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Anammox Microbial Biodiversity in the Karangkates Reservoir Malang and Its Activity as an Ammonia-Rich Waste Degradation

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Nitrogen is an important element that plays a role in the cycles in the world. The nitrogen cycle is the most important cycle for living things after the carbon cycle. One of the newest processes in the nitrogen cycle is the anaerobic ammonia oxidation (anammox) process. Anammox is a process when nitrite is used as an electron acceptor in the conversion of ammonium to nitrogen gas with the help of anammox microbes. This study aims to isolate anammox bacteria from sediments from Karangkates reservoir using the Bottle Batch Reactor method, as well as testing the activity of anammox bacteria in reducing excess ammonia in synthetic wastewater containing ammonia using colorimetry method. This study also identifying the biodiversities of anammox microbes insediment sample using Next Generation Sequencing (NGS) testing. The stages in this research are: (1) Preparation of sediment samples and water samples from Karangkates reservoir, (2) water sample parameters analysis, (3) identification of microbial biodiversity involved in nitrogen cycle using Next Generation Sequencing (NGS), (4) cultivation of samples in Bottle Batch Reactor and (5) analyse the performance microbes in removing ammonia. The NGS analysis obtained Anammox microbes from the Kuenen species, namely *Candidatus kuenenia stuttgartiensis* with an abundance of 0.07%. Apart from the plantomycetes phylum, it also found several other microbes which usually involved in the nitrogen cycle, such as *Proteobacteria* 40%, *Bacteroidetes* 4%, *Planctomycetes* 6%, *Chloroflexi* 1%, *Nitrosomonas* 0.03%, *Nitrosospira* 0.1%, *Nitrospiraceae* 0.5% and *Pseudomonas* 0.2%. The average of ammonia removal in this study was 18 % mL/Day.

Keywords: Anammox; *Candidatus kuenenia stutgartiensis*; nitrogen

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Environmental pollution is an unavoidable problem in developing and developed countries. One form of environmental pollution that often occurs in developing countries is water pollution [1]. Water pollution materials in the environment that are difficult to remove, and many are found, one of which is ammonia. As a nitrogen-rich substance its presence alongside other substances containing phosphorus can cause eutrophication [2]. In addition to having adverse impacts on health, eutrophication also has an impact on decreasing water quality. Water pollution can be triggered by several things, one of which is the influence of urbanization and industrialization [3-4]. The fact that the aquaculture industry can cause pollution in the waters to increase, this is due to waste disposal that is not handled properly.

High levels of ammonia in a body of water can have effects that can be detrimental to organisms. The process of removing high levels of nitrogen in waste can be carried out both traditionally and modernly. The process of removing ammonium levels in wastewater is traditionally carried out using nitrification and denitrification methods [5-6], but ammonia-rich waste treatment processes traditionally require considerable energy and costs [7]. The removal of nitrogen levels in waste in modern times can be carried out by physical or biological processes [8]. Physically process the removal of ammonium levels in wastewater is carried out by adsorption, but the process of removing ammonium levels in waste is biologically more effective and cost-effective [9]. Since the discovery of the anaerobic autotrophic bacteria ammonia oxidation (anammox) by Mulder et al. (1995), ammonia waste treatment methods have begun to shift from heterotrophic denitrification to anammox processes [7].

The anaerobic ammonium oxidation process (anammox) was widely developed because of its cost-effective, energy-saving process and its results are effective in reducing ammonium levels in wastewater

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that has a high ammonia concentration [9]. Research on the anammox method in Indonesia is still very limited, unlike in developed countries, one of which is Japan,which has done a lot of research. The results of a review of anammox bacteria obtained that the distribution of anammox bacteria can be found in natural environments and artificial environments such as reservoirs, rivers, wetlands, rice fields, estuaries, everglades and groundwater [11]. Therefore, it will be very potential to be found in Indonesia because most of Indonesia's territory is water area. Karangkates reservoir is used as a place where anammox bacteria can be found in Indonesia, because the upper reaches of the Karangkates reservoir are usually used in agriculture, fisheries and facilities for drainage. As the main discharge of rivers that emptied into reservoirs, Karangkates reservoirs receive water with various discharges and qualities ranging from rainwater to wastewater, where the waste contains household waste as well as from industrial waste [12].

