Microbial community response to Ciprofloxacin toxicity in sponge membrane bioreactor

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Abstract

This study aims to offer insights into how ciprofloxacin (CIP) impact bacterial community structures in the Sponge-MBR process when CIP is spiked into hospital wastewater. We found that the CIP toxicity decreased richness critical phylotypes such as phylum class δ-, β-, ɣ-proteobacteria, and Flavobacteria that co-respond to suppress denitrification and cake fouling to 37% and 28% respectively. Cluster analysis shows that the different community strutures were formed under the influence of CIP toxcicity. CIP decreased attached growth biomass by 2.3 times while increasing the concentration of permeate nitrate by 3.8 times, greatly affecting TN removal by up to 26%. Ammonia removal was kept stable by inflating the ammonia removal rate ($p<0.003$), with the wealthy *Nitrospira* genus guaranteeing the nitrification activity. In addition, we observed an increasing richness of *Chloroflexi* and *Planctomycetes*, which may play a role in fouling reduction in the Sponge-MBR. Therefore, if the amount of antibiotics in hospital wastewater continues to increase, it is so important to extend biomass retention for denitrification recovery.

Keywords: Antibiotics; Sponge membrane bioreactor; Hospital wastewater; Microbial community; Denitrification; Membrane fouling.

1. Introduction

Conventional activated sludge (CAS) was not designed to completely remove xenobiotics from wastewater treatment (Patel et al., 2019), and membrane bioreactors (MBR) have been suggested as a promising alternative technology thanks to their flexibility in controlling solids retention time (SRT) (Bui et al., 2018; Zheng et al., 2019). Recently, the Sponge-MBR, a hybrid process, has emerged to upgrade the performance of the MBR (Deng et al., 2014). The Sponge-MBR has a narrow footprint because it possesses a unique integration of bio-carriers (Sponge) with traditional low-pressure microfiltration to reduce excess nitrogen and fouling. The application of a Sponge-MBR with appropriate SRT could be a viable option to overcome the adverse effects of micropollutants in hospital wastwater s robustness has attracted attention for hospital wastewater treatment regarding antibiotics removal (Nguyen et al., 2016). Nguyen et al. (2016) reported that adding 20% more bio-carriers (v/v) into the MBR energetically activated the simultaneous nitrification-denitrification (SND), improving total nitrogen removal from 9% to 16%. Moreover, high degrees of antibiotics were eliminated: Norfloxacin 86%, Ofloxacin 93%, CIP 70%, Tetracycline (approximately 100%), and Trimethoprim 93% at a solid retention time of 20 days (Nguyen et al., 2017). However, Nguyen et al. (2019) found that daily exposure to Ciprofloxacin antibiotics (20-200 µg/L) were suspected of causing biomass lethality and reducing denitrification to 3-12% instead of the normal 35%. In other words, antibiotics strongly interfere with the SND process when the toxicity exceeds the tolerance of the microbial community.

Ciprofloxacin (CIP) is one of the most potent synthetic antibiotics currently being prescribed worldwide (Tchesnokova et al., 2019). and has been regularly reported in hospital effluent: 12.5–19.7 µg/L (Turkey) (Aydin et al., 2019), 0.6–53.3 µg/L (Vietnam) (Lien et al., 2016), 17.9 – 46 µg/L, and 3–87 µg/L (Switzerland) (Hartmann et al., 1998; Kovalova et al., 2012), 32–99 µg/L (Brazil) (Martins et al., 2008), 3.6–101 µg/L (Sweden) (Lindberg et al., 2004) and 2.2–236.6 µg/L (India) (Diwan et al., 2010). Moreover, CIP use is poised to rise since global consumption is estimated to increase by 15% between 2015 and 2030 (Klein et al., 2018). Considering the concentrations currently being seen, high levels of antibiotics present in on-site hospital treated wastewater might pose a risk to the microbial community and impair future treatment efficiency.

Tracking microbial communities using next-generation sequencing has enabled insight into microbial profiles and their metabolism (Goodwin et al., 2016). However, identifying key microbial members involved in the Sponge-MBR treating antibiotics has yet to be explored. For instance, denitrifying communities in suspended growth of activated sludge are commonly associated with phylum *Firmicutes, Bacteroidetes,* and *class β-, α-, δ- and γ-proteobacteria* (Heylen et al., 2006; Thomsen et al., 2007; Ju et al., 2014; Zhang et al., 2016; Gonzalez-Martinez et al., 2016). Whether such phylotypes are present when applying the attached growth process to hospital wastewater treatment is unknown, although attached growth is considered a core function directly related to nitrogen removal. For nitrification, the co-existence of *Brocadia anammoxidans* and *Nitrospira* were highlighted regarding nitrification activity at limited oxygen conditions (Van Kessel et al., 2015). This state creates a distinctive DO gradient along with the sponge-inward depth (Guo et al., 2010), therefore, it is important to explore the effects of

antibiotics on the nitrifying community in the Sponge-MBR. Under long SRT, the fraction of persistent soluble microbial products (SMP) in MBR occurs largely due to biomass decay and cell lysis. Miura et al. (2007) have successfully demonstrated the potential of phylum *Chloroflexi's* contribution to biodegradation glucose and N-acetyl glucosamine as SMP-like cell wall peptidoglycan is extremely useful for fouling reduction. Recent studies by Deng et al. (2016b, 2014) have shown that the sponge-MBR obtains lower concentrations of EPS and SMP in mixed liquor than the MBR. This issue of the relationship between *Chloroflexi* and the sponge carriers and their combined potential to reduce fouling should be addressed.

Bacterial activity, particularly when bacteria is exposed to CIP, is vastly decreased in aerobic granular sludge, partial-nitritation, conventional activated sludge (CAS), and the MBR process. Amorim et al. (2014) found that antibiotics greatly influenced TN removal and destroyed aerobic granular sludge. With dosages from 250 ng/L to 0.9 mg/L, antibiotics caused toxicity to *ammonia-oxidizing bacteria (AOB)* populations and increased effluent NH_4^+-N via partial-nitritation and conventional MBR processes (Gonzalez-Martinez et al., 2014; Meng et al., 2015). The family *Nitrosomonadaceae (AOB)* and genus *Nitrospira* saw suppression at 100 to 5000 µg/L in CAS (Zhang et al., 2019).

Such evidence implies that increased antibiotics in wastewater reduce the positive effects of autotrophs. Antibiotics are also believed to inactivate the function of biological wastewater treatment, damage symbiotic relationships, and stimulate the antibiotic resistant pathogenic bacteria. However, to date, discharge antibiotic regulations have yet to be established in hospitals worldwide (Patel et al., 2019). Currently, there is no standard technology or even guidance for

the removal of antibiotics in wastewater. To mitigate these adverse effects and develop effective operation strategies in the long-term, it is vital to understand the behavior of how bacterial communities respond to antibiotic toxicity. Therefore, this study investigates the changes in microbial communities for control and spiked CIP; and offers insights into how CIP impacts organic, nitrogen removal and fouling in the Sponge-MBR process. The outcomes derived from this research provide insight data to improve on-site hospital wastewater treatment.

