The stable performance of partial nitrification with the high salinity waste-brine

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Partial nitrification (PN) treatments on waste-brine were carried out in this study. Stable PN performance was obtained during continuous operation for 752 days, with a maximum nitrogen loading rate (NLR) of 3 $kg^{-N}\,m^{-3}\,day^{-1}$ and ammonium conversion rate (ACR) of 1.4 $kgNm^{-3}day^{-1}$. The ratios of NO_2^--N/ (NO_2^--N+NO_3^--N) (NR) were always above 97%, and BOD removal efficiencies were also stable at around 70% even if a sharp increase in NLR was applied during the stable period. Additionally, bacterial consortia analysis showed ammonium-oxidizing bacteria were the dominant microorganisms, which provided evidence for the long-term stable performance of this PN reactor. During the experiment, sludge setting properties deteriorated due to the absence of a biomass carrier. The stable performance of partial nitrification from waste-brine demonstrated the feasibility of the operation strategy in this study.

Keywords: Anammox, Brine, Nitrogen Removal, Partial Nitrification, Salinity.

1. Introduction

The natural gas and iodine, which are valuable natural resource, are produced from underground brine. The brine contains methane, iodine, and ammonium. After recovery of methane and iodine from this brine water, the remaining water (waste-brine) also contains high concentration of ammonium. It is important to note that the salinity of waste-brine was almost as high as seawater. Recently, nitrogen pollution has become a major concern in environment protection and increasing efforts have been directed at improving and discovering techniques for reducing the amount of nitrogen in wastewater [1]. Historically, sequential

nitrification and heterotrophic denitrification have typically been applied for nitrogen removal, as well as the removal of organic matter, from waste water. Because this treatment approach involves huge amounts of energy and chemical costs, development of a cost-effective and economical ammonium removal processes is required. A novel and promising biological process of anaerobic ammonia oxidation (Anammox) provides an alternative to traditional processes [2].

$$\mathbf{NH_4^+ + 1.31NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2H_2O} \quad (1)$$

Partial nitrification can produce a suitable influent to an anammox reactor for NH_4-N removal from waste-brine. Thus, partial nitrification of ammonium to nitrite is the principal factor for successful application of a shortcut nitrogen-removal system in combination with the anammox process [3].

In this study, the treatment performance of a PN reactor which was operated for 752 days was evaluated and the effect of free ammonia (FA) and free nitrous acid (FNA) were also investigated. Microscope observation and DNA analysis were applied to evaluate the characteristics of sludge and the bacteria shift in our reactor.

2. Materials and Methods

2.1. *Experimental setup and operational strategy*

The experiment was carried out in a laboratory-scale rectangular reactor with an effective volume of 5 L (Figure 1). The cross-sectional areas of the downdraft and updraft parts of the reactor were 110×110 mm and 110×30 mm, respectively, and the height of effluent port was 320 mm (total volume 6.2 L). A settling tank with volume of 2.5 L was used for sludge sedimentation and recycling. The air flow rate was changed along with increments in the nitrogen loading rate (NLR). Temperature was maintained at 26 ± 4°C throughout the study and pH was controlled at 7.6 ± 0.1 by addition of acid (1N HCl) and alkali (1N NaOH) solution. The dominant bacterial species in the consortium was identified as the Anammox bacterium KU2, and the initial MLSS was 4500 mg L^{-1}. The reactor was fed with the TN concentration of 180-200 mg L^{-1}.

