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Original Research Article

Characterization of Anammox Bacteria from Marine Water and Sediment Samples

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Abstract

Anammox activity of water and sediment taken from marine ecosistem around Semarang were investigated in anammox media agar and batch reactor. Effect of increasing salinities (3% to 5%, 7% and 9%) and decreasing salinities (3% to 2%, 1% and 0%) were investigated. Water and sediment taken from marine ecosistem around Semarang city showed a positive result. Increasing salinity up to 9% will have a positive influence on the activity of anammox bacteria. Whereas, the decrease in salinity will negatively affect anammox bacteria. The ratio of ammonium:nitrite for anammox process ranges from 0.89 to 1.22 and ammonium removal rate varies from 0.08 to 0.59 mg-N/liter hour.

Keywords: Compost; grass waste; banana leaf waste; cotton waste

1. Introduction

Biological nitrogen removal is usually achieved by a sequence of nitrification and denitrification processes. During nitrification, ammonia is biologically oxidized to nitrate via nitrite which is then reduced to nitrogen gas during the denitrification process. The nitrifies use ammonia or nitrite as an energy source, oxygen as an electron acceptor and carbon dioxide as a carbon source. During denitrification, microorganisms utilize nitrite and or nitrate as electron acceptors and organic matter as carbon and energy source. Therefore, nitrification and denitrification, known as conventional biological nitrogen removal processes, are proceeding slowly and are relatively expensive, referring to energy requirement for oxygen supply, alkalinity requirement and an external carbon source.

In the past few years, several alternatively biological nitrogen removal processes have been developed, including partial nitrification, denitritation, anaerobic ammonia oxidation (the Anammox process), and its combined systems. The anammox process involves the oxidation of NH_4+ with NO_2- as electron acceptor to produce dinitrogen gas (van de Graaf et al., 1995). Anammox activity has been identified in natural freshwater and terrestrial environments (Schuber et al., 2006; Zhu et al., 2011). In addition to freshwater environments, anammox bacteria have been detected in marine water and sediments (Dale et al., 2009; Rich et al., 2008; Schmid et al., 2007).

Anammox bacteria is identified using different methods. The unculturable Gram-negative anammox bacteria were first isolated via density gradient centrifugation (van de Graaf et al., 1995; Strous et al., 2002). The anammox bacteria are characterized by slow growth rate and low biomass yield (Strous et al., 1998), making this process difficult for practical wastewater treatments (Zhang et al., 2008). It is also reported that Anammox bacteria have a slow growth rate with doubling time around 10 days (Banihani et la., 2012).

Parameters of wastewater, such as pH, salinity, substrate, temperature fluctuates depending on the treated raw material that may change during a day in industrial production. Anammox process should work properly for changes of the parameters. Several important physiological parameters of anammox bacteria such as pH, salinity, Dissolved Oxygen, biomass yield, growth rate have been determined by (Strous et al., 1998; 1999; Dalsgaard et al., 2005). Rønning (2013), Luo et al., (2015) and Dong & Tollner (2003) whom have observed the effects of salinity on anammox process.

In recent years, there has been an increasing amount of research on anammox process. However, such research in Indonesia is still very rare. Inventory of anammox bacteria in various habitats in Indonesia is still urgently needed. Effect of salinity on growth and activity of anammox bacteria is also necessary in order to determine the stability of the anammox process on treating fluctuated saline wastewater.

2. Methodology

2.1. Water and Sediment Samples Collection

Water and sediment samples were taken at two estuary of large rivers (Banjir Kanal Timur – BKT and Banjir Kanal Barat – BKB) and coastal Semarang (Pantai Marina – PM) in Semarang. Water and sediment sampling points were all located in the middle of the estuary or coastal with a depth of more than 1.5 m. Water samples were taken at a depth of about 50 cm from the surface of the water. Sediments were taken from the river or the beach using plastic cans. Then, water and sediment samples are inserted into plastic boxes with volume of 15 liters that have been given an ice cube to maintain the temperature and later be tested in the laboratory. To simplify the naming of inoculant, the code 'A' is used for samples from water from Pantai Marina - PM_A, 'B' (Water from Banjir Kanal Timur – BKT_A), 'C' (Water from Banjir Kanal Barat – BKB_A), 'D' (sediment from Pantai Marina - PM_S), 'E' (sediment from Banjir Kanal Timur – BKT_S), 'F' (sediment from Banjir Kanal Barat - BKB_S).

