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Biological Characteristics of Anaerobic Baffled Reactor in Operation

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We developed an anaerobic baffled reactor (ABR) for the energy-saving and efficient anaerobic treatment of nitrogen-containing pollutants in aquaculture wastewater. The ABR was operated with sludge from the secondary sedimentation tank of an urban sewage treatment plant for 150 d. When the total nitrogen load was 0.150 kg/m^3·d, 90.20 and 99.83% of NH_4^+–N and NO_2^-–N were removed, respectively, and 84.51% of chemical oxygen demand decreased. The activated sludge in the ABR was analyzed to investigate biological properties, including extracellular polymers, specific anaerobic ammonium oxidation activity, cytochrome C, hydrazine dehydrogenase activity, polysaccharide (PS), and extracellular protein (PN). When the activated sludge was treated, the ratio of PN to PS (PN/PS) in extracellular polymers increased 55 times, specific anaerobic ammonium oxidation activity increased 2 times, and hydrazine dehydrogenase activity increased 1.4 times, while cytochrome C concentration decreased. The results revealed that the anaerobic ammonium oxidation activity was significantly improved. The morphology of the activated sludge observed with an electron scanning microscope became appropriate for treating the sludge. Although the microbial species in the sludge were not diverse, the dominant bacterial community was identified, which contributed to the increased granularity.

1. Introduction

Wastewater from animal farming and living sources seriously pollutes water sources. For example, the wastewater generated from pig urine, feces, and pigpen-washing water contains a considerable amount of organic matter and an extremely high concentration of ammonia (NH_4^+). Such wastewater discharged without treatment pollutes the environment considerably. Excessive NH_4^+–N causes the eutrophication of water bodies, which disrupts the ecological balance and threatens human health.(1)

Sensors and sensor technology play a crucial role in monitoring, controlling, and optimizing wastewater treatment processes, including an anaerobic baffled reactor (ABR). ABRs are

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specialized reactors designed to treat wastewater anaerobically through a series of compartments to foster the growth of anaerobic microorganisms. A pH meter and temperature, dissolved oxygen, conductivity, and oxidation-reduction potential sensors are used to ensure anaerobic microbial processes. By using sensors and sensor technology, ABRs can have stability, efficiency, and adaptability for effective wastewater treatment.

At present, the aerobic activated sludge treatment method is mainly used to treat polluted water in China. Although the method has a high removal rate for pollutants, a high infrastructure construction cost, a large land area, and high operating costs are required to apply the method to treat urban sewage.(2) However, anaerobic nitrogen-containing sewage treatment technology has been continuously improved to have treatment facilities with low energy consumption, a small land area, and convenient management. As the concern about energy shortage increases, anaerobic wastewater treatment technology has been paid more attention than ever before.

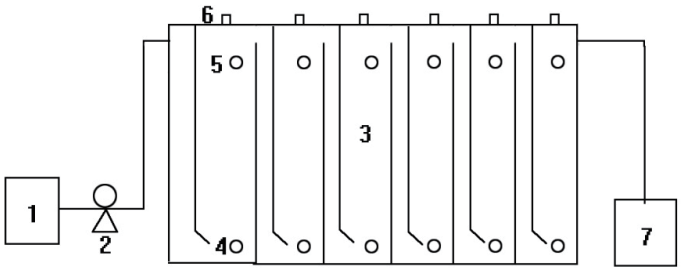
An ABR is an energy-saving and efficient wastewater treatment system.(3) Owing to its simple structure, low cost, high stability, excellent sludge interception ability, and convenient operation and management, the ABR has been studied and employed widely.(4) An ABR consists of small compartments in series, each of which is a closed reactor. After wastewater is input into the ABR, it is baffled by a peristaltic pump. The substrates and loads in the compartments are different, which produces methanogens and acidogenics easily for effective phase separation.

In this study, we monitored and analyzed the biological properties of the activated sludge in the anaerobic oxidation of NH_4^+. The changes in the concentrations of NH_4^+–N, nitrite nitrogen (NO_2^-–N), nitrate nitrogen (NO_3^-–N), and chemical oxygen demand (COD) were monitored to investigate how anaerobic ammonia-oxidizing bacteria were enriched and activated in the ABR.

2. Methods

2.1 ABR

The ABR used was made of plexiglass. Its outer wall thickness was 5 mm, and the inner baffle thickness was 3 mm. Its dimensions were 563 mm in length, 130 mm in width, and 450 mm in height, with an effective volume of 25.28 L (Fig. 1). The ABR comprised six compartments. The width of the first compartment was 1.5 times larger than those of the others. Each compartment consisted of a downflow chamber and an upflow chamber. The width of the downflow chamber was 1/3 of that of the upflow chamber. The bottom had a guide angle of 45° to baffle wastewater between the downflow and upflow chambers. The ABR was sealed and covered with a dark cloth to ensure an anaerobic and lightproof operation. There was an exhaust hole on the top to collect gas produced by the reaction of the ABR in a balloon. On the side, holes were made for collecting water and mud samples. A constant-flow peristaltic pump was used to supply water to the ABR. The hydraulic retention time (HRT) was set to 48 h.



(a)

(b)

Fig. 1. (Color online) ABR used in this study. (a) Structure of ABR. (1. Water inlet, 2. peristaltic pump, 3. reactor, 4. mud inlet, 5. water inlet, 6. exhaust port, and 7. water outlet.) (b) ABR (left) with and (right) without cloth.

2.2 Activated sludge

The activated sludge used in the experiment was collected from the secondary sedimentation tank of the sewage treatment plant of Longyan Longjin Water Purification Co., Ltd., China. The filled amount of the sludge was about 60% of the total volume of the reactor. The ABR was started for an HRT of 48 h. The composition of synthetic wastewater used in the experiment is shown in Table 1.

