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Environmental Contamination and Toxicology

Hematological Values of Rainbow Trout, *Oncorhynchus mykiss* W., Exposed to Premetallized Dyes

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Premetallized dyes are important chemicals used in the textile industry. From the chemical point of view they are composed of a metal atom to which one or more dye molecules, generally acid, are linked together forming a coordination complex with some affinity for protein and polyamide fibers. From the dyeing point of view, they are acid dyes and, as such, they are classified in the Color Index.

The environmental problems posed by dyes are rather moderate in their impact compared with those caused by other chemicals such as pesticides, detergents, industrial oils, heavy metals, etc. Dyes have been shown to be mildly to moderately toxic to aquatic organisms. The damage to the environment does not only depend on the amount of dyes disposed of but also on ecotoxicological properties of the products and the characteristics of their transport in the medium. Some dyes are soluble while others are not, so their toxicity and disapperance from the environment can be highly variable, but evaluations of their sublethal or chronic effects on aquatic organisms are not available in the literature.

Toxicity tests carried out in our laboratory in a previous study demonstrated that the 48 hr LC50 values for rainbow trout of the metal complex dye (C.I. Acid Violet 66) and the azoic base utilized in its synthesis (C.I. Acid Red 217) were 8.2 mg/L and 71.04 mg/L, respectively (Riva et al. 1990). It was also shown that, following treatment with sublethal concentrations, accumulation of the chemicals in bile and fish tissues occurred (Riva 1989).

In order to observe the direct or indirect physiological effects, if any, due to the premetallized dyes on rainbow trout, Oncorhynchus mykiss, a hematological study was undertaken after treating the fish with sublethal doses of C.I. Acid Violet 66 and C.I. Acid Red 217.

MATERIALS AND METHODS

Sexually immature) rainbow trout, Oncorhynchus mykiss (ca 10 to 15 cm long, were obtained from a local hatchery and maintained in well-aerated flowing freshwater (total hardness = 316 mg/L; pH = 8 ± 0.1; temperature = 14 ± 1 ºC) for at least two weeks before experiments. Fish were fed a commercial fish diet at a rate of 1% live weight per day during the acclimation period as well as during treatment. The OECD 305 method (1981) was followed and, according to this standard, the doses were the lowest possible and detectable. Specimens for experimentation were placed in 20 L tanks (6 fish in each tank and 7 tanks for each dose). Metal-complex dye solutions were prepared by adding 0.82,

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1.64 and 4.1 mg/L of the commercial dye. These concentrations correspond to 1/10, 1/5 and 1/2 of the LC 50 at 48 hr for the trout (Riva et al. 1990). In order to remove fish excrement and to maintain a relatively constant concentration of the toxicant in the treatment, solutions were replaced every 2 days. To compare the hematological effects of the metal-complex dye (C.I. Acid Violet 66) with an atom of chromium and the azo compound (C.I. Acid Red 217),some groups of fish were exposed to 0.58, 1.17 and 2.93 mg/L of C.I. Acid Red 217 for the same treatment periods which were 70, 42 and 14 days, respectively, for the low, medium and high doses of both compounds.

After caudal severance the blood was collected in hematocrit tubes and blood samples were analyzed for hematocrit, hemoglobin concentration, erythrocyte number, erythrocyte sedimentation rate (ESR), leucocrit, leucocyte number, plasma glucose, plasma lactate and differential leucocyte count. General hematological procedures were followed. Blood cell counts were estimated using an improved Neubauer hemocytometer. Hemoglobin was measured using the cyanmethemoglobin method with Drabkin reagent (Snieszko 1961). Hematocrit values were read and expressed as the volume of RBC per 100 mL, leucocrit was determined following the McLeay & Gordon method (1977), and the methods of Lowe-Jinde & Niimi (1983) and Niimi & Lowe-Jinde (1984) were used for the differential counts. The RBC indices of mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), and mean corpuscular volume (MCV) were calculated.

Blood smears for differential cell ratios were fixed in methanol and later stained with May Grunwald - Giemsa. A minimum of 1000 cells were enumerated under oil immersion. Erythrocytes were characterized as mature and immature, and leucocytes were characterized as lymphocytes, neutrophil granulocytes and thrombocytes.

