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Performance Optimization of Microbes from Shrimp Pond Sediment by Adding EM4 in Nitrification Process for the Treatment of Wastewater Containing High Ammonia Concentration

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In liquid wastes, especially domestic wastewater, many organic substances are disposed causing water quality degradation, one of them is ammonia. Liquid wastes containing ammonia can be treated using an activated sludge system. One of the active sludge that can be used is shrimp pond sediment. This experiment investigated the performance of microbes in shrimp pond sediments with the addition of EM4 in the nitrification process for the treatment of wastewater with high ammonia concentration in a 8 L batch reactor capacity. The results showed that the addition of shrimp pond sediment as the active sludge could remove high ammonia level almost completely and there was known interaction between time and variation of shrimp pond quantity (p value <0,05) to the decreasing of ammonia level. Efficiency of decreasing the concentration of ammonia up to 100% could be reached on the 15th day in each treatment. The addition of EM4 could shorten the period of the ammonia decreasing level by 50%.

Key words: activated sludge, ammonia, EM4, nitrification, shrimp pond sediment

Dalam limbah cair, terutama air limbah rumah tangga, banyak zat organik dibuang menyebabkan degradasi kualitas air, salah satunya adalah amonia. Limbah cair yang mengandung amonia dapat diolah dengan menggunakan sistem lumpur aktif. Salah satu lumpur aktif yang bisa digunakan adalah sedimen tambak udang. Percobaan ini meneliti kinerja mikroba pada sedimen tambak udang dengan penambahan EM4 dalam proses nitrifikasi untuk pengolahan limbah cair dengan konsentrasi amonia tinggi dalam kapasitas reaktor batch 8 L. Hasil penelitian menunjukkan bahwa penambahan sedimen tambak udang sebagai lumpur aktif dapat menghilangkan kadar amonia tinggi hampir seluruhnya dan diketahui adanya interaksi antara waktu dan variasi jumlah tambak udang (p value <0,05) terhadap penurunan kadar amonia. Efisiensi penurunan konsentrasi amonia hingga 100% dapat dicapai pada hari ke 15 pada setiap perlakuan. Penambahan EM4 dapat mempersingkat lama penurunan tingkat amonia hingga 50%.

Kata kunci: ammonia, EM 4, lumpur aktif, nitrifikasi, sedimen tambak udang

In liquid wastes, especially domestic wastewater, many organic substances are mixed and cause a decrease in water quality, one of them ammonia. In general, ammonia already exists in waters derived from urine and fish feces, but in a small concentration and can decompose naturally. According to the Regulation of the Minister of Environment Number 5 Year 2014, the standard of ammonia levels for businesses or activities that do not have a fixed standard is 5 mg L^{-1}. The presence of too high ammonia concentrations can be detected from a very strong odor (Plog *et al.* 1996).

With high concentrations of ammonia the water can be toxic to fish, humans and also can disrupt the ecosystem in the waters. Ammonia in the nitrification process will turn into nitrate and nitrite which can also harm health. Nitrate and nitrite with too high concentration can cause diarrhea, gastrointentinal disorders, until death if not given help (Soemirat 1994).

Wastewater containing ammonia can be processed using activated sludge system for the ammonia nitrification process. The active mud utilizes biological waste treatment in the presence of microorganisms decomposing organic substances within the sludge. The consortium of microorganisms in shrimp pond sediments is known to have the ability to break down ammonia such as *Nitrosomonas* sp. in the process of nitrification. Sediments of shrimp ponds are formed from the remains of shrimp feed and filth that settles on the bottom of the pond (Burford *et al.* 1998).

Microbes in shrimp pond sediments are expected to have the ability to decompose organic substances ie the ammonia content in water. However, it is necessary to add microbiological fertilizer in the form of Effective

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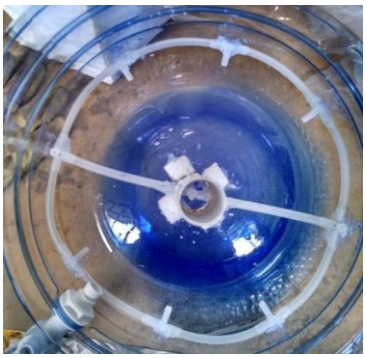

Fig 1 Interior design of a nitrification reactor.

