Bacterial Biofilms and Suspended Matter in the Sewer System: Examination at Various Scales

J. Houhou1,2*, B.S. Lartiges1,3, C. Mustin4, J. Ghanbaja5, Z. Khalil6

1University of Lorraine, LIEC (Laboratoire Interdisciplinaire des Environnements Continentaux), UMR CNRS 7360, 54501 Vandoeuvre-les-Nancy, France
2Environmental Health Research Laboratory (EHRL), Faculty of Sciences V, Lebanese University, Nabatieh, Lebanon
3University of Toulouse (Paul Sabatier), Geosciences Environnement Toulouse (UMR CNRS-UPS-IRD), 14 Av. E. Belin, 31400 Toulouse, France
4University of Lorraine, LIEC (Laboratoire Interdisciplinaire des Environnements Continentaux), UMR CNRS 7360, Campus Sciences, B.P. 70239, F-54506 Vandoeuvre-les-Nancy, France
5Institut Jean Lamour, CNRS-Université de Lorraine, Boulevard des Aiguillettes, BP 70239, 54506 Vandoeuvre-Les-Nancy, France
6Inorganic and Organometallic Coordination Chemistry Laboratory, Faculty of Sciences I, Lebanese University, Lebanon

Abstract: The nature and architecture of suspended matter (SM) and bacterial biofilms collected in Greater Nancy sewer system were investigated along two years. Samples were taken from three sewer sections from upstream to downstream. Samples characterizations were conducted at millimetric scale using Confocal Laser Scanning Microscope (CLSM) and nanometric scale using Transmission Electron Microscopy (TEM). Several physicochemical and bacterial parameters were studied such as dissolved organic carbon (DOC), total bacteria, total suspended solid (TSS), volatile matter (VM) and particle size distribution of SM. The concentration of DOC decreases along the sewer from upstream to downstream. No clear evolution was found in total bacterial number along sewer. TSS was mainly composed of VM, and was found to decrease along the sewer. The temporal evolution of wastewater quality at a given sampling site shows no obvious change in total bacterial number. DOC and TSS concentrations were highest at midday, and decreases to reach their lowest value at 6h00. The volume size distribution of SM evolves from a multimodal distribution at upstream to a monomodal one at downstream of the sewer system. This decrease of particle size along sewer is likely related to the settling of SM and not to the degradation of suspended organic matter. Microscopic investigations indicated that SM and biofilm have similar composition (bacterial cells, extracellular polymeric substances (EPS), cellulose fibers, cell lysis detritus, organo-mineral detritus and mineral particles). Nevertheless, they have different 3D architectures. In SM, cellulose fibers form a skeleton for bacterial aggregates and biomass formation, within which bacteria may biodegrade fibers. The structural quantification revealed an open and patchy SM with 0.8 as porosity and 45% as coverage surface. Whereas, biofilms were more compact and denser than SM (porosity 0.55, coverage surface 92%). Water channels were identified through the biofilm depth with a decrease of areal porosity from the top to the bottom. Cellulose fibers were embedded in EPS matrix and bacterial division participates to biofilm growth. Viruses including bacteriophages were encountered within biofilms which may be considered as a potential reservoir of pathogenic viruses, especially during combined sewer overflows. Exchanges between SM and biofilms have been proven. On one hand by the transfer of cellulose fibers from sewage to biofilms. On the other hand by the detachment of some parts from mature biofilms and their transport in sewage.

Keywords: Suspended matter, sewer biofilm, TEM, CLSM, cellulose fiber, particle size, sedimentation

1. Introduction

Until recently, the sewer system was considered as a drainage system used essentially to evacuate wastewater from cities. It is now recognized as a biophysicochemical reactor in which sewage, bacterial biofilms and sediments may interact to provide a primary stage of wastewater treatment system. Thus, the concentrations of organic matter (OM), chemical oxygen demand (COD), and dissolved oxygen (DO) in sewage were found to evolve during wastewater transport. In addition, the formation of hydrogen sulfide and its reaction with dissolved heavy metals illustrate such a role.

