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Acute and subchronic toxicity of metal complex azo acid dye and anionic surfactant oil on fish *Oreochromis niloticus*

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Abstract

The acute toxicity study of metal complex dark green azo acid dye, anionic surfactant oil and their mixture determined the 96 hr LC_{50} and fish behaviours. Subchronic toxicity determined haematology parameters and concentrations of copper and chromium in blood. The 96 hr LC_{50} was determined by probit analysis and subchronic toxicity was conducted in 90 days. No mortalities were observed in control and anionic surfactant oil treatments. The 96 hr LC_{50} value of mixture was 26.7 mg l^{-1} (95% CL = 20.7 - 46.8) and that of metal complex dark green azo acid dye was not met as the percentage of dead was below 50% of tested organisms. In a treatment of anionic surfactant oil and that of mixture observed behaviours were respiration response, uncoordinated movement, loss of equilibrium, erratic posture and loss of responsiveness. Subchronic toxicity indicated fluctuations in number of erythrocytes, leukocytes and thrombocytes in all chemical treatments. Erythrocyte morphology such as anisocytosis, erythrocytes hypertrophy, karyolysis, cytoplasm vacuolation, ghost cell were observed in fish blood in all chemical treatments. An inverse relation was observed between total copper and chromium concentration in blood. However, the toxicity effect was chemical dose dependent and length of exposure.

Key words

Behaviour, Chromium, Copper, Haematology, Nile tilapia

Introduction

Textile industries use synthetic dyes including metal complex dark green azo acid dye for direct or hand dyeing of silk and cotton yarn. The metals vary but may include copper and chromium (Christie, 2007). Even dispersal of dye is facilitated by addition of anionic surfactants (Chen *et al*., 2010). Solubility of acid azo dye in water is enhanced by sulphonate present in anionic surfactants (Cserhati *et al*., 2002; Christie, 2007). Some azo dyes and anionic surfactants have hydrophobic components that interact with non-polar molecules in compound mixtures (Cserhati *et al*., 2002; Freeman, 2004). Commonly, untreated dye effluents are discharged into domestic wastewater systems and their potential to negatively impact aquatic biota has raised environmental concern.

Toxicity of dyes depend on the physico-chemical composition of water as well as other substances present in water (Svobodova *et al*., 1993). Anionic surfactants can cause direct toxic effects to some aquatic organisms (Arslan-Alaton and Erdinc, 2006). These contaminants can disrupt fish sensory, hormonal, neurological and metabolic systems and can cause behaviour as changes (Scott and Sloman, 2004; Kane *et al*., 2004). Chemical toxicity and environmental stress may induce a variety of combative responses such as number of erythrocyte, thrombocytes and leucocytes (Heath, 1995). Abnormal cell morphology may also result due to chemical exposure (Bahari *et al*., 1994).

Nile tilapia, *Oreochromis nilotica*, was selected as a standard tropical species for this study. Although not endemic to

Thailand or Southeast Asia, this species is widespread in the region and has been employed commonly in toxicological studies in other countries (Campos-Mendoza *et al*., 2004; Abdel-Tawwab *et al*., 2007).The present study examined the effect of acute and subchronic concentrations of metal complex dark green azo acid dye and anionic surfactant oil, individually and in combination on the behaviour, haematology and accumulation of copper and chromium, over a period of 90 days, in *O. niloticus*.

