


Simultaneous nitrification and denitrification (SND) bioaugmentation with *Pseudomonas* sp. HJ3 inoculated for enhancing phenol and nitrogen removal in coal gasification wastewater

Weiwei Ma, Yuxing Han, Wencheng Ma, Hongjun Han , Chunyan Xu and Hao Zhu

ABSTRACT

A simultaneous nitrification and denitrification (SND) bioaugmentation system with *Pseudomonas* sp. HJ3 inoculated was established to explore the potential of simultaneous phenol and nitrogen removal in coal gasification wastewater (CGW). When the concentration of influent chemical oxygen demand (COD) and total phenols (TPh) was $1,765.94 \pm 27.43$ mg/L and 289.55 ± 10.32 mg/L, the average removal efficiency of COD and TPh at the stable operating stage reached $64.07\% \pm 0.76\%$ and $74.91\% \pm 0.33\%$, respectively. Meanwhile, the average removal efficiency of $\text{NH}_4^+\text{-N}$ and total nitrogen (TN) reached $67.96\% \pm 0.17\%$ and $57.95\% \pm 0.12\%$, respectively. The maximum SND efficiency reached 83.51%. Furthermore, SND bioaugmentation performed with good nitrification tolerance of phenol shock load and significantly reduced toxic inhibition of organisms. Additionally, the microbial community analysis indicated that *Pseudomonas* sp. HJ3 was the predominant bacterium in the SND bioaugmentation system. Moreover, the indigenous nitrogen removal bacteria such as *Thauera*, *Acidovorax* and *Stenotrophomonas* were enriched, which further enhanced the nitrogen removal in the SND bioaugmentation system. The results demonstrated the promising application of SND bioaugmentation for enhancing simultaneous phenol and nitrogen removal in CGW treatment.

Key words | bioaugmentation, biotoxicity, coal gasification wastewater, phenol degradation, *Pseudomonas* sp. HJ3, simultaneous nitrification and denitrification

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INTRODUCTION

Coal gasification wastewater (CGW) is a typical refractory industrial wastewater with high concentration of phenolic compounds, heterocyclic and polycyclic aromatic hydrocarbons, long chain alkanes and ammonia (Jia *et al.* 2016). Among these compounds, phenolic compounds are the main organic pollutants, accounting for about 30%–60% of the chemical oxygen demand (COD) content (Wang *et al.* 2014). In addition to phenolic compounds, ammonia is also one of the worst contaminants, resulting in a negative effect on CGW treatment (Zhao *et al.* 2013). Ammonia is usually treated by the conventional anoxic–oxic (A/O) nitrogen removal process, which requires nitrifying bacteria and denitrifying bacteria working in separate strictly aerobic and anaerobic conditions, thus increasing process complexity and energy consumption (Liu *et al.* 2015). What is more,

the nitrification process is excessively inhibited by a variety of toxic refractory compounds (Kulkarni 2012; Zhao *et al.* 2014). Additionally, inadequate biodegradable carbon sources for denitrifying bacteria also lead to a low total nitrogen (TN) removal efficiency (Li *et al.* 2011). Therefore, enhanced nitrogen removal is of great importance for CGW treatment, as well as the elimination of toxic compounds to inhibit nitrification and denitrification.

Fortunately, simultaneous nitrification and denitrification (SND) is highlighted to be an attractive nitrogen removal strategy in industrial wastewater treatment (Herrero & Stuckey 2015). Recently, some bacteria have been reported to be capable of heterotrophic nitrification and aerobic denitrification, which can convert ammonia aerobically into nitrogen gas using organic substrates as carbon

source and energy (Yang *et al.* 2018), such as *Pseudomonas stutzeri* (Zhu *et al.* 2014), *Paracoccus versutus* (Zhang *et al.* 2015), *Alcaligenes* sp. TB (Chen *et al.* 2016), *Zobellella taiwanensis* (Lei *et al.* 2016) and so on. These bacteria can simultaneously achieve nitrification and denitrification in a micro-oxygen reactor. Lu *et al.* (2017) isolated a heterotrophic nitrifying–denitrifying bacterium *Serratia* sp. LJ-1 that performs simultaneous ammonia and nitrate removal in aerobic conditions. Moreover, *Serratia* sp. LJ-1 presents much higher tolerance to phenol toxicity than that of the autotrophic nitrifying bacteria. Additionally, some heterotrophic nitrifying–denitrifying bacteria have 40% reduction of carbon source demand and 63% improvement of biological nitrogen removal efficiency (Yoo *et al.* 1999). Based on the above advantages, it is feasible to build a bioaugmentation system using high-efficiency SND bacteria for enhancing organics and nitrogen removal in a variety of complex industrial wastewaters. Compared with traditional biological nitrogen removal processes, the SND bioaugmentation system has many advantages: (i) nitrification and denitrification can take place simultaneously in the aeration tank, so it is unnecessary to construct additional advanced treatment systems for organics removal (Yang *et al.* 2018); (ii) the resistance capability to fluctuations can be greatly improved because the maximum growth rates of SND bacteria are higher than those of autotrophic nitrifying bacteria (Chen *et al.* 2015). Presently, efficient bioaugmentation processes have been achieved by immobilizing specific functional microorganisms on various types of support material, such as polyurethane foam cubes (Du *et al.* 2017a), mycelial pellets (Ma *et al.* 2015a) or microbial cellulose (Yu *et al.* 2011), thereby achieving an excellent removal ability for organic contaminants and nitrogen *in situ*. However, limited studies have explored the potential capability of SND bioaugmentation for simultaneous phenol and nitrogen removal in CGW treatment (Zhao *et al.* 2014; Ma *et al.* 2017).

In this study, an SND bioaugmentation system with heterotrophic nitrifying–aerobic denitrifying bacterium *Pseudomonas* sp. HJ3 inoculated was established to evaluate the potential capability of simultaneous phenol and nitrogen removal in CGW. Meanwhile, phenol tolerance and biotoxicity reduction of the SND bioaugmentation system were assessed. Moreover, the interaction between *Pseudomonas* sp. HJ3 and the indigenous microbial community was analyzed. Based on above results, the study aims to provide useful guidance for practical application of bioaugmentation technology for enhancing phenol and nitrogen removal in CGW treatment.

MATERIALS AND METHODS

Wastewater and inoculum

The wastewater in this study was obtained from a full-scale CGW treatment plant located in Inner Mongolia, China. This wastewater had been pretreated by phenol and ammonia recovery processes. In addition, trace elements were added into the wastewater to provide a balanced feed for microbial growth (Wang *et al.* 2011). The main characteristics of the wastewater were as follows: COD of 1,600–1,800 mg/L, total phenol (TPh) of 250–300 mg/L, $\text{NH}_4^+\text{-N}$ of 170–200 mg/L, TN of 200–240 mg/L, pH of 7.0–7.2.

The activated sludge was obtained from an aerobic tank located at a full-scale CGW treatment plant in Inner Mongolia, China. The ratio of mixed liquor volatile suspended solids (MLVSS) and mixed liquor suspended solids (MLSS) was around 0.72.

Pseudomonas sp. HJ3 strain was isolated and characterized with heterotrophic nitrification and aerobic denitrification efficiency in a laboratory study. The cell morphology and a neighbor-joining tree of strain HJ3 are shown in Figure S1 (Supplement). The 16S rDNA nucleotide sequence of strain HJ3 has been submitted to GenBank nucleotide sequence databases with the accession number KY441413. *Pseudomonas* sp. HJ3 strain was inoculated in 200 mL Luria Bertani (LB) medium and then incubated for seven days in a rotary shaker at 120 rpm and 30 °C for further experimental study.

