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# Microbial Characterization of Excessive Growing Biofilm in Sewer Lines Using Molecular Technique

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**Abstract** For elucidating excessive growth of biofilm that subsequently leads to the clogging problem in a small town's sewer lines of Wisconsin, the FISH method was employed. At the beginning of the simulated experiments, β-subclass proteobacteria prevailed in runs fed with industrial wastewater, while γ-subclass proteobacteria dominated in runs with domestic wastewater. However, the bacterial community structure changed significantly over six weeks; *Cytophaga-Flavobacterium* (CF)-group bacteria dominated in most runs fed with the small town's wastewater regardless of their source, while CF-group decreased strongly in run fed with domestic sewage from another city (Madison). It was also microscopically confirmed that most of those clogging materials was toilet tissue, which in turn may lead to vigorous growth of cellulose-degrading CF-group bacteria. This dominant presence of CF-group bacteria in the small town's sewer indicates that the main constituent of biofilm, toilet tissue (cellulose) in sewage, might have induced the unique pattern of their microbial community structure. Therefore, it suggests that molecular technique is useful for monitoring the clogging problems in sewer lines.

**Key words:** *Cytophaga-Flavobacterium* group, fluorescent *in situ* hybridization (FISH), sewer clogging

Attached microbial communities are often found on the surfaces of natural and engineered aquatic systems. Although they can be used in beneficial applications such as wastewater treatment systems, there are many problems associated with growth of biomass on structural surfaces, so-called biofilms. These problems include reduced heat transfer efficiency within heat exchangers, increased frictional resistance in pipes, deterioration of drinking water quality [8], and plugging of pipes [22, 24]. Because of such unwanted growth of biomass, costs for operating the involved systems increase significantly [6]. From an ecological point of view, biofilms are held together by extracellular polymeric substances (EPS) that allow the microorganisms to form stable aggregates of different cells, leading to synergistic microconsortia. This facilitates the sequential degradation of substances not easily biodegradable [10] because of their cometabolic activity.

A small town with a population of few thousands located in Wisconsin has been experiencing excessive growth of biomass in two sections of sewers influenced by wastewaters discharged from two facilities manufacturing precision metal stamping. Because of the rapid increase of biomass in the sewers, crews have cleaned the sewers frequently during warmer months. In order to determine the clogging causing materials that grow on the inner surface of sewer lines in the small town, the bacterial community structure was analyzed. Since the analysis of bacterial community composition grown on the clogging causing materials provides a clue about its chemical compounds [2], the direct identification method of bacteria, namely fluorescent *in situ* hybridization (FISH), has been applied to characterize bacterial populations not only in natural environments [1, 12], but also in activated sludge [3, 15, 16, 25], drinking water [18], and biofilm [18, 20]. In terms of bacterial populations in an aquatic environment, most bacteria are Gram-negative, and many of them belong to the class Proteobacteria and *Cytophaga-Flavobacterium* (CF) group [1]. Thus, the α, β, and γ-subclasses of proteobacteria and the CF-group bacteria in sewer plus biofilm were identified in the present study, using FISH along with monitoring conventional water quality parameters such as COD, nitrogen, phosphorus, suspended solids (TSS/VSS), and pH.

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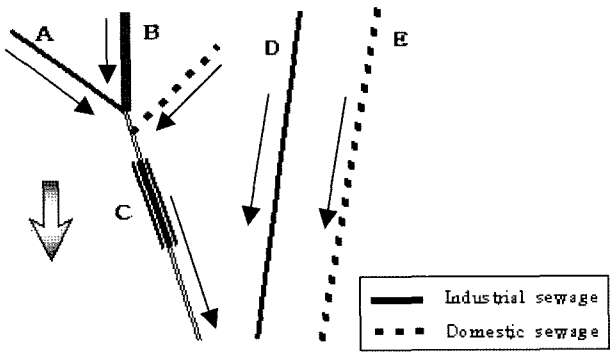


Site A : Sewage from Facility I
Site B : Sewage from other industry
Site C : Clogging site, domestic sewage mixed with site A&B
Site D : Sewage from Facility II
Site E : Domestic sewage

