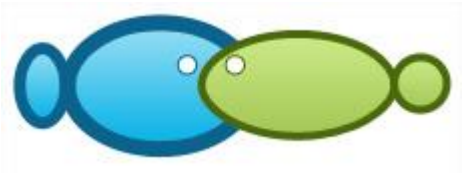

Isolation and identification of biofilm bacteria from bamboo in traditional vannamei shrimp ponds as an ammonia degradation factor

[1]Diani E. Tyas, [2]Haeruddin, [2]Pujiono W. Purnomo

[1] Master Program in Coastal Resources Management, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang, Central Java, Indonesia; [2] Department of Aquatic Resources, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang, Central Java, Indonesia. Corresponding author: D. E. Tyas, dianiestiningtyas@gmail.com

Abstract. The development of shrimp culture ponds, both intensively and extensively has both positive and negative impacts in the community. Such a negative impact is the ammonia waste produced that pollutes the environment and can cause even the death of aquatic biota. One way to reduce this waste is to utilize the bacterial community that exists on bamboo biofilms. The purpose of this study is to isolate and identify bacteria on bamboo biofilms and analyze the ability of these bacteria to degrade ammonia. The bacterial isolation method used was poured plate. The bacterial identification was carried out biochemically and molecularly (16S rRNA), and the analysis of ammonia was conducted using the Nessler method. The results of sample isolation showed 3 bacterial isolates (A1, A2, and A3) in the form of coccus and bacilli. Biochemical identification shows that the 3 isolates can produce the cytochrome oxidase enzyme and catalase enzymes, with positive results in the oxidase and catalase tests. The molecular identification results show that isolate A1 has a similarity of 94.35% with *Bacillus subtilis*, the sample code A2 has a similarity of 99.22% with *Marinobacter hydrocarbonoclasticus*, and the sample code A3 has a similarity of 91.16% with bacteria included in the genus *Sphingomonas*. According to the results of the analysis of ammonia, *Bacillus subtilis* reduced ammonia levels by 2.03%, *Marinobacter hydrocarbonoclasticus* by 4.12% and *Sphingomonas* did not reduce ammonia levels.
Key Words: 16S rRNA, aquaculture, bioremediation, isolate, *Litopenaeus vannamei*.

Introduction. Shrimp is one of the main export commodities of fisheries in Indonesia. This commodity can significantly improve the economy in most regions of Indonesia, such as Java, Kalimantan, Sumatra, Sulawesi and Irian Jaya, because the market demand for shrimp is large. Therefore, shrimp farming is increasingly growing. Cultivation techniques continue to be developed, ranging from traditional to intensive. According to Waikhom et al (2018), in many countries, shrimp farming, especially vannamei shrimp (*Litopenaeus vannamei*), is cultivated semi-traditionally and intensively. Each cultivation technique has its uniqueness. Traditional shrimp culture is characterized as dependent on natural food, with seedlings stocked in a density of 2-5 per m^2, in a limited land carrying capacity (Chamberlain 2011; Supono 2017). Intensive cultivation is characterized by high stocking densities and the use of additional feed with a sufficient crude protein content. The crude protein content of artificial feed is up to 40%, so that the pellet decomposition process produces inorganic nitrogen in the form of NH_3 and NH_4^+, which are toxic for shrimp (Romadhona et al 2016).

Ammonia is the main product of protein catabolism in crustaceans and can cause 60% to 70% nitrogen excretion with small amounts of amino acids, urea, and uric acid. Therefore, ammonia in shrimp ponds can be toxic because shrimps cannot convert ammonia into less toxic carbamoyl phosphate compounds, so they are very susceptible to ammonia toxicity effects (Schuler et al 2010). One toxicity effect is damage to the gills, occurring because ammonia blocks the transfer of oxygen from the gills to the blood. In addition, ammonia can cause the breakdown of the mucus-producing membrane, thus

damaging the internal intestinal lining (Ogbonna & Chinomso 2010). Other than being able to cause effects on aquatic biota, ammonia produced in these ponds will further pollute the environment if waste management is not applied. This is in accordance with Suwoyo et al (2019), who stated that only 25-30% of nitrogen content in ponds is assimilated into meat, while the remaining 75% is wasted and becomes a dangerous pollutant for the balance of the ecosystem.

