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Role of Biofilm in Rainwater Tank

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Abstract

In order to establish the role of biofilm in rainwater tank, it was investigated the phylogenetic distribution of the bacteria present in an operating rainwater tank. Most of the bacteria were closely related to fresh water, soil, and biofilm bacteria found in natural environments. The high proportion of proteobacteria indicates the generally clean oligotrophic nature of the tank water. To better understand the environmental conditions in rainwater tanks and the development of biofilms therein, the changes in biofilm cells and the bacterial community were investigated during biofilm development. We confirmed that the biofilm development process takes place in three stages: an initial stage characterized by the colonization of different populations, an intermediate stage characterized by a limited number of dominant populations utilizing similar resources, and a late/mature stage characterized by mature biofilms of a complex spatial structure. It was investigated microbial behaviour after inoculation of the bacterium, *Pseudomonas aeruginosa*, in pilot and full-scale rainwater tanks with different surface-to-volume (S/V) ratios. Ninety-nine percentage of the inoculated *P. aeruginosa* had been removed from the water phase. The faster removal rate in pilot and full-scale tank was due to its higher S/V ratio. From the results, several recommendations for tank design and management were suggested.

Keywords: bacterial composition, bacterial community, biofilm, biofilm development, CLSM, DGGE, microbial quality, *P. aeruginosa*, rainwater tank, surface-to-volume ratio

1. Introduction

Almost about one billion people in developing country suffer from water problem. Accordingly, rainwater harvesting is becoming now one of the major alternatives to tackle water scarcity and spreading to not only in developing country but also urban and remote rural communities

in developed country in the world. Rainwater management system has an advantages such as simple technology, low cost and low-energy consuming, but rainwater use is limited by uncertainty about rainwater quality, and especially its microbial quality [1, 2].

Krampitz and Holländer [1] concluded that tank cleaning was contra-productive and Deutsches Institut für Normung (DIN; in English, the German Institute for Standardization) recommend that people do not clean the rainwater tank <10 years. About 13% of all Australian households use rainwater tanks as a source of drinking water [3]. This study was motivated by those questions why the water quality was poorer after cleaning tank and why they are safe in spite of using untreated rainwater.

In the case of roof-harvested water, contamination could mainly occur on the roof collection system or in the storage facility [4]. The contaminant input is limited only from catchment area, and its management is very important for water quality in rainwater tank. Most of the contaminants come into the rainwater tank which is removed by sedimentation [5] and sludge generated thereby is able to lower the water quality by resuspension. Application of simple design such as sludge drain, calm inlet, intermediate wall, and baffle can control the contaminant in rainwater tank to the certain level [6].

Biofilm is one of the factors influencing the rainwater quality in tank. Many researches showed that presence of biofilm includes negative effects, such as biofouling in filter and biocorrosion and biocontamination in drinking water distribution networks, but also positive effects such as biofilm reactors for the degradation or production of chemical substances in wastewater treatment process [7–9]. It has been suggested that biofilm may have a function of self-cleaning of the tank and regulation of the microbial quality in rainwater [1, 2]. Although biofilm might have a positive impact on stored rainwater quality, only few studies investigated bacterial composition and distribution, its development and role in this particular environment. Through the research on these characteristics of biofilm in rainwater tank, it is possibly suggested a better information to improve the rainwater system in management and design perspectives

In this chapter, to establish the role of biofilm in rainwater tank, (1) it was investigated the kinds of bacteria that inhabit rainwater tanks, (2) the changes in the biofilm cells and the bacterial community during biofilm development, (3) the microbiological characteristics of rainwater in two tanks with different S/V ratios to identify how the internal design features of storage tanks affect the microbial quality of rainwater, and then (4) suggested design and maintenance guideline for rainwater tank.

2. Method and materials

2.1. Study sites

This study was carried out at Seoul National University in Seoul, Korea (**Figure 1**). In order to investigate the microbial community and how biofilms are developed and in operating

rainwater tank, the choice of sites for sample collection was made mainly with regard to the availability of the rainwater facility.

The system 1 was built in November 2003 and consists of a 200 m³ concrete storage tank located underground and a 2098 m² roof catchment area. The harvested rainwater supplies to the toilets of 167 households and a garden [10]. In this system, the study about microbial community was performed.

The system 2, which was constructed in October 2005, comprises a 250 m³ main storage tank, a 27 m³ smaller extra tank, and a 4 m³ supply tank located underground. The catchment area is a concrete roof surface with a total area of 2828 and 824 m² terrace. Rainwater collected from the roof of one part of the building (960 m²) flows into the main tank and that from the roof of another part of the building (1868 m²) and the aforementioned terrace flows into the extra tank. When the water in the extra tank reaches 1.2 m in depth, it is pumped into the main tank. About 1000 full-time staff and students occupy the building, and the amount of water used each day to flush the toilet is approximately 60–90 m³. In this system, study about biofilm development process in rainwater tank was performed.

System 3 and system 4 were installed at Buddle-gol, Seoul National University in October 2007, which collects rainwater from the valley of Mount Gwanak. Tank 3 was designed a concrete square with a storage volume of 20 m³. Tank 4 was assembled from polypropylene units with 95% pore space and has a storage volume of 20 m³. In these systems, study about microbial behaviour by full-scale spike test described was performed.

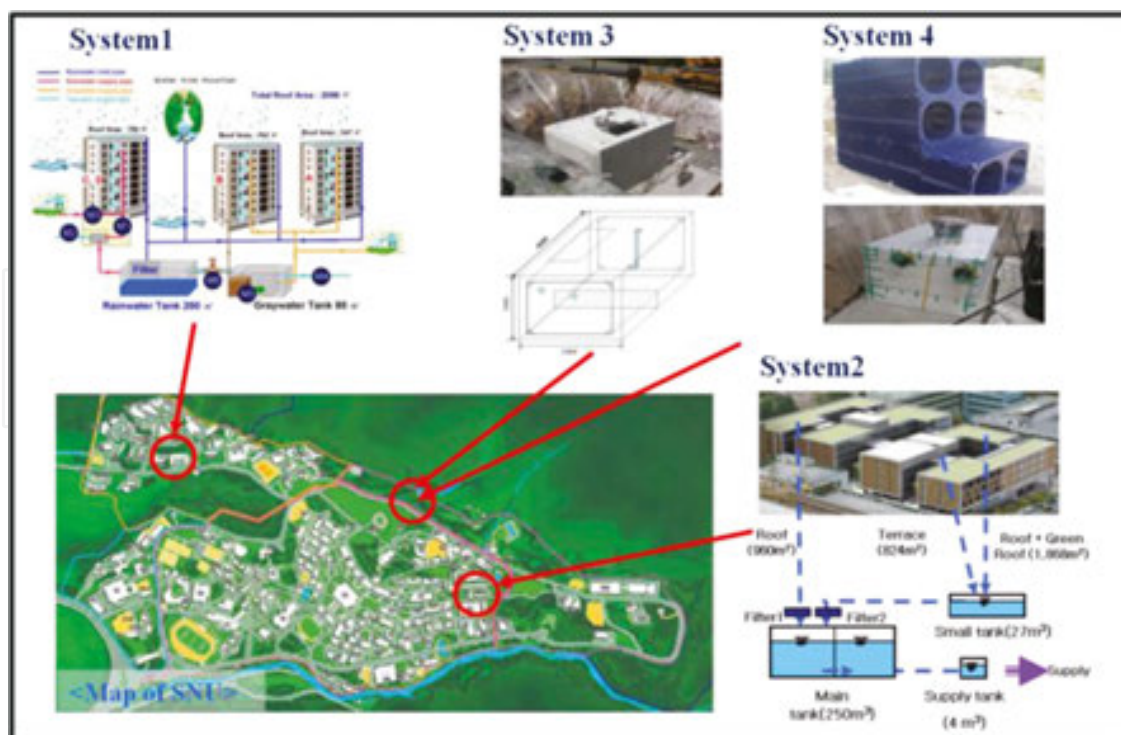


Figure 1. Schematic diagram of the four study sites in SNU campus.

2.2. Sampling sketches

2.2.1. Sampling and sample preparation for PCR-DGGE

In the system 1, the rainwater from the roof flows through a filter (VF6 type with a mesh size of 0.65 mm and a capacity of 70.5 L/s) at first, and then enters the main tank through a calm inlet. Inside the tank, the $W \times L \times H$ ratio changes from $7.4 \times 15.4 \times 2$ to $3.7 \times 30.8 \times 2$ due to the installation of a baffle.

The sampling points were indicated in **Figure 2**. Rainwater of 1.5 L was sampled at a depth of 50 cm in the tank and was carried directly to the lab in a sterile water bottle. Biofilm was collected from 0.04 m² of the wall surface in the tank and placed in a sterile tube containing 20 ml of distilled water.