**Fig. 1.** Sampling sites of the industrial and domestic sewages.

## MATERIALS AND METHODS

### Conditions of Simulated Sewer Experiments

To determine which sewer has the potential for excessive biomass growth, their wastewater was sampled in 5 sites for six weeks (Fig. 1). Five samples were taken weekly for six weeks: Site A - Facility I wastewater; site B - industrial wastewater without Facility I wastewater; site C, where the clogging problem occurs at a distance of 1.5 km - industrial (sites A and B) and domestic wastewater; site D - Facility II wastewater; and site E - domestic sewage. The experimental conditions are summarized in Table 1. For comparison, domestic sewage collected in the influent channel to the primary sedimentation tank in the Nine Springs Wastewater Treatment Plant (WWTP) in Madison, Wisconsin was also tested (Run 11). At the beginning of the experiment, 490 ml of raw sewage at each site or their mixtures (Table 1) and 10 ml of biofilm as an inoculum (sewer slime) that has caused the clogging of sewer lines were added in each plastic container of size 21×29×9(h) cm. This biofilm was obtained from site C and contained a significant amount of graphite discharged by Facility I. After physically removing most part of the graphite and homogeneous mixing in a mixer, it was added to each run. For 6 weeks, 50 ml of corresponding raw sewage or their mixture were added daily to each plastic container. The containers were partially closed during tests to minimize evaporation. Although this type of setup may not closely simulate the environment of sewer lines, it was thought that bacterial populations grown on corresponding raw sewage could represent their unique characteristics.

### Physicochemical Parameters

The conventional water quality parameters such as pH, total suspended solids (TSS), volatile suspended solids (VSS), total COD, soluble COD, TKN, ammonia, organic nitrogen, nitrite/nitrate, total phosphorus, and pH were analyzed as described by the APHA [4].

### Characterization of Precipitates and Biofilms

In order to compare the elemental composition of precipitates with that of biofilm, the raw wastewater taken from site A (affected by Facility I) and domestic sewage from site E, and biofilm from site C where sewer clogging has frequently occurred were analyzed; approximately 2 l of the raw wastewater taken from sites A and E were centrifuged at 8,000 rpm for 10 min. Then, the residual solids from sites A and E and the biofilm from site C were filtered with 0.45 μm filter, dried at 105°C for 2 h, and analyzed using a scanning electron microscope equipped with an energy dispersive X-ray detector (JOEL, JSM 6100).

Furthermore, to calculate the proportions of bacterial biomass to entire biofilm, the total bacterial cell surfaces on 10 randomly chosen scanning electron micrographs of biofilm were manually measured using millimeter paper. These results were expressed in ratio (%) of total bacterial surfaces to total biofilm surfaces.

### Characterization of Bacterial Community Structure

The analysis of bacterial communities in each run and biofilm was performed using FISH [15, 18] at the beginning

**Table 1.** Experimental conditions.

| Run no. | Sample | Temp., °C | Remarks |
|---|---|---|---|
| 1 | A | 20±4 | Only sewage discharged from Facility I |
| 2 | B | 20±4 | Other industrial sewage |
| 3 | C | 20±4 | Domestic sewage mixed with sample A+B |
| 4 | D | 20±4 | Only sewage discharged from Facility II |
| 5 | E | 20±4 | Only domestic sewage |
| 6 | A+B+E | 20±4 | Mixed sewage of A+B+E in volume ratio of 1:0.5:0.5 |
| 7 | A+B+E | 8±4 | The same sewage mixture as Run 6, but incubated at 8°C |
| 8 | A+B+E | 20±4 | The same as Run 6, but only supernatant of A's added |
| 9 | B+E | 20±4 | Mixed sewage of A+B+E in volume ratio of 1:1 |
| 10 | A+D+E | 20±4 | Mixed sewage of A+D+E in volume ratio of 1:0.5:0.5 |
| 11 | Comparison sample | 20±4 | Domestic sewage obtained from the Nine Springs WWTP, Madison, WI. |

