The identification of plankton, water quality, blood cell, and histology in culture pond of tilapia *Oreochromis niloticus* which infected by viral nervous necrosis (VNN)

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Abstract. Currently, Viral Nervous Necrotic (VNN) is not only attacking the marine fish but also the freshwater fish like tilapia (*Oreochromis niloticus*). The aims of study to identify the type of plankton, water quality status, blood cell status, also histology of VNN infected tilapia obtained in culture ponds. The methods included plankton identification and water quality analysis from the infected fish pond in the Krakal, Blitar. The quality of blood cells and the histology of tilapia infected by VNN observed using a microscope with Hematoxylin-Eosin staining. The result show plankton in a fish pond of infected tilapia includes 3 divisions: Chlorophyta, Cyanophyta, and Bacillariophyta and 2 phyla: Arthropoda, and Rotifera. The values of erythrocyte, hematocrit, and hemoglobin were smaller than normal tilapia, however, the leukocyte and macronuclei values of VNN-infected fish were higher than normal fish. The fish histology shows the vacuolation in the brain and eyes tissue. The water quality of the culture pond have the temperature, pH, turbidity, DO, CO₂, NOₓ, PO₄, TOM in the range of 30-32°C; 7.0-9.0; 25cm; 6.082–7.44mg/L; 3.98–9.08mg/L; 1.039–1.139 mg/L; 0.051-0.054mg/L; and 11.377-13.905mg/L, respectively. VNN causing high leukocyte and macronuclei and the damaging in brain and eyes tissue in infected tilapia.

1. Introduction

Viral Nervous Necrosis (VNN) is an RNA virus that commonly attacks marine fish commodities. However, the virus has been found to attack not only marine fish such as groupers but also tilapia cultivated in many cultivation centers in East Java, Indonesia. The VNN outbreak caused tilapia to die in January-March 2016 and 2017 in the village of Krakal, district of Wlingi, Blitar. Tilapia is one of the main commodities of the fisheries sector in Indonesia because it is easy to breed [1]. Tilapia can be found in various regions in Indonesia such as in Blitar, East Java.

The environment is one of the factors that should be considered in cultivation activities. One of the processes of the aquatic environment management can be seen from the quantity and quality of water [2]. The contaminated environment and poor water quality will trigger the increase of VNN infection [3]. In addition, VNN will infect stressful fish due to high stocking density and high water temperatures in cultivation systems [4].

VNN is a virus that has genetic materials in the form of single-stranded RNA. This virus attacks the grouper fish in the larvae and juvenile stadia and can cause a high mortality rate, up to 100% [5]. One of the symptoms is spinning fish. Some fish are seen sleeping or dying at the bottom. Also, some fish
become abnormal [6]. The clinical symptoms of Blitar tilapia are similar to the clinical symptoms of VNN infected snapper. The study aims to determine the types of plankton, water quality status, blood cell status, and histology of VNN infected tilapia obtained in culture ponds.

2. Methodology
Fish samples were taken at the time of the disease outbreak, in January-March 2016-2017 in Krakal, Wlingi district, Blitar. The research methods used were plankton identification using microscopes and quality analysis of water from the infected fish pond. Plankton samples were taken by filtering 25 L water samples using plankton net no. 25. The samples were put into the bottle film and preserved with Lugol’s (3 drops). The water quality parameters were observed including turbidity, temperature, pH, DO, CO₂, NO₃, PO₄, and TOM. The observation of water quality was done 3 times and the interval was one week. Water sampling used Wrinkle bottle and a 600ml bottle.

PCR assay with VNN target for DNA length band of 294 base pairs (bp) was performed to confirm the infected fish. The quality of blood cells and the histology of digestive systems infected by VNN were observed using microscopes with Hematoxylin and Eosin (HE) staining. Blood taking was done 3 times, on day-5, day-9, and day-14. The fish were placed with head on the left before the syringe was rinsed with Na-citrate 3.8% as an anticoagulant. Blood samples were taken with a syringe needle injected in the musculus at the midline of the body of the anal fin. Blood samples were taken, inserted into 1.5 ml tubes to observe blood image for the measurement of Erythrocyte, Leucocytes, Hemoglobin, Hematocrit, Micronucleus, and Macronucleus.

