Chronic malachite green toxicity in Nile tilapia: Pathological and hematological studies with special reference to quantitative histopathological assessment

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Abstract: Although it is not approved in many countries, malachite green is considered one of the most effective treatments for some fish diseases. This study aimed to investigate the pathological and hematological effects of chronic malachite green (MG) toxicity in Nile tilapia. Sixty fish used to determine 96hrs LC_{50} of MG, the obtained result was 0.76 mg/L. Forty fish were used to induce chronic toxicity, twenty fish were exposed to 0.076 mg/L for 6 weeks and other fish as control. Gills, hepatopancreas, posterior kidney and spleen were the most affected organs during chronic MG exposure. Proliferative interlamellar hyperplasia with fusion in gills, hydropic degeneration of the hepatic cells, renal tubular and hemopoietic tissue necrosis and splenic lymphocytic necrosis and depletion were recorded as histopathological changes. Modified quantitative microscopic assessment was used in this study to monitor tissue damage. Long term MG exposure induced deleterious effect on blood parameters including anemia and leukopenia.

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1. Introduction

Nowadays, Aquaculture is an important and rapidly expanding industry in many countries. Freshwater fish, primarily Nile tilapia, is cultivated on large scale for the local market. With the expanding scale production of Nile tilapia, problems arose with occasional mass mortalities caused by fungal and protozoal infections. Therefore, malachite green inevitably used to overcome these problems (Saglam et al., 2003).

Although not approved by the Food and Drug Administration (FDA) in the aquaculture industry, MG has been used since the early 1930s to combat ecto-parasites and to control fungal infection of fish eggs, fingerlings and adult fish because of anti-parasitic and anti-microbial properties (Diggles, 2001and Gieseker et al., 2006). Once in the body, malachite green appears to be reduced, to some extent, to leucomalachite green (Alderman, 1985; Saglam et al., 2003 and Srivastava et al.,2004)

The dye malachite green (C23H5N2) is used as bath for treatment protozoal ecto-parasites of fish (Alvárez-Pellitero, 2004). It is now known to be highly toxic to mammalian cells and a liver tumour promoter (Panandiker et al.1993).

Histopathology has revealed that MG caused detrimental effects in liver, gills and kidney. It caused sinusoidal congestion and necrosis of liver of *Heteropneustes fossilis* (Srivastava et al., 1998[a]) and severe damage to gills resulting in necrosis of lamellar cells in rainbow trout (Gerundo et al., 1991) and *Heteropneustes fossilis* (Srivastava et al., 1998[b]). The dye caused hyperplasia of epithelial cells in the proximal convoluted tubules and shrinkage of glomeruli in the kidney of *Heteropneustes fossilis* (Srivastava et al., 1998[b]).

Malachite green also affects hematological parameters: decreases in haematocrit values and anaemic responses have been reported in rainbow trout and Clarias gariepinus (Tanck et al., 1995 and Musa and Omoregie, 1999). Malachite green also had immunosuppressive effect on rainbow trout (Yonar and Yonar, 2010).

From literature, toxicological effect of MG has been considerably studied in many fish species; however, the local farmed species, Nile tilapia did not. Therefore, this study aimed to investigate the pathological and hematological effects in Nile tilapia following chronic exposure to MG and to monitor tissue damages using quantitative histopathological assessment.

2. Material and Methods

2.1 Fish:

A total number of 100 apparently healthy Nile tilapia fish (40-60 g) was used to determine 96 hrs LC_{50} and investigation of chronic MG toxicity. Fish was kept in prepared glass aquaria (90×50×35 cm). These aquaria were supplied with chlorine-free tap water and Oxygen at 21.5±2 0C. Fish were fed on a commercial diet containing 25% crude protein as described by Eurell et al. (1978).

2.2 Determination of 96 hrs LC50 of MG

Sixty fish were used in this experiment. Fish were divided into 6 equal and exposed to water containing (0,0.2, 0.6, 1.0, 1.4, and 1.8 mg/L) up to 96 hrs. Water and MG were renewed daily. The calculation of LC_{50} is done according to the formula of Reed and Munch (1938).

