Changes of microbial biofilm communities during colonization of sewer systems

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ABSTRACT

Coexistence of sulfate-reducing bacteria (SRB) and methanogenic archaea (MA) in anaerobic biofilms developed in sewer inner pipe surfaces favours the accumulation of sulfide (H$_2$S) and methane (CH$_4$) as metabolic end products, causing severe impacts on sewerage systems. In this study we investigated the time-course of H$_2$S and CH$_4$ production and emission rates during different stages of biofilm development in relation to changes in the composition of microbial biofilm communities. The study was carried out in a laboratory sewer pilot plant that mimics a full-scale anaerobic rising sewer using a combination of process data and molecular techniques (e.g. qPCR, DGGE and 16S rRNA gene pyrotag sequencing). After two weeks of biofilm growth, H$_2$S emission was notably high (290.7±72.3 mg S-H$_2$S l$^{-1}$ day$^{-1}$) whereas emissions of CH$_4$ remained low (17.9±15.9 mg COD-CH$_4$ l$^{-1}$ day$^{-1}$). This contrasting trend coincided with a stable SRB community and an archaeal community solely composed of methanogens derived from the human gut (i.e. *Methanobrevibacter* and *Methanosphaera*). In turn, CH$_4$ emissions increased after one year of biofilm growth (327.6±16.6 mg COD-CH$_4$ l$^{-1}$ day$^{-1}$) coinciding with the replacement of methanogenic colonizers by species more adapted to sewer conditions (i.e. *Methanosaeta* spp.). Our study provides data that confirm the capacity of our laboratory experimental system to mimic the functioning of full-scale sewers both microbiologically and operationally in terms of sulfide and methane production, gaining insight on the complex dynamics of key microbial groups during biofilm development.
INTRODUCTION

Wastewater collection systems, or sewers, consist of an underground network of physical structures-installations composed of pipelines, pumping stations, manholes and channels that convey wastewaters from its source to the discharge point, usually a wastewater treatment plant (WWTP). Sewer systems thus prevent direct contact of urban population to faecal material and potential microbial pathogens, greatly reducing the spread of infectious diseases. Sewers have traditionally only been considered as hydraulic transport systems for sewage although they are in fact “reactors” where complex physicochemical and microbial processes take place. Wastewater microorganisms are diverse and abundant and they are exposed to a wide range of both inorganic and organic substrates as well as changing conditions along their transport through sewers (1). In this regard, wastewater transport through the pipes facilitates the formation of microbial biofilms that grow attached to the inner surface of sewer pipes (2). Different factors such as high surface area, low flow velocity near pipe walls and nutrient availability may favour microbial colonization of pipe surfaces and biofilm growth. Formation of fully functional biofilms occurs in different steps, from surface conditioning, adhesion of microbial “colonizers”, initial growth and glycocalyx formation followed by secondary colonization and growth (3).

Anaerobic conditions in sewer pipes favour the accumulation of both sulfide (H₂S) and methane (CH₄) as end products of different microbial metabolisms, i.e. anaerobic respiration of organic matter by sulfate-reducing bacteria (SRB) and methanogenic archaea (MA), respectively. Both compounds have detrimental effects on the sewer system with different consequences on both the installation and its surroundings (2). Accumulation of H₂S in the sewer atmosphere causes malodour in the whole system,
health hazards due to the well-known toxicity of H2S, and corrosion of both the inner surface of pipes and the inlet zones of WWTPs (4, 5). H2S accumulation also impacts the structural integrity of the sewerage by microbial-mediated corrosion processes, which severely affect the performance and cost of downstream processes at the WWTPs (2, 6). Remediation or replacement of corroded pipes require high economic investment for large systems, ranging from several hundreds to several thousands € per m depending on pipe diameter and location depth (7). On the other hand, build-up of CH4 in sewers results from the activity of MA that colonize inner pipe surfaces and develop within the biofilm matrix under strict anaerobic conditions (8–10). In addition of being explosive at low concentrations, CH4 is a major greenhouse gas with a lifespan of about 12 years and a global warming potential of roughly 21–23 times higher than that of carbon dioxide (11). Recent reports suggest that CH4 emissions from sewers contribute significantly to the total greenhouse gas footprint of wastewater systems (12, 13). Accordingly, different mitigation strategies have been used to reduce H2S and CH4 production in sewers (14–24).

Although competition between SRB and MA has been reported in some environments such as freshwaters (25), sediments (25) and WWTPs (26), CH4 production in sewers containing high sulfate concentrations was first detected by Guisasola and co-workers (8). Assuming that SRB and MA may compete for the same substrates (e.g. complex organic matter, acetate, hydrogen) their co-occurrence in sewer systems is probably the rule rather than the exception, especially considering the large amount of organic matter in wastewater and the prevalence of anaerobic conditions in many sections of the sewer networks. In biofilms, this co-existence may be explained by mass transfer processes of required substrates (e.g. sulfate and organic matter) into the biofilm matrix, which result in a physicochemical stratification along its thickness. Very
recently, Sun and co-workers (10) investigated the stratification pattern of SRB and MA in sewer biofilms thicker than 800 µm, locating the former closer to the biofilm surface and locating the latter in greater abundance at deeper, highly anaerobic layers.

