Bioremediation the Toxic Effect of Mercury-Exposure in Nile Tilapia (*Oreochromis Niloticus*) by using *Lemna gibba L*

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Abstract: The effect of mercury (Hg) toxicity, its impact on histopathological changes, the median lethal concentration (LC_{50}-96 h) and the bioremediation effect of *Lemna gibba L* to Nile tilapia, *Oreochromis niloticus*, were investigated through semi-static acute toxicity test developed with mercury chloride ($HgCl_2$).). Fingerlings (2.76±0.21 cm and 0.51±0.12 g) were kept during 96 hours in 5-liter glass aquaria, according to the following mercury concentrations, set up in three replicates: 0.00 (control), 0.037, 0.185, 0.370, 0.740, 0.925 mg Hg L^{-1}. The value of LC_{50}-96h was estimated in 0.240 mg Hg L^{-1}.
This study indicated that:1) Hg poisoning caused structural damage in the fish organs ,2) *Lemna gibba L* (weed and extract) were effective in removing Hg from water and reducing Hg bioaccumulation in liver and muscular tissues of fish , 3) The addition of *Lemna gibba L*-extract reduced significantly ($P<0.05$) the Hg level uptake as compared to fish exposed to Hg alone and 4) Addition of *Lemna gibba L* remediated the toxic effect of Hg and provided protection against the degenerative action of Hg. **(*Oreochromis Niloticus*) by using *Lemna gibba L***. Journal of American Science 2011;7(3):336-343]. (ISSN: 1545-1003). http://www.americanscience.org .

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1. Introduction:

The aquatic environment is constantly exposed to various pollutants, and the group of heavy metals has been the focus of many studies with deep concern. Some particular heavy metals, such as mercury (Hg), are especially included in investigations due to their high toxicity. This element is classified as one of the most toxic metals, which are introduced into the natural environment by human interferences (Buhl, 1997). Inorganic mercury is the most common form of the metal released in the environment by industries, presenting a stronger acute effect on fish tissues than that of the organic form of mercury (Sunderland and Chmura, 2000). Some papers have reported situations where high mercury levels were detected in water, mainly nearby gold extraction locations (Maurice-Bourgoin *et al.*, 2000; Dolbec *et al.*, 2001) and industrial zones (Kime, 1998; Sunderland and Chmura, 2000). Consequently, aquaculture is vulnerable to this pollutant, since supplied ponds water generally comes from rivers, dams or other sources that can possibly be contaminated by mercury. Fish contaminated by Hg suffers pathological alterations, with consequent inhibition of metabolic processes, hematological changes, and decline in fertility and survival (Micryakov and Lapirova, 1997).

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. Metals interact with legends in proteins particularly, enzymes and may inhibit their biochemical and physiological activities (Passow *et al.*, 1961).

Metal bioaccumulation can occur via complexation, coordination, chelating, ion exchange and other processes of greater or lesser specificity. It 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. The reduction of toxic elements like cadmium and mercury 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 (Hiemesh & Mahadevaswamy, 1994).

The use of aquatic plants in water ecosystems and terrestrial plants in hydroponic systems has high potential to clean up the metal contaminated water through phytoextraction and

phytostabilization. Phytostabilization utilizes the plant production of compounds, which immobilize contaminants at the entrance of roots. An example of this method is where root exudates cause the precipitation of metals and reducing their bioavailability. Phytodegradation (also known as phytotransformation) is the enzyme-catalysed metabolism of contaminants, typically organics, within plant tissues. The enzymes are usually dehalogenases, oxygenases and reductases (Black, 1995).

Biosorption potential of *Prosopis juliflora* seed powder (PJSP) for lead (Pb) from aqueous solution has been investigated by Jayaram and Prasad (2009) where they found that the maximum Pb (II) adsorbed was found to be 40.322 mg/g and the adsorption process was spontaneous and exothermic in nature. Removal of certain heavy metals from waste water by *Lemna gibba L.* has been reported by Kwan & Smith (1991); Buckley (1994); Miranda & IIangovan (1996) and Wafaa (2007).

