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Role of effective microorganisms on hematological and biochemical indices of cultured *Oreochromis niloticus* exposed to lead, copper, and cadmium under temperature variations

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ABSTRACT

The freshwater environment suffers from a combination of stressors; pollution and global warming. Multiple effects of copper sulfate [$CuSO_4$], cadmium chloride [$CdCl_2$], and lead nitrate Pb [NO_3]$_2$ were studied on Nile tilapia under three temperatures ranges, compared to bioremediation using effective microorganisms (EMs). The fish were divided into eight groups, with each group exposed to three temperatures (24, 28, and 32°C). Water physicochemical parameters were measured, and fish hematological, physiological, and biochemical changes were considered. Water quality parameters revealed a significant increase in both electrical conductivity and total dissolved solids in the EM/Cu fish group in the Cu fish group at 32°C. The chemical oxygen demand levels indicated a remarkable fluctuation with a slight decrease in the control group (at 28°C) while reduced in the control and EM. The results were highly significant incomplete blood cells, total red blood cell count, hemoglobin concentration (Hb), hematocrit, mean corpuscular Hb, mean corpuscular Hb concentration, and total protein (g/dl) in the EM group and control group. It can be concluded that using EM in fish farms (1:1000) could help fish adapt to different temperatures and reduce the effects of toxic pollutants.

1. INTRODUCTION

It is known that Nile tilapia fish is considered the most valuable fish in aquaculture systems [1]. Recently, many troubles appeared due to the increasing intensive farming systems from spreading infectious diseases [2]. The misuse of available chemotherapies leads to resistance and restriction of chemical treatment choices [3]. Therefore, looking for other options to decrease disease infections in aquaculture became essential. One of the options is improving water quality that will control aquatic infections [4]. Otherwise, aquacultural systems could be enhanced by dietary additions; vitamins and minerals, plant extracts, and probiotics. Such supplements will improve feed utilization, digestibility, and growth performance, enhancing fish immunity [5]. Effective microorganisms (EMs) were recently introduced as a probiotic formulation into aquaculture. EMs consist of a mixture of aerobic and anaerobic beneficial bacteria; yeasts, lactic acid bacteria, actinomycetes, and photosynthetic bacteria [6].

Although trace metals are found naturally in the aquatic environment, their ground concentrations increase in the environment, particularly in agricultural and industrial fields [7]. There are two types of metals; Zn and Cu and non-essential (Pb and Cd) metals that lead to fish toxicity by disrupting physiological properties [8], biochemical activities [9], and growth and reproduction, which finally cause death [10]. The present study aimed to evaluate the effects of EM on fish hematological, physiological, and biochemical changes and copper and cadmium impact on water quality and blood parameters of *O. niloticus* with different temperatures under experimental conditions.

2. MATERIALS AND METHODS

2.1. Fish

The following research was conducted in strict accordance with the guidelines of the Ethical Committee, National Research Centre, Egypt,

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on the care and use of animals for scientific purposes. Three hundred and eighty-four fish (Nile tilapia *Oreochromis niloticus*), 90–110 g in weight, were obtained from the National Research Centre farm (Nubaria, Egypt). Fish were transferred to the biotechnology and biodiversity conservation laboratory, National Research Centre, in large plastic water containers with battery aerators as an oxygen source with dechlorinated tap water (24.5 ± 2.1°C and pH 7.2–8.2). Fish were supplied by a commercial fish diet (35% protein) during the experiment.

2.2. Water Quality Parameters

The physical parameters were determined during the experiment by following the procedures in the standard methods to examine water and wastewater [11].

2.3. Determination of Lead, Copper, and Cadmium Toxicities 96 h LC_{50}

A lethal concentration test of $CuSO_4 \cdot 5H_2O$ after 96 h (96 h LC_{50}) of exposure was conducted, referring to Litchfield and Wilcoxon [12] and Alkobaby and Abd El-Wahed [13]. In contrast, a static bioassay test was done to determine 96 h LC_{50} of cadmium ($CdCl_2.H_2O$) to Nile tilapia fish following Garcia-Santos *et al*. [14]. The $Pb(NO_3)_2$ concentrations were based on the 96 h LC_{50} of $Pb(NO_3)_2$ for *O. niloticus*, which was previously determined to be 44 mg/L according to Ullah *et al*. [15]. For both metals' toxicity calculations, the following ranges were tested: 0 (control), 10, 15, 20, 25, 30, and 35 mg/L, respectively. Dead fish numbers were counted every 12 h. The mortality rate was calculated at the end of 24, 48, 72, and 96 h.

