Original Research

Development of a Hybrid Partial-Nitrification and Anammox Reactor with Stirrer

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Abstract

This study developed the hybrid partial-nitrification and anammox (HPNA) reactor to increase efficiency in ammonia nitrogen (NH_4^+-N) removal. The HPNA reactor contained a fixed bed filled with biofilm carrier and a fluidized bed with mechanical stirrer to maximize withholding the sludge. The reactor achieved a total nitrogen (TN) removal efficiency of 70% and NH_4^+-N removal efficiency of 84%. The maximum nitrogen loading rate (NLR) of 6.1 kg-N/m³/d and nitrogen removal rate (NRR) of 4.7 kg-N/m³/d were achieved with a short hydraulic retention time (HRT) of 3 h. After 160 days of operation, both the mixed liquid suspended solids (MLSS) and the mixed liquor volatile suspended solids (MLVSS) increased in the lower part (from 3.7 to 14.7 g/L and 1.8 to 10.9 g/L, respectively) and the upper part (from 3.7 to 11.8 g/L and 1.9 to 6.7 g/L, respectively). The proteins (PN)/ polysaccharides (PS) ratio increased from 0.96 to 1.1 in the lower part but decreased to 0.7 in the upper part. Bacterial community profiles generated from 16S rRNA sequences further showed that the ammonium-oxidizing bacteria (AOB) *Nitrosomonas* and the anammox bacterium *Brocadiales* were enriched in the HPNA reactor.

Keywords: anammox, partial-nitrification, mechanical stirring, sludge characteristics, bacteria community

Introduction

Eutrophication, which has negative and irreversible effects on aquatic organisms [1], has been a worldwide problem for more than a century. It is mainly caused by the voluminous discharge of large animal farm wastewater, the use of nitrogen fertilizer, and the generation of landfill leachate, with ammonium nitrogen (NH_4^+-N) being the main fraction generated. Thus, the efficient removal of NH_4^+-N through a number of ways has been extensively studied.

The anaerobic ammonium oxidation (anammox) process combined with a partial-nitrification process to treat wastewater with a high concentration of NH_4^+-N has gained interested in recent years. Conventionally, this combined process is carried out in a system with two different reactors, such as the SHARON-anammox process [2]. However, in recent years, several studies combining partial-nitrification with anammox in a single reactor have also been reported, such as the single nitrogen removal reactor using an anammox and partial-nitrification (SNAP) system [3] and partial nitrification-

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anammox (PN/A) reactor [4]. The single-stage process generally has a higher volumetric nitrogen removal rate and lower capital costs than a system with two reactors, since no additional nitrification reactor is required for ammonium oxidation. The traditional up-flow anaerobic sludge blanket (UASB) reactors efficiently cultivate large granules with a diameter that could reach 0.7-1.5 mm [5, 6], but easily causes granular flotation [7, 8] leading to granule washout, which then eventually deteriorates the capacity of the reactor [9, 10]. UASB reactors with fixed biofilms or flowing filler have the ability to catch the suspended sludge, yet they have difficulty achieving a high nitrogen removal rate (NRR) [3, 11, 12].

In response to these technical issues, an innovative hybrid reactor was previously designed that combined fluidized and fixed beds for anammox treatment [13]. Using this system, the nitrogen loading rate (NLR) of the reactor could be increased to 27.3 kg-N/m^3/d with total nitrogen removal efficiency of 75%. However, the same reactor also required both nitrite (NO_2^--N) and NH_4^+-N as substrates.

In this study, the hybrid partial-nitrification and anammox (HPNA) reactor was developed that aimed to treat synthetic wastewater with a high concentration of ammonia nitrogen and maximize withholding the sludge. The fixed bed was designed to reduce the washout of the suspended sludge. The continuous stirring was designed to form shear stress and let the bubbles out of granules, which then eliminated flotation. It was hypothesized that AOB were active on the outer layers of the biofilm (or granule), producing a suitable amount of nitrite for the anammox organisms active in the inner layers.

