Denitrification processes and microbial communities in a sequencing batch reactor treating nanofiltration (NF) concentrate from coking wastewater

Enchao Li and Shuguang Lu

ABSTRACT

A biological denitrifying process was employed for the treatment of nanofiltration (NF) concentrate with high conductivity, which was generated from coking wastewater in a sequencing batch reactor (SBR). The results showed that the average removal efficiencies of chemical oxygen demand (COD), total nitrogen (TN) and nitrate were 47.6%, 61.1% and 94.6%, respectively. Different microbial communities were identified by sequencing the V1-V3 region of the 16S rRNA gene using the MiSeq platform, showing that the most abundant bacterial phylum in the SBR system was Proteobacteria, with the subclasses β-Proteobacteria and α-Proteobacteria being dominant. The key microorganisms responsible for denitrification belonged to the genera Thauera, Hyphomicrobium, Methyloversatilis, Hydrogenophaga, Ignavibacterium, Rubrivivax and Parvibaculum. Quantitative real-time polymerase chain reaction was used to assess the absolute abundance of microbial genera, using 16S rRNAs and denitrifying genes such as narG, nirS, nirK, nosZ, in both SBR start-up and stable operation. The abundances of narG, nirK and nosZ were lower during stable operation than those during the start-up period. The abundance of nirS at a level of 10^4–10^5 copies/ng in DNA was much higher than that of nirK, thus being the dominant functional gene in nitrite reduction.

Key words | coking wastewater, denitrification, microbial community, NF concentrate

INTRODUCTION

Nitrogen compounds from industrial wastewaters discharged into the environment can cause serious problems such as eutrophication of rivers and lakes. In addition, nitrates can also form nitrosamines and nitrosamides, which are potentially carcinogenic (Oh et al. 2005). Therefore, the removal of nitrogen from wastewater is of high importance and is gaining increasing attention within the wastewater treatment field.

Nanofiltration (NF) has been proven to be a successful tool for wastewater treatment and reclamation, as it has high retention of multi-valent ions and organic matters at low operating pressure (Shahmansouri & Bellona 2015). NF membranes can effectively remove chemical oxygen demand (COD) and other pollutants such as total nitrogen (TN) from biological wastewater treatment effluent and have been successfully applied to the management of the contaminated effluents derived from various industries (Li et al. 2016). However, one significant drawback of NF is the generation of streams of wastewater concentrate, which can have a volume of up to 10–50% of the in-feed water. Due to the high salinity and persistence of contaminants, the need for further treatment prior to disposal of NF concentrate (NFC) is one of the main challenges in the implementation of membrane technology.

Coking wastewater is generated during high-temperature coal carbonization, coal gas purification and the refining of chemical products with large volumes being produced globally, causing notable loss of water in China and other regions. The treatment processes used for coking wastewater generally employ biological activated sludge treatments involving anoxic–oxic, anaerobic–anoxic–oxic processes (Lu et al. 2010). NF technologies (Jin et al. 2015) have been found to be highly effective in the treatment of coking wastewater. However, highly polluted coking waste-water NFC containing high concentrations of dissolved salts and nitrates will be generated at the same time and, with the
increasingly stringent environmental regulations worldwide, untreated NFC discharge is prohibited.

Currently, the removal of nitrates from NFC using biological denitrification is a major focus in wastewater treatment research. Compared to physico-chemical treatment methods, biological treatment offers a highly efficient and cost-effective method for treating industrial wastewaters with high salinity levels, using either anaerobic or aerobic processes. Denitrification, the process of nitrate reduction to nitrite then to molecular nitrogen, is performed by a functional group of heterotrophs that use oxidized nitrogen as an electron acceptor in respiration (Zhou et al. 2011). High salinity in wastewaters such as NFC is known to affect the performance of biological processes, potentially by unbalancing osmotic stress across cell walls, leading to plasmolysis when water is lost from microbial cells and eventually the failure of biological treatment systems (Zhang et al. 2012). In general, microbial communities always change notably after an acclimation period in saline wastewater, due to the depletion of non-halophiles and the abundant growth of halophiles (Cortés-Lorenzo et al. 2014). Thus, the well adapted microorganisms with abundant microbial communities could overcome the effects of high salinity, resulting in a better denitrification performance.

