Microbial communities in biological denitrification system using methanol as carbon source for treatment of reverse osmosis concentrate from coking wastewater

Enchao Li, Xuewen Jin and Shuguang Lu

ABSTRACT

A biological denitrifying process using methanol as a carbon source was employed for the treatment of reverse osmosis concentrate (ROC) from coking wastewater in a sequencing batch reactor (SBR). The results showed that the average removal efficiencies of chemical oxygen demand (COD), total organic carbon, total nitrogen and nitrate were 81.4%, 83.7%, 90.6% and 92.9%, respectively. Different microbial communities were identified on the MiSeq platform, showing that the most abundant bacterial phyla were *Proteobacteria* and *Bacteroidetes*, the sum of which, in this study, accounted for almost over 92%. The key genera responsible for denitrification were *Hyphomicrobium, Thauera* and *Methyloversatilis*. Quantitative real-time polymerase chain reaction was used to quantify the absolute abundances of microbial genera by using 16S rRNAs and denitrifying genes, such as *narG, nirS* and *nirK*, during both start-up and stable operations in the SBR. *nirS* was much more abundant than *nirK*, thus became the main functional gene to execute nitrite reduction. The high removal efficiency of COD and nitrate suggests that a biological denitrifying process using SBR is an effective technique for treating ROC from coking wastewater.

Key words | coking wastewater, denitrification, microbial community, reverse osmosis concentrate

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INTRODUCTION

Coking wastewater is a complex industrial wastewater generated from coal coking plants (Li *et al.* 2010), which contains high concentrations of inorganic and organic pollutants, such as ammonia, nitrate, nitrogenous heterocyclic compounds, polycyclic aromatic hydrocarbons (PAHs) and other toxic compounds (Zhu *et al.* 2009). Traditionally, biological treatment is the main technology for coking wastewater treatment due to its low cost and maximal mineralization of pollutants. However, the effluents of common biological treatments, including anoxic–oxic (A/O) or anaerobic–anoxic–oxic (A/A/O) and sequencing batch reactors (SBRs), contain substantial amounts of chemical oxygen demand (COD), nitrate and total nitrogen (TN), resulting in failure to meet current effluent discharge standards.

In order to carry out further advanced wastewater treatment and reduce wastewater discharge, the reverse osmosis (RO) process has been used successfully for the treatment of effluents after biological treatment (Jin *et al.* 2013). However, the process can generate highly polluted reverse osmosis concentrate (ROC) at the same time. High concentrations of dissolved salts, organic contaminants and nitrate are the major characteristics of ROC produced during advanced coking wastewater treatment. Nitrate and nitrogen compounds from ROC that are discharged into the environment can cause serious problems, such as

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deterioration in the quality of water resources and eutrophication of rivers and lakes. Thus, the discharge of ROC must meet the requirements of the National Discharge Standard (GB16171-2012) in China, in which TN should be below 20 mg/L. Therefore, the removal of nitrate from ROC is important.

In comparison to physicochemical treatment methods, biological denitrification, in which the nitrate is reduced to harmless N_2 gas under anoxic conditions, is a potential approach to treating nitrate-contaminated ROC from coking wastewater (Nancharaiah & Venugopalan 2011). Biological denitrification is conducted by denitrifying microbes that use nitrate as a terminal electron acceptor, and organic and inorganic substances as electron donors, as well as energy sources for sustaining microbial growth.

In addition, the extent and overall rate of the denitrification process largely depend on the biodegradability of the influent wastewater or external carbon sources. In some industrial wastewater treatment plants where wastewater is treated with limited degradable organic matter, external carbon sources, such as glucose and acetate, are added into an anoxic reactor to facilitate denitrification. Methanol is the most commonly employed external carbon source and has the advantages of: (1) being easily assimilated by denitrifying bacteria, (2) having the potential for complete denitrification without nitrite accumulation, (3) having high nitrate removal efficiency, (4) possessing low operational costs and (5) being broadly available in various industrial wastewaters. A previous study has also reported a methanol-feeding denitrification system of coking wastewater, in which nitrate removal efficiency achieved 99% (Vazquez *et al.* 2006).

High-throughput sequencing technology has been successfully used to investigate the dynamics of microbial communities in both domestic and industrial wastewater treatment processes (Fernandes *et al.* 2013). Since these populations are associated with efficient pollutant removal, understanding community structures in coking wastewater is important. Some studies (Ma *et al.* 2015) have focused on revealing the abundance, diversity and distribution in full-scale coking wastewater treatment systems corresponding to the degradation of phenols, COD, TN, PAH and CN^-. Additionally, applications of quantitative real-time polymerase chain reaction (qPCR) have given further insight into features of denitrifying communities, such as nitrate reductase (Nar) and nitrite reductase (Nir) used for wastewater treatment in biological denitrification systems (Heylen *et al.* 2006). However, few studies have investigated further correlation between denitrification process and the microbial communities in ROC from coking wastewater, which is always regarded as a 'black box' model.

In this study, a laboratory-scale SBR was used to investigate the denitrification with methanol as an external carbon source of ROC for 90 days. The removal of COD, total organic carbon (TOC), TN and nitrate was evaluated for each stage. Concomitantly, microbial community compositions and abundances in sludge samples were analyzed using high-throughput sequencing. The study also quantified the absolute abundance of functional genes involved in nitrate removal and investigated the ecological associations among these functional genes in SBR. This study highlights the feasibility of the denitrifying process by SBR and provides insights into the diverse microbial groups that have the potential to degrade nitrate in ROC from coking wastewater.

