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RESEARCH ARTICLE

Effect of nitrobenzene on the performance and bacterial community in an expanded granular sludge bed reactor treating high-sulfate organic wastewater

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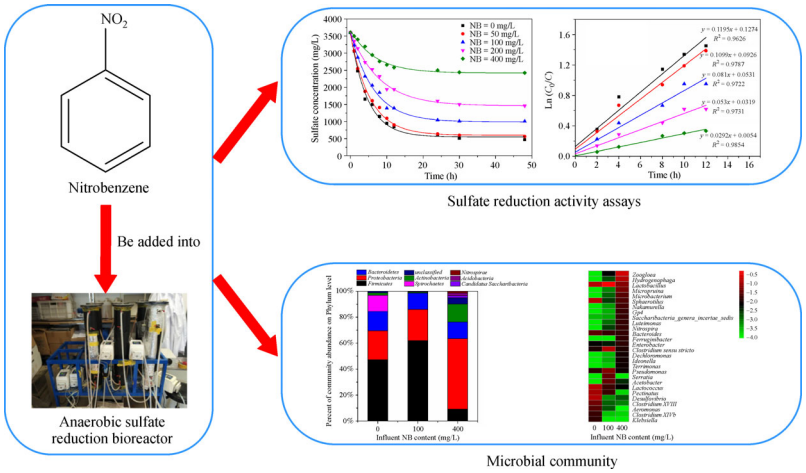
HIGHLIGHTS

- Less than 50 mg/L nitrobenzene brought little effect on anaerobic sulfate reduction.
- Kinetics of sulfate reduction under different nitrobenzene contents was studied.
- Increased nitrobenzene contents greatly changed the bacterial community structure.
- Genus *Desulfovibrio* played the key role in anaerobic sulfate reduction process.

GRAPHIC ABSTRACT



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ABSTRACT

Nitrobenzene (NB) is frequently found in wastewaters containing sulfate and may affect biological sulfate reduction process, but information is limited on the responses of sulfate reduction efficiency and microbial community to the increased NB contents. In this study, a laboratory-scale expanded granular sludge bed reactor was operated continuously to treat high-sulfate organic wastewater with increased NB contents. Results successfully demonstrated that the presence of more than 50 mg/L NB depressed sulfate reduction and such inhibition was partly reversible. Bath experiments showed that the maximum specific desulfuration activity (SDA) decreased from 135.80 mg SO_4^{2-}/gVSS/d to 30.78 mg SO_4^{2-}/gVSS/d when the NB contents increased from none to 400 mg/L. High-throughput sequencing showed that NB also greatly affected bacterial community structure. *Bacteroidetes* dominated in the bioreactor. The abundance of *Proteobacteria* increased with NB addition while *Firmicutes* presented an opposite trend. *Proteobacteria* gradually replaced *Firmicutes* for the dominance in response to the increase of influent NB concentrations. The genus *Desulfovibrio* was the dominant sulfate-reducing bacteria (SRB) with absence or presence of NB, but was inhibited under high content of NB. The results provided better understanding for the biological sulfate reduction under NB stress.

1 Introduction

Wastewater generated from industries of chemical and pharmaceutical synthesis is typically characterized with high concentrations of sulfate as well as bulky toxic organic compounds. Although sulfate itself is a kind of innocuous compound, excessive discharge of sulfate-containing wastewater could destroy the aquatic ecosystems of the receiving water bodies by affecting the natural

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sulfur cycle (Kuscu and Sponza, 2009b). The sulfate reduction process that the ubiquitous sulfate-reducing bacteria (SRB) utilize sulfate in huge amounts to get energy and produce the sulfide which could lead to incidences of acidification, blackening and unpleasant rotten-egg odor in natural water bodies or sediments. In addition, hydrogen sulfide from SRB could also contribute to the biogenic sulfide corrosion of concrete or metals in engineered systems (Huang et al., 2012). Hence, many industrial sections are embracing efficient treatment technologies for wastewater containing high-sulfate and high chemical oxygen demand (COD). Due to its eco-friendly and cost-competitive performance, anaerobic biological treatment of sulfate-rich wastewaters has drawn great attentions.

It is common that chemical and pharmaceutical wastewater ubiquitously consists of plenty of hazardous ingredients, e.g. nitrobenzene (NB), chlorobenzene and their derivatives, which could seriously affect the sulfate reduction and COD removal of anaerobic system (Zhang et al., 2015a). The degradation process of NB in anaerobic condition can produce intermediate products, such as phenylamine, which are toxic and their toxic inhibition to SRB should not be ignored (Kuscu and Sponza, 2009a). Sulfate, as a common electron acceptor, might in turn affect the anaerobic transformation of refractory nitro-aromatic compounds (NACs) (Ismail and Pavlostathis, 2010). For instance, Huang et al. (2012) showed that in anaerobic systems, organic substances could be transformed to volatile fatty acids and then serve as electron donors for a few contaminants, including both NACs and sulfate. Therefore, NACs are able to compete for substrate with sulfate. The antagonisms between toxic hazardous compounds and sulfate may deteriorate the sulfate reduction performance as well as COD removal efficiency. Therefore, to explore the effect of NACs on sulfate-reducing process in the sulfate reducing system makes sense to practical engineering application.