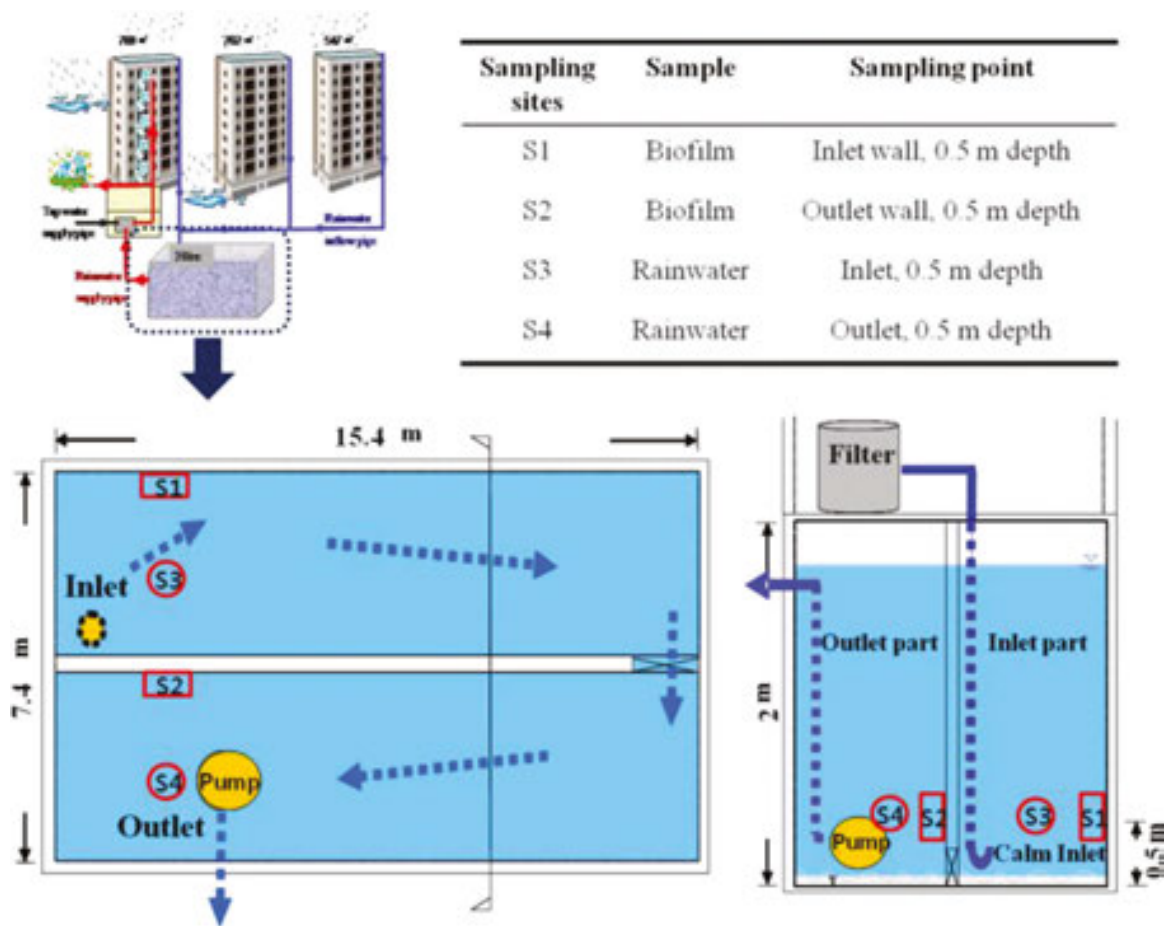


Figure 2. Schematic diagram and description of the sampling points in system 1.

Water samples in system 3 and system 4 were collected for physicochemical monitoring and after spiking test at a depth of 1.3 m from the bottom, around the point of supply in each tank (**Figure 3**). Three replicate samples were taken on four different occasions between May and August 2010.

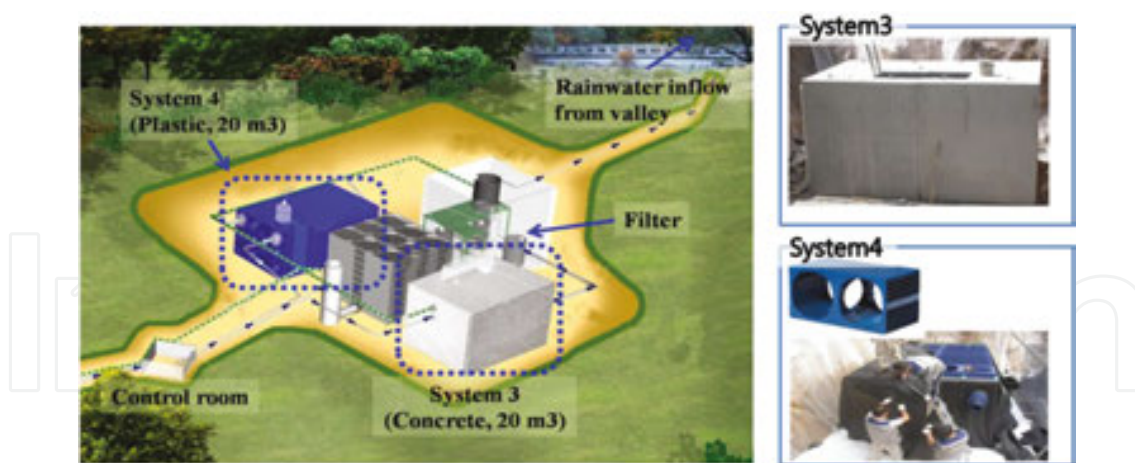


Figure 3. Schematic diagram of system 3 and system 4 used in full-scale experiments.

2.2.2. Coupon preparation and sampling

2.2.2.1. Characteristics of biofilm development on the surface

To study the biofilm formation on the surface, $3 \times 8 \times 0.5$ cm acrylic coupons were prepared (**Figure 4**). These coupons were immersed at the inlet and outlet of the system 2 tied to an acrylic support placed in the middle of the tank at a depth of 2 m from the bottom (**Figure 5**).

Figure 5 shows the plans and cross-sectional views of the tank, and sampling points in system 2. To minimize the influence of flow velocity during the experiment period, no rainwater was supplied, but overflow was permitted.

To evaluate biofilm growth, several tests were carried out with quantify the biofilm development on the surface, following the schedule presented in **Table 1**.



Figure 4. Picture for coupon prepared and set up in the tank.

Water samples were collected from the inlet and outlet portions of the tank once a month in two 1 L sterile screw-cap containers. The samples were taken at a depth of 2 m from the bottom of the tank to collect data on the coupons' environment. The samples were transported to the laboratory and analysed within 30 min of collection.

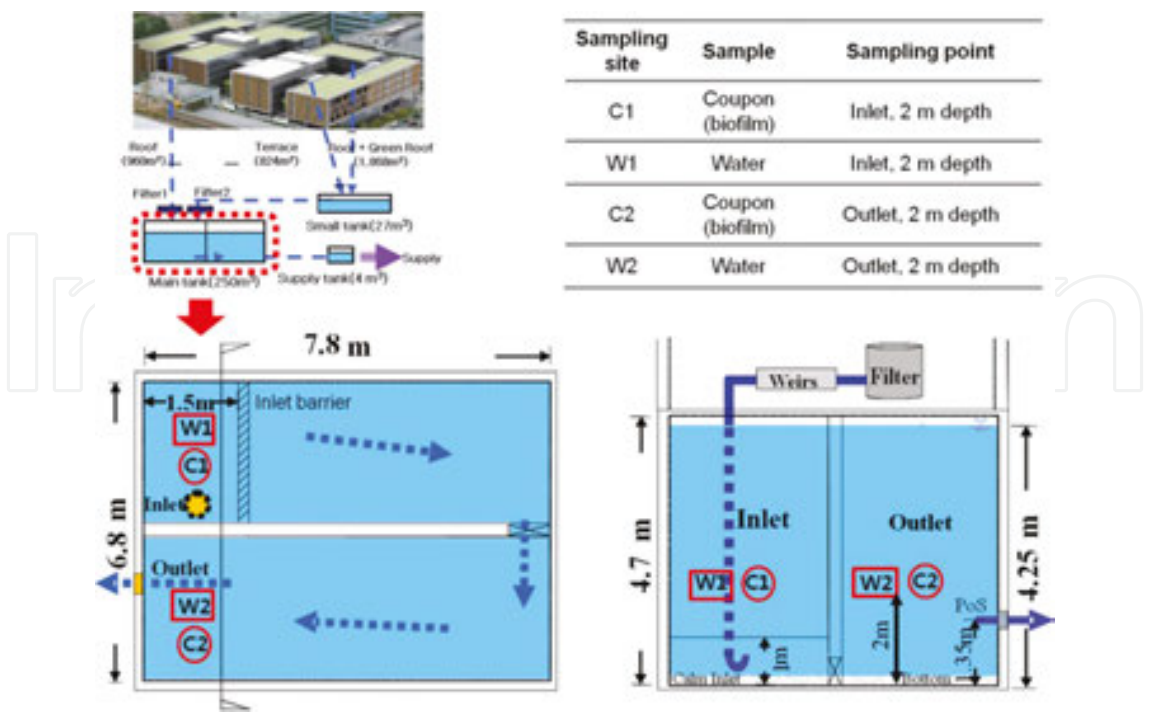


Figure 5. Schematic diagram and description of the sampling points in system 2.

Weeks (after immersion in the tank)	0	1	4	5	8	9	12	15	16
Coupon samples for HPC		✓	✓			✓		✓	
Coupon samples for CLSM		✓		✓		✓		✓	
Coupon samples for PCR-DGGE		✓		✓		✓		✓	
Water samples for pH, turbidity, EC, DO, Temp., SS, TN, TP, TOC, HPC	✓		✓		✓		✓		✓
Water samples for PCR-DGGE									✓

CLSM, confocal laser scanning microscopy; EC, electric conductivity; HPC, heterotrophic plate count; SS: suspended solids.

Table 1. Experimental schedule for coupons and water sampling in system 2.

