, was rapidly recycled mainly to sulfate as previously observed in freshwater wetlands, lakes and rivers (Jørgensen, 1990; Blodau et al., 2007; Heitmann et al., 2007; Berg et al., 2019). We speculate that here, in the freshwater sediments, hydrogen sulfide was reoxidised biologically by microbial action and abiotically with Fe(III) to elemental sulfur, polysulfide and thiosulfate, which was disproportionated to sulfide and sulfate (Pester et al., 2012). The occurrence of biotic cryptic sulfur cycling is also in line with the remarkable increase in the relative abundance of Sulfiturimonas in the freshwater sediment samples after incubating with DMS. We suggest Sulfiturimonas, a chemoautotrophic sulfur-oxidizing taxon, ubiquitous to sediments, oxidized hydrogen sulfide produced via DMS degradation to thiosulfate and sulfate or completely to sulfate (Grote et al., 2008; Lahme et al., 2020). Sulfiturimonas can utilize nitrate as the electron acceptor via autotrophic denitrification, which was previously demonstrated to occur in anoxic ecosystems, where sulfide and nitrate profiles overlap such as in the Baltic Sea, Mariager Fjord (Denmark) and the Black Sea (Brettar and Rheinheimer, 1991; Brettar et al., 2006; Jensen et al., 2008; Zhang et al., 2009; Fuchsmann et al., 2012). However, the freshwater growth medium in our incubations contained only 88 μM nitrate, which was likely consumed rapidly according to a previous study on Medway Estuary sediments (Shen et al., 2019). Hence, the Sulfiturimonas species in our incubations perhaps used iron oxides as the electron acceptor as it was found in high concentrations in the Medway Estuary sediments (Spencer, 2002). Alternatively, they might have used manganese oxides that were previously proven in a Sulfiturimonas isolate from the Black Sea water column (Henkel et al., 2019). Since methane production took place in the DMS-amended freshwater samples but did not reach the theoretical conversion rates, it is likely that cryptic sulfur cycling led to simultaneous degradation of DMS via methanogenesis and sulfate reduction, which produced hydrogen sulfide as an end product, contributing to the sulfur cycle in the freshwater sediment incubations.

The high relative abundance of Sulfiturimonas in DMS-amended brackish and marine sediments suggests that DMS contributed to the cryptic sulfur cycling also in these incubations, as Sulfiturimonas were previously linked to cryptic sulfur cycling in marine sediments and waters (Yao and Miller, 1996; Holmkvist et al., 2011; Callbeck et al., 2018). Although the control incubations exhibited an increased relative abundance of Sulfiturimonas, the increases observed in the DMS-amended sediments were greater. It is possible that, in the control incubations, the Sulfiturimonas species utilized hydrogen sulfide produced by SRB and the degradation of sulfur-containing amino acids, and assimilated the internal carbon and CO₂ in the sediments (Han and Perner, 2014).

**Fate of CO₂ during DMS degradation**

DMS degradation by both methanogens and SRB gives rise to CO₂, the most important greenhouse gas. Interestingly, we observed lower CO₂ concentrations in our DMS-amended sediments than expected stoichiometrically. This discrepancy may be attributed to the substantial increase in the relative abundance of Sulfiturimonas, which could fix CO₂ while oxidizing the hydrogen sulfide produced through DMS degradation or thiosulfate produced via cryptic sulfur cycling (Sievert et al., 2008). We calculated the HS⁻ production based on the stoichiometry of DMS-dependent methanogenesis (Eq. 2) and the methane concentrations in the incubations, which estimated the HS⁻ concentrations to be ~23 μmol g⁻¹ wet sediment, ~19.5 μmol g⁻¹ wet sediment and ~26.5 μmol g⁻¹ wet sediment in freshwater, brackish and marine incubations respectively. Sulfur-oxidizing bacteria, such as Sulfiturimonas, couple hydrogen sulfide or thiosulfate oxidation to CO₂ reduction with a stoichiometry of 1:0.5 or 1:2 to produce elemental sulfur or sulfate respectively (Klatt and Polerecky, 2015). Therefore, it is plausible to assume that the missing CO₂ in our incubations (~23 μmol g⁻¹ wet sediment, ~20.5 μmol g⁻¹ wet sediment and ~29 μmol g⁻¹ wet sediment in freshwater, brackish and marine incubations respectively) was used to oxidize hydrogen sulfide or thiosulfate and produce elemental sulfur and sulfate.

It could also be argued that low CO₂ concentrations in our incubations were due to the activity of hydrogenotrophic methanogens, which use hydrogen and CO₂ to produce methane; however, this is unlikely as our DMS-amended sediments were dominated by obligate methylotrophic methanogens and we did not detect hydrogenotrophic methanogens in these samples by mcrA sequencing. Alternatively, in the DMS-amended sediments, methylotrophic methanogens might have assimilated CO₂ into biomass or used it as a carbon source for mixotrophic growth as was recently shown for Methanococcosoides methylutens in marine sediments (Yin et al., 2019).

