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Field Application of Humic Acid Against the Effect of Cadmium Pollution on Cultured Tilapia *Oreochromis niloticus*

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Abstract: The role of humic acid in protecting *Oreochromis niloticus* from the harmful effects of cadmium exposure was studied. The exposure of fish to 15 ppm cadmium increased the concentration of cadmium in liver, kidney, musculature and gills. Cadmium exposure significantly decreased the red blood cells count, hemoglobin concentration, packed cell volume (PCV), total protein, albumin, globulin concentrations increased. The cadmium exposure increased also lipid peroxidation, reduced glutathione concentration in the liver, kidney, musculature and gills. The fish were treated with 15, 30 and 50 mg/l of humic acid through the pond water during cadmium exposure. The concentration of 15 mg/l had no significant effect on the cadmium exposed fish while the treatment of fish with 30 and 50 mg/l humic acid significantly reversed the effects of the cadmium toxicity.

Key word: Humic acid · Cadmium -*Oreochromis niloticus* · Lipid peroxidation · Reduced glutathione

INTRODUCTION

Cadmium is an extremely toxic heavy metal which is widely used in mining, metallurgical operation, electroplating industries manufacturing vinyl plastics, electrical contacts, metallic and plastic pipes. Most aquatic organisms have the capability of concentrating metals by feeding and metabolic processes, which can lead to accumulation of high concentrations of metals in their tissues. The reduction of toxic elements like cadmium in aquatic environments is needed by any acceptable method. The most widely used technique for the removal of toxic elements involves the process of neutralization and metal hydroxide precipitation [1] Chemicals can effectively remove certain toxic elements from industrial wastes or polluted media, but is usually costly. However, there are some cheap chemicals which are also free from undesirable side effects. In recent years, the remobilization of metals by synthetic anthropogenic chelating (binding) agents (Humic acid) has received much attention [2]. The literature reported number of chelators that have been used for chelate-induced hyperaccumulation [3]. Metal bioaccumulation can occur via complexation, coordination chelation, ion exchange and other processes of greater or lesser specificity [2]. Bioaccumulation processes are sometimes due to active metal accumulation by living cells. In other cases, bioaccumulation is a strictly aggressive process in which metal ions are sequestered by metal binding site in the interior of the cell. The removal of toxic elements from contaminated water has potential advantages over the conventional treatment process ion exchange, precipitation, membranes, etc. [4]. In spite of the amount of data published on the effect of waterborne exposure of cadmium and humic acid singly, information on the effects of Cd/humic acid mixture on aquatic organisms are limited and not uniform. Therefore the present study investigate the clinical picture, short and long- term bioassays of cadmium toxicity and evaluate the influence of humic acid on the bioaccumulation of cadmium in tissues with monitoring blood parameters, with serum and tissue enzymes and some physiological parameters in cultured Tilapia *Oreochromis niloticus*. Besids, the role of humic acid in elemination of Cd levels in fish tissues.

MATERIAL AND METHODS

Fish: Atotal of 100 healthy fish of Nile tilapia (*Oreochromis niloticus)* weighing 100 ±10 g were collected from the earthen ponds in private fish farm, at

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Kafr El Sheikh governorate The fish were distributed randomly in 5 cement fish ponds (3x8x1meter) Each pond contained 20 fish and supplied with filtrated water. The fish acclimated for 2 weeks. Fish were fed frequently a diet containing 25% crude protein (CP) at a rate of 3% of live body weight twice daily for 45 days according to Eurell *et al.* [5]

Humic Acid: Canada- Humex: liquid extract - from Egyptian Canadian for Humate Technologies & Agricultural consultancy.

Clinical Examination: Clincal examination for *O.niloticus* exposed to cadmium alone performed according to descriptions of Austin and Austin [6].

Sampling: Blood was collected from the caudal vein of five fish in each group after 15 and 45 days of the beginning of the experiment. The liver, kidneys, musculature and gills were also collected from five fish of each group after the period. The blood sample was divided into two portions. The first portion was kept as a whole blood in heparinized tubes for hematological examination. Serum was separated from the second portion for biochemical analysis.