Analysis of variance (ANOVA) was used to test for statistically significant differences among the means of replicates and treatments.

RESULTS AND DISCUSSION

No fish displayed any apparent toxicity symptoms (overturning, erratic movement, increased coughing, flared operculum, etc.) during the experiment. Effects of dyes on blood parameters of rainbow trout are presented in Tables 1, 2 and 3. The results in untreated control fish were similar to the values given for this species and other salmonidae (Blaxhall and Daisley 1973; Wedemeyer and Yasutake 1977; Miller et al. 1983), while the results in treated fish showed some variations in various parameters. We found that hematocrit, hemoglobin concentration, erythrocyte sedimentation rate and plasma glucose increased significantly ($p<0.05$, * in tables) after different concentrations of Acid Violet 66 exposure, while changes in RBC as well as WBC counts, leucocrit and plasma lactate were not significant ($p>0.05$) (Table 1). The comparation of parameters indicated a significant correlation ($p<0.01$) between RBC and WBC counts ($r=0.841$, $p<0.01$) for the dose 0.82 mg/L , and between hematocrit and ESR for 1.64 mg/L dose ($r=-0.869$, $p<0.01$) and for 4.1 mg/L dose ($r=-0.961$, $p<0.01$). The calculated parameters MCHC, MCH and MCV did not show a statistically significant effect of dose and duration of exposure.

In fish exposed to three Acid Red 217 concentrations, blood parameters showed little or no change from controls. The exceptions were hematocrit which increased and ESR which decreased significantly ($p<0.05$, * in tables) after 14 d exposure with the high dose (2.93 mg/L) (Table 1), but the parameters MCHC, MCH and MCV did not show significant differences. There was a good correlation between hematocrit and ESR with coefficients

Table 1. Influence of the dyes C.I. .Acid Violet 66 and C.I. Acid Red 217 on hematological parameters in rainbow trout, Oncorhynchus mykiss

Parameters	Control a	Dye Exposure 70 Days		Dye Exposure 42 Days		Dye Exposure 14 Days	