The polyurethane filler was prepared with a density of 0.008–0.015 g/cm³, surface area of 3,000 cm²/g, pore size of 2.0–2.5 mm and porosity >96%.

SND performance assessment of *Pseudomonas* sp. HJ3

The Luria Bertani medium for consortium enrichment contained 10.00 g/L of tryptone, 3.00 g/L of beef extract, 10.00 g/L of NaCl, at pH 7.2. The heterotrophic nitrification medium (HNM) for the heterotrophic nitrification study contained 7.90 g/L of Na_2HPO_4 , 1.50 g/L of KH_2PO_4 , 0.10 g/L of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.20 g/L of NH_4Cl , 0.20 g/L of phenol as sole carbon source, trace element solution 2.00 mL, at pH 7.2. The denitrification medium (DM1/DM2) for the aerobic denitrification study contained 7.90 g/L Na_2HPO_4 , 1.50 g/L KH_2PO_4 , 0.10 g/L $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.36 g/L KNO_3 (0.27 g/L of KNO_2), 0.20 g/L of phenol as sole carbon source, trace element solution 2.00 mL, at pH 7.2. All solid mediums were prepared with addition of 1.5% agar. All the above mediums were sterilized for 20 min at 0.11 MPa, 121 °C.

Measures of 100 mL HNM, 100 mL DM1 and 100 mL DM2 were added into 250 mL Erlenmeyer flasks, respectively, and then inoculated with 10% pre-culture of *Pseudomonas* sp. HJ3 cell suspensions. The three mediums were cultivated for 48 h at 30 °C and 120 rpm in a rotary shaker. All samples were periodically taken and centrifuged at 8,000 rpm to measure phenol, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations. All of the above experiments were conducted in triplicate.

The SND bioaugmentation reactor setup and operation

The SND bioaugmentation reactor made of a polyvinyl chloride (PVC) column with a working volume of 2.5 L was established. R1 was inoculated with 50% (V_0/V) of pre-cultured *Pseudomonas* sp. HJ3 suspension and activated sludge with MLSS of 3,000 mg/L. Polyurethane fillers were added into R1 for steady biofilm formation, which was also conducive to immobilizing the *Pseudomonas* sp. HJ3 strain in the reactor. Meanwhile, R2 was seeded with the same proportion of polyurethane fillers and activated sludge without *Pseudomonas* sp. HJ3 inoculated as a control under the identical operating conditions. The bacterial suspension, activated sludge and polyurethane fillers in the two reactors were suspended and mixed by a mixing device (JJ-1A, Skyray Instrument Co., Ltd, China) and a micro-pore aeration device (YXLY, LANXESS Co., Ltd, Germany). Dissolved oxygen (DO) concentration range was controlled at 0.5–1.0 mg/L using the micro-pore aeration device. Sludge residence time (SRT) was controlled at 25 d. The two reactors were operated with two cycles a day, of which one cycle of 12 h consisted of 10 min for feeding, 630 min for aeration, 60 min for settling, and 20 min for withdrawal. Considering that high-strength CGW might lead to a destabilization of the SND bioaugmentation system, stepwise increase of influent COD load was adopted at the initial operating stage. When influent COD and $\text{NH}_4^+\text{-N}$ reached about 1,800 mg/L and 200 mg/L, respectively, the concentration of COD and $\text{NH}_4^+\text{-N}$ in the effluent decreased significantly and a biofilm was formed on the surface of the fillers, indicating that the reactor had achieved stable operation. The temperature was maintained at 25–27 °C. All samples were periodically taken and centrifuged at 8,000 rpm to measure the concentration of COD, TPh, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$ and TN. All of the above data were measured in triplicate. The SND efficiency was calculated as follows:

$$r_{\text{SND}} = \left(1 - \frac{\text{NO}_x^- \text{ produced}}{\text{NH}_4^+ \text{ removal}} \right) \times 100\% \quad (1)$$

where NO_x^- produced is production of $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$, and NH_4^+ removal is decrease of $\text{NH}_4^+\text{-N}$ (Third et al. 2003).

Phenol shock load study

At the stable operating stage, R1 and R2 were operated at the influent phenol concentration of about 500 mg/L. This loading was continued for four days before switching back to normal loading. Then the influent phenol concentration was further increased to about 1,000 mg/L for four days. TPh and $\text{NH}_4^+\text{-N}$ concentrations were analyzed during the shock load stage.

Biotoxicity assessment

The raw CGW was assessed at five dilution ratios (5%, 10%, 20%, 30%, 40%), and the treated effluent of R1 and R2 was assessed at five dilution ratios (10%, 25%, 50%, 75%, 100%). A measure of 0.2 mL of pre-cultured *Tetrahymena thermophila* (*T. thermophila*) cells (cell density of about 1×10^5 cells/mL) were put into the 96-well microplate, then 0.8 mL of a diluted water sample or negative control (culture medium) was added. The *T. thermophila* was originally obtained from the Chinese Academy of Sciences (Beijing, China). Each diluted water sample and negative control was conducted in three replicates. After 24 h exposure, the cell density of *T. thermophila* was determined at 490 nm using a microplate luminometer (ELx800, BioTek, USA). The EC_{50} value was calculated by logistic fit using Origin 8.5 software (Origin Lab, USA). The toxic unit (TU) was calculated as the reciprocal of EC_{50} to reflect the toxicity level.

Microbial community analysis

The biofilm samples were collected from R1 and R2 at the stable operating stage (65 d) to analyze the microbial community via high-throughput sequencing. Total DNA was extracted using a DNA extraction kit (E.Z.N.ATM Mag-Bind Soil DNA Kit) according to the manufacturer's instructions. The extracted DNA was polymerase chain reaction (PCR)-amplified using the V3–V4 region of 16S rDNA gene universal primers with 341F (CCTACGGGAGGCAG-CAG) and 805R (GACTACHVGGGTATCTAATCC). The PCR procedure was conducted as described by Jia et al. (2015). The obtained PCR products were sequenced using MiSeq Illumina by Sangon Biotech Co., Ltd (Shanghai, China). The resulting sequences were clustered into operational taxonomic units (OTUs) with a 97% similarity threshold.

Analytical methods

The concentrations of COD, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$ and TN were determined according to *Standard Methods* (APHA 1998). TPh concentration was determined using the Folin phenol spectrophotometry method. DO concentration was measured with an oxygen meter (LDO10103, HACH, USA) and pH value was determined with a pH meter (pHS-3C, Leici, China).