The relative abundances of microbial communities and their functional genes are of high importance in exploring and allowing a better understanding of the mechanisms of denitrification, especially in high salinity NFC derived from coking wastewater treatment. Recently, high-throughput sequencing technology has been shown to be a highly efficient tool for identifying and analyzing the complete profile of microbial diversity (Zhu et al. 2017). Additionally, applications of quantitative real-time polymerase chain reaction (qPCR) have given further insight into denitrifying communities in wastewater treatment due to the high taxonomic diversity in biological denitrification systems (Araki et al. 2006). So far most previous studies (Kim et al. 2011) have 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). To the best of our knowledge, there is still a lack of research examining biological denitrifying processes and microbial community structure dynamics for treatment of NFC produced from coking wastewater.

Therefore, a laboratory-scale sequencing batch reactor (SBR) was used to investigate the denitrification of NFC from coking wastewater in this study. The research aims to (1) evaluate the denitrifying performance of SBR systems in NFC treatment and (2) analyze the microbial community using high-throughput sequencing and assess the abundance of denitrifying genes (narG, nirK, nirS, and nosZ) by qPCR to better understand the biological mechanisms involved in NFC denitrifying processes.

**MATERIAL AND METHOD**

**Status of coking wastewater treatment procedures**

The rate of coking wastewater production at the Baoshan Iron & Steel Co., Ltd (Shanghai, China) was 150 m³/hour. In the first phase, a two-step biological denitrification procedure was adopted (A1-A2-O1-A3-O2; anaerobic 1, anoxicic 2, aerobic 1, anoxic 3, aerobic 2). In the second stage, an advanced treatment procedure was employed (ultrafiltration + NF + reverse osmosis). During the entire procedure, 10 m³ of NFC was produced per hour. The NF membranes used in the procedure were NF270-400 (DOW Group, USA). NFC characteristics are shown in Table 1.

**SBR and operational conditions**

Experiments were performed using a laboratory-scale cylinder SBR with a working volume of 5.0 L. The SBR was operated with a 24 hour cycle period, where each cycle included five phases: filling (5 min); anoxic reaction (6.0 hours); aerobic reaction (12.0 hours); settling (30 min); decanting (25 min); and idle phase (5 hours). A time controller was used to automate the reactor and a mechanical stirrer was used to provide constant liquid mixing at 60 rpm during the aerobic reaction phase. During 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 COD/NO3-N ratio was set at 8/1, with sodium acetate added as the carbon source for NFC denitrification at the beginning of filling phase. The solution pH was 7.0 ± 0.1 at the start of each anoxic reaction cycle and the temperature was maintained at 25 ± 2°C during the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Parameter</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.8–8.6</td>
<td>BOD5/COD</td>
<td>0.03–0.1</td>
</tr>
<tr>
<td>Conductivity (μS/cm)</td>
<td>9,450–11,250</td>
<td>TN (mg/L)</td>
<td>49–66</td>
</tr>
<tr>
<td>Cl⁻ (mg/L)</td>
<td>500–2,100</td>
<td>Nitrate (mg/L)</td>
<td>23–41</td>
</tr>
<tr>
<td>COD (mg/L)</td>
<td>318–496</td>
<td>Nitrite (mg/L)</td>
<td>0–1</td>
</tr>
<tr>
<td>BOD₅ (mg/L)</td>
<td>15–33</td>
<td>Ammonia (mg/L)</td>
<td>0–4</td>
</tr>
</tbody>
</table>