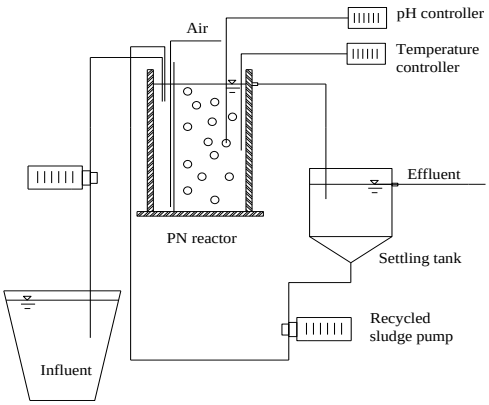

Fig. 1. Schematic of the PN reactor

2.2. *Analytical methods*

The influent and effluent samples were analyzed immediately or stored in the refrigerator at 4 °C until the analyses were carried out. NH_4^+-N was analyzed by the modified phonate method using o-phenyl phenol (OPP) (Kanda, 1995). NO^{2-} N and nitrite (NO_3^--N) were measured using a colorimetric method. The pH and DO values were determined using a pH meter (B-211, Horiba, Kyoto, Japan) and a DO meter (D-55, Horiba, Kyoto, Japan), respectively. MLSS analysis was performed by drying the samples at 105°C in an evaporating dish.

(1) SEM observation

The surface and inner parts of the Anammox granules were observed using scanning electron microscope (SEM). Samples were first washed in a 0.1 M phosphate buffer solution (pH 7.4) for 5 min. The samples were then hardened for 90 min in a 2.5% glutaraldehyde solution prepared with the phosphate buffer solution. Next, samples were washed in the buffer solution three times for 10 min each and fixed for 90 min in a 1.0% OsO4 solution prepared using the phosphate buffer solution. After washing samples three times for 10 min each in the buffer solution, they were dewatered for 10 min each in serially graded solutions of ethanol at concentrations of 10, 30, 50, 70, 90 and 95%. SEM observations were conducted using a scanning electron microscope (JEOL, JSM-5310LV, and Japan).

(2) DNA analysis

DNA extraction and Polymerase Chain Reaction (PCR) amplification

The granular sludge sample was ground with a pestle under liquid nitrogen. Meta-genom DNA was extracted using an ISOIL kit (Wako, Osaka, Japan) in accordance with the manufacturer's instructions. Amplification of the 16S rRNA gene was performed with Phusion High-Fidelity DNA polymerase (Finnzymes,

Espoo, Finland) using conserved eubacterial primers 6F (forward primer: 5'-GGAGAGTTAGATCTTGGCTCAG-3') (Tchelet, 1999) and 1492r (reverse primer: 5'-GGTTACCTTGTTACGACT-3') (Lane, 1991). PCR was carried out using the following thermo cycling parameters: 30 s initial denaturation at 98 °C, 25 cycles of 10 s at 98°C, 30 s at 51 °C, and 20 s at 72 °C, and 5 min final elongation at 72 °C. The amplified products were electrophoresed on a 1% agarose gel, and the excided fragments were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

3. Results and Discussion

3.1. *The stable performance of the PN reactor*

The operational parameters and treatment results are summarized in Table 1. Figure 2 shows the daily changes in NLR and AOR. table PN performance was obtained during continuous operation from day552-752, with a maximum nitrogen loading rate (NLR) of 4.2 kgN $m^{-3}day^{-1}$ and ammonium oxidation ratio (AOR) of 2.1 $kgNm^{-3}day^{-1}$.

Table 1. The performance of the PN reactor under various operational conditions

Items	Period I	Period II	Period III
Time (d)	0-226	226-552	552-752
HRT (h)	24	3	4.2
NLR	0.05-1.5		
InfluentTN (mg/L)	180-200	180-200	150-200
EffluentTN (mg/L)	60-130	0-150	50-150
Sludge concentration(mg-MLSS/L)	4500-17900	9000	32000

(1) Period I

During this period I (0–226 days), the reactor was operated to cultivate ammonia-oxidizing bacteria (AOB) and to inhibit nitrite-oxidizing bacteria (NOB). Initially, the reactor was started at a low NLR of 0.05 $kgNm^{-3}day^{-1}$ (200 mg/L of NH_4^-N), with a hydraulic retention time (HRT) of 24h. Air was supplied to the reactor at a flow rate of 0.2 L/min for controlling the reactor DO concentration to inhibit NO_3^--N production. Effluent NH_4^-N and NO_3^--N concentrations were decreased gradually in the PN reactor which demonstrated the occurrence of nitrification as well as the inhibition of NOB in the reactor.