Several studies indicated the evolution of bacterial biofilms in sewage pipes and its implication in the wastewater treatment. Bacterial biofilms uptake DO, OM, ammonium and nitrate from bulk sewage. These solutes are converted to biomass leading to biofilms growth and inducing a change in biofilms structure. Moreover, the physicochemical conditions in the sewer (flow velocity, anoxic and septic conditions) may influence the biofilm architecture. Mature biofilms may detach from pipe walls and re-suspend into sewage, increasing the amount of suspended matter (SM) in wastewater.

Past studies showed the evolution of SM in sewer network. Suspended organic matter may undergo fermentation and hydrolysis to give dissolved matter, the mineralogical nature of suspended minerals evolves along sewer, and SM may also be attached to sewer biofilms or settle as sediments.

Few studies have been carried out to characterize the uptake of suspended organic matter by bacterial biofilms and its transformation to biomass. Nevertheless, it still unclear how these OM are structured as solid matter within biofilms. The main objective of this work was to characterize the
architecture of sewer biofilms and SM, in order to improve the knowledge about their components, their architectures, and to underline the exchange between biofilms and SM. Thus, transmission electron microscopy (TEM) on resin-embedded samples and confocal laser scanning microscopy (CLSM) were used. TEM imaging at nanoscale gives information about the spatial distribution of microorganisms within the biofilm matrix. CLSM allows samples characterization at microscale under hydrated conditions, using a non-destructive optical sectioning within samples\cite{22,23}, allowing the reconstruction of the 3D structures of samples. Moreover, some physicochemical and biological parameters of sewage were characterized such as dissolved organic carbon (DOC), total bacterial number, total suspended solid (TSS), volatile matter (VM) and the particle size distribution of SM.

2. Experimental Section

1) Study Area

Samples were collected from the sewer network of Greater Nancy urban community, a city of about 270 000 inhabitants located in north-eastern France, lies on both banks of Meurthe river with a total catchment area of 193 Km\(^2\) (144 km\(^2\) on left bank). The sewage collecting system comprises 950 km of pipes, 250 km of them being man-entry sewer (pipe diameter ≥ 1.2 m).

The flow of wastewater in sewer pipes is turbulent (Re = 4 × 10\(^5\)) with 0.4 m/s as average velocity. Three sewer sections, each with 5 sampling sites from upstream to downstream of the sewer network, were selected for this study. These sites represent the various lands in the urban community (residential, commercial, industrial, high school, and hospital). On a general basis, the upstream sampling sites only receive domestic wastewater from a separated sewer, impacted either by hospital or industrial wastewaters. Whereas, the downstream sampling sites mainly receive sewage from high density residential areas and small businesses through a combined sewer.

2) Sample collection and preparation

Eight sampling campaigns of Grab-samples of sewage and biofilms were conducted in dry weather conditions. Sewage characteristics such as temperature, pH, conductivity, dissolved oxygen concentration, redox potential and turbidity were measured immediately upon sampling and illustrated in a previous work\cite{20}. Part of the sewage was filtered on site, and the filtrate was stored at 4°C in 65 mL glass bottles until DOC analysis using a Dohrmann 190 analyzer. 100 mL of wastewater were stored in a sterile bottle at 4°C to enumerate the total bacteria. About 1 L of sewage was taken to measure the SM size distributions. Two 24h campaigns were conducted in two different sampling sites in order to evaluate the temporal variations of wastewater characteristics.

Furthermore, five biofilm samples were collected from different manholes by gentle scraping of collectors inner walls, and each biofilm was divided into two aliquots in hermetic tubes and stored at 4°C for microscopic investigations.

About 20 L of sewage were brought back to the laboratory in a polyethylene jerrycans. After gentle end-over-end mixing of the jerrycans, 20 mL of sewage corresponding to biofilm sampling points were taken and allowed to settle in a test tube for 2 h. Settleable fractions were then aliquoted in close hermetic tubes and stored at 4°C for SM microscopic observations. 1 L of sewage was centrifuged in polyethylene bottles at 15000g for 30 min (Sorvall EvolutionRC). The mass of the TSS was then determined by drying an aliquot of centrifuged sewage at 105°C for 12 h, and the VM mass was obtained by further heating the same samples at 550°C for 4h. All samples were analyzed in triplicate.