2.3. Physicochemical characteristics

The various physicochemical parameters of the rainwater, such as temperature (Sension 1, Hatch, Japan), pH (Sension 1, Hatch, Japan), dissolved oxygen (DO) (ProODO, YSI, USA), electric conductivity (EC) (Sension 378, Hatch, Japan), turbidity (2100P, Hatch, Japan), suspended solid (SS) total nitrogen (TN) (HS-TN-L kit, Humas, Korea), total phosphate (TP) (HS-TP-L kit, Humas, Korea), and total organic carbon (TOC) (V CPH kit, Shimadzu, Japan) were measured.

2.4. Enumeration of bacteria

The heterotrophic bacteria were quantified using the conventional microbiological culture method. Faecal coliform tests were carried out through membrane filtration procedure, Standard method 9222D (APHA, 1998).

2.5. PCR-DGGE analysis

2.5.1. DNA extraction

The sample of rainwater and the detached biofilm sample in the PBS were separately passed through a filter, and genomic DNA was isolated with a water RNA/DNA purification kit (Norgen, Canada) according to the manufacturer's instructions.

2.5.2. Polymerase chain reaction (PCR)

The EUB 341F-GC and PRUN518R primer pair, comprising universal primers specific to bacteria, was used [11] for PCR, which was performed with a thermal cycler (GeneAmp PCR System 9700, Perkin Elmer). The PCR conditions are described in **Table 2**.

Primer set	Operation temperature and thermal cycler time (Temp., Time)					Cycles
	Initial denaturation	Denaturation	Annealing	Elongation	Final extension	
EUB 341F-GC, PRUN518R	94°C,	94°C	55°C	72°C	72°C	33
EUB 341F, PRUN518R	15 min	45 s	45 s	45 s	7 min	

Table 2. Reaction conditions for the PCR.

2.5.3. Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed using a D-Code system (Bio-Rad, USA). The 8% polyacrylamide gel contained a series of denaturant concentrations ranging from 30 to 60% (formamide and urea). The gels were run at 70 V for 11 h in a 1 × TAE buffer at 60°C. After electrophoresis, the gels were stained with ethidium bromide in a 1 × TAE buffer for 15 min and then destained in DDW (Deionized distilled water) for 20 min. The DGGE gels were visualized with a UV transilluminator (302 nm) mounted with a digital camera to capture photographs of them.

2.5.4. Re-amplification of the DGGE bands and sequencing

The DNA bands on the DGGE gels were excised under UV transillumination using sterile scalpels and then soaked overnight in 50 µL of sterile DDW at 4°C. Two µL of DNA solution was used for re-amplification with the same primer pair without a GC clamp. The reaction conditions for the PCR were the same as those described in **Table 2**. The PCR products were purified using a kit (AccuPrep PCR purification kit, Bioneer, Korea) and then sequenced using EUB341F (for bacteria) and F984 (for actinomycetes) in an automatic DNA sequencer (ABI

Prism 3730 XL DNA Analyzer, PE Applied Biosystems). The DGGE band sequences were compared with 16S rDNA sequences obtained through a BLAST search from the database of the DNA Data Bank of Japan (<http://blast.ddbj.nig.ac.jp/top-e.html>).

2.6. CLSM analysis

To observe the thickness of the biofilm via CLSM, two coupons from each part of the tank were sampled in sterile Petri dishes, and a BacLight Live/Dead bacterial viability kit (L-7012, Molecular Probes, USA) was employed to stain the live and dead cells. Photographs of two random locations on each coupon were taken with a Carl Zeiss LSM510 microscope. The CLSM images were analysed with an Image Structure Analyzer (ISA) [12].

2.7. Spike test

2.7.1. Pilot-batch tanks

To investigate the behaviour of microbial populations in spike tests in pilot-scale batch tanks with different S/V ratios, 200-liter (L) polyethylene (PE) tanks were filled with 100 L of rainwater. The S/V ratios were set to 10 and 50 m⁻¹ by installing acrylic plates (50 × 20 × 0.2 cm) (Figure 6). To ensure that a sufficient amount of biofilm attached to the tank walls before

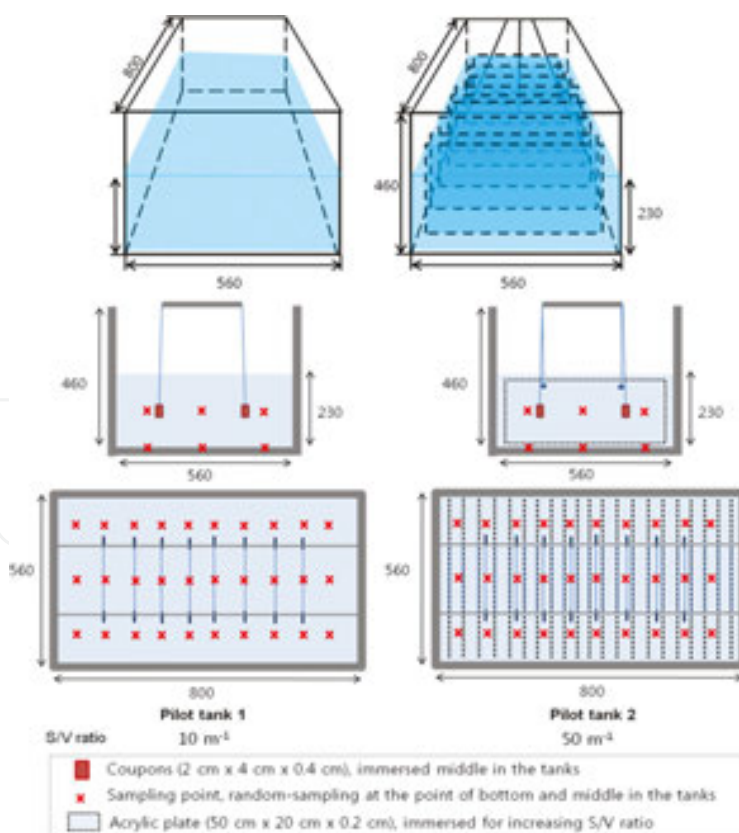


Figure 6. Schematic diagram for the pilot-scale batch experiments.

the spike test, the tanks were filled with 100 L of rainwater and stored for 4 weeks. Ten litres of rainwater per day were then replaced, and the retention time was controlled at 10 days. The water was stored in the dark at room temperature (20°C).

2.7.2. Full-scale tanks

Two full-scale rainwater tanks of system 3 and system 4 were employed to investigate the behaviour of the microbial populations in spike tests carried out with different S/V ratios (**Figure 3**). The S/V ratio was 2 m⁻¹ in system 3 and 15 m⁻¹ in system 4. The retention time was 10 days. The difference of tank material between two tanks was assumed to be negligible because material for biofilm formation primarily affects in the initial steps [13] and the two rainwater tanks used in this study had been in operation for 3 years.

2.7.3. Bacteria preparation and inoculation

Pseudomonas aeruginosa (KCTC #1636), a ubiquitous environmental bacterium that forms biofilms on wet surfaces such as those of rocks and soil, was used in the spike tests. The *P. aeruginosa* were grown to an exponential phase (OD₆₀₀ = 1.2, containing approximately 5×10^7 CFU/mL) in Luria-Bertani (LB) broths and washed twice with phosphate-buffered saline (PBS) (centrifuge at 8000 rpm, 4°C for 10 min). *P. aeruginosa* was put into the tanks at a final concentration in rainwater of about 5×10^5 CFU/mL for the pilot tests and 1.3×10^4 CFU/mL for the full-scale tests.

2.7.4. Sampling

Fifty microlitres of water samples were taken in duplicate from the bottom and middle sections of the two pilot tanks, and coupons were tested randomly every day for 8 days (**Figure 6**). In the full-scale test, 1 L rainwater samples were taken in duplicate from the two tanks every day for 10 days. The pH value in both pilot tanks was 7.1 ± 0.1 ; the DO was 7.9 ± 0.5 mg/L in Pilot Tank 1 and 6.7 ± 0.4 mg/L in Pilot Tank 2.

3. Results and discussions

3.1. Composition and distribution of bacteria in an operating rainwater harvesting tank

3.1.1. Physicochemical conditions in rainwater tanks create a distinct microbial habitat

The turbidity, EC, SS and VSS were lower at the outlet than at inlet, and the DO was slightly lower at the inlet than at the outlet. The TN and total phosphorous were 4.9 ± 0.4 and 0.08 ± 0.04 mg/L at the inlet but decreased at the outlet to 4.4 ± 0.2 and 0.05 ± 0.01 mg/L. The COD was 1.9 ± 1.12 and 0.9 ± 0.01 mg/L, and the TOC was 0.78 ± 0.03 and 0.26 ± 0.15 mg/L at the inlet and outlet, respectively. The values of the parameters were better at the outlet than at the inlet.

Because the rainwater tank under study is installed underground, the lack of sunlight and the average water temperature of as low as 19°C led to the absence of photosynthetic microbes

such as algae. The nutrient input depended on rainfall. Rainwater tanks indicated an oligotrophic environment, as the concentration of dissolved organic matter in these habitats is commonly <10 mg/L [14]. The inflow and outflow of rainwater in such tanks change according to the precipitation and rainwater usage. Thus, rainwater tanks constitute a unique habitat for microbes.

3.1.2. Bacterial composition and distribution

The bacterial composition in the rainwater and biofilm samples showed different tendencies. Seventeen species were identified from the selected DGGE bands (Figure 7). According to the standard phylogenetic classification of prokaryotes, the species belonged to 13 genera, 10 families, 8 orders, 5 classes and 3 phyla. Proteobacteria accounted for 88% of the species identified, with the remainder being Bacteroidetes and Firmicutes.

