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Abstract: Shale gas wastewater (SGW) with complex composition and high salinity needs an economical and efficient method of treatment with the main goal to remove organics. In this study, a coupled system consisting of ozonation and moving-bed-biofilm submerged membrane bioreactor (MBBF-SMBR) was comprehensively evaluated for SGW treatment and compared with a similar train comprising ozonation and submerged membrane bioreactor (SMBR) without addition of carriers attaching biofilm. The average removal rates of MBBF-SMBR were 77.8% for dissolved organic carbon (DOC) and 37.0% for total nitrogen (TN), higher than those observed in SMBR, namely, 73.9% for DOC and 18.6% for TN. The final total membrane resistance in SMBR was 40.1% higher than that in MBBF-SMBR. Some genera that specifically contribute to organic removal were identified. Enhanced gene allocation for membrane transporters and nitrogen metabolism was found in MBBF-SMBR biofilm, implying that this system has significant industrial application potential for organics removal from SGW.

Keywords: Moving-bed-biofilm submerged membrane bioreactor (MBBF-SMBR); Shale gas wastewater (SGW); Ozonation; Microbial community; Submerged Membrane bioreactor (SMBR)

1. Introduction

The extraction of shale gas, one of the unconventional energy resources, is expected to raise the global technically recoverable gas resources by over 40% [1]. In particular, horizontal drilling and hydraulic fracturing (HF) techniques have promoted shale gas development [2]. However, HF causes some environmental problems, the most serious being arguably the production of hazardous shale gas wastewater (SGW). In the drilling and completion of horizontal wells, abundant flowback and produced water (FPW) returns to the surface as SGW [3]. In HF operations, pollutants including suspended solids, salt, organic chemicals, naturally occurring radioactive materials (NORMs), and heavy metals contaminate the SGW [4], resulting in environmental risks if this stream is not properly purified before discharge or reuse.

Typical techniques that have been applied to treat SGW include basic separation technologies, adsorption, advanced oxidation, low pressure membrane filtration, and desalination technologies [5]. Pre-treatment for desalination steps is usually required, the complex organic matters limiting the efficiency and durability of desalination. For example, organics cause membrane fouling and limit the application of high-pressure-driven membrane technologies in SGW desalination [6]. Thousands of organic compounds have been detected in SGW and can originate from the shale formation, chemical reactions underground, or from HF additives [7]. Low molecular weight hydrophilic organic compounds, which are considered readily or inherently biodegradable, often represent the majority of dissolved organic matter (DOM) in SGW [7, 8]. Therefore, biological treatment may have great potential as an economical method

for organic matter removal and pre-treatment of the SGW for subsequent polishing.

Some researchers have utilized biological methods to treat FPW from oil or gas production, by applying: MBR and its variant methods [9-11]; microbial mats [12]; aerobic sludge granulation (ASG) [13]; biologically active filtration (BAF) [14, 15]; plant-microbial synergism [16]. The reported organic carbon removal rates were generally high by MBR and its variant methods for treating FPW with different quality [9-11]. MBR operates with a large amount of biomass, an important advantage when treating effluents with complex organic matters, and needs small footprint, thus it is suitable for on-site reuse. High salinity, refractory biodegradation of SGW, and the toxicity of some HF additives are major challenges in its application [8]. Advanced oxidation, especially ozonation, has been used as pretreatment to enhance organics removal in biological processes by pre-oxidizing refractory organic compounds and toxic substances [17, 18]. Ozonation coupled with BAF removed 83.2% DOC in SGW treatment [17]. Moreover, the coupling of ozonation and MBR has been proven to reduce membrane fouling and improving MBR performance [19], but this process has not yet been explored in SGW treatment.

MBBF-SMBR combines moving-bed-biofilm reactor (MBBR) with submerged membrane bioreactor (SMBR) [20] and it has several potential advantages: lower membrane fouling because of the interacting forces between the membrane and the carriers [21]; higher resistance of attached biomass to overloading and toxic compounds [22]; higher nitrogen removal from simultaneous nitrification and denitrification [23]. Considering the relatively low concentration of organic compounds and the medium-high

salinity of the SGW from the Sichuan Basin [5], in this study a MBBF-SMBR is applied for the biodegradation of organic matter and compared with a conventional SMBR. Ozonation is chosen as a pretreatment option to increase the biodegradability of SGW. Except for the addition of carriers, the two systems were operating in similar conditions.

Therefore, this study aims at evaluating the efficacy and feasibility of MBBF-SMBR and SMBR following ozone-based pre-oxidation for organics removal from SGW in the Sichuan Basin. Specifically, the main objectives of the present study are: (a) to evaluate and compare the removal of organic matters from SGW for the two systems; (b) to investigate and compare the component of membrane fouling; (c) to analyze difference of the microbial community composition and predicted function in the treatment of SGW.

2. Materials and methods

2.1 Pre-treatment of shale gas raw water (RW)

The SGW was obtained from the Weiyuan shale gas play (Sichuan, China). It was stored in plastic containers in the dark at room temperature and was coagulated and precipitated to remove large particles and colloids before other experiments. The resulting effluent is referred to as raw water (RW).

Pre-ozonation was applied to improve the biodegradability of the RW. In each batch, 900 mL RW was added into a 1000 mL glass bottle and treated with 100 mg ozone. Ozone, produced from dry oxygen (99%, v/v), was injected from the bottom through a gas diffuser with inlet concentration of 17±3 mg/L, for 60 min of reaction time and 0.1 L/min of flow rate (see supplementary material). The temperature was 20

±1 °C, maintained by a temperature-controlled water bath (HH-1, Xinrui Instrument Company, China) [24]. The concentration of ozone produced from laboratory ozone generator (Beijing Tonglin Co., Ltd., China) and that of the off-gas from the reaction bottle were measured by the indigo method. Before the bio-treatment, the water was settled for 2 h to sediment flocs formed after ozonation and the residual ozone in water was quenched by water bath heating for 30 min at 50 °C.

2.2 Experimental set-up and operation

Fig.1 presents the experimental setup of the biological processes. Both the MBR and the BFMBR tanks were made of glass with an effective volume of 300 mL. The water temperature of each bioreactor was maintained at 20-25 °C. An aeration disk was installed at the bottom to supply O_2 for microorganism growth at the flow rate of 20 mL/min: the dissolved oxygen (DO) concentration was kept at 4-6 mg/L in both MBBF-SMBR and SMBR. The submerged membrane module comprised hollow-fiber membranes made of polyvinylidene fluoride (PVDF). Their active filtration area was 5.2 cm^2. Virgin polyurethane cubes (side length: 10 mm) were used as carriers with volume fraction of 20% in the MBBF-SMBR.