Despite these findings, little information is available on the colonization dynamics and activity of SRB and MA relating to biofilm development in sewer systems. Particularly, processes behind early biofilm colonisation by SRB and MA in sewer pipes are still not fully understood. In this regard, a better understanding of how these processes take place and how they affect H2S and CH4 production rates during biofilm development is necessary to design effective biofilm-control strategies for the commissioning of sewers. This information could be crucial to the development and application of optional control-methods to reduce odour, corrosion and global warming issues generated by sewer biofilms.

The aim of this study was to investigate the initial stages of microbial biofilm development in sewer systems with a special focus on the interactions between SRB and MA. Biological activities and phylogenetic community structure during the colonisation phase were investigated using a combination of molecular techniques (DGGE, qPCR and massive parallel sequencing of 16S rRNA genes from target groups) and process data (H2S and CH4 production). The work was carried out using a laboratory sewer pilot plant fed with wastewater that reproduced a full-scale anaerobic pressured sewer. Microbial community composition was compared with biofilm from a full-scale sewer to validate the data obtained from our laboratory experiments.

MATERIALS AND METHODS

Anaerobic sewer biofilm reactor system. The study was carried out in a specially designed pilot system previously validated, the SCORe-CT method (27), that mimics...
H2S and CH4 production capacity of full-scale rising main sewers by reproducing its main characteristics including: i) hydraulic features: hydraulic retention times (HRT), turbulence and Area-to-Volume ratio (A/V), and ii) wastewater characteristics associated with real sewage. The laboratory system consisted of 3 airtight reactors (R1, R2, R3) each of them mimicking a section of an anaerobic sewer pipe (Fig. S1). Each reactor had a volume of 0.75 l, 80 mm of diameter and a height of 149 mm. The system was fed with fresh sewage (domestic fresh sewage collected in the upstream sections of the sewer network in the municipality of Girona, close to its source in households) by a peristaltic pump (Masterflex model 7518-10). Sewage was collected on a weekly basis and kept at 4ºC to minimize variation in its composition. Wastewater contained 26.5±2.6 mg S-SO4\(^{2-}\) l\(^{-1}\) and 0.1±0.1 mg COD-CH4 l\(^{-1}\). Volatile Fatty Acids (VFA) and soluble and total Chemical Oxygen Demand (COD) concentration were 42.3±8.3 mg COD-VFA l\(^{-1}\), 325.8±40.8 mg soluble COD l\(^{-1}\) and 672±93.2 mg total COD l\(^{-1}\) respectively. Sewage was heated to 20ºC before entering the reactors. Magnetic stirrers (Heidolph Mr Hei-MixS) were used to ensure homogeneous conditions and to produce a shear within the reactors. Wastewater was pumped 15 times a day in uneven periods (between 1 and 3 hours). During these intervals, wastewater was transferred from the storage tank to R1, then from R1 to R2 and, finally, from R2 to R3 in order to simulate the HRT pattern observed in a full-scale rising main used as a reference sewer pipe, the Radin collector (42.101843 N 3.131631 E, L’Escala municipality, Spain). The Radin anaerobic pipe is 2,930 m long and has 0.5 m of diameter with an HRT between 3–7 hours.

Plastic carriers (Anox Kaldnes, Norway) of 1 cm diameter were clustered on three stainless steel rods inside each reactor to increase biofilm growth surface area and to provide easily extractable biofilm samples. Taking into consideration reactor wall and
carriers, the total biofilm growth area in each reactor was 0.05 m² (Area/Volume ratio of 65 m² m⁻³). The system was operated continuously for 48 weeks. Colonization period was monitored during the first 12 weeks after start-up of the system. In addition, characterization of mature biofilms was undertaken during the 12th month after start-up. Microbial community composition of mature biofilms was compared to biofilm extracted from the upstream reference section of the Radin sewer pipe. Biofilm sample from the full-scale sewer pipe was obtained from a sewer air scour valve that was constantly in contact with the flowing wastewater. The valve was disassembled and the biofilm grown on its surface was scrapped using a sterile spatula and collected in a sterile Falcon tube containing 5 ml of Phosphate Buffered Saline (PBS, NaCl 137 mM; KCl 2.7 mM; Na₂HPO₄ 10 mM; KH₂PO₄ 1.8 mM), into which the collected biomass was resuspended. The sample tube was maintained at 4°C in a portable icebox until arrival to the laboratory (1 hour after collection) where it was immediately frozen at –20°C until DNA extraction.

H₂S generation, CH₄ production and VFA production/consumption in the laboratory system were monitored as the wastewater was transported through the system. Liquid phase sampling from R3 and off-line chemical analyses were done weekly during Normal Functioning tests (NF) for the determination of sulfur species (sulfate, sulfide, sulfite and thiosulfate), CH₄, COD and VFA. Sampling hours covered all of the HRT range (3h–7h). Also, 10 batch tests (BT) were performed to monitor H₂S and CH₄ production by biofilms. Batch tests were carried out once every 1–2 weeks. During BT the continuous operation of the reactors was stopped. The feed pump was activated for 10 minutes to ensure each reactor was filled with fresh sewage. After that, the feed was stopped and liquid samples were withdrawn every hour for a 3-hour period using a 10 ml syringe connected through a sampling port fitted with a valve and Tygon tubing.
Samples were analysed for sulfur species, CH₄, VFA and COD as described below. Using linear regression, H₂S and CH₄ production rates were calculated from the sampling-point data. A special 6-hour batch test was run in order to investigate changes of methane production depending on sulfate presence in R1 and R3. Samples were analyzed every hour over a 3-hour period for sulfur species, and every hour for a 6-hour period for methane in order to determine changes of methane production when sulfate was totally reduced to sulfide.