**Table 2.** Variations (mean value) of conventional water quality parameters in raw sewages over time in each run.

| | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | Run 6–8 | Run 9 | Run10 | Run 11 |
|---|---|---|---|---|---|---|---|---|---|
| pH | 6.6–8.4 (7.7) | 7.3–9.1 (7.8) | 6.8–8.5 (7.5) | 7.2–8.5 (7.7) | 7.1–8.0 (7.5) | 7.2–7.5 | 7.7 | 7.6 | 7.5 |
| TSS (mg/l) | 20–135 (64.8) | 50–365 (137.8) | 20–345 (188.3) | 32–165 (69.5) | 85–678 (297.2) | 128 | 183 | 148 | 248 |
| VSS (mg/l) | 13–57 (30.7) | 35–315 (121.3) | 13–148 (98.3) | 13–130 (41.3) | 75–643 (265.3) | 103 | 170 | 113 | 203 |
| Total COD (mg/l) | 18–184 (94.5) | 88–801 (297.5) | 41–456 (260.8) | 78–268 (140.7) | 264–865 (635) | 217 | 347 | 303 | 587 |
| Soluble COD (mg/l) | 6–78 (44.8) | 4–131 (59.2) | 9–57 (38.5) | 56–220 (116.8) | 111–445 (275.8) | 96 | 114 | 115 | 239 |
| TKN (mg/l) | 7–49 (18.8) | 24–49 (35.7) | 16–27 (19.7) | 4–16 (10.3) | 30–56 (47) | 24 | 39 | 20 | 51 |
| $NH_4^+$-N (mg/l) | 0–5 (3.2) | 16–32 (23.8) | 5–13 (10.2) | 1–11 (3.7) | 20–36 (28.2) | 12 | 20 | 8 | 36 |
| Organic N (mg/l) | 3–46 (15.7) | 5–25 (12) | 5–15 (9.7) | 0–13 (6.5) | 10–24 (18.5) | 12 | 18 | 12 | 15 |
| $NO_2/NO_3^-$ (mg/l) | 20–158 (75.5) | 0–4 (3) | 14–62 (32.5) | 40–371 (196.7) | 1–5 (3.5) | 8 | 1 | 27 | 1 |
| TP (mg/l) | 0–50 (13.2) | 2–7 (5) | 2–36 (14.7) | 0–3 (0.5) | 1–5 (2.3) | 14 | 3 | 15 | 8 |

prior to the addition of biofilm and at the end of the experiment. The mean values for each group-specific bacteria and total cell counts were calculated from the counts of 15 randomly chosen fields using epifluorescence microscopy (Zeiss, Axioplan 2), and the results were expressed in ratio (%) of the number of individual group-specific bacteria to the number of total bacteria [11]. The total cell counts in each run were enumerated biweekly.

## Results and Discussion

### Physicochemical Parameters

Ranges and average values of pH, total suspended solids (TSS), volatile suspended solids (VSS), total COD, soluble COD, TKN, ammonia, organic nitrogen, nitrite/nitrate, and total phosphorus for raw sewages added to each run over 6 weeks are summarized in Table 2. Sites A and D (affected by Facilities I and II) presented a lower concentration of TSS, VSS, and total and soluble COD values than other sewage. By contrast, TSS and VSS values at site E (domestic sewage) were generally greater than other sewage samples but varied significantly, and their total and soluble COD represented the greatest values among the five samples (sites A to E). Other industrial wastewater (site B) had the second highest total COD values. Considering the medium strength COD is 500 mg/l [23], the sample from site C, where clogging problem frequently occurred, represented the lowest strength of soluble COD (38.5 mg/l) and a rather weak strength of total COD (261 mg/l) compared with those of domestic sewage in site E (soluble COD, 276 mg/l; total COD, 635 mg/l). Furthermore, the differences between soluble COD and total COD in other industrial wastewater (site B) and sites C and E where domestic sewage are present at least in part were greater than those in sites A and D. It implies that those small town's municipal wastewater may contain much more insoluble organic materials.

Regarding the supplies of N and P, samples from Facilities I and II (Runs 1 and 4) had the lower TKN and ammonia values but higher organic nitrogen and nitrite/nitrate concentrations than other sewage. However, site A (Run 1) had higher total phosphorus concentrations than other sewage for the first two weeks. It suggests that the sporadically jumped value of phosphorus with higher organic nitrogen and nitrite/nitrate concentrations in sewage discharged from Facilities I and II may lead to excessive biomass growth when available C-source might concurrently be present. The COD:N:P ratio for the six-week average of raw sewages in each site was 100:99:13 (site A), 100:13:1.7 (site B), 100:20:6 (site C), 100:147:0.3 (site D), and 100:7.9:0.4 (site E). Depending on the balance of nutrient supplies, sewer from sites B and C may indicate the significant potential for the biomass growth, whereas sewers from Facilities I and II (sites A and D) are limited by the carbon source.