Microorganisms 4 (EM4) as a media optimization step to the microorganism consortium of shrimp pond sediment. EM4 is a useful mixture of microbial cultures that function to reduce pollutant parameters and increase nutrient ammonia (Fitria *et al.* 2008). Thus in this study several experiments were conducted to determine the performance of microbes in shrimp pond sediments with the addition of EM4 for water treatment containing high concentration of ammonia so that the results expected from this research can be beneficial to the community.

MATERIALS AND METHODS

Time, Place, and Research Variables. This research started from February 2017 to September 2017, located in Microbiology Laboratory, Wastewater Treatment and Water Treatment Center (BTPAL), Agency for Assessment and Application of Technology (BPPT) Serpong, Tangerang Selatan, Banten. The variables in this study are as follows: Independent variables that are value variations in this study was the composition of sediment shrimp ponds; Dependent variable which is variable of research center point that were pH as well as the concentrations of COD, ammonia, nitrite and nitrate; Control variables which are controlled and constant variables were aerator, EM4 concentration, mixture volume in the reactor.

Sediment Take. The preliminary treatment in this research was sediment sampling in shrimp ponds of High School of Fisheries (Sekolah Tinggi Perikanan or STP), Serang, Banten. The selected shrimp farm was a pond that had been at least one month after seed stocking, so that the sediment had been collected and was expected to have many microbial decomposition of ammonia in it. Sediments were taken at the center of the pond, where the sediments conical in the middle. Sediment samples were taken and placed in an ammonia container. Further water containing ammonia was made by adding the parent ammonia to the aquades to an ammonia concentration of 100 mg L^{-1}.

Initial Treatment of EM4. Effective Microorganism 4 (EM4) needed to be activated before being mixed into domestic wastewater as a starter, by mixing EM4 and aquades. 1:20 comparison was required for EM4 and aquades, then the mixture was fermented at room temperature for 5-7 d. It is necessary to activate the microorganisms contained in EM4 from dormant conditions, so that the active microorganisms return and can work optimally when mixing with ammonia solution (Higa and Widiana 1994).

Reactor Design. The reactor made was a reactor that can support the working system of the batch reactor and was assisted by the aerator. The batch reactor was a simple reactor system with the working principle of inserting reactants (tambak sediments) into the reactor, then left for several days to be tested for quality within a given time range. The reactor was simply designed that of gallon measuring 12 liters, then a small hose was connected to the aerator and affixed at the bottom of the gallon. The small hose formed a circle and was given a hole every 8 cm. In the diameter of the circle was installed with a connecting hose and given a hole in the middle to drain the air from the aerator. A PVC pipe with a diameter of 1.75 cm and a height of 8 cm was given at the center of the gallon. At the bottom of the PVC pipe many small hollows were made up to 2 cm. The perforated hose and PVC pipe in the center were aimed to drain the air in all directions so that aeration is evenly distributed in each section (Figure 1).

Research Procedure. Ammonia solution with concentration of 100 mg L^{-1} was fed into gallons vertically along with diluted EM4 and shrimp pond sediments with the following variations based on the results from our preliminary study:

a. Control (K0): Ammonia solution (100 mg L^{-1}) 8000 mL,

b. Control 1 (K1): Ammonia solution (100 mg L^{-1}) 7600 mL + 400 mL Em4,
c. Control 2 (K2): Ammonia solution (100 mg L^{-1}) 7500 mL + 500 mL sediment of shrimp ponds,
d. Treatment 1 (P1): Ammonia solution (100 mg L^{-1}) 7500 mL + 400 mL EM4 + 100 mL sediment of shrimp ponds,
e. Treatment 2 (P2): Ammonia solution (100 mg L^{-1}) 7300 mL + 400 mL EM4 + 300 mL sediment of shrimp ponds,
f. Treatment 3 (P3): Ammonia solution (100 mg L^{-1}) 7100 mL + 400 mL EM4 + 500 mL sediment of shrimp ponds,
g. Treatment 4 (P4): Ammonia solution (100 mg L^{-1}) 6900 mL + 400 mL EM4 + 700 shrimp pond sediment.