Materials and Methods

Oreochromis niloticus, commonly known as Nile tilapia (n=360) weighing 13.9 to 15.4 g were obtained from Khon Kaen University Fisheries Farm and used for experiment. Metal complex dark green azo acid dye (53) and anionic surfactant oil, used in this study, were obtained from a local dye shop (Chonnabot District, Khon Kaen Province, Thailand). Metals detected in the dye, during preliminary analyses, were total copper (1.1 mg kg^{-1}), aluminum (3.7 mg kg^{-1}), nickel (0.03 mg kg^{-1}) iron (336.5 mg kg^{-1}) and chromium (5314 mg kg^{-1}) (APHA, 2005). Aromatic amines (n = 24) were not detected in azo colourant (Azo Dyes European Standard EN 14362-1, 2012). Water solubility of azo dye was 64.3 g l^{-1}. Concentrations of phenol, sulfate (as SO_4^{2-}) and alkyl benzene sulphonate in anionic surfactant oil were < 0.001, 553.8 and 3.6 mg l^{-1}, respectively (APHA, 2005). Solubility of anionic surfactant oil in water was greater than 1000 mg l^{-1}. Total copper and chromium concentration in anionic surfactant oil were 0.002 and 0.049 mg l^{-1}, respectively (APHA, 2005).

The experimental set up followed a completely randomized design (Gomez and Gomez, 1984). Acute toxicity test consisted of 12 treatments, each with 3 replicates. Each continuously aerated tank contained 50 l of dechlorinated water and 15 fish (5 kg m^{-3}). The subchronic study was conducted over 90 days and consisted of 6 treatments, each with 3 replicates. Each aerated tank contained 270 l of dechlorinated water and 20 fish.

In acute toxicity study fish were exposed to 5, 10, 30, 40 and 90 mg l^{-1}, metal complex dark green azo acid dye 20 mg l^{-1} anionic surfactant oil and 25, 30, 50, 60 and 110 mg l^{-1} mixture of metal complex dark green azo acid dye each with 20 mg l^{-1} anionic surfactant oil. These concentrations were derived from an earlier preliminary acute toxicity study (Amwele *et al*., 2013). Concurrently, some fish were placed in water tanks with no added chemicals. These were considered as control. Fish mortalities were monitored for 96 hr in control and treatment tanks, the latter after the initiation of chemical exposures. Qualitative assessment of fish behaviour was described during acute exposures to metal complex dark green azo acid dye and anionic surfactant oil (ASTM international standard guide (E1711-12), 2013). Fish behaviour was observed three times daily at 06:00 – 07:00 a.m., 12:00 – 13:00 p. m. and 16:00 – 17:00 p.m. at 1, 2, 3 and 4 days (24, 48, 72 and 96 hr) of exposure.

Subchronic toxicity concentrations were as follows: 3 and 6 mg l^{-1} metal complex dark green azo acid dye, 5 mg l^{-1} anionic surfactant oil and mixtures of metal complex dark green azo acid dye containing 8 and 11 mg l^{-1}, each with 5 mg l^{-1} of anionic surfactant oil. These concentrations were derived from the results of acute toxicity study. Fish not exposed to metal complex dark green azo acid dye and/or anionic surfactant oil but otherwise given similar treatment were considered as control. Blood samples were collected at 30 day interval from fish (n = 5) in each tank. Blood was drawn by puncturing caudal vessel and blood was kept in heparinated vials. Total erythrocyte counts (10^6 mm^3) were made within 12 hr, using a Newbauer hemocytometer (Natt and Herrick, 1952). Erythrocyte morphology was evaluated by semiquantitative method (Wiess, 1984). Nikon digital sight camera, DC in 12V, connected to Nikon eclipse E200 microscope was used to capture images at 100 X lens. Hematocrit was determined by a micro-hematocrit method (Goldenfarb *et al*., 1971). Total leukocytes and thrombocytes were counted (cells μl^{-1}) in relation to erythrocyte counts from randomly selected monolayer fields (100 X) of stained blood smears and calculated per unit volume, using the formula of Tavares-Dias and Moraes (2006).

Pooled blood samples from three replicates (0.6 ml) were kept at -20 °C immediately after they were collected for further metal analysis. Total copper and chromium were analyzed (APHA, 2005) and estimated by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES).

The LC_{50} value was determined by probit analysis using SPSS software 11.5 program. Corrected fish mortality rates were calculated by Abbot's formula (Abbott, 1925) as mentioned. Corrected mortality (%) = (X – Y / X) x 100; where, X = percentage of living fish in control sample and Y = percentage of fish living in treatment samples. All parameters were analyzed using one way ANOVA to determine significance at $P<0.05$.