2.2. Anammox Bacteria Isolation and Selection

Samples of water (3 samples) and sediment (3 samples) which were about 5 ml to 45 ml, were added to anammox liquid medium with a salt (NaCl) concentration of 3% in sterilized erlenmeyer flask. The flasks were incubated in the rotary shaker for 24 hours at room temperature. Then, liquid from the erlenmenyer flasks was used as inoculation to the anammox agar media that contains salinity of 3% in the petri dish. After 48 hours of incubation, the microorganism in the petri dish was observed. Furthemore, colonies in petri dish were transferred to anammox agar slant tube. Gram stained was performed for all bacteria samples.

2.3. Effect of Salinity on Anammox Bacteria Growth

Isolate showing a presence of anammox were then selected for further observation. The selected isolate (5 ml) was put into 45 ml of medium anammox liquid with different salinity (0, 1, 2, 3, 5, 7 and 9% of NaCl) in erlenmeyer 100 ml. The erlenmeyers were then incubated for 4 days in an incubator shaker at room temperature (25°C) with a speed of 100 rpm. The growth of the bacteria was observed by OD 560 at all concentrations for 0, 6, 12, 24, 48, 72 and 96 hours. Furthermore, to determine the inhibitor type of salinity, the bacterial culture that has been grown on anammox liquid media with different salt concentrations was transferred back in anammox media with initial salt concentration (3%). Then, the growth of bacteria was observed with OD 560 for 0, 6, 12, 24, 48, 72 and 96 hours.

2.4. Media Anammox

Used anammox media was as follows (Uyanik et al., 2011):

246 mg/l $NaNO_2$, 189 mg/l $(NH_4)_2$ SO_4, 50 mg/l NO^{3-}, 1250 mg/l $KHCO_3$, 25 mg/l KH_2PO_4, 200 mg/l $MgSO_4.7H_2O$, 6.25 mg/l $FeSO_4$, 6.25 EDTA, 300 mg/l $CaCl_2.2H_2O$, 1.25 (ml/l). Trace element. Trace element solution contains 15000 mg/L EDTA, 430 mg/l $ZnSO_4.7H_2O$, 990 mg/l $MnCl_2.2H_2O$, 250 mg/l $CuSO_4.5H_{20}$,

220 mg/l $NaMoO_4$, 190 mg/l $NiCl_2.6H_{20}$, 14 mg/l $H3BO_4$, 210 mg/l $NaSeO_4$, 50 mg/l $NaWO_4.2H_2O$, and 240 Mg/L $CoCl_2.6H_2O$.

2.5. Batch Reactor

Three (acrylic) cylindrical batch reactors (Reactor PM, BKT and BKM) with working volume of 9 liter in parallel were used in this study (Figure 1). About 9 liters of water taken from the site location were added to each reactor. The reactors were inoculated with sediment obtained from the bottom part of the site location. Nitrogen sources for anammox bacteria, NH_4Cl and $NaNO_2$ were added up to 80 mg/l concentration of NH^{4+} - N and NO^{2-} -N. The reactors were incubated at room temperature of 25^{0}C. The internal pump was applied in the reactors to obtain homogeneous water. Ammonium, nitrite, nitrate, pH and DO were monitored for three days.

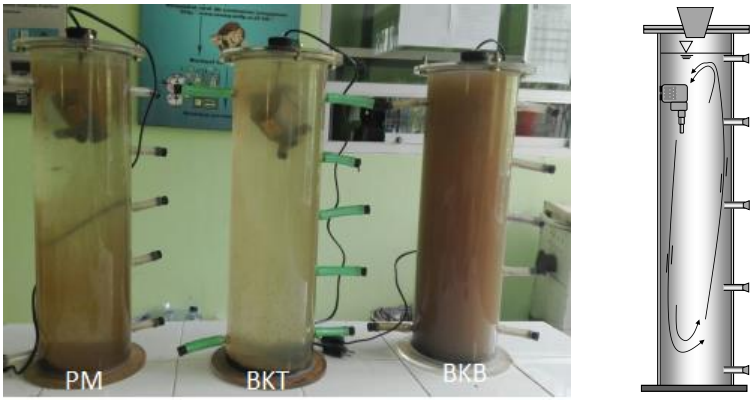


Figure 1. Batch reactors

2.6. Physicochemical Analyses

Immediately after taking the samples, Dissolved Oxygen (DO), pH, salinity, and temperature was determined in situ with calibrated Water Quality Checker (Horriba U 52 Japan). The samples for laboratory analyses were transported immediately to the laboratory. The samples were tested for, ammonium nitrogen (DEV E5 DIN 38406), nitrite (DEV D28 DIN 38405), nitrate (APHA 1995).