3. Results and Discussion
3.1. Tilapia disease analysis
The results of morphological observations and the behavior of tilapia indicate the presence of symptoms. They showed that the fish were infected by VNN. With regard to the visible symptoms of fish swimming on the surface, the movement was weak and skewed. The eyes bulged and the skin contained mucus. The body looks pale and some fish were found to have body color darkening. There was a change in swimming habits of VNN infected fishes such as swirling and whirling or abdominal position above because of swelling of bubbles [7]. Sometimes, VNN infected tilapia showed vertical swimming habits (in the bottom of the pond or spinning swiftly or stomping to the surface of the water). Detection of VNN on tilapia by PCR assay on primer 294 bp revealed that the brain, kidney, liver, intestine, and flesh were positively infected by VNN.

3.2. Water Quality Parameters on Tilapia Maintenance Pond
Table 1 shows that the water quality in the tilapia pond is still good for the growth of aquatic organisms, such as plankton and tilapia. The results of the measurement of the water quality parameters for 3 weeks are still in the range of quality standard for aquaculture. However, the pH and temperature measurements show that the ideal water quality condition for the retention of VNN infection in tilapia is in the pH range of 2-9 and temperature of 30-32 C.

3.3. Plankton Analysis
The identification of plankton in tilapia ponds from week 1 to 3 obtained phytoplankton and zooplankton. Phytoplankton consists of 3 divisions of Chlorophyta (2 genus), Cyanophyta (1 genus) and Bacillariophyta (7 genus). Zooplankton consists of 3 phylum including Arthropod (2 genus) and Rotifera (1 genus). Cyanophyceae is able to survive in non-light condition and indicates the presence of high organic matter content. This alga is able to take CO₂ and phosphorus in low concentration conditions in waters [8]. The abundance of zooplankton has increased and decreased due to the factors, including growth, death, vertical distribution and different migration as well as changes in water quality [9].

The calculation results of phytoplankton and zooplankton diversity index show that the ponds of tilapia belong to the low moderate H’ criteria. Based on the calculation of phytoplankton dominance index and zooplankton at week 1 and 2, there is dominant phytoplankton from the division of Cyanophyta (genus Merismopedia), while there is no dominant zooplankton in the waters. Each type of phytoplankton has a different response to the nutrient types dissolved in water bodies. This
condition can cause the phytoplankton community in a water body to have structure and dominance of different types with other water bodies [13].

### Table 1. Results Measurement of Water Quality Parameters on Tilapia Maintenance Pond.

| No | Parameter | Unit | Average | Quality standard |
|----|-----------|------|---------|------------------|
| 1  | Temperature | °C  | 31      | 25 – 32 (*)      |
| 2  | Turbidity  | cm  | 25      | 30 – 40 (*)      |
| 3  | pH         | -   | 7       | 6.5 – 8.5 (*)    |
| 4  | DO         | mg/L | 6.757  | ≥ 3 (*)          |
| 5  | CO₂        | mg/L | 6.32   | 1 – 10 (**)      |
| 6  | NO₃        | mg/L | 1.119  | 10 (***)         |
| 7  | PO₄        | mg/L | 0.052  | <0.2 (***)       |
| 8  | TOM        | mg/L | 12.640 | <50 (***)        |

Note: (*) [10]; (**) [11]; (***) [12]