Material and Methods

Configuration of HPNA Reactor

The lab-scale HPNA reactor with an effective volume of 7.5 L was made of acrylic resin and operated in a continuous-upflow mode (Fig. 1). Its internal diameter was 120 mm with a length/diameter ratio of 5.5. The HPNA reactor had two major beds or zones. The first was the fluidized bed in the lower part (0-250 mm from the bottom) and the other was the fixed bed in the upper part (250-600 mm from the bottom). In the upper part, a porous polyester pile fabric material (Ohyapile, Japan) was used as biomass carrier. The diameter and length of each pile fabric strip were 1 mm and 9 mm, respectively. In the lower part, a mechanical stirrer (Z-2200, Tokyo Rikakikai, Japan) with six stainless steel blades was installed from the top of the reactor and the length of each blade was 30 mm. Each lower and higher parts of the HPNA reactor were fixed with gas-liquid-solid separators (GSS). An aerator was positioned at the bottom of the reactor and dissolved oxygen (DO) was measured by a DO meter (OM-51, Horiba, Japan).
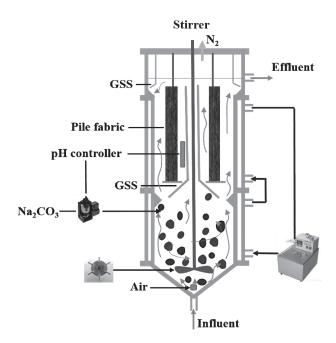

Fig. 1. Schematic of HPNA reactor. Seed sludge and feeding media.

The enriched anammox granular sludge (accounting for 75% of mixed liquid suspended solids (MLSS)) was collected from a 6.0 L hybrid reactor fed with synthetic wastewater [13, 14]. The reactor was seeded with 20 g-MLSS of Anammox sludge to reach an initial concentration of 2.7 g-MLSS/L. On the other hand, the nitrifying sludge was collected from a lab-scale fill-and-draw activated sludge reactor treated with ammonia nitrogen containing synthetic wastewater. This was added to the HPNA reactor with 3.75 g-MLSS, producing an initial concentration of about 0.5 g-MLSS/L.

The reactor was treated with ammonium sulfate as the only nitrogen source. The composition of synthetic wastewater contained 50-820 mg/L NH_4^+-N, 1 g/L $NaHCO_3$, 125-500 mg/L $KHCO_3$, 54 mg/L KH_2PO_4, 700 mg/L $CaCl_2$, 700 mg/L KCl, 500 mg/L $MgSO_4$, 500 mg/L $NaCl$, 9 mg/L $FeSO_4 \cdot 7H_2O$, 5 mg/L EDTA free acid, and 0.25 mg/L $CuSO_4 \cdot 5H_2O$ [15].

HPNA Reactor Operation

The HPNA reactor was operated at a constant temperature of 30±1ºC by a water jacket and a pH level of 7.3-7.5 by adding Na_2CO_3 (100-150 g/L) through a pH-controller (NPH-6900, Nissin, Japan). The aeration rate was mediated according to the concentration of nitrogen components in effluent. The HPNA reactor was operated under continuous stirring of 100 rpm.

Analytical Procedures

Concentration of NH_4^+-N was measured using ortho-phenyl phenol through a modified phonate method [16]. Concentrations of NO_2^--N and nitrate (NO_3^--N), 30-min

sludge volume indices (SVI$_{30}$), MLSS, and mixed liquor volatile suspended solids (MLVSS) were measured following the Standard Methods [17]. The granular sludge size was measured by a grainsize analyzer system (LA-920, Horiba, Japan). The morphology of sludge was observed by an electron microscope (Nikon Eclipse E600, Japan) with a digital camera (Nikon 4500, Japan). Extracellular polymeric substances (EPS) were extracted from sludge by formaldehyde plus NaOH [18], then the extracellular proteins (PN) and the polysaccharide (PS) content were determined according to the Lowry method [19].

