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The analysis of microbial community in the biodegradation, electron transfer based on sulfur metabolism integrated (BESI®) process for reverse osmosis concentrate (ROC) treatment by 454-pyrosequencing

Chao Wei[a], Wenjie He[b], Li Wei[a,*], Chunying Li[c], Dong Wei[a], Jun Ma[a]

[a]*State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin 150090, China, emails: wei8592@gmail.com (C. Wei), weilihit@126.com (L. Wei), yaoyuan_chenmo@163.com (D. Wei), ma_junhit@126.com (J. Ma)*
[b]*Tianjin Waterworks Group Co., Ltd., Tianjin 300040, China, email: HeWenjie8535@163.com*
[c]*School of Energy and Civil Engineering, Harbin University of Commerce, Harbin 150028, China, email: heart.li@163.com*

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ABSTRACT

In this study, a laboratory-scale biodegradation, electron transfer based on sulfur metabolism integrated (BESI®) process was used on the treatment of petrochemical reverse osmosis concentrate (ROC). ROC is a type of saline wastewater with low biodegradability. In the operational days, the chemical oxygen demand (COD) and total organic carbon removal efficiencies on average were 79.18 and 79.39%, respectively. The removal efficiencies of ammonia nitrogen and total nitrogen on average were 79.84 and 83.60%, respectively. High-throughput pyrosequencing was applied on the analysis of the microbial community in activated sludge and biofilm samples. The functional phylotypes sulfate-reducing bacteria (SRB) were detected in anaerobic reactor, and they participated in the COD removal and sulfate reduction. The genera *Hyphomicrobium*, *Azoarcus*, *Thauera*, *Paracoccus*, and *Nitrospira* were detected in the BESI® process. These genera contributed to the nitrogen transformation, and they played different roles in each reactor of the integrated process.

Keywords: BESI® (biodegradation, electron transfer based on sulfur metabolism integrated); Reverse osmosis concentrate (ROC); 454-pyrosequencing; Sulfate-reducing bacteria (SRB); Denitrification

1. Introduction

Reverse osmosis (RO) is one of the most effective means for removing a wide range of micropollutants, including dissolved solids, organic and ionic matters, so it is considered as the ultimate barrier for removing dissolved contaminants in a multibarrier approach [1,2]. RO has been successfully applied in the desalination of seawater and industrial wastewater because of its advantages of technical, operational maturity, and the lowest specific energy requirements of the developed desalination technologies, so the number of RO plants has been steadily increasing overtime in the last three decades [3–5].

*Corresponding author.

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One significant disadvantage is that the concentrate produced in RO process is equivalent to 5–25% of the influent flow. The high water quality of RO permeate is achieved, while most pollutants retained in the RO concentrate, leading to potential health and ecological risks. Soluble solids, toxic and non-biodegradable pollutants were concentrated in RO concentrate [6]. The salinity, dissolved organic matter content, and bio-toxicity of reverse osmosis concentrate (ROC) are several times higher than those in RO influent. The concentrate contains large quantity of hazardous substances, especially in petrochemical ROC. Because of the characteristic of accumulation to micropollutants in RO process, and meanwhile the RO method has taken great development in recent decades, large quantity of petrochemical concentrate with pollutants is produced every day. As a consequence, further treatment of the concentrate is necessary [7].

Nowadays, multiple treatments for ROC have been investigated, which include thermal evaporators, crystallizers, brine concentrators, spray dryers, electrochemical oxidation, and electro-oxidation. Advanced oxidation processes are also used single or together for removing the organics present in the ROC [8–10]. All these processes focus on physical and/or chemical methods; the exploration of biodegradation on ROC is weak and deficient. Compared to other approaches of wastewater treatment, biological treatment has the advantages of lower treatment costs with no secondary pollution.

In anaerobic environment, the sulfate could be reduced to sulfide by SRB, and the organic could be removed at the same time. The sulfate reduction could lead to a low sludge yield, and the minimal chemical oxygen demand (COD) requirement in sulfate-reducing bacteria is 2 g of COD consumed per gram of SO_4^{2-}-S reduced. In a closed anaerobic environment, sulfide generated from sulfate reduction tends to dissolve in water as pH increases [11,12]. The sulfide could serve as electron donor in denitrification. Under oxygen-limiting circumstances, sulfur is the major end-product of the sulfide oxidation, whereas sulfate formed under sulfide-limiting circumstances [13–15]. According to these theories, the BESI® process was designed and developed on the treatment of saline petrochemical wastewater containing sulfate. It consists of an anaerobic activated sludge reactor, an anoxic activated sludge reactor, and an aerobic biofilm reactor in this research. The COD was removed by SRB in anaerobic reactor, and the sulfate was reduced to sulfide through this process simultaneously. When nitrate exists in wastewater, the sulfide generated in anaerobic reactor is able to serve as electron donor for autotrophic denitrification, which realizes the function of nitrogen removal [16]. The ammonia nitrogen could be nitrified to nitrate in aerobic reactor, and the nitrate was recirculated to anoxic reactor for denitrification. Thus, the organic carbon, nitrate, and sulfate could be simultaneously removed in BESI® process. In this process, the escape of H_2S was controlled, and the effluent does not contain sulfide. The BESI® process is adequate for the treatment of saline industrial wastewater containing large amounts of sulfate. Sulfate and sulfite-laden wastewater could offer low-cost sulfur sources to drive BESI® process for wastewater treatment.

Through the analysis of microbial community, the relationship between the performance of reactors and microbial community structure were well understood. It is necessary to understand the microbial community structures in different reactors. High-throughput pyrosequencing has shown promise for the capture of the microbial taxa, and this method can generate enormous amounts of DNA reads through a massively parallel sequencing-by-synthesis approach. This technology has been widely used to analyze the microbial community in various environmental samples [17–20]. The aim of the study was to detect the performance of the BESI® process on the treatment of ROC. In order to optimize the understanding of the relationship between the process's performance and the microbial communities, the 454-pyrosequencing was applied on the analysis of microbial communities in the integrated process.