A bacterium can be bred using the isolation method. According to Singleton & Sainsbury's (2006) bacterial isolation is an act of taking bacteria from their place of origin and then growing them in an artificial medium that aims to be bred to obtain a pure culture. Previous research conducted by Wijanarka (2018), isolation was carried out using anammox media to obtain theresults of the growth curve of a gram-negative bacteria which is considered to be annamox bacteria, but from the study experienced difficulties in isolation because the method used was still not produced pure isolates or only limited to testing its activity in high salt levels, so it needs to use a new method i.e. the Batch Reactor method [14].

The Batch Reactor method is expected to later attach anammox microbes in the career of anaerobic reactors which will make it easier to analyze the types of anammox microbes attached to careers. Therefore, it is necessary to isolate and characterize to produce anammox bacteria, and can produce pure cultures of anammox bacteria from Indonesian waters. Based on the background that has been presented, this study aims to advance research and apply the anammox process, especially its activities can be used as a degradation agent for ammonia-rich waste in Indonesia.

EXPERIMENTAL

Chemicals and Materials

This research was carried out using micro pipette size 100 – 1000 µl, analytical balance, UV-Vis spectrophotometer, aluminum foil, shaker waterbath, thermometer, beaker glass (250mL and 50 mL), pH meter and stirrer.

This research used water samples and sediment samples from karangkates reservoir, $CaCl_2$ *p.a*, $MgCl_2$ *p.a*, NH_4HCO_3 *p.a*, KH_2PO_4 *p.a*, Na 2-EDTA.$2H_2O$ *p.a*, $ZnSO_4.7H_2O$ *p.a*, $CoCl_{2.6}H_2O$ *p.a*, $MnCl_2.4H_2O$ (*p.a*), $CuSO_4.5H_2O$ *p.a*, $Na_2MoO_4.2H_2O$ *p.a*, NiCl2. $6H_2O$ *p.a*, $NaSeO_3$ *p.a*, H_3BO_4 *p.a*, $FeCl_{2.4H\ 2}O$ *p.a* [15] ethanol 70%, NaCl 30%, Brusine Sulfate, H_2SO_4 concentrated, Sulfanilamide *p.a*, Naphtyl Ethylen-diamine dihydratedrochloride, NaOCl 70% aquades and nitrogen gas.

Procedures

Preliminary Test

Sample testing is carried out at the sampling location using nitrite test kit, nitrate test kit, ammonia test kit, and pH meter.

Next Generation Sequencing Test

Biodiversity testing using Next Generation Sequencing (NGS) begins with DNA extraction. Extraction is carried out by taking sediment samples (Metro River), then samples are isolated using the Nucleon Spin Soil Kit from Macherey-Nagel. Furthermore, the process of determining DNA concentration is carried out using the Nano Drop Spectrophotometer and Qubit Fluorometer. After it was known the DNA concentration, then carried out library preparation using Kits from Oxford Nanopore Technology. After that, DNA sequencing is carried out using GridiION which is operated by MinKNOW software version 20.06.9. Basic sorting is done using Guppy application version 4.0.11 with high accuracy [16] and FASTQ file quality is visualized using NanoPlot [17]. The last stage is that the reading of the sequencing results is filtered and classified using the centrifuge classifier [18] which previously already had a bacterial index and archaea downloaded from the centrifuge website and the final reading results were visualized using Pavian and Krona diagrams.

Bacterial Culture Using Bottle Batch Reactor

This bacterial culture method refers to the study [14] which is by means of 10 grams of sediment samples being included in an anaerobic bottle reactor. Coupled with a solution of synthetic media ± $^3/_4$ of the volume of the bottle. Then put the K1 caldnes career media with a diameter of 10 mm based on HDPE (High-density Polyethilene) into the bottle. Then the bottleis closed using a rubber cap. Then injected with nitrogen gas for ± 2 minutes. Next, the bottle that already contains the sample and the media is put in a shaker waterbath.