2.4. Experimental Design

Fishes were assigned to eight groups, each with three different temperatures duplicated. Fishes were placed in 150 l of glass aquaria (8 fish/group), as summarized in Figure 1. The experiment lasted 2 weeks.

2.5. Plasma Analyses

By the end of the experiment, blood samples were centrifuged at 3000 rpm for 5 min to allow plasma separation and subjected to some biochemical analyses. Using enzymatic-colorimetric methods through commercial kits (Biodiagnostic Co, Egypt), the following studies were conducted:

1. Total protein (g/dl) was measured, referring to Bradford [16]
2. Aspartate aminotransferase (AST, EC. 2.6.1.1) (u/l) and alanine aminotransferase (ALT, EC. 2.6.1.2) (u/l) were measured colorimetrically using transaminase kits following the method of Reitman and Frankel [17]
3. Creatinine (mg/dl) was recorded according to Henry *et al*. [18]
4. Uric acid (mg/dl) was determined using an enzymatic reaction adopted by Barham and Trinder [19].

2.6. Hematological Measures

The blood samples were transferred to Eppendorf tubes containing EDTA as an anticoagulation agent [20]. Then, the whole blood samples were used for assessment of red blood cell (RBC) counts, hemoglobin (Hb), hematocrit (Hct) levels, and white blood cell count using an automated technical analyzer (Celltac α MEK- 6400J/K). The mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentration levels (MCHC) were calculated, referring to Dacie and Lewis [21].

2.7. Statistical Analyses

The study results were represented as mean ± SE. Data were statistically analyzed utilizing analysis of variance (F-test) and *Duncan's* multiple range test to determine differences in means as expressed by different case letters in the descending order A, B, C, and D at $P < 0.05$ using SAS (Statistical Analysis System) version 9.1 [22]. A two-way analysis of variance (ANOVA) fixed effect model with interaction effects [23]. was applied for each studied variable using treatment and temperature as independent variables.

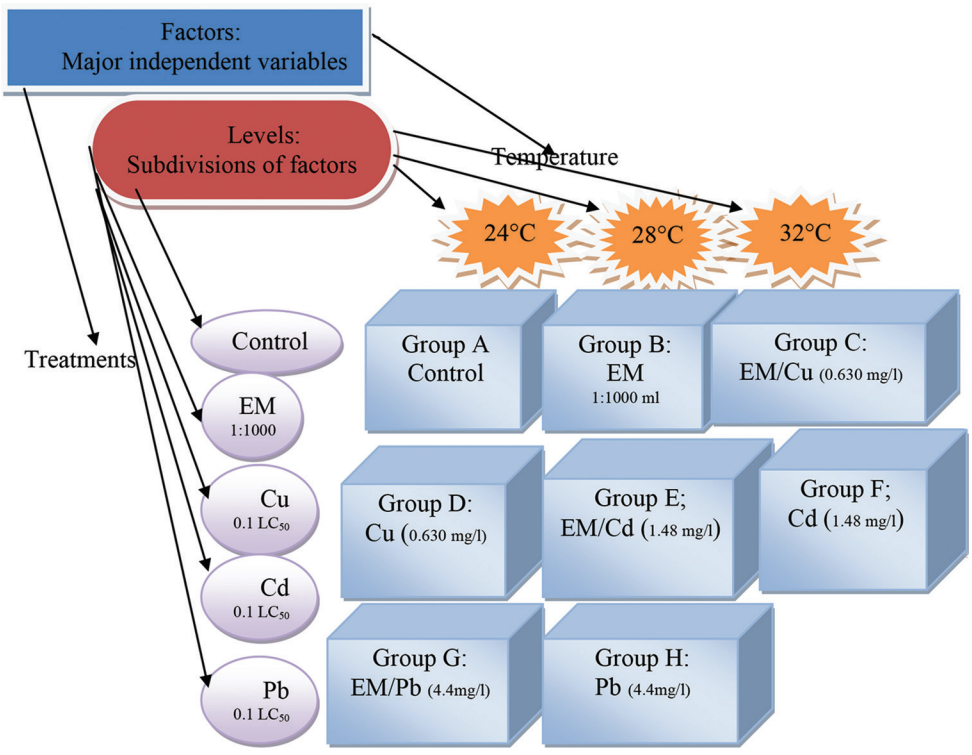

Figure 1: Experimental design.

3. RESULTS

3.1. Acute Toxicity

The 96 h LC_{50} values of both metals Cu, Pb, and Cd for Nile tilapia fish are 6.30 mg CuSO4/L, 14.8 mg/L of $CdCl_2$, and 44 mg/L of Pb $(NO_3)_2$ for *O. niloticus*. Then, the used concentrations in experimental groups are 1/10 LC_{50} of fish species (4.4 mgPb/l), (0.630 mg Cu/l), and (1.48 mg Cd/l).

