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Comprehensive Evaluation of a Full-Scale Combined Biological Process for the Treatment of Petroleum Refinery Wastewater using GC-MS and PCR-DGGE Techniques

Xiaoli Dai[1,2], *Chunmao Chen*[1], *Yu Chen*[1], *Shaohui Guo*[1,*]

[1] State Key Laboratory of Heavy Oil Processing, Beijing Key Laboratory of Oil and Gas Pollution Control, China University of Petroleum, Beijing 102249, China
[2] Environmental Protection Research Institute of Light Industry, Beijing Key Laboratory of Remediation of Industrial Pollution Sites, Beijing 10089, China
[*]E-mail: cupgsh@163.com

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A full-scale biological process, combining recirculated biofiltration, hydrolysis acidification, anoxic/oxic, anoxic/oxic and membrane bioreactor treatment (RBF-HA-A/O/A/O-MBR), was used for treating petroleum refinery wastewater (PRW). A 25-day field monitoring experiment was conducted to evaluate its running performance. The results showed that the removal efficiencies of COD_{Cr}, BOD_5, TOC, TN, NH_4^+-N were 96%, 100%, 98%, 56.24% and 100%, respectively. However, TN was reduced to 61.09 mg/L from 139.60 mg/L, which was failure to meet the industrial wastewater discharge requirements (20 mg/L). Gas chromatography-mass spectrometry (GC–MS) analysis showed that the main organic compounds in the influent of the A/O-MBR process were low-concentration and bio-refractory heterocyclic compounds, esters, organic acids, aldehydes and ketones, alkanes, alcohols. It was believed that insufficient effective carbon source limited the nitrogen removal efficiency. The microbial community structural analysis based on the polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) technology revealed that the predominant bacteria in different reactors were different. The results showed that the microorganisms of organics degradation were diverse, however, the amount of nitrifying bacteria and denitrifying bacteria were rarely detected or even undetected. Our results suggested that this biological treatment process was effective for organic removal but needs to be improved to increase the TN removal efficiency.

Keywords: Petroleum refinery wastewater; Full-scale biological system; RBF-HA-A/O/A/O-MBR; Running performance

1. INTRODUCTION

Petroleum refinery wastewater (PRW) is produced by refining crude oil and processing fuel, lubricants and petrochemical intermediates [1]. PRW usually contains high concentration of aromatic

fractions, salts, oil, volatile phenols, etc. It is characterized by complex compositions, high toxicity and bio-refractory [2, 3], which can lead to serious ecological pollution. PRW needs to be treated before being discharged into water bodies.

The conventional PRW treatment process includes a sequence of mechanical and physicochemical methods, followed by biological treatment of integrated activated sludge treatment units. Among them, biological treatment is the key process for ensuring that the effluent meets the discharge requirements. However, when the BOD_5/COD (B/C) ratio for untreated wastewater is below 0.3, simple biological treatment processes are defined as inappropriate [4, 5]. To meet the discharge standards required by legislation, combined biological technologies are widely developed to treat bio-refractory wastewater [6, 7]. Wu et al. [8] achieved a COD removal rate exceeding 85.4 % with hydrolysis acidification-anoxic/oxic process treatment of petrochemical wastewater. Zhang et al. [9] used anaerobic baffle reactors (ABRs), sequencing batch reactors (SBRs), and sand filters to treat high-salinity oily wastewater. Here, to treat high-salinity PRW, a combined biological process, including recirculating biological filter (RBF)-hydrolysis acidification (HA)-anoxic/oxic(A/O)-anoxic/oxic/membrane reactor (A/O-MBR) technology (Figure 1), is industrialized by a wastewater treatment plant in China (Table 1).

RBF is used in the pretreatment process of biological treatment units to improve biodegradability and decrease organic load. Charles et al. [10] demonstrated that the preaeration at the start of anaerobic digestion can increase of biogas production. HA is also an important pretreatment technology, which has been widely used as pretreatment process of industrial wastewater. It can improve the biodegradability of wastewater by decomposing dissolved colloidal organic pollutants into small molecular substances using anaerobic or anoxic metabolism of microorganisms [8, 11]. A/O process is often used to remove organics and biological nitrogen in wastewater. In fact, HA-A/O process is a conventional process for treating various industrial wastewater [3, 12]. A/O-MBR process is usually used for further treat organics and biological nitrogen in wastewater to make wastewater meet discharge requirements [13, 14]. Xia et al. [15] obtained high removal efficiencies of conventional pollutants and total nitrogen of artificial wastewater by an A/O-MBR process.

In this study, a full-scale biological treatment process including RBF-HA-A/O-A/O-MBR was evaluated in treating PRW. The running performance and microbial community structure were studied to explore an appropriate biological treatment process of refinery wastewater with the characteristics of enriched dissolved bio-refractory organics, high salinity and low B/C ratio. For this purpose, a 25-day field monitoring experiment was conducted. Gas chromatography-mass spectrometry (GC–MS) was used to further characterize the organic compounds changes in PRW. Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) technology was used to detect the dominant species in each biological process.

2. EXPERIMENTAL

2.1. Wastewater Characterization

The refinery wastewater treatment plant is located in Guangdong Province in southern China. The wastewater was collected from the influent and effluent of various biological treatment units over

25 days. The characteristics of PRW are shown in Table 1. In this study, chemical oxygen demand (COD_{Cr}), biochemical oxygen demand (BOD_5), total organic carbon (TOC), total nitrogen (TN) and ammonia nitrogen (NH_4^+-N) were mainly analyzed.