2.7. Chemicals

All chemicals used were of analytical grade without any further purification. To prepare all the reagents and calibration standards, double glass distilled water was used. All the experiments were carried out in duplicate.

3. Results and Discussion

3.1. Characterization of Water and Sediment Samples

Water and sediment samples originating from marine environments usually contain microorganisms that have the ability to be metabolically active under saline conditions. The characterization of the samples for parameters such as pH, temperature, DO, Ammonium, nitrite and nitrate is summarized in Table 1. It can be seen that the ammonium concentration in all three samples were less than 5 mg/l. Samples of BKT and BKB, that were fed with untreated domestic wastewater of surrounding settlement, contained lower ammonium concentration. Those low ammonium concentration, presumably caused by nitrification process using the available oxygen. Nitrification can still proceed at dissolved oxygen level of about 4 mg/l.

Tabel 1. Characterization of the used sediment/water samples

Sample	**Salinity**	**pH**	**T**	**DO**	**NH_4^+-N**	**NO_2^--N**	**NO_3^--N**
	(%)		**(°C)**	**(mg/l)**	**(mg/l)**	**(mg/l)**	**(mg/l)**
PM	2.67	7.65	32	3.8	4.08	0.78	4.08
BKT	3.03	7.68	32.6	4.8	1.87	0.68	3.81
BKB	2.79	7.72	33.1	5	1.65	0.83	4.35

3.2. Identification Anammox Bacteria from Several Inoculum

After 48 hours of incubation, colonies in petri dishes can be seen in **Figure 2**. Results of visual observation of the colonies in petri dishes are summarized in **Table 2**.