MATERIALS AND METHODOLOGY

Status of coking wastewater treatment procedures

The rate of coking wastewater production in a steel plant (Baosteel Company, Shanghai, China) was 150 m^3/h. The first phase used 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), and in the second stage, the advanced treatment procedure, namely membrane technology (ultrafiltration + nanofiltration + RO), was used. During the entire procedure, 20 m^3 of ROC was produced per hour with a water production rate of 65%. The RO membranes used in the procedure were TML20-400, supplied by Toray Group (Japan). The characteristics of ROC are shown in Table 1.

SBR and operational conditions

The experiments were performed using a laboratory scale cylinder SBR with a working volume of 5.0 L. The 24-h operational cycle of the SBR system included the following five

Table 1 | Characteristics of ROC from coking wastewater

Parameter	Range	Parameter	Range
pH	7.6–8.8	COD (mg/L)	168–324
Conductivity (μs/cm)	15,980–18,950	BOD_5 (mg/L)	19–26
Cl^- (mg/L)	2,800–5,200	BOD_5/COD	0.06–0.15
SO_4^{2-} (mg/L)	500–1,500	TN (mg/L)	138–203
F^- (mg/L)	60–105	NO_3^--N (mg/L)	102–152
Turbidity (NTU)	0.9–2.1	NO_2^--N (mg/L)	0.1–1
TOC (mg/L)	43–69	NH_4^+-N (mg/L)	0.8–3.1

phases: filling (5 min), anoxic reaction (6 h), aerobic reaction (12 h), settling (30 min), decanting (25 min) and idle phase (5 h). The COD:NO_3-N ratio was set at 8:1 by adding methanol as the external carbon source for denitrification into ROC 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 and the temperature was maintained at 25 ± 2 °C during the entire operational period. Inoculated sludge was obtained from the anoxic stage of coking wastewater. As the coking wastewater treatment plant (Shanghai, China) has been operating over 5 years, the inoculated sludge has better performance for nitrate removal. For biomass acclimatization, ROC was mixed with coking wastewater effluent at the sequencing change of the ratios from 60:40 to 80:20 and 100:0. After 30 days of acclimatization, the experiments were carried out for another 60 days. During the experiment, the mixed liquor suspended solids (MLSS) concentration in the reactor was kept at $3{,}220 \pm 240$ mg/L. The sludge retention time (SRT) was 20 days. Three SRT cycles were conducted in total operation.

Analytical methods

COD, TOC, nitrate, nitrite, ammonia, TN and MLSS were monitored according to *Standard Methods* (APHA 2005). The solution pH and conductivity were measured using a DR1900 portable spectrophotometer (HACH, USA).

DNA extraction, library preparation and sequencing

Total community DNA was extracted from all samples using the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. PCR amplification of the 16S rRNA gene V1–V3 variable region was performed using a 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 denaturation at 95 °C for 20 sec, annealing at 58 °C for 30 sec, 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 an 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, San Diego, CA, USA).

Sequence pre-processing

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

QIIME analysis

QIIME version 1.7 was used to perform operational taxonomic unit (OTU) clustering and alpha and beta diversity

analyses. Reference-based OTU clustering and de novo OTU clustering were done 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) following retraining against the aforementioned Greengenes reference sequences and chimera checking was performed using USEARCH with standard options implemented as in QIIME against the Greengenes reference alignment. Alpha diversity analysis was performed using the OTU, Shannon index, Goods' coverage and Simpson index.

qPCR analysis

Quantitative analysis of extracted DNA from all samples was determined using the Qubit® dsDNA HS Assay Kit (Invitrogen, USA). Subsequently, according to the sample concentration, samples were diluted to the same concentration of 1.0 ng/μl. Primers used for qPCR and thermal programs are shown in Table 2. Amplification reactions were performed in 20 μl volumes, with the reaction mixture containing 10 μl of SYBR Premix Ex Taq TM (Takara, Japan), 2.0 μM of each primer, 10 ng of total DNA and RNase-free water. PCR products from strains were cloned with the pUCm-T vector (Sangon Biotech, China) and transformed into *Escherichia coli* Top10. Isolated cloned plasmids were identified by sequencing and copy numbers were calculated based on mass concentration and average molecular weight. Ten-fold serial dilutions of the plasmids of known copy numbers were used as standard DNA to measure standard curves.

RESULTS AND DISCUSSION

Changes in COD and TOC during denitrification of ROC using methanol as a carbon source

In the advanced treatment process of coking wastewater, the conductivity of the RO feed was 58–361 μs/cm, which completely achieved the water quality standards for recycled cooling water. As the desalinization ratio of the RO membrane was maintained at 99.3%, the conductivity of ROC reached 17,539 ± 885 μs/cm. ROC from coking wastewater is a typical industrial wastewater with high conductivity, TN and nitrate (Table 1). Thus, it is essential to employ a biological means to remove nitrogen by a denitrification process.

The BOD_5/COD in raw ROC from coking wastewater was 0.06–0.15, with poor biodegradability, thus, it cannot be used as the internal carbon source of denitrification. Consequently, methanol is added before the SBR anoxic stirring reaction process, playing the role of a carbon source for microbes during denitrification. In the environment of ROC with high conductivity, enough methanol is added to achieve C:N (carbon:nitrogen) value of 8:1, hence ensuring

Table 2 | Primers used for qPCR and thermal programs in this study

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

the growth, reproductive and metabolic functions of the microbes are stable. Ge *et al.* (2012) reported that when the C:N ratio was below 6:1 in SBR using methanol as external carbon source, nitrite accumulation took place; when C/N ratio was equal or greater than 8, the nitrite concentration in the effluent was less than 2 mg/L, achieving complete conversion of nitrate to molecular nitrogen.

