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DUMBO CATFISH (*CLARIAS GARIEPINUS*) MICROPLASTIC EXPOSURE ON ABNORMALITIES AND LEVEL OF BLOOD COMPONENT USING A COMPLETELY RANDOMIZED DESIGN

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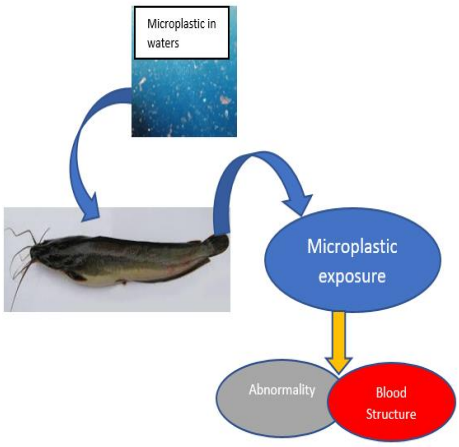
Graphical abstract



Abstract

Recently, researchers studied the increase in environmental contamination, particularly the increase in abnormalities and blood levels in aquatic creatures. Microplastics (<5mm) are formed as a result of the decomposition of macroplastics and mesoplastics in water. Blood components and blood levels used to determine the condition or health state of dumbo catfish (Claries gariepinus) by counting the number might change in response to environmental factors. A completely randomized design (CRD) with one factor and three treatment levels was used in this study. The results showed the rearing time increased from 0 to 30 days and that the doses of commercial fish feed and microplastic varied. The results showed the highest erythrocyte level of 2.78(x106) cell/mm3 in 15 days of rearing time by adding mixed commercial feed and microplastics of 15%. While the highest level of monocytes and lymphocytes by mixed commercial feed and microplastics of 15% on 30 days rearing time were 20.14x103) cell/mm3 and 89.61(x103) cell/mm3, respectively. Meanwhile, the number of leukocytes reached highest level of 23.28(x103) cell/mm3 by adding mixed commercial feed and microplastics of 15% on 30 days of rearing time. Highest level of hemoglobin of 5.58 g/dL was achieved by adding the mixed commercial feed and microplastic of 15% and rearing time of 15 days. The amounts of erythrocyte, leucocyte, hemoglobin, and differential leucocyte components in blood were analyzed to determine whether fish underwent a stress reaction after consuming microplastic. The four parameter values did not show any signs of stress in the fish.

Keywords: Microplastic, blood structural, abnormality, dumbo catfish, aquaculture.

1.0 INTRODUCTION

Indonesia is one of the countries that have the potential in the field of fisheries because of its geographical position which has very wide waters. This can be seen from the diversity of fish found in vast waters, such as oceans, rivers and lakes, and ponds that can be managed and utilized to improve the community's economy. According to FAO (2018), growth of development and population provides the

growth of industrialisation, which has a direct and indirect impact on the expansion of the fishing industry. As an experiment, dumbo catfish has promising potential for the freshwater fisheries sector and animal protein needs from fish.

This encourages various efforts to produce quality dumbo catfish, but in reality, the dumbo catfish consumed is not necessarily healthy because it is exposed to various wastes and garbage in the form of flakes floating in the water. According to Yona and As'adi (2020), plastic is one of the wastes that can degrade into 3 different types namely, mesoplastics (25 mm), mesoplastic (5-25 mm), and microplastic (<5 mm).

The types of plastic commonly used are polyethylene terephthalate (PET) and polypropylene (PP) [1]. Polyethylene (PE) plastic waste is widely found in society and industry, both in the form of HDPE (High-density polyethylene) and LDPE (Low-density polyethylene) which are hazardous wastes, difficult to decompose and can cause various diseases [2]. In addition, microplastics that are quite common in water are polystyrene. The types of microplastics found in water can be seen in Figure 1

Figure 1 Types of microplastic found in waters: a). Pellets, b). Fragments, and c). Fiber

Microplastics in the aquatic environment cannot be simply eliminated because plastic is a very persistent material. Microplastics are floating in the water and accidentally consumed by fish that are looking for food. It accumulates in the fish's body and causes physical and chemical damage such as damage to internal organs and blockage of the digestive tract, as carcinogenic, and endocrine disorders [3, 4]. The primary reaction, secondary response, and tertiary response are the three categories into which fish response mechanisms to stressors in the body are separated [4]. The brain directly receives the primary responses that take place when fish are under stress. The brain-sympathetic-chromaffin network and the hypothalamus-pituitary-interrenal (HPI) networks, which induce the production of stress hormones, catecholamines, and circulating cortisol, are the first steps in the initial stress response in fish. Fish reproductive may be hampered by elevated plasma cortisol's inhibitory action on sex hormones like testosterone, estradiol, and gonadotrophin.

Secondary responses include those that are hematologic, metabolic, and Heat Shock Protein related. While tertiary responses are related to erformance of all fish organs such as changes in growth, disease resistance, overall metabolism for activity, decreased reproductive capacity and ending in fish survival (Urbinati *et al*., 2019). Overview of brain sympathetic-chromaffin and hypothalamic-pituitary-interrenal network activation pathways due to stress responses (primary, secondary, and tertiary) in fish can be seen in Figure 2.

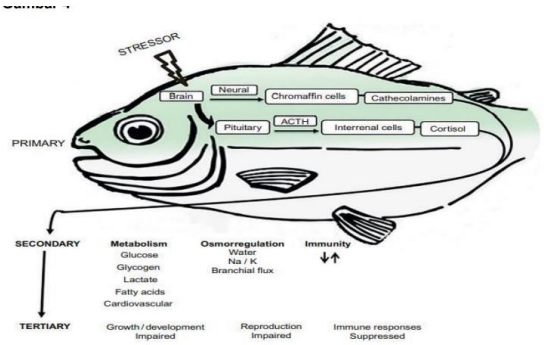


Figure 2 Stress response pathways and mechanisms in fish [5]

Quantitatively, the average amount of microplastic ingested by fish can increase as the fish grows [5]. Especially predatory or carnivorous fish, such as dumbo catfish, which are categorized as all-eating eaters who will eat easily and in large quantities of microplastics. Microplastics can be a potential threat to health if they accumulate in organisms, but there is no indication that can observe visually in unhealthy dumbo catfish.

