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Denitrification with glucose as an external carbon source and investigation of microbial communities in a sequencing batch reactor treating reverse osmosis concentrate produced by a coking wastewater treatment plant

Enchao Li[a,b], Shuguang Lu[a,*]

[a]*State Environmental Protection Key Laboratory of Environmental Risk Assessment and Control on Chemical Process, East China University of Science and Technology, Shanghai 200237, China, Tel. +86 21 64250709; Fax: +86 21 64252737; email: lvshuguang@ecust.edu.cn (S. Lu)*
[b]*Central Research Institute (R&D Center), Baoshan Iron & Steel Co., Ltd., Shanghai 201999, China, Tel. +86 21 26641820; Fax: +86 21 26646063; email: lienchao@baosteel.com*

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ABSTRACT

In this study, a biological denitrifying process with glucose as an external carbon source was employed for the treatment of reverse osmosis (RO) concentrate with a conductivity of 17,539 ± 851 μs/cm generated from coking wastewater using a sequencing batch reactor (SBR). The average chemical oxygen demand and nitrate removal efficiencies during 60 d of stable SBR operation were 79.2% and 92.8%, respectively. Different microbial communities were identified by sequencing the V1–V3 region of the 16S rRNA gene on the MiSeq platform. The most abundant bacterial phyla in the SBR were *Proteobacteria* and *Bacteroidetes*, which could be responsible for biological denitrification of the RO concentrate. The core genera that played an important role in nitrate reduction were *Thauera, Hyphomicrobium, Flavobacterium,* and *Methyloversatilis,* accounting for 5.4%–8.0%, 2.0%–8.6%, 1.2%–1.6%, and 0.8%–3.4%, respectively, throughout the stable operational period. The quantitative real-time PCR was used to quantify the absolute abundances of the denitrifying genes *narG*, *nirS*, *nirK*, and *nosZ* during the entire operational period. The abundances of *narG*, *nirK*, and *nosZ* were lower during stable operation than start-up. Among these genes, *nirS* played relatively more important role than *nirK* in the reduction of nitrite to nitric oxide.

Keywords: Coking wastewater; RO concentrate; Denitrification; Microbial community; SBR

1. Introduction

Wastewater containing high concentration of nitrate can have adverse consequences on the environment, for example, toxicity to aquatic life, oxygen depletion, and eutrophication in the receiving water bodies [1]. Since nitrate is easily transported to groundwater and surface waters, nitrate contamination of water resources is a significant environmental concern worldwide [2]. Therefore, many countries have legislated stringent effluent standards and requirements for nitrate-rich wastewater discharge.

Coking wastewater [3] is a complex industrial wastewater comprising pollutants such as ammonia, nitrate, nitrogen heterocyclic compounds, and inorganic salts. In China, the reverse osmosis (RO) process has been successfully used for the treatment of secondary effluents derived from coking wastewater to reduce the amount of wastewater discharged [4]. However, this process can generate highly polluted RO concentrate, and the high concentrations of nitrate and salinity are major characteristics of the RO concentrate produced during coking wastewater treatment.

Biological denitrification is considered as the most appropriate technology for the treatment of nitrate-containing wastewater. Biological denitrification is a respiratory

* Corresponding author.

process, in which heterotrophic bacteria consume organic substances as carbon sources and nitrate as the terminal electron acceptor [5]. Particularly, denitrification is an anoxic process, in which the nitrate is reduced to nitrite and subsequently to nitrogen gas by heterotrophic denitrifying bacteria in accordance with the following sequence: $NO^-_{3(aq)} \rightarrow NO^-_{2(aq)} \rightarrow NO_{(g)} \rightarrow N_2O_{(g)} \rightarrow N_{2(g)}$. Although denitrification is widely employed in the removal of nitrogen from wastewater, it is still not common in the treatment of saline nitrate-rich wastewater such as RO concentrate from coking wastewater. A major obstacle in applying biological denitrification is its high salinity in the RO concentrate, since high salt concentration leads to plasmolysis and significant reduction of cell activity in microorganisms [6].

In addition, the RO concentrate from coking wastewater contains mainly organic substances which are difficult to degrade and unable to serve as the carbon source for the denitrification process. Therefore, external organic carbon such as methanol, ethanol, and glucose should be supplied for denitrification [7]. As an external carbon source for denitrification, glucose provides the advantages of: (1) being a carbonaceous organic matter with small molecule, (2) being environmentally benign, and (3) having lower operational cost [8].

The adaptation of activated sludge and the utilization of salt-tolerant organisms in biological denitrification treatments have already been confirmed the possibility [9]. However, it is still unclear about the microbial community structures and functional microorganisms of the denitrifying process and their relationship with the change in environmental conditions; therefore, it is necessary for further investigation in order to understand the complex interactions in the reactors. Advanced high-throughput sequencing (or next-generation sequencing) can generate a significant number of DNA sequences, and have been used for analysis of mixed cultures from wastewater [10]. By applying this technology, it was found that the clone library used before is less adequate in reflecting the complete profile of a complicated microbial community, even for simple lab-scale reactors. Thus, an extensive ecological study was used to identify the community structures of a coking wastewater treatment process in revealing the abundance, diversity, and distribution of corresponding species presented in coking wastewater activated sludge [11]. Additionally, application of quantitative real-time PCR (qPCR) has provided further insight into denitrifying communities during wastewater treatment because of the high taxonomic diversity in biological denitrification systems [12]. Previous studies [13] have generally targeted the gene clusters that encode key enzymes involved in the denitrification pathways such as nitrate reductase (Nar), nitrite reductase (Nir), and nitrous oxide reductase (Nos). However, to the best of our knowledge, only limited studies have examined biological denitrifying processes using glucose as an external carbon source, and the microbial community structure dynamics during the treatment of RO concentrate generated in the coking wastewater treatment process is less reported.

