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Haematological Alterations in *Oreochromis niloticus* Following Chronic Exposure to Coir Industry Dye Effluent Containing Malachite Green

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Abstract

Synthetic dyes such as malachite green, widely used in the coir industry, pose significant ecological risks due to their high toxicity, chemical stability, and persistence in aquatic environments. The discharge of untreated dye effluents into nearby water bodies severely affects aquatic organisms, particularly fish. The present study evaluated haematological alterations in *Oreochromis niloticus* fingerlings exposed for 10, 20, and 30 days to 1/10-1/5 of the 96-h LC_{50} of malachite green effluent. Haematological parameters, recognised as reliable biomarkers of physiological and metabolic stress, were analysed to evaluate toxic effects. Significant reductions were observed in Red Blood Cell (RBC) count, haemoglobin (Hb), Packed Cell Volume (PCV), and Mean Corpuscular Haemoglobin Concentration (MCHC) compared to the control group. The degree of decline was proportional to both concentration and exposure duration. In contrast, White Blood Cell (WBC) count, Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Volume (MCV) increased significantly, indicating immune activation and stress response to toxic exposure. These findings demonstrate that exposure to malachite green-containing effluent induces anaemia and compromises oxygen transport capacity in *O. niloticus*.

Major Findings: The study highlights the ecological risks associated with coir dye effluent containing malachite green discharge and emphasises the urgent need for effective wastewater treatment and sustainable industrial practices to protect aquatic biodiversity.

Keywords: Aquatic Toxicology, Coir Dye Effluent, Environmental Pollution, Haematological Parameters, Malachite Green, *Oreochromis niloticus*

Abbreviations: Haemoglobin (Hb), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Lethal Concentration 50% (LC_{50}), Mean Corpuscular Volume (MCV), Packed Cell Volume (PCV), Red Blood Cell (RBC) Count, White Blood Cell (WBC) Count.

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1. Introduction

The fisheries sector is vital to the national economy. It provides a sustainable source of animal protein, supports livelihoods, and improves food security[1]. However, rapid industrialisation and population growth have increased the release of chemical pollutants into aquatic environments. This has led to serious ecological damage[2]. Coir dye industry effluents, specifically malachite green, are of particular concern because they contain high concentrations of organic and inorganic compounds, which degrade water quality and pose serious risks to both aquatic organisms and human health[3]. Fish, as permanent residents of aquatic ecosystems, are constantly exposed to these contaminants. This makes them good indicators of environmental pollution. The buildup of harmful

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substances in their tissues can cause physiological, biochemical, and genetic changes. Thus, they are useful models for ecotoxicological studies[4]. Among different species, *O. niloticus*, or *Nile tilapia*, stands out as a model organism. It is economically important, adaptable, and easy to maintain in the lab. This fish is the second most farmed globally after carp, prized for its rapid growth, varied diet, and ability to thrive in different environmental conditions[5]. In addition to aquaculture, *O. niloticus* helps with food security by providing high-quality protein and essential micronutrients[6], aids rural livelihoods[7], and acts as a key species for evaluating the toxicity of industrial waste.

Water pollution remains one of the most critical global threats to aquatic biodiversity and human health[8]. In recent years, environmental toxicology has shifted from studying acute lethality to examining sublethal and chronic effects, which, though less apparent, can disrupt homeostasis and reduce organismal resilience[9]. Both natural and anthropogenic pollutants—including pesticides, fertilisers, and industrial dyes—have been shown to adversely affect the physiology, metabolism, and haematological parameters of fish[10]. Coir dye effluents are a significant source of aquatic contamination, largely attributed to the presence of malachite green, a synthetic triphenylmethane dye ($C_{23}H_{25}N_2Cl$) known for its intense colouration, high solubility, and chemical stability, which make it resistant to biodegradation[11]. Exposure to malachite green-contaminated water in aquaculture systems has been reported to inhibit protein synthesis, deplete glycogen reserves, impair oxygen utilisation, and elevate blood glucose and chloride levels in fish[12]. Furthermore, several studies have documented haematological disturbances such as reductions in red blood cell count, haemoglobin content, hematocrit value, and monocyte percentage, alongside elevated white blood cell counts in species like *O. niloticus*, *Clarias gariepinus*, and *Heteropneustes fossilis*[13,14].

Haematological parameters are widely recognised as sensitive biomarkers for assessing the physiological and pathological status of fish under stress[15]. Parameters such as erythrocyte and leukocyte counts, haemoglobin concentration, hematocrit, and derived indices (MCV, MCH, MCHC) provide valuable insights into the effects of toxicants, diseases, and environmental fluctuations[16,17]. Malachite green, due to its lipophilicity, persistence, and potential carcinogenicity in mammals, is of particular concern for aquatic ecosystems and food safety[18]. Despite extensive research on the toxicological effects of malachite green in various fish species, comprehensive data on chronic haematological disruptions induced by industrial coir dye effluents containing malachite green remain scarce, particularly in economically important species such as *O. niloticus*. Therefore, the present study aims to evaluate the haematological alterations in *Nile tilapia* exposed to sublethal concentrations of coir dye effluent containing malachite green. This investigation provides insight into the physiological responses of fish under chronic dye exposure and contributes to understanding the broader ecological and aquacultural implications of industrial effluent contamination.

2. Materials and Methods

2.1 Experimental Fish, Acclimatisation, and Husbandry Conditions

Healthy fingerlings of *O. niloticus* (*Nile tilapia*), measuring approximately 8–10 cm in length, were procured from a certified local hatchery. The fish were acclimated to laboratory conditions for two weeks before experimentation, during which they were closely monitored to ensure normal behaviour and overall well-being. During the exposure period, fish were maintained under semi-static conditions with regular renewal of test solutions prepared using tap water. All experimental groups, including the control, were kept under identical water renewal and husbandry conditions throughout the study. Water quality parameters were monitored regularly, with the pH maintained at 8.7 and the temperature at 29°C. Fish were fed twice daily, in the morning and evening, during the experimental period; however, to minimise the influence of feeding on haematological and biochemical parameters, feeding was discontinued one week before blood sampling.

2.2 Effluent Collection and Preparation

The dye effluent used in the study was collected from a coir dyeing unit located at Cherthala, Alappuzha, following the coir treatment process, and was representative of industrial effluent. Samples were collected in clean polyethene containers, transported to the laboratory, and stored at room temperature for a period of up to six months as stock effluent until use. Before experimentation, the effluent was characterised for basic physicochemical parameters, including pH (8.7) and temperature (29°C).

The effluent contained malachite green; however, the exact concentration of the dye (mg L^{-1}) was not determined.

For toxicity experiments, the effluent was treated as a stock solution and diluted volumetrically with freshwater. Exposure concentrations were expressed as mL L^{-1}, indicating the volume of effluent added per litre of test water. The LC_{50} value of 186 mL L^{-1} was obtained from an unpublished acute toxicity test conducted, based on statistical analysis. Accordingly, the LC_{50} concentration was prepared by adding 186 mL of dye effluent to 1 L of water. Sub-lethal concentrations (1/5, 1/7, and 1/10 of the LC_{50}) were prepared by adding proportional volumes of effluent to the test medium.