In the field of fisheries, hematological parameters are very important to determine a condition or state of the fish's body so that it can be used as an indicator of the innate immune system and regulation of immune system function in fish. The hematological parameters of the fish in question are red blood cells (erythrocytes), white blood cells (leukocytes), hemoglobin, leucocyte differential, and hematocrit [6].

In addition, Ho *et al*. (2018) also reported that hematology can be used to assess disease states associated with blood disorders, infectious diseases, the immune system and lipoprotein metabolism, glucose regulation, and liver and kidney function [7]. Therefore, fish hematology confirms the fish condition with analysis of erythrocytes, leucocytes, hemoglobin, and differential leucocytes. The reduced number of erythrocytes in the body causes anemia in fish. If the erythrocyte profile decreases, it will cause the fish to lack oxygen and can interfere with the body's metabolic system, so that the body's immunity decreases and it is susceptible to disease (Figure 3).

Figure 3 shows the shape of the erythrocytes present in a healthy fish. It showed that fish exposed to microplastics and has digestive system disorders, reduced hunting ability, depletion of glycogen in the liver, significant changes in gene expression that are

affected by estrogen receptors, single cell necrosis, and abnormal changes in the blood level of fish [8]. In the long term, exposure to microplastics can cause structural and functional changes in the gut of fish, which can lead to significant damage in the early stages of life, thereby affecting reproduction, population size, and survival of organisms [9].

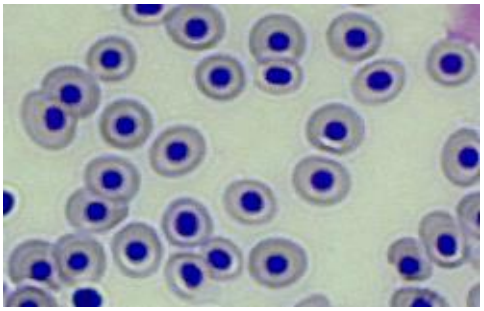


Figure 3 Erythrocytes form in fish blood [10]

Figure 4 showed the hemoglobin form in the fish blood. Hemoglobin concentration can be seen based on the color density of the red blood cell nucleus. The functions of hemoglobin are to transport oxygen, metabolic waste, hormones, and nutrients to be distributed throughout the body. Microplastic exposure has been widely studied by several researchers. where the effect of microplastics on fish digestion can increase the potential for fish mortality [11].

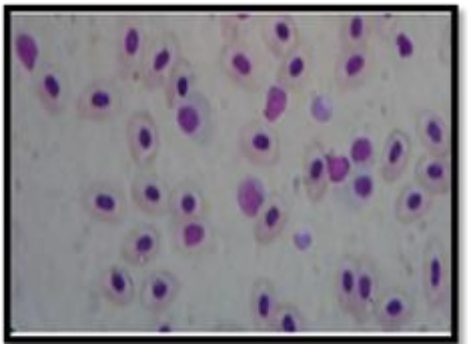


Figure 4 Hemoglobin form in fish blood [12]

Low hemoglobin (Hb) levels can be used as an indicator of low feed protein content, feed deficiency, or fish infection. High levels of hemoglobin (Hb) indicate fish in a state of stress. Measurement of hemoglobin (Hb) levels in fish blood was carried out using the Sahli hemometer method [12].

Abnormalization and changes in fish blood level are parameters that are often measured to determine fish health conditions. The threat of danger will also occur to humans who consume fish, so concrete and continuous data on microplastics is an interesting topic to be used as a reference for policymakers to carry out environmental management to control plastic waste management activities in waters.

Figure 5 illustrated the leucocytes in fish, which consist of 7 types, namely 3 types of eosinophil granulocytes and one type each of neutrophil granulocytes, lymphocytes, monocytes, and platelets. Neutrophils and monocytes are strong phagocytic leukocytes. Phagocytosis by neutrophils is carried out by approaching the particle to be phagocytized by releasing pseudopods in all directions around the particle, then the pseudopods unite with each other to carry out phagocytosis. One neutrophil can phagocytize 5-20 bacteria. Monocytes are stronger because they can phagocytize larger particles. Lymphocytes are not phagocytic but play a role in the formation of antibodies [13,14].

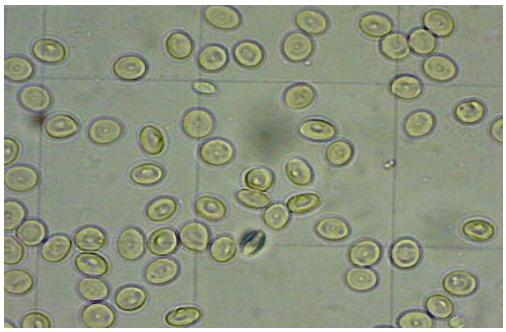


Figure 5 Leucocyte form in fish blood [10]

Differential leukocytes are a unit of white blood cells that consist of two groups, namely granulocytes and agranulocytes. The granulocyte group consists of neutrophils, heterosinophils, eosinophils, and basophils, while the granulocyte group consists of lymphocytes and monocytes [15]. According to Baratawidjaja (2010), the normal percentage of white blood cells is 70% polymorphonuclear neutrophils, 2-5% polymorphonuclear eosinophils, 0.4% polymorphonuclear basophils, 5.3% monocytes, and 30.0% lymphocytes [16].