3.2. Water Quality

Water quality parameters were measured in all experimental groups. Dissolved oxygen levels ranged from 5 to 10 mg/l**.** Analysis results of "electrical conductivity (EC) and total dissolved solids (TDS)" revealed a significant decrease in the EM group when compared to other fish groups (at 24°C and 28°C) except in the control group. Meanwhile, there is a considerable increase in both EC and TDS in the Cu group when compared to other experimental groups (at 32°C). The chemical oxygen demand (COD) levels indicated a remarkable fluctuation with a slight decrease in the control and EM groups at 32°C. Meanwhile, the levels were reduced in the control compared to other experimental groups at 28°C. Still, after being exposed to 24°C, the control group significantly decreased compared to other experimental groups except in the EM group [Table 1].

3.3. Hematological Parameters

The combined impact of different stressors: Temperature, trace metals (Pb, Cu, and Cd), and EM were studied in Nile tilapia *O. niloticus*. Results of blood parameters analysis revealed that the highest value of RBCs count was recorded significantly in the fish control group when compared to other groups, especially when subjected to 24°C and that the value was recorded significantly in the control group and Pb group when compared to other groups, except the EM/Pb group exposed to 28°C. Meanwhile, the lowest levels were recorded significantly in the EM/Cu group when compared to other groups exposed to 32°C, as shown in Table 2. Moreover, Hb levels were found significantly in the EM/Pb fish and Pb groups compared to other groups exposed to 24°C. Other blood parameters such as Hct were analyzed, and results showed that the lowest levels were significant in the EM/Cu group when compared to other groups, except in the control group exposed to 32°C. While the lowest levels were substantial in EM/Cu group when compared to the Cu group after being exposed to 24°C. Meanwhile, the levels were non-significant among all fish groups exposed to 28°C, as presented in Table 2. Referring to WBCs results, it was noted that there is a significant increase in cell counts in both Cu and Cd groups when compared to other experimental groups except in EM/Cd subjected to 24°C. At the same time, there was a significant increase in cell count in the EM/Cd group compared to other fish groups exposed to 28°C. While after exposure to 32°C, the significant increase in cell count in the Cd group compared to other experimental groups except in EM/Cd.

Other blood parameters, MCV, MCH, and MCHC, are represented in Table 3, and significant differences in values were recorded. The lowest MCV levels were found significantly in the control group, EM/Pb group, and Pb group compared to other fish groups exposed to 24°C and 28°C. The highest levels were recorded in the Cu group, EM/Cd group, and Cd group compared with other experimental groups subjected to 32°C. The MCH results showed the lowest levels in the control fish group compared to other groups except in the Cu and Cd groups subjected to 24°C. At the same time, the highest levels were found significantly in the EM/Cd fish group compared to other groups except for the EM/Pb group and the EM group exposed to

Table 1: Water physicochemical properties in all experimental groups.

Experimental groups	DO (mg/l)			TDS (mg/l)		EC (µS/cm)		COD (mg/l)	
	24°C	28°C	32°C	24°C	28°C	32°C	24°C	28°C	32°C	24°C	28°C	32°C
Control	8.20±0.11^{AB}	8.20±0.11^{AB}	8.30±0.15^{AB}	234.33±3.93^{C}	153.00±5.19^{DE}	166.00±19.92^{DE}	396±22.27^{C}	246.33±4.91^{DE}	272±27.15^{D}	138±29.14^{C}	142.67±17.52^{C}	155.33±13.38^{B}
E.M	7.80±0.06^{AB}	8.03±0.08^{AB}	8.26±0.13^{AB}	132.67±8.74^{D}	124±8.71^{E}	123.0±9.50^{E}	220±15.27^{D}	207.33±13.38^{E}	218.33±19.22^{D}	161±42.79^{BC}	324.66±37.54^{AB}	99.30±12.34^{B}
E.M. and Cu	10.26±1.06^{A}	9.80±1.08^{A}	9.50±1.26^{A}	336.67±5.24^{A}	429.33±6.74^{A}	253.33±20.71^{C}	591.33±24.87^{A}	720±9.45^{A}	389±31.81^{C}	244.67±27.72^{B}	291.30±27.40^{AB}	297.32±45.41^{A}
Cu	6.96±2.45^{AB}	6.30±2.06^{B}	7.40±2.77^{AB}	277.67±13.28^{B}	237.67±2.84^{C}	424.33±18.22^{A}	473±16.19^{B}	393.67±4.63^{C}	659.67±45.88^{A}	330.33±49.26^{AB}	292.31±49.68^{AB}	314±44.61^{A}
E.M. and Cd	8.45±0.30^{AB}	8.13±0.37^{AB}	8.10±0.15^{AB}	267.00±10.15^{B}	241.67±13.83^{C}	251.67±13.04^{C}	448.33±16.19^{B}	413.33±26.03^{C}	433.33±28.59^{C}	341.66±27.72^{A}	348.33±24.21^{AB}	362.33±35.43^{A}
Cd	8.36±0.30^{AB}	8.20±0.35^{AB}	8.20±0.23^{AB}	278.33±14.14^{B}	246.67±15.32^{C}	204.67±4.67^{D}	474.33±13.34^{B}	424.30±28.20^{BC}	379±17.52^{C}	310.00±26.46^{AB}	363.33±46.67^{A}	333.33±24.21^{A}
E.M. and pb	6.26±0.88^{B}	7.10±1.30^{AB}	6.20±1.21^{AB}	153.33±6.22^{D}	289±13.74^{B}	337.33±10.10^{B}	244.30±13.61^{D}	470.67±7.98^{B}	537±8.18^{B}	250.30±25.87^{AB}	278.88±32.92^{AB}	275.67±42.78^{A}
Pb	5.50±1.60^{B}	5.67±1.50^{B}	5.60±0.87^{B}	150±4.50^{D}	159±9.64^{D}	269±14.22^{C}	254.33±12.44^{D}	269.67±14.94^{D}	445±20.03^{C}	239.31±10.73^{B}	249.33±11.60^{B}	311.67±22.01^{A}