The biological denitrification experiment in the SBR with methanol as the carbon source continued for 90 days. The first 30 days were the acclimatization stage, and for the next 60 days, the reactor operated stably, whilst the sludge was discharged on time. Figure 1 illustrates the change of COD and TOC in the SBR influent and effluent of ROC from coking wastewater in discrete stages. During the 60 days of stable operation, the value of COD in raw ROC was 168–324 mg/L, with an average concentration of 242.6 mg/L. After the carbon source was added, the COD concentration of the SBR influent was 1,320–1,720 mg/L with the average concentration of 1,529.1 mg/L. The COD concentration of the SBR effluent was 219–366 mg/L with the average value of 284.8 mg/L. Therefore, the average COD removal rate was 81.4%. Apparently, the use of this external carbon source (methanol) seems to be nearly complete, with residual COD in effluent being mostly refractory organics. In addition, during the 60 days of stable operation, the value of TOC in raw ROC was 43–69 mg/L. The TOC concentration (including carbon source methanol) in the SBR influent was 269–388 mg/L with the average concentration of 331.7 mg/L. TOC concentration in the SBR effluent was 35–74 mg/L with the average value of 54.1 mg/L. Therefore, the average TOC removal rate was 83.7%, which was slightly higher than that of COD. The above results indicate that after acclimation, the microorganisms quickly adapted to the complex environment of the ROC, hence COD and TOC could be effectively degraded in the SBR process.

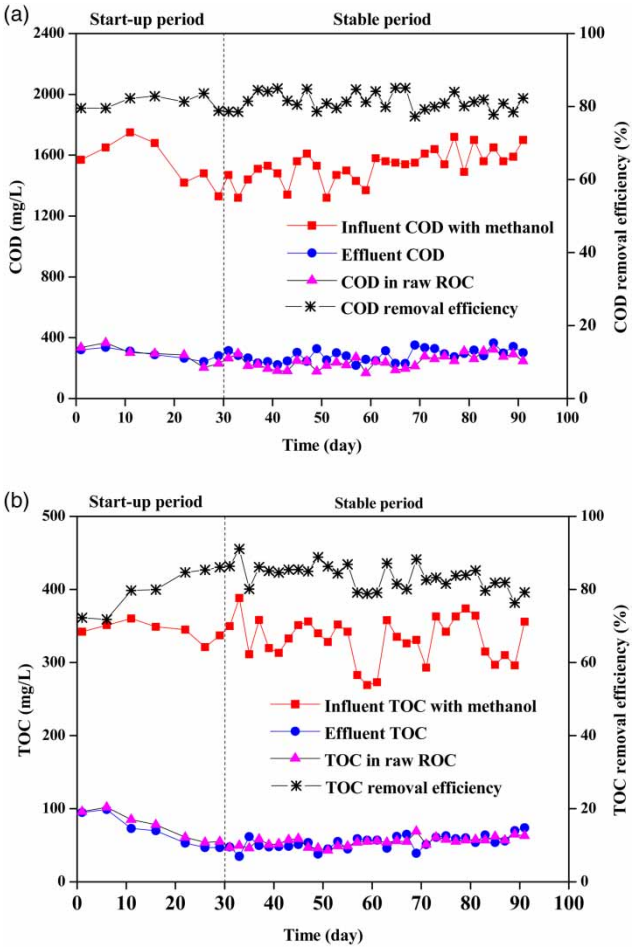

Figure 1 | Performance of (a) COD and (b) TOC in the SBR during the entire operation.

Changes in the nitrogen element during denitrification of ROC using methanol as a carbon source

During the stable operation stages, four nitrogen forms (TN, nitrate, nitrite and ammonium) were evaluated to characterize the SBR influent and effluent of the ROC (Figure 2).

During the 60 days of stable operation, the TN concentration in the SBR influent of the ROC was 138–203 mg/L with the average concentration of 162.4 mg/L. TN concentration in the SBR effluent was 11–19 mg/L with the average value of 15.3 mg/L. Thus, the average TN removal rate was 90.6%. The high ratio C:N is required to improve denitrification efficiency because a portion of nitrogen will be utilized for microbial growth and incorporated into microbial cells. The effluent TN met the requirements of the National Discharge Standard (GB16171-2012) in China (TN <20 mg/L). Nitrate concentration in the SBR influent was 102.1–151.5 mg/L with the average concentration of 115.5 mg/L. Nitrate concentration in the SBR effluent was 5.7–10.4 mg/L with the average value of 8.3 mg/L. Therefore, the average nitrate removal rate was 92.9%. In addition, nitrite concentration in the SBR influent of the ROC