Testing the Activity of Anammox Bacterial in Reactor Bottles

Testing of bacterial activity is carried out by taking media in a vial reactor. Then the inoculation water is centrifuged for 20 minutes at a speed of 1, 500 rpm.

After that, ammonia, nitrite and nitrate levels and nitrogen gases in the sample were tested. Testing is carried out a week 2 times for 2 months. Bacterial activity is seen from the reduction of ammonia and nitritelevels and the addition of nitrate and nitrogen gas levels in the sample.

RESULTS AND DISCUSSION

Sampling Location

Sampling was carried out at the Karangkates reservoir located in Karangkates Village, Sumber Pucung, Malang Regency, East Java. Karangkates Reservoir has an inundation area of 790 ha, 31 m deep, a surface height of 297 m and a water storage capacity of 343,000,000 m^3 [19]. The selection of sampling there was based on the abundance of floating cage cultivation, fish markets, agriculture, and tourist attractions [20]. This is proven directly in the field by researchers when sampling. So Karangkates reservoir have the potential to be a source of anammox microbial communities. Sampling was carried out in the benthic zone where the zoneis the bottom zone of the reservoir which is usually inhabited by microbes degrading the rest of living things [20]. The sampling location in karangkates reservoir precisely on the metro water translucent and the Berantas river water can be seen in Figure 2.

Sampling was carried out at 2 location points in the upper reaches of the Karangkates Reservoir, namely on the Berantas River and the Metro River. At the predetermined point, sampling is carried out in the form of sediment and water above the sediment. Sediment samples are the targets of microbial isolation of anammox indigent, while water samples are analyzed to determine the potential presence of anammox microbes in the sediment. The results of the water quality analysis showed that two samples from different points showed similar results (Table 1), namely the pH value (7), ammonia (5-10 mg / L), nitrites (0 mg / L), nitrates (10-25 mg / L). When viewed from the existing results, it shows the potential for anammox microbes.

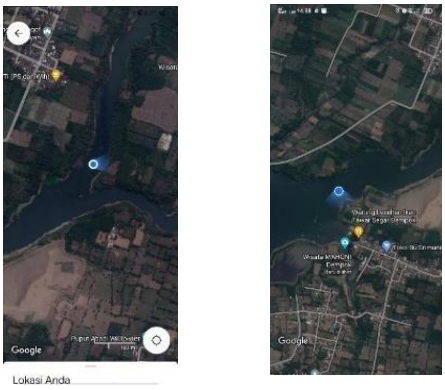

Figure 1. Sampling Location at Karangkates Reservoir: (a) Berantas River and (b) MetroRiver.

Table 1. Testing of Karangkates Reservoir Water Sample Parameters.

Location	pH	Sediment Base Water Quality		
		Ammonia (mg/L)	Nitrites (mg/L)	Nitrate (mg/L)
Translucent Berantas River	7	5	0	10
Metro River Breach	7	10	0	25

Microbial Biodiversity at Karangkates Reservoir

The results of microbial diversity at the Karangkates Reservoir using NGS obtained a varietyof microbes that read, namely 2549 with different abundances. NGS results showed that Karangkates Reservoir was dominated by Proteobacter phylum by 41% and in other Phylums such as *Firmicutes* & *Acidobakter* 20%, *Planctomycetes* 6%, *Actinobacter* & *Bacteroidetes* 4%, *Chloroflexi* 1% and 33 other Phylum < 1%.

Anammox microbes that have been found in artificial ecosystems are species of the genus *Candidatus* including *Cadindatus jettenia, Cadindatus brocadia, Cadindatus kuinenia stuttgartensis, Cadindatus scalindua, and Cadindatus anammoxoglobus*. In addition to being found in artificial ecosystems, anammox microbes can also be found in soil and agricultural samples. Anammox microbes in soil samples are usually dominated by microbes of the genus Brocadia and Kuenenia [21]. The presence of microbes in the Karangkates Reservoir (metro river) sample was seen using next generation sequencing (*NGS*). Metro river samples were chosen because in the initial parameter tests had high ammonia and nitrate levels compared to eradicated river samples. The NGS results show that there is an *anammox* genus, *Candidatus kuenenina stuttagartiensis* with an abundance of 0.07% of all microbes in the sediment. *Candidatus kuenenina stuttagartiensis* is a microbe of the phylum planto-mycetes and belongs to the microbial species anammox [22].