Data are represented as means of eight samples±S.E. Statistical significant differences (P<0.05) are shown with different capital letters in the same column. EC: Electrical conductivity, TDS: Total dissolved solid, COD: Chemical oxygen demand.

Table 2: Hematological parameters of *Oreochromis niloticus* in experimental groups.

Experimental groups	WBCs ($\times10^3$/mm^3)			RBCs ($\times10^6$/mm^3)			Hb (g/dl)			Ht (%)		
	24°C	28°C	32°C	24°C	28°C	32°C	24°C	28°C	32°C	24°C	28°C	32°C
Control	13.26±2.87^{C}	11.46±0.63^{D}	10.20±0.90^{C}	1.23±0.19^{A}	1.26±0.06^{A}	1.07±0.06^{A}	5.40±1.04^{B}	5.70±0.17^{AB}	4.90±0.75^{A}	13.46±2.38^{AB}	14.26±0.97^{A}	11.03±0.77^{BC}
E.M	38.70±10.27^{BC}	56.86±25.77^{B}	15.13±0.77^{C}	0.64±0.08^{BC}	0.71±0.02^{C}	1.18±0.06^{A}	4.97±0.72^{B}	5.26±0.55^{AB}	5.90±0.68^{A}	13.30±1.89^{AB}	16.13±2.36^{A}	13.73±0.61^{AB}
E.M. and Cu	21.63±9.46^{C}	47.00±9.04^{BC}	19.23±11.78^{C}	0.42±0.07^{C}	0.70±0.13^{C}	0.37±0.15^{C}	3.43±0.75^{B}	3.90±0.50^{B}	1.90±0.81^{B}	9.93±1.57^{B}	13.13±1.71^{A}	5.83±2.33^{C}
Cu	75.60±12.65^{A}	59.86±15.92^{B}	58.30±14.43^{B}	0.88±0.15^{B}	0.67±0.05^{C}	0.70±0.15^{B}	5.43±1.04^{B}	3.76±0.66^{B}	4.26±0.71^{A}	18.86±3.58^{A}	13.36±1.68^{A}	13.67±2.51^{AB}
E.M. and Cd	64.07±19.60^{AB}	86.23±1.47^{A}	74.20±17.25^{AB}	0.59±0.17^{BC}	0.77±0.03^{BC}	0.75±0.17^{B}	3.20±1.25^{B}	3.86±0.17^{B}	4.30±1.30^{A}	12.06±3.70^{B}	15.43±0.24^{A}	14.90±3.70^{AB}
Cd	82.40±2.37^{A}	35.76±15.66^{BC}	104.63±19.85^{A}	0.77±0.02^{B}	0.78±0.33^{BC}	0.93±0.01^{AB}	4.16±0.17^{B}	4.40±1.67^{B}	6.00±0.68^{A}	15±0.25^{AB}	13.96±5.92^{A}	18.60±1.18^{A}
E.M. and Pb	46.05±1.80^{BC}	20.93±0.73^{CD}	16.73±0.20^{C}	0.88±0.01^{B}	1.12±0.009^{AB}	1.02±0.04^{AB}	9±0.15^{A}	7.30±0.29^{A}	5.73±0.23^{A}	12.20±0.11^{B}	13.18±0.04^{A}	12.20±0.06^{B}
Pb	42.43±1.64^{BC}	20.56±1.07^{D}	13.56±0.55^{C}	0.83±0.01^{B}	1.20±0.02^{A}	1.13±0.09^{A}	7.76±0.29^{A}	5.80±0.15^{AB}	5.86±0.20^{A}	11.43±0.12^{B}	12.0±0.06^{A}	12.13±0.27^{B}