Bacterial Community Analysis

The bacterial community structure was analyzed using the pyrosequencing approach. Sludge samples were taken from HPNA reactor at days 14 and 160. Universal primers 6F (5'-ACTCCTACGGGAGGCAGCAG-3') and 1492r (reverse primer: 5'-GGTTACCTTGTTACGACT-3') targeting the V4 region of 16S rRNA gene were used for polymerase chain reaction (PCR) amplification at *Majorbio* Bio-pharm Technology Corporation (Shanghai, China). Discussion on the community composition only focused on the abundant taxa, occurring at >1 % of the bacterial community [20, 21].

Results and Discussion

Nitrogen Removal Performance

In this study, the newly designed HPNA reactor was operated for 168 days. The performance of the reactor, sludge characteristics, and community structure were analyzed in detail.

Rapid Startup of HPNA Reactor

The HPNA reactor rapidly started up within 28 days (phase 1 in Fig. 2). During this period, the reactor was operated without aeration to improve the activity of the anammox community. The synthetic wastewater that had a total nitrogen (TN) concentration of about 100 mg/L (NO$_2^-$-N to NH$_4^+$-N ratio of 1.0) was pumped into the reactor simultaneously for anammox reaction. The reactor was operated at a fixed hydraulic retention time (HRT) of 3.3 h. On the 8th day, TN removal efficiency and NRR reached 60% and 0.63 kg-N/m³/d (Fig. 2b and c), respectively, indicating that anammox already had some activity. Previous studies also reported that the simultaneous nitritation-anammox reactor initiated with anammox produced a slightly higher and more stable NRR than initiating with partial nitrification [22].

From the 9th day to the 28th day, the HPNA reactor was operated under an intermittent aeration with a time interval of 15 min (bulk DO in this period was under 0.2 mg/L). As shown in Fig. 2a, the influent NH$_4^+$-N

concentration increased from 80 mg/L to 110 mg/L, with NO$_2^-$-N concentration gradually decreasing to zero, while HRT decreased from 7.2 h to 2.3 h. On day 28, the removal rate of NH$_4^+$-N reached 79% (Fig. 2b). NLR and NRR reached 1.2 and 0.7 kg-N/m³/d, respectively (Fig. 2c). Chu et al. reported that when operated at influent ammonium concentration of 500 mg-N/L, a nitrogen volume loading rate of 0.25 kg-N/m³/d (HRT, 2d) was achieved in an SBR reactor after 60 days

Fig. 2. HPNA reactor performance at different times. Variations of influent and effluent nitrogen. concentrations along with HRT a), nitrogen removal efficiency b), and value of NLR, NRR, and DO c).

[23]. Kwak et al. also reported that a UASB reactor with biological filter could further achieve an NLR of 0.3 kg-N/m³/d along with HRT of 4 h after 46 days [24]. Compared with previous studies in these single-stage nitritaion-anammox reactors, this HPNA reactor had a relatively short startup period (28 days) with high adaptability to the rapid increase in NLR (0.4-1.2 kg-N/m³/d) and short HRT (7.2-2.4 h).

Stable operation and NLR Increasing Period

From day 29 to 168, the HPNA reactor was operated under continuous aeration. The feed medium was changed to contain only NH_4^+-N to initiate simultaneous partial nitrification and anammox reaction (phase 2 in Fig. 2a). The concentration of NH_4^+-N in influent increased from 250 to 830 mg/L, while the NH_4^+-N in effluent was always lower than 100 mg/L. NO_2^--N in effluent was kept under 20 mg/L, suggesting a proper control of aeration. The average TN and NH_4^+-N removal efficiencies reached 70% and 84%, respectively (Fig. 2b). A maximum NRR of 4.7 kg-N/m³/d was obtained with a maximum NLR of 6.1 kg-N/m³/d (Fig. 2c). HRT significantly varied (2.2 to 8.6 h) to optimize nitrogen removal efficiency and NRR. The value of DO was also detected during the whole operation period. As influent ammonium concentration and NLR increase, DO should be improved gradually to maintain the stable NH_4^+-N treatment. It has been reported that DO under 1 mg/L wouldn't inhibit anammox activity [22]. In this study, DO was kept at a relatively low value (0.2-0.5 mg/L) in order to reduce the anammox inhibition and ensure enough NO_2^--N concentration.