Daily H₂S and CH₄ emissions (calculated from NF test data) were also determined after one year of biofilm development to detect changes in activity between early and mature stages of biofilm development in the system.

Chemical analysis. Dissolved sulfide was measured continuously in R1 and R3 using an UV–VIS spectrometer probe (Messtechnik, GmbH, Austria) (28). For the analysis of dissolved sulfur species, 1.5 ml of wastewater was filtered through disposable Millipore filter units (0.22 μm pore size) and added to 0.5 ml preserving solution antioxidant buffer (SAOB) (29). Samples were analysed within 24 h in an ion chromatograph (IC) with UV and conductivity detector (Dionex ICS-5000). VFA were measured by gas chromatography (Thermo Fisher Scientific, coupled with FID detector). For CH₄ samples, 5 ml of sewage were filtered through disposable Millipore filter units (0.45 μm pore size) and injected into vacuumed glass tubes, with the help of a hypodermic needle attached to plastic syringe. After reaching liquid-gas equilibrium inside the tubes, the samples were analysed by gas chromatography (Thermo Fisher Scientific, coupled with FID detector). COD analyses were performed using a standard photometric test kit with commercially available reagent (LCK 114, Hach Lang). Absorbance readings were obtained using LCK 314 cuvette test in a
DR2800 Hach Langue spectrometer. During start up, Anox Kaldnes plastic carriers were regularly withdrawn to quantify changes in biomass content as a result of microbial biofilm formation. Biomass attached to each carrier was suspended in MilliQ water by vortexing (IKA, genius 3) until complete detachment (∼2 min). Concentration of total and volatile suspended solids (TSS, VSS) was analysed using standard methods 2510D (30). Biomass content was referred to carrier surface using volatile suspended solids values.

**DNA extraction.** DNA was extracted from biofilm biomass collected in reactor R1 and from sewage at different week intervals during the study period. Biomass attached to each carrier was suspended in 5 ml 1× PBS by vortexing (IKA, Genius-3). Suspended biomass from carriers and samples of wastewater (45 ml) were centrifuged at 11,000 rpm for 5 min at 25°C in an Eppendorf Centrifuge 5804R equipped with a F-34-6-38 rotor (Eppendorf, Hamburg, Germany). DNA was then extracted from pelleted biomass using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to manufacturer’s instructions. Genomic DNA concentrations of biofilm samples were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

**PCR amplification and 16S rRNA gene fingerprinting.** The microbial composition of biofilms formed on carrier surfaces was studied by combining specific amplification of 16S rRNA gene fragments and fingerprinting by denaturing gradient gel electrophoresis (DGGE) (31). Bacterial and archaeal 16S rRNA gene fragments were amplified from DNA extracts using primer pairs 357F-GC/907R (32) and 109(T)F/515R-GC (33), respectively. PCR amplifications (final volume of 50 μl) contained 10 μl of Buffer MgCl₂ (15 mM), 1 μl of dNTPs (10 mM), 2 μl BSA, 1 μl of
each primer (10µM), 0.25 µl of Taq Polymerase and 2 µl of DNA sample. DNA extracts were diluted with sterile MQ water to a final concentration of 10–50 ng µl⁻¹ to avoid inhibition of amplification reactions. Sequences of the different primer pairs used during the study and PCR conditions are summarized in Supplementary Tables S1 and S2, respectively.

DGGE analyses were run in an INGENY phorU-2 DGGE system (Ingeny International BV, Netherlands). Samples were loaded onto 6% polyacrylamide gels and run with 1× TAE buffer using a 30–70% (bacterial 16S rRNA) and a 30–50% (archaeal 16S rRNA) linear denaturing gradients of urea-formamide (100% denaturant agent contained 7M urea and 40% deionized formamide). A molecular ladder composed by a mixture of known SSU rRNA gene fragments was loaded in all gels to allow inter-gel comparison of band migration. Electrophoreses were run overnight at 60°C and at a constant voltage of 120 V. After electrophoresis, gels were stained for 30 min with 1× SYBR Gold nucleic acid stain (Molecular Probes Inc.) in 1× TAE buffer, rinsed and visualized under UV radiation. DGGE fingerprints were analyzed using GelCompar II (Applied Maths, Belgium). For sample comparison, a presence-absence matrix was used to calculate similarities between patterns and statistical analysis based on hierarchical cluster analysis was performed with the Dice distance and the UPGMA grouping algorithm.

DNA from excised bands of wastewater samples was eluted as previously described (34). DNA was then amplified using the same primer pairs (without GC clamp) and PCR conditions as before but sizing down the number of PCR cycles up to 20. PCR products were directly sent for sequencing on both strands to Genoscreen (Lille, France). Sequences were checked for chimeras using Uchime (35), aligned using
BioEdit (36), manually curated and then compared for the closest relatives in NCBI sequence database (http://www.ncbi.nlm.nih.gov/blast/) using the Basic Local Alignment Search Tool (BLAST) (37). Bacterial and archaeal 16S rRNA gene sequences obtained in DGGE fingerprintings were deposited in GenBank under accession numbers KR080151-KR080166.