RESULTS AND DISCUSSION

The SND properties of *Pseudomonas* sp. HJ3 strain

As plotted in Figure 1(a), phenol and ammonia were degraded significantly by *Pseudomonas* sp. HJ3 at 48 h. Phenol removal efficiency reached $90.12\% \pm 0.14\%$. Meanwhile $90.26\% \pm 0.09\%$ of $\text{NH}_4^+\text{-N}$ was removed with a maximum removal rate of about 2.10 mg/L/h, which was lower than that of *Pseudomonas stutzeri* YG-24 (8.75 mg/L/h)

and *Zobellella taiwanensis* DN-7 (17.3 mg/L/h) (Li et al. 2015; Lei et al. 2016). Because the carbon source used in these studies is citrate and succinate, they are more easily utilized by cells than phenol, which can accelerate cell growth rate and result in a higher $\text{NH}_4^+\text{-N}$ removal rate. Furthermore, the nitrate (DM1) and nitrite (DM2) were respectively used as sole nitrogen source to evaluate the aerobic denitrification ability of strain HJ3. As shown in Figure 1(b) and 1(c), phenol as denitrification carbon source was significantly degraded in the aerobic denitrification process, which performed with a similar trend to the heterotrophic nitrification process. As shown in Figure 1(b), $\text{NO}_3^-\text{-N}$ removal efficiency was about $73.38\% \pm 0.11\%$. The corresponding maximum $\text{NO}_3^-\text{-N}$ removal rate reached about 1.51 mg/L/h, which was similar with that of *Acinetobacter junii* YB (Yang et al. 2015). Meanwhile, nitrite was slightly accumulated at 9–21 h, and then decreased gradually to 0.47 ± 0.09 mg/L at 30 h. When nitrite was sole nitrogen source, the $\text{NO}_2^-\text{-N}$ removal efficiency was $50.26\% \pm 0.18\%$ (Figure 1(c)) and the corresponding maximum removal rate was about 0.98 mg/L/h. Meanwhile, no obvious accumulation

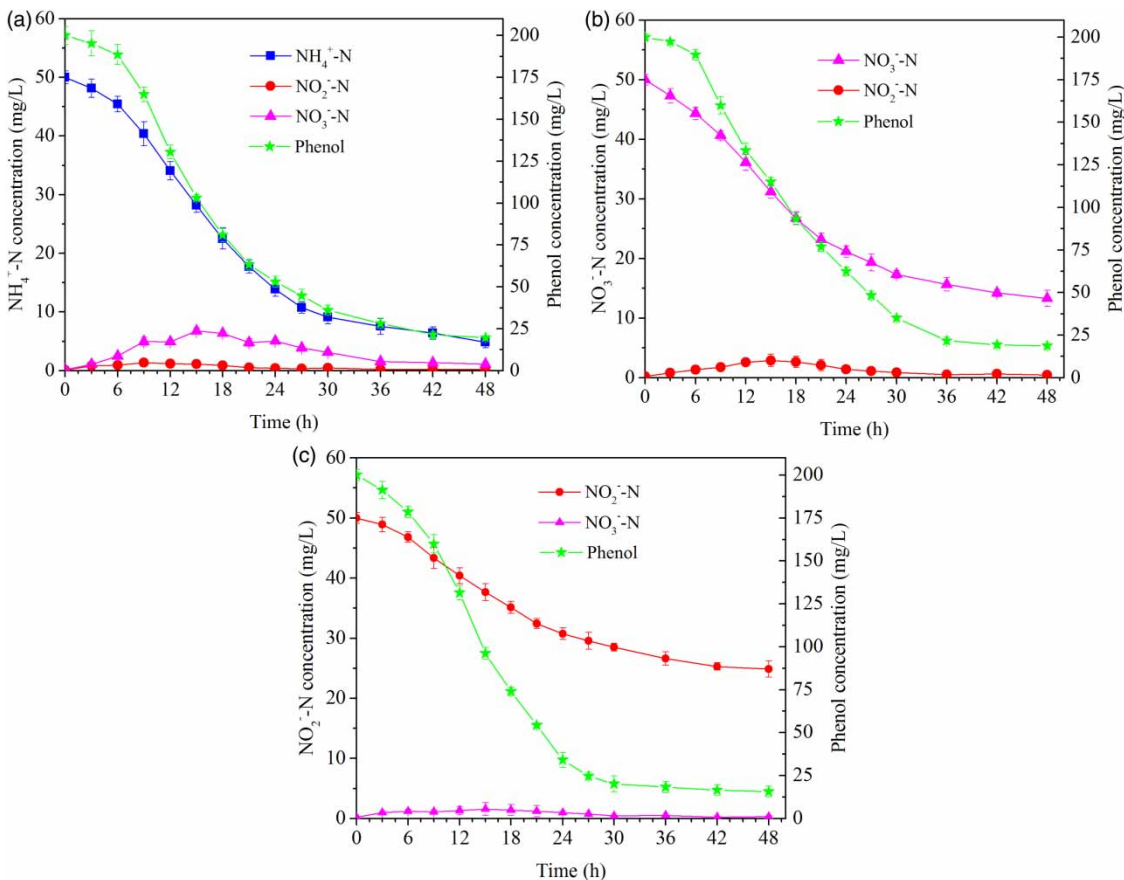


Figure 1 | Degradation efficiency of phenol and nitrogen with (a) ammonia, (b) nitrate and (c) nitrite as sole nitrogen source, respectively.

of nitrate was detected. The above results illustrate that *Pseudomonas* sp. HJ3 could utilize phenol as the carbon source for the heterotrophic nitrification and aerobic denitrification processes.

Phenol and nitrogen removal performances in SND bioaugmentation system

As shown in Figure 2, R1 and R2 had similar behavior with regards to COD, TPh, $\text{NH}_4^+\text{-N}$ and TN removals at the influent COD concentration of 545.81 ± 17.12 mg/L. The average removal efficiencies of COD, TPh, $\text{NH}_4^+\text{-N}$ and TN in R1 (0–20 d) were $58.20\% \pm 1.03\%$, $64.86\% \pm 0.54\%$, $53.40\% \pm 0.27\%$ and $44.11\% \pm 0.38\%$, respectively. This indicated that *Pseudomonas* sp. HJ3 was not effective because the biofilm attached to the filler was not mature during the start-up stage. With COD concentration increasing to $1,120.33 \pm 24.65$ mg/L, the concentrations of effluent COD, TPh, $\text{NH}_4^+\text{-N}$ and TN gradually decreased in R1 (21–40 d), and the

corresponding average removal efficiency reached $60.54\% \pm 0.32\%$, $70.79\% \pm 0.54\%$, $54.10\% \pm 0.13\%$ and $46.79\% \pm 0.61\%$, respectively. In addition, COD and TPh average removal efficiency in R2 was maintained at $52.51\% \pm 0.15\%$ and $58.58\% \pm 0.76\%$, respectively. However, $\text{NH}_4^+\text{-N}$ and TN average removal efficiencies were only $42.92\% \pm 0.24\%$ and $5.64\% \pm 0.45\%$, respectively. It has been reported that the activities of nitrifying and denitrifying bacteria are inhibited due to the toxicity of high-concentration refractory compounds (Joo et al. 2006; Kim et al. 2008). When influent COD and TPh concentrations increased to $1,765.94 \pm 27.43$ mg/L and 289.55 ± 10.32 mg/L, COD and TPh average removal efficiency in R1 in the stable operating stage (55–70 d) reached $64.07\% \pm 0.76\%$ and $74.91\% \pm 0.33\%$, respectively. Meanwhile, the average removal efficiency of $\text{NH}_4^+\text{-N}$ in R1 reached $67.96\% \pm 0.17\%$ at the stable operating stage, which was increased by about 44.09% over that of R2. This suggested that R1 with *Pseudomonas* sp. HJ3 bioaugmentation could enhance phenol and other