The results of next generation sequencing (*NGS*) metro canal sediment samples obtained several microbes involved in the nitrogen cycle such as microbes Ammonia Oxidizing Bacteria (AOB), Nitrite Oxidizing Bacteria (NOB), Anammox and Denitrification Bacteria (DB). These results are grouped in Table. 3.

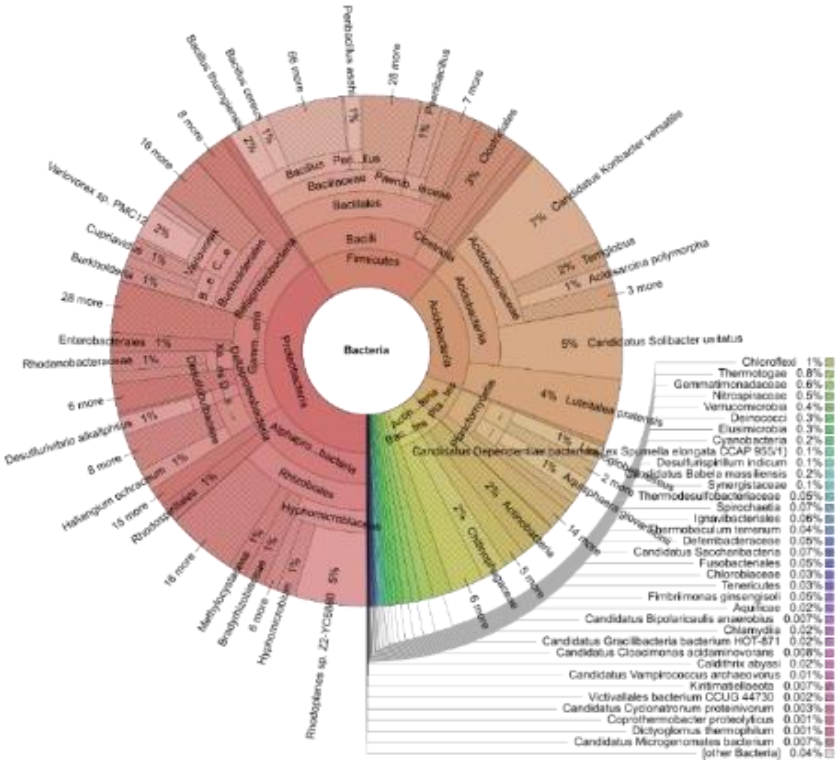

Figure 2. NGS (Next Generation Sequencing) Results of Sediment Samples of Weiran Karangkates.

Table 2. Microbial Communities table in the Nitrogen Cycle.

No.	Types of Microbes	Microbe
1.	Anammox	- *Genus Kuenina* - *Proteobacteria* - *Bacteroidetes* - *Planctomycetes* - *Chloroflexi*
2.	*Ammonia Oxidizing Bacteria* (AOB)	- *Nitrosomonas* - *Nitrobacter* - *Nitrosospira*
3.	*Nitrite Oxidizing Bacteria* (NOB)	- *Nitrospiraceae*
4.	*Denitrification Bacteria* (DB)	- *Rhodanobacter* - *Proteobacteria* - *Betaproteobacteria* - *Rhodocyclaceae*

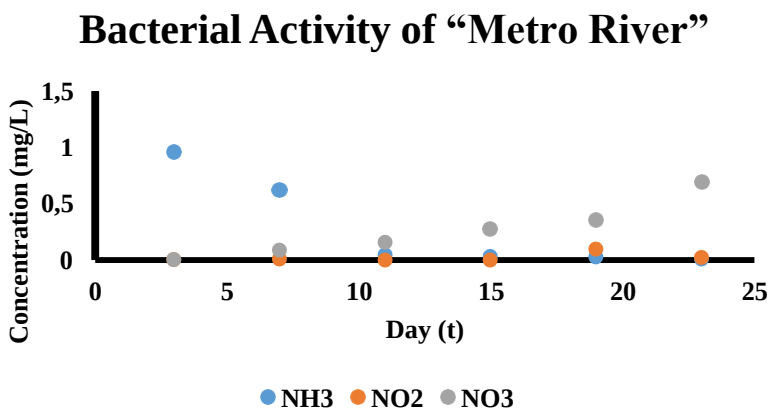

Figure 3. Graph of Microbial Activity.