The SMBR and the MBBF-SMBR were started for acclimation of the biofilm and the sludge. The bioreactor of MBBF-SMBR was firstly filled by the carriers at a filling ratio of approximately 20%, and then inoculated with the activated sludge mixed liquor, which was taken from a municipal wastewater treatment plant (Chengdu, China), up to half of the bioreactor's effective volume. Afterwards, the bioreactor was fed with municipal wastewater from the same treatment plant, and run in batch mode to allow con-

tact with the biomass. Then, the wastewater was fed for 2 days in continuous mode. At last, the bioreactor was fed with the mixture of municipal wastewater and pre-ozonized RW in continuous mode: the proportion of pre-ozonized RW was increased gradually up to 100% while the biofilm growth stabilized. In the continuous stage, the feed water was circulated between the feed tank and the bioreactor [15]. The acclimation of SMBR was the same as MBBF-SMBR, except for the addition of carriers. The detailed start-up strategy is found in supplementary material.

After the biofilm was acclimated, the SMBR and the MBBF-SMBR were operated in continuous mode. The operating conditions of the two systems were similar. The average membrane flux was 12 L $m^{-2}h^{-1}$ (LMH). The nominal hydraulic retention time (HRT) was 48 h while the nominal solid residence time (SRT) was 30 d. The experiment lasted 110 days. Days 1 to 50 were the start-up period, while days 51 to 111 were the formal experimental operation period. In the following description, the first day of the formal experimental operation is described as day 0.

2.3 Analytical methods

2.3.1 SGW quality analysis

Water samples were collected from the feed and the effluent during the experiments. DO, pH, and turbidity were measured with a DO meter (JPBJ-610L, INESA, Shanghai, China), a pH meter (PB-10, Sartorius Scientific Instruments Co, Ltd., Gottingen, Germany), and a turbidimeter (TL2310, Hach Company, Loveland, USA), respectively. An Ultrameter II 6PFC (Myron L Company, Carlsbad, USA) portable multifunctional meter was utilized to determine the TDS. The DOC concentration was de-

termined with a total organic carbon analyzer (TOC-L CPH, Shimadzu, Japan). Total nitrogen (TN) was quantified by alkaline potassium persulphate digestion-UV spectrophotometric method using the special reagent LH-NT (Lianhua Environmental Protection Technology Co., Ltd., Lanzhou, China). A UV–Vis spectrophotometry (Orion AquaMate 8000, Thermo Fisher Scientific Inc., MA, USA) was used to measure UV absorbance at 254 nm.

A three-dimensional fluorescence excitation emission matrix (3D EEM) spectrofluorometer (F-7000, Hitachi, Japan) was used to characterize the DOM fraction in the feed, effluent, and membrane fouling layer. Samples were filtered and diluted to a UV_{254} of 0.05 to avoid inner filter effect. In this study, the EEM spectra were collected by scanning emission wavelengths from 200 nm to 550 nm at 1 nm increments and excitation wavelengths from 200 nm to 400 nm at 5 nm increments. The excitation and emission slit width was set at 5 nm and the scanning speed was set at 12000 nm/min. The EEM spectrum of deionized (DI) water was collected as the blank and was subtracted from all the sample spectra to remove the influence of Raman scattering.

2.3.2 UF membrane fouling analysis

The surface morphology and elemental composition of the membrane fouling layers were investigated by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) (Regulus 8230, Hitachi, Tokyo, Japan). Before SEM-EDS analysis, the membrane samples were coated with ~2 nm of gold using a magnetron sputter (MSP-2S, IXRF Systems, USA). The method of filtration resistance analysis was described in detail in previous study [25].

2.3.4 Biological analysis

To assess the bacterial populations in the two bioreactors and analyze the function of the microorganisms, the RW, pre-ozonized RW, suspended sludge, and biofilm attached on carriers at the end of the experiment were collected and analyzed. DNA extraction, polymerase chain reaction (PCR) amplification, and Illumina Miseq sequencing were conducted in the same way as in previous study [25]. Analyses of the alpha diversity (e.g., Chao, Shannon, Simpson, ACE, and Coverage), principal component (PCA), bacterial community composition, and metabolic functional prediction were performed using the online Majorbio I-Sanger Cloud Platform (www.i-sanger.com).

3 Results and discussion

3.1 Contaminant removal performance

3.1.1 Organic matters and nitrogen removal

The characteristics of RW, pre-ozonized RW, and the effluent of SMBR and MBBF-SMBR are listed in Table 1. The organic matter removal rates of SMBR and MBBF-SMBR treatments are presented in Fig 2. The DOC removal rates were as high as 87.8% (SMBR) and 87.9% (MBBF-SMBR), but as the running time increased, the DOC removal rate decreased to 59.5% (SMBR) and 70.1% (MBBF-SMBR), with the MBBF-SMBR showing an obviously higher removal of DOC after 45 days. Overall, the average DOC removal rates were 73.9% (SMBR) and 77.8% (MBBF-SMBR). In the middle of the experiment, the DOC of the influent stream decreased significantly, which may be caused by the volatilization of a fraction of organic matter due to frequent opening of the container in the summer or degradation by anaerobic bacteria in the raw

water. The DOC contents of the mixture liquid were 39.61 mg/L in SMBR and 36.94 mg/L in MBBF-SMBR, higher than in the influent, indicating some accumulation of organic matter and possibly some toxic materials, such as biocides used as additive in the hydraulic fluid, which may inhibit the biological activity. The removal of UV_{254} was low both in the SMBR and in the MBBF-SMBR process, thus that the UV_{254} values of the effluent were sometimes higher than those of the influent water. Organic matters containing aromatic chromophores or unsaturated bonds (primarily humic substances) are related to the value of UV_{254} [26]. Considering the high DOC removal, some humic matter with high light absorption at 254 nm may have been produced by microorganisms as a byproduct of the degradation of other parent substances. As for TN, the MBBF-SMBR showed higher removal rates, which may be due to simultaneous nitrification and denitrification processes occurring on the carriers [23]. Because of the influence of high salinity and pH value to nitrifying bacteria [27, 28], the average value of TN removal rate were only 18.6% (SMBR) and 37.0% (MBBF-SMBR), lower than that observed with low salinity wastewater in other studies (>60%) [29].

Compared with other systems treating high salinity wastewater, the MBBF-SMBR system showed a high removal rate of organic matters. According to Lester's study [30], the removal rate of dissolved chemical oxygen demand decreased from 90% to 60% when the TDS increased from 1500 mg/L to 45000 mg/L in their synthetic hydraulic fracturing flowback stream treated with an activated sludge mixed liquor. Wang [31] used an aerobic sludge (AS) system to treat FPW, and removed about 72% organics with TDS of 16087 mg/L, lower than that observed in this study using the

MBBF-SMBR.