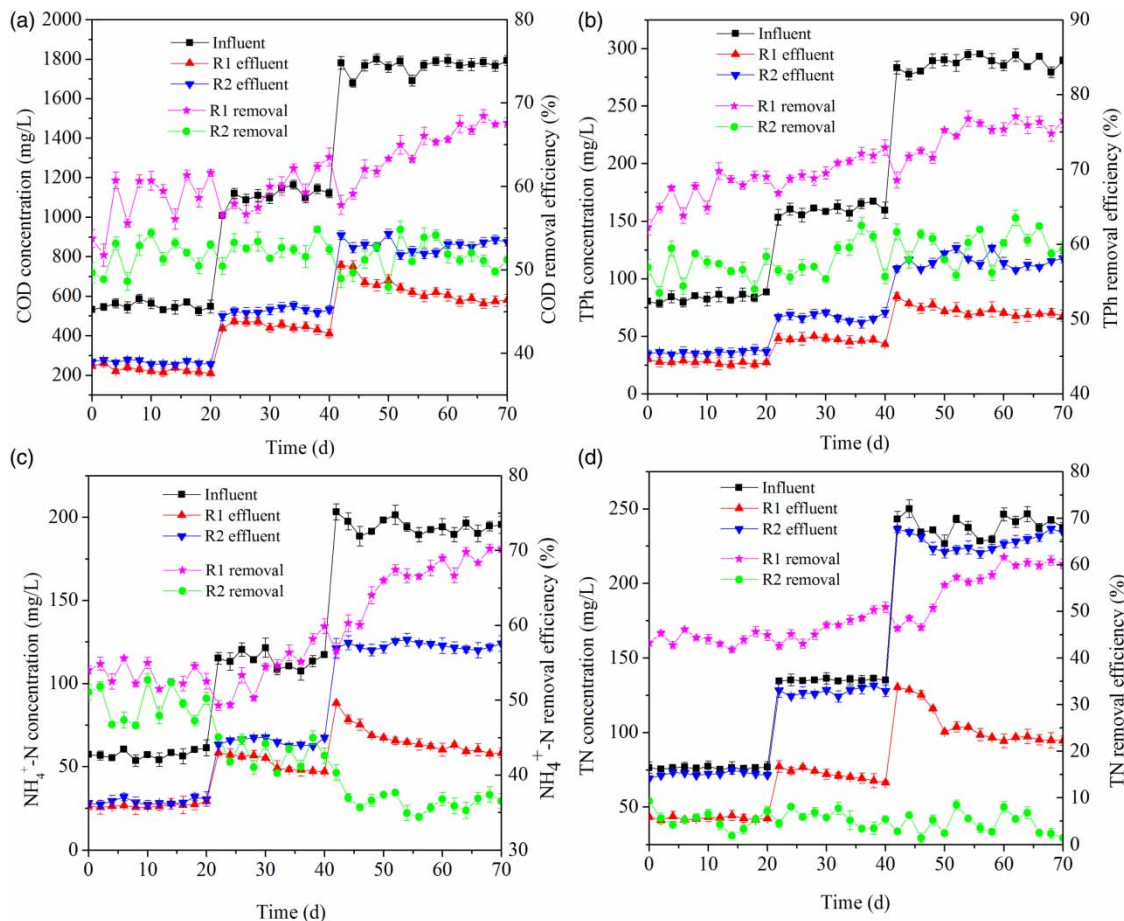


Figure 2 | Removal efficiencies of (a) COD, (b) TPh, (c) $\text{NH}_4^+\text{-N}$ and (d) TN in R1 and R2.

organics degradation in CGW treatment. Moreover, the removal of ammonia by heterotrophic nitrifying-aerobic denitrifying bacteria and the utilization of the organic carbon source were coupled. It was reported that electrons generated by the degradation of phenolic compounds can be transferred to ammonia monooxygenase to simultaneously remove ammonia. It is worth noting that an obviously higher TN removal efficiency ($57.95\% \pm 0.12\%$) was achieved in R1, while only $4.62\% \pm 0.11\%$ of TN was removed in R2, indicating that in R2 without *Pseudomonas* sp. HJ3 inoculated it was difficult to achieve the denitrification process. In the bioaugmentation system, bacteria are attached to the fillers to form a stable biofilm, which supplies a long residence time to retain large amounts of the inoculated strain in the reactor (Du et al. 2017b). Moreover, fillers can provide a gradient change of DO concentration from the outside to inner spaces, which can provide an anoxic microenvironment for denitrification (Körner & Zumft 1989). Accordingly, the SND bioaugmentation system could significantly strengthen simultaneous phenol and nitrogen removal in CGW treatment.

Nitrogen transformation and SND efficiency in SND bioaugmentation system

In order to further explore the SND mechanism in the bioaugmentation system, nitrogen transformation and SND efficiency were investigated. As plotted in Figure 3, $\text{NH}_4^+\text{-N}$ was significantly removed with a removal efficiency of $70.26\% \pm 0.13\%$ in R1. Meanwhile, $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ as the nitrification intermediates increased, but gradually decreased after 8 h. The reason for the accumulation and consumption of nitrification products might be that the ammonia was sufficient at the bacterial rapid growth stage, so *Pseudomonas* sp. HJ3 performed with stronger heterotrophic nitrification than aerobic denitrification, which resulted in the accumulation of nitrification products. With the decrease of ammonia concentration, the aerobic denitrification was stronger than the heterotrophic nitrification, so that the nitrification products were reduced. Moreover, SND efficiency reached 83.51%, indicating that $\text{NH}_4^+\text{-N}$ was effectively reduced to nitrogen gas in the SND bioaugmentation system. By contrast, the removal efficiency of $\text{NH}_4^+\text{-N}$ in R2 was only $38.32\% \pm 0.09\%$, and 19.76 ± 3.96 mg/L of $\text{NO}_2^-\text{-N}$ and 40.34 ± 4.31 mg/L of $\text{NO}_3^-\text{-N}$ were accumulated, resulting in an SND efficiency of only 17.79%. Evidently, the lower SND efficiency indicated that

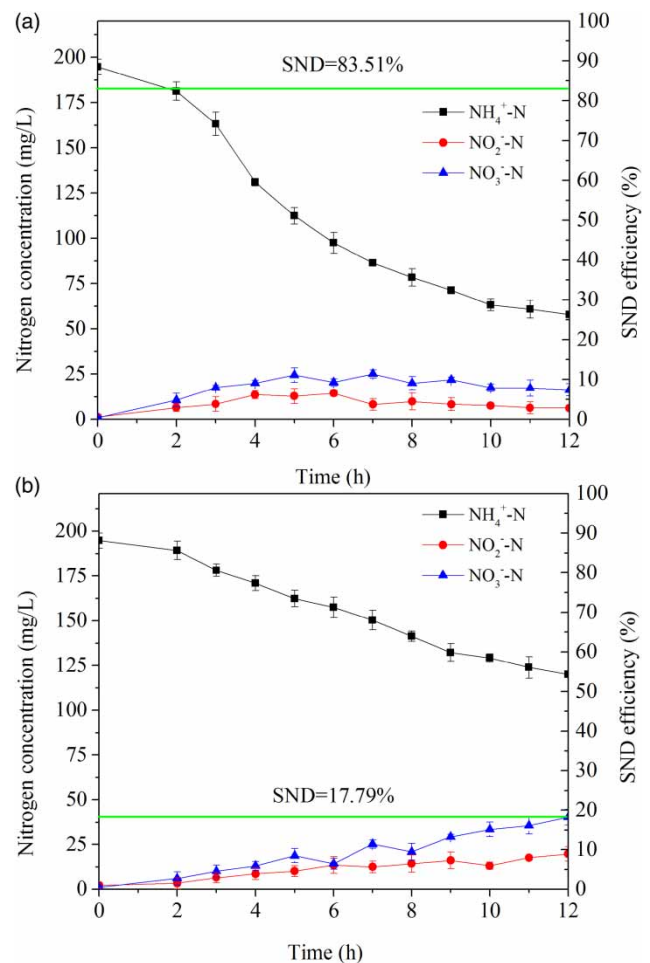


Figure 3 | The variations of $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations and SND efficiencies in a typical operating cycle of (a) R1 and (b) R2.

nitrogen removal in R2 possibly depended on microbial assimilation or filler absorption rather than denitrification. Moreover, the large accumulation of nitrification products may aggravate the potential toxicity to surrounding bacteria (Bancroft et al. 1979; Hao et al. 2016).

