https://doi.org/10.1051/e3sconf/202567708003

Development of anammox and AOB biofilm on honeycomb plastic media for application in partial nitritation-anammox reactor

Zulkarnaini Zulkarnaini[1*], *Viska* Clariza Bernida[1], *Yenni* Yenni[1], *Shinta* Silvia[1], *Zaini* Zaini[2], *Micko* Tomas[2], *Dewi* Nilawati[3], and *Shahrul* Ismail[4]

[1]Department of Environmental Engineering, Faculty of Engineering, Universitas Andalas, Padang 25175, Indonesia
[2]Department of Electrical Engineering, Faculty of Engineering, Universitas Andalas, Padang 25175, Indonesia
[3]Research Center for Environmental and Clean Technology, The National Research and Innovation Agency (BRIN), KST Samaun Samadikun, Bandung 40135, Indonesia
[4]Faculty of Ocean Engineering Technology and Informatics, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

Abstract. The purpose of this research was to cultivate anammox and ammonium-oxidizing bacteria (AOB) and analyze the performance of nitrogen removal using honeycomb plastic media in two reactors intended for use in a single partial nitrite-anammox reactor. Substrate was continuously supplied to the reactors, containing ammonium and nitrite of 180 mg-N/L for anammox and ammonium 180-500 mg-N/L for partial nitritation at HRT 12 hours. The reactors were operated until anammox and AOB biofilm growth in the media within 64 and 37 days, respectively. The reactors' performance was calculated based on ammonium conversion efficiency (ACE), nitrogen removal efficiency (NRE), and nitrogen removal rate (NRR). The anammox process can be directly initiated, achieving ACE, NRE, and NRR of 65.24%, 51.69%, and 0.10 kg-N/m³·d, respectively. Similarly, partial nitritation was successfully achieved faster with ACE and ΔNO_2^--N/ΔNH_4^+-N ratio of 51.14% and 0.6. Developing anammox bacteria and AOB using honeycomb plastic media could be a fast-startup strategy for single-stage nitritation-anammox reactors in wastewater nitrogen removal.

1 Introduction

Wastewater generally contains ammonium; therefore, a partial nitritation process is needed to support nitrogen removal by the anammox process. Partial nitritation is the process of partial oxidation of ammonia by ammonium-oxidizing bacteria (AOB) to nitrite under aerobic conditions. In recent years, partial nitritation has been considered a crucial mechanism, as it provides a sufficient substrate for the anammox process. Since wastewater typically has low nitrite content, partial nitritation is often combined with the anammox process to completely remove nitrogen from the wastewater, known as partial nitritation-anammox (PN/A). This combination can be carried out in a single reactor. The PN/A process has been successfully applied to treat complex wastewater, including landfill leachate, sewage, and anaerobic digestion effluent [1].

Compared with conventional methods, PN/A offers significant advantages. Xu et al. (2020) reported the concentration of ammonium nitrogen in the waste underwent a significant decline, dropping from the previous level of 519.1 mg/L to 6.9 mg/L using the partial nitritation-anammox process. Furthermore, an equally important finding was an increase in the average NRE, which reached 98.1% [2]. Jin et al. (2008) reported a high nitrogen removal efficiency, with NRE and ACE of 97% and 97.3%, respectively [3]. These results indicate that the up-flow biofilter (UBF) reactor with *Candidatus Brocadia anammoxidans* inoculum was able to effectively address nitrogen management issues, especially in removing nitrogen from wastewater.

Wastewater treatment through a biofilter system using plastic fiber media has quite good stability against fluctuations in concentration or processing load. The type of biofilter media commonly used is plastic media with a honeycomb-like design. The advantages of using this honeycomb plastic media include a surface area of 150–240 m^2/m^3 volume, a large cavity volume compared to other media, and a lower potential risk of clogging in smaller media. The use of honeycomb media demonstrated its effectiveness in removing nitrite (NO_2^-), ammonia (NH_4^+), and nitrate (NO_3^-) levels, with reductions of 97.23%, 85.80%, and 21.92%, respectively [4].