In this study, a lab-scale sequencing batch reactor (SBR) process was used to investigate the denitrification of RO concentrate from a coking wastewater treatment plant. The aims of this study were to: (1) evaluate the denitrifying performance of an SBR system during RO concentrate treatment, and (2) analyze the microbial community using a high-throughput sequencing technique, and assess the abundance of denitrifying genes (*narG*, *nirK*, *nirS*, and *nosZ*) by qPCR to understand the biological mechanisms involved in the RO concentrate denitrifying process.

2. Materials and methods

2.1. Coking wastewater treatment procedures

The rate of coking wastewater production in a steel plant (Baosteel Company, Shanghai, China) was 150 m^3/h. In the first phase, the two-step biological denitrification procedure, namely A_1-A_2-O_1-A_3-O_2 (anaerobic 1-anoxic 2-aerobic 1-anoxic 3-aerobic 2), was adopted, while in the second stage the advanced treatment procedure, namely membrane technology (ultrafiltration + nanofiltration + RO), was employed. During the entire procedure, 20 m^3 of RO concentrate was produced per hour with a water production rate of 65%. The desalination rate of the RO membrane was maintained above 99.3%, with the conductivity of the water produced and RO concentrate being 58–361 and 13,200–18,950 μs/cm, respectively. The RO membranes used in the procedure were TML20-400 (Toray Group, Japan). The characteristics of the RO concentrate from coking wastewater are shown in Table 1.

2.2. Sequencing batch reactor and operational conditions

The experiments were performed using a laboratory scale cylinder SBR with a working volume of 5.0 L (Fig. 1). The 24-h operational cycle of the SBR system included the following six phases: filling (5 min), anoxic reaction (6 h), aerobic reaction (12 h), settling (30 min), decanting (25 min), and idle phase (5 h). A time controller was used to automate the reactor, while a mechanical stirrer was used to constantly mix the liquid at 60 rpm during the anoxic reaction phase. In the aerobic reaction phase, aeration was provided to the base of the reactor using an air pump at a rate of 0.2 L/min. The chemical oxygen demand (COD)/NO_3–N ratio was set at 8/1 by adding

Table 1
Characteristics of RO concentrate from coking wastewater

Parameter	Range
pH	6.9–8.8
Total dissolved solids (mg/L)	7,950–9,500
Conductivity (μs/cm)	15,900–18,950
Cl^- (mg/L)	3,550–5,890
SO_4^{2-} (mg/L)	560–1,450
F^- (mg/L)	65–125
T-CN (mg/L)	0.1–2.9
Total organic carbon (mg/L)	36–85
COD (mg/L)	150–360
BOD_5 (mg/L)	15–40
Total nitrogen (mg/L)	120–230
NO_3^-–N (mg/L)	95–180
NO_2^-–N (mg/L)	0.1–1.8
NH_4^+–N (mg/L)	0.2–5

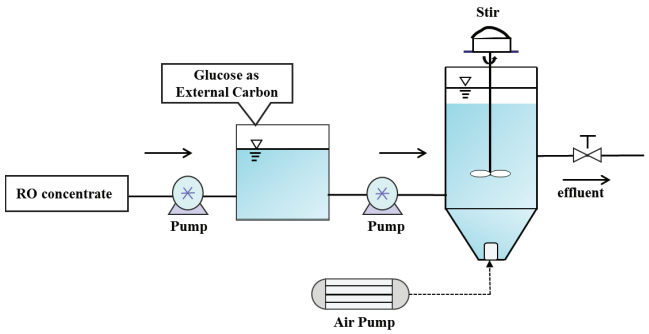

Fig. 1. Schematic diagram of sequencing batch reactor.

glucose as the external carbon source for denitrification into the RO concentrate at the beginning of the filling phase. The solution pH was maintained at 7.0 ± 0.1 at the beginning of every anoxic reaction cycle, whereas the temperature was maintained at 25°C ± 2°C during the entire operational period. The seed sludge taken from the secondary sedimentation tank in Shanghai Baosteel Coking Wastewater Treatment Plant was inoculated into the SBR at the start of the acclimatization period. This coking wastewater treatment plant has been operating over 5 years. For biomass acclimatization, the RO concentrate was mixed with coking wastewater effluent by gradually increasing the ratios to 60:40, 80:20, and 100:0. After 30 d of acclimatization, the experiments were carried out for another 60 d. During acclimation, sludge was not discharged until 31st day of operation. The sludge retention time (SRT) was maintained as 20 d by wasting 250 mL of mixed liquor in the reactor each day during the stable period. A 60-d stable operation is equal to three SRT periods. During the experiment, the mixed liquor suspended solid (MLSS) concentration in the reactor was kept at 4,360 ± 190 mg/L.

2.3. Analytical methods

The COD, nitrate, nitrite, ammonia, and MLSS were monitored according to the Standard Methods (APHA) [14]. The solution pH and conductivity were measured using a DR1900 portable spectrophotometer (Hach, USA).

2.4. DNA extraction, library preparation, and sequencing

The total community DNA was extracted from all samples using the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's instructions. PCR amplification of the 16S rRNA gene V1–V3 variable region was performed using Phanta UC Super-Fidelity DNA Polymerase for Library Amplification (Vazyme, Nanjing, China) and universal primers (27F: AGAGTTTGATCCTGGCTCAG, 534R: ATTACCGCGGCTGCTGG). The amplification program consisted of an initial denaturation heating cycle at 95°C for 2 min, 20 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 30 s, extension at 72°C for 2 min, and a final elongation cycle at 72°C for 5 min. Amplifications were separated by gel electrophoresis and purified using AxyPrep™ PCR Clean-Up Kit (Axygen Biosciences, CA, USA).

For Illumina sequencing, the DNA library was constructed using Illumina® TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The library was then sequenced on an Illumina MiSeq platform (Illumina).

