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Impact of organic matter on biofilm growth and microbial community diversity

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ABSTRACT

Biofilm in secondary water supply system (SWSS) may affect tap water biological stability. The growth of biofilm and the diversity of biofilm microbial community are partly influenced by organic matter content. In this paper, the impact of organic matter content on biofilm bacteria growth and biofilm density was studied. The diversity and variation process of biofilm microbial community were also analyzed by Metagenomic sequencing. Biofilm bacteria growth and compactness were positive correlate with organic matter content. Biofilm grew with tap water of low organic matter content (permanganate oxygen consumption, viz., COD_{Mn} <3 mg/L) was of higher diversity level than that in the condition of COD_{Mn} >3 mg/L. It may because of the proportion of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* changed in low COD_{Mn} concentration condition in comparison to that in high COD_{Mn} concentration condition. *Proteobacteria* and *Firmicutes* were dominant at phylum level in biofilm in different organic matter content in this experiment. Pathogenic bacteria including *Pseudomonas*, *Acinetobacter, Ledionella* and *Flavobacterium* could grow with tap water that met the Chinese drinking water standards (COD_{Mn} <3 mg/L).

Keywords: Biofilm; Organic matter content; Tap water; Microbial community diversity

1. Introduction

Biofilm formation and detachment are subjected to nutrient availability and flow conditions [1]. Biofilm in SWSS have caused continuous problems in maintain tap water quality especially in biological stability in recent years. It may lead to health problem with opportunistic pathogens [2]. It is necessary to get a better understanding of the impact of organic matter content on biofilm bacteria growth and its microbial community diversity. Consequently, both in engineered and natural systems, the developments of biofilm are of great interest with respect to the degradation of organic matter. Shen et al. found that biofilm had a denitrification rate of 13.54 ± 0.90mg N/(L h) at 25°C when starch was served as organic matter [3]. Biofilm degradation of organic matter was also used in water treatment system and its removal efficiency of BOD_5 was as high as 83 ± 9% [4]. However, little research has been done on the dependence of the biofilm growth and biofilm microbial community diversity on the organic matter content of SWSS in residential buildings.

Early as 1981, Characklis reported experimental results relating total bacteria count to organic matter content using rotating annular reactors. The total bacteria count increased with both increasing shear stress and increasing organic matter content [5]. Ndiongue et al. [6] and Moussard et al. [7] also found the level of organic matter was a key controlling factor of bacteria growth, and it was positively correlated with total bacteria count. Kwok et al. indicated a significant dependence of the biofilm density on organic matter supply in biofilm airlift suspension reactors that an increasing biofilm density with decreasing organic matter content [8]. But there appears to be no consensus on the correlation of biofilm bacteria and organic matter content. Sherr et al. did not find a

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significant relationship between bacteria abundance and total organic matter content in water in Northeast Pacific Ocean off Oregon, USA [9].

Additionally, due to different water qualities, there is relatively less stress appears on microbial community diversity of the biofilm in tap water system. It should be mentioned that organic matter content is not only an important parameter with respect to biofilm bacteria growth. The diversity of the microbial community in the biofilm has also been related to biofilm growth by several authors. Nature of the growth and reproduction of bacteria depends on the microbial diversity present in bioreactors [10]. Microbial community structure influences the degradation rate of organic matter and the length of the growth cycle [11,12]. Generally, microbial community diversity gets influenced by the operating conditions, e.g. organic matter content, hydraulic retention time (HRT), operating pH, etc. S. Venkata et al. [13] correlated the operation data with the microbial community profile to help to understand the process happening in the bioreactor.

Metagenomics method has had the greatest impact within the last few years and was used in this study, which aimed to access the genomic potential of an environmental habitat either directly or after enrichment for specific communities [14,15].

The objective of this study was to investigate the influence of organic matter on biofilm bacteria growth and microbial community diversity with rotating annular reactors simulating SWSS conditions in residential buildings. The purpose of this paper is to present a deeper understand of the impact of COD_{Mn} on tap water biofilm and biological safety in SWSS.

