

## TREATMENT OF HIGH-STRENGTH RARE-EARTH AMMONIA WASTEWATER WITH A TWO-STAGE ANAEROBIC AMMONIUM OXIDATION (ANAMMOX) PROCESS

WENJIE Z.<sup>1,2\*</sup>  
YUHAN D.<sup>1,2</sup>  
DUNQIU W.<sup>1,2</sup>  
HENG C.<sup>3</sup>  
YUE J.<sup>4</sup>

<sup>1</sup>Guangxi Key Laboratory of Environmental Pollution Control  
Theory and Technology

Guilin University of Technology, Guilin 541004, China

<sup>2</sup>Collaborative Innovation Center for Water Pollution Control  
and Water Safety in Karst Area  
Guilin University of Technology, Guilin 541004

<sup>3</sup>Key Laboratory of Experimental Marine Biology, Institute of Oceanology  
Chinese Academy of Sciences, Qingdao 266071, China

<sup>4</sup>Guangxi Key Laboratory of New Energy and Building Energy Saving  
College of Civil Engineering and Architecture  
Guilin University of Technology, 12, Jiangan Road, Guilin, 541004, China

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\*to whom all correspondence should be addressed:  
e-mail: 2010053@glut.edu.cn

### ABSTRACT

In this study, a two-stage anaerobic ammonium oxidation (anammox) system—including a partial nitrification system with a biological selector (PNBS) and a granular activated carbon-based granule anammox process (GAP) —was used for the treatment of real high-strength rare-earth ammonia wastewater (HRAW). A nitrogen removal rate of 89% on average was achieved at the end of the study with the influent total nitrogen concentration of 2200 mg l<sup>-1</sup>. The nitrogen-loading rate (NLR) of 17 kg N/(m<sup>3</sup>·d) was achieved in the PNBS, and a reduced NLR of 6 kg N/(m<sup>3</sup>·d) was maintained in the GAP. To our knowledge, this is the highest NLR applied to a two-stage anammox system. A genetic analysis of the sludge samples revealed that a *Nitrosomonas sp.* was enriched in the PNBS reactor, while, *Kuenenia stuttgartiensis*, *Uncultured bacterium clone KIST-JY001*, and *Uncultured anoxic sludge bacterium KU2* were enriched in the GAP reactor.

**Keywords:** high-strength; high rate; granule; selector; nitrite inhibition

### 1. Introduction

In the manufacturing of rare-earth concentrates, many chemical reagents are used. Therefore, rare-earth wastewater contains a large number of complex pollutants (Xiaoqi and Kristian, 2014), among which high-strength ammonia is deemed the main contaminant (Tingsheng *et al.*, 2014). High-strength rare-earth ammonia wastewater (HRAW) is characterized by hardness, high alkalinity, the presence of fluoride, high sodium, high salinity, and a difficulty to deal with. China is the largest exporter of rare-earth elements, but discharge of untreated HRAW has caused serious water pollution. Surplus ammonia in water even threatens the safety of the water supply in some areas. HRAW treatment at a reasonable cost is an important topic of research on the sustainable development of the rare-earth industry.

Physicochemical systems are conventional processes that are used for the treatment of HRAW, but they are not preferable in most cases because shortcomings such as secondary pollution and a high running cost are frequently reported (Huang *et al.*, 2009). In general, biological nitrogen removal processes are

recommended for removal of nitrogen from wastewater. Among biological nitrogen removal processes anaerobic ammonium oxidation (anammox) (Mulder *et al.*, 1995; Kuenen, 2008; Kartal, 2010) has become more popular due to several advantages over a nitrification+heterotrophic denitrification process: e.g., reduced oxygen supply, low carbon requirements, and lower sludge production (van Graaf *et al.*, 1997). The anammox process may be suggested as a way to treat HRAW from environmental and economic points of view.