This study was a developmental study using dumbo catfish as a sample and as a measuring parameter. A completely randomized Design (CRD) was used method in this study. The advantage of using the CRD method is that it is easy to design and implement, easy to analyze data, and flexible in terms of the number of treatments. There are suitable alternative nonparametric analyzes. The design treatment was carried out randomly on the variables used. Random treatment can be done in several ways to get the results of a random research floor plan, one of which can be done by using an application and doing a draw manually. The general form for the additive linear model of Completely Randomized Design (CRD) is as follows,

$$Yij = \mu + \tau\tau i + \epsilon ij \quad (1)$$

where

Yij = Response or observation value of the i-th treatment and j-th repetition

$\tau\tau$i = Effect of the i-th treatment

ϵij= Effect of experimental error on the I-th treatment and j-th repetition

This study was a developmental study using dumbo catfish as a sample and as a measuring parameter. The level of blood components performed consists of leukocytes (lymphocytes, monocytes, and neutrophils), erythrocytes, and hemoglobin as a parameters of microplastic exposure in dumbo catfish. According to Thummbancha and Srisapoome (2016), Abnormalization at the level of blood components caused by exposure to microplastics can have an impact on the health and immune system of the organism. The results of this study can provide ongoing information on the impact of microplastics on aquatic biota.

2.0 METHODOLOGY

The samples of dumbo catfish were put into 12 aquariums and given fish feed and microplastics regularly in varied doses (0%, 5%, 10%, 15%).

Preparation of Dumbo Catfish

Prepared dumbo catfish (*C. gariepinus*) were measured in the size of 10-15 cm, then adapted for 7 days in the laboratory. The fish was put into the maintenance aquarium according to a predetermined stocking density, namely 1 fish per 2 liters of water. Each aquarium contains 19 dumbo catfish, and during the adaptation stage, dumbo catfish fry is given commercial feed which is added to water to form a wet paste. This aims to familiarize the fish with the research treatment. Feeding was carried out ad libitum as much as 3% of the biomass with a commercial protein content of 40% [17].

Manufacturing of Microplastics for Fish Feeding

The microplastic used for research is a type of polystyrene. Microplastics for research were made by crushing styrofoam using a wet blender for 3-4 repetitions. The crushed microplastic is then dried in the sun. After drying, the microplastic powder was then sieved using a flour sieve with a diameter of 22 cm with a mesh size of 80 mesh (177 mm). In general, the size of flour particles that can be filtered by a sieve is at least 80 mesh [18], which is in accordance with SNI No. 76222:2011 [19]. Microplastics in the form of styrofoam powder are then stored in plastic jars. The microplastics that have been obtained are then tested by the SEM-EDS to determine the exact size and shape of the microplastics used.

Supplementation or exposure to microplastics given in vivo to dumbo catfish using microplastic admixture according to predetermined doses, as tabulated below,

Table 1 Composition feed supplement for fish feed

Code	Microplastic, %	Commercial Fish Feed, %
A	5	95
B	10	90
C	15	85
K	0	100

The commercial fish feed and microplastics are crushed using a small amount of hot water to speed up the process of forming the feed into a wet paste. The feed is then given to the dumbo catfish according to the treatment.

Provision of Microplastic Exposure to Test Animals

The exposure of microplastics is carried out by oral method 2 times a day, namely at 07.00-09.00 a.m. in the morning and 15.00-16.00 p.m. in the afternoon [20]. Feeding is carried out ad libitum. After feeding the aquarium fish, dissipate it so that the remaining feed and feces do not accumulate in the rearing medium.

Scanning Electron Microscope-Energy Dispersive X-ray Spectroscopy (SEM-EDS) Analysis

The SEM-EDS (Scanning Electron Microscope-Energy Dispersive X-ray Spectroscopy) test is a test to see the characteristics and size of small particles. The sample tested was Styrofoam powder using the FESEM set-up for EDS High Vacuum

Hematology Analysis

Hematological observations were made on normal fish and fish that had been given supplemental feed from microplastics after 15 days and 30 days of rearing. Fish blood is taken with a 1 ml syringe that has been given an anticoagulant through the caudal fin at the angle of 45 degrees. Furthermore, the calculation of erythrocytes, hemoglobin, leukocytes, and leukocyte differentials was carried out which will be explained as follows.

Erythrocyte Analysis

Rahma *et al*. (2015) in his journal explained the steps for calculating fish blood erythrocytes, namely fish blood taken using a syringe that has been mixed with an anticoagulant and put into a tube [21]. Then, blood was taken using an erythrocyte pipette until it reached a scale of 0.5 µL. Then it was diluted with Hayem's solution in an erythrocyte pipette until it showed 101 µL. The blood that has been mixed is then homogenized in a pipette and then the mixture is taken as much as 20 µL and put in a hemocytometer and then covered with a covered

glass. Discard the first 2 drops before being put into the hemocytometer, so that the solution is completely homogeneous. The number of erythrocytes was counted under a microscope in all erythrocyte boxes, then calculated using the following formula,

$$N = n \times 104 \quad (2)$$

where:

N = number of red blood cells in 1 m^{m3} of blood

n = number of red blood cells contained in 80 small boxes

Leukocyte Analysis

Fish blood was taken as much as 5 ml/fish in the caudal artery area using a 1 ml syringe which had been mixed with EDTA anticoagulant. Blood was taken using a leukocyte tomato pipette as much as 0.5 µL, then added 11 µL of Turk's solution and homogenized slowly so that the blood and solution mixed well. The first two drops of the resulting mixture were discarded, then the third drop was placed in the hemocytometer box and covered with a cover glass, then observed under a light microscope [22]. Total leukocytes are calculated using the following formula:

$$N = n \times 50 \quad (3)$$

where:

N = the number of white blood cells in 1 m^{m3} of blood

n = the number of white blood cells contained in 64 boxes.

