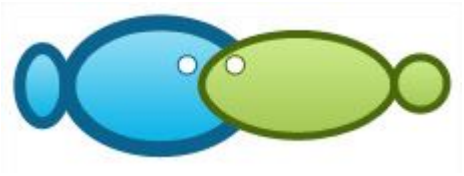

Oreochromis niloticus immune responses examination: a case study of environmental toxicology

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Abstract. For freshwater fish, Tempe liquid waste (TLW) toxicity is not considered harmful, since it does not cause death and/or physical changes to the fish. In this context, this study aimed to examine the effect of TLW on the immune response and survival of *Oreochromis niloticus*. As a case of environmental toxicology, this study consisted of 4 treatments that supported three replications each, with various concentrations of TLW (0%, 5%, 10%, and 15%), which was introduced to *O. niloticus* water media. The parameters, including immune responses (white blood cell - WBC, red blood cell - RBC, hematocrit, and hemoglobin) and survival rate (SR), were evaluated in order to determine the effect of TLW contamination. All data were analyzed via the SPSS program to provide statistical analysis. The results indicated that *O. niloticus* secreted copious mucus and lost its body pigment in response to TLW treatment as a stress response and avoidance behavior. In contrast, the immune parameters were mostly unaffected after TLW treatment, such as red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) ($p > 0.05$), except for white blood cells (WBC). White blood cells level of *O. niloticus* decreased significantly after 30 days of treatment in T3, while T1 and T2 had no distinction from T0. Furthermore, there was a considerable decline in lymphocytes, neutrophils, and phagocytosis activity for T2 and T3. Meanwhile, monocytes and basophils were normal in all treatments as the control group. The best treatments were found at T0 and T1 in SR data, reaching 87%. Therefore, TLW with a high concentration was not suitable for tilapia survival. According to the research findings, *O. niloticus* immune responses may have been influenced by TLW contamination, which resulted in a decrease in SR.

Key Words: immunology, phagocytosis, Tempe wastewater, tilapia.

Introduction. Tempe, an authentic Indonesian food, was made of fermented soybean with tasteful flavor and great digestibility (Nout & Kiers 2005; Nurdini et al 2015; Hartini et al 2018). In fact, there has been a significant increase in Tempe production (Nout & Kiers 2005; Gunawan-Puteri et al 2019), particularly in the Sanan Region of Malang, East Java, Indonesia, which at the same time increases waste products, both solid and liquid. For instance, the household-scale industry is estimated to produce the liquid Tempe wastes 200 to 300 L per day from 300 kg of soybeans (Hikma et al 2014). According to Harahap (2013), TLW contains 28.17 to 34.69 mg L^{-1} of ammonia (NH_3) released into the Berantas river, East Java, Indonesia. Furthermore, NH_3 is hazardous in aquatic habitats at values ranging from 0.53 to 22.8 mg L^{-1} (Li et al 2015). As a result, the release of TLW might establish an environmental threat (Puspawati & Soesilo 2018). They have two primary forms coming in unionized ammonia (NH_3) and ionized ammonium (NH_4^+) (Randall & Tsui 2002; Shin et al 2016), which are primarily determined by pH, temperature, and salinity (Dutra et al 2016; Pedrotti et al 2018).

Ammonia's acute and chronic toxicity to freshwater and marine fish has been reviewed (Person-Le Ruyet et al 1995; Camargo et al 2005; Ying et al 2018). Excessive NH_3 leads to the decrease in fish growth level (Li et al 2014; Sun et al 2014), tissue destruction (Smart 1976; Rama & Manjabhat 2014), immunity suppression (Chen et al 2016), energy metabolism (Sinha et al 2012), and cause mortality to aquatic animals

(Pedrotti et al 2018). As a result, the accumulation of NH_3 in the fish body causes damage to the organism by raising its levels in the blood and tissues (Barbieri & Bondioli 2015). According to Sinha et al (2012), the exposure to NH_3 impacts the fish's metabolic conditions and nutrition status, which breaks down tissue contraction. Besides NH_3, TLW also has a high concentration of biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), and pH, 950 mg L^{-1}; 1,534 mg L^{-1}; 309 mg L^{-1}; and 5, respectively (Dutra et al 2016). The small amounts of BOD and COD have had no discernible effect on the river's water quality (Amirkolaie 2008). However, toxic levels of BOD and COD would break fish respiration, leading to mortality (Kumari et al 2011).

