Stratified Microbial Structure and Activity in Sulfide- and Methane-Producing Anaerobic Sewer Biofilms

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ABSTRACT

Simultaneous production of sulfide and methane by anaerobic sewer biofilms has recently been observed, suggesting that sulfate-reducing bacteria (SRB) and methanogenic archaea (MA), microorganisms known to compete for the same substrates, can coexist in this environment. This study investigated the community structures and activities of SRB and MA in anaerobic sewer biofilms (average thickness of 800 μm) using a combination of microelectrode measurements, molecular techniques and mathematical modelling. It was seen that sulfide was mainly produced in the outer layer of the biofilm, between 0 - 300 μm, which is in good agreement with the distribution of SRB population as revealed by cryosection - fluorescence in situ hybridization (FISH).
SRB have a higher relative abundance of 20% on the surface layer, which decreased gradually to below 3% at a depth of 400 μm. In contrast, MA mainly inhabited the inner layer of the biofilm. Their relative abundances increased from 10% to 75% at depths of 200 μm and 700 μm, respectively, from the biofilm surface layer. High throughput pyrosequencing of 16S rRNA amplicons showed that SRB in the biofilm were mainly affiliated with five genera: *Desulfobulbus*, *Desulfomicrobium*, *Desulfovibrio*, *Desulfatiferula* and *Desulforegula*, while about 90% of the MA population belonged to the genus *Methanosaeta*. The spatial organization of SRB and MA revealed by pyrosequencing were consistent with the FISH results. A biofilm model was constructed to simulate the SRB and MA distributions in the anaerobic sewer biofilm. The good fit between model predictions and the experimental data indicates that the coexistence and spatial structure of SRB and MA in the biofilm resulted from the microbial types, their metabolic transformations and interactions with substrates.

**INTRODUCTION**

Sewer biofilms comprise complex multi-species microflora with a typical thickness of only about one millimeter (1). According to the electron donors and electron acceptors present in the wastewater, different carbon transformation processes can occur in close proximity in the sewer biofilms. Domestic wastewater normally contains a significant concentration of sulfate (ca. 100–1000 μM) but negligible nitrite and nitrate (2, 3). Therefore, under anaerobic conditions (normally occurs in pressure sewers fully filled with wastewater), sulfate reduction carried out by the sulfate-reducing bacteria (SRB) could be an important terminal electron accepting process in the sewer biofilms. The sulfate reduction activity in anaerobic sewers is important as the production of sulfide produced can be transferred to the gas phase of partially-filled gravity sewers and cause
extensive corrosion of concrete sewer pipes (4, 5). Also, the emission of sulfide from
sewers can cause odor problems to the surrounding area and pose health risks to sewer
workers (6, 7). Apart from sulfate reduction, methanogenesis by the respiration of
methanogenic archaea (MA) could also be a key terminal process in anaerobic sewer
biofilms (8, 9). Guisasola and colleagues found that methanogenesis accounted for more
than 70% of the chemical oxygen demand (COD) loss in laboratory anaerobic sewer
biofilm reactors (9). A recent report suggests that methane emissions from sewers
contribute significantly to the total greenhouse gas footprint of wastewater systems (10).

Under anaerobic conditions, both sulfate reduction and methanogenesis can
potentially occur in the same system while competing for the same electron donors,
primarily hydrogen and acetate. In the presence of adequate sulfate concentrations, SRB
will typically outcompete MA due to kinetic and thermodynamic advantages (11-13).

However, the coexistence of SRB and MA has been observed in anaerobic sewer
biofilms in the presence of sulfate. Guisasola et al. (9) hypothesized the coexistence of
SRB and MA in sewer biofilms was due to the penetration limitation of sulfate into the
biofilms, resulting in a stratified biofilm structure, with SRB being predominant in the
outer zone, nearer to the wastewater, while MA inhabit the inner zone, nearer the sewer
pipe. However, to date this hypothesis has not been verified. A few studies have
investigated the vertical distribution of SRB in oxic-anoxic sewer biofilms (biofilms
attached on gravity sewer pipe with the presence of oxygen or nitrate in wastewater), but
studies on the SRB distribution in the anaerobic sewer biofilms is scarce (14). In addition,
the distribution of MA in sewer biofilms and their interaction with SRB have not been
explored yet. Similarly, the phylogenetic diversity of SRB and MA in the anaerobic
sewer biofilms is rarely reported. These fundamental information could provide a better
understanding of the sulfate reduction and methanogenesis processes in sewer systems,
which would be useful for sewer management. Therefore, the aims of this study are to
investigate the community structures of both SRB and MA and to determine their spatial
arrangement in anaerobic sewer biofilms.