This study utilized a honeycomb as a carrier, a method commonly employed in wastewater treatment in Indonesia. This structured media is typically made from PVC (polyvinyl chloride) sheets. This construction approach enables the production of structured media at a

* Corresponding author: zulkarnaini@eng.unand.ac.id

lower cost per unit surface area compared to injection molding methods. PVC, as a resin material, is relatively more affordable and has superior mechanical properties compared to PP or HDPE. Although initially hydrophobic, PVC tends to become wet or exhibit good moisture retention properties within one to two weeks [4]. In this context, the development of efficient wastewater treatment technologies is closely aligned with the Sustainable Development Goals (SDGs) and disaster management, as water pollution and limited infrastructure can exacerbate environmental risks during disaster events. The purpose of this study was to cultivate and analyze the performance of anammox and partial nitritation reactors with honeycomb plastic media.

2 Materials and methods

2.1 Inoculum and media preparation

The anammox reactor was cultivated using anammox granules belonging to *Candidatus Brocadia fulgida* that have been cultivated in the environmental microbiology laboratory since 2021 at ambient temperature [5]. For the Partial nitritation reactor, AOB were obtained from mud collected from the Panjalinan estuary in Padang City, Indonesia.

The carrier for anammox and AOB biofilm growth was honeycomb plastic media. This medium has dimensions of 30 x 30 x 30 cm and a hole diameter of 3.8 mm, with a surface area of 150-240 m²/m³.

2.2 Substrate solutions

The substrate was prepared using groundwater in a 20 L plastic tank. The composition of the substrates for the anammox and partial nitritation processes was presented in Table 1.

Table 1. Composition of substrates for anammox and partial nitritation reactor.

Chemical	Anammox	Partial Nitritation
$Mg.SO_4.6H_2O$	300 mg/L	300 mg/L
$CaCl_2.7H_2O$	180 mg/L	180 mg/L
KH_2PO_4	27.2 mg/L	27.2 mg/L
Trace Element I	1 mL	1 mL
Trace Element II	1 mL	1 mL
NH_4HCO_3	180 mg-N/L	500 mg-N/L
$NaNO_2$	180 mg-N/L	-

2.3 Reactor configuration and operation

The reactor configuration consisted of an aquarium with an effective volume of 10 L and a honeycomb plastic carrier for bacterial growth (Fig. 1). The anammox and partial nitritation reactor schemes were illustrated in Fig. 2. The substrate was continuously supplied with a hydraulic retention time (HRT) of 12 hours after each inoculum was added to separate reactors. Anammox reactor was operated in an anaerobic condition, while partial nitritation was supplied with aeration using an aquarium blower. Effluent was extracted from the reactor through a membrane filter automatically using a water level sensor.

Fig. 1. Aquarium and honeycomb plastic.

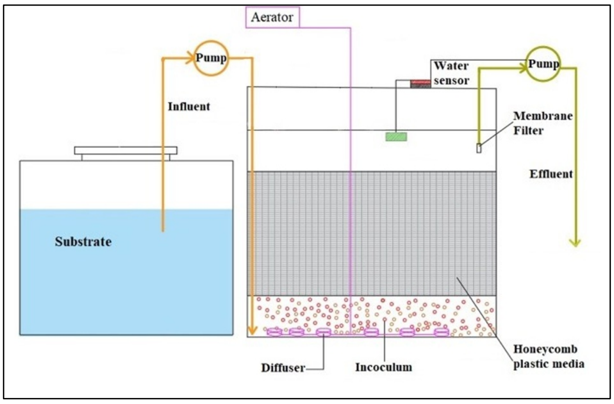

Fig. 2. Schematic reactor for cultivation of anammox and AOB.

2.4 Analysis sample and data

Influent and effluent samples were collected twice a week for analysis. Ammonium, nitrite, and nitrate were analyzed using Nessler's method, spectrophotometry, and ultraviolet screening spectrophotometry, based on standard methods [6]. Temperature and pH were measured using a pH meter (Hanna 98107) and a DO meter.