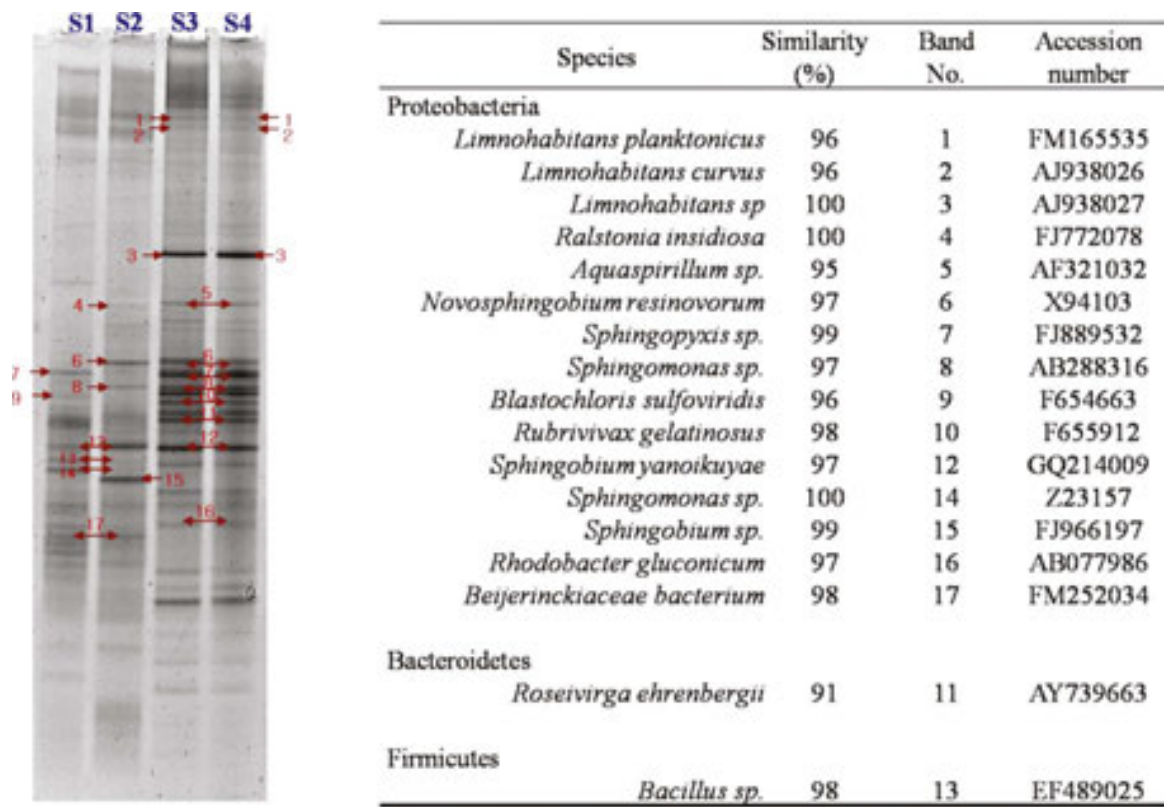


Figure 7. DGGE profiles at each sampling point and closest identified phylogenetic relatives found in the DGGE bands.

The DGGE profiles showed a clear difference between the planktonic bacterial community and the community in the biofilm (Figure 7). The bacterial composition tended to differ across the biofilm samples, but was similar across the rainwater samples. *Rubrivivax gelatinosus*, *Roseivirga ehrenbergii*, *Limnohabitans sp.*, *Aquaspirillum sp.* and *Rhodobacter gluconicum* were identified only in the rainwater, whereas *Sphingomonas sp.*, *Sphingobium sp.*, *Ralstonia insidiosa*, *Blastochloris sulfovirdis*, *Bacillus sp.* and *Beijerinckiaceae bacterium* were found in the biofilm. Some species,

such as *Sphingopyxis* sp., *Sphingomonas* sp., *Novosphingobium resinovorum* and *Sphingobium yanoikuyae* were found in the both rainwater and biofilm samples.

The bacterial composition in the biofilm differed according to the location. *Sphingopyxis* sp. (Band No. 7) and *Blastochloris sulfoviridis* (Band No. 9) were detected in the inlet samples, whereas *Ralstonia insidiosa* (Band No. 4), *Novosphingobium resinovorum* (Band No. 6), *Sphingomonas* sp. (Band No. 8), *Sphingobium* sp. (Band No. 15) were found only in the outlet samples. *Sphingobium yanoikuyae* (Band No. 12), *Bacillus* sp. (Band No. 13), *Sphingomonas* sp. (Band No. 14), and *Beijerinckia bacterium* (Band No. 17) were detected in both locations. Similar bacterial composition indicated at the inlet and outlet rainwater samples.

The samples contained mostly nonpathogenic proteobacteria. Many of the bacteria identified were closely related to fresh water, soil and biofilm bacteria found in natural environments [15–20]. Eighty-eight percentage of the identified bacteria were proteobacteria. It have been reported that proteobacteria are consistently more abundant at pristine sites, whereas Firmicutes and Actinobacteria are dominant at polluted sites [21]. Though estimates were made in terms of detection ratio only in this study and the species were not quantified, the results still indicate the clean oligotrophic nature of the tank water.

The bacterial composition in the biofilm was different from that in the rainwater. It is known that biofilm formation provides an advantage for bacteria that exist in oligotrophic environments [22]. Some of the species identified in the biofilm in this study, such as *Bacillus* sp., *Sphingomonas* sp. and *Sphingobium* sp., have been demonstrated to degrade certain contaminants and to act as bio-control agents [17, 18, 23]. These species may be relatively sensitive to nutrients in oligotrophic conditions and thus tend to develop a biofilm to survive. Therefore, in oligotrophic rainwater tanks, microbial species possibly remain constant in rainwater tank through biofilm formation.

3.1.3. Self-purification possibility of rainwater tanks and implications for rainwater quality

Bacterial communities in nature play a key role in the production and degradation of organic matter and many types of environmental contamination, and the cycling of nitrogen, sulphur, and many metals [24]. In addition, the sorptive capacity of biofilm for dissolved organic matter and metals has been widely demonstrated in sewage and marine systems [25, 26]. Thus, biofilm formation in rainwater tanks seems not only to promote the survival of bacteria, but also serves as a natural filter by removing contaminants and bacteria from rainwater.

3.2. Characteristics of biofilm development in rainwater tank

3.2.1. Physicochemical and microbial conditions in rainwater

The temperature of the stored rainwater ranged from 16 to 22°C, and the pH was around 7. At the inlet and outlet of the tank, the turbidity was 2.9 ± 1.6 and 2.1 ± 1.0 NTUs, respectively; the SS count was 3.2 ± 1.8 and 1.3 ± 0.8 mg/L, respectively; the TOC was 1.56 ± 0.54 and 0.91 ± 0.97 mg/L, respectively; and the TP count was 0.07 ± 0.04 and 0.04 ± 0.01 mg/L, respectively. Thus,

the particle and nutrient parameters of the rainwater, namely turbidity, SS, TOC, and TP, at the outlet of the tank were slightly lower than those at the inlet.

Figure 8 shows the number of viable cells at the inlet and outlet of the tank during the experimental period. The difference between the two sites was significant ($P < 0.05$): the number of viable cells at the inlet was triple that at the outlet (3×10^5 versus 1×10^5 CFU/mL, respectively).

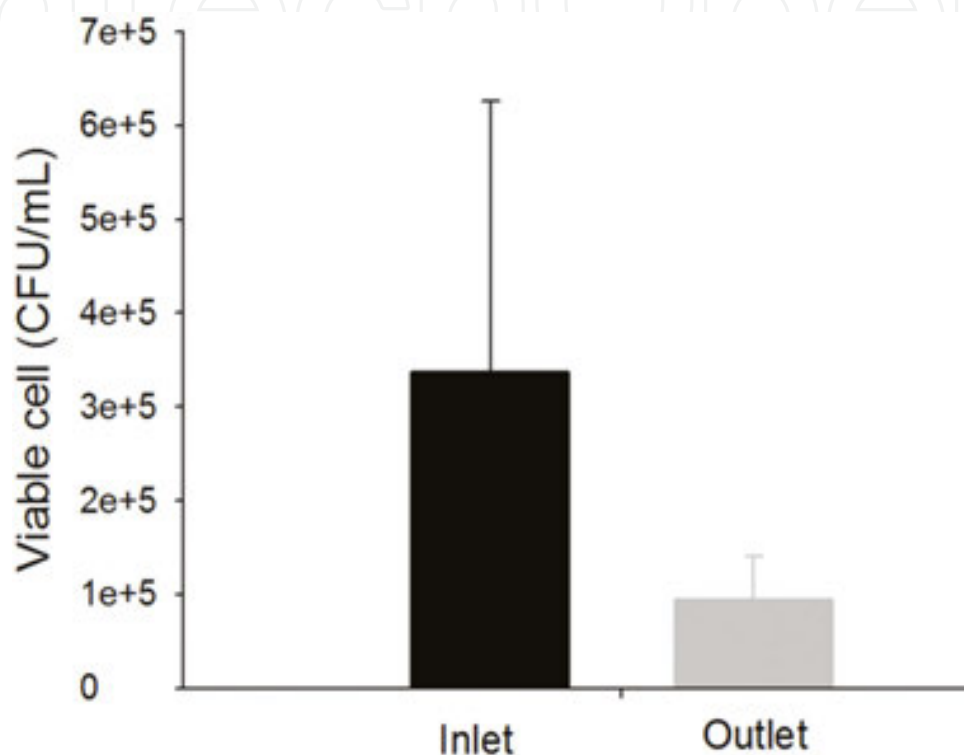


Figure 8. Comparison of the viable cell quantities at the tank inlet and outlet. The difference between them is statistically significant (Student's t-test; $P < 0.05$, $n = 12$).

