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Performance evaluation and microbial community analysis of the function and fate of ammonia in a sulfate-reducing EGSB reactor

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Abstract Ammonia is widely distributed in sulfate-reducing bioreactor dealing with sulfate wastewater, which shows potential effect on the metabolic pathway of sulfate and ammonia. This study investigates the sulfate-reducing efficiency and microbial community composition in the sulfate-reducing EGSB reactor with the increasing ammonia loading. Results indicated that, compared with low ammonia loading (166–666 mg/L), the sulfate and organic matter removal efficiencies were improved gradually with the appropriate ammonia loading (1000–2000 mg/L), which increased from 63.58 ± 3.81 to $71.08 \pm 1.36\%$ and from 66.24 ± 1.32 to $81.88 \pm 1.83\%$, respectively. Meanwhile, with the appropriate ratio of ammonia and sulfate (1.5–3.0) and hydraulic retention time (21 h), the sulfate-reducing anaerobic ammonia oxidation (SRAO) process was occurred efficiently, inducing the accumulation of S^0 (270 mg/L) and the simultaneous ammonia removal (70.83%) in EGSB reactor. Moreover, the key sulfate-reducing bacteria (SRB) (*Desulfovibrio*) and denitrification bacteria (*Pseudomonas* and *Alcaligenes*)

were responsible for the sulfate and nitrogen removal in these phases, which accounted for 3.66–5.54 and 3.85–9.13%, respectively. However, as the ammonia loading higher than 3000 mg/L (phases 9 and 10), the sulfate-reducing efficiency was decreased to only $28.3 \pm 1.26\%$ with the ammonia removal rate of $18.4 \pm 3.37\%$ in the EGSB reactor. Meanwhile, the predominant SRB in phases 9 and 10 were *Desulfomicrobium* (1.22–1.99%) and *Desulfocurvus* (4.0–5.46%), and the denitrification bacteria accounted for only 0.88% (phase 10), indicating the low nitrogen removal rate.

Keywords Ammonia loading · Sulfate reducing · SRAO · Microbial community · Denitrification bacteria

Introduction

Industrial wastewater from the process of monosodium glutamate manufacturing, tanning, pharmacy, and papermaking contained high concentration of ammonia, sulfate, and organic matters (Rodrigues et al. 2008; Vaiopoulou et al. 2005; Wang et al. 2008; Wang and Banks 2007). The wastewater discharging into the environment can pose various damage to the environment, including sulfur unbalance in nature (Silva et al. 2002), lake eutrophication, and river acidification (Lamers et al. 1998; Moore et al. 2000).

On account of the characteristic of this wastewater, previous studies mainly focus on the simultaneous removal sulfate and ammonia (Fdz-Polanco et al. 2001b; Fdz-Polanco et al. 2001c; Rikmann et al. 2012; Yang et al. 2009; Yuan et al. 2015b; Zhang et al. 2009), named sulfate-reducing anaerobic ammonia oxidation (SRAO) process. This process was first

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assumed by Fdz-Polanco et al. (2001a), and then proposed the equation describing the two-stage process (Liu et al. 2008; Rikmann et al. 2014) (Eqs. 1 and 2).

$$NH_4^+ + SO_4^{2-} \rightarrow NO_2^- + S^0 + 2H_2O \qquad (1)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (2)$$
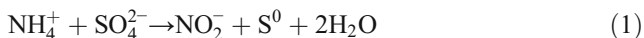
Obviously, the ratio of sulfate and ammonia is regarded as one of the most important parameter for SRAO process, and then affects the removal pathway of sulfate (Yuan et al. 2015a). Besides, the SRAO process usually occurred under inorganic conditions, and autotrophic bacteria was the predominant microorganism. However, the organic matter in sulfate-reducing bioreactor could inhibit the autotrophic bacteria activity (Giustinianovich et al. 2016; Montalvo et al. 2016), and then may influence the efficiency of sulfate removal. Hence, the performance of sulfate reducing and ammonia removal should be evaluated under organic condition, especially with the variation of ammonia loading.

Generally, ammonia exists in the wastewater mainly in the forms of ionic ammonia (NH_4^+) and free ammonia (FA) (NH_3), and the neutral FA can cross cell membrane and inhibit the activity of microorganisms (Calli et al. 2005). Some studies have demonstrated clearly that FA in wastewater usually affects fermentation and methane production process, and impedes the degradation of protein (Gallert and Winter 1997; Hansen et al. 1998; Koster and Lettinga 1988). Similarly, in sulfate-reducing reactor, FA may change the activity of sulfate reduction bacteria (SRB) and then influence the process of sulfate reduction, and moreover, FA could influence the microbial community in the bioreactor (Rikmann et al. 2017; Sui et al. 2016), including functional microorganisms. To the best of our knowledge, previous studies mainly discuss the operation of sulfate reduction reactor with low concentration (Ontiveros-Valencia et al. 2012; Zhou et al. 2014), whereas seldom focus on the influence of FA in sulfate-reducing reactor under high concentration of ammonia condition, including SRAO process.

In addition, with the coexistence of sulfate and ammonia, ammonia was converted to nitrite and nitrate through sulfate-reducing process. The nitrite and nitrate were mainly removed by anammox and denitrification bacteria, which may determine the microbial community composition in bioreactor. However, the existence of nitrite in bioreactor could affect the activity of anammox and then restrict nutrient removal (Raudkivi et al. 2017; Zekker et al. 2017; Zekker et al. 2016). Moreover, the nitrite and nitrate might severely reduce the activity of SRB (Ontiveros-Valencia et al. 2012; Yang et al. 2009). Therefore, it is also necessary to investigate the nitrogen forms in the sulfate-reducing bioreactor. On the other hand, the predominant SRB in sulfate reduction bioreactor under different ammonia loadings is still uncertain, as well

as the microbial community dynamics. Meanwhile, in the process of sulfate and nitrogen metabolism, the correlation between denitrifying bacteria and SRB is still indeterminately. Consequently, it is necessary to investigate and discuss the performance of sulfate reduction reactor with the variations of ammonia loading and examine the microbial community structure shifts and search the critical factor during this process.