Several kinds of anaerobic bioreactors have been reported for sulfate-rich wastewater treatment, e.g., anaerobic filters, anaerobic sequencing batch reactors, up-flow anaerobic fixed-bed reactors, up-flow anaerobic sludge bed reactors and expanded granular sludge bed reactors (EGSB) (Guerrero-Barajas et al., 2014). By a high height to diameter ratio and recirculation of effluent, EGSB reactor is capable of increasing the transfer of substrates and biomass by high liquid up-flow velocity. Therefore, the resistance to shock loading of hazardous organic compounds was enhanced, resulting in the wide application of EGSB in the treatment of various wastewaters containing high sulfate and COD. Previous studies on the influences of NACs on anaerobic process mainly concentrated on the influence of the electron donor, e.g. H_2, acetate or propionate (Huang et al., 2012) and humic acid (Dunnivant and Reynolds, 2007), and the optimization of reductive conditions (Zhang et al., 2015a). Generally, these studies are under low concentration of sulfate (Bernardez et al., 2012; Huang et al., 2012), usually less than 1000 mg/L, which might not represent well the sulfate reduction performance for chemical and pharmaceutical wastewater. It is also noteworthy that NACs might bring about bacterial community shift, consequently decreasing the performance of anaerobic reactors. However, there are limited knowledges on how the EGSB reactors that treat wastewater with high concentrations of sulfate resist to NACs stress through shifts in its bacterial community structure.

To this end, NB, as a representative NAC, was spiked into an EGSB reactor treating high concentration of sulfate (3600 mg/L) wastewater with influent NB concentrations from 0 to 400 mg/L. Bacterial community by high-throughput sequencing, mainly desulfurization bacteria populations, as well as the removal efficiency of COD and sulfate was investigated. The results may provide better understanding for biological sulfate reduction under NB stress.

2 Materials and methods

2.1 Reactor setup and operation

The EGSB reactor applied in this study had a working volume of 1.96 L and operated at 35±1°C by a water bath (Fig. S1 in Supplementary Materials). A three-phase separator at the top of the reactor was installed to maintain the biomass from running off. A proper up-flow velocity (V_{up}) was provided by external hydraulic circulation. The EGSB reactor was previously operated for the treatment of high concentration of sulfate wastewater (COD 9000mg/L and SO_4^{2-} 3600 mg/L). The sulfate wastewater was synthetic wastewater in which Na_2SO_4 provided sulfate and glucose was the major carbon sources to keep sulfate reduction. The synthetic wastewater also contained NH_4Cl, KH_2PO_4, $NaHCO_3$ and trace elements. The ratio of C:N:P was kept at 200:5:1. $NaHCO_3$ was used to keep the pH of the influent to 7.0–7.5. The components of trace elements were prepared according to our previous study (Liao et al., 2013). In addition, the influent COD/SO_4^{2-} ratios was maintained at 2.5 based on the previous studies that the ratio should be no less than 2 to ensure the anaerobic sulfate reduction performance unaffected by lack of carbon source (Wei et al., 2007; Dar et al., 2008; Zhou et al., 2014; Lu et al., 2016). The reactor was operated with V_{up} (1.5 m/h), hydraulic retention time (HRT) (24 h) and pH (7.0–7.5) of influent according to previous parameters optimization experiments.

Afterwards, in this study, to explore the effect of NB on the sulfate reducing system, NB working as external stress was spiked into the EGSB reactor maintaining influent COD of 9000 mg/L and SO_4^{2-} of 3600 mg/L. The EGSB reactor was further successively operated for 156 days and was divided into nine stages with different NB contents

from 0 to 400 mg/L (final NB contents were 0, 5, 10, 20, 50, 100, 200, 400 and 100 mg/L).

2.2 Batch experiments

To assess the influence of NB concentration on sulfate reduction activity, the specific desulfuration activity (SDA) of the biomass was determined by batch experiments under different influent NB concentrations. For each stage, 100 ml sludge mixture was taken out from the reactor, centrifuged and washed with deionized water by three times. Then, 400 ml synthetic wastewater was fed to the sludge for batch experiment. Prior to the batch experiment, nitrogen gas was used to get rid of the air in each conical flask. Batch experiment was conducted by a digital water bath temperature oscillator (THZ-82, HongHua instrument plant, China), at 120 rpm and 35°C. The initial concentration of sulfate and COD were 3600 mg/L and 9000 mg/L, respectively. Sampling was taken at a predetermined time interval and then centrifuged before measurement.