2. Materials and methods

2.1. Characteristics of the wastewater

The characteristics of the ROC are shown in Table 1. The ROC was a kind of saline petrochemical wastewater that is toxic and has low biodegradability. The ROC has a low COD concentration and high sulfate concentration and salinity. The COD concentration of the ROC varied from 285.82 to 316.15 mg/L. And the ROC contained high sulfate concentration,

Table 1
Characteristics of the saline petrochemical ROC

	Concentrate	
TOC	95.99–124.71	mg/L
COD	285.82–316.15	mg/L
SO_4^{2-}-S	117.30–133.13	mg/L
NH_4^+-N	10.97–13.51	mg/L
TN	86.64–97.12	mg/L
Salinity	7.4–8.6	‰

in which the sulfate-S concentration varied from 117.30 to 133.13 mg/L. The salinity of the ROC varied from 7.4 to 8.6‰.

The organic pollutants of the ROC were analyzed by GC–MS, and the gas chromatograms are shown in Fig. 1. Some organics, for instance, sulfurous acid, 2-ethylhexyl nonyl ester, benzene, 1,3-bis(1,1-dimethylethyl)-, heptadecane, 2-methyl-, phenol, 2,4-bis(1,1-dimethylethyl)-, butylated hydroxytoluene, octadecane, bis(1,3-dimethylbutyl) methylphosphonate, hexadecane, 2,6,10,14-tetramethyl-, cyclopentanepropanoic acid, 2-methyl-3-oxo-, methyl ester, trans-(.+−.)-, heptane, 1,7-dibromo-, heptadecane, 3-methyl-, eicosane, 3,9-dimethyl-4,8-diaza-3,8-undecadiene-2,10-dione dioxime, heneicosane, nonadecane, 9-methyl-, hentriacontane, were detected. The low-degradability organics contained in the ROC were mainly long-chain alkane and polycyclic aromatic.

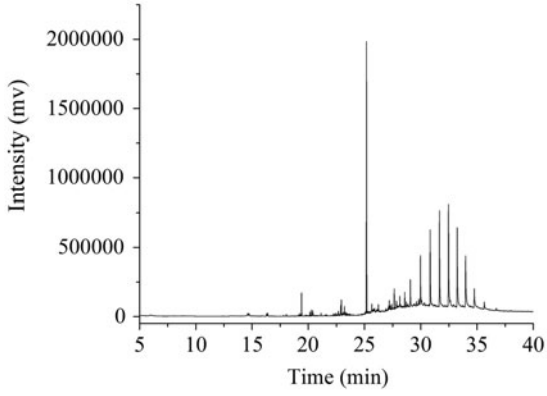

Fig. 1. The gas chromatograms of influent in the BESI® process.

2.2. Experimental setup and operation

A continuously fed synthetic glass upflow anaerobic sludge bed (UASB) reactor, activated sludge anoxic reactor, and biofilm aerobic reactor were used in sequence for our research (Fig. 2). The UASB and the anoxic reactors had a working volume of 18 and 16 L, respectively. The aerobic reactor had a working volume of 50 L, and a continuous air supply system was settled at the bottom of this aerobic reactor. Forty-eight (4 × 4 × 3) spherical plastic baskets with 0.1 m diameter were filled with polyurethane filters and placed in the aerobic reactor. The outlet of this aerobic reactor and the bottom of this anoxic were connected to a hose and a peristaltic pump, which were set as the reflux system, and the reflux ratio were settled at 50%. The anaerobic and anoxic reactors were operated at 37˚C to maintain the activity of anaerobic bacteria using thermostatic jackets. The aerobic reactor was operated at 30˚C using electric heaters, as we considered the proper culture temperature of nitrate bacteria.

In our research, the anaerobic sludge was obtained from industry wastewater treatment plant, and they have been acclimated at anaerobic condition for a long time. The tightness of the anaerobic reactor is good, and the reactor was also equipped with ORP probe to monitor the anaerobic condition. The influent of the anoxic reactor was composed of the effluent of the anaerobic reactor and the reflux from aerobic reactor.

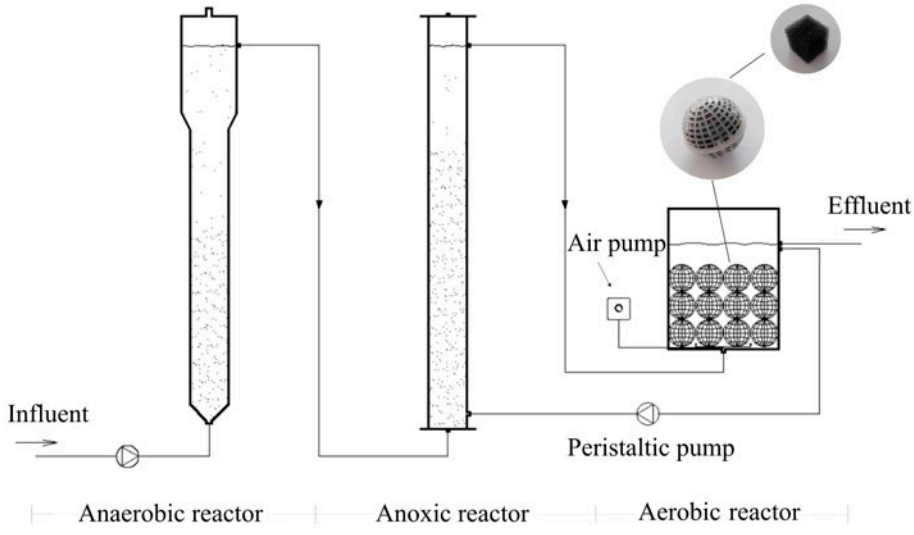

Fig. 2. Schematic diagram of the BESI® process.