3) Total bacteria enumeration

Total bacteria were enumerated in 35 wastewater samples using the most probable number (MPN) technique. The Luria Bertani (LB, Sigma\(^{®}\)) medium sterilized by autoclaving at 120°C during 20 min was used as growth medium. 10-fold serial dilutions in sterile NaCl 8%\(\text{w/w}\) were applied to 10 mL aliquots taken from wastewater. 25 µL aliquots of each dilution were then inoculated in 200 µL of culture medium which were introduced in sterile microplates wells. The number of replicates was 40 for each dilution. After inoculation, MPN microplates were incubated at 24°C during 48 hours. After incubation, the optical density of inoculated wells was measured at 620 nm (BioTek Instrument Inc.) and the MPN was determined using MPN calculator software 4.04(EPA).

4) Measurement of particle size distribution of suspended matter

SM particle size measurements were carried out immediately after the return to the laboratory using a Malvern MasterSizer particle size analyzer with a 1.2-600 µm particle size detection range. To avoid multiple scattering in the measurement cell, SM was diluted with the supernatant of centrifuged wastewater (15000g for 30 min, Sorvall\(^{®}\) EvolutionRC) to yield suspended solid concentrations varying from 1 to 9 mg/L. The dilution was obtained in a standard 1 L baffled reactor (90 mm diameter, 150 mm high), and the suspension was stirred with a 15 mm × 54 mm blade positioned at 1/3 of the height of the reactor. An adjustable-speed motor (Janke&Kundel RW 20 D2M) was used to provide stirring rates of 100 rpm, which corresponds to 135 s\(^{-1}\) as averaged velocity gradient G\cite{24}.

The agitated suspension was continuously withdrawn from the bottom of the reactor, and passed through the analyzer beam with a peristaltic pump located downstream the measurement cell, before being recycled to the reactor. Pumping flow rates of 35 mL/min, and a plastic tubing of 4.6 mm internal diameter, were selected. Size measurements were averaged over 1 s and taken every 2 s to allow a complete renewal of matter present in laser beam. Results obtained are given in percentage of particle volume versus floc diameter (µm).

5) Structure investigations of suspended matter and bacterial biofilm

The geometrical structures of bacterial biofilms and SM were characterized using a CLSM and TEM. Confocal microscopy is a non-destructive method, allowing in situ three-dimensional observation of hydrated samples at
microscale. It allows the examination of the complex interior-structure of stained samples with digital two-dimension image acquisitions in z-axis (optical sections), with a high resolution. Before observations, samples were stained with an Acridine-Orange buffered solution (Sigma-Aldrich, 22 μM AO, 5 mM EDTA, 0.15 M NaCl, 0.1 M phosphate-citrate buffer [pH 6]). Acridine orange behaves differently in the presence of DNA and RNA: it exhibits a maximum fluorescence emission at 525 nm (green) for an excitation wavelength of 502 nm when the dye fits between both strands of the double helix, whereas, when bound to RNA, it gives a maximum emission at 650 nm (red) when excited at 460 nm. This fluorochrome has also been widely used for visualizing mucopolysaccharid acids and glycolyzed proteins (27). The samples were stained with 100 μL of acridine orange buffered solution during 30 min at room temperature in the dark, and then washed 3 times with ultra-pure water (MilliQ plus) to remove excess of dye. Samples were placed in glass-bottom dishes (WillCo-Dish®, 0.22 mm), were observed with an inverted light microscope (Nikon TE 2000 U) equipped with a confocal head (Radiance 2100 Rainbow, Biorad). Objectives with magnification × 4, ×20, ×40 and ×60 were used to explore samples at different scales. The 488 nm and 457 nm lines of an argon ion laser, close to the excitation maxima of acridine orange, were used as light sources. The fluorescence signal of stained bacteria and exopolymers were recorded in the 530-560 nm frequency range and over 600 nm, respectively. Image stacks (512x512 pixel², corresponding to 344510 μm³) were generated using LaserSharp2000TM software (Bio-Rad Cell Science Division). During sample sectioning, the line by line excitation mode (Lambda-Strobing) was used to reduce bleed-through.