3.1.2 Fluorescence EEM spectra

The EEM fluorescence spectra of the RW and of the effluents are presented in Fig 2(c)-(f). In general, peaks in the excitation wavelength range from 200 to 250 nm and the emission wavelength range from 280 to 380 nm (Regions I: 280-330 nm; Regions II: 330-380 nm) are associated with simple aromatic proteins, such as tyrosine and tryptophan. Peaks located in the range of excitation wavelengths from 200 to 250 nm and the emission wavelengths from 380 to 550 nm represent fulvic acid-like substances (Region III). Peaks in the range from 250 to 400 nm excitation wavelengths and from 280 to 380 nm emission wavelengths are related to soluble microbial by-product-like material (Region IV). Peaks from 250 to 400 nm (excitation) and from 380 to 550 nm (emission) are related to humic acid-like organics (Region V) [32, 33]. The fluorescence regional integration (FRI) has been used for semi-quantitative analysis of DOM in SGW, and EEM fluorescence intensity is proved to be positively correlated with the real concentration of soluble microbial by-product-like material and aromatic proteins in produced water [34]. The distribution of FRI of DOM samples in the SMBR and MBBF-SMBR systems is shown in Fig 2 (g).

Soluble microbial by-product-like matters (region IV) was the dominant fraction in RW, and the ozonation removed most fluorescent fraction (75.9%) of the DOM. Soluble microbial by-product-like matters (region IV) and humic acid-like organics (Region V) increased after the SMBR unit (38.2%) or the MBBF-SMBR unit (70.6%). In particular, a significant peak appeared for soluble microbial by-product-like matters (region IV) in

the MBBF-SMBR effluent, indicating that the low molecular weight DOM produced by microorganisms in the bioreactor could not be rejected by membrane, and that the microbial activity in the MBBF-SMBR might be stronger than SMBR. This result is consistent with the rationalization discussed above about the values of UV_{254} and their apparent uniformity between influent and effluents streams.

3.2 Analysis of membrane fouling

3.2.1 Characteristics and elemental analysis of the cake layers

The fouling cake characteristics of SMBR and MBBF-SMBR membranes were analyzed with EDS, as shown in Fig 3. Representative SEM micrographs can be observed in the supplementary material file. Compared with the pristine membrane, the fouled membrane surface was covered by a thick layer of foulants. The surface of the sample from the SMBR was rougher, while the cake on the sample from the MBBF-SMBR was more porous. Numerous spherical particles were observed on the surface of the SMBR membrane, which may be silica (see supplementary material). According to the EDS analysis, organic foulants (C, O) were dominant, but inorganic elements (Si, Cl, I, Fe, Ca, Al, Na, K, Mg, Mn) were also observed on the surface. The proportion of Si was significantly larger for the membrane used in the SMBR, suggesting that inorganic pollution on SMBR may be more severe, and consistent with SEM images. As a consequence, the proportion of C and N elements was higher on the MBBF-SMBR membrane surface, which might be also partly due to a larger proportion of proteins attached on the MBBF-SMBR membrane surface. The membrane organic foulants, mainly including proteins and polysaccharides, were likely extracellular poly-

meric substance (EPS). The increased ratio of proteins from EPS was reported to be an important reason for decreasing membrane fouling in previous studies, which would be consistent with the trend observed in the two different reactors and the larger fraction of proteins found on the MBBF-SMBR membrane sample [35].

3.2.2 Organic composition of the cake layers

The EEM fluorescence spectra of the cake layers were acquired to analyze their organic composition (Fig 4(a) - (c)). Three peaks were observed on the SMBR membrane: Ex/Em of 280/350 nm in region IV; 225/350 nm in region II; 275/455 nm in region V. The peak in region V was not observed on MBBF-SMBR samples, suggesting that the macromolecular organic matters were degraded more significantly in the MBBF-SMBR system. Moreover, fouling components were lower for every region in the MBBF-SMBR sample with respect to SMBR membranes. The DOC values of the foulants were 110.9 mg/m^2 (SMBR) and 59.1 mg/m^2 (MBBF-SMBR), respectively, corroborating that the membrane fouling of SMBR was more significant and the resulting cake layer thicker and/or denser.

The fouling resistance was determined at the end of the experiments and shown in Fig 4(d). The final total membrane resistance for SMBR and MBBF-MBR were 12.50×10^{12} m^{-1} and 8.92×10^{12} m^{-1}, and the reversible portions were 8.38×10^{12} m^{-1} and 5.33×10^{12} m^{-1}, representing 67.0 % and 59.7 % of the total resistance, respectively. These data indicate that most fouling could be removed by physical cleaning with water, and that the fouling in SMBR was more significant and lightly more reversible than that in MBBF-SMBR. The porosity observed for the MBBF-SMBR cake layer (Fig 3(c)) and

the different composition in the two reactors, related to a larger EPS fraction and to an improved biodegradation in the MBBF-SMBR, may explain the lower resistance quantified for this system compared to the SMBR system.

3.3 Bacterial diversity

The Simpson, Shannon, chao1, abundance-based coverage estimator (ACE) and coverage are shown in Table 2 to evaluate the microbial richness and diversity of samples. The high coverage indicates that the analyses are robust and covered almost all sequences of samples. The microbial diversity (Shannon and Simpson indexes) and microbial richness (ACE and Chao 1 indexes) from bioreactors on day 0 of the experimental test were significantly higher than those on day 60. This result suggests that the accumulation of toxicity and salinity with the operating time inhibited the growth of some bacteria originated from domestic sewage from the municipal treatment plant. In addition, ozonation inactivated some bacteria, with the richness and diversity of pre-ozonized RW being the lowest among all samples. The PCA analysis (see supplementary material) suggested that the community composition of samples from the bioreactors became more similar and approached that of the pre-ozonized RW as the running time increased.