**Real-time quantitative PCR.** Real-time quantitative PCR (qPCR) assays were used to quantify gene copies of bacterial and archaeal 16S rRNA and dsrA functional genes. All qPCR reactions were run in a Stratagene MX3005P (Agilent Technologies). For all tests, qPCR standards contained a known number of target 16S rRNA genes. qPCR for bacterial genes contained 15 μl Brilliant III Ultra Fast SYBR Green qPCR Master Mix (Agilent Technologies), 400 nM each of forward (1048F) and reverse (1194R) primers (38), 1 μl of template and adjusted to a final volume of 30 μl with MB Grade sterile water. DNA sample stocks were diluted with water to a final concentration of 10-20 ng μl⁻¹. qPCR for archaeal 16S rRNA genes were carried out using the same conditions as for bacteria but using forward primer 806F (39) and reverse primer 915R (40) and reducing the number of cycles to 35. Quantification of SRB was based on the dissimilatory sulfate reductase subunit A (dsrA) gene according to Ben-Dov et al. (2007) (41). Primer sequences, reaction temperatures, R² values and amplification efficiencies for each qPCR reaction are compiled in supplementary tables S1 and S3. All qPCR analyses carried out followed the MIQE rules for quantitative PCR analyses (42) and all essential information has been included in this section.

**Pyrosequencing and phylogenetic analyses of microbial diversity.** DNA extracts from biofilms at early stages (weeks 1, 5 and 13), mature biofilms (one-year old), and from full-scale sewers were analysed through tag-encoded FLX-Titanium amplicon
pyrosequencing at the Research and Testing Laboratory (Lubbock, TX, USA). Briefly, genomic DNA from biofilm samples was used as a template in PCR reactions using universal bacterial (28F/519R) (33) and archaeal (341F/958R) (43, 44) primer combinations complemented with 454-adapters and sample-specific barcodes. Raw sequence datasets were pre-processed at RTL facilities to reduce noise and sequencing artefacts as previously described (45). Demultiplexing according to sample barcodes, sequence quality assessments, chimera detection and downstream phylogenetic analyses were conducted in MOTHUR (46). Bacterial and archaeal curated sequence datasets were then aligned in MOTHUR using the bacterial and archaeal SILVA reference alignments, respectively, available at the MOTHUR website (http://www.mothur.org). Taxonomic classification of bacterial sequences was carried out using the RDP taxonomy reference database with a cutoff value of 80% for valid assignments. Classification of archaeal sequences was carried out using the SILVA reference database and taxonomy files using the same cutoff as for bacteria (80%). Operational Taxonomic Units (OTUs, 97% cutoff) and representative sequences of each OTU were delineated and taxonomically assigned using MOTHUR. For community analysis, the number of sequences in each sample was normalized using a randomly selected subset of 1,500 sequences (for Bacteria) and 6,000 sequences (for Archaea) from each sample to standardize the sequencing effort across samples and minimize any bias due to a different number of total sequences. These normalized sequence datasets were then used in MOTHUR to calculate α-diversity indicators of richness (Chao1) and diversity (Shannon) and to calculate community similarity among sites (β-diversity) based on weighted UniFrac distance (47). Non-metric multidimensional scaling (nMDS) analysis was performed on the UniFrac similarity matrices to visualize patterns of community composition. Relative abundance of the most populated OTUs (OTUs with relative abundances ≥4%
of total sequences in at least one sample) across samples was visualized as bubble plots using bubble.pl (http://www.cmde.science.ubc.ca/hallam/bubble.php). Pyrosequencing data of this study have been deposited in the NCBI database via the Biosample Submission Portal (http://www.ncbi.nlm.nih.gov/biosample/) under accession number PRJNA279227.

After taxonomic classification of bacteria, sequences affiliated to class *Deltaproteobacteria* were selected and further grouped into 149 OTUs (97% cutoff). Representative sequences of each deltaproteobacterial OTU were delineated and assigned using MOTHUR and then compared for the closest cultured relative using BLAST. Phylogenetic trees were constructed in MEGA 5 (48) using representative sequences of abundant OTUs, defined as those having a relative abundance ≥ 4% of total deltaproteobacterial and archaeal sequences in at least one sample and closest cultured representative sequences.

**Statistical analyses.** Statistical analyses were carried out using SPSS (version 15.0; SPSS, Chicago, IL, U.S.A.). Normality of data was assessed by the Kolmogorov-Smirnov test for values obtained during batch test and inlet wastewater (sulfate and sulfur balance). Correlation between sulfate concentration in wastewater and sulfate reduction rates was assessed by the Pearson test.

**RESULTS**

**Differences in sulfide and methane production/emission between young and mature biofilms in laboratory and full-scale sewer systems.** Changes in microbial biomass were continuously monitored for 12 weeks after the beginning of the experiment to assess biofilm formation within bioreactors (Fig. 1A). The initial biofilm
growth was detected after stabilization of biomass content in the range between 2.1–3.5 mg VSS cm\(^{-2}\).