Materials and Methods

2.1. Wastewater

250 L of wastewater were collected twice a week from an emergency hospital with 1100 patients, staff, and visitors located in Ho Chi Minh City, Vietnam. The wastewater mainly came from the hospital's 900 beds. The influent was gathered by the hospital's wastewater treatment system based on the A/O process with a capacity of 1,000 m^3/day. Components of the raw wastewater included: COD (371± 68) mg/L, TKN (20.2±3.9) mg N/L, NH_4^+-N (8.1±3.1) mg N/L, TP (0.3±0.3) mg P/L, pH 6.5 ± 0.4 (mean ± standard deviation, n=24). Antibiotics in the hospital's wastewater were selected and are shown in Fig.1. These include: Amoxicillin (AMOX), Cefotaxime (CEF), Erythromycin (ERY), Norfloxacin (NOR), Ofloxacin (OFL), Ciprofloxacin (CIP), Sulfamethoxazole (SUL), Tetracycline (TET), Trimethoprim (TRI), and Vancomycin (VAN). The specific properties of these antibiotics (i.e., classification, molecular weight, formula, structure, solubility, pK_a, and Log K_{ow}) are denoted in Table S1.

2.2. Design and operating conditions

The seeding biomass was taken from a conventional MBR system. This system is an anoxic - MBR (MBR) with SRT of 20 days treating domestic wastewater. The seed sludge (mother community) was used for two identical laboratory-scale sponge-MBR reactors; a detailed schematic diagram is presented in Figure. S4 a, b. In brief, each system consists of a hollow fiber membrane module (polyvinylidene fluoride, Mitsubishi-Rayon, Japan); the active membrane surface area is 0.05 m^2 with a pore size of 0.4 µm. The sponges are made from polyurethane (CC-10B, Nisshinbo, Japan) with specific properties: density of 30 kg/ m^3, porosity of 98%, surface area over 3000 m^2 /m^3, and dimensions of 1 cm x 1 cm x 1 cm. Sponges occupied a working volume of 20% (v/v) in the reactor. In the reactor with spiked CIP, antibiotics were dosed into a feed tank (100 L) every three days. These concentrations were gradiated gradually from 20, 50, 100 to 200 µg/L in a month. The other Sponge-MBR was used as a control reactor using the same influent but a different feed tank. Both systems were operated with an apparent SRT of 20 days to preserve attached growth, a hydraulic retention time (HRT) of 8 h, a flux of 20 LMH, an average organic loading rate (OLR) of 1.1 kg COD/m^3d, and at an ambient temperature at 25-31°C. CIP and biomass interaction were increased from seed sludge (MBR, low antibiotics) to the Sponge-MBR (control, natural CIP of 45 ug/L) to the Sponge-MBR200 (spiked CIP of 200 ug/L).

2.3. Performance analysis

Conventional parameters included COD, total Kjeldahl nitrogen (TKN), NH_4^+–N, NO_2^-–N, NO_3^-–N, TN, and SVI were analyzed based on Standard Methods by American Public Health Association. The nitrogen balance, fouling resistance-in-series model, and antibiotics are from (Nguyen et al., 2017). Antibiotics in the sample were enriched by Oasis HLB cartridges (60 mg/3 mL) from Waters (Guyancourt, France) and analyzed by LC/MS/MS (Agilent1200 series). Biomass characteristics were determined as follows: (1) MLSS measurement (105°C) of attached and suspended growth, (2) observation by scanning electron microscope (SEM, Hitachi S-4800) on Sponge porosity (Figure S4 c,d) and (3) tracking the particle-size distribution (PSD) of suspended flocs by laser diffraction analysis (Horiba LA 950, Japan). The performance relative to each CIP dosage (i.e., 20, 50, 100 to 200 µg/L) has been extensively discussed previously by Nguyen et al. (2019). Part of previously published data belonging to the CIP of 200 µg/L was summarized to help interpret the microbial communities and compare them with control reactor data.

2.4. DNA extraction and sequencing analysis

454 pyrosequencing analysis was employed to compare each suspended sludge sample collected from the MBR, the Sponge-MBR, and the Sponge-MBR200. The attached biomass was further investigated in the added CIP reactor denoted as the Sponge-MBR200carrier. The biomass from the two reactors was collected for analysis at the same time. All samples were centrifuged at 11,000×g for 10 min, and the supernatant was decanted for DNA extraction. Genomic DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Cat. 6560-200, Solon, OH, USA). The extracted DNA was confirmed in gel-electrophoresis for 50 mins at 50 V, with 1%

agarose in tris-acetate-EDTA (TAE) buffer. The extracted DNA was purified with the MO BIO PowerClean DNA Clean-Up Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). Purified DNA was amplified by polymerase chain reaction (PCR). This study used fusion primers 27F/518R with different barcodes to target V1-V3 of 16S rRNA genes, as described previously (Yang and Jahng, 2016). Variable regions V4 and V5 were selected using fusion primers A519F/A958R for Archaea. The ITS2 (Internal Transcribed Spacer 2) was used for Eukarya detection. PCR was carried out using a thermocycler (T100 Thermal Cycler, Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the following conditions: initial denaturation (5 min at 95°C), 30 cycles of denaturation (30 s at 95°C), annealing (30 s at 55°C), extension (30 s at 72°C), and a final extension (7 min at 72°C). The PCR products were electrophoresed on a 1% (w/v) agarose gel and were purified by the QIAquick PCR Purification Kit (Qiagen, Cat. No. 28106, Germany). The concentration of the PCR amplicon was quantified using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). The pyrosequencing was carried out by ChunLab, Inc. (Seoul, Korea) using the 454 GS Junior Sequencing System (454 Life Science, Branford, CT, USA).

2.5. Processing of sequencing data

The obtained sequences were sorted via barcode sequences. Then, the linker, barcode, and PCR primers were trimmed before subsequent bioinformatics steps. Sequences less than 300 bp and less than the average quality score (value of 25 or less) were removed. The taxonomic classification is based on the EzTaxon-e database (https://www.ezbiocloud.net/taxonomy).

Sequences that could not be matched to the EzTaxon-e database at the species level (>97%) were considered chimeric artifacts and checked by the UCHIME program (Edgar et al., 2011).

2.6. Statistics Analysis and Bioinformatics

Student's t-tests were used to compare mean values of parameters with a significance level (p-value) of $\alpha = 0.05$. The alpha-diversity was calculated by mothur package (Schloss et al., 2009). Beta-diversity analysis obtained from the Fast UniFrac were used to describe the way that communities clustered (Hamady et al., 2010). Data analysis on influent antibiotics in the hospital was performed using R software. The change in dominant bacteria at the class level was obtained using a Circos software package (http://circos.ca/) (Krzywinski et al., 2009). The sequences of the most abundant bacteria, archaea, and eukarya were selected for multiple sequence alignment to construct a phylogenetic tree using Geneious software (Biomatters Ltd.) (Kearse et al., 2012).