2.3 Experimental Design and Sampling

For each treatment concentration (1/5, 1/7, and 1/10 of the LC_{50} value) and the control, a total of 30 fish were used. The experiment followed an independent sampling design. Fish were allocated into three subgroups corresponding to 10, 20, and 30 days of exposure. At each exposure period, six fish (n = 6) were randomly selected and sacrificed for blood collection. Thus, different individuals were used at each time point, and no fish was sampled repeatedly. Feeding was stopped one week before blood sampling to minimise potential dietary effects on haematological and biochemical parameters, while routine feeding was carried out twice daily during the exposure period.

2.4 Blood Collection and Sample Handling

At the end of each exposure period, blood samples were collected from ten randomly selected fish per treatment and control group. Peripheral blood was drawn from the caudal vein using a sterile 1 mL heparinised syringe fitted with a fine-gauge needle. Fish were handled carefully to minimise stress and prevent hemolysis. Approximately 50–100 µL of blood from each fish was transferred immediately into pre-chilled microcentrifuge tubes containing heparin as an anticoagulant. To minimise diurnal variations, all collections were conducted during early morning hours under uniform conditions.

2.5 Ethical Considerations

The experimental procedures were conducted in accordance with standard institutional guidelines for the care and handling of laboratory animals. The study involved commonly cultured fish species and did not require specific institutional ethical committee approval as per prevailing institutional and national regulations. All efforts were made to minimise stress and discomfort to the animals throughout the experimental period, including proper acclimatisation, maintenance of optimal water quality conditions, and humane handling during sampling.

2.6 Determination of Red Blood Corpuscle (RBC) Count

The total RBC count was determined using a Neubauer counting chamber[19]. Blood was drawn into an RBC pipette up to the 0.5 mark and diluted with Hayem's fluid to the 101 mark (1:200 dilution). The pipette was gently rotated to mix the sample, and a cover glass was placed over the chamber. A drop of diluted blood was charged into the chamber at a 45° angle and left for 2–3 minutes to allow cells to settle. RBCs were counted under a high-power objective in 80 small squares of the central grid, including cells on the upper and left boundary lines but excluding those on the lower and right lines. The total number of RBCs per cubic millimetre of blood was calculated using the following relationship:

$$\text{Area of small square} = \frac{1}{400} mm^2$$

$$\text{Depth of chamber} = \frac{1}{10} mm$$

$$\text{Volume of one small square} = \frac{1}{4000} mm^3$$

$$\text{Dilution factor} = 1{:}200$$

$$\text{Total RBC count (cell/mm}^3\text{)} = \frac{N \times 4000 \times 200}{80}$$

where **N** = number of cells counted in 80 small squares. The final values were expressed as the number of erythrocytes per cubic millimetre (million cells/mm^3) of blood.

2.7 Determination of White Blood Corpuscles (WBC) Count

The total WBC count was determined following the method of Blaxhall (1973)[20]. Blood was drawn up to the 0.5 mark of the WBC pipette, and the diluting fluid

was aspirated up to the 101 mark above the bulb, giving a dilution of 1:200. The contents of the pipette were thoroughly mixed by gentle shaking and allowed to stand for 2–3 minutes to ensure uniform dilution. A drop of the diluted blood was then placed on a clean Neubauer haemocytometer by holding the pipette at an angle of approximately 40°, and the chamber was allowed to stand for another 2–3 minutes to permit proper settling of the cells. The white blood cells were counted in the four large corner squares of the Neubauer counting chamber under a microscope. The total number of WBCs per cubic millimetre (mm^3) of blood was calculated using the formula:

$$\text{Total number of WBC} = \frac{\text{Number of cells} \times \text{dilution factor} \times \text{depth factor}}{\text{Area counted}}$$

2.8 Estimation of Haemoglobin (Hb)

Haemoglobin concentration was measured using the acid–haematin method with Sahli's haemocytometer[19]. Briefly, 0.02 mL of whole blood was drawn into a Sahli's pipette and transferred into a haemometer tube containing 0.1 N hydrochloric acid (HCl) up to the specified mark. The HCl lysed the red blood cells, releasing haemoglobin, which reacted to form brown-coloured acid haematin. The mixture was thoroughly stirred and allowed to stand for a few minutes to ensure complete reaction. Distilled water was then added gradually while continuously mixing until the colour matched that of the standard comparator. The final reading was recorded at the lower meniscus, and haemoglobin concentration was expressed in grams per decilitre (g/dL).

2.9 Determination of Packed Cell Volume (PCV) or Haematocrit Value

Haematocrit values were measured using Wintrobe's method, following the microhaematocrit procedure[21]. This method was selected due to the limited volume of blood available. The principle is based on the difference in specific gravity between red blood cells (1.090) and plasma (1.030), causing erythrocytes to settle at the bottom under centrifugal force. Blood samples anticoagulated with suitable agents were carefully drawn into Wintrobe tubes up to the 10 mL mark. The tubes were centrifuged at 3000 rpm for 30 minutes, resulting in the separation of blood into three layers: a lower column of packed red blood cells, a thin intermediate buffy coat of white blood cells, and an upper layer of plasma. The lengths of the packed red cell column (L_1) and the total blood column (L_2) were measured in millimetres, and the haematocrit value was calculated using the formula:

$$\text{Ht (\%)} = \frac{L1}{L2} \times 100$$

2.10 Determination of Mean Corpuscular Volume (MCV)

Mean corpuscular Volume (MCV) reflects the average size of individual red blood cells and is expressed in cubic microns (μm^3) or femtolitres (fL). It serves as an important indicator for assessing erythrocyte size and classifying different types of anaemia. MCV was calculated according to the formula[22].

$$\text{MCV (fL)} = \frac{\text{Ht (\%)}}{\text{RBC (million/mm}^3\text{)}} \times 10$$

where Ht denotes the haematocrit (packed cell volume), and RBC is the red blood cell count.

2.11 Determination of Mean Corpuscular Haemoglobin (MCH)

Mean Corpuscular Haemoglobin (MCH) indicates the average amount of haemoglobin present in a single red blood cell and is expressed in picograms (pg). It provides information about the oxygen-carrying capacity of individual erythrocytes. MCH was calculated using the formula[22].

$$\text{MCH (pg)} = \frac{\text{Hb (\%)}}{\text{RBC (million/mm}^3\text{)}} \times 10$$

where Hb is the haemoglobin concentration, and RBC is the red blood cell count.

2.12 Determination of Mean Corpuscular Haemoglobin Concentration (MCHC)

Mean Corpuscular Haemoglobin Concentration (MCHC) represents the average concentration of haemoglobin within a given volume of packed red blood cells and is expressed as a percentage. It serves as an indicator of the haemoglobin saturation level in erythrocytes. MCHC was calculated according to the formula[22].

$$\text{MCHC}(\%) = \frac{\text{Hb}\ (\%)}{\text{Ht}\ (\%)} \times 100$$

where Hb refers to the haemoglobin content and Ht (haematocrit) represents the packed cell volume.

3. Statistical Analysis

Haematological parameters (Hb, RBC, WBC, PCV, MCV, MCH, MCHC) of *O. niloticus* were measured in four groups: control (CTRL) and three sublethal concentrations (1/5, 1/7, 1/10 of 96-h LC_{50}). Statistical analyses were performed using R software[23] (version 3.4.0). Data were first tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. One-way analysis of variance (ANOVA) was applied to determine significant differences among groups. When significant effects were observed ($p < 0.05$), Dunnett's multiple comparison test was performed using the *multcomp* package[24] to compare each treatment group with the control. Highly significant reductions were observed in all exposed groups ($p < 0.001$). The results are presented as mean ± standard deviation (SD).

