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FAST START-UP MARINE ANAMMOX PROCESS USING INTENSIVE SHRIMP POND SOLID WASTE AS INOCULUM IN FILTER BIOREACTOR

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Abstract: Intensive shrimp culture waste contains high nitrogen, which can reduce water quality and environmental-carrying capacity. Anaerobic ammonium oxidation (anammox) is a potential and economical nitrogen removal process. However, limited information is available on the application of anammox process in aquaculture. The purpose of this study was to start up an anammox process in two filter bioreactors (FtBR) based on different inoculums. Run 1 used intensive shrimp solid waste, and Run 2 used intensive shrimp solid waste and granular of fresh anammox bacteria belonging to Candidatus Brocadia fulgida. Ammonium and nitrite concentrations of 70-100 mg N L^{-1} each were added to filtered and sterilized seawater as synthetic wastewater and flowed to the reactor with HRT 24-hour. After 120 days, the maximum ammonium conversion efficiency (ACE), nitrogen removal efficiency (NRE), and nitrogen removal rate (NRR) for Run 1 and 2 were 82.48%, 72.58%, 0.12 kg N $(m^3 \cdot d)^{-1}$ and 83.06%, 63.59%, 0.10 kg N $(m^3 \cdot d)^{-1}$, respectively. Experiment nitrogen stoichiometric ratios of NH_4^+:NO_2^-:NO_3^- were 1:1.40:0.12 and 1:1.13:0.14, which were close to the stoichiometry of the anammox process. Fast start-up of anammox process was achieved using intensive shrimp solid waste as inoculum. Anammox can be a new method for developing intensive shrimp culture wastewater treatment.

Keywords: Anammox, filter bioreactor, nitrogen removal, shrimp pond waste.
Abbreviations: Anaerobic ammonium oxidation (anammox), Ammonium Conversion Efficiency (ACE), Nitrogen Removal Rate (NRR), Nitrogen Removal Efficiency (NRE).

Introduction

Intensive shrimp farming, the primary method for increasing shrimp production, involves cultivating shrimp at high stocking densities (above 100 shrimp/m^2), significantly utilizing feed. However, the development of intensive shrimp farming causes high waste in the waters. The main content of shrimp pond waste that can reduce the environment's carrying capacity and impact environmental ecosystems is nitrogen and phosphate. The waste generated mainly comes from the feed with nitrogen retention of 22%, then about 78% will be discharged into the environment (Samocha *et al.,* 2017). Sustainable and environmentally friendly shrimp farming is needed to overcome the problem of shrimp pond waste to prevent pollution in the environment (Kumar *et al.,* 2015).

Anaerobic ammonium oxidation (anammox) could be a potential technology for nitrogen removal in aquaculture areas (Ismail *et al.*, 2022). Compared to conventional nitrification-denitrification, their high nitrogen removal capability reduces oxygen demand, and no additional carbon is required (Ni & Zhang, 2013). Full-scale wastewater treatment using an anammox process is currently widely practiced globally as a sustainable and cost-effective way (Lackner *et al.,* 2014). The following reaction is the anammox process (Lotti *et al.*, 2014):

$$1NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0{,}161NO_3^- + 0{,}986N_2 + 0.071CH_{1.74}O_{0.31}N_{0{,}20} + 2.002H_2O. \quad (1)$$

In aquaculture environments, *Candidatus Kuenenia* is predominant in bioaugmented zero water exchange aquaculture production systems (ZWEAPS), followed by *Candidatus Scalindua* (Nair *et al.*, 2020), and several studies have shown high nitrogen removal capabilities with good growth (Van Duc *et al.*, 2018).