		Ac. Violet 66 0.82 mg/L	Ac.Red 217 0.58 mg/l	Ac.Violet 66 1.64 mg/l	Ac.Red 217 1.17 mg/l	Ac.Violet 66 4.1 mg/l	Ac.Red 217 2.93 mg/l
Hematocrit (%)	26.33±3.34 (21--32)	30.51±2.26 (25–34)	32.00±3.10 (28–36)	*32.77±1.91 (28–37)	31.90±3.03 (26–38)	*34.28±2.05 (29–38)	*33.21±2.76 (28–39)
Hemoglobin (g /100ml)	5.74±0.54 (4.6–6.9)	6.61±0.43 (5.9–7.5)	5.74±0.53 (5.0–6.91)	*6.90±0.32 (6.2–7.4)	5.56±0.80 (4.26±7.28)	*7.15±0.31 (6.4–7.5)	5.30±0.45 (4.41–5.96)
Erythrocytes (10^6/ mm^3)	0.96±0.15 (0.74–1.2)	1.09±0.13 (0.88–1.35)	0.99±0.12 (0.79–1.3)	1.18±0.12 (0.97–1.4)	0.90±0.11 (0.72±1.16)	*1.23±0.61 (0.99–1.45)	0.97±0.12 (0.73–1.15)
ESR (mm h)	0.56±0.13 (0.35–0.69)	0.51±0.08 (0.39–0.70)	0.40±0.09 (0.25–0.58)	0.39±0.09 (0.29–0.54)	0.38±0.08 (0.22–0.58)	*0.34±0.08 (0.22–0.57)	*0.36±0.07 (0.23–0.53)
Leucocytes (10^4/ mm^3)	5.02±0.78 (3.83–6.19)	5.15±0.64 (4.15–6.68)	5.19±0.51 (3.92–6.21)	5.78±0.60 (4.66–6.99)	4.62±0.56 (3.58–5.84)	5.68±0.57 (4.39–7.02)	4.35±0.68 (2.99–5.58)
Leucocrit (%)	0.65±0.09 (0.49–0.79)	0.76±0.07 (0.60–0.90)	0.59±0.09 (0.41–0.74)	0.77±0.07 (0.64–0.90)	0.51±0.11 (0.25–0.77)	0.96±0.22 (0.71–1.49)	0.43±0.09 (0.24–0.66)
Glucose (mg /100 ml)	44.94±14.93 (27.4–69.3)	*83.45±22.12 (44.6–120.1)	52.22±13.41 (14.8–76.3)	*79.83±18.45 (45.2–106.5)	38.50±13.12 (11.0–76.3)	*98.24±15.96 (70.5–131.1)	59.84±18.05 (26.0–97.9)
Lactate (mg /100 ml)	11.34±3.94 (4.68±16.19)	14.70±5.93 (3.97-29.31)	16.65±10.19 (5.30–35.02)	10.92±6.18 (4.12–24.45)	13.04±4.78 (5.74–26.01)	16.42±4.44 (7.45–23.70)	18.41±7.29 (6.48–41.64)
MCHC (%)	22.00±2.32 (16.3–27.1)	21.71±1.32 (19.35–24.01)	18.09±2.45 (14.53–22.52)	21.11±1.04 (18.9–23)	17.52±2.60 (14.08–23.55	20.90±1.03 (19.2–23.8)	16.05±1.63 (13.08–20.76)
MCH (pg)	60.39±6.33 (50.4–77.30)	61.13±6.66 (52.39–73.02)	58.36±5.91 (49.21–70.58)	59.11±5.98 (50.2–74.6)	62.18±6.56 (51–73.22)	58.46±4.67 (48.2–66.5)	54.89±5.15 (47.3–66.6)
MVC (μm^3)	276.87±36.10 (205.2–347.8)	281.79±28.13 (221.40–339.5)	326.84±44.11 (204.56–399.14)	280.44±29.19 (226.7–336.4)	358.90±38.84 (267.6–431.5)	280.61±28.92 (206.7-332.3)	346.10±51.77 (259.6–491.0)