4. Results

4.1 Red Blood Corpuscles (RBC) Count

In the present study, the RBC count of *O. niloticus* showed a significant reduction under sublethal dye effluent exposure compared to the control. At 10 days, control fish exhibited markedly higher RBC counts, whereas all exposed groups showed a clear dose-dependent decline. A similar trend was observed at 20 and 30 days of exposure, with control values consistently remaining significantly higher than those of the treated groups. For all haematological parameters, one-way ANOVA followed by Dunnett's post hoc test showed significant differences ($p < 0.05$, often $p < 0.001$) between control and dye-exposed groups (Tables 1–14). Overall, prolonged exposure to dye effluent resulted in a persistent decline in RBC levels in *O. niloticus* (Figure 1).

4.2 White Blood Corpuscles (WBC) Count

The WBC count of *O. niloticus* showed a marked increase under sublethal dye effluent exposure compared to the control. In the control group, WBC levels remained relatively low and stable (2044 ± 473 to 3975 ± 1114), while exposure caused a significant, concentration-dependent rise. At the lowest sublethal concentration (1/10), WBC counts were markedly elevated compared to controls, with mean values of 16067 ± 2360 at 10 days and 14615 ± 1388 at 20 and 30 days, while higher concentrations (1/7 and 1/5) produced the greatest elevations after 30 days (23463 ± 3053 and 23127 ± 1778). The results indicated a clear dose- and time-dependent leukocytosis in fish exposed to malachite green effluent (Figure 2).

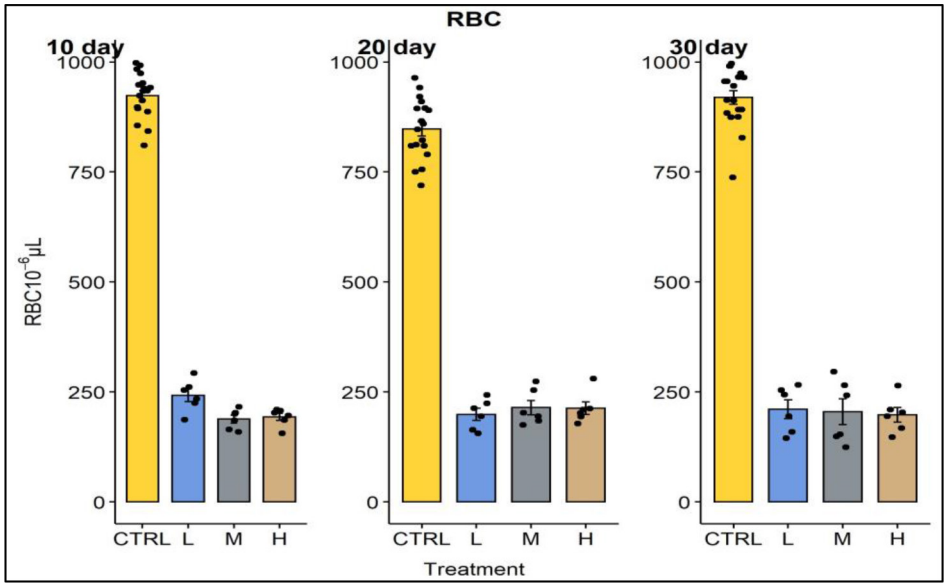


Figure 1. RBC count of *Oreochromis niloticus* after 10, 20, and 30 days of sub-lethal toxicant exposure.

Table 1. Effect of sublethal toxicant concentrations on RBC count of *Oreochromis niloticus* (ANOVA)

Duration		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
10 days	**Treatment**	3	4621117	1540372	868.9	<2e-16	***
	Residuals	32	56731	1773			
20 days	**Treatment**	3	3677644	1225881	389.3	<2e-16	***
	Residuals	32	100775	3149			
30 days	**Treatment**	3	4.60E+06	1533832	403.4	<2e-16	***
	Residuals	32	1.22E+05	3802			

Df = Degrees of freedom; ***Sum Sq*** = Sum of squares; ***Mean Sq*** = Mean square; ***F value*** = F-statistic; ***Pr(>F)*** = Probability value.
*** indicates a highly significant difference at $p < 0.001$.

Table 2. Dunnett's test showing RBC count differences in *Oreochromis niloticus* at sublethal toxicant concentrations versus control

Duration	Comparisons					Estimate	Std. Error	t value	Pr(>|t|)	
10 days	L	-	CTRL	==	0	-681.22	19.85	-34.32	<2e-16	***
	M	-	CTRL	==	0	-735.56	19.85	-37.06	<2e-16	***
	H	-	CTRL	==	0	-730.39	19.85	-36.8	<2e-16	***
20 days	L	-	CTRL	==	0	-648.78	26.45	-24.52	<2e-16	***
	M	-	CTRL	==	0	-633.61	26.45	-23.95	<2e-16	***
	H	-	CTRL	==	0	-635.11	26.45	-24.01	<2e-16	***
30 days	L	-	CTRL	==	0	-708.94	29.07	-24.39	<2e-16	***
	M	-	CTRL	==	0	-714.61	29.07	-24.59	<2e-16	***
	H	-	CTRL	==	0	-721.44	29.07	-24.82	<2e-16	***

L = Low concentration (1/10 of LC_{50}); M = Medium concentration (1/7 of LC_{50});
H = High concentration (1/5 of LC_{50}); CTRL = Control group.
The estimate represents the mean difference between treatment and control.
Std. Error = Standard error of the estimate; ***t*** value = ***t***-statistic; ***Pr***(>|***t***|) = Probability value.
*** indicates a highly significant difference compared to the control at $p < 0.001$ (Dunnett's test).

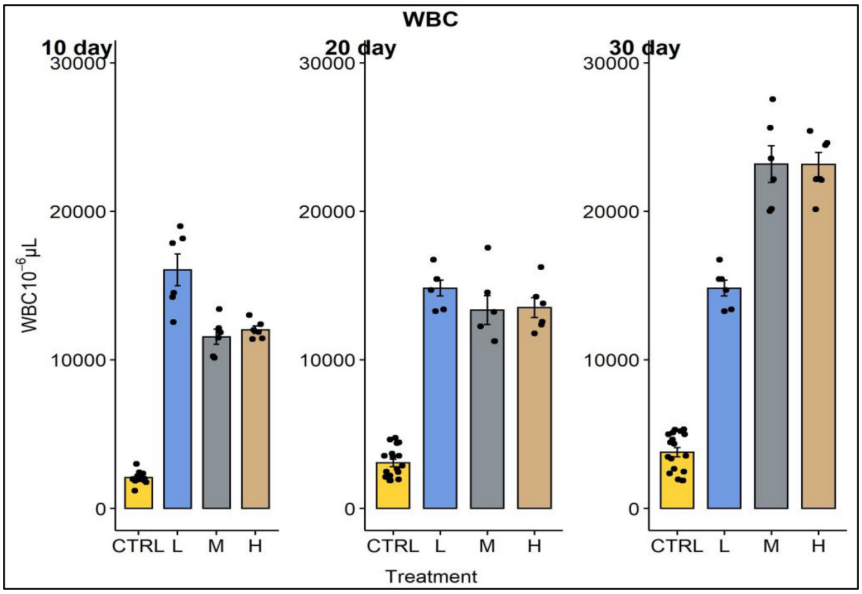


Figure 2. WBC count of *Oreochromis niloticus* after 10, 20, and 30 days of sub-lethal toxicant exposure.

