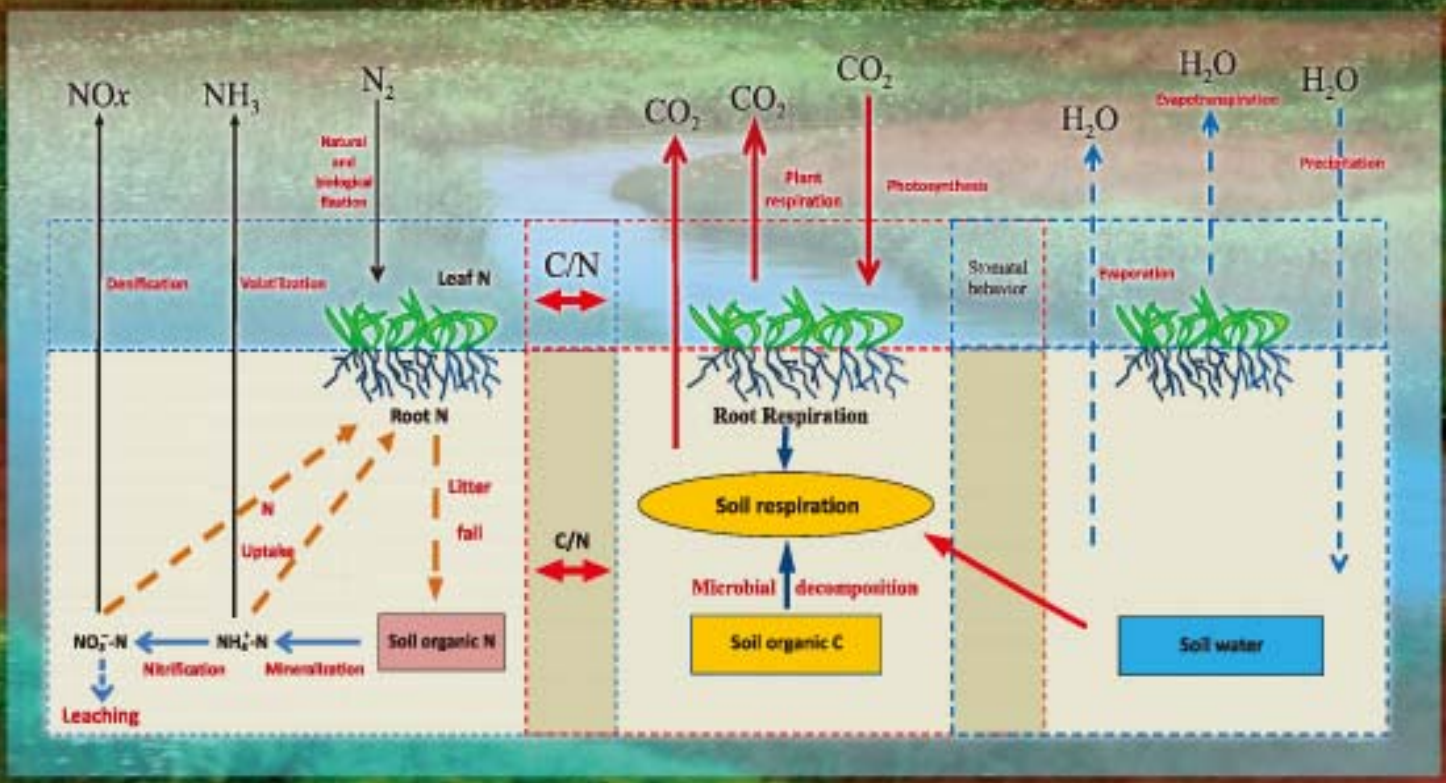
JES

JOURNAL OF ENVIRONMENTAL SCIENCES

ISSN 1001-0742
CN 11-2629/X

April 1, 2014 Volume 26 Number 4
www.jesc.ac.cn



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Science Press

ISSN 1001–0742 **Journal of Environmental Sciences** Vol. 26 No. 4 2014

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Serial parameter: CN 11-2629/X*1989*m*217*en*P*24*2014-4

Journal of Environmental Sciences 26 (2014) 717–725

Available online at www.sciencedirect.com

Journal of Environmental Sciences

www.jesc.ac.cn

Performance and microbial diversity of an expanded granular sludge bed reactor for high sulfate and nitrate waste brine treatment

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ARTICLE INFO

Article history:
Received 17 May 2013
revised 27 August 2013
accepted 29 August 2013

Keywords:
454-pyrosequencing
waste brine
denitrification
expanded granular sludge bed (EGSB) reactor

DOI: 10.1016/S1001-0742(13)60479-9

ABSTRACT

The disposal of waste brines has become a major challenge that hinders the wide application of ion-exchange resins in the water industry in recent decades. In this study, high sulfate removal efficiency (80%–90%) was achieved at the influent sulfate concentration of 3600 mg/L and 3% NaCl after 145 days in an expanded granular sludge bed (EGSB) reactor. Furthermore, the feasibility of treating synthetic waste brine containing high levels of sulfate and nitrate was investigated in a single EGSB reactor during an operation period of 261 days. The highest nitrate and sulfate loading rate reached 6.38 and 5.78 kg/(m^3·day) at SO_4^{2-}-S/NO_3^--N mass ratio of 4/3, and the corresponding removal efficiency was 99.97% and 82.26% at 3% NaCl, respectively. Meanwhile, 454-pyrosequencing technology was used to analyze the bacterial diversity of the sludge on the 240th day for stable operation of phase X. Results showed that a total of 9194 sequences were obtained, which could be affiliated to 14 phyla, including *Proteobacteria, Firmicutes*, *Chlorobi*, *Bacteroidetes*, *Synergistetes* and so on. *Proteobacteria* (77.66%) was the dominant microbial population, followed by *Firmicutes* (12.23%) and *Chlorobi* (2.71%).