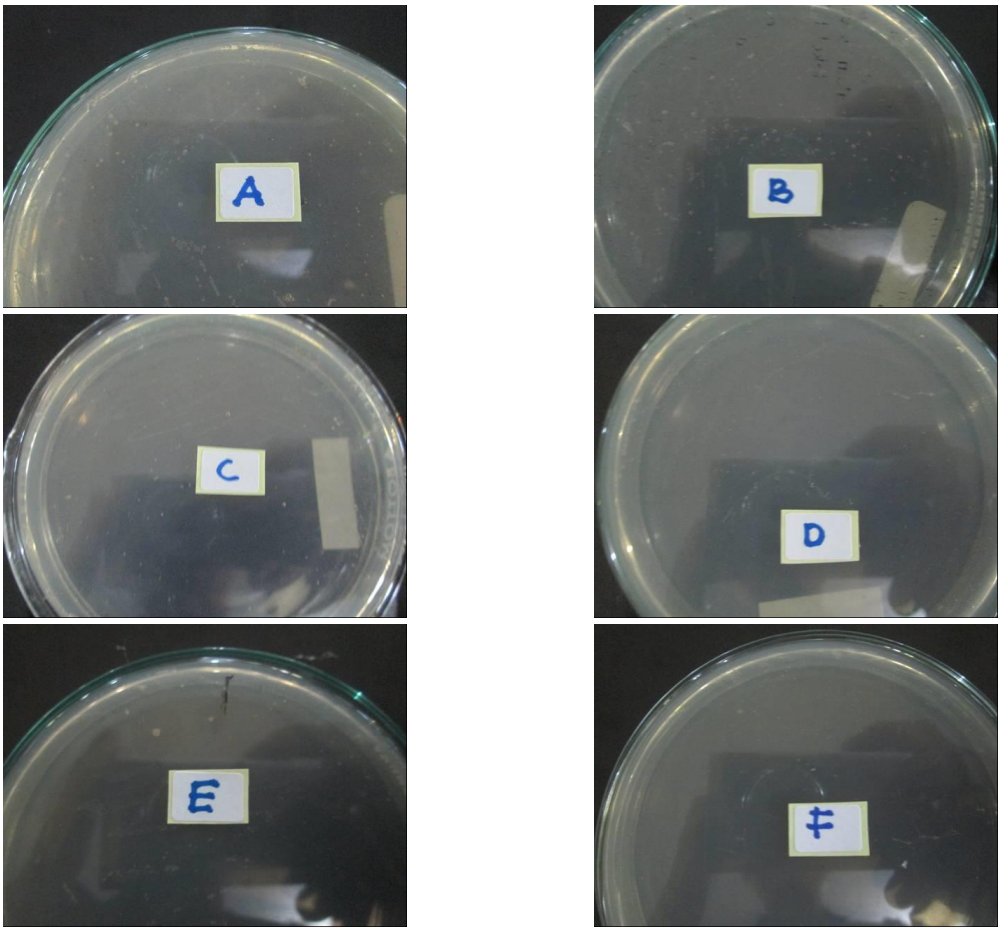


Figure 2. Colonies in petri dishes after 24 hours of incubation

Tabel 2. Characterization of colonies from different inoculate

	Inoculate	**Characterization**
1	A	- Small shiny colony - Diameter of colony is about 1 mm
2	B	- Amount of colony is more than that in sample A - Small shiny colony with about 1 mm diameter
3	C	- Similar to sample A
4.	D	- Yellowish colony, size smaller than 1 mm
5.	E	- diameter of colony is about 2 mm - There are two types: • E1 : colony surrounded by white halo • E2 : yellowish colony

	Inoculate	Characterization
6.	F	- Small, yellowish colony. - Diameter of colony is about 1 mm

To observe more details of colonies above, the colonies from those petri dishes were replanted to anammox slant tube, and the results are presented in the following **Figure 3**.

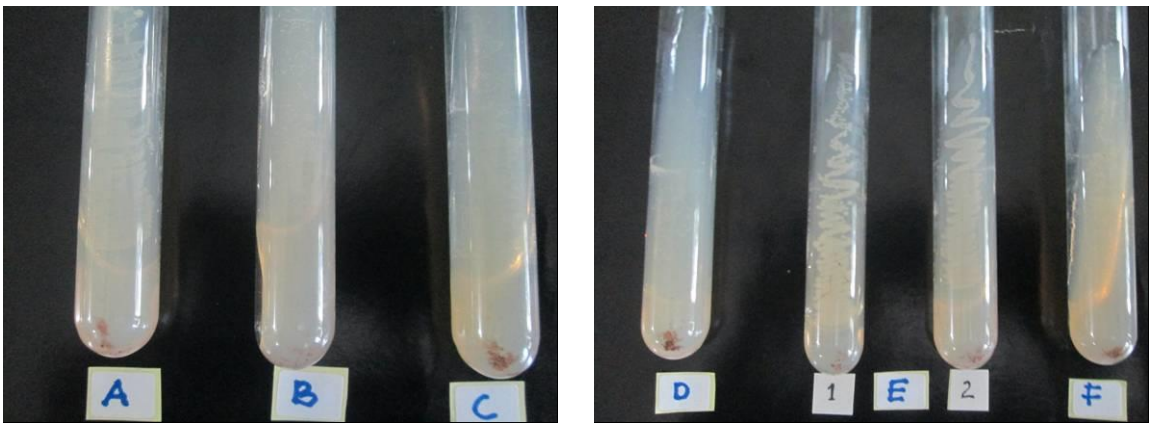
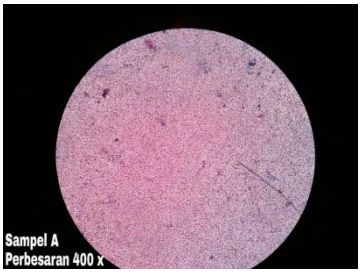
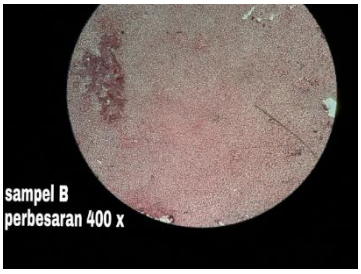


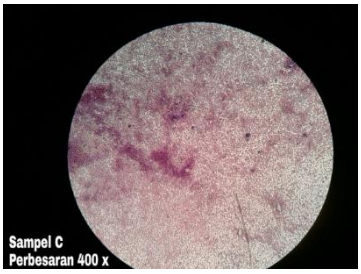
Figure 3. Photograph of slant tube agar media after 48 hours