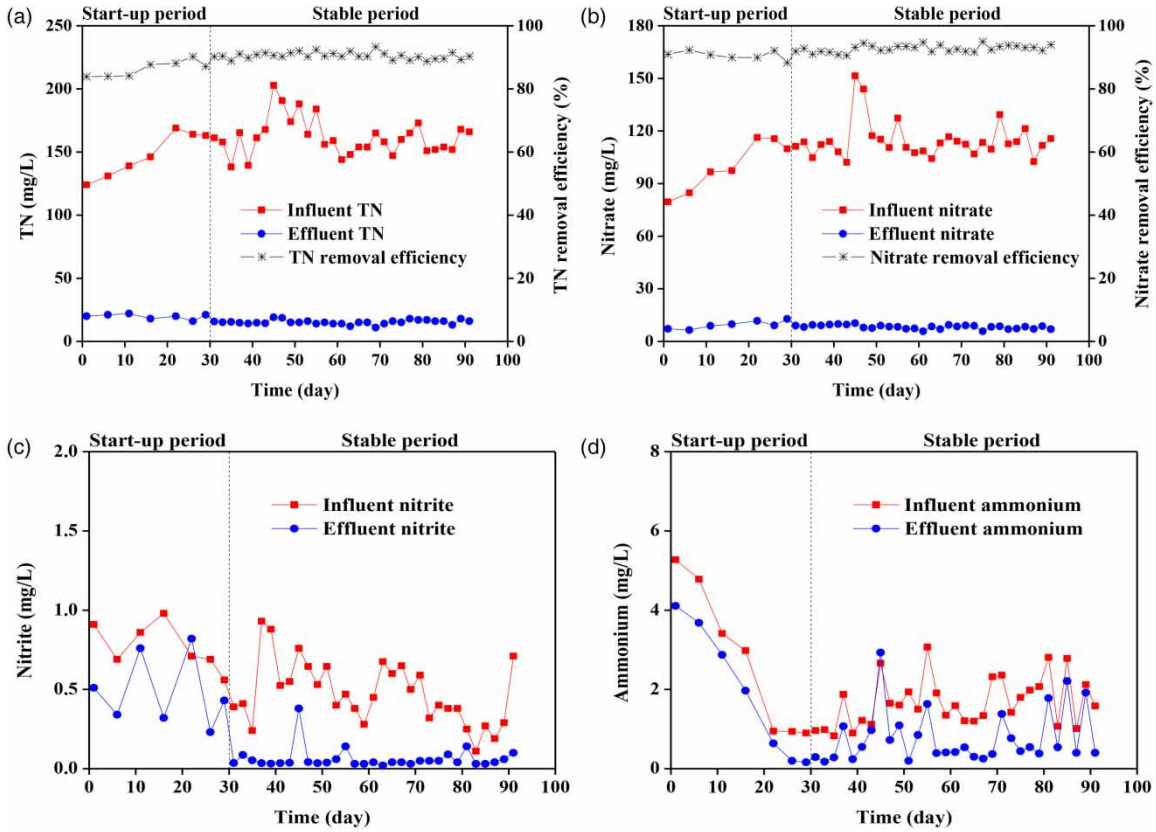

Figure 2 | Performance of (a) TN, (b) nitrate, (c) nitrite (TN) and (d) ammonium in the SBR during the entire operation.

was 0.11–0.93 mg/L with the average concentration of 0.48 mg/L. Nitrite concentration in the SBR effluent was 0.02–0.38 mg/L with the average value of 0.06 mg/L. The phenomenon of nitrite accumulation was not observed during the operation. It has been reported that when methanol is used as a carbon source and the C:N value is higher than 4, nitrite accumulation does not occur (Sun *et al.* 2009). Moreover, the ammonium concentration in both the SBR influent and effluent of the ROC was very low.

The above experimental results indicate that in the ROC from coking wastewater, when using methanol as a carbon source and C:N = 8, COD and TOC were effectively degraded in the denitrification process. The removal rates of TN and nitrate were kept stable, and the average values were 90.6% and 92.9%, respectively. Meanwhile, the nitrite accumulation phenomenon was not observed in the SBR reactor, implying that in an environment of ROC from coking wastewater with high conductivity, the denitrification process could be operated stably without the occurrence of inhibition.

Overall analysis of Illumina Miseq sequencing

The Miseq sequencing was used for the analysis of the bacterial 16S rRNA gene across the five samples of the activated sludge taken throughout various stages during the SBR operation. As shown in Table 3, a total of 1,444,364 effective sequences and 34,772 OTUs were retrieved from the biological denitrifying process, with 51,049–60,199 effective sequences identified for each sample. An RDP Classifier was used to assign these sequences into the different OTUs, with a nucleotide cutoff of 3%. For the SBR activated sludge samples taken on day 1 (ROC-1d), day 31 (ROC-31d), day 51 (ROC-51d), day 71 (ROC-71d) and day 91 (ROC-91d), 7,590, 6,702, 7,532, 7,355 and 5,593 OTUs, respectively, were identified. Moreover, the number of sequences and

Table 3 | Number of effective reads, OUT at 0.03 cutoff, Shannon index, Chao1, Good's coverage, and Simpson index during the entire SBR operation

Sample	Effective Sequence	OTU	Shannon Index	Chao1	Goods' coverage	Simpson Index
ROC-1d	55,616	7,590	8.93	24057.8	0.87	0.98
ROC-31d	58,061	6,702	8.37	20470.8	0.89	0.98
ROC-51d	51,049	7,532	9.11	26419.0	0.85	0.98
ROC-71d	56,249	7,355	9.42	24622.9	0.82	0.98
ROC-91d	60,199	5,593	6.73	16120.0	0.93	0.89

OTUs was much higher than that obtained from previous studies on other types of industrial wastewater (Fernandes *et al.* 2013). The statistical analysis and rarefaction curves based on the OTUs clustered from these MiSeq sequencing reads are shown in Figure 3. The coverage values of the five samples were 0.87, 0.89, 0.85, 0.82 and 0.93, respectively, indicating that the subtotal OTUs in SBR were measured during the research. For each community, the Shannon–Weaver index scores (H) were in the range of 6.73–9.42, corresponding to Simpson values ranging from 0.89 to 0.98 and Chao1 values from 16,120 to 26,419. These results confirm that the activated sludge samples from SBR contained an abundant diversity of bacterial genera.