2.5. Sequence pre-processing

FastQC [15] was used to assess the quality of raw reads, while the adapter sequences and low quality (<Q20) bases were trimmed using Cutadapt. The trimmed paired reads were merged into single contigs using the FLASH software. According to the designed barcode information, the reads were assigned to samples using this system.

2.6. QIIME analysis

QIIME [16] version 1.7 was used to perform operational taxonomic unit (OTU) clustering, and alpha and beta diversity analysis. Reference-based OTU clustering and de novo OTU clustering were performed with the pick_open_reference_otus method using default parameters. For reference OTU clustering and de novo OTU alignment, the 97% clustered Greengenes reference OTU NAST alignment was used. Taxonomy assignments were made using the Ribosomal Database Project (RDP) followed by retraining against the aforementioned Greengenes reference sequences. Chimera checking was performed using USEARCH with standard options implemented as in QIIME. Alpha diversity analysis was performed using the OTU, Shannon index, Goods' coverage, and Simpson index.

2.7. Quantitative real-time PCR analysis

Quantitative analysis of extracted DNA from all samples was conducted using the Qubit® dsDNA HS Assay Kit (Invitrogen, USA). Samples were diluted to 1 ng/μL, which is the same concentration as primers used for qPCR and thermal programs [17–19] (Table 2). Amplification reactions were performed in 20 μL volume, with reaction mixture containing 10 μL of SYBR Premix Ex Taq™ (Takara, Japan), 2 μM of each primer, 10 ng of total DNA, and RNase-free water. PCR products were cloned using a pUCm-T vector (Sangon Biotech, China) and transformed into *Escherichia coli* Top 10. Isolated cloned plasmids were identified by sequencing, and copy numbers were calculated based on mass concentrations and average molecular weight. Tenfold serial dilutions of plasmids of known copy numbers were used as reference DNA to measure standard curves.

3. Results and discussion

3.1. Denitrification performance of RO concentrate

The denitrification of RO concentrate produced from coking wastewater treatment plant using an SBR was performed for a total of 90 d, with 30 d of acclimatization and 60 d of stable operation. At the beginning of the acclimatization stage, the influent conductivity was 11,789 μs/cm, and then it gradually increased to 16,680 μs/cm until the 29th day. During the 60 d of stable operation, the average conductivity of the RO concentrate from coking wastewater was 17,539 ± 851 μs/cm (Fig. 2(a)).

Table 2
Primers used for qPCR thermal programs

Target gene	Sequence (5′–3′) of primer pairs	Thermal program
16S rRNA	1055F: ATGGCTGTCGTCAGCT	95°C for 30 s, followed by 45 cycles of 10 s at 95°C, 20 s at
	1392R: ACGGGCGGTGTGTAC	55°C and for 20 s at 72°C, and a cycle of 10 min at 72°C
narG	1960m2F: A(CT)GT(GC)GGGCAGGA(AG)AAACTG	95°C for 30 s, followed by 45 cycles of 10 s at 95°C, 20 s at
	2050m2R: CGTAGAAGAAGCTGGTGCTGTT	59°C and for 20 s at 72°C, and a cycle of 10 min at 72°C
nirS	nirS2F: TACCACCC(C/G)GA(A/G)CCGCGCGT	95°C for 30 s, followed by 45 cycles of 10 s at 95°C, 20 s at
	nirS3R: GCCGCCGTC(A/G)TG(A/C/G)AGGAA	60°C and for 20 s at 72°C, and a cycle of 10 min at 72°C
nosK	nirK583F: TCATGGTGCTGCCGCGKGACGG	95°C for 30 s, followed by 45 cycles of 10 s at 95°C, 20 s at
	nirK909R: GAACTTGCCGGTKGCCCAGAC	59°C and for 20 s at 72°C, and a cycle of 10 min at 72°C
nosZ	nos1527F: AGAACGACCAGCTGATCGACA	95°C for 30 s, followed by 45 cycles of 10 s at 95°C, 20 s at
	nos1527R: TCCATGGTGACGCCG TGGTTG	59°C and for 20 s at 72°C, and a cycle of 10 min at 72°C