Table 1. Characteristics of the PRW

Parameter	COD_{Cr}	BOD_5	TOC	NH_4^+-N	TN	NO_3^-
Concentration (mg/L)	2554	1198	610.93	81.20	139.60	8.43

2.2. Treatment Process

The total designed wastewater treatment capacity of the full-scale treatment plant was 3600 m^3/d. The design parameters of each process are shown in Table 2.

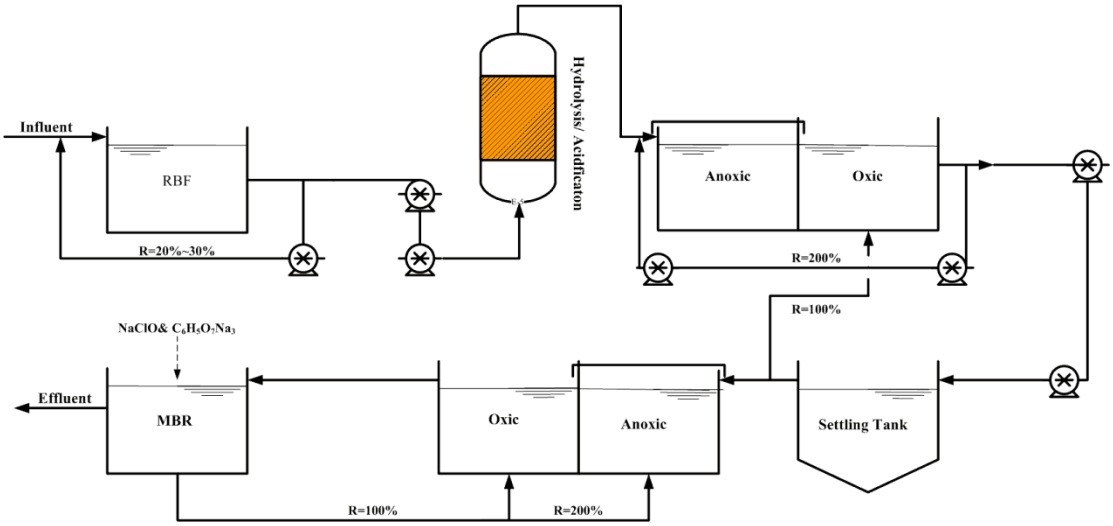

Figure 1. Flow diagram of the full-scale biological treatment process

Table 2. Design parameters of each process

Unit	Size	Number	Parameters
RBF	7 m×7.1 m×4 m	14	HRT: 14 h, DO: 2-4 mg/L
Hydrolysis Acidification	V = 10000 m^3	3	HRT: 30 h, DO: 0.2-0.5 mg/L
Primary Anoxic Tank	17 m×17 m×6 m V = 1700 m^3	2	HRT: 5 h, DO: 0.2-0.5 mg/L MLSS: 7000-9000 mg/L
Primary Oxic Tank	22 m×22 m× 6 m V = 3000 m^3	2	HRT: 10 h, DO: 2.0-5.0 mg/L, MLSS: 7000-9000 mg/L, Recycling ratio: 200%
Settling Tank	Φ=25 m×4 m V = 2000 m^3	1	Recycling ratio: 100%
Secondary	15 m×20 m×6 m	2	HRT: 6 h, DO: 0.2-0.5 mg/L,

AnoxicTank	V = 1800 m^3		MLSS: 5000-7000 mg/L
Secondary Oxic Tank	25 m×20 m×6 m V = 2700 m^3	2	HRT: 9 h, DO: 2.0-5.0 mg/L, MLSS: 5000-7000 mg/L
MBR	12.5 m×3.1 m×5.1 m V = 200 m^3	4	HRT: 4 h, DO: 2.0-5.0 mg/L MLSS: 10000-12000 mg/L, Recycling ratio: 300%

2.3. Wastewater Quality Analysis

The COD_{Cr} and TOC were measured according to the method of GB/T 11914-1989 and GB/T16488-1996. The BOD_5 was determined according to the standard of HJ 505-2009. NH_4^+-N was measured colorimetrically with Nessler's reagent. TN was determined by UV spectrophotometry according to the standards of GB/T 11894-89.

2.4. Organic Compounds Analysis

Organic compounds in PRW were analyzed by GC–MS. 1 mL pretreated samples were analyzed on a Thermo-Finnigan SSQ710 GC-MS with an HP-5MS elastic silica capillary column (60 m × 0.25 mm × 0.25 mm). The functions of GC–MS system were separation individual compounds from mixtures by gas chromatography-transfer separated compounds to the ionization chamber-ionization-mass analysis-detection the ions by an electron multiplier [16]. Details of the GC-MS operation procedures are given in Dai's work [17].

2.5. Microbial Community Structure Analysis

2.5.1. DNA Extraction and Detection

0.25 g activated sludge was collected for genomic DNA extraction. Powersoil DNA kit (MO-BIO, USA) was used for extraction DNA. Agarose gel (0.8% W/V) electrophoresis was used to detect genomic DNA.