2.3 Reagents and equipment

Chemical reagents in the experiments were made as follows.

(1) Potassium sodium tartrate solution: 50 g of solid potassium sodium tartrate was dissolved in 100 mL of distilled water and boiled to remove residual chlorine. After cooling, it was diluted to 100 mL.

(2) Nessler's reagent: Liquid A was prepared by dissolving 16.0 g of sodium hydroxide in 50 mL of distilled water and cooled. Liquid B was prepared by dissolving 7.0 g of potassium iodide and 10.0 g of mercuric iodide in 50 mL of distilled water. Liquid B was added to liquid A while stirring, and then the mixture was diluted to 100 mL with distilled water.

(3) Color developer: 325 mL of distilled water and 50 mL of phosphoric acid (15 mol/L) were mixed thoroughly. Then, 20.0 g of 4-amino benzenesulfonamide and 1.00 g of N-(1-

Table 1
Composition of synthetic wastewater used in the experiment.

Composition	Concentration
$NaHCO_3$	1250 mg/L
$MgSO_4{\cdot}7H_2O$	300 mg/L
$CaCl_2{\cdot}H_2O$	180 mg/L
KH_2PO_4	27 mg/L
$(NH_4)_2SO_4$	Add as needed
$NaNO_2$	Add as needed
Fe-EDTA	1 mL/L (EDTA : $FeSO_4{\cdot}7H_2O$ = 1 : 1)
Trace elements	1 mL/L (430 mg of $ZnSO_4{\cdot}7H_2O$, 240 mg of $CoCl_2{\cdot}6H_2O$, 990 mg of $MnCl_2{\cdot}4H_2O$, 314 mg of HBO_3, 250 mg of $CuSO_4{\cdot}5H_2O$, and 190 mg of $NiCl_2{\cdot}6H_2O$ in 1 L of water)

naphthyl)-ethylenediamine dihydrochloride were added and dissolved sequentially. The solution was diluted to 500 mL with distilled water.

(4) Ammonium sulfamate solution: 2 g of ammonium sulfamate was dissolved in a 1:4 acetic acid solution and then diluted to 100 mL.

(5) Thymol ethanol solution: 500 mg of thymol was dissolved in anhydrous ethanol and diluted to 100 mL.

(6) Silver sulfate sulfuric acid solution: 1 g of silver sulfate was dissolved in 100 mL of sulfuric acid (ρ20 = 1.84 g/mL).

(7) LH-DE-100 of Lianhua Technology, China was used to measure COD.

For electron microscopy analysis, a phosphate buffer was prepared by dissolving 3.39 g of potassium dihydrogen phosphate in 1 L of distilled water. 10 mL of 25% glutaraldehyde aqueous solution and 90 mL of phosphate buffer were mixed to make a glutaraldehyde fixative.

An extracellular-polymer-related reagent was prepared by dissolving 2 g of anthrone in 1 L of 80% sulfuric acid as an anthrone reagent. For protein detection, the Solarbo Lowry protein concentration kit (catalog number: PC0030) was used with the anthrone reagent.

As cytochrome C-related reagents, sodium phosphate buffer was prepared by mixing 39 mL of 0.02 mol/L NaH_2PO_4 solution and 61 mL of 0.02 mol/L Na_2HPO_4 solution. A hydrazine-dehydrogenase-activity-related reagent was obtained by mixing a sodium phosphate buffer at pH 7.0 and a potassium phosphate buffer prepared by mixing 94 mL of 0.05 mol/L K_2HPO_4 solution and 6 mL of 0.05 mol/L KH_2PO_4 solution at pH 8.0. The equipment used in the experiment is shown in Table 2.

2.4 Analytical method

2.4.1 Nitrogen and COD

NH_4^+–N was determined by Nessler's reagent spectrophotometric method. NO_2^-–N was determined by the N-(1-naphthyl)-ethylenediamine spectrophotometric method, and NO_3^-–N was determined by the thymol spectrophotometric method.[5] pH was determined using a pH meter, and COD was determined using a multiparameter water quality meter.

Table 2
Equipment used in this study.

Equipment	Model	Manufacturer
Vertical pressure steam sterilizer	YXQ-LS-30 S11	Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory
Electronic balance	AL204	Mettler-Toledo Instruments (Shanghai) Co., Ltd.
Biochemical incubator	SHP-250	Shanghai Senxin Experimental Instruments Co., Ltd.
Vacuum drying oven	D2F-6020	Shanghai Jinghong Experimental Equipment Co., Ltd.
Shaker	SPH-100B	Shanghai Shiping Experimental Equipment Co., Ltd.
Low-speed desktop refrigerated centrifuge	TDL-5000bR	Xiamen Baijia Biotechnology Co., Ltd.
Large-capacity high-speed refrigerated centrifuge	D-37520	Thermo Fisher Scientific
UV-visible spectrophotometer	UV-1800	Shanghai Mepda Instrument Co., Ltd.
Ultrasonic spectrophotometer	1510	Thermo Fisher Scientific Oy
Peristaltic pump	YZ1515X-A	Lange Constant Flow Pump Co., Ltd.
pH meter	UB-7	Sartorius Scientific Instruments (Beijing) Co., Ltd.
COD meter	5B-3B	Lanzhou Lianhua Environmental Protection Technology Co., Ltd.
Hitachi ion sputtering instrument	E-1045	Japan Calendar High-tech Co., Ltd. Naka Business Office
Hitachi scanning electron microscope	S-3400N	Japan Calendar High-tech Co., Ltd. Naka Business Office
Muffle furnace	SX-G07103	Tianjin Zhonghuan Experimental Electric Furnace Co., Ltd.