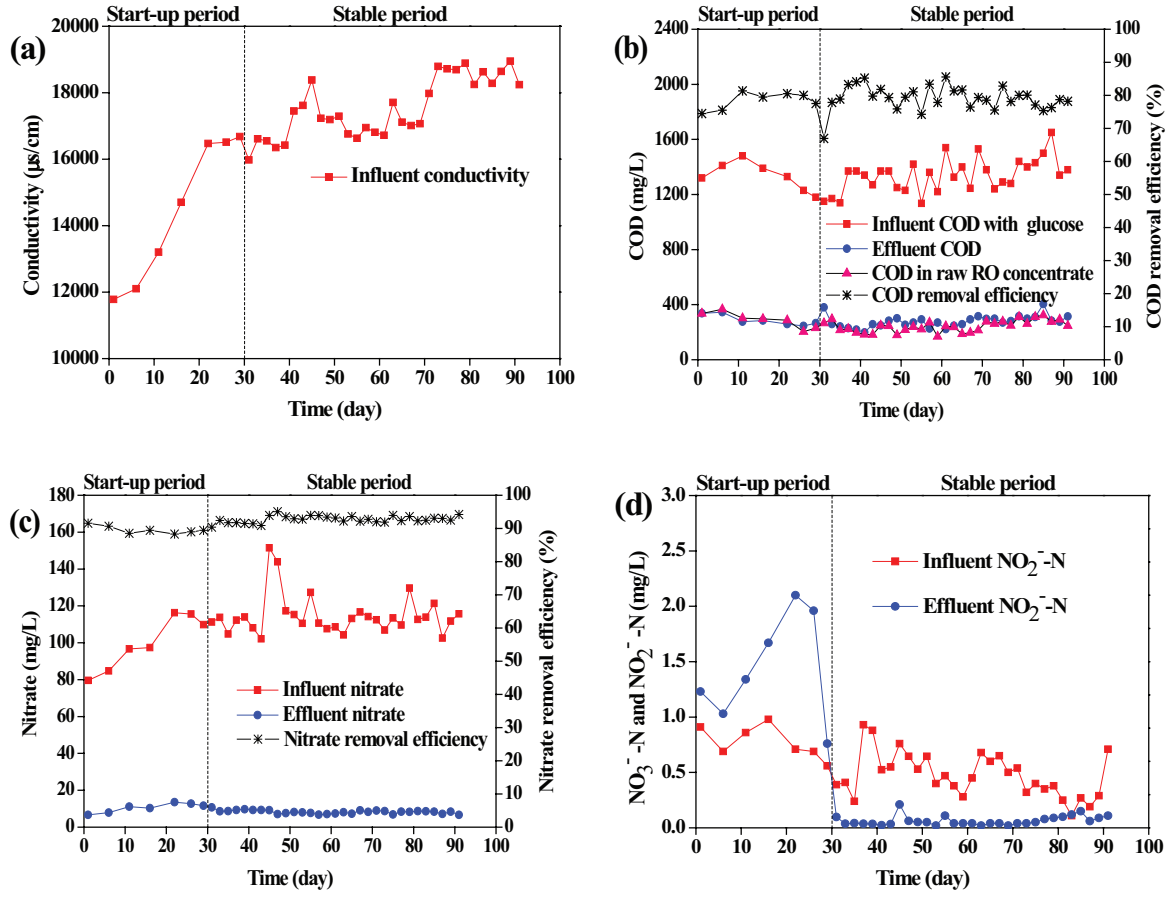

Fig. 2. Changes in (a) influent conductivity, (b) COD concentration and removal percentage; (c) nitrate concentration and removal percentage, and (d) nitrite concentration and removal percentage in the sequencing batch reactor during entire operation.

The amount of biodegradable organic carbon in the RO concentrate is generally limited [4]; thus, glucose was introduced into the RO concentrate as an external organic carbon at the beginning of the filling phase to facilitate denitrification under anoxic conditions in the SBR. The COD/NO_3–N ratio was set at 8/1 during the operation, which is higher than the stoichiometric value of 4.9 for complete denitrification (including bacterial growth). A higher COD/NO_3–N ratio was applied in order to increase the denitrifying activity and to maintain microorganism activity to eliminate surplus carbon sources. During the stable operation period, the COD in the raw RO concentrate was 168–324 mg/L, with an average of 242.6 mg/L (Fig. 2(b)). The influent COD concentration with glucose was between 1,135 and 1,650 mg/L, with an average concentration of 1,352.7 mg/L. Similarly, the effluent COD concentration varied between 198 and 406 mg/L, with an average concentration of 280.5 mg/L. The COD removal efficiency was among 67.0%–85.6%, with an average efficiency

of 79.2%, although the influent COD varied significantly. Apparently, the removed COD was mainly caused from glucose degradation rather than from refractory organic matters in RO concentrate. Sun et al. [20] observed that the removal efficiency of COD was 70% in a simulated leachate bioreactor when the COD/NO_3–N ratio was 9.08/1. Glucose, as a denitrification carbon source, would be mostly degraded by the end of the denitrification test [21], since glucose is a readily biodegradable carbon source that can be directly degraded by denitrifying microorganisms and stored as an energy source by microorganisms [22].

The changes in nitrate and nitrite concentrations in both the SBR influent and effluent throughout the entire operational period are shown in Figs. 2(c) and (d). The nitrate concentration in the influent ranged between 102.1 and 151.5 mg/L, with an average concentration of 115.5 mg/L, whereas the concentration of nitrate in the SBR effluent was between 6.7 and 10.7 mg/L, with an average concentration of 8.3 mg/L. The results showed that the high conductivity of the RO concentrate did not influence nitrate removal in the reactor. The average nitrate removal efficiency was 92.8%, and the effluent nitrate concentration was below 11 mg/L during stable operation, indicating that the heterotrophic carbon-oxidizing bacteria were not inhibited in the treatment of the real RO concentrate from the coking wastewater treatment plant. Jafari et al. [23] also reported that heterotrophic organisms performed well even at 5 g/L NaCl concentration and achieved an average nitrate removal of 92%. It is worth noting that the concentration of nitrite in the influent varied between 0.11 and 0.93 mg/L, whereas the concentration of nitrite in the SBR effluent was between 0.02 and 0.21 mg/L. When glucose was used as the external carbon source (COD/NO_3–N = 8/1), the accumulation of nitrite did not occur, which is consistent with the results previously reported in literature [21]. Significantly lower nitrite concentration in the SBR effluent indicated that heterotrophic denitrification was complete, and nitrate was converted to gaseous nitrogen in the saline RO concentrate.

The above results clearly indicate the effectiveness and efficiency of using glucose as an external carbon source in the SBR for denitrification of RO concentrate discharged from the coking wastewater treatment plant. Significant nitrate removal efficiency was achieved without the accumulation of nitrite in the SBR system, suggesting that the denitrifying microbial community quickly adapted to the complex environment of the RO concentrate after mature acclimatization using glucose as the only source of organic carbon. Consequently, a stable and active community of microflora suitable for efficient denitrification was formed without microbial metabolism inhibition caused by the high salt concentration.