2.5.2. PCR Amplification and DGGE Analysis

Genomic DNA was amplified by PCR in a peqSTAR 96 universal thermocycler (PEQLAB Biotechnology, Germany). 27F/1429R and 338F(with GC clamp)/518R were used as primers for two rounds of PCR, respectively. PCR amplification conditions are given in Dai's work [17]. DGGE analysis of the amplified DNA fragments was performed on 8% polyacrylamide gel with a linear denaturation gradient of 50-100%. Gel electrophoresis was performed on Dcode universal mutation detection system (Bio-Rad) at 60 °C, 120 V for 12 h. The gel after electrophoresis was stained with EB. Stained gel was detected by Gel Doc XR system (Bio-Rad, USA).

2.5.3. Sequencing Analysis

Selected DGGE bands were excised from the gel and dissolved in 150 µl sterile water. 1 µl eluted DNA was re-amplifed using the 338F(with GC clamp)/518R primers. The PCR products were connected with pMD@19-T plasmid, and the recombinant plasmid transferred into competent *Escherichia coli* DH5α. *E. coli* DH5α with recombinant plasmid were screened by blue and white spots, and the positive colonies were sequenced by Beijing Genomics institution. Plasmids and *E. coli* DH5α competent cells were purchased from TaKaRa, Japan.

3. RESULTS and DISCUSSIONS

3.1. Performances of the Biological Process

3.1.1. COD_{Cr}, BOD_5 and TOC Removal

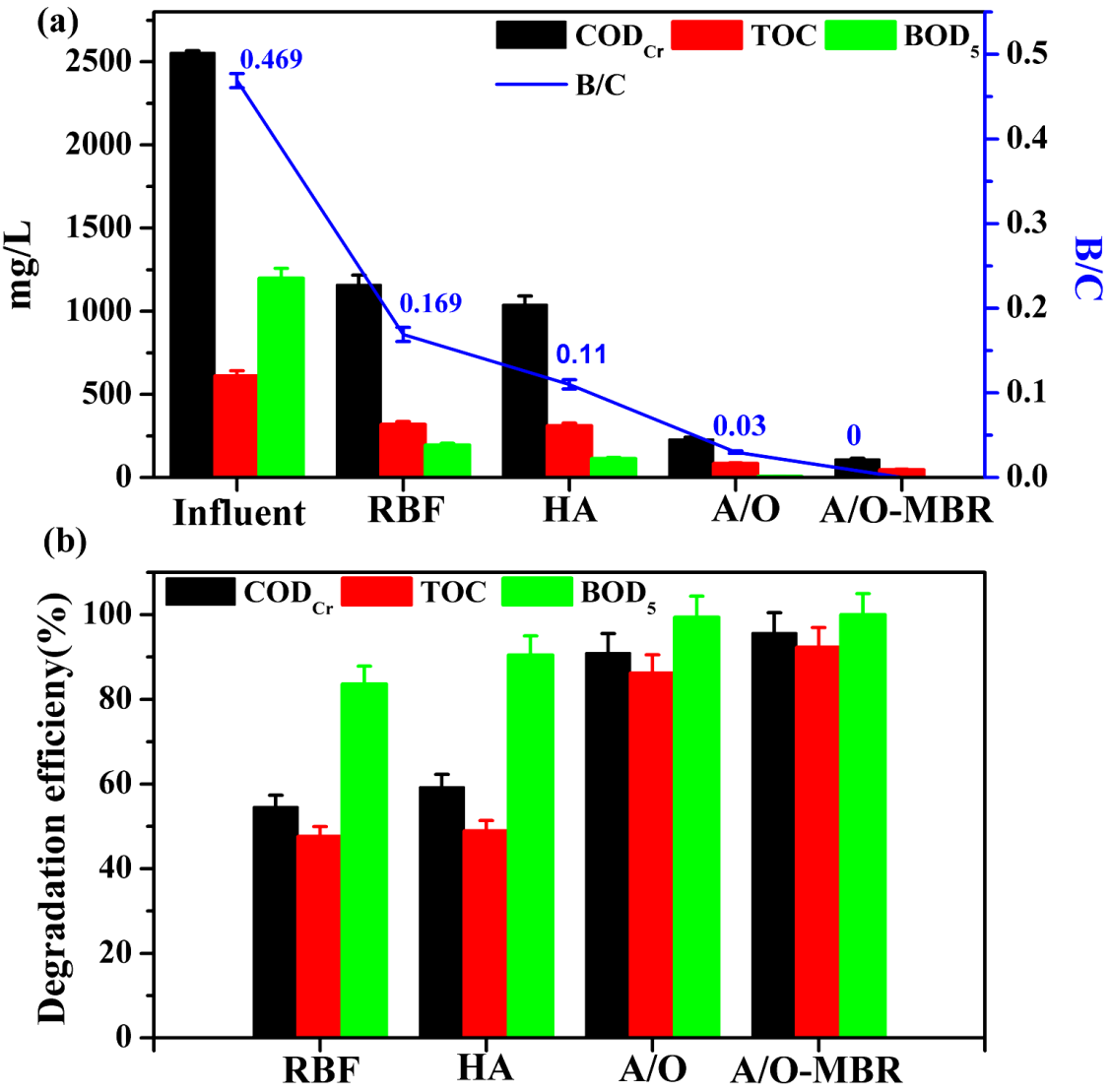

Figure 2. Change in COD_{Cr}, TOC, BOD_5 and B/C during the biological process

The $COD_{Cr,}$ BOD_5 and TOC of the wastewater were analyzed during the entire operation period (Figure S1, Figure S2 and Figure S3). Figure 2 plots the $COD_{Cr,}$ BOD_5 and TOC changes in influent and effluent of each process. The concentration of COD_{Cr}, BOD_5 and TOC of the wastewater declined persistently during the whole biological treatment process. The concentration of COD_{Cr} in effluent was finally reduced to approximately 109 mg/L from 2554 mg/L, the effluent BOD_5 was almost completely removed and TOC was persistently reduced from 610 mg/L to 46 mg/L. The treatment process removed 96% COD_{Cr}, 100% BOD_5 and 98% TOC. Compared to other biological processes, this one had obvious advantages in terms of organic pollutant removal [1, 18].