Tilapia (*Oreochromis niloticus*) may survive in various freshwater ecosystems, including lakes, reservoirs, and marshes (Lowe et al 2012; El-Sayed 2019). Because of their high demand and simplicity of growing, tilapia is a common aquaculture product in East Java, Indonesia (Mahmudi et al 2019). Studies on TLW's effect on freshwater fish are limited due to its presumed nature, which is not hazardous to *O. niloticus*. Whereas, the present study attemps to reveal that TLW released into the river can be a serious toxic to the immune, growth, and survival rate of *O. niloticus*.

Material and Method

Fish and TLW preparation. The TLW was obtained from from Sanan, Malang, East Java, Indonesia. The TLW was dissolved in water at varying doses, including 0% (T0), 5% (T1), 10% (T2), and 15% (T3). The study was conducted from June to August 2021 in the Laboratory of Fisheries, University of Muhammdiyah Malang, Indonesia. The twelve tanks (60 cm × 30 cm × 30 cm) were equipped with water recirculation for 4 treatments and 3 replications.

More than one hundred and eighty *O. niloticus* (8-10 cm) were purchased from an Indonesian fish farm in Tulungagung before being acclimated at 28°C with aeration in a tank. For two weeks before treatment, the fish were fed a commercial diet containing 3% of the fish's body weight every day.

Effect of TLW on the immune response. Erythrocyte count applied the method of Pal (2006) with some modifications. The fish blood was sucked by using erythrocyte pipette and dissolved in Hayem's solution. The liquid was put into a counting chamber and counted under a microscope. All erythrocytes were counted in 5 fields composed of 16 small areas on a hemocytometer:

$$N = n \times 10^4$$

where: N = erythrocyte total in 1 mL of blood;
n = erythrocyte total in the 80 areas of a hemocytometer.

A hemocytometer with an improved Neubauer Counting Chamber calculated the number of leukocytes. In the first step, fish blood was first diluted with Turk solution up to 20 times dilution, then placed in Counting Chamber. The leukocyte count was determined by using a microscope with a magnification of 10 × 40 times and calculated with the formula:

$$\text{Total numbers of WBCs present in 1 mm}^3\text{ of blood} = \frac{\text{The number of cells counted}}{\text{The number of 1 mm}^2\text{ counted}} \times \text{dilution}$$

The hemoglobin content of *O. niloticus* was determined by using the Sahli method, which involved dissolving 20 mm^3 of blood in 3.8 percent sodium citrate in a Sahli pipette (Barduagni et al 2003; Faatih et al 2017). The Sahli tube was then filled to the limit mark 2 with 0.1 N HCl solution while the fish blood was sucked using a 20 µL Sahli pipette. Afterward, the 20 µL of blood was transferred into the Sahli tube containing 0.1 N HCl. Following that, the mixture was pipetted 2–3 times and incubated for 3–5 minutes. The aquadest was then carefully dropped and stirred. The mixture's hue was then compared to the standard's. If the sample color is still darker than the standard, add distilled water until the color is the same. The Sahli tube scale measures hemoglobin in g dL-1.

A microcapillary tube was filled with fish blood and closed with paraffin to measure the hematocrit. The hematocrit (HCT) tube was spun at 5000 rpm in a centrifuge tube for five minutes, and then the HCT value was calculated using a ruler (Hudson et al 2008). Following the Svobodová et al (2008) procedure, a blood slide was allowed to dry in the air for a few seconds before being fixed in methanol for 5 minutes to count different leukocytes. The blood slide was stained for 30 minutes with Giemsa before being rinsed with distilled water and dried in the air. The slide was examined under a 1000x microscope.

The phagocyte's activity was measured by carefully cutting the hematocrit tube between erythrocytes and leukocytes. The leukocytes of 100µL were transferred into the microplate well and then *Aeromonas hydrophila* (10^5 cell mL^{-1}) with the same volume. The mixture was put on a slide and dried at room temperature after 20 minutes of incubation. Afterward, they were drained and dyed with 7 percent Giemsa for 10 minutes. The number of blood cells digesting *A. hydrophila* was used to calculate the phagocytosis activity percentage. (Andriawan et al 2019).

$$\text{Phagocytosis activities} = \frac{\text{Phagocyte cells}}{\text{WBCs Total}} \times 100$$

Moreover, the derived hematological including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), were measured employing standard formulation by Mostakim et al (2015):

$$MCV = (HCT \div RBC \text{ in millions}) \times 10\ \mu m^3;$$

$$MCH = (Hb \text{ in g} \div RBC \text{ in millions}) \times 10\ pg;$$

$$MCHC = (Hb \div HCT) \times 100g \text{ per } 100\ mL;$$

$$SR = N_t / N_0$$

where: SR = survival rate (%);
N_0 = initial number of fish;
Nt = final number of fish.