3.2. Overall analysis of Illumina MiSeq sequencing

MiSeq sequencing was utilized for analysis of the bacterial 16S rRNA gene V1–V3 region across 15 samples of activated sludge taken throughout various stages during SBR operation. A total of 784,368 effective sequences and 89,942 OTUs were retrieved from the biological denitrifying SBR process, with 43,953–58,019 effective sequences identified for each sample. An RDP classifier was used to assign these sequences to different OTUs, with a nucleotide cut off of 3% (Fig. 3). Sludge samples from days 1 and 31 were collected in which day 1 represented the seed sludge (ROG-1d), while day 31 represented sludge just after acclimatization (ROG-31d). Samples were also collected throughout the stable operation period on day 51 (ROG-51d), day 71 (ROG-71d), and day 91 (ROG-91d). On average, 6,359, 6,104, 6,785, 5,201, and 5,532 OTUs were identified for SBR activated sludge samples from days 1, 31, 51, 71, and 91, respectively (Table 3). The number of OTUs involved in this SBR system was significantly higher than that obtained from other industrial wastewater treatment plants [24]. For each sample, the Shannon index scores (*H*), which is commonly used to characterize species diversity of microbial communities and accounts for both abundance and evenness of the species present, were in the range of 7.01–8.93. Good's coverage analysis found that the results represented the majority of bacterial 16S rRNA sequences presented in each subsample, with coverage values ranging from 0.84 to 0.93 in each sample. In addition, the Simpson values varied from 0.93 to 0.98, and the Chao1 values from 13,856.2 to 27,436.5. When the Simpson values and Chao1 values were larger, higher alpha diversity was acquired. Overall, the data presented in Table 3 indicate that the activated sludge samples from the SBR in this study had an abundant diversity of bacterial genera.

The phylogeny-based weighted UniFrac analysis and principal coordinate analysis (PCoA) were performed using high-quality sequences in the SBR activated sludge samples. The results of the PCoA analysis, with a maximum variation of 57.92% (PC1) and 20.31% (PC2), are shown in Fig. 4. As evident in Fig. 4, samples from the inoculated sludge (ROG-1d) and sludge after 30 d of acclimatization (ROG-31d) were different. However, samples from ROG-51d, ROG-71d, and ROG-91d during the stable operation period appear to be clustered together, indicating that they had similar bacterial communities when employing the SBR process for denitrification of RO concentrate using glucose as the external carbon source.

3.3. Diversity and composition variation in the SBR bacterial community

A phylogenetic spectrum was used to characterize the microbial community structure and composition during the SBR operation. Fig. 5(a) shows the relative

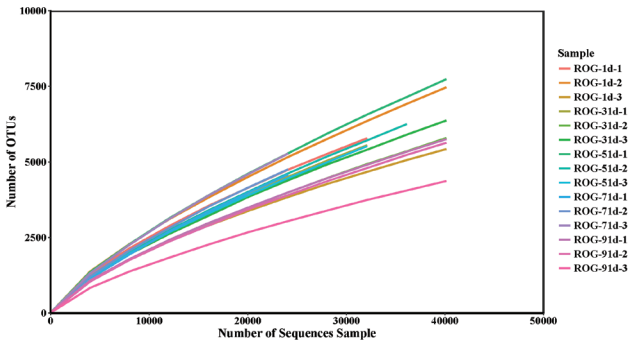

Fig. 3. Rarefaction curves at a dissimilarity level of 3% in the 16S rRNA gene sequences.

Table 3
Number of effective reads, OTU at 0.03 cut off, Shannon index, Chao1, Good's coverage, and Simpson index during entire sequencing batch reactor operation

Sample ID	Effective reads	OTU	Shannon index	Chao1	Good's coverage	Simpson index
ROG-1d-1	43,953	5,978	8.81	17,771.3	0.88	0.98
ROG-1d-2	55,616	7,590	8.93	24,057.8	0.87	0.98
ROG-1d-3	49,907	5,509	7.97	13,856.2	0.91	0.95
ROG-31d-1	44,290	5,699	8.23	19,953.5	0.87	0.96
ROG-31d-2	53,907	5,838	7.57	19,842.0	0.90	0.94
ROG-31d-3	57,848	6,774	8.09	24,054.2	0.89	0.96
ROG-51d-1	58,019	7,737	8.58	27,436.5	0.86	0.97
ROG-51d-2	56,016	6,633	7.84	22,647.1	0.88	0.95
ROG-51d-3	50,858	5,985	7.81	20,929.5	0.88	0.96
ROG-71d-1	47,382	4,716	7.33	17,069.2	0.86	0.93
ROG-71d-2	47,838	5,053	8.23	17,547.5	0.86	0.97
ROG-71d-3	52,778	5,833	8.54	21,547.8	0.84	0.97
ROG-91d-1	54,118	5,913	7.80	18,267.0	0.90	0.96
ROG-91d-2	54,590	5,844	7.68	18,793.5	0.90	0.95
ROG-91d-3	57,248	4,840	7.01	15,025.1	0.93	0.95

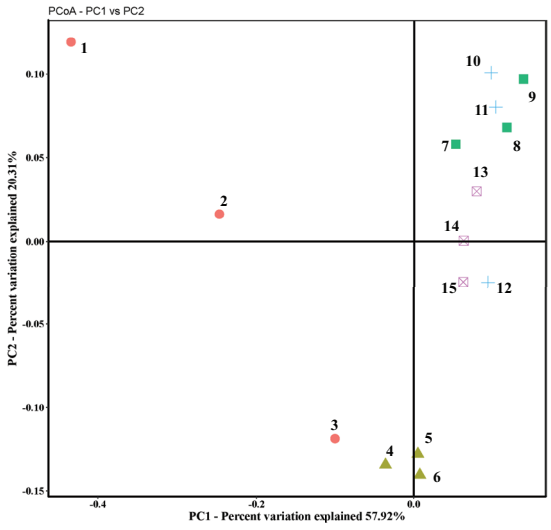

Fig. 4. Principal coordinate analysis (PCoA) based on the weighted UniFrac metric of 15 samples (1: ROG-1d-1, 2: ROG-1d-2, 3: ROG-1d-3, 4: ROG-31d-1, 5: ROG-31d-2, 6: ROG-31d-3, 7: ROG-51d-1, 8: ROG-51d-2, 9: ROG-51d-3, 10: ROG-71d-1, 11: ROG-71d-2, 12: ROG-71d-3, 13: ROG-91d-2, 14: ROG-91d-2, and 15: ROG-91d-3).