The first-order kinetic model was used to simulate the kinetics of sulfate reduction (Eswari et al., 2016). It was calculated as follows (Shen et al., 2016):

$$-\mathrm{d}C_t/\mathrm{d}t = kC_t, \quad (1)$$

where $\mathrm{d}C_t/\mathrm{d}t$ (mg/L/h) is the sulfate reduction rate, k is the reduction rate constant and t (h) is the reaction time. If the initial concentration of sulfate is C_0, the model can be expressed as follows:

$$\ln C_0/C_t = kt + b. \quad (2)$$

By plotting $\ln C_0/C_t$ versus t, the slope and intercept get corresponded to the values of k and b, respectively. And SDA of anaerobic sludge were calculated according to the values of k and VSS of sludge in each batch experiment.

2.3 Microbial structure analysis

Sludge samples were collected from the bottom of the bioreactor while each period reached a stable state. The collected sludge samples should immediately be fixed by 100% ethanol a 1:1 (v/v) and then stored at −20°C. DNA was extracted with FastDNA Soil Kit (MP Biomedicals, CA, USA) and thereafter amplified by the primers 27F/338R for bacteria (Luo et al., 2016) (Table S1). The volume of the PCR reactions was 50 μL, with 25 μL 2 × EasyTaq® PCR SuperMix (TransGen Biotech, Beijing, China), 2 μL of each primer (10 μM), 40 ng of template DNA (20 ng/mL) and 21 μL of ddH_2O. The amplification of DNA was conducted by the following steps: preheating, 95°C for 3 min; denaturation, 27 cycles of 95°C for 30 s; annealing, 55°C for 30 s; elongation, 72°C for 45 s, and at last, an extension at 72°C for 10 min. High-throughput sequencing was operated at Zhongyijinda Analytical & Testing Co., Ltd. (Yixing, Jiangsu, China) on Illumina Mi-seq platform. The DNA test sequences were split by Mothur (http://www.mothur.org/) according to the nucleotide bar code. Raw reads were denoised by Sickle software (https://github.com/najoshi/sickle) to remove sequences containing degenerate base or shorter than 20 bps. The sequencing reads were denoised by Mothur. Taxonomic classification of the bacterial sequences of different samples at the same sequencing depth was transmitted to RDP (http://rdp.cme.msu.edu/). 80% confidence intervals recommended by the RDP was used to strictly assign the sequences to different levels (Ye et al., 2011). The richness and diversity indices including operational taxonomic units (OTUs), Chaos, Shannon Index, abundance-based coverage estimation (ACE) and Simpson, were calculated by Mothur at 3% cutoff level.

2.4 Other chemical analysis

The concentrations of NB in the wastewater were measured by chromatography-mass spectrometer (GC-MS) (TRACE GC Ultra GC and ISQ MS, Thermal Fisher, USA). The GC/MS was equipped with a Thermo Scientific Trace GOLD GC Column (30 m in length, inner diameter 0.25 mm, and a coating thickness of 0.25 μm). Helium (purity 99.999%) was used as the carrier gas at a flow rate of 1.0 mL/min. The column temperature was first kept at 35°C for 2 min, raised to 150°C by 1°C min^{-1}, then increased to 280°C with 20°C min^{-1}, and lastly to 300°C for 10 min. Sulfate concentrations were measured by ion chromatography (ICS-1100, Thermal Fisher, USA) equipped with a self-regenerating suppressor and a conductivity detector. A DX Ionpac AS11-HC analytical column (4 mm × 250 mm) and an AG11-HC guard column (4 mm × 50 mm) were used. The unit was run by auto-suppression mode with 20 mM KOH eluent at a flow rate of 1.0 mL/min. The concentrations of soluble chemical oxygen demand and sulfide was measured by using the Standard Method.

3 Results and discussion

3.1 Performance of the EGSB reactor

Figure 1 shows the performances of EGSB reactor in terms of sulfate reduction efficiency with varied influent NB concentrations. The sulfate reduction efficiency was 80.5±2.1% without NB addition in Phase I with influent COD concentration of 9000 mg/L and sulfate of 3600 mg/L. Sulfate removal efficiency remained approximately 80% from phase II to V when influent NB concentration increased from 5 to 50 mg/L, indicating that low NB stress had little effect on sulfate reduction. However, there was an obvious decrease in sulfate reduction efficiency from 78.2% to 63.6% with the

