Garlic and Chlorella Biomodulate Lead Toxicity on Manganese Homeostasis in *Carassius gibelio* Bloch

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Environmental pollution negatively affects the aquatic ecosystems. Heavy metals are considered dangerous toxic elements for aquatic biota, as metallic elements or as salts which exhibit great stability. Among these, lead is toxic for all aquatic biotic components. Our research investigated the manganese distribution in tissue of Prussian carp fishes after lead intoxication, highlighted also the lead detoxifying potential of active principles from garlic and chlorella (powder). For our experiment 120 Prussian carp fingerlings, weighing 22-25g each, for 21 consecutive days, were divided according to the following treatments: C group (without treatment), E1 group (75ppm Pb in water as $Pb(NO_3)_2$ x ½H_2O), E2 group (75ppm Pb in water+2% freeze-dry garlic in feed), E3 group (75ppm Pb in water+2% freeze-dry chlorella in feed). At the end of the experimental period, tissue samples (gills, muscle myotomers–epaxial, heart, skin and scales, intestine, liver, brain, gonads, kidney) were collected after euthanasia with clove oil. Manganese concentration was analytical performed using AAS (atomic absorption spectrometry). Using fish as bioindicators of lead experimental contamination allowed us to obtain valuable informations about its ability to substitut/remove bioactiv minerals from animal tisssues. In the same time, we could emphasize the efficiency of natural antioxidants or chelators of edible plants (such as garlic) or algae (such as chlorella), to alleviat the lead impact on homeostasis of trace elements from tissues.

Keywords: fish, lead, manganese, garlic, chlorella

Industrial, economic and demographic development generates large quantities of wastes with different chemical composition: detergents, solvents, cyanides, heavy metals, organic acids, fats, dyes, washing agents, sulphides, ammonia, etc. Poor management of these wastes leads to environmental pollution and degradation. Pollution of industrial wastewater is the most massive and harmful category of pollution [1].

Heavy metals contamination of surface waters has negatively major effects on the environment for a very long period of time, affecting the water and aquatic biota, and also affecting the soil around the watercourse. Heavy metals have high molecular weight, some of them being generally toxic to living organisms even at extremely low concentrations [2]. The data presented in Table 1 show that for lead concentrations the ratio of sea-water to fresh-water is 1:100, indicating large amount of these metal in fresh-waters compared to sea-water. Also concentration of aluminum is 1:150 as a ration of sea-water to fresh-water, which is even higher then lead distribution. Contrary, the molybdenum is more present in sea-water compared to fresh-water, the ration being 20:1 in sea-water compared to fresh-water, like uranium 33:4; vanadium 5:1, or nickel 6:5 in sea-water compared to fresh-water [3].

In fact, lead is the most prevalent contaminant between heavy metals. Thus, lead levels in the aquatic environment and industrialized areas have been estimated to have three times higher concentration than in non industrial areas [4]. Pollution with heavy metals (like lead, zinc, mercury, iron, copper) and light metals (like aluminum and magnesium) influences the health status of humans and animals, in aquatic or terestial areas, due to low excretion and high accumulation in organs and tissues [5-7].

International organizations as US EPA (United Stated Environmental Protection Agency) and WHO (World Health Organization) have proposed and established regulations for maximum acceptable values of lead in drinking water and surface water used as drinking water resources to 10 mg/L and 50 mg/L [8]. In Romania, acording to Law No. 311/2004 [9] and Order No 161/16 february 2006 [10], the maximum limit of lead in drinking water and surface waters is 0.01 mg/L, being related with the quality of aquatic ecosystems.

The effects of heavy metal pollution are depending on the exposure period and the nature of the pollutant. Thus, acute or chronic diseases can occur after short-term exposure or after long-term exposure [11,12]. Toxic metals exert cumulative effects on different organs and systems, the effect being specific to the substance concerned. It is important to underline that these pollutants accumulate in human and animal tissues, with the possibility of insidious production of serious pathological alterations [13]. Some metals that are specific toxic to aquatic organisms and humans are mercury, cadmium, chromium, and lead, lead being considered the most important toxic heavy element in the environment [14]. In marine fish, the maximum acceptable toxicant concentration (MATC) for inorganic lead has been determined for several species, in different conditions, and results range from 0.04 mg/L to 0.198 mg/L [15].