Tissue homogenates were prepared from liver, kidneys, musculature and gills according to Combs *et al.* [7] for determination of lipid peroxidation, reduced glutathione. Another portion of each of the previous organs was digested as described by Cottenie [8] for determination of cadmium residues.

Tissue Cadmium Determination: Cadmium concentration was determined in the tissues according to Jackson [9].

Hematological Examination: Packed cell volume (PCV), hemoglobin (Hb) concentration and red blood cell (RBC) count were examined in the whole blood as described by Stoskopf [10].

Biochemical Analysis

a-Serum: Total protein level in serum was determined according to Cannon *et al.* [11] Serum albumin concentration was measured as described by Gustafsson [12]. Blood serum globulin was calculated by subtracting the concentration of albumin from that of the total protein and albumin/globulin ratio (A/G ratio) was calculated by dividing albumin concentration over that of globulin [13].

b- Tissue Homogenate: Malondialdehyde (MDA) in tissue homogenate was estimated according to Albro *et al* [14] as an expression of lipid peroxidation (Lpx) [15]. Reduced glutathione (GSH) level was measured according to Chanarin [16].

Statistical Analysis: Data were presented as mean±standard error (S.E.) and the significance of differences was estimated using ANOVA test as described by Snedecor [17].

RESULTS

Clinical Picture of *O. Niloticus* Exposed to Cadmium

Clinical Sings: *O.niloticus* exposed to cadmium showed slimy body with pale skin Fig. A., signs of restlessness, some fish suffered from asphyxia; increased opercularmovements, gluping atomsphoric air, swim near or on the water surface or even jumped outside water with stretched and expanded congested Fig. B. or pale and sticky gills. Finally loss of appetite, escape reflex and sluggish movements.

Post Mortum Examination: Post mortem lesions revealed inflammed, enlarged pale spleen and liver spotted with inflammatory patches Fig. C.

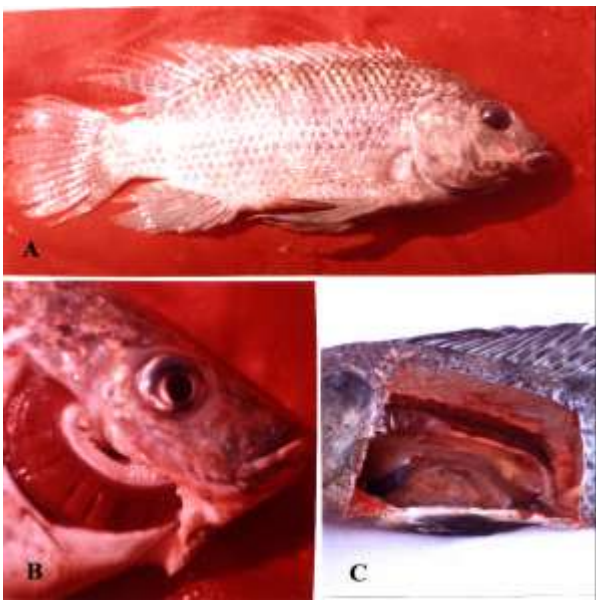

Fig. 1: *O.niloticus* exposed to cadmium showing: A. pale and slimy skin with erratic dorsal fin. B. congested gills. C enlarged pale liver spotted with inflammatory patches

Table 1: Cadmium exposure and humic acid treatment experimental design

Fish group*	Cadmium exposure concentration (ppm)	Humic acid treatment concentration (mg/l)
1st (negative control)	0	0
2nd (positive control)	15	0
3rd Treated group	15	15
4th Treated group	15	30
5th Treated group	15	50

* each group 20 fish

Table 2: Cadmium residues (μg/g wet weight) in the organs of *Oreochromis niloticus* exposed to 15 mg/L cadmium and treated with humic acid