2. Materials and methods

2.1. Reactors

Biofilms in SWSS in residential buildings were cultivated in rotating annular reactors (BAR) with continuous flow. BARs were constructed of polyvinyl chloride and consisted of a stationary outer cylinder and a rotating inner drum. Eighteen removable polyvinyl chloride slides (11.2 cm^2 per slide) were placed in recessed slots on the inside of the outer cylinder. The slides could be pulled from the reactor via port in the top of the reactor for sampling biofilm at any time without interrupting reactor operation. Mixing was established in the reactor through the rotation of the inner cylinder.

Rotation of the inner drum (50 revolutions per minute) simulated the hydraulic shear stress. The volume of the reactor was 700 mL. Influent to reactors was delivered using variable-speed peristaltic pumps and silicone tubing. Total flow rate to each reactor was 6 mL/min, making the hydraulic retention time was approximate 2 h. Residence time of 2 h could minimize planktonic growth of suspended heterotrophic organisms [16]. The whole experiment was performed at room temperature (20 ± 2°C).

Two reactors were performed over the course of the project. One reactor (R1) was operated with residential building tap water adding organic matter overall the experiment to provide approximate influent COD_{Mn} of 3–5 mg/L. The concentration of COD_{Mn} was determined according to Chinese drinking water standards [17]. The tap water supplied a continuous source of indigenous, heterotrophic bacteria in acclimated to a low-carbon SWSS environment. The mixed-population of heterotrophic bacteria in tap water also served as initial inoculum for biofilm in reactors. Humic substances were the organic matter utilized for experiment. A concentrated humic substances stock solution was prepared by adding commercial humic acid powder (humic substances used in this work were produced by Tianjin Zhiyuan chemical reagent co., LTD. It was chemical pure and its molecular weight was about 1000 D which was determined in the humic substances stock solution. Its mean UV_{254} were 0.023 cm^{-1} and 0.015 cm^{-1} corresponding to influent water with mean COD_{Mn} concentration of 3.56 mg/L and 1.59 mg/L, respectively) to sterile ultrapure water, adjusting the pH to 12 with 0.1 M sodium hydroxide solution, mixing for 24h. Then centrifuged at 10,000 g at 4°C to remove particulate matter. Following addition of humic substances stock solution to sterile ultrapure water the pH was adjusted to 7.5 using 1 M HCl. The other reactor (R2) was fed with residential building tap water without adding anything with initial COD_{Mn} of 0.5–3 mg/L to assess the influence of organic matter on biofilm growth. Influent flow to each reactor remained mean free residual chlorine concentration between 0.02 and 0.04 $mgCl_2/L$. Main water quality parameters of influent to each reactor were listed in Table 1.

Prior to operating, reactors, tubing and humic substances stock solution were sterile at the start of the experiment. Operation and sampling of the reactors followed aseptic technique throughout the experiment. The growth experiment was stopped when an obvious decline phase occurred. Total duration of the whole experiment was 70 d.

2.2. Reactor sampling

Samples were taken per three to five days during the operation of reactors, included samples of reactor influent and effluent for COD_{Mn} and biofilm used to calculate biofilm biomass including total bacteria count and biofilm density. COD_{Mn} analyses were performed according to analysis method [18] using titration in acid environment. The consumption rate of the organic matter was determined by calculating the difference concentration of COD_{Mn} between influent and effluent.

$$C_c = C_i - C_e \quad (1)$$

Table 1
Main water quality parameters of experimental influent water*

	R1	R2
COD_{Mn} concentration (mg/L)	3.56	1.59
Free residual chlorine (mg/L)	0.026	0.03
NO_3^- (mg/L)	0.32	0.18
TOC (mg/L)	2.18	1.06
Total bacteria count (CFU/mL)	36	28

*The values in Table 1 were mean values of these water quality parameters during the experiment.

Biofilm in reactors was sampled by removing a slide from the reactor, aseptically scraping biofilm from the slide with cotton swabs, then put them into test tube with sterile ultrapure water followed by ultrasonic cleaning at 40 kHz for 20 min using a ultrasonic cleaner (KQ-500B, Kunshan ultrasonic instrument co., LTD, Jiangsu, China) to slough biofilm off the cotton swabs, homogenizing at 20,000 rpm for 1 min using a tissue homogenizer (Model M37610-33, Barnstead International, IOWA, USA), then analyzing the sample for total bacteria count using plate culture method: a 1 mL aliquot of the sample or its decimal dilutions were mixed with melted nutrient agar medium and incubated for 24 h at 37°C.