3. Results and discussion

3.1. Diagnostic sponge treatment under antibiotic toxicity

Free antibiotics can greatly hamper efficient treatment of hospital wastewater. Fig. 1 shows the concentration distribution of the ten antibiotics (µg/L, n = 8). The average concentration was estimated at VAN (73.6±53.2), NOR (54.3±47.6), CIP (45.4±57.9), OFL (55.1±47.1), SUL (41.5 ± 59.4), TRI (16.2±19.5), ERY (6.3±5.9), TET (2.7± 3.8), AMO (2.0± 3.1), and CEF (0.3 ± 0.5). Fluoroquinolones (FQs) such as Norfloxacin (6.305-43.610), Ofloxacin (7.634-40.261), Ciprofloxacin (1.926-23.841) were detected in 100% of the collected raw influent sample (Nguyen et al., 2017) and are likely to increase in concentration in our one-year follow-up study

In particular, the CIP levels are very similar to those detected at several hospitals (42.8 μg/L) in Hanoi, Vietnam (Lien et al., 2016).

Insert Figure 1

Hospital wastewater treatment efficiency with a spiked CIP dosage is summarized in Table 1. Compared to the control reactor, the spiked CIP significantly decreased total MLSS by 26% (7563 ± 478 vs. 5592 ± 907 mg/L, $p<0.05$), respectively. However, it is possible that there is no biomass decrease in suspended growth, but rather a fractioning in the Sponge: attached growth in control was 2.3 times higher than the spiked CIP reactor (1504±641 vs. 1959±869 mg MLSS/L; $p<0.005$), respectively. Biomass in suspended growth is enhanced in the spiked CIP reactor (3059 ± 432 vs. 3653 ± 392 mg MLSS/L, $p>0.05$). The rationale for such a phenomena is that CIP can impact both suspended and attached growth; however, the surplus suspended biomass from the spiked CIP reactor could result from biomass detachment.

Insert Table 1

Using particle size distribution to observe floc size, we found an unexpected phenomenon (Fig. 6a). The suspended floc size peak belonging to the spiked CIP reactor frequently harbors two minor peaks: one peak constructed by a wide range of floc sizes (678-2301 μm), and the other by a smaller range (27–61 μm). The first peak could be attributed to biofilm detachment, while the second is associated with inherent suspended flocs. After analyzing the data, it was thought that the two minor peaks would merge into a single peak by the dynamic of aeration or self-decomposition, but currently there is little evidence. Perhaps future studies will address this point. It is therefore possible that detachment from carriers increased MLSS in suspended

biomass. The remnants of biofilms observed on the sponge surface confirmed the fact that CIP restricted biomass development in the attached growth (Fig. 6b).

The 3.8 times higher rate of nitrate released in permeate (1.5±1.5 vs. 5.7±3.4 mg/L; $p<0.00068$) implied that CIP significantly affects the nitrate reduction step, given a 26% lower TN removal (69 ± 12% and 43 ± 21%; $p<0.05$) and TN based on denitrification (37%) ((Table.1). There was no difference between ammonia removal (77±16 vs. 76 ±12%, $p = 0.98 > 0.05$), and the ammonia removal rate is likely to be improved under toxicity (0.09 ± 0.05 vs. 0.17±0.06 mg/g MLSS/h, $p<0.003$). In addition, the organic removal rate increased up to 36%. Such results implied that the nitrification process could withstand antibiotic stress. Moreover, CIP dosage seems to enhance sludge settleability ($p<0.001$) and decrease the mean size of suspended flocs (58±3 vs. 31±6 µm, $p<0.03$). Detachment of attached growth might also occur on the membrane leading to lower fouling, which decreased cake resistance by 28% on the membrane surface compared the control condition (Fig. 6c, d). In summary, the diagnostic performances are listed in Table 2. These results showed that added CIP into hospital wastewater has a great impact on biomass belonging to attached growth, membrane biofilms, narrow flocs size, and the undermining of the denitrification process.

Insert Table 2

3.2. Species richness and diversity

CIP injection is thought to corrupt deoxyribonucleic acid (DNA) synthesis and replication, which could logically reduce alpha diversity. However, our results suggest that these two Sponge-MBR reactors possess a higher number of OTUs, Chao1, and a higher diversity on the Shannon index

compared to seeding sludge (Table.3 and Fig.S1), resulting in more richness and diversity associated with gradients of antibiotics (i.e., Sponge-MBR200 > Sponge-MBR > MBR). Number OTUs in the Sponge-MBR200 approached 1.4 and 2.0 times higher than the Sponge-MBR and conventional MBR, respectively. High concentrations of antibiotics (Fig. 1) can induce a robust and sustained protective cell response, allowing them to withstand and increase cell numbers under CIP injection (Deng et al., 2019). This implies that richness and diversity could rebound from FQs.

Insert Table 3

Fig. 2 shows the community distance, which evolved from a common ancestor (MBR). High feed antibiotics (Fig. 1) extended the length of the phylogenetic relationships from seeding sludge resulting in a high richness and lineage diverse community (Table.3). Moreover, the distance of the community structure of Sponge-MBR200 (suspended sludge) is much farther from the Sponge-MBR200carrier (attached sludge). Previous studies have reported that the natural distribution of communities collected from suspended sludge is significantly different from the attached growth in the absence of exposure to antibiotics (Reboleiro-Rivas et al., 2015; Choi et al., 2017). However, the difference could also be taken into account for another reason; a spiked CIP might decrease the richness and diversity of the Sponge-MBR200carrier (Table.3) which negatively effects treatment performance. In such cases, a higher number of OTUs are generated from suspended growth than attached growth, possibly cause by detachment (Fig. 6 a,b).

Insert Fig. 2

3.3. Taxonomic structure response to feed antibiotics

The bacterial profile of the Sponge-MBR (suspended sludge) is almost the same as the Sponge-MBR200 (suspended sludge) and the Sponge-MBR200carrier (attached slugde) at the phylum level (Fig. 3). Acclimatization of seeding sludge to hospital wastewater appears to stimulate antibiotic susceptibility resulting in a bacterial profile very similar to the spiked CIP profile. The phylum *Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes,* and *Chloroflexi* accounted for 90% of OTUs. Notably, *Proteobacteria* and *Bacteroidetes* decreased slightly with an increased proliferation of *Chloroflexi, Planctomycetes, and Actinobacteria* in the Sponge-MBR200 (suspended sludge), and the Sponge-MBR200carrier (attached sludge). This implies that CIP also partly inhibits the growth of *Proteobacteria and Bacteroidetes* and enhances the abundance of minority groups.