2.3 Chronic MG toxicity

Forty Nile tilapia were randomly divided into 2 equal group; treated group exposed to $^{1}/_{10}$ (LC50) MG and control. The experiment extended to 6 weeks and the water and MG were renewed every 3 days. Fish were sacrificed on 2,4 and 6 weeks.

2.4 Histopathological studies

Tissue specimens (gills, hepatopancreas, posterior kidney and spleen) were rapidly fixed in 10% neutral buffered formalin. The fixed specimens were processed through the conventional paraffin embedding technique. Paraffin blocks were prepared, from which 5 microns thick sections were obtained. These sections were stained by Hematoxyline and Eosin (H&E) according to the method described by Culling (1983).

2.5 Quantitative histopathological assessment

A modified version of the quantitative histopathological assessment protocol described by Bernet et al. (1999) was used to evaluate histopathological alterations observed in the most affected organs. Examined organs were assessed according to four reaction patterns: circulatory disturbances (CD), degenerative and necrotic changes (D), progressive changes (PC) and inflammatory reaction (IR), these reaction patterns are made up of various alterations, concerning either functional units of the organ or an entire organ.

An importance factor (w) ranging from 1 to 3 was assigned to each alteration as a measure of how a specific alteration might affect fish health:1: minimal pathological importance, the lesion is easily reversible as exposure to an irritation ends; 2: moderate pathological importance, the lesion is reversible in most cases if the stressor is neutralized; while 3: marked pathological importance, the lesion is generally irreversible, leading to partial or total loss of the organ function. The significance of a lesion depends on its pathological importance, i.e., how it affects organ function and essentially the capability of the fish to survive.

Every alteration was also assigned a score value (a), ranging from 0 to 6. It was assigned based on the percentage, degree and extent of the alteration: (0): unchanged; (1 and 2): mild occurrence; (3 and 4): moderate occurrence and (5 and 6): severe occurrence (diffuse lesion).

Also prevalence (P) was assigned as the percentage of an alteration occurrence within all treated fish. Combining the information of the importance factor, score value and prevalence, a reaction pattern index and an organ index were calculated as:

Reaction pattern index ($I_{org\,rp}$)

$$I\ org\ rp = \sum org\ rp\ alt\ (w \times a \times p)$$

Organ index (I_{org})

$$I\ org = \sum I\ org\ rp$$

org = organ; rp = reaction pattern; alt = alteration; a = score value; w = importance factor and p = prevalence.

2.5 Hematological parameters

One ml of blood was collected from the caudal artery of each fish by using disposable tuberculin syringe into clean dry tube with citrated solution (0.1 ml of 3.8% sodium citrate solution / 1 ml of blood) to perform some hematological parameters: Total erythrocytic and leucocytic count (Kanaev, 1985),hemoglobin content and hematocrit Value according to Tietz (1976), mean corpuscular hemoglobin concentration (MCHC) as described by Hrubec et al. (2000).

2.7 Statistical analysis

Statistical analysis of the obtained data was by using the SPSS 11 computer program (SPSS Inc. Chicago, Illinois, USA); using analysis of variance (One-way ANOVA).

3. Results

3.1 96hrs LC50 of MG

96 hrs LC50 of MG was 0.76 mg/L as shown in table (1).

3.2 Chronic MG toxicity

3.2.1 Clinical signs and postmortem findings

The majority of fish in intoxicated group showed respiratory manifestation in the form of gasping, rapid opercular movements and crowdness at the oxygen source during the second week and onwards. Grossly, all fish exhibited mild ascitis and petechial hemorrhages allover body surface. These lesions were noticed during 3rd - 4th week then gradually disappeared. No mortalities were recorded during the chronic experiment. Internally, congested hepatopancreas, distended gallbladder with greenish bile and presence of ascetic fluid in the abdomen were evident.

Table (1): Mortality pattern during the estimation of 96hrs LC_{50}:

Group	MG mg/L	No. of fish	Number of dead fish				Total No. of dead fish	a	b	Σ a×b
			24hrs	48hrs	72hrs	96hrs				
1	0.2	10	0	0	0	0	0	0.2	0	0
2	0.6	10	0	1	1	2	4	0.4	2	0.8
3	1.0	10	1	2	2	2	7	0.4	5.5	2.2
4	1.4	10	2	8	0	0	10	0.4	8.5	3.4
5	1.8	10	10	0	0	0	10	0.4	10	4

$$Lc50 = \text{biggest dose} - \frac{\sum (a \times b)}{n}$$

a= difference between used doses. b= sum of dead fish in two consequent groups.
n= number of fish used in each group.