All treatment variations were incubated at 25 °C for 18 days and treated with mechanical aerators during the study. The parameters tested were COD, ammonia (Nh_3), nitrite (NO_2), nitrate (NO_3) and pH (APHA Standard Method 2012). The parameters were tested on days 0, 3, 6, 9, 12, 15, and 18. All treatments were duplicated so that there were 8 experimental units and 6 controls.

Characterization and Identification of Microbes. After testing was completed, the sediment samples were taken to characterize the bacteria contained therein. Samples were taken from the reactor to be cultured into Nutrient Agar (NA) medium using spread plate technique then incubated at 30 °C for 24 h. After forming several colonies, each colony was subcultured into selective media ie Steiner's medium (*Nitrosomonas* medium) and autotrophic nitrobacter medium (*Nitrobacter* medium). The petri dish was then closed tightly and incubated for 24 h at 30 °C. Bacteria grown in selective media were then tested for catalase and Gram staining before being observed under a light microscope to be identified. The catalase test was done by taking the culture of the sample by ose aseptically and then the culture was scraped on the object glass. Microbial cultures spilled with 1-2 drops of hydrogen peroxide (H_2O_2) were then observed. The presence of bubbles formed indicates positive catalase bacteria, if there are no bubbles including the negative catalase bacteria. Gram staining was also performed to morphologically characterize bacteria with the aid of a light microscope and determine the Gram positive or Gram-negative species of the bacterial isolate.

Data Analysis. The data of ammonia observation were analyzed by using analysis of variance or ANOVA (Analysis of Variance) method of Completely Random Design with 4 repetitions followed by Benferroni test with 5% confidence level using Minitab 16. While the observation data of nitrite, nitrate and COD were processed with Microsoft Excel 2013 qualitatively. All data is then presented in the form of a line graph using Microsoft Excel 2013.

Microbes Acclimatization. Acclimatization process in this research was done in such a way with experiment design and addition of aerator at each reactor. The shrimp pond sediment that had been taken was put into the reactor for the acclimation process. The sediment composition was used in accordance with the experimental design of 500 mL for the control reactor 2 (K2), 150 mL for the treatment reactor 1 (P1), 300 mL for the treatment reactor 2 (P2), 500 mL for the treatment reactor 3 (P3), and 700 mL for treatment reactor 4 (P4). The 5% activated 5% effective microorganisms (EM4) were also incorporated into the 400 mL reactor for each of the experimental reactors, while the ammonia water at concentrations of 30 mg L^{-1} was added to the reactor until each the reactor had a total volume of 8 liters. Acclimatization aimed to select and adapt microorganisms in shrimp pond sediments so that they were more accustomed to higher concentrations of ammonia compounds. This process was performed for ten days and at the end of the process it was known that ammonia had decreased its concentration perfectly from 30 mg L^{-1} to 0 mg L^{-1}.

Treatment of Ammonia High Water in Batch Reactors. After the acclimatization of the shrimp pond sediment was done and showed that the ammonia content could be derived, then the operation of the test reactor was started with the addition of ammonia solution 100 mg L^{-1} up to the total volume of the reactor to 8 L.

RESULTS

Characteristics of shrimp ponds in STP Serang can be seen in Table 1. The condition of the sediment showed a more acidic figure with its pH of 6.7 when compared with the shrimp pond water with its pH of 7.8.