The main obstacle to the development of anammox bacteria is the slow growth, with a doubling time of 10-12 days on a large scale and laboratory, and it cannot be isolated purely. The start-up of anammox process requires an appropriate inoculum for the rapid development of anammox bacteria (Zekker *et al.,* 2014). In the full-scale anammox reactor, a start-up with anammox granules from existing reactors can reduce the initial period for high-rate nitrogen removal (Liu *et al.*, 2020). Sludge from wastewater treatment facilities and the environment has been commonly used as inoculum to start the anammox process where no existing anammox reactor exists. The first identified fresh anammox bacteria in Indonesia was cultivated sludge from Lake Koto Baru, West Sumatra, using Filter Bioreactor (FtBR) belonging to *Candidatus Brocadia* and *Candidatus Anammoxoglobus* (Zulkarnaini, 2020b). Lulrahman *et al.* (2022) used sludge from the estuary Muaro Penjalinan, Padang City, Indonesia, to remove nitrogen in saline conditions using seawater in FtBR. A low nitrogen removal efficiency (NRE) of 20.268% was achieved in 143 days of operation. Marine anammox species corresponding to nitrogen removal in marine are mostly identified in the sea (Oshiki *et al.,* 2013; Sonthiphand *et al.*, 2014). Based on the above explanation, research is needed to develop anammox bacteria using inoculum from intensive shrimp pond sludge to be the basis for the large-scale anammox bioreactors in treating intensive shrimp aquaculture pond wastewater. This research aimed to analyze nitrogen removal performance in two bioreactor filters (FtBR) based on different inoculums.

Materials and Methods

Inoculum and Artificial Wastewater

One liter of sludge was collected from a sedimentation-intensive shrimp pond with a depth of 0.5 m below the soil surface. Artificial wastewater was made with seawater and continuously flowed into the reactor (Oshiki *et al.,* 2013; Wei *et al.,* 2016). The seawater was filtered using a sand filter and sterilized with UV before being used as artificial wastewater. The composition of the substrate in this study consisted of 70-100 mg N L^{-1} $(NH_4)_2SO_4$ and $NaNO_2$, $KHCO_3$ 500 mg L^{-1}, $MgSO_4$ 300 mg L^{-1}, $CaCl_2$ 180 mg L^{-1} and trace element I and II (Zulkarnaini *et al.,* 2021).

Reactor

This research used two filter bioreactors (FtBR, working volume 1.5 L) with different inoculum compositions. Both reactors were inoculated with 450 mL sludge from intensive shrimp pond sedimentation; Run 2 added 20 mL granular anammox bacteria belonging to *Candidatus Brocadia fulgida*, and Run 1

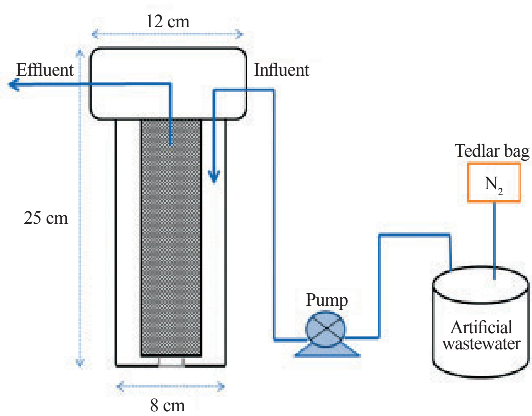

Figure 1: Schematic diagram of reactor operation

without. The anammox inoculum was derived from cultivation reactors that have operated for over two years (Putra *et al.,* 2020). The reactor was covered with aluminum foil to prevent light penetration and to inhibit photosynthetic bacteria and operated at room temperature (Zulkarnaini, 2020a). For anaerobic reactor operation, the artificial wastewater was flushed with nitrogen gas (N_2) for 30 minutes, and a tedral bag containing N_2 was connected to the influent tank. The artificial wastewater was delivered into reactors continuously with a hydraulic retention time of 24 hours. The string wound filter (0,5 µm pore size, 10-inch length) was useful as a carrier for anammox biomass and to prevent washout from the reactor.

Data Analysis

The anammox process was monitored by collecting samples from the influent and effluent every week. Nessler, spectrophotometry, and ultraviolet screening spectrophotometry methods were used to analyze the NH_4^+-N, NO_2^--N, and NO_3^--N concentrations. Reactor performance was calculated based on the nitrogen profile with parameters ammonium conversion efficiency (ACE, %), nitrogen removal efficiency (NRE, %), nitrogen loading rate (NLR, kg N $(m^3{\cdot}d)^{-1}$), and nitrogen removal rate (NRR, kg N $m^3{\cdot}d)^{-1}$) (Zulkarnaini *et al*., 2018).