The impact that occurs due to high ammonia levels in ponds requires handling and management. One way is to utilize the bacterial community in the bamboo (*Gigantochloa atter*) biofilm from the pond as an ammonia bioremediation agent. Biofilms are associations of microorganisms where cells attach on a surface encased in an extracellular matrix of polymeric substances produced by the bacteria itself (Jamal et al 2015; Azizah et al 2014). Biofilms can be used for the production of various chemicals (ethanol, butanol, lactic acid and succinic acid) or used for bioremediation (Berlanga & Guerrero 2016). Research on biofilm bacteria from bamboo is still rarely done. One of the studies on the identification of biofilm bacteria was carried out by Merina et al (2011) on the surface of seagrass leaves, finding *Bacillus* sp. and *Pseudomonas* sp. According to Solikhin (2016), *Bacillus cereus* bacteria isolated from traditional pond water can reduce the Total Ammonia Nitrogen (TAN) content by 22.9%. Based on several previous studies, biofilm bacteria in bamboo from traditional shrimp ponds can reduce ammonia levels in ponds. Therefore, it is necessary to isolate, identify and analyze the degradation of ammonia from biofilm bacteria on pond bamboo, so that the best species could be used as an ammonia degrading agent.

Material and Method

Materials. The material used in the study was biofilm bacteria from bamboo found in traditional shrimp ponds in the area of Tegal, Central Java, Indonesia. Zobell 2216E media, bacto-peptone, bacto-agar, yeast, selective media (indole media, sucrose media, maltose media, citrate media, urea media, SIM media, MR media, VP media) were used in biochemical tests, Paper oxidase and H_2O_2 3%, gram A, gram B, gram C, and gram D were used in the staining test for gram, distilled water, sterile sea water, Saponin, Chelex 20%, Phosphate Buffer Saline (PBS) were used in bacterial DNA extracts. Agarose, Tris-Borate-EDTA (TBE) buffer, ethidium bromide solution for electrophoresis, reverse primer (1429R), forward primer (27F), ddH_2O, mix PCR (promega) were used in the PCR test.

The equipment used in this study were petri dishes, bunsen, test tubes, test tube racks, electric scales, ose needles, incubators, laminary flow, slide glass, Erlenmeyer, tweezers used to scrap biofilm layers from bamboo, autoclaves, micropipettes, microtypes, aluminum foil to seal the Erlenmeyer, hot plate magnetic stirrer, and a cool box. Water Quality Control (WQC) was used to measure pond water pH, dissolved oxygen (DO), water temperature, and salinity. A spectrophotometer, centrifuge, and thermal cycler were used.

Methods. The method used in this study is an exploratory method with several stages. The first step is the sampling and isolation of biofilm bacteria from bamboo. It was followed by the identification of bacteria by biochemical and molecular tests using the 16S rRNA sequencing gene, and the degradation test for ammonia compounds.

Sampling and isolation of biofilm bacteria from bamboo. A sampling of biofilm bacteria from bamboo was carried out in traditional vannamei shrimp ponds in the Tegal area, in November 2019. The bamboo surface layer was scraped off using sterile dredging and placed into test tubes filled with seawater. The test tubes were put in a coolbox and transported to the Tropical Marine Biotechnology Laboratory, Diponegoro University, for the dilution and isolation of bacteria. The dilution process was carried out by shaking a sample of bacteria in the test tube and taking 1 mL, then inserting it into another test tube containing 9 mL of sterile seawater. Furthermore, 1 mL from the first dilution was taken and put into the second test tube. From the second tube, 1 mL is taken and put into the third tube, in the same way, and this was repeated for the 4^{th} tube. All tubes in the dilution process were filled with sterile seawater. Dilutions were

carried out in series of 10^{-2}, 10^{-3}, and 10^{-4}. 50 µL of samples from the serial dilution tubes 10^{-3} and 10^{-4} were isolated into a petri dish containing Zobell media using the Pour Plate technique. The isolated sample was then incubated for 3 days.

Biochemical identification of biofilm bacteria in bamboo. Identification of bacteria was conducted using purified isolates. Purification was carried out using the streak method. According to Sanders (2012), streak plates are used to isolate pure cultures of bacteria, or colonies from mixed populations with simple mechanical separation. Scratching technique was preformed by etching a bacterial colony with a different morphology on the surface of the bacterial growing media to obtain pure isolates. The pure isolate was then biochemically identified, with an oxidase test, catalase test, urea test, citrate test, motility test, indol test, confectionery test, MR-VP test and gram staining, which refers to bacterial identification guidelines from Bergey's Manual of Determinative Bacteriology (Bergey & Holt 1994).