To calculate the mean biofilm density, slides were pulled out of the reactors and weighed to determine the wet biomass (m_{WM}). Afterwards, the biofilm samples were dried at 105°C and weighed again (m_{DM}). The mean biofilm density was calculated by assuming an average wet biofilm density of 1.02 g/cm^3. The mean biofilm density (gDM/m^3) was determined by formula as below.

$$\rho_F = \frac{m_{DM}}{m_{WM} / \rho_W} \quad (2)$$

2.3. Metagenomic sequencing

2.3.1. DNA extraction

Biofilm was sampled from the reactors after being operated for 35 d, 65 d and 70 d, respectively. Genomic DNA was extracted using 50 mg of biofilm sampled from the reactor was used for total community DNA extraction using E.Z.N.A. Soil DNA Kit (OMEGA) as Shen et al. [19] described previously.

2.3.2. PCR amplification

Qubit2.0 DNA Kit (Life) was used to quantify genomic DNA precisely. The V3-V4 region of 16S rDNA was amplified by PCR with primer 341F (CCCTACACGACGCTCTTCCGATCTG) and primer 805R (GACTGGAGTTCCTTGGCACCCGAGAATTCCA) to conserved regions of the 16S rRNA genes. The PCR conditions were as follows: 94°C preheating for 3 min, 5 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 20 s, and extension at 65°C for 30 s, and a following 20 cycles as this processes at 94°C for 20 s, 55°C for 20 s, 72°C for 30 s and a final extension of 5 min at 72°C, respectively. PCR amplification repeated three times according to the above method.

2.3.3. Clone and sequencing of metagenomics

Clone library construction and metagenomic sequencing were carried out by Sangon Biotech (Shanghai) Co. Ltd. (Shanghai, China), sequencing was implemented on MiSeq Sequencing instrument (Illumina) with 2×300 standard mode.

2.4. Statistical analyses

The relationship between biofilm biomass and the consumption of COD_{Mn} were studied by Pearson correlation analysis with SPSS version 19.0 (SPSS, Inc., an IBM Company). The Metagenomic sequencing data was analyzed by Uclust (Vision1.1.579).

3. Results and discussion

3.1. Consumption of COD_{Mn} and biofilm growth

The reactor 1 (R1) was continuously fed with tap water adding humic substances and the reactor 2 (R2) was fed with tap water. The mean concentrations of COD_{Mn} of their influent water were 3.56 mg/L and 1.59 mg/L, respectively.

The results of the biofilm bacteria regrowth and COD_{Mn} consumption are shown in Fig. 1. The COD_{Mn} consumption was negative till the day after 38 days of startup of R1 and R2 was 50 d. This might be caused by the overfeeding of COD_{Mn} concentration of influent flow (bacteria in reactors and influent was in adaptive phase, total bacteria count of R1 and R2 were 34 CFU/cm^2 and 23 CFU/cm^2, respectively, Fig. 1) or fouling of the surfaces, the accumulation of organic matter on slides may also partly explain the decrease in COD_{Mn} [20]. A significant correlation (R1: R = 0.764, $p < 0.001$, R2: R = 0.748, $p < 0.001$) was found to exist between the consumption of COD_{Mn} (C_C) and biofilm bacteria growth.

C_C of high organic matter content (R1) increased after 15 d of operation with total number of bacteria entered an exponential phase (Fig. 1, days 25–34). The first stationary phase (days 35–36) of R1 was observed in 34 d with biofilm bacteria of 1.1×10^4 CFU/cm^2 and went up to 0 from then on as shown in Fig. 1. Subsequently, fraction of biofilm sloughed, i.e., entered the decline phase of biofilm bacteria growth and it lasted approximately 26 d (days 36–52, nearly a half of the entire biofilm microbial growth period of R1). This was due to the continuously supplying of high organic matter content which provided sufficient nutrient for a small amount of bacteria growth. The difference value between days 37 and 59 was the difference between the number of bacteria death and production during this period. The absence or trace amount of residual chlorine was also a reason for biofilm bacteria growth which was not enough to prevent the growth of biofilm bacteria [21]. As the reactors being operating, biofilm bacteria of R1 entered a new growth cycle and reached its second stationary phase in