Both experimental investigations and modeling analyses were conducted to achieve
the aims of this study. The experiments were carried out in an annular biofilm reactor
mimicking anaerobic sewer conditions, which was fed with real domestic wastewater.
Firstly, microelectrodes were applied to determine the spatial distribution of in situ
sulfide production activity within the biofilms. Although it would have been ideal to
determine the distribution of methane production activity using the same method, this
was difficult to perform due to the lack of suitable microelectrodes (15). Secondly, the
spatial distributions of the SRB and MA in the biofilms and their abundance at different
depths were determined by fluorescence in situ hybridization (FISH) after
cryosectioning the biofilm samples. This method has been used frequently to determine
the spatial distributions of microbial communities in biofilms or granules. However,
phylogenetic information is hardly revealed due to the limitation of oligonucleotide
probes used in FISH (16). Therefore, 16S rRNA gene amplicon pyrosequencing was
applied to further investigate the phylogenetic diversity. In previous studies of sewer
biofilms, the phylogenetic analysis is performed on the entire biofilm, and information
on the different genera at different biofilm depths is rarely reported (2, 14). In this study,
we determined the phylogenetic diversity in different layers of the sewer biofilms by
innovatively using pyrosequencing combined with cryosectioning. To our knowledge, to
date, this method has not been applied in any other studies related to biofilms and
granules. Finally, a mathematical model focusing on the interaction between SRB and
MA in the sewer biofilm was developed to evaluate and interpret the experimental
results.
MATERIALS AND METHODS

Reactor configuration, operation and monitoring. An annular biofilm reactor made of acrylonitrile butadiene styrene (ABS), one of the typical materials used for sewer pipes, was set up to mimic an anaerobic sewer pipe section (Fig. 1). The reactor consisted of an inner cylinder (of height 295 mm, and diameter of 130 mm) enclosed in an outer cylinder (of height 345 mm and inner diameter of 160 mm). Wastewater was filled in the gap between the two cylinders, with a volume of 3 L. Biofilms were grown on the walls of both cylinders in contact with the wastewater, resulting in a biofilm area to reactor volume (A/V) ratio of 119 m⁻¹. Mixing was established by the rotation of the inner cylinder driven by a motor at a speed of 200 rpm. The mixing is expected to create a uniform shear stress on the reactor walls so that biofilms grow relatively evenly on the wall. The average shear stress provided by the mixing was 2.11 N/m², which is typical in sewer systems (17). Eight removable ABS slides of width and length at 5 mm by 200 mm were mounted in recessed slots on the inside of the outer cylinder. The slides were removable via ports on the top of the reactor for biofilm sampling. The reservoir on the top of the reactor was used to ensure the reactor was full of wastewater during sampling. The reactor was operated in a temperature-controlled room (20 ± 2 °C). Domestic sewage, collected on a weekly basis from a local wet well (Brisbane, Queensland), was used as the feed for the reactor. The sewage compositions varied to a certain extent in terms of sulfate, Volatile fatty acid (VFA), and COD concentrations. The sewage typically contained sulfate at concentrations of 10-25 mgS/L, sulfide at < 3 mgS/L, Soluble COD (sCOD) at 200-300 mg/L, 50-120 mgCOD/L of VFAs and approximately 50 mgN/L of ammonium. Negligible amounts of sulfite, thiosulfate (<1 mgS/L), nitrate and nitrite (<1 mgN/L) were present. The sewage was stored in a cold room (4°C) to
minimize biological transformation, and was heated to 20±1°C prior to being pumped into the reactor (Fig. 1), which is consistent with the ambient temperature.

The sewage was fed to the reactor intermittently by a peristaltic pump (Masterflex 7520-47) to simulate the typical flow patterns of rising main sewers. For easier reactor monitoring, each day was divided into three identical 8-hour periods. Fig. S1 in Supporting Information (SI) shows the pumping patterns applied to the reactor for an 8-hour period and the hydraulic retention time (HRT) of sewage in the reactor. Every pumping event lasted for 3 min, delivering one reactor volume (3 L) of wastewater into the reactor. The HRT of the wastewater ranged between 30 minutes to 4 hours, which are in the range of HTR observed in a typical real sewer pipe (9).

Monitoring of the reactor performance was carried out during the eight-hour cycle periods every two weeks. Sulfide concentrations during the eight hour cycle were continuously monitored using the S::CAN VU-VIS spectrophotometer (Messtechnik GmbH, Austria), as previously described by Sutherland-Stacey et al. (18). In addition, samples were taken from the reactor before and after each pumping event and also at 2.5h, 5h, and 6.5h for the analysis of dissolved methane, sulfate, total COD (tCOD), sCOD and VFAs, using methods as described by Jiang et al. (3). Briefly, dissolved methane and VFA concentration were measured by gas chromatography. Sulfate was measured using ion chromatography. tCOD and sCOD were determined by means of COD cuvette tests (Merck, range 25–1500 mg L$^{-1}$). Detailed studies of the biofilm were carried out when the reactor reached pseudo steady-state conditions after about 10 months operation, as indicated by the relatively constant sulfide and methane profiles.

**Microelectrode analyses.** Hydrogen sulfide (i.e. molecular H$_2$S), pH, and dissolved oxygen in the biofilm were measured using microelectrodes (Unisense A/S, Denmark) with tip diameters of 10 μm, 25 μm and 100 μm, respectively. The sensors were
calibrated according to the manufacturer’s instructions. Hydrogen sulfide and pH profiles were measured to determine the total dissolved sulfide concentration as described in Kuhl et al. (19). Oxygen profiles were measured to confirm anaerobic conditions.