Molecular identification of 16S rRNA biofilm bacteria in bamboo

Bacterial DNA extraction. DNA extraction was carried out by the Chelex method (Walsh et al 2018). The first step was to insert the purified bacterial isolate into a tube containing 500 µL of saponin and 100 µL of ddH_2O or aqua bidestillata. It was left overnight. The next step was to centrifuge the tube for 15 min at 9000 rpm. After 15 minutes, the supernatant was removed from the centrifuge process. 1 mL of PBS solution was added and vortexed until it became homogeneous. Vortex tubes were centrifuged again for 10 minutes at the same speed. The supernatant was discarded and 100 µL of ddH_2O solution and 50 µL Chelex (20%) were added. The next step was to heat the tube in the heating block for 5 min at 95°C, and vortex afterwards. The heating process in the heating block for 5 min at 95°C was repeated. The last step was to centrifuge the heated tube 15 min and move 100 µL of supernatant into a new tube.

PCR (Polymerase Chain Reaction). The Polymerase Chain Reaction (PCR) process was carried out by making a mixture of 25 µL per sample containing 12.5 µL of master mix (Promega), universal primers (27F and 1429R) with 1.25 µL each, DNA template (2.5 µL) and ddH_2O (7.5 µL). PCR amplification was performed using a thermal cycler with the amplification program at the initial denaturation stage at 95°C for 3 min, then continued with 29 denaturation cycles at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. Finally, the final elongation process was carried out at 72°C for 7 min.

Electrophoresis. Agarose was prepared with a Tris-Borate-EDTA (TBE) solution (30 mL) and agarose (1% of a buffer solution). It was placed in a glass container. The glass container was heated at 250°C until the solution turned clear. After 15 min, the clear solution was placed into an agarose mold. The results of the PCR amplification were inserted into agarose wells with 3 µL micro pipets, then detected by electrophoresis at 100 volts and 400 amperes for 30 min. The next step was soaking the agarose gel in ethidium bromide solution for 30 min and the results were observed on camera. The PCR results were sent to PT Genetica Science for sequencing.

Ammonia compound degradation test. Ammonia degradation testing began by preparing a starter culture. 2.5 g of bacto-peptone and 0.5 g of yeast extract were weighed, then placed in an Erlenmeyer. Seawater that had been sterilized in an autoclave for 20 min at 121°C was added to reach a volume of 250 mL. Then, 125 mL of the media was transferred to another Erlenmeyer glass. One Erlenmeyer glass acted as a control medium and the other as a medium for bacterial inoculation. The control and treatment Erlenmeyer glasses were closed using aluminum foil bound with rubber band. The Erlenmeyers were sterilized in an autoclave at 121°C, 1 atm pressure for 1.5 h, until the autoclave was at 60°C. After the media was sterilized and cooled, bacterial inoculation was carried out in liquid media, by taking pure bacterial isolates that were present in a

1x1 cm Zobell solid (agar) medium and inserting them into the liquid media. Then the Erlenmeyer control glass and Erlenmeyer treatment glass were put on the rotary shaker for 3x24 h.

Bacterial isolates from the starter culture were transferred into an Erlenmeyer glass containing 125 mL sterile Zobell media. Each bacterial isolate was transferred into 3 Erlenmeyers (3 replicates). 10 mL of bacterial isolates from the starter cultures were transferred using a sterile measuring cup for each Erlenmeyer glass. Another Erlenmeyer glass acts as a control medium, so there were 4 Erlenmeyers for each bacterial isolate to be tested for ammonia degradation. Ammonia PA with a concentration of 10 ppm (12.5 mL) was added to the control and treatment. The Erlenmeyers were placed in the rotary shaker.

Testing for decreasing ammonia levels was carried out using the Nessler-Spectrophotometry method. Bacterial isolates with ammonia (10 ppm) were put into 25 mL containers. Then 1-2 drops of Seignette salt reagent were added to the 25 mL of sample. Seignette salt reagents are made from 50 g of potassium sodium tartrate tetrahydrate dissolved into 100 mL of NH_4^+ free aquadest. Afterwards, 0.5 mL of the Nessler reagent were added and the mix was shaken and left for 10 min. The yellow color that occurs due to reagents is measured by the spectrophotometer at a wavelength of 420 nm. Ammonia standard curves made with ammonia concentrations of 0.00, 1.0, 2.0, 3.0, 4.0, 5.0 ppm were used to calculate ammonia concentration in the sample.