Table 1 compares the performances of HPNA reactor with other single-stage reactors in recent studies. Comparing with the above partial-nitrification and anammox reactors, the performance of the HPNA reactor was superior, which might have benefited from the short HRT and mechanical stirring. A shorter HRT represented higher NLR, and even a higher flow rate, which efficiently transported NH_4^+ and NO_2^- to the top of the reactor, supplying substrate for the normal metabolisms of Anammox and ammonium-oxidizing bacteria (AOB) [26]. Meanwhile, mechanical stirring would form a fluid shear and thus enhance the mass transfer effect in the lower part [13]. Otherwise, the longtime stable operation and the relatively low concentration of effluent NH_4^+-N indicated that the HPNA reactor was quite qualified for the high influent ammonium concentration.
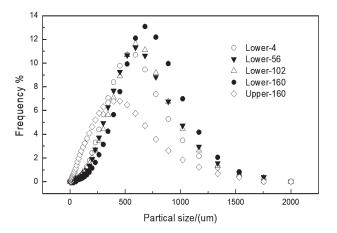

Fig. 3. Variations of SNA sludge size distribution during the experiment in the lower HPNA part on day 4 (Lower-4), day 56 (Lower-56), day 102 (Lower-102), and day 160 (Lower-160), and in the upper HPNA part on day 160 (Upper-160).

Characteristics of Granular Sludge

The granule diameter and distribution of SNA sludge in the lower and upper parts of the reactor were measured every 2 months during the whole operation period (Fig. 3). Table 2 summarizes the mean diameter and the corresponding distribution ratio. In the lower part of the reactor, the mean particle size of SNA sludge was maintained at 500-634 μm during the entire operation, which was higher than that in the upper part (420 μm on day 160). This result indicated that continuous stirring produced shear stress that was able to sustain the granular diameter of the sludge, allowing for easier removal of bubbles outside the SNA granules.

Fig. 4 shows the surface morphology of SNA sludge under the microscope, which differed significantly between the lower and upper parts. Obviously, the typical red anammox sludge dominated in the lower part (left), while the brown-yellowish AOB sludge was more apparent in the upper part (right). These two types of sludge formed the desirable SNA granules. Similar sludge colors were also reported during a simultaneous partial nitrification-anammox process in other up-flow biofilm reactors [22, 27]. These differences were mainly driven by the structure of HPNA reactor, where the lower fluid bed was optimum in generating anammox granules by stirring and the upper fixed biofilm helped withhold the easy-waste flocculent AOB.

Table 1. Final performance of different lab-scale single-stage reactors.

Reactor system	NLR (kg-N/m³/d)	NRR (kg-N/m³/d)	TN removals (%)	HRT (hour)
HPNA reactor	6.1	4.7	70	3.3
Single-stage system [25]	5.44	2.57	54.4	4
MBBR [4]	3.6	3.16	82	1.34
Up-flow biofilm reactor [22]	0.77	0.35	40	3

Table 2. Summary of the mean diameter and the distribution ratio of particles.

	Lower HPNA part				Upper HPNA part
	Day 4	Day 56	Day 102	Day 160	Day 160
Mean diameter (μm)	498±15.27	553±17.05	557±17.55	634±21.1	420±8.98
Distribution ratio	52.14%	49.75%	50.45%	42.89%	75.22%

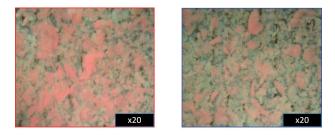Note: data of mean diameters are presented as mean with SE, n = 85

Fig. 4. Microscopic observation of SNA sludge on day 160 in the lower part (left) and in the upper part (right).