Before the microelectrode analyses, about 5 cm of the biofilm slide was removed from the reactor and mounted in a flow cell (as described in Gutierrez et al. (20)) containing 140 mL of 0.22 μm filtered wastewater and 20 mL of 300 mM phosphate buffer (added to ensure a stable pH of 7.0–7.5). Nitrogen gas (99.99% purity) was bubbled through the flow cell to ensure anaerobic conditions and to provide mixing. Microelectrodes were mounted on a micromanipulator and positioned on the surface of the biofilm using a dissecting microscope. The concentration gradients through the biofilm were obtained by moving the microelectrodes in increments of 25–100 μm. Steady-state profiles were obtained by incubating the biofilm for 1 h in the medium before measurements were made.

The local sulfide production rates were calculated from the total sulfide profiles based on Fick’s second law of diffusion. The calculation was carried out by a stepwise procedure as described by Gieseke and de Beer (21). Briefly, the production rate at point n can be calculated using following equations.

\[ r_n = \frac{J_{n-1,n} - J_{n,n+1}}{0.5(x_{n-1} + x_n) - 0.5(x_n + x_{n+1})} \]

\[ J_{n-1,n} = D_{eff} \frac{c_{n-1} - c_n}{x_{n-1} - x_n}, \quad J_{n,n+1} = D_{eff} \frac{c_n - c_{n+1}}{x_{n} - x_{n+1}}, \]

Where

\[ J_{n-1,n} \text{ and } J_{n,n+1} \text{ are flux between point } n-1 \text{ and } n, \text{ and point } n \text{ and } n+1, \text{ (mol/m²/s)}; \]
\[ x_{n-1}, x_n, \text{ and } x_{n+1} \text{ are depths of point } n-1, \text{ and } n \text{ and } n+1, \text{ (m)}; \]
\[ c_{n-1}, c_n, \text{ and } c_{n+1} \text{ are sulfide concentrations at point } n-1, \text{ and } n \text{ and } n+1, \text{ (mol/m³)} \]
$D_{eff}$ is effective diffusion coefficient of sulfide in the biofilm (m$^2$/s). The value used in this study was $1.39 \times 10^{-9}$ m$^2$/s (19) and this was based on the assumption that the diffusion coefficients within the biofilm were equal to the molecular diffusion coefficients.

**Fluorescence in situ hybridization.** Fluorescence *in situ* hybridization (FISH) was carried out to determine the distribution of SRB and MA in the biofilm. The sequences of all oligonucleotide probes used in this study are summarized in Table S1 (SI) and further detailed information is in probeBase (22). Due to a drawback of SRB probes which can detect other bacteria that are not SRB, in this study the SRB were determined by the overlapping fluorescence signal of the probes DELTA495a (CY3), DELTA495b (CY3) and DELTA495c (CY3) with probes SRB385 (CY5), SRB385Db (CY5) and DABAC 357 (CY5). Using this approach, most SRB in the phylum of the Deltaproteobacteria were detected, while discriminating the non-SRB targeted by these probes (16). SRB in other phyla were not detected by 16S rRNA pyrosequencing results, so probes for those phyla were not used. For FISH detection of MA, a combination of probes MSMX860 (CY5), MG1200b (CY5), MB1175 (CY5), MC1109 (CY5) and MC504 (CY5) were used to determine the total MA population in the biofilm. This combination of probes covers a wide range of MA in these ecosystems (23). SRB and MA were determined using different samples due to the different formamide concentrations required (35% for the SRB detection and 45% for the MA detection).

The probes EUB338mix (FITC) and ARC915 (CY3) were used to determine all bacteria and archaea in the biofilm respectively.

To conduct FISH analysis, the biofilm sampling slides were removed from reactor and cut to approximately 10 mm × 5mm. The biofilm samples on the small pieces were fixed with freshly prepared 4% paraformaldehyde solution for 2 h at 4°C.
biofilm sample was then embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) following the procedures described by Batstone et al. (24). The biofilm samples were then allowed to settle on the base of the OCT moulds and frozen at -20 °C. The frozen samples were then sectioned using a Research Cryostat (Leica CM3050 S) with a knife temperature of -20 °C, a cabinet temperature of -18 °C and a section size of 10 μm. The samples were divided into two groups and cryosectioned in two different directions. One group of samples was sectioned perpendicular to the substratum, to provide sections to visualize the arrangement of SRB and MA distributed through the depth of the biofilm. The other group of samples were sectioned parallel to substratum successively from the surface to the bottom of the biofilm. These samples were used to determine the relative abundance of SRB and MA at eight different depths within the biofilm. The cryosectioned samples were placed on Poly-L-Lysine coated microscope slides (Polysciences Asia Pacific, Inc.) and air dried for 6 – 10 h. The slides were then dehydrated for 3 min each in a 50%, 80% and 98% aqueous ethanol solution.

All in situ hybridizations were performed according to the protocol (25) in hybridization buffer at 46°C for 2–3 h. The buffer contained 0.9 M NaCl, 20 mM Tris hydrochloride (pH 7.2), 0.01% sodium dodecyl sulfate and formamide concentrations as previously mentioned. Subsequently, a stringent washing step was performed at 48°C for 15 min in 50 ml of washing solution comprising NaCl at a concentration dependent on the formamide concentration, and 20 mM Tris hydrochloride at pH 7.2. The slides were examined and recorded using a Zeiss LSM 510 confocal laser scanning microscope (CLSM) (Carl Zeiss, Jena, Germany) using three excitation channels (488 nm, green emission; 545 nm, red emission; and 633 nm, blue emission). The biofilm thickness was estimated by measuring the width of the biofilm sections cut perpendicular to the substratum. FISH images at eight different depth of the biofilm (0-10μm, 100-110μm,
200-210μm, 300-310μm, 400-410μm, 500-510μm, 600-610μm and 700-710μm) were analysed using DAIME version 1.3 (26) to determine the biovolume fractions of SRB and MA. About 20 confocal images of the biofilm sections were analyzed for each sample. The quantification results were calculated based on the average of two separately analyzed samples.