3.4. The Presence of Plankton and VNN in Tilapia Maintenance Pond

The phytoplankton identified in the digestive tract of tilapia are genus Nitzchia, navicula, Achnantes, Tabellaria, Surirella and Diatoma and genus Netrium. The zooplanktons identified in the digestive tract of tilapia are from genus Calanus and Keratella. The plankton contains several amino acids. The genus Nitzchia, Navicula and Achnantes contain amino acids, including L-aspartic acid and L-lysine [14]. Genus Calanus contains amino acids: lysine and arginine [15]. The genus Keratella has amino acid contents of L-aspartic acid, arginine, and L-lysine [16]. Protein is a virus capsid compiler. Betanodavirus utilizes the amino acid cysteine as a capsid; L-aspartic acid plays an important role in the cleavage of proteins in the Betanodavirus capsid. The amino acid arginine and lysine also play a role to bind the RNA genome into the inner layer of the capsid wall [17]. This suggests that amino acids contained in the host body (plankton) have corresponding amino acids the virus needed for reproductions. It can be indicated that genus Nitzchia, Navicula, Achnantes, Keratella and Calanus can be vectors against the spread of VNN on tilapia bodies by predation process, which can also be referred to as horizontal transmission.

3.5. Observation of Erythrocytes, Leucocytes, Hematocrit, Hemoglobin

The results of erythrocyte analysis can be seen in figure 1.

![Figure 1](image)

**Figure 1.** Amount of erythrocyte in tilapia.
The observation results on the first, second and third samples show a number of erythrocyte on VNN infected tilapia, which were 930,000 cell/mm³, 890,000 cell/mm³, dan 800,000 cell/mm³. However, the total of erythrocyte on healthy tilapia fish was 2,035,000 cell/mm³. The low number of erythrocyte is due to poor water quality in Krakal that affects the stress of tilapia. Erythrocytes have a nucleus and the number of variations depends on the species, stress condition, and temperature of the environment. The erythrocytes are in the range of 1.05 – 3.0 × 10⁶ cell/mm³ [18]. Decreasing erythrocyte is caused by water quality during the maintenance of the pond which is not in accordance with the quality standard. High levels of ammonia can cause fish stress, leading to diseases such as ectoparasite and VNN.

From the observations on first, second and third samples, it was found that the Hb levels on tilapia infected by VNN were 5 gr/100 ml, 4.5 gr/100 ml, dan 4 gr/100 ml. In a healthy fish, the total Hb is 6.7 gr/100ml. The results of hemoglobin analysis (Hb) can be seen in figure 2.

![Figure 2. Amount of Hemoglobin (Hb) in tilapia.](image)

The hemoglobin of the fish decreased due to the unbalanced ability to bind oxygen although the metabolism to produce energy is inhibited. Decreased hemoglobin is also seen in the decreased amounts of erythrocytes. Red blood cells decline is due to the lack of required oxygen levels. The low Hb causes the metabolism to decrease resulting in low energy production. This makes the fish weak and has no appetite. The fish were seen at the bottom or hanging below the water surface [19]. The results of hematocrit analysis can be seen in figure 3.

![Figure 3. Amount of hematocrit in tilapia.](image)
The results of the first, second and third fish samples indicated that the hematocrit in VNN infected tilapia were 15%, 14%, and 13%. However, the total of hematocrit in healthy fish is 27%.

![Figure 4. Amount of leukocytes in tilapia.](image)

The observation results show that the total hematocrit from the first week to the third week decreased. The low hematocrit is due to the number of red blood cells and low hemoglobin. Hematocrit has a strong correlation with the amount of hemoglobin. The lower red blood cells result in lower hemoglobin and hematocrit. Hematocrit will decrease in cases of anemia. It can be used as guidance for the low levels of protein, vitamin deficiency or infected fish [20].

Observations on leukocytes are shown in figure 4. Total leukocyte in tilapia infected by VNN on first, second and third samples were 189,000 cell/mm³, 176,000 cell/mm³ and 167,000 cell/mm³. The total of leukocytes of healthy fish was around 78,000 cell/mm³. The total of leukocytes was lower than the total red blood cells. The total leukocytes of every mm³ of teleost fish blood cells were around 20,000 cell/mm³ – 150,000 cell/mm³ [21].

3.6. Micronucleus (Mn)

The observations results on micronucleus were shown in figure 5.

![Figure 5. Amount of micronucleus in tilapia.](image)

Based on figure 5, it is known that the number of micronucleus in tilapia affected by VNN on first, second and third samples were 176.5 cells, 189.55 cells, dan 183.58 cells. However, the observation results of healthy tilapia fish show that the total average of micronucleus was 78.04 cells.
The increase of micronucleus is due to erythrocytes of chromosomal damage and disruption in DNA duplication. Micronucleus observations were performed to determine the chromosomal damage seen from erythrocyte and to detect genotoxicity in the waters (figure 6).