Nitrification tolerance characteristics under high phenol shock load

CGW is subjected to a high variability of phenol concentration due to fluctuations in the pre-treatment, which is known to inhibit nitrification (Fang et al. 2013). To evaluate the nitrification tolerance of the SND bioaugmentation system to phenol toxicity, the characteristic of nitrification efficiency was analyzed under phenol shock loads. As shown in Figure 4, when influent phenol concentration was increased from 300 ± 12.23 mg/L to 500 ± 14.32 mg/L, the performance of R1 was obviously disturbed. The removal

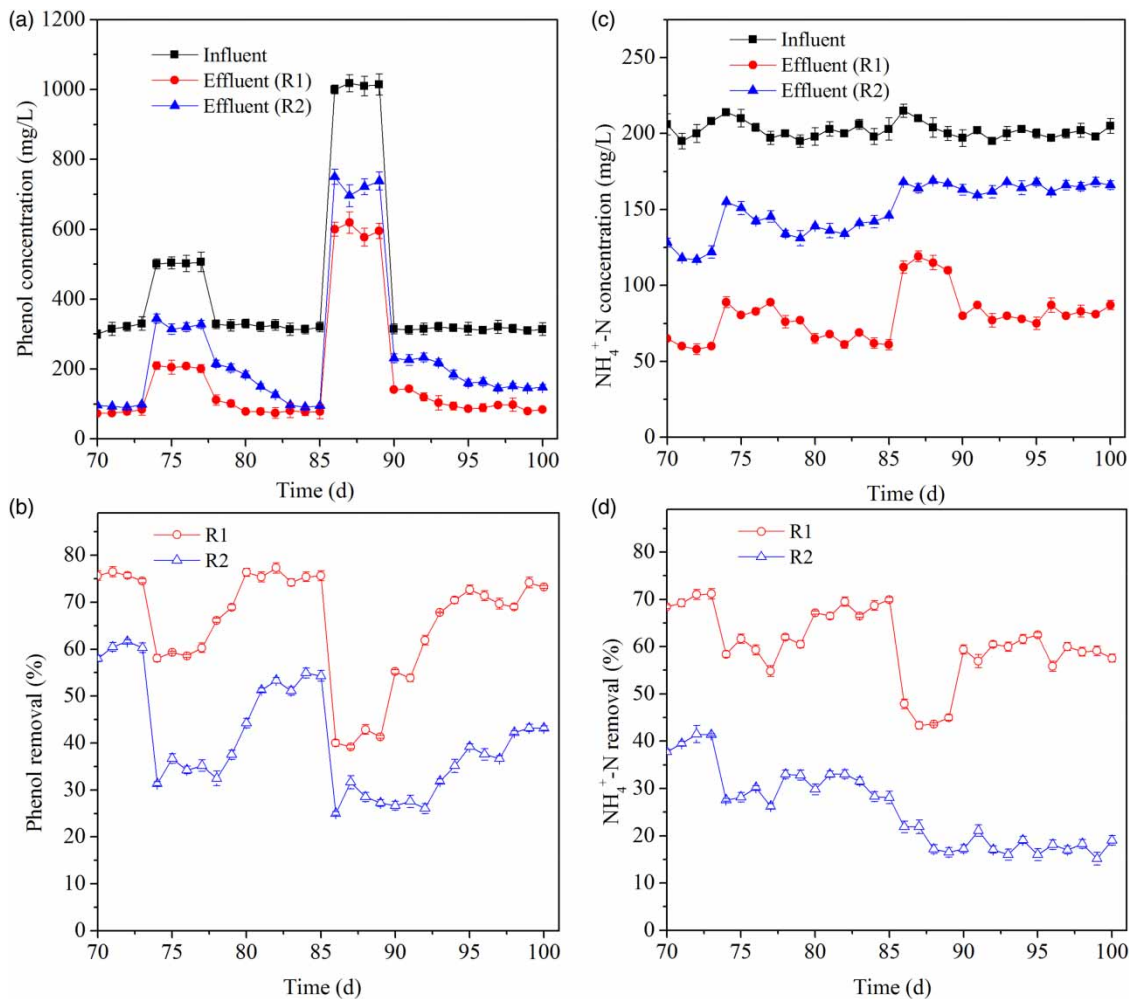


Figure 4 | Effect of high phenol shock loads on ammonia removal in R1 and R2.

efficiency of phenols decreased from $75.70\% \pm 1.03\%$ to $58.08\% \pm 0.31\%$. However, the system rapidly recovered to the initial level as the influent phenol concentration decreased to about 320 ± 16.40 mg/L. Meanwhile, $\text{NH}_4^+\text{-N}$ removal efficiency was $58.41\% \pm 0.26\%$ at a phenol concentration of 500 ± 14.37 mg/L, and gradually recovered to $74.21\% \pm 0.87\%$ as the phenol concentration decreased to 320 ± 16.40 mg/L within 4 d. When influent phenol concentration was further increased to $1,000 \pm 24.65$ mg/L, the removal efficiency of $\text{NH}_4^+\text{-N}$ in R1 rapidly decreased to $47.91\% \pm 1.01\%$ and gradually recovered to $60.51\% \pm 0.56\%$ with decreasing phenol concentration. By contrast, remarkable reductions of $\text{NH}_4^+\text{-N}$ and phenol removal efficiency were observed in R2. When phenol concentration increased to $1,000 \pm 24.65$ mg/L, the removal efficiency of $\text{NH}_4^+\text{-N}$ significantly decreased and could not be recovered to the initial level. Obviously, R2 performed with higher

sensitivity to phenol shocking. These results indicated that the SND bioaugmentation system performed with tremendous resistance and recovery capacity to phenol shocking, thereby resulting in an excellent performance for ammonia removal. In previous reports, the nitrification process by autotrophic nitrification bacteria could easily be inhibited at phenol concentrations as low as 10 mg/L (Pagga *et al.* 2006), while *Pseudomonas* sp. HJ3 was capable of heterotrophic nitrification and aerobic denitrification performed with higher phenol tolerance, which was favorable for the improvement of nitrification.

Biotoxicity reduction of CGW in SND bioaugmentation system

The results of the acute toxicity of influent and treated effluents of R1 and R2 are depicted in Table 1. The regression

Table 1 | Acute toxicity of *T. thermophila* exposed to raw influent and treated effluents from R1 and R2

Sample	24 h EC ₅₀ (%)	R ²	TU	Toxicity class
Influent	7.31	0.9978	13.68	Class IV high acute toxicity
Effluent				
R1	61.44	0.9922	1.63	Class III acute toxicity
R2	30.09	0.9997	3.32	Class III acute toxicity

analysis showed the R^2 value for each sample was higher than 0.99, indicating that *T. thermophila* was a suitable model organism for biotoxicity assessment of CGW. As shown in Table 1, the 24 h EC₅₀ value of the influent reached 7.31%. The acute TU value of the influent was 13.68, which could be classified as Class IV (high acute toxicity) according to the toxicity classification (Persoone et al. 2003). Apparently, CGW performs with strong acute toxicity to *T. thermophila* because of the high concentration of organic contaminants and ammonia that exist (Zheng et al. 2018). This indicates that the high toxicity of CGW has a negative effect on nitrogen removal. In contrast, the biotoxicity of CGW was significantly decreased through SND bioaugmentation treatment. As shown in Table 1, the 24 h EC₅₀ value of the R1 effluent was 61.44%, indicating that this effluent performed with slight toxic inhibition of *T. thermophila*. The acute TU value of the R1 effluent was only 1.63, which was effectively reduced by 88.08% compared with that of raw CGW. This suggests that a reduction in toxicity could be achieved in the SND bioaugmentation system. However, the effluent from R2 performed with relatively higher toxic effect on *T. thermophila*, and the corresponding 24 h EC₅₀ value and acute TU value were 30.09% and 3.32, respectively. This suggests that many toxic compounds were not degraded completely in R2, thus leading to a higher toxicity than the R1 effluent. Apparently, the SND bioaugmentation system significantly decreased the toxic inhibition of organisms owing to the efficient phenol and nitrogen removal in CGW.