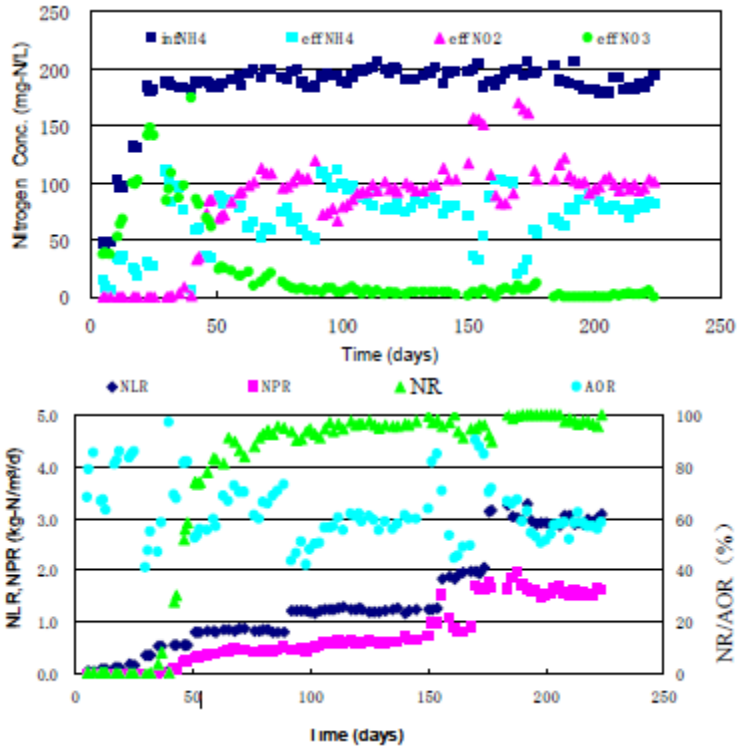

Fig. 2. Changes in total Influent NH4-N, effluent NH4-N NO2--N, NLR (TNLR) and AOR. (Period I)

(3) Period II

From day 226 to day 552, the influent NH_4^-N concentration was not changed and HRT was shortened to increase the NLR. The residual DO concentration in the reactor was under 0.1 mg/L. Varied operational conditions was adopted to check the stable performance. From day 276, the waste-brine was adopted as the substitution for synthetic wastewater. Negative effects were appeared in the first 100 running day. Also, the maximum NLR of 2.5 $kgNm^{-3}day^{-1}$ was obtained. The average effluent $NO^{2-}N$/ ($NO_2^-N+NO_3^-N$) ratio was always more than 99%.

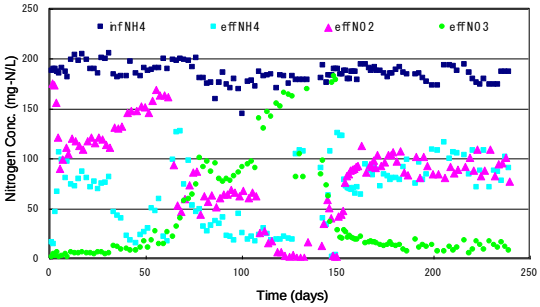

Fig. 3. Changes in total Influent NH4-N, effluent NH4-N NO2-N, NLR (TNLR) and AOR. (Period II)

Anthonisen et al. observed that inhibition of NOB began at a concentration of 0.1–1.0 mg-FA/L, while AOB became inhibited at 10–150 mg-FA/L[4]. The NOB growth can be selectively inhibited in a range of FNA of approximately 0.011–0.10 mg HNO_2-N/L.As shown in Figure 4, we confirm the fact that the production of NO_3^--N was decreased as well as the pH was higher than 7. The inhibition on AOB growth started at approximately 0.20 mg HNO_2-N/L and completely stopped at 0.60 mg HNO_2-N/L, while the inhibition on NOB growth started at approximately 0.021 mg HNO_2-N/L and completely stopped at approximately 0.033 mg HNO_2-N/L