Thus, the anoxic reactor could keep anoxic condition. The anoxic reactor was also equipped with ORP probe to monitor the anoxic condition. The laboratory-scale BESI® system was successfully operated for 100 d. Firstly, the integrated process was fed with 20% ROC and 80% nutrition containing glucose, NaCl, Na_2SO_4, KH_2PO_4, and urea, and this period lasted 10 d. Then, the ratios of ROC were adjusted to 40, 60, 80, and 100% progressively, and all the periods of them lasted 10 d. The HRTs of the research were determined by the processing efficiency and the performance of the process. At the acclimation stage, we took a lower water flow velocity and raised the load gradually according to the performance of the process. When the increase in the influent affected the performance, the influent velocity was determined, and the HRTs were calculated according to the volumes of the three reactors. After acclimation periods, the integrated system operated normally, and this period continued for 100 d. HRTs were 27, 12, and 37.5 h in the three reactors, respectively.

2.3. Analysis methods

The COD samples were detected using the potassium dichromate titrimetric method according to the Standard Methods for the Examination of Water and Wastewater [21]. Total organic carbon (TOC) and total nitrogen (TN) were detected using a TOC/TN analyser (Shimadzu TOC-5000A). Sulfate and ammonia nitrogen were analyzed using an ion chromatograph (HIC-20A super) according to standard method. Dissolved sulfide was measured using iodometric method with starch indicator [21].

Gas chromatography (GC, Agilent Technologies 7890A) coupled with mass spectrometry (MS, Agilent Technologies 7890A) was used to detect the decreasing progress of the organics. The buffer gas was highly pure nitrogen, and diluted samples were prepared using methyl tert-butyl ether (MTBE). The operational conditions of GC–MS were the following: The injector temperature was 250˚C, and the column initial temperature was maintained at 35˚C for 3 min. Then, the temperature was gradually increased to 280˚C at a rate of 10˚C/min and held for 5 min, and the ion source temperature of MS was 240˚C.

2.4. DNA extraction, PCR, and pyrosequencing

The microbial samples of day 100 were collected in the anaerobic, anoxic, and aerobic reactors. We collected the sludge from anaerobic and anoxic reactors and marked them as Sample 1 and Sample 2, respectively. Several blocks of fillers were taken out from the aerobic reactor, and they were shaken in deionized water. We collected the suspend solid in deionized water and marked it as Sample 3.

The DNA was extracted using the PowerSoil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the instruction, and the DNA was amplified using universal bacterial primer 8F (5′-3′ AGAGTTTGATCCTGGCTCAG) and 533R (5′-3′ TTACCGCGGCTGCTGGCAC) covering the V1 and V3 regions. Different ten-nucleotide barcode sequences and pyrosequencing adapters were added at the 5′ end of the universal bacterial primer. The PCR products were purified using the TaKaRa Agarose Gel DNA Purification Kit (TaKaRa, China) and quantified using NanoDrop. 454 pyrosequencing was carried out using the Roche 454 FLX Titanium platform at Majorbio.

2.5. Sequence processing

Initially, the base mismatches of sequencing primers were examined, and the sequences which they were no more than 2 bp were reserved. Then, the average base quality was examined, and when the average base quality in any continuous 50 bp read was less than 20 (error rate greater than 1%), the 50 bp read and the followed bases were removed. The containing ambiguous "N" and the followed bases were removed. Finally, the sequences shorter than 200 bp in length and containing repeat bases more than 10 bp were removed, and the chimeras generated in PCR amplification were filtered out to form high-quality sequences. The high-quality sequences were assigned to samples according to barcodes. The sequences were aligned using Mothur ver. 1.17.0 and clustered into operational taxonomic units (OTUs) at 90, 95, and 97% similarities. The OTUs (at 97% similarity) of the samples were used for coverage, Shannon (diversity), Chao (richness), ACE, Simpson, and rarefaction curve analysis. Taxonomic classification of the sequences was performed using the RDP Classifier of the Ribosomal Database Project (RDP), the National Centre for Biotechnology Information (NCBI) BLAST, and the Greengenes databases at 70% confidence threshold. The sequence data have been submitted to NCBI Sequence Read Archive database (Accession Numbers: SRR2374964, SRR2374997, and SRR2374998 for Sample 1, Sample 2, and Sample 3, respectively).

3. Results and discussion

3.1. Performance of the BESI® process

During the operational days, the influent concentration of TOC, COD, sulfate, ammonium nitrogen, and TN on average was 111.04 mg/L, 299.53 mg/L, 124.54 mg S/L, 12.15 mg/L, and 91.20 mg/L, respectively. The TOC concentration of influent and effluent varied from 95.99 to 124.72 mg/L. The total TOC removal efficiency on average was 79.39%, while the TOC concentration of effluent on average was 22.89 mg/L. On average, the effluent COD concentration of the aerobic reactor was 62.37 mg/L, and the total COD removal efficiency of this integrated process was 79.18%. After the treatment of this anaerobic reactor, the average effluent COD concentration was 146.99 mg/L. In anaerobic reactor, 152.54 mg/L COD on average was removed, and the average removal efficiency was 50.93%. The average sulfate concentration of the anaerobic reactor effluent was 76.48 mg S/L, so the concentration of sulfate reduced in anaerobic reactor was 48.06 mg S/L (Fig. 3). The effluent sulfide concentration of this anaerobic reactor varied from 24.88 to 34.31 mg S/L, and the average sulfide concentration was 29.46 mg S/L. In this anaerobic reactor, 61.30% reduced sulfate was converted into dissolved sulfide in water phase (Fig. 3). When COD was removed by SRB, the theoretical value required by COD to reduced SO_4^{2-}-S is two. When the ratio exceeds two, organic matter supplies excessive electrons for sulfate reduction; therefore, other bacteria groups convert the rest of the organic substances. When the ratio is below two, sulfate reduction would become dominant. In this anaerobic reactor, the average ratio of removed COD to reduced SO_4^{2-}-S was 3.17, so the concentration of removed COD by SRB was 96.24 mg/L.

On average, the ammonia nitrogen and TN concentrations of effluent were 2.45 and 14.96 mg/L, and the total removal efficiencies of them in the integrated process were 79.84 and 83.60%, respectively (Fig. 4). Thus, the integrated process is effective to nitrogen removal.