Overall analysis of microbial community in SND bioaugmentation system

The diversity and richness of the microbial community

The species coverage and diversity of biofilm samples in R1 and R2 at the stable operating stage (65 d) were analyzed by high-throughput sequencing. As shown in Table 2, good coverage was found in R1 (0.9987) and R2 (0.9969). This indicates

Table 2 | The diversity and richness of microbial community in R1 and R2 at the stable operating stage (65 d)

Sample	Shannon	ACE	Chao 1	Coverage
R1	5.96	1,401.85	1,378.36	0.9987
R2	6.86	1,544.53	2,359.22	0.9969

that the sequencing results reliably reflect the characteristics of the microbial communities. The ACE index and Chao1 index are commonly used to evaluate the richness of species in a microbial community. The two indexes of R1 are lower than that of R2, indicating that microbial richness decreased slightly after inoculation of *Pseudomonas* sp. HJ3. The Shannon diversity index not only presents the simple species richness, but also describes the abundance of each species distribution. The Shannon index of R1 is 5.96, which is also lower than that of R2. The possible reason for relatively lower richness and diversity of microbial community in the SND bioaugmentation system is that inoculated *Pseudomonas* sp. HJ3 was predominant, which resulted in the elimination of other bacteria in competition.

Bacterial community characteristics in SND augmentation system

In order to explore the diversification of the bacterial community, the microbial communities in an SND bioaugmentation system and a control without *Pseudomonas* sp. HJ3 inoculated were analyzed. As shown in Figure 5, Proteobacteria was the largest phylum in R1 and R2 with relative abundances of 70.53% and 75.41%, respectively. Bacteroidetes and Firmicutes were the secondary dominant phyla in R1 and R2 (data not shown). It has been reported that Proteobacteria and Bacteroidetes are closely correlated with nitrification and denitrification (Chen et al. 2018). Firmicutes are helpful for degradation of organic pollutants and tolerance to extreme conditions (Fang et al. 2017). Obviously, there was no significant difference ($P > 0.05$) at phylum level between R1 and R2.

However, the bacterial communities at the genus level in R1 and R2 were distinctively different. The detailed description of bacterial community composition at genus level is summarized in Figure 5. The relative abundance of *Pseudomonas* in R1 was 18.74%, which was obviously higher than that in R2, attributed to inoculation of the *Pseudomonas* sp. HJ3 strain suspension. As shown in Figure 6, although the relative abundance of *Pseudomonas* sp. HJ3 gradually decreased during the operating process, its relative abundance was maintained at 14.83% in R1 at 65 d, which

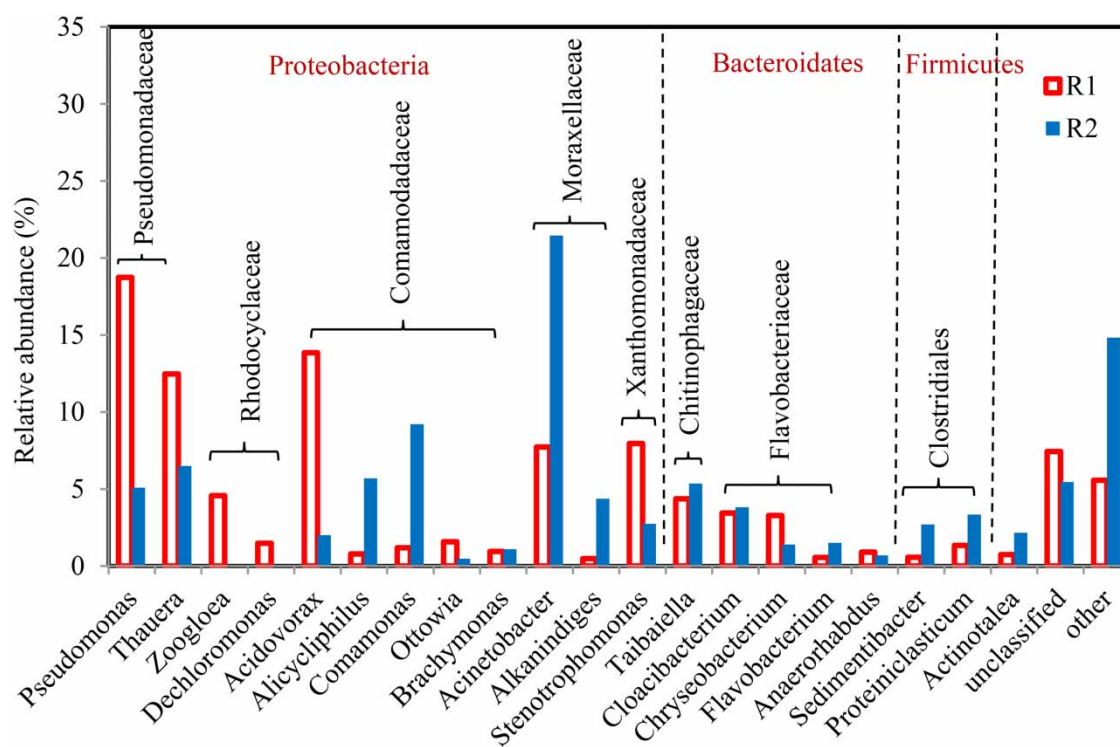


Figure 5 | The relative abundances of the microbial community at the genus level in R1 and R2 at the stable operation stage (65 d) (individual genera making up less than 1% of total composition in all samples are summed and indicated as 'other').

was significantly higher than that in R2. This indicates that *Pseudomonas* sp. HJ3 was the dominant strain in the SND bioaugmentation system, thereby facilitating the improvement of SND efficiency in the bioaugmentation system. Additionally, *Thauera* (12.47%), *Acidovorax* (13.85%) and *Stenotrophomonas* (7.96%) were the relatively higher abundance genera in R1 (Figure 5), which played a vital role in the nitrification and denitrification processes. Based on previous findings, *Thauera* are the main functional genus responsible for nitrogen removal and organic-compound degradation (Du et al. 2017b). *Acidovorax* are the common bacteria in wastewater treatment systems, which have been reported to be a novel aerobic denitrifying bacteria genus (Mergaert et al. 2001; Shen et al. 2013). In addition, the relative abundance of *Zoogloea* in R1 was 4.57%, which was significantly higher than that in R2. It has been found that *Zoogloea* species are favorable to the forming and holding of a biofilm (Gao et al. 2014). In contrast, *Acinetobacter* (21.46%), *Comamonas* (9.21%) and *Alicyclophilus* (5.70%) were dominant bacteria in R2, which are found to be capable of degrading phenolic compounds and polycyclic aromatic hydrocarbons in a wastewater treatment system (Ma et al. 2015b; Qu et al. 2015). However, indigenous nitrogen removal bacteria such