3.3.1 Bacterial community in SMBR and MBBF-SMBR systems

The bacterial community composition at the phylum level of RW, pre-ozonized RW, and samples obtained at different times (day 0 and day 60) from the two biological treatment systems is shown in Fig 5(a). A total of 43 bacterial phyla were identified. Seed sludge enriched the microbial community composition, so the quantity of phyla

was much larger than what previously detected in SGW [34, 36]. *Proteobacteria* was the most dominant bacterial in all samples (29.8%-87.9%), with the dominance becoming more obvious at the end of the experiments. In RW, *Desulfobacterota* (13.0%), *Bacteroidota* (10.7%), *Chloroflexi* (10.3%) were also major phyla. Because members of *Thermotogota* are anaerobic [37], this phylum was not found in other samples except RW. *WPS-2*, living in oxygen-rich environments [38], was a dominant phylum only in pre-ozonized RW (32.3%), possibly due to the large amount of oxygen produced by ozone decomposition. The inactivation effects of ozonation were obviously observed on *Actinobacteriota*, *Chloroflexi*, *Firmicutes*, *Acidobacteriota*, *Patescibacteria*, and *Verrucomicrobiota*, causing the content of these phyla to drop rapidly in pre-ozonized effluents. In samples obtained on day 0, *Actinobacteriota* (13.9%-27.5%), *Bacteroidota* (10.0%-11.8%), *Chloroflexi* (8.1%-17%) were the major phyla after *Proteobacteria*. In samples obtained on day 60, *Actinobacteriota* (3.2%-12.9%) was still a major phylum, but the third major group changed to *Gemmatimonadota* (2.1-6.2%). *Gemmatimonadota* was not found in significant fraction in RW and pre-ozonized RW, thus this phylum is hypothesized to originate from the seed sludge. These results indicate that the composition on the phylum level changed and adapted during the experiment.

The community compositions were further analyzed at genus level taxonomy (Fig. 5(b)). A total of 686 bacterial genera were recognized. *Norank_o__OPB41*, which was related to methanogenesis and is most likely anaerobic, was the major genus in RW. *Roseovarius*, which has been found in different oil reservoirs and water injection treatment systems [39], was also important in RW and especially pre-ozonized RW.

The dominant bacteria in the two bioreactors were similar, but very different compared to those observed in RW. Specifically, some bacteria related to the removal of nitrogen and other contaminants were detected, including *Denitromonas*, *norank_f__Xanthomonadaceae*, *norank_f__Gemmatimonadaceae*, *Paracoccus*, *Roseovarius*, *norank_f__JG30-KF-CM45*, *Iodidimonas*, *Nocardia*, *Erythrobacter*, *Defluviimonas*, *Mycobacterium*, *Paracoccus*, *Muricauda*, *Thalassospira* and *norank_f__Rhodococcus*. The summary of functions of these genera and and their reference sources can be found in supplementary materials. *Denitromonas* is a kind of denitrifying bacteria, and often discovered in hypersaline wastewater [40]. *Norank_f__Xanthomonadaceae*, *norank_f__Gemmatimonadaceae*, *Defluviimonas* and *Paracoccus* also have the ability of denitrification. *Roseovarius* (also found in RW) and *norank_f__JG30-KF-CM45* are nitrifying bacteria. Most of the nitrifying bacteria live in slightly alkaline environments. Possibly due to the consumption of alkalinity and the decrease of pH value during operation, the presence of denitrifying bacteria became much larger than that of nitrifying bacteria after 60 days. *Roseovarius* also belongs to sulfur-oxidizing prokaryotes, and has the ability to produce iodinated organic compounds [41]. *Iodidimonas* can oxidize organic iodine, and according to the EDS results of membrane fouling, the high iodine content in bioreactors might be their nutrient source. There might be a symbiotic relationship between *Roseovarius* and *Iodidimonas* in bioreactor systems. *Nocardia* is common filamentous bacteria in activated sludge and can degrade and assimilate recalcitrant aliphatic hydrocarbons [42]. It has been reported that *Pseudomonas* can degrade and detoxify phenolic compounds, including phenol, pentachlorophenol, and p-cresol

[43]. *Erythrobacter* contribute to the degradation of hydrocarbons, especially aromatic substances in saline oily wastewater [44]. *Defluviimonas*, *Mycobacterium*, *Paracoccus*, *Muricauda*, and *Thalassospira* are related to the degradation of PAHs. *Norank_f__Rhodococcus* can use hydroxylated derivatives of polychlorobiphenyls (HO-PCBs) as a source of carbon [45]. The MBBF-SMBR content of bacteria, mainly with the ability of denitrification and PAHs removal (*Denitromonas*, *Roseovarius*, *norank_f__Caldilineaceae*, *Defluviimonas*, *Mycobacterium*, and *Paracoccus*) was obviously higher than that in the SMBR. This different composition may be the principal reason for higher TN and DOC removal rates determined in the MBBF-SMBR system.

3.3.2 Bacterial functional prediction in SMBR and MBBF-SMBR systems

The metabolic functions of bacterial communities were predicted using the software package "Phylogenetic Investigation of Communities by Reconstruction of Unobserved States" (PICRUSt, v2.0.0) based on the Kyoto Encyclopedia of Genes and Genome (KEGG) Orthology database. 24 functional pathways from 5 categories are listed in Fig 6. Amino acid, carbohydrate, energy, and nucleotide metabolism were found to be related to the transformation of the main nutrients [46], while membrane transport was reported to play a crucial role in the survival of microbes in adverse environments, including the hypersaline environment [47].

The functional profile of ABC transporters was found at a higher relative abundance in biofilms than in the sludge, and it decreased from 3.27-3.79% on day 0 to 3.02-3.51% on day 60 of the experimental tests. Membrane transporters are related to the material exchange between bacteria and the environment, and might contribute to

biofilm formation [48]. They were also reported as directly or indirectly affecting factors connected to hydrocarbon metabolism [49]. Previous research found that with the increase of salinity the content of ABC transporters increased [50], the opposite of what generally observed in this study. It is hypothesized that other factors like the acidic environment may have slightly inhibited the expression of genes related to ABC transporters, causing their decrease after 60 days. Higher proportion of nitrogen metabolism was found in biofilm and it may be the cause of the higher TN removal rate in MBBF-SMBR. Overall, most of the pathways about metabolism were common among the eight samples, with some slight differences in the relative abundance of each metabolic pathway for the various samples. For example, the sequences related to nucleotide metabolism and alanine, aspartate and glutamate metabolism showed relative higher abundance in RW, and may suggest more energy consumption. Higher abundance of methane metabolism was found in RW indicating more anaerobic bacteria. The ability of starch and sucrose metabolism, citrate cycle (TCA cycle), glycolysis / gluconeogenesis, and amino sugar and nucleotide sugar metabolism (included in carbohydrate metabolism) were significantly lower after ozonation.

4. Conclusion

The results of this study indicated that the addition of biofilm attached to carriers significantly improved the removal efficiency of DOC and TN in ozonation-SMBR systems by 3.9% and 18.4%, respectively. This work demonstrated the feasibility and potential of MBBF-SMBR following ozonation for SGW treatment, and identified the accumulation of bacteria related to organic and nutrient removal. These functional bac-

teria were found in larger amount in MBBF-SMBR, causing higher organics removal rate. The functional genes related to membrane transport and nitrogen metabolism were enhanced in biofilm, which may be one of the reasons for the higher organic removal in the MBBF-SMBR system.