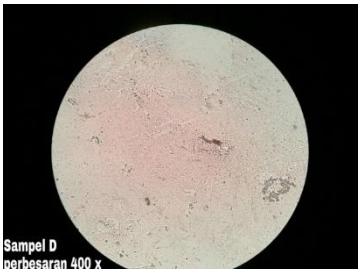
Purple = positive



Red = Negative



Red = Negative



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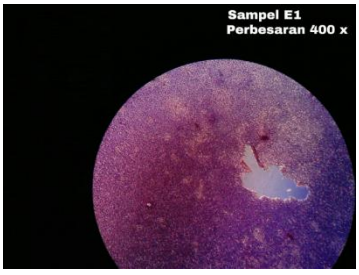

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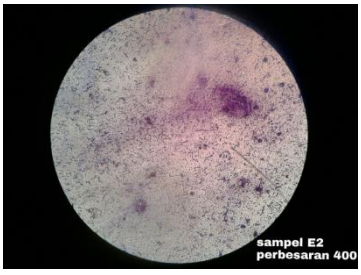
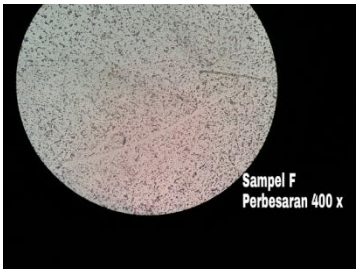


Purple = Positive



Purple = Positive

Figure 4. Photo of gram positive and negative staining for each isolates.

It can be seen that inoculate E, which was taken from sediment of Banjir Kanal Barat, shows the fastest growth among others. The characterization of isolated bacteria were followed by staining test that can be seen in **Figure 4**. Based on the observation of colonies and isolated bacteria on the agar media

petri dishes, the slant tubes and the staining results showed that E1 isolated baceria indicated a potential presence of Anammox bacteria. Therefore, the bacteria in the sample E was chosen for further study.

The effect of salinity on the growth of Anammox bacteria (taken from E slan tubes) was conducted in erlenmeyer tube and the optical density (OD) of medium was monitored for 4 days. OD profile of E inoculated bacteria which incubated at agar medium with different salinity can be seen at **Figure 5**.

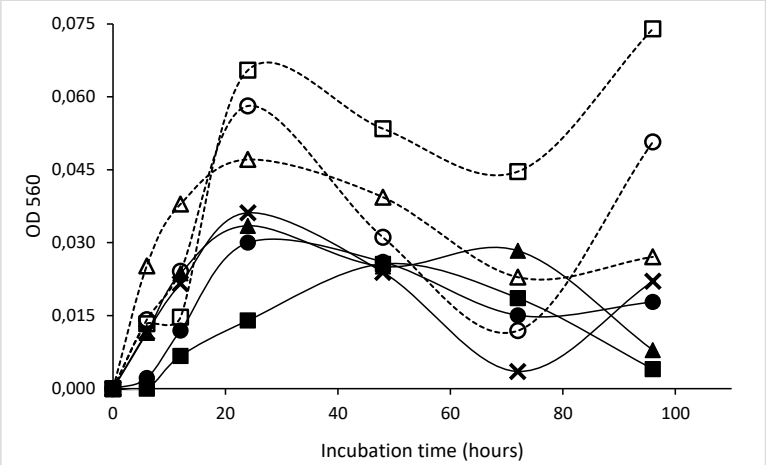


Figure 5. OD 560 profile at liquid agar medium with different salinity. Legends: closed squares, 0.00% salinity; closed circles, 1%; closed triangle, 2%; crosses 3%; open squares 5%; open circles 7%; open triangles 9%.

In phase I (hours 0 – 12), surprisingly, the most rapid growth of bacteria occurred in bacteria grown on media with 9% of salinity. Bacteria which were grown on salinity of 0.0 % and 1%, showed the slowest growth, they even needed adaptation time to start growing in the first 6 hours. In this phase, bacteria in 2 % to 7 % of salinity have relatively similar growth. In phase II (hours 12 – 24), the growth of bacteria in 5 % of salinity increase sharply and grown the fastest among others. The growth is almost double thegrowth in its previous salinity concentration (3%). Whereas, growth of inoculum in salinity of 0% was only 50% of that in its previous salinity concentration. It can be also found that all inoculum achieved the first maximum OD at hour 24, except for inoculum in 0% of salinity.