Results and Discussion

Nitrogen Profile

The nitrogen profile in Figure 2 shows the concentrations of NH_4^+-N, NO_2-N, and NO_3^--N in the experiment. The ammonium and nitrate effluents were relatively similar in the initial phase, even higher than the influent for 36 days. This was likely because the organic matter available in the sludge still supported the activity of denitrifying bacteria. After 43 days, there was a gradual increase in ammonium and nitrite consumption, from 4.29 mg-N/L^{-1} to 32.95 mg-N/L^{-1} until day 57. A rapid rise in influent nitrite occurred after day 62, causing the concentration of effluent nitrite to increase. The presence of nitrite in large quantities quickly can be a limiting factor for the activity of anammox bacteria due to shock loading and substrate inhibition (Kawagoshi *et al*., 2009). During this period, there was a fluctuation in consumption but it continued to increase gradually until the end. The highest ammonium consumption in Run 1 achieved 50.7 mg N L^{-1} and 71.44 mg N L^{-1} on day 113.

The nitrogen profile in Run 2 showed not much difference from Run 1. The concentration of ammonium and nitrite showed a significant decrease in the initial stage in 43 days. After that, it decreased until the end of the

$$ACE = \frac{[NH_4^+ - N]_{in} - [NH_4^+ - N]_{out}}{[NH_4^+ - N]_{in}} x100\% \quad (2)$$

$$NRE = \frac{[NH_4^+ - N]_{in} + [NO_2^- - N]_{in} - [NH_4^+ - N]_{out} - [NO_2^- - N]_{out} - [NO_3^- - N])_{out}}{[NH_4^+ - N]_{in} + [NO_2^- - N]_{in}} x100\% \quad (3)$$

$$NRR = \frac{[NH_4^+ - N]_{in} + [NO_2^- - N]_{in} - [NH_4^+ - N]_{out} - [NO_2^- - N]_{out} - [NO_3^- - N])_{out}}{HRT} \quad (4)$$