3.2.2 Histopathological lesions

Gills:

Circulatory disturbances were negligible, but mild telangiectasis appeared only on the 5th week of MG exposure due to rupture of retaining pillar cells (Fig. G1). The most dominant histopathological finding in gills was the progressive changes mainly interlamellar epithelial hyperplasia all over the chronic experiment leading to partial filamentous and complete lamellar fusion beside hyperplasia of goblet cells (Fig. G2) and lamellar necrosis noticed during the 4th week (Fig. G3). The inflammatory reaction was negligible, and appeared only on the 4th week as mild inflammatory cells infiltration within gill arches.

Hepatopancreas:

Microscopically, mild circulatory disturbance was noticed as congestion of blood vessels with hepatic sinusoidal dilatation especially after 2 weeks of malachite green exposure. The unique histopathological finding in the hepatopancreas was the degenerative changes, primarly hydropic degeneration of the hepatic cells that was detected all over the experiment (Fig. H).

Posterior kidney:

Circulatory disturbance was negligible and appeared only during the 2nd week of malachite green exposure as congestion. The most dominant histopathological finding in posterior kidney was the degenerative change as multifocal hydropic degeneration of tubular epithelium which began at 4th week then extended to the end beside intraepithelial hyaline droplets in proximal convoluted tubules (Fig. K1). Multifocal tubular and hemopoietic tissue necrosis was detected during the 2nd and 4th week (Fig. K2).

Spleen:

Congestion of the ellipsoids is the only detectable circulatory change during the 2nd week of exposure. Enlargement and activation of melanomacrophage centers (MMCs; Fig. S1) was noticed during the 2nd week. Multifocal lymphocytic cell necrosis and depletion was the most encountered lesion allover the experiment (Fig. S2).