Data are represented as means of eight samples±S.E. Means with the same letter for each parameter are not significantly different. Otherwise, they do. *Significant difference ($P<0.05$). WBC: White blood cell, RBC: Red blood cell, Hb: Hemoglobin, Ht: Hematocrit.

28°C; meanwhile, the levels were non-significant among all fish groups exposed to 32°C. The highest MCHC values were substantially in the Pb group compared to other experimental groups subjected to 24°C. At the same time, the highest levels were found significantly in the EM/Pb group in comparison to other groups subjected to 24°C. The highest levels were found significantly in the EM/Pb group and Pb fish group compared to other groups except in the control group exposed to 32°C.

3.4. Biochemical Analyses

Biochemical analysis results are represented in Table 4. Total protein results showed that the highest levels were significantly higher in the EM/Pb group compared to other fish groups exposed to 24°C, 28°C, and 32°C. The highest value of AST was markedly found in the EM/Pb group when compared to other fish groups exposed to 24°C but at higher temperatures (at 28°C); the highest level was recorded significantly in the Pb group and EM/Pb group when compared to other fish groups. At the highest temperature (at 32°C), the maximum level of AST was recorded significantly in the Pb group compared to other fish groups except in the EM/Pb group. The highest level of ALT was recorded significantly in the EM/Pb group when compared to other fish groups exposed to 24°C. The highest levels in the Pb group were found significantly compared to other fish groups subjected to 28°C. Still, at 32°C, the highest level was found significantly in the Pb and Cu groups compared to other fish groups.

Moreover, creatinine values represented the highest level was found significantly in the control group and Cd group when compared to other groups except in the EM/Cu group exposed to 32°C while at 24°C showed an increase significant in the Cu group in comparison to other groups except in the control group and EM/Cd group. In the analysis of uric acid, results showed that its highest level was recorded significantly in the Cu group compared to other fish groups exposed to 24°C. The lowest levels were found significantly in the EM and control groups exposed to 28°C and 32°C.

4. DISCUSSION

The results provided by this study comprise an effort to follow the impacts of EM on *O. niloticus* fish hematological, physiological, and biochemical changes as well as lead, copper, and cadmium effects on water quality and blood parameters. In terms of physicochemical parameters, water quality controls the environment of the whole aquatic system [24] and is considered an indicator of pollution [25-26]. The EC and TDSs are among the significant parameters affecting the distribution and survival of fishes [27]. Any variation in the water physicochemical properties (EC, pH, and TDS) alters trace metals' uptake by aquatic organisms [28]. In the present study, this may be imputed to high concentrations of organic and inorganic substances in the water of the EM/Cu group at 24°C and 28°C, while in the Cu group at 32°C, also decreased level in the EM group followed by the control group indicated the significant effect of the EM, Cu, Pb, and Cd sublethal concentrations may lead to a better impact of EM causing biodegradation of dissolved solids and uptake of trace metals. There was no detectable change in DO levels as it remains not <5 mg/l which lies in the recommended range for fish growth, as El-Sayed [29] mentioned. The COD level is usually used to detect oxygen equivalent of organic matter in samples liable to oxidation by chemicals. It is used in the following study to follow the impact of EM on water quality. The results exceeded permissible levels (10 mg/l) [30].

Table 3: Hematological parameters of *Oreochromis niloticus* in experimental groups.