In this study, the variation of sulfate reduction capability was investigated in an expanded granular sludge bed (EGSB) reactor under different ammonia loading conditions. Meanwhile, Illumina Mi-Seq high-throughput sequencing technology was used to compare the microbial community structure in the experimental process and explored the predominant SRB in EGSB reactor with different ammonia concentrations. Besides, statistical analysis was also conducted to reveal the ecological correlation between operational parameters (environment variables) and microbial community.

Materials and methods

Anaerobic reactor setup and process operation

This experiment was conducted in a cylindrical EGSB reactor with a working volume of 1.96 L with inner diameter of 60 mm and height of 91 cm. The EGSB reactor was fabricated with plexiglass with sampling points at the bottom and reflux point at the outlet (Miao et al. 2015). The inoculation sludge was collected from an anaerobic hydrolysis acidification reactor. Synthetic wastewater fed to the EGSB reactor contained COD of 9000 mg/L and SO_4^{2-} of 3600 mg/L. Detailed ingredients of the influent wastewater and trace element solution in this reactor are listed in Table S1. The bioreactor was totally operated for 165 days, which was divided into 10 phases with different influent NH_4^+ concentrations at 166, 250, 333, 666, 1000, 1500, 2000, 2500, 3000, and 4000 mg/L (Table 1). Each phase lasted for about 15–20 days before the bioreactor obtained a stable sulfate-reducing efficiency. Specific ammonia concentration and the NH_4^+/SO_4^{2-} ratio in different phases are shown in Table 1. The reactor temperature was kept 36 ± 2 °C using constant temperature circulating water bath (DC-0506), and the pH in bioreactor was measured with METTLER TOLEDO FE20-Five Easy Plus™. Meanwhile, the pH in EGSB reactor was measured using dissolved oxygen (DO) meter (HACA, HQ40D, USA), indicating the anaerobic environment in EGSB reactor (< 0.15 mg/L). Besides, the hydraulic retention time (HRT) was kept at 21 h, and the reactor reflux ratio was kept at 20:1 with the up-flow velocity of 0.9 m/h.

Table 1 The influent quality in different phases

Phase	Day	NH_4^+ (mg/L)	NH_4^+/SO_4^{2-}
1	1–12	166 ± 2	0.246
2	13–22	251 ± 2	0.370
3	23–36	330 ± 8	0.493
4	37–52	666 ± 4	0.987
5	53–68	1010 ± 9	1.481
6	69–86	1496 ± 17	2.222
7	87–102	2018 ± 41	2.963
8	103–124	2500 ± 64	3.704
9	125–146	3016 ± 103	4.444
10	147–165	3982 ± 78	5.926

Batch experiments

To determine the specific substrate consumption rate in different phases, batch experiments were conducted in the process of continuous operation of the reactor. Sludge mixed liquor (100 mL) sampled from the EGSB reactor in each phase was centrifuged for 5 min at 4000 rpm, and supernatant was discarded, and the liquor was then blended with deionized water centrifugation again (repeat twice). The batch experiment was determined in 1000-mL conical flasks with granular sludge (100 mL) and synthetic wastewater (400 mL), separately. At the beginning, the conical flask was blow off 10 min with high purity nitrogen gas and then sealed by butyl rubber plug. The experiment was performed at 35 ± 0.5 °C in a thermostat shaking bath with shaking rate 120 rpm. The concentration of sulfate, ammonia, and S^{2-} was measured in the filtered sample every 30 min.

Chemical analysis

The effluent wastewater was sampled every 2 days and then filtrated using a 0.45-μm glass fiber filter. The chemical oxygen demand (COD) was measured by potassium dichromate method, and the concentration of ammonia, nitrite, and nitrate in the wastewater was measured according to the standard methods by Nesslerization method, colorimetric method, and ultraviolet spectrophotometric screen method, respectively (Rice et al. 2012). The concentration of free ammonia (FA) was calculated by the formula as follows (Eq. 3) (Park and Bae 2009):

$$FA = \frac{17}{14} \frac{TAN \times 10^{pH}}{\left[\exp\left(6334 / (273 + °C)\right) + 10^{pH}\right]} \quad (3)$$

where TAN is total ammonia nitrogen concentration.

The concentration of SO_4^{2-} was measured by ion chromatograph (ICS-1100 AR, Thermo Fisher, USA) (details are shown in Method S1 of the Supplementary Materials).

Meanwhile, the effluent S^{2-} was measured by the iodometric method (Rice et al. 2012). The volatile fatty acid (VFA) of effluent was detected by gas chromatography (Agilent 7890A, USA) (details are shown in Method S2 of the Supplementary Materials). By stoichiometric equilibrium calculation, the elemental sulfur concentration was calculated according to the following equation (Eq. 4) (De Graaff et al. 2012):

$$\left[S^0\right] = \left[Influent\ S\right] - \left[SO_4^+\right] - 2 \times \left[S_2O_3^{2-}\right] - \left[HS^-\right] \quad (4)$$

DNA extraction

The activated sludge samples were harvested from each phase when the reactor reached a steady state under different concentrations of ammonia. The total genomic DNA was extracted depending on the procedures described in the FastDNA Soil Kit (MP Biomedicals, CA, USA). The DNA concentrations were measured with a microspectrophotometry (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, DE, USA), and its purity was documented based on the 260/280-nm absorbance ratio. Then, the DNA samples were stored in − 80 °C for the following molecular applications.