Note - The values represent the mean ±SD of 42 fish (a= 72 fish)

* Significantly different values at $p < 0.05$

Table 2. Blood cell ratios (%) of rainbow trout , Oncorhynchus mykiss, exposed to C.I. Acid Violet 66.

Parameter	Exposure Time (days) 1	2	3	4	8	10	14	28	36	42	56	70	x
Erythrocytes (%)													
Mature													
Control	82.1±4.5	80.3±5.1	79.7±5.3	83.7±3.2	80.7±5.0	85.4±3.6	81.1±6.7	79.7±5.6	84.1±1.3	82.5±6.2	78.5±9.0	79.4±7.6	83.29±5.2
0.82 mg/l				90.1±6.6	-	88.7±5.9	83.0±2.6	80.6±9.2	-	84.4±7.7	82.1±5.9	86.6±8.3	85.07±6.6
1.64 mg/l				85.6±2.2	88.5±4.9	83.7±8.1	81.9±5.9	90.5±6.3	92.6±5.9	90.1±9.0			87.56±6.04
4.1 mg/l	87.3±10	83.6±7.5	84.9±7.7	88.9±6.3	90.1±3.7	84.6±8.5	87.1±9.4						86.6±7.6
Immature													
Control	17.9±6.1	19.7±1.5	20.3±2.1	16.3±2.4	19.3±2.7	14.6±3.4	18.9±0.7	18.1±0.9	15.9±2.5	17.5±3.8	21.5±2.4	20.6±1.5	16.71±2.18
0.82 mg/l				9.9±5.1	-	11.3±4.4	17.0±4.0	19.4±3.9	-	15.6±4.5	17.9±6.3	13.4±7.1	14.93±5.04
1.64 mg/l				14.4±2.3	11.5±5.2	16.3±3.7	18.1±3.1	9.5±1.7	7.4±2.9	9.9±5.1			12.44±3.43
4.1 mg/l	12.7±3.3	16.4±5.5	15.1±4.3	11.1±3.7	9.9±2.5	15.4±7.0	12.9±6.2						13.35±4.64
Leucocytes (%)													
Lymphocytes													
Control	98.7±3.5	91.1±5.5	97.0±2.7	93.0±4.2	83.1±5.1	84.0±3.3	89.2±2.5	80.5±4.9	85.1±3.5	85.6±5.1	87.0±4.6	88.5±3.7	88.17±1.05
0.82 mg/l				92.0±4.7	-	90.4±7.9	82.5±6.1	80.3±5.9	-	81.4±9.9	79.7±9.6	78.9±10	83.6+2.75*
1.64 mg/l				84.3±7.2	81.5±6.9	80.4±8.3	88.0±5.9	87.3±6.5	86.6±7.8	81.4±6.9			84.9±2.07*
4.1 mg/l	84.5±4.4*	86.2±3.0*	84.3±2.3*	82.2±4.5*	80.6±3.1	81.2±5.5	82.2±6.1						84.45±2.13*
G. Neutrophiles													
Control	1.2±0.6	0.8±1.1	2.2±0.9	0.6±1.1	2.7±2.0	3.4±0.5	1.9±0.8	4.2±2.1	2.9±2.1	3.1±3.2	2.7±2.1	1.9±0.9	1.99±0.45
0.82 mg/l				0.9±0.5	-	0.8±0.3	0.5±0.4	1.1±0.9	-	0.9±0.5	2.1±0.9	1.6±1.1	1.13±0.66
1.64 mg/l				3.5±1.5	4.2±1.0	5.5±2.1	4.0±2.5	3.1±1.5	3.2±2.1	3.9±0.5			3.91±1.6
4.1 mg/l	1.7±0.9	2.2±1.3	4.5±4.0	3.6±2.2	4.4±3.1	5.6±4.0	6.2±3.9						4.03±2.77
Thrombocytes													
Control	10.1±0.9	8.1±0.7	10.8±0.1	6.4±1.3	14.2±3.1	12.6±3.6	8.9±2.0	15.3±1.8	12.0±2.4	11.3±2.5	10.3±3.3	9.6±3.5	9.84±2.6
0.82 mg/l				14.6±2.0*	-	13.0±0.6	8.2±1.0	6.7±2.1	-	8.5±1.0	9.7±1.2	6.2±3.7	9.56+1.65
1.64 mg/l				12.2±2.7*	14.3±7.2	14.1±1.5	8.0±2.7	9.6±3.3	10.2±4.5	14.7±3.1			12.16±3.86
4.1 mg/l	13.7±0.7*	11.6±1.5*	12.2±0.3*	14.2±3.8*	15.0±6.1	13.2±8.1	11.6±7.7						12.93±5.6

Note: The values represent the mean ±SD of 6 fish at each sample interval
* Significantly different values at $p<0.05$

Table 3. Blood cell ratios (%) of rainbow trout, Oncorhynchus mykiss, exposed to C.I. Acid Red 217