Table 3. Effect of sublethal toxicant concentrations on WBC count of *Oreochromis niloticus* (ANOVA)

Duration		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
10 days	**Treatment**	3	1.19E+09	397188283	276.5	<2e-16	***
	Residuals	32	4.60E+07	1436311			
20 days	**Treatment**	3	1.07E+09	355062198	164.9	<2e-16	***
	Residuals	32	6.89E+07	2152806			
30 days	**Treatment**	3	2.76E+09	920567296	285.5	<2e-16	***
	Residuals	32	1.03E+08	3224315			

Df = Degrees of freedom; Sum Sq = Sum of squares; Mean Sq = Mean square; F value = F-statistic; Pr(>F)=Probability value.
*** indicates a highly significant difference among treatments at $p < 0.001$ (one-way ANOVA).

Table 4. Dunnett's test showing WBC count differences in *Oreochromis niloticus* at sublethal toxicant concentrations versus control

Duration	Comparisons					Estimate	Std. Error	t value	Pr(>|t|)	
10 days	L	-	CTRL	==	0	13990	565	24.76	<2e-16	***
	M	-	CTRL	==	0	9486	565	16.79	<2e-16	***
	H	-	CTRL	==	0	9961	565	17.63	<2e-16	***
20 days	L	-	CTRL	==	0	11775.5	691.7	17.02	<2e-16	***
	M	-	CTRL	==	0	10289.7	691.7	14.88	<2e-16	***
	H	-	CTRL	==	0	10449.5	691.7	15.11	<2e-16	***
30 days	L	-	CTRL	==	0	11057.9	846.5	13.06	<2e-16	***
	M	-	CTRL	==	0	19408.6	846.5	22.93	<2e-16	***
	H	-	CTRL	==	0	19372.8	846.5	22.89	<2e-16	***

L = Low (1/10 LC_{50}); **M** = Medium (1/7 LC_{50}); **H** = High (1/5 LC_{50}); **CTRL** = Control.
Estimate = mean difference vs control; Std. Error = standard error; t value = t-statistic; Pr(>|t|) = p-value.
*** = highly significant vs control ($p < 0.001$, Dunnett's test).

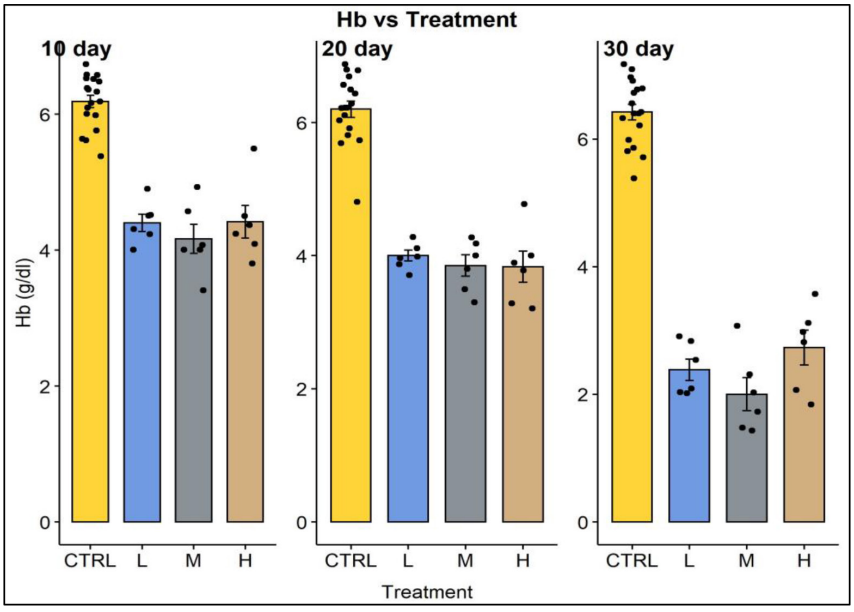


Figure 3. Haemoglobin (Hb) levels of *Oreochromis niloticus* after 10, 20, and 30 days of sub-lethal toxicant exposure.

4.3 Haemoglobin Content

Haemoglobin (Hb) content in *O. niloticus* decreased progressively with increasing concentrations and exposure durations of malachite green dye effluent. At 10 days, control fish showed normal Hb levels (6.19 ± 0.39 g/dl), while exposed groups recorded significant reductions (4.17 ± 0.52 to 4.42 ± 0.58 g/dl). Similar trends were observed at 20 and 30 days, with Hb dropping to 2.00 ± 0.64 g/dl at 1/7 concentration after 30 days, compared to 6.46 ± 0.51 g/dl in the control. These results indicate a dose- and time-dependent anaemic effect of malachite green effluent, reflecting haematological stress and impaired oxygen transport in *O. niloticus* (Figure 3).

4.4 Packed Cell Volume (PCV)

PCV in *O. niloticus* was assessed following sublethal exposure to malachite green dye for 10, 20, and 30 days. Control fish maintained relatively stable PCV levels throughout the experimental period, indicating normal haematological status. In contrast, dye-exposed fish exhibited a clear concentration- and duration-dependent reduction in PCV, with greater declines observed at higher exposure concentrations. Overall, PCV values of exposed fish were consistently lower than those of the control group throughout the study period (Figure 4).

4.5 Mean Corpuscular Volume (MCV)

MCV expressed in femtoliters (fL) was calculated using hematocrit and red blood cell count values to evaluate erythrocyte size alterations in *O. niloticus* following sublethal exposure to malachite green for 10, 20, and 30 days. The control group exhibited stable MCV values ranging from 25.0 ± 2.4 to 26.0 ± 2.8 fL, indicating normal erythrocyte

Table 5. Effect of sublethal toxicant concentrations on Hb content of *Oreochromis niloticus* (ANOVA)

Duration		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
10 days	**Treatment**	3	31.408	10.469	54.93	1.02E-12	***
	Residuals	32	6.099	0.191			
20 days	**Treatment**	3	47.94	15.98	71.54	2.79E-14	***
	Residuals	32	7.15	0.223			
30 days	**Treatment**	3	149.24	49.75	166.6	<2e-16	***
	Residuals	32	9.55	0.3			

Df = Degrees of freedom; *Sum Sq* = Sum of squares; *Mean Sq* = Mean square; *F value* = F-statistic; *Pr*(>F) = Probability value.
*** indicates a highly significant difference among treatments at $p < 0.001$ (one-way ANOVA).