Furthermore, MacBiophotonics ImageJ software (NIH, Bethesda, Maryland, USA) was used to calculate the areal porosity, the average porosity and the coverage rate (%) of SM and bacterial biofilm. Optical sections were thresholded and transformed to binary images before the calculation of structural parameters. For each optical section, areal porosity was calculated as the ratio of the void area to the total area of the image. The coverage rate of sample to the CLSM field view was determined after z projection of all optical sections; it was calculated as the ratio of full pixels to the total area of field view.

Bacterial biofilm and SM structures were also characterized at the nanoscale using a Philips CM20 TEM. Resin-embedded samples were prepared for TEM observation. The samples, first fixed with glutaraldehyde and osmiumtetroxide, were passed through stepwise acetone dehydration, before being impregnated in a graded series ofepoxy resin monomer in acetone solutions, and finally polymerized in molds at 60 °C for 24 h. Sections (100 nm thick) were cut on a ReichertOM U2 ultramicrotome with a diamond knife, stained with lead citrate and uranyl acetate, and placed oncozer gridds for TEM examination.

3. Results and Discussion

1) Enumeration of total bacteria

Total bacteria in sewage were enumerated using the MPN method. Figure 1 illustrates the evolution of the total bacterial number along the three sewer sections (Fig. 1a, 1b and 1c) and for one diurnal cycle (Fig. 1d). Interestingly, no significant evolution of bacterial number was noticed from the upstream to the downstream of all sewers sections. In fact, this can be related to the absence of limiting factor for bacterial growth in sewage. Several studies showed that the food/microorganisms ratio is relatively high in wastewater and the concentrations of OM and DOC does not influence the bacterial growth (1, 3).

The total number of bacteria along sewer sections ranges between 10² and 10³ bacteria/mL (Fig. 1a, 1b and 1c). These values are similar to those found in literature (29, 30).

(5.6 × 10⁶ and 2.8 ± 1.8 × 10⁷ bacteria/mL). The lowest total bacterial number was obtained at the CHU sampling site (Fig. 1a), which can be attributed to bacterial lyse by the antibiotics discharged in the sewer system from the hospital. Moreover, monitoring of total bacteria for one diurnal cycle (Fig. 1d) showed a weak evolution. Total bacterial number was 2 × 10⁵ bacteria/mL at 08h15. It decreases to reach 2 × 10⁴ bacteria/mL at midday. Then, it increases to reach the initial value at 14h00. At evening (18h00), the total bacterial number decreases to reach 1.6 × 10⁴ bacteria/mL, to increase later to reach 2.7 × 10⁵ bacteria/mL at 22h00. The raise of total bacterial number at midday and at night may correspond to the use of toilets after lunch and dinner.

2) Spatial evolution of suspended and dissolved organic matter

Figure 2 illustrates the spatial evolution of DOC (Fig. 2a, 2b and 2c), TSS and VM (Fig. 2d, 2e and 2f) from upstream to downstream of the three sewer sections. Values showed that the highest concentrations of DOC (gray column) are in the upstream of sewers, whereas the lowest concentrations are in the downstream. A single clear decrease was found along the sewer in the section 1 (Fig. 2a). Two reasons can explain the decrease of DOC concentrations. On one hand, DOC may be assimilated by the bacterial biofilm, leading to the growth of biofilm biomass evoked in previous studies (1, 3). On the other hand, the decrease of DOC along the sewer may correspond only to a simple dilution by clear water, and not to a biophysicochemical process.

In fact, the identification and the quantification of water sources in sewer using isotopic signature of water molecules (δ²H and δ¹⁸O) proves the infiltration of clear water (shallow aquifer) and the percolation of rainwater within sewer system through pipe crucks (41, 42). Impoverishment of stable isotopes in wastewater (inset Fig. 2a and 2b) appears clearly along sewer and gives a dilution factor between each point (31). Thus, dilution factors may be used to correct the measured concentration of DOC (Fig. 2a and 2b white column).