**16S rRNA gene amplicon pyrosequencing.** 16S rRNA gene amplicon pyrosequencing was conducted to investigate the phylogenetic diversity of SRB and MA at different layers in the biofilm. Biofilms on a 10 mm × 5 mm piece of slide were quickly removed from the reactor and embedded in OCT compound and then frozen at -20 °C in a OCT mould. The frozen samples were then cryosectioned successively from the surface to the bottom of the biofilm with a section size of 150 μm, using the cryostat as described above. The sectioned biofilm samples were then placed separately in 1 ml eppendorf tubes containing 0.5 ml of phosphate buffered saline (PBS, containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄) for DNA extraction.

Genomic DNA was extracted using the FastDNA SPIN Kit for soil according to the manufacturer's instructions (Q-Bio gene, Australia). The quantity and quality of the extracted DNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Rockland, DE) and agarose gel (0.8%, wt/vol) electrophoresis. The primers 926f (5'-AAACTYAAAKGAATTGACGG-3') and 1392r (5'-ACGGGCCTGTGTAC-3') (27) containing multiplex identifiers and LibL adaptor sequences (not shown) were used to generate amplicons. The PCR reaction tube (50 μL) contained 5 μL of 10X PCR buffer, 1 μL of 10 mM dNTPs, 1 μL each of 10 mM primers, 4 μL of 25 mM MgCl₂, 1.5 μL of 10 mg/ml Bovine Serum Albumin (BSA), 0.2 μL of 5.5 Units/μL Fisher Biotech Taq DNA Polymerase and 2.5 μL of 20 ng/μL DNA sample. The PCR reaction was conducted under the following conditions: 95 °C for 3
minutes, followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 74 °C for 30 seconds, and a final elongation step at 74 °C for 10 minutes. The pyrosequencing of amplicons was carried out according to Roche 454 protocols using a Roche 454 GS FLX sequencer (Roche, Switzerland). The sequence data was analysed through the ACE Pyrosequencing Pipeline (https://github.com/minillinim/APP) in a local implementation. Firstly, the sequencing reads were split according to the barcode in QIIME v1.8.0 (28). Then, De-multiplexed sequences were trimmed to 250 bp length and de-noised using ACACIA (29). Sequences with 97% similarity were assigned to one operational taxonomic unit (OTUs) by CD-HIT-OTU (30) and aligned by Pynast (31). Each sequence was then assigned to the taxonomy with the BlastTaxonAssigner in QIIME through the greengenes database (2013 Aug release). Sequences that were assigned to the classes of Clostridia and Deltaproteobacteria (containing most of the mesophilic SRB) and those assigned to the domain Archaea (containing the methanogens) were also compared with other sequences previously deposited in GenBank (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) and genus level classification were assigned (if >98% identity were obtained). Finally a non-normalized OTU table was generated by QIIME. Then, Normaliser (https://github.com/minillinim/Normaliser) was used to construct a centroid normalized OTU table.

**Biofilm modelling.** A multispecies one-dimensional biofilm model was constructed to simulate the microbial structure and biological reactions in the anaerobic sewer biofilm, employing the software AQUASIM V2.1d (32). The biofilm model was developed to evaluate the experimental results according to Sharma et al. (33) and Guisasola et al. (34). The biological reaction model is schematically summarized in SI Fig. S2 with definition of model components summarized in Table S2 (SI).
Briefly, the biological model consisted of four types of microbial processes: hydrolysis, fermentation, sulfate reductions and methanogenesis. Glucose is used in the reaction to represent fermentable substrates (e.g. sugars and/or other carbohydrates), in the same way as used previously (34). Three fermentation products were considered in the model, namely hydrogen, acetate and propionate. Sulfate reductions were carried out with the three electron donors, i.e. hydrogen, acetate and propionate. Given the fact that SRB tend to outcompete acetogenic bacteria for propionate utilisation and that propionate concentrations in real sewage were always lower than 10 mgCOD/L, propionate was considered as an electron donor only for sulfate reduction (34). While sulfate reduction using fermentable substrates (e.g. sugars or other carbohydrates) is also possible, it was not considered in the model (34). The use of these substrates by SRB would otherwise be accounted for by the use of the fermentation products from these substrates. Both hydrogenotrophic and acetoclastic pathways for methanogenesis were included in the model. The stoichiometric matrix for microbial processes and the kinetic expressions of processes were shown in Table S3 (SI) and Table S4 (SI), respectively. All model parameters were obtained from the literature and are presented in Table S5 (SI).

Nucleotide sequence accession number. The 16S rRNA gene sequence data were deposited into NCBI Short Read Archive (SRA) under accession numbers SRR1560806, SRR1560807, SRR1560808, SRR1560809, SRR1560810.