However, the B/C of the wastewater declined continuously during the whole biological treatment. According to the process design, HA improved the ratio of B/C, however, after the process treatment, the ratio decreased to 0.11, indicating that HA was ineffective. It could be inferred that oxygen (DO=1.8 mg/L) inhibited the growth of microorganisms, playing the role of hydrolysis acidification. Generally, the wastewater is bio-refractory when the ratio of BOD_5/COD_{Cr} is below 0.3. However, the effluent of HA can be treated by the A/O process, which decreased COD_{Cr} from 1040 mg/L to 230 mg/L, showing that anoxic hydrolysis could also transform nondegradable organic compounds of wastewater into degradable substances. This finding is consistent with previous research results [1].

3.1.2. TN and NH_4^+-N Removal

The bar charts in Figure S4 and Figure S5 show the changes in TN and NH_4^+-N during the biological treatment process. As shown in Figure 3a, TN declined persistently during the whole biological treatment process and NH_4^+ began to decrease after the RBF process. In the RBF process, the NH_4^+-N concentration slightly increased. This may be due to cell lysis and organic nitrogen degradation under aerobic conditions [19]. TN in effluent was finally reduced to approximately 61.094 mg/L from 139.6 mg/L, and NH_4^+-N was almost completely removed from the effluent (Figure 3b). The treatment process removed 56.24% TN and 100% NH_4^+-N (Figure 3b). The results showed that advanced treatment is recommended for TN removal.

According to the process design, the A/O and A/O-MBR processes should be the main contributors to TN removal. As shown in Figure 3, the A/O process decreased TN from 110.70 mg/L to 67.13 mg/L (Figure 3a), the removal efficiency was 39.56% (Figure 3b). This process was effective for TN removal. However, in the A/O-MBR process, TN only slightly decreased to 61.09 mg/L from 67.13 mg/L (Figure3a). This denitrification efficiency of A/O-MBR process was low compared to the A/O process. TN in effluent of anoxic and oxic processes was 65.46 mg/L and 62.66 mg/L (Figure 3a), and NO^-_3 in the effluent of anoxic and oxic processes was 10.67 mg/L and 10.11 mg/L.