Results and Discussion

Fish mortalities did not occur among control, 20 mg l^{-1} anionic surfactant oil and 5 mg l^{-1} metal complex dark green azo acid dye at all exposure periods to 96 hr. Mortality in fish exposed to 10 and 30 or 40 mg l^{-1} metal complex dark green azo acid dye was 7 and 9% after 96 hr. Exposure to 90 mg l^{-1} metal complex dark green azo acid dye resulted in 4 % mortality after 96 hr. Thus, it was not possible to calculate 96 hr LC_{50} for metal complex dark green azo acid dye or anionic surfactant oil, based on the chemical concentrations and time periods examined. However, the concentration of 5, 10, 30, 40 and 90 mg l^{-1} metal complex dark green azo acid dye 96 hr LC_{50} examined in this study, indicates to be relatively non toxic. In contrast with exposure to mixtures of metal complex dark green azo acid dye with anionic surfactant oil at 25 mg l^{-1} mortality was 18% after 72 hr and 100% after 48 hr at 30, 50, 60 and 110 mg l^{-1} concentration. Therefore, calculated 96

hr LC_{50} (95%CL) for mixtures of metal complex dark green azo acid dye with anionic surfactant oil was 26.7 mg l^{-1} (20.7 - 46.8), indicating the mixture to be moderately toxic as it was well within the toxicity range of 10-100 mg l^{-1} as proposed by Leblanc (2004).

Fish mortality was dose dependent during exposure to mixture of metal complex dark green azo acid dye with anionic surfactant oil. The mixture of metal complex dark green azo acid dye with anionic surfactant oil was more toxic to fish than metal complex dark green azo acid dye or anionic surfactant oil alone.

Control fish swam slowly within the deeper portions of the tanks, with periodic interruptions of station holding throughout the 96 hr observation period. Propulsion was provided mostly by lateral movement of the caudal peduncle and tail. Equilibrium was maintained and median fins were erect. Behaviours changed dramatically on exposure to concentrations of metal complex dark green azo acid dye and the mixtures of metal complex dark green azo acid dye with anionic surfactant oil. After approximately 90 and 8 min exposure to metal complex dark green azo acid dye and the mixtures of metal complex dark green azo acid dye with anionic surfactant oil, respectively, fish descended to the bottom of tanks where they exhibited minimal activity. After 179 min of exposure to metal complex dark green azo acid dye, fish that survived began to show similar swimming behaviour than control fish. Fish exposed to mixtures of metal complex dark green azo acid dye with anionic surfactant oil and anionic surfactant oil swam to the water's surface repeatedly within about 16 and 71 min of exposure, where they remained for several minutes at the time gulping air which was assumed to represent respiratory stress. Swimming became uncoordinated; equilibrium was unstable and surfacing was more frequent in fish exposed to the mixture and anionic surfactant oil after approximately 20 and 169 min exposure, respectively. These behaviours were more prolonged with exposure time. Fish were also relatively unresponsive to tactile stimuli. After about 47 min, the fish surviving exposure to the mixtures of metal complex dark green azo acid dye with anionic surfactant oil displayed erect median fins, slowly moved their pectoral fins, partially regained equilibrium and remained stationary in water. Similar behaviour was observed in anionic surfactant oil after 3 hr. After 2 and 4 hr, fish exposed to mixtures of metal complex dark green azo acid dye with anionic surfactant oil and anionic surfactant oil regained responsiveness and resumed similar swimming behaviour to that of control fish for the duration of exposures. Swimming behaviour could not be observed due to dark colour of water when the concentration of dye was greater than 30 mg l^{-1}.