Additionally, pH, TSS, VSS, and COD were analyzed in each run at Weeks 0 (initial), 4 (intermediate), and 6 (final) just before adding fresh sewage mixtures (data except soluble COD not shown). However, it was extremely difficult to take representative samples, because of the inhomogeneous distribution of biomass and different evaporation rates in each run; therefore, the results expressed in concentration were not so reliable, as shown in Fig. 2: The value of

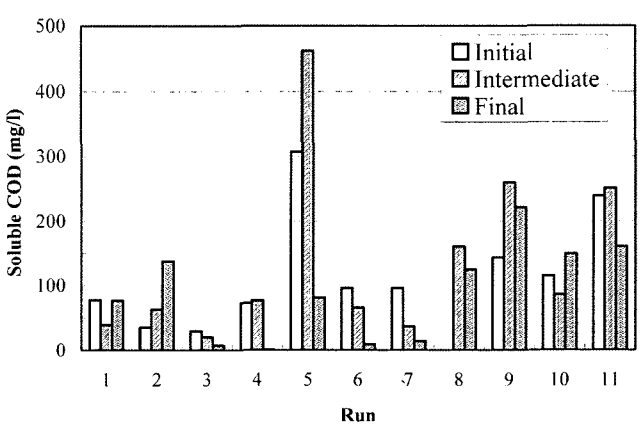


**Fig. 2.** Soluble COD variations in each run for 6 weeks.

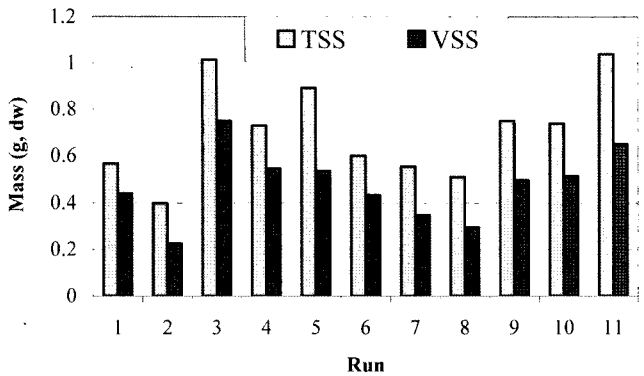


**Fig. 3.** Total TSS and VSS accumulated for 6 weeks, determined by calculating the entire biomass in each container.

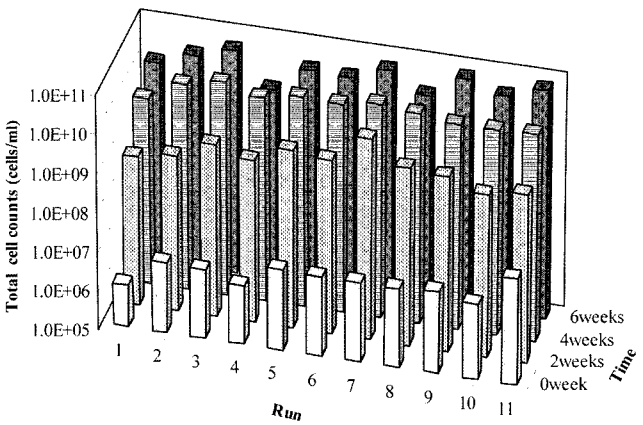


**Fig. 4.** Variations of total cell counts over time (0–6 weeks) in each run, determined by counting DAPI-stained cells using epifluorescence microscopy in 11 runs.

soluble COD over the course of time (0, 4, and 6 weeks) varied widely, even in the same run. Consequently the final biomass (VSS & TSS) was measured using the entire content in each container after 6 weeks (Fig. 3). The Run 11 (comparison run) fed with Madison's WWTP raw sewage had the highest TSS, followed by Run 3 (site C) and Run 5 (site E) fed with municipal wastewaters. In terms of VSS, Run 3, with the clogging causing sample added, had the greatest, followed by Run 11. It implies that the clogging material may consist of organic compounds. TSS and VSS were not different between Run 9 (sites B+E) and Run 10 (sites A+D+E), implying no difference due to the kind of industrial wastewater. However, among the same sewage mixtures (Runs 6–8), TSS and VSS were greater at 20°C (Run 6) with site A's raw sewage, probably because of larger enzyme activities of bacteria due to increased temperature than at 8°C (Run 7). Therefore, temperature appears to be one of the main factors leading to vigorous biomass growth in that small town's sewer lines, in support of claims by crews that the clogging occurred only in warmer months. Furthermore, the evaluation of conventional water quality parameters showed no significant differences between runs related to the clogging problem.

### Characterization of Precipitates and Biofilm

The elemental compositions of precipitates and biofilm are summarized in Table 3. It appears that biofilm from site C was affected by Facility I wastewater, since the elemental composition of the biofilm resembled those of Facility I. By contrast, precipitates in domestic sewage at site E had higher calcium and silicon contents, but lower phosphorus content, than precipitate from Facility I wastewater, thus indicating that excessive biomass growth in site C, where facility I wastewater contained sporadically high phosphorus and nitrite/nitrate flows (Table 2), might have occurred.