RESULTS

Performance of the anaerobic sewer reactor. The typical sulfide and methane profiles in the sewer biofilm reactor during an 8-hour operation cycle is shown in Fig. 2A and B. Sulfide and methane were produced simultaneously in the reactor and concentrations of
sulfide (13.0-18.6 mgS/L) and methane (9.3-14.9 mg/L) at the end of each pumping cycle varied according to the HRT. During the 8-h cycle, the tCOD was decreased by 17% and nearly 86% of the sulfate was reduced. Table 1 shows the average daily transformation of COD, VFAs, sulfur species and methane at pseudo steady state, calculated based on the concentration differences at the beginning and end of each pumping cycle. The tCOD was consumed by 688.2 ± 29.2 mg/day, with productions of sulfide and methane at 123.9 ± 11.1 mgS/day and 103.4 ± 3.2 mg/day, respectively. Similar daily sulfate consumption and sulfide production indicated that sulfide was the major product of sulfate reduction. The sCOD and propionate were also consumed in the reactor while acetate accumulated. The COD balance was calculated assuming that all hydrogen produced due to fermentation was consumed during the experiment. The COD utilization per gram of sulfide and methane formed is assumed to be 2 gCOD/gH2S-S and 4 gCOD/gCH4, respectively (9). Therefore, sulfidogenesis accounted for 36.0 ± 2.4 % of the tCOD loss in the wastewater while methanogenesis accounted for 60.0 ± 4.3 % (Table 1).

**Distribution of sulfide production within the biofilm.** The micro-scale sulfide, pH and oxygen levels were measured throughout the depth of the biofilm (Fig. 3). A significant increase of sulfide concentration is seen from the biofilm surface to ca. 250 μm into the biofilm. The pH remained constant throughout the depth of the biofilm, due to the buffering capacity of the system. Negligible levels of oxygen were detected within the biofilm. The *in situ* sulfide production rates were calculated based on the sulfide profiles according to Fick’s law of diffusion (Fig. 3), which indicated that sulfide was mainly produced in the region that extended from the biofilm surface to a depth of about 300 μm into the biofilm. Though sulfide concentration was the highest below the depth
of 300 μm, the calculated sulfide production under that depth only accounted for less than 10% of the total production.

Spatial distributions of SRB and MA populations as determined by FISH. FISH of the biofilm sections cut perpendicular to the substratum show the localization of SRB and MA (Fig. 4A and B). SRB (white in Fig. 4A) were mainly situated at the outer layer (0-300 μm) of the biofilm while MA (purple in Fig. 4B) were mainly located in the inner layer (below 250 μm). Fig. 4 C-F shows typical FISH images of the biofilm sections cut parallel to the substratum at depths of 100 μm and 700 μm. Accordingly, SRB were detected in much higher abundance in the biofilm section at the depth of 100 μm in comparison to the 700 μm deep section (Fig. 4C vs. Fig. 4D). In contrast, there was hardly any MA at the depth of 100 μm whereas MA were dominant at the depth of 700 μm (Fig. 4E vs. Fig. 4F). The relative abundances of SRB and MA at different depths show that SRB accounted for about 20% of the total population at the surface and at 100-μm into the biofilm and the percentage decreased continuously to lower than 3% at the depth of 400 μm (Fig. 5). This distribution of SRB is consistent with the profile of the in situ sulfide production rate (Fig. 3). In contrast to the SRB distribution, the MA were detected at below 3% abundance at the surface and at the depth of 100 μm, and increased to 10% at 200 μm, 60% at 500 μm and then 75% at the depth of 700 μm (Fig. 5).

Biofilm community structure as determined by 16S rRNA sequence analysis. The 16S rRNA gene sequence analysis was applied to five layers of the biofilm, successively from the surface to the bottom of the biofilm (Layer 1 to Layer 5). The sequence reads for each layer are shown in SI Table S6. The thickness of each layer was 150 μm. The results revealed that the SRB detected in the biofilm were mainly affiliated with five genera and their proportions of the total SRB detected were: Desulfohabitus at 33%.
Desulfobulbus, Desulfomicrobium and Desulfovibrio were also observed in the inner layers 4 and 5, but SRB in these inner layers only accounted for less than 10% of the total SRB detected in the biofilm. About 90% of the MA population belonged to the genus of Methanosaeta, which use acetate as substrate rather than hydrogen. The other 10% of the MA population mainly belonged to five genera: Methanospirillum, Methanomethylovorans, Methanobrevibacter, Methanobacterium, Candidatus Methanomethylophilus. The heatmap (Fig. 6B) also demonstrates that MA was mainly located in the inner layer of the biofilm. Interestingly, Methanobrevibacter, Methanomethylovorans, and Methanospirillum actually showed higher abundance in the outer layer than in the inner layer. However, these accounted for about 5% of the total MA population detected in the biofilm, thus having only a minor effect on the overall MA distribution in the biofilm.