Decreased Ammonia Concentrations in Batch Reactors. Based on the result of absorbance determination of ammonia standard solution a linear regression was got y = 0.0049 + 0.9358x, with R2 = 0.9995. The results of the efficiency of decreasing the ammonia concentration in the batch reactor for eighteen days can be seen from Figure 2. Based on the graph in Figure 2 it can be explained qualitatively that the zero control (K0) containing only the ammonia solution of 101.92 mg L^{-1} and the control one (K1)

Parameter	Shrimp Pond	
	Result of Analysis	Quality Standards
Water		
pH	7.8	7.5-8.5*
Temperature	29.4 °C	28.5-31.5 °C*
		5 (mg L-1) **
		0.01 (mg L-1)*
		0.5 (mg L-1)*
Ammonia (NH_3)	0.1 (mg L-1)	5 (mg L-1) **
Nitrite (NO_2)	0.2 (mg L-1)	0.01 (mg L-1)*
Nitrate (NO_3)	1.57 (mg L-1)	0.5 (mg L-1)*
Sediment		
pH	6.7	

Source Description:
* = SNI 01-7246-2006
** = Ministry of Environment of the Republic of Indonesia Regulation No. 5/2014

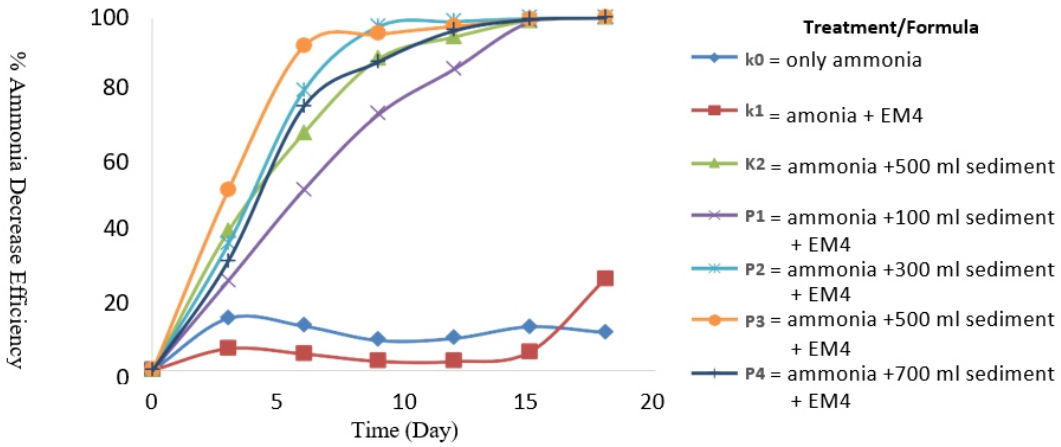

Fig 2 Curve of ammonia decrease efficiency.

The above efficiency values were calculated using the following equation:

$$Efficiency\ Value(\%) = \left(\frac{H_0 - H_n}{H_0}\right) \times 100\%$$

Description:
H_0 = Value on Day 1, H_n = Value on Day n
(Irmanto and Suyata 2009)

containing an ammonia solution of 87.13 mg L^{-1} with the addition of 400 mL EM4 5% showed an efficiency in ammonia concentration decrease of only 10.66% and 25.87%, respectively, hence the ammonia concentration on the 18th day became 91.05 mg L^{-1} and 64.59 mg L^{-1}, respectively. It shows that K0 and K1 reactors did not have good ammonia decreasing abilities. At reactor K2 containing an ammonia solution of 97.25 mg L^{-1} with addition of 500 mL of shrimp pond sediment could be seen to be able to decrease of ammonia concentration up to 99% starting at day 14 hence the concentration of ammonia on the 18th day had become 0.063 mg L^{-1}. While at reactor P1, P2, P3 and P4 showed that the ammonia concentration decreased almost 100% so that the ammonia concentration on the 18th day became 0.04 mg L^{-1}, 0.12 mg L^{-1}, 0.11 mg L^{-1}, and 0.14 mg L^{-1}, respectively. Thus, K2, P1, P2, P3 and P4 reactors have positive results in lowering ammonia concentrations.

Result data in P1, P2, P3 and P4 test reactor were analyzed by ANOVA variety to know the real difference in each treatment. The result of variance analysis showed that variation of volume and time had significant effect ($p<0,05$). There was a real interaction

between the two factors so that the analysis test was continued with Bonferroni test (Cleophas and Zwinderman 2011). The average of ammonia concentration on volume variation with time can be seen in Figure 3. The line graph in Figure 3 shows the depletion of ammonia concentration in reactor P1, P2, P3 and P4 along with the length of operation of the reactor.