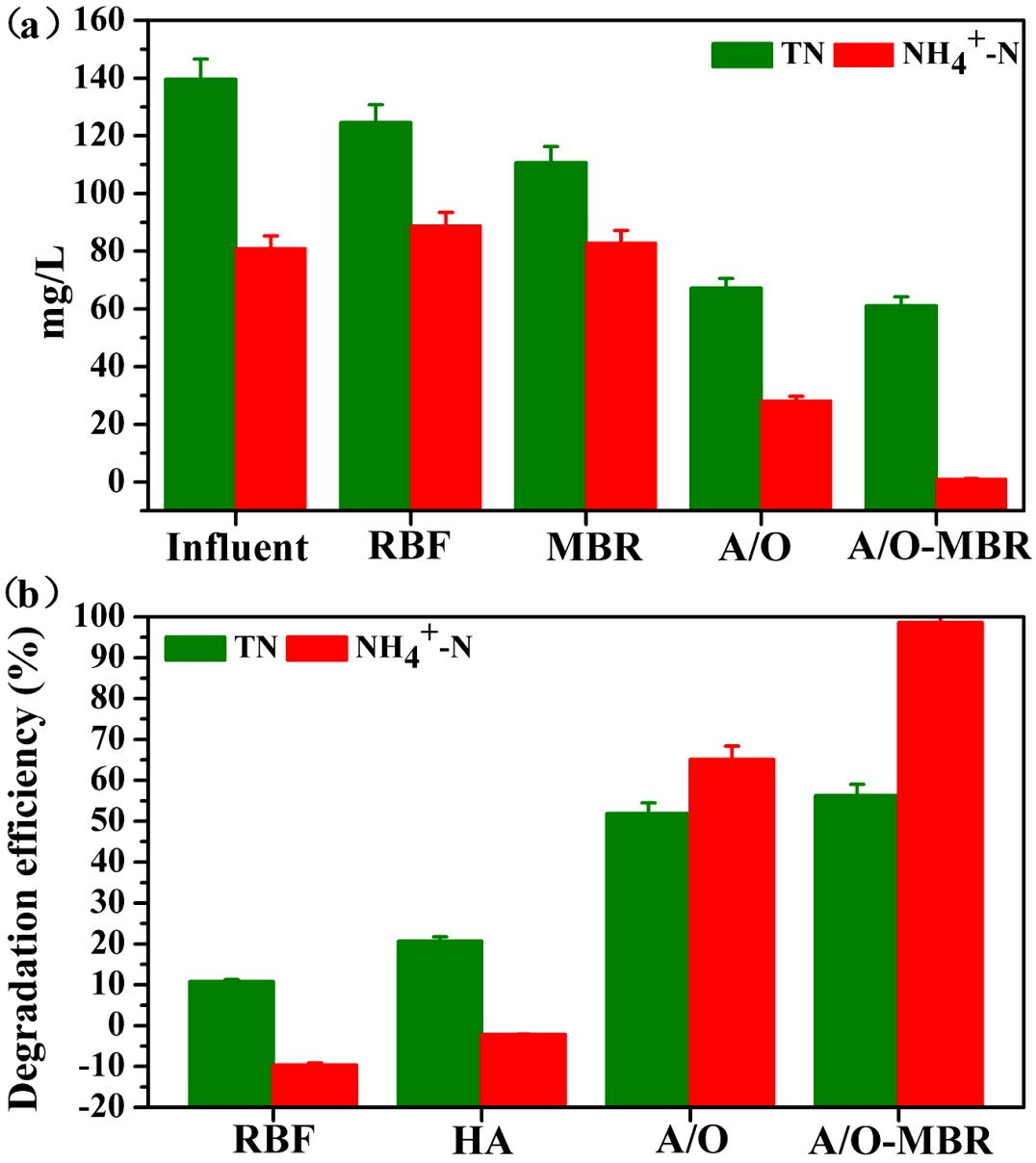

Figure 3. Change in TN (a) and NH_4^+-N (b) during the biological process

The results showed that the anoxic process was ineffective for denitrification, which may be due to the lower ratio of C/N (TOC/TN=1.25). Julián Carrera et al. [20] showed that the C/N ratio for effective denitrification was higher than 4.2. NH_4^+-N in the effluent of anoxic and oxic processes was 13.83 mg/L and 1.09 mg/L, showing that NH_4^+-N was almost completely removed during the oxic nitrification process.

3.2. Organic Pollutants Removal

The organics in influent and effluent of each biological process were analyzed by GC-MS (Figure S6). The top 8 types of organics detected in influent were organic acids, esters, alcohol, heterocyclic compounds, alkanes, aromatic hydrocarbons, aldehydes and ketones and phenols (Table

3), which was consistent with those reported in previous studies [1, 8]. The most dominant category in the influent was organic acids, accounting for 50.94% of the total number of compounds.

Small molecular organic acids, alcohols, phenols, alkanes, heterocyclic compounds, aromatic hydrocarbons, ketones and aldehydes were effectively degraded by RBF process (Figure S6). The relative molecular mass and carbon numbers of the above organic compounds increased (Table 3). Esters were the most dominant fraction in the RBF effluent, showing that the effluent was more complex and bio-refractory. After HA treatment, the composition of the effluent was no significant change. It could be inferred that HA did not improve the wastewater biodegradability, which was consistent with the above studies (Figure 2). After pretreatment, organic acids, esters, alcohols and aromatic hydrocarbons were significantly removed during the A/O process. However, bio-refractory organic compounds were still reserved in effluent, and heterocyclic compounds became the most dominant fraction, accounting for 38.87%. Moreover, TOC in the effluent of A/O process was approximately 84 mg/L, indicating that the concentration of organics in effluent was low. After A/O-MBR treatment, the compositions of the effluent showed significant changes (Figure S6). Organic acids, esters, heterocyclic compounds and alkanes were the dominant fractions, and alcohol, aromatic hydrocarbons, aldehydes and ketones were almost completely removed. Moreover, TOC was decreased to 46 mg/L, BOD_5 could not be detected, and C:N:P was 283:158:1 [11]. The results showed that the wastewater was not suitably treated biologically, and advanced oxidation technologies needed to be used to meet the strict discharge requirements (GB 18918–2002).

Table 3. Organics in influent and effluent of each biological process

Organics		Acids	Esters	Alcohol	Heterocyclic compounds	Alkanes	Aromatic hydrocarbons	Aldehydes and ketones	Phenols
Number of peaks	Influent	28	11	11	13	5	9	9	3
	RBF effluent	12	20	8	9	3	5	1	
	HA effluent	13	18	8	9	7	2	1	
	A/O effluent	5	10	7	20	8	1	7	
	A/O-MBR effluent	7	13	5	10	17	1	4	
Carbon number	Influent	C_2-C_{20}	C_{10}-C_{20}	C_5-C_{15}	C_5-C_{11}	C_8-C_{14}	C_8-C_{11}	C_6-C_{15}	C_6-C_{10}
	RBF effluent	C_6-C_{20}	C_9-C_{20}	C_9-C_{17}	C_6-C_{16}	C_{10}-C_{12}	C_{14}-C_{19}	C_{12}	
	HA effluent	C_6-C_{20}	C_9-C_{20}	C_9-C_{17}	C_6-C_{16}	C_{10}-C_{15}	C_{14}-C_{19}	C_{12}	
	A/O effluent	C_{14}-C_{20}	C_{15}-C_{26}	C_{10}-C_{20}	C_4-C_{22}	C_{10}-C_{27}	C_{16}	C_9-C_{21}	
	A/O-MBR effluent	C_8-C_{20}	C_{16}-C_{30}	C_5-C_{27}	C_4-C_{22}	C_{11}-C_{26}	C_{25}	C_{10}-C_{21}	
Relative molecular mass distribution	Influent	60-312	156-310	86-224	86-191	108-268	106-142	82-220	94-154
	RBF effluent	142-294	156-334	140-244	113-244	136-168	186-256	180	
	HA effluent	142-294	156-334	140-244	113-244	136-168	186-256	180	
	A/O effluent	244-296	240-372	152-312	102-385	138-378	210	152-358	
	A/O-MBR effluent	178-312	266-438	86-416	102-346	156-364	344	156-318	
Relative abundance(%)	Influent	50.94	12.31	10.19	8.59	4.21	0.96	5.09	7.65
	RBF effluent	15.57	39.72	16.35	16.79	6.55	3.11	1.26	
	HA effluent	16.21	34.03	15.89	20.6	9.84	2.14	1.29	
	A/O effluent	12.94	10.56	12.67	38.87	12.49	0.68	11.79	
	A/O-MBR effluent	27.42	19.24	4.79	24.49	19.56	0.48	2.32	