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Figure 1(a, b, c): evolution of total bacterial number along the sewer sections 1, 2 and 3 respectively. (d) Evolution of total bacterial number during a diurnal cycle. Black arrows indicate the release of storm water.

Figure 1(a, b, c): evolution of dissolved organic carbon (DOC) along the sewer in sewer sections 1, 2 and 3, respectively. Insets in Fig. a and b show the evolution of δ¹⁸H-H₂O and the dilution of wastewater along the sewer by groundwater infiltration (Gray column: measured DOC, white column: corrected DOC by dilution factors). (d, e and f) evolution of total suspended solid (TSS: gray column) and volatile matter (VM: white column) along sewer. Furthermore, TSS and VM concentrations follow the same trend of DOC (Fig. 2d, 2e and 2f). On one hand, the decrease of TSS and VM concentrations can be related to the degradation of particulate OM along sewer[5,32,33]. On the other hand, this decrease may come from the sampling protocol. In fact, grab-samples were taken from the wastewater system, and SM may settle down along sewer, leading to a decrease in TSS and VM concentrations[31]. SM in stored rainwater was allowed to settle down leading to the decrease in TSS and VM concentrations[35].

Table 1: Size of largest particles (µm) obtained at each sampling-site

| Site 1 | Size | Site 2 | Size | Site 3 | Size |
|--------|------|--------|------|--------|------|
| CL     | 167  | PR     | 161  | UGC    | -    |
| CHU    | 33   | PVP    | 130  | SP     | 29   |
| JB     | 68   | RA     | 136  | SLU    | 32   |
| PV     | 54   | BL     | 160  | SFN    | 82   |
| RM     | 49   | LO     | 57   | JV     | 43   |

Figure 3: Temporal evolution of dissolved organic carbon (DOC) (a) total suspended solid (TSS: column gray) (b) and volatile matter (VM: white column) along sewer.

In all sampling sites, VM represents 80 ± 9% of TSS. This result is similar to those found in literature for suspended solid collected in dry weather[32].

3) Temporal evolution of suspended and organic matter

Figure 3 illustrates the temporal evolution of DOC (Fig. 3a), TSS and VM (Fig. 3b) in wastewater for one diurnal cycle. DOC concentration increases from the morning to the midday to reach 160 mg/L, unlike TSS and VM. After 14h00, DOC concentration decreases to reach 12 mg/L at 06h15. TSS and VM had similar trend than DOC. Lowest concentrations of DOC, TSS and VM were measured at 16h00 and after midnight. These low values were due to the dilution of sewage by discharged storm water in the sewer system from retention basin, as mentioned above[31]. SM in stored rainwater was allowed to settle down leading to the decrease in TSS and VM concentrations[35].

4) Suspended matter size distribution

The particles sizes of SM were measured along the three sewer sections using Malvern MasterSizer particle size analyzer (Table 1). Values range between 29 and 167 µm, which are similar to those illustrated in literature[36,37]. Average values of particles sizes obtained at each sewer section were 47 ± 27, 74 ± 53, and 129 ± 42 µm.
volatile matter (VM: while column) (b) for one diurnal cycle. Black arrows indicate the release of stormwater.

Figure 4: a) Relationship between larger particles (mode µm) and the concentration in TSS for samples collected at sewer sections 1 (●), 2 ( ■ ), and 3(▲ ▲ ). (b and c) Examples of particle size distribution of suspended matter in upstream (b) and downstream (c) of sewer system.

The particle sizes in all sewer sections appear to be proportional to the concentration of TSS (Fig. 4a). Moreover, the size distribution of SM evolves along the sewer system. In upstream (Fig. 4b), the volume size distribution is multimodal and suspended particles have different ranges of particle sizes (3-6, 20-30, and 130-160 µm), corresponding to different types of discharged matters in the sewer upstream such as toilet paper, excrement and vegetalespecies [34]. The volume size distributions become bimodal in the middle of the sewer (30-50 and 100-140 µm). Whereas, it is monomodal (40-60 µm) in downstream, with a slight skewness toward smaller particle sizes (Fig. 4c).