**Mathematical modeling.** Mathematical modeling was performed to describe the microbial distribution and the sulfide concentration profiles within the biofilms. The model-predicted relative abundances of SRB and MA fit well with the experimentally results as determined by FISH (Fig. 7A and B). The SRB abundance was 19% at the surface and decreased gradually to below 5% at the depth of 400 μm. The abundance of MA was lower than 5% at the surface and at 100 μm, increased to 65% at the depth of 500 μm and then gradually rose up to 80% at 700 μm. These results are consistent with the experimental data. The model-predicted sulfide concentration profiles within the biofilms also matched well with the data measured by microelectrode. The good agreement between the model-predicted results and the experimental data indicated that
The spatial structure of SRB and MA in the anaerobic sewer biofilms resulted from the microbial types, their metabolic transformations and interactions with substrates.

**DISCUSSION**

**The distribution of SRB and MA in anaerobic sewer biofilms.** This study investigated the distribution of SRB and MA in the sewer biofilms through both experimental and simulation analysis. The stratified distribution of SRB and MA in the biofilm was confirmed and verified using two independent molecular techniques, i.e. FISH and pyrosequencing, as well as microelectrode measurements and mathematical modelling. All the results are highly consistent. The results show that SRB were mainly located in the outer layer of the biofilm while MA was mainly situated in the inner layer. The distribution of in situ sulfide production activity was consistent with the distribution of the SRB population. The high sulfide concentration in the inner layer of the biofilm is mainly due to the diffusion transport mechanism. While the sulfide production activity in the inner layer of the biofilm is much lower than that in the outer layer, in the absence of a sulfide sink in this layer, any sulfide produced will accumulate to a level higher than that in the outer layer, providing a concentration gradient for the sulfide produced to be transferred out of the biofilm.

Under anaerobic conditions, SRB and MA are known to compete for the same substrates (primarily acetate and hydrogen) for metabolism. In sulfate-rich environments SRB can normally out-compete MA and this is commonly attributed to the different affinities for substrates of the two populations. The affinity constant for hydrogen of SRB is considered to be around five times lower than that of MA (35, 36). The difference is even stronger in the case of acetate (12, 37). However, the coexistence of SRB and MA are observed in some systems under sulfate-limiting conditions or even in
sulfate non-limiting conditions where other factors play a role. These included mass
transfer limitations (38), differences in microbial colonization and adhesion properties
(39, 40) or variable sulfide toxicities (41, 42).

In anaerobic sewers, sulfate is normally not depleted, particularly in networks with
relatively short HRT. The stratified distribution of SRB and MA suggests that mass
transfer limitation plays an important role for the coexistence of SRB and MA in sewer
biofilms. We used model simulation to determine the average concentrations of sulfate
and soluble biodegradable COD in the sewer biofilm (Fig. 8). Sulfate could penetrate
into the outer layer of the biofilm. In these conditions SRB outcompeted MA due to their
higher affinity to acetate and hydrogen, resulting in a relatively higher abundance of
SRB in the outer layer. However, the modelling result showed that sulfate was almost
consumed in the outer layer due to the high sulfate reduction activity, and thus could not
reach the inner layer (Fig. 8). As a result, SRB activity and growth was limited in the
deeper layers of the biofilm. On the other hand, soluble biodegradable COD (including
propionate, acetate or hydrogen and soluble COD which could be fermented to these
products) was not totally consumed by SRB in the outer layer of the biofilm and it was
able to penetrate into the inner layers, providing substrate for methanogenesis.
Consequently, the co-existence and stratification of these populations is largely a result
of the mass transfer of substrates into the biofilm.

The domination of MA in cores of anaerobic granules or at the inner layers of
anaerobic biofilms has previously been attributed in some studies to better attachment
characteristics of MA (40, 43). However, this cannot be a main reason in the case of
anaerobic sewer biofilms. During the startup of the sewer reactor, the sulfate reducing
activity increased much faster than the methanogenic activity in the first several weeks
(data not shown), indicating that at the beginning, more SRB were attached on the
substratum than MA, and that these were the pioneering colonizers of the biofilm. Variations of sulfide toxicities to SRB and MA are also considered as a reason for the coexistence of SRB and MA in some studies (41, 42). However, in our system, the sulfide concentration is far below toxic threshold levels to either group of microorganisms. It has been reported that sulfide concentrations of above 300 ppm are required to induce 50% inhibition of the growth of most SRB and MA (44).

The spatial arrangement of SRB and MA in sewer biofilms revealed in this study is of practical importance. Chemicals such as nitrate, oxygen, magnesium hydroxide and sodium hydroxide are often added to sewers to control the emission of hydrogen sulfide in sewers (6). As MA mainly inhabit in the inner layer of the biofilms, they are likely to be protected from being exposed to chemicals added for in-sewer sulfide and methane mitigation. Jiang et al. (45) found that sewer biofilms were capable of methanogenesis after nitrate dosing for four weeks. To explain this they suggested that nitrate was not able to fully penetrate into the biofilm and it failed to reach the MA in the deeper layer. This is supported by a complete suppression of methane production after they increased the nitrate-dosing rate. Similar results were also observed by Ganigué et al. (46), where they found methane was produced by the sewer biofilms after oxygen treatment and attributed it to the partial penetration of oxygen. Consequently, given the spatial distribution of MA in sewer biofilms, full penetration of chemicals into biofilms is required to completely control methane production. This should be an important consideration for methane abatement strategies in sewers. Due to the difficulty in obtaining intact biofilm from real sewers, it remains to be the verified if the biofilm developed in our laboratory reactor fully represents that in real sewers, despite of the use of realistic wastewater and shear conditions. Therefore, the implications discussed above need to be verified in real sewer systems.
Phylogenetic diversities of SRB and MA and their hypothesized functions. This study innovatively used pyrosequencing coupled with cryosection to investigate the phylogenetic diversity of SRB and MA in anaerobic sewer biofilms. Pyrosequencing can provide more detailed phylogenetic information than FISH. Together with cryosectioning, the phylogenetic information at different depths in the biofilms was investigated. However, it is worthwhile to note, due to a significant quantity of biomass required for pyrosequencing analysis, the biofilm sections needed for this purpose was much thicker than those for FISH (150 μm vs. 10 μm in this study). Consequently, the spatial resolution of the method was limited to layers of this size. However, this approach was successful and revealed the microbial diversity of both SRB and MA at five depths of the biofilm, allowing us to attempt to reconstruct the possible metabolic transformations in different regions of the sewer biofilm.