The surfacing behaviour displayed by fish on exposure to anionic surfactant oil and their mixture with dye was likely a response to chemical inhibition of respiration (ASTM international standard guide (E1604-12), 2013). Other locomotive responses observed in fish exposed to anionic surfactant oil and its mixture with dye in this study were similar to those reported by Sloman and Wilson (2006), in fish exposure to anionic surfactant. They suggested that anionic surfactant enters brain directly through olfactory system and causes accumulation of acetylcholine elicit cholinergic toxicity due to continuous stimulation of cholinergic receptors via nerve system.

Mortalities were not recorded for control fish or those exposed to 3 mgl^{-1} dye over the 90 - day experimental period. Mortalities occurred, after 15 days, in fish exposed to in 5 mgl^{-1} surfactant oil (15%), 3 and 6 mgl^{-1} dye (13 and 3%) and 8 mgl^{-1} mixture of dye and surfactant oil (7%). Mortalities increased over the 90 days exposure in 5 mgl^{-1} surfactant oil (83%), 11 and 8 mgl^{-1} dye and oil mixture (73 and 57%, respectively) and 6 mgl^{-1} dye (20%). During subchronic exposure fish mortality depended on exposure period to all the treatments.

Erythrocyte counts did not differ significantly among most treatments until 90 days of exposure (Table 1). Except the erythrocyte counts in fish exposed to 5 mg l^{-1} anionic surfactant oil for 90 days were not significantly different from control but less ($P<0.05$) than those exposed to 11 mg l^{-1} mixture (Table 1). The failure to find a consistent pattern of change in erythrocyte concentrations on exposure to textile dye is not consistent with earlier observations of Sharma *et al*. (2006) in *Poecilia reticulate* and Poornima *et al*. (2011) in *O. mossambicus*. Haematocrits (%) of treated fish did not change in relation to control until 90 days when those exposed to dye, in the presence or absence of surfactant oil, tended to increase although increases were mostly not significant (Table 1). Morphology of erythrocytes in control fish did not change after 30, 60 and 90 days in contrast to that of tilapia exposed to 5 mg l^{-1} anionic surfactant oil, 3 and 6 mg l^{-1} metal complex dark green azo acid dye, 8 and 11 mg l^{-1} of where mixture anisocytosis, karyolysis, cytoplasmic vacuolation and ghost cells were observed (Fig. 1).

Similar changes in erythrocyte morphology were reported by Soni *et al*. (2006) in *Gambusia affinis* fish exposed to untreated and treated textile dye wastewater. Erythrocytes hypertrophy, karyolysis, anisocytosis, vacuolation, ghost cell and fish mortality were indication of toxicity due to metal complex azo acid dye and anionic surfactant oil.

Leukocyte counts did not differ significantly ($P>0.05$) among all treatments at 30 and 90 days of exposure (Table 2). Only at 60 day, the number of leukocytes in control fish were significantly ($P<0.05$) less than those exposed to anionic surfactant oil (Table 2). Leukocyte count increased with chemical concentration, similar findings were reported by Afag and Rans (2009) in *Cirrhinus mrigala* exposed to acid leather brown dye and they suggested that leukocytes responded due to intoxication as protective reaction against foreign substance that enters the fish's body. This corresponds with an increase in leukocytes count relative to total copper and chromium concentration detected in fish blood.