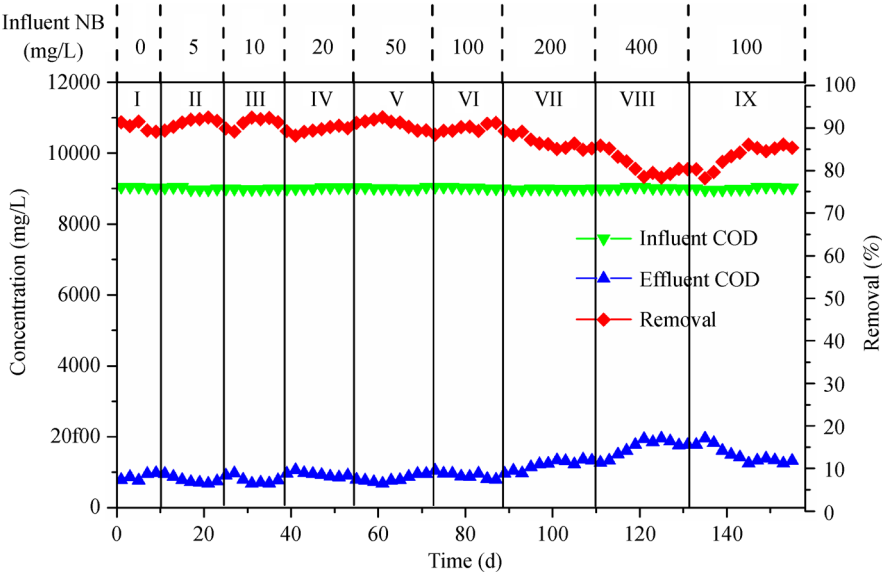

Fig. 1 SO_4^{2-} removal performances of EGSB reactor with different influent NB concentrations.

increase of influent NB from 50 to 100 mg/L in phase VI, which might be caused by the toxicity of NB to SRB, restraining the sulfate-reduction related enzymes. Only 20.3±1.2% reduction was achieved with influent NB content further up to 400 mg/L, indicating that sulfate reduction process was severely inhibited under this condition. Finally, the influent NB content was further reduced to 100 mg/L and a partly recovery of sulfate reduction was observed, with sulfate reduction efficiency recovered from 20.3% to 40.6%, which suggested that the effect of NB on sulfate reduction is recoverable.

Figure 2 shows the changes of COD as well as its removal efficiency in EGSB reactor with increased NB contents. The COD removal showed a similar trend with the sulfate reduction. The COD removal efficiency kept between 88% and 93% in the initial six stages until influent NB concentration was raised to 200 mg/L. Furthermore, the COD removal efficiency slightly dropped to 79.5% with influent NB at 400 mg/L. Finally, influent NB concentration was reduced to 100 mg/L, leading to a partly recovery of COD removal increased to 85.2%.

From the above, it was proposed that when EGSB bioreactor was used to treat high-sulfate organic wastewater, the influent NB content should be less than 50 mg/L in order for good performance. The tolerance concentration of SRB to NB was higher than that in the research by Huang et al. (2012) in which presence of 250 μmol/L NB (30.78 mg/L) inhibited sulfate reduction. The cultivated bacterial communities in the EGSB reactor have high tolerance to shock load and toxicity, helping to reduce the adverse effect of NB. In addition, the transfer of substrates and biomass by high liquid up-flow velocity in EGSB enhanced the resistance to shock loading of NB. The sulfate reduction efficiency was extremely low when the influent NB increased to 400 mg/L, which may mainly be caused by the toxicity inhibition of NB. In addition, it have been reported that the toxic effect was not only caused by NB, but also by aniline, the intermediate products of NB degradation, and some other toxic intermediates (Sponza and Kuscu, 2011). However, the inhibition of increasing NB concentration on COD was less compared to that on sulfate, which showed that the inhibition of NB to sulfate reduction was more serious than to organic matters degradation. In the last period, with the weakening of NB stress, both sulfate and COD removal efficiency were recovered, suggesting that the inhibition of NB to microbial organisms was partly reversible, but the sulfate reduction efficiency was only 63.6% of that in Stage VI, which meant some SRB cannot survive in high-NB wastewater. Previous study has indicated that the presence of NB did not destroy cells of functional microorganism, but inhibited transformation and synthesis of some related enzymes (Ji et al., 2010), so the performances of the bioreactor could be recovered after the concentration of NB was reduced.

3.2 Sulfate reduction in batch experiments

Figure 3 displays the time course profiles of sulfate removal efficiency and first-order kinetics model of sulfate reduction process. As shown in Fig. 3(a), sulfate concentrations decreased with the contact time and NB contents. The reduction efficiency of sulfate was found as the highest without NB addition with a removal efficiency of 87.0%. In the experiment of 50 mg/L NB, the sulfate removal efficiency was 84.4%, which meant 50 mg/L NB had little effect on sulfate reduction. However, in the batch experiments with 100, 200 and 400 mg/L NB added, the