The concentrations of NH_4^+–N and NO_2^-–N and the COD removal rate were calculated as

$$X = \frac{C1 - C2}{C1}. \tag{1}$$

Here, $C1$ and $C2$ are the corresponding index concentrations in the inlet and outlet wastewaters, respectively.

2.4.2 Suspended solid concentration (*SS*) and volatile suspended solid concentration (*VSS*)

The sludge volume index (*SVI*) indicates the looseness and coagulation properties of the sludge. To measure *SVI*, the activated sludge sample was placed in a 20 mL measuring cylinder. A mud layer scale was calculated as the ratio of the sludge concentrations after 5 and 30 min (SVI_5 and SVI_{30}, respectively).[6] The sludge sample was dried in an oven at 105 °C until the weight difference (M_1) before and after drying became less than 0.005 g. The sludge was transferred into a crucible and dried to a constant weight. The crucible containing the anaerobic activated sludge was dried to a constant weight recorded as M_2. The crucible was placed in a cold muffle furnace at 600 °C for 6 h. Then, it was weighed as M_3.

SS and *VSS* were calculated as

$$SS\ (\text{mg/L}) = (M_2 - M_1) \times 1000/V, \tag{2}$$

$$VSS\ (\text{mg/L}) = (M_3 - M_1) \times 1000/V. \tag{3}$$

Here, V represents the volume of anaerobic activated sludge.

2.4.3 Scanning electron microscopy (SEM)

Small amounts of anaerobic activated sludge samples were taken from the six compartments and rinsed three times with phosphate buffer. The rinsed samples were placed in 2.5% glutaraldehyde fixative at 4 ℃ for 24 h. After fixation, the sludge samples were rinsed three times with isothermal phosphate buffer for 10 min. Subsequently, they were dehydrated and rinsed with increasing ethanol concentration (50, 70, 80, 90, 95, and 100%) for 15 min each, consecutively.[7] The dehydrated samples were washed with anhydrous ethanol twice and then put in a vacuum drying oven at 50 ℃ for longer than 24 h. After drying, an appropriate amount of sludge was mounted on the stage for gold spraying using the Hitachi ion sputtering machine. A Hitachi SEM system was used for scanning and photographing the sludge samples.[6]

2.4.4 Extracellular polymers (EPS)

10 mL of the anaerobic activated sludge was centrifuged at 3200 rpm for 30 min. The supernatant was used for free-EPS determination. The precipitate was resuspended in 9 mL of boiling 0.9% NaCl solution for 1 h and centrifuged at 3200 rpm for 30 min. After centrifugation, the supernatant was retained for bound-EPS determination. The polysaccharide and protein concentrations were determined in the free and bound EPS. The anthrone-sulfuric acid method was used to determine the polysaccharide concentration in EPS with a standard glucose solution (0.1 mg/L) as a reference. The Lowry method was used to determine the protein concentration in EPS with a standard protein concentration as a reference.[7]

2.4.5 Specific anaerobic ammonium oxidation activity (*SAA*)[1]

85 mL of the culture medium (simulated wastewater) for anaerobic ammonium oxidation was placed in six 110 mL serum bottles. Then, 10 mL of the anaerobic activated sludge was moved into the serum bottles. pH was adjusted to 7.5 with 0.1 mol/L HCl. The sludge in the serum bottle was aerated with argon gas for 10 min to remove oxygen, then sealed and stored. The sealed serum bottle was shaken at 180 rpm using a lightproof shaker at 35 ± 1 ℃. Subsamples were periodically collected using a syringe to determine the concentrations of NH_4^+–N and NO_2^-–N. The remaining solution was placed in the serum bottle to check if pH was maintained in the optimal range of 7.5−7.8. The sludge in the serum bottle was taken to measure *VSS*. The degradation slopes of NH_4^+–N and NO_2^-–N of the *VSS* of anaerobic activated sludge were calculated to determine the anaerobic ammonia oxidation activity in each compartment (mg-TN/g-VSS/h).

2.4.6 Cytochrome C[7]

5 mL of the anaerobic activated sludge taken from each compartment was centrifuged at 3000 rpm and 4 ℃ for 5 min. The supernatant was washed twice and suspended in sodium phosphate buffer. It was ultrasonicated at 800 W and 4 ℃ for 20 min. It was then centrifuged at 8000 rpm and 4 ℃ for 15 min. 4 mL of the supernatant was placed in a clean test tube. Then, 0.1 mL of pyridine was added and vortex-mixed. 0.1 mL of 1 mol/L NaOH solution was added and vortex-mixed again. A small amount of sodium dithionite crystals and 0.01 mL of distilled water were added to each sample to reduce hemoglobin. The treated sample in a glass cuvette was scanned at a wavelength of 500 to 600 nm to set a baseline. The peak at 557 and 541 nm and the difference in the troughs were used to calculate the cytochrome C concentration in μmol/g·VSS. A digestion coefficient of 20.7 was used in Eq. (4).

$$\text{Cytochrome C (mmol/L)} = (ABS_{557} - ABS_{541})/20.7 \quad (4)$$

2.4.7 Hydrazine dehydrogenase activity[8]

10 mL of the anaerobic activated sludge collected from each compartment was centrifuged at 5000 rpm and 4 ℃ for 30 min. The supernatant was discarded, and the precipitate was washed twice with sodium phosphate buffer and resuspended in 20 mL of the same buffer. The precipitate was ultrasonicated at 225 W and 4 ℃ for 30 min to break the cells and then centrifuged at 15000 rpm and 4 ℃ for 30 min to remove cell debris. The supernatant was stored at 4 ℃ for the determination of cell hydrazine dehydrogenase activity. 0.1 mL of supernatant, 0.42 mL of potassium phosphate buffer, 0.25 mL of 50 μmol/L cytochrome C, and 0.333 mL of 500 μmol/L hydroxylamine solution were added to a dark brown liquid chromatography bottle and mixed. Argon was infused into the mixed solution to remove oxygen at 35 ℃ for 20 min. Distilled water was used as a blank control, and the cytochrome C concentration measured at 550 nm was used for the baseline. Enzymatic activity was measured using cytochrome C. The enzyme was missed with an oxidized cytochrome C solution, and the absorbance change at 550 nm was monitored to determine the enzymatic activity. The enzymatic activity is expressed as the amount of cytochrome C per milligram of protein, while the unit of the reduced cytochrome C is μmol/mg·pro/min.