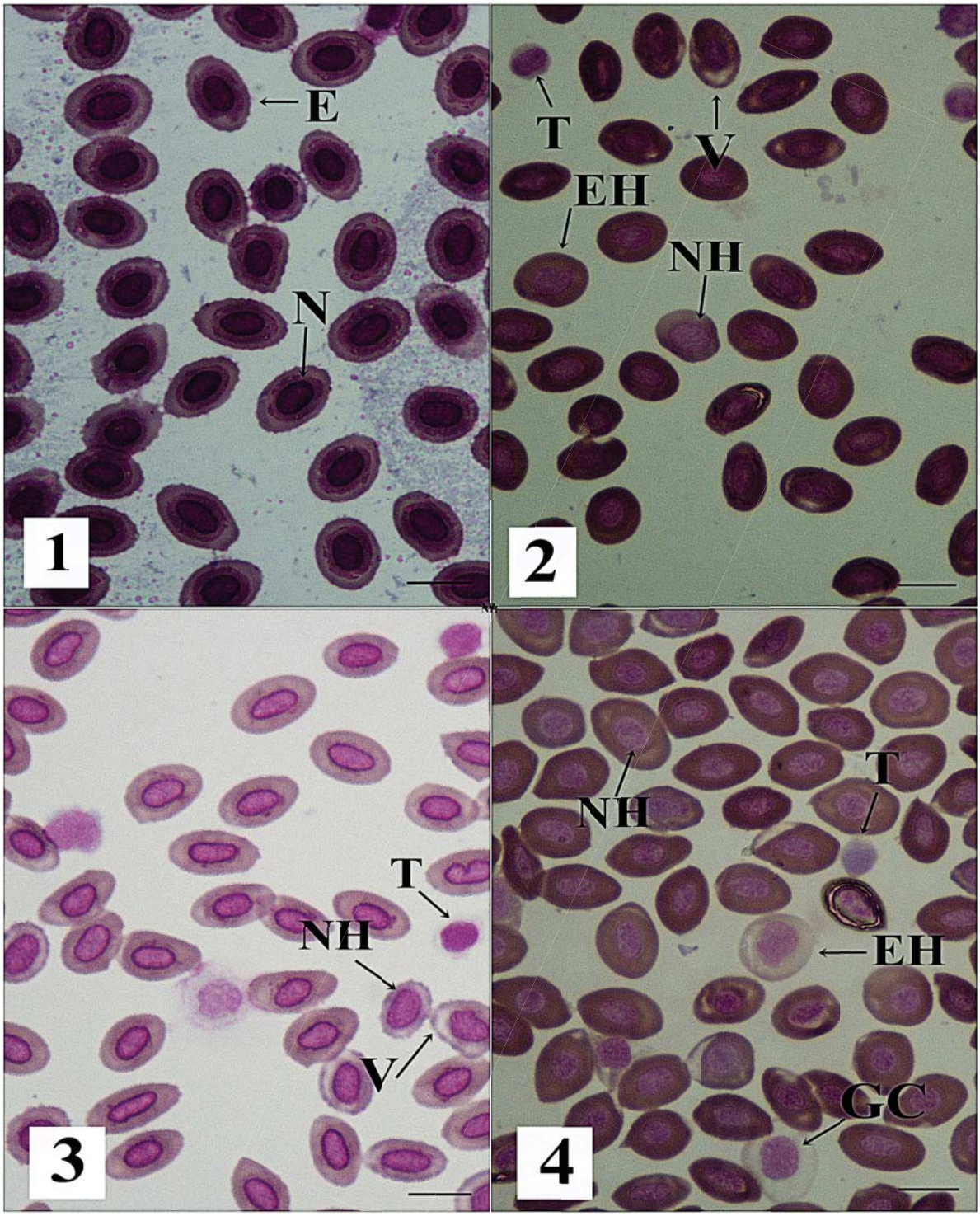

Fig. 1 : Erythrocyte morphology for fish *O. niloticus* exposed to control (1), Anionic surfactant oil (2), metal complex dark green azo acid dye (3) and mixture of metal complex dye with anionic surfactant oil (4): cytoplasm vacuolation (V), dense nucleus (N), erythrocytes (E), erythrocyte hypertrophy (EH), nucleus hypertrophy (NH), ghost cell (GC) and thrombocytes (T). Wright-geimsa stain, bar = 10 μm

Table 1 : Erythrocytes (x10^6/mm^3) and haematocrit (%) count in fish *O. niloticus* exposed to different treatments