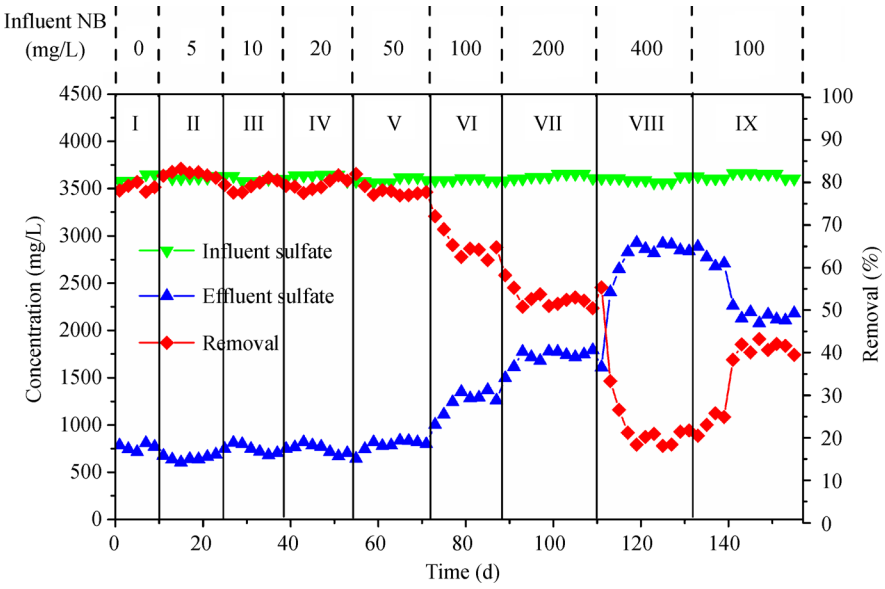

Fig. 2 COD removal performances of EGSB reactor with different influent NB concentrations.

reduction efficiency of sulfate was found as 72.0%, 59.5% and 32.7%, respectively. These results were in consistent with those of continuous experiment, which indicated that more than 50 mg/L NB could depress sulfate reduction.

According to Eq. (1), the slope k could be obtained by plotting $\ln C_0/C$ versus t, which corresponded to reduction rate constant. The experimental data with different NB concentrations were all in accord with the first-order kinetic model during the initial 12 h (Fig. 3(b)). The reduction rate constant was 0.1195 gSO_4^{2-}/h without NB addition, higher than those at NB contents of 50 mg/L (0.1099 gSO_4^{2-}/h), 100 mg/L (0.081 gSO_4^{2-}/h), 200 mg/L (0.053 gSO_4^{2-}/h) and 400 mg/L (0.0292 gSO_4^{2-}/h). Moreover, it can be seen from Fig. 3b that coefficient (R^2) in batch experiments were all above 0.96, indicating that this model could well reflect the initial stage of real sulfate reduction process.

The SDA were calculated by the values of k and the measured VSS of sludge which are presented in Table 1. A maximum SDA of 135.80 mg SO_4^{2-}/gVSS/d was obtained without NB addition, higher than that of Lopes et al. (2007), in which the maximum SDA was 57 mg SO_4^{2-}/gVSS/d. This may because the higher initial sulfate concentration in this study stimulated the growth of bacteria (Lopes et al., 2010). Once it was fed with 50 mg/L NB, the SDA decreased to 116.91 mg SO_4^{2-}/gVSS/d. The SDA kept steadily dropping to 84.12 and 59.36 mg SO_4^{2-}/gVSS/d with NB content of 100 and 200 mg/L, respectively. The lowest SDA was 30.78 mg SO_4^{2-}/gVSS/d with 400 mg/L NB which was only 22.66% of that without NB fed, indicating that sulfate reduction process was severely inhibited. In the batch experiments with different NB concentrations, the toxicity was mainly caused by NB, aniline, and some other toxic intermediates (Sponza and Kuscu, 2011). The results of batch experiments further confirmed the inhibition of NB on SRB.

3.3 Richness and diversity of bacteria communities

Table 2 estimates the OTUs as well as richness and diversity of microbial communities without NB and at NB contents of 100 and 400 mg/L. The richness of the bacteria communities was represented by the Chao values and ACE while that of the diversity was characterized by the Shannon and Simpson values. To compare all samples fairly with the same sequence depth, the sequence of each sample was normalized to 51323 reads. Both the rarefaction curves and Shannon index were gradually close to the plateau (Figures not shown), which suggested the current sequencing depth was reasonable and adequate (Chen et al., 2017). Although the Shannon index did not decrease linearly with the increased NB contents, the samples with the NB concentrations equal to and less than 100 mg/L exhibited higher values than that with NB content of 400 mg/L (Table 2). Similar tendency could be observed for the values of OTU, Chao and Ace, which suggested no more than 100 mg/L influent NB made no difference on the richness and diversity of the bacterial communities while 400 mg/L NB content had a significantly impact. The Simpson values showed a negative tendency as a lower value represents more diversity. Apart from the microbial diversity, the Shannon index could also indicate the community evenness, which played a great role in resisting high environmental stress, including salinity stress (Wittebolle et al., 2009). As the study was conducted under high concentration of sulfate (3600 mg/L), the relatively higher Shannon diversity indexes compared with other researches (Chen et al., 2017)