	Liver		Kidneys		Musculature		Gills	
Organ								
Group	15 days	45 days	15 days	45 days	15 days	45 days	15 days	45 days
Control	0.067±0.002 A	0.041±0.002 A	0.057±0.002 A	0.043±0.002 A	0.034±0.001 A	0.031±0.001 A	0.073±0.003 A	0.061±0.002 A
Cadmium	0.967±0.070 aB	6.270±0.170 aB	0.964±0.031 aB	3.205±0.128 aB	0.315±0.016 aB	1.276±0.067 aB	0.525±0.021 aB	1.276±0.073 aB
Cadmium+15 mg/l humic acid	0.903±0.091 a	5.940±0.164 aC	0.933±0.029 aC	2.895±0.106 aC	0.305±0.013 aC	1.168±0.072 aC	0.511±0.020 aC	1.210±0.039 aC
Cadmium+30 mg/l humic acid	0.789±0.043 ab	3.690±0.107 abc	0.711±0.022 abcD	1.764±0.081 abcD	0.155±0.006 abcD	0.671±0.012 abcD	0.350±0.010 abcD	0.914±0.028 abcD
Cadmium+50 mg/l humic acid	0.767±0.038 ab	2.950±0.105 abc	0.635±0.019 abcd	1.492±0.067 abcd	0.114±0.005 abcd	0.432±0.013 abcd	0.266±0.008 abcd	0.764±0.023 abcd

Each value represents mean ±S.E.; N=5.

Small letters a, b, c and d in the same column represent a significant change against capital letters A, B, C and D respectively by LSD using ANOVA at P= 0.05

Table 3: Some hematological prarameters in *Oreochromis niloticus* exposed to 15 mg/L cadmium and treated with humic acid

	RBC count (x 10^6/mm^3)		Hemoglobin (g/dl)		PCV (%)	
Parameter Group	15 days	45 days	15 days	45 days	15 days	45 days
Control	2.20±0.08	2.20±0.10 A	7.20±0.21	7.32±0.30 A	24.50±1.32	24.60±1.41 A
Cadmium	2.05±0.09	1.70±0.07 aB	6.72±0.23	5.52±0.17 aB	20.17±1.40	17.62±1.29 aB
Cadmium+15 mg/l humic acid	2.05±0.08	1.80±0.08 aC	6.75±0.20	5.83±0.19 aC	21.21±1.35	19.65±1.26 aC
Cadmium+30 mg/l humic acid	2.10±0.09	2.10±0.06 bc	6.85±0.21	6.76±0.21 bc	23.56±1.61	23.44±1.05 bc
Cadmium+50 mg/l humic acid	2.15±0.07	2.15±0.08 bc	7.09±0.20	7.18±0.24 bc	24.22±1.25	24.40±1.19 bc

Each value represents mean ±S.E.; N=5.

Small letters a, b, c and in the same column represent a significant change against capital letters A, B and C respectively by LSD using ANOVA at P= 0.05

Table 4: Blood serum proteins levels in *Oreochromis niloticus* exposed to 15 mg/l cadmium for 15 and 45 days and treated with humic acid

	Total protein (g/dl)		Albumin (g/dl)		Globulin (g/dl)		A/G ratio	
Parameter								
Group	15 days	45 days	15 days	45 days	15 days	45 days	15 days	45 days
Control	4.70±0.25 A	4.75±0.28 A	1.55±0.09 A	1.63±0.08 A	3.15±0.12 A	3.12±0.15 A	0.58±0.04 A	0.59±0.03 A
Cadmium	3.50±0.20 aB	3.15±0.14 aB	0.94±0.05 aB	0.88±0.04 aB	2.56±0.13 aB	2.27±0.10 aB	0.39±0.02 aB	0.32±0.02 aB
Cadmium+15 mg/l humic acid	3.66±0.23 aC	3.30±0.15 aC	1.04±0.06 aC	0.96±0.05 aC	2.62±0.11 aC	2.34±0.10aC	0.42±0.02 aC	0.37±0.02 aC
Cadmium+30 mg/l humic acid	4.42±0.20 bc	4.35±0.22 bc	1.36±0.07 bc	1.47±0.07 bc	3.06±0.14 bc	2.88±0.13 bc	0.50±0.03 b	0.52±0.03 bc
Cadmium+50 mg/l humic acid	4.60±0.20 bc	4.64±0.25 bc	1.50±0.08 bc	1.56±0.08 bc	3.10±0.16 bc	3.08±0.13 bc	0.54±0.05 bc	0.55±0.04 bc