Performance of reactors was calculated based on nitrogen compounds composition with parameters ACE, NRE, and NRR:

$$ACE = \frac{[NH_4^+\text{-}N]_{in} - [NH_4^+\text{-}N]_{out}}{[NH_4^+\text{-}N]_{in}} \times 100\% \quad (1)$$

$$NRE = \frac{[NH_4^+\text{-}N]_{in} + [NO_2^-\text{-}N]_{in} - [NH_4^+\text{-}N]_{Out} - [NO_2^-\text{-}N]_{Out} - [NO_3^-\text{-}N]_{Out}}{[NH_4^+\text{-}N]_{in} + [NO_2^-\text{-}N]_{in}} \times 100\% \quad (2)$$

$$NRR = \frac{[NH_4^+\text{-}N]_{in} + [NO_2^-\text{-}N]_{in} - [NH_4^+\text{-}N]_{out} - [NO_2^-\text{-}N]_{out} - [NO_3^-\text{-}N]_{Out}}{HRT} \quad (3)$$

Where:
$[NH_4^+\text{-}N]_{in}$ = influent concentration ammonium
$[NO_2^-\text{-}N]_{in}$ = influent concentration nitrite
$[NH_4^+\text{-}N]_{out}$ = effluent concentration ammonium
$[NO_2^-\text{-}N]_{out}$ = effluent concentration nitrite
$[NO_3^-\text{-}N]_{Out}$ = effluent concentration nitrate
HRT = hydraulic retention time (hour)

3 Results and discussion

3.1 Nitrogen profile of anammox cultivation

The nitrogen profile includes ammonium, nitrite, and nitrate. The performance of the anammox process was observed, as evidenced by a simultaneous reduction in ammonium and nitrite, and an increase in nitrate, under anaerobic conditions.

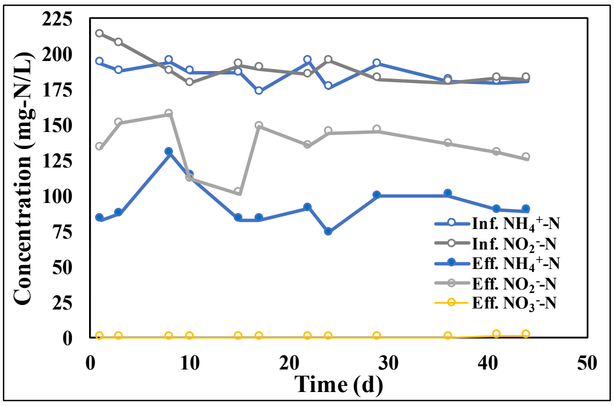

Fig. 3. Nitrogen concentration of the anammox process.

Strous et al. (1999) reported that the anammox process remains stable until the ammonium concentration reaches 1 g-N/L [7]; however, other studies have reported that high ammonium levels can interfere with the anammox process. Dapena-Mora et al. (2007) Dapena-Mora et al., (2007) observed that the ammonium concentration value that can inhibit the anammox process was 770 mg-N/L [8]. The study's results showed variations in ammonium concentration in the influent from the beginning to the end of the measurement, ranging from 173.2018 mg-N/L to 194.2075 mg-N/L. Figure 3 shows that the ammonium concentration decreased directly at the beginning of operation from 192.93 mg-N/L to 82.81 mg-N/L. The concentration fluctuated until the end of cultivation on the 44th day, reaching a maximum of 89.18 mg/L. Ammonium acts as a substrate in the anammox process, being converted into nitrogen gas under anaerobic conditions. The reduction of ammonium in the effluent indicates that the anammox process has occurred.

Nitrite is essential for the growth of anammox bacteria. Concentrations that are too low (<50 mg/L) will slow down the growth of anammox bacteria, and excessive concentrations (>280 mg/L) will result in the loss of anammox activity [9]. The nitrite concentration in the effluent gradually decreased from 133.48 mg-N/L to 125.70 mg-N/L. These results indicate that the anammox process was effective in removing nitrite as an electron acceptor and converting it into nitrogen gas along with ammonium.

The presence of nitrate in the effluent provides evidence that the nitrate formed during the anammox process conforms to the stoichiometry of the anammox reaction, which produces a small amount of nitrate under anaerobic conditions. The range of effluent concentrations measured from start to finish ranged from 0.21 mg-N/L to 0.97 mg-N/L.

3.2 Performance of nitrogen removal

Nitrogen removal performance can be observed from the calculation of ACE, NRE, and NRR. At the beginning of the operation, high performance was achieved, with rates of 57.08%, 46.72%, and 0.09 kg-N/m³·d, respectively. The result fluctuated until day 17 and tended to stabilize toward the end of cultivation, with values of 50.69%, 40.55%, and 0.07 kg-N/m³·d, respectively, as shown in Fig. 4.