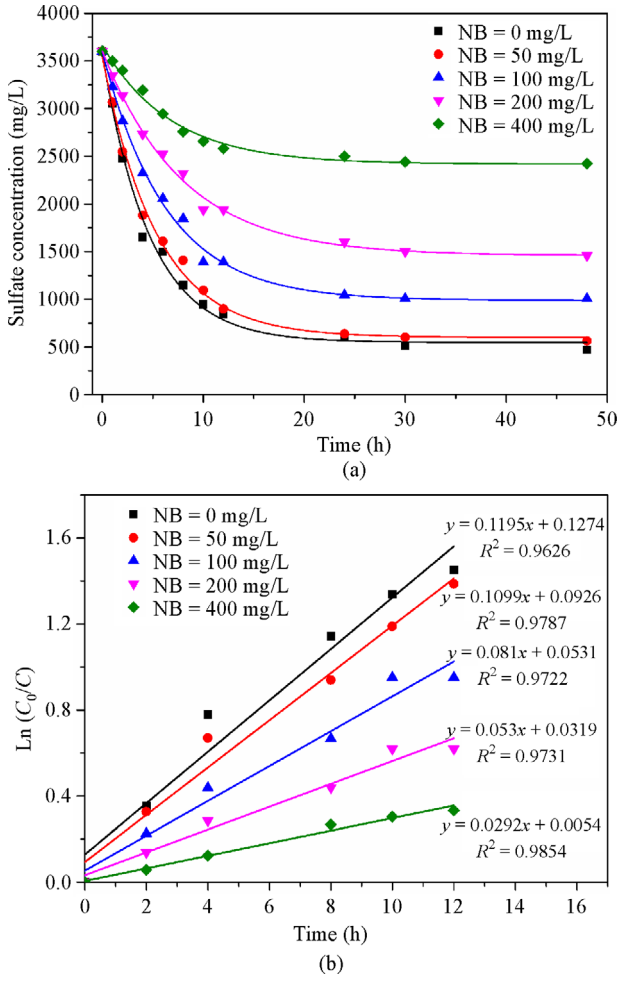

Fig. 3 Sulfate removal with different NB concentrations in batch experiments (a) efficiency and (b) first-order kinetics model.

Table 1 Batch experiments parameters with different NB concentrations

Influent NB (mg/L)	VSS(g/L)	SDA(mg SO_4^{2-}/gVSS/d)
0	21.12	135.80
50	22.56	116.91
100	23.11	84.12
200	21.43	59.36
400	22.77	30.78

Table 2 Change of bacteria community biodiversity with increased influent NB concentrations

Influent NB (mg/L)	Sequence number	OTUs	Ace	Chao	Shannon	Simpson
0(S1)		362	437	445	3.752	0.0503
100(S2)	51323	364	424	435	3.964	0.0455
400(S3)		290	354	361	3.102	0.1190

were helpful for resisting against the high-sulfate environment.

3.4 Bacterial community shift

To better understand the bacterial community structure under different concentrations of NB, taxonomic affiliation at phylum level and genus level were analyzed without NB and at NB contents of 100 and 400 mg/L. As shown in Fig. 4, a total of eight frequently detected bacterial phyla were identified, including *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, *Candidatus Saccharibacteria*, *Nitrospirae*, *Spirochaetes*, which accounted for 95.07%–99.54% of the total reads. In all samples, *Proteobacteria and Firmicutes* were the two predominant phyla and their average relative abundances were 33.69% and 39.48%, followed by *Bacteroidetes* (13.37%), which agrees with the study by Lee et al. (2012) demonstrating that *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were the most frequently detected phyla in anaerobic bioreactors. The relative abundance of *Proteobacteria* kept at about 23% at without NB addition and NB contents of 100 mg/L, but seriously up to 54.48% when the NB content further raised to 400 mg/L. Contrary to that of *Proteobacteria*, the relative abundance of *Firmicutes* decreased with higher stress of NB concentrations. The abundance of *Firmicutes* took up 47.24% without NB addition and increased to 62.07% at 100 mg/L influent NB whereas severely decreased to 9.13% when NB concentration arrived 400 mg/L. *Proteobacteria* gradually replaced *Firmicutes* for the dominant bacteria on phylum level, which indicates *Proteobacteria* could tolerant the toxicity of NB and survive in highly toxic environment.

The predominant genera in the bioreactor included *Lactobacillus*, *Zoogloea*, *Sphaerotilus*, *Lactococcus*, *Clos-*

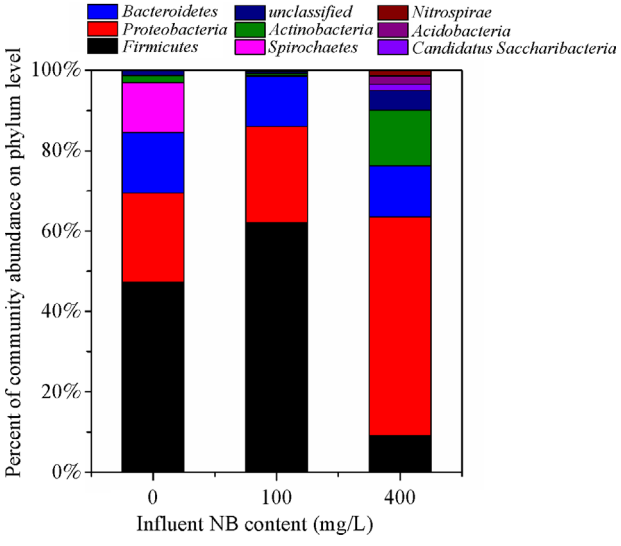

Fig. 4 Taxonomic classification on phylum level in EGSB reactor with different influent NB contents.