Appendix A. Supplementary data

E-supplementary data for this work can be found in e-version of this paper online.

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Figure captions

Fig 1. Schematic diagram of the SMBR and MBBF-SMBR.

Fig 2. Organic compounds removal: (a) DOC influent concentration (right axis), effluent concentration (right axis), and removal rates (left axis) as a function of time during SMBR and MBBF-SMBR treatment. (b) Average values of DOC, UV_{254}, and TN removal rates (n=21). 3D EEM fluorescence spectra of: (c) RW; (d) pre-ozonized RW; (e) effluent of SMBR; (f) effluent of MBBF-SMBR. (g) FRI distribution of RW, pre-ozonized RW, effluent of SMBR, and effluent of MBBF-SMBR.

Fig 3. EDS analysis results for fouled membrane from (a) SMBR, (b) MBBF-SMBR (magnification: 1000× and 5000×).

Fig 4. Composition of the membrane fouling layer. 3D-EEM fluorescence spectra of membrane fouling from (a) SMBR and (b) MBBF-SMBR. (c) FRI distribution of membrane fouling in the SMBR and MBBF-SMBR. (d) Fouling resistance parameters, including membrane resistance (R_m), reversible resistance (R_r), and irreversible resistance (R_{ir}) at the end time of SMBR and MBBF-SMBR tests.

Fig 5. Bacterial community composition of: RW; pre-ozonized RW; samples (sludge and biofilm) obtained from SMBR and MBBF-SMBR on day 0 and day 60. Analysis at: (a) phylum (relative abundance＞1%); (b) genus level (relative abundance＞2%). (“SMBR” and “MBBF-SMBR” refer to the sludge samples from SMBR and MBBF-SMBR systems, while “Biofilm” refers to the biofilm samples from the MBBF-SMBR system).

Fig 6. Bacterial functional traits and categories of: RW; pre-ozonized RW; samples

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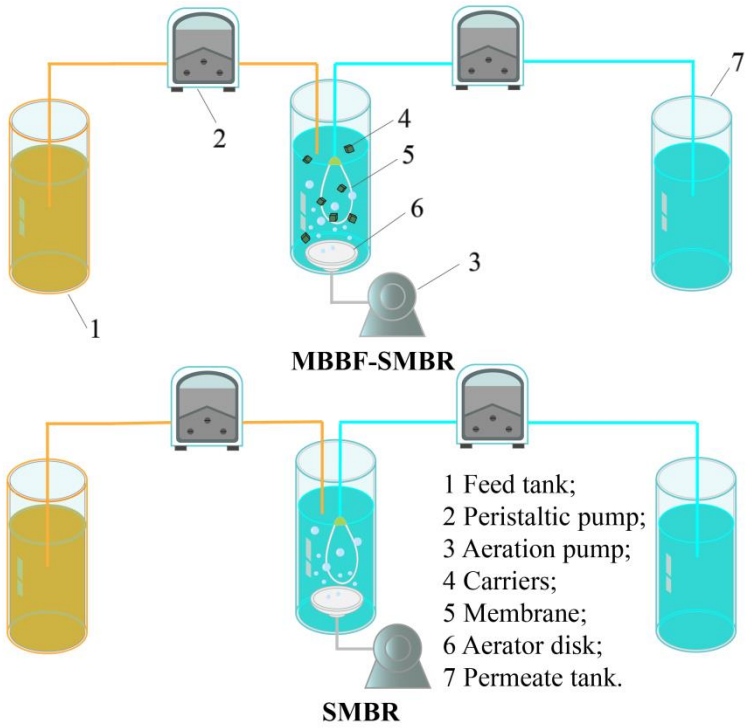
Table 2. Estimates of richness and diversity for operational taxonomic units (OTUs) definition of 97% similarity for RW, pre-ozonized RW, and for samples (sludge and biofilm) obtained from SMBR and MBBF-SMBR on day 0 and day 60 of the experimental test. ("SMBR" and "MBBF-SMBR" refer to the sludge samples from SMBR and MBBF-SMBR systems, while "Biofilm" refers to the biofilm samples from the MBBF-SMBR system).



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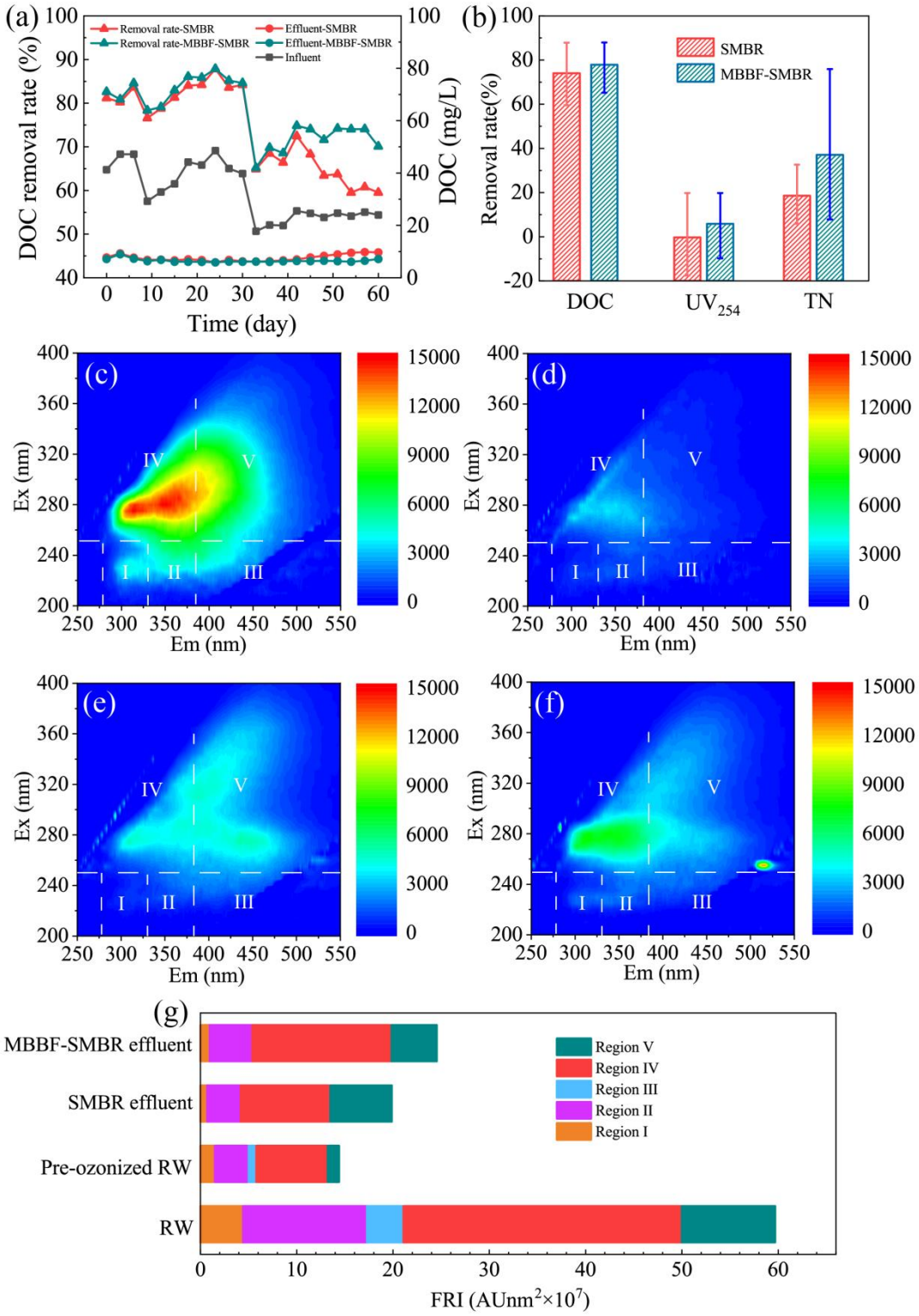