There were more than 100 operating installations based on the anammox process worldwide as of 2014 (Lackner *et al.*, 2014). According to the survey of full-scale installations by Lackner *et al.* (2014), aeration control is reported to be one of the main operational difficulties. Aeration control is heavily dependent on a dissolved oxygen (DO) sensor, but a failure of the DO signal often happens, which leads to a system crash. Recently, one partial nitrification system with a biological selector (PNBS) with a high nitrite production rate, 8.25 kg N/(m<sup>3</sup>·d), was used for the treatment of high-strength ammonia-containing wastewater (Yue *et al.*, 2015). In a PNBS, a biological selector instead of aeration control is used to select ammonia-oxidizing bacteria over nitrite-oxidizing bacteria. Therefore, the DO sensor is not crucial in a PNBS, and an increase in the nitrogen-loading rate (NLR) is expected. Reducing the environmental footprint is a research issue of applications of the anammox process to treatment of HRAW. A PNBS with a high potential NLR is proposed here for treatment of HRAW as a pretreatment at the anammox stage.

The aim of this study was to devise a two-stage anammox system for HRAW treatment. This paper presents two separate stages: the first one is a PNBS and the second one is a granular activated carbon-based granule anammox process (GAP). Genetic analysis was employed to characterize the microbial population of the sludge.

## 2. Materials and Methods

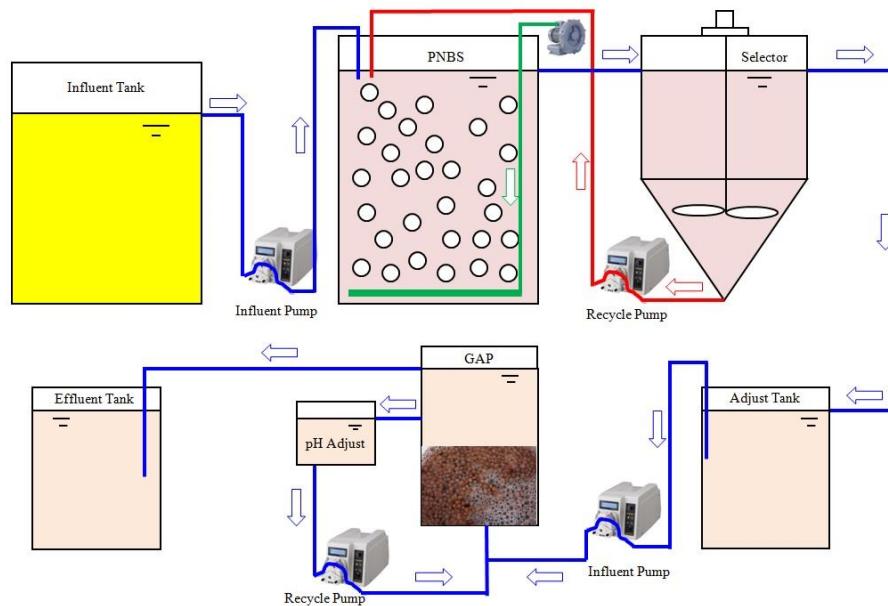
### 2.1. HRAW

HRAW used in this study was obtained from the storage tank of a rare-earth-producing plant in the city of Hezhou, Guangxi, China. At this plant, daily discharge of HRAW is ~1000 m<sup>3</sup>/d. Characteristics of this wastewater are as follows: pH 7.8, suspended solid 200–500 mg l<sup>-1</sup>, NH<sub>4</sub><sup>+</sup> nitrogen 2000–2500 mg l<sup>-1</sup>, while concentrations of NO<sub>2</sub><sup>-</sup> nitrogen and NO<sub>3</sub><sup>-</sup> nitrogen are negligible. HRAW contains high concentrations of nitrogen-containing pollutants, mainly in the form of NH<sub>4</sub><sup>+</sup> nitrogen. Consequently, nitrogen removal is necessary before discharge.

### 2.2. Reactors

A diagram of the PNBS and GAP is shown in Fig. 1. The PNBS reactor used in this study is the same as the one used by Yue *et al.* (2015). The pH level was maintained at 7.1–7.2 by addition of an alkali solution (0.5 mol l<sup>-1</sup> NaHCO<sub>3</sub> and 0.5 mol l<sup>-1</sup> Na<sub>2</sub>CO<sub>3</sub>). Reactor temperature was maintained at 30±1 °C by means of one heater.