Diversity and compositional variation in the SBR bacterial community

The phylogenetic spectrum was used to characterize the microbial community structure and composition during the SBR operation. On the level of the phylum, 34 main phyla, as listed in Figure 4, were selected. The analytical results show that the seed sludge (ROC-1d) contained a relative abundance of 84.4% *Proteobacteria*, 7.4% *Planctomycetes*, 2.4% *Acidobacteria*, 1.8% *Bacteroidetes* and 0.7% *Chloroflexi*. After 30 days of acclimatization (ROC-31d), the abundance of *Proteobacteria* increased gradually from 84.4% to 94.1%. The relative abundance of *Planctomycetes* was 1.2% (ROC-31d), which was a significant decrease due to its inability to effectively adapt to the ROC during

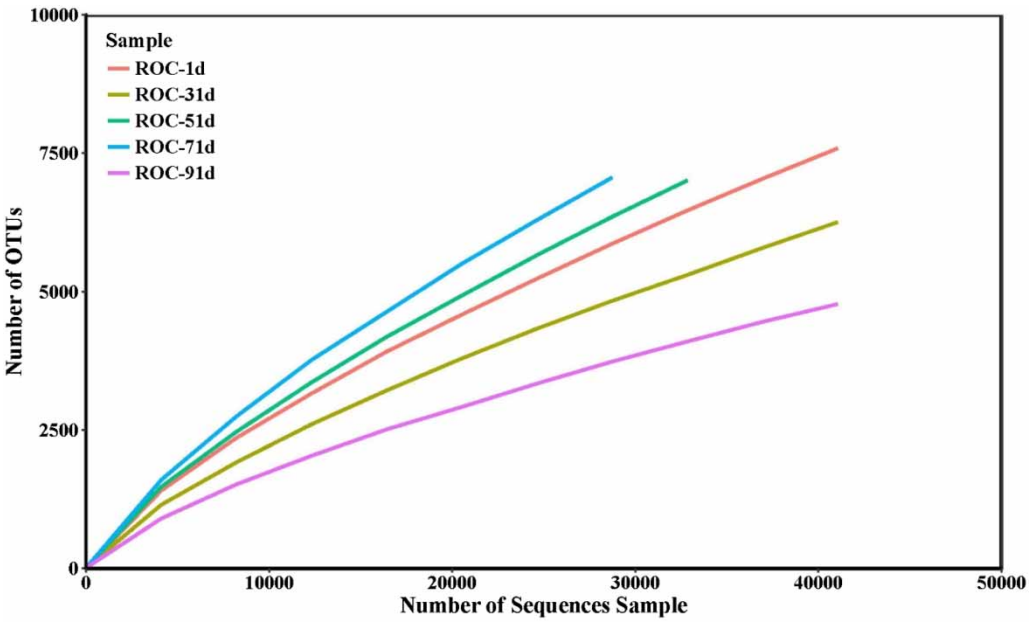

Figure 3 | Rarefaction curves at a dissimilarity level of 3% in the 16S rRNA gene sequences.