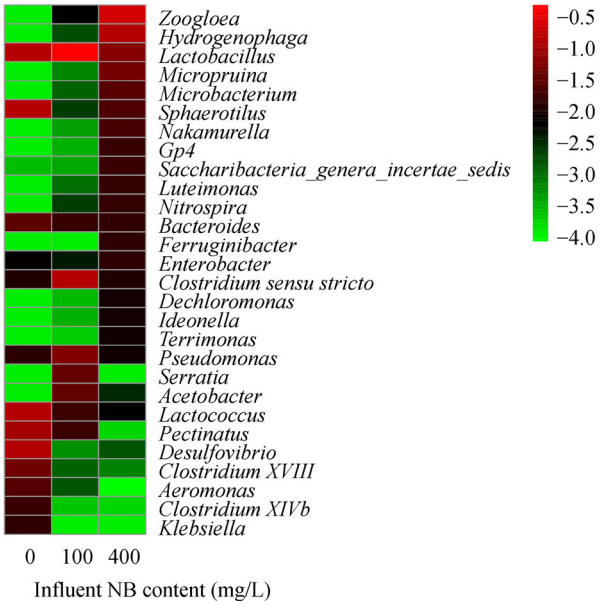

Fig. 5 Taxonomic classification on genus level in EGSB reactor with different influent NB contents.

tridium sensu stricto, *Desulfovibrio*, *Hydrogenophaga*, *Pectinatus* and *Pseudomonas* (Fig. 5 and Table S2). NB shock brought about significant bacterial community shift at genus level since *Lactococcus*, *Lactobacillus*, *Sphaerotilus* and *Desulfovibrio* were dominant without NB addition, while *Lactobacillus* and *Clostridium sensu stricto* were prevalent under 100 mg/L NB. With the content of NB up to 400 mg/L, the predominant community was replaced by *Zoogloea*, *Lactobacillus* and *Hydrogenophaga*, accounting for 19.88%, 5.47% and 11.64%, respectively. It is noted that *Lactobacillus* was the only genus that were prevalent under all the three samples. *Lactobacillus* belonged to lactic acid producing bacterium and played a major role in spore-forming process, so it can bear extreme environments and maintain high relative abundance under different NB stress (Badiei et al., 2012). Genus *Zoogloea* and *Hydrogenophaga* was enhanced by influent NB concentration. The high relative abundance of *Zoogloea* was linked to the cleavage of the aromatic compounds (Braga et al., 2015) and ensured high removal performance of COD (Li et al., 2014). *Hydrogenophaga* has been reported to be able to degrade some organic compounds, including 2,4,6-trinitrotoluene (Zhang et al., 2015b), 2-methylnaphalene (Folwell et al., 2016) and 4-aminobenzenesulfonate (Gan et al., 2011). It is noteworthy that 2,4,6-trinitrotoluene and NB have similar structure, both containing nitro and benzene. Thus, *Hydrogenophaga* may involve in degradation of NB in anaerobic process. Moreover, some *Hydrogenophaga* were found to carry the functional genes of 3,4-dioxygenase, which was a primary enzyme involved in aromatic ring ortho cleavage, so it is expected that some *Hydrogenophaga* played an important function in degradation of aromatic ring and other aromatic metabolites (Qiu et al., 2013), including NB. The relative abundance of *Sphaerotilus*, *Lactococcus*, *Pectinatus* and *Desulfovibrio* experienced a significant decrease along with the increase of NB contents from 0 to 400 mg/L. *Sphaerotilus* were frequently detected under aerobic environment and their dominance in bioreactor ensured high removal performance for COD (Li et al., 2014). The genus *Lactococcus* and *Pectinatus* are responsible for fermentation, which are able to produce lactic acid from various carbon sources, including glucose (Peng et al., 2013). According to Fig. 5, the relative abundance of *Pseudomonas* were 1.56% and 5.19% at NB concentration of 0 and 100 mg/L, but could hardly be detected when influent NB increase to 400 mg/L. Interestingly, as is