The daily profile of H\(_2\)S measured using an UV–VIS spectrometer showed a gradual increase of H\(_2\)S production during the first 12 weeks of biofilm development in R1 and R3 (Fig. S2). The higher H\(_2\)S production rate determined in R1 compared to R3 was probably related to the low sulfate in the wastewater arriving at R3. H\(_2\)S and CH\(_4\) production rates were calculated for the same time period to assess the activity of recently formed biofilms. Figure 1B shows the H\(_2\)S production capacity within reactors in batch test experiments. H\(_2\)S production increased immediately after the start-up of the system. After the second week of operation, the capacity of the biofilm to produce H\(_2\)S stabilized between 3.5–7.7 mg S-H\(_2\)S l\(^{-1}\) h\(^{-1}\).

Sulfate reduction rates were between 3.2–7.7 mg S-SO\(_4^{2-}\) l\(^{-1}\) h\(^{-1}\), which were positively related to H\(_2\)S production rates in each reactor (Fig. S3). Differences in H\(_2\)S production showed a good correlation with the sulfate concentration in inlet wastewater (Pearson correlation index R=0.881, \(p=0.02\)). Interestingly, from week 8 to week 12, H\(_2\)S production in reactor R1 was higher than in R2 and R3. Regarding CH\(_4\) production, low rates were detected in all reactors during these early stages of development (0.08±0.11, 0.12±0.16 and 0.16±0.16 mg COD-CH\(_4\) l\(^{-1}\) h\(^{-1}\) in R1, R2 and R3, respectively).

Sulfide emission was measured weekly for 24 h to evaluate the impact of SRB activity in the system, mimicking an accurate representation of the full-scale sewer conditions. After the second week of operation, H\(_2\)S emission ranged between 195.7 and 388.8 mg S-H\(_2\)S l\(^{-1}\) day\(^{-1}\) (Fig. S4A), representing 78.6%±14.0% of the inlet sulfate. Therefore, some SO\(_4^{2-}\) was still present in the effluent wastewater (75.3±33.0 mg S-SO\(_4^{2-}\) l\(^{-1}\) day\(^{-1}\)) because not all sulfate in the influent wastewater was reduced within the
On the other hand, CH$_4$ emissions were very low (between 0 and 8.7 mg COD-CH$_4$ l$^{-1}$ day$^{-1}$) for the first 6 weeks (Fig. S4B) but increased to values as high as 44.5 mg COD-CH$_4$ l$^{-1}$ day$^{-1}$ from week 8 to week 12.

A 6-hour batch test experiment was carried out during week 14 (Fig S5) to assess if CH$_4$ production was limited by the presence of sulfate. For the first 4 h, the CH$_4$ production rate in R3 was twice that in R1 (R1=0.37 and R3=0.88 mg COD-CH$_4$ l$^{-1}$ h$^{-1}$). Remarkably, CH$_4$ production increased after 4 hours of testing (R1=1.06 and R3=2.07 mg COD-CH$_4$ l$^{-1}$ h$^{-1}$) coinciding with the reduction of all sulfate available.

High variability of VFA production rates was observed due to the simultaneous production and consumption of these compounds during batch test experiments (Fig S6A). Nevertheless, VFA production rates were remarkably low for the first two weeks of biofilm development. Furthermore, the concentration of VFA exiting the system was higher in comparison to values measured in inlet wastewater (Fig S6B).

Comparison of H$_2$S and CH$_4$ emissions measured after one year of biofilm development with those calculated during the first three months of operation in the laboratory suggested similar activity of SRB but clear differences in methanogenesis. After one year of growth, emission of H$_2$S by laboratory biofilms were slightly different (204.7±14.6 mg S-H$_2$S l$^{-1}$ day$^{-1}$) from that measured at the initial stage (316.5±61.0 mg S-H$_2$S l$^{-1}$ day$^{-1}$). This discrepancy may have been caused by differences in sulfate concentration of the inlet wastewater between the two periods (26.7±2.5 mg S l$^{-1}$ and 16.0±1.0 mg S l$^{-1}$ during the first weeks and after one year, respectively). Regardless of these differences in absolute values, mature biofilms performed better when these concentrations were compared in relative terms (around 80% and 100% of SO$_4^{2-}$ reduced to H$_2$S during initial weeks and after one year of operation, respectively).
To determine if the high production of H₂S and CH₄ in mature biofilms under laboratory conditions were similar to emissions of these compounds under natural conditions (e.g. full-scale sewers) we calculated the daily production of both compounds in both systems. Whereas full-scale sewers discharged 4.56 g S-H₂S day⁻¹ m⁻² laboratory systems produced 1.58 g S-H₂S day⁻¹ m⁻². Similar values were obtained for CH₄ production; whereas the full-scale sewer produced 4.24 g COD-CH₄ day⁻¹ m⁻² laboratory systems emitted 1.65 g COD-CH₄ day⁻¹ m⁻².

Changes in the composition of microbial communities during biofilm development. DGGE fingerprints showed compositional differences over the study period between the bacterial community in the inlet wastewater and that of biofilms grew in R1 (Fig. 2A). Even though several bands were consistently detected at different time intervals, the variation in the banding pattern suggested changes in the composition of bacterial communities during biofilm development. Hierarchical clustering of samples according to Dice similarity index clearly segregated wastewater samples from laboratory biofilms. Moreover, biofilm samples clustered according to date of collection (e.g. developmental stage). Less variation between wastewater and biofilm samples was observed for archaeal communities although similar clustering of biofilm samples according to date was distinguished (Fig. 2B). A total of 16 of the 23 excised bands (9 and 7 bands from the bacterial and archaeal wastewater communities, respectively) (Fig. S7) yielded good quality sequences. Differences in bacterial closest relatives identified and band patterns showed high variability of wastewater bacterial communities. On the
other hand, closest relatives of the identified archaea were less diverse, belonging to Methanobrevibacter smithii and Methanosphaera stadtmanae (Table S4).