bacterial community abundance on the phylum level. The analytical results showed that the seed sludge (ROG-1d) contained a relative abundance of 65.2% *Proteobacteria*, 15.7% *Planctomycetes*, 6.7% *Acidobacteria*, and 3.9% *Bacteroidetes*. After 30 d of acclimatization (ROG-31d), the abundance of *Proteobacteria* increased gradually from 65.2% to 93.7%. The relative abundance of *Planctomycetes* was 1.8% (ROG-31d), which significantly decreased because of its inability to effectively adapt to the RO concentrate environment during the sludge acclimatization period. Similar reduction in the abundance of *Planctomycetes*, along with a salinity gradient, was also observed in a membrane bioreactor with high saline wastewater in previous studies [25]. In addition, on day 31 (ROG-31d), the abundances of *Acidobacteria* and *Bacteroidetes* decreased to 2.8% and 1.5% in the sludge, respectively, while the phyla *WPS-2*, *Fusobacteria*, and *Deferribacteres* completely disappeared. Previous studies [26] reported that most bacterial phyla such as *Acidobacteria* and *Bacteroidetes* decreased sharply with the increase of salinity in an SBR, leading to the eventual disappearance of some bacterial phyla on day 31. During the acclimatization period, the nitrate removal percentage in the RO concentrate increased gradually, and the relative abundance of the major bacteria phyla changed in response to the hypersaline environment of the RO concentrate.

During the stable operation period, *Proteobacteria* were the most prevalent phyla present in the microbial community, accounting for 74.9%, 78.4%, and 88.7% of ROG-51d, ROG-71d, and ROG-91d, respectively. *Proteobacteria* are prominent phylum in coking wastewater and other industrial wastewater treatment systems, although the degree of dominance varies considerably, ranging from 65% to 91% with relative proportions in various coking wastewater treatment plants [11]. Moreover, *Proteobacteria* are a common and predominant group in saline wastewater treatment bioreactors [27]. *Bacteroidetes* were the second most abundant phyla, contributing approximately 21.7% (ROG-51d), 18.9% (ROG-71d), and 7.4% (ROG-91d) to the microbial communities in this study. It is reported [28] that *Bacteroidetes* are one of the most abundant bacterial groups present in wastewater and marine environments, and appear to play a key role in the degradation of organic contaminants. Compared with previous studies [11], the sum of *Proteobacteria* and *Bacteroidetes* in this study accounted for a higher percentage during the stable operation period, even reaching 97.3% in ROG-71d. This may be due to the fact that these two dominant phyla are important contributors to both nitrate removal and glucose degradation in the RO concentrate from coking wastewater. *Chlorobi* are the main dominant

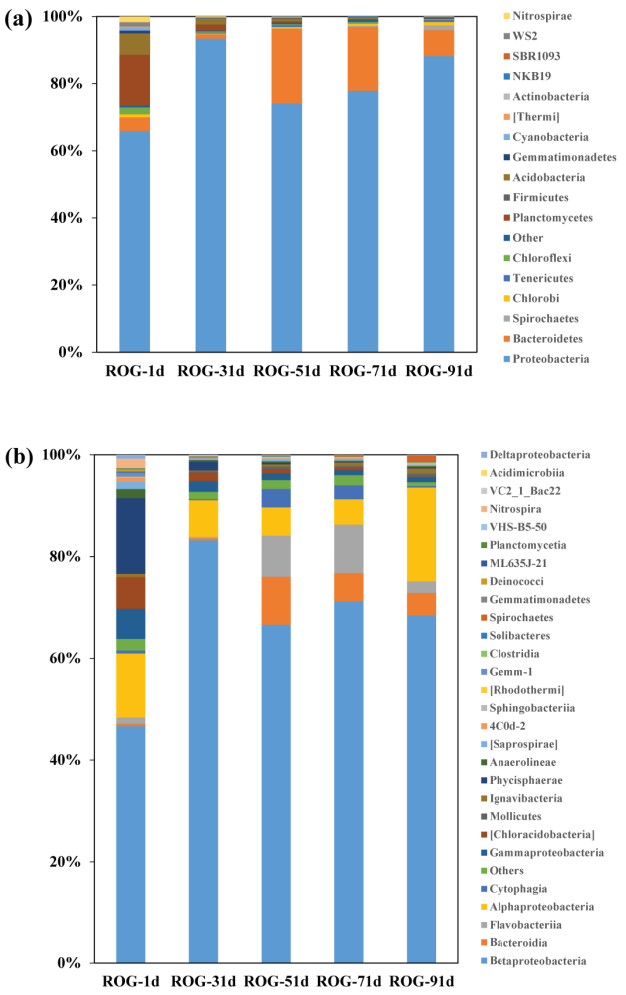

Fig. 5. Taxonomic classification of bacterial 16S rRNA genes on days 1, 31, 51, 71, and 91 (relative abundance >0.01%), showing (a) phylum level and (b) class level (the average values of the three subsamples were calculated to represent the value of the corresponding sample).

phyla in the RO concentrate, and their relative abundances in ROG-51d, ROG-71d, and ROG-91d were 0.4%, 0.6%, and 0.9%, respectively. They have been found to be widespread in wastewater treatment bioreactors [29], and play a role in the nitrogen removal process. Another dominant phylum was *Spirochaetes*, with an abundance of 0.1% (ROG-51d), 0.3% (ROG-71d), and 1.4% (ROG-91d). Delbes et al. [30] found that *Spirochaetes* activity in the bacterial community increased when glucose was added to an anaerobic digestion system, indicating a functional role of *Spirochaetes* in glucose fermentation. The abundances of *Acidobacteria* were 0.9% (ROG-51d), 0.5% (ROG-71d), and 0.1% (ROG-91d), as found in a previous study using a granular sludge bed reactor with an autotrophic nitrogen removal process [31]. Overall, the abundances of the dominant phyla exhibited a high level of tolerance to salinity, maintaining a high nitrate removal efficiency of 92.8% in the RO concentrate.