Hemoglobin Analysis

Measurement of hemoglobin (Hb) levels was carried out using the Sahli method. This method is to convert hemoglobin into the form of hematin acid by hydrochloric acid. Fish blood was taken using a Sahli pipette to a scale of 20 m^{m3}, and the tip of the pipette that had been used to collect fish blood was cleaned with tissue paper. The blood is taken and then transferred to a hemoglobin tube containing 0.1 N HCl and allowed to stand for 3-5 minutes so that the hemoglobin reacts with HCl to form hematin acid. After that, the blood is stirred, and added distilled water drop by drop until the color is the same as the standard color. The scale reading is done by looking at the height of the solution surface which is matched with a hemometer which means the amount of hemoglobin in grams per 100 ml of blood [23].

Differential Leucocyte Analysis

The differential leukocytes were measured by the number of lymphocytes, neutrophils, and monocytes. According to Arsal *et al*. (2014), the first thing to do was to soak the object glass in a 70% alcohol solution so that the grease and dirt contained in the object glass faded [22]. Fish blood dripped on the object's glass. Furthermore, another glass object is placed on top of the blood drop to form an angle of about 30o, then pulled until the blood spreads along the edge of the first object glass. The blood samples were then air-dried and stained using Giemsa's solution (1:20) for 15-20 minutes, then rinsed with running water and observed under a microscope. The calculation of the number of lymphocytes, neutrophils, and monocytes according to Wooton and Smith (2014), is as follows [24].

$$\% \text{ Lymphocytes} = L/100 \times 100\% \quad (4)$$

$$\% \text{ Monocytes} = M/100 \times 100\% \quad (5)$$

$$\% \text{ Neutrophils} = N/100 \times 100\% \quad (6)$$

Where:

L is the number of lymphocytes in blood

M is the number of monocytes in blood

N is the number of neutrophils in blood

Water Quality Analysis

Water quality analysis can support the results of fish diseases that were not caused by water conditions. The parameters were temperature, pH, dissolved oxygen (DO), ammonia, nitrate, and nitrite.

A Completely Randomized Design (CRD)

This research method uses an experimental method by treatment in the form of feed with exposure to microplastics in animals test animals in the form of catfish (C. gariepinus). The research design that will be used in this study is a Completely Randomized Design (CRD) consisting of 4 treatments with 3 replicates in each treatment and total number of treatments is 12 maintenance aquariums.

Catfish fingerlings (C. gariepinus) were reared in aquariums measuring 30x50x30 cm with a water level of 25 cm. Every 2 liters of water was filled with 1 test animal so that there were 19 catfish fry in each aquarium, which had 19 catfish (C. gariepinus) fry. The dose of microplastic exposure in fish feed refers to the research by Ding *et al*. (2018), A (5%), B (10%), C (15%) and K (0%) which were modified using catfish.

The modified test animals used catfish (C. gariepinus). Dosage amount fish feed with a FR (feeding rate) of 3% (Arief *et al*., 2014). Calculation of feed dose using 3% FR multiplied by the total biomass of fish in the aquarium. The treatment doses used in this study is as tabulated in Table 2.

Table 2 The used treatment doses

Code	Treatment Doses
A	Treatment of giving microplasics (5%) mixed with commercial feed
B	Microplasma treatment (10%) mixed with commercial feed
C	Treatment of microplasma (15%) mixed with commercial feed
K	Treatment of catfish fry feed that is not given exposure to microplastics in the feed.

3.0 RESULTS AND DISCUSSION

SEM-EDS Analysis

The sample tested was styrofoam powder using the SEM-EDS High Vacuum, which resulted in the size and shape of the styrofoam powder below 5 mm using the crushing method. Using a wet blender with a ratio of 1:2 for water and styrofoam. SEM-EDS resulted in Figure 6.

Based on the results of the SEM-EDS test, the used styrofoam powder that has a size of 197-519 µm, was in the microplastic category. As known, based on the size of plastics are divided into 3 types, microplastic (> 2.5 mm), mesoplastic (2.5 – 5 mm), and microplastic (< 5 mm) [25, 26].

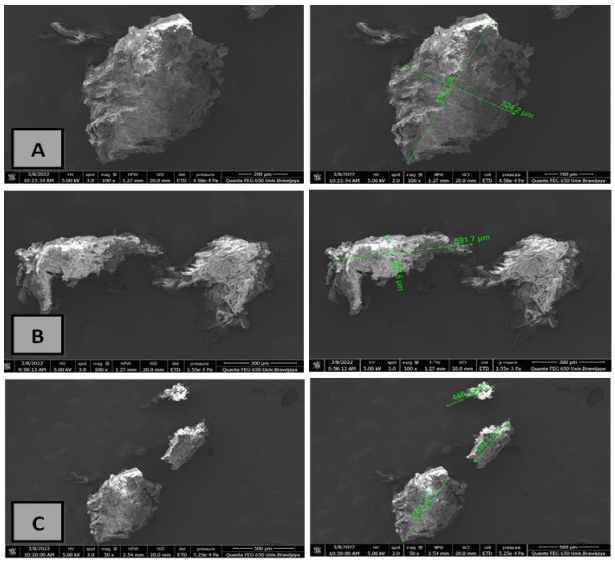


FIgure 6 Various forms of styrofoam powder and their sizes with 100x and 50x magnification; A and B 100x magnification and C 50x magnification

Microplastics have properties with a significant affinity for heavy metal ions and organic pollutants which carry contaminant properties in the environment, especially the aquatic environment [27]. Figures 6 and 7 illustrated the content of elements of C and O in the sample microplastic A and B, respectively.