Illumina Mi-Seq sequencing and bioinformatics analysis

Illumina Mi-Seq sequencing

The extracted DNA of sludge sample was amplified with a set of primers targeting the hypervariable V1–V2 region of the 16S rRNA gene. The forward primer is 5′-AGAG TTTGATYMTGGCTCAG-3′, and the reverse primer is 5′-TGCTGCCTCCCGTAGGAGT-3′. The polymerase chain reaction (PCR) was performed in a 50 μL mixture containing 25 μL 2 × EasyTaq® PCR SuperMix (TransGen Biotech, Beijing, China), 2 μL of each primer (10 μM), 21 μL of double-distilled H_2O (dd H_2O), and 40 ng of DNA template. The PCR amplifications were run under the following conditions: 95 °C for 5 min; followed by 35 cycles consisting of 95 °C for 30 s, 55 °C for 30 s, and 72°Cfor 40 s; and completed with a final extension at 72 °C for 10 min.

The PCR product was purified using TaKaRa MiniBEST DNA Fragment Purification Kit Ver. 4.0 (TaKaRa, Japan). DNA samples with different barcodes were mixed in a mixture of amplicons and sent to Jiangsu Zhongyijinda Analysis and Detection Ltd. (Jiangsu, China) for sequencing on Illumina Mi-Seq Platform. All sequencing data obtained in this study were deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under accession number PRJNA378950.

Bioinformatics analysis

Primitive data of all sludge samples were split with Mothur software (http://www.mothur.org/) based on nucleotide barcode (Zhang et al. 2012). Then, the generated sequences were denoised by Mothur software (Miao et al. 2015). In order to compare all the samples effectively, all samples were kept at the same sequencing depth (33,745 reads). Operational taxonomic units (OTUs) were subsequently defined by de novo strategy at 97% identity cutoff by using UCLUST algorithm in QIIME (Edgar 2010). Most abundant sequences were used to represent each OTU and were aligned by using PyNAST (Caporaso et al. 2010). Then, the taxonomic classification was performed using RDP classifier via Silva SSU database (Release123) with a confidence threshold of 80% (Cole et al. 2009). After that, the Chao 1 and Shannon index were calculated for each sample using Mothur software.

In order to evaluate microbial community structure evolution at genus level and functional microorganisms, the principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) were carried out using the software PAST (v3.14, http://folk.uio.no/ohammer/past/). Cluster analysis was performed using Bray-Curtis distance with the number of bootstrap set as 999. Furthermore, canonical correspondence analysis (CCA) was calculated and visualized by Past 3.14 software to facilitate understanding the ecological correlation between microbial community and operation parameters. In order to evaluate the significant correlation, the Person test was calculated with SPSS 20.0 software.

Results

Ammonia loading effects on the bioreactor performance

As the influent ammonia ranged from 166 to 666 mg/L (phases 1–3), the removal rate of COD and sulfate in EGSB reactor showed slightly fluctuation and maintained at 63.58 ± 3.81 and $66.24 \pm 1.32\%$ (Fig. 1a), respectively. Meanwhile, the NH_4^+-N loading removal rate was less than 0.5 kg/(m^3 day), which indicated that the EGSB reactor maintained a low and stable ammonia removal rate. However, with the ammonia loading reached to 666 mg/L, sulfide ion in effluent was gradually decreased to less than 300 mg/L, and meanwhile, sulfur elemental was increased to more than 100 mg/L. Specially, the ammonia removal rate was increased rapidly, from 40.21 to 58.39% (Fig. 1b). Moreover, NO_3^--N was evidently accumulated with the concentration of 50 mg/L at phase 4. These results indicated that the simultaneous sulfate and ammonia removal was occurred in this phase. In addition, batch experiments have also demonstrated that the EGSB reactor maintained a high degradation rate of sulfate (Fig. 2a) with the ammonia removal rate in reactor of 27.35%

at phase 4 (Fig. 2b). Thus, though the SRAO process was occurred at phases 1–4, it was obviously inefficient with the NH_4^+-N loading removal rate reached to only 0.7 ± 0.06 kg/(m^3 day) at the end of phase 4.

Subsequently, with the continuous increase of ammonia concentration (phases 5 to 7), the sulfate reduction ability of EGSB reactor was obviously improved (from 71.08 to 81.88%), whereas the improvement of sulfate reduction efficiency did not induce the increase of sulfide contents. As shown in Fig. 1a, the concentration of S^{2-} in effluent dropped sharply from 378 ± 13 mg/L at phase 5 to 56 ± 8 at phase 7. Correspondingly, S^0 in the reactor rose rapidly from 76 to 270 mg/L. Meanwhile, the ammonia removal rate increased from 38.62 to 70.83%, indicating that the simultaneous sulfate and ammonia removal may occur efficiently in this phase. These above results demonstrated that the simultaneous ammonia and sulfate removal in SRAO process promotes the generation of S^0 (Eq. 1). This phenomenon has also been demonstrated by batch experiments (Fig. 2), in which the bioreactor maintained a high ammonia removal efficiency and low concentration of sulfide in effluent at phases 5 (1000 mg/L) and 7 (2000 mg/L) than at phase 4 (666 mg/L). Due to the occurrence of SRAO process, the ammonia loading removal rate increased to 2.2 kg/(m^3 day), higher than previous phases. Nevertheless, it should be noted that the continuous accumulation of nitrite and nitrate nitrogen occurred during these phases. At the end of phase 7, the effluent NO_2^--N and NO_3^--N were accumulated to 52.3 and 117.4 mg/L, respectively. In general, nitrate and nitrite were removed through denitrification process and consume VFA in the EGSB reactor. As a result, the effluent VFA was decreased from 48 ± 3 to 20 ± 2 mg/L (Fig. 1c). Moreover, due to the lack of VFA, the EGSB reactor obtained a poor removal efficiency of nitrate and nitrite.