Differences in microbial community structures could also be identified at the class level (Fig. 5(b)). Within the *Proteobacteria* population, β-*Proteobacteria* was the most dominant class, accounting for approximately 46.0% (ROG-1d), 84.0% (ROG-31d), 68.0% (ROG-51d), 72.1% (ROG-71d), and 69.2% (ROG-91d). During operation, the relative abundance of α-*Proteobacteria* increased to 6.8%, 5.1%, 4.7%, and 18.2% on days 31, 51, 71, and 91, respectively. The relative abundance of γ-*Proteobacteria* was relatively stable, at 2.2% (day 31), 1.5% (day 51), 1.1% (day 71), and 0.6% (day 91). The β-*Proteobacteria* class had the highest distribution, followed by α-*Proteobacteria* and γ-*Proteobacteria*, which is consistent with a previous investigation on community structure of *Proteobacteria* in coking wastewater treatment [11]. Manz et al. [32] reported that the β-*Proteobacteria* and α-*Proteobacteria* classes were highly versatile in their pollutant degradation capacity and could be detected in various biotreatment systems. Furthermore, they found that denitrifying bacteria such as *Thauera*, *Methyloversatilis*, *Thiobacillus*, and *Rubrivivax* belong to the β-*Proteobacteria* class [33], while denitrifying bacteria such as *Hyphomicrobium* and *Novispirillum* are from the α-*Proteobacteria* class. Throughout the experiment, the relative abundance of *Flavobacteria* was lower in the sludge samples of ROG-1d (1.2%) and ROG-31d (0.3%), but significantly increased in the sludge samples of ROG-51d (7.8%) and ROG-71d (9.6%) during the stable operation period. This value decreased to 2.1% in the ROG-91d sample, indicating that *Flavobacteria* could endure the hypersaline nitrate-degrading RO concentrate environment. Zhang et al. [10] reported that the abundance of *Flavobacteria* increased with the increasing salinity in wastewater. All these changes indicate a multi-phase adaption to the hypersaline RO concentrate environment. In the present study, high nitrate removal efficiency and high COD degradation was potentially dependent on these bacterial phyla. In addition, *Phycisphaerae* and *Anaerolineae* decreased gradually until the relative abundance was below 0.3% for both bacterial groups during the stable period, suggesting a poor adjustment to the RO concentrate environment.

Thirty of the most dominant bacterial groups were identified at the genus level in the investigation of the dynamics and variation within the microbial community in RO concentrate treatment (Fig. 6). The major genus detected in sludge samples were *Thauera*, *Hyphomicrobium*, *Flavobacterium*, and *Methyloversatilis*, accounting for 5.4%–8.0%, 2.0%–8.6%, 1.2%–1.6%, and 0.8%–3.4%, respectively, throughout the stable operational period. The genus *Thauera* is a Gram-negative bacteria in the family *Rhodocyclaceae* of class β-*Proteobacteria*, widely known as an important nitrate-reducing bacteria in wastewater treatment systems [34]. They are capable of biodegrading aromatic, phenolic, and other heterocyclic organic pollutants in coking wastewater, and can use organic matter such as glucose as electron donors in the denitrifying reaction [35]. *Hyphomicrobium*, *Flavobacterium*, and *Methyloversatilis*, detected in this system, are important genera that have denitrifying abilities under anoxic conditions in industrial wastewater [36]. Consistent with the findings reported in previous studies [33], using both culture-dependent and culture-independent methods, populations related to *Thauera*, *Hyphomicrobium*, *Flavobacterium*, and *Methyloversatilis* have been identified in various methanol- and acetate-feeding denitrification systems. The same genera are also identified in denitrification systems of RO concentrate using glucose as a carbon source. Additionally, *Rubrivivax*, *Hydrogenophaga*,

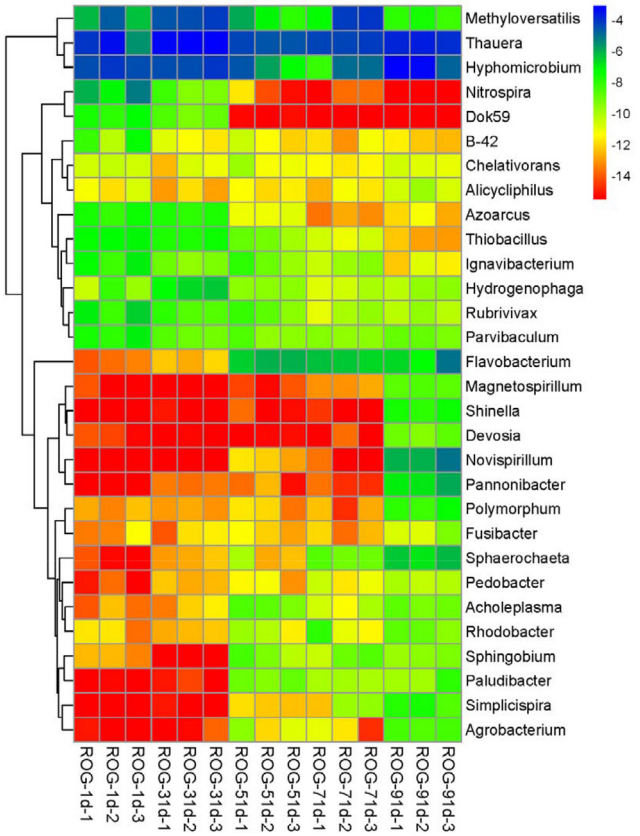

Fig. 6. Heatmap of relative abundances of the 30 most abundant bacterial genera (values are calculated as $\log_{10}$) during the entire sequencing batch reactor operation.