The GAP reactor used in this study is the same as that described by Zhang *et al.* (2014). The GAP reactor was filled with red granular activated carbon-based granules, which had an average settling velocity of 200 m h<sup>-1</sup> (5 cm s<sup>-1</sup>) (Wenjie *et al.*, 2015). The reactor was operated in up-flow mode, with the influent introduced at the bottom by a peristaltic pump (BT100-2J, LongerPump, China). A recycling pump (BT600-2J, LongerPump, China) was used to dilute the influent with treated wastewater. Reactor temperature was maintained at 35±1 °C using a water bath. A portion of the effluent was collected in a 5-L container (with a mixer and heater) for recycling. The pH level was adjusted by an online pH controller (TPH/T-10, Tengine, China) using 0.5 mol l<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub>. The reactor was enclosed in a black-vinyl sheet to inhibit the growth of photosynthetic bacteria and algae.



**Figure 1.** Diagram of PNBS and GAP reactor

### 2.3. Analytical methods

$\text{NO}_2^-$ -N,  $\text{NH}_4^+$ -N and TN were measured by the colorimetric method according to Standard Methods (APHA, 1995).  $\text{NO}_3^-$ -N (of the original sample) was determined by calculation of the difference of TN and the sum of  $\text{NO}_2^-$ -N and  $\text{NH}_4^+$ -N. The pH was measured by using a pH meter (9010, Jenco, USA), and dissolved oxygen (DO) was measured by using a DO meter (6010, Jenco, USA).

### 2.4. Microbial community analysis

#### 2.4.1. DNA extraction and PCR amplification

Meta-genomic DNA was extracted using an ISOIL kit (Wako, Osaka, Japan) according to the manufacturer's instructions. Amplification of the 16S rRNA gene was performed with Phusion High-Fidelity DNA polymerase (FINNZYMES, Finland) using conserved eubacterial primers 6F (forward primer: 5'-GGAGAGTTAGATCTTGGCTCAG-3') and 1492r (reverse primer: 5'-GGTTACCTTGTTACGACT-3'). PCR was carried out according to the following thermocycling parameters: 30 s initial denaturation at 98 °C, 25 cycles of 10 s at 98 °C, 30 s at 51 °C, 20 s at 72 °C and 5 min final elongation at 72 °C. The amplified products were electrophoresed on a 1% agarose gel and extracted fragments were purified using Wizard SV Gel and PCR Clean-Up System (Promega, U.S.A.).

#### 2.4.2. Cloning and sequencing of the 16S rRNA gene

The purified fragments were ligated into the EcoRV site of pBluescript II KS+ (Stratagene, USA) and *Escherichia coli* DH10B was transformed using the constructed plasmids. White colonies including the insert were randomly chosen and the plasmids were extracted by the alkaline method. The nucleotide sequences were determined with a 3130xl genetic analyzer and a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The sequences determined in this study were compared with the sequences in the nr database using the basic local alignment search tool program (BLAST) on the NCBI web site.

#### 2.4.3. Denaturing gradient gel electrophoresis (DGGE)

Partial 16S rRNA gene was amplified by PCR with a eubacterial primer set, 357F (forward primer: 5'-CCTACGGGAGGCAGCAG-3') and 534R (reverse primer: 5'-ATTACCGCGTCTGCTGG-3').

The extracted meta-genomic DNA and cloned plasmids were used as templates for the sample and markers in the DGGE, respectively. The amplified fragments were purified and combined with the GC-clamp (5'-CGCCGCGCGCCCCGCGCCCGTCCCGCGCCCCGCCCCG-3') at the 5' termini by a second PCR

using a primer set, 357F with GC-clamp and 534R. The products were resolved by DGGE for 14 h at 90 V at 60 °C using the DCode system (Bio-Rad, Hercules, CA, U.S.A.). An 8% polyacrylamide gel with a 30–65% denaturing gradient was used, where 100% denaturant was defined as 7 M urea and 40% formamide. The gel was stained with SYBR-Gold (Invitrogen, U.S.A.) and visualized using the FLA-2000 system (Fuji Photo Film, Tokyo, Japan).