Finally, under high ammonia loading condition (3000 to 4000 mg/L), the EGSB reactor showed approximately 28.3% sulfate removal performance (Fig. 1a). Especially, the S^0 concentration was decreased to nearly zero, and the ammonia removal rate in EGSB reactor was decreased to 18.4%. These results indicated that the SRAO process was occurred inefficiently. Besides, the sulfate removal was mainly through sulfate-reducing process in phases 8–10 and resulting in the cumulative concentration of S^{2-} in effluent higher than that in phases 5–7 (Fig. 1a). On the other hand, the NH_4^+-N loading removal rate was less than 1 kg/(m^3 day) in phases 5–7, while the TN loading removal rate was 0.5 kg/(m^3 day) (Fig. S1). This result indicated that ammonia in EGSB reactor was mainly converted to nitrite and nitrate instead of N_2. Correspondingly, the nitrate and nitrite were accumulated in EGSB reactor. In addition, the reactor pH dropped quickly and fluctuated within the range of 7.0–7.5, and the FA in effluent accumulated of 127.4 mg/L at phase 10. Overall, high

Fig. 1 The performance of EGSB reactor during different periods. **a** COD and sulfate removal rate and effluent S^{2-} and S^0 concentration. **b** Variations of the effluent concentration of nitrogen and free ammonia. **c** pH and VFA in reactor
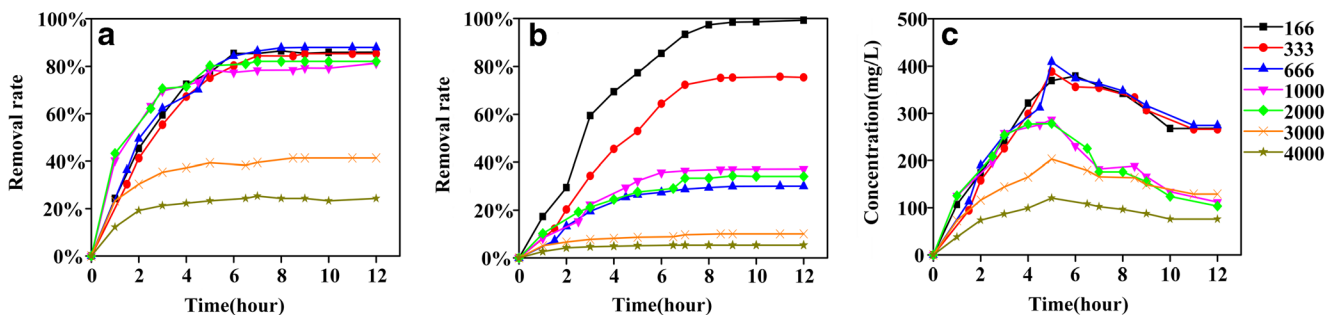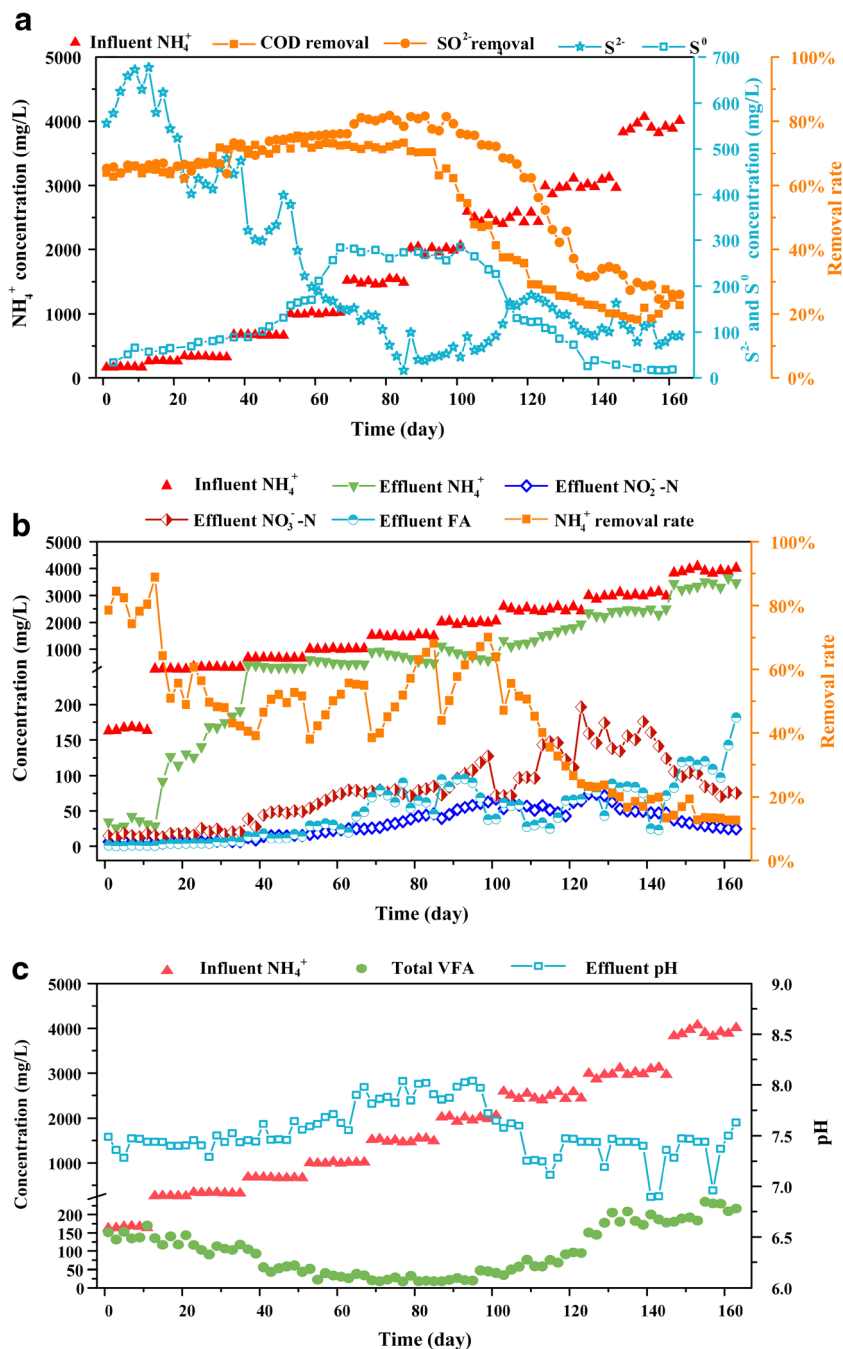