Table 6. Dunnett's test showing Hb content differences in *Oreochromis niloticus* at sublethal toxicant concentrations versus control

Duration	Comparisons					Estimate	Std. Error	t value	Pr(>|t|)	
10 days	L	-	CTRL	==	0	-1.7889	0.2058	-8.692	<1e-08	***
	M	-	CTRL	==	0	-2.0222	0.2058	-9.826	<1e-08	***
	H	-	CTRL	==	0	-1.7722	0.2058	-8.611	<1e-08	***
20 days	L	-	CTRL	==	0	-2.2	0.1538	-14.309	<1e-09	***
	M	-	CTRL	==	0	-2.35	0.216	-10.881	<1e-09	***
	H	-	CTRL	==	0	-2.3667	0.286	-8.276	<1e-09	***
30 days	L	-	CTRL	==	0	-4.0389	0.2576	-15.68	<2e-16	***
	M	-	CTRL	==	0	-4.4222	0.2576	-17.17	<2e-16	***
	H	-	CTRL	==	0	-3.6889	0.2576	-14.32	<2e-16	***

L = 1/10 LC_{50}; **M** = 1/7 LC_{50}; **H** = 1/5 LC_{50}; **CTRL** = control.
Estimate = mean difference vs control; Std. Error = standard error; t = t-statistic; Pr(>|t|) = p-value.
*** = highly significant vs control (p < 0.001, Dunnett's test).

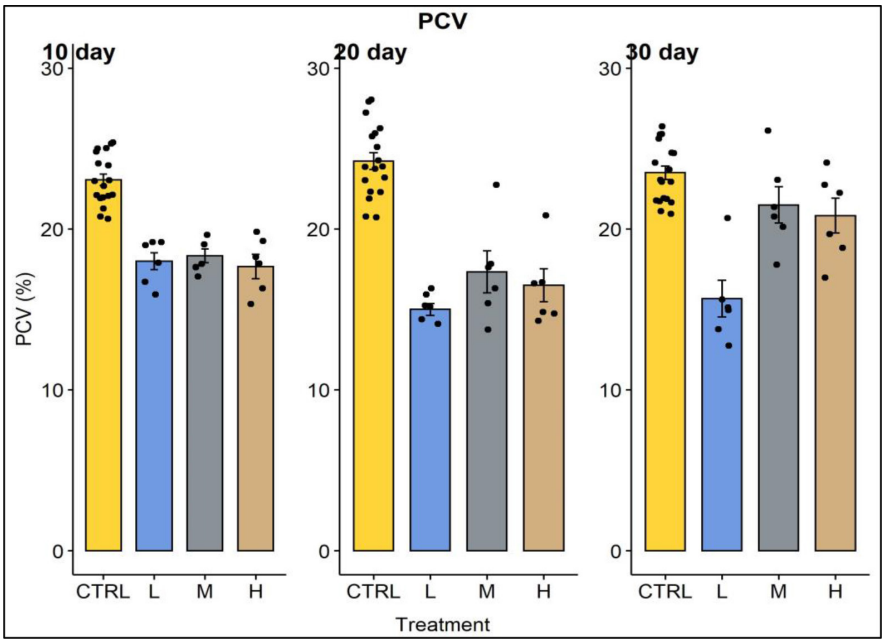


Figure 4. Packed Cell Volume (PCV) of *Oreochromis niloticus* after 10, 20, and 30 days of sub-lethal toxicant exposure.

Table 7. Effect of sublethal toxicant concentrations on PCV of *Oreochromis niloticus* (ANOVA)

Duration		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
10 days	**Treatment**	3	231.36	77.12	35.45	2.74E-10	***
	Residuals	32	69.61	2.18			
20 days	**Treatment**	3	584.8	194.94	36.28	2.07E-10	***
	Residuals	32	171.9	5.37			
30 days	**Treatment**	3	278.6	92.86	17.88	5.36E-07	***
	Residuals	32	166.2	5.19			

Df = Degrees of freedom; ***Sum Sq*** = Sum of squares; ***Mean Sq*** = Mean square; ***F value*** = F-statistic; ***Pr***(>F) = Probability value.
*** indicates a highly significant difference among treatments at $p < 0.001$ (one-way ANOVA).

Table 8. Dunnett's test showing PCV differences in *Oreochromis niloticus* at sublethal toxicant concentrations versus control

Duration	Comparisons					Estimate	Std. Error	t value	Pr(>|t|)	
10 days	L	-	CTRL	==	0	-5.0556	0.6953	-7.271	<1e-05	***
	M	-	CTRL	==	0	-4.7222	0.6953	-6.792	<1e-05	***
	H	-	CTRL	==	0	-5.3889	0.6953	-7.751	<1e-05	***
20 days	L	-	CTRL	==	0	-9.222	1.093	-8.44	< 1e-06	***
	M	-	CTRL	==	0	-6.889	1.093	-6.304	1.32E-06	***
	H	-	CTRL	==	0	-7.722	1.093	-7.067	< 1e-06	***
30 days	L	-	CTRL	==	0	-7.833	1.074	-7.292	<1e-04	***
	M	-	CTRL	==	0	-2	1.074	-1.862	0.1904	
	H	-	CTRL	==	0	-2.667	1.074	-2.482	0.0523	.

L = 1/10 LC_{50}; **M** = 1/7 LC_{50}; **H** = 1/5 LC_{50}; **CTRL** = control.
Estimate = mean difference vs control; Std. Error = standard error; t = t-statistic; Pr(>|t|) = p-value.
*** = $p < 0.001$; . = $p > 0.05$ vs control (Dunnett's test).

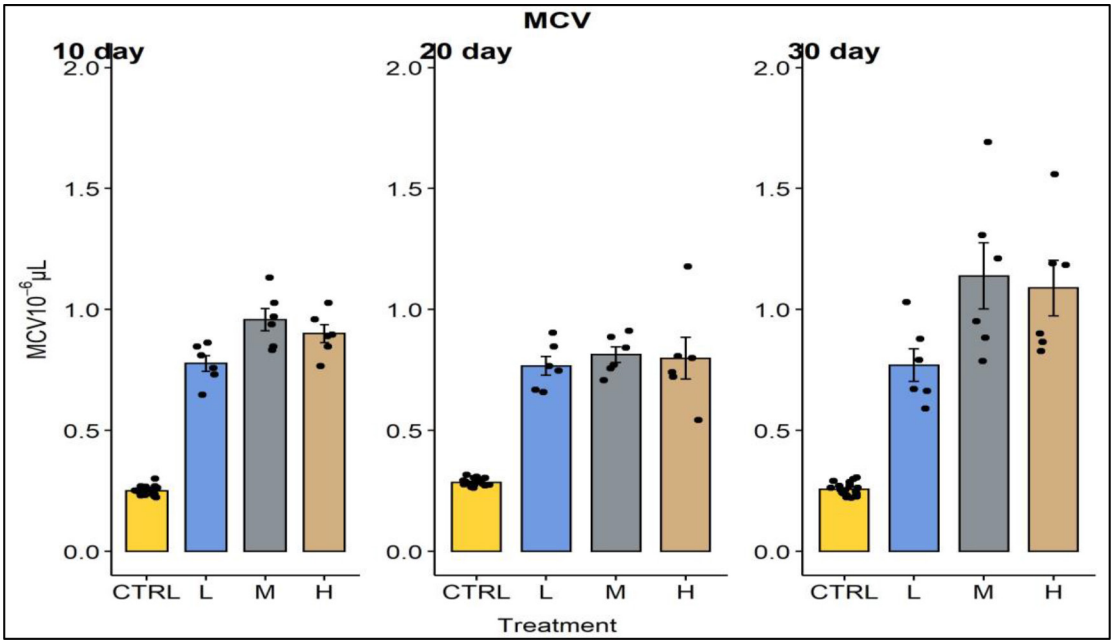


Figure 5. Mean corpuscular volume (MCV) of *Oreochromis niloticus* after 10, 20, and 30 days of sub-lethal toxicant exposure.