MLSS, MLVSS, SVI_{30}, and EPS were measured only at the beginning (day 18) and the end of the whole period (day 160) (Table 3), since the stable operation of the reactor would be influenced by sampling. It was reported that sludge with SVI_{30} ranging from 50 to 100 mL/g-MLVSS, indicated good settling property [28]. In this study, the SVI_{30} of SNA sludge was maintained at 63 mL/g-MLVSS in the upper part, and was reduced to 49 mL/g-MLVSS in the lower part. This further indicated better settling ability of both fixed bed and fluid bed. Generally, the floatation of Anammox granules could result in lower settling ability and washing out of the granular sludge [8, 9]. Thus, the maintained settling property of the sludge in this study was attained by mechanical stirring, which in turn eliminated flotation [10]. Besides, both MLSS and MLVSS had remarkably increased after operating for

160 days. The MLVSS/MLSS ratio, which represents sludge activity, increased from 49% on 18 day to 74% and 57% on day 160 in the lower and upper parts, respectively. This was similar to the MLVSS/MLSS ratios reported in other studies, which range from 69-78% in an anammox process [29, 30]. The higher SNA sludge concentrations in the lower part resulted from the maintained granules with good settling properties.

At the end of the operation after 160 days, both proteins and polysaccharides in the lower and upper parts increased from 58 to 108 and 84 mg/g-MLVSS, and from 60 to 99 and 123 mg/g-MLVSS, respectively (Table 3). High contents of extracellular polymer substrates (EPS), which mainly contained PN and PS, indicate dense structure of the granules [31], since EPS was primarily responsible for the structural and functional integrity of the aggregates and essential to their physicochemical and biological properties [32]. Moreover, larger amounts of PN in EPS could lead to better aggregation of anammox sludge [33]. Similarly, studies also found that the secretion of extracellular protein from anaerobic granules was stimulated under high hydrodynamic shear force [34]. In this study, the SNA granular sludge in the lower part accumulated much more PN, which was probably due to the high hydrodynamic shear force generated by continuous stirring, suggesting a favorable aggregation.

The proteins-to-polysaccharides ratio (PN/PS) is usually used to evaluate granular stability, where a lower PN/PS ratio indicate higher strength and better

Table 3. Summary of the MLSS, MLVSS, sludge volumetric index (SVI30), and EPS contents in the lower and upper parts of the HPNA reactor on days 18 and 160.

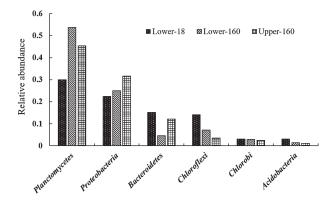|	Lower HPNA part (on day 160)	Upper HPNA part (on day 160)	Lower HPNA part (on day 18)
MLSS (g/L)	14.7	11.8	3.7
MLVSS (g/L)	10.9	6.7	1.8
MLVSS/MLSS (%)	74	57	49
SVI_{30} (mL/g-MLVSS)	49	63	60
EPS (mg/g-MLVSS)			
Proteins(PN)	108	84	58
Polysaccharides(PS)	99	123	60
PN/PS	1.1	0.7	0.96

Fig. 5. Relative abundance of major phyla in three samples, which were harvested from the lower part on day 18 (Lower-18) and day 160 (Lower-160), and the upper part on day 160 (Upper-160); the abundance is presented as the percentage of the different phyla among the total reads in a sample (only prevalent populations taking >1% of the community are shown).

settling ability [35]. Here, the HPNA reactor maintained relatively low levels of PN/PS ratio (0.7-1.1), indicating a strong structure of SNA granular and a favorable settling ability of HPNA reactor.