Fig. 2 Batch experiment in different phase. **a** The reduction rate of sulfate. **b** The removal rate of ammonia. **c** The concentration of S^{2-}

ammonia loading affected the efficiency of sulfate reduction and altered the nitrogen removal rate in EGSB reactor.

Ammonia loading effects on microbial community structure

After high-throughput sequencing technology and bioinformatics analysis, the number of the obtained high-quality sequences was normalized at the same sequencing depth (33,745 sequences for each sample). First, the Chao1 and Shannon indices were calculated to analyze the microorganism richness and diversity in different phases. As shown in Table S2, from phases 4 to 7, the Chao1 index decreased gradually, which showed that richness of bacterial communities decreased along with the increase of ammonia loading. However, the EGSB reactor in phases 9 and 10 had higher Chao1 indices, showing opposite changing trends. Furthermore, granular sludge had the higher Shannon index during phases 4 and 7, suggesting that the bacterial community was distributed more evenly. Conversely, at phase 9 and 10, the Shannon indices was lower than other phases. Based on the above results, the richness of microbial community decreased as the concentration of ammonia ranged from 666 to 2000 mg/L, but the higher richness and lower biodiversity were obtained when the ammonia concentration reached 3000 mg/L or higher.

In addition, the taxonomic of microbial community has been presented at phyla and genus level. As shown in Fig. S3, eight different phylum were detected in these samples altogether. The predominant bacteria were mainly *Bacteroidetes* and *Firmicutes*, which account for more than 60% of the total abundance. It should also be noted that the abundance of *Proteobacteria* increased from phases 5 to 7, which ranged from 18.08 to 29.04%. Under high ammonia loading stress, however, the abundance of *Proteobacteria* decreased gradually. Meanwhile, *Spirochaetes*, hardly be detected in other phases, accounted for 7.05 and 5.98% of the total abundance at phases 9 and 10, respectively. In order to further analyze the microorganisms in EGSB reactor (including unclassified bacteria), the relative abundance of OTUs higher than 1% was selected from each sample (Fig. S5). As the ammonia loading ranged from 1000 to 2000 mg/L, the relative abundance of OTU4694, OTU6953, OTU3520, and OTU5632 was increased gradually, which was affiliated with *Firmicutes*. Especially, the relative abundance of these OTUs was reached to 42.59% in phase 7. However, as the ammonia loading increased to 4000 mg/L, these OTUs were decreased to 6.27, 6.68, 4.54, and 4.25%, respectively. Besides, the applied ratio of sulfate and ammonia promoted the growth of OTU557, OTU8138, OTU9874, and OTU1686. Under high ammonia loading condition (phases 9 and 10), OTU7731 and OTU5904 were increased to 7.03 and 7.51%, respectively.

At genus level, a total of 26 genera (relative abundance > 1% in each sample) were selected from each samples (Fig. 3a), which showed high biodiversity during the whole operation. However, the abundance of most of major genera showed apparently diverse changing trends with the ammonia loading increase. For instance, *Lactobacillus* gradually disappeared as the ammonia loading increased, whose abundance decreased to 0.18% in phase 10. In the present study, the EGSB reactor had different predominant sulfate-reducing and denitrification bacteria with the variation of ammonia. However, anammox bacteria had not been detected during the whole operation period, indicating that the anammox process did not take place in the bioreactor and the main nitrogen metabolism was denitrification process. To obtain the similarity of microbial community structure, PCoA and NMDS were further analyzed at genus level. As shown in Fig. 3c, d, the microbial community structure was presented a succession tendency. Especially, the microbial community structure from phases 4 to 7 tended to cluster together, while it tended to cluster together at phases 9 and 10. This result had also been demonstrated by cluster analysis (Fig. 3b), which showed the high similarity of microbial community structure from phases 4 to 7.