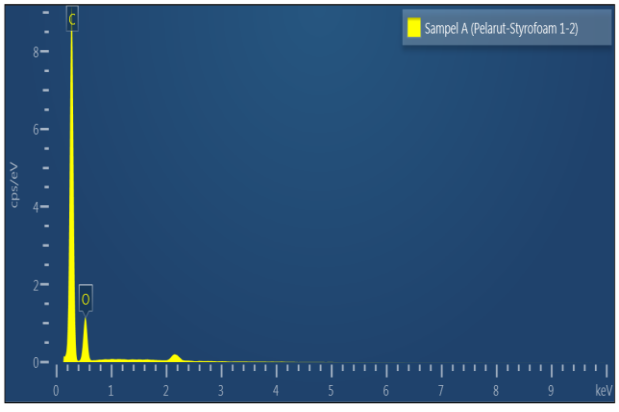


Figure 7 Content of elements C and O in the sample solvent –styrofoam in a ratio of 1:2 (Sample A)

SEM-EDS resulted in the two peaks that pointed to two types of elements, namely element C (carbon) and O (oxygen). The dominating element in the sample with a solvent and styrofoam ratio of 1:2 was consist of element C at 81.45% while element O at 18.55% (Figure 7).

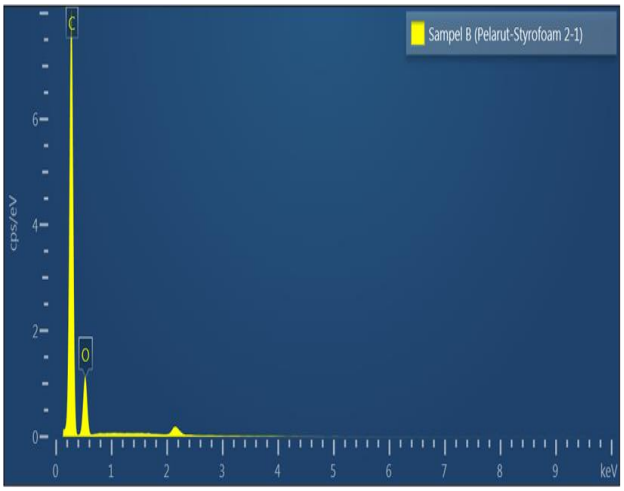


Figure 8 Content of elements C and O in the sample solvent –Styrofoam in a ratio of 2:1 (Sample B)

Regarding to the ratio of solvent and Styrofoam of 2:1, the results obtained that element C dominated with a value of 80.41%, while element O of 19.86% (Figure 8). These results illustrated a normal condition, that Styrofoam in general is indeed composed of several chemicals, such as benzene and styrene, which have element C followed by many functional groups to form poly bonds and then called polystyrene. This is supported by the research of Zong *et al.* (2021), who stated that microplastic polystyrene was synthesized from the chemical styrene, and benzene was also found [28]. In addition, polystyrene is said to be able to reduce the soluble properties of organic materials as seen from ultraviolet radiation in adsorption experiments on several compounds [29].

Changes in Blood Level

Samples of Fish blood were carried out 3 times for 0 days, 15 days, and 30 days for the experiment of K (commercial feed without microplastic mixture) and mixed doses of commercial feed and microplastic, namely A (5%), B (10%), and C (15%). Blood parameters observed were erythrocytes, leukocytes, hemoglobin, and leukocyte differential consisting of neutrophils, monocytes, and lymphocytes.

A: Erythrocytes

The number of red blood cells describes a state of health, and the low value of erythrocyte indicates a fish is in a state of stress or there is a foreign object enter to its body [30]. Based on the observation

results, the erythrocyte values were presented in Figure 9.

Based on Figure 9, the lowest average erythrocyte value in dumbo catfish reached of 0.65±0.24 (x10⁶) cells/m^{m3} on the first day of maintenance (H0) with treatment B. While the highest mean levels of erythrocytes in dumbo catfish were in the control treatment with the 30th day of maintenance of 1.04±0.12 cells/m^{m3} and followed by treatment B with a value of 0.93±0.11 cells/mm3. Then a test was carried out with one-way ANOVA analysis using SPSS statistics 25 to see and determine the effect of exposure to microplastics mixed through feed on the erythrocyte levels of dumbo catfish. The pattern of increased erythrocyte levels is presented in Figure 8 as follows.

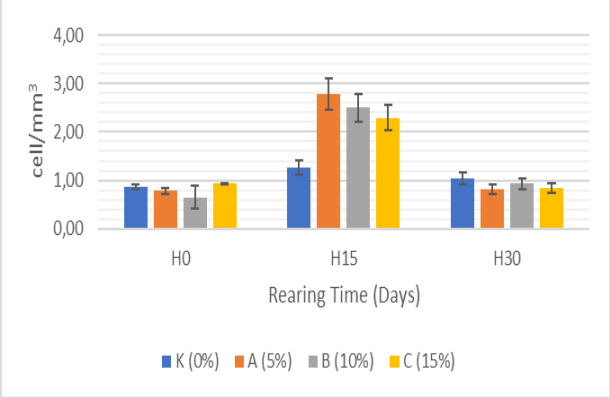


Figure 9 Erythrocyte Value of Dumbo catfish (*Clarias gariepinus)*

The results of the one-way ANOVA analysis are calculated, which shows that exposure to microplastics with different doses did not have a significant effect (P>0.05) on the erythrocyte levels of dumbo catfish at each rearing time, 0 day, 15 days and 30 days. However, in this study, it can be seen that the levels of erythrocytes in treatment A (5%), B (10%) and C (15%) with the rearing time of the 15 days increase significantly from 1.26–2.29 (x10⁶) cell/mm³. Then it decreased again at the 30th day of further rearing time to 0.83 (x10⁶) cell/mm³.