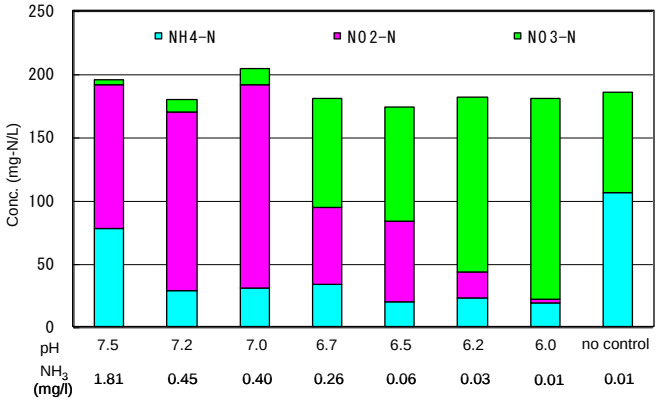

Fig. 4. The production of NH4-N, NO2-N and NO3-N under different pH.

(3) Period III

After obtaining satisfactory PN treatment results for the subsequent Anammox reactor, the influent flow rates and TN concentration were simultaneously adjusted to get the suitable ratio of NO_2^--N/ NH_4-N. A maximum NLR of 3 kgN $m^{-3}day^{-1}$ and the stable NPR of 1.4 kgN $m^{-3}day^{-1}$ were achieved on day 600 with an influent NH_4^-N concentration of 200 mg/L. The DO

concentrations in the reactor were maintained near to 0 mg/L in this period. A stable effluent NO_2^-–N/NH_4–N ratio of 1.1 ± 0.2 was also steadily maintained which was suitable for the subsequent Anammox process.

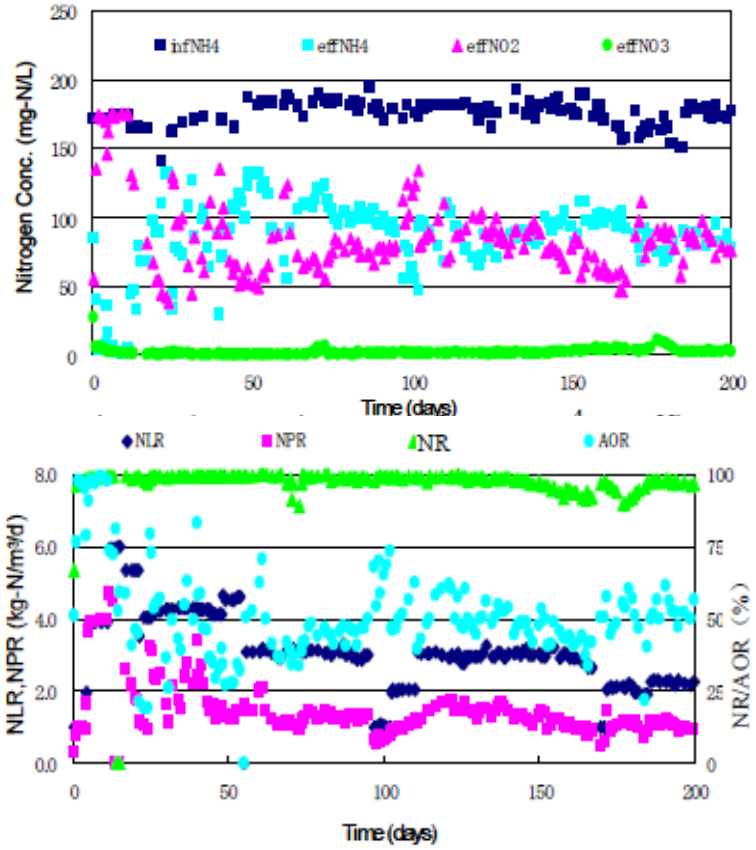

Fig. 5. Changes in total influent NH4-N, effluent NH4-N, NO2-N, NLR (TNLR) and AOR. (Period III)