BOD₅ – 5-day biochemical oxygen demand.
entire operational period. Inoculated sludge added to the SBR at the start of the acclimatization period was obtained from the anoxic stage of coking wastewater. The coking wastewater treatment plant (Shanghai, China) had been in operation for a period of 5 years at the point of sludge collection. For biomass acclimatization, NFC was mixed with coking wastewater effluent at varying ratios of 60:40, 80:20 or 100:0. For each ratio, the process ran for 10 days, while the acclimation period lasted for 30 days. During acclimation, sludge was not discharged until day 31 of operation and the sludge retention time (SRT) was 20 days, with three SRT cycles conducted during the stable period. During the experimental period, the mixed liquor suspended solids (MLSS) concentrations in the reactor were kept at 2.02 ± 0.11 g/L.

**Chemical analysis**

COD, nitrate, nitrite, ammonia and MLSS were monitored using *Standard Methods* (APHA 2005). 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 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 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.

**QIIME analysis**

QIIME version 1.7 was used to perform OTU clustering, alpha and beta diversity analysis. 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 RDP Classifier following retraining against the Greengenes reference sequences. Chimera checking was performed using USEARCH with standard options implemented as in QIIME, against the Greengenes reference sequences. Alpha diversity analysis was performed using the Chao1, Shannon, OTU, goods’ coverage and Simpson metrics.

**qPCR analysis**

Quantitative analysis of extracted DNA from all samples was determined using the Qubit® dsDNA HS Assay Kit (Invitrogen, USA). Subsequently, according to sample concentrations, samples were diluted to 1 ng/μl, the same concentration as the primers used for qPCR and thermal programs (Table 2). Amplification reactions were performed in a volume of 20 μl, with the reaction mixture containing 10 μl of SYBR Premix Ex Taq TM (Takara, Japan), 2 μM of each primer, 10 ng of total DNA, and RNase-free water. PCR products were cloned using 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. Ten-fold serial dilutions of plasmids of known copy numbers were used as reference DNA to measure standard curves.

**RESULTS AND DISCUSSION**

**Assessment of the denitrification of NFC**

The denitrification process for NFC discharged from coking wastewater treatment plants using SBR lasted for 90 days in total, of which the acclimatization stage was
30 days and the stable operation period was 60 days. It is of note that the conductivity of NFC in the SBR ranged 10,050–11,800 μs/cm.

The value of COD in raw NFC in SBR was 318–496 mg/L, with an average concentration of 387.6 mg/L during the stable operation period (Figure 1). The BOD₅/COD ratio in NFC from coking wastewater was 0.03–0.1. This suggests that COD in NFC contained mainly organic substances which were difficult to degrade and therefore not able to serve as the carbon source for denitrification processes. Therefore, sodium acetate was added as a microbial carbon source prior to the onset of the SBR anoxic phase for biological denitrification. During the stable operation period, the influent COD concentration in the SBR was 645–975 mg/L, with an average of 755.2 mg/L. The COD concentration in the effluent was 316–465 mg/L, with an average of 396.1 mg/L and an average COD removal rate of 47.6%. The external carbon source was almost completely consumed, with residual COD in the effluent consisting mostly of refractory organics. It appears that COD in NFC is not readily degraded and not consumed in the denitrification process. Furthermore, following acclimatization, the heterotrophic denitrifying microorganism in SBR systems may have high suitability and tolerance to the saline NFC from coking wastewater with continual growth activity.

During the stable operation, the influent TN was in the range of 49–66 mg/L with an average concentration of 58.8 mg/L (Figure 2). The SBR effluent TN concentration was 17–28 mg/L with an average of 22.9 mg/L and successful TN removal of 61.1%. Conversely, the average influent nitrate was 31.8 mg/L, whereas the average effluent nitrate was 1.7 mg/L. The nitrate removal rate was 94.6%, with a maximum of 97.7%, suggesting that the denitrification of NFC from coking wastewater was markedly stable and complete in the SBR system. In addition, the average organic nitrogen concentration of influent was 25.1 mg/L, with average organic nitrogen effluent concentration of 20.1 mg/L. The organic nitrogen removal rate reached 19.8%, suggesting that anoxic-aerobic processes in the