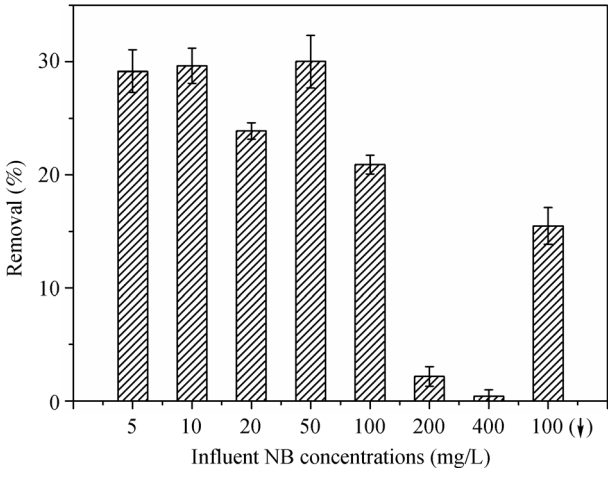

Fig. 6 NB removal performances of EGSB reactor with different influent NB concentrations.

shown in Fig. 6, the NB removal was about 30% with influent NB content less than 100 mg/L. While the content increased higher than 100 mg/L, the removal was significantly decreased and only account for 0.4% in the condition of 400 mg/L. Since *Pseudomonas* had both the oxidative and reductive ability to degrade NB (Liu et al., 2013), which might be a possible reason for the decrease of NB degradation efficiency when influent NB concentration reached 400 mg/L.

3.5 SRB populations in the bioreactor

SRB can use multifarious substrates (mainly via the hydrogen) with sulfate as the electron acceptors (Mikheev et al., 1990) and exert a key role during various environmental pollutants biodegradation. According to whether they can oxidize acetate or not, SRB include two groups, the non-acetate oxidizers group and the acetate oxidizers group (Kuscu and Sponza, 2009b). The non-acetate oxidizers group includes genera *Desulfovibrio*, *Desulfobulbus* and *Desulfotomaculum*, while the acetate oxidizers group contains genera *Desulfobacter*, *Desulfobacterium*, *Desulfosarcina* and *Desulfococcus* (Parker et al., 1999). Bacteria in non-acetate oxidizers group usually utilize hydrogen, lactate, ethanol, pyruvate and some fatty acids as electron donors, converting sulfate into hydrogen sulfide while the genera in acetate oxidizers group mostly mainly oxidize acetate, reducing sulfate to sulfide (O'Reilly and Colleran, 2006).

As shown in Fig. 5 and Table S2, the majority of sequences in all communities belonged to 28 genera, among which genus *Desulfovibrio* (*Proteobacteria*) were the dominant SRB that can reduce SO_4^{2-} to S^{2-} with organic matters as electron donors and probably the major genus contribute to the sulfate removal. *Desulfovibrio* belong to the hydrogen-utilizing bacteria and have higher affinity for sulfate than SRB which utilize acetate, propionate or butyrate as carbon source (Borglin et al., 2009). Abundant *Desulfovibrio* were commonly observed in many wastewater treatment systems where they are involved in degradation of organic pollutants (O'Reilly and Colleran, 2006). In this study, both the genus *Desulfovibrio* and *Clostridium* (*Clostridium sensu stricto*, *Clostridium XVIII*, *Clostridium XlVb*) were detected with certain abundance (Fig. 5). The co-existence of genus *Clostridium* and *Desulfovibrio* in anaerobic processes has been reported previouly (Boonchayaanant et al., 2008). Since bacteria in genus *Clostridium* was capable of cooperating with SRB for sulfate reduction, bacteria in genus *Clostridium* would therefore rather to grow syntrophically in coculture with *Desulfovibrio*. In addition, *Desulfovibrio* may exert a great role in NB degradation as it has been reported that *Desulfovibrio* were capable of reducing 2,4-DNT and TNT (Yang et al., 2009), which have similar structure with NB.

Further from Fig. 5, the relative abundance of *Desulfovibrio* accounted for 11.76% without the addition of NB, but decreased significantly to 0.05% and 0.14% when NB contents reached 100 mg/L and 400 mg/L. The low abundance of genus *Desulfovibrio* may be one of the major reasons for the poor performance of sulfate removal under high stress of NB. Besides, the observed decrease in the relative abundance of *Desulfovibrio* could not be attributed to the decrease in sulfate availability as the COD/SO_4^{2-} ratio was maintained at 2.5. Hence, toxicity inhibition of NB may be the major cause of the decrease of *Desulfovibrio* and therefore reducing the sulfate removal of the anaerobic system.

4 Conclusions

Effect of influent nitrobenzene concentrations on the performance and bacterial community in an EGSB reactor treating high high-sulfate organic wastewater were investigated. More than 50 mg/L NB could depress sulfate reduction and such inhibition was partly reversible. The sulfate removal process showed a good fit with the first order kinetic model under different NB contents, and achieved a maximum SDA of 135.8 $mgSO_4^{2-}$/gVSS/d without NB addition. *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were the three predominant phyla and the average relative abundance of them was 33.69%, 13.37% and 39.48%. With the increase of influent NB concentration, *Proteobacteria* gradually replaced *Firmicutes* as the dominant microorganisms on phylum level. Genera *Desulfovibrio* were the bacteria contributed to the sulfate reduction and the relative abundance of *Desulfovibrio* decreased significantly with high stress of NB.

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