Insert Fig. 3

The communities changed sharply below the subphylum level (Fig. 4). For instance, the MBR showed the dominant 35% *Gamma- (γ-proteobacteria*) approximate 18984 reads, which decreased after hospital wastewater treatment. In contrast, *Alpha-(α-proteobacteria)* were greatly increased. The *α-proteobacteria* were the most prevalent in the Sponge-MBR (28%) (suspended slugde), the Sponge-MBR200 (35%) (suspended slugde), and the Sponge-MBR200carrier (31%) (attached slugde), respectively. As reported, both *α- and γ-* branches could persist against FQs undergoing treatment in aerobic granular sludge (Amorim et al., 2014), SBR (Kong et al., 2017), AO/MBR (Wen et al., 2018). However, our results show that antibiotics seem to stimulate the growth of the α- instead of the *γ* -branch and lead to *α-proteobacteria,* which is considered the

most resistant phylotype in all biological wastewater treatment processes. On the other hand, the richness of *Delta-(δ-proteobacteria)* and *Flavobacteria* decreased in the Sponge-MBR200 (suspended slugde) and the Sponge-MBR200carrier compared to the Sponge-MBR. A total of four dominant members were suppressed under the added CIP condition (Fig. 4). The lack of *ɣ-proteobacteria, δ-proteobacteria*, *Flavobacteria,* and *Acidimicrobiia,* may have a severe impact on nitrogen removal in the added CIP reactor (Section 3.1)

Insert Fig. 4

Pyrosequencing data have generated many uncultured microorganisms, and a nucleotide-based phylogenetic analysis was used to identify the sequences homology with available sequences in the NCBI Database (Fig. 5). To counter the effects of antibiotics, bacteria expend energy to survive and replicate. As a result, their main function is the production of EPS, and they are well-known for their drug resistance and as denitrifiers. Moreover, it was reported that *Euryarchaeota* which belong to the domain *Acharea* can co-existence with *Thaumarchaeota* under oxytetracycline and spiramycin stress in a UASB/anoxic/oxic reactor (Zhang et al., 2015). Our study confirmed that *Euryarchaeota* (class *Methanobacteria)* can also persist in hospital wastewater and in added CIP conditions. Because *Euryarchaeota* thrive in strictly anaerobic conditions; sponge carriers, in this case, could be an important habitat for their growth.

Insert Fig. 5

3.4. Nitrification

Tracking slow-growing autotrophs can lead to a better understanding of nitrification activity under the influence of xenobiotics. One of the top vital phyla, *Nitrospirae (NOB*), is still

researched because of its involvement in the nitrogen cycle. In this study, *Nitrospirae* accounted for 0.12% of the phyla in Sponge-MBR200 and 0.31% in the Sponge-MBR200carriers. Both values were higher than in the Sponge-MBR (0.02%) (Fig. 3). These results indicate that *Nitrospirae* increases with increasing CIP at 200 µg/L in hospital wastewater. These finding are consistent with Yi et al. (2017) where *Nitrospirae* adapted to CIP in an SBR process with added concentrations up to 2 mg/L (Yi et al., 2017). Current knowledge suggests that the genus *Nitrospira (Comammox)* itself can perform complete nitrification (Van Kessel et al., 2015), hence, the high levels of *Nitrospirae* in the Sponge-MBR200carrier might contribute to a stable amonia removal rate (77%) that in turn increased the nitrification rate up to 88% (Table 1). *Candidatus Brocadia anammoxidans* has also been detected in the Sponge-MBR200 (Fig. S2). The co-existence of *Brocadia* and *Nitrospira* was associated with nitrification activity at low oxygen concentrations (Van Kessel et al., 2015). Because of this, the anoxic/anaerobic zone in sponge carriers could provide critical habitat to select slow-growing organisms (as K-strategists) to enhance diversity in the Sponge-MBR200.

3.5. Denitrification and resistant bacteria

In the spiked CIP reactor, the higher values of nitrates released in permeate imply that denitrification has an important impact, because denitrified communities constructed by the existence of phylum *Firmicutes, Bacteroidetes, class β, α-, δ - and ɣ-proteobacteria* are frequently reported. (Heylen et al., 2006; Thomsen et al., 2007; Ju et al., 2014; Zhang et al., 2016; Gonzalez-Martinez et al., 2016). The control reactor provides high-efficiency nitrogen removal that contributes to the presence of *class β-, δ - branch,* and *Flavobacteria*

(*Bacteroidetes*). This group (*class β-, δ - branch,* and *Flavobacteria*) includes the families *Comamonadaceae* (Mcilroy et al., 2016), *Bdellovibrionacea* (Grossmann et al., 2007), and *Flavobacteriaceae* (Kundu et al., 2014) that have essential roles for denitrification in the Sponge-MBR (Fig. S3). As reported, those groups harbor denitrifying gene clusters and ammonification systems to perform the SND process. Thus, the *β-, δ -branch, and Flavobacteria* are possibly related to improving nitrogen removal in the control reactor. In contrast, the CIP spiked reactor lost various strains responsible for denitrification, such as *Firmicutes (*Fig. 3), *Flavobacteria, δ, β, and ɣ-branch* (Fig. 4), reducing its capacity for denitrification (Table 2).

α-*Proteobacteria, Sphingobacteria, Chitinophagia*, and *Actinobacteria* are resistant phylotypes in the Sponge-MBR200 (Fig. S3). Interestingly, this group is related to the denitrifying communities such as genus *Amaricoccus* (Maszenan et al., 2000); class *Sphingobacteria* (Chen et al., 2015) discovered in SBR's anaerobic/oxic/anoxic process (Ginige et al., 2004; Kondo et al., 2009). Although the above groups (*-Proteobacteria, Sphingobacteria, Chitinophagia,* and *Actinobacteria*) could partially improve denitrification, the efficacy is much lower than in the control condition where *Flavobacteria, δ, β, and ɣ- branch* are simultaneously found.

3.6. Pioneer bacterial species responsible for biofouling

Fig. 6c shows the re-analyzed results from the spiked CIP reactor from Nguyen et al. (2019) with CIP suppressing the trans-membrane pressure (TMP) evolution. This shows that with fouling the biofilm thickness inside the cake layer expands the anoxic zone, which provides appropriate conditions for the selection of specific bacterial development, especially those as *Firmicutes, class ɣ-proteobacteria,* and *Flavobacteria* (Lim et al., 2012; Zhang et al., 2014; Takada et al.,

2018; Takimoto et al., 2018). The Sponge-MBR200 retained a low richness of *Firmicutes, Flavobacteria, δ, β, and ɣ-proteobacteria* and this may have caused the Sponge-MBR200 to have a 28% lower cake layer resistance (R_c) than the control (Fig. 5d), decreased attached growth (Fig. 6b, Table 1), and a suspended flocs structure (Fig. 6a, Table 2). Thus, these findings emphasized that those *Firmicutes, Flavobacteria, δ, β, and ɣ-proteobacteria* co-responded to suppress denitrification and fouling.