Variations in bacterial and archaeal abundance in R1 biofilms were assessed by qPCR during the study period devoted to monitoring biofilm development. Although bacterial 16S rRNA gene copies were always higher than archaeal 16S rRNA copies, both genes showed similar trends in increase of copies for the first two weeks of growth followed by a steady state that suggested a balanced composition of biofilm communities for the rest of the study period (Fig. S8). Remarkably, dsrA gene abundance showed a similar time course as bacterial 16S rRNA genes (Fig. S8) suggesting a similar growth dynamics of SRB for the first two weeks of experiment.

Composition of microbial communities from R1 and full-scale sewer biofilms were assessed by massively parallel sequencing to determine whether or not H₂S and CH₄ production rates measured over time were related to compositional changes of bacterial and archaeal biofilm communities. Bacterial and archaeal 16S rRNA gene libraries were constructed using pyrotags from different samples collected during the study period (Week 1, Week 5, Week 13, One year and full-scale Sewer). Relative contribution of bacterial phyla changed during biofilm development (Fig. 3A). Furthermore, the composition of the bacterial community in one-year-old biofilms was clearly different from that of the full-scale sewer system (Fig. 3A). Sequences affiliated to bacterial classes Bacilli, Fusobacteria and Gammaproteobacteria progressively decreased during biofilm maturation. It is noteworthy that no sequences affiliated to these classes were identified in the bacterial community from the full-scale sewer biofilm. In turn, sequences affiliated to class Betaproteobacteria were prevalent in the full-scale sewer biofilm and in R1 samples collected at the first stages of biofilm development (20–26%
of total sequences) but they showed less representativeness after one year of operation (4.9% of total sequences). On the other hand, sequences affiliated to classes Synergistia and Deltaproteobacteria increased during biofilm colonization, reaching similar relative abundances as those found in full-scale sewer biofilm. Concerning archaeal communities, no archaea other than methanogens were identified in pyrotag libraries from biofilms samples. Specifically, archaeal sequences affiliated to three main genera: Methanosphaera, Methanobrevibacter and Methanosaeta. Whereas sequences affiliated to Methanosphaera (relative abundances ranging from 10 to 23%) and Methanobrevibacter (76–86%) were prevalent during the first weeks of biofilm development (Fig. 3B), the archaeal community in one-year-old biofilms was mainly dominated by sequences affiliated to genus Methanosaeta, which were also prevalent in the biofilm collected from the full-scale sewer (Fig. 3B).

Grouping sequences into OTUs (97% cutoff) resulted in 1,283 and 137 OTUs for Bacteria and Archaea, respectively (Table S5). OTU delineation allowed us to identify potentially those OTUs (i.e. species) that may make a relevant contribution in the development and activity of sulfidogenic and methanogenic biofilms. Because of the high diversity of the sample and nutrient availability in the system, OTUs were considered relevant in terms of abundance if their relative abundance was ≥4% in at least one sample. Whereas the relative abundance of some OTUs increased only at the end of the incubation period (OTU-B1, -B6 and -B7), that of others clearly decreased during this time (OTU-B3, -B8, -B10, -B12, and -B20) (Fig. 4A). One of the most prevalent OTUs in early stages of biofilm development (OTU-B3, >10% of total sequences) showed a 100% sequence identity to Macellibacteroides fermentans, a fermentative member of the Porphyromonadaceae (Bacteroidetes) (49). Other common OTUs identified during this period (e.g. OTU-B8 and OTU-B20) were rare in mature
and full-scale sewer biofilms. In turn, most prevalent OTUs in full-scale sewer biofilm were rare in the laboratory system with the exception of OTU-B1 (83% sequence identity to *Rikenella microfusus* strain Q-1, an obligate anaerobic fermentative microorganism) (50). The bacterial community in the biofilm collected from the full-scale sewer was composed mainly of microorganisms affiliated to class *Betaproteobacteria* (OTU-B2, -B14, and -B18), phyla *Synergistetes* (OTU-B4, -B5 and -B13) and *Chloroflexi* (OTU-B9) (Fig. 4A). Only OTU-B6 affiliated to class *Deltaproteobacteria* having a 99% sequence identity to *Desulfobacter postgatei* strain 2ac9.

In order to study the phylogenetic structure of the SRB community during biofilm development in more detail, sequences affiliated to class *Deltaproteobacteria*, which includes most of the sulfate reducers known to date, were retrieved and grouped into OTUs that were then used to construct a phylogenetic tree (Fig. S9A). Whereas abundant OTUs in the first weeks of incubation (OTU-D3 and OTU-D4) were phylogenetically related to *Desulfobulbus propionicus* strain DSM2032 (Fig. S10), the composition of the SRB community changed as biofilm developed. After one year of operation, the community was mainly dominated by OTU-D1 (36% of total deltaproteobacterial sequences) which showed a 99% sequence identity to *Desulfobacter postgatei* strain 2ac9 (Fig. S9A and Table S6). Although this OTU was also present in biofilms collected from a full-scale sewer, the deltaproteobacterial community in natural conditions was more diverse than that grown under laboratory conditions.