3.3. Microbial Community Diversity Analysis

PCR-DGGE is developed for microbial community analysis without culture, by sequencing-isolation the PCR products of 16S rDNA fragments. The separation principle of DGGE is that the electrophoretic mobility of partially fused double stranded DNA molecules in polyacrylamide gel with linear gradient of DNA denaturant is relatively low [21]. 16S rDNA fragments of microbial community were amplified using 338F (with GC-clamp)/518Rprimers. Then, bacterial species were distinguished by comparing the migration distance of the PCR products in polyacrylamide gel. Each DGGE band is usually a separate species. The number, abundance and position of DGGE bands in different processes were different (Figure 4), indicating that each process may play a different role in PRW treatment.

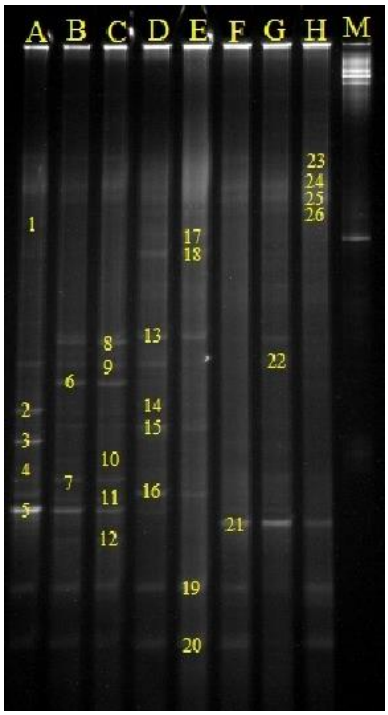

Figure 4. DGGE patterns of samples taken from the active sludge of each process (HA: RBF, hydrolysis acidification T-205, hydrolysis acidification T-206A, A/O, A/O-MBR, M: marker)

Table 4. Sequencing results of bacterial DGGE gel bands

Band	Relatives	Accession number	Similarity	Affiliation
1	*Acidovorax* sp. CPO 4.0017	KC902440.1	98%	*Gammaproteobacteria*
2	*Soehngenia* sp. L35B_140	JF946902.1	100%	*Sarcina*
3	*Comamonadaceae* bacterium B1-08	JF754519.1	99%	*Betaproteobacteria*
4	*Bradyrhizobiaceae* bacterium GJW-30	HF970589.1	94%	*Alphaproteobacteria*
5	*Clostridiales* bacterium De1161	HQ183782.1	100%	*Firmicutes*
6	*Desulfomicrobium* sp.	JX548546.1	100%	*Deltaproteobacteria*
7	*Bacteroidales* bacterium M6	KC769129.1	99%	*Bacteroides*
8	*Mesotoga* sp. VNs100	KC800693.1	100%	*Thermotogae*
9	*Acidobacteria* bacteriumD199A	KC845239.1	100%	*Acidobacteria*
10	*Clostridium* sp.PACOL4_36	GQ257695.1	94%	*Firmicutes*

11	*Rhodocyclaceae* bacterium MBfR_NS-150	JN125706.1	98%	*Betaproteobacteria*
12	*Soehngenia* sp. B4119	HQ133183.1	100%	*Sarcina*
13	*Pseudomonas* sp. clone S2P1061	KF145944.1	100%	*Gammaproteobacteria*
14	Acidobacteria *bacterium SH2*	KC715858.1	100%	*Acidobacteria*
15	Uncultured bacterium ZBAF2-55	HQ682030.1	100%	
16	Uncultured bacterium OX G09	FN429550.1	100%	
17	*Rhodospirillales* bacterium Agri_anode2_47	JN540116.1	98%	*Alpha proteobacterium*
18	*Acidobacteria* bacterium SH6	KC715862.1	95%	*Acidobacteria*
19	*Thauera* sp. BC0187	KC166840.1	95%	*Betaproteobacteria*
20	*Gluconacetobacter* sp. T61213-21-1a	B778532.1	100%	*Rhodospirillales*
21	*Geobacter* sp. KS-54	EU809806.1	91%	*Deltaproteobacteria*
22	*Acidobacteria* bacterium S2-047	KF182983.1	99%	*Acidobacteria*
23	*Nitrosomonas nitrosa* strain S12	KF483596.1	98%	*Betaproteobacteria*
24	*Verrucomicrobia* bacterium	JF410432.1	99%	*Verrucomicrobium*
25	*Acidobacteria* bacterium	JQ402332.1	99%	*Acidobacteria*
26	*Parvularcula* sp.REV_R1PII_12F	FJ933486.1	100%	*Alpha proteobacterium*