Figure 9 showed seen that each treatment tends to the normal range of erythrocyte value, which is around 0.65-2.78 (x10⁶) cells/mm³. The results are in accordance with Ekyall et.al. (2019) that the erythrocyte level is 0.65-1.54 (x10⁶) cells/mm³ [31]. The range of erythrocyte values was included in the range of normal erythrocytes. Normal dumbo catfish erythrocyte values were in the range of 2.00–3.00 (x 10⁶) cells/mm³ [32]. The increase of erythrocyte value in 15 days was reached due to the response of the fish's body to microplastic exposure, which was given through the feed mixture. The increase of erythrocyte value after exposure to microplastics is a challenging test in this study as showing fish stress. Fish stress was caused by increasing in erythrocytes value. This phenomenon illustrated the effect of microplastic given to feed mixture that occurs due to exposure from outside the fish's body [33].

B. Leukocytes

Leukocytes are blood components that play an important role in fighting foreign objects that enter the fish's body. Based on the observations, the leukocyte values were presented in Figure 10 below.

Based on Figure 9, the lowest average leukocyte values were found in treatment B (10%) with a a rearing time of 30 days of 12.90 (x10³) cells/mm³. While the highest average leukocyte levels in African dumbo catfish were in treatment C (15%) with 30 days of maintenance. Then a test was carried out with one-way ANOVA analysis using SPSS statistics 25 to see and determine the effect of exposure to microplastics mixed through feed on leukocyte levels of dumbo catfish.

The results of the one-way ANOVA analysis were calculated, which shows that exposure to microplastics with different doses did not have a significant effect (P>0.05) on the erythrocyte levels of dumbo catfish at each rearing time of 0 days, 15 days, and 30 days. However, leukocyte values in treatment C (15%) described a gradual increase in leucocyte value that start from 15 days until 30 days. The increase in the average number of leukocyte values is achieved due to leukocytes responding to the presence of foreign bodies from outside the body of the dumbo catfish. This was supported by the statement of Ding et.al. (2018) that an increase in the number of leukocyte levels can occur due to the body's response to bad environmental conditions, stress factors, and disease infections [34]. The increase in the average number of leucocyte values of dumbo catfish was presented in Figure 10 as follows,

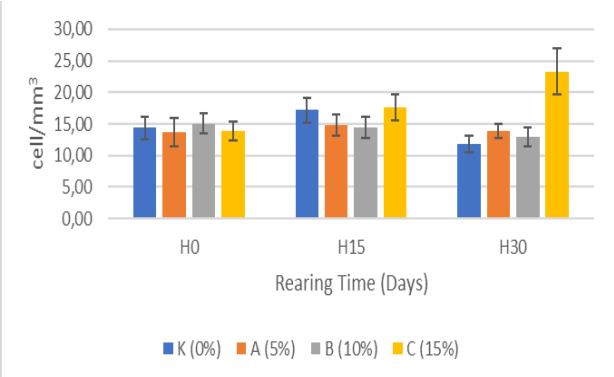


Figure 10 Leukocyte Value of Dumbo catfish (*Clarias gariepinus*)

The average number of leukocyte levels in African dumbo catfish in each of these treatments was in the range of normal fish leukocyte levels and some were in the range of high leukocyte levels, which ranged

from 11.84 – 23.28 (x10^3) cells/mm^3. Normal leukocyte value was achieved in the range of 3.39 to 14.20 (x10^3) cells/mm^3 [35]. Moreover, an increase in the number of leukocytes is called leukocytosis, while a decrease is called leucopenia. This leukocytosis can occur in a healthy or sick state and is usually physiological or pathological [36].

C Hemoglobin

Based on the observation results, the hemoglobin value is presented in Figure 11 as follows. Based on Figure 11, it illustrated that the average hemoglobin values during the rearing time of 15 days to 30 days were reached in the range of 2.18-2.52 g/dL. The highest hemoglobin value using treatment A was 5.58 g/dL. Meanwhile, the lowest hemoglobin value was found by using treatment A of 2.18 g/dL with a rearing time of 0 days. The same value was achieved by rearing time of 30 days, which showed the lowest hemoglobin value of 3.31 g/dL. The value changes of hemoglobin are related to the balance of blood plasma osmolarity [37].

The results of the one-way ANOVA analysis are presented in Appendix 4 which shows that exposure to microplastics with different doses had a significant effect ($P<0.05$) on the 0th day of maintenance for each treatment, namely treatment A (5%), B (10 %) and C (15%). However, after further ANOVA testing, the results obtained were not significantly different between treatments, namely on the 10th day of maintenance. Furthermore, for the 15th and 30th days of maintenance, based on the results of one-way ANOVA analysis, it was shown that treatment with different doses of microplastic exposure did not have a significant effect ($P> 0.05$).

Changes in hemoglobin values indicated that the fish is under stress. If the hemoglobin level of fish decreases, the fish will experience anemia. Several factors that affect low hemoglobin are protein content in the feed, water quality, and the presence of pathogen infection. Decreased hemoglobin value cause hypoxia to the fish, so metabolic processes will be disrupted [32,38]. Hemoglobin concentration can be seen based on the color density of the red blood cell nucleus. In general, hemoglobin levels are proportional to the number of erythrocytes [29]. The concentration of hemoglobin in the blood has a positive correlation with the number of erythrocytes. If the number of erythrocytes is higher, the hemoglobin level will also be higher and vice versa [35]. The pattern of increase and decrease in the average hemoglobin level is presented in Figure 11.