The rainwater tank used in this study was designed with an internal wall in the inlet section and a baffle in the middle to improve sediment efficiency. Ryu [6] reported that such design factors as inlet barrier and baffles can affect the removal of the particles that come into a rainwater tank. Hence, the slight differences in the physicochemical characteristics identified at the tank inlet and outlet in this study appear to be due to these design factors. In addition, the physicochemical conditions appear to influence the microbes in the water, as can be seen in the different microbial numbers.

3.2.2. Comparing cell dynamics on coupon: cell number

To compare the biofilm development at the tank inlet and outlet, we also investigated the number of viable cells on the coupons immersed at each site (**Figure 9**). The two sites exhibited a similar number of cells until the fourth week. At week 9, however, the number at the inlet was 2.5 times higher than that at the outlet (2.7×10^5 and 1.1×10^5 CFU/cm², respectively),

suggesting there was greater biofilm growth at the former. After 15 weeks, the corresponding figures were 3.6 CFU/cm² at the inlet and 3.0 CFU/cm² at the outlet, and the difference in cell numbers between the two sites had been reduced to 1.2 times.

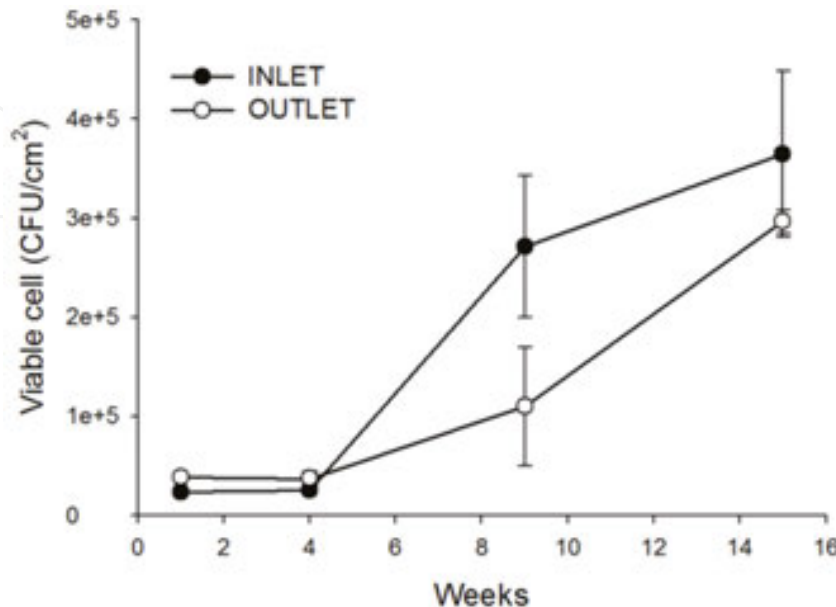


Figure 9. Number of viable cells on the coupons immersed at the tank inlet and outlet for 15 weeks.

Mature biofilm development may take anywhere from several hours to several weeks, depending on the system [27]. Biofilm formation is one possible survival strategy for bacteria, and one of the advantages of bacterial adherence is the greater availability of nutrients attached to the surface [28]. Geesey et al. [29] reported high rates of biofilm development in oligotrophic environments. In the current study, biofilm formation was observed on the coupons after 1 week of immersion. Considering the oligotrophic nature of rainwater tanks [2], it appears that biofilms develop within 1 week in this environment.

In this study, cell number in outlet site would be smaller than that in inlet part because of attachment to the existing biofilms on the wall in inlet part and sedimentation with small particles. The difference in biofilm formation between inlet and outlet part would be results of nutrient concentration and planktonic cell number in rain water flowing from inlet part. The influence of flow velocity and the substratum effect was most likely excluded in this study because, during the experiment period, a coupon of identical material was placed in the middle of the tank and to minimize the effect of water flow, the water flow was controlled without supply by overflowing.

3.2.3. Biofilm thickness

The CLSM images of the biofilm thickness on the coupons exhibited similar viable cell patterns (**Figure 10**). At the tank inlet, the thickness was $4.5 \pm 0.1 \mu\text{m}$ at the end of the first week, increasing to $48.4 \pm 1.3 \mu\text{m}$ at week 9 and then decreasing to $25.0 \pm 2.8 \mu\text{m}$ at week 15.

Apilanez et al. [30] demonstrated that once biofilm has attained a certain weight, which can be related to a certain thickness, detachment occurs. The development of greater biofilm thickness can thus lead to earlier, and a great extent of detachment. Several processes can lead to detachment: erosion or shearing, and sloughing and abrasion. Donlan [31] wrote that when biofilm increases in thickness, its rate of erosion also increases. The detached biofilm possibly settles at the bottom of the tank, but it also provides a way for cells to migrate and colonize a less populated area.

At the tank outlet, in contrast, a biofilm thickness of $5.7 \pm 0.7 \mu\text{m}$ was seen at the end of the first week and that thickness continued to increase until it reached $29.5 \pm 2.0 \mu\text{m}$ at week 15. No detachment phase was observed at the outlet in this study. It seems that because the nutrient concentration is lower at the outlet, biofilm development is slower at that site.

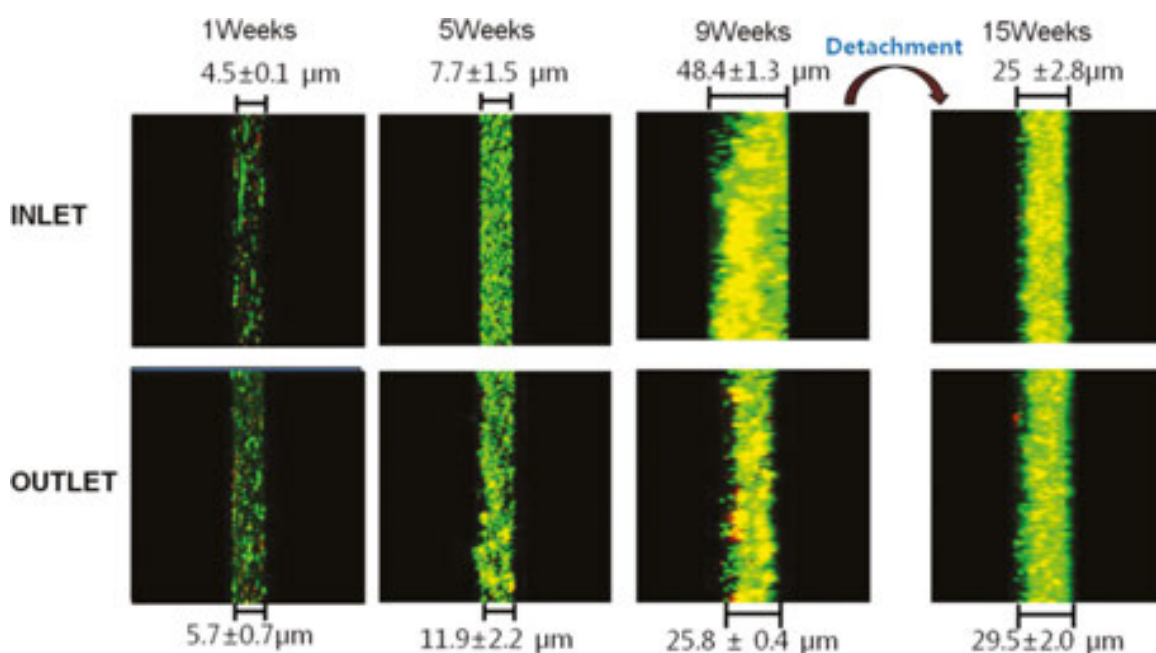


Figure 10. Comparison of biofilm thickness obtained by CSLM (average \pm standard deviation, $n = 4$).

3.2.4. Dynamics in bacterial community

Two sets of biofilms displayed changes in their DGGE banding patterns and number of bands as they developed (**Figures 11 and 12**). Differences were apparent between the inlet and outlet samples, both in the individual samples of a specific age and in the overall pattern of bacterial community development. The biofilm at the inlet exhibited a greater number of bands in the earliest sample (1 week), displaying a decrease by the fourth week and then increasing again. At the outlet, there were also a greater number of bands after 1 week, a decrease by the ninth week, and then a slight increase. The band patterns appeared similar between the initial two stages (weeks 1 and 4) and later two stages (weeks 9 and 15), and seemed to simplify over time as the biofilm developed.