The work of Vadivelu et al. indicates that nitrite oxidation could be selectively inhibited by FNA [5]. The inhibition on AOB growth started at approximately 0.10 mg HNO_2-N/L and completely stopped at 0.40 mg HNO_2-N/L, while the inhibition on NOB growth started at approximately 0.011 mg HNO_2-N/L and completely stopped at approximately 0.023 mg HNO_2-N/L. This indicates that the NOB growth can be selectively inhibited in a range of FNA of approximately 0.011–0.10 mg HNO_2-N/L. Compared with the low DO concentration in this reactor, the FNA concentration was considered as the main factor to inhibit NOB activity except for the startup period. Therefore, it is essential to control FA and FNA within the appropriate range during operation.

In this study, the control of FA and FNA was also regarded as the operational strategy. This study researched the time courses of FA and FNA concentrations during the entire experimental period (Figure 6).

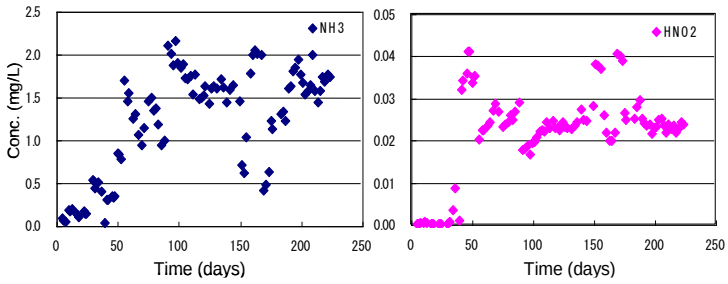

Fig. 6. Time courses of FA and FNA concentrations during experimental period.

As shown in Figure 6, during the first 100 days, the FA and FNA concentrations fluctuated, accompanied with significant changes in the ACR. In the successive operational period, ACR was stable and the FA and FNA concentrations were approximately 1.5-2.0 mg/L and 0.02-0.03 mg/L, respectively. The accumulation of NO_3^--N was negligible, indicating that aerobic ammonium oxidizing reactions were dominant, due to the successful control of FA and FNA, in accordance with the findings by Anthonisen et al.

3.2. *Sludge characteristics of PN reactor*

The morphology and size distribution of the sludge taken from the PN reactor on the 180 day are shown in Figure 7 (a) to (b). As the experiment progressed, there were decreasing trends in sludge size and frequency, which may be due to the absence of a biomass carrier in the PN reactor. The collision and friction among particles would be strengthened without the buffering function of the biomass carrier. Consequently, the sludge diameter tended to decrease progressively.

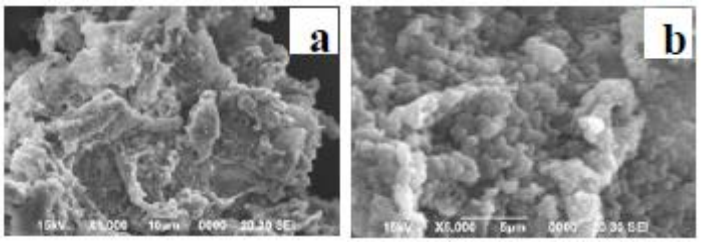

Fig. 7. The sludge morphology and size taken from the PN reactor on the 180 day.

Table 2 shows the results of inorganic components analysis of sludge 167 day. Fe and Si in the inorganic components were accounted for more than 60%, but P, Ca, Na, Al, Mg, K, S were accounted for less than 40%. In the brine, iron

concentration was 2.5 mg/L which was 10 times of the iron in seawater. Therefore, iron was the most occupied in the PN sludge.