Each value represents mean±S.E.; N=5. Small letters a, b, c and in the same column represent a significant change against capital letters A, B and C respectively by LSD using ANOVA at P= 0.05

Table 5: Lipid peroxidation (nM malondialdehyde/mg protein) in the organs of *Oreochromis niloticus* exposed to 15 mg/L cadmium and treated with humic acid

	Liver		Kidneys		Musculature		Gills	
Organ								
Group	15 days	45 days	15 days	45 days	15 days	45 days	15 days	45 days
Control	8.34±0.31A	8.20±0.36A	9.46±0.32A	9.51±0.26A	7.15±0.18A	7.21±0.23A	5.27±0.17A	5.35±0.23A
Cadmium	12.51±0.59aB	15.41±0.53aB	14.33±0.62aB	16.13±0.52aB	9.34±0.25aB	11.55±0.43aB	7.83±0.23aB	9.47±0.38aB
Cadmium+15 mg/l humic acid	12.35±0.57aC	14.62±0.54aC	14.02±0.50aC	15.71±0.57aC	9.22±0.41aC	11.35±0.59aC	7.38±0.19aC	9.18±0.29aC
Cadmium+30 mg/l humic acid	9.63±0.49bc	9.45±0.44bc	10.66±0.51bc	10.51±0.43bc	7.85±0.31bc	8.27±0.38bc	5.67±0.15bc	6.04±0.28bc
Cadmium+50 mg/l humic acid	9.42±0.40bc	9.13±0.48bc	9.85±0.56bc	9.80±0.61bc	7.31±0.32bc	7.45±0.36bc	5.51±0.17bc	5.70±0.18bc

Each value represents mean ±S.E.; N=5.Small letters a, b, c and in the same column represent a significant change against capital letters A, B and C respectively by LSD using ANOVA at P= 0.05

Table 6: Reduced glutathione concentration (mM/mg protein) in the organs of *Oreochromis niloticus* exposed to 15 mg/L cadmium and treated with humic acid

	Liver		Kidneys		Musculature		Gills	
Organ								
Group	15 days	45 days	15 days	45 days	15 days	45 days	15 days	45 days
Control	26.34±1.17A	26.95±1.36A	21.50±1.05A	21.65±1.13A	11.27±0.63A	11.51±0.66A	23.57±1.36A	23.40±1.72A
Cadmium	32.73±1.64aB	47.02±2.35aB	27.64±1.16aB	34.54±1.47aB	15.73±0.82aB	19.42±0.70aB	29.93±1.28aB	36.84±1.35aB
Cadmium+15 mg/l humic acid	32.15±1.37aC	46.55±2.22aC	26.78±1.24aC	33.07±1.53aC	15.27±0.84aC	19.06±0.65aC	28.45±1.23a	36.16±1.52aC
Cadmium+30 mg/l humic acid	29.26±1.63bc	31.24±1.31bc	23.65±1.17bc	25.03±1.43bc	12.76±0.95bc	13.31±0.72bc	25.67±1.44b	30.10±1.63bc
Cadmium+50 mg/l humic acid	27.78±1.12bc	29.51±1.43bc	22.10±1.25bc	23.42±1.35bc	11.64±0.87bc	12.21±0.80bc	24.80±1.40b	24.38±1.57bc

Each value represents mean ±S.E.; N=5.

Small letters a, b, c and in the same column represent a significant change against capital letters A, B and C respectively by LSD using ANOVA at p= 0.05

Cadmium Residues in *Oreochromis niloticus* Tissues: Cadmium residues were significantly icreased in *O. niloticus* tissues comparing to control group, humic acid with either concentrations 30 and 50 mg/L significantly reduce cadmium residues in fish as shown in Table 2.