Introduction

In the last few decades, ion-exchange resins have been extensively utilized to remove nitrate from drinking water (Clifford and Weber, 1986; Ghurye et al., 1999; Malika et al., 2010). Especially selective resins have also been developed to remove nitrate from water (Liu and Clifford, 1996; Saba et al., 2006; Song et al., 2012). To reduce the operational costs of water treatment, spent IX resin should be regenerated and reused (Gu et al., 2003; Batista et al., 2000). However, the regeneration process usually produces harmful waste brines with high levels of nitrate and sulfate. Van der Hoek et al. (1988) reported that the nitrate and sulfate accumulated up to 500 and 5000 mg/L in their brine recycling system, respectively. Liu et al. (1996) found that sulfate in the recycled brine reached a maximum level of 16,000 mg/l. The disposal of waste brines has become a major challenge that hinders the wide application of ion-exchange resins in the water industry. Nevertheless, there have been few studies about the treatment of these wastewaters. Bae et al. (2002) used two up-flow sludge blanket reactors (USBR) at 3% NaCl to treat waste brines with high concentrations of nitrate (600–1700 mg/L) and sulfate (500–2500 mg/L). The average removal efficiency obtained was 90% for nitrate and 50% for sulfate. However, the processing efficiency was low and the adoption of the two reactors required excess equipment and controlling units, thus raising the operation and installation costs.

The expanded granular sludge bed (EGSB) reactor ex-

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hibits higher up-flow velocity, intensified hydraulic mixing and enhanced wastewater-biomass contact compared to the USBR. Other advantages include the dilution of the wastewater at the point of entry to the bioreactor, the formation of biomass granules with good settling property, and the improvement of substrate diffusion from the bulk liquid to the liquid/granule interface (Chou et al., 2008). Furthermore, Chen et al. (2008) found it was feasible to achieve simultaneous sulfate/nitrate/COD removal in a single EGSB reactor. They analyzed the microbial community structure in the granules with single-strand conformation polymorphism and denaturing gradient gel electrophoresis. Nevertheless, their influent was different from the waste brines mentioned above.

The recently launched GS-FLX-titanium sequencer based on pyrosequencing allows for quick and inexpensive analysis of microbial diversity in different samples in a single run without the need for cloning (Liu et al., 2008). Other existing approaches are inferior to this novel technology. For example, Sanger sequencing has low depth of coverage and post-Sanger sequencing can rarely detect minority organisms in a community (Krause et al., 2008; Petrosino et al., 2009). Pyrosequencing has been widely used to analyze the microbial community in various environmental samples such as marine water, soil, human distal intestine, wastewater treatment plant influent, and active sludge (Kwon et al., 2010; Ye et al., 2011). In this study, the waste brine containing high concentrations of sulfate and nitrate was treated in a single EGSB reactor. The bacterial diversity of granular sludge from the EGSB reactor was determined by 454-pyrosequencing technology. The identification of the microbial compositions could provide a theoretical basis for waste brine disposal.

1 Materials and methods

1.1 Experimental equipment and operational conditions

The schematic diagram of the bench-scale EGSB reactor used in this study is shown in **Fig. 1**. The Plexiglas EGSB reactor was 50 mm in diameter and 85 cm in height, giving a total volume of 1.96 L and a working volume of 1.18 L. A peristaltic pump was used to introduce influent at the column bottom of the reactor. A gas-washing device was used to collect the generated H_2S and N_2 gas at the column top. A three-phase separator was installed at the reactor top to keep the biomass within the reactor. Excess sludge was discharged from the bottom of the EGSB reactor. The liquid up-flow velocity was controlled by inner recirculation. The EGSB reactor was operated under mesophilic conditions (35 ± 1°C) (Chen et al., 2008) and its temperature was maintained by a water bath.

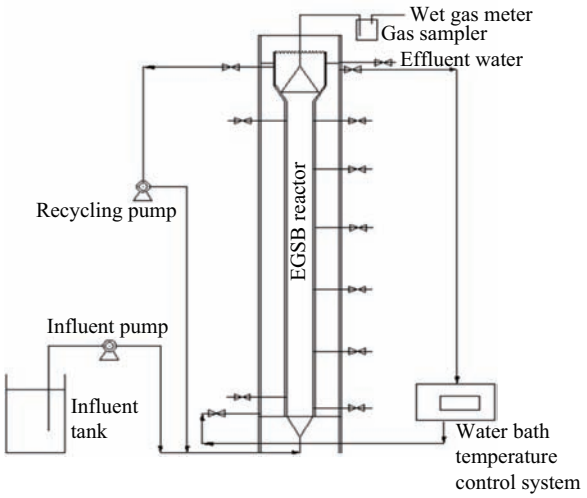

Fig. 1 Experimental apparatus and process flow chart of the expanded granular sludge bed (EGSB) reactor operated at (35 ± 1)°C.

1.2 Synthetic waste brine

The influent synthetic wastewater was prepared to simulate typical waste brine regenerated by ion-exchange adsorption of nitrate from wastewater. The synthetic waste brine preparation was as follows: the COD:SO_4^{2-} mass ratio at 2:1 was regulated by adding ethanol and sodium sulfate and the nitrate nitrogen was added in the form of sodium nitrate. Nitrogen and phosphorus were supplied as NH_4Cl and $K_2HPO_4{\cdot}3H_2O$ respectively in order to obtain a COD/N/P ratio of 200/5/1 in the tap water-based influent solution (Chen et al., 2008). Sodium bicarbonate (1.5 ± 0.3 g/L) was added to maintain the influent pH at 8.0 ± 0.3. Sodium chloride (30 g) and trace element solution (1 mL) were added per liter synthetic wastewater. Trace element solution consisted of (g/L): 0.50 H_3BO_3, 0.50 $NiCl_2{\cdot}6H_2O$, 1.50 $FeCl_3{\cdot}7H_2O$, 0.50 $AlCl_3{\cdot}6H_2O$, 5.00 $MnCl_2{\cdot}4H_2O$, 1.00 $NaSeO_3{\cdot}5H_2O$, 0.50 $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$, 0.50 $CuSO_4{\cdot}5H_2O$, 0.50 $ZnCl_2$, 0.50 $CoCl_2{\cdot}6H_2O$, 50.00 EDTA and 37% HCl solution (5 mL/L).