3. Results and Discussion

3.1 Changes in concentrations of wastewater quality indicators

Before the operation of the ABR, the NH_4^+–N and NO_2^-–N concentrations were 4.85 and 14.20 mg/L, respectively. As anaerobic NH_4^+-oxidizing bacteria in the ABR were enriched, the anaerobic NH_4^+ oxidation activity increased. After the ABR was operated for 150 d, the concentrations of NH_4^+–N and NO_2^-–N reached 148.5 and 152.17 mg/L, respectively. The concentrations and removal rates of NH_4^+–N, NO_3^-–N, NO_2^-–N, COD, and pH are shown in Fig. 2.

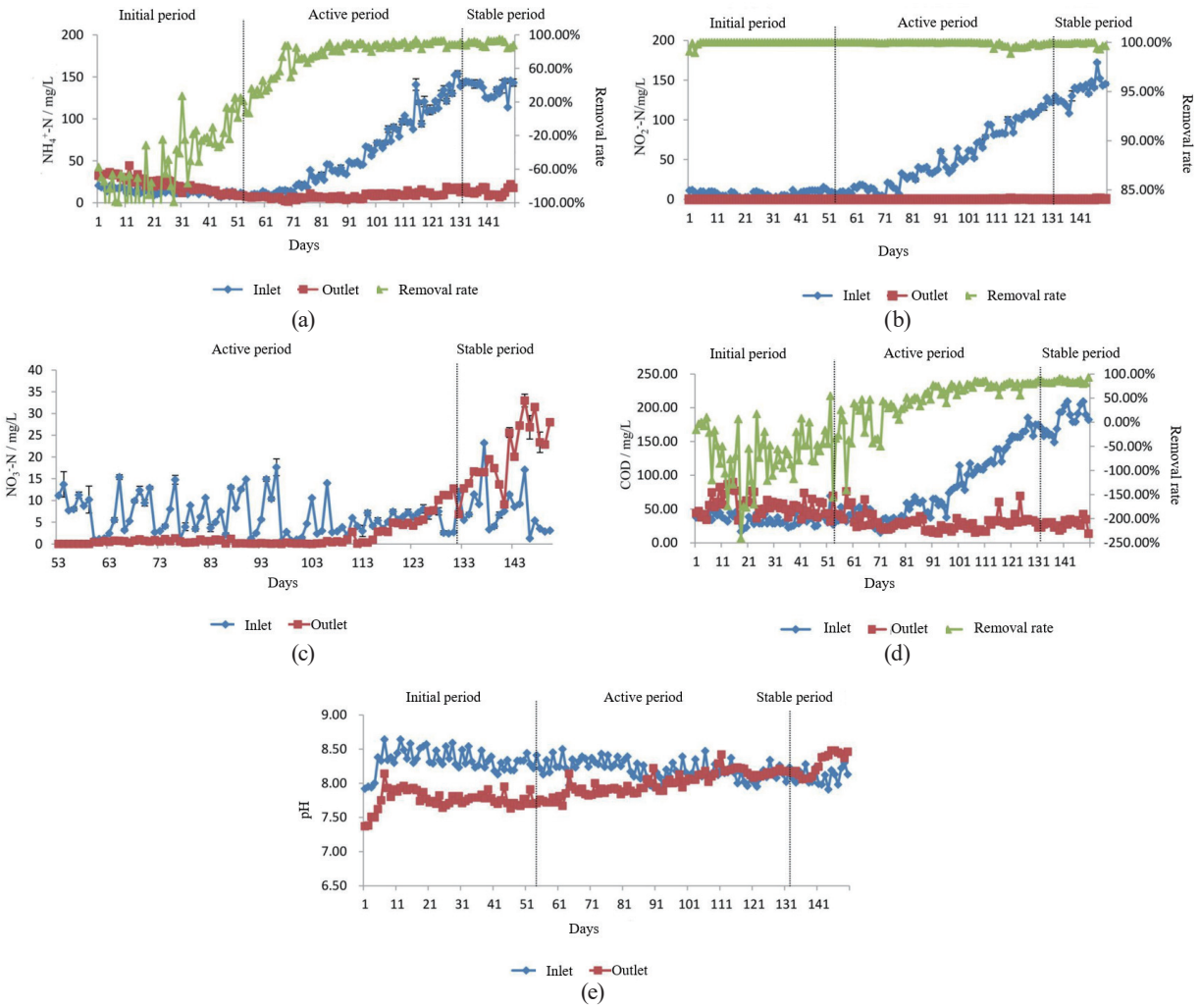


Fig. 2. (Color online) Concentrations and removal rates of (a) NH4$^+$–N, (b) NO_2^-–N, (c) NO_3^-–N, (d) COD, and (e) pH in each period of experiment.