In phase III (hour 24 – 72), all OD decreased, indicating a shortage of substrate for inoculum. OD in erlenmeyer tubes with salt content higher than 3%, during phase IV (hour 72 – 96), began to increase a second time. Whereas, OD in erlenmeyer tubes with low salinity continue decreasing. It can be concluded that, inoculum, which was grown on a higher salinity, will grow faster than the growth in the original salinity. Meanwhile, the growth would decline if the inoculum was inoculated on the lower salinity. High salinity is a 'catalyst' for inoculum, while a low salinity is inhibitor for the inoculum. Rønning (2013) also observed an adaptation to higher salt concentration from freshwater anammox bacteria. Yi et al. (2011) stated that the freshwater anammox population could adapt to NaCl Concentration of 3%. Whereas, the result of Dapena-Mora et al. (2007) showed that the concentrations of NaCl up to about 9% do not affect the anammox activity. The anammox activity would decreased to about 50 % of the normal activity at concentrations higher than 13.5 %.

Furthermore, to determine the inhibitor type of salt on anammox bacteria, the bacteria, which was grown in different salt concentration medium, further was re-inoculated on anammox liquid medium with a salt concentration of 3% (the initial concentration). the result of OD 560 can be shown in **Figure 6**.

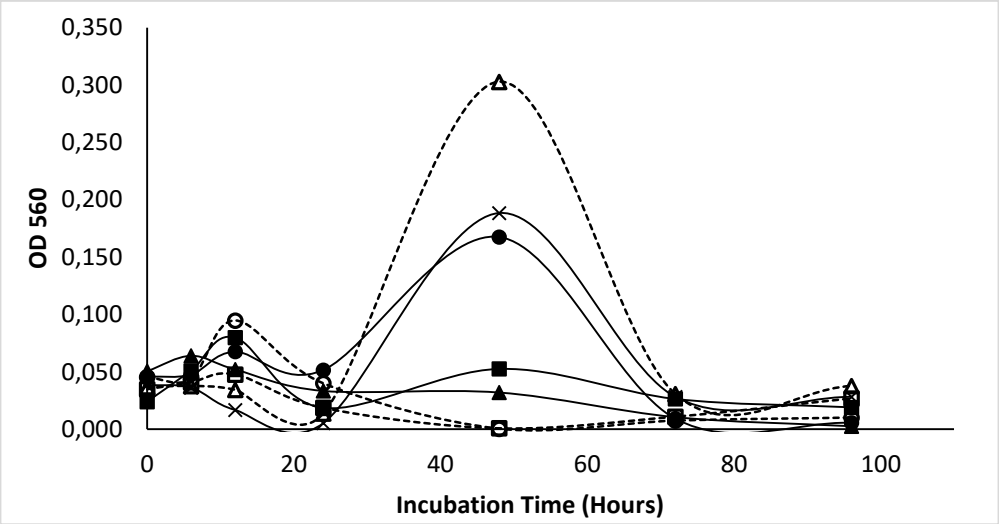


Figure 6. OD 560 profile of liquid agar medium after inoculated back at 3% salinity. Legends: closed squares, 0,00% salinity; closed circles, 1%; closed triangle, 2%; crosses 3%; open squares 5%; open circles, 7%; open triangles 9%.

Bacteria growth in salinity concentrations of 3% and 9% declined in the first 24 hours but immediately increased after that. Maximum OD was achieved in 48 hours, while bacteria with salinity concentration below 3% of have already peaked at 6 and 12 hours. It is rather difficult to draw conclusions from these results. However, based on an increase in OD on all the tube, it can be concluded that the salinity ranging 0 – 9 % is a reversible inhibitor for anammox bacteria. A reversible inhibitory property of salinity on anammox bacteria was also proved by Rønning, (2013). After anammox bacteria was incubated on salinity level of 4.5%, their activity still can be recovered.