1.3 Experimental procedure

The seed sludge was domesticated from the digested sludge (mixed liquor volatile suspended solids (MLVSS): 22.178 g/L) of the Jiangxinzhou Urban Sewage Treatment Plant, Jiangsu, China, which was filtered through 0.2 mm Tyler mesh to eliminate most grit. The reactor was started up with the influent SO_4^{2-} concentration at 1000 mg/L and liquid up-flow velocity at 2.5 m/hr, and ethanol and sodium sulfate were added to maintain the COD:SO_4^{2-} mass ratio at 2:1. During 145 days of operation, the influent SO_4^{2-} concentration of the synthetic wastewater gradually increased from 1000 to 1400, 2000, 3000 and 3600 mg/L. Each concentration gradient and operation duration are given in **Table 1**, with corresponding steps recorded as I, II, III, IV and V.

Table 1 Continuous operational parameters of the expanded granular sludge bed reactor

Phase	Time (day)	HRT (hr)	Influent sulfate concentration (mg/L)	COD/SO_4^{2-} ratio (*W/W*)	SO_4^{2-}-S/NO_3^--N ratio (*W/W*)	Salinity (wt%)
I	1–14	22	1000	2:1	–	3
	15–31	15	1000	2:1	–	3
II	32–51	15	1400	2:1	–	3
III	52–75	15	2000	2:1	–	3
IV	76–111	15	3000	2:1	–	3
V	112–145	15	3600	2:1	–	3
VI	146–158	15	3600	2:1	4/1	3
VII	159–174	15	3600	2:1	2/1	3
VIII	175–199	15	3600	2:1	4/3	3
IX	200–221	15	3600	2:1	1/1	3
X	222–241	15	3600	2:1	4/3	3
XI	242–261	15	3600	2:1	6/5	3

Starting from the 146th day, keeping COD/SO_4^{2-} (2/1), sulfate concentration (3,600 mg/L) and HRT (15 hr) constant, the synthetic wastewater gradually received nitrate at different SO_4^{2-}-S/NO_3^--N ratios, and the SO_4^{2-}-S/NO_3^--N ratios were set to 4/1, 2/1, 4/3, 1/1, 4/3, 6/5, corresponding to the phases VI, VII, VIII, IX, X, XI, respectively. The MLVSS concentration was controlled to be from 30 to 40 g/L during the whole experiment process.

1.4 Chemical analysis

Ion chromatography (Dionex ICS-1100) was employed to measure the concentrations of nitrate, nitrite and sulfate in the collected liquor samples following 0.45-μm filtration. Sample separation and elution were performed using an IonPac AG23 AS23 4 mm analytical column with carbonate/bicarbonate eluent (4.5 mmol/L Na_2CO_3/0.8 mmol/L $NaHCO_3$ at 1 mL/min) and a sulfuric regeneration (H_2SO_4, 50 mmol/L at 1 mL/min). Wastewater COD, MLSS and MLVSS were analyzed according to China NEPA standard methods (1997).

1.5 DNA extraction, PCR amplication and pyrosequencing

Sludge samples were composite samples, which were taken from the upper, middle and bottom part of the reactor on the 240th day during stable operation of phase X. DNA extraction was the same as previously described (Liao et al., 2013)

Primer sets 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and A518R (5′-ATTACCGCGGCTGCTGG-3′) (Ionescu et al., 2012) were used for bacterial sequences. The V1-V3 region of the 16S rDNA gene was amplified by PCR from GA DNA using primers adapted for the Roche-454 Titanium kit. Then DNA samples with different barcodes were mixed in equal concentration and sequenced by a Roche 454 FLX Titanium sequencer (Roche, Nutley, NJ, USA) at Beijing Genome Institute (Shenzhen, China). The pyrosequencing methodology employed was the same as previously described (Davis et al., 2011). The pyrosequencing results were deposited into the NCBI sequence reads archive database (accession number SRA059328).

1.6 Post-run analysis

The raw reads treatment with the Pyrosequencing Pipeline Initial Process (Cole et al., 2009) of the Ribosomal Database Project (RDP) was the same as previously described (Liao et al., 2013). After raw reads were denoised and their chimera were removed according to the step reported by Zhang et al. (2011), the number of selected bacterial sequences was 9194 for all the following analyses.

Taxonomic classification of the bacterial sequences of samples was carried out using the RDP classifier. A bootstrap cutoff of 50% suggested by the RDP was applied to assign the sequences to different taxonomy levels.

The normalized sequence set of samples was aligned by Infernal (Nawrocki and Eddy, 2007) using the bacteria-alignment model in Align module of the RDP. By applying Complete Linkage Clustering, sequences were assigned to phylotype clusters at three cutoff levels of 1%, 3% and 5%. On the basis of these clusters, the rarefaction curve, Shannon index and Chao1 richness were calculated using the relevant RDP modules, including Rarefaction and Chao1 Estimator.

2 Results and discussion

2.1 Performance of EGSB reactor for removal waste brine containing high concentrations of sulfate

The removal efficiencies of sulfate and COD during the startup stage are shown in **Fig. 2a**. During the first 15 days of phase I, the average removal efficiency of COD

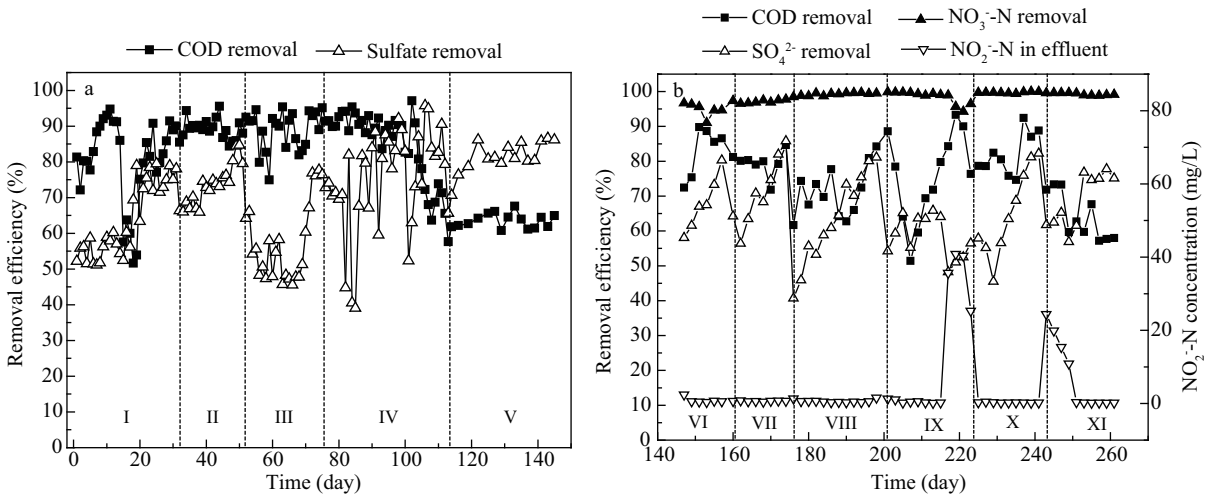

Fig. 2 Changes of the relative parameters in the effluent from the EGSB reactor. (a) SO_4^{2-} and COD during the startup stage; (b) COD, SO_4^{2-}, NO_3^--N and NO_2^--N for different SO_4^{2-}-S/NO_3^--N ratios.