3.2. Microbial diversity

The rarefaction analysis of the bacterial communities derived from the anaerobic (Sample 1), anoxic (Sample 2), and aerobic (Sample 3) samples is

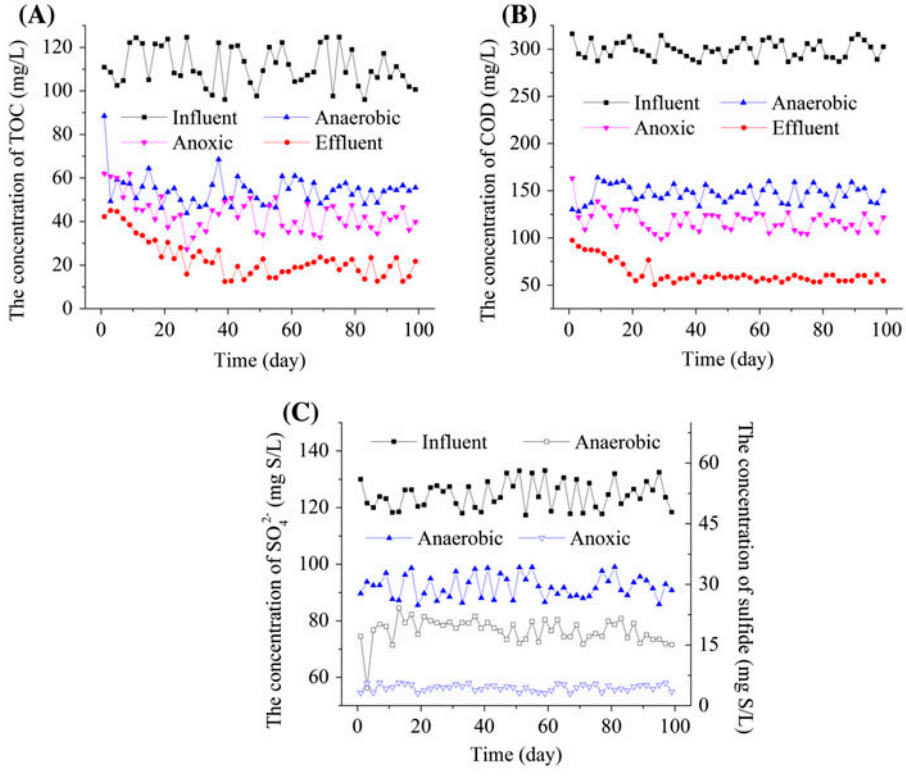

Fig. 3. (A) The organic removal and sulfur transformation efficiencies of the BESI® process, (B) TOC and COD removal efficiencies of each reactor, and (C) the sulfate concentration of influent and anaerobic effluent (represented by black lines and symbols), and the sulfide concentration of anaerobic and anoxic effluents (represented by blue lines and symbols).

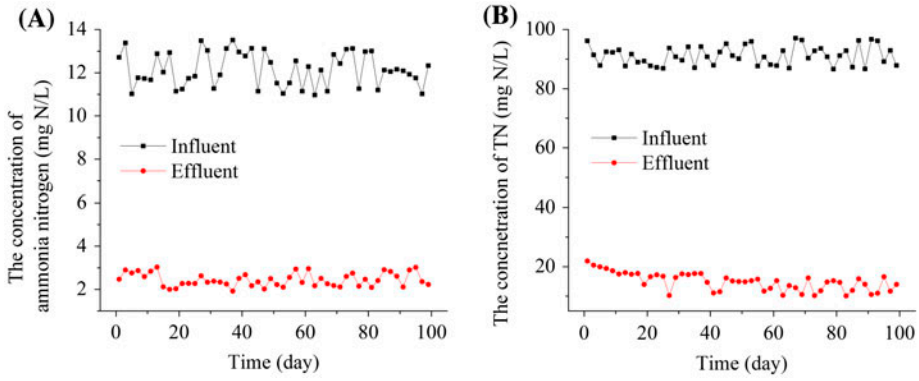

Fig. 4. The nitrogen removal efficiencies of the BESI® process: (A) the ammonia nitrogen concentration of influent and effluent and (B) the TN concentration of influent and effluent.

depicted as having a 97% similarity. At 3% genetic distance, the three curves approached saturation, indicating that the sequencing nearly covered all the OTUs in the three samples (Fig. 5). The coverage indexes of the three samples approached 99%, which indicated that the recovered sequences well represent the microbial diversity in the three samples. The well-distributed rank-abundance curves showed that the distribution of OTUs derived from Sample 2 was the widest of the three samples, which indicated that the microbial diversity of Sample 2 was the highest in the three samples. The OTUs derived from Sample 1 was wider than those of Sample 3, which indicated that the microbial diversity of Sample 1 was higher than that of Sample 3. In addition, the values of the ACE, Chao, and Shannon indices further supported this result (Fig. 5, Table 2).

The unique and shared OTUs were represented by a Venn diagram, and the results showed that 287, 381, and 574 OUTs were unique to Sample 1, Sample 2, and Sample 3, respectively. A total of 509, 172, 50 OTUs were shared by Sample 1 and Sample 2, Sample 2 and Sample 3, and Sample 2 and Sample 3, respectively. Two hundred twenty-four OTUs were common for the three samples. As the results shown, there were greater differences of microbial community between Sample 1 and Sample 3, but Sample 2 shared a lot of OTUs with Sample 1 or Sample 3 (Fig. 6).