In the present study, short and long-term bioassays were designed to evaluate the influence of *Lemna gibba L-* plant and/or its extract on the reduction of mercury in water as well as to investigate the reducing effect of *Lemna gibba L* on some histopathological alterations induced by Hg exposure on Nile tilapia (*Oreochromis niloticus*).

2. Materials and Methods:

Fish culture management

Healthy *Oreochromis niloticus* fingerlings were collected in Marsh 2010, from ponds of the Central Laboratory for Aquaculture Research at Abbassa, Abo-Hammad, and Sharkia, Egypt (belonging to a single population) .They were collected locally and confined to large plastic aquaria bearing tap water for up to 7 days in the laboratory for acclimation.

Mercury chloride

Technical grade mercury chloride (99% purity) was obtained from El-Nasr Chemical Company (Cairo, Egypt) and prepared in aquatic solution to provide the required concentrations of mercury. Control test without mercury was performed.

Determination of LC_{50}

Acute Toxicity Assays

The stock solution (370 mg Hg L^{-1}) was prepared by dissolving a calculated quantity of active ingredient (0.5 g $HgCl_2$ in 1,000 mL of dechlorinated tap water). A series of five concentrations of Hg was prepared by adding a calculated volume from the stocky solution into test containers, considering the equivalent on mercury (Hg). Therefore, nominal concentrations were: 0.037, 0.185, 0.370, 0.740, and 0.925 mg Hg L^{-1} (range determined by preliminary tests). One container was kept as unexposed control group. Test was carried out with three simultaneous replicates. No food was supplied during the experiment. Test solutions were replaced by fresh ones of the same respective concentrations every 24 h until 96 h of testing, according to the renewal method recommended in APHA (1998).

The bioassay was conducted in Marsh 2010, Laboratory, Department of Veterinary Hygiene and Management, Faculty of Veterinary Medicine, Cairo University, with controlled conditions of water temperature (24.40±2.25 ^{o}C) and photoperiod (10L: 14D cycle). The used fish species was Nile tilapia, *Oreochromis niloticus*. Fingerlings with a mean weight of 0.51±0.12 g and mean total length of 2.76±0.21 cm. The acclimatization period was of 7 days, in a 50-L glass aquarium. During this period, fish were fed a dry commercial food (pellets with 25% of crude protein). Afterwards, fingerlings were transferred to 5-L glass aquaria, which were internally covered with a plastic film to prevent contamination by residues from previous experiments. Plastic film was also placed on the top of the aquarium to prevent evaporation. Air pumps and individual air stone diffusers provided aeration. The experiment was carried out at a stocking density of 10 fish/aquarium.

Mortalities were recorded at 24, 48, 72 and 96 h of exposure, and dead fish were removed regularly from the test solutions. The data obtained were statistically analyzed using the Trimmed Spearman Karber method (Hamilton *et al.*, 1977) for estimating the median lethal concentration (LC_{50}), and 1/100 of the LC_{50}-96 h was taken as the safe Hg concentration (Sprague, 1971).

The tested weed

The duckweed species used was *Lemna gibba L* which was taken from Ganabiet-Tersa drain, Giza, Egypt. The duckweed was acclimatized to the laboratory conditions for one week before starting the experiments.

Plant extracts

Dried plant materials were extracted twice with 50% and 100% methanol as well as 50% and 100% acetone in v/v proportions (200 ml/5g plant) for 2 hrs with constant stirring. The collected filtered extracts were dried in a rotary evaporator (Büchi: Rotavapor-R114 and water bath B-481) at 40^{o}C under reduced pressure (Ghobrial *et al.*, 2009).

Mercury reduction

Tilapias were distributed randomly in 50-Litres rectangular fiberglass aquaria filled with well-aerated tap water (pH 6.5–7.0) at a rate of 10 fish / aquarium. Dissolved oxygen in each tank was maintained at close to saturation by aeration. The temperature in each aquarium was maintained at 24±1.5°C by means of thermostats. The photoperiod was 10L: 14D cycle. These aquaria were divided into five groups with three replicates each per group. The first group was free from Hg and *Lemna gibba L* and maintained as a control. The second group was exposed to 0.0 6 mg of $HgCl_2$ only (Equivalent to 1/4 96 h LC_{50}). The third, fourth and fifth groups were exposed to 0.0 6 mg $HgCl_2$ L^{-1} and 0.1, 1 and 0.1 plus 1 g L^{-1} extract, plant and extract plus plant of *Lemna gibba L*, respectively .