According to Australian and New Zealand Environment and Conservation Council (ANZEC), Canadian Council of Ministers of the Environment (CCME), EC (European Commission), and US EPA (US Environmental Protection Agency) the water quality standards for the protection of

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Table 1
HEAVY AND LIGHT METALS IN SEA AND FRESH NATURAL WATERS [3]

Metal or Metalloid	Sea-water	Fresh-water
	Concentration (mg/m^3)	
Aluminum (Al)	2	300
Antimony (Sb)	0.2	0.2
Beryllium (Be)	0.006	0.3
Cadmium (Cd)	0.1	0.1
Chromium (Cr)	0.3	1.0
Cobalt (Co)	0.02	0.2
Copper (Cu)	0.3	3
Lead (Pb)	0.03	3
Manganese (Mn)	0.2	8
Mercury (Hg)	0.03	0.1
Molybdenum (Mo)	10	0.5
Nickel (Ni)	0.6	0.5
Silver (Ag)	0.04	0.3
Tin (Sn)	0.004	0.009
Uranium (U)	3	0.4
Vanadium (V)	2.5	0.5
Zinc (Zn)	5	15

aquatic life are sumarized by International Lead Associations [16-19] (table 2).

In living cells, pathotoxicity of metalic lead involves ionic mechanism and oxidative stress. Many researchers have shown that oxidative stress in living cells is caused by critical balance between specific free radicals generation and antioxidants defense, leading to a disorder of redox signaling and control having as result molecular damage. Lead intoxication favors the ROS (reactive oxygen species) generation, while antioxidants prevent ROS formation. In very high concentrations ROS induce cell membranes impaired and also DNA proteins and lipids damage, specific to oxidative stress [20]. Chelation therapy and new therapeutic strategies (like nanoencapsulation), are used to treat lead intoxication effects on central nervous, hematopoietic, hepatic and renal system [21].

Today, the ability of herbal antioxidants to provide protection against lead induced oxidative stress is well known [22-25]. Thus microalgae chlorella contains a (1,3)-β-D-glucan, a powerful scavenger of free radicals. By its antioxidant protective components Chlorella detoxifies the organism from harmful chemicals (pesticides, drugs, heavy metals). Experimental studies showed that chlorella treatment significantly reduces oxidative stress caused by lead exposure. Also, garlic shows protective antioxidative and chelation ability, provided by its organo-sulphur compounds (diallyl tetrasulfide), sulphur-containing amino acids (S-allyl cysteine and S-allyl mercaptocysteine) and compounds with free carboxyl and amino groups [26-28]. Essential metals such as manganese has also been shown to have antioxidant properties, leading to ability to monitor the activity of free radicals in living organisms.

Manganese and superoxide dismutases form antioxidant defence metalloenzymes. Mitochondria are cellular organelles responsible for energy production, and 90% of the body's oxygen consumption takes place here, mitochondria being vulnerable to attack of free radicals. Mangan superoxid-dismutase is a metalloenzyme which transform free radical superoxide produced by mitochondrial activity into hydrogen peroxide which is then transformed into water by other enzymes. But, essential trace elements deficiency such as manganese, zinc, copper, chromium, calcium and magnesium enhance lead absorbtion. Consequently, as we have shown in previous works [29-31], lead can interfere with tissue calcium, zinc, copper and iron. This may be due to disruption of ceruloplasmin and ferritin production, very important copper and iron-binding proteins. Displacing zinc, copper,

Table 2
SURVEY OF WATER QUALITY STANDARDS FOR THE PROTECTION OF AQUATIC LIFE

Location	Reference	Acute (μg/L)	Chronic (μg/L)	Notes
Australia/New Zealand	ANZECC		2	Hardness 20 mg/L
			6.5	Hardness 50 mg/L
			37.8	Hardness 200 mg/L
			4.4	Seawater
Canada	CCME		1	Hardness 0-60 mg/L
			2	Hardness 60-120 mg/L
			4	Hardness 120-180 mg/L
			7	Hardness >180 mg/L
European Union	EC	14.25	1.2*	EU generic (worst case)
			2.4	DOC 2 mg/L
			6	DOC 5 mg/L
			12	DOC 10 mg/L
		14.25	1.3	Seawater
United States	U.S. EPA	10.8	0.4	Hardness 20 mg/L
		30.1	1.2	Hardness 50 mg/L
		136	5.3	Hardness 200 mg/L
		210	8.1	Seawater
*bioavailable EQS				

Note: EQS-Environmental Quality Standard; DOC- Dissolved Organic Carbon manganese, iron and chromium, lead can interfere with the function of most of essential metallo-enzymes.