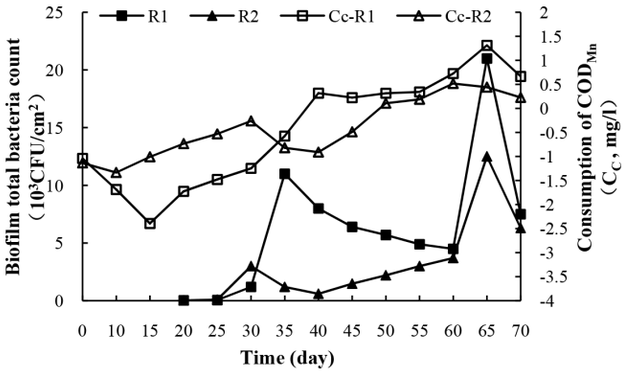

Fig. 1. Total bacteria of biofilm developed in different reactors and the consumption of COD_{Mn} in reactors.

days 65 with biofilm bacteria number of 2.1×10^4 CFU/ cm^2. Biofilm bacteria of R2 met its maximum total number of bacteria for the first time in days 30, 3×10^3 CFU/cm^2, which was a time higher than that number in R1. A downward trend of bacteria number of R2 was observed soon after this reaching a minimum value of 6×10^2 CFU/cm^2 and it started to below the biofilm bacteria number of R2 obviously at that time. This might be account for the higher concentration of COD_{Mn} in effluent of R1 and the consumption of COD_{Mn} in R1 was in excess of R2 apparently. The second time that the maximum biofilm bacteria number of R2 appeared in 65 d with number of 1.25×10^4 CFU/cm^2.

Biofilm bacteria growth period of R1 and R2 were approximately 35 and 20 d, respectively. The first time R1 reached its maximum biofilm biomass was 5 d longer than R2 with about 8×10^2 CFU/cm^2 more on bacteria number. The second time the maximum value of bacteria number was observed after respectively 5 and 25 d (2.1×10^4 CFU/ cm^2, 1.25×10^4 CFU/cm^2). This find led to the conclusion that the growth of biofilm biomass is significantly influenced by the concentration of COD_{Mn} under SWSS in residential buildings condition. Biofilm biomass has a relatively higher value and shorter time to reach the maximum total bacteria count with high COD_{Mn} concentration. The maximum numbers of biofilm bacteria of both reactors that achieved for the second time increased greatly compared to the first time, 90% and 317%, respectively. The mean biofilm densities were measured for the first time at the moment the first stationary phase appeared with 5.2 Kg/m^3 of R1 and 9.9 Kg/ m^3 of R2. The highest mean biofilm densities were achieved on days 68 of R1 (10.1 Kg/m^3) and days 65 of R2 (15.8 Kg/m^3). This fact led to similar results that have been reported earlier [22]. It is conceivable that biofilm detachment and sloughing led to biofilm compaction due to permanent bacteria production. Biofilm bacteria grew with low COD_{Mn} concentration (< 3 mg/L) had shorter growth period than that with high COD_{Mn} concentration (> 3 mg/L) so that biofilm in R2 was easy to sloughing and entering new growth period. Finally, these processes and the low concentration of COD_{Mn} in continuous inlet would yield higher biofilm densities.

3.2. Biofilm microbial community diversity

Biofilm samples were taken and analyzed for three times over the experiment with Metagenomic sequencing method, day 35, day 65 and the exfoliated biofilm during the experiment. At the phylum level, variations of top eight microorganism populations of R1 and R2 were analyzed, respectively. As shown in Fig. 2, *Proteobacteria* and *Firmicutes* (*Proteobacteria*: 80.27 vs. 53.97% in 35d, 64.45 vs. 44.75% in 65d, 45.68 vs. 32.43% in exfoliated biofilm, *Firmicutes*: 14.41 vs. 37.28% in 35 d, 32.74 vs. 47.95% in 65 d, 9.17 vs. 33.91% in exfoliated biofilm) constituted two common dominant phyla in both R1 and R2, which accounted for more than respectively 91 and 54% of the total sequencing number on the slide biofilm and exfoliated biofilm in both two reactors. The proportion of *Proteobacteria* in R1 was quite higher than that in R2 in each sample time. Liu et al. reported that *Proteobacteria* was the most dominant phylum in drinking-water distribution system and its biofilm sample which was affected by organic matter [23]. *Firmicutes* was found to be