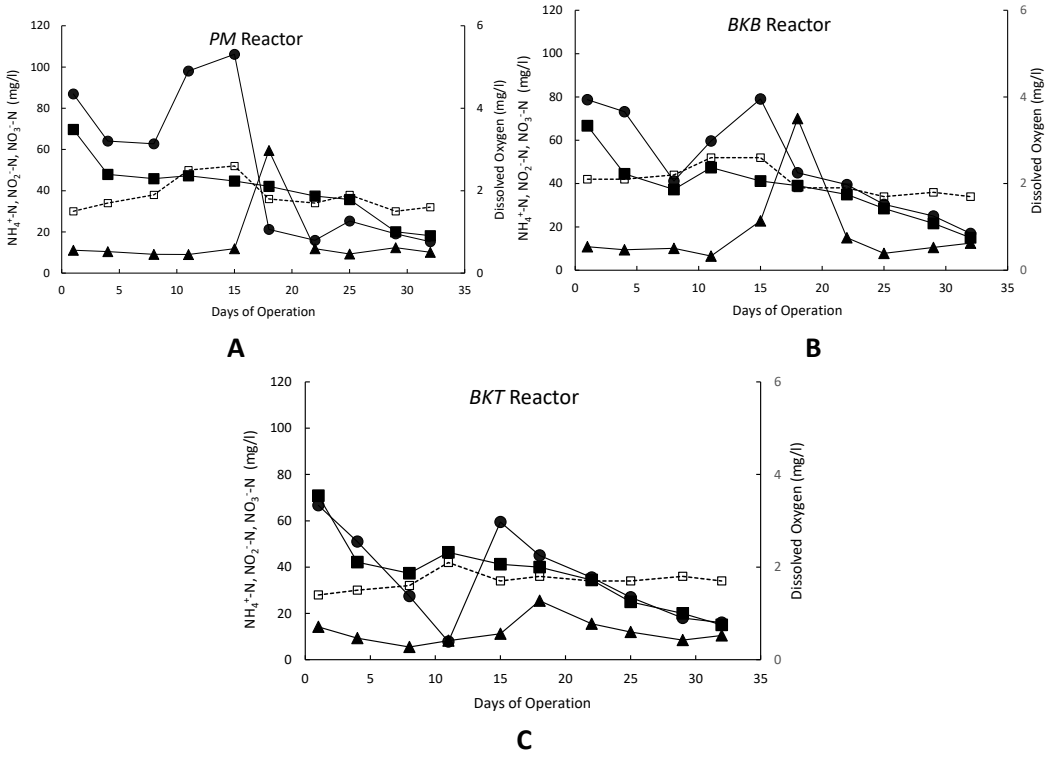


Figure 7. Progress of ammonium, nitrite, nitrate and dissolved oxygen in the reactors (A. Pantari Marina Reactor; B. Banjir Kanal Timur Reactor and C. Banjir Kanal Barat Reactor). Legends: closed squares, Ammonium; closed circles, Nitrite; closed triangle, Nitrate; open squares DO.

Banihani et al. (2012) summarized various bioreactors, that have been used for the enrichment of the Anammox bacteria. In this study, batch reactor was used to maximize the biomass concentration. The result of ammonia, nitrite and pH monitoring can be seen in **Figure 7**. At the beginning of incubation, ammonium and nitrite in the all reactors has decreased at relatively similar rate (days 1-8). In those period, nitrate concentration remain constant, close to zero. Anammox process is characterized by a decrease in ammonium and nitrite, but nitrate remains constant. Surprisingly, the anammox activity probably started at the beginning of incubation. The presence of anammox activity at the beginning of incubation time was also found at batch reactor (Chamchoi & Nitisoravut, 2007). This is also in line with the results of test in agar media, where OD which presenting their growth, also started to increase at the early hour.

Dissolved oxygen level was about 1.7 mg/l in all reactor. The anammox process is sensitive to oxygen (dissolved oxygen = DO). Concentrations of dissolved oxygen as low as 0.064 mg/l would inhibit the process and dissolved oxygen higher than 2.3 mg/l have been reported to be toxic to the process (Rønning, 2013). One of the latest presented specific values regarding oxygen tolerance of anammox in the literature is of the specie Kuenenia stuttgartiensis, with tolerant levels of dissolved oxygen ranging from 0-200 µM (0-6.4 mg/l) (Kartal et al., 2012). There is also a report on level of dissolved oxygen in medium fed to anammox bacteria which is 2-5 mgO2/L with no inhibitory effect (Li et al., 2012).

A possible explanation of the data above is that nitrificaton and denitrification process proceed simmultaneously. The DO level of about 1.7, the nitrification process, which results in a decrease in ammonium and nitrite, is still possible.