![Image](image_url)

**Figure 6.** Observation results of Micronucleus on Interphase, Prophase, Metaphase, Anaphase, Telophase, and MN.

Micronucleus were conducted from acentric fragments that fail to join the cells during the process of cell divisions. They can also be formed from the chromosome left behind or not carried away in the process of mitosis or occurs due to complex chromosome configuration at the time of the anaphase process. Micronucleus criteria include a diameter of less than one-fifth of the nucleus (10μm) located in the cytoplasm and out of the nucleus, no contact with a nucleus [22].

3.7. Histopathological Analysis of Brains Tilapia Infected by VNN

VNN infects the tilapia brain organs, directly attacking the fish receptors because VNN is a virus that does not have envelopes. Then, VNN is spread to the brain tissue through the blood circulation. The VNN is also stable in extreme environments, and the difference is that if the environment is appropriate, then this VNN will be endemic. VNN attacks the brain through blood circulation [23]. VNN is directly attached to the host's receptor, then introducing genetic material into the host cell or intracellular infection by leaving the protein coat outside the cell [24]. In the histopathological observation of infected tilapia, VNN used pieces of brain tissue of tilapia that have been prepared and colored by using HE staining. They were then observed using a microscope as shown in figure 7.

![Image](image_url)

Figure 7(A) shows the healthy tilapia brain tissue, the structure of brain tissue that is still good, regular and does not show any damage. The existence of healthy fish here was used as a comparison in the observation of normal brain tissue and that which has been damaged. The histopathology of healthy fish brains indicates a normal state of the histological structure without any indication of damages and defects. In the molecular layer, the granular layer and pyramid layer appear normal and there is no sign of necrosis and no apparent hemorrhage occurs [25]. For the first observation, the tilapia showed disruption to He, Va and Ko (figure 7B). The second observation showed the disruption of He, Va and Ne (figure 7C). The third observation showed disruption to He, Ne and Va (figure 7D). He of VNN infected tilapia brain tissue was observed in all observations (figure 7B, 7C, and 7D) which occurred in neuronal cells/nerve. Ko was found in the first observation (figure 7B) occurring on brain nerve cells of tilapia.
Figure 7. Histopathology of Tilapia Brain Tissue. (A) Healthy brain tissue. (B) The first observation shows the Kongesti(Ko), Vacuolization (Va) and Hemorrhage (He). (C) The second observation shows the damage of Va, He and Necrosis(Ne). (D) The third observation shows He., Ne., and Va.

Congestion can be seen microscopically in the erythrocytes collected in blood vessels. The fish brains exhibited neurological signs, capillary congestion, and venoms clearly visible in the cerebellar valvula and medulla oblongata, which are associated with dissociation of fiber edema in nerves [26]. Damage to Ne was found in the second and third observations (figure 7C and 7D)). Cells experienced necrosis that caused the cells to be destroyed so that only empty space was left in the brain tissue or vacuolization that occurred due to cell damage. This is due to VNN infection through the bloodstream and into the brain, causing damage to the tissues. Damage to vacuolization (Va) was found in the 1st observation (figure 7B), the second observation (figure7C), and the third observation (figure 7D) characterized by the appearance of a nucleus that appeared enlarged and bubbly and not eosinophils (redness) as a result of changes in fluid balance in the cell due to increased amount of fluid. Vacuolization occurred due to cell damage, then remained empty in the brain tissue.

4. Conclusion
The identified plankton consist of Chlorophyta (2 genus), Cyanophyta (1 genus) and Bacillariophyta or Chrysophyta (7 genus) and Arthropoda (2 genus) and Rotifera (1 genus) phyla. Parameters of measured water quality support the planktons growth and aquaculture activities as well as the presence of VNN in tilapia ponds. The hematology and micronucleus status of VNN-induced tilapia show that the deterioration of the condition became worse, which could cause fish death.

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