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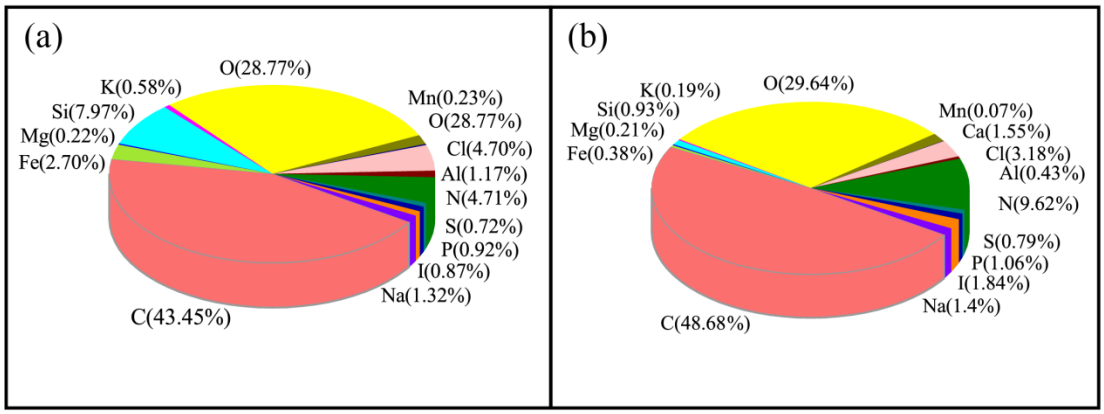

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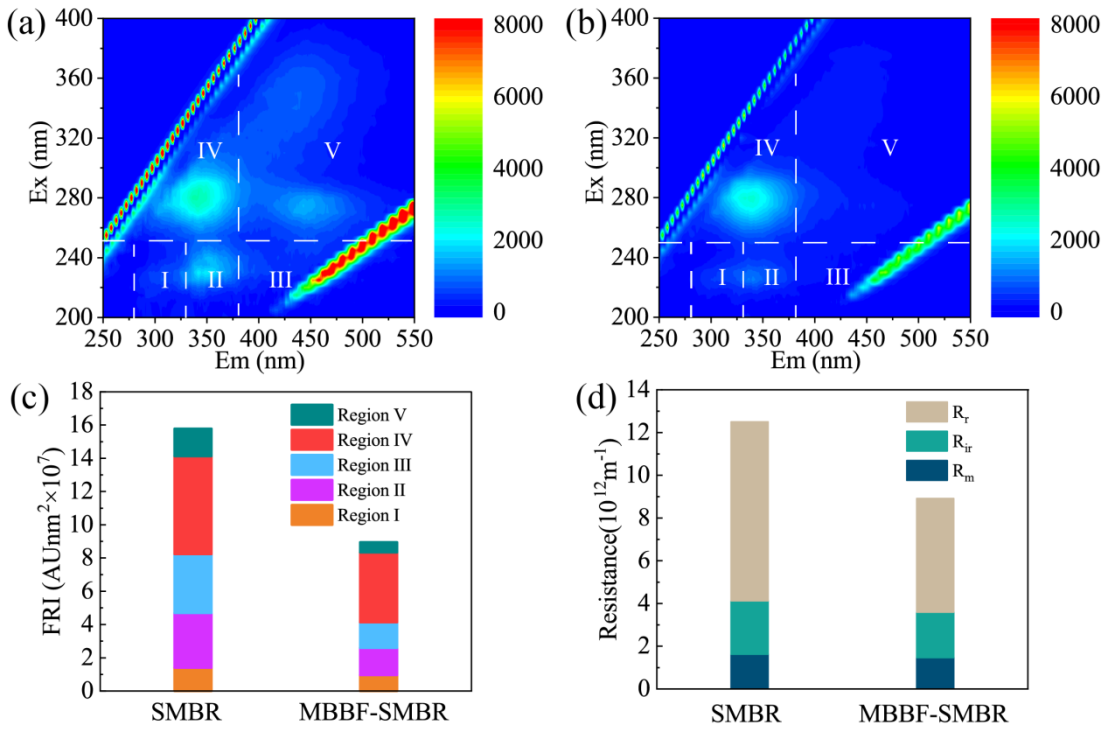