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence (5’-3’) of primer pairs</th>
<th>Thermal program</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>1055F: ATGGCTGTCTGTCAGCT</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>1392R: ACGGCGGTGTGTTAC</td>
<td></td>
</tr>
<tr>
<td>narG</td>
<td>1960m2F: A(CT)GT(GC)GGCAGGA(AG)</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>AAACGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2050m2R: CGTAAAGAAGCTGTGCTGTTTT</td>
<td></td>
</tr>
<tr>
<td>nirS</td>
<td>nirS2F: TACACCC(C/G)GA(A/G)</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>CCGCGGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>nirS3R: GCCGCCGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(A/G)TG(A/C/G)AGGAA</td>
<td></td>
</tr>
<tr>
<td>nirK</td>
<td>nirK583F: TCATGGTGCTCCGCGKGACGG</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>nirK909R: GAACTTGCGTGGCCAGAC</td>
<td></td>
</tr>
<tr>
<td>nosZ</td>
<td>nos1527F: AGAACGACCAGCTATCGACA</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>nos1527R: TCCATGGTGACGCCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGTTGG</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1 | The changes of COD in influent and effluent water of the SBR along with operational time.
SBR converted some organic nitrogen to ammonia. Moreover, the concentration of nitrite in effluent was 0.09–0.87 mg/L. This shows that nitrite accumulation could not occur in the presence of sufficient sodium acetate as a carbon source. Meanwhile, the concentration of ammonia in effluent was below 3 mg/L.

In this study, stable and efficient denitrification in NFC discharged from coking wastewater treatment plant in the SBR system using sodium acetate as the sole carbon source was achieved. In the stable operational period, the removal efficiencies for COD, TN and nitrate in this system were 47.6%, 61.1% and 94.6%, respectively. Additionally, nitrite was lower than 0.87 mg/L due to the denitrification process. Significant nitrate removal efficiency was achieved in the SBR system, suggesting that the denitrifying microbial community present in the SBR were highly stable in the treatment of saline NFC after mature acclimatization. The results of this study are consistent with those of Jafari et al. (Jafari et al. 2015) who found that heterotrophic microorganisms could achieve an average nitrate removal of 92–99% in saline wastewater.

### Bacterial community diversity and composition variation in SBR

To analyze microbial community diversity in the NFC denitrification process, sludge samples were taken from SBR and the sequencing of the 16S rRNA gene was performed using the MiSeq platform. The community richness and diversity indices Good’s coverage, Shannon and Chao1 were calculated for five samples, at a cutoff level of 3% (Table 3). The values of the diversity indices reveal that the majority of communities were accounted for by Illumina MiSeq pyrosequencing.

The dynamics of main bacterial phyla and classes during SBR operation are shown in Figure 3. To assess the bacterial community dynamics throughout the denitrifying process, sludge samples were collected from day 1 (NFC-1d) and day 31 (NFC-31d), where NFC-1d represents the seed sludge and NFC-31d represents sludge immediately post-acclimatization. Samples were also collected throughout the stable operation period on day 51 (NFC-51d), day 71 (NFC-71d) and day 91 (NFC-91d) for analyses.

The analytical results (Figure 3(a)) showed the seed sludge (NFC-1d) contained a relative abundance of 65.2% Proteobacteria, 15.7% Planctomycetes, 6.7% Acidobacteria, and 3.9% Bacteroidetes. After 30 days of acclimatization (NFC-31d), the relative abundance of major phyla was 87.6% for Proteobacteria, 4.2% for Acidobacteria, 2.9% for Planctomycetes and 2.2% for Bacteroidetes. The relative abundance of Planctomycetes significantly decreased due to its inability to effectively adapt to the environment of NFC during the sludge acclimatization process. The removal rate of nitrate and nitrogen in NFC increased gradually and after full acclimatization the relative abundance of the major bacteria phyla changed.