Table 2. The Composition of the PN sludge

inorganic component	ratio (wt %)	inorganic component	ratio (wt %)
Fe	39.81	Si	22.98
P	8.42	Ca	7.34
Na	5.63	Al	5.02
Mg	3.24	K	3.19
S	2.80		

The initial sludge volume index (SVI) value of the sludge was 123 mL/g, and then decreased to 62.7 mL/g after 110 days of operation, which indicated the improvement of sludge settling properties. The low SVI value was concluded to be due to the increase in sludge density, as nitrifiers often form cell aggregates in enrichment cultures. However, an obvious increase in SVI value occurred from the 150th day on, with a maximum value as high as 162 mL/g on day 184, causing abundant sludge accumulation in the settling tank. This phenomenon was attributed to either of two reasons. One is the absence of a biomass carrier. it reported that the attachment and detachment motion of biofringe carriers can improve the settling ability of sludge[6]. The other possibility is nitrogen overload, which arose from the sharp increase of influent substrate concentration and flow rate. High NLR evidently needed higher aeration to support sufficient dissolved oxygen for ammonium oxidation, which may have resulted in excessive turbulence in the system [7].

An improvement in the settling characteristics was achieved by using biofringe material as a biomass carrier in the reactor. The swimming motion enhances detachment of excess biomass, which causes formation of a dense sludge floc [8-9]. Thus, sludge with good settling characteristics was formed. The SVI value decreased gradually to 80 mL/g, while MLSS decreased concurrently, which indicates an increase in attached sludge on the biofringe material.

3.3. DNA analysis

After long-term operation, adaptation could either lead to the acclimation of the existing bacterial population to the new conditions or a significant shift in the microbial community, so the changes in the bacterial populations were monitored. Biomass samples were taken from the reactor on day 37 and day 89 and analyzed for the presence of nitrifying bacteria and the composition of the microbial community. As listed in Table 3, during the steady operational phase, DNA analysis showed that almost bacteria hybridized with Parvularcula sp.CC-

MMS-1 distributed throughout the biofilm, namely most of bacteria were consisted of Parvularcula sp. CC-MMS-1 positive beta-subclass ammonium-oxidizing bacteria (micrographs are not shown). However, for the sample on day 89 after long terms of running of reactor, the DNA analysis clearly showed that the dominant bacteria were unclearly.

The explanation for these findings is that the bacterial established a new equilibrium in the population, leading to the uniform distribution of bacterial population.

In this way, the nitrite oxidizers, existing in the oxygen-limited reactor, were eliminated under high FA concentration condition for the further potential steady operation of partial nitrification process. Actually the acclimation of the bacteria to the operational conditions might be a problem since FA influence might be reduced significantly after long time for the acclimatization of biomass. However, this phenomenon did not significantly appear in this study, which could be interpreted as absolutely change of bacteria populations. In the high ammonium conditions, the most expected microbial group, representing mostly by members of the AOB and holding a critical role in the reactor, was developed and could be more potential for partial nitrification in this reactor.