After the sludge was loaded into the reactor and operated for 24 h, a large amount of sludge floated in each compartment, preventing the ABR from operating normally. The sludge barely settled, so aerobic bacteria and single-cell organisms (paramecium) existed in the sludge. Their activities caused the anaerobic activated sludge to float. When the ABR was sealed and not operated for 3 d, the sludge began to sink. After 7 d of sealing, there was no floating sludge, and then the synthetic wastewater was introduced to operate the ABR. Figure 2 shows that the NH_4^+–N concentration was higher in the ABR outlet wastewater than in the inlet wastewater, indicating that NH_4^+ was not removed and NO_2^-–N was consumed in large quantities with a removal rate of 98.9%. In the initial stage of ABR operation, bacteria in the sludge did not survive. In the sludge, unicellular organisms gradually disappeared, and heterotrophic bacteria decomposed the lysed bacteria. In addition, the cellular debris of unicellular organisms and N-containing organic matter in the sludge produced a large amount of NH_4^+–N. At this stage,

the density and activity of anaerobic NH_4^+-oxidizing bacteria in the sludge were low, so it was impossible to decompose NH_4^+. With a large amount of organic matter, denitrification occurs in the ABR, consuming a large amount of NO_2^-–N.[9] In this period, COD was significantly higher in the outlet wastewater than in the inlet wastewater. As the organic matter was not completely decomposed, COD in the outlet wastewater was higher. In the initial period, the operation of the ABR was unstable, and the COD fluctuated considerably. The pHs of the inlet and outlet wastewaters were 8.3 and 7.8, respectively. The optimal pH of an anaerobic ammonium oxidation is between 7.7 and 8.2,[10] which was similar to the measured pH in the ABR.

In 54−132 d, the removal rate of NH_4^+–N increased, indicating that the activity of anaerobic ammonia-oxidizing bacteria also increased. Owing to the rapid expansion of the bacterial population, the concentrations of NH_4^+–N and NO_2^-–N rapidly increased. After 70 d, the total nitrogen load increased 10 times from 0.014 to 0.150 kg/m^3·d. The average removal rates of NH_4^+–N and NO_2^-–N were 85.42 and 99.87%, respectively. In the active period, the anaerobic ammonium oxidation in the ABR continued to increase, while denitrification continued to decrease. NO_2^-–N was used as an electron acceptor by anaerobic ammonium-oxidizing bacteria so the removal rate remained high in this period. NO_3^-–N was a product of an anaerobic ammonium oxidation. The NO_2^-–N concentration in the inlet wastewater fluctuated. NO_3^-–N in the inlet wastewater was oxidized from NO_2^- in the synthetic wastewater. During 53−115 d, the NO_3^-–N concentration in the outlet wastewater remained low (0−1 mg/L). After 115 d, the NO_3^-–N concentration began to increase. After 115 d, the anaerobic ammonium oxidation in the ABR increased. In this period, COD was stabilized, and the removal rate continued to increase. pH in the outlet wastewater continued to rise, gradually approaching that of the inlet wastewater. The results indicated that the anaerobic NH_4^+ oxidation in the ABR gradually intensified.

The ABR was operated relatively stably during the stable period (133−150 d), and the removal rates of NH_4^+–N, NO_2^-–N, and COD became stable. The maximum removal rates of NH_4^+–N, NO_2^-–N, and COD were 95.01, 99.98, and 92.56%, while the average removal rates in the three periods were 90.20, 99.83, and 84.51%, respectively. At this stage, pH was higher in the outlet wastewater than in the inlet wastewater owing to the anaerobic ammonium oxidation, which consumed H^+ and increased pH.[11,12] The NH_4^+–N, NO_2^-–N, and NO_3^-–N production ratio during 145−150 d was 1:1.23:0.23, which was close to the stoichiometric values of anaerobic ammonium oxidation of 1:1.32:0.26,[13] which showed the stable operation of the ABR.

3.2 Properties of anaerobic activated sludge

3.2.1 *SVI*, *SS*, and *VSS*

The measured *SVI*, *SS*, and *VSS* in the sludge are presented in Tables 3 and 4. In the active period, the ratio of SVI_{30} to SVI_5 was close to 1, indicating that the anaerobic activated sludge more settled to the bottom than in the initial period. From the initial to the stable period, the ratio of *VSS* to *SS* decreased. This was contrary to the expected increasing ratio with the continuous improvement of treatment. Such a result was due to a large volume of the ABR. The degree of treatment of the anaerobic activated sludge in each compartment was not consistent, and the

Table 3
SVI measurement results.

Compartment	Initial period			Active period		
	SVI_5	SVI_{30}	SVI_{30}/SVI_5	SVI_5	SVI_{30}	SVI_{30}/SVI_5
1	80.00	39.00	0.49	9.00	9.00	1.00
2	80.00	39.00	0.49	13.5	13.40	0.99
3	80.00	39.00	0.49	12.00	11.20	0.93
4	80.00	39.00	0.49	18.00	16.00	0.89
5	80.00	39.00	0.49	13.50	11.30	0.84
6	80.00	39.00	0.49	8.00	7.30	0.91

Table 4
SS and *VSS* measurement results.

Compartment	Initial stage			Active period			Stable period		
	SS	*VSS*	*VSS/SS*	*SS*	*VSS*	*VSS/SS*	*SS*	*VSS*	*VSS/SS*
1	81455	26175	0.32	13926	4396	0.32	27380	11780	0.43
2	81455	26175	0.32	36900	10900	0.30	42110	11540	0.27
3	81455	26175	0.32	27080	7985	0.29	19910	5130	0.26
4	81455	26175	0.32	40815	12265	0.30	24220	6290	0.26
5	81455	26175	0.32	25835	7855	0.30	39970	10470	0.26
6	81455	26175	0.32	18935	5765	0.30	18090	4870	0.27

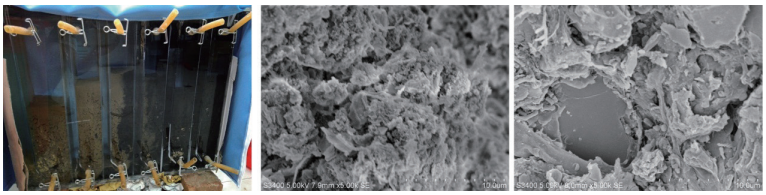
sludge was hardened in the treatment process. Therefore, *VSS* and *SS* decreased, while in the first compartment with a relatively high degree of treatment, they increased. This showed that in the first compartment, anaerobic ammonia-oxidizing bacteria were more enriched than in the other compartments.