On day 3 and day 6 the P3 reactor showed a marked difference in the decrease of ammonia concentration between the test reactors. This showed that the P3 reactor had the ability to lower the ammonia concentration faster than the other reactors. The ability of this P3 reactor with shrimp pond sediment volume to be added as much as 500 mL could be assumed as the best composition in the 8 L capacity test reactor, while for 3 day operation time on each test reactor showed a significant decrease among other days. Thus the 3rd day was suggested to be the most optimum time for the bacteria in the reactor started to decrease the concentration of ammonia.

Concentrations of Nitrite and Nitrate in the Batch Reactors. From the nitrite test using the standard method of BPPT IP No. 5 (2012), nitrite values were obtained as described in Figure 3. From Figure 3 it could ben seen that the nitrite concentration increased on day 4 and decreased thereafter until day 16. According to the nitrification process, nitrite is converted to nitrate. Therefore in this study nitrate concentration measurement was also conducted. The results of nitrate concentration measurement using standard method from lab BPPT IP No. 6 (2012) were shown in Figure 4. The graph in Figure 4 showed that during 16 days time the new nitrate concentration increased after the 4th day. The 16th day nitrate concentration in P1, P2, P3, and P4 was each 101.25 mg L^{-1}, 75.25 mg L^{-1}, 79.05 mg L^{-1}, and 82.3 mg L^{-1}, respectively. The concentrations of the four reactors were close to the ammonia concentration at the start of the operation. Thus, it can be assumed that ammonia had been converted to nitrate.

Characterization of Nitrifying Microbes in the Batch Reactor. Sampling for isolation was taken from the P3 reactor because the reactor showed a decrease in the ammonia concentration faster than the other reactors. Prior to the sampling of the P3 reactor, the reactor was recharged with 50 mg L^{-1} ammonia as the feeding for *Nitrosomonas* sp., *Nitrobacter* or *Nitrospira* sp. bacteria to be active again. After the 5th day of feeding, the sample was taken for inoculation with the general medium of nutrient agar media (NA) by the spread plate method on two petri dishes. From the isolation results in two NA media petri dishes three different colonies were visible visually. Subsequently each colony was transferred to in two petri dishes of bacterial nitrification selective media such as Stanier's medium for *Nitrosomonas* sp. bacteria and Nitrobacterial Autotrophic medium for *Nitrobacter* sp. (Atlas 2010).

There are three different colonies in NA media that grow in Stainer's medium (*Nitrosomonas* medium), but there was no colony at all in the Autotrophic Nitrobacter medium for *Nitrobacter* sp.. To ensure that the bacteria *Nitrobacter* sp. present or not in the medium, the sample was recovered from within the P3 reactor to be isolated directly to the selective medium of nitrobacter autotrophic medium with an incubation time of 48 h. Apparently the results showed that bacteria did not grow on the autotrophic selective nitrobacterial medium. Furthermore, to ensure bacteria that grow on Stainer's media selective media is indeed the bacterium *Nitrosomonas* sp., it was then morphologically observed under a microscope with Gram staining and activity test by catalase test on the three colonies formed. While the results of Gram staining seen in Figure 5 showed that the three colonies were Gram negative by showing the red color in the microscope. The results of the observations can be seen in Table 2 and Table 3. The three colonies showed positive results in the catalase test by generating air bubbles after the addition of hydrogen peroxide (H_2O_2). It shows that the three colonies could produce catalase enzymes that could break down H_2O_2 into oxygen and water. These three colonies were then labeled as *Nitrosomonas* sp1., *Nitrosomonas* sp2., and *Nitrosomonas* sp3.