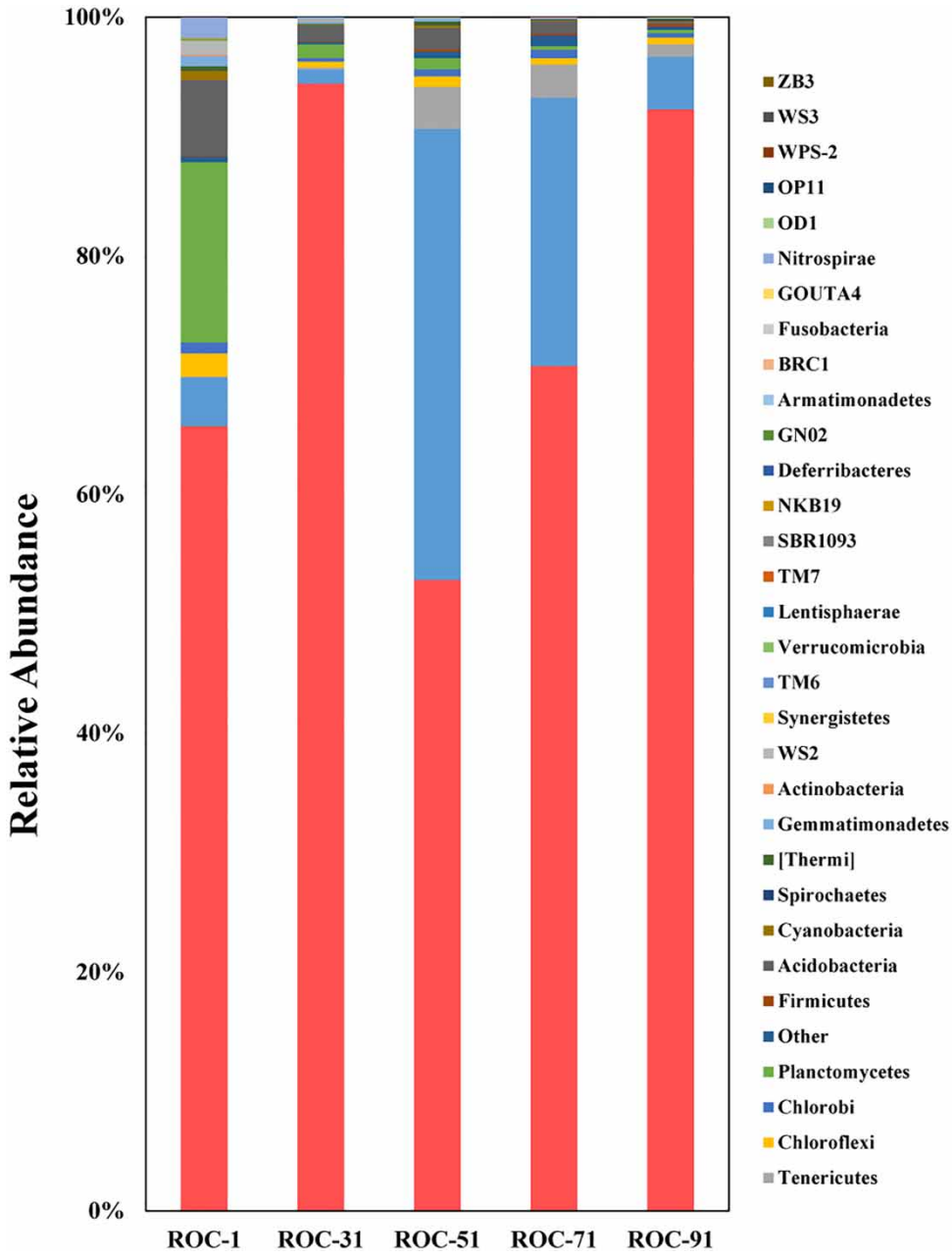

Figure 4 | Taxonomic classification of bacterial 16S rRNA genes on phylum level (relative abundance >0.01%).

the sludge acclimatization period. Sanchez *et al.* (2013) found *Planctomycetes* at lower proportions in high saline wastewater and seawater. In addition, on day 31 (ROC-31d), the abundances of *Acidobacteria* and *Chloroflexi*, decreased to 1.3% and 0.6% in sludge, respectively, whilst the phyla *GN02*, *Lentisphaerae* and *TM6* completely disappeared. Previous studies (Zhao *et al.* 2016) have also found a similar reduction in the abundance of *Acidobacteria* along with a salinity gradient, which was also observed in a membrane bioreactor with high saline wastewater. During the acclimatization period, the conductivity in SBR increased gradually, and the relative abundances of the major bacterial phyla changed in response to the hypersaline environment of ROC.

During the stable operation period, *Proteobacteria* were by far the largest phylum in the microbial community, accounting for 62.2%, 77.7% and 93.7% in ROC-51d, ROC-71d and ROC-91d, respectively. Belonging to Gram-negative bacteria and classified as part of the heterotrophic community, *Proteobacteria* played important roles in removing COD, nitrate and nitrite from the wastewater treatment system. Previously, *Proteobacteria* was the dominant phylum with 65–91% in various coking wastewater treatment plants. Moreover, *Proteobacteria* were also a highly common and predominant group in saline wastewater treatment bioreactors (Ma *et al.* 2015). *Bacteroidetes* were the second most abundant phylum, accounting for 31.3% (ROC-51d), 22.2% (ROC-71d) and 3.7% (ROC-91d) of the microbial communities. Previous studies (Cortes-Lorenzo *et al.* 2014) have reported that the phylum *Bacteroidetes* is one of the most abundant heterotrophic bacterial groups in high saline water environments, playing a key role in the degradation of particulate organic matter. The sum of *Proteobacteria* and *Bacteroidetes* in this study accounted for almost over 92% during the stable operation period, even reaching 97.5% in ROC-91d. Their numerical dominance in the stable period seemed to indicate that *Proteobacteria* and *Bacteroidetes* played important roles in nitrate removal and the biotransformation of organics, especially methanol degradation in ROC from coking wastewater. Moreover, a previous study (Lu *et al.* 2014) reported that bacterial strains isolated from denitrifying bioreactors are closely related to *Hyphomicrobium*, *Thauera* and *Paracoccus* spp. in *Proteobacteria*. In real wastewater denitrifying systems, most of the denitrifying genus are taxonomically affiliated with *Proteobacteria* (59%) and *Bacteroidetes* (16%). *Tenericutes* was the main dominant phylum in ROC, with relative abundances in ROC-51d, ROC-71d and ROC-91d of 1.6%, 3.0% and 0.7%, respectively. *Tenericutes* have been found in different natural and artificial saline environments (He *et al.* 2015). Another dominant phylum was *Acidobacteria*, classified as a heterotroph with an abundance of 1.7% (ROC-51d), 1.1% (ROC-71d) and 0.1% (ROC-91d). *Acidobacteria* not only could consume carbon and nitrite resources, but also produced glue-like extracellular polymers for microbial aggregation (Ebrahimi *et al.* 2010). The abundances of *Chlorobi* were 0.9% (ROC-51d), 0.5% (ROC-71d) and 0.1% (ROC-91d) in the growth of heterotrophic bacteria in the nitrogen removal process. Overall, the abundances of the dominant phyla exhibited a high level of tolerance to salinity and maintained a high nitrate removal efficiency of 92.9% in ROC treatment.