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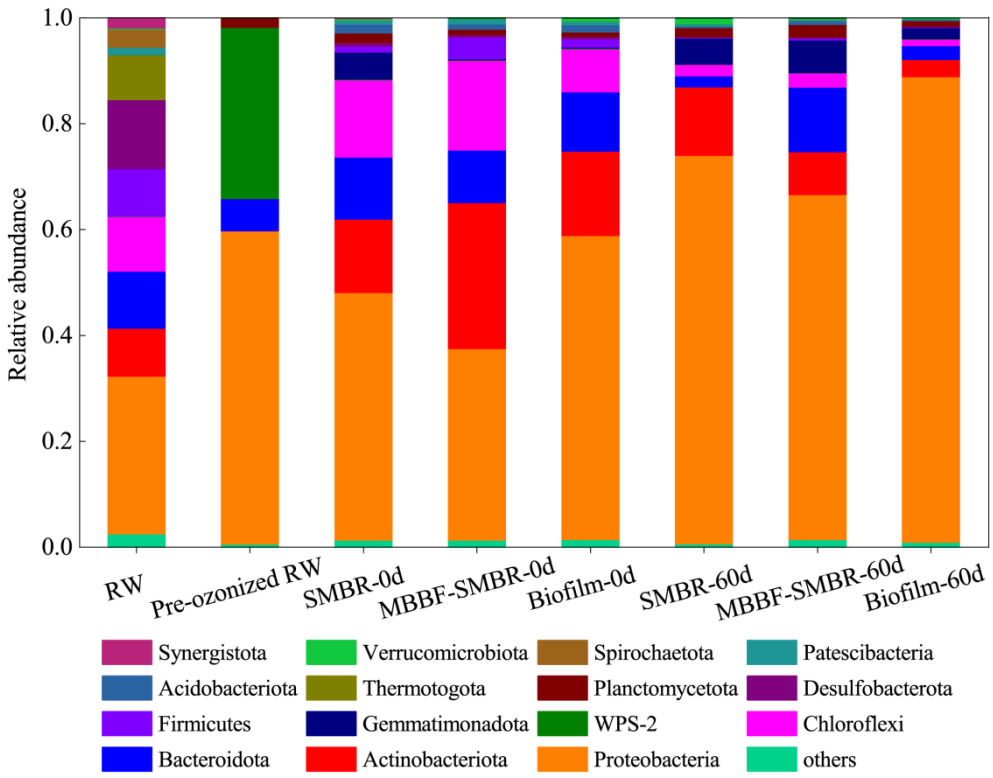
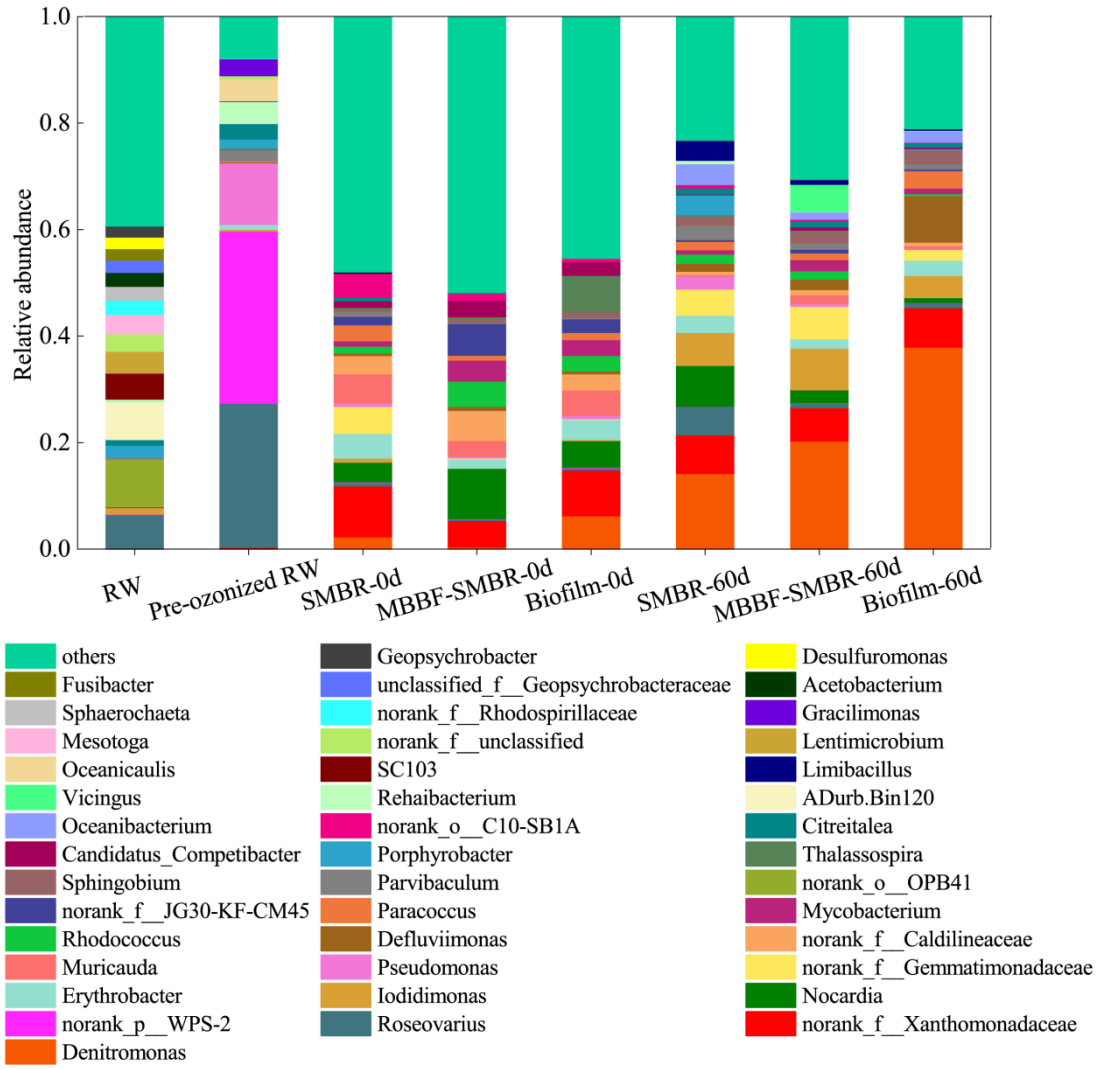

1.0
0.8
0.6
0.4
0.2
0.0
Relative abundance
RW
Pre-ozonized RW
SMBR-0d
MBBF-SMBR-0d
Biofilm-0d
SMBR-60d
MBBF-SMBR-60d
Biofilm-60d
Synergistota
Verrucomicrobiota
Spirochaetota
Patescibacteria
Acidobacteriota
Thermotogota
Planctomycetota
Desulfobacterota
Firmicutes
Gemmatimonadota
WPS-2
Chloroflexi
Bacteroidota
Actinobacteriota
Proteobacteria
others


1.0
0.8
0.6
0.4
0.2
0.0
Relative abundance
RW
Pre-ozonized RW
SMBR-0d
MBBF-SMBR-0d
Biofilm-0d
SMBR-60d
MBBF-SMBR-60d
Biofilm-60d
others
Geopsychrobacter
Desulfuromonas
Fusibacter
unclassified_f__Geopsychrobacteraceae
Acetobacterium
Sphaerochaeta
norank_f__Rhodospirillaceae
Gracilimonas
Mesotoga
norank_f__unclassified
Lentimicrobium
Oceanicaulis
SC103
Limibacillus
Vicingus
Rehaibacterium
ADurb.Bin120
Oceanibacterium
norank_o__C10-SB1A
Citreitalea
Candidatus_Competibacter
Porphyrobacter
Thalassospira
Sphingobium
Parvibaculum
norank_o__OPB41
norank_f__JG30-KF-CM45
Paracoccus
Mycobacterium
Rhodococcus
Defluviimonas
norank_f__Caldilineaceae
Muricauda
Pseudomonas
norank_f__Gemmatimonadaceae
Erythrobacter
Iodidimonas
Nocardia
norank_p__WPS-2
Roseovarius
norank_f__Xanthomonadaceae
Denitromonas