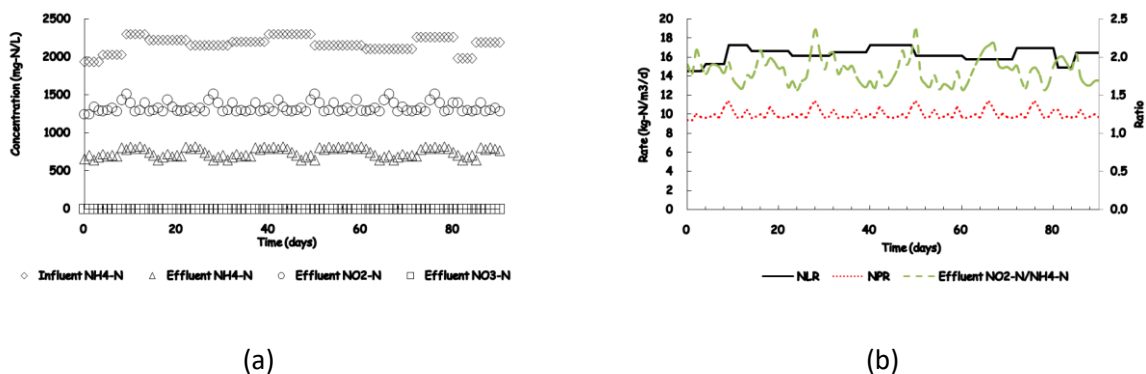
#### 2.4.4. Cloning and sequencing of 16S rDNA

The purified DNA fragments were ligated into the EcoRV site of pBluescript II KS + (Stratagene, USA). *Escherichia coli* DH5 $\alpha$  was transformed using the constructed plasmids. The plasmids were extracted from the clones carrying them by the alkaline method. The DNA fragments were sequenced using a 3130xl genetic analyzer and BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The sequences determined in this study were compared with those in the nr database by the basic local alignment search tool (BLAST) program available on the NCBI website.

### 3. Results and Discussion

#### 3.1. Performance of the PNBS reactor

In this study, synthetic wastewater was used to start the PNBS reactor at an NLR of 0.5 kg-N/(m<sup>3</sup>·d) (data not shown). The start-up period of the PNBS reactor was completed within 30 days, during which time the NLR was increased to 15 kg-N/(m<sup>3</sup>·d) with the influent NH<sub>4</sub><sup>+</sup> nitrogen concentration of 2000 (mg N)/l and hydraulic retention time (HRT) of 3.2 h. Under these conditions, the effluent NO<sub>2</sub><sup>-</sup> nitrogen and NH<sub>4</sub><sup>+</sup> nitrogen concentrations were 1100 and 830 (mg N)/l, respectively. Overall, the reactor could operate with a stable effluent NO<sub>2</sub><sup>-</sup> nitrogen to NH<sub>4</sub><sup>+</sup> nitrogen ratio of > 1.32 during treatment of synthetic wastewater. After the start-up period, the influent was changed to raw HRAW. Fig. 2 shows the time course of NH<sub>4</sub><sup>+</sup> nitrogen, NO<sub>2</sub><sup>-</sup> nitrogen, and NO<sub>3</sub><sup>-</sup> nitrogen processed by the PNBS as well as the NLR, nitrite production rate (NPR), and the ratio of effluent NO<sub>2</sub><sup>-</sup> nitrogen to NH<sub>4</sub><sup>+</sup> nitrogen. This ratio above 1.32 is considered an achievable goal for the PNBS reactor to provide an appropriate substrate for the next reactor (GAP).