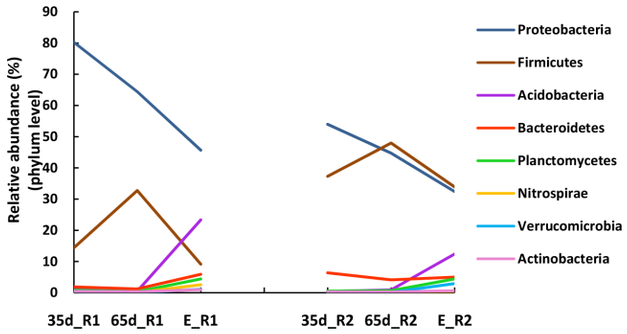

Fig. 2. Variations of biofilm microbial community at phylum level with different organic matter content (only top 8 phyla were shown which accounted for more than 91% of sequences identified).

with higher amount in surface water biofilm than that in groundwater biofilm [24]. This experiment was essentially the same with their result. It could come to the conclusion that *Proteobacteria* and *Firmicutes* was positively correlated with COD_{Mn} concentration. The number of *Acidobacteria, Planctomycetes, Nitrospirae, Verrucomicrobia and Actinobacteria* increased significantly after the biofilm sloughing. *Acidobacteria* was associated with the nitrification and *Actinobacteria* grew well in condition with chloramine residual maintained 1.6–2.18 mg/L in the lab-scale system [25]. As the experiment influent contained tiny nitride, *Acidobacteria and Actinobacteria* might not grow well in slide biofilm and were easy to exfoliate. In addition, *Acidobacteria* was also reported as *biodegradation* organism of various organic matters [26]. Thus, it could degrade other death microbial cell in exfoliated biofilm to make itself grow. Phylum of Bacteroidetes had the same increase trend with those five phyla only in R1 (COD_{Mn} concentration is 3–5 mg/L); its number changed a little during the experiment with COD_{Mn} concentration under 3 mg/L. It probably due to the organic matter content as Regueiro *et al*. reported previously [27]. The high organic matter content could cause an increase in *Bacteroidetes.*

Further at the genus level, the evolution of biofilm microbial community structure of R1 and R2 was analyzed (shown in Fig. 3). Genus of *Pseudomonas, Bacillus, Cupriavidus, Comamonas* and *Rhizobium* grew well in slide biofilm in both R1 and R2. However, each bacteria number of these genera presented a sharp decrease when the

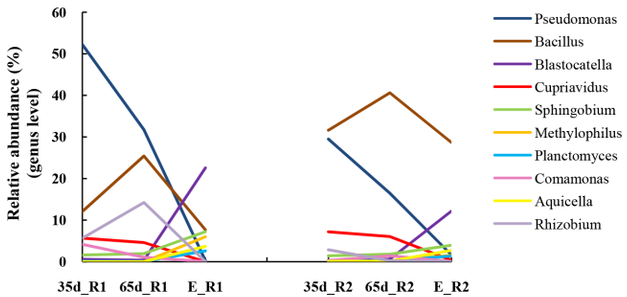

Fig. 3. Variations of biofilm microbial community at genus level with different organic matter content (only genera with a relative abundance greater than 1% were shown).

biofilm sloughed. In contrast, genus of *Blastocatella, Sphingobium*, *Methylophilus, Planctomyces* and *Aquicella* grew slowly or even stopped growing in slide biofilm while those increased obviously in exfoliated biofilm in both R1 and R2. Moreover, there was an apparent difference in the proportions of genera in slide biofilm and exfoliated biofilm in both two reactors. The dominant two bacteria genera in slide biofilm in R1 and R2 were same; they were *Pseudomonas* (52.2 vs. 29.49%) and *Bacillus* (12.1 vs. 31.63%). *Rhizobium* (5.68 %) occupied the third place in slide biofilm in R1 while that in R2 was *Cupriavidus* (7.20%). As the biofilm sloughed, the predominant genera in exfoliated biofilm in both R1 and R2 changed to *Blastocatella* (22.57 vs. 12.19%), *Bacillus* (7.66 vs. 28.68%) and *Sphingobium* (7.22 vs. 3.97%). Genus of *Pseudomonas* was found to be involved in the nutrient cycling such as denitrification [28]. Since tap water had quite little nitrides (<0.02 mg/L), bacteria number of *Pseudomonas* decreased as the time went. *Bacillus* was Gram-positive bacteria belonging to the phylum of *Firmicutes.* Besides, it was reportedly produced large amount of endospores for survival under stressful condition [28]. That might be the reason for the high growth capacity of *Bacillus. Rhizobium* was a genus grew slowly for long periods in soil and it might stimulate metal uptake of the nodulated plant [29]. In this study, organic matter content in R2 was lower than R1 and might also be difficult to be used by bacteria. Louie et al. demonstrated genus of *Cupriavidus* was of great biodegradation capability for recalcitrant compounds and xenobiotic [30] which probably was the reason of the result obtained in this experiment. Genus of *Blastocatella* was found to be aerobic, chemoorganotrophic bacteria with strictly respiratory type of metabolism [31]. *Sphingobium* had been reported to be a DBP degradation genus [32].