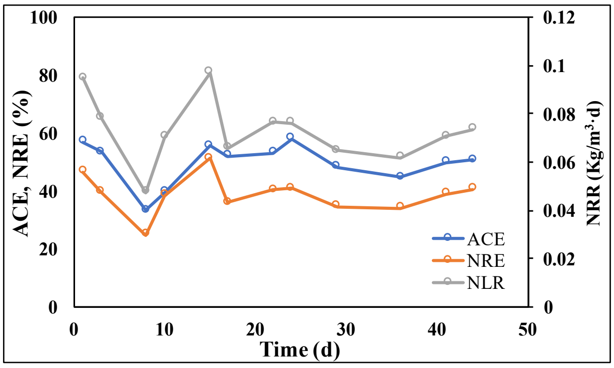

Fig. 4. Performance of nitrogen removal in anammox cultivation.

Previous research has shown that the ACE value reached 97.3% and the NRE reached 97% at 12-hour HRT using an UBF reactor [3]. Ma et al. (2013) also demonstrated that the anammox process could remove ammonium up to 92% of the ACE and 94% of the NRE using a UASB reactor [10]. This experiment achieved an NRR of 0.10 kg-N/m³·d, with an NLR of 0.20 kg-N/m³·d at a 12-hour HRT. Additionally, NRE and ACE are directly proportional to NRR, increasing with time.

Since the initial phase of operation, nitrogen removal was evident from the simultaneous removal of ammonium and nitrite, as well as the production of nitrate. This phenomenon indicates the occurrence of the anammox process. In addition, a key aspect of the anammox process can be seen through NRR. The NRR consistently increased along with NLR of 0.20 kg-N/m³·d. At the beginning of the experiment, the NRR was 0.09 kg-N/m³·d. The low NRR level in the initial phase of operation was caused by the slow growth of anammox bacteria compared to other types of bacteria, such as AOB. During the test period, the NRR consistently increased until it reached a maximum value on the 15th day, which was 0.10 kg-N/m³·d. On day 8, the NRR decreased to 0.05 kg-N/m³·d, but then it gradually increased until it reached a value of 0.09 kg-N/m³·d on day 64.

Previous research has shown NRR of 5.72 kg-N/m³·d with an NLR of 6 kg-N/m³·d using a UASB reactor [10]. Jin et al. (2008) demonstrated that the anammox process can be achieved in a short operating time of 31 days, with an NRR of 0.3 kg-N/m³·d [3]. Meanwhile, Trigo et al. (2006) operated the anammox process using a membrane sequencing batch reactor (MSBR) reactor using nonwoven media with an HRT of 7.5 hours with NRR of 710 mg/L·d (0.71 kg-N/m³·d) [11]. Based on previous research, the NRE and NRR values in this study were lower but are expected to increase due to the slow growth of anammox bacteria gradually.

3.3 Partial nitritation process

In the PN reactor, a partial nitritation process occurs by oxidizing ammonium to nitrite and prevents the formation of nitrate by controlling DO. Figure 5 showed the nitrogen profile during reactor operation for cultivating AOB by the PN process.

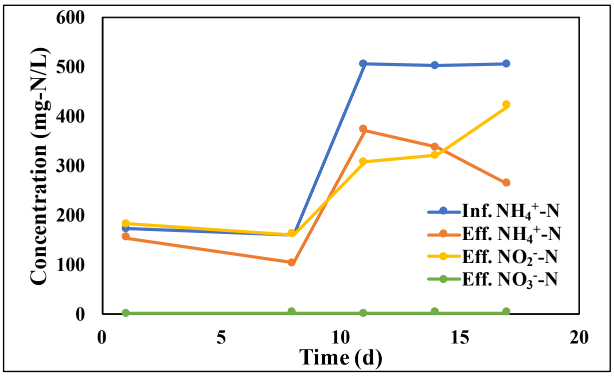

Fig. 5. Nitrogen profile of the partial nitritation process.

The ammonium concentration measured in the influent ranged from 160.4710 mg-N/L to 504.8377 mg-N/L. Ammonium tended to decrease through the partial nitritation process, resulting in a nitrite production of 262.32 mg-N/L at the end of the experiment. Without additional nitrite in the influent, the concentration increased gradually from 180.99 mg-N/L to 420.73 mg-N/L in the effluent, which indicates the partial nitritation process produced nitrite.