During the period of stable operation, Proteobacteria was the most prevalent phylum present in the microbial community, accounting for 73.8%, 81.7% and 91.9% in

### Table 3 | Microbial community richness and diversity indices of the sludge samples

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Effective reads</th>
<th>OTU*</th>
<th>Shannon</th>
<th>Chao1</th>
<th>Goods’ coverage</th>
<th>Simpson</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFC-1d</td>
<td>49,825</td>
<td>6,359</td>
<td>8.6</td>
<td>18,561.8</td>
<td>0.89</td>
<td>0.97</td>
</tr>
<tr>
<td>NFC-31d</td>
<td>52,913</td>
<td>6,461</td>
<td>8.4</td>
<td>21,130.7</td>
<td>0.88</td>
<td>0.97</td>
</tr>
<tr>
<td>NFC-51d</td>
<td>53,874</td>
<td>7,387</td>
<td>8.8</td>
<td>25,651.5</td>
<td>0.86</td>
<td>0.98</td>
</tr>
<tr>
<td>NFC-71d</td>
<td>48,431</td>
<td>5,776</td>
<td>8.7</td>
<td>21,961.6</td>
<td>0.83</td>
<td>0.97</td>
</tr>
<tr>
<td>NFC-91d</td>
<td>51,308</td>
<td>5,754</td>
<td>7.9</td>
<td>18,249.4</td>
<td>0.89</td>
<td>0.96</td>
</tr>
</tbody>
</table>

*OTUs were defined at a 97% sequence similarity cutoff level.
NFC-51d, NFC-71d and NFC-91d, respectively. These findings show that Proteobacteria was the dominant population in SBR system, being favorable for the denitrification process of NFC, with Bacteroidetes being the second most abundant phylum, contributing approximately 18.7% (NFC-51d), 13.5% (NFC-71d) and 4.8%
(NFC-91d) respectively to the microbial communities. The sum of these two most dominant phyla accounted for over 90% of the biological population during the stable operation period, which may be because Proteobacteria and Bacteroidetes are the important contributors for both nitrate removal and COD degradation in NFC from coking wastewater. Proteobacteria are a widespread and highly diverse phylum, with Ma et al. (Qiao et al. 2015) finding Proteobacteria to be the dominant phylum (65–91%) in various coking wastewater treatment plants. Additionally, the predominance of Proteobacteria in denitrifying communities has also been found in an acetate-fed denitrification reactor (Yoshie et al. 2006). Bacteroidetes as the second phylum in NFC could degrade particulate organic matter, especially high molecular weight compounds and represent one of the most abundant heterotrophic groups observed in marine environments (Diezvives et al. 2012). Acidobacteria are the third most dominant phylum, whose relative abundance in NFC-51d, NFC-71d and NFC-91d was 2.7%, 1.6% and 0.5%, respectively. Previously, Acidobacteria have also been found to be widespread in wastewater (Deng et al. 2016) playing an essential role in facilitating the reduction of nitrate and nitrite. Another dominant phylum were Planctomycetes, with an abundance of 1.0% (NFC-51d), 0.5% (NFC-71d) and 0.6% (NFC-91d), indicating some function in nitrogen removal. Studies have reported that this group of bacteria not only has a widespread distribution and abundant diversity in nature but also account for about 30–50% N2 production in ocean (Ward 2003). Overall, the dominant phyla exhibited tolerance to high salinity, maintaining a high nitrate removal efficiency of 94.6% in NFC.