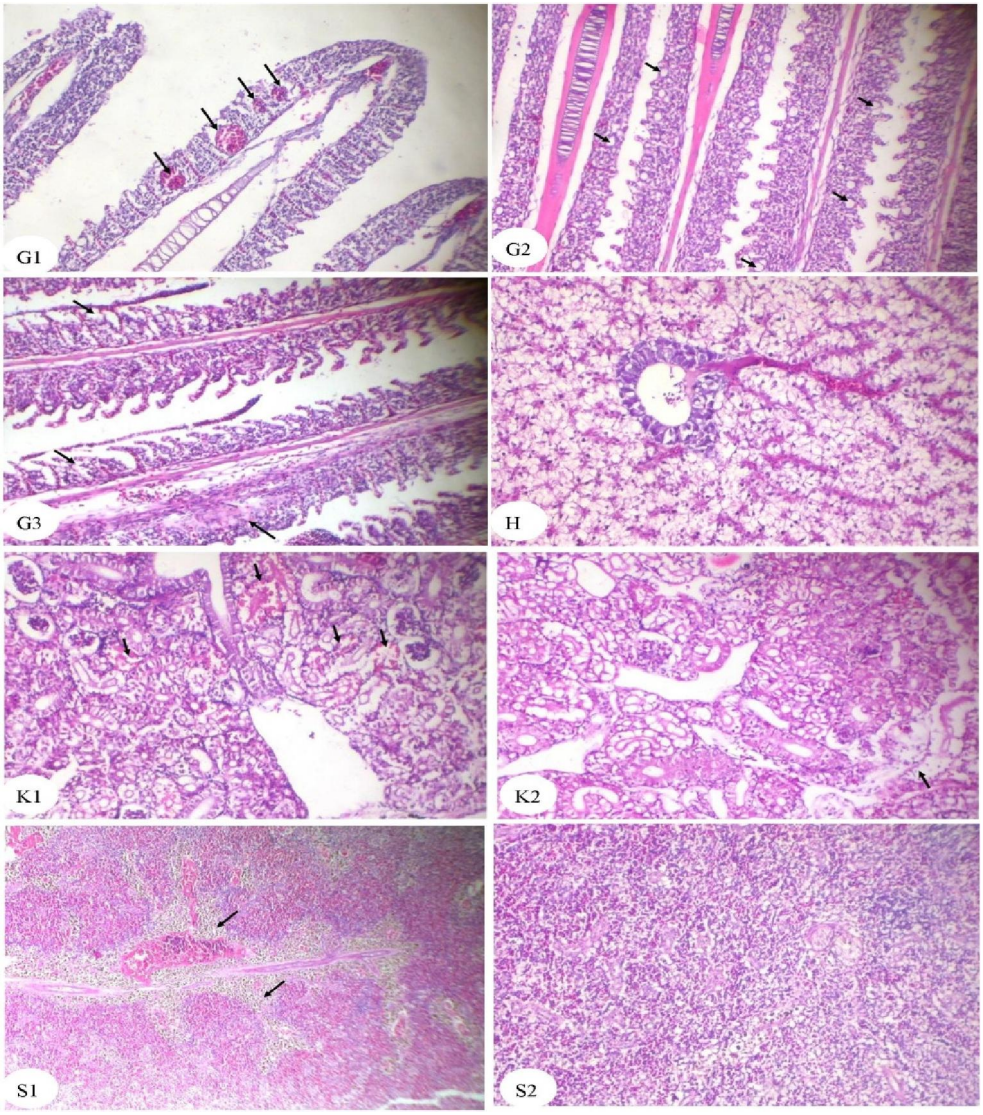

Fig. (G1): Gills of a Nile tilapia fish during 5th week of chronic MG toxicity showing diffuse lamellar fusion and telangiectasis (arrows).H&E(X400).

Fig. (G2): Gills of a Nile tilapia fish during 5th week of chronic MG toxicity showing diffuse inter-lamellar epithelial hyperplasia with complete lamellar fusion and goblet cells hyperplasia (arrows). H&E (400).

Fig. (G3): Gills of a Nile tilapia fish during 7th week of chronic MG toxicity showing diffuse inter-lamellar epithelial hyperplasia and multifocal lamellar necrosis (arrows). H&E (400).

Fig. (H): Hepatopancreas of a Nile tilapia fish during 4th week of chronic MG toxicity showing congestion of blood vessels with hepatic sinusoidal dilatation and diffuse hydropic degeneration. H&E (X400).

Fig. (K1): Posterior kidney of a Nile tilapia fish during 3rd week of chronic MG toxicity showing; hydropic degeneration of the renal tubules; hyaline droplets inside the proximal convoluted tubules (arrows).H&E (400).

Fig. (K2): Posterior kidney of a Nile tilapia fish during 6th week of chronic MG toxicity showing focal tubular and interstitial necrosis (arrow) beside multifocal hydropic degeneration of the tubular epithelium. H&E (X250).

Fig. (S1): Spleen of a Nile tilapia fish during 2nd week of chronic MG toxicity showing congestion of ellipsoids and enlargement of MMCs (arrows). H&E (X160).

Fig. (S2): Spleen of a Nile tilapia fish during 4th week of chronic MG toxicity showing lymphocytic necrosis and depletion. H&E(X250).

3.2.3 Quantitative histopathological Assessment

Quantitative assessment of the histopathological changes in Nile tilapia exposed to chronic MG toxicity (0.076 mg/L) was summarized in tables (2; 3; 4 and 5). The proliferative alteration pattern was higher in gills; however degenerative and necrosis pattern was the higher reaction pattern in hepatopancreas, posterior kidney and spleen. The organ index values were higher in gills followed by hepatopancreas, spleen then posterior kidney.