3.3. Microbial community

Microbial compositions at the phylum level were shown in Fig. 7 and Table 3. *Proteobacteria* was the most dominant (average abundance > 10%) phylum in the three samples, accounting for 37.02, 53.28, and 33.42% in Sample 1, Sample 2, and Sample 3, respectively. In Sample 1, the phyla *Firmicutes* (20.69%), *Planctomycetes* (12.05%), and *Chloroflexi* (10.13%) with *Proteobacteria* were dominant (total accounting for 79.90%) in the bacterial communities of Sample 1, followed by a few other abundant (average abundance > 1%) phyla, including *Actinobacteria* (7.13%),

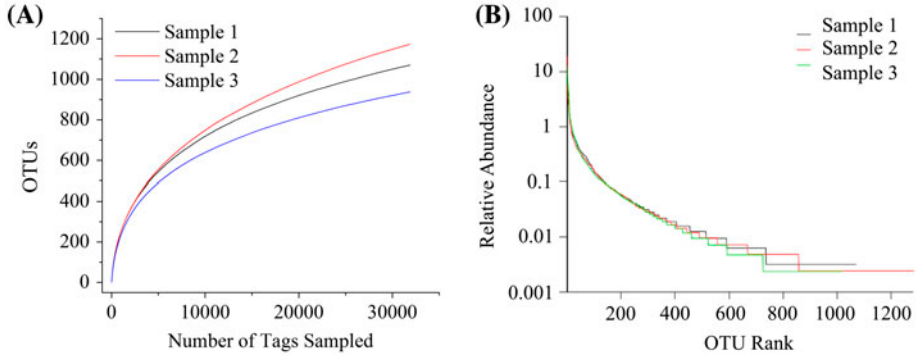

Fig. 5. Rarefaction analysis of the different samples: (A) rarefaction curves are depicted at 3% dissimilarity level and (B) rank-abundance shows pyrosequencing abundance of different samples.

Table 2
Diversity indexes of the three samples

	Valid sequence	Trimed sequence	Coverage index	ACE	Chao	Shannon	Simpson
Sample 1	40,248	33,819	0.990	1,437.042	1,448.306	4.852	0.025
Sample 2	48,062	44,646	0.990	1,795.187	1,769.189	4.638	0.050
Sample 3	46,341	43,341	0.993	1,342.897	1,343.619	4.658	0.032

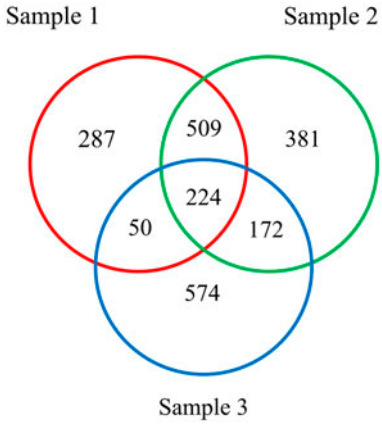

Fig. 6. Venn diagram shows unique and shared OTUs between different samples.

Bacteroidetes (2.93%), *Synergistetes* (1.98%), and *Cyanobacteria* (1.91%). The microbial communities and the dominant phyla of the three samples were different. In Sample 2, the dominant phylum *Proteobacteria* accounted over 50%. The other abundant phyla were *Firmicutes* (9.13%), *Actinobacteria* (8.87%), *Planctomycetes* (8.70%), *Synergistetes* (5.21%), *Chloroflexi* (4.48%), and *Bacteroidetes* (1.99%). The abundance of phyla *Proteobacteria* (33.41%) and *Planctomycetes* (32.78%) was approximate and was dominant in Sample 3, followed by a few other abundant phyla, including *Actinobacteria* (11.63%), *Acidobacteria* (5.21%), *Nitrospirae* (4.16%), *Chloroflexi* (3.26%), *Firmicutes* (1.71%), *Gemmatimonadetes* (1.62%), and *Armatimonadetes* (1.42%). The phyla *Firmicutes* (20.69%), *Actinobacteria* (7.13%), *Bacteroidetes* (2.94%), and *Synergistetes* (1.98%) were major groups in the anaerobic reactor, which have wide ecological niches in both natural and industrial environments. Most of the close relatives of the OTUs are chemoorganotrophic, and some of them exist in contaminated environments containing complex organic matters [17].

The genera (bacteria count > 100) were shown by hierarchical heatmap (Fig. 8). The most abundant genus in Sample 1 was *Pirellula* (9.05%), and the other

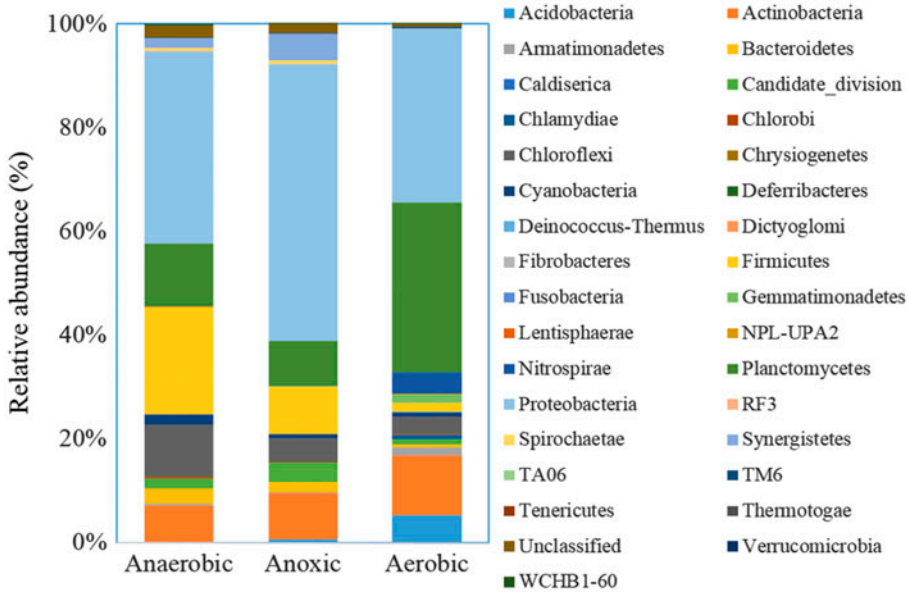

Fig. 7. Microbial compositions at the phylum level. Color-coded bar plot showing the microbial phylum relative abundance across the three samples.