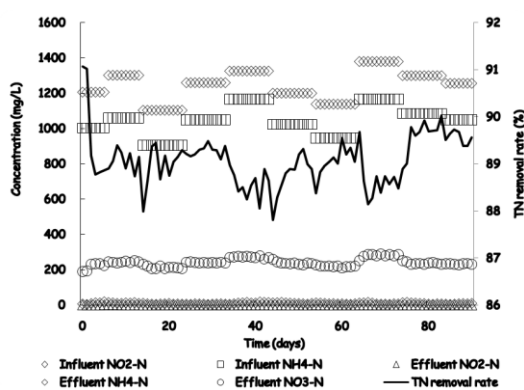
**Figure 2.** Time courses of nitrogen concentrations treating by PNBS (a), nitrogen loading rate (NLR), nitrite production rate (NPR), and ratio of effluent NO<sub>2</sub><sup>-</sup>-N/NH<sub>4</sub><sup>+</sup>-N (b)

As shown in Fig. 2a, NO<sub>2</sub><sup>-</sup> nitrogen accumulation started immediately since the introduction of raw HRAW, with the influent NH<sub>4</sub><sup>+</sup> nitrogen concentration of 1934–2300 mg l<sup>-1</sup>. This result indicates that the sludge in the reactor had been acclimated well to the artificial wastewater. HRT was maintained at 3.2 h. The effluent NO<sub>2</sub><sup>-</sup> nitrogen concentrations were 1200–1600 mg l<sup>-1</sup>, and the average conversion efficiency of ammonia to nitrite was ~70% (Fig. 2 b). No NO<sub>3</sub><sup>-</sup> nitrogen production was detected in the effluent during the whole operation period; this finding indicates that the activity of nitrite-oxidizing bacteria was successfully inhibited.

As shown in Fig. 2b, NLR and NPR on average were 17 and 10 kg N/(m<sup>3</sup>·d), respectively, when raw HRAW was the influent. To our knowledge, this is the highest NLR and NPR to be successfully used with HRAW to date. Li *et al.* (2010) reported that an NLR of 5.7 kg N/(m<sup>3</sup>·d) was achieved with a swim-bed reactor. In our study, the NLR is almost three times this value. Therefore, the PNBS reactor—as demonstrated here—may find an effective application to treatment of naturally occurring high-strength wastewaters.

### 3.2. Performance of the GAP reactor

The influent total nitrogen (TN) concentration was increased to 2200 mg l<sup>-1</sup> with the nitrogen removal efficiency above 89% and the NLR at 6 kg N/(m<sup>3</sup>·d) (process hydraulic retention time of 8.8 h) with artificial wastewater (data not shown). After the NLR of 6 kg N/(m<sup>3</sup>·d) was achieved with the artificial feed influent, an adjusted nitrite to ammonium molar ratio of 1.2 was set by means of the PNBS effluent (Fig. 3). DO of HRAW treated by PNBS was zero in this study. Therefore, DO control was not considered further in the GAP reactor. The pH level was controlled well in the GAP reactor; thereafter, the influent pH was not controlled either during the study. The GAP reactor was operated at an NLR of 6 kg N/(m<sup>3</sup>·d), and the TN removal efficiency did not decrease and remained at ~89%. No obvious change was observed since introduction of the PNBS effluent. The results showed that the sludge of the GAP reactor adapted well to the PNBS effluent.



**Figure 3.** Time courses of nitrogen concentrations treating by GAP

As shown in Fig. 3, the influent TN concentration was 2200 (mg N)/l, with the nitrogen removal efficiency above 89% and the NLR at 6 kg N/(m<sup>3</sup>·d). High concentration of NO<sub>2</sub><sup>-</sup> nitrogen is reported to inhibit the activity of anammox bacteria. There is still debate regarding the maximal tolerable concentration (Strous *et al.*, 1999; Kimura *et al.*, 2010; Lotti *et al.*, 2012). We proceeded with the experiments even though the concentration suitable for anammox growth is unknown. In this study, the NO<sub>2</sub><sup>-</sup> nitrogen concentration of 150 (mg N)/l was used by diluting the PNBS effluent with treated HRAW. Yamamoto *et al.* (2011) used tap water to dilute the high-strength digest liquor. Nevertheless, this practice is forbidden by law for wastewater treatment in China. In our study, good performance was achieved in the GAP reactor when the NO<sub>2</sub><sup>-</sup> nitrogen concentration was below 150 (mg N)/l when diluted with treated wastewater. The results showed that the GAP reactor used here has a high potential for real-life wastewater treatment.