### Characterization of Bacterial Community Structure

Variations of total cell counts over time in 11 runs are shown in Fig. 4. The initial number of total bacteria stained with DAPI in each run varied from $1.2\times10^6$ (Run 1; Facility I wastewater) to $55.1\times10^6$ cells/ml (Run 11; Madison's WWTP raw sewage). At the beginning of the experiments, the total cell counts were higher in solely municipal wastewater (Runs 5 and 11) and combined wastewater with municipal sewage (Runs 6–10) than those in Facilities I and II

**Table 3.** Comparative analysis of elemental composition in precipitates and biofilm determined by SEM-EDS. Results are expressed in %.

| Element | Sample no.1<br>Precipitate from site A (Facility I wastewater) | Sample no. 2<br>Biofilm from site C | Sample no. 3<br>Precipitate from site E (domestic sewage) |
|---|---|---|---|
| Ca | 21.76 | 25.32 | 37.78 |
| Si | 5.05 | 11.31 | 22.21 |
| P | 40.10 | 22.59 | 11.33 |
| Fe | 10.79 | 19.02 | 10.12 |
| Al | 15.14 | 7.39 | 8.96 |
| S | 0 | 2.81 | 4.81 |
| Mg | 2.68 | 6.50 | 3.46 |
| Na | 1.56 | 0 | 1.32 |
| K | 2.29 | 1.63 | 0 |

wastewater (Runs 1 and 4). The total cell counts in other industrial wastewater (Run 2) was relatively high ($6.2\times10^6$ cells/ml). After 6 weeks, a comparatively minimal value was obtained from Run 4 (Facility II wastewater; $0.7\times10^{10}$ cells/ml) and a maximum value from Run 11 (Madison's WWTP raw sewage; $7.6\times10^{10}$ cells/ml), followed by Run 10, Run 7, and Run 3 where the clogging problem occurred. Generally, the numbers of total bacteria were greater in mixed sewages (Runs 6–10) than in Facility I and II wastewater (Runs 1 and 4), and it appears that there is a synergistic effect of sewage mixture on biomass growth.

Overall, the numerical difference of total cell counts between runs fed with the small town's wastewater over 6 weeks was not too critical to distinguish the biofilm forming potential of each other. There was also no difference in total cell counts between the small town's wastewater and Madison's WWTP raw sewage, although they represent different biofilm-forming potential *in situ*. This is probably due to the fact that biofilm consists not only of bacterial cell biomass, but also EPS [6]. The measured total bacterial cell surfaces on 10 randomly chosen scanning electron micrographs to total biofilm surface (Fig. 6e/f) reached only 16–32% (data not shown), similar to the report that the cell biomass constitutes only a minor fraction of the organic matter in the biofilms [13]. Therefore, the total cell counts may not appear to clearly indicate the biofilm-forming potential in each run.

### Comparison of *In Situ* Bacterial Community Structure in Sewages and Biofilm

The proportions of the α-, β-, and γ-subclasses of proteobacteria and the CF-group bacteria to total cell counts (DAPI) at the beginning of the experiment (0w) are shown in Fig. 5. While the β-subclass proteobacteria was the dominant group in Facilities I and II wastewater samples (Run 1: 56%, Run 4: 27%) and other industrial wastewater (Run 2: 35%), the γ-subclass proteobacteria was more abundant than β-subclass proteobacteria in mainly municipal sewage (Run 5: 63% and Run 11: 36%) and domestic sewage mixed with wastewater from Facilities I and other industry (Run 3: 41%). These results are in good agreement with other studies that β-subclass proteobacteria dominated in oligotrophic freshwater, where metabolic activities of bacteria were concurrently determined [1]. It was further noted that γ-subclass proteobacteria grew well in such nutrient-rich environment [19], and then these made up the major parts of fast growing and cultivated bacteria on nutrient media [16, 25].

In contrast to the numerical domination of β- and γ-subclass proteobacteria in raw wastewater, the CF-group bacteria accounted for 43% of total bacterial populations in biofilm (Run 12). As reported in other studies on its prevalent occurrence of reed biofilm in lakes [5] and biofilm in rivers [20], CF-group bacteria made up substantial proportions in biofilms. Their dominant presence in biofilm is probably due to their activities in the decomposition of biofilm-forming materials [5, 11, 20]. The α-subclass proteobacteria in biofilm accounted for 5%, β-subclass proteobacteria 31%, and γ-subclass proteobacteria 16%. Many filamentous bacteria belonging to β-subclass proteobacteria were also observed in the biofilm. It was morphologically identified as *Sphaerotilus natans*, of which sheathed filaments represent false branching, and is frequently found in sewage and biofilm [7] according to the key of Eikelboom [9] and Jenkins *et al.* [14].