Data analysis. DNA sequencing results and phylogenetic trees were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) version X. Bacterial species names are obtained by entering the results of sequencing into the National Center for Biotechnology Information (NCBI) on the Basic Local Alignment Search Tool (BLAST) menu to be matched with DNA in GenBank. Phylogenetic tree construction is based on genetic kinship distance by the Neighbor-Joining method (Nurkanto & Agusta 2015) using 1000 bootstrap replications.

The concentration of ammonia compounds that can be degraded can be calculated based on the formula of Clesceri et al (1989):

$$AO=(AK-AP)/AK \times 100$$

Where: AO - concentration of oxidized ammonia; AK - ammonia concentration in the control media (mg L^{-1}); AP - ammonia concentration in bacteria inoculated media (mg L^{-1}).

Results and Discussion

Isolation of biofilm bacteria in bamboo. Bacterial isolation from bamboo biofilms resulted in 3 species of different shapes and colors. The isolates obtained were coded A1, A2, and A3. The colony color obtained in A1 was milky white with yellow in the middle, A2 was milky white, and A3 was clear white. The cell forms were bacilli for A1 and A2, and coccus for A3. Isolation and purification produced 3 pure isolates namely A1, A2 and A3. The amount of isolate produced is related to the dilution process carried out before bacterial inoculation. The dilution process of the sample will reduce the amount of bacteria produced. Tiered dilution aims to reduce the amount of microbes present in the liquid. The higher is the dilution, the less microbes are produced. This was confirmed by Blaize et al (2020), who state that the dilution technique is a simple technique used to obtain the desired concentration, systematically reducing the known or unknown entities (solutes, organisms, etc.).

Biochemical identification of biofilm bacteria in bamboo. The results of the identification of biochemical-forming bacteria biofilm are presented in Table 1.

Based on the results of the bacterial test, A1 showed negative results on the indol, citrate, motility, MRVP, urea, and maltose tests, but showed positive results on the catalase, gram staining, and oxidase tests. A2 showed negative results on the indol,

citrate, MRVP, urea, maltose, and gram staining tests, but showed positive results on the motility, catalase and oxidase tests. A3 showed negative results on the indol, motility, citrate, MRVP, urea, maltose, and gram staining tests, but showed positive results on the catalase and oxidase tests.

Table 1

Biochemical test results of bamboo biofilm bacteria

Biochemical test	*Sample code*		
	A1	*A2*	*A3*
Indol	-	-	-
Citrate	-	-	-
Motility	-	+	-
MRVP	-	-	-
Urea	-	-	-
Sucrose	+/g(-)	+/g(-)	+/g(-)
Maltose	-	-	-
Oxidase	+	+	+
Catalase	+	+	+
Gram staining	+	-	-

Note: (+) - positive; (-) - negative; +/g(-) - positive and no gas.

A1 is a bacterium that can produce catalase and cytochrome oxidase enzymes and has gram-positive properties. Based on biochemical tests, it was found that A1 was from the genus *Bacillus*. Khusro et al (2014) proved that the cell wall of *Bacillus subtilis* is characterized by the ability of metal absorption when gram staining. Moreover, *Bacillus subtilis* has facultative aerobic or anaerobic properties, heterotroph, positive catalase, and negative indole production. Lu et al (2018) also proved through biochemical tests that bacteria identified as *Bacillus* were unable to ferment lactose and maltose, but could ferment glucose. This bacterium is also negative in indole and methyl red formation tests.

A2 can produce catalase and cytochrome oxidase enzymes and are gram-negative. Based on biochemical tests, it was found that A2 was from the genus *Marinobacter*. Gauthier et al (1992) stated that the *Marinobacter hydrocarbonoclasticus* strain SP.17 has a gram-negative, rod-shaped, motile character because it has a single flagellum, without urea activity. In addition, ornithine, phosphate, gelatin, and esculine are not detected. Ng et al (2014) mentioned that biochemical testing of the genus *Marinobacter* isolated from seawater showed negative results on indole production, lactose fermentation. In addition, some could hydrolyze starch and belong to gram-negative bacteria.

A3 has properties that can produce catalase and cytochrome oxidase enzymes and are gram-negative, but they cannot move, as proved by the negative motility tests. Based on biochemical tests, it was found that A3 belonged to the genus *Sphingomonas.* This is confirmed by Nandy et al (2013), who state that bacteria from the genus *Sphingomonas* are gram-negative, include aerobic bacteria, without spores, and produce yellow or white pigment in colonies. Motility is not detected at temperatures over 37^{o}C. This is confirmed by Zhou et al (2019), who state that the genus *Sphingomonas* is a member of the Sphingomonadaceae family in the Alphaproteobacteria class, being found in various environments, such as water, soil or plants. The cells of this genus are gram-negative, do not form spores, are non-motile or motile with a single flagellum.