Fish were fed frequently on a diet containing 25% crude protein at a rate of 2-3% of live body weight twice daily for 7 and 25 days. Siphoning three quarters aquariums was done every day for waste removal and replacing it by an equal volume of water containing the same concentration of Hg and *Lemna gibba L.* Dead fish were removed and recorded daily.

Hg Residue

Preparation and Analysis of Mercury

Water Samples

The analysis of water samples was carried out according to A.P.H.A. (1992). The water samples were filtered through 0.45µl membrane filter. The required volume (100 ml) of the filtrate was collected to measure mercury levels in water samples by using Air/Acetylene Flame Atomic Absorption Spectrophotometer (UNICAM 696 AA Spectrometer).

Fish Samples

The measurement of the mercury concentration in examined fish samples was carried out at minimal temperature for all fish samples where 0.5 gram macerated fish tissues was digested according to the technique described by Diaz-Ravina *et al*. (1994). About 5 ml stannous chloride solution were added to the obtained solutions to reduce mercury to elemental form and then analyzed by using Flameless Atomic Absorption Spectrophotometer equipped with "MHS" mercury hydride system "Cold Vapour Technique".

Histopathological examination

Tissue specimens from fresh NileTilapia were taken (liver and muscles) and fixed in 15 % buffered neutral formalin. They were processed to obtain five micron thick paraffin sections then stained with Hematoxylin and Eosin (Bancroft *et al.*, 1996) and examined under light microscope.

Statistical analysis

The obtained data were subjected to analysis of variance according to Snedecor & Cochran (1982). Differences between means were done at the 5% probability level, using Duncan's new multiple range test (Duncan, 1955).

Exposure Time (hour)	*LC50* (mg Hg L^{-1})	*95% Confident Limit* (mg Hg L^{-1})
24	**0.48**	**0.38 ñ 0.46**
48	**0.39**	**0.32 ñ 0.53**
72	**0.29**	**0.24 ñ 0.33**
96	**0.24**	**0.19 ñ 0.24**

3. Results:

Table 1. Mean mortality (%) of Nile tilapia, *Oreochromis niloticus,* and expressed according to different exposure times to mercury concentrations.

Concentration (mg Hg L^{-1})	*Time* (hour)			
	24	48	72	96
0.00 (control)	10	10	12	15
0.037	0	15	22	15
0.185	3.33	20	30	40
0.370	28.6	40	90	90
0.740	92.3	100	100	98
0.925	100	100	100	100

Three replicates

Table 2.*LC50* and Confident Limit

The value of $_{LC50\text{-}96h}$ was estimated in 0.240 mg Hg L^{-1}.

Table 3. LC50 of mercury in Tilapias fish species.

Reference	*Species*	*LC_{50}-96h*
Ishikawa *et al.* (2007)	*Oreochromis niloticus*	0.220
Charuwan-Somsiri (1982)	*Oreochromis niloticus*	3.710
Ramamurthi *et al.* (1982)	*Tilapia mossambicus*	0.739
Present study	*Oreochromis niloticus*	**0.240**

Histopathological alterations

The histopathology of different Tilapia tissues revealed that there are several histopathological changes in different Tilapia organs as shown in our figures.