Genus level analysis can provide further information on microbial adaptation. Thirty of the most dominant bacterial groups were identified for the investigation of the dynamics and variations in ROC (Figure 5). The major genus detected in the sludge samples were *Hyphomicrobium*, *Thauera* and *Methyloversatilis*, accounting for 5.0–42.7%, 3.7–10.6% and 0.8–14.6%, respectively, throughout the stable operational period. *Hyphomicrobium* belong to *Proteobacteria* and are facultative methylotrophs with the versatility and ability to utilize toxic waste compounds in wastewater (Fesefeldt *et al.* 1998). It has been reported that *Hyphomicrobium* was quite efficient in the removal of both methanol and nitrate in the denitrification process. The genus *Thauera* is a Gram-negative organism in the phylum *Proteobacteria* and widely known as an important nitrate-reducing bacterium in wastewater treatment systems (Mao *et al.* 2013). Thus, *Thauera* has emerged as an important genus for its metabolic versatility and the remediation of environmental pollutants under aerobic conditions. Liu *et al.* (2006) assessed the quantitative dynamics of denitrifying *Thauera* in bioreactor, and their study suggested that bacterial groups may be functionally important in removing nitrate and COD under denitrifying conditions. *Methyloversatilis* has been identified in various methanol-feeding denitrification systems and belongs to *Proteobacteria* (Baytshtok *et al.* 2009). *Hyphomicrobium*, *Thauera* and *Methyloversatilis* were detected in this system and are important genera, having denitrifying ability under anoxic conditions in ROC. In accordance with a previous report (Jia *et al.* 2016), an anoxic–aerobic process was used to treat gasification wastewater with COD and TN removal efficiencies of 90% and 21%, respectively; *Hyphomicrobium*, *Thauera* and *Methyloversatilis* were the major bacterial genera in the bioreactor. Additionally, *Acholeplasma*, *Thiobacillus*, *Rubrivivax*, *Parvibaculum* and *Ignavibacterium* were found to be dominant in the SBR system and contributed significantly to both denitrification and COD removal.

Quantitative abundances of denitrifying genes in SBR

It is well known that nitrate reductase (Nar) and nitrite reductase (Nir) are responsible for converting nitrate to

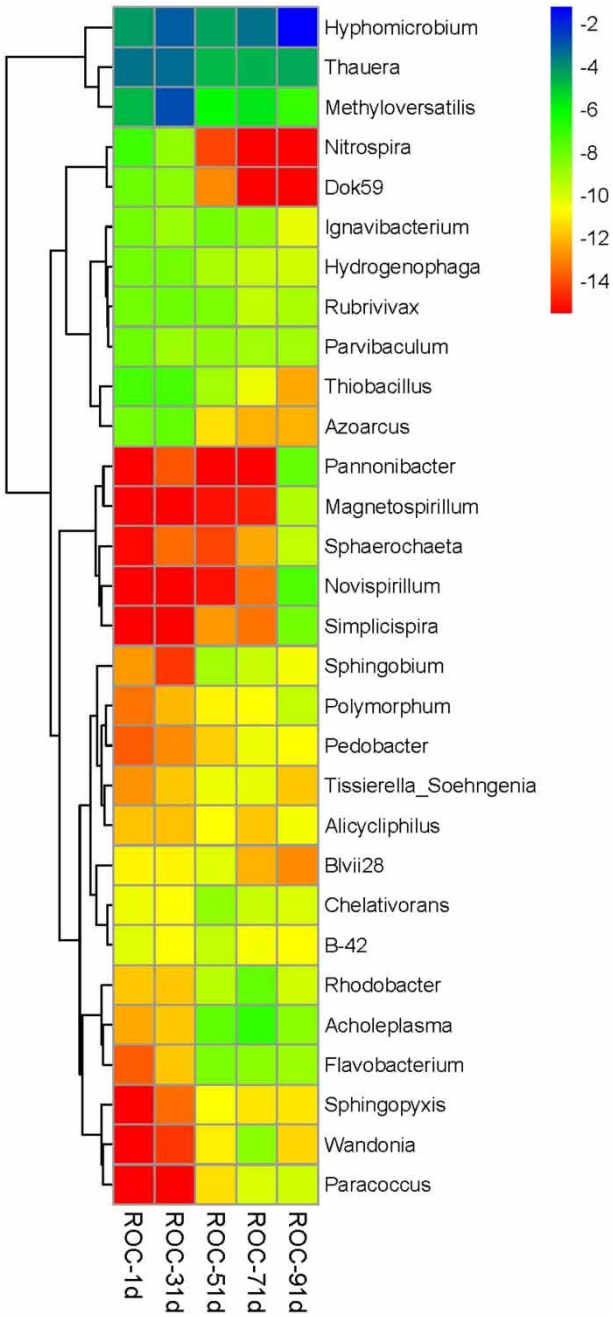

Figure 5 | Relative abundance of the 30 largest bacterial genera (values are calculated as $\log_2$) during the entire SBR operation.

nitrite and nitrite to nitrous oxide, respectively. In this study, the 16S rRNA, *narG*, *nirS* and *nirK* gene copy numbers were detected during the entire SBR operation period; these denitrifying bacteria were generally spread across a number of genera of *Proteobacteria*.