Molecular identification of 16S rRNA biofilm bacteria in bamboo. The results of PCR visualization in Figure 1 show that the isolation PCR amplification was successful because the resulting DNA band size on the 1500 bp marker had the length of the marker to be achieved. The results of matching sequence results with the GenBank database are presented in Table 2.

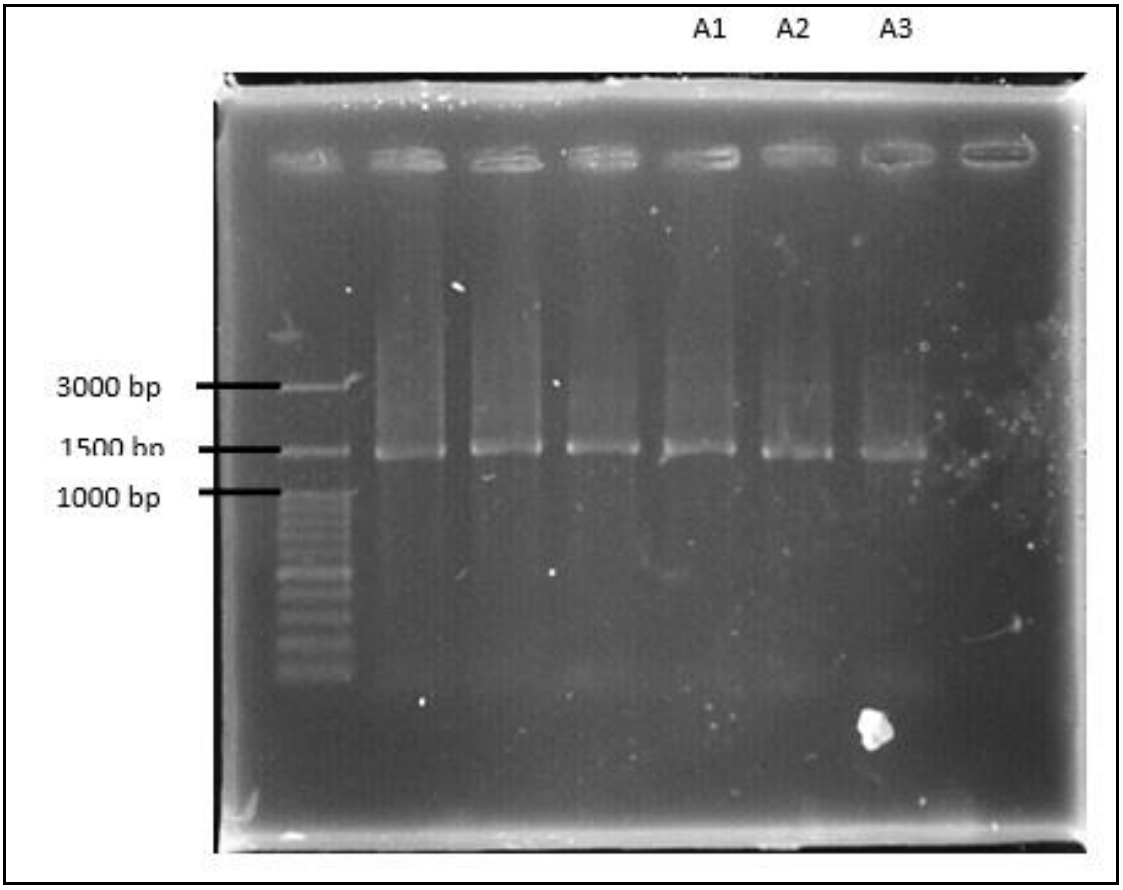

Figure 1. Electrophoresis results of 16S rRNA gene PCR products from isolates of biofilm bacteria in bamboo. A2, A1 and A3 isolates are in the 4th, 5th, and 6th place, respectively.