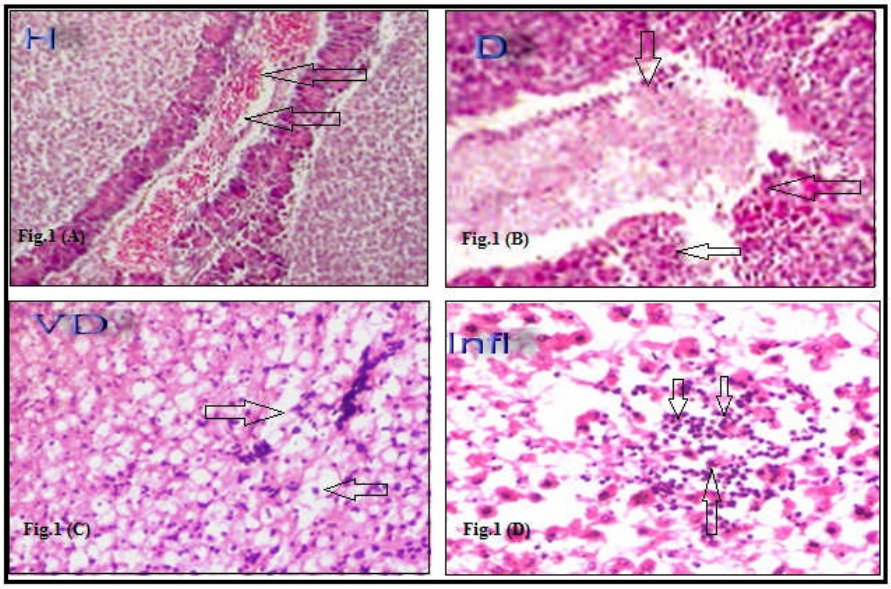

Figure 1.

A. Liver of tilapia treated with mercury showing intravascular haemolysis is seen in blood vessels and sinusoids (arrows) (H & E X 400).

B. Liver of tilapia treated with mercury showing degeneration of the hepatocytes with nuclear pyknosis and necrosed hepatocytes (arrows) (H & E X 400).

C. Liver of tilapia treated with mercury showing dissociation of hepatocytes with individual hepatocellular necrosis and focal mononuclear cell aggregation (H & E 400 X). (arrows) (H & E X 400).

D. Liver of tilapia treated with mercury showing lecucytic infiltration (arrows) (H & E X 400).

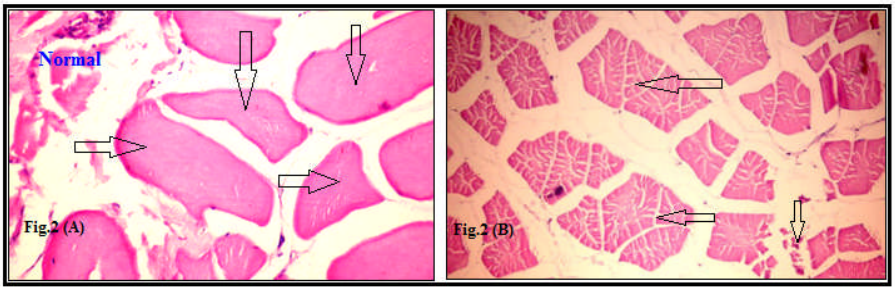

Figure 2.

A. Muscles of tilapia treated with mercury showing healthy muscular tissue (H & E X 400).

B. Muscles of tilapia treated with mercury showing hyalinised muscles tissue (arrows) (H & E X 400).

Table 4: Changes in mercury residue in water (mg Hg L^{-1}), liver and muscles (mg Hg g^{-1} dry weigh) of Nile tilapia *(O. niloticus)* exposed to Hg with and without *Lemna gibba L* plant

Group	Water	Liver		Muscles	
Days		**7**	**25**	**7**	**25**
Control (metal free water)	0.002±0.0002[a]	0.003±0.001[a]	0.0040±0.001[a]	0.0021±0.0004[a]	0.008±0.0007[a]
Mercury alone (0.06 mg) L^{-1}	0.060±0.009[b]	0.0550±0.008[b]	0.0588±0.0054[b]	0.0485±0.001[b]	0.097±0.0018[b]
Mercury (0.06 mg)+0.1g ext. L^{-1}	0.04 ±0.027[b]	0.0 32±0.009[b]	0.0200±0.008[b]	0.0143±0.0086[a]	00.068±0.018[b]
Mercury (0.06 mg)+1g P L^{-1}	0.038 ±0.008[c]	0.050±0.015[c]	0.039 ±0.002[c]	0.0180±0.008[b]	0.0383±0.007[b]
Mercury (0.06 mg)+0.1g ext. L^{-1}+1g P L^{-1}	0.0120 ±0.005[d]	0.0210 ±0.001[d]	0.0020 ±0.005[d]	0.0026±0.0006[a]	0.0021±0.0006[a]