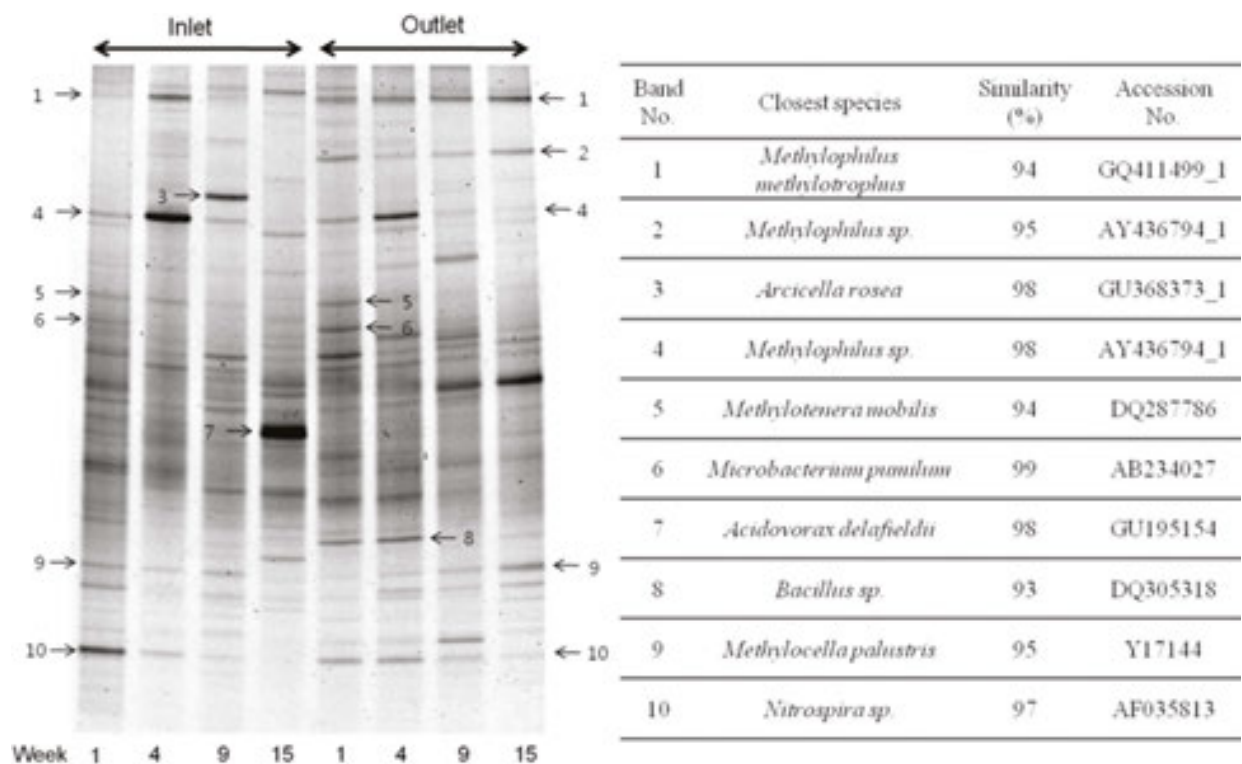


Figure 11. DGGE profiles at each sampling time point and the closest phylogenetic relatives found in the DGGE bands.

Following a high number of bands at the initial stage, reductions occurred later, possibly arising from the competitive dominance of a few populations. The biofilms at the inlet and outlet demonstrated a reduction in the number of bands after the first sample date. The populations that were initially detected may still have been present in later biofilms, but the rapid growth of other populations made them more difficult to detect. As biofilm matures, the number of available microhabitats may increase (for example, from the formation of an anaerobic pocket within the biofilm), thereby supporting a greater number of bacterial populations.

Despite the overall differences in banding patterns, a number of bands appeared at the same position in the DGGE gels in almost all of the samples, including *Methylophilus methylotrophus* (Band No. 1), *Methylocella palustris* (Band No. 9) and *Nitrospira sp.* (Band No. 10), although their intensity differed. *Methylophilus sp.* (Band No. 2) was found only at the outlet site at all stages, whereas *Methylostenura mobilis* (Band No. 5), *Microbacterium pumilum* (Band No. 6) and *Bacillus sp.* (Band No. 8) were identified in the earlier samples (week 1 and/or week 4). Some bands, such as *Nitrospira sp.* (Band No. 10), faded over time.

In the initial stage of biofilm formation, free-swimming bacteria attach to the surface through hydrophobic and electrostatic interactions and through the use of flagella [32]. In this study, for example, both *Methylostenura mobilis* (Band No. 5) and *Microbacterium pumilum* (Band No. 6) were detected in the earlier samples. The former is mobile by means of a single flagellum [33], and the latter is non-motile [34]. Initial colonization on the surface may not be entirely random,

in that certain bacterial species may have greater colonization aptitude than others, such as greater mobility.

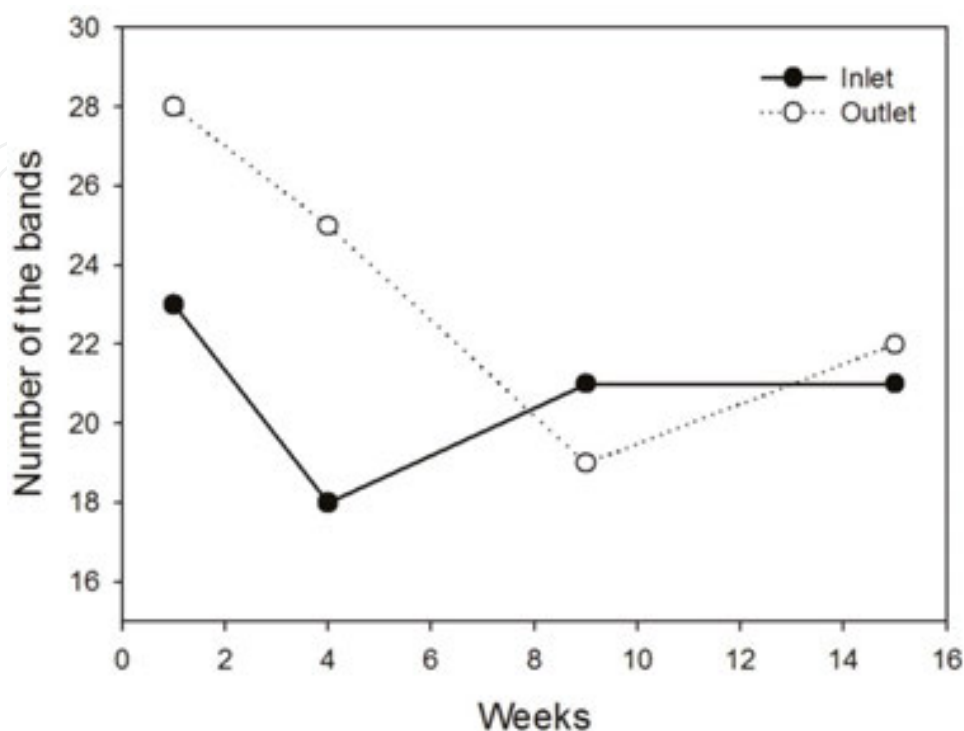


Figure 12. Changes in the number of DGGE bands in the biofilm during the experimental period.

Rank-abundance distributions provide insights into both richness and evenness. In this study, the rank-abundance plots displayed a trend towards a geometric distribution (**Figure 13**), and linear regression was performed to examine changes in the pattern of evenness during biofilm development (**Table 3**). Lower slope values indicate greater evenness, and higher values indicate greater dominance by certain populations. At the tank inlet, the slope value was -0.113 at the end of the first week. It then increased steeply after 4 weeks, decreased after the ninth week, and then increased again. At the outlet, in contrast, the slope value increased sharply at the 9-week sample and then exhibited a decrease at the last sample.

The pattern of biofilm development seems to follow three major stages. Jackson et al. [35] suggested an initial stage characterized by the colonization of different populations, an intermediate stage characterized by a limited number of dominant populations utilizing similar resources, and a late or mature stage characterized by mature biofilm of a complex spatial structure that facilitates greater diversity through increased variation in habitat and available resources. However, in the current study, this characterization appeared to apply only up until the mature stage, after which detachment occurred. Following detachment, biofilm development appeared to return to the intermediate and/or mature stage, and the process was then repeated. In this study, this pattern/cycle was confirmed at the tank inlet site, where biofilm development was more rapid throughout the experimental period than at the

outlet site, most likely due to the difference in nutrient concentrations. As the biofilm development process was faster at the tank inlet, more sludge from the detachment was also seen at this site.

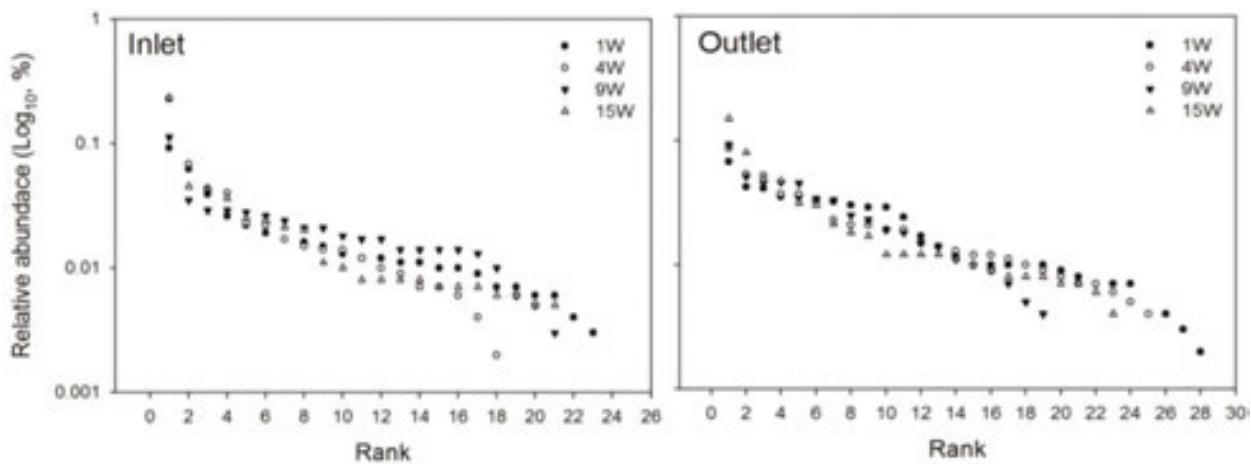


Figure 13. Rank-abundance distributions of bacteria in different periods of biofilm development.