Table 9. Effect of sublethal toxicant concentrations on MCV of *Oreochromis niloticus* (ANOVA)

Duration		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
10 days	**Treatment**	3	3.663	1.2209	275.3	<2e-16	***
	Residuals	32	0.142	0.0044			
20 days	**Treatment**	3	2.322	0.7741	81.75	4.32E-15	***
	Residuals	32	0.303	0.0095			
30 days	**Treatment**	3	5.444	1.8148	52.76	1.74E-12	***
	Residuals	32	1.101	0.0344			

Df = Degrees of freedom; ***Sum Sq*** = Sum of squares; ***Mean Sq*** = Mean square; ***F value*** = F-statistic; ***Pr***(>F) = Probability value.
*** indicates a highly significant difference among treatments at $p < 0.001$ (one-way ANOVA).

Table 10. Dunnett's test showing MCV differences in *Oreochromis niloticus* at sublethal toxicant concentrations versus control

Duration	Comparisons					Estimate	Std. Error	t value	Pr(>|t|)	
10 days	L	-	CTRL	==	0	0.52722	0.03139	16.79	<2e-16	***
	M	-	CTRL	==	0	0.70889	0.03139	22.58	<2e-16	***
	H	-	CTRL	==	0	0.65056	0.03139	20.72	<2e-16	***
20 days	L	-	CTRL	==	0	0.48111	0.04587	10.49	<1e-10	***
	M	-	CTRL	==	0	0.52778	0.04587	11.51	<1e-10	***
	H	-	CTRL	==	0	0.51278	0.04587	11.18	<1e-10	***
30 days	L	-	CTRL	==	0	0.51389	0.08743	5.878	3.69E-06	***
	M	-	CTRL	==	0	0.88222	0.08743	10.09	< 1e-06	***
	H	-	CTRL	==	0	0.83222	0.08743	9.518	< 1e-06	***

L = Low concentration (1/10 of LC_{50}); M = Medium concentration (1/7 of LC_{50}); H = High concentration (1/5 of LC_{50}); CTRL = Control group.
The estimate represents the mean difference between treatment and control.
Std. Error = Standard error; ***t*** value = ***t***-statistic; ***Pr***(>|***t***|) = Probability value.
*** indicates a highly significant difference compared to the control at $p < 0.001$ (Dunnett's post hoc test).

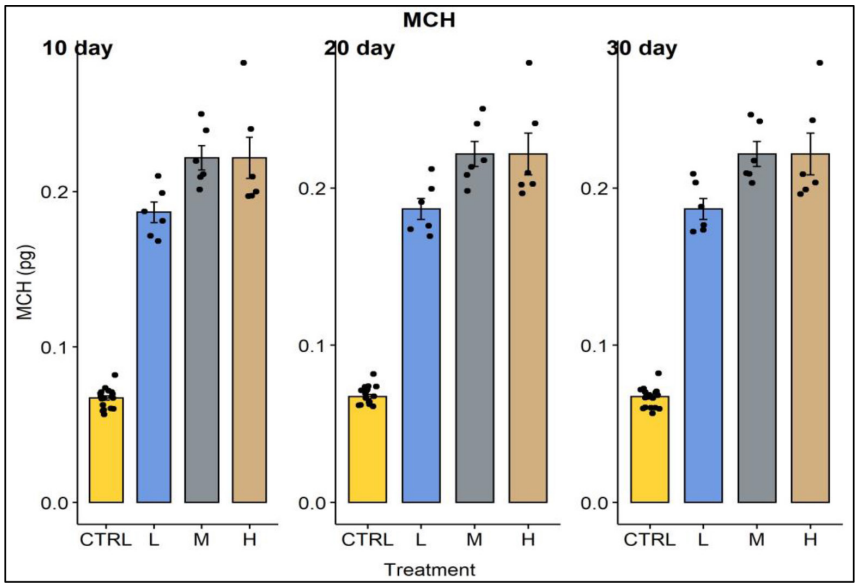


Figure 6. Mean corpuscular haemoglobin (MCH) of *Oreochromis niloticus* after 10, 20, and 30 days of sub-lethal toxicant exposure.

Table 11. Effect of sublethal toxicant concentrations on MCH of *Oreochromis niloticus* (ANOVA)

Duration		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
10 days	**Treatment**	3	0.18837	0.06279	221.7	<2e-16	***
	Residuals	32	0.00906	0.00028			
20 days	**Treatment**	3	0.12518	0.04173	176.2	<2e-16	***
	Residuals	32	0.00758	0.00024			
30 days	**Treatment**	3	0.024914	0.008305	38.42	1.02E-10	***
	Residuals	32	0.006917	0.000216			

Df = Degrees of freedom; ***Sum Sq*** = Sum of squares; ***Mean Sq*** = Mean square; ***F value*** = F-statistic; ***Pr***(>F) = Probability value.
*** indicates a highly significant difference among treatments at $p < 0.001$ (one-way ANOVA).

Table 12. Dunnett's test showing MCH differences in *Oreochromis niloticus* at sublethal toxicant concentrations versus control

Duration	Comparisons					Estimate	Std. Error	t value	Pr(>|t|)	
10 days	L	-	CTRL	==	0	0.119444	0.007932	15.06	<2e-16	***
	M	-	CTRL	==	0	0.154444	0.007932	19.47	<2e-16	***
	H	-	CTRL	==	0	0.154444	0.007932	19.47	<2e-16	***
20 days	L	-	CTRL	==	0	0.133889	0.007254	18.46	<2e-16	***
	M	-	CTRL	==	0	0.108889	0.007254	15.01	<2e-16	***
	H	-	CTRL	==	0	0.107222	0.007254	14.78	<2e-16	***
30 days	L	-	CTRL	==	0	0.045	0.006931	6.493	< 1e-04	***
	M	-	CTRL	==	0	0.028333	0.006931	4.088	0.000781	***
	H	-	CTRL	==	0	0.068333	0.006931	9.86	< 1e-04	***

L = 1/10 LC_{50}; **M** = 1/7 LC_{50}; **H** = 1/5 LC_{50}; **CTRL** = control.
Estimate = mean difference vs control; Std. Error = standard error; t = t-statistic; Pr(>|t|) = p-value.
*** = $p < 0.001$ vs control (Dunnett's test).

morphology throughout the experimental period. In contrast, treated groups showed a clear concentration- and time-dependent increase in MCV. Fish exposed to the lowest concentration (1/10 of 96-h LC_{50}) demonstrated moderate elevations (75.0 ± 9.3 to 77.0 ± 15.1 fL), whereas higher concentrations (1/7 and 1/5 of 96-h LC_{50}) produced marked increases, reaching 114.0 ± 34.5 and 112.0 ± 30.2 fL, respectively, after 30 days of exposure. The progressive elevation in MCV suggests a macrocytic response, likely associated with compensatory erythropoiesis, the release of immature erythrocytes into circulation, or structural alterations in red blood cells under toxic stress conditions. These findings indicate that malachite green exposure induces significant haematological disturbances in *O. niloticus*, particularly affecting erythrocyte volume in a dose- and duration-dependent manner (Figure 5).