In this paper, we have proposed to investigate the manganese tissue distribution in selected organs of Prussian carp fish before and after lead intoxication and to highlight the detoxifying potential of the garlic and chlorella active principles.

Experimental part

One year juvenile fish were harvested from a local fish farm and acclimated under controlled laboratory conditions for 2 weeks. Then, healthy specimens weighing 22-25 g were selected by gravimetric measurements and randomly distributed in four 60 L capacity aquariums (30 individuals/aquarium) with aeration equipment as follow: the control (C) group was maintained in fresh water Pb free, the others three groups receiving 75 ppm Pb into the water as $Pb(NO_3)_2$ x ½H_2O (E1 - Pb), 75 ppm Pb into water+2% freeze-dry garlic in feed (E2 - Pb+garlic), (75 ppm Pb into the water + 2% freeze-dry chlorella in feed (E3-Pb+chlorella). During the acclimatization and experimental period, the fish were fed 2 times a day with a pelleted commercial product; having 12 h light and 12 h dark regime; with special attention of water quality: temperature, *p*H, dissolved oxygen, hardness, nitrites, nitrates, to avoid potential alterations of tested substance toxic action. A Hanna Hi 9145 Water Resistant Oxygen meter has been used to check water temperature and dissolved oxygen; and a Termatest kits -was used for pH, NO^-_2, NO^-_3, water hardness. Once a week, the water from each aquarium was changed and the corresponding amount of the contaminant was added to the replaced one. Tissue samples (gills, muscles myotome-epaxial, heart, skin and scales, intestine, liver, brain, gonads, kidney) were collected after a 12 h starving period, and fish euthanasia with clove oil. Manganese tissue concentrations were analytically determined using atomic absorption spectrometery (AAS). Statistical analysis was performed using SPPS IBM 22 software. Data were reported as a significant level at $p<0.05$. Testing differences between means was realized by ANOVA completed with post-hoc Tukey test.

Results and discussions

Manganese biochemical functions can be summarized as follows: it is an essential component of some enzymes (ie. pyruvate carboxylase), but also an activator for others (ie. phosphate transferases, phosphate dehydrogenases); it is involved in oxidative phosphorylation processes; in cholesterol and fatty acid synthesis; manganese activates several enzymes that influence the carbohydrates metabolism; it is cofactor for enzymes involved in cartilage and bone tissues production; and also synthesis of hemoglobin requires manganese. Thus, manganese is included in an essential trace element required for cellular functions regulation and control. It is readily absorbed from the gastro-intestinal tract, gills, fins and skin of fish and crustacea, liver and kidneys being manganese-rich tissues [32, 33]. Mean manganese concentrations of up to 17 mg/kg dry body weight have been found in tissues (liver, kidney, whole body) from a variety of reptiles and mammals. In tissues of marine and freshwater fish, manganese tends to range from < 0.2 mg/kg to 19 mg/kg dry weight [34, 35].

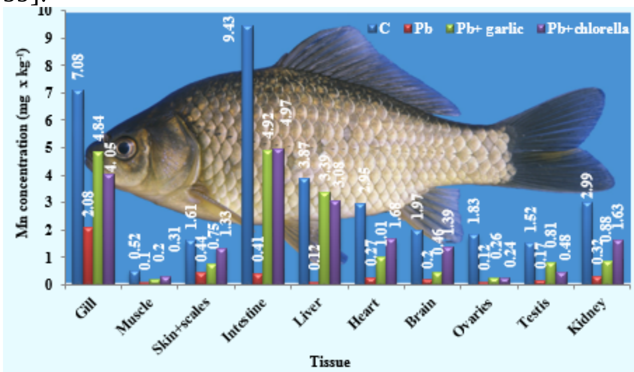

Fig. 1. Influence of water-borne lead exposure on manganese in fish tissues (average values)