**Conclusion**

Our study addresses a major knowledge gap in the diversity of microbial populations underpinning anaerobic DMS degradation in anoxic sediments, which has so far only been characterized by cultivation-dependent methods. Here, we demonstrated that DMS degradation is a potentially important pathway to produce methane.
along the sulfate gradient of anoxic estuarine sediments and this process is carried out by recognized but distinct methylotrophic methanogens in different parts of the estuary, suggesting niche partitioning of DMS-degrading methanogens driven by sulfate concentration. In our incubations, we used DMS concentrations higher than the in situ concentrations and perhaps reflecting this, we observed a prolonged lag phase in methane production and higher methane yields than natural sediments produce. However, given the total global area of estuaries, we argue that the DMS-dependent methane production in estuarine sediments may be a globally significant process. Moreover, this process is likely important in other sulfate-rich ecosystems such as in saltmarsh and marine sediments, where DMS concentrations can be orders of magnitude higher than in estuarine sediments. In addition to the methane production, DMS affects the sulfur cycle in sediments either due to its use as a growth substrate by SRB or via a cryptic sulfur cycle. Our study highlights the importance of further work to reassess the microbial diversity and pathways of methylotrophic methanogenesis in marine and marine-influenced sediments in order to better understand the methane and carbon cycles in the environment.

**Experimental procedures**

**Site locations and sediment sampling**

The Medway Estuary is a macrotidal estuary located in Kent, South East England. Sediment was sampled using Perspex corer tubes (3.5 cm in diameter) at low tide from three locations along the natural salinity gradient of the estuary, namely Maidstone, (freshwater site, salinity 0.3%), Medway Bridge Marine (brackish site, salinity 6%) and Grain (marine site, salinity 32%) in November 2018 (Fig. 1). Sediment samples were transferred to the laboratory the same day for incubations set up and a small portion of sediment from each location was kept at −80 C until further analysis.

**Microcosm set-up**

Five replicated incubations were established per location using 5 g of sediment and 40 ml of ASW, which contained 0.32 M NaCl, 10 mM MgSO₄·7H₂O, 8.8 mM NaN₃, 3.1 mM CaCl₂·2H₂O, 10 mM MgCl₂·6H₂O, 9 mM Trizma base, 0.21 mM K₂HPO₄·3H₂O, trace element and vitamin solutions (Wyman et al., 1985; Wilson et al., 1996). Full strength ASW was used for marine sediment incubations, while it was diluted to 50% and 1% with sterile distilled water for brackish and freshwater sediment incubations respectively. The sulfate concentrations in the incubation bottles were adjusted according to in situ salinities of each location (0.1, 14 and 29 mM for freshwater, brackish and marine sediments respectively) and were amended with DMS as the carbon and energy source. Additionally, two sets of control incubations were set up: without DMS to monitor endogenous methane production and with twice-autoclaved sediment samples as abiotic controls to monitor the sediment adsorption of DMS. All incubations were set up in 120 ml serum bottles, sealed with butyl rubber stoppers and flushed with oxygen-free nitrogen for 15 min. The incubation bottles were kept at −21 C in the dark to avoid the photochemical destruction of DMS (Brimblecombe and Shooter, 1986). The amount of DMS provided to the bottles was increased gradually to avoid DMS toxicity. Initially, all incubations were amended with 1.6 μmol DMS g⁻¹ wet sediment. When the first addition of DMS was depleted, 4 μmol DMS g⁻¹ wet sediment was amended and then the additions were increased to 8 μmol DMS g⁻¹ wet sediment. All incubations were terminated when the methane production entered the stationary phase. The methane concentration measured in the stationary phase showed the total amount of methane produced, whilst the summation of individual DMS injections minus DMS remained in the bottles at the end of the incubation period showed the total DMS consumed. Three millilitre of water phase from each microcosm was transferred to gas-tight vials (3 ml Exetainer, Labco, UK) using a gas-tight syringe, and fixed using 24 μl of the bactericide ZnCl₂ 50% wt./vol., for the measurement of the total dissolved inorganic carbon (ΣDIC) as below. Then, each serum bottle was opened, the water and sediment phases were separated, and placed at −20 C until further analysis.

**Analytical measurements**

DMS and MT were measured in 100 μl of headspace gas sampled with a gas-tight syringe (Hamilton, UK) using a gas chromatograph (Agilent Technologies, 6890A Series, USA) fitted with a flame photometric detector and an Agilent J&W DB-1 column. Helium was used as the carrier gas at 200 C. DMS standards were prepared by the dilution of ≥99% DMS (Sigma-Aldrich, USA) in distilled water previously made anaerobic.