The 1-26 DGGE bands were sequenced and then aligned with the bacterial sequences shown in the gene bank (Table 4). The predominant bacterial species (brighter band 2, 3 and 5) in the RBF (A) were *Soehngenia* sp., *Comamonadaceae* bacterium and *Clostridiales* bacterium, all of which can degrade hydrocarbons [22-24], The predominant bacterial species in the HA (B and C) were the *Clostridiales* bacterium (band 5), *Desulfomicrobium* sp. (band 6), *Mesotoga* sp. VNs100 (band 8). They can degrade small organic molecules to produce short-chain fatty acids under anaerobic conditions [24, 25]. However, the ratio of B/C was only reduced from 0.169 to 0.11 in the HA process, showing that the HA process may be inhibited. In the A/O process, the predominant bacterial species in the anoxic process were *Acidobacteria* bacterium D199A (band 9), *Pseudomonas* sp. clone S2P1061 (band 13), and *Acidobacteria* bacterium SH6 (band 18), and the predominant bacterial species in the oxic process were the *Pseudomonas* sp. clone S2P1061, *Rhodospirillales* bacterium Agri_anode2_47 and *Acidobacteria* bacterium SH6. Among the above genera, *Pseudomonas* sp. play an important role in nitrification and denitrification [26, 27], and *Acidobacteria* sp. is hydrolytic-acidogenic bacteria that can promote the hydrolysis of bio-refractory organics [28, 29], which was consistent with the performance of the biological process. In the process of A/O-MBR, the bacterial abundance decreased significantly, and the predominant bacterial species were all *Geobacter* sp. KS-54. It could be inferred that microbial activity and diversity were inhibited by low concentrations of bioavailable carbon sources.

4. CONCLUSIONS

This paper investigated the performance of a full-scale combined biological treatment process, including RBF, hydrolysis acidification tank, A/O and A/O-MBR. The process was operated at a low ratio of B/C after the RBF process, although it attained high organic substance removal efficiency. The removal efficiencies of COD_{Cr}, BOD_5, and TOC were 96%, 100% and 98%. However, the process had a poor performance regarding nitrogen removal. Although NH_4^+-N was almost completely removed,

the TN was just reduced to 61.09 mg/L from 139.60 mg/L, which cannot meet the discharge requirements of industrial wastewater. The organics in effluent were mainly composed of organic acids, esters, heterocyclic compounds and alkanes which were low in concentration, complex in composition and difficult to biodegrade. The predominant bacteria in the biological process are those exhibiting organic degradation capabilities, however, the nitrifying bacteria and denitrifying bacteria were rarely detected or even undetected. Further studies are warranted to analyze the limiting factors for improving the performance of the biological process.