The results of this study showed that dumbo catfish were treated with a mixture of microplastics from 0 days to 30 days, which had an average hemoglobin value of 2.18 – 5.58 g/dL. The average hemoglobin level in this study was below normal. According to the statement of Banaee et.al. (2019) healthy and normal dumbo catfish are fish with hemoglobin levels ranging from 12-14 g/dL [40]. In addition, normal hemoglobin levels in fish blood generally range between 9-13 g/dL [40].

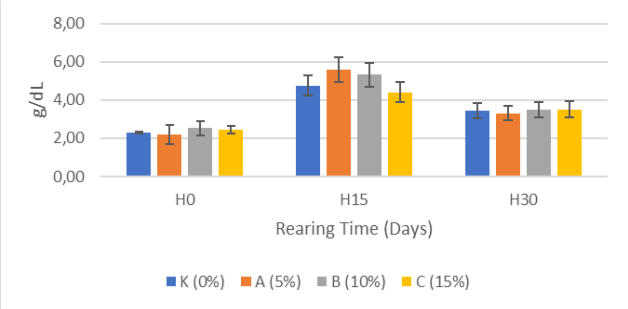


Figure 11 Hemoglobin value of dumbo catfish (*Clarias gariepinus*)

D. Leucocyte Differential

Leukocyte differential is a unit of white blood cells that consists of two groups, namely granulocytes and agranulocytes [25]. Leucocyte differential observation is used to determine differences in the percentage of leukocyte cell components. The components of leukocyte cells include 3 cells, namely lymphocytes, monocytes, and neutrophils.

(1) Lymphocytes

Based on Figure 12, the average value of the lymphocyte count of dumbo catfish given microplastic exposure via the feed mixture that shows the lowest number of lymphocyte cells of 35.33% was used by treatment C (15%) and rearing time of 15 days. Meanwhile, the highest lymphocyte values using treatment C and a rearing time of 30 days. The average value of lymphocyte values on 0 days was reached in the range of 72.79-78.70%, which was included in the category of normal lymphocyte value of dumbo catfish.

This is in accordance with the statement of Chen *et al*. (2020) that normal lymphocyte levels in healthy fish are 60.20-81.00% [33]. Meanwhile, on the 15th day of maintenance in each treatment, the lymphocyte levels decreased and were included in the category of abnormal lymphocyte counts. The decrease in lymphocyte levels is thought to be due to fish responding to exposure to microplastics as foreign bodies that want to enter the fish's body. This is in accordance with the statement of Preanger et. al. (2016) that the decrease in the percentage of dumbo catfish (*Clarias gariepinus*) lymphocytes used as a sample experienced stress and the result of the environment where the dumbo catfish was bred was in an unfavorable condition [10].

Based on the results of the one-way ANOVA test were calculated, there was no significant difference between the treatments and at each rearing period ($P>0.05$). However, when compared descriptively, the lymphocyte levels in the African dumbo catfish

on day 15 responded spontaneously to the microplastic exposure given so that the decrease in lymphocyte levels was seen to be two-fold lower compared to day 0. Then it went back up on the 30th day and was included in the normal category which was suspected because the dumbo catfish was starting to get used to this exposure as food. an increase in the percentage of lymphocytes as a sign of the success of the immune system in developing a cellular (non-specific) immune response [40]. The pattern of changes in lymphocyte levels of dumbo catfish treated with microplastic exposure through the feed mixture is presented in Figure 12.

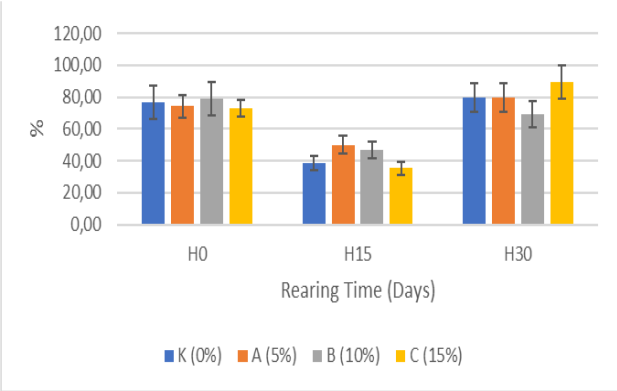


Figure 12 Lymphocyte Value of Dumbo catfish (*Clarias gariepinus*)

(2) Monocytes

Figure 13 illustrated the average amount of dumbo catfish (*Clarias gariepinus*) MonocyteCellsl. The value of the lymphocyte of dumbo catfish given microplastic exposure caused by feed mixture. It shows that the lowest number of monocyte cells was using treatment A (5%) of 10.15% with a rearing time of 0 days. (H0). Then for the highest number of monocyte levels, namely in treatment A (5%) but with a rearing time of 30 days (H30). Then a test was carried out with one-way ANOVA analysis using SPSS statistics 25 to see and determine the effect of exposure to microplastics mixed through feed on monocyte levels of dumbo catfish.

Based on the results of the one-way ANOVA test calculated, there was no significant difference between the treatments and at each rearing period (P>0.05) for the monocyte content parameter for the fish given the feed mixed with polystyrene microplastics. However, monocyte levels during the study were in the above-normal range, of 10.15 to 21.45%. Meanwhile, the normal level of monocytes in fish is 0.1% of total leukocytes [10]. Monocyte levels in this study are also in line with the statement of Muntasiroh et. al. (2018) that monocyte values in the range of 6.69%- 0.98%, observed the dumbo catfish (*Clarias gariepinus*) were still in a normal state [41]. Monocytes play a role in phagocytosis activity after being in the tissue. The pattern of changes in monocyte levels is presented in Figure 13 below.

The occurrence of changes in monocyte cells is the same as with lymphocytes where the increase in blood cells in fish was a response to experiencing stress caused by changes in environmental conditions.