DISCUSSION

Shrimp Pond Characterization. The shrimp farms used in the study were eighty days after seed stocking. During the period of eighty days the sediment formed was quite a lot and it was expected that the ammonia degradation microbes had been in sufficient quantities. According to Suwoyo et al (2016), as time increases after seed stocking there will be an increase in sediment formation which is the accumulation of shrimp feces, dead organisms (either shrimp or plankton), shrimp feed residues containing organic ingredients, high, and mud particles carried by the flow of water supply from the sea. Sediment collection from shrimp ponds at STP Serang was done at the center of

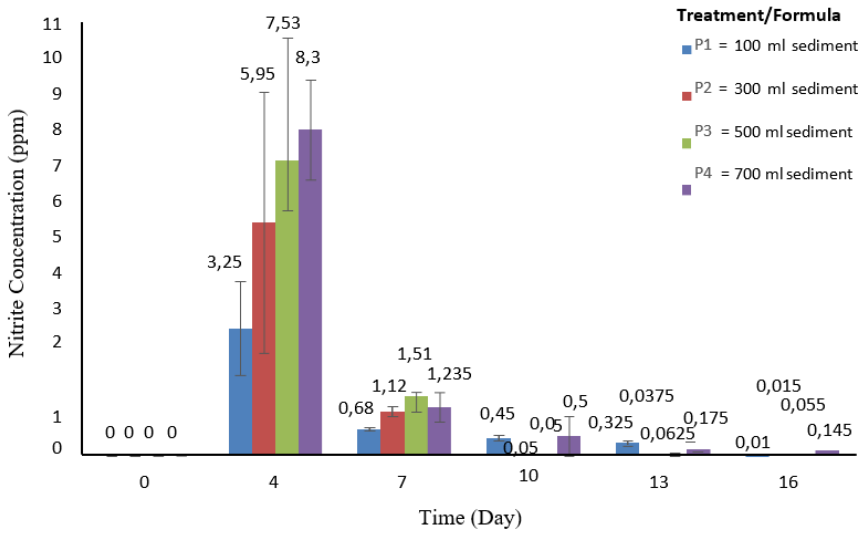

Fig 3 Curve of nitrite concentration.

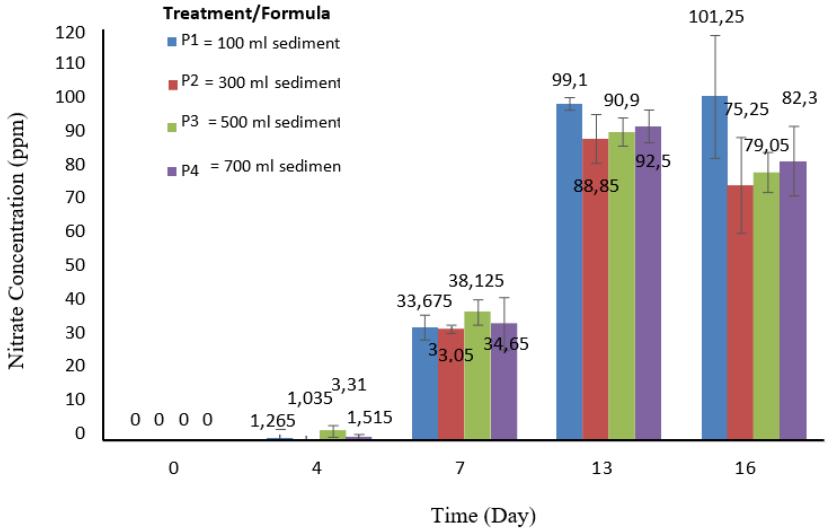

Fig 4 Curve of nitrate concentration.