Insert Fig. 6

Additionally, a high richness of minority phylum (Fig. 3) might play an essential role in fouling reduction. One of the most advantageous features of the Sponge-MBR is its long-term low fouling rate compared to the MBR. Previous results showed that added bio carriers decrease effluent concentration extracellular polymeric substances (EPS) more in SMP than in MBR during prolonged SRT (Deng et al., 2016, 2014). Unfortunately, insights into SMP sorption and biodegradation mechanisms by bio carriers have not been fully explored, while profiles of bacterial communities regarding fouling are still overlooked. This study shows that an abundance of *Chloroflexi, Planctomycetes, Acidobacteria, and Actinobacteria* in the Sponge-MBR is higher than in the MBR, which can aid in biodegradation of metabolic products and slow down the TMP jump when spiked CIP and control conditions are taken into account (Fig. 3). Because CIP partly inhibits the growth of *Proteobacteria* and *Bacteroidetes* (as an r-strategists microorganism) (section 3.3) resistant bacteria synthesizes more EPS, while high SMP compounds are generated in the mixed liquor. Those metabolite products, cell death, and SMP can be rich substrate sources for the proliferation of symbiotic populations. This increase in

minority phylotypes is due to the consumption of excess soluble substrates which indirectly reduce fouling. Previous studies reported that the metabolic versatility of *Chloroflexi* and *Planctomycetes* has contributed to a decrease of persistent SMP and EPS in conventional MBR, and that *Chloroflexi* is strongly related to fouling reductions (Miura et al., 2007; Wang et al., 2015). *Planctomycetes* are especially resistant to β-lactams, quinolone nalidixic acid, and glycopeptide vancomycin (Cayrou et al., 2010). The addition of 20% more sponges to the MBR process not only allows them to become an excellent habitat for slow-growing microorganisms, but also enhances symbiotic populations for more effective treatment under prolong SRT and stressful conditions.

3.7. Environmental Implications

In developing countries, environmental pollution increases the rate of bacterial infection, which in turn leads to an increase in the variety and concentration of antibiotics discharged in hospital wastewater. Since antibiotics significantly inhibit biomass growth, a proper SRT operation can be the first step to enhancing antibiotic sorption and degradation. Possessing enough biomass might also accelerate denitrification recovery. Bioaugmentation using denitrifier culture is a second viable method for reestablishing a symbiotic relationship to remove organic matter and nitrogen. Importantly, improving the overall biological removal function of CAS or MBR is an urgent need in hospital wastewater treatment. The advantage of the MBR process has been demonstrated in its ability to suppress a higher number of antibiotic resistance genes (1.5-7.3

log) than CAS (0.8-3.4 log) (Li et al., 2019). Additionally, Sponge-MBR systems could benefit urban hospitals with their saving space footprint. Sponge-MBR systems with appropriated SRT could be an option to overcome many adverse effects from micropollutants. However, short HRT could be a disadvantage for complete antibiotic removal as even the release of high surplus antibiotics can stimulate the spread of antibiotic-resistant bacteria in outside treatment plants. Currently, regulations on discharged antibiotic concentrations have not been universally established, and this has created a barrier for setting technology treatment standards. Consequently, the integration of tertiary treatment after the MBR process, such as low-cost biochar-based filtration systems, should be considered to enhance antibiotic removal in urban and rural hospitals.

4. Conclusions

The spiked CIP in hospital wastewater decreased the attached growth biomass by 2.3 times which have reduced TN removal by 26%. CIP caused a 3.8 times higher release of nitrates in permeate compared to control conditions while decreasing total MLSS up to 26%. The lack of *Firmicutes, Flavobacteria, δ, β, and ɣ-proteobacteria* directly co-responds to suppress denitrification (37%) and cake fouling (28%) in the Sponge-MBR. Ammonia removal reaches 77 ± 16% and the removal rate is increased to 0.17 ± 0.06 mg/gMLSS.h at CIP dosage of 200 µg/L. The Sponge-MBR possesses an excellent habitat for developing slow-growing microorganisms such as *Nitrospira (Comammox)* and symbiotic populations such as (*Chloroflexi, Planctomycetes, and Actinobacteria);* for improving organics and nitrification; and

for playing a pivotal role in fouling reduction. The application of Sponge-MBR with appropriate SRT could be a viable option to overcome the adverse effects of micropollutants in hospital wastwater.

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Table 1. Comparison of performance status between the Sponge-MBR (control) and the Sponge-MBR with added CIP of 200µg/L (added CIP). Sample number for each reactor condition (n = 12).

Parameters	Reactor condition	Feed (mg/L)	Permeate (mg/L)	Efficiency (%)	Removal rate (mg/gMLSS.h)
COD (mg/L)	Control	366 ± 65	14 ± 8	96 ± 3	0.14 ± 0.03
	Added CIP	373 ± 65	21 ± 14	94 ± 4	0.19 ± 0.06
NH_4^+-N (mg/L)	Control	6.8 ± 3.4	1.4 ± 0.6	76 ± 12	0.09 ± 0.05
	Added CIP	9.5 ± 2.3	2.3 ± 1.8	77 ± 16	0.17 ± 0.06
TKN (mg/L)	Control	21.0 ± 3.4	4.7 ± 2.1	77± 11	0.27 ± 0.08
	Added CIP	19.3 ± 4.2	4.9 ±1.7	74 ± 10	0.34 ± 0.14
TN (mg/L)	Control	21.8 ± 3.2	6.7 ± 2.5	69 ± 12	0.25 ± 0.08
	Added CIP	20.1 ± 4.3	11.1 ± 4.4	43 ± 21	0.21 ± 0.14
NO_2^--N (mg/L)	Control	0.5 ± 0.7	0.6 ± 0.7	-	-
	Added CIP	0.5 ± 0.3	0.4 ± 0.7	-	-
NO_3^--N (mg/L)	Control	0.3 ± 0.2	1.5 ± 1.5	-	-
	Added CIP	0.3 ± 0.2	5.7 ± 3.4	-	-
Biomass (mg/L)	Control	7562 ± 478$_{Total}$ / 4504 ± 641$_{Carriers}$ / 3059 ± 423$_{Suspended\ growth}$			
	Added CIP	5592 ± 907$_{Total}$ / 1959 ± 869$_{Carriers}$ / 3653 ± 392$_{Suspended\ growth}$			
SVI (mL/g)	Control	180 ± 6			
	Added CIP	134 ± 41			

Remarks: Data are given as average values and standard deviations.