Experimental groups	MCV (fento litre)			MCH (pg/cell)			MCHC (g/dl)		
	24°C	28°C	32°C	24°C	28°C	32°C	24°C	28°C	32°C
Control	119.93±4.68^{C}	113.16±2.77^{B}	112.10±0.91^{C}	53.0±4.27^{D}	47.50±2.11^{C}	49.10±3.73^{A}	46.46±3.81^{C}	40.16±1.87^{C}	47.36±2.03^{AB}
E.M	163.30±13.93^{B}	199.23±5.85^{A}	113.20±2.07^{C}	63.63±3.15^{BC}	62.43±1.90^{AB}	49.06±3.97^{A}	33.93±1.47^{D}	30.70±0.75^{DE}	43.63±3.60^{B}
E.M. and Cu	209.80±6.40^{A}	188.63±10.18^{A}	155.03±2.71^{B}	64.30±3.51^{BC}	56.63±5.00^{BC}	52.93±1.67^{A}	29.70±1.33^{D}	30.00±1.50^{DE}	32.43±0.64^{C}
Cu	201.67±11.91^{A}	196.63±12.21^{A}	198.17±5.48^{A}	61.43±3.75^{CD}	54.83±6.02^{BC}	62.00±2.55^{A}	30.53±0.98^{D}	27.76±1.50^{E}	31.33±0.58^{C}
E.M. and Cd	204.90±2.54^{A}	167.00±1.04^{A}	188.16±7.26^{A}	71.23±3.88^{B}	67.0±2.77^{A}	64.63±0.33^{A}	33.43±1.61^{D}	31.33±0.48^{DE}	34.46±1.32^{C}
Cd	194.23±2.34^{A}	166.56±6.86^{A}	194.16±11.55^{A}	54.46±0.88^{CD}	54.66±0.97^{BC}	62.03±6.63^{A}	28.83±1.30^{D}	32.33±2.09^{D}	29.67±2.10^{C}
E.M. and Pb	139.67±1.20^{C}	130.03±25.53^{B}	119.26±0.86^{C}	84.86±2.62^{A}	62.60±1.56^{AB}	43.80±18.96^{A}	64.13±1.04^{B}	54.36±1.04^{A}	53.0±1.85^{A}
Pb	139.0±1.73^{C}	103.33±2.96^{B}	110.23±1.17^{C}	92.10±1.65^{A}	51.90±2.97^{C}	52.40±1.62^{A}	70.07±0.85^{A}	45.56±0.39^{B}	50.46±1.71^{A}

Data are represented as means of eight samples±S.E. Means with the same letter for each parameter are not significantly different. Otherwise, they do. *Significant difference (P<0.05). MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration.

Variations in fish blood parameters are known as metal-induced stress, and so they are always used as indicators of heavy metals toxicity [31]. Fisheries biologists use hematological parameters to assess environmental health and control any pathological operations in fish [32]. Hematological indicators are helpful tools for assessing fish status [32]. Stress can alter the hematological indices of fish by downstream effects on many physiological operations [33,34]. In this study, RBCs count was lower than the optimum levels previously stated by Hrubec *et al*. [35] who detected much higher counts (2.31 × 10^6 cells/mm^3) and Hb levels (8.2 g/dl) and Hct (33%). Estimation of MCV, MCHC, and MCH is valuable parameters for ammonia content in many animals [36]. In this research, MCV values were average in both EM, control, EM/Pb, and Pb groups at 24°C. The value was also at the normal range in the EM/Cd, Cd, control, EM/Pb, and Pb groups exposed to 28°C and the EM/Pb, Pb, EM/Cu, EM, and control groups exposed to 32°C, reflecting the impact of EM on fish immunity. Our results for MCH and MCHC were higher than the reference level reported by Hrubec *et al*. [35] who examined such parameters in hybrid tilapia, and results were more elevated in intensive aquaculture systems (115–183 μm^3 for MCV, 28.3–42.3 pg for MCH, and 22–29 g/dl for MCHC). Moreover, the change in MCH and MCHC could be attributed to RBCs hemolysis and the reduction of RBCs production in the hemopoietic tissues under the action of the bioaccumulated heavy metals. Moreover, our findings were confirmed using the two-way ANOVA that showed a significant impact of EM, Pb, Cu, and Cd in various temperatures in all experimental groups except for Hb levels at 24°C and 28°C. Low levels of RBCs and Ht refer to hemolysis due to Hg toxicity [37]. Our results agree with those obtained previously by Çiftçi *et al*. [38] who noted that RBC levels decreased when *O. niloticus* was treated with CuSO4 and Pb $(NO_3)_2$. Furthermore, Al-Asgah *et al*. [39] found that RBC, Hb, and Hct levels lowered when *O. niloticus* was subjected to various levels of cadmium chloride for 10, 20, and 30 days.

Significant increases in WBCs levels in the Cu and Cd groups compared to the other experimental groups may be related to fish adaptation to afford pollution. Removal of cell debris resulting from tissue damage may also increase WBCs. This result was in agreement with other studies [40], which indicated the presence of a defensive response of fish against toxic invasions, indicated by high WBC levels.