Bacterial Activity

Testing the activity of anammox bacteria that have been isolated in the Bottle Batch Reactor with ammonia-rich growth media. Activity testing is carried out by taking a portion of bacterial culture water that has been several days in a shaker bottle. Testing is expected to find out howthe development of insulating microbes in reducing ammonia and nitrite levels in the media used by microbes to produce nitrogen and nitrate gases. The test was carried out using a UV- Vis Spectrophotometer that corresponded to each test. The results of the activity test are shownin Figure 3 and Figure 4.

The curve has shows that a high level of ammonium on day 3. this is caused by the lysis process of aerobic and heterotrophic microbes that die because of changes in environmental conditions. On day 7 there is a decrease in ammonium levels and an increase in nitrite levels and an increase in nitrate levels are possible due to the activity of AOB microbes (*Nitrosomonas*), and the activity of denitrifying microbes (*Pseudomonas*) that dominate in culture sediments.

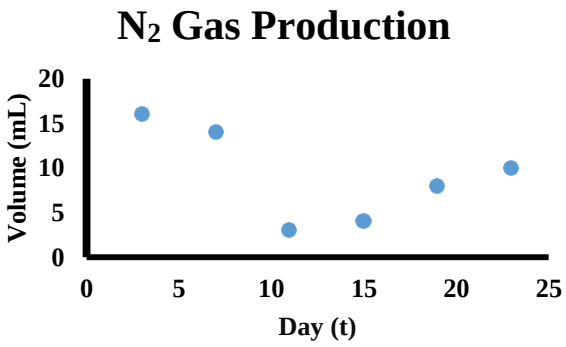

Figure. 4 Graph of Gas Production N_2.

On days 11-15 there is a decrease in ammonium levels, an increase in nitritelevels and an increase in nitrate levels. On the day of the sting, it is possible to have the activityof the anammox *microbe Candidatus kuenenia stutgartiensis* which begins to live because thistype of anammox *candidatus* microbe begins to undergo cell division on days 8-11 in the culture sediment. On the 19th day there was an increase in nitrite levels, the increase was due to the activity of denitrifying microbes (*Pseudomonas*) which converted nitrates to nitrites under anaerobic conditions. However, on day 19, high nitrate levels were still possible due to the activity of anammox microbes that utilize nitrite from denitrifying microbes. On day 23, ammonium and nitrite levels decreased while nitrate levels increased. On day 23 this is possible in culture sediments dominated by anammox microbes. The average anammox bacteria produced from the Karangkates Reservoir manage to reduce ammonia by 18% mL/Day.

To produce nitrogen gas in the sample, the highest day 3 was obtained, which was 16. Meanwhile, on days 7-11, nitrogen gas production gradually decreases due to microbial activity in the bottle experiencing a decreased phase. Meanwhile, on days 15-23 gas production rose again due to the activity of anammox microbes that had grown. However, this gas measurement cannot be ascertained to be only in the form of nitrogen gas because of the limited tools to check the gas.

CONCLUSION

Technological developments in the field of waste treatment are rapidly developing, one of which is the use of anammox bacteria in the treatment of ammonia-rich waste. In Indonesia, there is very little research on anammox bacteria, especially on the discovery of anammox bacteria native to Indonesia. In this study, it has succeeded in finding the microbe Anammox from the phylum Planctomycetes and is included in the Species of Kueninena, namely the microbe *Candidatus kuenenina stuttagartiensis.* Apart from the plantomycetes phylum, this study managed to find several other anammox microbes such as *Proteobacteria, Bacteroidetes, Planctomycetes* and *Chloroflexi.* As well as found microbes in the nitrogen cycle such as *Nitrosomonas, Nitrobacter, Nitrosospira, Nitrospiraceae* and *Pseudomonas*. The average of ammonia removal in this study was 18 % mL/Day.

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