Table 2. Diagnostic performance of reactor under CIP toxicity

Parameters	Control	Added CIP	Diagnostics CIP toxicity at 200 µg/L
Total biomass (mg/L)	7563±478	5592±907	Inhibited bacteria growth 26% ($p<0.05$)
Attached biomass (mg/L)	4504 ±641	1959±869	CIP decreased biomass 56% ($p<0.05$)
Average flocs size (um)	58±3	31±7	CIP decreased in floc size ($p<0.03$)
Ammonia removal (%)	77 ±16	76 ±12	Less effect nitrification process ($p>0.05$)
TN-Denitrification (%)	44	6.7	CIP inhibited on denitrification process.
TN removal (mg/L)	69±12	43±21	Fluctuation and lower removal ($p<0.05$)
Cake layer (%)	64	36	Low membrane fouling (TMP < 20kPa)
SVI (mL/g)	180 ± 6	134 ± 41	High sludge settleability ($p<0.001$)

Table 3. Richness and diversity of bacterial phylotypes

Sample	Valid reads	OTUs	Chao1	Shannon	Goods Lib. Coverage
Conventional MBR[a]	52920	1313	1333.78	3.70	99.63
Sponge-MBR[b]	53646	1887	1931.43	5.50	99.46
Sponge-MBR200[c]	63776	2611	2631.56	5.51	99.57
Sponge-MBR200 carrier	52512	2266	2293.50	5.33	99.42

Remarks: [a] Conventional A/O-MBR systems treating domestic wastewater; [b]Sponge-MBR treating hospital wastewater without spiking CIP dosage; [c]Sponge-MBR treating hospital wastewater with added CIP of 200 μg/L included suspended growth (Sponge-MBR200) and attached growth (Sponge-MBR200 carrier).

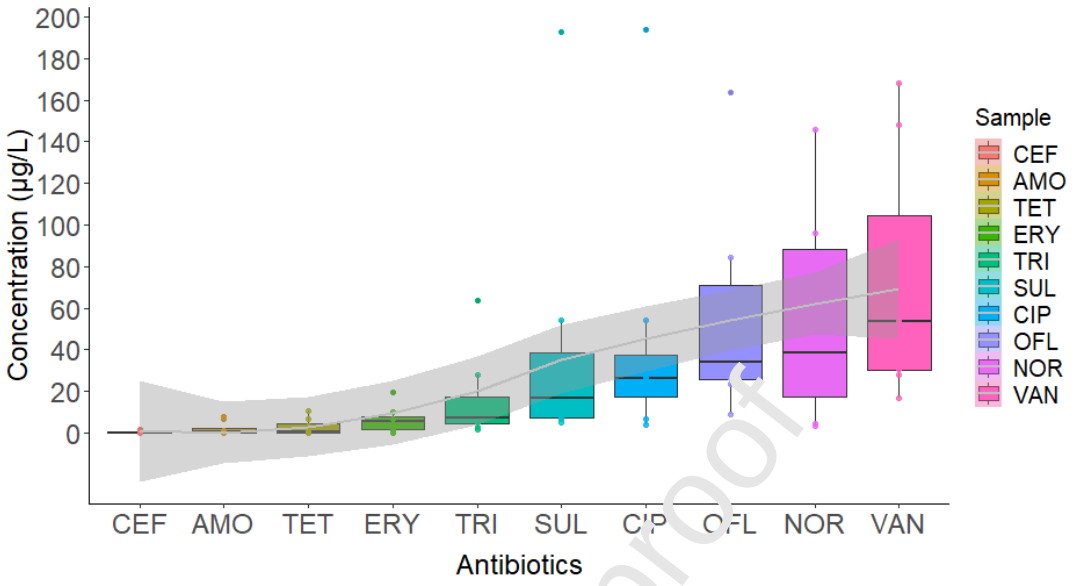

Fig. 1. Box plot of influent antibiotics from hospital wastewater (n=8, the overall antibiotic trends shown conditional means with a 95% confidence level interval)

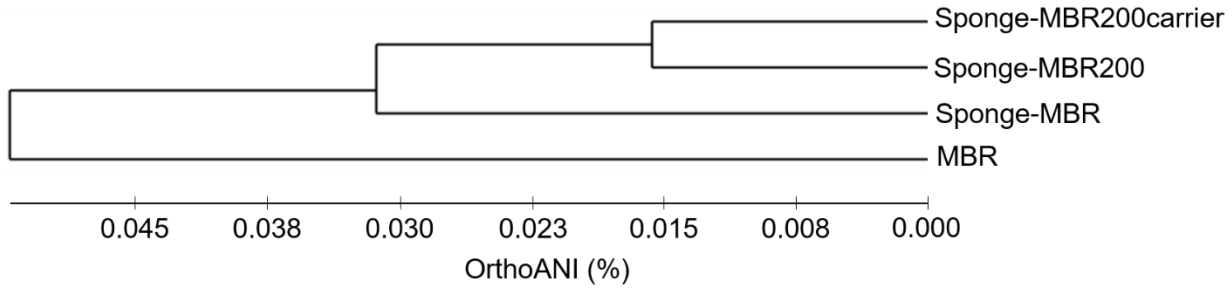

Fig. 2. The dendrogram analysis of the bacteria communities was represented by Unweighted pair group method with arithmetic mean (UPGMA). A higher CIP volumne contained in treated wastewater resulted in a greater phylogenetic relationship distance. The branching patterns of suspended growth (i.e., Sponge-MBR200) is distant from the attached growth (Sponge-MBR200carrier); spiked CIP could cause an imbalance in the distribution of community structures in the Sponge-MBR200.

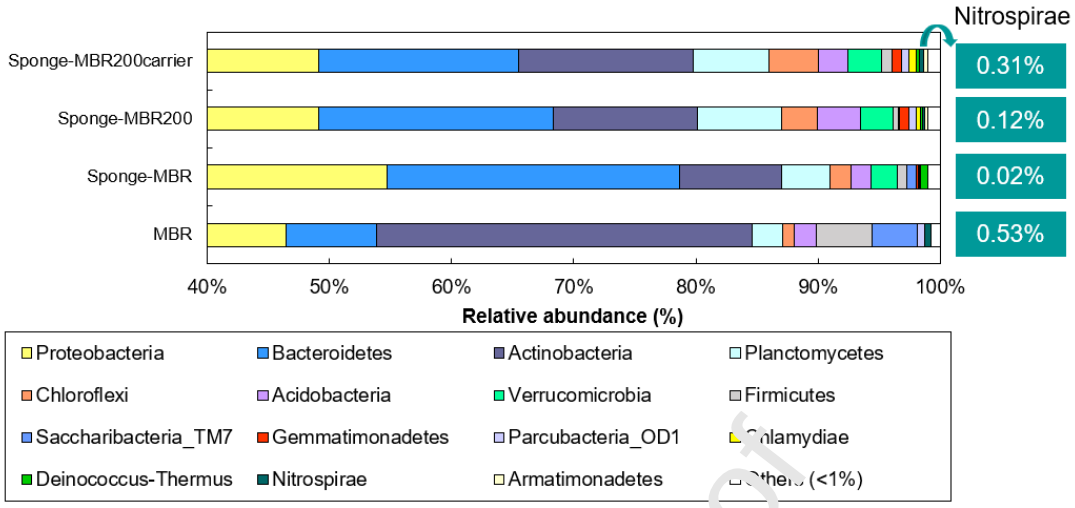

Fig. 3. Taxonomic composition of bacterial communities at the phylum level, *Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes, and Chloroflexi* accounted for 90% of the two Sponge-MBR.