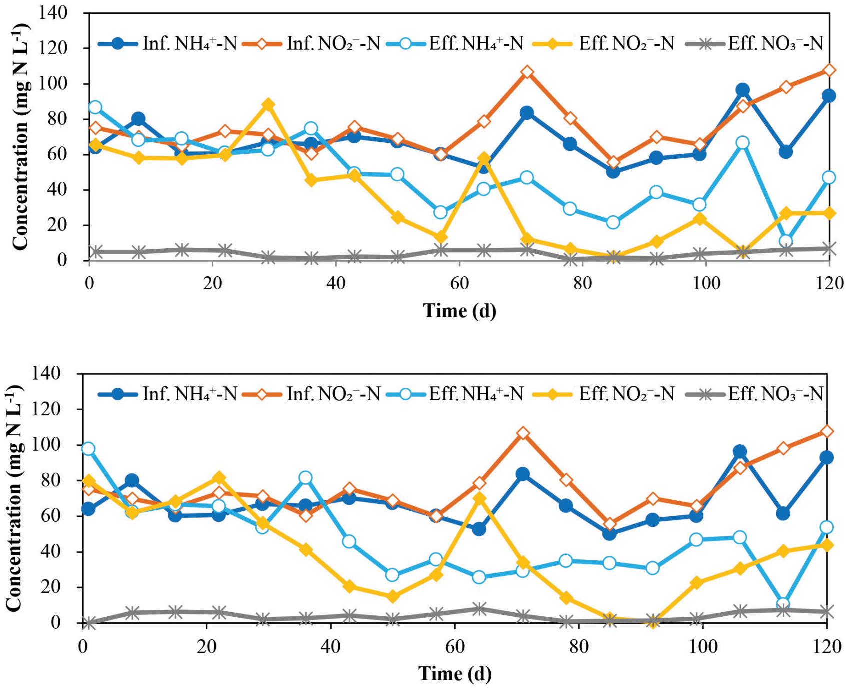

Figure 2: Profile of nitrogen in Run 1 and Run 2

experiment, although there were fluctuations. Maximum consumption of ammonium and nitrite was 51.06 mg N L^{-1} and 57.83 mg N L^{-1}, achieved on day 113. Both reactors showed low ammonium consumption in the first 36 days, and only nitrite was consumed. Conditions like this indicate a low anammox population. The increased development of anammox bacteria was indicated by the increased consumption of ammonium and nitrite until the end of the research. The nitrate concentration tended to increase until the final period of the study. The presence of nitrate is one of the products of the anammox process, which indicates that the anammox bacteria are working, but decreased or absent nitrate indicates a denitrification reaction that also occurs in the reactor with the available organic carbon (Kawagoshi *et al.,* 2010).

The results of the nitrogen removal performance in Figure 3 for Run 1 and 2 show almost the same trend. The maximum nitrogen removal for Run 1 and 2 with parameters ACE, NRE, and NRR were 82.48%, 72.58%, 0.12 kg N $(m^3{\cdot}d)^{-1}$ and 83.06%, 63.59%, 0.10 kg N $(m^3{\cdot}d)^{-1}$ at NLR 0.16 kg N $(m^3{\cdot}d)^{-1}$, respectively. The substrate source had the same concentration in both reactors, causing the same NLR value. At the initial stage of the reactor for up to 36 days, negative NRR was obtained because nitrogen removal was not observed clearly. This condition was the acclimatization phase of anammox bacteria to the substrate and reactor condition. The acclimatization process and the inoculum of anammox bacteria determine the NRR level at the reactor's initial stage (Kindaichi, Awata, Suzuki *et al.,* 2011). During the initial stage of the anammox process using sludge for start-up, it is common to observe a higher concentration of ammonium in the effluent than the influent. This phenomenon is known as the sluggish phase, caused by the conversion of organic nitrogen present in the seeding sludge into ammonium by bacteria living within the sludge (Chen *et al.,* 2012). Additionally, the increase in ammonium concentration observed during this phase can also be attributed to environmental changes, such as cell lysis and breakdown of organic nitrogen, leading to the release of ammonia from