was 85.81% with a maximum of 94.77%. When HRT was reduced from 22 to 15 hr, the removal efficiency of COD dropped to 57.51%, indicating that increasing the organic loading rate would influence the performance of the reactor. Nevertheless, COD removal efficiency was recovered after about one week, which showed that the EGSB had a good resistance to great variations in loading rate. With the increase in the influent sulfate concentration at phases II and III, the average COD removal efficiency remained 89.06%. However, during the last week of phase IV, it decreased to about 70%, and the average removal efficiency of COD was only 63.33% at phase V. The sulfate removal efficiency dropped when the concentration of influent sulfate increased, and then gradually increased. The total trend of sulfate removal efficiency increased during the 145 days of operation. The maximum sulfate removal efficiency (95.71%) was achieved at day 106 in phase IV.

COD/SO_4^{2-} ratio is a major factor that affects sulfate reduction reactions. The COD/SO_4^{2-} ratio of 0.67 is a minimum requirement for sulfate reduction (Lens et al., 1998). Complete sulfate reduction was reported at COD/SO_4^{2-} ratio of 10 and 5, whereas it strongly deteriorated at a COD/SO_4^{2-} ratio of 0.5 in a methanol-fed upflow anaerobic sludge blaket (Vallero et al., 2003). The sulfate removal efficiency decreased when the substrate/SO_4^{2-}-S ratio became less than 6, because sulfate-reducing bacteria (SRB) faced strong competition for substrate with methane producing bacteria in an ethanol-fed EGSB reactor (Smul et al., 1999). It was also found that SRB became a predominant microbial group when the influent COD/SO_4^{2-} ratio was maintained at less than or equal to 1.3, whereas methane producing bacteria became predominant when the influent COD/SO_4^{2-} ratio was maintained at greater than or equal to 2.0 (Chen, 1999; O'Reilly and Colleran, 2006). So the influent COD/SO_4^{2-} ratio was fixed at 2 in this study. The average COD removal efficiency reached over 85% at phases I, II, and III, and the maximum value was 95.58%, at a fixed COD/SO_4^{2-} ratio of 2, while at phases IV and V the COD removal efficiency gradually decreased. On the contrary, the sulfate removal efficiency gradually increased at phases I and II, then dropped at phase III, and finally continued to increase at phase IV and V. The average removal efficiency of COD and sulfate reached 82.78% and 69.64% at phases I–V, respectively. However, Li et al. (2012) reported that total sulfate removal efficiency was 65% in a two-stage USBR at HRT of 34 hr, and the total COD removal efficiency was 51% at HRT of 38 hr at COD/SO_4^{2-} ratio of 2.1. Chaiprapat et al. (2011) reported that sulfate and COD removal efficiencies of the USBR could be sustained at 27.37% ± 2.55% and 33.1% ± 1.0%, respectively, at pH of 8 and COD/SO_4^{2-} ratio of 2. This indicated that, in treating high sulfate wastewater, the EGSB reactor performs better than the USBR. The reasons are as following: firstly, the dilution degree of the wastewater is greatly increased through the inner recirculation of the EGSB reactor. Secondly, the high up-flow velocity is beneficial to keep sufficient contact between wastewater and biomass.

2.2 Treatment performance of waste brine containing sulfate and nitrate

To research the treatment performance of waste brine containing high levels of sulfate and nitrate in the single EGSB reactor, ethanol was used as the sole carbon source and electron donor, and the COD/SO_4^{2-} mass ratio of 2 was maintained; namely the influent sulfate concentration of 3600 mg/L remained unchanged, while the influent nitrate nitrogen concentration changed, that is, the SO_4^{2-}-S/NO_3^--N ratio (**Table 1**) was altered. The corresponding removal efficiency was investigated and the results are shown in **Fig. 2b**.

As can be seen from **Fig. 2b**, at phase VI, two days

after 300 mg/L nitrate nitrogen was added into the reactor, the nitrate nitrogen removal efficiency reached 96.68%, and the accumulated nitrite nitrogen was just 2.71 mg/L. However, in the following 10 days, nitrite nitrogen concentration decreased below 1.00 mg/L, and the average nitrate nitrogen removal efficiency was 94.48%; while the average COD removal efficiency increased from 63.33% to 83.08%, which indicated that adding nitrate can promote the removal efficiency of COD. However, the average sulfate removal efficiency decreased from 80.49% to 67.95%. Notably, just two days after adding nitrate, it dropped from 86.15% to 58.06%.

At phase VII and phase VIII, the influent nitrate nitrogen increased to 600 mg/L and 900 mg/L respectively, corresponding to the SO_4^{2-}-S/NO_3^--N ratios of 2/1 and 4/3. At this time, the COD removal efficiency gradually decreased while the sulfate removal efficiency gradually increased, and reached the maximum (85.80%). Moreover, the effluent nitrite nitrogen concentration was less than 1.5 mg/L, and the nitrate nitrogen removal efficiency gradually increased to 99%. At phase IX, corresponding to the SO_4^{2-}-S/NO_3^--N ratio of 1/1, the influent nitrate nitrogen concentration reached 1200 mg/L, although the nitrate nitrogen removal efficiency decreased slightly, and the effluent nitrite nitrogen accumulated concentration achieved 40.80 mg/L. At the same time, the sulfate removal efficiency decreased, and was less than 66%, while COD removal efficiency increased to 93.34%. This indicated that nitrate inhibited the reduction of the sulfate, and promoted the reduction of the COD at the SO_4^{2-}-S/NO_3^--N ratio of 1/1 and COD/SO_4^{2-} ratio of 2/1. This result was similar to the reported literature (Telang et al., 1997; Hubert and Voordouw, 2007).