In this study, the oxygen removal from water was not conducted, so the water is still contained by oxygen. The DO depletion and an anerobic condition were expected to occur during ammonium oxidation. Internal mixing using aquarium pump only stirred the liquid part, while the solids remain settled at the bottom of reactor. The liquid presumably was aerobic, whereas the sediment was anaerobic. Nitrate could be reduced to nitrogen gas via denitrification process at the bottom of reactor, so that no nitrate accumulation was detected in reactors.

Other explanation why in DO level of about 1.7 mg/l, ammonium and nitrite decrease while nitrate reamains constant indicated that anammox process occured at unstrictly anaerobic conditions. Ni & Zhang (2013) stated that anammox bacteria, being strictly anaerobic and autotrophic, are difficult to enrich. Low concentrations of dissolved oxygen might also trigger partial nitrification to occur simultaneously to an anammox process in a microbial community where both of the organisms are present. During the second phase (days 8-18), in all reactors, DO concentration rose up to about 2.2 mg/l. Leakage was likely to occur so that oxygen can easily get into reactor. Along with the increasing of DO, nitrite concentration in PM reactor and BKT reactor incrased double, about 100 mg/l. The trend continued until day 15. This high concentration of nitrite can be toxic for biological process.

As with salinity, the literatures show a huge diversity for the values in which the levels of nitrite are inhibitory or toxic to the anammox process. Concentrations ranged from 5 to 40 mg NO_2^- - N mg/l have been reported as strongly inhibiting (Dapena Mora et al., 2007), and a level of 100 mg NO_2^- - N mg/l has been reported as complete, irreversible inhibiting (Jin et al., 2012). But Lotti et al. (2012) claimed that anammox bacteria are able to maintain their activity up to concentrations as high as 300 mg NO_2^- - N mg/l. Ammonium oxidation during nitritation process produces nitrite and reduce the concentration of ammonium. However, in this phase, there wasn't any downward trend in the concentration of ammonium. This can be explained that, under these conditions, actually, ammonium reduction occured, but at the same time ammonification, which result ammonium, also might proceed. In other words, at this second stage, both ammonification and nitrification processes occured. Hwang et al. (2005) and Waki et al. (2007) also found that ammonium was increased during anammox process. Slightly different from the other two reactors, in BKT reactor, increasing nitrite concentration also occurred when the DO level was more than 2 mg/l. Accumulation of nitrite concentration in the BKT reactor is the lowest among other reactors.

To prevent or reduce leakage of oxygen on the lid, on day 15, the reactor was covered more tightly. On day 18, the concentration of oxygen decrease to 1.7 mg / l, along with a decreasing concentration of nitrite and increasing nitrate concentration in all reactors. In the third period, (days 18-25), ammonium and nitrite, as well as nitrate decreased. Reduction of ammonium and nitrite at low DO conditions, can be explained as an anammox process. Whereas, the decline in nitrate was caused by denitrification process. So at this stage, the anammox process takes place simultaneously with the denitrification process. The simultaneous process of anammox and denitrification also were found by (Dong and Tollner, 2003). Even, most investigations described that denitrifiers contributed more on nitrogen removal than the anammox bacteria (Zhang et al., 2008).

During last stage (day 25 - 32), the DO concentration remained at around 1.7 mg/l. Concentration of ammonium and nitrite decreased, as nitrate remained constant which indicates that anammox process continued. The ratio of the average concentration of ammonium or nitrite at this last stage is 1.22, 0.99 and 0.89 for the reactor PM, BKT and BKB respectively. The average rate of ammonium removal at those ratios are 0.10, 0.59 and 0.08 mg-N/liter hour. Rønning, (2013) found that the average ammonium : nitrite ratio in a continuous anammox reactor was 0.75 with maximum recorded ammonium removal rate was 2.08 mg-N/liter hour.

4. Conclusions

Water and sediment taken from marine ecosystem around Semarang city showed positive anammox activity. Increasing salinity up to 9% will have positive influence on the activity of anammox bacteria. Whereas the decrease in salinity would negatively affect anammox bacteria. The ratio of ammonium: nitrite for anammox process ranges from 0.89 to 1.22 and ammonium removal rate varies from 0.08 to 0.59 mg-N/liter. Hour.

Acknowledgement

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