ACKNOWLEDGEMENTS
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SUPPLEMENTARY MATERIAL

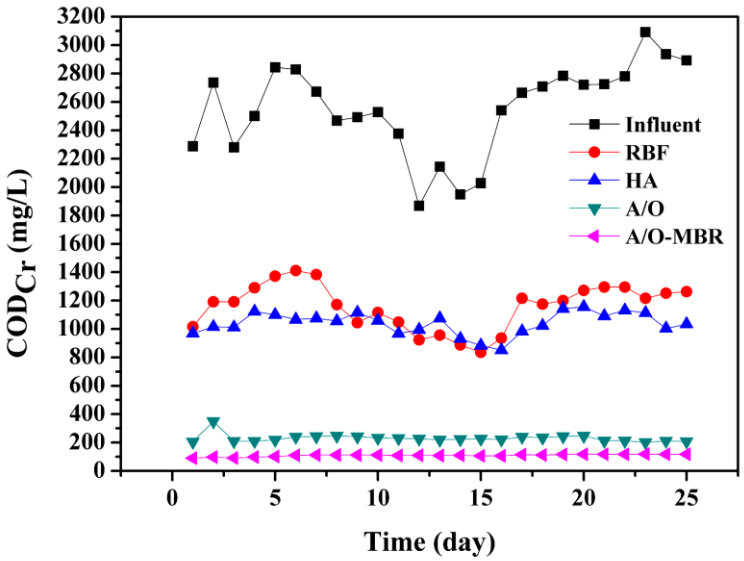

Figure S1. Changes of COD_{Cr} during biological treatment process

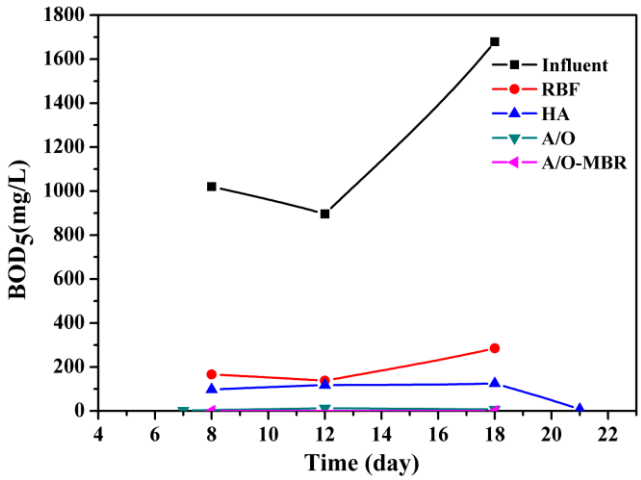

Figure S2. Changes of BOD_5 during biological treatment process

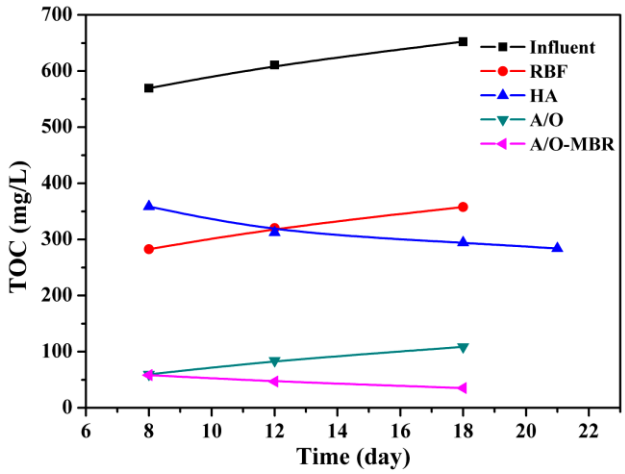

Figure S3. Changes of TOC during biological treatment process

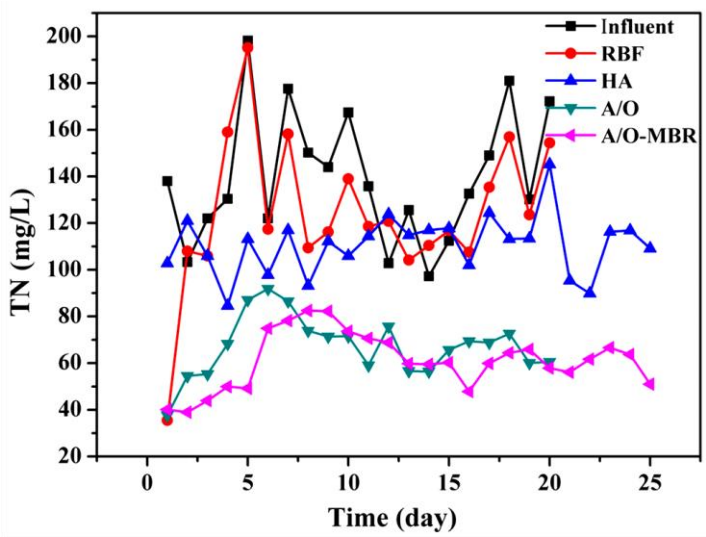

Figure S4. Changes of TN during biological treatment process

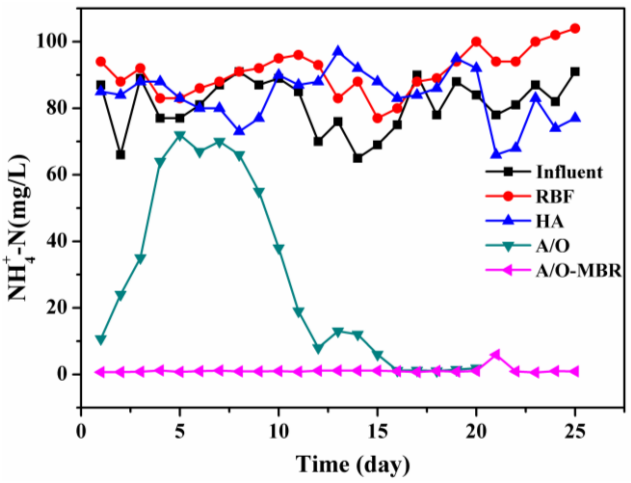

Figure S5. Changes of NH_4^+-N during biological treatment process

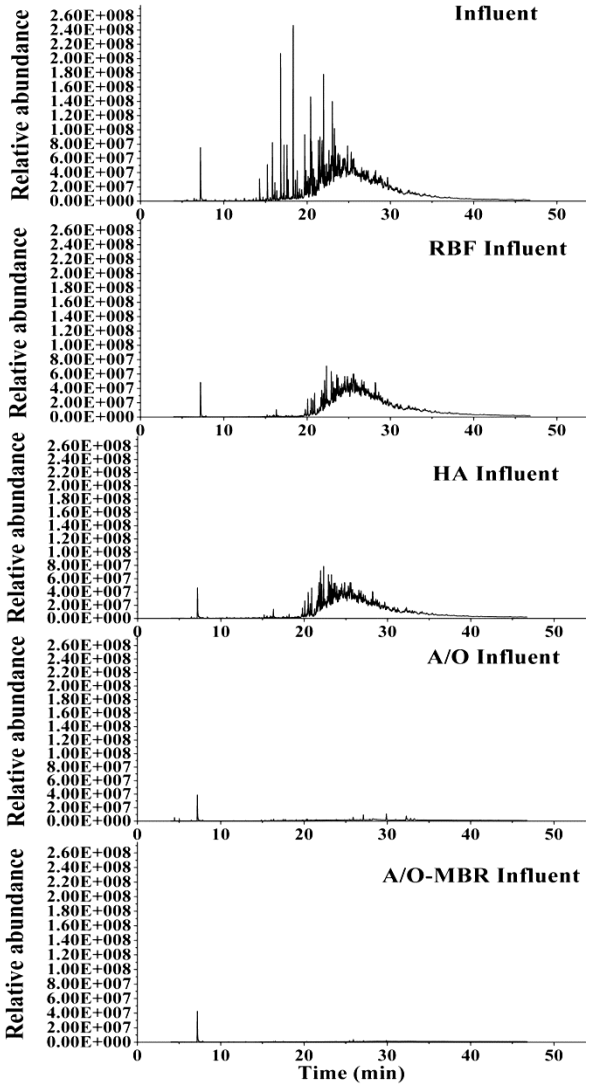

Figure S6. Total ion chromatogram of biological treatment of influent and effluent from various biochemical treatment processes

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