Table 3. The DNA analysis of bacteria taken from PN sludge of day 37 and day 89

Taxon	Accession	day 37		day 89	
		Identity (%)	Number of clones	Identity (%)	Number of clones
Uncultured bacterium clone SS-57	AY945873	92-93	14/76	93	4/27
Parvularcula sp. CC-MMS-1	EU346850	91-92		92	
Uncultured alpha proteobacterium clone F1_08f	EF123358	91-92		92	
Uncultured bacterium clone Np77	AM295541	98	8/76	98	1/27
Nitrospira marina	X82559	98		98	
Nitrospira marina strain Nb-295	L35501	96		96	
Nitrococcus mobilis ATCC 25380	L35510	97	1/76	97	1/27
Nitrosococcus oceani ATCC 19707	CP000127	99	3/76	99	1/27
Nitrosococcus oceani strain AFC27	AY690336	99		99	
Nitrosomonas sp. ls425	AJ621029	99	2/76	99-100	12/27
Nitrosococcus mobilis Nc2	AF287297	99		98-100	
Uncultured *Roseovarius* sp. clone B217	EU360291	96	7/76	–	–
Roseobacter sp. 812	DQ120726	96			
Roseovarius tolerans strain Ekho Lake-172	Y11551	95-96			
Iodide-oxidizing bacterium SE-1	AB159209	95-96			
Iodide-oxidizing bacterium SE-1	AB159209	97-98	3/76	–	–
Iodide-oxidizing bacterium RB-2A	AB159203	96-97			
Uncultured *Roseobacter* 253-13	AJ294351	96-97			
Iodide-oxidizing bacterium A-6	AB159200	99	2/76	–	–
Roseovarius sp. 2S5-2	AB114422	99			
Uncultured *Roseobacter* sp. clone DE4.1	AY588949	94	1/76	–	–
Iodide-oxidizing bacterium WAI-2	AB159208	90	3/76	–	–
Iodide-oxidizing bacterium Q-1	AB159207	90			
Uncultured gamma proteobacterium clone MKC19	EF173350	96	6/76	96	3/27
Lysobacter taiwanensis strain CL-1358	DQ314555	95		95-96	
Uncultured gamma proteobacterium clone C-CS3	AY622233	98	2/76	–	–
Rhodanobacter spathiphylli type strain: B39	AM087226	97			
Rhodanobacter spathiphylli type strain: B39	AM087226	94	1/76	–	–
Roseovarius aestuarii strain SMK-122	EU156066	97	1/76	97	2/27
Marinobacter sp. NK-1	AB026946	98	1/76	98	1/27
Rubrimonas cliftonensis strain:0Ch317	D85834	94	5/76	–	–
Uncultured alpha proteobacterium clone SM1C11	AF445668	93			
Uncultured soil bacterium clone PK_V	EF540436	96	3/76	–	–
Rhodobacter sphaeroides ATCC 17029	CP000578	95			
Rhodobacter vinaykumaraii strain JAJA249	AM600642	95	1/76	–	–
Pseudomonas pseudoalcaligenes strain: 14	AB276371	99	1/76	–	–
Arenibacter latericius KMM 426T	AF052742	97	1/76	–	–
Alteromonas sp. D	AB004313	95	1/76	–	–
Sinorhizobium sp. NH-14	EF486317	95	1/76	–	–
Uncultured *Alcanivorax* sp. clone XJ40	EF648121	94	1/76	–	–
Colwellia sp. BSi20045	DQ060391	94	1/76	–	–
Uncultured bacterium clone WLB16-200	DQ015862	97	1/76	–	–
Uncultured bacterium clone MKC25	EF173356	98	1/76	–	–
Uncultured bacterium clone ELB19-216	DQ015834	96	1/76	–	–
Uncultured alpha proteobacterium clone GuBH2-AG-	AJ519653	94	1/76	–	–
Uncultured bacterium clone 21B42	DQ925896	91	1/76	–	–
Uncultured Rhodobacteraceae bacterium isolate EG7	AM691097	88	1/76	–	–
Uncultured bacterium clone Asc-s-95	EF632661	–	–	99	2/27

4. Conclusion

In this study, we showed that the anammox process is reliable at the salinity of 30 g/L. A maximum nitrogen loading rate (NLR) of 3 kgN $m^{-3}day^{-1}$ and

ammonium conversion rate(ACR) of 1.4 $kgNm^{-3}day^{-1}$. According to the DNA analysis results, during the steady operational phase, DNA analysis showed that almost bacteria hybridizedwith Parvularculasp. CC-MMS-1 distributed throughout the biofilm. It was also confirmed in this study that the sludge settling properties of the sludge could be effectively improved by using the biofringe material as a biomass carrier.

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