Thus, the sizes of SM evolve along the sewer to give smaller aggregates. This evolution may be related to the physical disaggregation, chemical and bacterial decomposition of SM during their transport, and to the settling down of large particles. At the end, smaller homogeneous particles remain suspended in wastewater.

5) Microscopical speciation of suspended matter

Nine suspended matter from downstream sampling points were collected. Their architectures have been explored at micrometer and nanometer scales using CLSM and TEM, respectively. Figure 5a illustrates a 2D image of a SM floc characterized by CLSM(objective × 4, zoom 1.5). Whereas, figures 5b, 5c and 5d (objective × 60, zoom 3) are projections along z-axis (central image), y-axis (bottom image), and x-axis (right image) of image stack recorded by the confocal microscope.

Figure 5 shows a typical 2D observation of SM which is composed of elongated fibers (in blue) with a diameter range between 10 µm and 20 µm, forming a skeleton-like structure at which bacterial clusters (in green) were aggregated. Cluster diameters range from 20 µm to 250 µm. Skeleton fibers may correspond to cellulose fibers from toilet paper discharged in the sewer. This hypothesis is proven after the observation of disaggregated toilet paper using an optical microscope, showing fibers with 10-25 µm as diameter. This may be related to the local flushing down of the toilet paper. Such habit can certainly impact the sewer functioning as it was established that about 15% of sewer solids correspond to toilet paper in UK [38].

Furthermore, figure 5b shows a detailed characterization of a cluster from figure 5a. This cluster is composed of bacterial cells (green), embedded in an EPS matrix (red), forming a biomass using cellulose fibers as substratrum.

Some SM contains filamentous bacteria (Fig. 5c) with a rod shape (diameter 1.5-2 µm/ length 2-4 µm). This observation was previously illustrated by Æsøy [17], showing the growth of filamentous bacteria (sphaerotilus natans) in a synthetic wastewater. Structure quantification of SM (Fig. 5c) gives 45% as coverage surface and 0.8 as areal porosity.

Moreover, figure 5d shows an aggregate with a completely different structure, comparing to those observed. It appears more compact than that illustrated in figure 5c, with 78% as surface coverage of 0.5 as areal porosity. Moreover, this SM contains fungi within their structure (inset Fig. 5d), suggesting a detachment from bacterial biofilm [39,81]. Thus, biofilm may be detached from collector walls and released into wastewater [41,43].

Figure 5: (a) 2D optical image of a suspended matter floc (objective × 4, zoom 1.5). (b) Detailed investigation of a zone from figure a. xy, xz and yz projections of CLSM stack optical sections (objective × 60, zoom 1.7). Blue: cellulose fiber, green: bacteria and red: extracellular polymeric substances (EPS). (c) xy, xz and yz projections of CLSM stack optical sections (objective × 60, zoom 3), showing
cellulose fibers (blue) surrounded by filamentous bacteria (red). (d) xy, xz and yz projections of CLSM stack optical sections (objective × 60, zoom 3). Detached and suspended fragment from bacterial biofilm contains bacteria, EPS and fungi (inset Fig. d).

Structural characterizations of resin-embedded SM by TEM (25 observations) confirm and complete CLSM investigations. Figure 6 illustrates a typical example of obtained micrographs. SM mainly contain cellulose fibers and microbial biomass (Fig. 6a). The cellulose matrix trapped dead and living bacteria, cell lyses material and organo-mineral particles. Detailed observations at nanometric scale showed an interaction between microorganisms and cellulose fibers (Fig. 6b). Bacterial cell biodegrade cellulose fibers and the resulting detritus are spread in the bacteria/cellulose interaction zones. Thus, in addition to their role as skeleton, cellulose fibers can be used as carbon source by adherent bacterial cells to ensure their growth.