In our experiment higer manganese concentrations have been recorded in control group fish in intestine (9.43 mg/kg wet weight), gills (7.08 mg/kg wet weight), liver (3.87 mg/kg wet weight) and kidney (2.99 mg/kg wet weight). The distribution of Mn in fish organs varied as: intestine > gilss > liver > kidney > heart > brain > ovaries > skin > testis Ã muscle. Acording to IAEA, tolerable concentrations for Mn in fish must not exceed 11.00 mg/kg dry weight

Table 3
FISH TISSUE MANGANESE CONCENTRATION - STATISTICAL SIGNIFICANCE OF LEAD TOXICITY

Tissue	*Number*	*Sum*	*Average*	*Variance*	*SD*
Mn (mg/kg wet weight)					
Gill	4	18.05	4.51	4.27	1.79
Muscle	4	1.13	0.28	0.03	0.15
Skin+scales	4	4.13	1.03	0.28	0.46
Intestine	4	19.73	4.93	13.56	3.18
Liver	4	10.46	2.61	2.87	1.46
Heart	4	5.91	1.47	1.29	0.98
Brain	4	4.02	1.00	0.67	0.71
Ovaries	4	2.45	0.61	0.66	0.70
Testis	4	2.98	0.74	0.33	0.50
Kidney	4	5.82	1.45	1.33	1.00
Group					
C	10	33.77	3.37	7.78	2.64
Pb	10	4.23	0.42	0.35	2.46
Pb+freeze-dry garlic	10	17.52	1.75	3.52	2.45
Pb+chlorella	10	19.16	1.91	2.60	2.53
Surce of variance					*p*
Between tissues					p<0.05
Between treatments					p<0.05

[36]. Lower levels of Mn have were found in pike'oragns by Rajkowska and Protasowicki [37], but not in bream's gills and skind where Mn content exceed the concentrations that we have registered. Lead toxicity caused changes in manganese homeostasis ($p<0.05$) (table 3, fig. 1), reaching values of 2.08 mg/kg for gills, 0.41 mg/kg wet weight for intestine, 0.12 mg/kg wet weight for liver and 0.32 mg/kg wet weight for kidney. As such, dietary and drinking manganese availability and absorption is reduced by the presence of large amounts of lead in water. This means that, Pb suppresses the translamellar and transduodenal transport of Mn, hence its low concentrations which have been registered in the other analyzed tissues.

Freeze-dry garlic and chlorella powder addition in fish feed determined the gradual diminishing of the lead effect on manganese transport and tissue uptake, the maximum efficiency of garlic and chlorella showed in intestinal Mn level (4.97 mg/kg – chlorella and 4.82 mg/kg – garlic). There were no differences in chelating/antioxidant effect between garlic and chlorella for ovaries and muscle. Mn concentration in the ovary was 0.24-0.26 mg/kg, around 5 mg/kg in intestine and 0.2-0.3 mg/kg in muscle for the groups protected with garlic and chlorella in feed. However, in hepatic tissue, garlic closely followed by chlorella raised the Mn level to the concentration of control group (3.39 mg/kg and 3.08 mg/kg, respectively). Comparing the effects of bioactive principles of chlorella and garlic, the chlorella seems to be more efficient in counteraction of lead antagonistic effect to Mn in kidney, brain, heart skin and muscle, while garlic powder showed evident effect in gills and liver. However, regardeless of the analyzed organ, the active components from garlic and clorella tend to restore manganese tissue levels in gonads and muscle.

Conclusions

Our results suggest that:

- lead intoxication disturbs manganese homeostasis, causing tissue depletion and preventing the utilization of manganese in fish organism;
- the freeze-dry garlic and chlorella provide protective effect on lead, inducing manganese tissue mobilization.
- chlorella powder was more efficient in kidney, brain, heart and skin, while garlic powder - in gills and liver;
- both increased the Mn level in hepatic tissue of control fish, but showed no differences in chelating/antioxidant potency between garlic and chlorella for intestine, ovaries and muscle.

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Manuscript received: 21.11.2017