Key microorganisms involved in sulfate and nitrogen metabolism

In this study, we specially analyzed the key microorganisms involved in sulfate reducing and denitrification bacteria at genus level. Key microorganisms were picked out based on the review of SRB (Liu et al. 2015) and denitrification bacteria (Lu et al. 2014), respectively. As ammonia increased from 133 to 666 mg/L, all detectable SRBs into the bioreactor belonged to *Desulfovibrio* and *Desulfomicrobium* (Fig. 4a), and the total abundance of the SRB ranged from 0.80 to 2.41%. In phase 5, however, the abundance of *Desulfovibrio* increased to 9.26%, and the other SRB accounted for only 0.68%. This result showed that ammonia promoted the growth of *Desulfovibrio*. However, as the ammonia increased to 2000 mg/L, the relative abundance of *Desulfovibrio* decreased sharply (3.66–5.54%); especially at phases 9 and 10, it decreased to only 1.26%. At phases 9 and 10, the predominant SRB changed to *Desulfocurvus* (account for 4.0–5.46%) and *Desulfomicrobium* (account for 1.22–1.99%). Obviously, the *Desulfocurvus* might adapt better to the high ammonia loading condition than the other SRBs. Besides, cluster analysis (Fig. S4a) showed that the sulfate-reducing bacteria have similar community composition under high ammonia loading condition.

On the other hand, the main denitrification bacteria involved in nitrogen metabolism are listed in Fig. 4b. The denitrification bacteria was mainly responsible for nitrite and nitrate removal. As ammonia concentration ranged from 166 to

Fig. 3 **a** Microbial community composition at genus level (relative abundance > 1%). Classical clustering (**b**), principal coordinate analysis (PCOA) using Bray-Curtis distance (**c**), and non-metric multidimensional scaling (NMDS) (**d**) at genus level
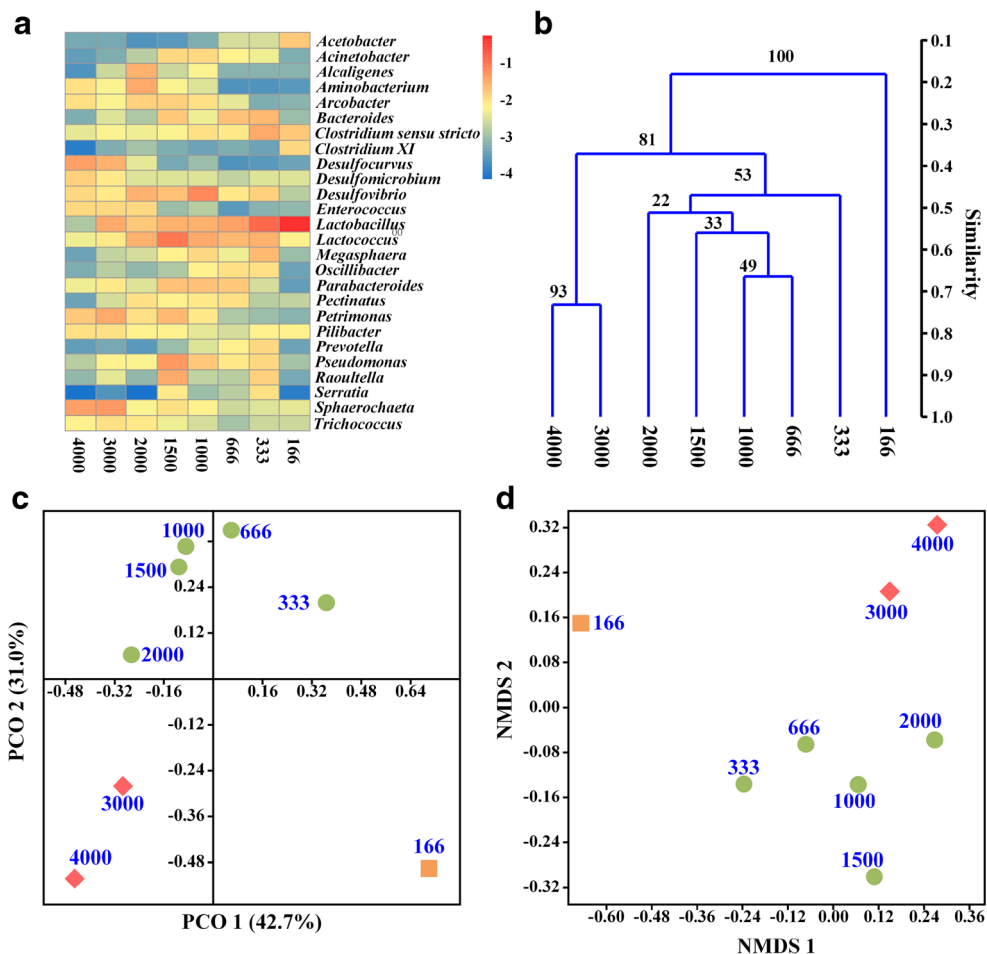
1500 mg/L, the most predominant denitrification bacteria were classified into the genus of *Pseudomonas* and *Acinetobacter*. In phase 6, the relative abundance of *Pseudomonas* increased to 6.61%, and the total denitrification bacteria accounted for 9.13% in this EGSB reactor. In phase 7, however, the abundance of *Pseudomonas* decreased to only 0.86%, and the predominant denitrification bacteria was

turned to *Alcaligenes* (account for 4.15%). In spite of the fact that the predominant denitrification bacteria changed, the abundance of the denitrification bacteria still accounted for 5.43%. These results demonstrated that the EGSB reactor obtained a high efficiency of denitrification process at phases 5–7. With the termination of SRAO process (phases 9 and 10), the abundance of denitrification bacteria gradually decreased,