**Table 1.** Treatment performances of PNBS-GAP process.

Parameters	Influent (mg l <sup>-1</sup> )	Effluent (mg l <sup>-1</sup> )	Removal Rate (%)
pH	7.8	7.5-7.6	7.5-
NH <sub>4</sub> -N	2000-2500	5-10	99
NO <sub>2</sub> -N	-	0.2-2	99
NO <sub>3</sub> -N	-	206-280	-
TN	2000-2500	210-290	89-90
SS	200-500	60-90	70-80

As shown in Table 1, the PNBS-GAP process showed good performance on treatment of HRAW. We achieved the TN removal rate of 89–90% with influent  $\text{NH}_4^+$  nitrogen at 2000–2500  $\text{mg l}^{-1}$ . The residual nitrogen in the effluent was mostly in the form of  $\text{NO}_3^-$  nitrogen. Because there is no requirement for  $\text{NO}_3^-$  nitrogen, this quantity in treated water can meet the Chinese discharge standards for industrial wastewater.

In this study, an NLR of 15  $\text{kg N}/(\text{m}^3\cdot\text{d})$  was attained in the PNBS reactor during treatment of HRAW. In contrast, an NLR of only 6  $\text{kg N}/(\text{m}^3\cdot\text{d})$  was used in the GAP reactor. An extremely high NLR, up to 26–76.7  $\text{kg N}/(\text{m}^3\cdot\text{d})$  is possible for one anammox reactor (Tsushima *et al.*, 2007; Tang *et al.*, 2011). In our study, the floating granules became serious when NLR was increased further. Thereafter, a reduced NLR was applied during treatment of HRAW in this study. Control of floating granules is a research issue during the development of a high-rate anammox reactor (Dapena-Mora *et al.*, 2004; Chen *et al.*, 2010; Chen *et al.*, 2014).

### 3.3. The microbial community

**Table 2.** Homology search results for 16S rRNA gene sequences (PNBS)

Phylum	Taxon	Identity (%)	Number of clones	Accession
$\beta$ -proteobacteria	<i>Nitrosomonas sp. ENI-11</i>	98-100		AB079053
	<i>Nitrosomonas europaea ATCC 19718</i>	98-99	12	AL954747
	<i>Ammonia-oxidizing bacterium NS500-9</i>	98-99		AY135356
	<i>Nitrosomonas sp. ENI-11</i>	95	10	AB079053
	<i>Nitrosomonas europaea ATCC 19718</i>	95		AL954747
	<i>Nitrosomonas eutropha</i>	98	1	AY123795
Bacteroidetes	<i>Uncultured soil bacterium clone bacnit28</i>	96		EU861887
	<i>Uncultured bacterium clone KD8-75</i>	95	2	AY218692
	<i>Sphingobacteriaceae bacterium BR5-29</i>	94		EU370957
	<i>Uncultured bacterium clone KD3-77</i>	91		AY218616
	<i>Uncultured bacterium clone sls1367</i>	91	1	EU244084
	<i>Sphingobacteriaceae bacterium BR5-29</i>	90		EU370957
	<i>Uncultured bacterium clone NR.4.002</i>	90		EF494312
	<i>Uncultured Cytophagales bacterium clone 17</i>	88	1	AF361198

Table 2 shows the main results of homology search for 16S rRNA gene sequences in the microbial community of the PNBS reactor (day 90). The genetic analysis revealed that the share of *Nitrosomonas sp.* was 85% at the genus level. *Nitrosomonas sp. ENI-11*, *Nitrosomonas europaea ATCC 19718*, and *Ammonia-oxidizing bacterium NS500-9* were dominant strains in the PNBS reactor. *Uncultured soil bacterium clone bacnit28*, *Uncultured bacterium clone KD8-75*, *Sphingobacteriaceae bacterium BR5-29*, *Uncultured bacterium clone KD3-77*, *Uncultured bacterium clone sls1367*, *Sphingobacteriaceae bacterium BR5-29*, *Uncultured bacterium clone NR.4.002*, and *Uncultured Cytophagales bacterium clone 17* were identified as coexisting bacteria in the PNBS reactor. Clones of 37 clones of bacterial members (27%) of the community had relatively low sequence identity with the BLAST database. They shared 95% sequence identity with *Nitrosomonas sp. ENI-11* and *Nitrosomonas europaea ATCC 19718*. We assumed that these bacteria may have contributed to the high NLR used in this study.