Table 2

The result of matching sequence results with GenBank database

No	*Original code*	*Species identification (BLAST)*	*Access number*	*Sequence length (bp)*	*Ident (%)*	*Query cover (%)*
1	A1	*Bacillus subtilis*	KC438378.1	1040	94.35	99
2	A2	*Marinobacter hydrocarbonoclasticus*	KY425613.1	1153	99.22	99
3	A3	*Sphingomonas* sp.	JQ659449.1	802	91.16	100

The results of sequencing data matching with the GenBank database (Table 2) on the BLAST menu showed that A1 had a 94.35% similarity with *Bacillus subtilis*, with a sequence length of 1040 bp. A2 had a 99.22% similarity with *Marinobacter hydrocarbonoclasticus*, with a sequence length of 1153 bp. A3 had a 91.16% similarity with the genus *Sphingomonas*, with a sequence length of 802 bp. Matching results were converted to a phylogenetic tree (Figure 2).

The phylogenetic analysis in Figure 2 shows that A1 (code 5 27F) is in the same branch with *Bacillus subtilis*. A2 (code 4 1429F) is in the same branch with *Marinobacter hydrocarbonoclasticus*, and A3 (code 6 27F) is in the same branch as the genus *Sphingomonas*, without being specific to one species. This is consistent with the results of DNA similarity produced from BLAST. The higher the genetic level produced is, the closer are the evolutionary and kinship relationships.

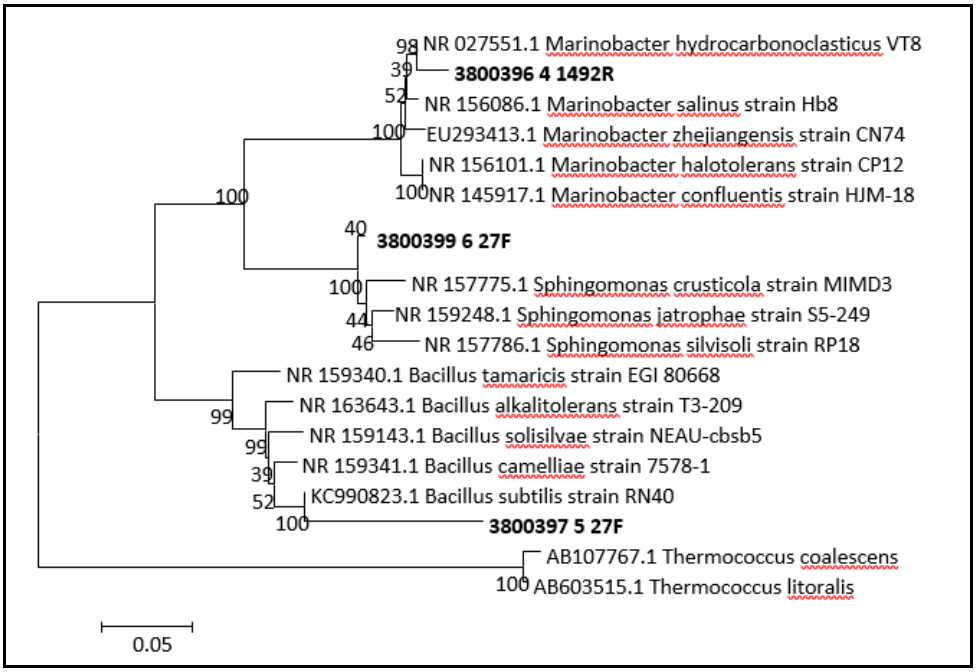

Figure 2. Phylogenetic tree of 3 isolates of biofilm bacteria from bamboo identified with the 16S rRNA gene, by Neighbor-Joining method with 1000 bootstrap replication.

The ability of biofilm bacteria in bamboo degrades ammonia. Ammonia reduction results can be seen in Table 3.

Table 3

Ammonia degradation by bacteria

No	*Sample name*	*Ammonia decline (%)*
1	*Bacillus subtilis*	2.03
2	*Marinobacter hydrocarbonoclasticus*	4.12
3	*Sphingomonas*	-

Based on the decrease in ammonia values, the 3 bacteria have different abilities. *B. subtilis* can reduce ammonia levels by 2.03%, *Marinobacter hydrocarbonoclasticus* by 4.12%, and the *Sphingomonas* genus bacteria cannot reduce ammonia levels. The daily decrease in ammonia values can be seen in Figure 3 for *B. subtilis*, Figure 4 for *M. hydrocarbonoclasticus,* and Figure 5 for *Sphingomonas.*