Statistical analysis. ANOVA (One-way analysis of variance) and the Duncan test were applied to evaluate significant differences among treatments using SPSS (version 17, USA). Results were displayed as the mean±SD, $p < 0.05$ was chosen as the significance level for every test.

Results

Stress response and avoidance behavior. Figure 1 indicates the unfavorable effect of 30 days of TLW exposure to *O. niloticus*. In all treatments, TLW degraded O. niloticus color and increased mucus production. Based on those findings, the current study hypothesized that *O. niloticus* engaged in avoidance behavior to ensure survival following TLW administration.

Figure 1. *O. niloticus* pre-treatment with TLW (left) and post-treatment with TLW (right).

The immunity responses. The innate immune system is a no-specific defense that serves as a defense system for infectious diseases and toxins. Various blood parameters

are often examined in this scenario to stress circumstances and environmental concerns. The purpose of this study was to determine the influence of TLW on RBC, WBC, Hb, HCT, and erythrocyte indices, including MCV, MCH, and MCHC, in *O. niloticus* (Table 1). The present study found that T2 showed the highest level of Hb (8.33±0.58 g mL^{-1}), followed by T1, T3, and the control group (7.67±0.58 g mL^{-1}, 7.67±0.58 g mL^{-1}, and 6.67±1.15 g mL^{-1}, respectively). Only T2 exhibited a statistically significant difference from the control group (T0) compared to T1 and T3, while T1 and T3 did not. Moreover, *O. niloticus* levels of RBC and HCT were not affected by treatment ($p > 0.05$). As a result, no significant hemoglobin and RBC level affected the calculation of MCH and MCHC, which was proved with the same notation in all treatments.

As for *O. niloticus* WBC dropped significantly by T3 to 54.93 10^3 mm^{-3}, which was the greatest concentration of TLW. The present investigation was based on the hypothesis that *O. niloticus* WBC decreased due to the high level of environmental stress. Meanwhile, T1 and T2 (77.3±1.20 × 10^3 mm^{-3} and 71.73±0.90 × 10^3 mm^{-3}, respectively) generated the same level as the control group ($p > 0.05$). In the further analysis, the higher concentration of TLW (T3) also declined monocyte, lymphocyte, neutrophil, and basophil level (0.67±0.57, 3.33±1.16, 79±7.93, and 6±2.00, respectively) (Table 2). It is related to WBC results that also decreased by the higher concentration of TLW. Regarding those findings, the present study assumed that the addition of TLW could only influence WBC in high quantity, but it did not affect Hb and HCT.

Moreover, all treatments presented the same tendency regarding macrophages' phagocytic activity for pre-treatment ($p>0.05$), while after treatment, it was higher in treated fish than in control fish. The phagocytic activity in treated fish was 36.53%, 51.15%, and 45.03% on average (T1, T2, and T3, respectively), while the fish control group (T0) was only 23.77%.

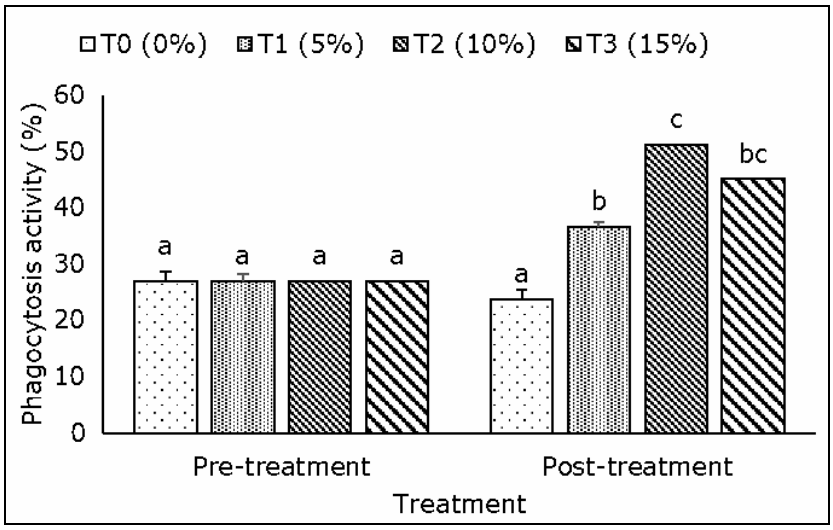

Figure 2. Index phagocytes with various treatments.