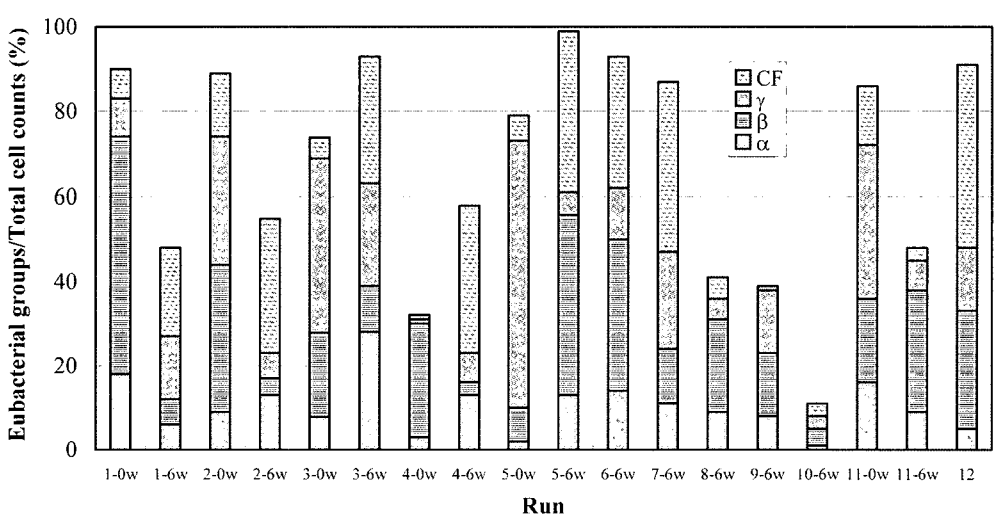


**Fig. 5.** Changes of bacterial community structure in sewage from the initial sample (0w) to 6-weeks sample (6w) at different sites (Runs 1–5: small town raw sewage; Runs 6–10: mixed sewage; Run 11: for comparison, Madison's WWTP raw sewage; Run 12: biofilm) expressed in ratio (%) of the number of individual group-specific bacteria [α-, β-, and γ-subclass and *Cytophaga-Flavobacterium* (CF)] to the total cell counts.

After 6 weeks of biofilm culturing with daily addition of raw sewage, the changes of bacterial community structure were compared with the structure in the initial sample (Fig. 5). The proportion of CF-group bacteria increased greatly in most runs fed with the small town's sewers, independent of the kind of the wastewater. Their ratio to total cell counts increased from 7% to 21% in Run 1, from 15% to 32% in Run 2, from 5% to 30% in Run 3, from 1% to 35% in Run 4, and from 6% to 38% in Run 5, similar to its proportion in biofilm (43%). On the contrary, the proportion of CF-group bacteria in Run 11 (comparison run) decreased greatly from 14% to 3%, although the same biofilm from the small town's sewer as an inoculum was added at the beginning of the experiment. These changes indicate the different characteristics of raw sewage at the Madison's WWTP (Run 11) from those of the small town's. Therefore, it suggests that the small town's wastewater containing large amounts of specific organic materials is preferentially decomposed by CF-group bacteria. It is noted that the CF-group bacteria are involved in the mineralization of more slowly degradable macromolecules such as proteins, carbohydrates, and pesticides [11].

To elucidate the specific organic materials causing the dominance of CF-group bacteria, the biofilm was dissected and observed under a phase-contrast microscope (Fig. 6a/b/c) and SEM (Fig. 6d/e/f). The large amounts of toilet tissue fiber that makes the backbone of the biofilm were observed (Fig. 6a). The origin of filaments was confirmed to be toilet tissue fiber by observing under a microscope (Fig. 6a/b). Moreover, these micrographs indicate that the biovolume