4.6 Mean Corpuscular Haemoglobin (MCH)

MCH values in the control group remained stable throughout the experimental period, measuring 0.07 ± 0.007 pg (10 days), 0.07 ± 0.004 pg (20 days), and 0.07 ± 0.005 pg (30 days), indicating normal haemoglobin content per erythrocyte. In contrast, exposed groups exhibited a marked increase in MCH at 10 days (0.18 ± 0.016 to 0.21 ± 0.037 pg) and 20 days (0.18 ± 0.011 to

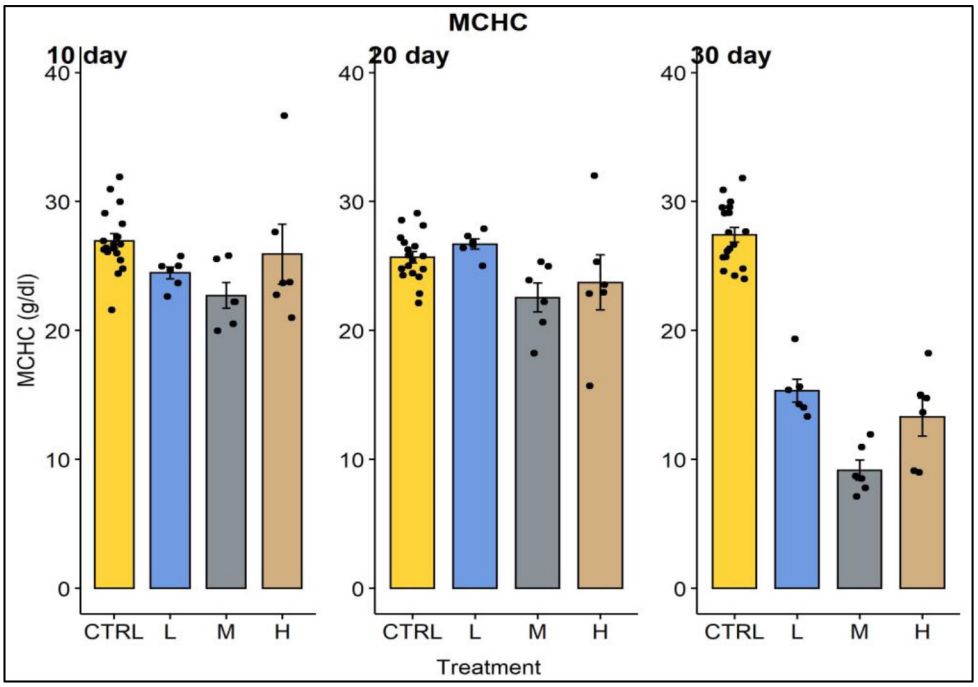


Figure 7. Mean Corpuscular Hemoglobin Concentration (MCHC) of *Oreochromis niloticus* after 10, 20, and 30 days of sub-lethal toxicant exposure.

Table 13. Effect of sublethal toxicant concentrations on MCHC of *Oreochromis niloticus* (ANOVA)

Duration		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
10 days	**Treatment**	3	90.96	30.321	3.233	0.0351	*
	Residuals	32	300.11	9.378			
20 days	**Treatment***	3	70.98	23.661	3.175	0.0373	*
	Residuals	32	238.48	7.452			
30 days	**Treatment**	3	2095.7	698.6	109.1	<2e-16	***
	Residuals	32	204.9	6.4			

Df = Degrees of freedom; ***Sum Sq*** = Sum of squares; ***Mean Sq*** = Mean square; ***F value*** = F-statistic; ***Pr***(>F) = Probability value. *indicates significant difference at $p < 0.05$; *** indicates highly significant difference at $p < 0.001$ (one-way ANOVA).

Table 14. Dunnett's test showing MCHC differences in *Oreochromis niloticus* at sublethal toxicant concentrations versus control

Duration	Comparisons					Estimate	Std. Error	t value	Pr(>|t|)	
10 days	L	-	CTRL	==	0	-2.483	1.444	-1.72	0.2463	
	M	-	CTRL	==	0	-4.236	1.444	-2.934	0.0178	*
	H	-	CTRL	==	0	-1.033	1.444	-0.715	0.8454	
20 days	L	-	CTRL	==	0	1.021	1.287	0.793	0.802	
	M	-	CTRL	==	0	-3.119	1.287	-2.424	0.06	.
	H	-	CTRL	==	0	-1.946	1.287	-1.512	0.347	
30 days	L	-	CTRL	==	0	-12.091	1.193	-10.14	<1e-10	***
	M	-	CTRL	==	0	-18.252	1.193	-15.3	<1e-10	***
	H	-	CTRL	==	0	-14.126	1.193	-11.84	<1e-10	***

L = Low (1/10 LC_{50}), M = Medium (1/7 LC_{50}), H = High (1/5 LC_{50}); CTRL = Control.
Estimate = mean difference vs control; Std. Error = standard error; ***t*** = ***t***-value; ***Pr***(>|***t***|) = ***p***-value.
*** ***p*** < 0.001; ***p*** < 0.05; . ***p*** ≥ 0.05 (Dunnett's test).

0.21 ± 0.024 pg), corresponding to reduced RBC counts and moderate declines in haemoglobin concentration. However, by 30 days, MCH values declined to 0.10 ± 0.015–0.14 ± 0.028 pg, although they remained higher than control levels. The observed pattern suggests an initial compensatory increase in haemoglobin content per erythrocyte in response to dye-induced erythrocyte depletion, followed by altered erythropoietic activity during prolonged exposure, indicating progressive haematological stress in the exposed fish (Figure 6).

4.7 Mean Corpuscular Haemoglobin Concentration (MCHC)

MCHC in *O. niloticus* was analysed over 10, 20, and 30 days of sublethal malachite green dye exposure. Control values remained stable (25.69 ± 2.03 to 27.09 ± 2.28 g/dl), indicating normal haemoglobin concentration. Exposure caused a concentration- and time-dependent decline, with moderate reductions at 1/10 concentration (24.47 ± 1.37 to 26.36 ± 0.96 g/dl) and more pronounced decreases at 1/7 and 1/5 concentrations, reaching 9.83 ± 1.79 and 13.79 ± 3.60 g/dl at 30 days. These results indicate dose- and duration-dependent hypochromia, impaired haemoglobin synthesis, and erythrocyte fragility under toxic stress (Figure 7).

5. Discussion

Aquatic contamination arising from human activities poses a serious threat to aquatic ecosystems, as industrial and agricultural pollutants—including coir dye effluents containing malachite green—can induce marked physiological disturbances in fish[25]. Haematological parameters are widely recognised as sensitive indicators of such toxic stress, as they reflect alterations in erythrocyte indices as well as changes in leukocyte profiles and immune function[26]. Establishing baseline haematological values under normal conditions is therefore essential for accurately identifying toxicant-induced deviations. Because fish blood parameters respond rapidly to fluctuations in water quality, they serve as reliable biomarkers for evaluating pollution and pesticide exposure and for assessing overall fish health[27,28]. In particular, several studies on *O. niloticus* exposed to chemical contaminants have reported reductions in erythrocyte indices and associated anaemic conditions following prolonged sublethal exposure, underscoring the importance of haematological assessment in monitoring aquatic ecosystem health[27,28].