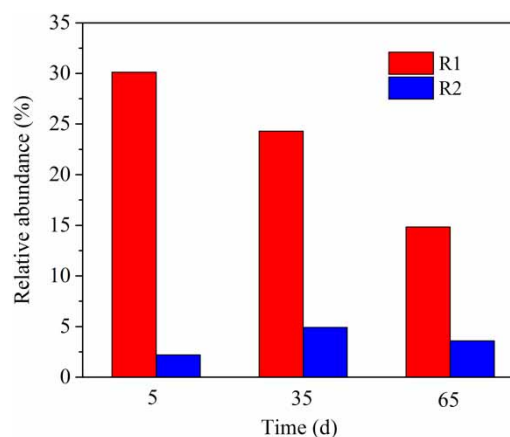


Figure 6 | The relative abundance of *Pseudomonas* strain HJ3 in R1 and R2 during the operating process.

as *Thauera* and *Acidovorax* accounted for only a small proportion in R2, which was a possible reason for the lower nitrogen removal efficiency in R2. It has been reported that a bioaugmentation system can change inoculated bacteria abundance and reform the indigenous microbial community structure (Zhao et al. 2016; Zhang et al. 2018). On the one hand, the enrichment of indigenous nitrogen removal bacteria might compete with *Pseudomonas* sp. HJ3, resulting

in a decrease in the abundance of *Pseudomonas* sp. HJ3. On the other hand, SND bioaugmentation with *Pseudomonas* sp. HJ3 inoculated would stimulate the growth of indigenous nitrogen removal bacteria, thereby offering a large biological nitrogen removal potential. Therefore, the interaction of *Pseudomonas* sp. HJ3 with indigenous nitrogen removal bacteria enhanced the nitrogen removal in the SND bioaugmentation system.

CONCLUSION

The SND bioaugmentation system with *Pseudomonas* sp. HJ3 inoculated achieved efficient simultaneous removal of phenols and nitrogen in CGW. The corresponding average removal efficiencies of COD, TPh, $\text{NH}_4^+\text{-N}$ and TN at the stable operating stage reached $64.07\% \pm 0.76\%$, $74.91\% \pm 0.33\%$, $67.96\% \pm 0.17\%$ and $57.95\% \pm 0.12\%$, when the concentration of influent COD, TPh and $\text{NH}_4^+\text{-N}$ was $1,765.94 \pm 27.43$ mg/L, 289.55 ± 10.32 mg/L and 187.25 ± 14.07 mg/L, respectively. Meanwhile, the maximum SND efficiency reached 83.51%. Moreover, the SND bioaugmentation reactor exhibited a better capability to resist fluctuations in influent phenol loading and reduce the toxic inhibition of organisms. Additionally, SND bioaugmentation with *Pseudomonas* sp. HJ3 inoculated altered the microbial community structure and established a new bacterial community balance *in situ* for enhancing nitrogen removal.

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SUPPLEMENTARY MATERIAL

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REFERENCES

APHA 1998 *Standard Methods for the Examination of Water and Wastewater*, 20th edn. APHA, Washington, DC, USA.

- Bancroft, K., Grant, I. F. & Alexander, M. 1979 Toxicity of NO_2^- : effect of nitrite on microbial activity in an acid soil. *Applied and Environmental Microbiology* **38** (5), 940–944.
- Chen, Q., Ni, J. R., Ma, T., Liu, T. & Zheng, M. S. 2015 Bioaugmentation treatment of municipal wastewater with heterotrophic-aerobic nitrogen removal bacteria in a pilot-scale SBR. *Bioresource Technology* **183**, 25–32.
- Chen, J., Gu, S., Hao, H. & Chen, J. 2016 Characteristics and metabolic pathway of *Alcaligenes* sp. TB for simultaneous nitrification–aerobic denitrification. *Applied Microbiology and Biotechnology* **100** (22), 9787–9794.
- Chen, H., Li, A., Wang, Q., Cui, D., Cui, C. W. & Ma, F. 2018 Nitrogen removal performance and microbial community of an enhanced multistage A/O biofilm reactor treating low-strength domestic wastewater. *Biodegradation* **29** (3), 285–299.
- Du, C., Cui, C. W., Qiu, S., Shi, S. N., Li, A. & Ma, F. 2017a Nitrogen removal and microbial community shift in an aerobic denitrification reactor bioaugmented with a *Pseudomonas* strain for coal-based ethylene glycol industry wastewater treatment. *Environmental Science and Pollution Research* **24** (12), 11435–11445.
- Du, C., Cui, C. W., Qiu, S., Xu, S. W., Shi, S. N., Sangeetha, T. & Ma, F. 2017b Microbial community shift in a suspended stuffing biological reactor with pre-attached aerobic denitrifier. *World Journal of Microbiology Biotechnology* **33** (7), 148.
- Fang, F., Han, H., Zhao, Q., Xu, C. & Zhang, L. 2013 Bioaugmentation of biological contact oxidation reactor (BCOR) with phenol-degrading bacteria for coal gasification wastewater (CGW) treatment. *Bioresource Technology* **150**, 314–320.
- Fang, D. X., Zhao, G., Xu, X. Y., Zhang, Q., Shen, Q. S., Fang, Z. Y., Huang, L. P. & Ji, F. Y. 2017 Microbial community structures and functions of wastewater treatment systems in plateau and cold regions. *Bioresource Technology* **249**, 684–693.
- Gao, C., Wang, A., Wu, W. M., Yin, Y. & Zhao, Y. G. 2014 Enrichment of anodic biofilm inoculated with anaerobic or aerobic sludge in single chambered air-cathode microbial fuel cells. *Bioresource Technology* **167** (3), 124–132.
- Hao, T. W., Mackey, H. R., Guo, G., Liu, R. L. & Chen, G. H. 2016 Resilience of sulfate-reducing granular sludge against temperature, pH, oxygen, nitrite, and free nitrous acid. *Applied Microbiology and Biotechnology* **100** (19), 8563–8572.
- Herrero, M. & Stuckey, D. C. 2015 Bioaugmentation and its application in wastewater treatment: a review. *Chemosphere* **140**, 119–128.
- Jia, S. Y., Han, H. J., Zhuang, H. F., Hou, B. L. & Li, K. 2015 Impact of high external circulation ratio on the performance of anaerobic reactor treating coal gasification wastewater under thermophilic condition. *Bioresource Technology* **192**, 507–513.
- Jia, S. Y., Han, H. J., Zhuang, H. F. & Hou, B. L. 2016 The pollutants removal and bacterial community dynamics relationship within a full-scale British Gas/Lurgi coal gasification wastewater treatment using a novel system. *Bioresource Technology* **200**, 103–110.