Differences on microbial community structure could also be identified at the class level (Figure 3(b)). Within the Proteobacteria population, β-Proteobacteria was the most dominant class, accounting for approximately 46.0% (NFC-1d), 74.9% (NFC-31d), 58.6% (NFC-51d), 71.6% (NFC-71d) and 77.1% (NFC-91d). Studies have reported that β-Proteobacteria play an important role in the degradation of organic matter and the removal of nitrous oxides from the activated sludge process in denitrifying reactors, with denitrifying bacteria such as Thauera, Methyloversatilis, Thiomicrotum and Rubrivivax belonging to the β-Proteobacteria class (Lu et al. 2014). The percentage abundance of α-Proteobacteria ranged from 8.1% to 13.1% during the stable operation period and for γ-Proteobacteria decreased notably from 6.0% (NFC-1d) to 1.1% (NFC-91d). β-Proteobacteria had the highest distribution followed by α-Proteobacteria and γ-Proteobacteria, consistent with other studies showing β-subdivisions to be more abundant than other classes of Proteobacteria in coking wastewater (Qiao et al. 2015). It is also reported that α-Proteobacteria can use short-chain fatty acids for biological denitrification in anaerobic industrial wastewater (Jena et al. 2016). Many facultative strains such as the genus Hyphomicrobium belong to α-Proteobacteria, some of which can utilize acetate as the sole source of carbon and energy, which may contribute to nitrate and nitrite degradation observed in the present study. Throughout the experiment, the relative abundance of Flavobacteria was lower in the sludge samples of NFC-1d (1.2%) and NFC-31d (0.07%), but significantly increased in the sludge samples of NFC-51d (11.1%) and NFC-71d (8.7%) during the stable operation period, although this decreased to 2.0% in NFC-91d sample. Previous studies also have shown that Flavobacteria exist in hyper-saline industrial wastewater and are capable of degrading organic matter (Lu et al. 2014). In addition, Chloracidobacteria and Phycisphaerae decreased gradually until the relative abundance was below 1% for both bacterial groups by the end of the operational period, implying poor adjustment to the NFC environment.

For the investigation of dynamics and variation within the microbial community in NFC, thirty of the most dominant bacterial groups were identified at the genus level (Figure 4). Genus-level analysis provides further insight into microbial adaptation in NFC environments. As NFC from coking wastewater is regarded as an extreme environment with high conductivity, nitrate and TN concentrations, the denitrifying microorganisms should be notably different from those in domestic wastewater. The dominant genera were found to be Thauera, Hyphomicrobium and Methyloversatilis, which accounted for 4.8–10.7%, 2.9–5.5% and 1.3–3.6%, respectively, throughout the stable operational period. Thauera is known to be widely distributed in a variety of industrial wastewater treatment plants and is not only capable of degrading aromatic and heterocyclic organic pollutants, but is also one of the bacterial genera known to use organic matter such as acetates as electron donors in denitrifying reactions (Hao et al. 2015). Hyphomicrobium in NFC is an important genus for denitrification and is widely distributed in industrial wastewaters (Layton et al. 2000). Consistent with reports in the literature, Methyloversatilis was found to be an important bacterial genus for effective denitrification using organic matter as a carbon source (Wang et al. 2011). In the denitrifying system present in this SBR assessment, Thauera, Hyphomicrobium and Methyloversatilis formed a dominant community structure. Thus, the high nitrate removal efficiency (94.6%) observed in NFC may be due to the activity...
of this denitrifying genus. Additionally, *Novispirillum*, *Azospirillum*, *Simplicispira*, *Wandonia* and *Paracoccus* were found to be dominant in the SBR system.

Quantitative abundances of denitrifying genes in SBR

In general, the enzymes nitrate reductase (Nar), nitrite reductase (Nir) and nitrous oxide reductase (Nos) are considered the main indicators used for denitrification processes in wastewater treatment (Lu et al. 2014). The 16S rRNA, *narG*, *nirS*, *nirK*, and *nosZ* gene copy numbers were detected during the entire SBR operation period.