3.2.2 Morphology of anaerobic activated sludge

In earlier periods, sludges in compartments 1 and 2 changed from black to brown-yellow. To explore whether the brown-yellow sludges changed in morphology, SEM was used to observe the morphology (Fig. 3).

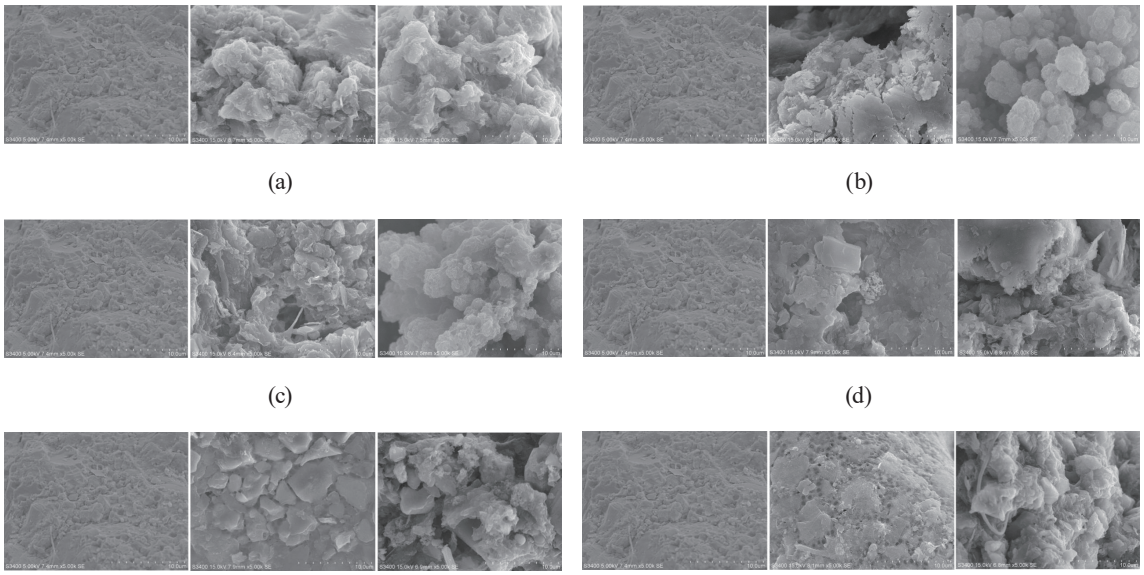
Figure 4 shows that the brown-yellow sludge had looser particles than the black one, and a large number of filamentous bacteria dwelled on the surface. The color change of the sludge from black to brown-yellow presented granulation in the treatment. To explore the relationship between the sludge morphology and the nitrogen load, subsamples were collected on the 0th, 68th, and 150th days. The SEM images of the sludge in each compartment at a magnification of 515× for each collection day are shown in Fig. 4.

In the sludge acclimation process, the sludge in each compartment showed granular shapes as the total nitrogen load on the ABR increased. Filamentous bacterial communities were observed on the surface, which promoted the formation of granular shapes. After 150 d of treatment, the sludge changed its color from black to red, proving that the anaerobic NH_4^+-oxidizing bacteria were enriched (Fig. 5).



(a) (b) (c)

Fig. 3. (Color online) SEM images of sludges in initial period (24 d): (a) in ABR, (b) brown-yellow sludge, and (c) black sludge.

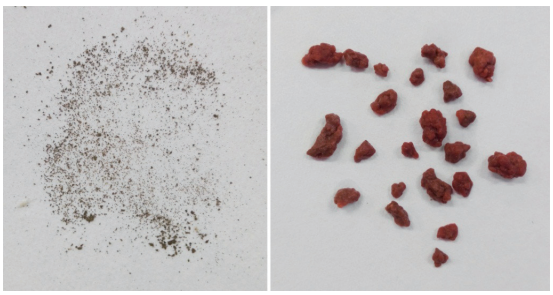


(a) (b)

(c) (d)

(e) (f)

Fig. 4. SEM images of anaerobic activated sludge in compartments on (left) 0th, (middle) 68th, and (right) 150th days. Compartments (a) 1, (b) 2, (c) 3, (d) 4, (e) 5, and (f) 6.



(a) (b)

Fig. 5. (Color online) Comparison of sludge colors observed (a) before (0 d) and (b) after treatment for 150 d.

3.3 Biological characteristics of anaerobic activated sludge

3.3.1 EPS

EPS refers to high-molecular-weight polymers secreted by microorganisms (mainly bacteria) under certain environmental conditions. It determines the physical and chemical properties of the cell surface and plays an important role in the formation and stability of granular sludge.[13] The sludge samples were taken on the 69th day in the initial period and the 150th day in the stable period to measure EPS. With the increase in total nitrogen load, the polysaccharide concentration decreased, while the protein concentration increased. The ratio of protein to polysaccharides was significantly greater in the stable period than in the active period. The higher the ratio of protein to polysaccharides, the worse the sedimentation of the sludge.[14,15] However, in this study, the sedimentation of the sludge increased with the ratio of protein to polysaccharides. The ratio was higher in the stable period than in the active period (Tables 5 and 6).[1]

The polysaccharide and protein concentrations in compartment 5 in the stable period were lower than those in the other compartments. As there was no filler in the ABR, the anaerobic activated sludge was compacted during the treatment process. The compaction affected the treatment degree of the sludge most significantly in compartment 5. Therefore, the polysaccharide and protein concentrations were lower in compartment 5 than in the other compartments. In general, the extracellular protein-to-polysaccharide ratio (PN/PS) was 55 times higher in the stable period than in the active period, indicating that granular sludge was formed and stabilized during the treatment process.