SRB detected in this anaerobic sewer biofilm were mainly affiliated with five genera: *Desulfobulbus*, *Desulfovibrio*, *Desulfomicrobium*, *Desulforegula* and *Desulfatiferula*. The first four genera have also been found in aerobic/anoxic wastewater biofilms, with *Desulfobulbus*, *Desulfovibrio*, *Desulfomicrobium* appearing in higher abundances (2, 14, 47, 48). Also, *Desulfobulbus* and *Desulfovibrio* are reported to be numerically important in anaerobic methanogenic-sulfidogenic aggregates (40). *Desulfatiferula* is a newly defined genus by Cravo-Laureau et al. (49) and members are mesophilic, Gram-negative sulfate-reducing bacteria.

SRB can use many different compounds as electron acceptors besides acetate and hydrogen (37, 50, 51). In the studied sewer biofilm, *Desulfobulbus* spp., with 98.4% sequence identity to *Desulfobulbus propionicus*, are a well-known propionate-utilizing SRB (2, 51, 52). As SRB tend to outcompete acetogenic bacteria for propionate utilisation due to their stronger affinity for this carbon substrate (36, 53), the high
fraction of *Desulfobulbus* in the SRB population explained the low propionate concentration in the effluent (<1 mgCOD/L). *Desulfovibrio* spp. can use hydrogen, formate, lactate, pyruvate and many other organic compounds to reduce sulfate (54). It has been suggested that *Desulfovibrio* is an important member of the hydrogen-utilizing bacteria in wastewater biofilms (2, 48). *Desulfomicrobium* spp. are also able to use various substrates such as hydrogen, acetate and lactate (55, 56). It is recognized that hydrogen, acetate and propionate are important electron donors for sulfate reduction in sewer systems (33, 34). However, in this study, we also observed the proliferation of SRB which normally grow on large molecular organic substrates rather than hydrogen, acetate and propionate. *Desulforegula* and *Desulfatiferula* are known to use long-chain fatty acids and long-chain alkenes to reduce sulfate (49, 57). Also, some *Desulfovibrio* spp. are known to use amino acids and many other organic compounds as electron donors (54, 58). It is thought that SRB can be outcompeted by very fast growing fermentative (acidogenic) bacteria for the large molecular organic substrates (50, 59). However, since fermentable COD or sCOD is abundant in sewer systems and they would not be totally used by fermentative bacteria (Fig. 7), the coexistence of SRB using large molecular organic substrates with fast growing fermentative bacteria is possible. From an ecological viewpoint, it is interesting to understand how different SRB, which use different electron donors, compete for sulfate when it is limiting. However, to date, only a few studies have addressed this competition for sulfate (51). The coexistence of different SRB in our biofilm seems to indicate that their affinities to sulfate are similar.

Though SRB mainly inhabited the outer layer of the sewer biofilm, small amounts of *Desulfobulbus, Desulfomicrobium, Desulfovibrio* were also observed in the inner layers (Fig. 6). Since sulfate did not penetrate here, the SRB in the inner layers probably grew by fermenting organic matter. *Desulfobulbus* species can ferment lactate and ethanol.
Desulfovibrio and Desulfomicrobium species grow by fermenting pyruvate to form acetate, carbon dioxide and hydrogen as products (51, 54, 55). In comparison, Desulforegula and Desulfatiferula were not detected in the inner layers, as these SRB can hardly ferment organic matter (49, 57).

Of the MA, about 90% of the population was Methanosaeta, which is an obligate acetoclastic methanogen. Therefore, acetate was likely the main substrate for methanogenesis in anaerobic sewer systems. Since acetate could be simultaneously produced and consumed in the sewer system, the accumulation of acetate is probably due to the production rate of acetate being higher than its consumption rate under the tested condition. Currently, the only genera known to use acetate for methanogenesis are Methanosarcina and Methanosaeta. However, Methanosarcina failed to inhabit in the anaerobic sewer biofilms, which is consistent with the finding that usually only one aceticlastic methanogen dominates such anaerobic environments (60). It is likely Methanoseata outcompeted Methanosarcina due to differences in their affinities for acetate. Methanosaeta is a superior acetate utilizer in that it can use acetate at concentrations as low as 5–20μM, while Methanosarcina requires a minimum concentration of about 1 mM (61). The acetate concentration in the wastewater was about 0.6 mM and would therefore not favor the growth of Methanosarcina.