When the influent nitrate nitrogen concentration decreased from 1200 to 900 mg/L with SO_4^{2-}-S/NO_3^--N ratio increasing from 1/1 to 4/3 at phase X, both the nitrate nitrogen and sulfate removal gradually rose again. Moreover, the effluent nitrite nitrogen concentration dropped back to under 0.500 mg/L, while the corresponding COD removal efficiency declined slightly. Meanwhile, the high nitrate and sulfate loading rate was 6.38 kg/(m^3·day) and 5.78 kg/(m^3·day) respectively, and the corresponding removal efficiency was 99.97% and 82.26%. However, Bae et al. (2002) reported that the average removal efficiency obtained was 90% for nitrate and 50% for sulfate using two USBRs at 3% NaCl to treat waste brines. This further indicated that treating waste brines by applying the EGSB reactor had some advantages compared with the USBR. The reason lies in the structural characteristics of the EGSB reactor. Specific reasons need in-depth study.

At phase XI, in which the SO_4^{2-}-S/NO_3^--N ratio declined from 4/3 to 6/5 again, namely the influent nitrate nitrogen concentration increased from 900 to 1000 mg/L, the nitrate nitrogen, sulfate and the COD removal efficiencies all declined again, and the effluent nitrite nitrogen concentration increased to about 24.39 mg/L. The above analysis indicated that the optimal SO_4^{2-}-S/NO_3^--N ratio was 4/3.

Moreover, as can be seen from **Fig. 2b**, both the COD and sulfate removal efficiencies had a downward trend when the influent nitrate concentration increased in every instance, and then slowly recovered, increasing after some days' operation. This indicated that there was an inhibition of sulfate reduction by nitrate as reported previously (Mohanakrishnan et al., 2008; Grigoryan et al., 2008). There are two possible mechanisms: competition for nutrients and inhibition of the sulfate reduction pathway. Firstly, nitrate addition stimulates resident heterotrophic nitrate-reducing bacteria and nitrate-reducing and sulfide-oxidizing bacteria, which compete with SRB for the same carbon sources (Grigoryan et al., 2008). Secondly, studies using pure cultures of *Desulfovibrio vulgaris* have revealed that nitrite produced by nitrate-reducing bacteria is a strong inhibitor of SRB, and may block dissimilatory sulfite reductase and consequently down-regulate the upstream genes of the sulfate reduction pathway (Haveman et al., 2004).

2.3 Microbial diversity and phyla distribution analysis of pyrosequencing

A total of 12557 16S rRNA sequence reads were generated by the pyrosequencing of duplicate samples from the suspended granular sludge in the EGSB reactor. After filtering the low quality reads using the RDP Initial Process in Pyrosequencing Pipeline and trimming the adapters, barcodes and primers, there were 11276 effective reads for the sample. After denoising, filtering out chimeras, and removing the archaeal sequences, the library size of the sample was normalized to 9194 sequences, to conduct the downstream analyses.

Rarefaction analysis was employed to standardize and compare observed taxon richness between samples and to identify whether the sample was unequally sampled. Within rarefaction curves, distance values of 0.03, 0.05, 0.07 and 0.1 are generally accepted as points at which differentiation occurs at the species, genus and family/class level, respectively (Stackebrandt and Goebel, 1994; Bowman et al., 2012). As there are some debates about these distinctions, particularly the 0.03 cutoff for novel isolates, a more stringent 0.01 distance value was also calculated for the rarefaction curve (**Fig. S1**, Supporting materials). A rarefaction curve that reaches a plateau reflects a habitat that has been sampled to saturation with regard to species diversity in that ecosystem (Hughes and Hellmann, 2005). **Figure S1** shows that the slopes of the GA sample tended to be flat at levels of 0.03 and 0.05 cutoff, thus the sequences analysis was an accurate representation of the bacterial diversity. This result supports the usefulness of pyrosequencing as a tool for deep coverage of bacterial diversity as a function of 16S rRNA gene sequences in aquatic ecosystems. The corresponding numbers of OTUs,

the Chao1 and Shannon index (H') are summarized in **Table 2**, which also showed the sample from the EGSB reactor had rich diversity. Community diversity showed more richness than that reported by Ye et al. (2011) and Zhao et al. (2012), who observed that there were 494 OTUs in nitrification reactor sludge and 644 OTUs in an anaerobic sludge at level of 3% cutoff, respectively. However, the GA sample from the EGSB reactor displayed considerably less richness compared with active sludge from sewage treatment plants (Zhang et al., 2011; Hu et al., 2012), where 1183–4120 OTUs are in a sludge sample at 3% cutoff level and H' varied from 6.3 to 7.3.

The 9194 selected effective bacterial sequences were assigned to different taxa levels (from genus to phylum) using the RDP Classifier at 50% threshold. **Table 3** shows that the unclassified sequence portions of the total community increased from the phylum level to the genus level, and were similar to the result reported by Zhang et al. (2011). For example, 15.28% and 46.23% of sequences in this study could not be assigned to any taxa at families and genera levels. Zhang et al. (2011) found that the samples from 14 sewage treatment plants contained 20%–43% (family) and 32%–57% (genus) unclassified taxa respectively.

As shown in **Table S1**, there were 14 different phylogenetic groups at the phylum taxonomic rank. Notably, the predominant bacterial phylum was *Proteobacteria* (7140 sequences), which accounted for 77.66% of the total effective bacterial sequences (**Table S1** and **Table 3**). This is similar to the analytical results of bacterial communities in soil (Roesch et al., 2007) and active sludge (Zhang et al., 2011; Hu et al., 2012), in which *Proteobacteria* were also the most dominant community. The other dominant phyla were *Firmicutes* (12.23%), *Chlorobi* (2.71%) and *Bacteroidetes* (1.94%). These four groups were dominant (94.54%) in bacterial communities of the granular sludge sample in this study, which was different from a few previous studies on activated sludge

Table 2 OTUs, Chao 1 (LCI95 and UCI95) and Shannon index (H')

Level	OTUs	Chao1	LCI95-UCI95	H'
99%	2419	4214	3962–4505	6.70735 ± 0.00034
97%	1047	1429	1337–1549	5.7609 ± 0.00027
95%	645	923	840–1039	5.05381 ± 0.00026

OTUs: operational taxonomic units.