In the present study, the WBCs level, as indicated in the two-way ANOVA test, was within the normal range as shown by Hrubec *et al*. [35] except in the control fish group at 24°C and control, EM/Pb and Pb groups at 28°C, meanwhile, except in the control group, EM group, EM/Cu group, EM/Pb group, and Pb group exposed to 32°C. Hrubec *et al*. [35] stated a reference range of WBCs in healthy hybrid tilapia of 21.559–154.690 × 10^3cells/mm^3 with a median value of 75.659 × 10^3cells/mm^3. Kotsanis *et al*. [41] proved a significant decrease in WBCs number in *Oncorhynchus mykiss* treated with arsenic due to a decrease in lymphocytes level. They also confirmed that the decrease in WBCs levels during acute and sublethal treatments might be due to the extensive toxic impact on the kidney, which is the primary site of hematopoiesis, thrilling immunosuppression and could be attributed to primary or secondary variation in hemopoietic organs. Another possible cause may be inhibition of white blood cell maturation and then liberation from tissue reservoirs by the impact of trace metals, Datta *et al*. [42].

Using EM in our research affected fish hematology and helped to enhance immunity; this appeared in high levels of RBCs count in the EM group and control group at 32°C, also high levels of WBCs count recorded in the Cd group at 24°C and 32°C followed by EM/Cd at the same temperature. High levels of WBCs count were detected in the Cu group at 24°C but reduced in EM/Cu at the same temperature. Such results may indicate bioremediation of pollutants using EM, also increasing WBCs count.

They have indicated that chronic exposure to arsenic influenced the structure of head kidneys identified by relief in leukocyte number. Such results agreed with Shalaby [43] findings that sublethal levels of Cd lead to significant increases in ALT and AST in carp after 7 and 25 days. High ALT and AST levels may be correlated with hepatocellular injury or cellular damage in the spleen or liver [44]. The ALT values in our findings, especially at 24°C, 28°C, and 32°C, were less than Chen *et al*. [45] (74.7–118.8 u/l), and this may be due to metal concentration and exposure time of EM in the experiment that may need to prolong to get a better impact on treated fish. Concerning AST results, they agreed with Chen *et al*. [45] (170.2–278.0 u/l) except in control at 24°C while in the control and EM/Cu groups exposed to 28°C results decreased.

Moreover, results of AST decreased except in the Cd group, EM/Pb group, and Pb group exposed to 32°C. Similar effects of AST and ALT were mentioned by Öner *et al*. [46]. They detected significant variation in liver transaminase activities after treatment with heavy metals (Zn, Cu, Cd, Cr, and Ag) for 30 days.

Creatinine and uric acid are rough indicators of animal kidney functions. Hrubec *et al*. [35] found that the physiological reference range of healthy tilapia fish elevated in intensive production systems to 0.1–0.5 mg/dl. Such findings match our results in the EM and EM/Cu groups at different temperatures compared to other fish groups expressing the impact of EM on fish immunity. Uric acid

Table 4: Biochemical parameters of *Oreochromis niloticus* in experimental groups.