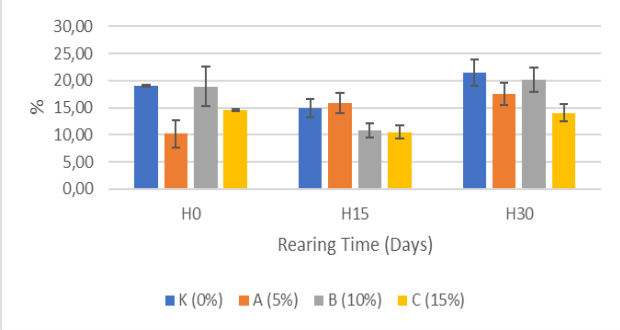


Figure 13 Monocyte values of dumbo catfish (*Clarias gariepinus*)

It can also occur due to the contamination of foreign bodies. Monocytes and lymphocytes are very similar in shape. The difference is that monocytes have a large and lobulated nucleus [34[. The role of monocytes is very important as the main phagocytic cell to destroy various invading pathogens and plays a role as antigen-presenting cells (APC) whose function is to present antigens to lymphocyte cells [35, 42]

(3) Neutrophils

Based on Figure 14, it was illustrated that the lowest average leukocyte values were found at 35.33% using treatment C (15%) and a rearing time of 15 days Meanwhile, the highest average leukocyte values in dumbo catfish were found in treatment C (15%) with the 30th day of maintenance of 89.61%. Then a test was carried out with one-way ANOVA analysis using SPSS statistics 25 to see and determine the effect of exposure to microplastics mixed through feed on leukocyte levels of African dumbo catfish. The pattern of changes in neutrophil levels is presented in Figure 14 as follows.

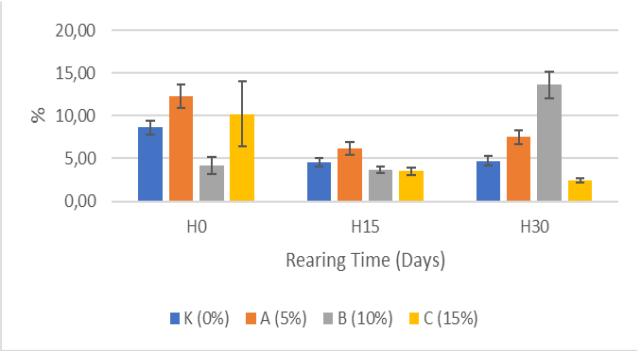


Figure 14 Neutrophil values of dumbo catfish (*Clarias gariepinus*) during the study

Based on the results of the one-way ANOVA test that was calculated, there was no significant difference between the treatments and at each rearing period (P>0.05) for the neutrophil content parameter for the fish given the feed mixed with polystyrene microplastics. The neutrophil levels in this study decreased from the 15th day of rearing time and increased on the 30th day of rearing time. The decrease on the 15th day was suspected because the fish experienced stress due to exposure to microplastics and then increased on the 30th day of rearing time as one of the successful formations of the fish's immune system and began to get used to exposure to these microplastics as food. However, the neutrophil values increased significantly in the range of 35.33% to 89.61%. Meanwhile, the average total number of normal neutrophils in dumbo catfish is 3.25 – 6.40% [41].

Increased production of white blood cells will increase neutrophils in the peripheral blood and exceed the normal limit of the percentage of neutrophils. Neutrophils are a type of white blood cell that are markers of inflammation or inflammation. Neutrophils will work when there is a signal from the hormone cytokine which indicates the location of inflammation in the body [30]. Neutrophil cells increase in pathogen and-infected tissue. Neutrophil cells increased activity during infection as a response to chemicals stimulated by immunostimulants [42].

Water quality analysis has been analyzed to support the results of fish diseases that were not caused by water conditions. The parameters were temperature, pH, dissolved oxygen (DO), ammonia, nitrate, and nitrite. Table 3 showed the data on water quality after the study by adding microplastic to the fish feed. All parameters are in the range of standard water quality. It is concluded that the cause of unhealthy dumbo catfish after the study is the exposure of microplastics in the fish blood.

Table 3 Data of water quality after treatment by adding the microplastic in the fish feed

Parameters	Water quality				Standard
	K	A	B	C	[19]
Temperature, ^{0}C	25-27.9	25-27.8	25-28.7	25-28.6	25-30
pH	6.8-7.91	6.7-7.89	6.7-7.86	6.8-7.89	6.5-8.0
DO, mg/L	3.8-6.0	4.3-6.2	4.5-6.7	3.9-5.9	>3
Ammonia, mg/L	0	0	0	0	0
Nitrate, mg/L	0	0	0	0	0
Nitrite, mg/L	0	0	0	0	0

4.0 CONCLUSION

In this study, the effect of microplastics in the fish feed was analyzed deeply, which caused the change in the blood level of fish. Scanning electron microscope–energy dispersive system (SEM-EDS) is used to analyze the pictures and composition of microplastics. The blood of dumbo catfish was analyzed through the parameters of the erythrocytes, leucocytes, hemoglobin, and differential leucocytes. It showed that the increase in rearing time from 0 to 30 days achieved the highest erythrocyte value on rearing time of 15 days and a dose of a mixture of commercial feed and microplastics of 15%. Meanwhile, the number of monocytes and lymphocytes reached the highest value on the mixture of commercial fish feed and microplastics at 15% and a rearing time of 30 days. Leucocytes and hemoglobin produced the highest value on treating of mixture commercial fish feed and microplastics of 10% and a reaching time of 15 days.

The amounts of erythrocyte, leucocyte, hemoglobin, and differential leucocyte components in the blood were analyzed to determine whether fish underwent a stress reaction after consuming microplastic. The four parameter values did not show any signs of stress in the fish.

Conflicts of Interestf

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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