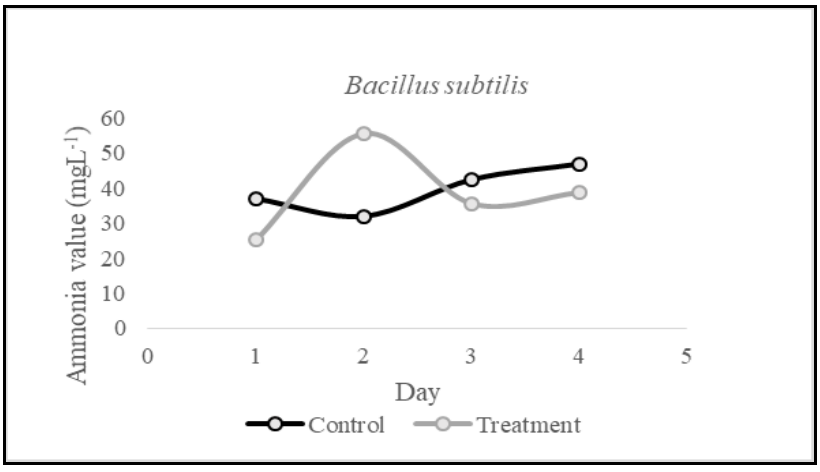

Figure 3. Changes in ammonia value by *Bacillus subtilis*.

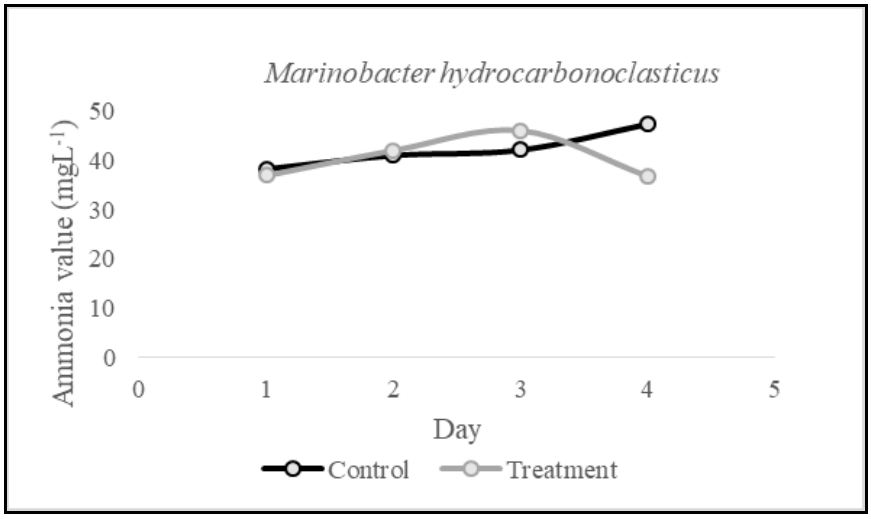

Figure 4. Changes in ammonia value by *Marinobacter hydrocarbonoclasticus*.

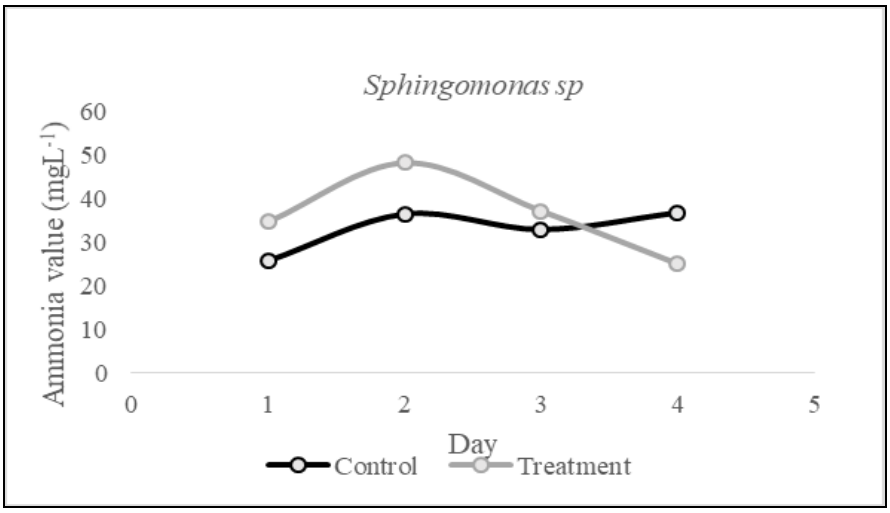

Figure 5. Changes in ammonia value in *Sphingomonas* sp.