Treatments	30days		60days		90days	
	Erythrocytes (x10^6 mm^{-3})	Haematocrit (%)	Erythrocytes (x10^6 mm^{-3})	Haematocrit (%)	Erythrocytes (x10^6 mm^{-3})	Haematocrit (%)
Control	2.7±0.6^a	26.0±3.8^a	2.7±0.2^a	28.0±1.0^a	2.7±0.2^{bc}	28.0±3.3^{ab}
Anionic surfactant oil 5 mgl^{-1}	2.6±0.3^a	26.7±2.3^a	3.4±0.5^a	30.3±2.1^a	2.5±0.2^c	25.7±1.1^b
Metal complex dark green azo acid dye 3 mgl^{-1}	2.4±0.2^a	26.6±2.3^a	2.9±0.7^a	28.7±4.9^a	3.1±0.2^{ab}	29.7±2.5^{ab}
Metal complex dark green azo acid dye 6 mgl^{-1}	2.5±0.5^a	24.0±4.3^a	2.9±0.7^a	29.0±2.6^a	3.0±0.3^{ab}	30.3±1.5^{ab}
Mixture of metal complex dark green azo acid dye with anionic surfactant oil 8 mgl^{-1}	2.5±0.5^a	25.3±0.5^a	2.9±0.4^a	28.0±1.0^a	3.2±0.2^{ab}	32±.32.3±6.7^{ab}
Mixture of metal complex dark green azo acid dye with anionic surfactant oil 11 mgl^{-1}	2.8±0.5^a	27.3±2.9^a	3.4±0.4^a	30.0±2.6^a	3.4±0.4^a	35.7±8.1^a

Different letters ^{a,b,c} on the column indicate that the means are significantly different in comparison test ($p<0.05$). Values are mean ± SD

Table 2 : Leukocytes count (cells µl^{-1}) for fish *O. niloticus* exposed to different treatments

Treatments	30days	60days	90days
Control	4120±3714^a	4345±684^{ab}	4375±3788^a
Anionic surfactant oil 5 mgl^{-1}	4580±3029^a	6847±912^a	2455±0^a
Metal complex dark green azo acid dye 3 mgl^{-1}	4528±4351^a	4717±2161^{ab}	3633±157^a
Metal complex dark green azo acid dye 6 mgl^{-1}	2840±2217^a	3290±1628^b	2396±830^a
Mixture of metal complex dark green azo acid dye with anionic surfactant oil 8 mgl^{-1}	2613±1497^a	3765±1255^b	2702±936^a
Mixture of metal complex dark green azo acid dye with anionic surfactant oil 11 mgl^{-1}	3340±0^a	4070±2311^{ab}	3854±954^a

Different letters ^{a,b} on the column indicate that the means are significantly different in comparison test ($p<0.05$). Values are mean ± SD

Table 3 : Thrombocytes count (cells µl^{-1}) for fish *O. niloticus* exposed to different treatments

Treatments	30days	60days	90days
Control	7897±6621^a	7505±6943^b	7657±413^b
Anionic surfactant oil 5 mgl^{-1}	33587±10638^a	15800±10946^a	16367±2835^{ab}
Metal complex dark green azo acid dye 3 mgl^{-1}	28563±22926^a	14622±10236^{ab}	18621±1850^{ab}
Metal complex dark green azo acid dye 6 mgl^{-1}	22720±32900^a	11750±8140^{ab}	25396±2415^{ab}
Mixture of metal complex dark green azo acid dye with anionic surfactant oil 8 mgl^{-1}	35607±11442^a	6693±724^b	15132±936^{ab}
Mixture of metal complex dark green azo acid dye with anionic surfactant oil 11 mgl^{-1}	16650±5768^a	5567±964^b	39090±848^a

Different letters ^{a,b} on the column indicate that the means are significantly different in comparison test ($p<0.05$). Values are mean ± SD

Thrombocyte numbers also did not differ significantly among all treatments at 30 and 60 days of exposure (Table 3). However, those of control fish at 60 and 90 days were significantly less ($P<0.05$) than of fish exposed to 5 mg l^{-1} anionic surfactant oil and 11 mg l^{-1} mixtures of metal complex dark green azo acid dye with anionic surfactant oil, respectively (Table 3). Harvey (2001) suggested that increased thrombocyte counts were occasionally observed due to hemolysis of erythrocyte, as expressed by erythrocyte morphology anisocytosis, karyolysis, cytoplasmic vacuolation and ghost cells.