Hematological Studies: There was significantly decreased RBCs count, Hemoglobin concentration and PCV% in samples of 45 days than that of 15 days in *O.niloticus* exposed cadmium as shown in Table 3.

Serum Biochemical Components: The total protein, albumin, globulin levels and Albumin/globulin (A/G) ratio, were significantly decreased in the serum of *Oreochromis niloticus* fish after 15 and 45 days of exposure to cadmium comparing with control group as shown in Table 4.

Lipid Peroxidation (Lpx) and Antioxidants of Tissues: There was significant elevation of lipid peroxidation and reduced glutathione in liver, kidney,musculature and gills of cadmium exposed *O.niloticus* As shown in Tables 5 and 6.

DISCUSSION

Humic substances from soil and water are major controlling materials for metal speciation, pollutant binding and nutrient availability. An understanding of metal ion binding and competition remains difficult because of the complexity of humic substances. Humic substances are proposed to be irregular polymers with a number of chemically unidentical acidic functional groups having a variable electric potential [18]. The degree of acidity, or acid strength, of the colloid is dependent on the nature of the reactive group involved and associated structures on the molecule [19]. The carboxyl and phenolic hydroxyl groups are considered to be the dominant metal binding sites, although other functional groups (e.g., amines, thiols) might be involved to some extent (Interactions between metal ions and humic substances are of major importance in the migration and redistribution of potentially toxic metal ions. To describe metal binding with humic substances, it is necessary to develop models in which various simplifications are made because of the complex heterogeneous nature of humic materials and the variable electrostatic interaction between functional groups [20, 21].

Concerning the concentration of cadmium in fish tissues it is observed that cadmium concentration in liver, kidney, musculature and gills was significantly higher in fish exposed to cadmium for 15 and 45 days than control group and the elevation in cadmium concentration is more drastic at the end of 45 days exposure. Humic acid treatment at 30 and 45 mg/l concentrations significantly decreased the concentration of cadmium in tissues but not to the level of control. Cadmium demonstrated a tendency to preferably associate with larger, humified and less soluble organic materials such as HA [22]. Present results indicate that humic acid is effective in removing Cd from water reducing Cd bioaccumulation in fish. Particulate organic matter can scavenge metal from water and help to reduce metal from fish. These results are in agreement with Santachi [23] who found that any agent that can remove Cd from water helps to reduce the bioaccumulation of this metal in fish. Cd accumulation in liver, gills and musculature of fish exposed to Cd alone was higher than that of Humic acid. These results suggest that Humic acid could chelate Cd ions producing a stable complex, thus reducing the chance for metal uptake by tissues. Besides the Humic acid groups eliminated more amount of Cd from the body through feces and urine. The formation of Cd-Humic acid complex in water and elimination of more amount of Cd evidently reduced the metal burden in tissues.

Regarding hematological parameters, cadmium exposure for 45 days significantly diminished RBC count, PCV and hemoglobin concentration in *Oreochromis n-iloticus* in comparison with control. Treatment with humic acid at concentrations 30 and 50 mg/l significantly elevates these parameters near the control values. The reduction of these parameters in Nile tilapia, *O niloticus* exposed to cadmium might be due to the destruction of mature RBCs and the inhibition of erythrocyte production due to reduction of haemosynthesis that affected by pollutants [24].Also, the decrease in RBCs count may be attributed to haematopathology or acute haemolytic crisis that results in severe anemia in most vertebrates including fish species exposed to different environmental pollutants [25] or may be the decrease in the RBCs may be attributed to reduction of growth and other food utilization parameters which results in sever aneamia [26]. Also, Gill and Epple [27] found a significant reduction in the RBCs, Hb and Hct in American eel *(Anguilla rostrata*) after exposure to 150 ug Cd/L. Karuppasamy *et al*. [28] found a significant decrease in total erythrocyte count, haemoglobin content, haematocrit value and mean corpuscular haemoglobin concentration in air breathing fish, *Channa punctatus* after exposure to sublethal dose of Cd (29 mg Cd/L).The addition of Humic acid improved

the haematological parameters (RBCs, Hb and Hct) which indicating to the capability of Humic acid to chelate Cd from the water and fish. Subsequently, the Cd pollution was reduced. These results are in agreement with James *et al*. [29] who observed that *Oreochromis mossambicus* exposed to copper along with Humic acid showed a significant improvement in blood parameters