Table 3
The abundance of phyla (bacterial count > 200) in the three samples. Arranged according to the alphabetic order

Phylum	Abundance		
	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)
Acidobacteria	0.03	0.65	5.21
Actinobacteria	7.13	8.87	11.63
Armatimonadetes	0.28	0.20	1.42
Bacteroidetes	2.94	1.99	0.66
Candidate_division	1.90	3.71	1.02
Chlamydiae	0.00	0.01	0.78
Chloroflexi	10.13	4.48	3.26
Cyanobacteria	1.91	0.97	0.95
Firmicutes	20.69	9.13	1.71
Gemmatimonadetes	0.00	0.01	1.62
Nitrospirae	0.00	0.01	4.16
Planctomycetes	12.05	8.70	32.78
Proteobacteria	37.02	53.28	33.41
Spirochaetae	0.56	0.63	0.02
Synergistetes	1.98	5.21	0.03
Unclassified	2.26	1.46	0.39

abundant genera in Sample 1 were *Peptostreptococcaceae_Incertae_Sedis* (6.37%), *Methylocystis* (2.96%), *Planctomyces* (1.30%), *Erysipelothrix* (1.20%), *Nocardioides* (1.19%), *Hyphomicrobium* (1.14%), and *Dietzia* (1.08%) (Table 4). In Sample 1, the SRB (accounting for 1.85%) were detected as abundant functional phylotypes. The COD could be removed by SRB, while the sulfate was reduced to sulfide in anaerobic reactor. Over half of reduced COD in the anaerobic reactor came from SRB. The other organics in the anaerobic reactor were removed by methanogens. Compared with SRB, methanogens have a much narrower spectrum of substrate. From the thermodynamic point of view, the reduction of sulfate to sulfide by SRB released more energy than the production of methane by methanogens, thereby enabling SRB to outcompete methanogens.

The most dominant genus was *Azoarcus* (19.03%) in Sample 2, and the other abundant genera were *Pirellula* (4.29%), *Methylocystis* (3.29%), *Nocardioides* (1.43%), *Dietzia* (1.13%), and *Anoxynatronum* (1.04%). In Sample 3, the most dominant genus was *SM1A02* (15.28%), and the other abundant genera were *Planctomyces* (8.74%), *Gordonia* (5.88%), *Nitrospira* (4.16%), *Blastocatella* (3.57%), *Legionella* (1.80%), *Thiobacillus* (1.55%), *Urania-1B-19_marine_sediment_group* (1.50%), *Hyphomicrobium* (1.43%), and *Thioalkalivibrio* (1.37%) (Table 4). The *Anaerolineae* (accounting for 7.44%) was an abundant group in anaerobic reactor, which shares common physiological and morphological traits, such as anaerobic growth on carbohydrates [22,23]. The *Clostridium* genus was detected in all the three samples, and it is a member of the *Clostridia* class; the class was dominant in Sample 1, which accounted for 18.48 and 8.02% in Sample 1 and Sample 2, respectively. The *Clostridia* class could be related to the biodegradation of the organic pollutants [17] (Fig. 8 and Table 5).

The three samples shared certain genera, while the abundance of the three samples was different. The abundance of shared genera was quite different between Sample 1 and Sample 3, but Sample 2 shows the mutual characteristics with Sample 1 or Sample 3, and the microbial diversity of Sample 2 was higher than that of the other two samples. The microbial communities in the three samples were primarily related to the environmental parameter of dissolved oxygen in the three reactors. Some genera were exclusively detected in one sample, or the abundance of them was much higher than that of them in the other two samples. These genera included *Peptostreptococcaceae_Incertae_Sedis* (6.37%), *BD1-7_clade* (0.89%), and *Dethiosulfatibacter* (0.54%) in Sample 1, *Aminobacterium* (0.62%) in Sample 2, *Planctomyces* (8.74%), *Gordonia* (5.88%), *Nitrospira* (4.16%), *Blastocatella* (3.57%), *Legionella* (1.80%), *Urania-1B-19_marine_sediment_group* (1.50%), *Thioalkalivibrio* (1.37%), *Nitriliruptor* (0.95%), *Mycobacterium* (0.73%), *Sphaerobacter* (0.56%), and *Nocardia* (0.52%) in Sample 3. The genera *Pirellula* (accounting for 9.05 and 4.29% in Sample 1 and Sample 2, respectively), *Fastidiosipila* (0.90 and 0.44%) and *Thauera* (0.84 and 0.65%) were detected in Sample 1 and Sample 2, and the abundance of them in Sample 1 was higher than that of them in Sample 2. The genera *Methylocystis* (2.96 and 3.29%), *Nocardioides* (1.19 and 1.43%), *Anoxynatronum* (0.56 and 1.04%) *Rhodobacter* (0.47 and 0.62%), *Leucobacter* (0.47 and 0.64%) *Anoxynatronum* (0.56 and 1.04%), and *Thermovirga* (0.33 and 0.82%) were detected in Sample 1 and Sample 2, and the abundance of them in Sample 1 was lower than that of them in Sample 2. Some of these genera were also detected in Sample 3, but the abundance of them was much lower than that of them in the other two samples. The genera *Azoarcus* (19.03 and 0.32%), *SM1A02* (0.41 and 15.28%), and *Legionella* (0.14 and 1.80%) were detected in Sample 2 and Sample 3, and the abundance of them was much higher than that of them in Sample 1. Meanwhile, the *uncultured*, *unclassified*, and *uncultured_norank* accounted for a large proportion, which most likely play a significant yet unknown or less understood role.