Genus of *Bacillus* is a member of phylum *Firmicutes*; genus of *Blastocatella* is a member of phylum *Acidobacteria*; and genus of *Planctomyces* belongs to phylum *Planctomycetes.* Other seven genera were members of phylum *Proteobacteria* which were detected in biofilm with a relative abundance greater than 1% (shown in Fig. 3). This was in consistent with the results obtained above that phylum of *Proteobacteria* was common dominant phylum in both R1 and R2. Various genera of phylum *Proteobacteria* existed in biofilm might also contribute to the dominant position of *Proteobacteria.* Since each genus had its own characteristics, they could adapt to different conditions together.

The Venn figure reflecting the difference among different times in R1 and R2 was depicted (Fig. 4). There were 4470, 4305 and 4324 OTUs in slide biofilm in 35 d, 65 d and in exfoliated biofilm in R1, respectively. In comparison to R1, there were 4254 and 4348 OTUs in slide biofilm in 35 d and 65 d, respectively, 4431 OTUs in exfoliated biofilm in R2. The total shared richness in R1 and R2 during the experiment was 447 and 773, respectively. It indicated that 326 OTUs in R2 were absent in R1, thereof 29 and 41 belonged to *Proteobacteria* and *Firmicutes*. Therefore, it could be concluded that the microbial species diversity of R2 was higher than that of R1. That may because of the proportion of *Proteobacteria, Firmicutes* and *Bacteroidetes* changed in low COD_{Mn} concentration condition in comparison to that in high COD_{Mn} concentration condition. Phyla of *Proteobacteria, Firmicutes* and *Bacteroidetes* were dominant phyla in most mirco-polluted water. The sum of the proportions of *Firmicutes* and *Bacteroidetes* in R2 was much higher than that in R1 for all of 6 samples and the sum of their proportions increased as the sampling time increased. However, the proportion of *Proteobacteria* in each reactor decreased while the proportions of *Firmicutes* and *Bacteroidetes* increased. Thus, the total proportion of those three dominant phyla in R2 was higher than that in R1, namely the microbial community diversity of R2 was higher than that of R1. An ecosystem with rich species can maintain system stability and balance due to its stronger anti-disturb ability against external environment [33]. In brief, the rich diversity of R2 might be a reasonable explanation for the higher total shared richness in R2 from the beginning to the end.

Some genera of pathogenic bacteria which were listed by World Health Organization (WHO) [34] were detected (Fig. 5). *Pseudomonas* accounted for more than 50% in slide biofilm with high COD_{Mn} concentration (R1) and it was hard to exfoliate (it accounted for only 0.25% in