Sample	Inlet		Outlet	
	Slope	r ²	Slope	r ²
1 week	-0.113	0.90	-0.106	0.96
4 weeks	-0.194	0.89	-0.105	0.97
9 weeks	-0.111	0.84	-0.150	0.98
15 weeks	-0.141	0.81	-0.123	0.88

Table 3. Regression statistics for rank-abundance distributions of bacteria in the biofilm at different sites and stages of development ($P < 0.05$; n varies by sample).

3.3. The effect of biofilms on microbial quality in rainwater tanks

3.3.1. *P. aeruginosa* removal in water

The persistence of the *P. aeruginosa* cells inoculated into the pilot tanks resulted from the interaction between the cell growth and death rates and from that among the attachment, detachment, and sedimentation processes. The total number of inoculated cells in the water samples and on the bottoms and walls of the tanks decreased in both tanks (Figure 14). Cell death contributed more to the observed cell decline than did growth in the tanks due to low-nutrient conditions.

Figure 15 shows the removal rate of *P. aeruginosa* from the water of the two pilot tanks. Ninety-nine percent of the inoculated *P. aeruginosa* was removed after 4 days in Pilot Tank 2 and after 5 days in Pilot Tank 1. The faster removal rate in Pilot Tank 2 was due to its higher S/V ratio.

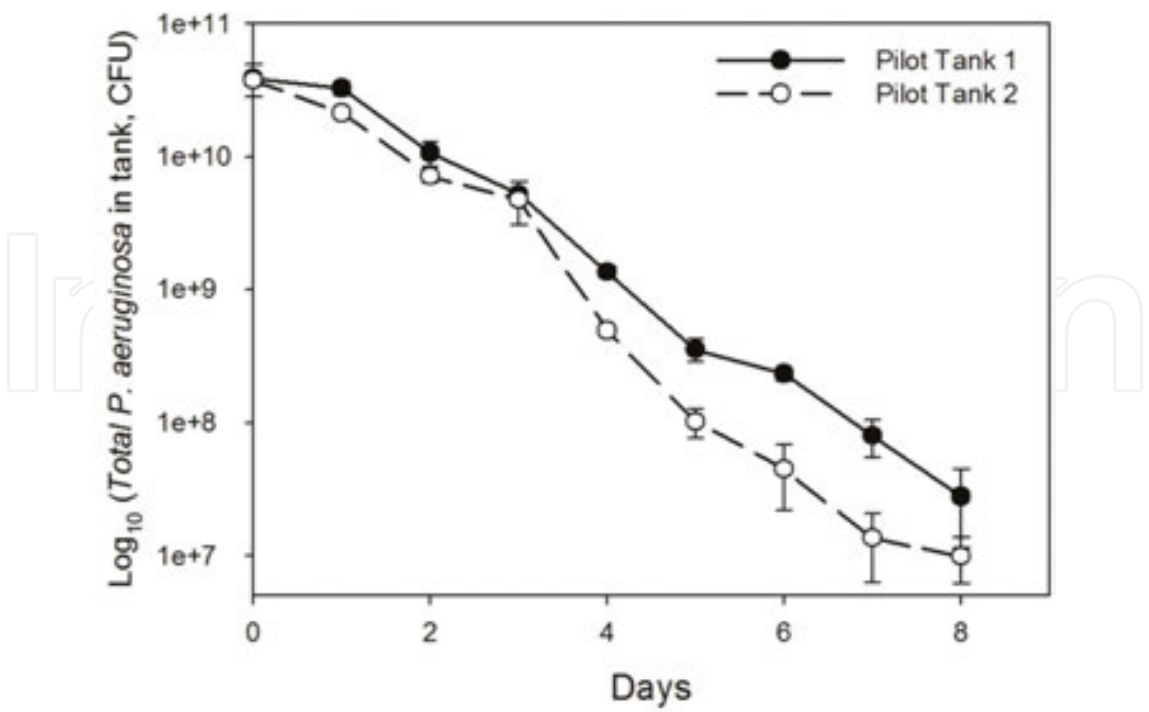


Figure 14. Total amount of *Pseudomonas aeruginosa* inoculated in pilot-scale tanks. . [Student’s t-test; $P < 0.05$ except Day 0 ($P = 0.55$) and 3 ($P = 0.15$)].

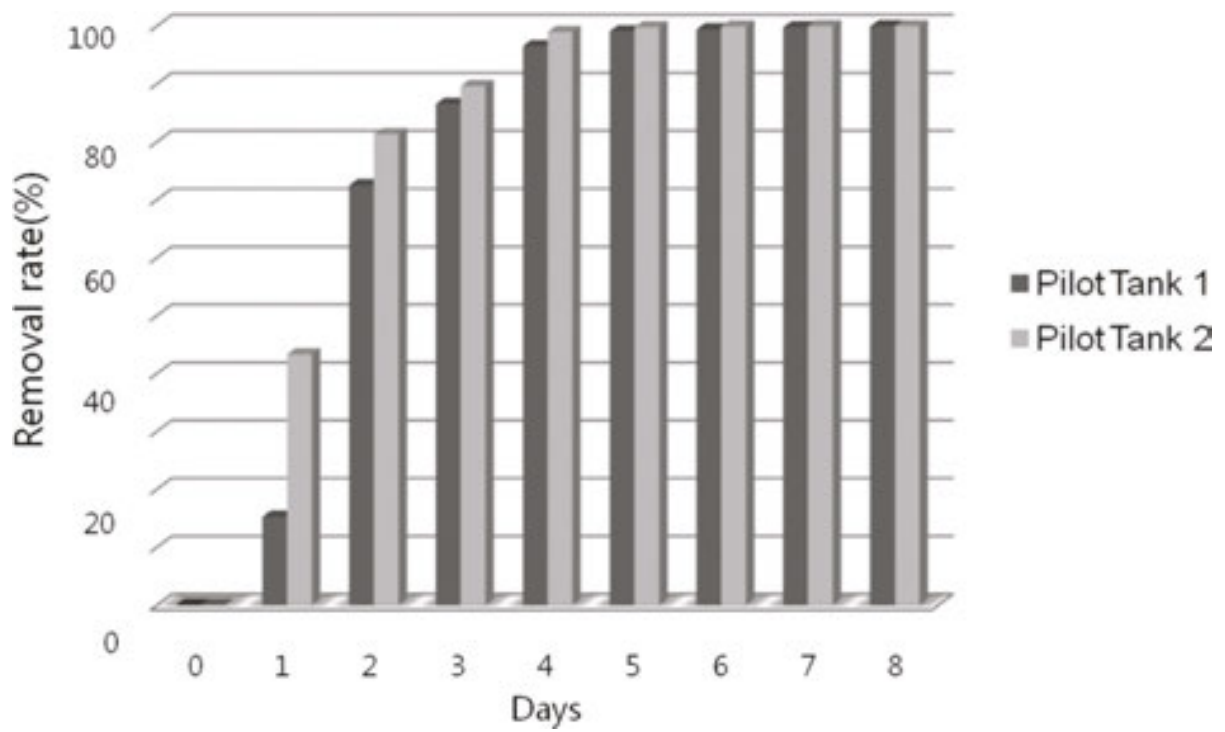


Figure 15. Removal rate of *Pseudomonas aeruginosa* inoculated in the water of the two pilot tanks.

3.3.2. Microbial behaviour of *P. aeruginosa* put into the rainwater tanks

The number of *P. aeruginosa* in the water decreased by 3–4 log units, indicating that the death, attachment and sedimentation processes dominated the overall dynamics (**Figure 16A**). The removal rate of *P. aeruginosa* in the water phase was $-0.57 \log_{10} \text{ cells ml}^{-1} \text{ day}^{-1}$ ($r^2 = 0.93$) in Pilot Tank 1 and $-0.74 \log_{10} \text{ cells ml}^{-1} \text{ day}^{-1}$ ($r^2 = 0.98$) in Pilot Tank 2. A faster removal rate was shown in the tanks with higher S/V ratios.