- Joo, H. S., Hirai, M. & Shoda, M. 2006 Piggery wastewater treatment using *Alcaligenes faecalis* strain No. 4 with heterotrophic nitrification and aerobic denitrification. *Water Research* **40** (16), 3029–3036.
- Kim, Y. M., Park, D., Lee, D. S. & Park, J. M. 2008 Inhibitory effects of toxic compounds on nitrification process for cokes wastewater treatment. *Journal of Hazardous Materials* **152** (3), 915–921.
- Körner, H. & Zumft, W. G. 1989 Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. *Applied and Environmental Microbiology* **55** (7), 1670–1676.
- Kulkarni, P. M. 2012 Effect of shock and mixed loading on the performance of SND based sequencing batch reactors (SBR) degrading nitrophenols. *Water Research* **46** (7), 2405–2414.
- Lei, Y., Wang, Y. Q., Liu, H. J., Xi, C. W. & Song, L. Y. 2016 A novel heterotrophic nitrifying and aerobic denitrifying bacterium, *Zobellella taiwanensis* DN-7, can remove high-strength ammonium. *Applied Microbiology and Biotechnology* **100** (9), 4219–4229.
- Li, H. Q., Han, H. J., Du, M. A. & Wang, W. 2011 Inhibition and recovery of nitrification in treating real coal gasification wastewater with moving bed biofilm reactor. *Journal of Environmental Sciences* **23** (4), 568–574.
- Li, C., Yang, J., Wang, X., Wang, E., Li, B., He, R. & Yuan, H. 2015 Removal of nitrogen by heterotrophic nitrification–aerobic denitrification of a phosphate accumulating bacterium *Pseudomonas stutzeri* YG-24. *Bioresource Technology* **182**, 18–25.
- Liu, Y., Hu, T., Song, Y., Chen, H. & Lv, Y. 2015 Heterotrophic nitrogen removal by *Acinetobacter* sp. Y1 isolated from coke plant wastewater. *Journal of Bioscience & Bioengineering* **120** (5), 549–554.
- Lu, H., Weng, Z., Wei, H., Zhou, J., Wang, J., Liu, G. & Guo, W. 2017 Simultaneous bisphenol F degradation, heterotrophic nitrification and aerobic denitrification by a bacterial consortium. *Journal of Chemical Technology & Biotechnology* **92** (4), 854–860.
- Ma, F., Sun, Y. L., Li, A., Zhang, X. N. & Yang, J. X. 2015a Activation of accumulated nitrite reduction by immobilized *Pseudomonas stutzeri* T13 during aerobic denitrification. *Bioresource Technology* **187**, 30–36.
- Ma, Q., Qu, Y. Y., Shen, W. L., Zhang, Z. J., Wang, J. W., Liu, Z. Y., Li, D. X., Li, H. J. & Zhou, J. T. 2015b Bacterial community compositions of coking wastewater treatment plants in steel industry revealed by Illumina high-throughput sequencing. *Bioresource Technology* **179**, 436–443.
- Ma, W., Han, Y., Ma, W., Han, H., Zhu, H., Xu, C., Li, K. & Wang, D. 2017 Enhanced nitrogen removal from coal gasification wastewater by simultaneous nitrification and denitrification (SND) in an oxygen-limited aeration sequencing batch biofilm reactor. *Bioresource Technology* **244** (Pt 1), 84–91.
- Mergaert, J., Boley, A., Cnockaert, M. C., Müller, W. R. & Swings, J. 2001 Identity and potential functions of heterotrophic bacterial isolates from a continuous-upflow fixed-bed reactor for denitrification of drinking water with bacterial polyester as source of carbon and electron donor. *Systematic and Applied Microbiology* **24** (2), 303–310.
- Pagga, U., Bachner, J. & Strotmann, U. 2006 Inhibition of nitrification in laboratory tests and model wastewater treatment plants. *Chemosphere* **65** (1), 1–8.
- Persoone, G., Marsalek, B., Blinova, I., Törökne, A., Zarina, D., Manusadzianas, L., Nalecz-Jawecki, G., Tofan, L., Stepanova, N., Tothova, L. & Kolar, B. 2003 A practical and user-friendly toxicity classification system with microbiotests for natural waters and wastewaters. *Environmental Toxicology* **18** (6), 395–402.
- Qu, Y., Ma, Q., Deng, J., Shen, W., Zhang, X., He, Z., van Nostrand, J. D., Zhou, J. & Zhou, J. 2015 Responses of microbial communities to single-walled carbon nanotubes in phenol wastewater treatment systems. *Environmental Science & Technology* **49** (7), 4627–4635.
- Shen, Z. Q., Zhou, Y. X. & Wang, J. L. 2013 Comparison of denitrification performance and microbial diversity using starch/poly(lactic acid) blends and ethanol as electron donor for nitrate removal. *Bioresource Technology* **131**, 33–39.
- Third, K. A., Burnett, N. & Cord-Ruwisch, R. 2003 Simultaneous nitrification and denitrification using stored substrate (PHB) as the electron donor in an SBR. *Biotechnology & Bioengineering* **83** (6), 706–720.
- Wang, W., Ma, W., Han, H., Li, H. & Yuan, M. 2011 Thermophilic anaerobic digestion of Lurgi coal gasification wastewater in a UASB reactor. *Bioresource Technology* **102** (3), 2441–2447.
- Wang, W., Zhang, J., Wang, S., Shen, J. & Pan, S.-L. 2014 Oxygen-limited aeration for relieving the impact of phenolic compounds in anaerobic treatment of coal gasification wastewater. *International Biodeterioration & Biodegradation* **95**, 110–116.
- Yang, L., Ren, Y.-X., Zhao, S.-Q., Liang, X. & Wang, J.-P. 2015 Isolation and characterization of three heterotrophic nitrifying–aerobic denitrifying bacteria from a sequencing batch reactor. *Annals of Microbiology* **66** (2), 737–747.
- Yang, J. X., Zhao, B., An, Q., Huang, Y. S. & Guo, J. S. 2018 Bioaugmentation with *A. faecalis* strain NR for achieving simultaneous nitrogen and organic carbon removal in a biofilm reactor. *Bioresource Technology* **247**, 871–880.
- Yoo, H., Ahn, K. H., Lee, H. J., Lee, K. H., Kwak, Y. J. & Song, K. G. 1999 Nitrogen removal from synthetic wastewater by simultaneous nitrification and denitrification (SND) via nitrite in an intermittently-aerated reactor. *Water Research* **33** (1), 145–154.
- Yu, D. Y., Yang, M. Y., Bo, G. & Liu, W. C. 2011 Disposal of domestic sewage by heterotrophic nitrification–aerobic denitrifier bacteria immobilized with bacterial cellulose membrane. *Fresenius Environmental Bulletin* **20** (2), 398–404.
- Zhang, Y., Shi, Z., Chen, M., Dong, X. & Zhou, J. 2015 Evaluation of simultaneous nitrification and denitrification under controlled conditions by an aerobic denitrifier culture. *Bioresource Technology* **175**, 602–605.
- Zhang, S. S., Sun, X. B., Wang, X. M., Qiu, T. L., Gao, M., Sun, Y. M., Cheng, S. T. & Zhang, Q. J. 2018 Bioaugmentation with

- Diaphorobacter polyhydroxybutyrativorans* to enhance nitrate removal in a poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-supported denitrification reactor. *Bioresource Technology* **263**, 499–507.
- Zhao, Q., Han, H., Xu, C., Zhuang, H., Fang, F. & Zhang, L. 2013 Effect of powdered activated carbon technology on short-cut nitrogen removal for coal gasification wastewater. *Bioresource Technology* **142**, 179–185.
- Zhao, Q., Han, H. J., Hou, B. L., Zhuang, H. F., Jia, S. Y. & Fang, F. 2014 Nitrogen removal from coal gasification wastewater by activated carbon technologies combined with short-cut nitrogen removal process. *Journal of Environmental Sciences* **26**, 2231–2239.
- Zhao, X. Y., Yang, J. X., Bai, S. W., Ma, F. & Wang, L. 2016 Microbial population dynamics in response to bioaugmentation in a constructed wetland system under 10°C. *Bioresource Technology* **205**, 166–173.
- Zheng, M., Han, Y., Xu, C., Han, H. & Zhang, Z. 2018 Discrimination of typical cyclic compounds and selection of toxicity evaluation bioassays for coal gasification wastewater (CGW) based on toxicity mechanism of actions (MOAs). *Science of The Total Environment* **644**, 324–334.
- Zhu, Q. S., Guo, S. H., Guo, C. M., Dai, D., Jiao, X. K., Ma, T. Q. & Chen, J. F. 2014 Stability of Fe–C micro-electrolysis and biological process in treating ultra-high concentration organic wastewater. *Chemical Engineering Journal* **255**, 535–540.

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