Table 3 16S rDNA phylotype distribution by taxonomic phylum, class, order, family and genus (the top five abundant and unclassified bacteria were selected in each catagory). The abundance is presented in terms of percentages of the total sequences in a sample.

Phylotype (phylum)	No. of sequences		Phylotype (family)	No. of sequences	
Proteobacteria	7140	77.66%	*Rhodocyclaceae*	3864	42.03%%
Firmicutes	1124	12.23%	*Rhodobacteraceae*	1222	13.29%
Chlorobi	249	2.71%	*Helicobacteraceae*	514	5.59%
Bacteroidetes	178	1.94%	*Campylobacteraceae*	354	3.85%
Synergistetes	134	1.46%	*Clostridiaceae 2*	335	3.64%
Unclassified phylum	162	1.76%	Unclassified family	1405	15.28%
Phylotype (class)	**No. of sequences**		**Phylotype (genus)**	**No. of sequences**	
Betaproteobacteria	4483	48.76%	*Thauera*	2594	28.21%
Alphaproteobacteria	1343	14.61%	*Wolinella*	388	4.22%
Epsilonproteobacteria	938	10.2%	*Arcobacter*	344	3.74%
Clostridia	842	9.16%	*Alkaliphilus*	334	3.63%
Erysipelotrichia	272	2.96%	*Erysipelothrix*	208	2.26%
Unclassified class	246	2.68%	Unclassified genus	4250	46.23%
Phylotype (order)	**No. of sequences**				
Rhodocyclales	3864	42.03%			
Rhodobacterales	1222	13.29%			
Campylobacterales	901	9.8%			
Clostridiales	838	9.11%			
Erysipelotrichales	272	2.96%			
Unclassified order	987	10.74%			

A bootstrap cutoff of 50% suggested by the RDP was applied to assign the sequences to different taxonomy levels.

using pyrosequencing (Zhang et al., 2011), microarray (Xia et al., 2010) and cloning (Snaidr et al., 1997). They found the four dominant groups were *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. The possible reason is that the samples of granular sludge and activated sludge were collected from different environments, therefore they showed different characteristis. However, the specific resons need further indepth research. The dominant groups were followed by a few other phyla, including *Synergistetes* (1.46%), *Tenericutes* (0.88%), *Chloroflexi* (0.58%), *Deferribacteres* (0.49%), *Spirochaetes* (0.25%), *Deinococcus-Thermus* (0.02%), *Actinobacteria* (0.01%), *Thermotogae* (0.01%), and *Planctomycetes* (0.01%).

There were 22 classes and 33 orders detected in the total bacterial population. The most dominant bacterial classes were *Betaproteobacteria* (4483 sequences), *Alphaproteobacteria* (1343 sequences) and *Epsilonproteobacteria* (938 sequences), which accounted for 48.76%, 14.61% and 10.20% of the total effective bacterial sequences, respectively. Moreover, *Betaproteobacteria* was the most dominant *Proteobacteria*, followed by *Alphaproteobacteria, Epsilonproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria*. This is similar to the results of a study using pyrosequencing (Roesch et al., 2007; Zhang et al., 2011). The predominant phyla order was *Rhodocyclales* (3864 sequences), and the next most abundant were *Rhodobacterales* (1222 sequences) and *Campylobacterales* (901 sequences), accounting for 42.03%, 13.29% and 9.80%.

There were 52 families and 76 genera detected (**Tables S2** and **S3**), and *Rhodocyclaceae*, *Rhodobacteraceae*, *Helicobacteraceae* were the most dominant families, accounting for 42.03%, 13.29% and 5.59% of the total effective bacterial sequences, respectively. At the same time, the sequence number of unclassified family was 1405, accounting for 15.28%; whereas the sequence number of unclassified genus was as high as 4250, accounting for 46.23% of the total effective bacterial sequences, *Thauera* was the most dominant bacterial genus detected, accounting for 28.21%. The following major (average abundance >1%) genera were found, including *Wolinella*, *Arcobacter*, *Alkaliphilus*, *Erysipelothrix*, *Chlorobaculum*, *Sulfurovum*, *Tissierella*, and *Denitratisoma*. Meanwhile, the abundances of other genera were < 1%, such as *Desulfovibrio*, *Acetoanaerobium*, *Desulfuromonas*, *Azomonas*, *Zymobacter*, *Nitratifractor*, *Desulfococcus* and so on. *Thauera* and *Denitratisoma* played an important role in denitrification of high strength nitrate in terms of the references reported by Mao et al. (2008, 2010). Furthermore, *Desulfovibrio*, *Desulfuromonas*, *Desulfococcus* and *Sulfurovum* played a significant part in desulfating in terms of the references reported by Chen et al. (2008) and Tang et al. (2009).

3 Conclusions

High sulfate removal efficiency (80%–90%) was achieved at the influent sulfate concentration of 3600 mg/L and 3% NaCl after 145 days in a single EGSB reactor. Based on the above-mentioned research, nitrate added into the reactor with different SO_4^{2-}-S/NO_3^--N mass ratios was investigated, and the optimal SO_4^{2-}-S/NO_3^--N ratio was 4/3. Meanwhile, the bacterial population was investigated with 454-pyrosequencing technology at phase X. Sequence analyses showed that *Proteobacteria* (77.66%) was the dominant bacterial phylum; *Betaproteobacteria* (48.76%) and *Rhodocyclales* (42.03%) were the most abundant taxonomic class and order. The predominant bacterial family and genus were *Rhodocyclaceae* (42.03%) and *Thauera* (28.21%). Furthermore, *Thauera* and *Denitratisoma* played an important role in denitrification of high strength nitrate, and *Desulfovibrio*, *Desulfuromonas*, *Desulfococcus* and *Sulfurovum* played a significant part in desulfating in the EGSB reactor.

Acknowledgments

The work was supported by the National Natural Science Foundation of China (No. 51178215, 51378251), the Jiangsu Provincial Natural Science Foundation (No. BK2011032, BK20130575), the Jiangxi Provincial Natural Science Foundation (No. 2010GQC0106) and the State Key Laboratory of Pollution Control and Resource Reuse Foundation (No. PCRRF12006).