The sulfides generated in anaerobic reactor are able to serve as electron donor for autotrophic

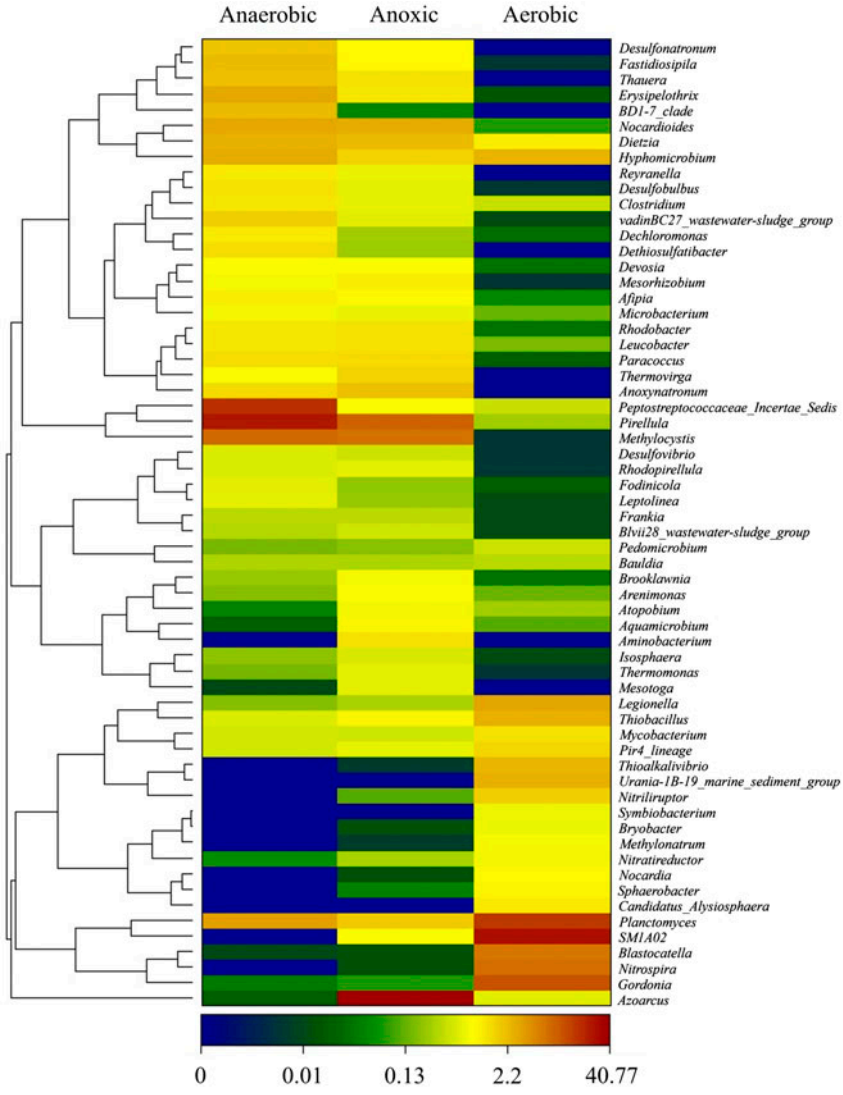

Fig. 8. Relative abundance of genera in Sample 1, Sample 2, and Sample 3. The heatmap color-coded bar plot depicts the relative abundance of each sample. The relative abundance for microbial genera is indicated by color intensity from low (blue) to high (red) with the legend indicated at the bottom.

denitrification. The ammonia nitrogen and TN contained in the ROC could be removed in the BESI® process. Through the analysis of 454-pyrosequncing, the *Hyphomicrobium* genus was detected in the anoxic reactor, and the presence of this genus might play important role in the biodegradation of nitrogenous organic compounds in water [24]. In anoxic reactor, the *Azoarcus* genus was the first dominant genus, which contributed to heterotrophic denitrification, and this genus has been proved to have the sulfide-oxidizing ability under denitrifying condition [25]. Some other denitrification-related phylotypes were also detected in anoxic reactor, which include genera *Thauera* and *Paracoccus*, and they accounted for 0.65 and 0.73%, respectively. *Paracoccus* was confirmed to have high efficiency in denitrification. The end-products of denitrification by *Thauera* were CO_2 and N_2 when oxidizing lactate with nitrate [24]. *Nitrospira* is the most important nitrite-oxidizing bacteria (NOB), and it is adapted to live under significant substrate limitation [26,27], which make *Nitrospira* groups could flourish in the ROC biodegradation reactor. In the aerobic reactor, the ammonia nitrogen could be oxidized to nitrate, and the nitrate was refluxed to the anoxic reactor, which could provide substrate for denitrification. These genera contributed to the nitrogen transformation and realized the function of nitrogen removed in the integrated process.

Table 4
The abundance of genera (bacterial count > 100) in the three samples, arranged according to the alphabetic order

	Abundance		
Genus	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)
480-2_norank	0.34	0.46	0.29
AKYG1722_norank	0.00	0.00	0.35
AKYH478_norank	0.00	0.00	0.71
A0839_norank	0.67	0.45	0.00
Afipia	0.41	0.45	0.00
Aminobacterium	0.00	0.62	0.00
Anoxynatronum	0.56	1.04	0.00
Aquamicrobium	0.00	0.47	0.00
Arenimonas	0.00	0.40	0.00
Armatimonadetes_norank	0.00	0.00	1.42
Atopobium	0.00	0.36	0.00
Azoarcus	0.00	19.03	0.33
BD1-7_clade	0.89	0.00	0.00
Blastocatella	0.00	0.00	3.57
Brooklawnia	0.00	0.37	0.00
Bryobacter	0.00	0.00	0.38
Candidate_division_BRC1_norank	0.00	0.27	0.94
Candidate_division_TM7_norank	1.51	0.81	0.00
Candidate_division_WS3_norank	0.00	2.60	0.00
Candidatus_Alysiosphaera	0.00	0.00	0.65
Chlamydiaceae_norank	0.00	0.00	0.65
Chloroplast_norank	0.38	0.00	0.00
Clostridium	0.00	0.36	0.00
Clostridium	0.47	0.31	0.00
Dechloromonas	0.44	0.00	0.00
Desulfobulbus	0.49	0.29	0.00
Desulfonatronum	0.77	0.46	0.00
Dethiosulfatibacter	0.54	0.00	0.00
Devosia	0.31	0.41	0.00
Dietzia	1.08	1.13	0.60
Elioraea	0.00	0.00	0.24
Enhygromyxa	0.00	0.00	0.03
Erysipelothrix	1.20	0.61	0.00
FW34_norank	0.00	0.59	0.26
Fastidiosipila	0.90	0.44	0.00
GR-WP33-30_norank	0.00	0.00	1.15
Gordonia	0.00	0.00	5.88
Hyphomicrobium	1.14	0.78	1.43
Isosphaera	0.00	0.25	0.00
JG30-KF-CM45_norank	0.95	0.95	0.53
KCM-B-112_norank	0.00	0.00	5.96
KI89A_clade_norank	0.00	0.00	0.35
Legionella	0.00	0.00	1.80
Leucobacter	0.47	0.64	0.00
Litorilinea	0.00	0.00	0.27
ML635J-40_aquatic_group_norank	0.99	0.35	0.00
MLE1-12_norank	0.00	0.00	0.31
MNG7_norank	0.50	0.68	0.00
MSB-1E8_norank	0.00	0.00	1.15
Mesorhizobium	0.00	0.53	0.00
Mesotoga	0.00	0.30	0.00