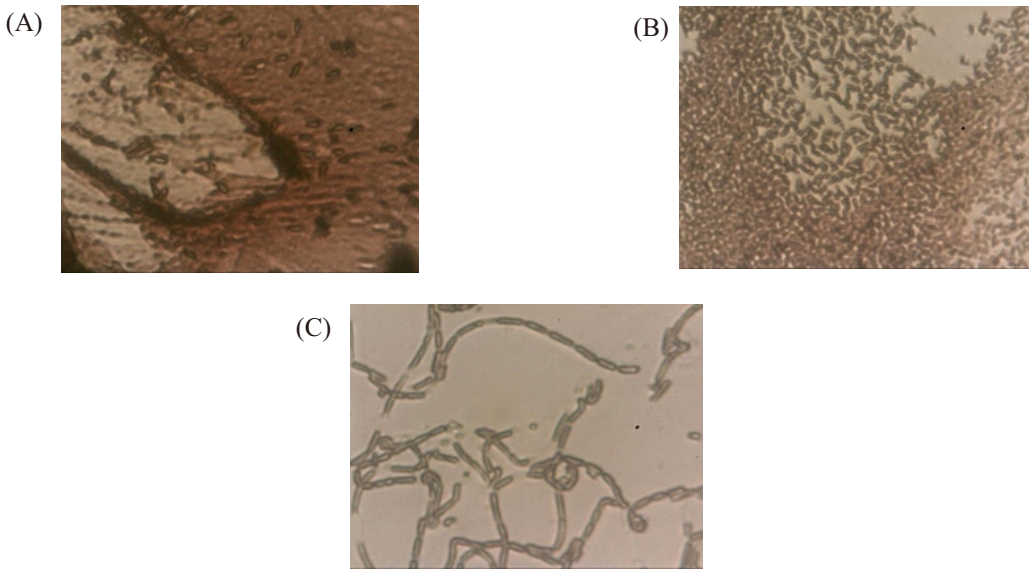

Fig 5 Identification Using Light Microscope with 100x Enlargement after Gram Staining (A) *Nitrosomonas* sp3., (B) *Nitrosomonas* sp2., and (C) *Nitrosomonas* sp1.

Table 2 Morphological characterization of the microbes in the batch reactor

Colony Observation	*Nitrosomonas* sp*1.*	*Nitrosomonas* sp2.	*Nitrosomonas* sp3.
Morphology			
Colour	White	Yellowish	Yellowish White
Shape	Round	Irregular	Irregular
Margin	Filamented	Undulate	Lobate
Elevation	Flat	Flat	Umbonate
Under Light Microscope			
Gram Staining	Negative	Negative	Negative
Cell form	Rod (Streptobacillus)	Rod (Bacillus)	Rod (Diplobacillus)

Table 3 Catalase test on the microbes in the batch reactor

Colony Observation	*Nitrosomonas* sp*1.*	*Nitrosomonas* sp2.	*Nitrosomonas* sp3.
Catalase Test	Positive	Positive	Positive

the pond due to the shrimp pond sunken form towards the middle and also to avoid the different sediment composition when the taking was done at different points.

High concentrations of nitrate in the shrimp ponds of STP Serang can stimulate the growth of natural food for shrimp such as clekap, plankton and moss (Sawyer *et al.* 2008). Sediment pH values were known to be more acidic than the pH value of shrimp pond water the longer the age of the pond, then the sediment deposition was more increased due to the decomposition process from the remaining shrimp feed (Suwoyo *et al.* 2016).

Decreased Ammonia Concentrations on Batch Reactors. The systems in the K0 and K1 reactors were not able to reduce the ammonia concentration well because the reactors were intended as the negative controls. The K0 reactor containing only ammonia solution with the aid of the aerator had no ability in reducing ammonia concentration since no bacteria culture was added. Whereas the K1 reactor added with EM4 as a biofertilizer also had not been seen to have the ability to reduce ammonia concentration well. This could be possible because the nitrogen element in the form of ammonia in the test reactor was too high so that in the absence of cultures of ammonia oxidizing bacteria had caused the bacteria in EM4 could not work to break down the substrate in the reactor well by themselves. Bacteria in EM4 will work well as biofertilizer if the nutrients in the substrate are well met which can break down the substrate optimally (Javaid and Bajwa 2010).

Each treatment reactor similarly contained shrimp pond sediment so that it could be seen that there were ammonia decomposing bacteria in the sediment. According to Burford *et al.* (1998), in shrimp pond sediments there are bacteria capable of processing the nutrients accumulated in the sediment. Shrimp pond sediments contain bacteria that can recycle nitrogen especially using the nitrification process to convert ammonia (NH_3) to nitrite (NO_2^-) by the aid of *Nitrosomonas* sp bacteria. and *Nitrobacter* sp. or *Nitrospira* sp. in converting nitrites to nitrates (NO_3^-) under aerobic conditions, while the conversion of nitrate to nitrogen was done by denitrifying bacteria in anaerobic conditions in the sediment.