Community Analysis

Analysis of Community Structure under Phylum Level

Summary of the community relative abundances at the phylum level are shown in Fig. 5. The major phyla observed were *Planctomycetes*, accounting for 30-54% of the total reads, and *Proteobacteria* (22-32%), *Bacteroidetes* (5-15%), and *Chloroflexi* (4-14%). The 4 phyla accounted for more than 80% of the total reads in each sample. Other abundant phyla (2-6%) included *Chlorobi* and *Acidobacteria*. The sample
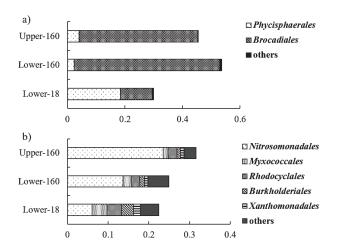

Fig. 6. Relative abundance of genera within the phyla; the genera under phylum *Planctomycetes* a) and the genera under phylum *Proteobacteria* b).

taken from the lower part on day 18 was used as the control. After 160 days of operation, *Planctomycetes*, which included anammox species, dominated in both the lower and upper parts and increased to 54% and 45% of the community, respectively, compared to the control of 30%. *Proteobacteria* also increased to 25% and 32% from 22% in the control, while *Bacteroidetes* decreased from 15% to 5% and 12%, respectively. The phylum of *Bacteroidetes* possessed the ability to digest and grow on a variety of complex substrates such as cellulose, chitin, and agar [36]. Its decrease might be due to a lack of available organic matter. *Chloroflexi* was reported to utilize cellular compounds derived from dead biomass and metabolites from anammox bacteria [37]. This phylum was also detected in other single-stage nitritation-anammox reactors, and found to gradually increase toward the end of the reactor operations [23]. However, in this study, *Chloroflexi* decreased to 7% and 4% in the lower and the upper parts, respectively. This could indicate that the dominant bacteria in the HPNA reactor performed higher activities.

Analysis of Community Structure under Genus Level

The two most abundant phyla were further analyzed (Fig. 6). At the genus level, *Brocadiales* (Anammox bacteria) and *Phycisphaerales* accounted for more than 99% of the total *Planctomycetes* reads (Fig. 6a). *Phycisphaerales* was observed to account for 18% in the control, but decreased to 2% in the lower part and 5% in the upper part. This genus belonging to the class of *Phycisphaerae* was isolated from a marine alga in 2008 [38] and reported to account for 20% in a one-stage nitritation-anammox reactor [23], but its function remains unknown. In this study, *Phycisphaerales* was replaced over time by the dominant *Brocadiales*, which largely increased from 11% in the control to 51% in the lower part and 41% in the upper part.

Fig. 6b) showed that *Nitrosomonadales* (AOB bacteria) was also the most dominant taxa in *Proteobacteria*, accounting for 6%, 14%, and 24% in the control, the lower HPNA part, and the upper HPNA part, respectively. The relative abundances of *Burkholderiales*, *Rhodocyclales*, *Myxococcales*, and *Xanthomonadales* decreased after the operation period. Members of *Burkholderiales* and *Rhodocyclales* are heterotrophic denitrifying bacteria and could degrade and utilize organic compounds [39]. *Xanthomonadales* belonging to γ-*Proteobacteria* are capable of degrading aromatic compounds, and *Myxococcales* belonging to δ-*Proteobacteria* play an important role in transforming organics [40]. The reduction of the four genera indicated that the SNA sludge had fine activity, thus the heterotrophic bacteria failed to use organics, which were released by dead bacteria, for metabolism.

The dominant anammox in the lower part and the dominant AOB in the upper part showed that

the sectionalized HPNA reactor was suitable for generating and maintaining SNA sludge. The high abundance of anammox and AOB also benefited from the limitation in oxygen, which was reported in [27].

Conclusions

The HPNA reactor was quickly started up with 28 days and stably operated for 168 days at high influent ammonium concentration (700-800 mg/L), with a final NRR of 6.1 kg-N/m^3/d and an ammonia nitrogen removal efficiency of 83%. Meanwhile, a short HRT of 3.3 h was achieved. The SNA granule sludge in this reactor was well kept and had a favorable settling property due to continued stirring. Community analysis showed that *Brocadiales* was dominant in the lower part and *Nitrosomonadales* was prior in the upper part. HPNA reactor is efficient for sludge enrichment and co-culture of AOB and Anammox bacteria, which has guiding significance for practical engineering.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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