(*Continued*)

Table 4 (*Continued*)

Genus	Abundance		
	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)
Methylocystis	2.96	3.29	0.00
Methylonatrum	0.00	0.00	0.43
Microbacterium	0.00	0.31	0.00
Mycobacterium	0.00	0.00	0.73
Nitratireductor	0.00	0.00	0.41
Nitriliruptor	0.00	0.00	0.95
Nitrospira	0.00	0.00	4.16
Nocardia	0.00	0.00	0.52
Nocardioides	1.19	1.43	0.00
OCS155_marine_group_norank	0.00	0.00	0.40
Paracoccus	0.54	0.73	0.00
PeM15_norank	1.03	1.13	0.00
Pedomicrobium	0.00	0.00	0.24
Peptostreptococcaceae_Incertae_Sedis	6.37	0.43	0.00
Pir4_lineage	0.00	0.32	0.89
Pirellula	9.05	4.29	0.00
Planctomyces	1.30	0.88	8.74
Reyranella	0.45	0.32	0.00
Rhodobacter	0.47	0.62	0.00
Rhodopirellula	0.00	0.29	0.00
Run-SP154_norank	2.19	1.76	0.00
S0134_terrestrial_group_norank	0.00	0.00	0.39
SB-1_norank	0.00	0.27	0.00
SHA-109_norank	1.45	0.55	0.00
SM1A02	0.00	0.41	15.28
SM1D11_norank	0.00	0.00	0.59
SPOTSOCT00m83_norank	0.00	0.00	0.05
SRB2_norank	0.73	0.00	0.00
Sh765B-TzT-29_norank	0.00	0.00	0.91
Sphaerobacter	0.00	0.00	0.56
Subgroup_6_norank	0.00	0.00	0.61
Symbiobacterium	0.00	0.00	0.39
Thauera	0.84	0.65	0.00
Thermomonas	0.00	0.29	0.00
Thermovirga	0.33	0.82	0.00
TK10_norank	0.00	0.00	0.50
TM6_norank	0.00	0.00	0.37
Thioalkalivibrio	0.00	0.00	1.37
Thiobacillus	0.00	0.47	1.55
Unclassified	18.50	15.59	6.87
Urania-1B-19_marine_sediment_group	0.00	0.00	1.50
env.OPS_17_norank	0.00	0.00	0.28
WCHB1–60_norank	0.40	0.00	0.00
uncultured	24.26	18.84	7.18
uncultured_norank	1.32	1.41	5.13
vadinBC27_wastewater-sludge_group	0.68	0.28	0.00
vadinHA17_norank	0.00	0.35	0.00

Table 5
The abundance of class (bacterial count > 200) in the three samples, arranged according to the alphabetic order

Class	Abundance		
	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)
Acidimicrobiia	0.09	0.55	1.16
Acidobacteria	0.01	0.04	4.94
Actinobacteria	6.13	6.74	8.61
Alphaproteobacteria	25.27	26.61	10.10
Anaerolineae	7.44	1.99	0.25
Armatimonadetes_norank	0.27	0.20	1.42
Bacteroidia	2.31	1.15	0.02
Betaproteobacteria	4.99	21.05	3.22
Caldilineae	1.67	1.42	0.55
Candidate_division_BRC1_norank	0.28	0.27	0.94
Candidate_division_TM7_norank	1.51	0.81	0.02
Candidate_division_WS3_norank	0.06	2.60	0.01
Chlamydiae	0.00	0.01	0.78
Clostridia	18.48	8.02	1.25
Coriobacteriia	0.11	0.65	0.15
Deltaproteobacteria	1.98	1.33	2.78
Erysipelotrichia	1.37	0.70	0.06
Gammaproteobacteria	4.65	4.28	17.05
Gemmatimonadetes	0.00	0.01	1.62
Holophagae	0.03	0.60	0.26
JG30-KF-CM66	0.00	0.01	0.53
Nitriliruptoria	0.00	0.06	0.96
Nitrospira	0.00	0.01	4.16
Phycisphaerae	0.12	0.59	16.92
Planctomycetacia	11.91	8.09	15.56
SHA-109	1.45	0.55	0.02
SM1D11	0.00	0.00	0.59
Spirochaetes	0.56	0.63	0.02
Synergistia	1.98	5.21	0.03
TK10	0.03	0.03	0.50
Thermoleophilia	0.66	0.81	0.72
Thermomicrobia	0.95	0.98	1.14
Unclassified	2.42	1.53	0.73

4. Conclusion

The BESI® process is effective for petrochemical ROC treatment. High-throughput 454-pyrosequncing provides sufficient sequencing for the analysis of the microbial community. The SRB existed in anaerobic reactor, and they participated in organic removal; the sulfide generated from sulfate-reducing bacteria also contributed to denitrification. The genera, which contributed to the nitrogen transformation, were also detected. Thus, the integrated process realized the function of denitrification in anoxic reactor. The analysis of microbial community is helpful to understand the mechanisms of organics degradation and nitrogen reduction in the integrated system.

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