The present study revealed a significant decline in RBC counts in *O. niloticus* exposed to dye effluent. Control fish consistently maintained higher RBC levels, whereas all exposed groups exhibited marked reductions in a concentration- and duration-dependent manner. The decrease was evident across exposure periods, indicating a persistent haematological effect of the dye effluent. This dose- and time-dependent decrease aligns with previous reports of malachite green and other pollutants, such as microplastics, silver nanoparticles, arsenic, copper, cadmium, and pesticides, which induce erythrocyte

destruction, anaemia, and haematological stress in fish[29-32]. The reduction in RBCs may result from oxidative damage, impaired erythropoiesis, or toxicant-induced stress, though transient increases have been reported under certain conditions due to splenic contraction or adrenergic stimulation[33,34]. Overall, these findings confirm RBC counts as sensitive biomarkers of aquatic toxicant exposure and highlight the ecological risks of malachite green in fish.

Exposure of *O. niloticus* to malachite green dye effluent caused a significant, concentration- and time-dependent increase in WBC counts, with the highest values observed at the greatest concentrations and longest exposure, indicating a stress-induced immune response. Similar leukocytosis has been reported in fish exposed to pollutants or immune stimulants, reflecting mobilisation of the immune system under chemical stress[35,36]. Conversely, some toxicants can decrease WBC counts, depending on type, concentration, and exposure duration[37]. These findings confirm that WBC counts are sensitive biomarkers of haematological and immunological stress, and the observed leukocytosis highlights the ecological risks of malachite green effluents in aquaculture.

Haemoglobin (Hb) levels in *O. niloticus* exhibited a progressive decline following exposure to dye effluent. Similar Hb declines have been reported in *Nile tilapia* and other species exposed to microplastics, silver nanoparticles, malachite green, and various pesticides[30,38]. These findings reinforce that haemoglobin is a sensitive biomarker of haematological stress, and the observed dose- and duration-dependent decline highlights the ecological risks of malachite green and the importance of monitoring fish blood parameters.

A negative correlation was observed between malachite green dye effluent exposure and Packed Cell Volume (PCV) in *O. niloticus*, with higher concentrations and longer exposures causing a significant reduction. Similar decreases in PCV, haemoglobin, and RBC counts have been reported in *Nile tilapia* and other species exposed to pollutants like profenofos, indicating common hematological disruption[29,39]. In contrast, dietary supplements such as selenium or *Ulva fasciata* extract can restore PCV and erythrocyte health, mitigating toxicant effects[40,41]. These findings confirm that PCV is a sensitive biomarker of haematological stress under environmental contamination.

Malachite green dye effluent exposure led to a significant, dose- and time-dependent increase in Mean Corpuscular Volume (MCV) in *O. niloticus*, with the highest values recorded at the greatest concentrations and longest exposure periods. The enlargement of erythrocytes likely represents a compensatory mechanism to maintain oxygen transport when RBC production or survival is compromised, indicating haematological stress. Similar MCV changes have been observed in *Labeo rohita* exposed to fenvalerate and *Clarias batrachus* exposed to arsenic, often linked to the release of immature RBCs or increased RBCs per plasma volume[32]. Studies in *Nile tilapia* under heavy metal exposure or poor water quality report comparable disruptions, while interventions such as biofloc systems can help maintain normal erythrocyte morphology[42]. These findings reinforce that MCV is a sensitive indicator of compensatory erythropoiesis and adaptive responses in fish exposed to aquatic pollutants[43].

In *O. niloticus* and other fishes, anemia under toxic or stress conditions typically presents as reduced RBC, Hb, and PCV, often accompanied by altered red cell indices[44,45]. Increased MCV with decreased MCHC and variable MCH has been associated with erythrocyte swelling, hemolysis, and impaired erythropoiesis in fish under metal, arsenic, cadmium or infectious stress[45,46]. Elevated MCV and MCH with reduced MCHC have been specifically linked to macrocytic anaemia and swelling of erythrocytes due to toxic injury to hematopoietic organs and RBC membranes[46]. *In Nile tilapia* exposed to microplastics or pesticides, similar profiles are reported: ↓RBC, ↓Hb, ↓PCV with ↑MCV and ↑MCH, indicating anaemia accompanied by larger erythrocytes and morphological abnormalities suggestive of hemolysis and stressinduced release of immature cells[44]. Infection with *Shewanella putrefaciens* in *O. niloticus* also caused ↓Hb, ↓RBC, ↓MCHC, with ↑MCV and ↑MCH, interpreted as anaemia with compensatory changes in erythropoiesis and stress response[47].

Exposure to sublethal concentrations of malachite green dye effluent significantly altered the hematological profile of *O. niloticus*, manifesting as anemia and leukocytosis, which reflect physiological stress and compromised oxygen transport; similar erythrogram disturbances (decreased RBCs, haemoglobin, and hematocrit with altered leukocyte counts) and erythrocyte abnormalities have been reported in *Nile tilapia* exposed to pesticides, microplastics, and other contaminants, indicating disrupted erythropoiesis, hemolysis, and immune modulation[44,47]. Moreover, industrial effluents and complex pollutant mixtures typically contain

heavy metals, pesticides, hydrocarbons, and microplastics that can act additively or synergistically to intensify oxidative stress, lipid peroxidation, and inflammatory responses, as shown for heavy metal-rich industrial effluents in *O. niloticus* and for combined microplastics–engine oil or metal–pesticide exposures that exacerbate oxidative, hematological, and immunotoxic effects compared with single toxicants[48,49]. Such combined toxicant effects amplify disturbances in metabolism, growth, and reproduction via oxidative damage, endocrine disruption, and neurotoxicity, ultimately impairing fish health and threatening aquatic biodiversity[50].

6. Conclusion

Exposure to sublethal concentrations of malachite green dye effluent induces significant haematological alterations in *O. niloticus*, including anaemia and leukocytosis, reflecting physiological stress and impaired oxygen transport. These findings highlight the toxic impact of synthetic dyes on fish health, metabolism, and overall physiological functioning. Such haematological disturbances can compromise essential biological processes, including respiration, feeding, growth, and reproduction, ultimately reducing fish productivity and threatening aquatic biodiversity. The results underscore the urgent need to control the discharge of untreated industrial effluents, regulate the indiscriminate use of hazardous chemicals, and promote eco-friendly alternatives to safeguard aquatic ecosystems. However, further studies integrating biochemical and histopathological endpoints are required to elucidate the underlying mechanisms of malachite green-induced hematotoxicity. Therefore, monitoring haematological parameters in cultured *O. niloticus* may serve as a practical early warning tool for detecting dye effluent contamination, particularly in coir-producing regions where malachite green discharge is prevalent.

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8. Conflicts of interests

Authors have declared that no competing interests exist.

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Author Contributions

Conceptualization, B.V.R and A.U.A.; methodology, B.V.R and A.U.A.; software, B.V.R.; validation, B.V.R and A.U.A.; formal analysis, B.V.R and A.U.A.; investigation, B.V.R and A.U.A.; resources, B.V.R and A.U.A.; data curation, B.V.R and A.U.A.; writing—original draft preparation, B.V.R.; writing—review and editing, B.V.R.; visualization, B.V.R and A.U.A.; supervision, A.U.A. All authors have read and agreed to the published version of the manuscript**.**

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