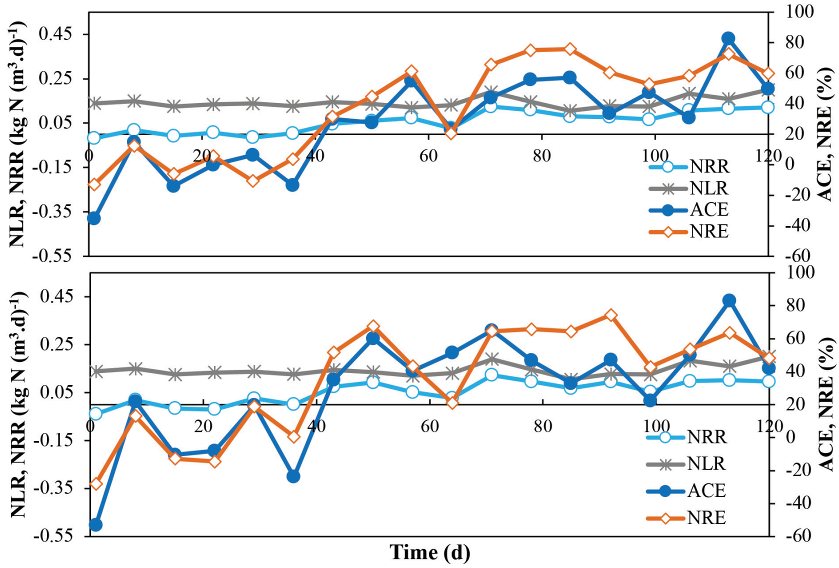

Figure 3: Nitrogen removal performance in Run 1 and Run 2

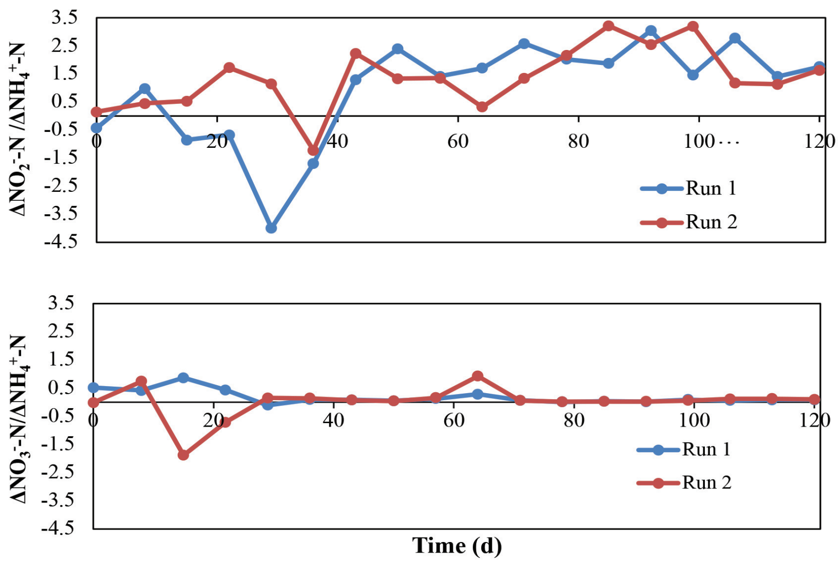

Figure 4: ΔNO_2^--N/ΔNH_4^+-N and ΔNO_3^--N/ΔNH_4^+-N in Run 1 and Run 2

dead aerobic and heterotrophic bacteria (Wang *et al.,* 2013).

Negative efficiency performance was obtained in the first 26 days and then, after 43 days, showed positive results that reflected the activity of anammox at the exponential stage. The significant decrease in NRE on day 64 was due to the increasing influent concentration of ammonium and nitrite in the reactor due to shock loading. In the study of marine anammox bacteria at seabed waste disposal sites, it was found that nitrite residues can reduce the performance of anammox (Kawagoshi *et al.,* 2009). The nitrogen concentration in the influent was decreased for recovery.

As with addition of fresh anammox bacteria as inoculum in Run 2, there was no effect in profile and performance of nitrogen removal with Run 1. *Candidatus Brocadia fulgida* is a fresh anammox species and cannot adapt to high salinity. The measured salinity during this study was 32-33 ppt. The sudden change in the cultivation environment caused this type of bacteria not to survive because salinity directly affects the performance and population of anammox bacteria (Gonzalez-Martinez *et al.,* 2018). Although some freshwater anammox bacteria can adapt to high salinity conditions, their activity and population will decrease (Gonzalez-Silva *et al.,* 2017). In the bio-augmented shrimp pond, freshwater anammox bacteria, *Candidatus Kuenenia,* were also found (Nair *et al.,* 2020). Candidatus Kuenenia, representing the freshwater anammox habitat group, was found in the marine aquaculture zone (Li & Gu, 2016). They are primary and possible components of anammox activity contributing to the nitrogen cycling in shrimp pond sediments of subtropical aquaculture ecosystems (Amano *et al.,* 2011).

In both reactors, the results obtained in 120 days were not in a stable phase but in an activity elevation stage where there was a drastic or significant decrease in both ammonium and nitrite concentration in the effluent. They continued toward steady-state/stable conditions, and the nitrate concentration in the effluent tended to increase (Lulrahman *et al.*, 2022).

Nitrogen stoichiometric ratios were 1:1.40:0.12 and 1:1.13:0.14, which were close to the stoichiometry of the anammox process 1:1.32:0.26 (Strous *et al.,* 1998). In research on other marine anammox bacteria, slightly different stoichiometric results were obtained (Kawagoshi *et al.*, 2010) with a ratio 1:1.07 within 320 days on enriched seawater-related samples, coastal sediment 1:1.28:0.24 in 393 days (Kindaichi, Awata, Suzuki *et al.*, 2011), and on the cultivation of the Hiroshima bay sediment was 1:1.21:0.15 in 306 days (Kindaichi, Awata, Tanabe *et al.*, 2011).

Conclusion

The anammox process occurred in both bioreactors with high nitrogen removal performance using intensive shrimp pond waste as inoculum. High nitrogen removal was achieved in a short time between 50-57 days. The anammox process could be a new technology for shrimp farming wastewater treatment.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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