The addition of EM4 to reactor P1, P2, P3 and P4 showed the time difference of ammonia concentration decreasing. Similarly, if compared between reactor treatments with K2 reactor which was not added EM4, the rate difference of ammonia concentration decrease would be seen and indicated on the gradient difference from each line in graph of Figure 2. It showed that addition of EM4 also had an effect to improve microbial performance in shrimp pond sediments to decompose ammonia in the reactor, which could accelerate the decrease of ammonia concentration. This was clearly evident from the comparison of the graph line of K2 (control 2 which is only filled with 500 g of shrimp pond sediments without additional EM4) with the graph line of P3 (treatment 3 filled with 500 g of shrimp pond sediment with an additional 400 mL of EM4 solution). At the P3 treatment the efficiency of ammonia concentration decreased by more than 90%

on day 6, while on the same day in the new K2 the efficiency of ammonia concentration decreased by about 65% and the efficiency of the decrease was almost 99% on day 14 (Figure 2). Our results were in accordance with the results indicated in previous studies using EM4 for the treatment of wastewater from various sources (Jasmiati *et al.* 2010; Munawaroh *et al.* 2013; Pitriani 2015).

Concentrations of Nitrite and Nitrate in Batch Reactors. Based on the nitrogen cycle of the nitrification process, the decreased ammonia concentration is converted to nitrites by the bacterium *Nitrosomonas* sp. and then nitrite is converted to nitrate by the bacterium *Nitrobacter* sp. or *Nitrospira* sp. (Atlas and Bartha 1993; Widiyanto 2005). The increase of nitrite concentration on the 4th day was followed by its decrease thereafter until the end of the study was possible because the bacteria *Nitrosomonas* sp. in the reactor began to break down the ammonia into nitrites so that nitrites increased on the 4th day, while the decline that occurs was possible because after that day the nitrites had been converted to nitrate by bacteria *Nitrobacter* sp. or *Nitrospira* sp. in the shrimp pond sediments. According to Prosser (2005), the decomposition of ammonia into nitrites under aerobic conditions performed by *Nitrosomonas* sp. experienced the following reaction:

$$NH_2 + O_2 + 2H^+ + 2e^- \text{------>} NH_2OH + H_2O$$
$$NH_2OH + H_2O \text{------>} NO_2^- + 5H^+ + 4e^-$$

Ammonia is oxidized to hydroxylamine by oxygen in endergonic reactions, subsequently hydroxulamine is oxidized to nitrite by the aid of oxygen in water by exergonic reactions.

New nitrate concentrations increased after the 4th day showing that the nitrate was formed only after nitrite was formed. According to Prosser (2005), nitrites oxidized to nitrates in aerobic conditions by *Nitrobacter* sp. or *Nitrospira* sp. by catalyzing nitrite with oxidoreductase as the following reaction:

$$NO_2^- + H_2O \text{------>} NO_3^- + 2H^+ + 2e^-$$

The nitrate concentration that continued to increase until day 16 showed that in the reactor there were no denitrifying bacteria that convert nitrates to nitrogen in the form of gas. Denitrification is a process that requires anaerobic conditions. While the reactor was aerated with the aerator hence the nitrate in the reactor was high because there was no anaerobic condition to perform the process of denitirification. The nitrate concentration on the last day of the study within each of the four reactors was close to the concentration of ammonia at the beginning of the operation. It could then be assumed that ammonia had almost all converted to nitrate.

Characterization of Nitrifying Microbes in the Batch Reactor. It was known that the nitrate test showed the presence of nitrate compound in the reactor meaning that there was the nitrification process in converting nitrite to nitrate which should be done by *Nitrobacter* sp. bacteria. However after isolation, *Nitrobacter* sp. did not grow in the selective media of autotrophic nitrobacterial media. This was possible because the bacteria *Nitrobacter* sp. which was in the reactor could not be cultured (unculturable). According to Koops and Roser (2001), some nitrifying bacteria can not be cultured on the media due to their prolonged development or the bacteria lose nutrients to the other bacteria in the media. Molecular techniques are hence required to ensure the presence of these unculturable bacteria.

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