In turn, abundant archaeal OTUs (>4% of total sequences) were all affiliated to methanogenic lineages. Particularly, OTU-A1, which showed a 99% sequence
similarity to *Methanosaeta concilii*, was only detected in mature biofilms and in biofilms from the full-scale sewer (Fig. 4B and Fig. S9B). In turn, OTU-A2 and OTU-A3 were mainly detected during the first weeks of biofilm growth. Both OTUs had a 100% sequence identity to *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, respectively. Finally, OTU-A4 (showing a 99% sequence similarity to *Methanobrevibacter acididurans*) was detected at low relative abundances in all pyrotag libraries analyzed.

Richness and diversity metrics calculated for the bacterial biofilm communities increased during the experimental period (Table S7). However, the bacterial community in the biofilm from the full-scale sewer was less rich and diverse than that from biofilms under laboratory conditions. In turn, richness of archaeal community showed an opposite trend, clearly decreasing during the 13 weeks of incubation but remained at similar level in mature biofilms (Table S7). Despite these changes in richness, the archaeal diversity remained fairly constant from the start-up to the end of the monitoring period and decreased in mature biofilms. Moreover, both richness and diversity of archaeal biofilm community in the full-scale in-sewer biofilm were higher than the levels estimated from biofilms after one year of operation under laboratory conditions.

To easily compare bacterial and archaeal biofilm communities, samples were distributed in a nMDS 2D ordination space according to their similarity based on the weighted UniFrac distance (Fig. S11). The ordination segregated biofilm samples collected at early stages of development (Weeks 1, 5 and 13) from those collected in mature stages from the lab-scale and from the biofilm samples from the full-scale sewer. It is noteworthy that bacterial and archaeal communities in mature biofilms (*i.e.*
DISCUSSION

Sulfide and methane production rates during biofilm formation. In this study we investigated the association between H\textsubscript{2}S and CH\textsubscript{4} production and the corresponding biofilm development stage in a laboratory-scale anaerobic sewer pilot plant. H\textsubscript{2}S production rates suggested a fully adapted and functional SRB community after two weeks of biofilm colonization. The low production of H\textsubscript{2}S for the first two weeks may have been a consequence of the low abundance of SRB in young biofilms after the experimental set up (Fig. 4 and Fig. S8). In turn, the higher H\textsubscript{2}S production in reactor R1 compared to R2 and R3 may have resulted from the system design, considering that the bioreactors were connected in series and that wastewater that entered R2 and R3 contained only trace amounts of sulfate because of its consumption in R1.

Methane production rates measured in batch tests were minimal for the first 12 weeks probably because reactors were filled with fresh wastewater (containing high sulfate) just before the start-up of each batch test. The differences in CH\textsubscript{4} production and emission rates might be a consequence of biofilm adaptation to each reactor conditions, which mainly varied in terms of sulfate concentration and HRT. During normal functioning, the low quantity of sulfate in the R1 effluent could have promoted active methanogenesis in R2 and R3 whereas conditions in R1 (high sulfate and organic matter), in turn, favoured SRB over MA (25, 51). Results from 6-hour batch test experiment confirmed a stimulation of CH\textsubscript{4} production after 3–4 hours of wastewater retention in the system (when sulfate was depleted), especially in R3 where sulfate concentration was already low (Fig. S4). These results point to a spatial segregation of
microbial communities responsible for H₂S and CH₄ production along the length of the anaerobic sewer although no direct evidences of this differential distribution were obtained. Further work is then needed to validate if both composition and activity of SRB and MA communities in sewer biofilms vary along length in full-scale sewer systems.

**Sulfide and methane emissions by mature biofilms.** Comparison between H₂S emissions from young and from mature biofilms showed a decrease as a consequence of the lower amount of sulfate available in the influent wastewater. Notwithstanding this, the relative amount of sulfate reduced to H₂S increased in mature biofilms (from ≈ 80% to 100%). Concerning CH₄ emission, several factors could account for its increase in mature biofilms (from 17.9±15.9 to 327.6±16.6 mg COD-CH₄ l⁻¹ day⁻¹), namely: i) the low sulfate concentration in the inlet wastewater after one year of experiment favouring a higher methanogenic activity, ii) the high consumption rate of sulfate by SRB in mature biofilms stimulating CH₄ production, or iii) a change in the composition of the methanogenic community over time towards species more adapted to local conditions resulting in a higher production of CH₄.

**Compositional changes of microbial communities.** DGGE fingerprints showed differences in the overall composition of bacterial and archaeal communities between inlet wastewater and biofilm samples. Despite the inherent limitations of the PCR-DGGE approach (52), similarity analysis of both bacterial and archaeal communities based on DGGE band patterns grouped samples according to sampling date (i.e. stage of biofilm development) showing that the structure of microbial biofilm communities progressively adapted to local conditions in the system. The fact that both bacterial and
archaeal communities showed a similar clustering pattern suggest potential interactions (e.g. synergy, competition) that deserve further investigation.