Fig. 4 Relative abundance of SRB (**a**) and denitrifying bacteria (**b**) in different phase
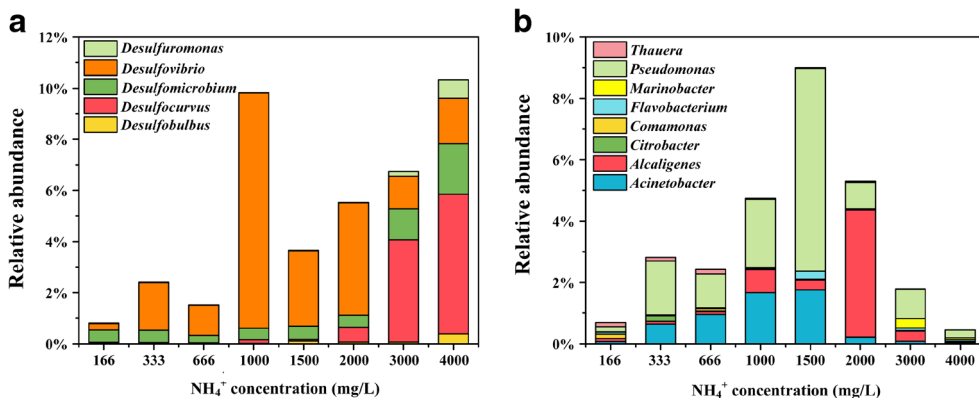
Fig. 5 Ordination plots generated by canonical correspondence analysis (CCA) showing the relationship between microbial community compositions (at genus level, relative abundance > 1%) and environment parameters. Correlations between environmental variables and CCA axes were represented by the length and angle of *arrows* (environmental factor vectors). *VFA* volatile fatty acid, NH_4^+ R NH_4^+ removal rate, *COD R* COD removal rate, SO_4^{2-} R SO_4^{2-} removal rate, NH_4^+ NLR NH_4^+ nitrogen loading removal rate, *FA* free ammonia
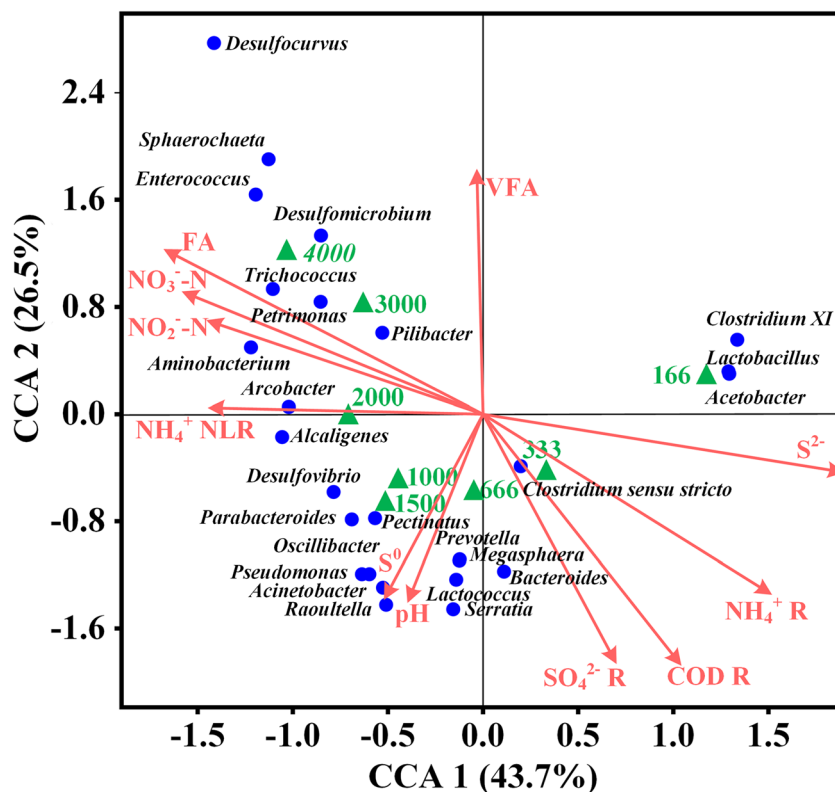
especially at phase 10 (account for 0.88%). Cluster analysis also showed that denitrification bacteria tended to cluster together at low ammonia loading (Fig. S4b).

Correlations between operational parameters and microbial community

CCA was further applied to investigate the correlation between operational parameters and microbial community structure (at genus level) (Fig. 5). Concerning the variation of bacteria at genus level, the principal component 1 (PC1) explained 43.7% of the variation of species-environment relation, the PC1 and PC2 together explained 70.2% (Fig. 5), demonstrating the reliability of the CCA results. CCA analysis revealed that the abundance of *Desulfovibrio* was related to ammonia loading removal rate (NH_4^+ NLR) and S^0. The denitrification bacteria (*Pseudomonas* and *Acinetobacter*) were quantitatively significantly correlated with S^0 (Person test, $p < 0.01$), and the abundance of the denitrification bacteria *Alcaligenes* showed significant correlations with NH_4^+ NLR (Person test, $p < 0.05$). On the other hand, the microbial community structure at phases 5 and 6 was correlated with S^0, and it was also positively correlated with NH_4^+ NLR at phase 7 (2000 mg/L), which indicated that the EGSB reactor may obtain a high removal rate for sulfate and ammonia in phases 5–7. In addition, the abundance of *Desulfomicrobium* and *Desulfocurvus* had positive correlation with FA levels, which indicated that FA has no obvious inhibition on these SRB.