Table 3 shows the main results of homology search for 16S rRNA gene sequences in the microbial community of the GAP reactor (day 90). After treatment of HRAW, *Kuenenia stuttgartiensis*, *Uncultured bacterium clone KIST-JJY001*, and *Uncultured anoxic sludge bacterium KU2* became dominant in the reactor and constituted 70% at the genus level. According to the 16S rRNA analysis, *Uncultured bacterium clone Dok53*, *Uncultured planctomycete clone 5GA Pla HKP 48*, *Uncultured bacterium clone KIST-JJY024*, *Uncultured bacterium clone Dok55*, *Uncultured bacterium clone KIST-JJY012*, *Uncultured bacterium clone Dok23*, *Uncultured bacterium clone delph2B11*, *Uncultured candidate division TM7 bacterium clone EMP2*, *Uncultured bacterium clone: AnSal-09*, which have often been detected as common co-existent anammox

bacteria, constituted the majority of the clones (30%). The function of co-existent anammox bacteria is still largely unknown. *Uncultured bacterium clone KIST-JJY024* and *Uncultured bacterium clone FN-11* are well known to form netlike structures, which may contribute to the aggregation process or biofilm formation.

**Table 3.** Homology search results for 16S rRNA gene sequences (GAP)

Taxon	Identity (%)	Number of clones	Accession
<i>Kuenenia stuttgartiensis</i>	98-99	14	CT573071
<i>Uncultured anoxic sludge bacterium KU2</i>	98-99		AB054007
<i>Uncultured bacterium clone KIST-JJY001</i>	96-100	8	EF515083
<i>Uncultured bacterium clone Dok53</i>	99	1	FJ710771
<i>Uncultured planctomycete clone 5GA Pla HKP 48</i>	97	1	GQ356194
<i>Uncultured bacterium clone KIST-JJY024</i>	99	3	EF594056
<i>Uncultured bacterium clone Dok55</i>	99		FJ710774
<i>Uncultured bacterium clone KIST-JJY012</i>	99-100	3	EF584532
<i>Uncultured bacterium clone Dok23</i>	99-100		FJ710742
<i>Uncultured bacterium clone delph2B11</i>	96	1	FM209162
<i>Uncultured candidate division TM7 bacterium clone EMP2</i>	96	1	AM936584
<i>Uncultured bacterium clone: AnSal-09</i>	99	1	AB434261

In the PNBS and GAP system, the energy consumption is mainly attributable to aeration and the pump. In the anammox process, only a half of ammonium should be oxidized to nitrite. Therefore, in comparison with the conventional nitrification + denitrification process, the consumption of energy by aeration is reduced by 60%. The total energy consumption of the anammox process involving PNBS and GAP for treatment of HRAW is estimated at 1.5–2.0 kW·h<sup>-1</sup>·kg<sup>-1</sup> N<sub>removal</sub>. In this figure, the proportion of energy consumed by the pump is approximately 0.5–0.7 kW·h<sup>-1</sup>·kg<sup>-1</sup> N<sub>removal</sub>, and the share of aeration energy (consumed by the blower) is ~1.0–1.3 kW·h<sup>-1</sup>·kg<sup>-1</sup> N<sub>removal</sub>.

#### 4. Conclusions

Nitrogen removal efficiency above 89% was achieved along with the influent TN concentration of 2000–2500 (mg N)/l by means of a PNBS and GAP for treatment of HRAW. The treated HRAW can meet the Chinese discharge standards for industrial wastewater. Thus, the PNBS-GAP process holds promise for application to industrial treatment of HRAW.

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