6) Microscopy speciation of sewer biofilms
TEM and CLSM observations of bacterial biofilms showed that they have similar composition than SM (EPS, bacteria, cellulose fibers). Whereas, they have very different structures and 3D architectures.

The structures of three bacterial biofilms were characterized following 12 CLSM observations. Figures 7a, 7b, 7c (objective × 40, zoom 1) and 7d (objective × 40, zoom 1.5) illustrate typical projections at z-axis (central image), y-axis (bottom image), and x-axis (right image) of optical sections. CLSM observations (Fig. 7a) showed that bacterial biofilms were mainly composed of a large amount of EPS matrix (in red) in which bacterial cells are enclosed (in green) and Biofilm surface is covered by filamentous cells (inset Fig. 7a).

The coverage surface of sewer biofilm is 92% and its porosity is 0.55. Thus, biofilm is more compact and denser than suspended matter (coverage surface 45%, SM porosity 0.79). Furthermore, large amount of cellulose fibers was identified within the biofilm and deposited at its surface (Fig. 7b). Figure 7c (x and y projections) shows fibers in the depth of the biofilm. Thus, cellulose fiber may settle from sewage on the biofilm surface and they are later embedded by the EPS matrix during biofilm growth.

Detailed CLSM investigations (Fig. 7d) identified several holes within the biofilm, corresponding to water channels with an average width of 30 ± 15 µm at the top of the biofilm. The size of these holes decreases with depth, leading to a more compact and denser biofilm near the pipe wall. Water channels are involved in the nutrition and matter transfer within the biofilm. Open channels are connected to the bulk liquid, and can enhance the rate of substrate diffusion by facilitating convection when the mass transfer boundary layer closely follows the irregular biofilm surface.

Figure 6: TEM micrographs of thin stained sections of resin-embedded suspended matter. (a) Non-altered cellulose fibers (1), bacterial cells (2), cell lyses material (3) and inorganic matter (4). (b) Biodegraded cellulose fiber (1), bacteria (2), bacteria/cellulose interaction zones (3) and biodegradation detritus (4).

Figure 7: Typical xy, xz and yz projections of CLSM optical sections of bacterial biofilm stained with Acridine-Orange. Green: bacteria, red: EPS and blue: cellulose fibers. (a) Observation using objective × 20 and zoom 1. Biofilm surface covered by filamentous cells (inset Fig. a, biofilm stained by live/dead fluorochrome). (b) objective × 20, zoom 1, cellulose fibers deposit at biofilm surface. (c) objective × 20, zoom, cellulose fibers embedded in EPS matrix. (d) objective × 40, zoom 1.5, water channels within the biofilm.

Moreover, figure 8 illustrates the depth profile of biofilm areal porosity. At the biofilm top, areal porosity is high (0.8), with a patchy layer at the contact surface with wastewater. Areal porosity decreases with depth to reach 0.35, giving a denser biofilm. Marjaka (2003) has found that bacterial biofilm was composed of four layers: the bulk water phase, the heterogeneous non-dense layer, the dense layer and the porous bed (substratum). Several factors may influence the structure of biofilm in sewer system such as PO4 and flow velocity. Studying the nitrification capacity of sewer system, Åsby (1998) showed that biofilm grown under anoxic condition was brownish, fluffy, porous, uneven
and contained gas bubbles\(^{17}\). While, in septic condition biofilm was black, grainy, compact and thin.

The similar composition of SM and sewer biofilms suggests that there is an equilibrated exchange between these two compartments. SM may deposit at the surface of biofilms, thus contributing to the biofilm development. Whereas, under shear stress and at mature state, some parts of the biofilm may be detached and suspended in wastewater\(^{41}\).

Few studies have been carried out to examine the co-localisation of microorganisms and viruses in sewer biofilms. Biofilms was used as biological barriers to remove viruses from wastewater. Viruses were adsorbed to bacterial cells and EPS\(^{53}\). Moreover, a previous study showed that \(\phi X174\), MS2 and B40-8, and poliovirus-1 phages (mean size 24-30 nm) are able to attach an artificial biofilm in a drinking-water distribution system\(^{54}\). Some phages had polysaccharide-degrading enzymes, leading to cells lysis and a rapid destruction of the biofilm\(^{55}\).