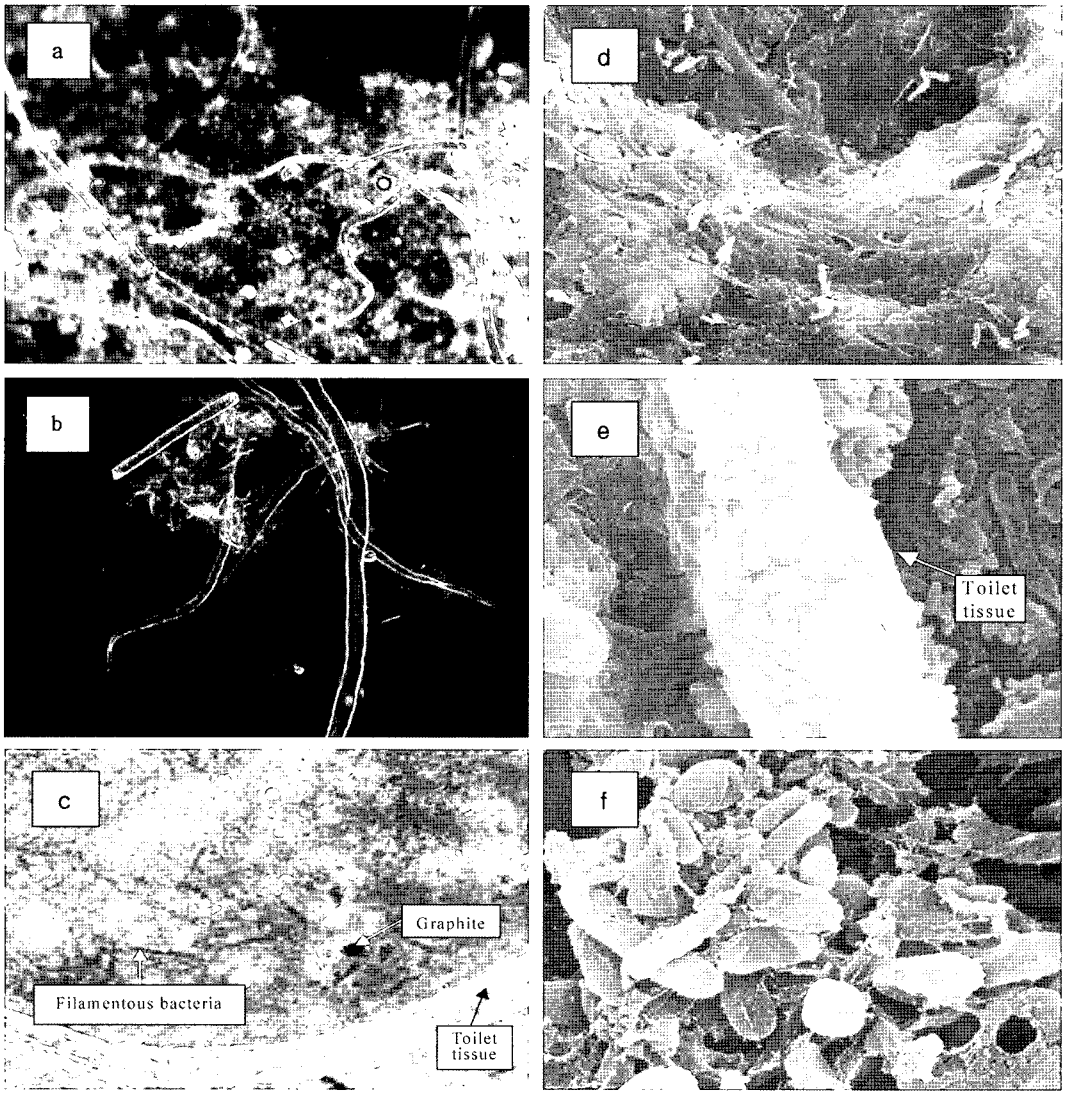


**Fig. 6.** Phase-contrast (a/b/c) and SEM (d/e/f) micrographs of biofilms occurred in the small town's wastewater.
Phase-contrast micrographs of biofilm (a; ×100), dissolved toilet tissue sample (b; ×100), detailed part of a (c; ×630), and SEM (d; ×2,500, e; ×4,500, f; ×15,000).

of toilet tissue fiber and EPS may be much greater than bacterial biovolume in biofilm (Fig. 6e/f), since the total bacterial surface reached only 16–32% of entire biofilms. A lot of wood fibers has been reported to be frequently observed in raw sewage of the small town [21], hence the accumulation of toilet tissues available as a carbon source in the sewer probably induced the excessive growth of cellulolytic bacteria and formation of EPS [13] including extracellular enzymes to degrade toilet tissue composed of cellulose. Moreover, filamentous organisms such as *Sphaerotilus natans* occurring frequently in those biofilms along with viscosity of EPS may have facilitated the aggregation of suspended particulates [10], including graphite particles, discharged from Facilities I and II (Fig. 6c). In addition, the relatively high concentration of nitrite/nitrate and total phosphorus in Facilities I and II wastewaters (Table 2) may contribute to proliferation of cellulolytic bacteria belonging to CF-group bacteria and consequently increases of biomass, especially during the warmer months, probably because of increased bacterial metabolic activities.