Experimental groups	Total protein (g/dl)			ALT (U/l)			AST (U/l)			Creatinine (mg/dl)			Uric acid (mg/dl)		
	24˚C	28˚C	32˚C	24˚C	28˚C	32˚C	24˚C	28˚C	32˚C	24˚C	28˚C	32˚C	24˚C	28˚C	32˚C
Control	3.10±0.52^{C}	3.25±0.09^{C}	1.54±0.12^{F}	3.33±0.96^{D}	3.36±0.14^{C}	3.26±0.82^{D}	3.50±0.06^{E}	10.16±1.01^{D}	2.80±0.25^{D}	1.07±0.10^{AB}	0.99±0.06^{AB}	1.13±0.20^{A}	5.20±0.50^{CD}	2.03±0.73^{C}	1.76±0.09^{D}
E.M	2.30±0.02^{D}	2.16±0.02^{E}	3.38±0.13^{C}	4.91±0.34^{D}	6.45±0.18^{C}	6.26±0.08^{D}	255.30±65.90^{C}	209.50±52.97^{B}	5.36±0.78^{D}	0.71±0.01^{BC}	0.88±0.04^{BC}	0.75±0.03^{BC}	4.90±0.70^{CD}	3.26±0.75^{C}	1.73±0.06^{D}
E.M. and Cu	3.18±0.15^{C}	2.19±0.03^{E}	1.38±0.06^{F}	6.96±3.81^{D}	5.40±0.46^{C}	6.83±1.62^{CD}	144.86±20.36^{D}	103.13±20.29^{C}	50.96±7.10^{CD}	0.74±0.05^{BC}	0.91±0.29^{AB}	0.99±0.01^{AB}	6.96±0.39^{B}	6.93±0.28^{A}	3.46±0.20^{C}
Cu	2.10±0.17^{D}	2.11±0.02	1.51±0.12^{F}	7.67±1.57^{D}	3.30±1.08^{C}	10.60±0.70^{CD}	210.30±11.99^{CD}	191.40±27.35^{BC}	72.10±12.07^{CD}	1.24±0.40^{A}	0.99±0.11^{AB}	0.68±0.18^{BC}	8.66±1.12^{A}	5.10±0.26^{B}	3.50±0.11^{C}
E.M. and Cd	2.34±0.07^{D}	2.39±0.06^{D}	2.63±0.13^{E}	16.78±1.17^{C}	31.37±10.11^{B}	12.58±1.33^{C}	158.80±23.16^{D}	243.33±54.93^{B}	113.07±24.47^{C}	1.17±0.09^{AB}	1.24±0.04^{A}	0.70±0.10^{BC}	3.86±0.38^{D}	6.56±0.87^{AB}	3.80±0.90^{C}
Cd	1.91±0.03^{D}	2.49±0.03^{D}	2.91±0.01^{D}	10.26±1.36^{CD}	35.25±3.78^{B}	58.60±5.02^{A}	188.53±40.41^{CD}	202.76±13.70	295.93±74.32^{B}	0.15±0.01^{D}	0.88±0.04^{BC}	1.13±0.09^{A}	5.86±0.30^{BC}	5.73±0.14^{AB}	5.90±0.06^{B}
E.M. and Pb	5.83±0.17^{A}	5.60±0.06^{A}	4.48±0.06^{A}	66.53±5.86^{A}	38.23±2.83^{B}	32.83±1.52^{B}	687.90±10.35^{A}	352.23±2.46^{A}	361.10±2.47^{AB}	0.50±0.04^{CD}	0.54±0.01^{C}	0.62±0.02^{C}	4.30±0.24^{CD}	6.69±0.42^{A}	7.13±0.29^{A}
Pb	4.87±0.19^{B}	3.98±0.11^{B}	3.86±0.08^{B}	41.0±1.45^{B}	59.67±1.44^{A}	61.36±1.41^{A}	449.46±6.27^{B}	394.73±6.36^{A}	417.96±9.32^{A}	0.17±0.03^{D}	0.71±0.04^{BC}	0.60±0.05^{C}	3.62±0.34^{D}	5.75±0.13^{AB}	8.08±0.14^{A}

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase.

values showed a significant increase in the Cu group in comparison to other groups subjected to 24°C and in the Pb group and EM/Pb group subjected to 32°C, which could be attributed to modulation of metabolism in association with the toxic stress in both Cu and Pb exposed groups. Fluctuations in blood urea indicate declining liver functions and failure of gill osmoregulatory capability, Mensinger *et al.* [47]. The uric acid level was 1.6–2.3 mg/dl when Abdel-Tawwab and Wafeek [48] measured in an experiment for 6 weeks in *O. niloticus* treated by Cd. Our results were slightly higher than previous ones except in the control group at 28°C.

In comparison, in both EM and control groups at 32°C, results were expected. This may refer to the experimental period of EM application, which needs to prolong to favor metabolism modulation under toxic stress of the Pb, Cu, and Cd fish groups. Plasma proteins are known to play a key role in maintaining osmolarity, buffer capacity, and pH of blood, besides acting as carriers of various nutrients, metabolites, and metal ions. They also have great importance in the body's defense mechanisms concerning their globulin fraction, Yousuf *et al*. [49]. The highest values of total proteins were recorded significantly in the EM/Pb group in comparison to other groups when subjected to 24 °C, 28°C, and 32°C. It is a good indication of the impact of EM with temperature variation as tool for adapting to climate change and as biodegradation of impact of some heavy metals.

5. CONCLUSION

The investigated set of biomarkers proved to be efficient and reliable in biomonitoring the role of EM as a probiotic in enhancing biochemical parameters and water quality of cultured Nile tilapia; *O. niloticus*. The association between biochemical and hematology examination proved to be an efficient tool for the assessment of fish response to heavy metals toxicity (copper, cadmium, and lead) as well as EM treatment with different temperatures on *O. niloticus* showed a significant decrease in the EM group with biochemical analysis for uric acid, AST, and ALT. Therefore, the following research recommends using EM in fish farms at the proposed dilution ratio of 1:1000, which plays an excellent role in helping fish adapt to different temperatures and reduce the effects of toxic pollutants.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

The research described herein was performed on Mozambique tilapia (Oreochromis niloticus). This study was conducted in strict accordance with the guidelines of the Ethical Committee, National Research Centre, Egypt, on the care and use of animals for scientific purposes.

11. DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

12. PUBLISHER'S NOTE

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