Table (2): lesions in the gills of experimentally exposed Nile tilapia to chronic waterborne MG (0.076 mg/ liter) and their importance, score, Prevalence and index values:

Exposure time	Reaction pattern	Alterations	Importance factor (W)	Score value (a)	Prevalence (P) (n=5)	Index	
						Reaction pattern (I rp)	Organ (I org)
2nd week	CD	-	-	-	-	-	8.0
	D	-	-	-	-	-	
	PC	Interlamellar epithelial hyperplasia with lamellar fusion	2	5	80%	8.0	
	IR	-	-	-	-	-	
4th week	CD	-	-	-	-	-	16.4
	D	-Lamellar necrosis	3	2	60%	3.6	
	PC	Hyperplasia of goblet cells Interlamellar epithelial hyperplasia with lamellar fusion	2 2	3 5	60% 80%	11.6	
	IR	Inflammatory cells within gill arch	1	2	60%	1.2	
6th week	CD	Telangiectasis	1	2	60%	1.2	9.2
	D	-	-	-	-	-	
	PC	Interlamellar epithelial hyperplasia with lamellar fusion	2	5	80%	8.0	
	IR	-	-	-	-	-	

Abbreviations: (CD) circulatory disturbances; (D) degenerative changes and necrosis; (PC) progressive changes; (IR) inflammatory reaction.

Table (3): lesions in the hepatopancreas of experimentally exposed Nile tilapia to chronic waterborne MG (0.076mg/ liter) and their importance, score, Prevalence and index values:

Exposure time	Reaction pattern	Alterations	Importance factor (W)	Score value (a)	Prevalence (P) (n=5)	Index	
						Reaction pattern (I rp)	Organ (I org)
2nd week	CD	Congestion with sinusoidal dilatation	1	2	80%	1.6	9.6
	D	Acute cellular swelling (hydropic deg)	2	5	80%	8.0	
	PC	-	-	-	-	-	
	IR	-	-	-	-	-	
4th week	CD	Congestion with sinusoidal dilatation	1	1	60%	0.6	6.6
	D	Acute cellular swelling (hydropic deg)	2	5	60%	6.0	
	PC	-	-	-	-	-	
	IR	-	-	-	-	-	
6th week	CD	Congestion with sinusoidal dilatation	1	2	40%	0.8	4.4
	D	Acute cellular swelling (hydropic deg)	2	3	60%	3.6	
	PC	-	-	-	-	-	
	IR	-	-	-	-	-	

Abbreviations: (deg.) degeneration.

Table (4): lesions in the posterior kidney of experimentally exposed Nile tilapia to chronic waterborne MG (0.076 mg/ liter) and their importance, score, Prevalence and index values:

Exposure time	Reaction pattern	Alterations	Importance factor (W)	Score value (a)	Prevalence (P) (n=5)	Index	
						Reaction pattern (I rp)	Organ (I org)
2nd week	CD	Congestion	1	3	40%	1.2	6.0
	D	Hyaline droplets inside P.C.T Tubular and interstitial necrosis	1 3	3 2	40% 60%	4.8	
	PC	-	-	-	-	-	
	IR	-	-	-	-	-	
4th week	CD	-	-	-	-	-	6.0
	D	Acute cellular swelling of the R.C.T Tubular andinterstitial necrosis	2 3	3 2	60% 40%	6.0	
	PC	-	-	-	-	-	
	IR	-	-	-	-	-	
6th week	CD	-	-	-	-	-	4.8
	D	Acute cellular swelling of the R.C.T	2	3	80%	4.8	
	PC	-	-	-	-	-	
	IR	-	-	-	-	-	

Abbreviations: (R.C.T) renal convoluted tubules; and (P.C.T) proximal convoluted tubules

Table (5): lesions in spleen of experimentally exposed Nile tilapia to chronic waterborne MG (0.076 mg/ liter) and their importance, score, Prevalence and index values

Exposure time	Reaction pattern	Alterations	Importance factor (W)	Score value (a)	Prevalence (P) (n=5)	Index	
						Reaction pattern (I rp)	Organ (I org)
2nd week	CD	Congestion	1	2	40%	0.8	8.0
	D	Lymphocytic necrosis and depletion	3	2	60%	3.6	
	PC	-	-	-	-	-	
	IR	Activation and enlargement of MMCS	2	3	60%	3.6	
4th week	CD	-	-	-	-	-	7.2
	D	Lymphocytic necrosis and depletion	3	3	80%	7.2	
	PC	-	-	-	-	-	
	IR	-	-	-	-	-	
6th week	CD	-	-	-	-	-	3.6
	D	Lymphocytic necrosis and depletion	3	2	60%	3.6	
	PC	-	-	-	-	-	
	IR	-	-	-	-	-	

Abbreviations: (MMCS) melanomacrophage centers.