As shown in Figure 5, the quantity of 16S rRNA detected showed the trend of an initial increase, followed by a more constant quantity overall with the copy numbers of 16S rRNA being $8.15 \times 10^4$ copies/ng on NFC-1d, $6.69 \times 10^5$ copies/ng on NFC-31d, $9.37 \times 10^5$ copies/ng on NFC-51d, $11.9 \times 10^5$ copies/ng on NFC-71d, and $7.26 \times 10^5$ copies/ng on NFC-91d. NFC-1d had the lowest quantity, with NFC-31d, NFC-51d, NFC-71d and NFC-91d being similar in magnitude, indicating that hyper-saline NFCs with the addition of sodium acetate promoted steady concentrations of the dominant bacterial communities after acclimatization.

*NarG*, a membrane-bound nitrate reductase enzyme, catalyzes the reduction of nitrate into nitrite during the SBR denitrification process, with *narG* concentration being highest initially at NFC-31d at $6.85 \times 10^3$ copies/ng. Abundance gradually decreased and became stable during the later period of stable operation with quantities of $4.35 \times 10^2$ copies/ng at NFC-51d, $9.74 \times 10^1$ copies/ng at NFC-71d, and $1.77 \times 10^2$ copies/ng at NFC-91d.

*NirS* and *nirK* are structurally different, but functionally similar to nitrite reductases and are crucial enzymes for the transformation of nitrate into nitrogen (Zumft 1997). During the whole operation process, the quantity of *nirS* showed the trend of an initial increase, followed by generally stable abundances, with $9.52 \times 10^4$ copies/ng at NFC-1d, $2.27 \times 10^5$ copies/ng at NFC-31d, $1.14 \times 10^5$ copies/ng at NFC-51d, $6.81 \times 10^4$ copies/ng at NFC-71d, and $6.81 \times 10^4$ copies/ng at NFC-91d, suggesting that NFC promoted *nirS* containing microbial growth. The abundance of *nirK* showed a similar trend, with the quantity at NFC-31d being highest at $6.34 \times 10^3$ copies/ng, declining to overall magnitudes of $1.73 \times 10^3$ copies/ng at NFC-51d, $1.58 \times 10^3$ copies/ng at NFC-71d, and $1.81 \times 10^3$ copies/ng at NFC-91d.
NFC-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 may be more active in the second process of denitrification (reduction of nitrite to nitric oxide) in the SBR. Previous studies have also reported that nirS and nirK were found to be prevalent in β-Proteobacteria and α-Proteobacteria, respectively, with equal frequency for both genes in γ-Proteobacteria (Heylen et al. 2006).

NosZ reduces N₂O into N₂ and completes the final step of denitrification. The trend in change of nosZ quantities was consistent with that of nirS and nirK, with 1.91×10^3 copies/ng at NFC-1d, 2.59×10^3 copies/ng at NFC-51d, 3.21×10^2 copies/ng at NFC-51d, 1.96×10^2 copies/ng at NFC-71d, and 1.79×10^2 copies/ng at NFC-91d, indicating some interplay between nirS and nirK.

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

CONCLUSIONS

The experimental results in this study clearly indicated the effectiveness and efficiency of SBR processes for the denitrification of NFC discharged from coking wastewater treatment plants using sodium acetate as an additional carbon source. Significant TN and nitrate removal rates were achieved, without the accumulation of nitrite observed in bio-reactors. Proteobacteria and Bacteroidetes were the dominant phyla in NFC denitrification, with the main genera being Thauera, Hyphomicrobium and Methyloversatilis. Furthermore, the absolute abundance of the denitrifying genes narG, nirS, nirK and nosZ were validated by qPCR throughout the entire operational period. Among these genes, the nitrite reductase nirS played a more important role in nitrite reduction than nirK.

ACKNOWLEDGEMENTS

This study was financially supported by grants from the National Natural Science Foundation of China (No. 41373094 and No. 51208199) and the Natural Science Foundation of Shanghai (16ZR1407200).

REFERENCES

APHA 2005 Standard Methods for the Examination of Water and Wastewater. American Public Health Association (APHA), Washington, DC, USA.


First received 25 May 2017; accepted in revised form 29 August 2017. Available online 11 September 2017.