![Figure 8: Areal porosity profile from the top to the bottom of bacterial biofilm](image)

Figure 8: Areal porosity profile from the top to the bottom of bacterial biofilm

Forty five TEM micrographs were carried out on five resin-embedded biofilm. Figure 9a illustrates a typical micrometer scale observation. The distribution of bacterial cells is heterogeneous\(^{21}\), with a much denser bacterial zone in the left of micrograph. Some bacteria had an elongated shape, with 1-3.5 \(\mu\)m of length and 0.5-1.5 of diameter. Circular shape bacteria were also observed, but they may correspond to a vertical slice of elongated bacteria. Several microorganisms with division septum were identified, showing bacterial cells in division phase, and the contribution of cell division into biofilm growth. Wanner and Gujer (1985) showed that bacterial cells grow within the biofilm and displace other biofilm components perpendicularly to the substratum \(^{50}\). In addition, Chen (2003) indicated that biofilm in gravity sewer contained a high ATP level and was very active\(^{51}\).

TEM observations at the nanometric scale (Fig. 9b) showed that the space between bacterial cells contained EPS matrix and some mineral particles\(^{21}\). A previous chemical speciation of these biofilms showed that they contain heavy metal-bearing particles including alloys and metal fragments, oxidized metals and sulfides\(^{10}\). These formers diffuse from bulk water into biofilm matrix\(^{52}\). TEM investigations of bacterial cytoplasm indicate the presence of polyphosphate grains and ovoid white bubbles that may be lipid reserves (Fig. 9b).

![Figure 9: Typical TEM micrographs of bacterial biofilm, thin stained sections of resin-embedded biofilm.](image)

Figure 9: Typical TEM micrographs of bacterial biofilm, thin stained sections of resin-embedded biofilm. (a) micron scale showing a heterogeneous bacterial distribution (1) and microorganisms in division phase (2), space between cells contains EPS (3), mineral particles (4), cell detritus (5) and chain viruses (6). (e) Viruses in contact with bacteria wall. Some of these viruses are hexagonal bacteriophages (inset Fig. e). Bacteria cytoplasm contains lipid reserves (8).

Thus, bacterial biofilm may be considered as a micro-niche in which viruses are concentrated. In our case, sewer biofilm may concentrate viruses originated from human excretion such as Levivirus and Allolevivirus and may be considered as a potential source of river pollution by pathogenic viruses, especially during combined sewer overflows\(^{56}\).

4. Conclusion

The results of the present study indicate that the concentrations of DOC decrease from the upstream to the downstream of sewer pipes. This decrease may be due to the assimilation of DOC by sewage bacteria or to a simple dilution by the infiltration of unpolluted water (groundwater or rainwater percolation) into collectors through wall crucks. The decrease of TSS, VM and particle size distribution along the sewer is originated mainly from the sedimentation of SM towards downstream, even if cellulose biodegradation
by attached clusters was shown by TEM. The total number of bacterial cells in sewage doesn’t evolve along the sewer, due to a high food/microorganisms ratio in wastewater.

TEM and CLSM observations indicate that bacterial biofilms and SM have similar composition (EPS, bacteria, cellulose fibers, inorganic particles). Whereas, they have very different structure and 3D architecture. SM is a patchy biological aggregate (SM porosity 0.8) in which cellulose fibers are skeleton for bacterial adhesion and cluster formations. In contrast, sewer biofilm structure appears more compact and denser (biofilm porosity 0.55) than SM, and cellulose fibers are embedded within biofilm EPS matrix. Furthermore, a dynamic exchange was identified between SM and biofilms. SM may deposit at the surface of biofilms and contribute to the biofilm development. Likewise, under shear stress and at mature stage, some part of biofilms may be detached and suspended into wastewater. Moreover, TEM investigations of sewer biofilms showed that they entrap and concentrate viruses circulating in sewage and may be considered as a potential source of river pollution by pathogenic viruses, especially during combined sewer overflow.

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