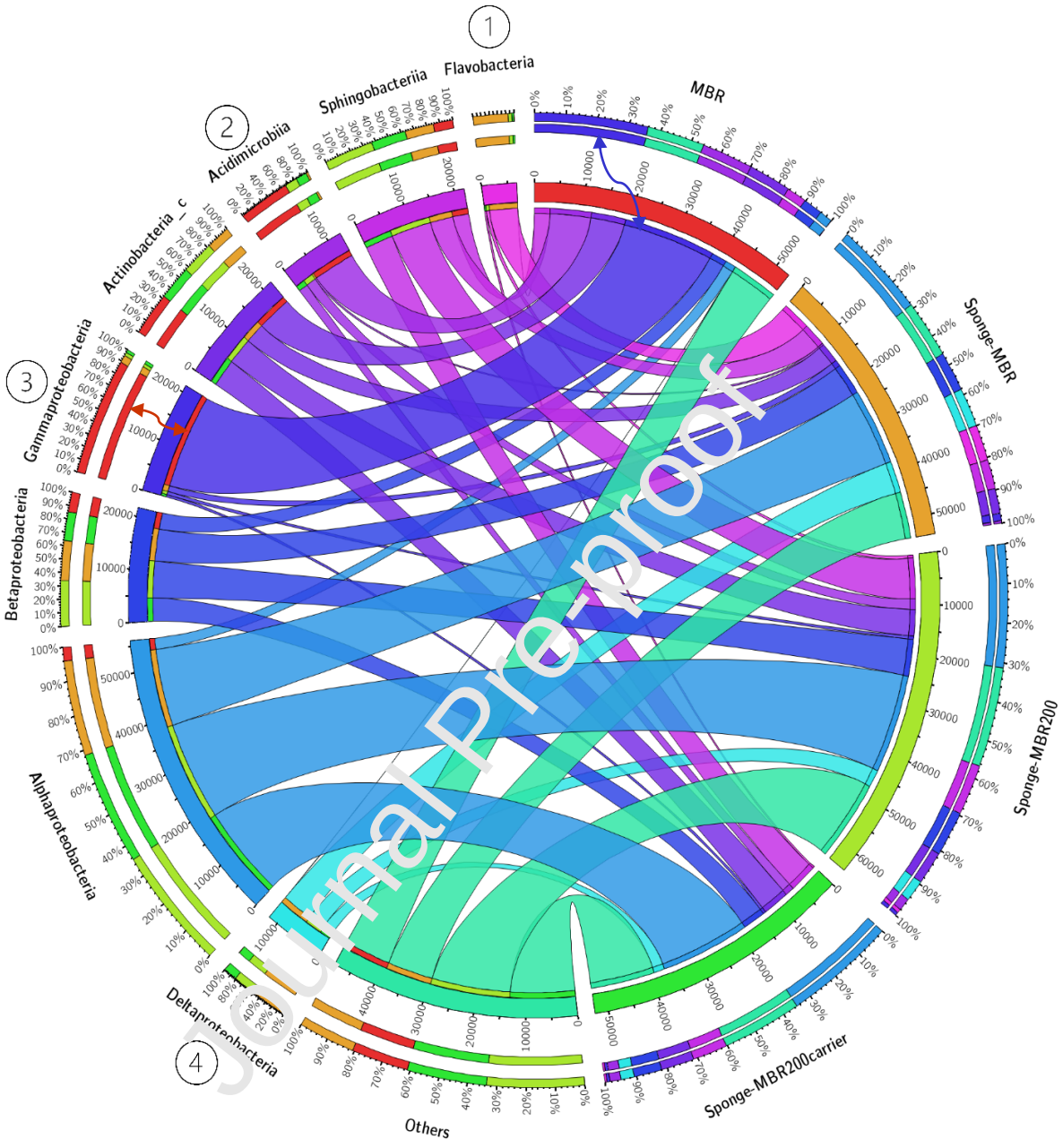

Fig. 4. Distributions of bacterial communities at the class level. The right side of the circle indicates name samples while the left side of the circle denotes name phylotypes. The number sequence read was normalized into a percentage (%). The outer ring includes several cells with different colors linked to the inner ring's color to quantify the amount of phylotype present. A total of four dominant members were suppressed under the spiked CIP condition.