3.2.4 Hematological parameters:

Regarding some blood parameters in Nile tilapia after chronic exposure to MG (0.076 mg/L) revealed that PCV; RBCs; MCHC; And TLC recorded a significant decrease during the 2nd and 4th weeks. While all these blood parameters were not significantly different during the 6th week (Table 6).

Table (6): Hematological effect chronic MG toxicity in Nile tilapia.

Period	Group	PCV %	RBCs x 10^6	MCHC %	WBCs x 10^3 µ
2nd week	control	5.933 ± .5686 [a]	4.400 ± .6245 [c]	31 ± 4 [ef]	34 ± 3 [h]
	exposed	3.267 ± .4041 [b]	2.467 ± .3215 [d]	23 ± 5 [g]	20 ± 3 [i]
4th week	control	5.533 ± .8505 [a]	3.867 ± .3215 [c]	32 ± 1 [e]	35 ± 1 [h]
	exposed	4.000 ± .4359 [b]	2.900 ± .5568 [d]	26 ± 1 [fg]	22 ± 1 [i]
6th week	control	5.267 ± .4726 [a]	4.533 ± .5508 [c]	35 ± 4 [e]	36 ± 2 [h]
	exposed	5.000 ± .2646 [a]	4.033 ± .2082 [c]	34 ± 2 [e]	33 ± 2 [h]

Means with the same letter(s) of the same parameter are not significantly different at $p \geq 0.05$. - Data are represented as Mean ± SD SD = Standard deviation. -Number of observation in each mean =5

4-Discussion:

Although MG is not approved in the world as a treatment for diseased fish, the compound is highly effective, readily available and relatively inexpensive. Most of the toxicological interests in MG and its major metabolite leucomalachite green have focused on the dealing with both clinical and experimental aspects produced by this compound (Marlasca et al., 1992 and Allen et al., 1994)). Defining MG effects in fish and other aquatic species is of considerable importance, because MG will persist in the aquatic environment for a long time and may pass via the food chain from there to untreated fish intended for human consumption (Sudova et al., 2007). MG may affect the aquatic life and cause detrimental effects in liver, gill, kidney, intestine and gonads (Kumar et al., 2005).

In the present study, it was found that the 96 hours LC50 of MG in Nile tilapia was 0.76 mg/L. This value is similar to the toxicity threshold for MG of approximately 1.0 mg/L reported by (Srivastava, et al., 1994 and Srivastava et al., 1995) in a freshwater catfish and Shu Perng (2009) in rainbow trout. While (Hanan, 2001) estimated 96hrs LC50 as 0.075 mg/L in Nile tilapia fingerlings. This difference may be due to variation in age and size.

In the present study, fish were exposed to MG equal to $^1/_{10}$ LC50 (0.076 mg/L), the clinical signs were in the form of respiratory manifestations which may be attributed to decrease of the gills surface epithelium, repeated exposure to MG lead to hyperplasia of the epithelium at the base of secondary lamellae which resulted in complete lamellar fusion of the majority of the lamellae in addition to marked epithelial proliferation of the apices of the gill filaments as acclimatization trying to prevent the toxic substance absorption, so that interfere with the gas exchange function of the gills leading to respiratory distress (Kumaraguru et al., 1982). The behavioral patterns exhibited by the fish are similar to those recorded by (Omoregie et al., 1998) in rainbow trout and Nile tilapia.

Mainly, the more pronounced change in gills was interlamellar epithelial hyperplasia which consider as defense mechanism that reduces the branchial superficial area in contact with the external milieu. This mechanism increases the diffusion barrier to the pollutant (Fernandes and Mazon, 2003). Hyperplasia may in some situations represent an adaptation by the organism to protect underlying tissues from any irritant. (Kumaraguru et al., 1982). Circulatory disturbances only appeared in the 6th week of exposure as mild telangiectasis. Exposure to pollutants leads to rupture the retaining pillar, or pilaster cells, which normally join the dorsal surface of secondary lamellae to the ventral one. The result will be dilation of the lamellar capillary and pooling of the blood, leads to the telangiectasis which is characteristic pathological change of the gill associated with physical or chemical trauma (Robert, 2001). Lamellar necrosis may be due to direct effect of MG, these results agreed with (Gerundo et al., 1991). Gills are important not only for gaseous exchange but also for osmoregulation and excretion of toxic waste products (Robert, 2001), thus any harm in the gills leads to impairment of such vital functions revealing respiratory distress, impaired osmoregulation and retention of toxic wastes.