MA that use other substrates such as hydrogen or methylated compounds only accounted for less than 10% of the total MA population in the sewer biofilm. The hydrogen-utilizing MA mainly belonged to genera of Methanobrevibacter, Methanospirillum and Methanobacterium. Theoretically, the relative contribution of acetate and hydrogen in methanogenesis is close to 2:1, given the fact that the fermentation of hexose yields 4 H₂, 2 acetate, and 2 CO₂ and that 4 H₂ are required to...
reduce CO₂ to methane (62). One possible reason for the low abundance of hydrogenotrophic MA in the sewer biofilms was the low hydrogen concentration in the system. This can be explained in that hydrogenotrophic MA was out-competed by the hydrogen-utilizing SRB, which have higher affinity and lower threshold values for hydrogen (35, 36). In addition, at 20°C, homoacetogenesis might occur, which could also outcompete methanogenesis for hydrogen (63). It is interesting to note that although there were more SRB in the outer layer of the biofilm, the hydrogen-utilizing MA were more abundant in the outer layer as opposed to the inner layers (Fig. 5). Though hydrogen was largely consumed by SRB, still more hydrogen was available in the outer layer, due to H₂-producing bacteria having a higher abundance in the outer layer (data not shown). *Methanomethylovorans* and *Candidatus Methanomethylophilus* are known to use methylated compounds such as methanol, methanethiol and dimethyl sulfide for methanogenesis (64-66). Their low abundance could be explained by the relatively low concentrations of these substrates in the wastewater (67, 68).

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List of Tables and Figures

TABLE 1 Daily transformation of COD, VFAs, sulfur species and methane in the sewer biofilm reactor.

FIG 1 Schematic of the laboratory-scale anaerobic, annular biofilm reactor.

FIG 2 Sulfide (A) and methane (B) profiles in the sewer biofilm reactors during a typical 8-hour cycle. The vertical solid lines at the bottom of the graphs indicate the pumping events in the 8-hour cycle.

FIG 3 Profiles of measured total dissolved sulfide, oxygen, pH and calculated sulfide production rate in the biofilm. Negative depths in the profile represent the distance from the biofilm surface into the wastewater.

FIG 4 FISH images of different sections of the sewer reactor biofilm. (A) and (B) are images of the biofilm sections cut perpendicular to the substratum with SRB in white (in A) and MA in purple (in B). Arrows indicate the biofilm surface. (C) and (D) are images of biofilm sections cut parallel to the substratum at the depth of 100 \( \mu \text{m} \) and 700 \( \mu \text{m} \), respectively, with SRB in white, archaea in red and other bacteria in green, blue and yellow. (E) and (F) are images of biofilm sections cut parallel to the substratum at the depth of 100\( \mu \text{m} \) and 700 \( \mu \text{m} \), respectively, with MA in purple, other archaea in red and bacteria in green. The scale bar is 50 \( \mu \text{m} \).

FIG 5 The SRB (A) and MA (B) proportions of total microorganisms (bacteria and archaea) detected by FISH within the sewer biofilms.

FIG 6 Heatmap displaying the distribution of the predominant SRB (A) and MA (B) in different biofilm layers from the biofilm surface to the bottom (Layer 1 to Layer 5).

FIG 7 Comparison of model-predicted results with the experimentally measured data: (A) relative abundance of SRB and MA, (B) sulfide concentration profiles in the biofilm.

FIG 8 Model-predicted sulfate and fermentable COD profiles in the biofilm.
**TABLE 1** Daily transformation of COD, VFAs, sulfur species and methane in the sewer biofilm reactor.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Unit</th>
<th>Daily transformation$^1$</th>
<th>ΔCOD (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total COD</td>
<td>mgCOD</td>
<td>-688.2 ± 29.2</td>
<td>-100.0 ± 0.0 %</td>
</tr>
<tr>
<td>Soluble COD</td>
<td>mgCOD</td>
<td>-362.5 ± 12.7</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>mgCOD</td>
<td>+49.4 ± 17.2</td>
<td></td>
</tr>
<tr>
<td>Propionate</td>
<td>mgCOD</td>
<td>-76.5 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Sulfate</td>
<td>mgS</td>
<td>-123.5 ± 12.8</td>
<td></td>
</tr>
<tr>
<td>Sulfide</td>
<td>mgS</td>
<td>+123.9 ± 11.1</td>
<td>36.0 ± 2.4 %</td>
</tr>
<tr>
<td>Dissolved methane</td>
<td>mg</td>
<td>+103.4 ± 3.2</td>
<td>60.0 ± 4.3 %</td>
</tr>
<tr>
<td>COD balance</td>
<td></td>
<td></td>
<td>-4.0 ± 2.0 %</td>
</tr>
</tbody>
</table>

1. “+” refers to production and “-” refers to consumption.
A

Biofilm surface

Desulfobulbus
Desulfromicrobium
Desulfovibrio
Desulfitaterula
Desulforegula

Layer 5
Layer 4
Layer 3
Layer 2
Layer 1

High
Low

B

Biofilm surface

Methanoaeta
Methanospirillum
Methanomethylophilus
Methanobrevibacter
C. Methanobacterium

Layer 5
Layer 4
Layer 3
Layer 2
Layer 1

High
Low