A1 was able to reduce ammonia levels by 2.03%. In previous studies, *B. subtilis* had the ability to degrade several compounds. According to Qiao et al (2017), the genus *Bacillus* is a facultative aerobic microbe that belongs to gram-positive bacteria. *B. subtilis* is also reported to have the ability to biodegrade aflatoxin B_1 and ochratoxin A through biotransformation. Research by Watanakij et al (2020) proved that the extracellular fraction of *B. subtilis* BCC 42005 has the potential to degrade aflatoxin B_1. *B. subtilis* is a species whose distribution is extensive, because it can be isolated from both terrestrial and aquatic environments. According to Polonca (2019), *B. subtilis* is a type of cosmopolitan bacteria that can be found almost everywhere in the world. It is widely used in microbiological research because it is considered as a model organism easy for studying biofilms (Gingichashvili et al 2017). This is due to its ability to form clearly segmented three-dimensional colony biofilms. According to Ryan-Payseur & Freitag (2018), *B. subtilis* has the capacity to form biofilms in their life cycle. The ability of *B. subtilis* to degrade ammonia can occur because it has enzymes for degrading organic waste. It can produce enzymes that can break down various proteins, carbohydrates and lipids derived from animals and vegetables into their constituent units (Olmos & Paniagua-Michel 2014). The enzymes can also degrade organic waste from shrimp and fish. The bacterium can also produce antibiotics, mainly from peptides.

A2, identified as *M. hydrocarbonoclasticus*, was able to reduce ammonia levels by 4.12%. According to Li et al (2013), *M. hydrocarbonoclasticus* isolated from soil in

Yancheng City, China, is a halophilic bacterium that can grow in concentrations of NaCl ranging from 20 to 120 g L^{-1}. The bacterium can grow on various carbon sources and has considerable denitrification ability (94.2% of nitric denitrification and 80.9% of nitrogen denitrification in 48 h). Ammonia degradation results produced are not as high as in previous studies. This is possible because of the lack of carbon sources in the media used. The ability of denitrification of these bacteria is influenced by carbon sources, because cells use carbon sources as electron donors and will gradually reduce the nitrate content to N_2, thereby eliminating organic matter and nitrates simultaneously. Research conducted by Mounier et al (2014) proved that *M. hydrocarbonoclasticus* SP 17 can form biofilms (oleolytic biofilms) on various hydrophobic substrates, including hydrocarbons, alcohols, fatty acids, triglyceride fatty acids, and wax esters. The biofilm formed is thought to degrade hydrophobic organic compounds, especially lipids and hydrocarbons.

A3, identified as a bacterium of the genus *Sphingomonas*, was unable to degrade ammonia. This is indicated by the percentage value generated negatively, the ammonia value in the treatment media increasing every day. Even so, the bacteria of this genus have the ability to degrade aromatic hydrocarbons. This is in accordance with the research of Zhou et al (2016), who state that members of the genus *Sphingomonas* isolated from petroleum-contaminated soil can degrade polycyclic aromatic hydrocarbons. Polycyclic aromatic hydrocarbons are dangerous compounds because they are toxic, genotoxic, mutagenic and/or carcinogenic.

Conclusions. Biofilm bacteria found in bamboo from shrimp ponds are *Marinobacter hydrocarbonoclastus*, *Bacillus subtilis*, and bacteria from the genus *Sphingomonas*. Bacteria that can degrade ammonia are *Marinobacter hydrocarbonoclasticus* and *Bacillus subtilis*. However, the rate of reduction of ammonia was still below 10%.

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Authors:
Diani Estining Tyas, Coastal Resources Management, Department of Aquatic Resources, Faculty of Fisheries and Marine Science, University of Diponegoro, Jl. Prof. Soedarto SH, Kampus Undip Tembalang, 50277 Semarang, Central Java, Indonesia, e-mail: dianiestiningtyas@gmail.com
Haeruddin, Department of Aquatic Resources, Faculty of Fisheries and Marine Science, University of Diponegoro, Jl. Prof. Soedarto SH, Kampus Undip Tembalang, 50277 Semarang, Central Java, Indonesia, e-mail: haeruddindaengmile@gmail.com
Pujiono Wahyu Purnomo, Department of Aquatic Resources, Faculty of Fisheries and Marine Science, University of Diponegoro, Jl. Prof. Soedarto SH, Kampus Undip Tembalang, 50277 Semarang, Central Java, Indonesia, e-mail: purnomopoed@gmail.com

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