3.3.2 *SAA*

SAA was an important indicator for the denitrification of NH_4^+ by anaerobic ammonium-oxidizing bacteria.[16] In this study, *SS*A was measured on the 69th and 150th days (Table 7). The *SAA* of the six compartments was higher in the stable period than in the active period. This indicated that the denitrification by the anaerobic ammonia-oxidizing bacteria in each compartment increased during the treatment. The degree of acclimation of compartments 1, 2, and 3 in the active period was higher than that of compartments 4, 5, and 6. Therefore, the *SAA* of the first three compartments was higher than that of the last three compartments, and the anaerobic activated sludge in the first three compartments was more granulated. When the oxygen concentration becomes lower than 0.5% of air saturation, the activity of anaerobic ammonia oxidizing bacteria is completely inhibited.[17] Therefore, the *SAA* of the first three compartments was lower than that of the last three compartments. After the anaerobic activated sludge was treated, the *SAA* of the stable period increased more than twice that of the active period.

Table 5
Polysaccharide concentration in EPS in each compartment of ABR.

Period	Compartment	Polysaccharides (mg/g·VSS)		
		FPS	CPS	PS
Active period	1	1.86	423.81	425.67
	2	0.63	57.57	58.20
	3	0.76	94.57	95.33
	4	0.52	42.48	43.00
	5	0.87	110.86	111.73
	6	1.12	194.97	196.10
Stable period	1	0.80	4.76	5.56
	2	0.76	5.24	6.00
	3	1.57	7.65	9.22
	4	1.37	10.24	11.61
	5	0.78	2.72	3.50
	6	1.83	8.75	10.57

Table 6
Polysaccharide concentration in EPS in each compartment of ABR.

Period	Compartment	Proteins (mg/g·VSS)			PN/PS
		Free protein	Bound protein	Total protein	
Active period	1	0	13.27	13.27	0.08
	2	0	2.42	2.42	0.41
	3	0	1.53	1.53	0.65
	4	0	2.01	2.01	0.50
	5	0	2.53	2.53	0.40
	6	0	2.85	2.85	0.35
Stable period	1	0	55.78	55.78	10.03
	2	0	100.27	100.27	16.71
	3	4.00	121.14	125.14	13.57
	4	1.42	262.32	263.74	22.72
	5	2.30	4.78	7.08	2.02
	6	14.67	318.28	332.94	31.49

Table 7
Results of *SAA* determination.

Stage	Compartment	NH_4^+–N removal rate (mg/L)	NO_2–N removal rate (mg/L)	TN removal rate (mg-TN/h)	*SAA* (mg-TN/g-VSS/h)
Active period	1	2.94	1.42	4.37	6.79
	2	1.30	2.43	3.74	3.57
	3	1.90	2.68	4.57	3.73
	4	2.00	2.38	4.38	3.17
	5	1.34	2.10	3.44	3.04
	6	1.72	2.34	4.06	9.79
Stable period	1	7.07	0.18	7.25	9.15
	2	4.63	0.36	5.00	4.24
	3	3.95	0.32	4.27	6.21
	4	4.54	0.17	4.71	15.32
	5	5.07	0.16	5.24	7.81
	6	4.44	0.20	4.64	11.34

3.3.3 Cytochrome C

Anaerobic ammonium-oxidizing bacteria contain abundant cytochrome C. When ther are the dominant species in the sludge, the color is dark red. Therefore, the amount of anaerobic ammonium-oxidizing bacteria in the ABR can be determined from the cytochrome C content. We measured cytochrome C in the sludge taken on the 69th and 150th days (Table 8). The cytochrome C concentration was higher in the active period than in the stable period. This indicated that the degree of acclimation of anaerobic activated sludge was low in the early ABR operation.

3.3.4 Hydrazine dehydrogenase activity

Van de Graaf *et al.* proposed a metabolic model of anaerobic ammonium-oxidizing bacteria using the ^{15}N tracer method.[18] In the anaerobic ammonium oxidation, nitrite is reduced to hydroxylamine, which then reacts with ammonia to form hydrazine, which is finally converted into nitrogen gas. Van de Star *et al.* studied the conversion of hydroxylamine by anaerobic ammonium-oxidizing bacteria. Hydrazine dehydrogenase is an enzyme used by anaerobic ammonium-oxidizing bacteria. Hydrazine dehydrogenase reduces hydrazine to ammonia and releases electrons from hydrazine.[19] Therefore, the activity of hydrazine dehydrogenase reflects the strength of the anaerobic ammonium oxidation. Thus, we analyzed the activity of hydrazine in the sludges taken on the 69th and 150th days (Table 9). The enzymatic activity was also measured using the enzyme solution. In compartments 1 to 4, the activity of hydrazine dehydrogenase was higher in the stable period than in the active period. The enzymatic activity in compartments 5 to 6 was lower in the stable period than in the active period, and the difference in compartment 5 was larger. During the treatment in the ABR, the protein content increased rapidly, decreasing the enzymatic activity per unit of time. The activity of hydrazine dehydrogenase was 1.4 times higher in the stable period than in the active period.

Table 8
Cytochrome C concentration (μmol/g·VSS).

Period	Compartment					
	1	2	3	4	5	6
Active period	1.62	0.71	0.68	0.28	0.61	0.54
Stable period	0.11	0.16	0.30	0.18	0.14	0.37

Table 9
Hydrazine dehydrogenase activity.