Respecting the serum protein, cadmium exposure for 15 and 45 days significantly declined the levels of total protein, albumin and globulin as well as A/G ratio comparing with control. Decreases in serum protein concentration and the albumin/globulin ratio in the blood may indicate some liver dysfunction. When exposed to stressors, the gills become "leaky" to water and ions, often resulting in osmoregulatory imbalances [30]. So the decline in serum total protein, albumin and globulin may be also due to a degree of haemodilution under the stress of pollution. The A/G ratio is an index used to track relative changes in the composition of serum or plasma [31].A drop in A/G ratio can indicate a shift from albumin production to globulin proteins in response to stress. Treatment with 30 and 50 mg/l humic acid in the aquarium water significantly elevates the values of these parameters to nearly the values of control. These may be due to that presence of humic acid as a chelating agent reducing cadmium level from water as well as fish tissues improving physiological status and enhancing immune response of fish.

Respecting lipid peroxidation, cadmium exposure for 15 and 45 days significantly increased the lipid peroxidation in the liver, kidney, musculature and gills of *O. niloticus*. Similar results were recorded by Gupta *et al.* [32]. They found that cadmium increased hepatic lipid peroxidation in Atlantic croaker and *Clarias batrachus* fish respectively. On the other hand our result disagree with that observed by Tort *et al*. [33] they found that liver thiobarbituric acid reactant substances in rainbow trout remained unaffected by cadmium. Lipid peroxidation has been suggested to be one of the primary mechanisms of cell injury by xenobiotics [34] The stimulatory effect of xenobiotics on lipid peroxidation have been attributed to impairment of cystolic protective systems including low molecular weight antioxidant suchas ascorbic acid [35] and cystolic enzyme such as glutathione peroxidase [36] catalase Humic acid at concentrations 30 and 50 mg/l significantly decreased the lipid peroxidantion in tissues. The effect of humic acid in decreasing lipid peroxidation indicates that it protect the fish from the oxidative stress produced by the cadmium exposure.

The exposure of fish to cadmium for 15 and 45 days significantly increased the reduced glutathione levels in the liver, kidney, musculature and gills. Regarding the effect of cadmium of tissue reduced glutathione levels, our findings agree with that obtained by Allen [37] found that cadmium elevated reduced glutathione concentration in the kidney of Oreochromis niloticus fish. The de novo synthesis of glutathione is accomplished by two critical enzymes namely, γ-glutamyl-cysteine synthetase and glutathione synthetase. The first enzyme catalyzes the formation of γ-Glutamyl-cysteine from glutamic acid and cysteine while the second enzyme catalyzes the formation of reduced glutathione from glutamyl-cysteine and glycine [38]. γ-Glutamyl-cysteine synthetase is the rate limiting enzyme in glutathione synthesis in aquatic organisms [39]. The increase in the concentration of reduced glutathione that occur in the present study after exposure of fish to cadmium may be due to induction of the synthesis of the rate limiting enzyme, γ-glutamyl-cysteine synthetase as previously described by Zalups and Lash [40]. The treatment with 30 or 50 mg/l of humic acid significantly decreased reduced glutathione levels in the liver, kidney, musculature and gills nearly to the control level indicating the improvement of the fish health and physiology.

From the present study, it was concluded that humic acid is a promising tool for controlling cadmium pollution in aquaculture at short and long term, whereas it improve hematological, physiological and immunological state of *O. niloticus,* exposed to cadmium, especially when it used at a concentration of 30 mg/L. Also, it significantly reduces Cd level in fish tissues including musculsture.

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