As shown in Figure 6, the quantity of 16S rRNA detected showed a trend of 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 ROC-1d, 3.53×10^5 copies/ng on ROC-31d, 4.62×10^5 copies/ng on ROC-51d, 4.97×10^5 copies/ng on ROC-71d and 3.56×10^5 copies/ng on ROC-91d. The change in copy numbers indicated that the hyper-saline ROC promoted steady concentrations of the dominant bacterial communities after acclimatization.

NarG, a membrane bound nitrate reductase enzyme, was the key gene for converting nitrate to nitrite, which consumed nitrate and provided substrate nitrite for converting NO_2^- to NO (Lu *et al.* 2014). The *narG* concentration on ROC-1d was 6.85×10^3 copies/ng, then gradually increased to 1.31×10^5 copies/ng on ROC-31d and became stable during the later period of stable operation with quantities of 4.54×10^3, 5.47×10^3 and 4.21×10^3 copies/ng at ROC-51d, ROC-71d and ROC-91d, respectively. These results illustrate that the high abundance of *narG* had promoted the reduction of nitrate to nitrite, ensuring the performance of denitrifying nitrogen removal.

The *nirS* and *nirK* genes express cytochrome cd1-containing and copper-containing nitrite reductase, respectively, which catalyze the reduction of nitrite to nitric-oxide. *NirS* and *nirK* were found to be prevalent in *Betaproteobacteria* and *Alphaproteobacteria*, respectively; *nirS* and *nirK* were found at equal frequency in the Gammaproteobacteria

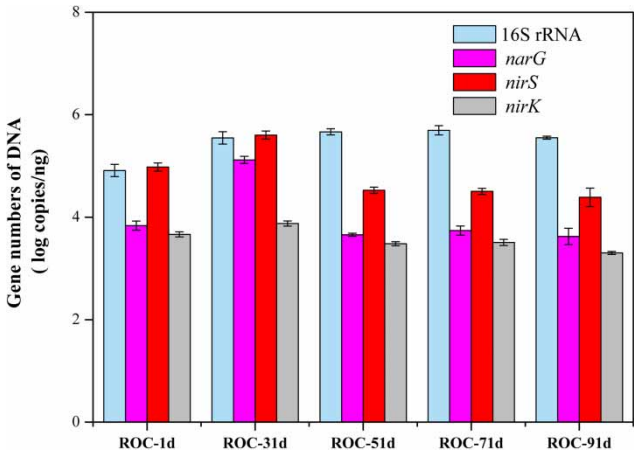

Figure 6 | Quantitative abundances of denitrifying 16S rRNA genes, *narG*, *nirK* and *nirS* in the SBR.

(Heylen *et al.* 2006). During the whole operational process, the quantity of *nirS* showed a trend of an initial increase, followed by generally stable abundances with 9.52×10^4 copies/ng (ROC-1d), 3.99×10^5 copies/ng (ROC-31d), 3.35×10^4 copies/ng (ROC-51d), 3.19×10^4 copies/ng (ROC-71d) and 2.45×10^4 copies/ng (ROC-91d). The abundance of *nirK* showed a similarly changing pattern with the quantity on ROC-31d being the highest at 7.52×10^3 copies/ng, and declining to overall similar magnitudes of 3.03×10^3 copies/ng (ROC-51d), 3.21×10^3 copies/ng (ROC-71d) and 2.01×10^3 copies/ng (ROC-91d). As expected, *nirS* and *nirK* genes are often used as nitrite reduction markers to study the denitrifying bacterial community. Several studies (Petersen *et al.* 2012) have reported that *nirS* and *nirK* bearing microorganisms take up different niches in the environment or have different habitat preferences. During the stable operation period, the quantity of *nirS* was higher than that of *nirK* by 10–100 orders of magnitude, indicating that the denitrifying bacterial genera containing *nirS* had played an important role in the denitrification process of ROC from coking wastewater. This result was consistent with those reported by previous studies of the quantity of *nirS* being higher than that of *nirK* by 1–4 orders of magnitude during the treatment of wastewater (Chen *et al.* 2016). Higher *nirS* gene abundance indicates that *nirS* not only played a dominant role in nitrite reduction, but also was a primary contributor to NO greenhouse gas production in SBR. The abundances of *narG*, *nirS* and *nirK* involved in the denitrification process were enriched in the stable operation, suggesting that under complex environmental conditions, the denitrifying community may adapt to the hyper-saline ROC after acclimatization. Overall, this suggests that gene analysis may be relied upon to assess the relative dominance of the denitrifying microbial populations in ROC treatment from coking wastewater.

CONCLUSIONS

In this study, stable and efficient denitrification was achieved by using an SBR process to treat ROC discharged from a coking wastewater treatment plant. The removal efficiencies of COD, TOC, TN and nitrate in this system were 81.4%, 83.7%, 90.6% and 92.9%, respectively. Miseq sequencing was utilized to investigate microbial communities and structures in SBR. The results showed that *Proteobacteria* and *Bacteroidetes* were the dominant phyla, with *Hyphomicrobium*, *Thauera* and *Methyloversatilis* being the predominant clusters at the genus level. Furthermore, real-time PCR was used to validate the absolute abundance of 16S rRNAs and denitrifying genes, including *narG*, *nirS* and *nirK*, during the complete operational period. Dominance of nitrate reductases genes in SBR may contribute immensely to the high nitrate removal. Among the denitrifying genes, *nirS* might play a more important role in nitrite removal than *nirK*. This study provides clear understanding about microbial community dynamics and structures during denitrification in ROC from coking wastewater.

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