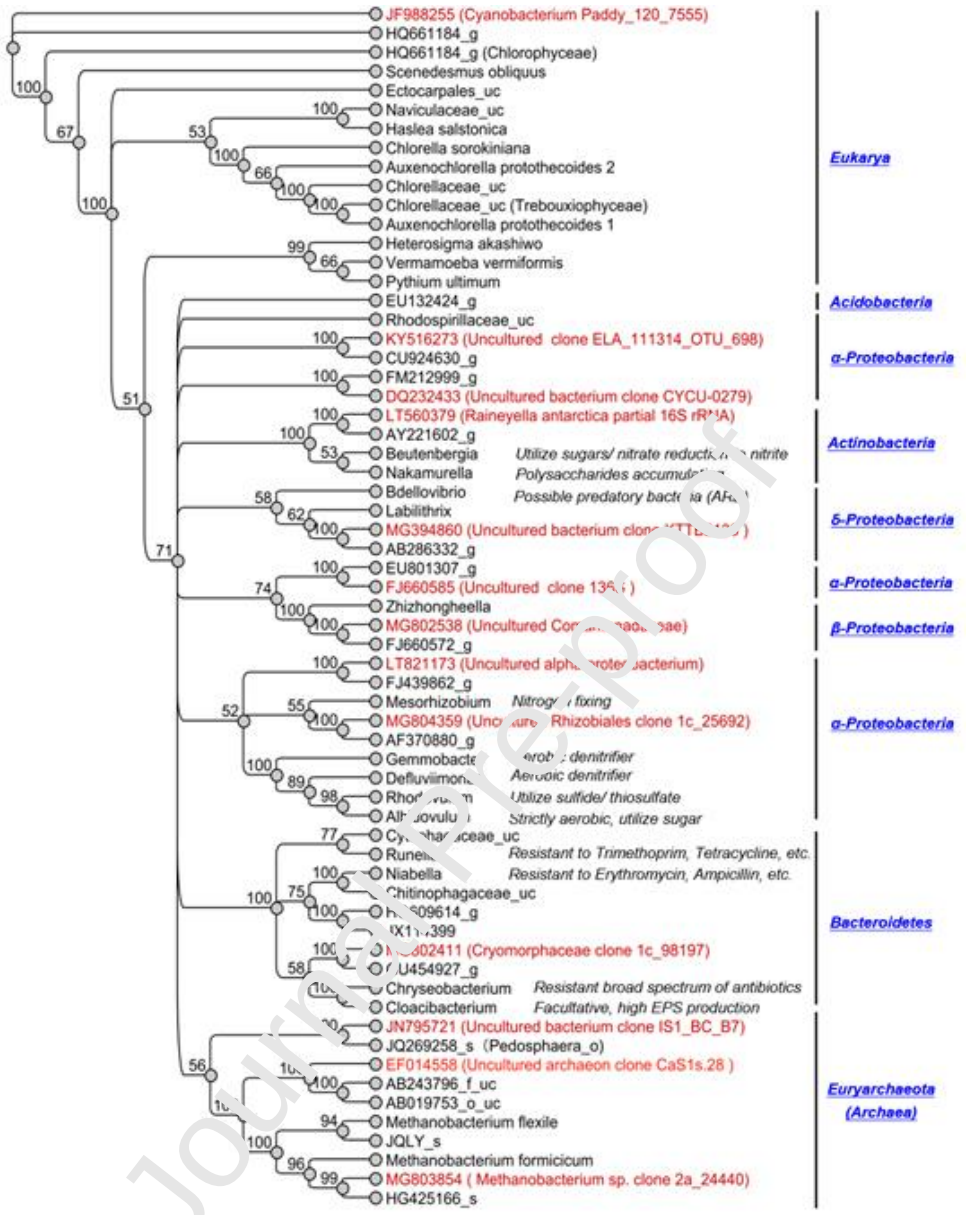

Fig. 5. Phylogenetic tree based on the sequences read. The molecular phylogenetic analysis was peformed using 1000 bootstrap replications. The phylogenetic tree includes eukarya, bacteria, and archaea. Bacteria are mainly involved in the production of EPS. They also have a denitrification function, and a well-known history of drug resistance. The archaea detected in Sponge-MBR (21 sequencing reads) and Sponge-MBR200 (15 sequencing reads) were constructed by phylum Euryarchaeota while no Archaea were found in the MBR. Similarly, Eukarya was found in 118 and 141 of the Sponge-MBR200, and the Sponge-MBR, respectively. Sequences were identified from Genbank using BLAST, the relationship of an unknown sequence similar to public sequences is show in red.

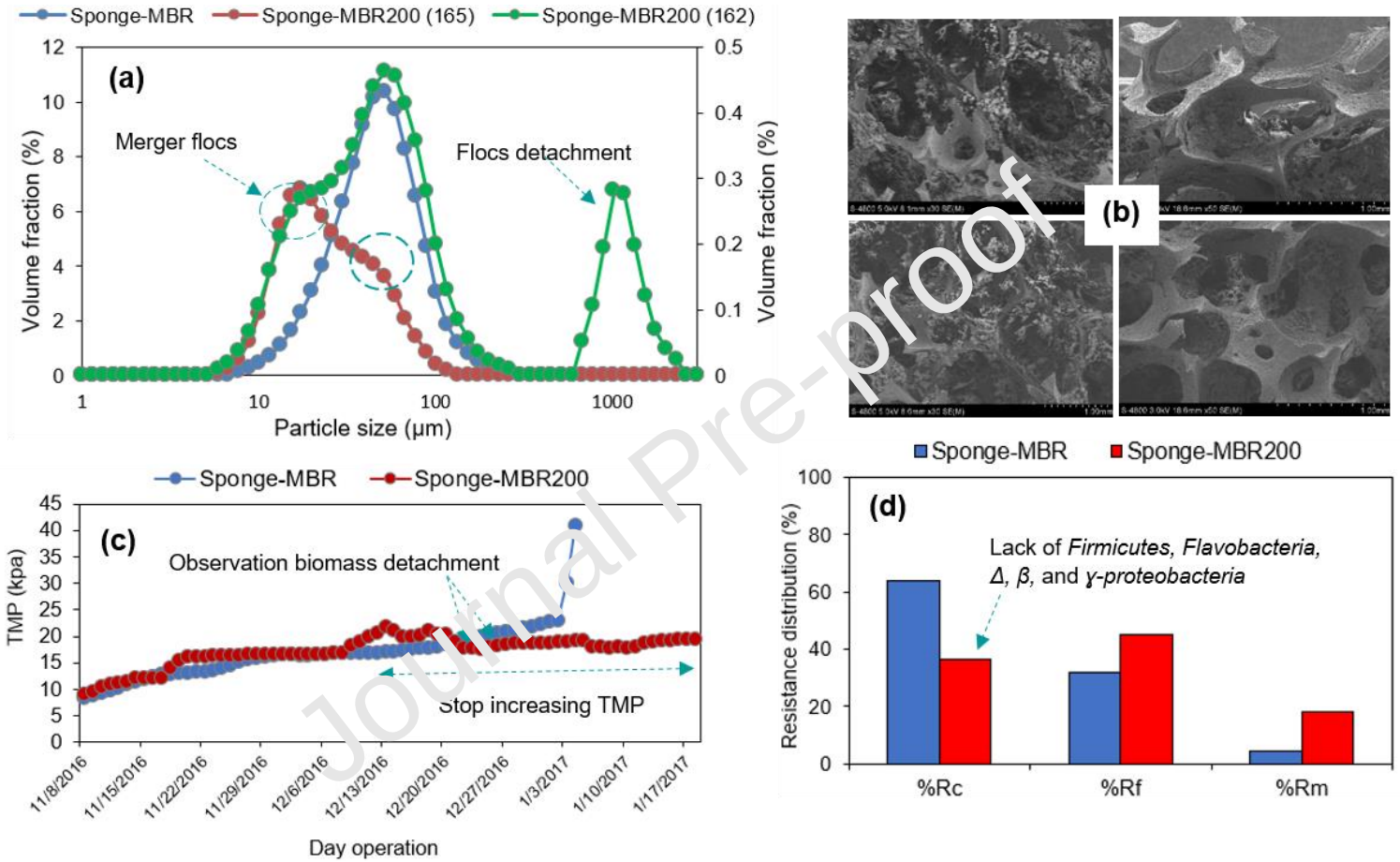

Fig. 6. a) The detachment governed the effect of floc size, cake fouling (R_c) and biomass in attached growth. The peak (circle) indicated that flocs detachment in Sponge merged to suspended floc at days 165 and 162; (b) Surface biofilm of the carrier in control (left) and spiked CIP (right); c) The evolution of trans-membrane pressure by time; and (d) Membrane resistance properties refer to cake layer (R_c), fouling (R_f), and membrane (R_m).

Graphical abstract