Discussion

In the present study, the impact of ammonia loading on both the sulfate-reducing efficiency and bacterial community were thoroughly investigated in an EGSB reactor. Meanwhile, the study evaluated the function and fate of ammonia in the sulfate-reducing bioreactor, and then gave suggestions to simultaneous sulfate and ammonia removal. At the preliminary phase (1–4), low ammonia loading has no obvious impact on the EGSB reactor performance, which maintained a stable sulfate-reducing efficiency (Fig. 1a). Moreover, with the poor efficiency of SRAO process, the ammonia removal rate was less than 50%. Thus, under low ammonia loading condition (< 666 mg/L), ammonia had no positive or negative effect on sulfate-reducing process, and discharged in the effluent. These results were mainly because the SRAO process cannot occur efficiently under low ratio of NH_4^+/SO_4^{2-} (< 1) conditions (Rikmann et al. 2014).

Under the appropriate ratio of NH_4^+/SO_4^{2-} conditions (1.481–2.963) (phases 5–7), the sulfate-reducing efficiency was improved to 81.88% with the increase of ammonia loading. Meanwhile, the decrease of sulfide in effluent indicated that the higher efficiency of SRAO process was obtained under suitable ammonia loading condition, corresponding the S^0 increased gradually (Fig. 1a). Different from the SRAO process in inorganic reactor (Prachakittikul et al. 2016; Rikmann et al. 2016), the EGSB reactor contained a high concentration of organic matter in this study, which inhibited the

accumulation of anammox bacteria (Ni et al. 2012; Tang et al. 2010). Consequently, due to the lack of anammox bacteria (Fig. 3a), ammonia was mainly converted to nitrite instead of nitrogen gas. As the inhibitor of SRB, NO_2^- in the reactor may lead to the decrease of SRB (Yang et al. 2009). However, the abundance of SRB has increased promptly (Fig. 4a), especially for *Desulfovibrio*. Previous studies have reported that *Desulfovibrio* used not only SO_4^{2-} but also NO_2^- and NO_3^- as electron acceptors (Krekeler and Cypionka 1995). Thus, in phases 5–7, *Desulfovibrio* selectively used NO_2^- and NO_3^- as electron acceptor (Zhou et al. 2014), and then accumulated in the bioreactor. Meanwhile, other types of SRB may have been constrained by nitrite (Fig. 4a).

In addition, due to the existence of nitrite and organic matter, the abundance of denitrification bacteria increased gradually (Fig. 4b). Moreover, *Desulfovibrio* and denitrification bacteria were responsible for the nitrite and nitrate removal in the EGSB reactor. From phases 5 to 6, the abundance of *Desulfovibrio* decreased rapidly, and conversely, the abundance of denitrification bacteria increased. In the EGSB reactor, substrate competition between *Desulfovibrio* and denitrification bacteria may exist at phases 5–7. Particularly, with the ammonia loading of 2000 mg/L (phase 7), the predominant denitrification bacteria *Acinetobacter* (Chen et al. 2015) and *Desulfovibrio* both contribute to nitrite and nitrate removal. Based on the above results, both the sulfate and the nitrogen (including ammonia and total nitrogen) removal rate increased (Fig. S1), indicating the simultaneous sulfate and ammonia removal under an appropriate ratio of NH_4^+/SO_4^{2-} condition (1.481–2.963).

With the further rise of ammonia loading, the ratio of NH_4^+/SO_4^{2-} increased so much that it beyond the optimal range of sulfate-dependent anaerobic ammonia oxidation (Cai et al. 2010; Zhang et al. 2009). Due to the inefficient of SRAO process, the abundance of *Desulfovibrio* and denitrifying bacteria decreased sharply (Fig. 4). However, the abundance of SRB increased under high ammonia loading condition, especially for the predominant SRB of *Desulfocurvus*. The result proved that *Desulfomicrobium* and *Desulfocurvus* have a high tolerance of ammonia loading, whereas the high abundance of SRB has no capacity to effectively reduce sulfate at phases 9 and 10. Thus, future studies should also to explore the sulfate-reducing process at transcription and protein level with high ammonia loading.

In addition, the concentration of FA exceeds 100 mg/L (Fig. 1b), which might inhibit most of bacteria (Calli et al. 2005; Gutierrez et al. 2009). Thus, the Shannon indices decreased to 4.97 at phase 10 (Table S2), indicating the decrease of microbial diversity. Nevertheless, the species richness of microbial communities was gradually higher with the Chao 1 index increase. Therefore, it is inferred that the growth of bacteria, with low tolerance of FA, was inhibited at phases 9 and 10, whereas the bacteria with high tolerance of FA were

gradually accumulated. On the other hand, CCA analysis result demonstrated that the microbial community composition was significant influenced by FA at phases 9 and 10. Previous studies has also reported that FA showed its contribution to the changes of the bacterial communities in nitrite accumulation reactor (Sui et al. 2016) and anaerobic digestion reactor (Dai et al. 2016).

With the coexistence of sulfate and ammonia in wastewater, nutrient successful removal is difficult to achieve under organic condition. In fact, an appropriate ratio of NH_4^+/SO_4^{2-} would largely enhance the sulfate reducing and ammonia removal efficiency. In this study, the appropriate ratio of NH_4^+/SO_4^{2-} (1.5–3.0) has promoted the removal efficiency in EGSB reactor, and correspondingly, SRB and denitrification bacteria were mainly responsible for sulfate and nitrogen removal. Unlike the performance of EGSB reactor in appropriate condition, the accumulation of FA could reduce the sulfate and nitrogen removal efficiency with high ammonia loading. Overall, under appropriate ratio of sulfate and ammonia condition, the simultaneous sulfate and nitrogen (including ammonia) removal could also be achieved with the existence of organic matter.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was conducted without the use of human or animal participants by any of the authors.

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