	Exposure Time (days)												
	1	2	3	4	8	10	14	28	36	42	56	70	x
Erythrocytes (%)													
Mature													
Control	85.4±2.2	82.7±4.6	89.5±3.1	87.5±4.9	90.0±2.7	86.4±6.1	84.4±5.4	87.1±7.1	84.5±5.0	79.9±5.2	81.4±7.2	82.7±5.9	83.29±4.95
0.58 mg/l				79.9±8.8	-	84.5±9.2	83.7±9.2	89.5±10.1	-	86.1±10.5	84.7±7.5	89.6±6.8	85.43±8.16
1.17 mg/l				89.4±8.2	88.5±10.2	86.6±6.1	81.5±10.3	87.2±6.1	88.4±9.4	82.9±10.5			86.35±8.68
2.93 mg/l	88.3±5.1	84.9±6.8	86.9±6.8	84.5±3.7	86.3±6.1	90.1±3.7	82.9±7.3						86.27±5.28
Immature													
Control	14.6±1.7	17.3±1.5	10.5±2.1	12.5±2.9	10.0±1.3	13.6±3.5	15.6±3.9	12.9±2.4	15.5±4.3	20.1±2.0	18.6±4.7	17.3±2.5	16.70±2.73
0.58 mg/l				20.1±10.5	-	15.5±4.8	16.3±8.1	10.5±5.8	-	13.9±3.6	15.3±7.9	10.4±3.2	14.57±6.27
1.17 mg/l				10.6±5.1	11.5±6.6	13.4±4.8	18.5±7.7	12.8±4.0	11.6±6.6	17.1±4.7			13.64±5.63
2.93mg/l	11.7±8.8	15.1±8.1	13.1±7.6	15.5±9.9	13.7±7.3	9.9±4.4	17.1±8.2						13.70±7.67
Leucocytes (%)													
Lymphocytes													
Control	89.2±3.2	90.7±3.9	92.5±3.4	91.4±47	93.1±4.9	92.4±1.9	88.6±7.2	87.4±5.5	88.9±7.2	85.3±4.4	87.1±5.6	86.6±7.5	88.17±5.36
0.58 mg/l				85.3±5.6	-	88.2±5.3	83.4±7.7	85.6±6.9	-	83.8±4.1	86.1±7.8	89.2±9.2	85.94±6.65
1.17 mg/l				91.1±2.4	92.1±3.2	84.5±7.5	80.3±6.9	82.0±7.9	88.7±6.1	84.5±7.9			86.17±5.98
2.93 mg/l	82.3±2.5*	80.7±5.8*	85.6±3.2*	79.2±3.9*	78.7±10.5	78.5±9.9	80.1±8.4						80.73±8.45
G.Neutrophiles													
Control	0.9±0.6	1.2±0.9	0.6±1.2	1.1±1.1	0.9±1.3	1.2±0.8	2.3±1.5	2.2±0.7	1.1±0.8	3.1±2.0	2.6±1.9	2.9±2.1	1.99±1.25
0.58 mg/l				3.2±1.2	-	2.6±1.1	4.9±3.0	3.7±3.0	-	2.9±1.1	3.2±0.9	2.6±1.2	3.30±1.64
1.17 mg/l				2.1±0.6	1.1±0.9	2.1±2.0	4.5±3.0	5.7±3.1	4.3±3.5	3.9±2.2			3.38±2.18
2.93 mg/l	2.8±2.2	3.9±3.6	3.7±1.1	6.4±6.1	5.6±5.0	4.8±4.5	5.5±3.9						4.67±3.77
Thrombocytes													
Control	9.9±1.3	8.1±2.1	6.9±3.0	7.5±3.5	6.0±3.6	6.4±4.1	9.1±3.3	10.4±3.5	10.0±3.9	11.6±3.4	10.3±4.3	105±2.9	9.84±3.49
0.58 mg/l				11.5±2.9	-	9.2±3.4	11.7±2.5	10.7±4.1	-	13.3±6.2	10.7±3.5	8.2±4.1	10.75±3.81
1.17 mg/l				6.8±4.1	6.8±0.6	13.4±7.4	15.2±5.1	12.3±2.7	7.0±2.5	11.6±4.3			10.44±3.81
2.93 mg/l	14.9±3.5*	15.4±3.9*	10.7±0.7*	14.4±2.4*	15.7±7.3	16.7±10.3	14.4±9,6						14.60±7.28

Note: The values represent the mean ±SD of 6 fish at each sample interval
*Significantly different values at p< 0.05

r=-0.896, r=-0.928 and r=-0.943($p<0.01$) for doses of 0.58, 1.17 and 2.93 mg/Lof the Acid Red 217 dye, respectively.