Considering the potential of biomass growth between runs after 6 weeks, wastewaters from Facilities I and II (Runs 1 and 4) alone are not more favorable for bacterial growth than other domestic sewages. It was found that inactive cells with low ribosome content were not detectable with FISH [12, 18, 19]. Additionally, Fig. 5 shows that Facilities I and II wastewaters (Runs 1 and 4) or the other industrial wastewater (Run 2) represented lower detectability by FISH than the domestic sewage (Run 5; site E) and Facilities I and other industrial wastewater mixed with domestic sewage (Run 3; site C), since Runs 3 and 5 revealed high TSS/VSS values over the course of the experiment (Fig. 3). Also, the increased cell detectability by FISH during the experiments indicates the raw sewages added in Runs 3 and 5 provided better conditions for bacterial growth than those in other runs. Moreover, Run 10 composed of Facilities I and II wastewaters and domestic sewage had the lowest cell detectability by FISH among the 11 runs, followed by Run 9 (sites B+E) but their differences were remarkably large, even though these two runs represented no significant differences in TSS and VSS (Figs. 3 and 5). The lowest detectability by FISH in Run 10 with Facilities I and II wastewaters may suggest the strongly reduced metabolic activities of bacteria and consequently the reduced potential of biomass growth over 6 weeks under the given condition.

Among the combined wastewaters in Runs 6 through 10, domestic sewage mixed with Facility I and other industrial wastewater (sites A+B+E; Runs 6 and 8) provided more favorable conditions than the mixture of other industrial wastewater and domestic sewage (sites B+E; Run 9) and the wastewater mixed with Facilities I and II wastewater and domestic sewage (sites A+D+E; Run 10). However, when only the supernatant of Facility I wastewater was added (Run 8), the detectability by FISH was relatively lower, due to metabolic activities of bacteria [12]. Furthermore, the mixture of the small town's wastewater in Run 10 did not induce the dominant growth of CF-group bacteria, whereas Runs 6 and 7 did. When precipitates of Facility wastewater I were present in the feed wastewater, the cell detectability by FISH was greater (Run 6; 96%) than when only supernatant was added (Run 8; 41%), indicating that phosphorus and other precipitates contained in the metal stamping facility wastewater may provide a better condition for bacterial growth.

In summary, CF-group bacteria dominated in most runs fed with sewages from the small town, whereas those bacteria in Run 11 (comparison run) fed with raw sewage from the Madison's WWTP decreased significantly. The dominance of CF-group bacteria is most probably due to the presence of toilet tissue accumulated in the small town's sewer. The frequent occurrence of clogging problem in warmer months is most likely due to the fact that the increased temperature in summer may facilitate massive production of extracellular enzymes to degrade toilet tissue (cellulose), which belongs to not easily biodegradable substances [10]. Although the FISH method using group-specific oligonucleotide probes provided information not on the species level, but on a rougher scale, it appears to be a useful tool for differentiating the bacterial populations grown on clogging causing organic materials in sewers. The FISH method introduced in this study, one of the advanced molecular techniques normally used for the quantitative analysis of bacteria, provided more precise information on clogging problems associated with bacterial growth in sewer lines. By contrast, the determination of conventional water quality parameters, which have generally been used in environmental engineering, did not reveal any distinguished characteristics associated with such problems, probably due to difficulties of exact simulation.

Furthermore, as reported that the flow velocity should be greater than 1 m/sec to reduce the growth of biofilm due to the shear force [6, 24], the flow rate of sewage in the small town could also be a factor leading to accumulation of the above-mentioned compounds and excessive growth of biofilm inside the sewer lines. According to the Manning equation, $V=n^{-1} R^{2/3} S^{1/2}$, the minimum slopes (S) for gravity-flow sanitary sewers at n (roughness coefficient)= 0.013 are 0.0033, 0.0025, and 0.0019 while at n=0.015 0.0044, 0.0033, and 0.0026 for hydraulic mean radius (R) at 20.32 cm, 25.40 cm, and 30.48 cm, respectively [23]. The sections of sewers experiencing excessive biomass growth had lower slopes (0.002–0.0022) than other sewers (0.0028–0.004). Considering the slopes of the existing sewers (personal communication), it is likely that the velocity (V) in the sewer was so slow that excessive biomass growth might have occurred. Further detailed analysis and experiments are needed to confirm this slope issue.

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