Survival rate. The SR data showed a significant difference among treatments, particularly at week 1, 3, and 4. Overall, all treatments had SR decreases moderately until the end of the period presented in Figure 3. However, the T1 still could be determined as the recommended treatment to promote *O. niloticus* survival rate (86.67%). Meanwhile, T2 was the worst treatment for *O. niloticus* SR based on yield statistics. According to the research findings, *O. niloticus* could withstand low concentrations of TLW but no large concentrations.

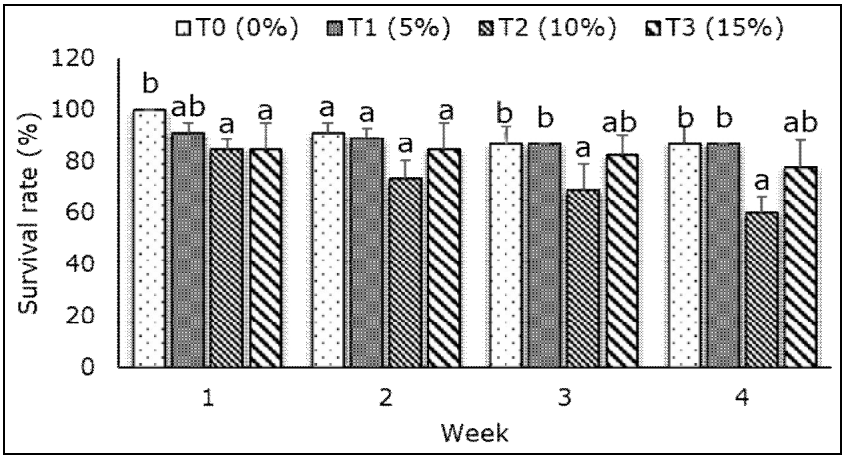

Figure 3. *O. niloticus* survival rate recorded within 4 weeks.

Table 1

Hematological change of *O. niloticus* exposed to different concentrations of TLW. Data are the mean of three replicates, and ± values are standard errors

Parameters	*Data sampling*				
	1st day	*30th day*			
	Control	*T0 (0%)*	*T1 (5%)*	*T2 (10%)*	*T3 (15%)*
RBC (10^6 mm^{-3})	7.8±0.33	8.0±0.03^a	8.1±0.01^a	8.3±0.01^a	8.4±0.02^a
WBC (10^3 mm^{-3})	82±0.45	72.43±0.45^b	77.3±1.20^b	71.73±0.90^b	54.93±0.58^a
Haemoglobin (g dL^{-1})	6.67±1.15	6.67±1.15^a	7.67±0.58^{ab}	8.33±0.58^b	7.67±0.58^{ab}
Haematocrit (%)	18.2±1.20	17.83±1.20^a	18.97±2.78^a	19.23±2.91^a	20.43±1.39^a
MVC	233.33±1.1	222.87±1.12^a	234.19±4.17^{ab}	231.68±9.16^{ab}	243.21±10.6^b
MCH	8.55±0.12	8.232±1.41^a	9.43±0.71^a	10.47±0.66^a	0.91±0.45^a
MCHC	21.97	37.41±6.48^a	40.43±3.12^a	43.31±1.89^a	37.54±3.21^a

Note: the different superscripts in the same row indicate significant differences between treatments ($p < 0.05$).

Table 2

Differential leucocytes of *O. niloticus* were exposed to different concentrations of TLW. Data are the mean of three replicates, and ± values are standard errors

Parameters	*Data sampling*			
	30th day			
	0%	*5%*	*10%*	*15%*
Monocyte	5.3±1.52^{ab}	5.7±0.57^b	4.0±0.10^{ab}	3.33±1.16^a
Limphocyte	183.6±9.86^c	184±6.92^c	116.3±13.57^b	79±7.93^a
Neutrophil	14±1.73^b	8±1.00^a	6±1.73^a	6±2.00^a
Basophil	2.33±1.15^{ab}	3.00±1.00^b	0.67±0.57^a	1.33±0.57^{ab}

Note: the different superscripts in the same row indicate significant differences between treatments ($p < 0.05$).