614

Fig 5. Bacterial community composition of: RW; pre-ozonized RW; samples (sludge and biofilm) obtained from SMBR and MBBF-SMBR on day 0 and day 60. Analysis at: (a) phylum (relative abundance＞1%); (b) genus level (relative abundance＞2%). ("SMBR" and "MBBF-SMBR" refer to the sludge samples from SMBR and MBBF-SMBR systems, while "Biofilm" refers to the biofilm samples from the MBBF-SMBR system).

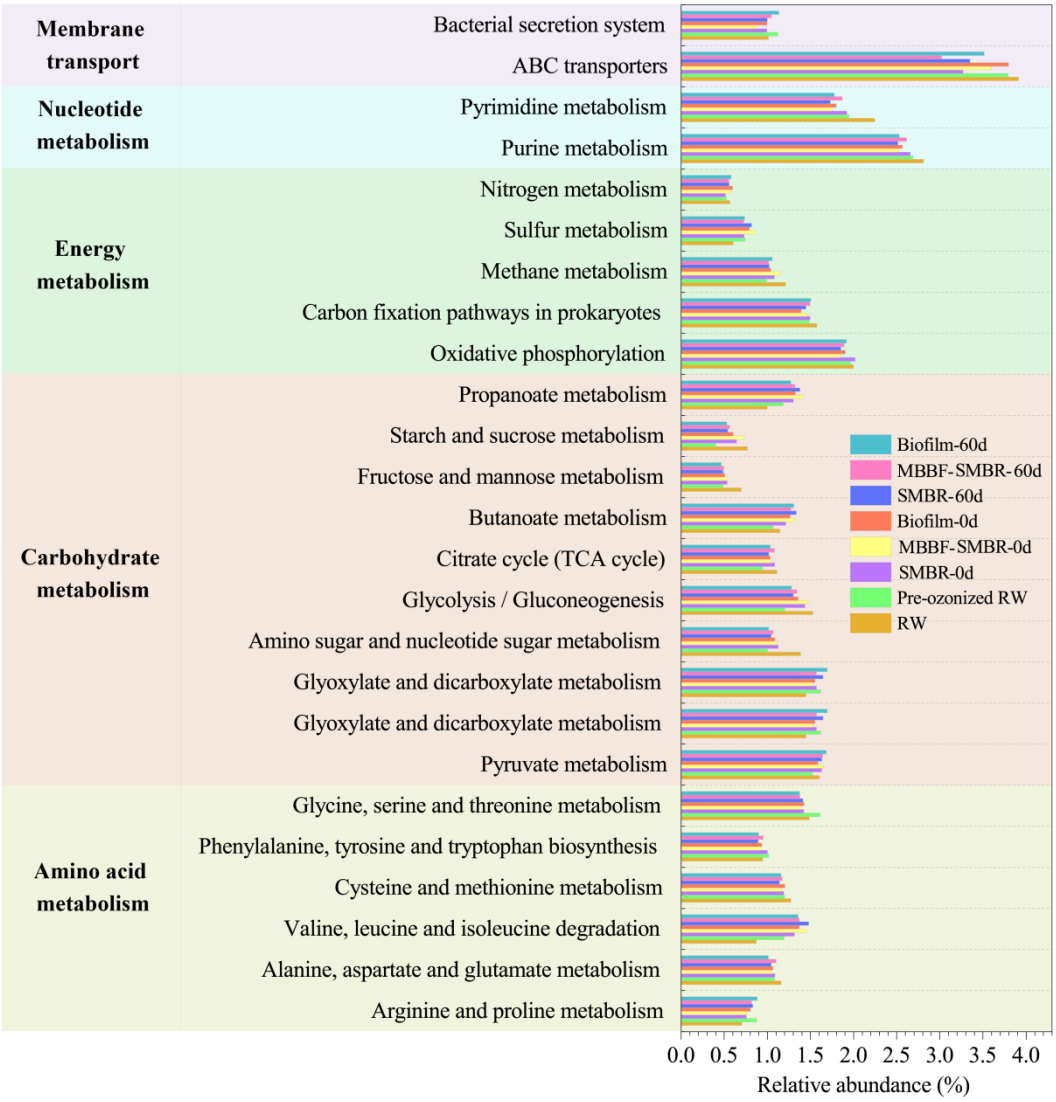


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Table 1. Characteristics of RW, pre-ozonized RW, effluent of SMBR, and effluent of MBBF-SMBR.

Parameters	RW	Pre-ozonized RW	SMBR	MBBF-SMBR
pH	7.28	7.77	5.65	5.23
TDS (g/L)	19.69	19.98	20.65	20.84
Electrical conductivity (mS)	32.34	32.73	33.28	33.79
Turbidity (NTU)	30	19.4	0.19	0.18
DOC (mg/L)	27.32	32.34	7.56	6.53
TN (mg/L)	64.51	46.56	37.77	29.45
UV_{254} (cm^{-1})	0.115	0.087	0.087	0.082

Table 2. Estimates of richness and diversity for operational taxonomic units (OTUs) definition of 97% similarity for RW, pre-ozonized RW, and for samples (sludge and biofilm) obtained from SMBR and MBBF-SMBR on day 0 and day 60 of the experimental test. ("SMBR" and "MBBF-SMBR" refer to the sludge samples from SMBR and MBBF-SMBR systems, while "Biofilm" refers to the biofilm samples from the MBBF-SMBR system).

Samples	Shannon	Simpson	Ace	Chao 1	Coverage
RW	4.24	0.03	264.89	263.33	1.00
Pre-ozonized RW	2.70	0.13	185.30	165.33	1.00
SMBR -0d	4.82	0.02	1203.11	1180.38	0.99
MBBF-SMBR -0d	4.91	0.02	1228.62	1247.05	0.99
Biofilm-0d	4.70	0.03	1198.41	1200.22	0.99
SMBR -60d	3.90	0.05	831.78	827.67	1.00
MBBF-SMBR -60d	4.10	0.06	1076.83	1161.83	0.99
Biofilm-60d	3.13	0.16	773.38	751.52	1.00