Methane and CO₂ in the headspace were measured using a gas chromatograph (Agilent Technologies, USA, 6890N Series) fitted with a flame ionization detector (at 300 C), Porapak q packed stainless steel column (Q 80/100; Supelco, USA) and hot-nickel catalyst to reduce CO₂ to methane (Agilent Technologies, USA; Sanders et al., 2007). Zero grade nitrogen (British Oxygen Company, BOC, UK) was used as the carrier gas at 30 C with a flow rate of 14 ml min⁻¹. The concentrations of both methane and CO₂ were calculated using certified...
gas mixture standards (100 ppm methane, 3700 ppm CO₂, 100 ppm N₂O, balance N₂, BOC) and all samples were corrected for the headspace-water partitioning using Henry’s law (Henry’s law constants based on Wiesenburg and Guinasso Jr., 1979). All the gas concentrations were calculated per gram of wet sediment. The total amount of CO₂ in the incubation bottles was calculated by the summation of the headspace CO₂ and the total dissolved inorganic carbon (ΣDIC). For ΣDIC measurements, a 1 ml headspace was created in the 3 ml sample vials containing the water phase using a two-way system, where a syringe was used to remove 1 ml of liquid while oxygen-free nitrogen was introduced into the headspace. 100 μl of 35% HCl was injected through the septa and the vials were shaken to ensure complete acidification. After equilibrium, the concentration of CO₂ in the headspace was measured against an inorganic carbon calibration series (0.1–8 mM) of sodium carbonate (Sigma-Aldrich; Trimmer et al., 2009; Shelley et al., 2017).

For the sulfate measurements, pore water collected from all original and DMS-incubated samples was filtered using 0.2 μm syringe filters (PTFE hydrophilic; Fisher Scientific, USA) and analysed using an ICS-5000 Dual Gradient RFIC Ion Chromatograph (Thermo Fisher Scientific, USA) equipped with a Dionex IonPac AS11-HC-4 μm column (2 × 250 mm) and a Dionex IonPac AG11-HC-4 μm guard column (2 × 50 mm). A gradient of 1.5–22 mM KOH with a flow rate of 0.35 ml min⁻¹ at 30 C was used as eluent for the analysis.

DNA Extraction, PCR, quantitative PCR

DNA was extracted from the samples collected when the incubations were terminated on days 37, 22 and 62 for freshwater, brackish and marine sediments respectively using the DNeasy PowerSoil kit (Qiagen, NL) following the manufacturer’s instructions. The 16S rRNA gene was amplified for total microbial diversity analysis. The mcrA and dsrB genes were amplified for the diversity analysis of methanogens and SRB respectively. Quantitative PCR (qPCR) of the mcrA and the dsrB genes was performed to enumerate the total methanogens and SRB respectively, using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Details about these methods can be found in Supporting Information.

High-throughput sequencing and sequence data analysis

PCR products were sequenced using the Illumina MiSeq platform (300 bp paired-end; Illumina, CA, USA). QIIME 2 (2019.1; Bolyen et al., 2019) on Queen Mary University of London (QMUL)’s Apocrita HPC facility, supported by QMUL Research-IT (King et al., 2017), was used for the sequence data analysis. Amplicon sequence variants were analysed using DADA2 and clustered into species-level OTUs (Callahan et al., 2016). For taxonomic classification, the Greengenes 13.8 database was used for the 16S rRNA samples at 99% similarity (DeSantis et al., 2006), while a custom database was used for mcrA (Wilkins et al., 2015). The dsrB sequences were taxonomically classified according to the phylogenetic framework established by Müller et al. (2015) and the recognized dsrB sequences according to the database by Pelikan et al. (2016) were retained for further analyses. Shannon (H) diversity indices were calculated using the data extracted from QIIME2. Average read abundances of each OTU were calculated using the read abundances of five replicates. Sequence datasets are publicly accessible at the National Center for Biotechnology Information (NCBI) Read Archive under the bioproject number PRJNA714116.

Statistical analysis

One-way ANOVA and pairwise PERMANOVA (9999 permutations) were conducted to test the statistical significance of the differences in diversity and abundance. Principal components analysis was carried out using the microbial relative abundance data and plotted to reduce the data complexity as well as to determine the underlying trends explaining the observed variation. Following this, Spearman’s correlation analysis was carried out to identify the factors that may have affected the relative abundances by correlating the first two principal components to the experimental variables (concentrations of utilized DMS, produced methane, initial sulfate and consumed sulfate). All statistical analyses were carried out using PAST (version 4.2; Hammer et al., 2001).

Acknowledgements

Stephania L. Tsola was funded by the Queen Mary University of London PhD studentships. We acknowledge the Warwick Integrative Synthetic Biology Centre (WISB) and Dr. Sarah Bennett for the sulfate analysis. We would like to thank Dr. Ian Sanders for fieldwork assistance, Dr. Kate Randall for qPCR discussions and P.K.H. Lee for providing the mcrA database and the bioinformatics documents for the analysis.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Appendix S1.** Supporting Information.