Sphaerochaeta, *Novispirillum*, and *Pannonibacter* were found to be dominant in the SBR system, and they provide important contributions to both denitrification and COD removal.

3.4. Quantitative abundances of denitrifying genes in SBR

The 16S rRNA, *narG*, *nirS*, *nirK*, and *nosZ* gene copy numbers were detected during the entire SBR operation period. In general, the enzymes nitrate reductase, nitrite reductase, and nitrous oxide reductase are considered as the main indicators used for denitrification processes in wastewater treatment.

According to Fig. 7, the quantity of detected 16S rRNA shows an initial increase, followed by a more constant quantity overall, with the copy numbers of 16S rRNA being 8.15×10^4 copies/ng on ROG-1d, 1.85×10^6 copies/ng on ROG-31d, 6.66×10^5 copies/ng on ROG-51d, 7.90×10^5 copies/ng on ROG-71d, and 9.89×10^5 copies/ng on ROG-91d. ROG-1d had the least quantity, while ROG-31d, ROG-51d, ROG-71d, and ROG-91d were similar in magnitude, indicating that hypersaline RO concentrate with added glucose promoted steady concentrations of the dominant bacterial communities after acclimatization.

NarG, which is a membrane-bound nitrate reductase enzyme, catalyzes the reduction of nitrate into nitrite during the SBR denitrification process, with *narG* concentration being the highest initially at ROG-31d at 6.85×10^3 copies/ng.

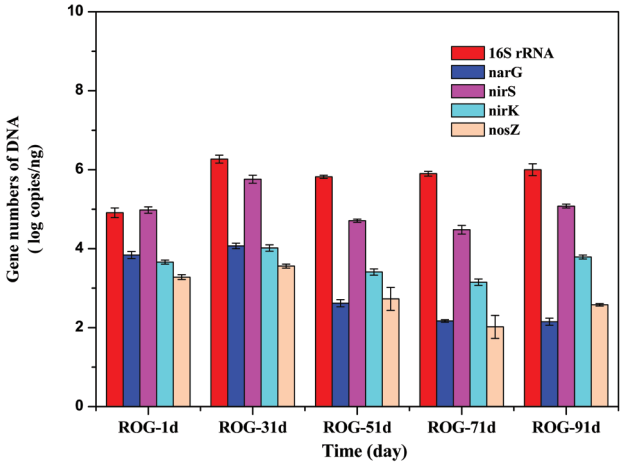

Fig. 7. Quantitative abundances of 16S rRNA, *narG*, *nirK*, *nirS*, and *nosZ* in SBR systems on days 1, 31, 51, 71, and 91 (the average values of the three subsamples were calculated to represent the value of the corresponding sample).

Abundance gradually decreased and became stable during the later period of stable operation, with 4.16×10^2 copies/ng at ROG-51d, 1.48×10^2 copies/ng at ROG-71d, and 1.40×10^2 copies/ng at ROG-91d.

NirS and *nirK* are crucial enzymes for the transformation of nitrates into nitrogen [37]. During the entire operation process, the quantity of *nirS* showed an initial increase, followed by generally stable abundances, with 9.52×10^4 copies/ng at ROG-1d, 5.81×10^5 copies/ng at ROG-31d, 5.10×10^4 copies/ng at ROG-51d, 3.02×10^4 copies/ng at ROG-71d, and 1.21×10^5 copies/ng at ROG-91d. This suggests that ROG promoted *nirS*-containing microbial growth. The abundance of *nirK* showed a similar trend, with the quantity at ROG-31d being the highest at 1.05×10^4 copies/ng, declining to overall magnitudes of 2.55×10^3 copies/ng at ROG-51d, 1.40×10^3 copies/ng at ROG-71d, and 6.21×10^3 copies/ng at ROG-91d. During the stable operation period, the quantity of *nirS* was higher than that of *nirK* by 10^1–10^2 orders of magnitude, indicating that the denitrifying bacterial genera containing *nirS* played an important role in the denitrification process of ROG. In addition, previous studies have also reported that the quantity of *nirS* was higher than that of *nirK* by 10^2–10^4 orders of magnitude in denitrification processes [38].

NosZ reduces N_2O into N_2, and completes the final step of denitrification. The trend in *nosZ* quantities is consistent with that of *nirS* and *nirK*, with 1.91×10^3 copies/ng at ROG-1d, 3.60×10^3 copies/ng at ROG-31d, 5.34×10^2 copies/ng at ROG-51d, 1.04×10^2 copies/ng at ROG-71d, and 3.38×10^2 copies/ng at ROG-91d.

The changing trends of *narG*, *nirS*, *nirK*, and *nosZ* show that ROG had an inhibitory effect on microbes containing these functional genes and that the gene quantity stabilized following acclimatization to conditions. The changes in quantity of the four functional genes strongly indicate that microbes may adapt to the hypersaline environment after acclimatization under complex environmental conditions. These changes indicate a multi-phase adaption to the hypersaline environments of ROG.

4. Conclusions

This study demonstrated that biological denitrification using glucose as an external carbon source in an SBR could stabilize the high saline RO concentrate produced from a coking wastewater treatment plant. The average COD removal efficiency of the system was 79.2%, while the average nitrate removal efficiency was 92.8% during stable operation. High-throughput sequencing was applied to investigate the denitrifying microbial communities in the SBR. *Proteobacteria* and *Bacteroidetes* were found to be the dominant phyla. The key microorganisms responsible for denitrification were from the genera *Thauera, Hyphomicrobium, Flavobacterium,* and *Methyloversatilis*. Furthermore, real-time PCR was used to validate the absolute abundance of 16S rRNAs and denitrifying genes, including *narG*, *nirS*, and *nirK* during the entire operational period. This study provides new insights to the understanding of the denitrifying processes of RO concentrate from coking wastewater.

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