Discussion

Stress response and avoidance behavior. *O. niloticus* reduced its color significantly after TLW exposure. The present study believed that those actions revealed an environmental acclimation because of the high ammonia level from TLW. Avoidance behavior is a defensive mechanism against an unsupported environment (Ragen et al 2016). It has been studied in many animals, such as land and water animal (Sweatt 2010; Thieltges & Poulin 2010; Lari & Pyle 2017). Many scientists have paid attention to similar results in different fish species in response to various toxicants (Hussain et al 2015), such as excessive mucus secretion (Iwama & Nakanishi 1996; Bols et al 2001),

color change (Nur et al 2019), tissue fasciculation (Croke & McDonald 1998), rejection of feeding (Alonso & Valle-Torres 2018), and respiratory distress (Shinn et al 2015; Olowolafe & Olufayo 2018). In a study by Nur et al (2019), 0.01 mg L^{-1} to 0.06 mg L^{-1} of mercury as a toxicant agent reduced the pigment of *Carassius auratus.*

In another study, avoidance behavior was shown by rainbow trout with avoiding and detecting toxicants by their olfactory function when exposed to 0.1 %, 1 %, and 10 % of oil sands process-affected water (OSPW) (Lari & Pyle 2017). Similar behavior was also shown by *O. niloticus* and *Clarias gariepinus* to 50 mg L^{-1}, 60 mg L^{-1}, 70 mg L^{-1}, 80 mg L^{-1}, 100 mg L^{-1}, and 120 mg L^{-1} copper. They showed unbalanced swimming, vertical position, motionless and loss of stability (Ezeonyejiaku et al 2011). Xu et al (2005) also proved that behavior changes were shown by *O. niloticus* toward 0.13 mg L^{-1}, 0.79 mg L^{-1}, and 2.65 mg L^{-1} of ammonia. Compared to other studies, the TLW usage negatively impacted *O. niloticus* pigment by few concentrations.

The immunity responses. The innate immune system comprises natural barriers, such as cellular and humoral units, against external materials, such as microbes and toxins (Andriawan et al 2019). The data reveals that the immune system of *O. niloticus* did not significantly differ from the control group ($p > 0.05$). Theoretically, RBCs, hematocrit, MCH, and MCHC have different functions on the immune mechanism. Generally, the primary role of RBCs plays a role in gas transportation to cells and tissues. However, they also showed other functions than oxygen delivery (Shen et al 2018), such as gene expression site (Morera et al 2011) and environmental stress (Lewis et al 2010). In some circumstances, exposing toxicants to the aquatic environment, such as dissolved nitrogen, inhibits RBC reduction (Manissery & Madhyastha 1993; Tilak et al 2007; Shin et al 2016). Meanwhile, the level of HCT presents some physiological conditions, including anemia, protein content, vitamin deficiency, and infection by infectious pathogens (Shabirah et al 2019). Somehow, this contradicts the current research findings that all treatments of TLW did not change *O. niloticus* RBC, HCT, MCH, and MCHC levels. Meanwhile, the previous study carried out by Harahap (2013) revealed that ammonia, nitrite, and nitrate were discovered at a high level.

Furthermore, Shin et al (2016) proved that 0.1 mg L^{-1} to 1.0 mg L^{-1} of Ammonia chloride (NH_4Cl) (Sigma, St. Louis, MO, USA) solution declined rockfish RBCs by two weeks of treatment at 24°C. Meanwhile, after 14 days of observation, ammonium nitrate (4 mg L^{-1} and 8 mg L^{-1}) decreased the Nile tilapia HCT % compared to the control group (Shokr 2015). The data of *C. gariepinus* MCH and MCHC levels evaluated by Oyeniran et al (2021) revealed that there was a climb significantly ($p < 0.05$) under abattoir wastewater influence. Based on those references, the present study assumed that the stability of *O. niloticus* RBC and HCT was due to the lactic acid bacteria (LAB) in TLW. According to Amaliah et al (2018), eight isolates were obtained from soybean soaking liquid waste, seven of which were LAB. Those bacteria help break down organic matter in water to improve water quality (Novik et al 2017). Therefore, further research needs to be conducted to examine the concentration of which TLW affects RBC and HCT. To sum up, TLW did not influence the number of *O. niloticus* RBC and their derivation during medium treatment.