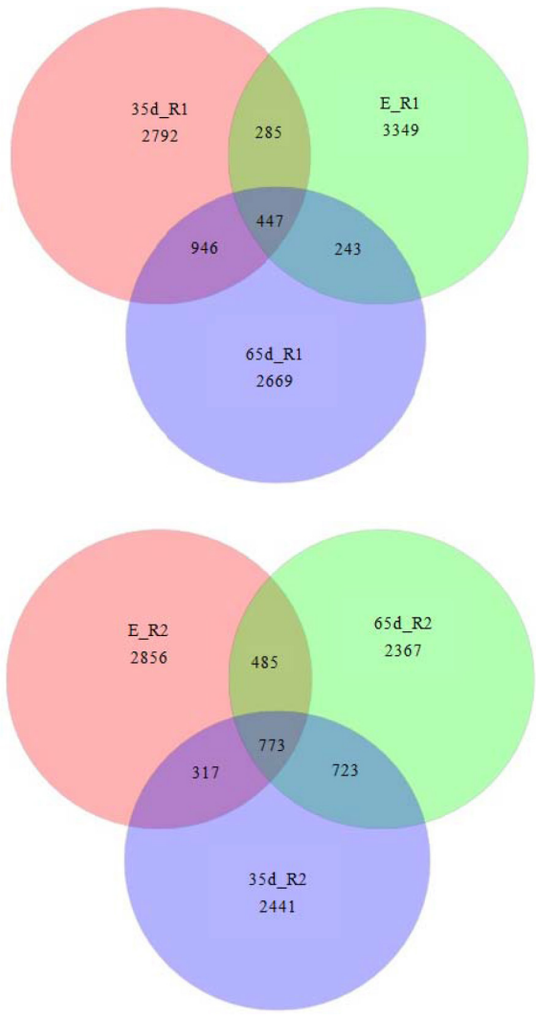

Fig. 4. Venn diagram (a: compared richness in different time in R1; b: compared richness in different time in R2).

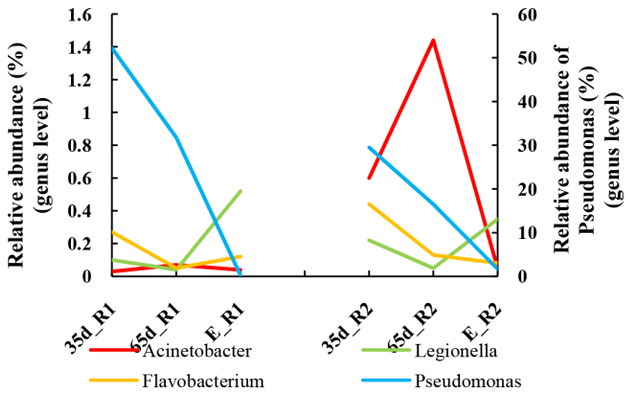

Fig. 5. Development of pathogenic bacteria that detected in biofilm in different organic matter content.

exfoliated biofilm over the experiment in R1). Meanwhile, the amount of Pseudomonas in slide biofilm in R2 was approximately half of that in R1 but was times higher in exfoliated biofilm in R2 than that in R1. All these results indicated that the growth of *Pseudomonas* and its activity were in positive correlation with organic matter content. Genus of *Acinetobacter, Legionella* and *Flavobacterium* were also pathogenic bacteria that have been detected in the experiment. *Legionella* could keep growing in exfoliated biofilm in both R1 and R2.

4. Conclusions

In summary, the present experiment studied the biofilm growth and its microbial community diversity in different organic matter content conditions. The results suggested that organic matter content had significant effect on biofilm growth including bacteria number and biofilm density. Tap water with qualified organic matter content according to Chinese drinking water standards (COD_{Mn} < 3 mg/L) might make the biofilm in SWSS stay biological stability within 50 days while that for unqualified tap water (COD_{Mn} > 3 mg/L) was 30 days. The organic matter content also influenced the diversity of biofilm microbial community. Phyla of *Proteobacteria* and *Firmicutes* were dominant in both biofilm cultivated with unqualified and qualified tap water (R1 and R2). Phylum of *Acidobacteria* kept growing in exfoliated biofilm and grew better in exfoliated biofilm with COD_{Mn} more than 3 mg/L. Reactor fed with low COD_{Mn} concentration has a higher total shared richness than that in reactor fed with high COD_{Mn}. The proportion of genera was associated with organic matter content. Pathogenic bacteria such as *Pseudomonas*, *Acinetobacter, Ledionella* and *Flavobacterium* could grow in tap water, although the tap water was conformed to standards.

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Symbols

C_c	—	consumption of COD_{Mn} (mg/L)
C_i	—	concentration of COD_{Mn} in the influent (mg/L)
C_e	—	concentration of COD_{Mn} in the effluent (mg/L)
m_{WM}	—	mass of the wet biofilm (g)
m_{DM}	—	mass of the dry biofilm (g)
ρ_W	—	density of the wet biofilm (g/m^3)
ρ_F	—	mean biofilm density (g/m^3)

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