A small concentration of nitrate was detected in the effluent, indicating the successful completion of the nitrification process. The process can be suppressed because the nitrification process occurs later after long-term operation. Nitrate concentrations during the experiment ranged from 0.0881 mg-N/L to 0.1767 mg-N/L.

3.4 Ammonium conversion of the partial nitritation process

The nitrite concentration in the PN Reactor was formed through a partial nitritation process by AOB bacteria originating from the mud of the Panjalinan estuary, Padang, Indonesia. The mud was used as *a starter* for AOB bacteria to oxidize some of the ammonium to nitrite. AOB bacteria require a certain amount of oxygen to synthesize ammonium into nitrite [12]. AOB bacteria oxidized some of the ammonium to form nitrite, which was used as an electron acceptor in the anammox process. Nitrite compounds are unstable because, during the formation of nitrite compounds, they will be oxidized to nitrate [13]. The theoretical ΔNO_2^--N/ΔNH_4^+-N ratio formulated was 1.146 [13]. The highest ratio ΔNO_2^--N/ΔNH_4^+-N in this study was 0.6, which was obtained at the end of the experiment (Fig. 6).

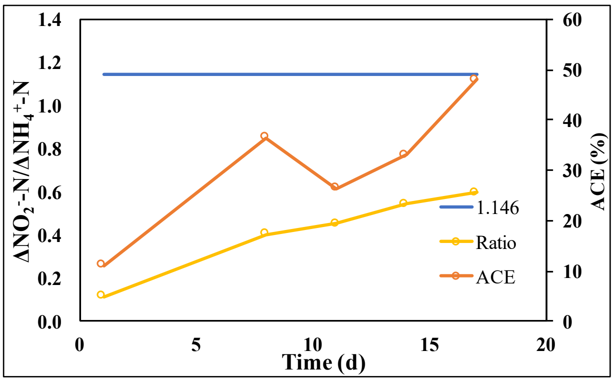

Fig. 6. Ratio ΔNO_2^--N/ΔNH_4^+-N during partial nitritation process.

3.5 Monitoring temperature, DO, and pH

The pH in the influent and effluent was in the range of 7.6-8.8, where anammox bacteria grow optimally at a pH between 6.7-8.3 [7]. In the anammox granular sludge system operating in continuous flow with an effluent pH ranging from 8.5 to 9.3, the anammox reaction continued to proceed stably. During the experiment, the pH remained in optimal conditions.

Various studies have recorded variations in the optimal pH value for nitritation. However, the optimal pH for AOB and nitrite-oxidizing bacteria (NOB) generally ranges from 7 to 8. Because AOB uses NH_4^+-N as a substrate, they tend to prefer an environment with slightly alkaline properties. In experiments with the AOB reactor, it was noted that the pH values of the influent and effluent ranged from 8.5 to 10.1. The pH remained at optimal levels throughout the experiment.

In this study, the anammox process was carried out at ambient temperature on a laboratory scale. The temperature recorded during sampling from the anammox reactor ranged from 27.4 to 31.5°C. Anammox reactors are mostly operated at high temperature ranges (≥ 30°C) and treat wastewater with high nitrogen concentration. The optimal temperature range for the growth of anammox bacteria ranges between 30 and 40°C. The activity of anammox bacteria metabolism enzymes decreases at temperatures lower than the optimal temperature; therefore, the absorption of nitrite by anammox bacteria stops, and the anammox process is inhibited due to the accumulation of nitrite in the reactor. Several reports indicate that when the temperature decreases in the range of 20 °C-30 °C, the nitrogen removal rate in the anammox reactor decreases significantly. Operating the reactor in a Wastewater Treatment Plant (WWTP) at higher temperatures (≥30°C) is uneconomical, while operating the reactor at lower temperatures (≤20°C) remains a challenge for researchers.

The temperature recorded during sampling from the AOB reactor ranged from 27.3 to 29.5°C. Based on the research conducted, it has been proven that both anammox and AOB bacteria can grow and adapt in areas with temperatures below optimum conditions, as evidenced by the occurrence of nitrogen removal during the experimental period. Data regarding temperature, DO, and pH during the experiment can be found in Fig. 7 and Fig. 8.