O. niloticus Hb and MVC increased by T2 (8.33±0.58 g mL^{-1}) and T3 (243.21± 10.6), respectively, after the exposure of TLW media within 30 days. Theoretically, the function of hemoglobin shows to be accommodated to the various metabolic requirements of animals and steady environmental changes (De Souza et al 2007). Meanwhile, the MCV measures the RBCs' average volume in a blood test (Ware 2020). The low MCV value indicates a high average RBC count in *O. niloticus* blood. However, some studies showed that fish Hb decreased post-exposed water pollution (Tilak et al 2007; Patrik Saoud et al 2014; Honda et al 2017). In environmental contamination, ammonia, nitrite, nitrate (0.80 ppm, 171.36 ppm, and 1075.10 ppm, respectively) could drop *Cyprinus carpio* Hb within 24 hours (Tilak et al 2007). The decrease of fish hemoglobin leads to anemia which generates high fish mortality (Honda et al 2017). In the case of MCV, Oyeniran et al (2021) elaborated that *C. gariepinus* MCV level elevated after abattoir wastewater treatment reaching 163.00±12.81 fL on the 14th day before its

considerable decline, accounting for 94.20±2.54 fL on the 30th day. Therefore, these findings could be related to De Souza et al (2007) review that fish could adapt to environmental changes by raising their total hemoglobin content or performing changes in the intrinsic oxygenation characteristics, involving the structure and accumulations of hemoglobin components.

In addition, the highest concentration of TLW dropped the WBC level of *O. niloticus* after 30 days of observation. Meanwhile, the TLW with 5% and 10% concentrations was still suitable for *O. niloticus*. It is assumed that the decrease of WBC was stimulated by contamination of TLW in water media. The TLW has been mentioned to contain a high contamination compound, such as ammonia (Harahap 2013). Therefore, leukocytes are commonly used to observe stress conditions because of environmental changes (Davis et al 2008). Leucocyte is a part of fish's nonspecific immune system and act as an indicator of a fish's health (Pereira et al 2012). A decline in fish nonspecific immunity could be linked to leukocyte numbers after its exposure to contaminants (Hedayati 2012; Ribas et al 2016). According to Thangam et al (2014), after 35 days of ammonia exposure, freshwater fish *C. carpio* showed a considerable drop in leukocyte count. Using 4 mg mL^{-1} and 8 g mL^{-1} of ammonium nitrate in the same research significantly decreased *O. niloticus* leukocytes post 21 days exposure (Shokr 2015). Based on those findings, the amount of *O. niloticus* leukocytes was still tolerant to TLW under 15% concentration, but it began deteriorating over 15% application.

Finally, differential leukocytes during the study went down considerably in T2 and T3 compared to the control group. Based on leukocyte evaluation, differential leucocyte decrease might be caused by the high ammonia concentration in TLW. This finding is in accordance with Shokr (2015) who introduced various dosages of ammonium nitrate (2 mg mL^{-1}, 4 mg mL^{-1}, and 8 mg mL^{-1}) could decrease *O. niloticus* differential leukocytes on day 7. According to Modrá et al (1998), differential leukocyte counts are critical indicators of a fish's health and can be used to assess the immune system in several circumstances. Therefore, the low differential leukocytes could lead to high mortality of fish due to their inability to adjust to environmental conditions.

Survival rate. After 30 days of observation, only T1 did not affect *O. niloticus* SR. The high dose of TLW reduced the number of *O. niloticus* by almost 20% compared to the control group. It is believed that the ammonia in TLW inclined the death of fish subjects. According to Kim et al (2017), ammonia is a poisonous substance that could harm fish.

Interestingly, T2 exhibited a lower amount of SR than T3, which could be due to several reasons, including temperature and pH, which increase ammonia's toxicity (Romano & Zeng 2013; Souza-Bastos et al 2017). Those findings suggest that the loss of *O. niloticus* SR in T2 may have environmental consequences.

Conclusions. According to the observations, TLW contamination drastically lowered the color of *O. niloticus* after 30 days. This is referred to as avoiding an uncomfortable environment. The present study found that Only WBC, hemoglobin, and MVC altered by 10% to 15% of TLW in the immunological parameters. Moreover, The T2 dramatically reduced O. niloticus SR compared to other TLW treatments, indicating TLW toxicity. In summary, TLW in *O. niloticus* water media affects blood assessment and environmental adaptability.

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Conflict of interest. The authors declare that there is no conflict of interest.

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