Herein, MG caused diffuse hydropic degeneration of the hepatic cells. MG acts as a respiratory enzyme poison, disturb the production or performance, or both, of intracellular respiratory enzymes; to inhibit RNA synthesis ;and to uncouple oxidative phosphorylation (Werth and Boiteaux, 1967), causing depletion of cellular ATP and subsequent decrease in ion pumping ATPases which lead to acute cellular swelling (Cheville, 1999). This result is slightly similar to those recorded by (Gerundo et al., 1991)

posterior kidney exhibited histopathological changes comprising acute cellular swelling of tubular epithelium, hyaline degeneration in proximal tubular epithelium which may be attributed to cytotoxic effect of MG and/or its metabolite that rendered the glomeruli more permeable even to blood protein

"albumin" and focal tubular and interstitial necrosis. Same lesions reported in kidney of *Heteropneustes fossilis* treated with MG by (Srivastava et al., 1998[b]). Spleen exhibited lymphocytic cells necrosis that may be attributed to direct cytotoxic effect of MG and/or its metabolite on lymphopoietic tissue, that may correlated with immune depressed (Roberts, 2001 and Yonar and Yonar, 2010). Activation of MMCs also recorded; the melanophores appeared heavily loaded with dark brown melanin pigment, and enlargement of the MMCs where large area of splenic parenchyma was replaced by melanophores contain golden brown pigment. These observations are slightly agreed with (Rehulka, 1977) who observed increased haemosiderosis in spleen after 14 days of MG treatment of fish., the circulating macrophages replete with particulate matter, possible microbile in origin, home selectively on the macrophage centers, hence the activation of the MMCs considered as line of defense (Robert, 2001).

The traditional methods for the evaluation of histopathological alterations are rather divergent. Alterations are described morphologically, and the extent of the alterations is assessed using a scale such as mild, moderate, and severe. The use of different methods and assessment scales as well as the inclusion of different histopathological alterations make it difficult to compare different studies. The aim of this study was to use a standardized health assessment protocol including a standardized quantitative histopathological assessment tool to identify the histopathological alterations in the organs of the fish and thereby also assess the effect of toxicants on the health status of fish.

Fish histopathology was used as a tool to monitor the health status of fish from a polluted ecosystem. Where alterations from the normal may not always be made apparent in various health assessment methods, histopathology will reflect damage caused by toxicants, especially at an early stage. It is important to know the effects of environmental toxicants before they affect higher levels of organization and histopathology makes this possible. Information obtained from such a study can be utilized by environmental managers, conservationists and the like for the implementation of ecosystem and environmental remediation and protection.

The hematological results of chronic exposure of Nile tilapia to MG revealed a significant decreased PCV; RBCs; MCHC; And TLC during the 2nd and 4th weeks as compared with the control group. This may be attributed to depletion of interstitial hemopoietic tissue in posterior kidney and white pulp depletion in spleen. This explanation was supported by El-Boushy (1994) and Robert (2001). These results are partially agreed with (Tanck et al. 1995; Srivastava et al. 1996 and Musa and Omoregie 1999) who reported RBCs and haematocrit reduction and Musa and Omoregie (1999) reported a reduction in white blood cell count in cat fish. While disagree with (Srivastava et al. 1996) who demonstrated increased TLC in cat fish. Also we completely disagree with (Alderman and Clifton-Hadley 1993) who observed elevated packed cell volume in rainbow trout. While all these blood parameters were not significantly different during the 6th week, this may be attributed to fish adaptation to MG exposure as recorded in histopathological examination.

Finally, chronic MG exposure cause detrimental effects on fish health at histopathological and hematological level. Modified quantitative histological tool considered as monitoring program during experimental evaluation of the histopathological effect of waterborne pollutants on fish.

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