Supporting materials

Supplementary data associated with this article can be found in the online version.

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Supporting materials

Performance and microbial diversity of an expanded granular sludge bed reactor for high sulfate and nitrate waste brine treatment

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Table S1 16S rDNA phylotype distribution by taxonomic phylum, class, and order.

Phylotype (phylum)	No. of sequences	%	Phylotype (order)	No. of sequences	%
Proteobacteria	7140	77.66	*Rhodocyclales*	3864	42.03
Firmicutes	1124	12.23	*Rhodobacterales*	1222	13.29
Chlorobi	249	2.71	*Campylobacterales*	901	9.80
Bacteroidetes	178	1.94	*Clostridiales*	838	9.11
Synergistetes	134	1.46	*Erysipelotrichales*	272	2.96
Tenericutes	81	0.88	*Chlorobiales*	249	2.71
Chloroflexi	53	0.58	*Desulfovibrionales*	195	2.12
Deferribacteres	45	0.49	*Synergistales*	134	1.46
Spirochaetes	23	0.25	*Flavobacteriales*	90	0.98
Deinococcus-Thermus	2	0.02	*Rhizobiales*	87	0.95
Actinobacteria	1	0.01	*Acholeplasmatales*	62	0.67
Thermotogae	1	0.01	*Anaerolineales*	52	0.57
Planctomycetes	1	0.01	*Xanthomonadales*	48	0.52
Unclassified *phylum*	162	1.76	*Deferribacterales*	45	0.49
Phylotype (class)	No. of sequences	%	*Sphingobacteriales*	31	0.34
Betaproteobacteria	4483	48.76	*Spirochaetales*	23	0.25
Alphaproteobacteria	1343	14.61	*Bacteroidales*	23	0.25
Epsilonproteobacteria	938	10.20	*Oceanospirillales*	18	0.20
Clostridia	842	9.16	*Desulfuromonadales*	13	0.14
Erysipelotrichia	272	2.96	*Pseudomonadales*	10	0.11
Chlorobia	249	2.71	*Nautiliales*	7	0.08
Deltaproteobacteria	242	2.63	*Caulobacterales*	4	0.04
Synergistia	134	1.46	*Syntrophobacterales*	3	0.03
Flavobacteria	90	0.98	*Chromatiales*	3	0.03
Gammaproteobacteria	90	0.98	*Lactobacillales*	3	0.03
Mollicutes	81	0.88	*Burkholderiales*	2	0.02
Anaerolineae	52	0.57	*Deinococcales*	2	0.02
Deferribacteres	45	0.49	*Actinomycetales*	1	0.01
Sphingobacteria	31	0.34	*Desulfobacterales*	1	0.01
Spirochaetes	23	0.25	*Thermotogales*	1	0.01
Bacteroidia	23	0.25	*Bacillales*	1	0.01
Bacilli	4	0.04	*Planctomycetales*	1	0.01
Deinococci	2	0.02	*Caldilineales*	1	0.01
Actinobacteria	1	0.01	Unclassified order	987	10.74
Thermotogae	1	0.01			
Planctomycetacia	1	0.01			
Caldilineae	1	0.01			
Unclassified class	246	2.68			

A bootstrap cutoff of 50% suggested by the RDP was applied to assign the sequences to different taxonomy levels. The abundance is presented in terms of percentages of the total sequences in a sample.

Table S2. 16S rDNA phylotype distribution by taxonomic family

Phylotype (family)	No. of sequences	%	Phylotype (family)	No. of sequences	%
Rhodocyclaceae	3864	42.03	*Ruminococcaceae*	6	0.07
Rhodobacteraceae	1222	13.29	*Flammeovirgaceae*	5	0.05
Helicobacteraceae	514	5.59	*Bradyrhizobiaceae*	5	0.05
Campylobacteraceae	354	3.85	*Clostridiales Incertae Sedis XII*	5	0.05
Clostridiaceae 2	335	3.64	*Caulobacteraceae*	4	0.04
Erysipelotrichaceae	272	2.96	*Geobacteraceae*	3	0.03
Chlorobiaceae	249	2.71	*Rhizobiales incertae sedis*	3	0.03
Clostridiales Incertae Sedis XI	239	2.60	*Lachnospiraceae*	3	0.03
Synergistaceae	134	1.46	*Syntrophaceae*	2	0.02
Desulfovibrionaceae	90	0.98	*Desulfomicrobiaceae*	2	0.02
Flavobacteriaceae	73	0.79	*Brucellaceae*	2	0.02
Acholeplasmataceae	62	0.67	*Clostridiales Incertae Sedis XIII*	2	0.02
Anaerolineaceae	52	0.57	*Trueperaceae*	2	0.02
Xanthomonadaceae	48	0.52	*Cytophagaceae*	1	0.01
Deferribacteraceae	45	0.49	*Syntrophobacteraceae*	1	0.01
Clostridiaceae 1	45	0.49	*Desulfohalobiaceae*	1	0.01
Porphyromonadaceae	23	0.25	*Desulfobacteraceae*	1	0.01
Halomonadaceae	18	0.20	*Burkholderiales incertae sedis*	1	0.01
Spirochaetaceae	17	0.18	*Alcaligenaceae*	1	0.01
Peptostreptococcaceae	15	0.16	*Eubacteriaceae*	1	0.01
Hyphomicrobiaceae	11	0.12	*Enterococcaceae*	1	0.01
Pseudomonadaceae	10	0.11	*Carnobacteriaceae*	1	0.01
Clostridiales incertae sedis	10	0.11	*Bacillales Incertae Sedis XII*	1	0.01
Cyclobacteriaceae	9	0.10	*Planctomycetaceae*	1	0.01
Phyllobacteriaceae	9	0.10	*Caldilineaceae*	1	0.01
Nautiliaceae	7	0.08	unclassified family	1405	15.28
Desulfuromonadaceae	6	0.07			

A bootstrap cutoff of 50% suggested by the RDP was applied to assign the sequences to different taxonomy levels.

The abundance is presented in terms of percentages of the total sequences in a sample.

Table S3. 16S rDNA phylotype distribution by taxonomic genus.