During the first weeks of biofilm development, the most abundant OTUs belonging to class Deltaproteobacteria (OTU-D3 and OTU-D4) were closely related to Desulfobulbus propionicus. Interestingly, this species has recently been identified by Sun and coworkers as the main SRB in outer layers of sewer biofilms (10). D. propionicus reduces sulfate via the incomplete oxidation of organic acids such as lactate, propionate, butyrate and ethanol to acetate (53), all of them available in the inlet wastewater. In turn, the SRB community in mature biofilms was mainly composed of a deltaproteobacterium closely related to Desulfobacter postgatei (OTU-D1), whereas sequences affiliated to SRB colonizers (i.e. OTU-D3 and OTU-D4) were rare after one year of incubation (Fig. S10).

Hydrogenotrophic methanogens (belonging to orders Methanomicrobiales or Methanobacteriales) may use H₂ generated in fermentative metabolisms or act as hydrogen scavengers in syntrophic growth with acetate-oxidising microorganisms (54–57). Also, acetate produced during fermentation of organic substrates by anaerobic heterotrophs within the biofilm matrix would be used by acetoclastic methanogens (Methanosarcinaceae and Methanosaetaceae) (58). The identification of sequences belonging to both groups of methanogens (hydrogenotrophic and acetoclastic) in our experimental system during the study period lends support to a progressive change of methanogenic pathways over time in relation to both local environmental conditions and the composition of the archaeal community at each stage of biofilm development.

*Methanobrevibacter smithii* and *Methanosphaera stadtmanae* (Methanobacteriales) are considered to be the prevalent methanogens in the human gut (59). In our study,
sequences belonging to both species were identified in DGGE fingerprints from inlet wastewater samples and in pyrotag libraries from the first weeks of biofilm development, suggesting that archaeal colonizers at early stages of biofilm development derive from human fecal material in wastewater. These human-derived methanogens were probably outcompeted later on by acetoclastic methanogens (e.g. Methanosaeta concilii), which would probably be more adapted to environmental conditions in the pilot plant. The time needed by these better-adapted methanogens to get established in the biofilm matrix is consistent with the low CH₄ production during the initial phases of biofilm development. During this first stage, methanogenesis was also probably inhibited by sulfate reducers, which lower the H₂ potential pressure below levels required by methanogens when sulfate is not limiting (60). Despite the well-known competitive interaction between SRB and MA, several studies have demonstrated that both groups coexist under certain conditions (60, 61). Particularly, Struchtemeyer and co-workers reported that low levels of sulfate may favour acetate consumption by MA rather than by SRB (62). In this regard, and although it is always risky to infer functional properties from phylogeny (63), sequences affiliated to both Deltaproteobacteria and MA identified in mature biofilms were closely related to species able to use acetate (i.e. D. postgatei and M. concilii, respectively). Accordingly, the increase in CH₄ production measured after one year of incubation might be explained by the establishment of acetoclastic methanogens in the biofilm favored by a greater availability of acetate in wastewater. Besides, the increase in CH₄ production could also been favored by the stratification of both groups within the biofilm matrix as recently reported (10) although in our case no measurements aimed to resolve the spatial organization of SRB and MA in the studied biofilms were carried out.
Altogether, this study provides data that confirm the capacity of our laboratory experimental system to mimic the functioning of full-scale sewers both microbiologically and operationally in terms of H₂S and CH₄ production, and composition of microbial communities during biofilm growth. Whereas H₂S emission was notably high during early stages of biofilm development, CH₄ emissions increased after biofilm maturation coinciding with a establishment of a methanogenic community better adapted to sewer conditions; for that reason, it is important to take into account that the management of sewer systems is really important from the first stages of sewer functioning. Although further research is needed to better resolve the dynamics of the bacterial communities in biofilms and to identify the key bacterial players involved in both nutrient transformations and potential syntrophic interactions that occur in these complex ecosystems, our results should be valuable when designing optimal strategies to mitigate H₂S and CH₄ emissions from sewer systems.

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MEGA5: Molecular evolutionary genetics analysis using maximum likelihood,

fermentans gen. nov., sp. nov., a member of the family Porphyromonadaceae


Captions to Figures

Figure 1. (A) Temporal changes of microbial biomass in reactors R1, R2 and R3. (B) Sulfide production rates determined in the batch tests on reactors R1, R2 and R3 and sulfate concentration in inlet wastewater (IW, grey area).

Figure 2. Negative images of DGGE gels of 16S rRNA gene fingerprints for Bacteria (A) and Archaea (B) from wastewater and biofilms grown in R1. Hierarchical clustering of samples based on Dice similarity indexes of the banding patterns are also shown. White arrows indicate biofilm samples used in further pyrosequencing analyses (Weeks 1, 5 and 13).

Figure 3. Relative abundance of sequences (%) affiliated to (A) main bacterial classes and (B) main archaeal genera in week 1, week 5, week 13, One Year and full-scale sewer biofilm samples.

Figure 4. Bubble plots of bacterial (A) and archaeal (B) OTUs showing their relative abundances across samples, their taxonomy affiliation (at Genus level) and the percentage identity to the first BLAST hit against reference sequence databases. Data values are proportional to radius and plotted in a logarithmic scale as indicated below the graph. Relative abundance (%) of each OTU at different sampling points is indicated next to the corresponding bubble (grey figures).
A

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