The hematologic parameters were studied to observe physiological effects due to premetallized dyes on rainbow trout, Oncorhynchus mykiss. The more common hematological parameters are influenced by biological factors. Changes of these values in rainbow trout have been attributed to the effects of seasons, diet, sex, and among strains. The results of this study are consistent with the values found by Wedemeyer and Yasutake (1977), Miller et al. (1983), Blaxhall and Daisley (1973), McCarthy et al. (1973) and Lowe-Jinde and Niimi (1983). Some parameters may be indicators of physiological stress but they also are influenced by capture, sampling and sample storage (Lowe-Jinde and Niimi 1983). The increased hematocrit levels may be attributed to an increase in size of the erythrocytes due to increased PCO2 in the blood, hypoxia or stressful procedures (Soivio et al. 1974). In the Acid Violet 66 exposure MCV did not vary, and a significant increase in hemoglobin concentration was observed which was reflective of the increase in hematocrit and RBC count. It is known that certain metals can affect blood parameters. Waiwood (1980) reported an increase in hematocrit values due to the action of copper. In our case it would be possible to assume that a relation between the presence of chromium (of the metal complex dye) and increase of hematocrit exists. The wide range reported for some hematologic parameters may be due to different techniques used. A correlation between hematocrit, hemoglobin and erythrocyte number was indicated (Snieszko 1961). The erythrocytes in suspension could affect the erythrocyte sedimentation rate, being that ESR is a non-specific reaction giving a measure of the presence and intensity of disease processes in the body (Blaxhall and Daisley 1973). The ESR decrease found in fish exposed to the high doses of dyes Acid Violet 66 and Acid Red 217 could be related to hematocrit increase, with high correlation coefficients.

The mature and immature erythrocyte percentages were comparable to that of controls in both dye treatments (Tables 2 and 3). An examination of the effects of Acid Violet 66 and Acid Red 217 on WBC ratios indicated significant differences ($p<0.05$) between control and exposed fish (Tables 2 and 3). In white cell differential ratios among fish exposed to both dyes lymphocyte percentage decreased and thrombocyte percentage increased at the highest exposure doses. McLeay and Gordon (1977) suggested leucocrit and WBC counts as useful methods for detection of sublethal effects on fish due to toxics. Fish exposed to Acid Violet 66 and Acid Red 217 revealed leucocytosis and leucopenia, respectively, during the first period of the treatment, and after the 4th day, a return to the levels of controls. We found that only the high dose of both compounds induced significant lymphopenia and an increase in thrombocytes. Acute exposure of trout to copper induces leucopenia, the majority of which is the result of lymphopenia, whereas, chronic exposure to the same metal results in significant neutrophilia (Dick and Dixon 1985). The leucopenia observed in trout after acute exposure can be attributed to a generalized stress response rather than to a specific cytotoxic action of copper. Leucopenia, caused by increased pituitary-interrenal activity (Donalson and Dye 1975), has been observed in fish exposed to a variety of acute stressors (McLeay and Gordon 1977), being composed mainly of lymphopenia (Ellis 1981). While neutrophilia has been reported in situations of acute stress (Weinreb 1958), it would appear to be more significant after chronic stress. McLeay (1973) observed neutrophilia in salmonidae exposed to sublethal levels of bleached kraft mill effluent for 25 days.Thrombocytes are involved in the blood clotting process, and an increase in these cells may be related to acute and chronic stress situations (Wedemeyer and McLeay 1981).

Other blood indicators of environmental stress are plasma glucose and plasma lactate. The present experimentation showed that in fish exposed to the Acid Violet 66 very high

levels of plasma glucose were reached during the first four days, while the Acid Red 217 dye treatment produced increases only after the first day and later decreased to control values.This increase of plasma glucose agrees with sensitivity of this parameter to low concentrations of the insecticide dieldrin (Silbergeld 1974), and with the glucose levels due to the presence of aromatic hydrocarbons (Zbanyszek 1984). The increase of glucose may result from activation of glycogen stores by adrenalin mediated through stress (Nakano and Tomlinson 1967), with a subsequent return to normal levels (Casillas and Smith 1977). Glucose and lactate reflect the changes in the carbohydrate metabolism under hypoxic and stress conditions (Soivio et al.1974, 1977).There is high variability in the literature for the plasma lactate. Our results concur with data presented for salmonidae, and in dye exposures no significant ($p>0.05$) differences for plasma lactate in relation to the untreated fish were observed. Our study shows that C.I. Acid Violet 66 and C.I. Acid Red 217 induce similar effects on blood in rainbow trout at the doses tested. These results suggest that it is not possible to establish a clear relation between the presence of chromium, in the metal complex dye, and the blood effect at least in these experimental conditions, as it was expected.

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