Phylotype (genus)	No. of sequences	%	Phylotype (genus)	No. of sequences	%
Thauera	2594	28.21	*Treponema*	2	0.02
Wolinella	388	4.22	*Desulfomonile*	2	0.02
Arcobacter	344	3.74	*Desulfomicrobium*	2	0.02
Alkaliphilus	334	3.63	*Geothermobacter*	2	0.02
Erysipelothrix	208	2.26	*Azovibrio*	2	0.02
Chlorobaculum	199	2.16	*Bosea*	2	0.02
Sulfurovum	122	1.33	*Daequia*	2	0.02
Tissierella	101	1.10	*Caulobacter*	2	0.02
Denitratisoma	99	1.08	*Methylarcula*	2	0.02
Acholeplasma	62	0.67	*Azomonas*	2	0.02
Soehngenia	57	0.62	*Zymobacter*	2	0.02
Desulfovibrio	52	0.57	*Anaerovorax*	2	0.02
Proteiniclasticum	45	0.49	*Truepera*	2	0.02
Stenotrophomonas	41	0.45	*Aminiphilus*	2	0.02
Denitrovibrio	40	0.44	*Thermovirga*	2	0.02
Proteiniphilum	22	0.24	*Leptolinea*	2	0.02
Chlorobium	22	0.24	*Bellilinea*	2	0.02
Prosthecochloris	17	0.18	*Paludibacter*	1	0.01
Levilinea	17	0.18	*Meniscus*	1	0.01
Acetoanaerobium	15	0.16	*Bizionia*	1	0.01
Thioclava	14	0.15	*Myroides*	1	0.01
Longilinea	13	0.14	*Nitratifractor*	1	0.01
Proteiniborus	10	0.11	*Desulfoglaeba*	1	0.01
Spirochaeta	9	0.10	*Desulfococcus*	1	0.01
Fontibacter	9	0.10	*Thiomonas*	1	0.01
Desulfocurvus	7	0.08	*Achromobacter*	1	0.01
Desulfuromonas	6	0.07	*Shinella*	1	0.01
Pannonibacter	5	0.05	*Stappia*	1	0.01
Fusibacter	5	0.05	*Pseudorhodobacter*	1	0.01
Fulvivirga	4	0.04	*Azotobacter*	1	0.01
Thioreductor	4	0.04	*Acetobacterium*	1	0.01
Serpens	4	0.04	*Vagococcus*	1	0.01
Blastobacter	3	0.03	*Alkalibacterium*	1	0.01
Pseudaminobacter	3	0.03	*Exiguobacterium*	1	0.01
Cucumibacter	3	0.03	*Sharpea*	1	0.01
Catellibacterium	3	0.03	*Rhodopirellula*	1	0.01
Clostridium XlVb	3	0.03	*Caldilinea*	1	0.01
Flavonifractor	3	0.03	unclassified genus	4250	46.23
Dethiosulfovibrio	3	0.03			

A bootstrap cutoff of 50% suggested by the RDP was applied to assign the sequences to different taxonomy levels. The abundance is presented in terms of percentages of the total sequences in a sample.

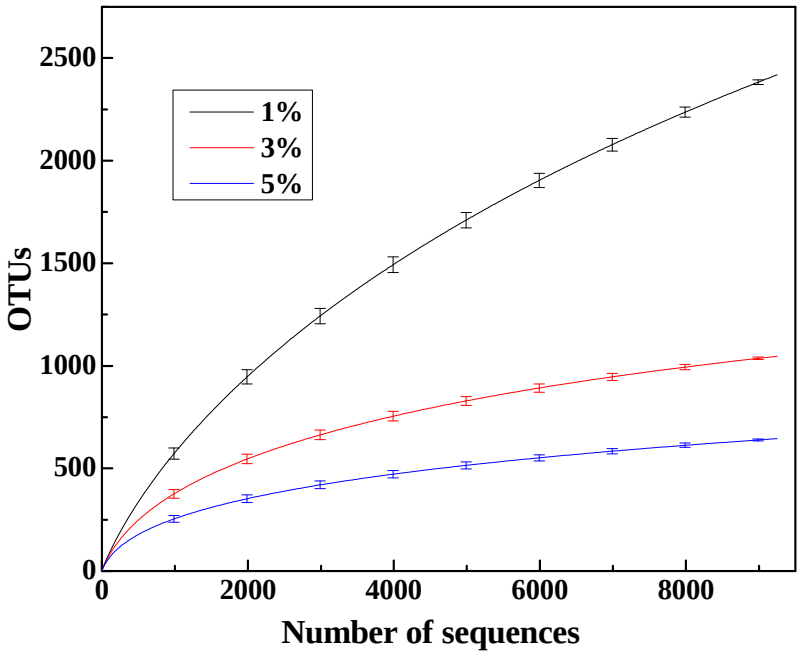

Fig. S1 Rarefaction curves of BN samples at cutoff levels of 1%, 3% and 5% . The rarefaction curve, plotting the number of observed OTUs as a function of the number of sequences, was computed using RDP Pyrosequencing Pipeline Rarefaction tool. The error bars show 95% confidence intervals. The samples were arranged descendingly based on the numbers of OTUs.

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Journal of Environmental Sciences (Established in 1989)

Vol. 26 No. 4 2014

Supervised by	Chinese Academy of Sciences	**Published by**	Science Press, Beijing, China
Sponsored by	Research Center for Eco-Environmental		Elsevier Limited, The Netherlands
	Sciences, Chinese Academy of Sciences	**Distributed by**	
Edited by	Editorial Office of Journal of	Domestic	Science Press, 16 Donghuangchenggen
	Environmental Sciences		North Street, Beijing 100717, China
	P. O. Box 2871, Beijing 100085, China		Local Post Offices through China
	Tel: 86-10-62920553; http://www.jesc.ac.cn	Foreign	Elsevier Limited
	E-mail: jesc@263.net, jesc@rcees.ac.cn		http://www.elsevier.com/locate/jes
Editor-in-chief	Hongxiao Tang	**Printed by**	Beijing Beilin Printing House, 100083, China

CN 11-2629/X **Domestic postcode: 2-580** **Domestic price per issue RMB ¥ 110.00**

ISSN 1001-0742