The same letter in the same column is not significantly different at P<0.05.
The first group was free from Hg and *Lemna gibba L* and maintained as a control.
The second groups were exposed to 0.06 mg of $HgCl_2$ only (Equivalent to 1/4 96 h LC_{50}).
The third was exposed to 0.06 mg Hg L^{-1} and 0.1 extract.
The fourth group was exposed to 0.06 mg Hg L^{-1} and 1 g L^{-1} *Lemna gibba L* plant.
The fifth group was exposed to 0.06 mg Hg L^{-1} + 0.1g extract L^{1} +1 g L^{-1} *Lemna gibba L* plant.

4. Discussion:

Physical and chemical variables analyzed in the test solutions showed no statistical differences among the range of five concentrations, neither between concentration range and control group. The average values for these variables were: temperature, 25.40±2.45 °C; pH, 7.20±0.50; electric conductivity, 83.58±2.04 ìS cm^{-1}; hardness, 44.96±1.30 mg $CaCO_3$ L^{-1}; alkalinity, 29.45±1.35 mg $CaCO_3$ L^{-1}; and total ammonia, 1.72±0.22 mg L^{-1}. All of these variables results were in conformity to the standards that are recommended in APHA (1998) for toxicity tests.

Mortalities recorded along 96-hours exposure are registered in Table 1. The values of LC_{50} determined for Nile tilapia in the present study, according to the different exposure times, are shown in Table 2. The LC_{50}-96h was compared with the results from other studies developed on mercury toxicity to fish, Table 3. Ramamurthi *et al.* (1982) and Charuwan-Somsiri (1982) estimated higher LC_{50}-96h for *Tilapia mossambica* and *Oreochromis niloticus*, respectively. The higher values obtained by Ramamurthi *et al.* (1982) and Charuwan-Somsiri (1982) may be attributed to some differences in standard techniques that were adopted in their experiments, such as the larger size of the test-organisms (3.5 cm) used by Charuwan-Somsiri (1982). According to Buhl (1997) and Boening (2000), older and larger aquatic organisms are more resistant to toxicants.

The LC_{50} determined in the present study (LC_{50}-96h, 0.240 mg Hg L^{-1}) was very similar to those reported to Ishikawa *et al.* (2007) who estimated similar LC_{50}-96h for *Tilapia Oreochromis niloticus* (LC_{50}-96h, 0.220 mg Hg L^{-1}) and other fish groups, such as *Cyprinus carpio* and *Roccus americanus* (Rehwoldt *et al.* 1972), *Varichorhinus barbatulus (V. barbatus)*, *Variocorhinus barbatulus (V. barbatus)* and *Zacco barbata* (Shyong and Chen, 2000), *Ptychocheilus lucius* (Buhl, 1997).

Histopathological alterations

Liver of tilapia treated with mercury showed degeneration of the hepatocytes with nuclear pyknosis in the majority of the cells as well as the accumulation of the metal binding proteins in their nuclei. Intravascular hemolysis is seen in blood vessels and sinusoids with necrosed hepatocytes (Figures 1: A, B, C, D).

Muscular tissues degeneration in muscle bundles with aggregations of inflammatory cells (leucocytic infiltration) between them with focal areas of necrosis , atrophy and edema of muscle bundles as well as splitting of muscle fibers and hyalinized muscles tissue were seen (Figure 2: A, B).

Histopathological biomarkers have been largely used in fish to identify and evaluate the toxic effects of pollutants exposure (Rabitto *et al*., 2005; Oliveira Ribeiro *et al*., 2006). The presence of necrosis is in fact one of the most visible damages in tissues affected by a pollutant (Rabitto *et al*., 2005). According to Manahan (1991) the occurrence of necrosis is also a consequence of enzymatic inhibition, damages in the cellular membrane integrity, and disturbances in the synthesis of proteins and carbohydrate metabolism.