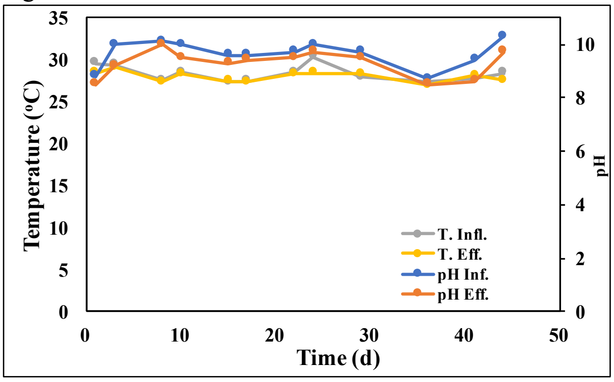

Fig. 7. Temperature and pH in the anammox reactor.

The study by Ma et al. (2013), which spans a temperature range of 16-30°C, suggests a wider temperature range, potentially reflecting a more comprehensive study that summarizes the effects of temperature over a broader range. The results of this study can provide insight into how microorganisms and biological processes adapt to more extensive temperature variations.

On the other hand, this study's focus on the temperature range of 27.4–31.5°C suggests a narrower and more specific temperature range. This temperature range may have been chosen based on more focused considerations of the specific environment or waste source being addressed. Research results within this narrower temperature range could provide more detailed insights into how microorganisms and biological processes respond to more limited temperature changes.

In both cases, this comparison demonstrates that temperature is a crucial factor in regulating the efficiency and effectiveness of biological processes in wastewater treatment. The wider temperature range in the study by Ma et al. (2013) provides a more comprehensive view of the response of microorganisms to temperature variations [10]. Meanwhile, this study, with its focus on a narrower temperature range, focuses on a more specific environmental setting. Both are valuable in providing insight into adaptation and the influence of temperature on the performance of biological processes.

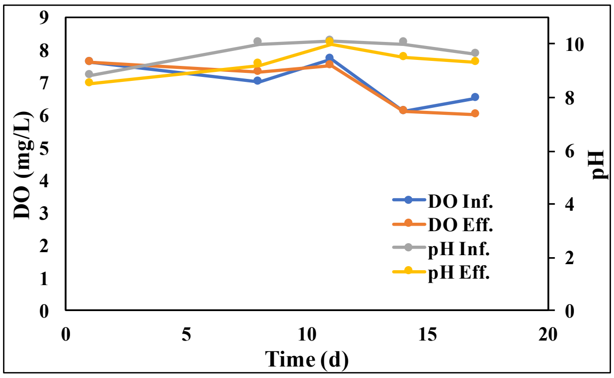

Fig. 8. DO and pH partial nitritation reactor.

The dissolved oxygen (DO) concentration in the PN reactor ranged from 6.0-8.0 mg/L, which indicates that the reactor was aerobic for the nitritation process by AOB. The DO concentration during the study in the PN reactor was shown in Fig. 8. Cema et al (2011) studied the effect of DO in MBBR. They observed that increasing biofilm thickness did not affect nitrogen removal even if the oxygen concentration was high or low [13]. Higher DO can result in higher nitrogen removal, but also causes higher nitrate production. Zubrowska-Sudol et al. (2011) investigated the impact of various aeration strategies on the deammonification process during operation of a pilot-scale MBBR. They concluded that an optimal aeration strategy was required by considering the DO concentration and the ratio between the non-aerated and aerated phase times.

4 Conclusion

This study successfully demonstrated the development of *anammox* and ammonium-oxidizing bacteria (AOB) biofilms on honeycomb plastic media for application in a partial nitritation–anammox (PN/A) system. The anammox reactor achieved stable nitrogen removal performance with an ammonium conversion efficiency ACE, NRE, and NRR of 65.24%, 51.69%, and 0.10 kg-N/m³·d, respectively, at HRT of 12 hours. Similarly, the partial nitritation reactor achieved an ACE of 51.14% and a ΔNO_2^-–N/ΔNH_4^+–N ratio of 0.6, indicating efficient conversion of ammonium to nitrite under controlled aerobic conditions.