Compartment	Active period		Stable period	
	μmol/min·mL	μmol/g-protein/min	μmol/min·mL	μmol/g-protein/min
1	0.47	6.66	0.72	2.44
2	0.58	4.40	0.97	1.87
3	0.49	1.96	0.56	1.94
4	0.38	2.89	1.12	1.50
5	2.22	12.82	0.94	28.15
6	0.56	2.82	0.52	0.72

4. Conclusions

The ABR was operated for treating the anaerobic activated sludge taken from a municipal sewage treatment plant. The sludge was anaerobically treated for 150 d at 25−35 ℃. In the three periods after beginning the operation, activity enhancement, and stable period, the total nitrogen load increased more than 10 times. The removal rates of NH_4^+–N, NO_2^-–N, and COD were 90.20, 99.83, and 84.51%, respectively. The ratio of NH_4^+–N and NO_2^-–N removal to NO_3^-–N production reached 1:1.23:0.23, which was close to 1:1.32:0.26, the conventional ratio in anaerobic ammonia oxidation. The pHs of the inlet and outlet wastewaters ranged from 7.8 to 8, which is in the optimal range of 7.7 and 8.2 for anaerobic ammonium oxidation. With the load increased, the ABR showed stability and surge resistance. When the total nitrogen load was increased, the ABR quickly reached the optimal removal rates of NH_4^+–N, NO_2^-–N, and COD. A large number of filamentous bacteria were observed on the surface of the anaerobic activated sludge, which was in granular form. The surface was uneven with a large number of pores. The biological properties of the anaerobic activated sludge indicated that the EPS concentration of the sludge increased 55 times, the anaerobic ammonia oxidation activity increased 2 times, and the hydrazine dehydrogenase activity increased 1.4 times. Such results showed the improved biological properties and reactions of the anaerobic ammonia oxidation bacteria. The anaerobic NH_4^+ oxidation was effectively carried out in the ABR. The anaerobic NH_4^+-oxidizing bacteria were sufficiently enriched in the ABR to treat a higher nitrogen load. The results of this study provide an important basis for aquaculture wastewater treatment using the ABR.

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References

1 Q. Sui, C, Liu, H. Dong, and Z. Zhu: J. Biosci. Bioeng. **113** (2014) 277. https://doi.org/10.1016/j.jbiosc.2014.02.017
2 W. Kang, H. Chai, Y. Xiang, W. Chen, Z. Shao, and Q. He: Sci. Rep. **7** (2017) 17497. https://doi.org/10.1038/s41598-017-17783-3
3 S. Nachaiyasit and D. C. Stuckey: Water Res. **33** (1999) 1559. https://doi.org/10.1016/S0043-1354(98)00371-6
4 W. P. Barber and D. C. Stuckey: Environ. Technol. **19** (1998) 489. https://doi.org/10.1080/09593331908616705

5 J. H. Guo, J. B. Hu, Z. Ju, H. F. Shui, J. Shui, C. F. Xi, F. Fa, W. Hui, and W. Hui : Water and Wastewater Monitoring and Analysis Methods (China Environmental Science Press, Beijing, 1991) 4th ed. (updated Chinese Edition).
6 American Public Health Association, American Water Works Association, and Water Environment Federation: Standard Methods for the Examination of Water and Wastewater, R. Baird, A. D. Eaton, E. W. Rice, and L. Bridgewater Eds. (American Public Health Association, Washington DC, 2005).
7 H. Chen, H. Y. Hu, Q. Q. Chen, M. L. Shi, and R. C. Jin: Bioresour. Technol. **211** (2016) 594. https://doi.org/10.1016/j.biortech.2016.03.139
8 X. Yin, S. Qiao, J. Zhou, and X. Quan: Bioresour. Technol. **196** (2015) 376. https://doi.org/10.1016/j.biortech.2015.07.096
9 N. Chanchoi and S.Nitisoravut: Chemosphere **66** (2007) 2225. https://doi.org/10.1016/j.chemosphere.2006.09.036
10 J. Wu, S. Cui, Q. Zhang, L. Zhang, J. Zhuang, F. Qian, and L. Yuan: JWPE **50** (2022) 103353. https://doi.org/10.1016/j.jwpe.2022.103353
11 M. Oshiki, Y. Masuda, T. Yamaguchi, N. Araki, and Z. Shaoqi: Chemosphere **213** (2018) 498. https://doi.org/10.1016/j.chemosphere.2018.09.055
12 S. Bagchi, R. Biswas, and T. Nandy: JIMB **37** (2010) 943. https://doi.org/10.1007/s10295-010-0743-4
13 J.-H. Tay, Q.-S. Liu, and Y. Liu: WST **46** (2002) 13. https://doi.org/10.2166/wst.2002.0540
14 Z. W. Wang, Y. Liu, and J.-H. Tay: Appl. Microbiol. Biotechnol. **69** (2005) 469. http://dx.doi.org/10.1007/s00253-005-1991-5
15 J. Quarmby and C. F. Forster: Water Res. **29** (1995) 2449. https://doi.org/10.1016/0043-1354(95)00083-W
16 C. Tang C, P. Zheng, Q. Mahmood, and J.-W. Chen: JIMB **36** (2009) 1093.
17 L. Yuan, R. Tang, H. Yao, Z.-H. Hu, Y. Wang, S. Yuan, and W. Wang: Desalin. Water Treat. **180** (2020) 156. https://doi.org/10.5004/dwt.2020.25071
18 A. A. van de Graaf, P. de Bruijn, L. A. Roberson, M. S. M. Jetten, and J. G. Kuenen: Microbiology **143** (1997) 2415. http://dx.doi.org/10.1099/00221287-143-7-2415
19 W. R. L. van der Star, M. J. van de Graaf, B. Kartal, C. Picioreanu, M. S. M. Jetten, and M. C. M. van Loosdrecht: Appl. Environ. Microbiol. **74** (2008) 4417. https://doi.org/10.1128/AEM.00042-08