Pandey *et al.* (1994) described alteration in liver and intestine of *Liza parsia* exposed to $HgCl_2$ (0.2 mg Hg L^{-1}) for 15 days. Similarly, Oliveira-Ribeiro *et al.* (2002) reported serious injuries in gill and olfactory epithelium of *Sal velinus alpinus* exposed to 0.15 mg Hg L^{-1}. According to Allen (1994), the exposure of *Orechromis aureus* to 0.5 mg Hg L-1 caused a raise in the number of

leucocyte and erythrocyte within 24 hours. Gill and Pant (1985) also related hematological anomalies in *Barbus conchonius* exposed to 0.18 mg Hg L-1 in acute test.

Hg Bioaccumulation

The highest bioaccumulation of mercury was observed in the organs mainly implicated in metal intoxication and so it was higher in the liver followed by muscles.

Addition of *Lemna gibba L*-extract to the Hg polluted media reduced significantly ($P<0.05$) the Hg level in aquarium's water as compared to that of Hg alone. Hg concentration in water exposed Hg alone was 0.06 mg Hg L^{-1} and declined significantly ($P<0.05$) to 0.04, 0.038 and 0.0120 mg L^{-1} with 0.1, 1 and 0.1 plus 1 g L^{-1} extract, weed and extract plus weed of *Lemna gibba L*, respectively. The highest amount of Hg residue was found in the liver after 7 days of exposure. The uptake of Hg in the liver of fish exposed to Hg alone was 0.0550 and 0.0588 mg g^{-1} dry weight for 7 and 25 days, respectively. It declined significantly to 0.0 32, 0.050 and 0.0210, 0.0200 , 0.039 and 0.0020 mg g^{-1} dry weight in fish group exposed to Hg with 0.1, 1 and 0.1 plus 1 g L^{-1} extract, weed and extract plus weed of *Lemna gibba L* at 7 and 25 days, respectively. Similar trends were observed in fish muscles.

The present results indicate that *Lemna gibba L* weed and extract are effective in removing Hg from water and reducing Hg bioaccumulation in Tilapia fish, Table 4. The addition of *Lemna gibba L*-extract reduced significantly ($P<0.05$) the Hg level in water and the metal uptake as compared to fish exposed to Hg alone. Hg concentration in water was 0.06 mg L^{-1} and it decreased significantly ($P< 0.05$). Hg accumulation in liver and muscles of fish exposed to Hg alone was higher than that of *Lemna gibba L*-extract treatment group.

These results suggest that *Lemna gibba L weed and/or* extract could chelate Hg ions producing a stable complex, thus reducing the chance for metal uptake by tissues. These results are in agreement with Santschi (1988) who reported that any agent that can remove Hg from water helps to reduce the bioaccumulation of this metal in fish.

The addition of *Lemna gibba L*-extract reduces the toxic effect of Hg in Tilapia fish which indicating the capability of *Lemna gibba L*-extract to chelate Hg from the media. Subsequently, the Hg toxicity was reduced. These results are in agreement with those recorded by Jayaram & Prasad (2009) who observed the biosorption potential of *Prosopis juliflora* seed powder (PJSP) for Pb (II) from aqueous solution at pH 6.0 and Kaoud *et al.,*(2011) who found the removal of cadmium (Cd) from aqueous solution by the addition of *Lemna gibba L*-extract and/or the plant. Findings in fish also indicated that degenerative changes were less when *Lemna gibba L*-extract or the weed and their extract were added to the rearing water.

The present study shows that addition of *Lemna gibba L*- weed and/or its extract to Hg contaminated media reduced significantly the Hg level in the water and helped to eliminate metal from the fish body and in turn improved the biochemical parameters as compared to fish exposed to Hg alone.

Finally, we could conclude that Hg poisoning cause structural damage in Tilapia liver and muscles. It is also demonstrated that *Lemna gibba L*-extract, weed or the weed plus the extract provided protection against the degenerative action of Hg and increased the chance of tissue regeneration.

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