These results confirm that honeycomb plastic media provide a suitable environment for simultaneous growth of anammox and AOB biofilms, enabling faster start-up and stable operation under ambient tropical temperatures (27–31°C). The use of this low-cost PVC-based structured media offers a practical and scalable approach for integrating anammox and partial nitritation in single-stage nitrogen removal systems. Further research is recommended to optimize reactor configuration and evaluate long-term performance under varying organic loads and wastewater compositions.

This research was supported by LPPM Universitas Andalas with Grand Number: 499/UN16.19/PT.01.03/PUJK/2025

References

1. A. Magrí, M. B. Vanotti, and A. A. Szögi, Anammox sludge immobilized in polyvinyl alcohol (PVA) cryogel carriers. Bioresour. Technol. **114**, 231 (2012). http://dx.doi.org/10.1016/j.biortech.2012.03.077
2. Y. Xu, Y. Xu, T. Li, G. Wang, and X. Dai, Two-step partial nitrification-anammox process for treating thermal-hydrolysis anaerobic digester effluent: Start-up and microbial characterization. J Clean Prod **252**, (2020). https://doi.org/10.1016/j.jclepro.2019.119784
3. R. C. Jin, P. Zheng, A. H. Hu, Q. Mahmood, B. L. Hu, and G. Jilani, Performance comparison of two anammox reactors: SBR and UBF. Chemical Engineering Journal **138**, (2008). https://doi.org/10.1016/j.cej.2007.06.038

4. G. P. Sali, A. Suprabawati, and Y. Purwanto, *Efektivitas teknik biofiltrasi dengan media sarang tawon terhadap penurunan kadar nitrogen total limbah cair.* Jurnal Presipitasi: Media Komunikasi Dan Pengembangan Teknik Lingkungan **15**, (2018). https://doi.org/10.14710/presipitasi.v15i1.1-6
5. Z. Zulkarnaini, P. S. Komala, R. P. Putra, M. Ali, N. Matsuura, K. Koike, J. Wang, P. C. Mawarda, and R. Yamamoto-Ikemoto, Cultivation of anammox bacteria from a tropical lake in Indonesia using a novel filter bioreactor to enhance nitrogen removal efficiency. Water Science and Technology **91**, 811 (2025). https://doi.org/10.2166/wst.2025.050
6. APHA, Standard methods for the examination of water and wastewater, 23rd ed. (American Public Health Association, Washington D.C., 2017)
7. M. Strous, J. G. Kuenen, and M. S. M. Jetten, Key physiology of anaerobic ammonium oxidation. Appl Environ Microbiol **65**, (1999). https://doi.org/10.1128/AEM.65.7.3248-3250.1999
8. A. Dapena-Mora, I. Fernández, J. L. Campos, A. Mosquera-Corral, R. Méndez, and M. S. M. Jetten, Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. Enzyme Microb Technol **40**, (2007). https://doi.org/10.1016/j.enzmictec.2006.06.018
9. R. C. Jin, G. F. Yang, J. J. Yu, and P. Zheng, The inhibition of the anammox process: A review. Chem. Eng. Jou. **197**, (2012). https://doi.org/10.1016/j.cej.2012.05.014
10. B. Ma, Y. Peng, S. Zhang, J. Wang, Y. Gan, J. Chang, S. S. S. Wang, S. S. S. Wang, and G. Zhu, Performance of anammox UASB reactor treating low strength wastewater under moderate and low temperatures. Bioresour. Technol. **129**, 606 (2013). https://doi.org/10.1016/j.biortech.2012.11.025
11. C. Trigo, J. L. Campos, J. M. Garrido, and R. Méndez, Start-Up of the anammox process in a membrane bioreactor. J Biotechnol 126, 475 (2006). https://doi.org/10.1016/j.jbiotec.2006.05.008
12. T. Lotti, R. Kleerebezem, C. Lubello, and M. C. M. M. van Loosdrecht, Simultaneous partial nitritation and anammox at low temperature with granular sludge. Water Res. 60, 1 (2014). https://doi.org/10.1016/j.watres.2014.07.047
13. G. Cema, E. Płaza, J. Trela, and J. Surmacz-Górska, Dissolved oxygen as a factor influencing nitrogen removal rates in a one-stage system with partial nitritation and Anammox process. Water Science and Technology **64**(5), (2011) https://doi.org/10.2166/wst.2011.449