After 60 days of exposure, total copper concentration in blood of control fish and those exposed to 6 mg l^{-1} metal complex dark green azo acid dye (0±0.004 µg l^{-1}) were significantly ($P<0.05$) less than those exposed to 5 mg l^{-1} anionic surfactant oil (112±0.002 µg l^{-1}), 3 mg l^{-1} metal complex dark green azo acid dye (3±0.002 µg l^{-1}), 8 mg l^{-1} mixture of metal complex dark green azo acid dye with anionic surfactant oil (38±0.002 µg l^{-1}) and 11 mg l^{-1} of the mixtures of metal complex dark green azo acid dye with anionic surfactant oil (98±0.004 µg l^{-1}). After 90 days of exposure, total copper concentration in control blood (382±0.002 µg l^{-1}) was not significantly different ($P>0.05$) than those exposed to 5 mg l^{-1} anionic surfactant oil (241±0.001 µg l^{-1}), 3 mg l^{-1} metal complex dark green azo acid dye (293±0.002 µg l^{-1}) and 6 mg l^{-1} (431±0.001 µg l^{-1}), 8 and 11mg l^{-1} mixture of metal complex dark green azo acid dye with anionic surfactant oil (388 and 438 µg l^{-1}), respectively.

After 60 days of exposure, total chromium in blood of control fish (72±0.003 µg l^{-1}) was significantly ($P<0.05$) less than

that of fish exposed to 5 mg l^{-1} anionic surfactant oil (126 μg l^{-1}), 3 mg l^{-1} metal complex dark green azo acid dye (123 μg l^{-1}) and 6, 8 or 11 mg l^{-1} mixtures of metal complex dark green azo acid dye with anionic surfactant oil (130, 130 or 130 μg l^{-1}), respectively. After 90 days of exposure, total chromium in the blood of control fish (129 μg l^{-1}) was significantly ($P<0.05$) less than that of fish exposed to 3 mg l^{-1} metal complex dark green azo acid dye (36 μg l^{-1}) and 6, 8 or 11mg l^{-1} mixtures of metal complex dark green azo acid dye with anionic surfactant oil (64, 36 and 26 μg l^{-1}) respectively but lower than that exposed to 5 mg l^{-1} anionic surfactant oil (465 μg l^{-1}).

Inverse relation between total copper and chromium concentration in blood could be due to competition for metal absorption through sodium pathway and metabolic reactions (Wood *et al*., 2012). Concentration of metal in blood might occur due to imbalance in route and rate of uptake, metabolism and excretion (Hodgson, 2004). High concentration of total copper and chromium compounds in blood potentially contribute to a subchronic effect on changes in number of erythrocytes, leukocytes and thrombocytes, in addition to morphological abnormality in erythrocytes. Total copper and chromium was also detected in control fish that was presumably derived from the commercial food fed during study. Commonly, fish food is supplemented with copper and chromium as both at low concentrations, are considered essential elements for animal health (Abdel-Baki *et al*., 2011). Concentration of total chromium in blood of fish exposed to anionic surfactant oil, metal complex dark green azo acid dye and their mixture after 60 and 90 days were higher than the range of basal requirement intake of 0.2-45μg per day, however, total copper concentration were lower than basal requirement for copper intake of 200 - 1300 μg per day for human consumption depends on their age (National Research Council, 2001).

The results of the present study strongly suggest that unmanaged discharge of effluent from textile industries, especially those that employ metal complex azo acid dyes and anionic surfactant, are likely to impose harmful effects on fish health. Further, excessive consumption of contaminated fish adversely affects human health.

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