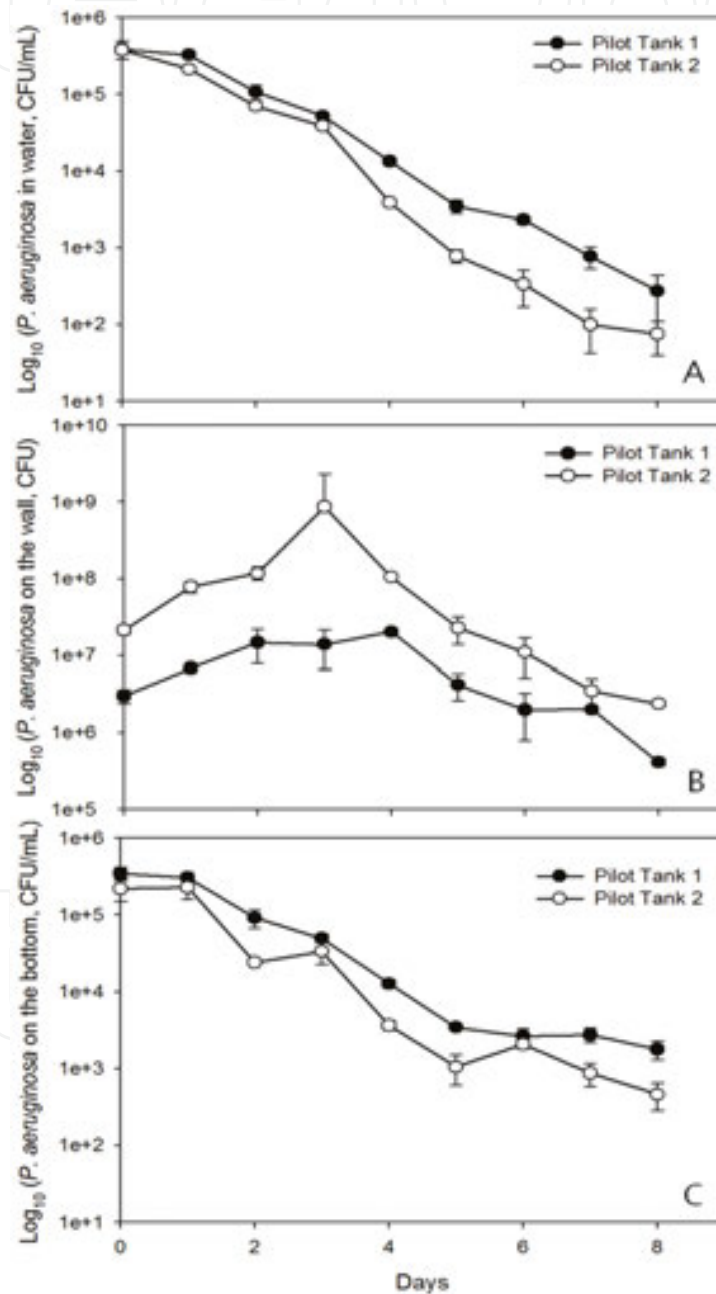


Figure 16. Behavior of *Pseudomonas aeruginosa* inoculated in (A) the water and on the (B) wall and (C) bottom of the 8 pilot-scale tanks. [Student's t-test; (A) $P < 0.05$ except Day 0 ($P = 0.51$); (B) $P < 0.1$ except Day 0 ($P = 0.17$) and 1 ($P = 0.14$); (C) $P < 0.01$ except Day 7 ($P = 0.10$)].

The number of attached *P. aeruginosa* cells increased over 4 days in Pilot Tank 1 and over 3 days in Pilot Tank 2 (**Figure 16B**). Their attachment to the biofilm on the wall was initially dominant, and more bacteria were attached in Pilot Tank 2 because of the higher S/V ratio.

After 4 days, the number of attached *P. aeruginosa* cells declined by 2–3 log units, indicating that the death or detachment processes were the dominant bacterial dynamics on the wall (**Figure 16B**). Established biofilms developed from indigenous river water bacteria have been shown to reduce the persistence of introduced *E. coli* and other enteric pathogens [36]. Banning et al. [37] showed that, under certain conditions, the presence of mixed-populated biofilms may limit the survival potential of enteric bacteria pathogens introduced into groundwater. In addition, biofilm dynamics changes and pathogen persistence are affected by increasing in nutrient levels. It was reported that a significant decrease in the survival rate of the *Campylobacter jejuni* strain in heterogeneous tap-water biofilms following the addition of serine, a carbon source favoured by *C. jejuni*, and a concurrent increase in the number of indigenous biofilm microflora [38]. These studies demonstrate that, under certain conditions, biofilms represent sites of intensified competition for limited nutrients. Therefore, for the biofilms in oligotrophic rainwater tanks, a decrease in *P. aeruginosa* cells may result from the nutrients competition with indigenous microbial communities.

Inoculated *P. aeruginosa* were found on the bottom in tanks and decayed over time (**Figure 16C**). More bacteria observed at the bottom of Tank 1, which had a lower S/V ratio, and more bacteria observed on the wall in Tank 2. The number of *P. aeruginosa* increased slightly on days 3 and 6 in Tank 2, probably due to detachment from the wall rather than bacterial regrowth, as this effect was not observed in Tank 1.

3.3.3. Microbial behaviour of *P. aeruginosa* put into the full-scale tanks

The number of *P. aeruginosa* in the water decreased by 1.5 log units in Tank 1 and by 2 log units in Tank 2 (**Figure 17**). The removal rate was $-0.604 \log_{10} \text{ cells ml}^{-1} \text{ day}^{-1}$ ($r^2 = 0.99$) in Tank 1 and $-0.854 \log_{10} \text{ cells ml}^{-1} \text{ day}^{-1}$ ($r^2 = 0.98$) in Tank 2. In line with the results of the pilot test, a faster removal rate was shown in Tank 2 due to its higher S/V ratio. Thus, it can be concluded that increasing the S/V ratio in rainwater tanks to a certain level is possibly effective to remove bacteria from rainwater.

In this study, the removal rates of *P. aeruginosa* were determined by calculating the slope and correlation coefficient (r^2) of the linear regression of the log-transformed cell concentration data according to the first-order decay equation. Crane and Moore [39] reviewed a variety of modified models of first-order decay kinetics and concluded that the simplest model is the most advantageous. As noted, the findings of the current study suggest that increasing the S/V ratio in rainwater tanks is an effective way of improving their microbial quality. Accordingly, additional research aimed at identifying which range of S/V ratios is most effective in improving such quality may benefit from modifying the first-order kinetics. The resulting information would help in the development of appropriate guidelines for the design of rainwater tanks.

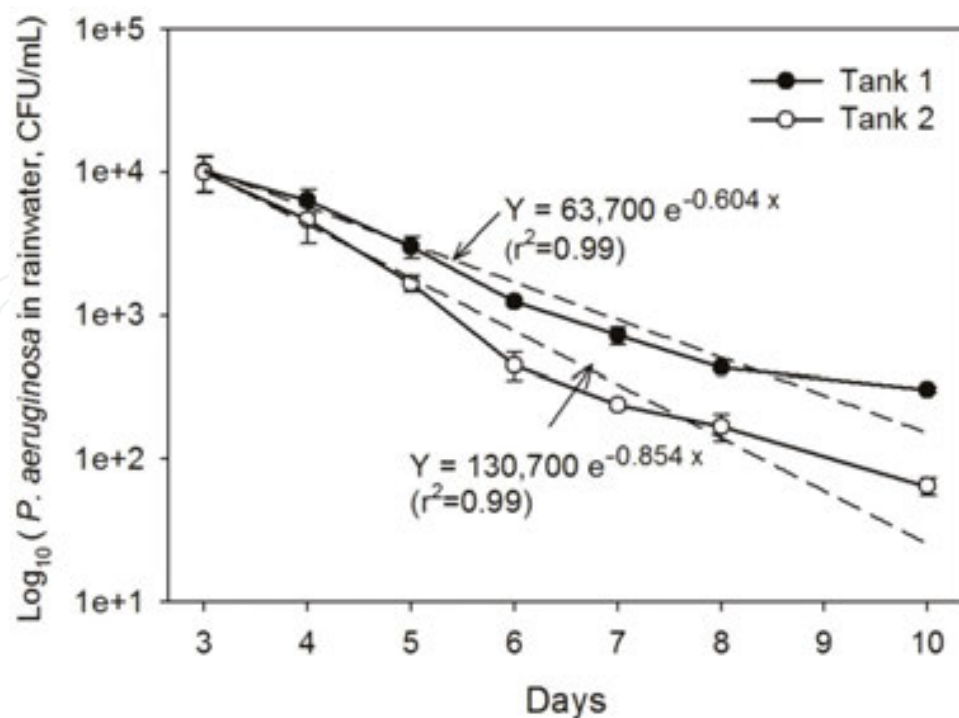


Figure 17. Number of *Pseudomonas aeruginosa* inoculated in the rainwater of the full-scale tanks. [Student's t-test; $P < 0.1$ except Day 0 ($P = 0.67$)].

3.3.4. Biofilm's role in rainwater tank

It has been suggested that rainwater tanks are unique ecosystems that support functional ecosystems comprising complex communities of environmental bacteria [2]. This study showed that a wider surface area for biofilm formation led to a higher removal rate of *P. aeruginosa* in rainwater. When opportunistic pathogens such as *P. aeruginosa* introduced to rainwater tanks with limited nutrient conditions, it seem to be removed due to their attachment to biofilms and die both naturally because of competition with indigenous microbial communities for nutrients.

4. Suggestion of a design and maintenance guideline for rainwater system

From this study, it was suggested the expected role of biofilms for improving water quality in rainwater tank. Contaminants including microorganisms in rainwater are possibly attached on biofilm, and the biofilms are grown by nutrient degradation and additional attachment. Then, followed sloughing and sedimentation processes, the rainwater quality seemed be sustained by certain level.

In addition, four recommendations were suggested for design and maintenance of RWH system as followed description.

1. There seems to be a unique microbial ecosystem which is able to control the microbial quality to the certain level by themselves. Thus, it is recommended that avoid mixing with chlorinated tap water which might disturb the microbial ecosystem in rainwater tank. When rainwater and tap water connected system designs, it is advisable to arrange separate supply tank without direct connection of tap water to the main storage tank.
2. Design of baffle and inlet barrier is recommended because they not only induce the sedimentation of inflow particle but also control bacterial quality in rainwater by increasing the surface for biofilm development.
3. Increasing the S/V ratio bacterial quality is possibly controlled by inducing more biofilm development. Therefore, it is recommended to consider the parameter of S/V ratio when rainwater tank is designed.
4. Frequent cleaning and/or disinfection of rainwater tank inside seems to be counterproductive because biofilm developed in rainwater tank improve the bacterial quality in rainwater tank by adhesion of bacteria in rainwater.

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